

Phase II study of bi-weekly irinotecan for patients with previously treated HER2-negative metastatic breast cancer: KMBOG0610B

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Abstract

Background A trial was conducted to evaluate the feasibility, efficacy, and safety of biweekly administration of irinotecan, a novel topoisomerase I inhibitor, for patients with metastatic breast cancer (MBC) previously treated with either anthracycline-based or taxane-based chemotherapy.

Methods Eligible patients were HER2-negative, had a performance status of 0 to 2, and had been treated previously with either anthracyclines or taxanes for MBC. Patients received irinotecan intravenously at 150 mg/m² on days 1 and 15 every 4 weeks. The primary end-point was feasibility, and the treatment was considered feasible if a patient was able to receive three administrations of irinotecan within the first 8 weeks, as pre-specified in the protocol.

Results Eighteen patients (median age 60 years) were enrolled. Fifteen patients received irinotecan more than 3 times within the first 8 weeks, with resulting feasibility of 83.3%. The median number of treatment cycles was 2 (range 1–16) during this period, and the relative dose intensity was 91.2%. Partial response was observed for one patient, so overall response rate was 5.6%. Nine patients (50.0%) had stable disease, and overall disease control was 50.0%. Median progression-free survival and overall survival periods were 3.2 and 9.6 months, respectively. The only grade 3/4 hematological toxicity was neutropenia (22.2%). Grade 3/4 non-hematological toxicities were anorexia (11.2%), diarrhea (11.2%), and fatigue (5.6%). No treatment-related death occurred.

Conclusions This study demonstrated that biweekly administration of 150 mg/m² irinotecan was feasible for

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patients with MBC treated previously with anthracyclines or taxanes.

Keywords Metastatic breast cancer · Irinotecan · Phase II trial · Topoisomerase I inhibitor · HER2-negative

Introduction

Metastatic breast cancer (MBC) remains an incurable disease, and the main objectives of treatment are prolongation of survival time and palliation. The standard first-line chemotherapy for MBC has been based on anthracyclines or taxanes [1, 2]. For patients with HER2-negative disease, there is limited consensus regarding the use of subsequent chemotherapy if treatment with anthracyclines and taxanes fails, whereas introduction of anti-HER2 therapy has resulted in improved survival of patients with HER2-positive MBC [3]. Anthracyclines and taxanes are the mainstays of adjuvant therapy for breast cancer, but after such therapy tumor cells often develop resistance to the drugs when the cancer recurs. To overcome the problem of refractory disease, drugs with different mechanisms of action would be of great value.

Irinotecan is an inhibitor of topoisomerase I, an enzyme necessary for DNA replication. The irinotecan metabolite, SN-38, binds to the topoisomerase I-DNA complex, preventing the enzyme from resealing the DNA during replication and transcription, and thereby producing DNA breaks, leading to apoptosis.

Irinotecan-based chemotherapy has been shown to be active against metastatic colorectal cancer and small-cell lung cancer. Similarly, this agent had clinical activity against MBC in phase II studies [4–6]. Previous trials involving patients with MBC have used two different irinotecan regimens (every 3 weeks or weekly on day 1 of each of 4 consecutive weeks, followed by a 2-week rest period), and moderate toxicity and promising efficacy have been observed. However, with regard to bi-weekly administration of irinotecan, no systematic studies have been reported in which the safety and efficacy of the regimen for patients with MBC were evaluated [5].

In this study, we investigated the feasibility and efficacy of irinotecan administered bi-weekly for patients with HER-2-negative MBC that had been treated previously with either anthracycline-based or taxane-based chemotherapy.

Patients and methods

Study design and patient selection

This was a multicenter single-arm phase II trial designed to evaluate the feasibility of a bi-weekly schedule of

irinotecan for women in whom MBC had recurred or progressed after anthracycline-based or taxane-based chemotherapy, or both. The trial was conducted by the Kinki Multidisciplinary Breast Oncology Group (KMBOG).

Eligible patients were female, aged ≥ 20 and ≤ 75 years, and had cytologically or histologically confirmed metastatic breast cancer that was HER2-negative (IHC 0–1 or IHC 2+ confirmed as FISH-negative). Other eligibility criteria included: prior treatment with at least one regimen containing an anthracycline or a taxane for advanced disease, or relapse during or within 6 months for adjuvant chemotherapy, an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤ 2 , and a life expectancy of more than 3 months. Within 14 days of study registration, patients had to have an absolute neutrophil count of $\geq 1,500/\text{mm}^3$ or a white blood cell count of $\geq 3,000$, platelets $\geq 100,000/\text{mm}^3$, and hemoglobin ≥ 8.0 g/dL. Also at baseline, creatinine had to be less than the upper limit of normal (ULN), total bilirubin had to be <1.5 mg/dL, and both AST and ALT had to be 2.5 times the ULN.

Patients who had undergone chemotherapy, immunotherapy, biological systemic anticancer therapy, and radiation therapy within ≤ 2 weeks, and hormonal therapy within 4 weeks of study registration were excluded. Previous administration of irinotecan was not permitted. Also excluded from participation were patients with other serious illnesses and medical conditions, for example uncontrolled infection, intestinal obstruction, persistent diarrhea, interstitial pneumonia, other malignancies, or CNS metastases that were still symptomatic. In addition, women who were pregnant, lactating, or unwilling to use adequate contraception were excluded.

The study was performed according to the precepts established by the Helsinki Declaration. The protocol was approved by the institutional review boards of all the participating centers, and each patient provided written informed consent to participate.

Treatment schedule

Irinotecan was administered at a dose of 150 mg/m^2 in normal saline or 5% dextrose as a 90-min infusion on days 1 and 15 every 4 weeks. Use of hematopoietic growth factors (G-CSF) was allowed for patients with febrile neutropenia. At the investigator's discretion, treatment with analgesics and antiemetics was permitted. Loperamide was also allowed for treatment of diarrhea during and between irinotecan administrations.

Administration of irinotecan on day 1 was delayed by 1 or 2 weeks for patients with diarrhea, $>$ grade 2 non-hematological toxicity, leucopenia, neutropenia, or a platelet count $<75,000$. On day 15, administration of irinotecan was skipped if the same toxicity occurred. If grade 4

hematological or grade 3 non-hematological toxicity was observed, the dose was reduced to 120 mg/m² from the next cycle onwards. If, after dose reduction to 120 mg/m², the same toxicity recurred, the treatment was discontinued. No dose re-escalation was allowed after dose reduction.

Treatment was continued until the appearance of grade 4 non-hematological toxicity, patient refusal, disease progression, patient removal for clinical reasons at the investigator's discretion, or death.

Patient evaluation

Pretreatment evaluations included complete medical history, physical examination, tumor evaluation, hematology, and biochemistry, and ECG. Patients were evaluated for toxicity every 2 weeks, in accordance with the National Cancer Institute Common Toxicity Criteria, version 3.0 (NCI-CTC v3.0). Radiological tumor assessments were performed every 2 months to confirm response until progression. The response rate (RR) was defined as the percentage of evaluable patients whose best overall response was classified as either complete response (CR) or partial response (PR) according to the Response Evaluation Criteria in Solid Tumors (RECIST).

Data analysis

Intention-to-treat analysis considering all patients was performed. The primary endpoint was feasibility, and the treatment was regarded as feasible if a patient was able to receive three infusions of CPT-11 within the first 8 weeks, as pre-specified in the protocol. Assuming expected feasibility of 80%, accuracy of 20%, and a threshold completion rate of 60%, a minimum of 16 evaluable patients were required. In consideration of this number, and possible ineligible patients and/or dropouts, the target number of patients for this study was set at 18.

Secondary endpoints included the objective response (CR + PR) rate, median progression-free survival, median overall survival, relative dose intensity, and toxicity. Progression-free survival was calculated as the time from the first day of treatment to the first day of documented progression or death. Survival time was defined as the time from the day of registration to the final date of confirmed survival or the date of death. Analysis was performed with SPSS version 16.0 for Windows (SPSS, Chicago, IL, USA).

Results

Patient characteristics

Eighteen patients were enrolled in the trial between April 2006 and December 2009 (Table 1). All patients were

evaluable for toxicity and response. Two-thirds of the patients (12/18) had previously received two or more chemotherapy regimens for advanced disease. In addition, more than three quarters of the patients (14/18) had received both anthracycline-based and taxane-based treatment, including an adjuvant setting. There were measurable lesions in all patients (involving the liver in 5 patients, lymph nodes in 8 patients, and lungs in 12 patients). Six patients had triple-negative breast cancer (TNBC).

Treatment

Eighteen patients received a total of 78 cycles of the therapy (1–18 cycles per patient). The relative dose intensity of irinotecan was 91.5%. Fifteen patients were administered irinotecan more than 3 times within the first 8 weeks, and the feasibility was 88.9%. The median number of treatment cycles was 2 (range 1–16) during this period. Over the course of the trial, 2 patients (11.1%) required dose reduction on day 1 of the second course because of toxicity (grade 3 diarrhea and anorexia, respectively), and treatment interruption was necessary for 7 patients.

Table 1 Patient characteristics (*n* = 18)

	<i>N</i> (%)
Median age (range)	59 (40–75) years
ECOG performance status	
0	3 (16.7)
1	14 (77.8)
2	1 (5.6)
Receptor status	
ER and/or PR positive	11 (61.1)
ER/PR negative	7 (38.9)
Previous treatment	
Anthracyclines	15 (83.3)
Taxanes	17 (94.4)
Both anthracyclines and taxanes	14 (77.8)
Number of chemotherapy regimens for metastases	
1	1 (5.6)
2	4 (22.2)
3 or more	13 (72.2)
Site of metastasis	
Lung	12 (66.7)
Bone	7 (38.9)
Liver	8 (44.4)
Lymph nodes	5 (27.8)
Chest wall	5 (27.8)

ER estrogen receptor, PR progesterone receptor, ECOG Eastern Cooperative Oncology Group

Table 2 Frequency of adverse events seen in >10% of patients, attributable to treatment

Adverse event	Highest grade/number of patients (%)		
	Grade 1–2	Grade 3	Grade 4
Fatigue	4 (22.2%)	1 (5.5%)	0 (0.0%)
Nausea	4 (22.2%)	1 (5.5%)	0 (0.0%)
Vomiting	2 (11.1%)	2 (11.1%)	0 (0.0%)
Anorexia	2 (11.1%)	2 (11.1%)	0 (0.0%)
Diarrhea	3 (16.5%)	2 (11.1%)	0 (0.0%)
Neutropenia	4 (22.2%)	3 (16.5%)	1 (5.5%)

Toxicity

Treatment-related toxicity was generally modest and manageable (Table 2). The most common grade 3 and 4 adverse events were neutropenia in 4 patients (22.2%), and febrile neutropenia occurred in one patient only. The other grade 3 adverse events were diarrhea ($n = 2$, 11.1%), anorexia ($n = 2$, 11.1%), and vomiting ($n = 2$, 11.1%). Only one patient experienced a grade 4 adverse event (neutropenia) during the course of the study. No instance of irinotecan-related death was observed.

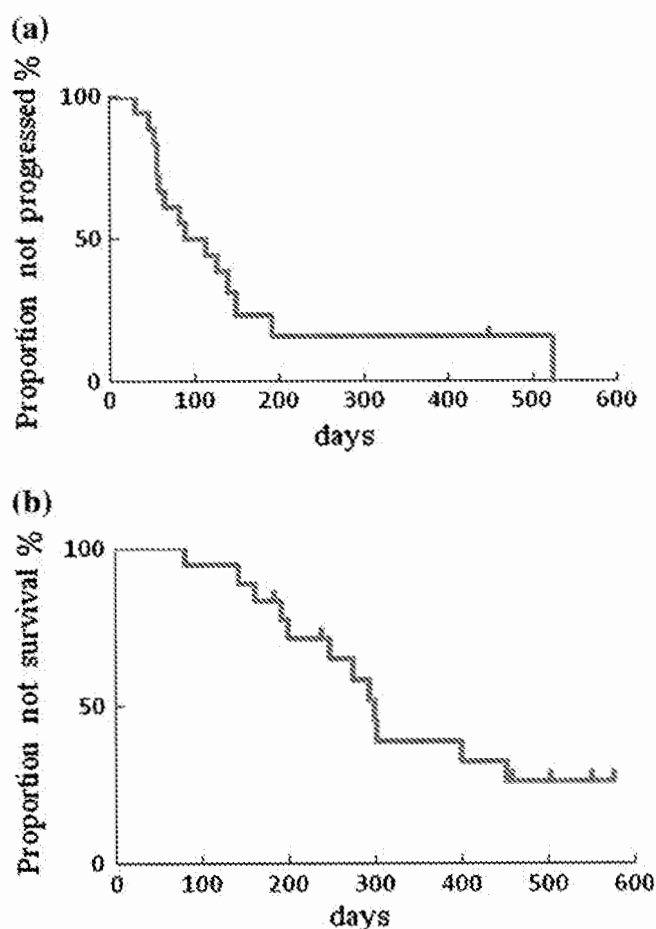
The primary reason for trial drug discontinuation was disease progression ($n = 15$, 83.3%), followed by toxicity, patient refusal, and deterioration of PS ($n = 1$, 5.6%, respectively). None of the patients had continued receiving irinotecan by March 2011.

Efficacy

The objective response rate was 5.6% (95% CI 0.1–27.3%). An additional 8 patients had SD for ≥ 4 months, giving a clinical benefit rate (CR + PR + SD ≥ 4 months) of 50.0% (95% CI 26.0–80.0%). The median progression-free survival time was 2.9 months (95% CI 0.7–5.2 months) and the median overall survival period was 10.0 months (95% CI 8.9–11.1 months) (Fig. 1).

Discussion

In this trial we demonstrated that biweekly administration of 150 mg/m² irinotecan was feasible for patients with MBC treated previously with anthracyclines or taxanes. Even in the trial for pre-treated patients in MBC, feasibility was 88.9% and toxicity associated with biweekly administration of irinotecan was predictable and manageable. The results also suggested efficacy was promising, with disease control of 50.0% (95% CI 26.0–80.0%) and median progression-free survival of 2.9 months (95% CI 0.7–5.2 months).

**Fig. 1** Progression-free (a) and overall (b) survival ($N = 18$)

Mono-chemotherapy with irinotecan for advanced MBC has also been investigated by another group, who used two different schedules: every 3 weeks or weekly on day 1 of each of 4 consecutive weeks, followed by a 2-week rest period [6]. Although the dose of irinotecan in that trial was equivalent to that in ours, both grade 3 and 4 hematological and non-hematological toxicity were more manageable for our bi-weekly schedule. In the other trial, weekly and tri-weekly regimens of irinotecan achieved response rates of 14 and 23%, respectively; the median response duration was 4.2 months, and the median OS was 8.6 months (95% CI 7.0–12.3 months). Although response to bi-weekly irinotecan was lower than that in the weekly or tri-weekly schedules, the PFS and OS were similar in both trials. Good tolerability of bi-weekly irinotecan may have contributed to this result, despite the lower response rate.

Recently, eribulin, a tubulin-targeting agent, has been shown to significantly improve overall survival in women with heavily treated MBC in comparison with other available cytotoxic therapy; the response rate was 12% (95% CI 9.4–15.5%), the median PFS 3.7 months (95% CI 3.3–3.9 months), and the median OS 13.1 months (95% CI

11.8–14.3 months) [7]. In several phase II trials, capecitabine, an orally available fluoropyrimidine, has also been shown to achieve a response rate of 15–28%, a median time to progression of 7.5–8.3 months, and a median OS of 10.1–15.2 months [8–11]. Although we are unable to reach any firm conclusion, because of the small size of this feasibility trial, the safety profiles are comparable with those of the other agents described above that had modest anti-tumor effects with good tolerability and survival.

Although a few phase II trials investigating bi-weekly irinotecan for other types of solid tumor were reported more than a decade ago [12], evaluation of feasibility was adopted as primary end point in this study. Recently, numerous patients with MBC received several lines of chemotherapy during their lifetimes, because of the increasing number of active compounds and prolongation of survival time. This study included many patients who had been intensively treated by use of other regimens, and we suppose that heavily-treated patient's profiles with advanced MBC are different from those of patients with other solid tumors. Moreover, several supportive medicines for management of toxicity from chemotherapy have been developed in the last decade, and assessment of the feasibility of bi-weekly irinotecan for this group of patients is meaningful. Given the different situation in the previous trials for other types of cancer, we reasonably evaluated the feasibility of bi-weekly irinotecan in pre-treated MBC and demonstrated that this regimen was quite feasible in this population.

Several studies have linked the *UGT1A1**28 allele to the severe toxicity of irinotecan. Patients homozygous for *UGT1A1**28 are at significantly high risk of severe adverse events, for example diarrhea and myelosuppression because of reduction of *UGT1A1* protein expression and SN-38 glucuronidation capacity [13, 14]. In addition, a single nucleotide polymorphism, *UGT1A1**6, has been proved to reduce the catalytic activity of *UGT1A1*, and to be associated with drug-induced severe toxicity in Asian populations [15, 16]. In this study, although *UGT1A1* genotypes were not analyzed, grade 3 diarrhea developed in only 2 patients and was manageable, suggesting that bi-weekly administration of 150 mg/m² irinotecan is feasible in unselected patients.

In conclusion, this study demonstrated that biweekly administration of 150 mg/m² irinotecan is feasible for patients with MBC who have already been heavily treated with anthracyclines and taxanes. Further trials to evaluate the efficacy of this promising regimen for MBC in this setting are warranted. Additionally, we are now conducting another phase II trial which investigates the efficacy and toxicity of irinotecan combined with trastuzumab for extensively pretreated patients with HER2-positive MBC.

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Assembly of nucleic acid-lipid nanoparticles from aqueous-organic monophases

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Abstract

Effective, reproducible, and scalable methods for DNA–lipid assembly are important for the success of non-viral vectors in *in vivo* gene therapy. We hypothesized DNA–lipid assembly would be optimal if started from a liquid monophase where both DNA and lipids separately form molecular or micellar solutions prior to mixing, without preexisting condensed lipid phases, thus allowing DNA–lipid assembly under conditions close to equilibrium. Previously, we found that mixing plasmid DNA, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC), cholesterol and a cationic lipid, 1, 2-dioleoyl-3-(trimethylammonio) propane (DOTAP) in 50% (v/v) aqueous ethanol spontaneously produced an optically transparent solution. Upon ethanol removal, DNA–lipid nanoparticles (Genospheres™) were formed. For comparison with well-known technologies, different DNA–lipid particles were prepared by interaction of plasmid DNA and stable or ethanol-destabilized lipid vesicles by combining the components in water or 30% (v/v) aqueous ethanol, respectively. Among the three studied DNA–lipid assembly methods, only Genospheres combined the properties of small size (less than or around 100 nm), high incorporation of both lipid and DNA, high degree of DNA protection (dye accessibility 5–12%), a narrow distribution of particle density and when immuno-targeted, the highest transfection efficiency in HER2-overexpressing cells *in vitro*. We conclude that the Genosphere assembly methodology offers advantages for the development of effective, scalable and targetable non-viral gene delivery vectors.

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Keywords: Non-viral gene delivery; DNA–lipid complex; Nucleic acid encapsulation; HER2 targeting

1. Introduction

Gene therapy is a potential therapeutic strategy for the treatment and cure of inherited diseases such as cystic fibrosis and diseases that are acquired, such as cancer. Lipid based vectors are seen as a

promising tool, overcoming many of the immunogenic side effects associated with viral carriers for gene delivery.

An ideal carrier should be small, have the ability to evade the RES system to reach remote diseased sites such as tumors and be able to deliver the intact genetic material intracellularly at the target cell. The carrier must also be easily modified in terms of altering composition and/or incorporation of targeting ligands to suit specific applications.

Presently, methodologies for DNA–lipid particle production include a liquid–liquid phase separation step, the presence of lipid solubilizing detergent, DNA interaction with pre-formed liposomes or a drying and rehydration step. We hypothesized that DNA–lipid assembly would be improved if occurring within a liquid monophase where both DNA and lipids were separately molecularly/micellarly soluble prior to mixing, without pre-existing condensed lipid phases, thus allowing

Abbreviations: DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; HEPES, 2-(4-[2-hydroxyethyl]piperazino)ethanesulfonic acid]; HBS, 5 mM HEPES, pH 7.4, 144 mM NaCl; PEDL, particles formed from ethanol-destabilized liposomes; PEG-DSPE, N-[ω-methoxy-(poly(oxyethylene)-α-oxy-carbonyl)]-DSPE (PEG mol. weight 2,000); PEG-DSG, 1,2-distearoyl-*sn*-glycerol 1-(methoxypoly(ethylene glycol) ether. (PEG Mol. weight 2,000)

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DNA–lipid assembly under conditions close to equilibrium. To affect the assembly, the organic solvent component should be removed without separation of the system into two liquid phases or to complete dryness.

Previously, we found that mixing plasmid DNA with neutral (POPC, Cholesterol) and cationic (DOTAP) lipids in an aqueous/organic solvent monophase where initially no lipid condensed phases were observed, spontaneously produced an optically transparent solution. Upon solvent removal, we observed that small DNA–lipid nanoparticles were formed (Genospheres™) [1]. In this study, we compared the DNA–lipid particles prepared by the monophase assembly (in particular, containing 50% (v/v) ethanol) with particles prepared by two well known methodologies; interaction of DNA with stable or 30% (v/v) ethanol-destabilized lipid vesicles as described in the literature by Felgner et al. [2] and Maurer et al. [3], respectively. The particles were characterized by: DNA compactness, lipid/DNA and cationic/neutral lipid proximity, DNA exposure; lipid lamellarity; compositional homogeneity; completeness of DNA and lipid incorporation; and in vitro transfection in a non-targeted or ligand-(antibody scFv fragment)-targeted variant. The data suggested that the Genospheres technology platform may offer advantages for the manufacture of pharmaceutical gene delivery formulations.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-(trimethylammonio)propane (DOTAP), 1-palmitoyl,2-oleoyl-3-*sn*-phosphatidylcholine (POPC), (*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-phosphatidylethanolamine) NBD-PE and *N*-CAP-carboxy-fluorescein-phosphatidylethanolamine (CFPE) were purchased from Avanti (Alabaster, AL) and used without further purification. Cholesterol (Chol) was purchased from Calbiochem (San Diego, CA). Stock solutions of lipids dissolved in ethanol (Gold Shield, Hayward, CA) were stored at –20 °C. *N*, *N*-di-³H methyl-*N,N*-diocadecylammonium bromide ([³H]-DDAB), [1 α , 2 α (n)-³H] cholesterol ([³H]-Chol) (Amersham Pharmacia Biotech, Piscataway, NJ) and phosphatidylcholine, 1- α -dipalmitoyl [dipalmitoyl-1-¹⁴C], (American Radiolabeled Chemicals, Inc., St. Louis, MO) were stored at –80 °C prior to use.

Purified Firefly luciferase and D-(–) luciferin were obtained from Roche (Indianapolis, IN). PicoGreen®, OliGreen®, YOYO-1®, EtHD-2 (ethidium homodimer-2), 1,1'-dioctadecyl-3,3',3'',3'''-tetramethylindocarbocyanine-5,5'-disulfonic acid (DiI₁₈(3)-DS), 1,1'-dioctadecyl-3,3',3'',3'''-tetramethylindocarbocyanine perchlorate (DiI(3)), bis-(3-propyl-5-oxoisoxazol-4-yl)pentamine oxonol (oxonol (VI)) and anti-fluorescein/Oregon Green rabbit polyclonal IgG Fab fragment were purchased from Molecular Probes (Eugene, OR). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA) while spermidine was obtained from Sigma (St. Louis, MO). Cell culture media was purchased from the UCSF cell culture facility (UCSF, San Francisco) and cells were obtained from ATCC (Rockland, MD). F5-PEG-DSPE conjugate was prepared from the purified anti-HER2 scFv, F5, by conjugation to the maleimide-activated PEG termini of the PEG-DSPE lipid anchor through an engineered c-terminal cysteine as described previously [4]. Other reagents of the highest possible grade were purchased and used without further purification.

2.2. Light scattering of lipid solutions of varying ethanol content

A DOTAP/POPC/Chol/PEG-DSPE (molar ratio 6/15/10/0.16) lipid solution was prepared from stock lipid solutions in ethanol. The mixture was filtered through a 0.2 μ m polycarbonate membrane (Whatman, NJ) at 60 °C to remove

any large particles that would interfere with the measurements. The filtrate was divided into four round-bottomed flasks and evaporated under vacuum; the resulting lipid films hydrated in the desired organic solvent/5% (w/w) dextrose solution followed by brief mixing. The lipid solutions were diluted to 2.1 mM phospholipid in their respective organic solvent containing dextrose solution, and the light scattering intensity was determined by photon correlation spectroscopy using a Nicomp C370 particle size analyzer (Particle Sizing Systems, Santa Barbara) at 632.8 nm at 23 °C and 55 °C by reading the scattering event frequency at constant gain. Alternatively, light scattering was measured at 90° angle using a fluorescence spectrometer (Fluorolog, Jobin Yvon Spex, NC) at $\lambda_{exc/em}$ =633 nm with 1 nm slit widths, using a stirred, thermostated cell holder (± 0.1 °C).

2.3. DNA–lipid particle preparation

A bacterial plasmid containing firefly luciferase gene under the control of early cytomegalovirus promoter (pCMV/luc⁺) and a penicillin resistance gene was constructed as described previously [5]. *E. coli* transfected with pCMV/luc⁺ were selected on an ampicillin agar plate and grown in LB medium with ampicillin (50 μ g/ml). The plasmid was extracted and purified using the EndoFree Plasmid Giga kit from Qiagen (Chatsworth, CA). The plasmid concentration was determined by absorbance at 260 nm (ϵ =6600 l mol^{–1} cm^{–1}) and purity by calculating the ratio $A_{260\text{ nm}}/A_{280\text{ nm}}$ (Shimadzu, UV160U). DNA is considered to be sufficiently protein free with an $A_{260\text{ nm}}/A_{280\text{ nm}}$ ratio of at least 1.85. An siRNA duplex that was shown to have specific gene inactivation of an adaptor protein Shc A [6], which has been implicated in many types of receptor mediated cell signaling was obtained from Dharmacon (Lafayette, CO) (AA-N19 mRNA Target 5'→3'; AACUACUUGGUUCGGUACAUGG). Phosphorothioate modified oligonucleotide was obtained from MWG-Biotech AG, NJ and had a sequence shown to have specific inhibition of up-regulated genes implicated in colon cancer (5'CACTGCCAATCTCATGGTGC 3') (LC Chen and NM Lee, California Pacific Medical Center Research Institute, personal communication). Both siRNA and oligo were supplied in a lyophilized form and were rehydrated with deionized water with concentrations determined by absorbance at 260 nm using the expressions that 1 O.D equates to 40 μ g/ml siRNA and 33 μ g/ml oligonucleotide.

From a plasmid DNA (pCMV/luc⁺, 4.5 kbp) stock solution of 2 mg/ml in TE, pH 8.0 buffer, plasmid DNA, siRNA or oligonucleotide was diluted to 0.4 mg/ml in 5 mM HEPES–Na buffer, pH 7.4 and used in the preparation of complexes. For fluorescence resonance energy transfer (FRET) studies, intercalating dyes were added to aliquots of the plasmid DNA solution at a concentration of 1 dye per 200 bp and incubated at room temperature for 1 hour to allow adequate time for complete dye binding before further processing. A stock lipid solution of DOTAP, POPC, and Chol dissolved in ethanol at a molar ratio of 6:15:10 was used in the preparation of the complexes. The lipid mixture was added to the DNA as described below at a ratio of 6 nmol DOTAP per 1 μ g of DNA. This corresponds to a 2-fold excess of cationic charge from DOTAP over the anionic phosphates of each nucleotide. Fluorescent lipid markers DiI(3), DiI(3)-DS and NBD-PE were added if required to the lipid mixtures prior to complex formation, at a molar ratio of 2:1 to the intercalating DNA dye in the final complex.

2.3.1. Lipoplex

The complexes made from mixing cationic liposomes and DNA together are commonly referred to as lipoplexes [7]. An aliquot of the lipid stock solution containing 3.1 μ mol (total lipid) lipid was brought to dryness under vacuum and re-hydrated with 0.5 ml 5 mM HEPES, pH 7.4. The lipid suspension was vortexed and sonicated to clarity (5 min) in a bath sonicator (Laboratory Supplies Company, Hicksville Inc., NY) to produce liposomes [5]. Stock DNA solution was diluted twice to a final volume of 0.5 ml in 5 mM HEPES (pH 7.4) and added to the liposome solution rapidly at room temperature. These lipoplexes were used within 1–2 h after preparation.

To validate the use of DiI₁₈(3) and DiI₁₈(3)-DS as appropriate FRET markers for cationic and neutral lipids, respectively, sonicated liposomes were prepared containing each dye separately in a 1:1 molar mixture with DOPE as described above. Lipoplex complexes were prepared by mixing liposomes with DNA in a ratio of two dyes per DNA phosphate. After incubation at room temperature for 30 min, the suspensions were briefly centrifuged (13,000 rpm for 5 min). An estimate of the amount of fluorescence lipid remaining in solution

was made by comparison of the fluorescence intensity of the solution before and after centrifugation, by dissolving known aliquots in 0.1% Triton X-100 and measuring intensities using (λ_{ex} 549 nm/ λ_{em} 566 nm).

2.3.2. Particles prepared from water/organic solvent mixtures

Aliquots (3.1 μmol) of the lipid mixture stock solution were diluted with appropriate volumes of ethanol and 5 mM HEPES, pH 7.4 buffer and ethanol to bring the concentrations of ethanol to 30% (v/v) or 50% (v/v), in a final volume of 0.5 ml. Alternatively, the lipid was dried and re-dissolved in the appropriate organic solvent/aqueous mixture. In separate tubes, aliquots of the nucleic acid stock solution (containing 100 μg) were mixed with appropriate volumes of 5 mM HEPES, pH 7.4 buffer and organic solvent to obtain 0.5 ml of a solution containing the desired water miscible solvent concentration. The nucleic acid and lipid solutions were pre-warmed at 60 °C, and the nucleic acid solution was added rapidly to the lipid solution of corresponding solvent concentration, pipetting up and down 4–5 times to ensure complete mixing. The mixtures were further incubated at 60 °C for 5 min, then removed from the water bath and allowed to reach room temperature. The mixture was dialyzed exhaustively against 5 mM HEPES, pH 7.4 buffer with or without 0.15 M NaCl (as indicated below) at room temperature, using dialysis tubing with a molecular weight cut-off of 6000–8000 Da.

2.3.3. HER2 targeted particles

HER2-targeted lipid–DNA particles were prepared using the method previously described for liposomes [4]. HER2-specific, highly internalizable single chain Fv was conjugated via a c-terminal cysteine group to a thiol-reactive amphiphilic lipid anchor maleimido-PEG-DSPE, and the micelle-forming conjugate (F5-PEG-DSPE) was purified by size exclusion chromatography. F5-PEG-DSPE was incubated with the DNA–lipid particles in HEPES–saline buffer (5 mM HEPES–Na, 144 mM NaCl, pH 7.4) at 37 °C overnight with typically the amount of conjugate equal to 5 μg antibody added per μmol of POPC in the sample. The antibody conjugate is presented in a solution also containing a proportion of cysteine quenched maleimide-PEG-DSPE [8,9] and therefore non-targeted control samples were heated in the same manner using an identical amount of additional PEG-DSPE (0.73 nmol PEG-DSPE per μmol PC in sample). Attachment of the antibody is achieved by spontaneous incorporation of the lipophilic (DSPE) portion of the conjugate into the outer lipid layer of the particles during the incubation. Allen and coworkers describe this technique in detail [10]. Genosphere samples were stored at 4 °C until use. To maintain sterility of the lipoplexes, DNA, sonicated liposomes, and F5-PEG-DSPE solutions were filter-sterilized and combined under aseptic conditions.

2.4. Particle size analysis

The volume-weighted mean and standard deviation (SD) of the particle diameter distribution was determined in 5 mM HEPES–saline pH 7.4 at 23 °C using quasielastic light scattering (Nicom C370 Particle Size Analyzer (Nicom Particle Sizing Systems, Santa Barbara, CA) using the solid particle mode and Gaussian analysis for chi of 3.0 or less, or SDP analysis for chi over 3.0. The measurement of particle sizes for the studies reported in (Fig. 3A, B) was made by a Beckman PCS Particle Sizer, using the volume weighted size and standard deviation analysis function.

2.5. PicoGreen dye accessibility and particle DNA quantification assay

Aliquots (100 μl) of the particle suspensions containing approximately 700 ng/ml of DNA were placed in a 96-well microtiter plate, diluted with 100 μl of HEPES–saline, pH 7.4, and mixed with 2.5 μl of the PicoGreen DNA intercalating dye stock solution (Molecular Probes, Inc., OR). DNA concentration standards in the range of 50–1000 ng/ml were prepared in the same manner using pCMV/luc⁺ plasmid solutions instead of the particle suspensions. To provide for 100% DNA-accessibility reference, a 2 $\times$ dissociation solution (10% (v/v) DMSO, 1% (w/v) Zwittergent 3–14 in HEPES–saline) was used as a particle suspension diluent instead of HEPES–saline, and the samples were incubated at 60 °C for 30 min to solubilize the lipid components of the particles. The samples were cooled down to the room temperature, mixed with PicoGreen

dye stock and incubated for 20 min in the dark. The fluorescence intensity of the samples was determined in quadruplicate using microplate fluorometer (FL-600A, Bio-Tek Instruments, Inc.) equipped with a 485/20 nm bandpass excitation filter and a 530/25 nm bandpass emission filter. The percent of dye-accessible DNA was determined as the fluorescence intensity ratio between non-dissociated particle samples and dissociated (solubilized) particle samples times 100%, and the total amount of DNA was quantified from the fluorescence intensity of the dissociated particle samples using the concurrently run standard curve. PicoGreen was used for plasmid DNA and siRNA quantification, whereas Oligreen was used for oligonucleotide determination in a similar manner as described above.

2.6. FRET studies

2.6.1. DNA compactness

The FRET efficiency between the DNA-intercalating fluorophores YOYO-1 (λ_{ex} 470 nm/ λ_{em} 509 nm) and EthD-2 (λ_{ex} 530 nm/ λ_{em} 620 nm) forming a donor–acceptor pair was used to characterize the compactness of the complexed DNA. Each dye was added to the DNA at the ratio of 1 dye per 200 bp. A parameter E reflecting the energy transfer efficiency was calculated from the measured fluorescence intensities (Fluorolog, Jobin Yvon Spex, NC) (I) as follows:

$$E = \frac{F}{F_0} - 1 \quad (1)$$

$$F_0 = \frac{(I_{\text{EthD-2}}^{620 \text{ nm}} + I_{\text{YOYO-1}}^{620 \text{ nm}})}{(I_{\text{EthD-2}}^{509 \text{ nm}} + I_{\text{YOYO-1}}^{509 \text{ nm}})} \quad (2)$$

$$F = \frac{I_{\text{YOYO-1}}^{620 \text{ nm}}}{I_{\text{YOYO-1}}^{509 \text{ nm}} + I_{\text{EthD-2}}^{620 \text{ nm}}} \quad (3)$$

i.e., F_0 was determined from the measurements when only one of the probes was present, and therefore, was indicative of the spectral intensities at zero FRET efficiency, and F was determined when both probes were present, and FRET may have occurred. The measurements were taken at an excitation wavelength of 470 nm; the superscripts and subscripts in formulas (2) and (3) refer to the emission wavelengths and the particular dye(s) measured, respectively.

2.6.2. Relative distances between DNA and cationic lipid or neutral lipid

YOYO-1 labeled DNA and either DiI(3) (cationic) or DiI(3)-DS (neutral) lipid probes were used. The FRET efficiency parameter (E) was determined as above, using the fluorescence intensities of the emission maxima of the respective probes (DiI(3), DiI(3)-DS at 566 nm).

2.6.3. Relative proximity of cationic lipid and neutral lipid within the complexes

The lipid probes NBD-PE (λ_{ex} 465 nm/ λ_{em} 530 nm) and DiI(3) (λ_{ex} 549 nm/ λ_{em} 566 nm) were used as a FRET donor/acceptor pair to characterize the proximity of cationic lipid and neutral dialkyl-lipid in the DNA–lipid particles and lipoplexes. The lipid dyes were added at 0.1 mol% total lipid and the parameter E was calculated as above.

In the FRET studies outlined above, the samples can become diluted during dialysis. To account for the fluorescence intensity variations due to particle dilution, the fluorescence intensity was normalized to the total amount of phosphate-containing compounds in the particle preparation [11]. Complexes prepared without fluorescent probes were used to correct for background scattering.

2.7. Particle lamellarity determination

The proportion of the carboxyfluorescein-labeled phospholipid headgroups (CFPE) exposed on the particle outer surface, i.e., whose fluorescence was quenched by a membrane-impermeable quencher, anti-fluorescein Fab' fragment was considered indicative of the particle lamellarity. This allows one to obtain structural information on the number of enclosed bilayers contained within the particle. DNA–lipid particles and unilamellar liposomes (control)

were prepared using an ethanolic solution of DOTAP/POPC/Chol (6:15:10 molar ratio) with CFPE (0.5 mol% of POPC content) as described above. The particle samples were diluted to approximately 5 μ M POPC in HBS, pH 7.4 and fluorescence intensity was measured at (λ_{ex} 497 nm/ λ_{em} 528 nm) before and after the addition of 12 μ l of 0.5 mg/ml anti-fluorescein/Oregon Green IgG stock solution per 0.6 ml of sample. No further quenching was seen above this ratio of anti-fluorescein IgG to CFPE. Quenching was expressed as the percentage drop in CFPE emission after addition of antibody.

2.8. Sucrose density gradient centrifugation

Sucrose gradients (0–13% v/w) in HEPES-buffered saline were reproducibly prepared using a precision dispensing system that consisted of two P-500 high precision programmable pumps, a GM-1 gradient mixer, a MV-8 valve, and an LCC-500 FPLC system controller (Amersham Pharmacia Biotech). The controller was programmed to dispense sequentially six linear 0–100% gradients of solvent B (13% sucrose in 5 mM HEPES–saline, pH 7.4) in solvent A (5 mM HEPES–saline, pH 7.4) into six Beckman Ultra-Clear 13 $\times$ 51 mm ultracentrifuge tubes at the total dispensed volume of 4 ml per tube. Sample aliquots (200 μ l) were carefully applied onto the top of the gradient solution, and the tubes were centrifuged at 192,000 $\times$ g for 10 h at 22 $^{\circ}$ C (Ultracentrifuge L8-70M, Beckman) using an SW50.1 rotor. After centrifuga-

tion, the tubes were illuminated from the top and photographed against a black non-reflective background to detect the location of particle bands by light scattering. The gradients were divided into 150 μ l fractions, starting from the top, and the fractions were analyzed for lipid content by ^{14} C and ^{3} H radioactivity measurements (for neutral and cationic lipid, respectively), particle size, and nucleic acid content (by PicoGreen or Oligreen). The sucrose solution densities of the fractions were calculated from the measurements of sucrose concentrations using a refractometer (Leica AR200, Leica Microsystems, IL.) and conversion to density by using tables found in [12].

2.9. In vitro transfection

HER2-overexpressing human breast adenocarcinoma cells SK-BR-3 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) fetal bovine serum, 100 μ g/ml of streptomycin sulfate and 100 U/ml of penicillin G at 37 $^{\circ}$ C, 5% CO₂. The cells were plated at a density of 150,000 cells per well in 12 well plates (Corning) and acclimated overnight in the above media without serum or antibiotic supplements. The final media volume in each well was 1 ml and each well received 1 μ g of pCMV/luc⁺ as either Lipofectamine 2000-DNA complex (using transfection conditions recommended by the Lipofectamine manufacturer), free DNA, or a DNA–lipid preparation. The cells were exposed to the DNA–lipid samples for 12 h, and Lipofectamine 2000 complexes for 6 h,

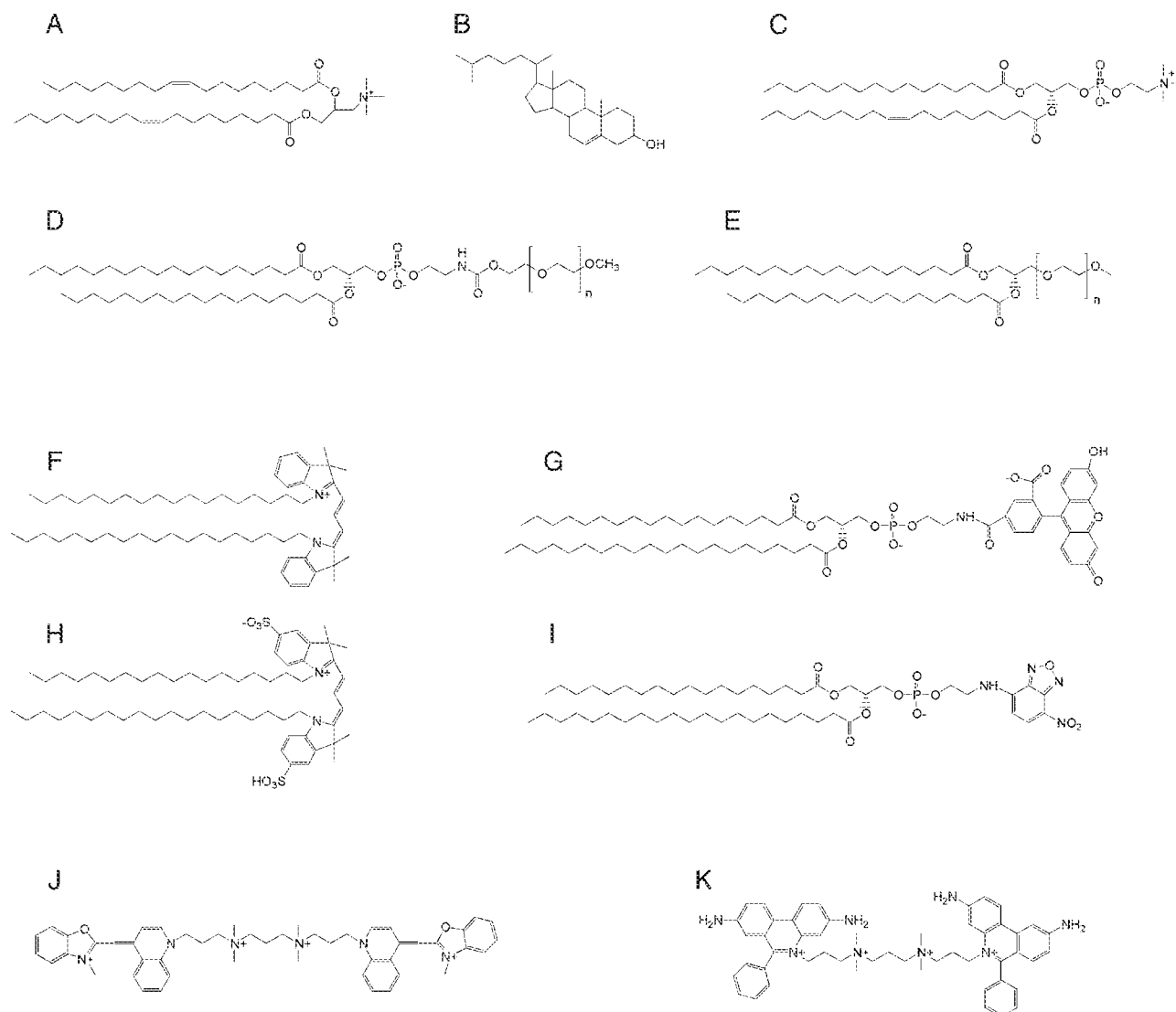


Fig. 1. The structures of fluorescent, non-fluorescent lipid components and DNA intercalators used in this study. Molecular structures denoted as follows; (A) DOTAP; (B) Chol; (C) POPC; (D) PEG-DSPE; (E) PEG-DSG; (F) DiIC₁₈(3); (G) CFPE; (H) DiIC₁₈(3)-DS; (I) NBD-PE; (J) YOYO-1 and (K) EthD-2.

after which time the media was aspirated, cells washed twice with PBS, and the supplemented growth medium added to a final volume of 1 ml per well. After further incubation for 24 h, the cells were washed with PBS twice, and then 1 ml PBS (with 3 mM EDTA) added to each well to detach cells. After 5 min cell suspension was thoroughly mixed and transferred to a clean Eppendorf tube and centrifuged at $14,500 \times g$ for 2 min. The supernatant was removed, and the pellet washed with PBS. After removal of PBS by centrifugation as above, 0.1 ml sodium phosphate 0.1 M was added and tubes quickly frozen at -80°C . Cellular disruption was achieved by 2 freeze–thaw cycles, and a clear cell lysate was obtained following centrifugation at $14,500 \times g$ for 30 s.

Luciferase in the supernatants was quantified by luminometry (Monolight 3010, Analytical Luminescence Laboratory). An aliquot (100 μl) of freshly prepared substrate solution containing 0.5 mM D(-)-luciferin, 30 mM Tris-HCl pH 7.8, 3 mM ATP, 15 mM MgSO_4 , 10 mM DTT, and 0.2% Triton X-100 was injected into the luminometer cuvette containing 20 μl of the cell lysate, and after a 2-s delay, the luminescence was read for 10 s. The luciferase standards were prepared by 10-fold serial dilutions of the 0.5 mg/ml firefly luciferase stock made with the cell lysis buffer to a typical concentration range of $5 \cdot 10^{-9}$ to $5 \cdot 10^{-5}$ mg/ml, and read in a similar manner. Protein concentration in the cell lysates was measured using a Micro BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. The amount of luciferase was expressed in ng luciferase per mg of cell lysate protein.

3. Results

3.1. Determination of suitable aqueous/organic solvent mixtures to prepare Genospheres

The structures of the fluorescent and non-fluorescent lipid components used in the present study as well as the fluorescent DNA binding molecules are shown in Fig. 1. Condensed lipid phases in solution tend to form particles whose presence may be detected by increased light scattering. The light scattering intensities of a lipid mixture, DOTAP/POPC/Chol/PEG-DSPE (6:15:10:0.16), dispersed in water/water-soluble organic solvent mixtures of varying organic solvent concentrations was measured (Fig. 2). The mixtures were measured at two temperatures, 23°C and 55°C . For each solvent tested, there was appreciable scattering intensity at lower organic solvent concentrations (less than 30% (v/v)) at both temperatures. However, at 55°C , in the case of isopropanol, ethanol and ethylene glycol dimethyl ether, higher organic solvent concentrations caused the scattering intensity to drop to the level of the background solvent, indicating the disappearance of condensed lipid phases and the formation of a molecular (true) or micellar solution.

We then prepared plasmid DNA/lipid complexes in aqueous/organic solvent mixtures using the above lipid formulation at such organic solvent concentrations, where condensed phases were absent, to determine if the organic solvent component in the aqueous/organic solvent mixture affected the properties of the resultant plasmid DNA–lipid particles. Subjecting the DNA–lipid particles to sucrose density gradient centrifugation assessed one such property, particle homogeneity. This technique allowed separation of lipid-rich (light) and DNA-rich (heavy) fractions, as well as the removal of free DNA, if any was present. After centrifugation, all preparations formed distinct light-scattering bands (Fig. 3A). The particles produced from 40% (v/v) isopropanol and 50% (v/v) ethanol had a similar density (1.016–1.02) and almost identical lipid/DNA ratios as

the starting material indicating the particles were homogeneous with respect to density and composition (Table 1). However, those particles made in 50% dioxane and 60% (v/v) ethylene glycol dimethyl ether, produced distinct bands that were lipid rich and/or heavier DNA rich bands signifying that these particular solvents may not be optimal for producing homogeneous complexes.

DNA-binding hydrophilic dyes, such as PicoGreen® greatly increase their fluorescence upon binding to DNA exposed to the solution. The increase in the dye fluorescence in the presence of DNA–lipid particles is proportional to the amount of “dye-accessible” DNA, i.e., the DNA that is incompletely encapsulated, free, or adsorbed on the particle surface. After the release

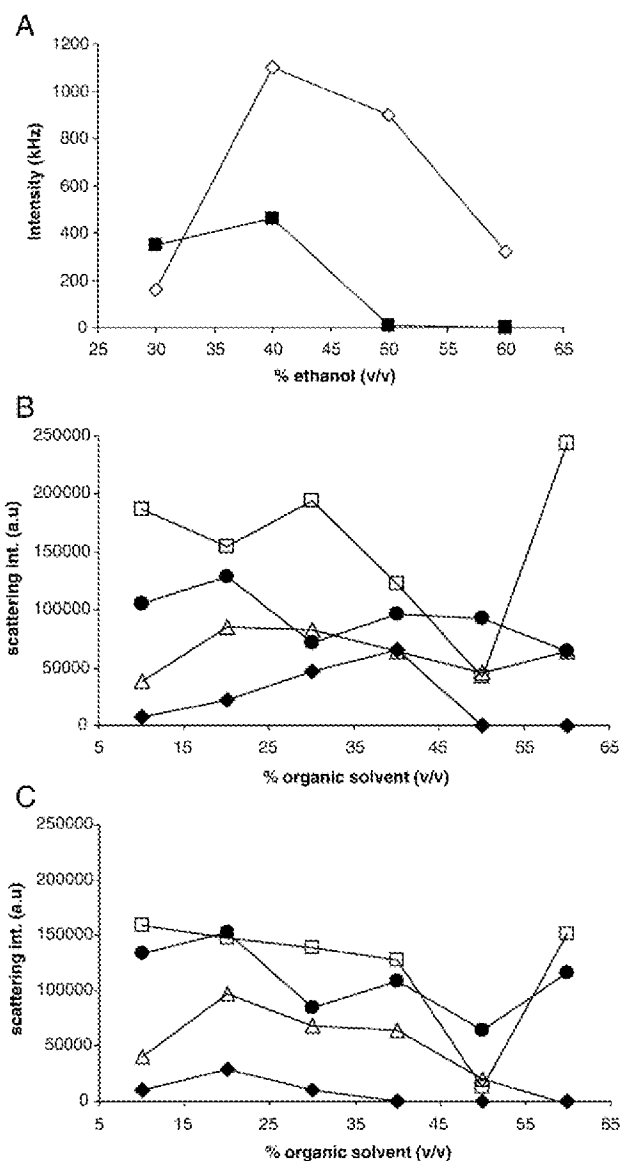


Fig. 2. The light scattering intensities of DOTAP/POPC/Chol/PEG-DSPE (molar ratio 6/15/10/0.16) lipid mixtures in 5% (w/w) dextrose with varying organic solvent concentrations. All measurements were made at a phospholipid concentration of 2.1 mM in (A) ethanol containing solutions at 23°C (open diamond) and 55°C (filled square) or at 23°C (B) and 55°C (C) using the following solvents, dioxane (open square), isopropanol (filled diamond), dimethylformamide (filled circles) and ethyleneglycol dimethyl ether (open triangle).

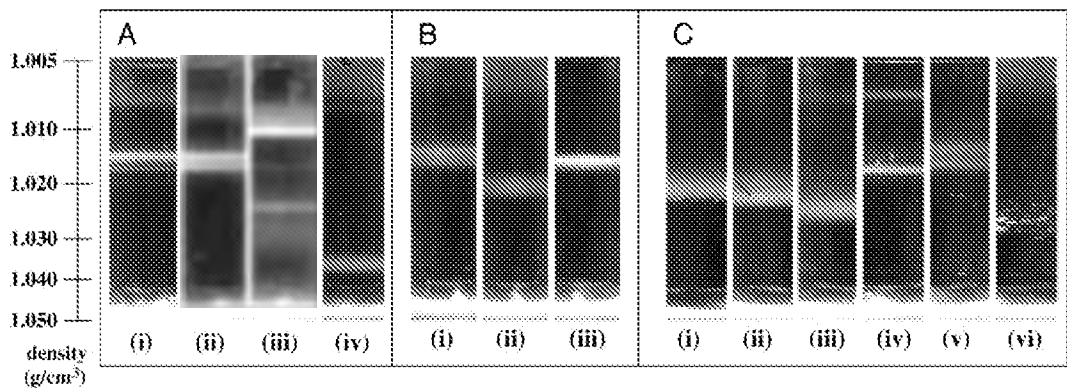


Fig. 3. Analysis of nucleic acid-lipid particles produced from various aqueous/organic-solvent mixtures using sucrose density centrifugation (0–13% (w/w) sucrose). (A) Complexes of formulation DOTAP/POPC/Chol/PEG-DSPE (molar ratio 6/15/10/0.16 nmol/nmol/nmol/nmol per μg plasmid DNA) prepared from 40% (v/v) isopropanol, 50% (v/v) ethanol, 50% (v/v) dioxane, and 60% (v/v) ethylene glycol dimethyl ether were applied to the top of each column (i–iv respectively) and tubes centrifuged at 192,000 g for 10 h at 22 °C. (B) Genospheres containing DOTAP/POPC/Chol with the molar ratio of 6/15/10 nmol/nmol/nmol of lipid per μg plasmid DNA, siRNA or oligonucleotide (i–iii) respectively were prepared and centrifuged as described above. (C) DNA-lipid complexes (DOTAP/POPC/Chol (6/15/10 nmol/nmol/nmol lipid per μg plasmid DNA) prepared from 0, 20, 30, 40, 50 and 60% (v/v) ethanol were applied to the sucrose gradients (tubes (i–vi) respectively).

of DNA from the complexes, e.g., by solubilization with a detergent, the fluorescence further increases, now being proportional to the total amount of DNA contained in the sample. The ratio of DNA-binding dye fluorescence between the native and solubilized particles is known as the dye accessibility and serves as the measure of completeness of the DNA encapsulation within the lipid [13]. Using this technique, we observed that the particles produced in either 40% (v/v) isopropanol and 50% (v/v) ethanol had relatively low dye accessibilities and when purified on the sucrose gradient the values were found to be close to the starting material (Table 1). However, with 50% (v/v) dioxane and 60% (v/v) ethylene glycol dimethyl ether we observed initially high dye accessibilities, which was reduced by purification on the gradient as a consequence of non-encapsulated DNA removal. In both cases the majority of DNA resided in the bottom fraction.

In order to determine if the use of an aqueous/organic solvent monophase was suitable for encapsulation of other nucleic acid types, we used one such monophase, a 50% (v/v) ethanol/water mixture with the above lipid formulation to separately entrap small double stranded RNA with 22 bp (siRNA) and short single stranded DNA with 20 bases (phosphorothioate modified antisense oligonucleotide). We then compared these Genosphere complexes with Genosphere particles that contained plasmid DNA (4.5 kbp) in a similar manner as above (Fig. 3B). We observed the main light scattering bands for the plasmid (i) and

oligo (iii) entrapped complexes to have similar densities ($\rho=1.014\text{--}1.017$ and $1.016\text{--}1.018$, respectively), while the siRNA (ii) sample was slightly more dense ($\rho=1.019\text{--}1.022$). Some free lipid was observed on the gradient in the case of the siRNA. The lipid/plasmid DNA and lipid/oligo ratios for their respective main bands were almost identical to the starting components (Table 2).

This technique facilitated DNA–lipid interaction in a medium that initially does not contain lipid condensed phases. We then compared the particles made from the Genosphere method, to particles produced by interaction of plasmid DNA and stable (lipoplex) or ethanol-destabilized lipid vesicles by combining the components in water or 30% (v/v) aqueous ethanol, respectively, using the same lipid formulation. The particles made from ethanol-destabilized liposomes will be designated as PEDL.

3.2. DNA compaction within the complexes

As an increase in DNA compactness brings distant nucleotide pairs closer to each other, the extent of FRET between the dyes (YOYO-1 and EthD-2), sparsely and randomly distributed along the DNA molecule would serve as a direct measure of the closeness of DNA base pairs and therefore of the DNA compactness [14]. Indeed, in the absence of DNA compacting agents, the FRET efficiency parameter (E) values of YOYO-1

Table 1
Characterization of DNA–lipid particles made from various aqueous/organic-solvent mixtures before and after purification by centrifugation on a sucrose density gradient

Solvent, % (v/v)	Size (nm $\pm$ S.D. nm)	Initial PC/DNA	Initial % Dye Accessibility	PC/DNA main band	% Dye Accessibility of main band	Density (g/cm ³)
Isopropanol, 40%	115.8 $\pm$ 46.9	14.6 $\pm$ 0.5	11.6 $\pm$ 0.4	13.3 $\pm$ 0.6	8.9 $\pm$ 0.3	1.016–1.017
Ethanol, 50%	116.2 $\pm$ 49.4	13.4 $\pm$ 0.5	9.5 $\pm$ 2.7	13.0 $\pm$ 0.8	8.5 $\pm$ 0.5	1.016–1.017
Dioxane, 50%	192.9 $\pm$ 58.2	14.1 $\pm$ 1.2	32.5 $\pm$ 2.6	8.8 $\pm$ 0.3	12.8 $\pm$ 0.8	1.016–1.017
Ethylene glycol dimethyl ether, 60%	552.5 $\pm$ 223.7	66.2 $\pm$ 2.7	44.2 $\pm$ 1.6	22.4 $\pm$ 1.4	38.7 $\pm$ 1.8	1.043–1.047

The formulation used was DOTAP/POPC/Chol/PEG-DSPE with the molar ratio of 6/15/10/0.16 nmol of lipid, respectively, per μg DNA. The refractive index of each fraction was measured and sucrose density (g/cm³) calculated as described above.

Table 2

Characterization of Genospheres made using different nucleic acid types, before and after purification by sucrose density gradient centrifugation

Nucleic acid	Initial PC/ nucleic acid	% dye accessibility (initial)	Size (initial) ^a	PC/nucleic acid (main band)	% dye accessibility (main band)	Size (main band) ^a	Density (g/cm ³)
Plasmid 5.5 kbp	13.3±0.4	3.5±0.2	123±5	13.4±0.5	6.1±0.3	122.5±47	1.014–1.017
siRNA 21 bp	13.9±0.2	18.6±1.1	148.8±57.6	8.4±0.4	17.5±0.9	128.7±62.1	1.019–1.022
Oligonucleotide 20 bases	14.0±1.0	13.8±0.6	105.7±43.4	20.4±1.9	14.5±0.2	117.4±54.5	1.016–1.018

The formulation used was DOTAP/POPC/Chol in the molar ratio of 6/15/10 nmol of lipid, respectively, per µg nucleic acid.

^a Size (nm±S.D. nm).

and EthD-2 dual-labeled plasmid DNA did not exceed 0.6 in the various water and water/ethanol mixtures tested, while in the presence of spermidine, a well-known DNA condensing agent [15], *E* values increased to 6–8 (Table 3). When the lipid mixture described above was added to DNA in the form of liposomes, the value was close to 2. Lower values were obtained when lipid is added in 30% (v/v) and 50% (v/v) ethanol, i.e., close to 1 in both cases indicating slightly less DNA compaction. After dialysis, the Genospheres and PEDL particles showed *E* values of 1 and 2.1, respectively. When the salt concentration was adjusted to a physiological concentration, all particles exhibited approximately equal and a rather modest degree of DNA compaction.

3.3. Validation of the use of cationic and neutral FRET lipid pairs

The structures of fluorescent lipid dyes DiI(3) and DiI(3)-DS (Fig. 1) suggested them as good position markers for cationic and neutral dialkyl lipids, respectively, when used as either a FRET-donor or acceptor in a multicomponent lipid assembly. In order to validate this suggestion, we studied the ability of these dyes to form liposomes and further form complexes with DNA in a manner characteristic for conventional cationic liposome. Small sonicated liposomes were prepared with each dye in combination with DOPE (1:1), and mixed with DNA at a ratio of 2 dyes per DNA phosphate, which is a common procedure for

making lipoplexes (1). Upon mixing, turbidity developed in the DiI(3)/DOPE–DNA liposome sample, and no apparent turbidity developed in the DiI(3)-DS/DOPE–DNA preparation. After centrifugation approximately 50% of the DiI precipitated from solution, indicating the formation of charge stoichiometric complex between the dye liposomes and the DNA, while none of the DiI-DS liposomes precipitated, as quantified by fluorescence measurements of the supernatants.

3.4. Determination of closeness of DNA to cationic and neutral dialkyl lipid in the particles

Similarly, we used the FRET efficiency parameter *E* between the DNA bound donor dye YOYO-1 and the fluorescently labeled DiI(3) (cationic) and DiI(3)-DS (neutral) lipid acceptor labels to characterize the proximity of the cationic or neutral dialkyl–lipid components of the particles to the DNA molecule (Table 4). In a lipoplex, both cationic and neutral lipid labels were at similar distances from DNA as indicated by the similar *E* values. When the lipids were combined with DNA in a 50% (v/v) ethanol monophase at 60 °C. The cationic lipid attained a significantly closer proximity to DNA (*E*=1.6) than did the neutral lipid (*E*=0.2), indicating that cationic lipid component assembled on the DNA molecule first. After the temperature decreased to ambient, neutral and cationic lipids showed the

Table 3

The FRET parameter *E* of dual labeled uncomplexed DNA, DNA–spermidine complexes and DNA incorporated into various lipid complexes in different aqueous media

Sample	aqueous ethanol	5 mM HEPES, pH 7.4	HBS, pH 7.4
DNA	0.3	0.6	N/A
DNA+Spermidine	8.4	6.2	N/A
Lipoplex	N/A	2.0	1.4
Genosphere	1.2	1.0	0.8
PEDL	1.0	2.1	1.3

EthD-2 and YOYO-1 were added to DNA (1:200 dyes per base pair for both fluorescent probes) and formulated into various cationic lipid-containing complexes as described above. *E* was measured in the various media and for comparison, also measured for uncomplexed and condensed DNA (with spermidine). The values shown for Genospheres and PEDL in ethanol/aqueous mixtures are for 50% and 30% (v/v), respectively. The *E* values for DNA and DNA with spermidine are those measured in 50% (v/v) ethanol and are similar to values obtained in 30% (v/v) ethanol. Spermidine and sodium chloride were added from concentrated solutions to give final concentrations of 10 mM and 144 mM, respectively. All measurements were taken at 23 °C.

Table 4

The FRET parameter *E* as measured using YOYO-1 bound DNA as the donor molecule and either DiI₁₈(3) (cationic) or DiI₁₈(3)-DS (neutral) as the acceptor molecules

Sample	60 °C in aqueous ethanol	22 °C in aqueous ethanol	22 °C in 5 mM HEPES, pH 7.4	22 °C in HBS, pH 7.4
<i>Lipoplex</i>				
Cationic	N/A	N/A	1.9	1.1
Neutral	N/A	N/A	1.7	1.3
<i>Genosphere</i>				
Cationic	1.6	1.5	2.3	1.7
Neutral	0.2	2.0	2.3	2.1
<i>PEDL</i>				
Cationic	0.7	2.0	3.2	2.3
Neutral	0.6	1.6	1.5	1.6

FRET measurements were taken at various stages during the preparation procedure of the complexes and after removal of the organic solvent by dialysis against 5 mM HEPES, pH 7.4. Sodium chloride was added from a stock solution to give a final concentration of 144 mM.

same proximity to DNA ($E=2.0$) suggesting that the neutral lipid components joined the cationic lipid in a DNA-enclosing lipid shell. After dialysis, E increased only slightly for both probes, suggesting that no major redistribution of the lipid components in relation to DNA occurred during the removal of ethanol. In contrast, when the lipids were combined with DNA at 60 °C in 30% (v/v) ethanol, cationic and neutral lipids were equidistant from DNA ($E=0.6$ – 0.7), suggesting the DNA first bound to the pre-formed cationic-neutral lipid condensed phase as a whole. Upon cooling to room temperature, the FRET efficiency between DNA label and both neutral and lipid component equally increased ($E=1.6$ – 2.0), indicating that structural rearrangements took place bringing the lipids closer to DNA; and upon removal of ethanol, the cationic lipid–DNA proximity further increased ($E=3.2$). This sequence of events is consistent with the proposed mechanism of “small multilamellar liposome” formation through DNA-induced rearrangements of ethanol-destabilized lipid vesicles [3]. Increasing the sodium chloride concentration to 144 mM caused the E parameter to become smaller, especially between cationic lipid and DNA in all cases. There was relatively little change in E with respect to DNA and neutral lipid. This indicates some further structural rearrangements occur upon exposure to physiological salt concentrations where the distance from cationic lipid and DNA become greater. The smallest changes in E upon adding salt are seen for Genospheres indicating they may be less susceptible structural changes induced by the ionic strength of the surrounding media.

3.5. Relative lipid distribution within the particles

To understand how cationic and neutral lipids were distributed relative to each other, we measured the efficiency of FRET between two lipid labels, a headgroup-labeled “neutral” lipid label NBD-CAP-PE serving as a donor, and a cationic lipid acceptor DiI_{C18}(3) (Table 5). In the absence of DNA, liposomes prepared by various routes (extrusion of an aqueous lipid dispersion, or ethanol removal from 30% (v/v) or 50% (v/v) ethanol mixtures) showed fairly efficient FRET

Table 5

FRET parameter (E) between cationic and neutral lipids within the various lipid assemblies measured in aqueous ethanol and buffer at 22 °C and 60 °C

Sample	60 °C in aqueous ethanol	22 °C in aqueous ethanol	22 °C in 5 mM HEPES, pH 7.4	22 °C in HBS, pH 7.4
Lipoplex	N/A	N/A	1.8	2.2
Lipoplex ‘minus’ DNA	N/A	N/A	1.2	1.6
Genosphere	0	1.7	2.4	2.7
Genosphere ‘minus’ DNA	0	0	1.1	1.4
PEDL	1.3	2.3	2.2	2.5
PEDL ‘minus’ DNA	0.8	1.7	2.2	2.5

The neutral donor NBD-PE and the cationic acceptor DiI_{C18}(3) molecules were incorporated into the formulations as previously described. For each of the particle-forming methods, replicas were made without DNA (i.e., ‘minus’ DNA) to provide information on how the presence of DNA affects the relative lipid distributions.

Table 6

Size and % dye accessibility of DNA–lipid complexes prepared using lipoplex, PEDL or Genosphere preparation procedures

Sample	Size (nm)	% Dye Accessibility
Lipoplex	216.9±100.6	61.4±1.3
Genosphere	66.4±32.8	4.6±0.8
PEDL	86.6±40.5	38.1±0.6

Complexes were prepared as described above using the lipid composition of DOTAP/POPC/Chol (6/15/10 μmol per μg DNA) with ethanol removal affected by dialysis against unbuffered saline.

between neutral and cationic lipid labels ($E=1.1$ – 2.2), and so did DNA–lipid particles prepared by different routes, suggesting that all these structures contained mixed lipid phases in which cationic and neutral components were not clearly separated. In the presence of 30% (v/v) ethanol, even at 60 °C, there was relatively efficient FRET between neutral and cationic lipid components ($E=0.8$), indicating the presence of a mixed lipid condensed phase, which is consistent with the light scattering measurements. In the presence of DNA a further increase in FRET ($E=1.7$) was observed, pointing to the compaction of the lipid phase upon interaction with DNA, possibly resulting from the exclusion of interdigitating ethanol molecules. In 50% (v/v) ethanol without DNA at 60 °C, as expected from the light scattering data, no detectable FRET was observed between the neutral and cationic lipid until the solvent was dialyzed away, and liposomes formed. In the presence of DNA at 60 °C and 50% (v/v) ethanol, the neutral-to-cationic lipid FRET was also undetectable, consistent with the observation that only cationic lipid was bound to DNA under these conditions. Upon cooling to ambient temperature, the neutral lipid joined the condensed lipid phase around the DNA molecule, and the neutral-to-cationic lipid FRET became efficient ($E=1.7$). Exposure to saline caused a small increase in E , roughly to the same extent for all lipid assemblies. We did, however, observe the presence of scattering material in 50% (v/v) at 22 °C ethanol (Fig. 2) but did not measure any detectable FRET, and this suggests that the particles responsible for the scattering in this case did not contain both of the probes; for example it is possible that cholesterol is less soluble at 22 °C in this solvent mixture.

3.6. Size and dye accessibility of particles

We compared the average particle size and dye accessibilities of lipoplexes, PEDL particles, and Genospheres having the same composition as those used for FRET proximity studies (Table 6). The average size of the particles produced using the lipoplex methodology was over 200 nm, consistent with a multilamellar structure [16]. The dye accessibility is high (>60%), suggesting that a substantial amount of DNA is either adsorbed on the surface of the lipid structure or is incompletely covered with lipid. Both Genospheres (50% (v/v) ethanol) and PEDL (30% (v/v) ethanol) preparations consisted of small particles (100 nm) resembling unilamellar liposomes by size, but differed in the amount of dye-accessible DNA. This indicates the particles assembled from the DNA and lipid components in solution provided for somewhat more complete

entrapment of DNA within the lipid than the particles formed by deformation of pre-existing vesicles.

3.7. Particle homogeneity of particles made from water/ethanol mixtures of varying ethanol concentrations

To assess particle homogeneity, DNA–lipid particles prepared from a range of ethanol concentrations (20, 30, 40, 50 and 60% (v/v)) were subjected to sucrose density gradient as described before (Fig. 3C). Particles prepared at 0% ethanol (lipoplex) and 20% (v/v) ethanol formed a single band with $\rho = 1.021$ – 1.022 . In a 30% (v/v) ethanol preparation, the band with $\rho = 1.025$ was accompanied by another band at $\rho = 1.007$ which was found to contain practically no DNA (“empty” liposomes). In a 40% (v/v) ethanol preparation, the main band with $\rho = 1.019$ was still accompanied by the “empty liposomes” band, while at 50% (v/v) ethanol, there was no “empty liposomes” band, and two closely spaced bands with $\rho = 1.014$ and $\rho = 1.019$ were detected. The preparation made at 60% (v/v) ethanol showed a diffuse light-scattering area at the top of the gradient, and a band of highly aggregated material having $\rho = 1.028$ – 1.030 .

To determine if the mode of particle assembly affected the particle size and composition, the gradients were fractionated and analyzed for total DNA (by PicoGreen assay), and for two lipid components, cationic (DOTAP) and neutral (POPC), using multiple radioactive labels, [^3H]-DDAB (cationic) and [^{14}C]-DPPC (neutral phospholipid), respectively. In addition, the main bands were analyzed for particle size and DNA dye accessibility (Table 7). The low density bands ($\rho = 1.007$ or less) were confirmed as having practically no DNA, while various small amounts of free DNA were found in the bottom fractions, consistent with the higher buoyant density of free DNA ($\rho > 1.050$). In the isolated main bands, the smallest particles (65–68 nm on average) with the most extensively encapsulated DNA (dye accessibility 11–12%) were observed in the preparations made in 40% (v/v) and 50% (v/v) ethanol. However, the highest level of incorporation of both DNA and lipid in the particles, reaching about 87–88% of the lipid and practically quantitative for DNA, was observed in the preparation made at 50% (v/v) ethanol. This finding was consistent

Table 7
Analysis of size, dye accessibility, lipid and DNA content of purified complexes from a sucrose gradient

Tube ID	% Ethanol	% D.A.	Size (nm)	% DOTAP	% POPC	% DNA
A	0	71.3 $\pm$ 4.4	450.4 $\pm$ 210.6	79.3	77.8	82.7
B	20	42.6 $\pm$ 3.4	104.6 $\pm$ 46.3	49.1	44.1	90.8
C	30	46.1 $\pm$ 3.9	177.6 $\pm$ 71.4	39.5	34.5	92.2
D	40	11.1 $\pm$ 1.6	67.8 $\pm$ 31.9	58.2	54.1	90.2
E	50	11.8 $\pm$ 0.7	64.9 $\pm$ 28.4	88.3	86.8	99.5
F	60	16.2 $\pm$ 0.8	N/A	21.6	16.8	91.2

Examination of the main bands taken from the sucrose gradients of the complexes prepared from 0 to 60% vol. ethanol. Size was measured by quasielastic light scattering; lipid was quantified by scintillation counting and DNA dye accessibility (% D.A.). Total DNA content was measured by PicoGreen analysis.

Table 8

The fluorescence quenching of CFPE molecules incorporated into the DNA–lipid assemblies by an anti-fluorescein IgG

Sample	% Quenched
Unilamellar liposomes	57.7
Lipoplexes	18.2
Prepared in 20% (v/v) ethanol	53.3
Prepared in 30% (v/v) ethanol	63.7
Prepared in 40% (v/v) ethanol	59.7
Prepared in 50% (v/v) ethanol	49.3
Prepared in 60% (v/v) ethanol	41.2

DNA–lipid complexes comprising of DOTAP/POPC/Chol (6:15:10 molar ratio) per 1 μg DNA were prepared which included CFPE (0.5 mol% of POPC content). Fluorescence was measured before and after the addition of anti-fluorescein/Oregon Green IgG.

with the absence of the “empty liposome” band on the gradient, and also with the fact that the particle composition (lipid/DNA ratios) closely approximated the theoretical value of 6.0 (DOTAP/DNA) and 15.0 (POPC/DNA) (Table 7). We did not find any differences in the composition or other properties between the particles in the closely spaced bands recovered from the 50% (v/v) ethanol preparation. In the preparation at 40% (v/v) ethanol only about 55–60% of the lipid was incorporated, resulting in heavier (more DNA-rich) particles and substantial amount of free lipid. In the preparation produced at 60% (v/v) ethanol, the particles were grossly aggregated, but DNA was well encapsulated (dye accessibility 16%) compared to lipoplexes (71%). These particles were also poor in lipid, containing only about one fifth of the added lipid material. This finding is consistent with the hypothesis that in the 60% (v/v) ethanol preparation, the DNA prior to encapsulation has partially aggregated due to the higher ethanol concentration resulting in lower surface area available for lipid assembly. The PEDL material (30% (v/v) ethanol) after centrifugation showed an increase in size (from 113 nm to 180 nm average), a moderate degree of DNA encapsulation (dye accessibility 46%), and substantial amount (approx. 60%) of lipid present in the non-DNA bound form. Thus, the amount of ethanol in the monophase used to assemble the DNA–lipid particles had a profound effect on the particle composition and properties of the particles assembled under the mutual solubility conditions. Preparation of complexes with a monophase consisting of 50% (v/v) ethanol showed the highest encapsulation of DNA and lipid, small size, and low level of DNA exposure to the environment.

3.8. Lamellarity of particles

Using a non-membrane penetrating antibody that recognizes and selectively quenches the carboxyfluorescein fluorophore (anti-fluorescein IgG) to approximately 7% of its fluorescence intensity [17], we estimated the proportion of EPE with a carboxyfluorescein headgroup exposed on the surface of the particles and used this as an indication of the degree of lamellarity of the particles (Table 3). We found that all particles prepared from aqueous ethanol solutions, except those formed at 60% (v/v) ethanol, had 49–64% of the CF-labeled lipid

exposed on the surface. This is in contrast with the lipoplex particles with contained less than 20% of the lipid on the outside of the particles. The particles formed at 60% (v/v) ethanol had somewhat increased amount of lipid exposed on the surface. This finding, along with the lower particle aggregation stability and higher buoyant density (Fig. 3C) suggested that, at this ethanol concentration, the particles assembled differently, perhaps due to the partial loss of molecular solubility of the DNA. As a control, 0.05 μm extruded liposomes of the same formulation were tested and gave a quenching value (58%) consistent with a unilamellar vesicle with a slight transmembrane lipid density asymmetry originating from the bilayer curvature.

3.9. *In vitro* transfection activity of particles made in 0, 30 and 50% (v/v) ethanol/water mixtures

To correlate the mode of particle assembly with their capacity to deliver functional DNA into living cells, we studied the levels of transgene (luciferase) expression by cultured cells exposed to the particles containing a luciferase-encoding plasmid. A human HER2-specific scFv antibody–lipid conjugate, F5-PEG-DSPE, was added to a portion of each set of particles. This ligand can induce internalization and has been used previously with anti-HER2 immunoliposomes for the targeted drug delivery to HER2 (c-erbB-2, neu) over-expressing cancers [4,18]. Lipofectamine 2000 was used as positive control to confirm the activity of the luciferase encoded plasmid (Table 9). All three DNA–lipid preparations (lipoplexes, PEDL and Genospheres) had the same lipid and DNA composition and were incubated with the cells for 12 h. The addition of an internalizable targeting ligand F5-PEG-DSPE conferred an increase of transfection activity in all cases, the highest activity enhancement occurring for Genospheres (over 20-fold). Thus, DNA packaging into lipid particles using Genosphere process did not adversely affect the transfection

properties of the plasmid and in fact enhanced it over the other DNA–lipid complexes tested.

4. Discussion

Non-viral vectors are generally prepared by the assembly of nucleic acids with carrier components, such as lipids. Lipids because of their amphiphilic nature have a propensity to self-assemble into more or less stable structures. Interactions of nucleic acids with self-assembled lipid structures depend in large part on the ability of the lipid molecules in the structures to undergo rearrangements as a result of the interaction. This ability is hindered by strong attraction between the assembled lipid molecules. Therefore, the lipid–nucleic acid complexes obtained from lipids self-assembled into bilayers are heterogeneous [19] and with time undergo slow changes known as “maturation” [20]. Reproducible assembly of particles can be aided by weakening the intermolecular interactions between the lipid molecules leading to a reduction or elimination of the kinetically limited component in the nucleic acid–lipid interactions. This has been accomplished by solubilizing the lipid with a dialyzable detergent [21] or through destabilizing preformed lipid bilayers with 30%–40% ethanol [3]. Alternatively, DNA in the form of its cationic lipid salt was extracted into organic phases, in which DNA would not be ordinarily soluble [22,23].

We explored the possibility of assembling the DNA–lipid particle in the environment where particle-forming lipids would not form a condensed phase beyond a micelle, and DNA would still be soluble by itself. We found that such conditions could be satisfied in a number of different organic solvent/water mixtures. For the purpose of establishing the feasibility of this approach, we initially chose to use plasmid DNA, a common vector for carrying an expressible transgene, and a combination of lipids containing a DNA-binding, transfection-enhancing cationic lipid DOTAP [24] a bilayer-forming neutral lipid, POPC, and a membrane-rigidifying lipid, cholesterol in the amount of 6/15/10 nmol/nmol/nmol per μg DNA. The average molecular weight of a DNA base is 330 g mol^{-1} , so one microgram of DNA corresponds to approximately 3 nmol of bases, i.e., 3 nmol of DNA phosphate. Genosphere particles were formed with 6 nmol cationic lipid per μg DNA, giving a $\text{N}(\text{CH}_3)_3^+/\text{PO}_4^-$ ratio of 2. This was enough cationic lipid to completely neutralize the DNA phosphate charge and ensure stability during formation of the particle. Therefore, Genospheres were constructed with a minimum of excess cationic lipid, which is known to endow lipid–DNA complexes with tropism toward the elements of capillary wall [25] and which may interfere with the ability of the complexes to reach the intended anatomical target.

Of the solvent mixtures tested, we found that 40% isopropanol and 50% (v/v) ethanol solutions were ideal in this respect, as they produced optically transparent lipid solutions (Fig. 2) and upon combining DNA and lipid in such mixtures, subsequently produced small, homogenous DNA–lipid particles (Fig. 3A, Table 1). We then sought to see if this methodology would entrap nucleic acids of varying size and chemical

Table 9
Luciferase expression in HER2 overexpressing human breast cancer cells SKBR3, transfected with DNA–lipid complexes prepared in 0, 30 and 50% (v/v) ethanol/water mixtures

Sample	Luc (ng/mg protein)	T/NT
F5-DSPE Genospheres	21.04 $\pm$ 4.1	15.8
Non-targeted Genospheres	1.33 $\pm$ 0.5	
F5-DSPE PEDL	5.3 $\pm$ 0.64	2.7
Non-targeted PEDL	1.95 $\pm$ 0.5	
F5-DSPE Lipoplex	1.8 $\pm$ 0.4	11.3
Non-targeted Lipoplex	0.16 $\pm$ 0.07	
Lipofectamine 2000	162.8 $\pm$ 49.1	
Plasmid DNA	0.003 $\pm$ 0.005	
Untreated cells	0.003 $\pm$ 0.001	

Particles that were made target specific by the addition of the highly internalizable human anti-HER2 scFv antibody F5 (F5-PEG-DSPE) conjugate were tested in parallel with non-targeted ones. The lipid formulation was DOTAP/POPC/Chol in the molar ratios 6/15/10 nmol, respectively, per μg DNA. Improved recovery of particles following sterilization by microfiltration, could be achieved by the addition of 2 mol% of a neutral PEG-lipid derivative (PEG-DSG) to the initial lipid formulations. PEG-DSG was included into the lipoplex formulation post DNA–lipid complexation under aseptic conditions by a procedure adapted from Hong et al. [5].

composition and found that it was relatively efficient at entrapping both small double and single stranded nucleic acid molecules (Fig. 3B, Table 2). In addition, entrapment by this method was also relatively insensitive to the chemical composition of the backbone linkages (phosphodiester of plasmid and siRNA versus phosphorothioate of oligonucleotide) and base composition (RNA vs. DNA) of the nucleic acid used. Also, the fact that the resultant particles had similar sizes, while the nucleic acid component size ranged from a single stranded 20 nucleotide oligo to 4.5 kbp plasmid indicates that the nucleic acid molecular size does not dictate the overall particle size.

Taking one such monophasic composition, i.e., 50% (v/v) ethanol, the particles ("Genospheres") were then compared to particles produced using stable liposomes and from ethanol-destabilized liposomes (PEDL) by the methods described by Felgner et al. [2] and Maurer et al. [3], respectively, using identical plasmid DNA and lipid compositions. While the components of these three types of formulations were identical, the resulting particles differed by their size, the degree of DNA exposure, homogeneity of the preparations, and the completeness of DNA and/or lipid incorporation.

It was of interest to assess the state of DNA condensation in the DNA–lipid particles obtained by different methods. DNA condensation has been observed in solution in many instances using cationic multivalent salts such as spermidine³⁺ and cobalt hexamine³⁺ [26]. Cationic polymers such as poly-L-lysine, [27], cationic peptides [28], polyethylenimine [29] and dimerizable surfactants [30] have been used to condense DNA in the formation of DNA–lipid particles. It has been suggested that charge neutralization of DNA by monovalent cationic lipids, also results in compaction of DNA within these types of complexes [31]. To measure the extent of DNA compaction within the different particles, we used a simple approach, measuring the FRET between an intercalating donor/acceptor fluorescent DNA label pair YOYO-1 and EthD-2 (Table 3), respectively. These bis-intercalating probes bind to DNA with sufficient affinity and YOYO-1 has been used previously as probe for DNA condensation [32], where it was shown that electronic interactions among YOYO-1 molecules at high dye/nucleotide ratios (1:50) led to a decrease in the probe quantum yield during condensation. We used a lower YOYO-1 dye/nucleotide ratio (1:200), where the fluorescence was unaffected by condensation. Also, it was shown that DNA bound YOYO-1 was not displaced by the binding of PEI or CTAB [32]. As the DNA is compacted, the sparsely distributed DNA-bound YOYO-1 and EthD-2 molecules become closer to each other, and the magnitude of FRET increases. Indeed, when both probes were bound to plasmid DNA the FRET parameter was 0.6 or less, compared to the simple sum of DNA-bound YOYO-1 or EthD-2 spectra recorded at the same concentrations. However, in the presence of spermidine, a known DNA-condensing agent, the FRET value increased to 6.0–8.0. The increase in EthD-2 emission intensity due to FRET after the addition of spermidine suggests that EthD-2 is also not displaced from DNA either in aqueous or aqueous ethanol mixtures.

Assuming that *E* values greater than 6 (DNA with large excess of spermidine) correspond to highly condensed plasmid,

and values less than 0.6 reflect an uncondensed state, it appears that DNA inside all of the studied lipid complexes was far from being highly condensed, but was compacted slightly with *E* values ranging from 0.8 to 2.1.

Differences in the mechanism of particle formation between Genospheres and PEDL were apparent when the proximity of cationic and neutral dialkyl-lipids to the DNA was studied using FRET between a DNA-bound probe and a fluorescent cationic lipid probe (DiI) or neutral lipid probe (DiI-DS) (Table 4). As a control, we demonstrated that when mixed with so called "helper" lipid (DOPE) in a liposome formulation DiI(3) behaved as a cationic lipid, by electrostatically interacting with DNA, forming large complexes and that DiI(3)-DS behaved as a neutral lipid by not reacting with the polyanionic DNA. This functional similarity produced further evidence for fitness of these the cationic and neutral DiI lipid dyes as probes for the position of cationic or the neutral dialkyl lipids used in the present FRET study in the structure of DNA–lipid complex assemblies.

The data suggests that during Genosphere formation, cationic lipid is first to assemble around the DNA, while neutral lipid remains in solution. When the solution is cooled, neutral lipid joins the lipid moiety around the DNA, and after ethanol removal both types of lipid are at equal distance from DNA in the resulting particle. During the formation of PEDL at 60 °C, both lipids are equidistant from DNA from the beginning, indicating that the lipid vesicle interacts with the DNA as a whole. When the temperature drops, the cationic lipid becomes closer to DNA. When DNA interacted with preformed stable liposomes, the relative distances of cationic and dialkyl-neutral lipids to DNA in the resulting complex were also very similar throughout the procedure. Thus, unlike Genospheres, the PEDL and liposome–DNA complexes are likely to be formed through deformation of the mixed condensed lipid phases without their dissociation into lipid components.

These conclusions were in agreement with the results of a FRET study measuring relative proximities of cationic (DiI_{C18} (3)) and dialkyl-neutral lipid (NBD-PE) in aqueous ethanol solutions in the presence or absence of DNA (Table 5). In 30% (v/v) ethanol at 60 °C (DNA absent), there was appreciable amount of FRET measured, indicating the presence of a mixed condensed lipid phase. In contrast, in 50% (v/v) ethanol at the same temperature (DNA absent) practically no FRET was detectable, indicating the cationic and neutral lipids were at significant distances from each other. This was consistent with the light scattering data indicating the absence of lipid condensed phases in 50% (v/v) ethanol. However, when DNA is added in the 50% (v/v) ethanol case (Genosphere), FRET was only measured when the ethanolic solution was cooled. This suggests that cationic and dialkyl-neutral lipids are spaced far from each other when DNA is present at 60 °C, but assume a closer proximity when cooled to 22 °C. Together with the results demonstrating at 60 °C in 50% (v/v) ethanol, cationic lipid is close to DNA and neutral lipid is not, we can see that Genosphere formation is a multi-step process with the cooling step importantly allowing the dialkyl-neutral lipid to condense on the DNA–cationic lipid complex in such a way that both types of lipid are equidistant from DNA. Removal of ethanol

caused both lipids to become even closer to DNA (Table 4) and to each other (Table 5).

Alternatively, the case of PEDL, FRET was measurable initially in the presence of DNA and increased upon cooling. This implies that both type of lipids are close to DNA during the initial mixing step and during cooling and subsequent ethanol removal, cationic lipid moves closer to DNA. This is similar to the lipoplex situation, where both lipids are equally spaced from DNA (Table 4) and are relatively close to each other in the final complex (Table 5). At the present, we can only account for the neutral dialkyl-lipid (POPC) distribution within the complexes as this was reported to us by the structurally similar DiI(3)-DS. However, the neutral lipid pool also contains cholesterol and its distribution is at this time unclear. This will be the subject of a future study. Also, we must point out that the described studies give information on relative DNA and lipid molecular organization at defined points during the particle assembly process, and as yet we have no data regarding rates of assembly for individual components of the complexes.

As controls for the FRET studies, “minus one” principle was used, where the matching liposomes, lipoplexes, or complexes having just one of the FRET pair members were used to assess a zero-FRET intensities, matching the actual environment of these probes in the samples containing both FRET members. This is because the fluorescent intensity of a probe may depend on the environment of the probe, including the aggregate state of the lipids surrounding the probe, and on the chemical composition of the probe environment. Therefore, by using the zero-FRET controls with identical lipid/DNA composition and method of preparation, the effects of lipid environment on the FRET probes were accounted for. In addition, we included the correction for light scattering/autofluorescence control, using similarly prepared complexes without any of the FRET pair labels.

Therefore, despite differences in the manner of the lipid attachment to DNA among the three complexation methodologies, the extent of DNA compaction and the relative proximity of the lipids to DNA and of the cationic lipids to neutral lipids in the resulting particles were similar. However, the particles differed by their size, lamellarity, and DNA dye accessibility. Genospheres and PEDL were relatively small with average particle diameter of approximately 66 nm and 87 nm, respectively, lipoplexes were considerably larger (greater than 200 nm) (Table 6). Accessibility of encapsulated DNA to small molecules, such as enzymes (e.g., DNase) is to be avoided if functionally intact nucleic acids are to be delivered. Therefore, the degree of DNA exposure to its environment is an important characteristic of a synthetic delivery vector and can be measured by a DNA-binding dye accessibility assay. Of all three types of DNA–lipid particles tested, Genospheres had the lowest dye accessibility (Table 6). Differences in the observed DNA accessibility may result from differences in the DNA encapsulation mechanisms leading to DNA exposure, or from the non-homogeneity of particle preparation, such as the presence of particle species with incomplete encapsulation and/or surface-bound DNA molecules.

Differences in the relative DNA/lipid content in the particles would result in different buoyant densities. Therefore, we employed ultracentrifugation on a sucrose density gradient to analyze the

homogeneity of the particles made by plasmid DNA–lipid complexation in aqueous solutions containing no ethanol or 20–60% (v/v) ethanol, with subsequent ethanol removal by dialysis (Fig. 3C and Table 7). The preparations showed at least one distinct, primary light-scattering band, and sometimes a second distinct band, corresponding to different particle densities. The lipid and DNA amounts in the main bands varied among the preparations made at different ethanol concentrations. While the amount of DNA incorporated into the particles of the main band was consistently above 80% (v/v), the amount of incorporated lipid in the particles produced in the presence of 20–40% (v/v) ethanol and at 60% (v/v) ethanol was quite incomplete (16–58%) resulting in “heavy” bands with higher DNA content, accompanied (in 30 and 40% (v/v) ethanol preparations), with a light band of “empty” liposomes that did not contain any significant amount of DNA. Unexpectedly, in the particles produced at 50% (v/v) ethanol, i.e., using the Genosphere method, the incorporation of both DNA and the lipid was practically complete (86–99%). The particles showed two closely spaced bands whose lipid and DNA compositions were the same. The narrow distribution of particle density and closeness of the composition of purified complexes to the relative composition of the original starting materials pointed to the homogenous nature of the Genosphere preparation.

Measurements of the proportion of the lipids exposed on the surface of the particles produced in aqueous ethanol solutions are consistent with the oligolamellar structure (Table 8). This is in contrast to the lipoplex particles with contain less than 20% of the lipid on the outside of the particles, thus indicating numerous bilayers, as described previously [16,33,34]. Particles produced using the Genosphere, PEDL and lipoplex methodologies had different levels of transfection activity in cell culture (Table 9). While the absolute levels of activity were lower than the positive control prepared using Lipofectamine 2000, optimized for *in vitro* gene transfer, these data show that small DNA–lipid particles containing lipid-protected DNA, can give similar levels of transfection activity to the large lipoplex complexes of a similar composition. In fact, Genospheres gave the highest activity, possibly due to the well-protected nature of the DNA encapsulation. Also, the particles of all three types could be rendered targetable by non-covalent association with an amphipathic ligand–lipopolymer conjugate in a manner similar to production of antibody-targeted immunoliposomes [4,10] and more than an order of magnitude enhancement of activity was observed for Genospheres. In light of the results from previous studies suggesting that larger non-targeted complexes are more active at delivering DNA *in vitro* [35], it was important to demonstrate that small particles made from a monophasic did not in some way render the resulting targeted particles inactive. The fact that the same lipid formulation when processed in such a way that produced small uniform particles (Genosphere), elicited higher transfection activity that that obtained from the sonicated liposome/DNA complex was surprising. As Genospheres are designed ultimately for systemic administration as an injectable therapeutic gene delivery vehicle, this finding is advantageous and important in light of the poor pharmacokinetic characteristics often associated with lipoplexes [36,37].

The lipid–DNA particles obtained by complexation in the presence of 50% (v/v) aqueous ethanol showed a number of beneficial properties (small, uniform size; uniform density; high degree of incorporation of both DNA and the lipid; and low DNA exposure), without the need for a large excess of cationic lipid. We hypothesize that these properties are a result of conducting the DNA–lipid complexation under the conditions where both lipids and DNA independently form molecular (true) or micellar solutions, therefore the assembly of the particles does not require rearrangements of pre-formed condensed phases (e.g., bilayers) that introduce a high activation energy, kinetically slow reaction step. Instead, we speculate that high efficiency of DNA entrapment is due to the high degree of utilization of the cationic lipid to augment the hydrophobic character of the DNA molecule, as a result of freer movement of molecularly- or micellarly-dissolved cationic lipid component at the early stage of Genosphere assembly. This is supported by our observations on the cationic versus neutral lipid proximity to DNA during the Genosphere assembly process. The existence of areas of mutual independent solubility of DNA and a cationic surfactant in alcohol–water solutions has been reported by Sergeyev et al [38]. The apparent long axis length of T4 phage DNA within a DNA–CTAB complex measured by fluorescence microscopy revealed a globule–coil–globule transition of DNA at elevated alcohol concentration. At intermediate ethanol concentrations 40–60% (v/v) the complex was solubilized due to the transfer of CTAB into solution, and DNA relaxed from a globule into a random coil conformation. At higher ethanol contents, the DNA compacted into a globule because of the loss of solubility, and at lower ethanol concentrations, the DNA was compacted by the assembling detergent molecules. This window of mutually independent solubility of DNA and CTAB is remarkably similar to the ethanol concentration window that produces Genospheres. We proposed that using 50% (v/v) ethanol to separately solubilize the particle components is ideal in this respect. Furthermore, while ethanol has an advantage of being quite acceptable in the pharmaceutical manufacturing process, it is very likely that other water-miscible organic solvents, such as isopropanol can be used instead of ethanol at concentrations providing for independent solubility of both nucleic acid and lipid components. Perhaps more important is the requirement that the organic solvent maintains complete water miscibility during its removal from the DNA–lipid mixture, leaving water behind. The systems that separate phases at increasing water content [23], even if at first homogenous, eventually lead to extraction of the DNA–lipid complex in the organic-rich phase, thus preventing efficient particle assembly.

By varying the concentration of ethanol in a water-organic monophasic, we were able to compare the assembly of DNA–lipid nanoparticles in the systems with identical DNA and lipid compositions in three different modes: DNA association with stable cationic liposomes (lipoplex process, no ethanol); DNA association with ethanol-destabilized liposomes (PEDL process, 30% (v/v) ethanol), and DNA association with molecularly or micellarly dissolved lipids (Genosphere process, 50% (v/v) ethanol). All three processes resulted in DNA–lipid particles with nearly identical DNA compaction, lipid–DNA proximity. However, it was only the Genosphere methodology that produced

particles that also were small, homogenous with respect to composition, contained well-protected DNA, and resulted in 90% or better incorporation of both DNA and the lipids. The underlying mechanism of this unexpected finding is not yet clear. A two-step assembly may be envisioned as a viable hypothesis. At the first (germination) step, upon mixing of DNA and the lipids in 50% (v/v) ethanol, uncoiled DNA molecules interact with at least a portion of the cationic lipid component to form partially compacted cationic lipid–DNA complexes, while neutral lipids are still in solution. At the second (deposition) step, upon cooling, the solubility of neutral and/or excess cationic lipid components decreases and causes them to self-assemble into neutral or mixed neutral-cationic lipid bilayers which are deposited around pre-formed cationic lipid–DNA complexes acting as colloidal germinis for the assembly. The deposition continues until the lipids are exhausted from solution, thus leading to uniform and nearly quantitative inclusion of both lipid and DNA into the particles. The fact that Genospheres gave the highest *in vitro* transfection activity with the most targeting enhancement of activity may be attributable to these features of high DNA protection and particle uniformity.

The Genosphere process also offers advantages in manufacturing, which is an important consideration for the clinical translation of any pharmaceutical technology. It is a one-pot process that takes place under near equilibrium conditions, without the need for mechanical shearing steps such as dispersion, membrane extrusion or microfluidization, to pre-formulate the lipids into liposomes or post-formulate the lipid–DNA complexes into a nano-sized format. It involves essentially two well-scalable operations, temperature-controlled solution mixing and ethanol removal; the latter can be conveniently scaled up using tangential flow ultrafiltration or hollow fiber dialysis, the procedures quite common in pharmaceutical manufacturing. The process does not use toxic solvents, such as chloroform or methanol, and resulting nanoparticle preparations are sterilizable by microfiltration.

Uniquely, DNA and lipid were separately solubilized in an identical aqueous-solvent monophasic, thereby avoiding areas of variable solvent concentrations during the mixing step. The mixing of two different solvents in such a process could place more critical importance on the rate of mixing so that non-uniformities of the resulting particles do not occur.

In this study, we focused on the general features and physico-chemical characterization of Genospheres and the Genosphere process. The studies of particle stability upon storage and in the presence of blood plasma, as well as of the effect of particle lipid composition on the surface charge, transfection properties, and antibody-directed targeting of Genospheres have been performed with encouraging results, have been reported separately [1]. In conclusion, we hope that the described Genosphere methodology for the assembly of nano-sized, targetable nucleic acid delivery systems will help to overcome current barriers in bringing nucleic acid-based therapeutics into medical practice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbarmem.2006.03.020.

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Efficacy of a toxicity-adjusted topotecan therapy in recurrent small cell lung cancer

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ABSTRACT: The present prospective multicentre trial investigated whether topotecan, given at a starting dose of 1.25 mg·m⁻² with individual dose adjustment, can improve safety in patients with relapsed/refractory small cell lung cancer without loss of efficacy.

Patients received topotecan intravenously on days 1–5, every 21 days, for up to six courses. In the absence of relevant haematotoxicities, topotecan was increased to 1.5 mg·m⁻² and reduced to 1.0 mg·m⁻² in case of severe haematotoxicities.

Of 170 recruited patients, 73.2% had stage IV disease and 63.4% had platinum-containing pre-treatment. Patients received a total of 521 courses. In 72.6% of those courses, the dose remained at 1.25 mg·m⁻²; in 9.1% it was reduced and in 18.3% it increased. Overall response rate was 14.1% including one complete response; 28.8% had stable disease. Median duration of response was 13.6 weeks and median survival was 23.4 weeks. Clinical benefit was obvious for sensitive as well as for refractory patients. Haematotoxicity of grade 3 or 4 was clearly lower compared with the standard dose of 1.5 mg·m⁻².

In conclusion, topotecan at a dose of 1.25 mg·m⁻² appears to be as effective as the dose of 1.5 mg·m⁻², but with reduced toxicity. Since patients with recurrent small cell lung cancer have a poor prognosis, they benefit especially from good tolerability.

KEYWORDS: Second-line therapy, small cell lung cancer, topotecan

Small cell lung cancer (SCLC) is considered to be among the most chemosensitive solid tumours [1]. With combination chemotherapy, such as platinum/etoposide or cyclophosphamide/doxorubicin/etoposide, objective response rates of 20–90% are observed, with a median survival of ~10 months. However, the majority of patients will experience tumour recurrence after successful therapy [2–4]. The prognosis with second-line treatment was analysed by HUISMAN *et al.* [5] from 21 published phase II studies, including 1,749 patients showing response rates of 20%. Response to second-line therapy most likely depends on response to first-line treatment and length of the treatment-free interval. Patients developing disease progression within 3 months after first-line therapy are classified as refractory. Patients with disease progression >3 months after the last treatment of first-line therapy, which has induced an objective response, are classified as sensitive [3]. Since refractory patients have a smaller chance of responding to any drug than sensitive patients, stratification between sensitive and refractory patients is necessary to describe the efficacy of a tested regimen. Topotecan (Hycamtin®; GlaxoSmithKline, Munich, Germany)

is a specific inhibitor of the nuclear enzyme topoisomerase I, which interferes with DNA replication and transcription. Inhibition of this enzyme produces lethal DNA damage [6]. Topotecan was reported to be effective as second-line treatment for SCLC [7]. In a randomised phase III study, topotecan (1.5 mg·m⁻² *i.v.*, days 1–5, every 21 days) was at least as effective as the three-drug regimen of cyclophosphamide 1,000 mg·m⁻², doxorubicin 45 mg·m⁻² and vincristine 2 mg (CAV). Response rate was 24.3% in the topotecan arm and 18.3% in the CAV arm. Median survival was 25 weeks for topotecan and 24.7 weeks for CAV. The present study demonstrated that topotecan has significant advantages in controlling disease-related symptoms [8]. In three studies, a total of 264 evaluable patients with recurrent SCLC were stratified as either refractory (126 patients) or sensitive (138 patients), according to their response to first-line therapy. All patients received *i.v.* topotecan 1.5 mg·m⁻² on days 1–5, every 21 days. The efficacy is summarised in table 1 [9–11]. The most frequent adverse event of the 1.5 mg·m⁻²·day⁻¹ regimen in all studies was neutropenia grade 3/4. VON PAWEL *et al.* [12] reported an incidence of grade 4 neutropenia in 67.3% of patients (32.5% of

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courses) in their phase II study [12] and 70.2% (38.7% of courses) of patients in their phase III study [8]. ARDIZZONI *et al.* [11] reported grade 4 neutropenia in 46.9% of courses. Grade 4 thrombocytopenia was also shown in 8–11% of courses [11, 12]. Infection associated with grade 4 neutropenia occurred in 28% of patients and 8.7% of courses in the topotecan arm of a previous comparative phase III trial, along with four treatment-related deaths (3.7% of patients) [8]. ARDIZZONI *et al.* [11] observed infection in 6% of courses and one treatment-related death. PEREZ-SOLER *et al.* [13] reported a lower incidence of haematological toxicity when topotecan was administered in a dose of 1.25 mg·m⁻² *i.v.* on days 1–5 in patients with SCLC refractory to etoposide/platinum. In 28% of courses, grade 4 neutropenia was observed, but there were no episodes of neutropenic fever and no treatment-related deaths [13].

The objective of this multicentre phase II trial was to evaluate prospectively the efficacy and safety of topotecan starting with a starting dose of 1.25 mg·m⁻² and with dose adjustment in accordance with toxicity. Patients with recurrent or refractory SCLC were stratified according to pre-treatment with a platinum-containing or platinum-free regimen and their response to pre-treatment.

PATIENTS AND METHODS

Patient eligibility

Patients with histologically and/or cytologically documented SCLC, which has recurred or progressed after first-line chemotherapy, were eligible. Further inclusion criteria were: bi-dimensional measurable disease (minimum size of lesion = 2 × 2 cm); male or female patients aged 18–75 yrs; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2; written informed consent according to the local institutional ethics committee requirements; leukocyte count ≥ 4,000 cells·μL⁻¹; platelet count ≥ 100,000·μL⁻¹; haemoglobin concentration ≥ 9.0 g·dL⁻¹; and creatinine clearance ≥ 60 mL·min⁻¹.

Exclusion criteria included: pre-treatment with a topoisomerase I inhibitor; more than one pre-treatment; pregnant or lactating females; history of neoplasm other than SCLC; serious concomitant medical conditions; dementia; surgery within 2 weeks before study entry; and participation in any other clinical study within 30 days before study entry.

The present study was approved by the ethical committee of each participating centre.

Descriptive statistical methods were used to assess response evaluation, dose intensity, time to progression and toxicity data. Survival estimates were performed using the Kaplan–Meier method.

Treatment

Topotecan was administered as a 30-min *i.v.* infusion at a starting dose of 1.25 mg·m⁻² for 5 consecutive days, repeated every 21 days for six cycles. One topotecan dose escalation of 0.25 mg·m⁻² was to be performed in the second cycle in the absence of haematological toxicities grade 3 or 4. Further dose escalations of 0.25 mg·m⁻² in the absence of haematological toxicities grade 3 or 4 was left to the discretion of the investigator.

A topotecan dose reduction of 0.25 mg·m⁻² was to be performed in case of the following: 1) grade 4 neutropenia lasting 7 days or longer, or complicated by fever or infection; 2) platelet count < 25,000·μL⁻¹, or neutrophil count < 1,500 cells·μL⁻¹ and platelet count < 100,000·μL⁻¹ on day 22; and 3) in case of grade 3 or 4 nonhaematological toxicity (except for nausea, vomiting and alopecia). In any case, the minimum topotecan dose had to be 1.0 mg·m⁻²·day⁻¹. No dose re-escalation was allowed.

Use of granulocyte colony-stimulating factor (G-CSF) was left to the discretion of the investigator. Duration of the treatment was based on response evaluation which was performed after a minimum of two courses. In case of complete response, treatment was continued for two additional courses. In case of partial response, or stable disease, treatment was continued until disease progression or severe toxicity was observed or for the planned treatment of six cycles. Patients were withdrawn from the current study in case of disease progression or incomplete haematological recovery 2 weeks after scheduled treatment or for other generally accepted reasons. Response and toxicity were evaluated according to the World Health Organization and National Cancer Institute–Common Toxicity Criteria (revised version, 1994) [14].

Study design

The objectives of the present study were to evaluate response rates, response duration, toxicities, dose-intensity, and median survival of relapsed SCLC treated with topotecan at a starting dose of 1.25 mg·m⁻²·day⁻¹. Patients were stratified according to pre-treatment with or without platinum, and according to response to pre-treatment (sensitive *versus* refractory).

According to given treatment, patients were grouped to a “safety”, “survival” or “response” collective. The objectives of the current study were analysed within the corresponding groups. Patients (n=170) who received at least one dose of study drug were evaluated by safety analysis. Patients (n=164)

TABLE 1 Efficacy of *i.v.* topotecan (1.5 mg·m⁻²·day⁻¹, on days 1–5) in second-line small cell lung cancer

First author [ref.]	Subjects n	CR	PR	OR %	SD %	Survival weeks*
ECKHARDT [9]						
Refractory	36 [†]		1	2.6	45	20.4
Sensitive	36 [†]	3	4	19.4	36	26.6
Total	74			10.8	40.5	NR
VON PAWEL [10]						
Refractory	41 [†]		1	2.4	NR	16.3
Sensitive	57 [†]	1	7	14.0	NR	25.7
Total	98			9.2	27	21.6
ARDIZZONI [11]						
Refractory	47 [†]	1	2	6.4	40	18.8
Sensitive	45 [†]	6	11	37.8	31	27.6
Total	92			21.7	35.9	21.6

CR: complete response; PR: partial response; OR: odds ratio; SD: stable disease; NR: not reported. *: median. †: patients who failed to respond or progressed within 90 days of first-line therapy were termed refractory; ‡: patients who relapsed after 90 days after first-line therapy were termed sensitive.

TABLE 2 Patients characteristics^a

Patients registered	
Assessable for response intention to treat	170 (100)
Assessable for survival	164 (96.5)
Assessable for toxicity	170 (100)
Age	
Median 61 yrs	
<60 yrs	66 (40.3)
>60 yrs	98 (59.7)
Sex	
Male	128 (78.0)
Female	36 (22.0)
ECOG performance status	
0 Karnofsky 100%	34 (20.7)
1 Karnofsky 80–90%	92 (56.1)
2 Karnofsky 60–70%	37 (22.6)
Extent of disease	
Limited disease	4 (2.4)
Extensive disease without distant metastasis	38 (23.2)
Extensive disease with distant metastasis	120 (73.2)
No data	2 (1.2)
Liver metastases	
Present	59 (36.0)
Absent	105 (64.0)
Brain metastases	
Present	46 (28)
Absent	118 (72)
Best response to first-line treatment	
Complete response	31 (18.9)
Partial response	95 (57.9)
Stable disease	9 (5.5)
Progression	29 (17.7)
Time to relapse after first-line therapy	
<3 months refractory	57 (34.8)
>3–<6 months sensitive	107 (65.2)
Time to progression after first-line therapy	
Median days	191
Prior anticancer treatment	
Prior radiotherapy	85 (51.8)
Prior surgery	17 (10.4)
First-line therapy	
Platinum-based	104 (63.4)
Nonplatinum-based	60 (36.6)
First-line chemotherapy regimen	
Carboplatin, etoposide, vincristin	48 (29.3)
Platinum, cisplatin or carboplatin, etoposide	36 (21.9)
Carboplatin, taxol, etoposide	7 (4.3)
Adriamycin, cyclophosphamide, vincristine	20 (12.2)
Epirubicin, cyclophosphamide, vincristine	20 (12.2)
Cyclophosphamide, etoposide, adriamycin	12 (7.3)
Other regimens	21 (12.4)
Number of measurable lesions	
1	69 (42.1)
2	50 (30.5)
3	33 (20.1)
>3	12 (7.2)

Data are presented as n (%), unless otherwise stated. ECOG: Eastern Cooperative Oncology Group. ^a n=170.

who had received at least one course of study medication and did not show any serious study deviation (*i.e.* no measurable disease) were evaluated for survival, demographic data, dose intensity and time to progression. Objective response evaluation was performed in patients (n=120) who received at least two courses of the study drug and had a tumour baseline status with re-evaluation (response collective). In 41 patients, clinical progressive disease or death was observed prior to response re-evaluation after the end of the second cycle. Clinical progressive disease, death or missing response data were regarded as progressive disease, according to a worst-case scenario (intention-to-treat).

Tumour response was judged by each individual centre. Median time to progression and survival were calculated according to the Kaplan-Meier product limit method.

RESULTS

Patient characteristics

A total of 170 patients were enrolled from 44 participating centres between February 1998 and June 1999. The last follow-up was performed in December 2002. It was possible to evaluate all patients for safety analysis. In total, 164 patients were evaluated for survival analysis (two patients did not complete a full course and four patients did not have a bi-dimensionally measurable lesion). Tumour response, as the primary end-point of the present study, was evaluated in all patients who received at least one dose of the study drug (n=170; intention-to-treat). Of these, 37 patients received less than two courses and seven patients were lost to follow-up. A total of 76.8% of patients had performance status ECOG 0–1. The majority of patients (73.2%) had distant metastatic disease and 63.4% of patients had received a platinum-based primary therapy. The overall response rate to primary therapy was 76.8%. In total, 34.8% of patients were refractory, whereas 65.2% of patients were sensitive to prior therapy. The median time to progression after first-line therapy was 191 days (table 2).

Dosing

A total of 514 topotecan courses were received by 164 patients. Of these, 22.6% of patients (n=37) received only one course of therapy, 2.4% (n=4) of patients received eight courses and 52 patients (31.7%) received two courses. The first cycle was administered to 164 patients and the sixth cycle to 36 patients. In 58.2% of courses (n=299), the starting dose was maintained. The topotecan dose had to be reduced to 1.0 mg·m⁻²·day⁻¹ in 8.9% (n=46) of courses. The targeted dose of topotecan (1.5 mg·m⁻²·d⁻¹) was reached in 32.9% (n=169) of courses. In the first course, the topotecan dose was administered as a starting dose of 1.25 mg·m⁻²·day⁻¹ to 98.2% of patients (n=161). This starting dose was maintained in 46.5% of patients (n=59) in course two. Dose escalation to 1.5 mg·m⁻²·day⁻¹ of topotecan was performed in 37.8% of patients (n=48), whereas in 15.7% (n=20) it was reduced to 1.0 mg·m⁻²·day⁻¹. Protocol deviations, according to dose modification in the second cycle, were noticed in 13.1% (no dose escalation to 1.5 mg·m⁻²) and 17.0% (no dose reduction to 1.0 mg·m⁻²) of patients, respectively. While the number of patients who received subsequent courses decreased, the percentage of patients who received an escalated dose rose from 46.7% (third cycle) to 58.3% (sixth

cycle). At the same time, the percentage of patients who received a reduced dose decreased from the third cycle (13.3%) to the sixth cycle (8.3%), with the exception of the fifth cycle (14.0%). The percentage of patients within the third to sixth cycles receiving the starting dose was between 30.2% (fifth cycle) and 40.0% (third cycle). Median dose intensity of all administered cycles was $1.25 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ (table 3).

Efficacy

Response to treatment

A total of 170 patients were evaluated for their response (intention to treat). The overall response rate was 14.1% with one complete response and 23 (13.5%) partial responses. Stable disease was observed in 25.9% and progressive disease in 60% of patients. No difference in response rates was seen between patients with or without platinum-containing pre-treatment, with overall response rates of 14.9 and 12.7%, respectively. However, patients who had been refractory to primary therapy achieved a lower response rate and a higher rate of disease stabilisation to topotecan therapy. The overall response rate was 8.6% in refractory patients *versus* 17.1% in sensitive patients and stable disease was observed in 36.2 and 20.7% of patients, respectively (table 4). Among all responding patients, the median time to response was 7.2 (range 2.9–14.7) weeks. There was no significant difference between patients with platinum-containing and platinum-free pre-treatment at 8.1 *versus* 6.6 weeks ($p=0.8952$), respectively. In refractory patients, time to response was significantly longer at 12.4 weeks *versus* 6.4 weeks ($p=0.0260$). As shown in table 5, median duration of response was 13.6 (3.0–47.9) weeks and was not significantly different among the subgroups. Median time to progression for all patients was 8.0 (0.1–53.6) weeks. An analysis according to stratification parameters showed no significant differences between sensitive *versus* refractory patients.

Survival

It was possible to evaluate 164 patients for survival. The median survival time was 23.4 weeks (95% confidence interval (CI) 19.0–27.0 (0.9–92.4) weeks) and mean survival was 28.3 weeks (fig. 1). Median survival time for patients with a

complete and partial response, stable disease or progressive disease was 43.4, 28.6 and 26.0 weeks, respectively. Stratified according to pre-treatment and response to pre-treatment, no differences in median survival time could be detected. Median survival time of 62 weeks was found in patients with an objective response to topotecan and nonplatinum-containing pre-treatment.

After 1 yr 25 patients were alive according to a 1-yr survival rate of 15.2%.

Toxicity

A total of 170 patients received at least one dose of topotecan and were evaluable for toxicity. Treatment delays of >14 days due to haematological toxicity were observed in five patients. Delays of >7 days due to toxicity or management reasons were performed in 11.7% of courses. Therapy had to be stopped in nine patients. All cases were associated with myelosuppression. One treatment-related death was observed. This patient, with poor performance status, declined hospitalisation despite grade 4 neutropenia and thrombocytopenia. Major side-effects were neutropenia and leukopenia, whereas anaemia and thrombocytopenia were less common. Grade 3 and 4 neutropenia occurred in 27.7 and 27.6% of patients, and in 31.5 and 6.9% of treatment courses, respectively. G-CSF was administered in 21.2% of patients in 11.5% of treatment courses. Febrile neutropenia was observed in 0.8% of courses. The incidence of grade 3 and 4 thrombocytopenia was 10.3% and 5.0% of treatment courses (23.5 and 13.5% of patients), respectively. In total, 10% of patients received a platelet substitution in 3.3% of treatment courses. Anaemia grade 3 and 4 was less common (5.5% and 0.6% of all courses, respectively); in 3.6% of courses, erythrocytes had to be substituted. No evidence of cumulative haematological toxicity was observed. Haematological toxicity among the subgroups is presented in table 6 demonstrating lower rates of neutropenia and thrombocytopenia in refractory patients. In patients without platinum-containing pre-treatment, grade 3 and 4 anaemia was more frequent. Nonhaematological toxicities of grade 3 and 4, which were observed in >1% of patients, included: pain (5.9%), infection (4.7%), nausea (1.8%) and fever (1.8%).

DISCUSSION

Despite the high chemosensitivity of SCLC, the majority of patients have a relapse after induction chemotherapy. The prognosis of patients with recurrent disease remains poor. The goals of chemotherapy in this patient population were to obtain maximum control of disease symptoms, prevent serious complications and increase survival without diminishing quality of life [1, 4, 5]. The current prospective study is the first trial in which an individual dose adjustment strategy with a reduced topotecan starting dose was evaluated in order to reduce toxicity, a procedure which is in close relationship to the clinical practice in relapsed SCLC.

A prospective analysis of the haematological toxicity profile of topotecan, administered at a dose of $1.25 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ on days 1–5 to pre-treated patients with ovarian cancer, revealed that the severity of topotecan-induced thrombocytopenia is maximal after the first cycle, but significantly decreases after the second cycle without dose reductions [15]. FIELDS *et al.* [16] performed a retrospective analysis of second-line patients with

TABLE 3 Dose intensity and administered courses in the study patients

Course	Administered dose $\text{mg}\cdot\text{m}^{-2}$			
	1.00	1.25	1.5	>1.5
1	2 (1.2)	161 (98.2)	1 (0.6)	0 (0.0)
2	20 (15.7)	59 (46.5)	48 (37.8)	1 (0.8)
3	10 (13.3)	30 (40.0)	35 (46.7)	1 (1.3)
4	5 (8.2)	22 (36.1)	34 (55.7)	2 (3.3)
5	6 (14.0)	13 (30.2)	22 (55.8)	2 (4.7)
6	3 (8.3)	12 (33.3)	21 (58.3)	1 (2.8)
7	0 (0.0)	1 (25.0)	3 (75.0)	0 (0.0)
8	0 (0.0)	1 (25.0)	3 (75.0)	0 (0.0)

Data are presented as n (%).

TABLE 4 Responses according to stratification

	All patients	Platinum pre-treatment		Response to pre-treatment [#]	
		Yes	No	Sensitive	Refractory
Subjects n	170	107	63	111	58
Complete response	0.6	0.9	0.0	0.9	0.0
Partial response	13.5	14.0	12.7	16.2	8.6
Overall response	14.1	14.9	12.7	17.1	8.6
Stable disease	25.9	25.2	27.0	20.7	36.2
Progressive disease	60.0	59.8	60.3	62.2	55.2
95% CI for response		30.8–50.1	27.6–52.8	28.8–47.5	31.7–58.5

Data are presented as %, unless otherwise stated. CI: confidence interval. [#]: missing data (n=1).

SCLC treated with the standard topotecan dose. In 110 patients, the dose was reduced to $\leq 1.25 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ due to haematological toxicity. Response rates and survival in the reduced dose population were similar to the results of the standard dose population [16].

The presented results demonstrate that treatment with the reduced topotecan starting dose of $1.25 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ and an individual dose adjustment is equi-effective to the efficacy of the standard dose of $1.5 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$. VON PAWEL and co-workers [8, 16] reported a median survival of 25 weeks for 98

patients receiving the *i.v.* regimen of $1.5 \text{ mg}\cdot\text{m}^{-2}\cdot\text{day}^{-1}$ in the phase II and 107 patients in the phase III study; in the present study, the median survival was 23.4 weeks. Of note, in these trials, mostly sensitive patients were included [8, 12].

The greater tolerability of this regimen is also reflected in the reduced rate of haematological toxicities compared with recently reported trials, where topotecan was given at a standard dose. In the current trial, grade 4 neutropenia and thrombocytopenia occurred in 6.9 and 5% of courses, respectively, and febrile neutropenia was observed in only 0.8%. In other studies using the standard *i.v.* dose of topotecan in relapsed SCLC, grade 4 neutropenia and thrombocytopenia were observed in 32.5–46.9% and 7.7–11.9% of courses, respectively [8, 11]. Febrile neutropenia occurred in 3.3–6% of *i.v.* topotecan courses. Nonhaematological toxicities were mild and did not influence patients' well-being. Notably, grade 3 and 4 neutropenia and thrombocytopenia is less frequent in refractory compared with sensitive patients. This is very important for refractory patients, since the clinical benefit does not seem to be associated with a higher toxicity and justifies therapy in this special patient group.

TABLE 5 Response duration, time to progression and survival

	Patients	Weeks
Response duration		
All	24	13.6 (3.0–47.9)
Platinum +PT	16	12.1 (3.0–47.9) [#]
Platinum -PT	8	19.4 (7.3–40.9)
Sensitive	19	15.0 (3.0–47.9) [†]
Refractory	5	13.0 (4.7–27.1)
Time to progression		
All	164	8.0 (0.1–53.6)
Platinum +PT	104	7.9 (2.1–53.6) [†]
Platinum -PT	60	9.6 (0.1–48.0)
Sensitive	107	8.0 (2.1–53.6) [‡]
Refractory	57	9.6 (0.1–41.3)
Survival		
All	164	23.4 (0.9–92.4)
Platinum +PT	104	23.4 (0.9–92.4) [†]
Platinum -PT	60	22.9 (1.1–83.1)
Sensitive	107	22.4 (0.9–92.4) [‡]
Refractory	57	23.7 (1.1–85.7)

Data are presented as n or median (interquartile range). Platinum +PT: platinum-containing pre-treatment; Platinum -PT: platinum-free pre-treatment. [#]: p=0.372 platinum +PT versus platinum -PT; [†]: p=0.339 sensitive versus refractory; [‡]: p=0.017 platinum +PT versus platinum -PT; [§]: p=0.472 sensitive versus refractory; [†]: p=0.878 platinum +PT versus platinum -PT; ^{**}: p=0.82 sensitive versus refractory.

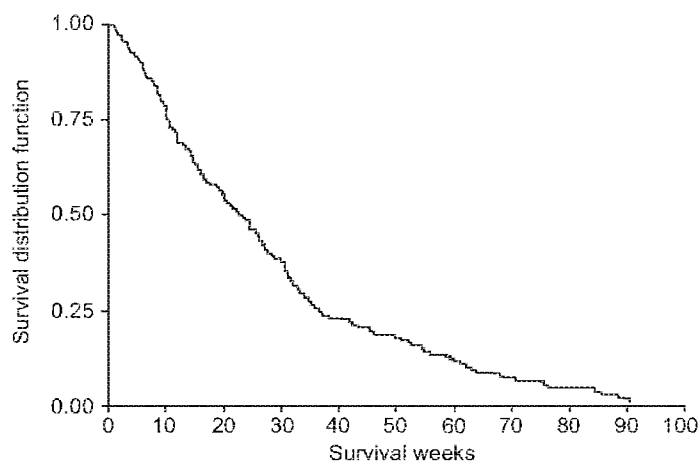
**FIGURE 1.** Kaplan-Meier plot of overall survival (n=164). Median survival: 23.4 weeks.

TABLE 6 Haematological toxicity

	All	Platinum pre-treatment		Response to pre-treatment	
		Yes	No	Sensitive	Refractory
Subjects	170	104	60	107	57
Leukopenia	44.1/17.6	44.3/16.3	45.5/21.2	47.7/22.4	38.6/10.5
Neutropenia	27.7/27.6	28.9/31.7	38.4/23.3	35.6/31.7	26.3/22.8
Thrombopenia	23.5/13.5	25.0/12.5	20.1/16.6	12.2/16.8	15.8/8.8
Anaemia	16.0/1.7	14.4/1.9	18.4/1.6	14.0/2.8	19.3/0

Data are presented as n or % of patients with grade 3/4 toxicity.

Median dose intensity of all administered cycles was $1.25 \text{ mg} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$. In 58.2% of all courses, the starting dose was maintained and in 32.9% of courses the dose was escalated to $1.5 \text{ mg} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$. Only 8.9% of courses had to be reduced due to haematological toxicity compared with phase II studies with a standard topotecan dosage, where dose reductions of 16–19% were reported [11, 12].

The overall response rate of 14.1% obtained in the present study is comparable to recently published reports using *i.v.* topotecan in the standard dosage (table 1). As expected, the response rate was higher in sensitive patients (17.1%) than in refractory patients (8.6%), and median time to response was longer for refractory patients (12.4 weeks) than for sensitive patients (6.4 weeks). This is in accordance with reports by ARDIZZONI *et al.* [11] and PEREZ-SOLER *et al.* [13]. In contrast, no difference was seen between patients with or without platinum-containing pre-treatment indicating the lack of cross-resistance to platinum. PEREZ-SOLER *et al.* [13] reported the responses to topotecan in etoposide-refractory patients and concluded that it may overcome etoposide resistance. Interestingly, in 36.2% of refractory patients and 20.7% of sensitive patients, stabilisation of disease was observed. As shown by CESANO *et al.* [17], stable disease represents a potential clinical benefit, since stable disease has the same survival benefit as a partial response *versus* progressive disease. Primary progression rate also provides support for a clinical benefit; it shows no difference between the four subgroups and is not higher in refractory patients.

Median duration of response was 13.6 weeks and median time to progression was 8.0 weeks for all patients. Patients with platinum-free pre-treatment had an advantage in median time to progression compared with patients with platinum-containing pre-treatment. Median time to progression was 9.6 weeks *versus* 7.9 weeks in these groups (table 5). The results concerning pre-treatment are difficult to compare with other studies, as the distinction between platinum-containing and platinum-free pre-treatment is not reported.

The median survival was 23.4 weeks and 1-yr survival was 15.2% without any differences between the subgroups. The survival data for all patients are comparable to reports using the standard topotecan dosage in phase II and III trials [9–11]. ARDIZZONI *et al.* [11] reported longer survival data for sensitive than for refractory patients (6.9 *versus* 4.7 months). In the

present study, survival data of refractory patients are similar to survival in sensitive patients (23.7 *versus* 22.4 weeks). High rate of disease stabilisation, especially in the refractory patients' group, may contribute to better median survival of refractory patients in the current study. This is in agreement with previous reports in which survival of patients with partial response was comparable with survival of patients with stable disease following topotecan therapy of SCLC and ovarian cancer [17]. Stable disease is a valid end-point provided that symptom palliation is achieved and treatment-related toxic side-effects are mild.

A meta-analysis of four multicentre trials treating patients with relapsed SCLC with topotecan at a starting dose of $1.5 \text{ mg} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$ suggests that dose reductions due to haematological toxicity ($\leq 1.25 \text{ mg} \cdot \text{m}^{-2} \cdot \text{day}^{-1}$) are not correlated with decreased efficacy. The response rate and median survival in the reduced dose population was 17.3% and 29.9 weeks, respectively, compared with 18.1% and 28.6 weeks in the standard dose population. These results support those of the present study, indicating that moderate topotecan dose reductions can counterbalance the haematological toxicity but are not associated with reduced efficacy [16].

To the best of the present authors' knowledge, this is the first prospective trial in relapsed small cell lung cancer which shows a positive impact on safety parameters of topotecan therapy at a reduced starting dose with an individual dose adjustment strategy. It is of special interest that this impact is in association with the same efficacy compared with standard topotecan regimens. These results demonstrate that patients with sensitive and refractory relapsed small cell lung cancer can benefit from chemotherapy and that treatment-related side-effects are manageable by individual dose titration.

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Confirmation Number:	5137
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Customer Number:	153749
Filer:	Mary Rucker Henninger/Richard King
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29	Non Patent Literature	NCT01770353_2013-08-09.pdf	109265	no	3
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Information:					

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56	Non Patent Literature	Hanna_2006.pdf	167865	no	6
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57	Non Patent Literature	Hare_2013.pdf	753899	no	12
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60	Non Patent Literature	Huber_2006.pdf	309745 5abb4ec02c5d37e28b17539c77d08e4cd7087c3c	no	7
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Information:					
Total Files Size (in bytes):			91621732		
<p>This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.</p> <p><u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.</p> <p><u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.</p> <p><u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.</p>					

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use HYCAMTIN safely and effectively. See full prescribing information for HYCAMTIN.

HYCAMTIN (topotecan hydrochloride) for Injection

Initial U.S. Approval: 1996

WARNING: BONE MARROW SUPPRESSION

See full prescribing information for complete boxed warning.

Do not give HYCAMTIN to patients with baseline neutrophil counts less than 1,500 cells/mm³. In order to monitor the occurrence of bone marrow suppression, primarily neutropenia, which may be severe and result in infection and death, monitor peripheral blood cell counts frequently on all patients receiving HYCAMTIN. (5.1)

RECENT MAJOR CHANGES

Dosage and Administration (2) 02/2014

INDICATIONS AND USAGE

HYCAMTIN for Injection is a topoisomerase inhibitor indicated for:

- metastatic carcinoma of the ovary after failure of initial or subsequent chemotherapy. (1)
- small cell lung cancer sensitive disease after failure of first-line chemotherapy. (1)
- combination therapy with cisplatin for Stage IV-B, recurrent, or persistent carcinoma of the cervix which is not amenable to curative treatment with surgery and/or radiation therapy. (1)

DOSAGE AND ADMINISTRATION

- Ovarian cancer and small cell lung cancer: 1.5 mg/m² by intravenous infusion over 30 minutes daily for 5 consecutive days, starting on day one of a 21-day course. (2.1)
- Cervical cancer: 0.75 mg/m² by intravenous infusion over 30 minutes on days 1, 2, and 3 followed by cisplatin 50 mg/m² by intravenous infusion on day 1 repeated every 21 days. (2.2)

See Dosage Modification Guidelines for patients with neutropenia or reduced platelets. (2.1, 2.2)

See Dosage Adjustment in Renal Impairment. (2.3)

DOSAGE FORMS AND STRENGTHS

4-mg (free base) single-dose vial. (3)

CONTRAINDICATIONS

- History of severe hypersensitivity reactions (e.g., anaphylactoid reactions) to topotecan or any of its ingredients (4)
- Severe bone marrow depression (4)

WARNINGS and PRECAUTIONS

- Bone marrow suppression: Administer HYCAMTIN only to patients with adequate bone marrow reserves. Monitor peripheral blood counts and adjust the dose if needed. (5.1)
- Topotecan-induced neutropenia can lead to neutropenic colitis. (5.2)
- Interstitial lung disease: HYCAMTIN has been associated with reports of interstitial lung disease. Monitor patients for symptoms and discontinue HYCAMTIN if the diagnosis is confirmed. (5.3)
- Pregnancy: Can cause fetal harm. Advise women of potential risk to the fetus. (5.4, 8.1)

ADVERSE REACTIONS

Ovarian and small cell lung cancer:

- The most common hematologic adverse reactions were: neutropenia (97%), leukopenia (97%), anemia (89%), and thrombocytopenia (69%). (6.1)
- The most common (>25%) non-hematologic adverse reactions (all grades) were: nausea, alopecia, vomiting, sepsis or pyrexia/infection with neutropenia, diarrhea, constipation, fatigue, and pyrexia. (6.1)

Cervical cancer (HYCAMTIN plus cisplatin):

- The most common hematologic adverse reactions (all grades) were: anemia (94%), leukopenia (91%), neutropenia (89%), and thrombocytopenia (74%). (6.1)
- The most common (>25%) non-hematologic adverse reactions (all grades) were: pain, nausea, vomiting, and infection/febrile neutropenia. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact GlaxoSmithKline at 1-888-825-5249 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- Do not initiate G-CSF until 24 hours after completion of treatment with HYCAMTIN. Concomitant administration can prolong duration of neutropenia. (7)
- Greater myelosuppression is likely to be seen when used in combination with other cytotoxic agents. (7)

USE IN SPECIFIC POPULATIONS

- Nursing Mothers: Discontinue nursing when receiving HYCAMTIN. (8.3)

See 17 for PATIENT COUNSELING INFORMATION.

Revised: 02/2014

FULL PRESCRIBING INFORMATION: CONTENTS*

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15 REFERENCES

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FULL PRESCRIBING INFORMATION

WARNING: BONE MARROW SUPPRESSION

Do not give HYCAMTIN[®] to patients with baseline neutrophil counts less than 1,500 cells/mm³. In order to monitor the occurrence of bone marrow suppression, primarily neutropenia, which may be severe and result in infection and death, monitor peripheral blood counts frequently on all patients receiving HYCAMTIN [see *Warnings and Precautions (5.1)*].

1 INDICATIONS AND USAGE

HYCAMTIN is indicated for the treatment of:

- metastatic carcinoma of the ovary after failure of initial or subsequent chemotherapy.
- small cell lung cancer sensitive disease after failure of first-line chemotherapy. In clinical studies submitted to support approval, sensitive disease was defined as disease responding to chemotherapy but subsequently progressing at least 60 days (in the Phase 3 study) or at least 90 days (in the Phase 2 studies) after chemotherapy [see *Clinical Studies(14)*].

HYCAMTIN in combination with cisplatin is indicated for the treatment of:

- Stage IV-B, recurrent, or persistent carcinoma of the cervix which is not amenable to curative treatment with surgery and/or radiation therapy.

2 DOSAGE AND ADMINISTRATION

Verify dose using body surface area prior to dispensing. Recommended dosage should generally not exceed 4 mg intravenously [see *Overdosage (10)*].

Prior to administration of the first course of HYCAMTIN, patients must have a baseline neutrophil count of >1,500 cells/mm³ and a platelet count of >100,000 cells/mm³.

2.1 Ovarian Cancer and Small Cell Lung Cancer

Recommended Dosage:

- The recommended dose of HYCAMTIN is 1.5 mg/m² by intravenous infusion over 30 minutes daily for 5 consecutive days, starting on day 1 of a 21-day course.
- In the absence of tumor progression, a minimum of 4 courses is recommended because tumor response may be delayed. The median time to response in 3 ovarian clinical trials was 9 to 12 weeks, and median time to response in 4 small cell lung cancer trials was 5 to 7 weeks.

Dosage Modification Guidelines:

- In the event of severe neutropenia (defined as <500 cells/mm³) during any course, reduce the dose by 0.25 mg/m² (to 1.25 mg/m²) for subsequent courses.
- Alternatively, in the event of severe neutropenia, administer G-CSF (granulocyte-colony stimulating factor) following the subsequent course (before resorting to dose reduction) starting from day 6 of the course (24 hours after completion of topotecan administration).
- In the event the platelet count falls below 25,000 cells/mm³, reduce doses by 0.25 mg/m² (to 1.25 mg/m²) for subsequent courses.

2.2 Cervical Cancer

Recommended Dosage: The recommended dose of HYCAMTIN is 0.75 mg/m² by intravenous infusion over 30 minutes daily on days 1, 2, and 3; followed by cisplatin 50 mg/m² by intravenous infusion on day 1 repeated every 21 days (a 21-day course).

Dosage Modification Guidelines: Dosage adjustments for subsequent courses of HYCAMTIN in combination with cisplatin are specific for each drug. See manufacturer's prescribing information for cisplatin administration and hydration guidelines and for cisplatin dosage adjustment in the event of hematologic toxicity.

- In the event of severe febrile neutropenia (defined as $<1,000$ cells/mm³ with temperature of 38.0°C or 100.4°F), reduce the dose of HYCAMTIN to 0.60 mg/m² for subsequent courses.
- Alternatively, in the event of severe febrile neutropenia, administer G-CSF following the subsequent course (before resorting to dose reduction) starting from day 4 of the course (24 hours after completion of administration of HYCAMTIN).
- If febrile neutropenia occurs despite the use of G-CSF, reduce the dose of HYCAMTIN to 0.45 mg/m² for subsequent courses.
- In the event the platelet count falls below 25,000 cells/mm³, reduce doses to 0.60 mg/m² for subsequent courses.

2.3 Dosage Adjustment in Specific Populations

Renal Impairment: No dosage adjustment of HYCAMTIN appears to be required for patients with mild renal impairment (Cl_{cr} 40 to 60 mL/min). Dosage adjustment of HYCAMTIN to 0.75 mg/m² is recommended for patients with moderate renal impairment (20 to 39 mL/min). Insufficient data are available in patients with severe renal impairment to provide a dosage recommendation for HYCAMTIN [see *Use in Specific Populations (8.6) and Clinical Pharmacology (12.3)*].

HYCAMTIN in combination with cisplatin for the treatment of cervical cancer should only be initiated in patients with serum creatinine ≤ 1.5 mg/dL. In the clinical trial, cisplatin was discontinued for a serum creatinine >1.5 mg/dL. Insufficient data are available regarding continuing monotherapy with HYCAMTIN after cisplatin discontinuation in patients with cervical cancer.

2.4 Instructions for Handling, Preparation, and Intravenous Administration

Handling: HYCAMTIN is a cytotoxic anticancer drug. Prepare HYCAMTIN under a vertical laminar flow hood while wearing gloves and protective clothing. If HYCAMTIN solution contacts the skin, wash the skin immediately and thoroughly with soap and water. If HYCAMTIN contacts mucous membranes, flush thoroughly with water.

Use procedures for proper handling and disposal of anticancer drugs. Several guidelines on this subject have been published.¹⁻⁴

Preparation and Administration: Each 4-mg vial of HYCAMTIN is reconstituted with 4 mL Sterile Water for Injection. Then the appropriate volume of the reconstituted solution is diluted in either 0.9% Sodium Chloride Intravenous Infusion or 5% Dextrose Intravenous Infusion prior to administration.

Stability: Unopened vials of HYCAMTIN are stable until the date indicated on the package when stored between 20° and 25°C (68° and 77°F) [see USP] and protected from light in the original package. Because the vials contain no preservative, contents should be used immediately after reconstitution.

Reconstituted vials of HYCAMTIN diluted for infusion are stable at approximately 20° to 25°C (68° to 77°F) and ambient lighting conditions for 24 hours.

3 DOSAGE FORMS AND STRENGTHS

4-mg (free base) single-dose vial; light yellow to greenish powder.

4 CONTRAINDICATIONS

HYCAMTIN is contraindicated in patients who have a history of severe hypersensitivity reactions (e.g., anaphylactoid reactions) to topotecan or to any of its ingredients. HYCAMTIN should not be used in patients with severe bone marrow depression.

5 WARNINGS AND PRECAUTIONS

5.1 Bone Marrow Suppression

Bone marrow suppression (primarily neutropenia) is the dose-limiting toxicity of HYCAMTIN. Neutropenia is not cumulative over time. In ovarian cancer, the overall treatment-related death rate was 1%. In the comparative trial in small cell lung cancer, however, the treatment-related death rates were 5% for HYCAMTIN and 4% for CAV (cyclophosphamide-doxorubicin-vincristine).

Neutropenia:

- Ovarian and small cell lung cancer experience: Grade 4 neutropenia (<500 cells/mm³) was most common during course 1 of treatment (60% of patients) and occurred in 39% of all courses, with a median duration of 7 days. The nadir neutrophil count occurred at a median of 12 days. Therapy-related sepsis or febrile neutropenia occurred in 23% of patients, and sepsis was fatal in 1%. Pancytopenia has been reported.
- Cervical cancer experience: Grade 3 and Grade 4 neutropenia affected 26% and 48% of patients, respectively.

Thrombocytopenia:

- Ovarian and small cell lung cancer experience: Grade 4 thrombocytopenia ($<25,000$ /mm³) occurred in 27% of patients and in 9% of courses, with a median duration of 5 days and platelet nadir at a median of 15 days. Platelet transfusions were given to 15% of patients in 4% of courses.
- Cervical cancer experience: Grade 3 and Grade 4 thrombocytopenia affected 26% and 7% of patients, respectively.

Anemia:

- Ovarian and small cell lung cancer experience: Grade 3/4 anemia (<8 g/dL) occurred in 37% of patients and in 14% of courses. Median nadir was at day 15. Transfusions were needed in 52% of patients in 22% of courses.

- Cervical cancer experience: Grade 3 and Grade 4 anemia affected 34% and 6% of patients, respectively.

Monitoring of Bone Marrow Function: Administer HYCAMTIN only in patients with adequate bone marrow reserves, including baseline neutrophil count of at least 1,500 cells/mm³ and platelet count at least 100,000/mm³. Monitor peripheral blood counts frequently during treatment with HYCAMTIN. Do not treat patients with subsequent courses of HYCAMTIN until neutrophils recover to >1,000 cells/mm³, platelets recover to >100,000 cells/mm³, and hemoglobin levels recover to 9.0 g/dL (with transfusion if necessary). Severe myelotoxicity has been reported when HYCAMTIN is used in combination with cisplatin [see *Drug Interactions (7)*].

5.2 Neutropenic Colitis

Topotecan-induced neutropenia can lead to neutropenic colitis. Fatalities due to neutropenic colitis have been reported in clinical trials with HYCAMTIN. In patients presenting with fever, neutropenia, and a compatible pattern of abdominal pain, consider the possibility of neutropenic colitis.

5.3 Interstitial Lung Disease

HYCAMTIN has been associated with reports of interstitial lung disease (ILD), some of which have been fatal [see *Adverse Reactions (6.2)*]. Underlying risk factors include history of ILD, pulmonary fibrosis, lung cancer, thoracic exposure to radiation, and use of pneumotoxic drugs and/or colony stimulating factors. Monitor patients for pulmonary symptoms indicative of interstitial lung disease (e.g., cough, fever, dyspnea, and/or hypoxia), and discontinue HYCAMTIN if a new diagnosis of ILD is confirmed.

5.4 Pregnancy

Pregnancy Category D.

HYCAMTIN can cause fetal harm when administered to a pregnant woman.

Topotecan caused embryoletality, fetotoxicity, and teratogenicity in rats and rabbits when administered during organogenesis. There are no adequate and well-controlled studies of HYCAMTIN in pregnant women. If this drug is used during pregnancy, or if a patient becomes pregnant while receiving HYCAMTIN, the patient should be apprised of the potential hazard to the fetus [see *Use in Specific Populations (8.1)*].

5.5 Inadvertent Extravasation

Inadvertent extravasation with HYCAMTIN has been observed; most reactions have been mild, but severe cases have been reported.

6 ADVERSE REACTIONS

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared with rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Ovarian Cancer and Small Cell Lung Cancer: Data in the following section are based

on the combined experience of 453 patients with metastatic ovarian carcinoma, and 426 patients with small cell lung cancer treated with HYCAMTIN. Table 1 lists the principal hematologic adverse reactions and Table 2 lists non-hematologic adverse reactions occurring in at least 15% of patients.

Table 1. Hematologic Adverse Reactions Experienced in ≥15% Ovarian Cancer and Small Cell Lung Cancer Patients Receiving HYCAMTIN

Hematologic Adverse Reaction	Patients (n = 879) % Incidence
Neutropenia	
<1,500 cells/mm ³	97
<500 cells/mm ³	78
Leukopenia	
<3,000 cells/mm ³	97
<1,000 cells/mm ³	32
Thrombocytopenia	
<75,000/mm ³	69
<25,000/mm ³	27
Anemia	
<10 g/dL	89
<8 g/dL	37

Table 2. Non-hematologic Adverse Reactions Experienced by ≥15% of Ovarian Cancer and Small Cell Lung Cancer Patients Receiving HYCAMTIN

Non-hematologic Adverse Reaction	Percentage of Patients With Adverse Reaction (879 Patients)		
	All Grades	Grade 3	Grade 4
Infections and infestations Sepsis or pyrexia/infection with neutropenia ^a	43	NR	23
Metabolism and nutrition disorders Anorexia	19	2	<1
Nervous system disorders Headache	18	1	<1
Respiratory, thoracic, and mediastinal disorders Dyspnea Coughing	22 15	5 1	3 0
Gastrointestinal disorders Nausea Vomiting Diarrhea Constipation Abdominal pain Stomatitis	64 45 32 29 22 18	7 4 3 2 2 1	1 1 1 1 2 <1
Skin and subcutaneous tissue disorders Alopecia Rash ^b	49 16	NA 1	NA 0
General disorders and administrative site conditions Fatigue Pyrexia Pain ^c Asthenia	29 28 23 25	5 1 2 4	0 <1 1 2

NA = Not applicable.

NR = Not reported separately.

^a Does not include Grade 1 sepsis or pyrexia.

^b Rash also includes pruritus, rash erythematous, urticaria, dermatitis, bullous eruption, and maculopapular rash.

^c Pain includes body pain, back pain, and skeletal pain.

Nervous System Disorders: Paresthesia occurred in 7% of patients but was generally Grade 1.

Hepatobiliary Disorders: Grade 1 transient elevations in hepatic enzymes occurred in

8% of patients. Greater elevations, Grade 3/4, occurred in 4%. Grade 3/4 elevated bilirubin occurred in <2% of patients.

Table 3 shows the Grade 3/4 hematologic and major non-hematologic adverse reactions in the topotecan/paclitaxel comparator trial in ovarian cancer.

Table 3. Adverse Reactions Experienced by ≥5% of Ovarian Cancer Patients Randomized to Receive HYCAMTIN or Paclitaxel

Adverse Reaction	HYCAMTIN (n = 112)	Paclitaxel (n = 114)
Hematologic Grade 3/4	%	%
Grade 4 neutropenia (<500 cells/mm ³)	80	21
Grade 3/4 anemia (Hgb <8 g/dL)	41	6
Grade 4 thrombocytopenia (<25,000 plts/mm ³)	27	3
Pyrexia/Grade 4 neutropenia	23	4
Non-hematologic Grade 3/4	%	%
Infections and infestations		
Documented sepsis ^a	5	2
Respiratory, thoracic, and mediastinal disorders		
Dyspnea	6	5
Gastrointestinal disorders		
Abdominal pain	5	4
Constipation	5	0
Diarrhea	6	1
Intestinal obstruction	5	4
Nausea	10	2
Vomiting	10	3
General disorders and administrative site conditions		
Fatigue	7	6
Asthenia	5	3
Pain ^b	5	7

^a Death related to sepsis occurred in 2% of patients receiving HYCAMTIN and 0% of patients receiving paclitaxel.

^b Pain includes body pain, skeletal pain, and back pain.

Table 4 shows the Grade 3/4 hematologic and major non-hematologic adverse reactions in the topotecan/CAV (cyclophosphamide-doxorubicin-vincristine) comparator trial in small cell lung cancer.

Table 4. Adverse Reactions Experienced by ≥5% of Small Cell Lung Cancer Patients Randomized to Receive HYCAMTIN or CAV

Adverse Reaction	HYCAMTIN (n = 107)	CAV (n = 104)
Hematologic Grade 3/4	%	%
Grade 4 neutropenia (<500 cells/mm ³)	70	72
Grade 3/4 anemia (Hgb <8 g/dL)	42	20
Grade 4 thrombocytopenia ($<25,000$ plts/mm ³)	29	5
Pyrexia/Grade 4 neutropenia	28	26
Non-hematologic Grade 3/4	%	%
Infections and infestations		
Documented sepsis ^a	5	5
Respiratory, thoracic, and mediastinal disorders		
Dyspnea	9	14
Pneumonia	8	6
Gastrointestinal disorders		
Abdominal pain	6	4
Nausea	8	6
General disorders and administrative site conditions		
Fatigue	6	10
Asthenia	9	7
Pain ^b	5	7

^a Death related to sepsis occurred in 3% of patients receiving HYCAMTIN, and 1% of patients receiving CAV.

^b Pain includes body pain, skeletal pain, and back pain.

Cervical Cancer: In the comparative trial with HYCAMTIN plus cisplatin versus cisplatin in patients with cervical cancer, the most common dose-limiting adverse reaction was myelosuppression. Table 5 shows the hematologic adverse reactions and Table 6 shows the non-hematologic adverse reactions in patients with cervical cancer.

Table 5. Hematologic Adverse Reactions in Patients with Cervical Cancer Treated with HYCAMTIN Plus Cisplatin or Cisplatin Monotherapy^a

Hematologic Adverse Reaction	HYCAMTIN Plus Cisplatin (n = 140)	Cisplatin (n = 144)
Anemia		
All Grades (Hgb <12 g/dL)	131 (94%)	130 (90%)
Grade 3 (Hgb <8-6.5 g/dL)	47 (34%)	28 (19%)
Grade 4 (Hgb <6.5 g/dL)	9 (6%)	5 (3%)
Leukopenia		
All Grades (<3,800 cells/mm ³)	128 (91%)	43 (30%)
Grade 3 (<2,000-1,000 cells/mm ³)	58 (41%)	1 (1%)
Grade 4 (<1,000 cells/mm ³)	35 (25%)	0 (0%)
Neutropenia		
All Grades (<2,000 cells/mm ³)	125 (89%)	28 (19%)
Grade 3 (<1,000-500 cells/mm ³)	36 (26%)	1 (1%)
Grade 4 (<500 cells/mm ³)	67 (48%)	1 (1%)
Thrombocytopenia		
All Grades (<130,000 cells/mm ³)	104 (74%)	21 (15%)
Grade 3 (<50,000-10,000 cells/mm ³)	36 (26%)	5 (3%)
Grade 4 (<10,000 cells/mm ³)	10 (7%)	0 (0%)

^a Includes patients who were eligible and treated.

Table 6. Non-hematologic Adverse Reactions Experienced by ≥5% of Patients with Cervical Cancer Treated with HYCAMTIN Plus Cisplatin or Cisplatin Monotherapy^a

Adverse Reaction	HYCAMTIN Plus Cisplatin (n = 140)			Cisplatin (n = 144)		
	All Grades ^b	Grade 3	Grade 4	All Grades ^b	Grade 3	Grade 4
General disorders and administrative site conditions						
Constitutional ^c	96 (69%)	11 (8%)	0	89 (62%)	17 (12%)	0
Pain ^d	82 (59%)	28 (20%)	3 (2%)	72 (50%)	18 (13%)	5 (3%)
Gastrointestinal disorders						
Vomiting	56 (40%)	20 (14%)	2 (1%)	53 (37%)	13 (9%)	0
Nausea	77 (55%)	18 (13%)	2 (1%)	79 (55%)	13 (9%)	0
Stomatitis-pharyngitis	8 (6%)	1 (<1%)	0	0	0	0
Other	88 (63%)	16 (11%)	4 (3%)	80 (56%)	12 (8%)	3 (2%)
Dermatology	67 (48%)	1 (<1%)	0	29 (20%)	0	0
Metabolic-Laboratory	55 (39%)	13 (9%)	7 (5%)	44 (31%)	14 (10%)	1 (<1%)
Genitourinary	51 (36%)	9 (6%)	9 (6%)	49 (34%)	7 (5%)	7 (5%)
Nervous system disorders						
Neuropathy	4 (3%)	1 (<1%)	0	3 (2%)	1 (<1%)	0
Other	49 (35%)	3 (2%)	1 (<1%)	43 (30%)	7 (5%)	2 (1%)
Infection-febrile neutropenia	39 (28%)	21 (15%)	5 (4%)	26 (18%)	11 (8%)	0
Cardiovascular	35 (25%)	7 (5%)	6 (4%)	22 (15%)	8 (6%)	3 (2%)
Hepatic	34 (24%)	5 (4%)	2 (1%)	23 (16%)	2 (1%)	0
Pulmonary	24 (17%)	4 (3%)	0	23 (16%)	5 (3%)	3 (2%)
Vascular disorders						
Hemorrhage	21 (15%)	8 (6%)	1 (<1%)	20 (14%)	3 (2%)	1 (<1%)
Coagulation	8 (6%)	4 (3%)	3 (2%)	10 (7%)	7 (5%)	0
Musculoskeletal	19 (14%)	3 (2%)	0	7 (5%)	1 (<1%)	1 (<1%)
Allergy-Immunology	8 (6%)	2 (1%)	1 (<1%)	4 (3%)	0	1 (<1%)
Endocrine	8 (6%)	0	0	4 (3%)	2 (1%)	0
Sexual reproduction function	7 (5%)	0	0	10 (7%)	1 (<1%)	0
Ocular-visual	7 (5%)	0	0	7 (5%)	1 (<1%)	0

Data were collected using NCI Common Toxicity Criteria, v. 2.0.

^a Includes patients who were eligible and treated.

^b Grades 1 through 4 only. There were 3 patients who experienced Grade 5 deaths with investigator-designated attribution. One was a Grade 5 hemorrhage in which the drug-related thrombocytopenia aggravated the event. A second patient experienced bowel obstruction, cardiac arrest, pleural effusion and respiratory failure which were not treatment related but probably aggravated by treatment. A third

patient experienced a pulmonary embolism and adult respiratory distress syndrome; the latter was indirectly treatment-related.

^c Constitutional includes fatigue (lethargy, malaise, asthenia), fever (in the absence of neutropenia), rigors, chills, sweating, and weight gain or loss.

^d Pain includes abdominal pain or cramping, arthralgia, bone pain, chest pain (non-cardiac and non-pleuritic), dysmenorrhea, dyspareunia, earache, headache, hepatic pain, myalgia, neuropathic pain, pain due to radiation, pelvic pain, pleuritic pain, rectal or perirectal pain, and tumor pain.

6.2 Postmarketing Experience

In addition to adverse reactions reported from clinical trials or listed in other sections of the prescribing information, the following reactions have been identified during postmarketing use of HYCAMTIN. Because they are reported voluntarily from a population of unknown size, estimates of frequency cannot be made. These reactions have been chosen for inclusion due to a combination of their seriousness, frequency of reporting, or potential causal connection to HYCAMTIN.

Blood and Lymphatic System Disorders: Severe bleeding (in association with thrombocytopenia) [see *Warnings and Precautions* (5.1)].

Immune System Disorders: Allergic manifestations, anaphylactoid reactions.

Gastrointestinal Disorders: Abdominal pain potentially associated with neutropenic colitis [see *Warnings and Precautions* (5.2)].

Pulmonary Disorders: Interstitial lung disease [see *Warnings and Precautions* (5.3)].

Skin and Subcutaneous Tissue Disorders: Angioedema, severe dermatitis, severe pruritus.

General Disorders and Administration Site Conditions: Inadvertent extravasation [see *Warnings and Precautions* (5.5)].

7 DRUG INTERACTIONS

G-CSF: Concomitant administration of G-CSF can prolong the duration of neutropenia, so if G-CSF is to be used, do not initiate it until day 6 of the course of therapy, 24 hours after completion of treatment with HYCAMTIN.

Platinum and Other Cytotoxic Agents: Myelosuppression was more severe when HYCAMTIN, at a dose of 1.25 mg/m²/day for 5 days, was given in combination with cisplatin at a dose of 50 mg/m² in Phase 1 trials. In one trial, 1 of 3 patients had severe neutropenia for 12 days and a second patient died with neutropenic sepsis.

Greater myelosuppression is also likely to be seen when HYCAMTIN is used in combination with other cytotoxic agents, thereby necessitating a dose reduction. However, when combining HYCAMTIN with platinum agents (e.g., cisplatin or carboplatin), a distinct sequence-dependent interaction on myelosuppression has been reported. Coadministration of a platinum agent on day 1 of dosing with HYCAMTIN required lower doses of each agent compared to coadministration on day 5 of the dosing schedule for HYCAMTIN.

For information on the pharmacokinetics, efficacy, safety, and dosing of HYCAMTIN at a dose of 0.75 mg/m²/day on days 1, 2, and 3 in combination with cisplatin 50 mg/m² on day 1 for cervical cancer, *see Dosage and Administration (2), Adverse Reactions (6), Clinical Pharmacology (12.3), and Clinical Studies (14).*

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category D [*see Warnings and Precautions (5.4)*].

HYCAMTIN can cause fetal harm when administered to a pregnant woman. In rabbits, a dose of 0.10 mg/kg/day (about equal to the clinical dose on a mg/m² basis) given on days 6 through 20 of gestation caused maternal toxicity, embryoletality, and reduced fetal body weight. In the rat, a dose of 0.23 mg/kg/day (about equal to the clinical dose on a mg/m² basis) given for 14 days before mating through gestation day 6 caused fetal resorption, microphthalmia, pre-implant loss, and mild maternal toxicity. A dose of 0.10 mg/kg/day (about half the clinical dose on a mg/m² basis) given to rats on days 6 through 17 of gestation caused an increase in post-implantation mortality. This dose also caused an increase in total fetal malformations. The most frequent malformations were of the eye (microphthalmia, anophthalmia, rosette formation of the retina, coloboma of the retina, ectopic orbit), brain (dilated lateral and third ventricles), skull, and vertebrae.

There are no adequate and well-controlled studies of HYCAMTIN in pregnant women. If this drug is used during pregnancy, or if a patient becomes pregnant while receiving HYCAMTIN, the patient should be apprised of the potential hazard to the fetus [*see Warnings and Precautions (5.4)*].

8.3 Nursing Mothers

Rats excrete high concentrations of topotecan into milk. Lactating female rats given 4.72 mg/m² IV (about twice the clinical dose on a mg/m² basis) excreted topotecan into milk at concentrations up to 48-fold higher than those in plasma. It is not known whether the drug is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from HYCAMTIN, discontinue breastfeeding when women are receiving HYCAMTIN.

8.4 Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

8.5 Geriatric Use

Of the 879 patients with metastatic ovarian cancer or small cell lung cancer in clinical trials of HYCAMTIN, 32% (n = 281) were 65 years of age and older, while 3.8% (n = 33) were 75 years of age and older. Of the 140 patients with Stage IV-B, relapsed, or refractory cervical cancer in clinical trials of HYCAMTIN who received HYCAMTIN plus cisplatin in the randomized clinical trial, 6% (n = 9) were 65 years of age and older, while 3% (n = 4) were 75 years of age and older. No overall differences in effectiveness or safety were observed between these patients and younger adult patients, and other reported clinical experience has not

identified differences in responses between the elderly and younger adult patients, but greater sensitivity of some older individuals cannot be ruled out.

There were no apparent differences in the pharmacokinetics of topotecan in elderly patients, once the age-related decrease in renal function was considered [see *Clinical Pharmacology* (12.3)].

This drug is known to be substantially excreted by the kidney, and the risk of toxic reactions to this drug may be greater in patients with impaired renal function. Because elderly patients are more likely to have decreased renal function, care should be taken in dose selection, and it may be useful to monitor renal function [see *Dosage and Administration* (2.3)].

8.6 Renal Impairment

No dosage adjustment of HYCAMTIN appears to be required for patients with mild renal impairment (Cl_{cr} 40 to 60 mL/min). Dosage reduction is recommended for patients with moderate renal impairment (Cl_{cr} 20 to 39 mL/min). Insufficient data are available in patients with severe renal impairment to provide a dosage recommendation for HYCAMTIN [see *Dosage and Administration* (2.3) and *Clinical Pharmacology* (12.3)].

10 OVERDOSAGE

Overdoses (up to 10-fold of the prescribed dose) occurred in patients treated with intravenous topotecan. The primary complication of overdose is bone marrow suppression. The observed signs and symptoms of overdose are consistent with the known adverse reactions associated with HYCAMTIN for intravenous use [see *Adverse Reactions* (6.1, 6.2)]. In addition, elevated hepatic enzymes and mucositis have been reported following overdose. One patient received a single dose of 40 mg/m² of intravenous topotecan and developed gastrointestinal toxicity, skin toxicity, and myelosuppression leading to septic shock. Another patient received a single dose of 35 mg/m² and experienced severe, reversible neutropenia.

There is no known antidote for overdose with HYCAMTIN. If an overdose is suspected, monitor the patient for bone marrow suppression and institute supportive-care measures (such as prophylactic G-CSF and antibiotic therapy) as appropriate.

11 DESCRIPTION

HYCAMTIN (topotecan hydrochloride) is a semi-synthetic derivative of camptothecin and is an anti-tumor drug with topoisomerase I-inhibitory activity.

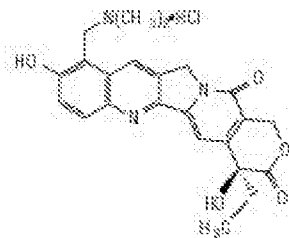
HYCAMTIN for Injection is supplied as a sterile, lyophilized, buffered, light yellow to greenish powder available in single-dose vials. Each vial contains topotecan hydrochloride equivalent to 4 mg of topotecan as free base. The reconstituted solution ranges in color from yellow to yellow-green and is intended for administration by intravenous infusion.

Inactive ingredients are mannitol, 48 mg, and tartaric acid, 20 mg. Hydrochloric acid and sodium hydroxide may be used to adjust the pH. The solution pH ranges from 2.5 to 3.5.

The chemical name for topotecan hydrochloride is (*S*)-10-[(dimethylamino)methyl]-4-ethyl-4,9-dihydroxy-1*H*-pyrano[3',4':6,7] indolizino [1,2-*b*]quinoline-3,14-(4*H*,12*H*)-dione

monohydrochloride. It has the molecular formula $C_{23}H_{23}N_3O_5 \cdot HCl$ and a molecular weight of 457.9.

Topotecan hydrochloride has the following structural formula:



It is soluble in water and melts with decomposition at 213° to 218°C.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Topoisomerase I relieves torsional strain in DNA by inducing reversible single-strand breaks. Topotecan binds to the topoisomerase I-DNA complex and prevents religation of these single-strand breaks. The cytotoxicity of topotecan is thought to be due to double-strand DNA damage produced during DNA synthesis, when replication enzymes interact with the ternary complex formed by topotecan, topoisomerase I, and DNA. Mammalian cells cannot efficiently repair these double-strand breaks.

12.2 Pharmacodynamics

The dose-limiting toxicity of topotecan is leukopenia. White blood cell count decreases with increasing topotecan dose or topotecan AUC. When topotecan is administered at a dose of 1.5 mg/m²/day for 5 days, an 80% to 90% decrease in white blood cell count at nadir is typically observed after the first cycle of therapy.

12.3 Pharmacokinetics

The pharmacokinetics of topotecan have been evaluated in cancer patients following doses of 0.5 to 1.5 mg/m² administered as a 30-minute infusion. Topotecan exhibits multiexponential pharmacokinetics with a terminal half-life of 2 to 3 hours. Total exposure (AUC) is approximately dose-proportional.

Distribution: Binding of topotecan to plasma proteins is about 35%.

Metabolism: Topotecan undergoes a reversible pH-dependent hydrolysis of its lactone moiety; it is the lactone form that is pharmacologically active. At pH ≤4, the lactone is exclusively present, whereas the ring-opened hydroxy-acid form predominates at physiologic pH. In vitro studies in human liver microsomes indicate topotecan is metabolized to an N-demethylated metabolite. The mean metabolite:parent AUC ratio was about 3% for total topotecan and topotecan lactone following IV administration.

Excretion: Renal clearance is an important determinant of topotecan elimination.

In a mass balance/excretion trial in 4 patients with solid tumors, the overall recovery of total topotecan and its N-desmethyl metabolite in urine and feces over 9 days averaged 73.4% ± 2.3% of the administered IV dose. Mean values of 50.8% ± 2.9% as total topotecan and

3.1% $\pm$ 1.0% as N-desmethyl topotecan were excreted in the urine following IV administration. Fecal elimination of total topotecan accounted for 17.9% $\pm$ 3.6% while fecal elimination of N-desmethyl topotecan was 1.7% $\pm$ 0.6%. An O-glucuronidation metabolite of topotecan and N-desmethyl topotecan has been identified in the urine. These metabolites, topotecan-O-glucuronide and N-desmethyl topotecan-O-glucuronide, were less than 2% of the administered dose.

Effect of Gender: The overall mean topotecan plasma clearance in male patients was approximately 24% higher than that in female patients, largely reflecting difference in body size.

Effect of Age: Topotecan pharmacokinetics have not been specifically studied in an elderly population, but population pharmacokinetic analysis in female patients did not identify age as a significant factor. Decreased renal clearance, which is common in the elderly, is a more important determinant of topotecan clearance [see *Dosage and Administration (2.3) and Use in Specific Populations (8.5)*].

Effect of Race: The effect of race on topotecan pharmacokinetics has not been studied.

Effect of Renal Impairment: In patients with mild renal impairment (creatinine clearance of 40 to 60 mL/min), topotecan plasma clearance was decreased to about 67% of the value in patients with normal renal function. In patients with moderate renal impairment (Cl_{cr} of 20 to 39 mL/min), topotecan plasma clearance was reduced to about 34% of the value in control patients, with an increase in half-life. Mean half-life, estimated in 3 patients with renal impairment, was about 5.0 hours. Dosage adjustment is recommended for these patients [see *Dosage and Administration (2.3)*].

Effect of Hepatic Impairment: Plasma clearance in patients with hepatic impairment (serum bilirubin levels between 1.7 and 15.0 mg/dL) was decreased to about 67% of the value in patients without hepatic impairment. Topotecan half-life increased slightly, from 2.0 hours to 2.5 hours, but these patients with hepatic impairment tolerated the usual recommended topotecan dosage regimen.

Drug Interactions: Pharmacokinetic trials of the interaction of topotecan with concomitantly administered medications have not been formally investigated.

In vitro inhibition studies using marker substrates known to be metabolized by human P450 CYP1A2, CYP2A6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E, CYP3A, or CYP4A or dihydropyrimidine dehydrogenase indicate that the activities of these enzymes were not altered by topotecan. Enzyme inhibition by topotecan has not been evaluated in vivo.

Cisplatin: Administration of cisplatin (60 or 75 mg/m² on day 1) before topotecan (0.75 mg/m²/day on days 1 to 5) in 9 patients with ovarian cancer had no significant effect on the C_{max} and AUC of total topotecan.

Topotecan had no effect on the pharmacokinetics of free platinum in 15 patients with ovarian cancer who were administered cisplatin 50 mg/m² (n = 9) or 75 mg/m² (n = 6) on day 2 after paclitaxel 110 mg/m² on day 1 before topotecan 0.3 mg/m² IV daily on days 2 to 6.

Topotecan had no effect on dose-normalized (60 mg/m²) C_{max} values of free platinum in 13 patients with ovarian cancer who were administered 60 mg/m² (n = 10) or 75 mg/m² (n = 3)

cisplatin on day 1 before topotecan 0.75 mg/m² IV daily on days 1 to 5.

No pharmacokinetic data are available following topotecan (0.75 mg/m²/day for 3 consecutive days) and cisplatin (50 mg/m²/day on day 1) in patients with cervical cancer.

Myelosuppression was more severe when HYCAMTIN was given in combination with cisplatin [see *Drug Interactions* (7)].

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity testing of topotecan has not been performed. Topotecan is known to be genotoxic to mammalian cells and is a probable carcinogen. Topotecan was mutagenic to L5178Y mouse lymphoma cells and clastogenic to cultured human lymphocytes with and without metabolic activation. It was also clastogenic to mouse bone marrow. Topotecan did not cause mutations in bacterial cells.

Topotecan given to female rats prior to mating at a dose of 1.4 mg/m² IV (about equal to the clinical dose on a mg/m² basis) caused superovulation possibly related to inhibition of follicular atresia. This dose given to pregnant female rats also caused increased pre-implantation loss. Studies in dogs given 0.4 mg/m² IV (about 1/4th the clinical dose on a mg/m² basis) of topotecan daily for a month suggest that treatment may cause an increase in the incidence of multinucleated spermatogonial giant cells in the testes. Topotecan may impair fertility in women and men.

14 CLINICAL STUDIES

14.1 Ovarian Cancer

HYCAMTIN was studied in 2 clinical trials of 223 patients given topotecan with metastatic ovarian carcinoma. All patients had disease that had recurred on, or was unresponsive to, a platinum-containing regimen. Patients in these 2 trials received an initial dose of 1.5 mg/m² given by intravenous infusion over 30 minutes for 5 consecutive days, starting on day 1 of a 21-day course.

One trial was a randomized trial of 112 patients treated with HYCAMTIN (1.5 mg/m²/day × 5 days starting on day 1 of a 21-day course) and 114 patients treated with paclitaxel (175 mg/m² over 3 hours on day 1 of a 21-day course). All patients had recurrent ovarian cancer after a platinum-containing regimen or had not responded to at least 1 prior platinum-containing regimen. Patients who did not respond to the trial therapy, or who progressed, could be given the alternative treatment.

Response rates, response duration, and time to progression are shown in Table 7.

436 **Table 7. Efficacy of HYCAMTIN Versus Paclitaxel in Ovarian Cancer**

Parameter	HYCAMTIN (n = 112)	Paclitaxel (n = 114)
Complete response rate	5%	3%
Partial response rate	16%	11%
Overall response rate	21%	14%
95% Confidence interval (<i>P</i> -value)	13 to 28% (0.20)	8 to 20%
Response duration ^a (weeks)	n = 23	n = 16
Median	25.9	21.6
95% Confidence interval	22.1 to 32.9	16.0 to 34.0
Hazard-ratio (HYCAMTIN:paclitaxel) (<i>P</i> -value)	0.78 (0.48)	
Time to progression (weeks)		
Median	18.9	14.7
95% Confidence interval	12.1 to 23.6	11.9 to 18.3
Hazard-ratio (HYCAMTIN:paclitaxel) (<i>P</i> -value)	0.76 (0.07)	
Survival (weeks)		
Median	63.0	53.0
95% Confidence interval	46.6 to 71.9	42.3 to 68.7
Hazard-ratio (HYCAMTIN:paclitaxel) (<i>P</i> -value)	0.97 (0.87)	

437 ^a The calculation for duration of response was based on the interval between first response and
438 time to progression.
439

440 The median time to response was 7.6 weeks (range: 3.1 to 21.7) with HYCAMTIN
441 compared with 6.0 weeks (range: 2.4 to 18.1) with paclitaxel. Consequently, the efficacy of
442 HYCAMTIN may not be achieved if patients are withdrawn from treatment prematurely.

443 In the crossover phase, 8 of 61 (13%) patients who received HYCAMTIN after paclitaxel
444 had a partial response and 5 of 49 (10%) patients who received paclitaxel after HYCAMTIN had
445 a response (2 complete responses).

446 HYCAMTIN was active in ovarian cancer patients who had developed resistance to
447 platinum-containing therapy, defined as tumor progression while on, or tumor relapse within
448 6 months after completion of, a platinum-containing regimen. One complete and 6 partial
449 responses were seen in 60 patients, for a response rate of 12%. In the same trial, there were no
450 complete responders and 4 partial responders on the paclitaxel arm, for a response rate of 7%.

HYCAMTIN was also studied in an open-label, non-comparative trial in 111 patients with recurrent ovarian cancer after treatment with a platinum-containing regimen, or who had not responded to 1 prior platinum-containing regimen. The response rate was 14% (95% CI: 7% to 20%). The median duration of response was 22 weeks (range: 4.6 to 41.9 weeks). The time to progression was 11.3 weeks (range: 0.7 to 72.1 weeks). The median survival was 67.9 weeks (range: 1.4 to 112.9 weeks).

14.2 Small Cell Lung Cancer

HYCAMTIN was studied in 426 patients with recurrent or progressive small cell lung cancer in 1 randomized, comparative trial and in 3 single-arm trials.

Randomized Comparative Trial: In a randomized, comparative, Phase 3 trial, 107 patients were treated with HYCAMTIN (1.5 mg/m²/day × 5 days starting on day 1 of a 21-day course) and 104 patients were treated with CAV (1,000 mg/m² cyclophosphamide, 45 mg/m² doxorubicin, 2 mg vincristine administered sequentially on day 1 of a 21-day course). All patients were considered sensitive to first-line chemotherapy (responders who then subsequently progressed ≥60 days after completion of first-line therapy). A total of 77% of patients treated with HYCAMTIN and 79% of patients treated with CAV received platinum/etoposide with or without other agents as first-line chemotherapy.

Response rates, response duration, time to progression, and survival are shown in Table 8.

Table 8. Efficacy of HYCAMTIN Versus CAV (cyclophosphamide-doxorubicin-vincristine) in Small Cell Lung Cancer Patients Sensitive to First-Line Chemotherapy

Parameter	HYCAMTIN (n = 107)	CAV (n = 104)
Complete response rate	0%	1%
Partial response rate	24%	17%
Overall response rate	24%	18%
Difference in overall response rates	6%	
95% Confidence interval of the difference	(-6 to 18%)	
Response duration ^a (weeks)	n = 26	n = 19
Median	14.4	15.3
95% Confidence interval	13.1 to 18.0	13.1 to 23.1
Hazard-ratio (HYCAMTIN:CAV) (95% CI) (P-value)	1.42 (0.73 to 2.76) (0.30)	
Time to progression (weeks)		
Median	13.3	12.3
95% Confidence interval	11.4 to 16.4	11.0 to 14.1
Hazard-ratio (HYCAMTIN:CAV) (95% CI) (P-value)	0.92 (0.69 to 1.22) (0.55)	
Survival (weeks)		
Median	25.0	24.7
95% Confidence interval	20.6 to 29.6	21.7 to 30.3
Hazard-ratio (HYCAMTIN:CAV) (95% CI) (P-value)	1.04 (0.78 to 1.39) (0.80)	

^a The calculation for duration of response was based on the interval between first response and time to progression.

The time to response was similar in both arms: HYCAMTIN median of 6 weeks (range: 2.4 to 15.7) versus CAV median 6 weeks (range: 5.1 to 18.1).

Changes on a disease-related symptom scale in patients who received HYCAMTIN or who received CAV are presented in Table 9. It should be noted that not all patients had all symptoms, nor did all patients respond to all questions. Each symptom was rated on a 4-category scale with an improvement defined as a change in 1 category from baseline sustained over 2 courses. Limitations in interpretation of the rating scale and responses preclude formal statistical analysis.

Table 9. Percentage of Patients With Symptom Improvement^a: HYCAMTIN Versus CAV in Patients With Small Cell Lung Cancer

Symptom	HYCAMTIN (n = 107)		CAV (n = 104)	
	n ^b	(%)	n ^b	(%)
Shortness of breath	68	(28)	61	(7)
Interference with daily activity	67	(27)	63	(11)
Fatigue	70	(23)	65	(9)
Hoarseness	40	(33)	38	(13)
Cough	69	(25)	61	(15)
Insomnia	57	(33)	53	(19)
Anorexia	56	(32)	57	(16)
Chest pain	44	(25)	41	(17)
Hemoptysis	15	(27)	12	(33)

^a Defined as improvement sustained over at least 2 courses compared with baseline.

^b Number of patients with baseline and at least 1 post-baseline assessment.

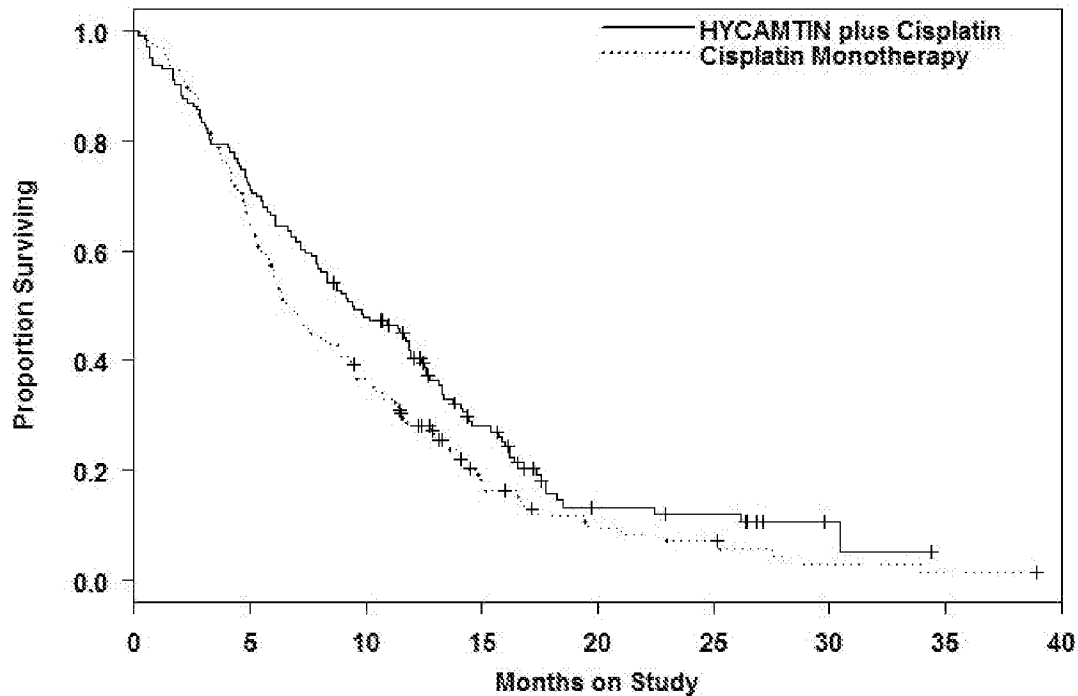
Single-Arm Trials: HYCAMTIN was also studied in 3 open-label, non-comparative trials in a total of 319 patients with recurrent or progressive small cell lung cancer after treatment with first-line chemotherapy. In all 3 trials, patients were stratified as either sensitive (responders who then subsequently progressed ≥ 90 days after completion of first-line therapy) or refractory (no response to first-line chemotherapy or who responded to first-line therapy and then progressed within 90 days of completing first-line therapy). Response rates ranged from 11% to 31% for sensitive patients and 2% to 7% for refractory patients. Median time to progression and median survival were similar in all 3 trials and the comparative trial.

14.3 Cervical Cancer

In a comparative trial, 147 eligible women were randomized to HYCAMTIN (0.75 mg/m²/day IV over 30 minutes $\times$ 3 consecutive days starting on day 1 of a 21-day course) plus cisplatin (50 mg/m² on day 1) and 146 eligible women were randomized to cisplatin (50 mg/m² IV on day 1 of a 21-day course). All patients had histologically confirmed Stage IV-B, recurrent, or persistent carcinoma of the cervix considered not amenable to curative treatment with surgery and/or radiation. Fifty-six percent (56%) of patients treated with HYCAMTIN plus cisplatin and 56% of patients treated with cisplatin had received prior cisplatin with or without other agents as first-line chemotherapy.

Median survival of eligible patients receiving HYCAMTIN plus cisplatin was 9.4 months (95% CI: 7.9 to 11.9) compared with 6.5 months (95% CI: 5.8 to 8.8) among patients randomized to cisplatin alone with a log rank *P*-value of 0.033 (significance level was 0.044 after adjusting for the interim analysis). The unadjusted hazard ratio for overall survival was 0.76 (95% CI: 0.59 to 0.98).

Figure 1. Overall Survival Curves Comparing HYCAMTIN plus Cisplatin versus Cisplatin Monotherapy in Cervical Cancer Patients



Number at Risk									
HYCAMTIN plus Cisplatin	147	104	68	32	16	8	2	0	0
Cisplatin Monotherapy	146	93	52	17	8	5	2	1	0

15 REFERENCES

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3. American Society of Health-System Pharmacists. ASHP Guidelines on Handling Hazardous Drugs. *Am J Health-Syst Pharm.* 2006;63:1172-1193.
4. Polovich M, White JM, Kelleher LO (eds.) 2005. Chemotherapy and Biotherapy Guidelines and Recommendations for Practice. (2nd ed) Pittsburgh, PA: Oncology Nursing Society.

16 HOW SUPPLIED/STORAGE AND HANDLING

HYCAMTIN for Injection is supplied in 4-mg (free base) single-dose vials.
NDC 0007-4201-01 (package of 1)

Storage: Store the vials protected from light in the original cartons at controlled room temperature between 20° and 25°C (68° and 77°F) [see USP].

17 PATIENT COUNSELING INFORMATION

17.1 Bone Marrow Suppression

Inform patients that HYCAMTIN decreases blood cell counts such as white blood cells, platelets, and red blood cells. Patients who develop fever, other signs of infection (e.g., chills, cough, or burning pain on urination), or bleeding while on therapy should notify their physician promptly. Inform patients that frequent blood tests will be performed while taking HYCAMTIN to monitor for the occurrence of bone marrow suppression.

17.2 Pregnancy and Breastfeeding

Advise patients to use effective contraceptive measures to prevent pregnancy and to avoid breastfeeding during treatment with HYCAMTIN.

17.3 Asthenia and Fatigue

Inform patients that HYCAMTIN may cause asthenia or fatigue. If these symptoms occur, caution should be observed when driving or operating machinery.

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GlaxoSmithKline

Research Triangle Park, NC 27709

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HYJ:xxPI

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use HYCAMTIN safely and effectively. See full prescribing information for HYCAMTIN.

HYCAMTIN (topotecan) for injection

Initial U.S. Approval: 1996

WARNING: BONE MARROW SUPPRESSION

See full prescribing information for complete boxed warning.

HYCAMTIN can cause severe myelosuppression. Administer only to patients with baseline neutrophil counts greater than or equal to 1,500 cells/mm³ and platelet count greater than or equal to 100,000/mm³. Monitor blood cell counts. (5.1)

RECENT MAJOR CHANGES

Contraindications, Bone Marrow Depression, removed (4) 06/2015

INDICATIONS AND USAGE

HYCAMTIN for injection is a topoisomerase inhibitor indicated for:

- metastatic carcinoma of the ovary after disease progression on or after initial or subsequent chemotherapy. (1.1)
- small cell lung cancer platinum-sensitive disease in patients who progressed after first-line chemotherapy. (1.2)
- combination therapy with cisplatin for Stage IV-B, recurrent, or persistent carcinoma of the cervix which is not amenable to curative treatment. (1.3)

DOSAGE AND ADMINISTRATION

- Ovarian cancer and small cell lung cancer: 1.5 mg/m² by intravenous infusion over 30 minutes daily for 5 consecutive days, starting on Day 1 of a 21-day course. (2.1, 2.2)
- Cervical cancer: 0.75 mg/m² by intravenous infusion over 30 minutes on Days 1, 2, and 3 repeated every 21 days in combination with cisplatin 50 mg/m² on Day 1. (2.3)
- Renal impairment: Dose reduce HYCAMTIN for injection in patients with moderate renal impairment (20 to 39 mL/min). (2.4)

DOSAGE FORMS AND STRENGTHS

4 mg (free base) lyophilized powder in single-use vial. (3)

CONTRAINDICATIONS

- History of severe hypersensitivity reactions to topotecan. (4)

WARNINGS AND PRECAUTIONS

- Bone marrow suppression: Administer HYCAMTIN only to patients with adequate bone marrow reserves. Monitor peripheral blood counts and adjust the dose as needed. (2.4, 5.1)

- Neutropenic enterocolitis: Fatal typhlitis can occur. (5.2)
- Interstitial lung disease (ILD): Fatal cases have occurred. Permanently discontinue for confirmed ILD. (5.3)
- Embryofetal toxicity: Can cause fetal harm. Advise women of potential risk to the fetus. (5.4, 8.1, 8.3)
- Extravasation and tissue injury: Severe cases have been reported. If extravasation occurs, immediately stop administration and institute recommended management procedures. (5.5)

ADVERSE REACTIONS

Ovarian cancer:

- The most common hematologic adverse reactions were: neutropenia (Grade 4: 80%), anemia (Grade 3/4: 41%), thrombocytopenia (Grade 4: 27%), and febrile neutropenia (23%). (6.1)
- The most common (>5%) non-hematologic adverse reactions (all grades) were: nausea, vomiting, fatigue, diarrhea, and dyspnea. (6.1)

Small cell lung cancer:

- The most common hematologic adverse reactions were: neutropenia (Grade 4: 70%), anemia (Grade 3/4: 42%), thrombocytopenia (Grade 4: 29%), and febrile neutropenia (28%).
- The most common (>5%) non-hematologic adverse reactions (all grades) were: asthenia, dyspnea, nausea, pneumonia, abdominal pain, and fatigue.

Cervical cancer (HYCAMTIN plus cisplatin):

- The most common hematologic adverse reactions were: neutropenia (Grade 3/4: 74%), anemia (Grade 3/4: 40%), and thrombocytopenia (Grade 3/4: 33%). (6.1)
- The most common (>25% and greater than or equal to 2% more than cisplatin alone) non-hematologic adverse reactions (all grades) were: pain, vomiting, and infection/febrile neutropenia. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact GlaxoSmithKline at 1-888-825-5249 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- Do not initiate G-CSF until 24 hours after completion of treatment with HYCAMTIN. Concomitant administration can prolong duration of neutropenia. (7)

USE IN SPECIFIC POPULATIONS

- Lactation: Discontinue breastfeeding. (8.2)

See 17 for PATIENT COUNSELING INFORMATION.

Revised: 06/2015

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FULL PRESCRIBING INFORMATION

WARNING: BONE MARROW SUPPRESSION

HYCAMTIN[®] can cause severe myelosuppression. Administer only to patients with baseline neutrophil counts of greater than or equal to 1,500 cells/mm³ and platelet counts greater than or equal to 100,000 cells/mm³. Monitor blood cell counts [see Warnings and Precautions (5.1)].

1 INDICATIONS AND USAGE

1.1 Ovarian Cancer

HYCAMTIN for injection, as a single agent, is indicated for the treatment of patients with metastatic carcinoma of the ovary after disease progression on or after initial or subsequent chemotherapy.

1.2 Small Cell Lung Cancer

HYCAMTIN for injection, as a single agent, is indicated for the treatment of patients with small cell lung cancer with platinum-sensitive disease who progressed at least 60 days after initiation of first-line chemotherapy.

1.3 Cervical Cancer

HYCAMTIN for injection in combination with cisplatin is indicated for the treatment of patients with Stage IV-B, recurrent, or persistent carcinoma of the cervix not amenable to curative treatment.

2 DOSAGE AND ADMINISTRATION

Verify dose using body surface area prior to dispensing. Recommended dosage should generally not exceed 4 mg intravenously [see *Overdosage (10)*].

2.1 Ovarian Cancer

Recommended Dose and Schedule

The recommended dose of HYCAMTIN is 1.5 mg/m² by intravenous infusion over 30 minutes daily for 5 consecutive days, starting on Day 1 of a 21-day course.

2.2 Small Cell Lung Cancer

Recommended Dose and Schedule

The recommended dose of HYCAMTIN is 1.5 mg/m² by intravenous infusion over 30 minutes daily for 5 consecutive days, starting on Day 1 of a 21-day course.

2.3 Cervical Cancer

Recommended Dose and Schedule

The recommended dose of HYCAMTIN is 0.75 mg/m² by intravenous infusion over 30 minutes daily on Days 1, 2, and 3 in combination with cisplatin 50 mg/m² on Day 1, repeated every 21 days.

2.4 Dose Modifications

Hematologic Toxicities

For single-agent use, dose reduce HYCAMTIN to 1.25 mg/m² for:

- neutrophil counts of less than 500 cells/mm³, or administer granulocyte-colony stimulating factor (G-CSF) starting no sooner than 24 hours following the last dose of HYCAMTIN.
- platelet counts less than 25,000 cells/mm³ during previous cycle.

For combination use with cisplatin, dose reduce HYCAMTIN to 0.60 mg/m² (and further to 0.45 mg/m² if necessary) for:

- febrile neutropenia (defined as neutrophil counts less than 1,000 cells/mm³ with temperature of greater than or equal to 38.0°C (100.4°F), or administer G-CSF starting no sooner than 24 hours following the last dose of HYCAMTIN.
- platelet counts less than 25,000 cells/mm³ during previous cycle.

Renal Impairment

For single-agent use, dose reduce HYCAMTIN to 0.75 mg/m² in patients with moderate renal impairment (creatinine clearance [Clcr] = 20 to 39 mL/min). Insufficient data are available in patients with severe renal impairment (Clcr less than 20 mL/min) to provide a dosage recommendation for HYCAMTIN [see Use in Specific Populations (8.6), Clinical Pharmacology (12.3)].

2.5 Preparation and Intravenous Administration

HYCAMTIN is a cytotoxic drug. Follow applicable special handling and disposable procedures.¹

Preparation and Administration

Reconstitute each 4-mg vial of HYCAMTIN with 4 mL Sterile Water for Injection, USP. Dilute the appropriate volume of the reconstituted solution in either 0.9% Sodium Chloride Intravenous Infusion, USP or 5% Dextrose in Water Injection, USP prior to administration.

Stability

Unopened vials of HYCAMTIN are stable until the date indicated on the package when stored between 20°C and 25°C (68°F and 77°F) [see USP] and protected from light in the original carton. Because the vials contain no preservative, contents should be used immediately after reconstitution.

Reconstituted vials of HYCAMTIN diluted for infusion are stable at approximately 20°C to 25°C (68°F to 77°F) and ambient lighting conditions for 24 hours.

3 DOSAGE FORMS AND STRENGTHS

For injection: 4 mg (topotecan free base) lyophilized powder in single-use vial for reconstitution; light yellow to greenish powder.

4 CONTRAINDICATIONS

HYCAMTIN is contraindicated in patients who have a history of severe hypersensitivity reactions to topotecan.

5 WARNINGS AND PRECAUTIONS

5.1 Bone Marrow Suppression

Bone marrow suppression (primarily neutropenia) is the dose-limiting toxicity of HYCAMTIN. Neutropenia is not cumulative over time. Severe myelotoxicity has been reported when HYCAMTIN is used in combination with cisplatin [see *Drug Interactions (7)*].

- The following data on myelosuppression are based on an integrated safety database from 8 trials (N = 879) using HYCAMTIN injection at 1.5 mg/m² by intravenous infusion over 30 minutes daily for 5 consecutive days, starting on Day 1 of a 21-day course in patients with ovarian cancer and small cell lung cancer and from one trial in patients with cervical cancer (N = 147) using HYCAMTIN 0.75 mg/m² by intravenous infusion over 30 minutes daily on Days 1, 2, and 3 repeated every 21 days in combination with cisplatin 50 mg/m² on Day 1.

Neutropenia

- Monotherapy: Grade 4 neutropenia (less than 500 cells/mm³) occurred in 78% of patients, with a median duration of 7 days and was most common during Course 1 of treatment (58% of patients). Grade 4 neutropenia associated with infection occurred in 13% of patients and febrile neutropenia occurred in 5% of patients. Sepsis occurred in 4% of patients and was fatal in 1% of patients. Pancytopenia has been reported.
- Combination with cisplatin: Grade 4 neutropenia occurred in 48% of patients.

Thrombocytopenia

- Monotherapy: Grade 4 thrombocytopenia (less than 25,000/mm³) occurred in 27% of patients, with a median duration of 5 days.
- Combination with cisplatin: Grade 4 thrombocytopenia occurred in 7% of patients.

Anemia

- Monotherapy: Grade 3 or 4 anemia (less than 8 g/dL) occurred in 37% of patients.
- Combination with cisplatin: Grade 3 or Grade 4 anemia occurred in 40% of patients.

Administer HYCAMTIN only to patients with a baseline neutrophil count of greater than or equal to 1,500 cells/mm³ and a platelet count greater than or equal to 100,000/mm³. Monitor peripheral blood counts frequently during treatment with HYCAMTIN. Refer to Section 2.4 for dose modification guidelines for hematological toxicities in subsequent courses. Do not treat patients with subsequent courses of HYCAMTIN until neutrophils recover to greater than 1,000 cells/mm³, platelets recover to greater than 100,000 cells/mm³, and hemoglobin levels recover to 9.0 g/dL (with transfusion if necessary).

5.2 Neutropenic Enterocolitis

Topotecan can cause fatal typhilitis (neutropenic enterocolitis). Consider the possibility of typhilitis in patients presenting with fever, neutropenia, and abdominal pain.

5.3 Interstitial Lung Disease

Interstitial lung disease (ILD), including fatalities, has occurred with HYCAMTIN. Underlying risk factors include history of ILD, pulmonary fibrosis, lung cancer, thoracic radiation, and use of pneumotoxic drugs and/or colony stimulating factors. Monitor patients for pulmonary symptoms indicative of ILD (e.g., cough, fever, dyspnea, and/or hypoxia), and discontinue HYCAMTIN if a new diagnosis of ILD is confirmed.

5.4 Embryofetal Toxicity

Based on animal data, HYCAMTIN can cause fetal harm when administered to a pregnant woman. Topotecan caused embryoletality, fetotoxicity, and teratogenicity in rats and rabbits when administered during organogenesis. Advise females of reproductive potential to use effective contraception during treatment and for at least 1 month after the last dose of HYCAMTIN. Advise women of the potential risk to a fetus [*see Use in Specific Populations (8.1, 8.3)*].

5.5 Extravasation and Tissue Injury

Extravasation with HYCAMTIN has been observed; severe cases have been reported. If signs or symptoms of extravasation occur, immediately stop administration of HYCAMTIN and institute recommended management procedures [*see Adverse Reactions (6)*].

6 ADVERSE REACTIONS

The following serious adverse reactions are described below and elsewhere in the labeling:

- Bone Marrow Suppression *[see Warnings and Precautions (5.1)]*
- Neutropenic Enterocolitis *[see Warnings and Precautions (5.2)]*
- Interstitial Lung Disease *[see Warnings and Precautions (5.3)]*
- Extravasation and Tissue Injury *[see Warnings and Precautions (5.5)]*

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared with rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Ovarian Cancer

Table 1 shows the Grade 3/4 hematologic and major non-hematologic adverse reactions in the topotecan/paclitaxel comparator trial in ovarian cancer.

Table 1. Adverse Reactions Experienced by ≥5% of Ovarian Cancer Patients Randomized to Receive HYCAMTIN or Paclitaxel

Adverse Reaction	HYCAMTIN (n = 112)	Paclitaxel (n = 114)
Hematologic Grade 3/4	%	%
Grade 4 neutropenia (<500 cells/mm ³)	80	21
Grade 3/4 anemia (Hgb <8 g/dL)	41	6
Grade 4 thrombocytopenia (<25,000 plts/mm ³)	27	3
Febrile neutropenia	23	4
Non-hematologic Grade 3/4	%	%
Infections and infestations		
Sepsis ^a	5	2
Respiratory, thoracic, and mediastinal disorders		
Dyspnea	6	5
Gastrointestinal disorders		
Abdominal pain	5	4
Constipation	5	0
Diarrhea	6	1
Intestinal obstruction	5	4
Nausea	10	2
Vomiting	10	3
General disorders and administrative site conditions		
Fatigue	7	6
Asthenia	5	3
Pain ^b	5	7

^a Death related to sepsis occurred in 2% of patients receiving HYCAMTIN and 0% of patients receiving paclitaxel.

^b Pain includes body pain, skeletal pain, and back pain.

Small Cell Lung Cancer

Table 2 shows the Grade 3/4 hematologic and major non-hematologic adverse reactions in the topotecan/CAV (cyclophosphamide-doxorubicin-vincristine) comparator trial in small cell lung cancer.

Table 2. Adverse Reactions Experienced by ≥5% of Small Cell Lung Cancer Patients Randomized to Receive HYCAMTIN or CAV

Adverse Reaction	HYCAMTIN (n = 107)	CAV (n = 104)
Hematologic Grade 3/4	%	%
Grade 4 neutropenia (<500 cells/mm ³)	70	72
Grade 3/4 anemia (Hgb <8 g/dL)	42	20
Grade 4 thrombocytopenia ($<25,000$ plts/mm ³)	29	5
Febrile neutropenia	28	26
Non-hematologic Grade 3/4	%	%
Infections and infestations		
Sepsis ^a	5	5
Respiratory, thoracic, and mediastinal disorders		
Dyspnea	9	14
Pneumonia	8	6
Gastrointestinal disorders		
Abdominal pain	6	4
Nausea	8	6
General disorders and administrative site conditions		
Fatigue	6	10
Asthenia	9	7
Pain ^b	5	7

^a Death related to sepsis occurred in 3% of patients receiving HYCAMTIN and 1% of patients receiving CAV.

^b Pain includes body pain, skeletal pain, and back pain.

Hepatobiliary Disorders in Ovarian and Small Cell Lung Cancer Patients Receiving HYCAMTIN: Based on the combined experience of 453 patients with metastatic ovarian carcinoma, and 426 patients with small cell lung cancer treated with HYCAMTIN, Grade 1 transient elevations in hepatic enzymes occurred in 8% of patients. Grade 3/4 elevations occurred in 4%. Grade 3/4 elevated bilirubin occurred in less than 2% of patients.

Cervical Cancer

In the comparative trial with HYCAMTIN plus cisplatin versus cisplatin in patients with cervical cancer, the most common dose-limiting adverse reaction was myelosuppression. Table 3 shows the hematologic and non-hematologic adverse reactions in patients with cervical cancer.

Table 3. Adverse Reactions Experienced by $\geq 5\%$ of Patients with Cervical Cancer Randomized to Receive HYCAMTIN plus Cisplatin or Cisplatin Monotherapy (Between-Arm Difference $\geq 2\%$)^a

Adverse Reaction	HYCAMTIN plus Cisplatin (n = 140) %	Cisplatin (n = 144) %
Hematologic		
Neutropenia		
Grade 3 (<1,000-500 cells/mm ³)	26	1
Grade 4 (<500 cells/mm ³)	48	1
Anemia		
Grade 3 (Hgb <8-6.5 g/dL)	34	19
Grade 4 (Hgb <6.5 g/dL)	6	3
Thrombocytopenia		
Grade 3 (<50,000-10,000 cells/mm ³)	26	3
Grade 4 (<10,000 cells/mm ³)	7	0
Non-hematologic^{b,c}		
General disorders and administrative site conditions		
Constitutional ^d	69	62
Pain ^e	59	50
Gastrointestinal disorders		
Vomiting	40	37
Stomatitis-pharyngitis	6	0
Other	63	56
Dermatology^f	48	20
Infection-febrile neutropenia^f	28	18
Cardiovascular^f	25	15

^a Includes patients who were eligible and treated.

^b Data were collected using NCI Common Toxicity Criteria, v. 2.0.

^c Grades 1 through 4 only. There were 3 patients who experienced deaths with investigator-designated attribution. The first patient experienced a Grade 5 hemorrhage in which the drug-related thrombocytopenia aggravated the event. A second patient experienced bowel obstruction, cardiac arrest, pleural effusion, and respiratory failure which were not treatment-related but probably aggravated by treatment. A third patient experienced a pulmonary embolism and adult respiratory distress syndrome; the latter was indirectly treatment-related.

^d Constitutional includes fatigue (lethargy, malaise, asthenia), fever (in the absence of neutropenia), rigors, chills, sweating, and weight gain or loss.

^e Pain includes abdominal pain or cramping, arthralgia, bone pain, chest pain (non-cardiac and non-pleuritic), dysmenorrhea, dyspareunia, earache, headache, hepatic pain, myalgia, neuropathic pain, pain due to radiation, pelvic pain, pleuritic pain, rectal or perirectal pain, and tumor pain.

^f High-level terms were included if the between-arm difference was $\geq 10\%$.

6.2 Postmarketing Experience

The following reactions have been identified during postmarketing use of HYCAMTIN. Because they are reported voluntarily from a population of unknown size, estimates of frequency cannot be made. These reactions have been chosen for inclusion due to a combination of their seriousness, frequency of reporting, or potential causal connection to HYCAMTIN.

Blood and Lymphatic System Disorders

Severe bleeding (in association with thrombocytopenia) [*see Warnings and Precautions (5.1)*].

Immune System Disorders

Allergic manifestations, anaphylactoid reactions.

Gastrointestinal Disorders

Abdominal pain potentially associated with neutropenic enterocolitis [*see Warnings and Precautions (5.2)*].

Pulmonary Disorders

Interstitial lung disease [*see Warnings and Precautions (5.3)*].

Skin and Subcutaneous Tissue Disorders

Angioedema, severe dermatitis, severe pruritus.

General Disorders and Administration Site Conditions

Extravasation [*see Warnings and Precautions (5.5)*].

7 DRUG INTERACTIONS

7.1 G-CSF

Concomitant administration with G-CSF can prolong the duration of neutropenia. If G-CSF is used, it should be started no sooner than 24 hours following the last dose of HYCAMTIN.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

Based on animal data, HYCAMTIN can cause fetal harm when administered to a pregnant woman. Topotecan caused embryoletality, fetotoxicity, and teratogenicity in rats and rabbits when administered during organogenesis at doses similar to the clinical dose [*see Data*]. There are no available human data informing the drug-associated risk. Advise pregnant women of the potential risk to a fetus. The background risk of major birth defects and miscarriage for the

indicated populations are unknown; however, the background risk in the US general population of major birth defects is 2% to 4% and of miscarriage is 15% to 20% of clinically recognized pregnancies.

Data

Animal Data: In rabbits, a dose of 0.10 mg/kg/day (about equal to the clinical dose on a mg/m² basis) given on Days 6 through 20 of gestation caused maternal toxicity, embryoletality, and reduced fetal body weight. In the rat, a dose of 0.23 mg/kg/day (about equal to the clinical dose on a mg/m² basis) given for 14 days before mating through gestation Day 6 caused fetal resorption, microphthalmia, pre-implant loss, and mild maternal toxicity. Administration of an intravenous dose of 0.10 mg/kg/day (about half the clinical dose on a mg/m² basis) given to rats on Days 6 through 17 of gestation caused an increase in post-implantation mortality. This dose also caused an increase in total fetal malformations. The most frequent malformations were of the eye (microphthalmia, anophthalmia, rosette formation of the retina, coloboma of the retina, ectopic orbit), brain (dilated lateral and third ventricles), skull, and vertebrae.

8.2 Lactation

Risk Summary

It is not known whether this drug is present in human milk; however, topotecan is excreted in rat milk at high concentrations [see *Data*]. Because many drugs are present in human milk and because of the potential for serious adverse reactions in nursing infants with HYCAMTIN, advise nursing mothers to discontinue breastfeeding during treatment with HYCAMTIN.

Data

Animal Data: Following intravenous administration of topotecan to lactating rats at a dose of 4.72 mg/m² (about twice the clinical dose on a mg/m² basis), topotecan was excreted into milk at concentrations up to 48-fold higher than those in plasma.

8.3 Females and Males of Reproductive Potential

Contraception

Females: Advise female patients of reproductive potential to use effective contraception during treatment with HYCAMTIN and for one month after the last dose. Advise females to contact their healthcare provider if they become pregnant, or if pregnancy is suspected, while taking HYCAMTIN [see *Use in Specific Populations* (8.1)].

Males: HYCAMTIN may damage spermatozoa, resulting in possible genetic and fetal abnormalities. Advise males with a female sexual partner of reproductive potential to use effective contraception during and for three months after treatment with HYCAMTIN [see *Nonclinical Toxicology* (13.1)].

Infertility

Females: HYCAMTIN may have both acute and long-term effects on fertility [see *Nonclinical Toxicology* (13.1)].

Males: Effects on spermatogenesis have been observed in animals administered HYCAMTIN. Advise males of the potential risk for impaired fertility and to seek counseling on fertility and family planning options prior to starting treatment [see *Nonclinical Toxicology* (13.1)].

8.4 Pediatric Use

Safety and effectiveness in pediatric patients have not been established.

8.5 Geriatric Use

Of the 879 patients with metastatic ovarian cancer or small cell lung cancer in clinical trials of HYCAMTIN, 32% (n = 281) were aged 65 years and older, while 3.8% (n = 33) were aged 75 years and older. Of the 140 patients with Stage IV-B, relapsed, or refractory cervical cancer in clinical trials of HYCAMTIN who received HYCAMTIN plus cisplatin in the randomized clinical trial, 6% (n = 9) were aged 65 years and older, while 3% (n = 4) were aged 75 years and older.

No overall differences in effectiveness or safety were observed between these patients and younger adult patients, and other reported clinical experience has not identified differences in responses between the elderly and younger adult patients.

8.6 Renal Impairment

The systemic exposure to both topotecan lactone and total topotecan increased in patients with moderate renal impairment (Cl_{cr} = 20 to 39 mL/min) compared with patients with normal renal function (Cl_{cr} greater than 60 mL/min). Reduce the dose of HYCAMTIN in patients with moderate renal impairment (Cl_{cr} = 20 to 39 mL/min). No dosage adjustment of HYCAMTIN is recommended for patients with mild renal impairment (Cl_{cr} = 40 to 60 mL/min) [see *Dosage and Administration* (2.4), *Clinical Pharmacology* (12.3)].

Insufficient data are available in patients with severe renal impairment (Cl_{cr} less than 20 mL/min) to provide a dosage recommendation for HYCAMTIN.

10 OVERDOSAGE

Overdoses (up to 10-fold of the prescribed dose) occurred in patients treated with intravenous topotecan. The primary complication of overdose is bone marrow suppression. The observed signs and symptoms of overdose are consistent with the known adverse reactions associated with HYCAMTIN for intravenous use [see *Adverse Reactions* (6.1, 6.2)]. In addition, elevated hepatic enzymes and mucositis have been reported following overdose. One patient received a single dose of 40 mg/m² of intravenous topotecan and developed gastrointestinal toxicity, skin toxicity, and myelosuppression leading to septic shock. Another patient received a single dose of

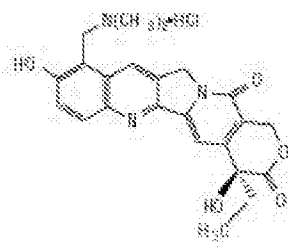
35 mg/m² and experienced severe, reversible neutropenia.

There is no known antidote for overdosage with HYCAMTIN. If an overdose is suspected, monitor the patient closely for bone marrow suppression and institute supportive-care measures (such as the prophylactic use of G-CSF and antibiotic therapy) as appropriate.

11 DESCRIPTION

HYCAMTIN (topotecan) is a semi-synthetic derivative of camptothecin and is an anti-tumor drug with topoisomerase I-inhibitory activity. The chemical name for topotecan hydrochloride is (S)-10-[(dimethylamino)methyl]-4-ethyl-4,9-dihydroxy-1*H*-pyrano[3',4':6,7]indolizino [1,2-*b*]quinoline-3,14-(4*H*,12*H*)-dione monohydrochloride. It has the molecular formula C₂₃H₂₃N₃O₅•HCl and a molecular weight of 457.9. It is soluble in water and melts with decomposition at 213°C to 218°C.

Topotecan hydrochloride has the following structural formula:



HYCAMTIN for injection is supplied as a sterile, lyophilized, buffered, light yellow to greenish powder available in single-dose vials. Each vial contains topotecan hydrochloride equivalent to 4 mg of topotecan as free base. The reconstituted solution ranges in color from yellow to yellow-green and is intended for administration by intravenous infusion.

Inactive ingredients are mannitol, 48 mg, and tartaric acid, 20 mg. Hydrochloric acid and sodium hydroxide may be used to adjust the pH. The solution pH ranges from 2.5 to 3.5.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Topoisomerase I relieves torsional strain in DNA by inducing reversible single-strand breaks. Topotecan binds to the topoisomerase I-DNA complex and prevents re-ligation of these single-strand breaks. The cytotoxicity of topotecan is thought to be due to double-strand DNA damage produced during DNA synthesis, when replication enzymes interact with the ternary complex formed by topotecan, topoisomerase I, and DNA. Mammalian cells cannot efficiently repair these double-strand breaks.

12.3 Pharmacokinetics

Following administration of HYCAMTIN for injection at doses of 0.5 to 1.5 mg/m² administered

as a 30-minute infusion to cancer patients, topotecan exhibited multiexponential pharmacokinetics with a terminal half-life of 2 to 3 hours. Total exposure (AUC) is approximately dose-proportional.

Distribution

Binding of topotecan to plasma proteins is approximately 35%.

Metabolism

Topotecan undergoes a reversible pH-dependent hydrolysis of its lactone moiety; it is the lactone form that is pharmacologically active. At pH ≤ 4 , the lactone is exclusively present, whereas the ring-opened hydroxy-acid form predominates at physiologic pH. *In vitro* studies in human liver microsomes indicate topotecan is metabolized to an N-demethylated metabolite. The mean metabolite:parent AUC ratio was about 3% for total topotecan and topotecan lactone following IV administration.

Excretion

Renal clearance is the primary route of topotecan elimination.

In a mass balance/excretion trial in 4 patients with solid tumors, the overall recovery of total topotecan and its N-desmethyl metabolite in urine and feces over 9 days averaged $73.4\% \pm 2.3\%$ of the administered IV dose. Mean values of $50.8\% \pm 2.9\%$ as total topotecan and $3.1\% \pm 1.0\%$ as N-desmethyl topotecan were excreted in the urine following IV administration. Fecal elimination of total topotecan accounted for $17.9\% \pm 3.6\%$ while fecal elimination of N-desmethyl topotecan was $1.7\% \pm 0.6\%$. An O-glucuronidation metabolite of topotecan and N-desmethyl topotecan has been identified in the urine.

Specific Populations

Gender: Plasma clearance of topotecan lactone in male patients was approximately 24% higher than that in female patients, largely reflecting difference in body size.

Age: Population pharmacokinetic analysis in female patients did not identify age as a significant factor. Decreased renal clearance, which is common in the elderly, is a more important determinant of topotecan clearance [see *Dosage and Administration* (2.4), *Use in Specific Populations* (8.5)].

Renal Impairment: In patients with mild renal impairment (Clcr = 40 to 60 mL/min), plasma clearance of topotecan lactone was decreased by 33% compared with patients with normal renal function (Clcr greater than 60 mL/min). In patients with moderate renal impairment (Clcr = 20 to 39 mL/min), plasma clearance of topotecan lactone was reduced by 65% compared with patients with normal renal function. Dosage adjustment is recommended for patients with moderate renal impairment. No dosage adjustment is required in patients with mild renal impairment [see *Dosage and Administration* (2.4), *Use in Specific Populations* (8.6)].

Hepatic Impairment: Plasma clearance of topotecan lactone in patients with hepatic impairment

serum bilirubin levels between 1.7 and 15.0 mg/dL) was decreased by 33% compared with patients with normal hepatic function (serum bilirubin levels less than 1.7 mg/dL).

Drug Interactions

Effects of Topotecan on Drug-Metabolizing Enzymes: *In vitro* inhibition studies using marker substrates for human P450 CYP1A2, CYP2A6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E, CYP3A, or CYP4A or dihydropyrimidine dehydrogenase indicate that the activities of these enzymes were not altered by topotecan.

Cisplatin: Administration of cisplatin (60 or 75 mg/m² on Day 1) before topotecan (0.75 mg/m²/day on Days 1 to 5) in 9 patients with ovarian cancer had no significant effect on the C_{max} and AUC of total topotecan.

Topotecan (0.3 mg/m² IV daily on Days 2 to 6) had no effect on the pharmacokinetics of free platinum in 15 patients with ovarian cancer who were administered cisplatin 50 mg/m² (n = 9) or 75 mg/m² (n = 6) on Day 2 after paclitaxel 110 mg/m² on Day 1. Topotecan (0.75 mg/m² IV daily on Days 1 to 5) had no effect on dose-normalized (60 mg/m²) C_{max} values of free platinum in 13 patients with ovarian cancer who were administered 60 mg/m² (n = 10) or 75 mg/m² (n = 3) cisplatin on Day 1.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity testing of topotecan has not been performed. Topotecan is known to be genotoxic to mammalian cells and is a probable carcinogen. Topotecan was mutagenic to L5178Y mouse lymphoma cells and clastogenic to cultured human lymphocytes with and without metabolic activation. It was also clastogenic to mouse bone marrow. Topotecan did not cause mutations in bacterial cells.

Topotecan given to female rats prior to mating at a dose of 1.4 mg/m² IV (about equal to the clinical dose on a mg/m² basis) caused superovulation possibly related to inhibition of follicular atresia. This dose given to pregnant female rats also caused increased pre-implantation loss. Studies in dogs given 0.4 mg/m² IV (about 0.25 times the clinical dose on a mg/m² basis) of topotecan daily for a month suggest that treatment may cause an increase in the incidence of multinucleated spermatogonial giant cells in the testes. Topotecan may impair fertility in women and men.

14 CLINICAL STUDIES

14.1 Ovarian Cancer

HYCAMTIN was studied in 2 clinical trials of 223 patients given topotecan with metastatic ovarian carcinoma. All patients had disease that had recurred on, or was unresponsive to, a platinum-containing regimen. Patients in these 2 trials received an initial dose of 1.5 mg/m²

given by intravenous infusion over 30 minutes for 5 consecutive days, starting on Day 1 of a 21-day course.

One trial was a randomized trial of 112 patients treated with HYCAMTIN (1.5 mg/m²/day × 5 days starting on Day 1 of a 21-day course) and 114 patients treated with paclitaxel (175 mg/m² over 3 hours on Day 1 of a 21-day course). All patients had recurrent ovarian cancer after a platinum-containing regimen or had not responded to at least 1 prior platinum-containing regimen. Patients who did not respond to the trial therapy, or who progressed, could be given the alternative treatment. The efficacy outcome measures were response rate, response duration, and time to progression.

The results of the trial did not show statistically significant improvements in response rates, response duration, time to progression, and overall survival as shown in Table 4.

Table 4. Efficacy of HYCAMTIN versus Paclitaxel in Ovarian Cancer

Parameter	HYCAMTIN (n = 112)	Paclitaxel (n = 114)
Overall response rate (95% CI)	21% (13% to 28%)	14% (8% to 20%)
Complete response rate	5%	3%
Partial response rate	16%	11%
Response duration ^a (months)		
Median (95% CI)	6.0 (5.1 to 7.6)	5.0 (3.7 to 7.8)
Time to progression (months)		
Median (95% CI)	4.4 (2.8 to 5.4)	3.4 (2.7 to 4.2)
HR (HYCAMTIN:paclitaxel) (95% CI)	0.76 (0.57 to 1.02)	
Survival (months)		
Median (95% CI)	14.5 (10.7 to 16.5)	12.2 (9.7 to 15.8)
HR (HYCAMTIN:paclitaxel) (95% CI)	0.97 (0.71 to 1.34)	

HR = hazard-ratio; CI = confidence interval.

^a The calculation for duration of response was based on the interval between first response and time to progression.

The median time to response was 7.6 weeks (range: 3.1 to 21.7) with HYCAMTIN compared with 6.0 weeks (range: 2.4 to 18.1) with paclitaxel. In the crossover phase, 8 of 61 (13%) patients who received HYCAMTIN after paclitaxel had a partial response and 5 of 49 (10%) patients who received paclitaxel after HYCAMTIN had a response (2 complete responses).

HYCAMTIN was active in ovarian cancer patients who had developed resistance to platinum-containing therapy, defined as tumor progression while on, or tumor relapse within 6 months after completion of, a platinum-containing regimen. One complete and 6 partial responses were seen in 60 patients, for a response rate of 12%. In the same trial, there were no

complete responders and 4 partial responders on the paclitaxel arm, for a response rate of 7%.

HYCAMTIN was also studied in an open-label, non-comparative trial in 111 patients with recurrent ovarian cancer after treatment with a platinum-containing regimen, or who had not responded to 1 prior platinum-containing regimen. The response rate was 14% (95% CI: 7% to 20%). The median duration of response was 22 weeks (range: 4.6 to 41.9 weeks). The time to progression was 11.3 weeks (range: 0.7 to 72.1 weeks). The median survival was 67.9 weeks (range: 1.4 to 112.9 weeks).

14.2 Small Cell Lung Cancer

HYCAMTIN was studied in 426 patients with recurrent or progressive small cell lung cancer in 1 randomized, comparative trial and in 3 single-arm trials.

Randomized Comparative Trial

In a randomized, comparative, Phase 3 trial, 107 patients were treated with HYCAMTIN (1.5 mg/m²/day × 5 days starting on Day 1 of a 21-day course) and 104 patients were treated with CAV (1,000 mg/m² cyclophosphamide, 45 mg/m² doxorubicin, 2 mg vincristine administered sequentially on Day 1 of a 21-day course). All patients were considered sensitive to first-line chemotherapy (responders who then subsequently progressed greater than or equal to 60 days after completion of first-line therapy). A total of 77% of patients treated with HYCAMTIN and 79% of patients treated with CAV received platinum/etoposide with or without other agents as first-line chemotherapy. The efficacy outcome measures were response rate and duration of response.

The results of the trial did not show statistically significant improvements in response rates, response duration, time to progression, and overall survival as shown in Table 5.

Table 5. Efficacy of HYCAMTIN versus CAV (cyclophosphamide-doxorubicin-vincristine) in Small Cell Lung Cancer Patients Sensitive to First-Line Chemotherapy

Parameter	HYCAMTIN (n = 107)	CAV (n = 104)
Overall response rate (95% CI)	24% (16% to 32%)	18% (11% to 26%)
Complete response rate	0%	1%
Partial response rate	24%	17%
Response duration ^a (months) Median (95% CI)	3.3 (3.0 to 4.1)	3.5 (3.0 to 5.3)
Time to progression (months) Median (95% CI)	3.1 (2.6 to 4.1)	2.8 (2.5 to 3.2)
HR (HYCAMTIN:CAV) (95% CI)	0.92 (0.69 to 1.22)	
Survival (months) Median (95% CI)	5.8 (4.7 to 6.8)	5.7 (5.0 to 7.0)
HR (HYCAMTIN:CAV) (95% CI)	1.04 (0.78 to 1.39)	

HR = hazard-ratio; CI = confidence interval.

^a The calculation for duration of response was based on the interval between first response and time to progression.

The time to response was similar in both arms: HYCAMTIN median of 6 weeks (range: 2.4 to 15.7) versus CAV median 6 weeks (range: 5.1 to 18.1).

Changes on a disease-related symptom scale in patients who received HYCAMTIN or who received CAV are presented in Table 6. It should be noted that not all patients had all symptoms, nor did all patients respond to all questions. Each symptom was rated on a 4-category scale with an improvement defined as a change in 1 category from baseline sustained over 2 courses. Limitations in interpretation of the rating scale and responses preclude formal statistical analysis.

Table 6. Percentage of Patients with Symptom Improvement^a: HYCAMTIN versus CAV in Patients with Small Cell Lung Cancer

Symptom	HYCAMTIN (n = 107)		CAV (n = 104)	
	n ^b	(%)	n ^b	(%)
Shortness of breath	68	(28)	61	(7)
Interference with daily activity	67	(27)	63	(11)
Fatigue	70	(23)	65	(9)
Hoarseness	40	(33)	38	(13)
Cough	69	(25)	61	(15)
Insomnia	57	(33)	53	(19)
Anorexia	56	(32)	57	(16)
Chest pain	44	(25)	41	(17)
Hemoptysis	15	(27)	12	(33)

^a Defined as improvement sustained over at least 2 courses compared with baseline.

^b Number of patients with baseline and at least 1 post-baseline assessment.

Single-Arm Trials

HYCAMTIN was also studied in 3 open-label, non-comparative trials in a total of 319 patients with recurrent or progressive small cell lung cancer after treatment with first-line chemotherapy. In all 3 trials, patients were stratified as either sensitive (responders who then subsequently progressed greater than or equal to 90 days after completion of first-line therapy) or refractory (no response to first-line chemotherapy or who responded to first-line therapy and then progressed within 90 days of completing first-line therapy). Response rates ranged from 11% to 31% for sensitive patients and 2% to 7% for refractory patients. Median time to progression and median survival were similar in all 3 trials and the comparative trial.

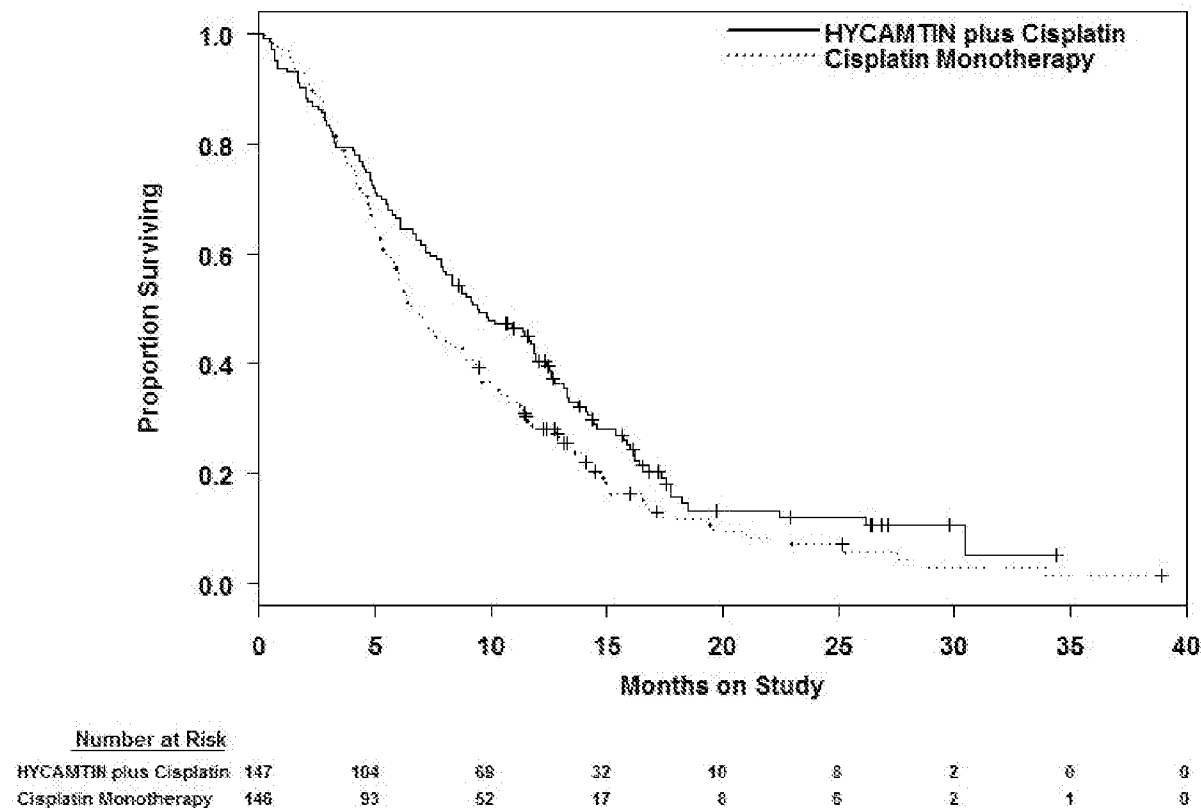
14.3 Cervical Cancer

In a comparative trial, 147 eligible women were randomized to HYCAMTIN (0.75 mg/m²/day IV over 30 minutes × 3 consecutive days starting on Day 1 of a 21-day course) plus cisplatin (50 mg/m² on Day 1) and 146 eligible women were randomized to cisplatin (50 mg/m² IV on Day 1 of a 21-day course). All patients had histologically confirmed Stage IV-B, recurrent, or persistent carcinoma of the cervix considered not amenable to curative treatment with surgery and/or radiation. Fifty-six percent (56%) of patients treated with HYCAMTIN plus cisplatin and 56% of patients treated with cisplatin had received prior cisplatin with or without other agents as first-line chemotherapy.

Median survival of eligible patients receiving HYCAMTIN plus cisplatin was 9.4 months (95% CI: 7.9 to 11.9) compared with 6.5 months (95% CI: 5.8 to 8.8) among patients randomized to cisplatin alone with a log rank *P*-value of 0.033 (significance level was 0.044 after adjusting for

the interim analysis). The unadjusted hazard ratio for overall survival was 0.76 (95% CI: 0.59 to 0.98).

Figure 1. Overall Survival Curves Comparing HYCAMTIN plus Cisplatin versus Cisplatin Monotherapy in Cervical Cancer Patients



15 REFERENCES

1. "OSHA Hazardous Drugs." OSHA. <http://www.osha.gov/SLTC/hazardousdrugs/index.html>.

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

HYCAMTIN for injection is supplied as a sterile, lyophilized, buffered, light yellow to greenish powder. HYCAMTIN for Injection is supplied in 4-mg (free base) single-dose vials.

NDC 0007-4201-01 (package of 1)

16.2 Storage and Handling

Store at controlled room temperature between 20°C and 25°C (68°F and 77°F) [see USP].

Protect from light in original carton. Handle and dispose of HYCAMTIN for injection consistent with recommendations for the handling and disposal of hazardous drugs¹.

17 PATIENT COUNSELING INFORMATION

- **Bone Marrow Suppression**

Inform patients that HYCAMTIN decreases blood cell counts such as white blood cells, platelets, and red blood cells. Advise patients to notify their healthcare provider promptly for fever, other signs of infection (e.g., chills, cough, or burning pain on urination), or bleeding. Inform patients that frequent blood tests will be performed while taking HYCAMTIN to monitor for the occurrence of bone marrow suppression.

- **Embryofetal Toxicity**

Advise patients to contact their healthcare provider if they become pregnant, or if pregnancy is suspected during treatment with HYCAMTIN. Advise females of reproductive potential to use effective contraception during treatment and for 1 month after the last dose of HYCAMTIN. Advise males with a female sexual partner of reproductive potential to use effective contraception during treatment and for 3 months after the last dose of HYCAMTIN [*see Warnings and Precautions (5.4), Use in Specific Populations (8.1, 8.3)*].

- **Lactation**

Advise nursing mothers to discontinue breastfeeding during treatment with HYCAMTIN [*see Use in Specific Populations (8.2)*].

- **Infertility**

Advise male and female patients of the potential risk for impaired fertility and possible family planning options [*see Use in Specific Populations (8.3)*].

- **Asthenia and Fatigue**

Advise patients that HYCAMTIN may cause asthenia or fatigue. These symptoms may impair the ability to safely drive or operate machinery.

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GlaxoSmithKline
Research Triangle Park, NC 27709

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HYJ:xxPI

Preclinical Activity of Nanoliposomal Irinotecan Is Governed by Tumor Deposition and Intratumor Prodrug Conversion

Ashish V. Kalra, Jaeyeon Kim, Stephan G. Klinz, Nancy Paz, Jason Cain, Daryl C. Drummond, Ulrik B. Nielsen, and Jonathan B. Fitzgerald

Abstract

A major challenge in the clinical use of cytotoxic chemotherapeutics is maximizing efficacy in tumors while sparing normal tissue. Irinotecan is used for colorectal cancer treatment but the extent of its use is limited by toxic side effects. Liposomal delivery systems offer tools to modify pharmacokinetic and safety profiles of cytotoxic drugs. In this study, we defined parameters that maximize the antitumor activity of a nanoliposomal formulation of irinotecan (nal-IRI). In a mouse xenograft model of human colon carcinoma, nal-IRI dosing could achieve higher intratumoral levels of the prodrug irinotecan and its active metabolite SN-38 compared with free irinotecan. For example, nal-IRI administered at doses 5-fold lower than free irinotecan achieved similar intratumoral exposure of SN-38 but with superior antitumor activity. Tumor response and pharmacokinetic modeling identified the duration for which concentrations of SN-38 persisted above a critical intratumoral threshold of 120 nmol/L as determinant for antitumor activity. We identified tumor permeability and carboxylesterase activity needed for prodrug activation as critical factors in achieving longer duration of SN-38 in tumors. Simulations varying tumor permeability and carboxylesterase activity predicted a concave increase in tumor SN-38 duration, which was confirmed experimentally in 13 tumor xenograft models. Tumors in which higher SN-38 duration was achieved displayed more robust growth inhibition compared with tumors with lower SN-38 duration, confirming the importance of this factor in drug response. Overall, our work shows how liposomal encapsulation of irinotecan can safely improve its antitumor activity in preclinical models by enhancing accumulation of its active metabolite within the tumor microenvironment. *Cancer Res*; 74(23); 7003–13. ©2014 AACR.

Introduction

Liposomal carriers have become clinically accepted in cancer therapy as delivery systems that can enhance the utility of existing anticancer drugs (1). The potential benefits of these macromolecular carriers include overcoming solubility issues for certain drug classes, protecting the drug from unwanted metabolism and extending the residence time in plasma and tissue. In particular, liposomes tend to preferentially accumulate in tumors as a result of an enhanced permeability and retention (EPR) effect. The EPR effect is attributed to the abnormal tumor vasculature permitting extravasation of macromolecules, as well as impaired lymphatic drainage that promote the retention of these molecules within the tumor microenvironment, thereby providing sustained release at the tumor site mimicking a metronomic dosing (2). Increased

tumor deposition via the EPR effect may also prevent drug resistance by overcoming the activity of multidrug resistant proteins (3, 4) and may offer possible means of improving safety aspects by reducing systemic exposure relative to tumor exposure (5). There are potential pharmacologic advantages of the EPR effect, particularly for antineoplastic agents that have to engage their target over a longer time period or have little binding activity; for example drugs of the camptothecin class with topoisomerase 1 enzyme (TOP1) as the primary target.

Irinotecan (CPT-11), a clinically approved camptothecin, is a prodrug that is activated by carboxylesterase (CES) enzymes, present primarily in liver and colon tissue to the active form, SN-38. (In the article, CPT-11 is used when referring to the prodrug levels in plasma or tumor samples following either free irinotecan or nal-IRI administration. SN-38 is used when referring to the active metabolite of CPT-11.) The active SN-38 can be subsequently inactivated through glucuronidation by members of the UDP glucuronosyltransferase family (6). The principal mechanism of action leading to cell death is through DNA damage after replication-fork collisions with transient drug-TOP1 cleavage complexes, thus emphasizing the time of drug exposure as important driver for cytotoxicity of camptothecins (7, 8). Recently, we described the development of a novel nanoliposomal formulation of irinotecan, nanoliposomal formulation of irinotecan (nal-IRI; also known as MM-398 or PEP02; ref. 9). nal-IRI features very high drug loading efficiency, a high drug payload, and marked *in vivo* drug retention that

Merrimack Pharmaceuticals, Inc., Cambridge, Massachusetts.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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also stabilizes the active lactone configuration of irinotecan. The pharmacokinetic (PK) properties of the encapsulated irinotecan were dramatically altered in the plasma of female rats, with a 344× increase in the area under the curve (AUC), an 8.5× decrease in the volume of distribution, and a 39.6× increase in the half-life of the total drug. Pharmacokinetic analysis in a clinical study confirmed these performance characteristics of nal-IRI in patients (10).

Plasma drug concentrations cannot readily be translated into therapeutic effect; a sufficient amount of active therapeutic agent must be transported to the tumor site of action (i.e., be available for uptake by cancer cells) to observe favorable drug activity (11). The transport of macromolecules across the tumor vasculature is a complex process depending on vessel perfusion, surface area, and permeability, as well as tumor and drug characteristics. Several studies have used mathematical models to understand liposomal drug delivery within solid tumors (12, 13). Of particular interest is work done by Hendriks and colleagues (14), where the authors constructed a computational model to describe the parameters that affect the tumor delivery of pegylated liposomal doxorubicin, the first liposomal anticancer agent to receive clinical approval. The study concluded that liposome PK and tumor permeability to liposomes (tumor deposition) were the most important parameters controlling liposomal drug delivery to tumors.

In the case of nal-IRI, the complex metabolism (15) and mechanism of action (8, 16) of free irinotecan, in addition to the above-mentioned parameters, may play a role in the overall liposomal irinotecan delivery within tumors. In this study, we describe a systems pharmacology approach to identify critical parameters that differentiate nal-IRI from free irinotecan with regard to *in vivo* activity. A mechanistic tumor PK model was developed and trained to describe CPT-11 and SN-38 levels observed in plasma and tumor, following administration of either nal-IRI or free irinotecan in tumor xenografts. A model sensitivity analysis was performed to identify the critical parameters driving *in vivo* activity, which were then experimentally confirmed by measuring these factors in multiple cell line and patient-derived xenograft models. The findings in this study highlight critical parameters that could serve as potential biomarkers to identify cancer indications and patient populations with an increased likelihood of nal-IRI responsiveness.

Materials and Methods

Materials and nal-IRI preparation

nal-IRI was prepared as previously described (9) using a lipid composition of DSPC, cholesterol, and PEG-DSPE (3:2:0.015, mol:mol:mol), an initial drug-to-lipid ratio of 500 g drug/mol phospholipid, and extrusion through 0.1 μm polycarbonate filters. The resulting preparations displayed a particle size of 111 nm (with polydispersity index of 0.04), and a drug load of 473 mg irinotecan-HCl/mmol phospholipid. All lipids were obtained from Avanti Polar Lipids Inc. Irinotecan hydrochloride was purchased from the pharmacy. Acetic acid, methanol, and acetonitrile were from EMD Chemicals Inc. Water and trifluoroacetic acid (TFA) were from J.T. Baker. Fetal bovine serum was from Tissue Culture

Biologicals and phosphate-buffered saline (PBS) was purchased from Life Technologies.

Cell culture

Cell lines [HT-29 (colon), SK-ES-1 (Ewing's sarcoma), A549 (lung), LoVo (colon), MDA-MB-231 (breast)] were obtained from the ATCC, whereas A2780 cells (ovarian) was obtained from Sigma-Aldrich. Cells from the ATCC and Sigma were received in 2010. All cells were authenticated before receipt and were propagated for less than 6 months after resuscitation. Cultures are regularly tested for *Mycoplasma*. All cell lines were cultured in humidified CO₂ atmosphere at 37°C using media recommended by the manufacturer.

Pharmacokinetic and tissue biodistribution study

Five-week-old female NOD/SCID mice were purchased from Charles River Laboratory. The care and treatment of experimental animals were in accordance with the Institutional Animal Care and Use Committee guidelines. Subcutaneous tumors were established by injecting 10 million HT-29 cells into the right flank of mice. When the average tumor volume reached approximately 200 mm³, mice were randomized into groups (*n* = 4/time point) that received a single intravenous (i.v.) dose of nal-IRI at 5, 10, 20, or 40 mg/kg. Following 1, 4, 8, 24, 48, 72, and 168 hours after a single dose, mice were sacrificed and perfused with PBS before harvest of tumor and other normal tissues.

Antitumor activity studies

Five-week-old female NOD/SCID mice were purchased from Charles River Laboratory. Subcutaneous tumors were established by injecting 10 million HT-29 and SK-ES-1 cells or 5 million A549 cells into the right flank of mice. Tumor growth was measured twice per week by calipers and calculated with formula: width² × length × 0.52. When the average tumor volume reached approximately 200 mm³, mice were randomized into treatment groups (*n* = 5–8/group) that received weekly i.v. dose of PBS (control), free irinotecan (50 mg/kg), or nal-IRI at various doses ranging from 1.25 to 20 mg/kg.

Tumor growth inhibition (TGI) was calculated with formula:

$$\text{TGI}(\%) = \left[1 - \frac{(V_{\text{treated}}(d_{\text{final}}) - V_{\text{treated}}(d_0))}{(V_{\text{control}}(d_{\text{final}}) - V_{\text{control}}(d_0))} \right] \quad (\text{A})$$

where V_{treated} and V_{control} represent the volumes of tumor at a given time point following treatment with drug or PBS, and d_0 and d_{final} represent first day and final day of treatment, respectively.

Characterizing tumors from cell-line and patient-derived xenografts

The cell line-derived xenografts (HT-29, SK-ES-1, A549, MDA-MB-231, LoVo, AsPC-1, and A2780) were established as described above. The patient-derived tumor models [CTG-0062 (colorectal), CTG-0079 (colorectal), CTG-0252 (ovarian), CTG-0288 (pancreatic), CTG-0158 (lung), and CTG-0283 (pancreatic)] were established by Champions Oncology using their Champions TumorGraft (CTG) technology. When the

average tumor volume reached approximately 300 mm³, mice were randomized into treatment groups ($n = 4/\text{group}$) that received single i.v. dose of either PBS or nal-IRI at 10 mg/kg. Before tumor collection, intracardial perfusion was performed to remove the blood components from the tumor compartment. Briefly, a butterfly needle (23G) connected to a 10-mL syringe filled with PBS is inserted into the left ventricle. Inferior vena cava is cut and animal is perfused with 10-mL PBS (within 1–2 minutes). The control tumors were harvested 24 hours after PBS administration and used for the irinotecan activation assay, whereas the treated tumors were harvested either 24 or 72 hours following nal-IRI treatment and used for high-performance liquid chromatography (HPLC) analysis.

HPLC quantification of CPT-11 and SN-38

Tumor and normal tissues were analyzed for CPT-11 and SN-38 concentrations using a modification of the method previously described (9). Briefly, tissues were weighed and homogenized for 2 minutes in 20% w/v water using a Tissue-Lyser (Qiagen). The homogenates were extracted by mixing 0.1 mL homogenate with 0.9 mL 1% acetic acid/methanol followed by 10 seconds vortexing and placing at -80°C for 1 hour. The samples were centrifuged at 10,000 rpm for 10 minutes at room temperature and supernatants collected for HPLC analysis (Dionex). The samples and standards (CPT-11 and SN-38) were analyzed using a C18 reverse phase column (Synergi Polar-RP 80A 250 $\times$ 4.60 mm 4 μm column). The drug metabolites were eluted running a gradient from 30% acetonitrile; 70% 0.1% TFA/H₂O to 68% acetonitrile; 32% 0.1% TFA/H₂O during a 13 minutes span at a flow rate of 1.0 mL/min. The

initial elute composition was restored after 14 minutes and continued for 6 minutes before the next injection. The CPT-11 peak was detected at approximately 7.7 minutes and the SN-38 peak eluted at approximately 8.4 minutes, using an in-line fluorescence detector excited at 372 nm and emitting at 556 nm.

Irinotecan activation assay

Tumor tissue lysates were prepared by homogenizing the tissue in 6% w/v 0.1 M Tris HCL/1% Triton X-100 solution (pH7.5) using a TissueLyser for 2 to 4 minutes. Protein concentration of lysates was measured using the BCA reagent (Thermo Scientific). Lysates (250 μg of protein) were mixed with an equal volume of 10 $\mu\text{mol/L}$ irinotecan and incubated at 37°C . Following 24 hours of incubation the reaction was terminated by adding an equal volume of 1% acetic acid/methanol and samples centrifuged at 10,000 rpm for 15 minutes. The supernatant was processed for HPLC quantification of CPT-11 and SN-38 as described above.

Statistical analysis

The statistical significance of differences between groups was analyzed with the one-way ANOVA test. Results were considered statistically significant at $P < 0.05$. The analysis was performed using GraphPad Prism 6.01.

Model development and simulation

Pharmacokinetic profiles of metabolites in plasma and tumor from free irinotecan and nal-IRI were described by using multi-compartmental models (Fig. 1B). The model equations are explained and summarized in the Supplementary

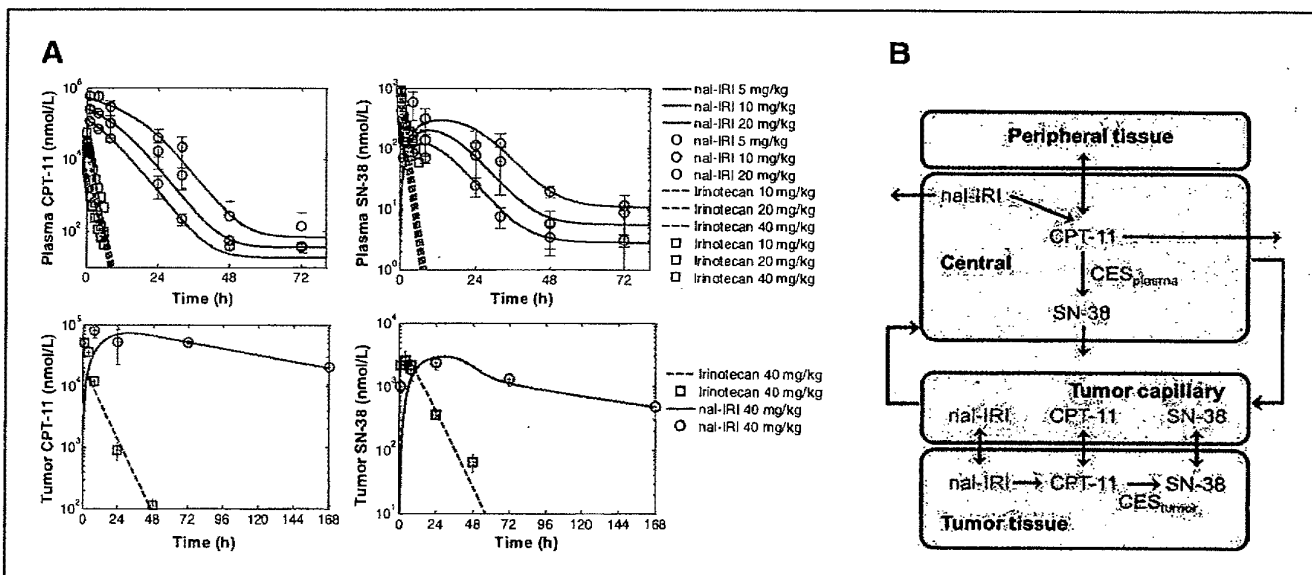


Figure 1. Pharmacokinetic profile of nal-IRI and free irinotecan. A, plasma and tumor PK of nal-IRI were compared with free irinotecan in HT-29 xenograft bearing mice. NOD/SCID mice bearing HT-29 tumors were treated with single i.v. dose of free irinotecan or nal-IRI. Plasma and tumors were collected at various intervals; CPT-11 and SN-38 were measured by HPLC analysis ($n = 4$ animals/time point). Plasma PK data for free irinotecan were taken from Kaneda and colleagues (35). Solid lines represent the model simulations for nal-IRI, whereas dashed lines represent the model simulations for free irinotecan. B, diagram of the mechanistic tumor pharmacokinetic model developed to describe the various steps in metabolism, pharmacokinetics and tumor deposition of nal-IRI.

Table 1. Summary of model parameters for plasma PK and tumor deposition models

Name	Value	Units	Description	Reference
Plasma PK model parameters				
<i>For free irinotecan</i>				
Cl_{CPT-11}	1.222e-4	L/min	Plasma clearance rate of CPT-11	Estimated
Cl_{SN-38}	2.138e-5	L/min	Plasma clearance rate of SN-38	Estimated
$k_{12,CPT-11}$	8.444e-5	1/min	Rate of CPT-11 transport from plasma to peripheral compartment	Estimated
$k_{21,CPT-11}$	4.213e-2	1/min	Rate of CPT-11 transport from peripheral to plasma compartment	Estimated
$k_{12,SN-38}$	2.656e-5	1/min	Rate of CPT-11 transport from plasma to peripheral compartment	Estimated
$k_{21,SN-38}$	3.44e-4	1/min	Rate of CPT-11 transport from peripheral to plasma compartment	Estimated
$V_{max,CES,p}$	2.263e-1	nmol/min	Maximum rate coefficient for CES enzyme in plasma compartment	Estimated
$K_{m,CES,p}$	2.67e5	nmol/L	Michaelis-Menten constant for CES enzyme in plasma compartment	Estimated
V_p	9.46e-5	L	Volume of plasma compartment	Estimated
V_{ph}	0.02	L	Volume of peripheral compartment	Fixed (14)
<i>For nal-IRI</i>				
$Cl_{nal-IRI}$	1.87e-7	L/min	Plasma clearance rate of nal-IRI	Estimated
Cl_{CPT-11}	1.634e-5	L/min	Plasma clearance rate of CPT-11	Estimated
Cl_{SN-38}	2.957e-6	L/min	Plasma clearance rate of SN-38	Estimated
$k_{12,CPT-11}$	1.619e-4	1/min	Rate of CPT-11 transport from plasma to peripheral compartment	Estimated
$k_{21,CPT-11}$	5.349e-7	1/min	Rate of CPT-11 transport from peripheral to plasma compartment	Estimated
$V_{max,Release,p}$	8.413e-6	nmol/min	Maximum rate coefficient for CPT-11 release from nal-IRI in plasma compartment	Estimated
$K_{m,Release,p}$	2.04	nmol/L	Michaelis-Menten constant for CPT-11 release from nal-IRI in plasma compartment	Estimated
$V_{max,CES,p}$	5.943e-2	nmol/min	Maximum rate coefficient for CES enzyme in plasma compartment	Estimated
$K_{m,CES,p}$	1.198e-5	nmol/L	Michaelis-Menten constant for CES enzyme in plasma compartment	Estimated
V_p	1.12e-3	L	Volume of plasma compartment	Estimated
V_{ph}	0.02	L	Volume of peripheral compartment	Fixed (14)
Tumor deposition model parameters				
Q_{tumor}	2.119e-6	L/min	Blood flow rate to tumor	(14)
$PS_{nal-IRI}$	7.858e-5	L/min/kg	Tissue permeability coefficient of nal-IRI	Estimated
PS_{CPT-11}	1.851e-3	L/min/kg	Tissue permeability coefficient of CPT-11	Estimated
PS_{SN-38}	2.687e-2	L/min/kg	Tissue permeability coefficient of SN-38	Estimated
$\sigma_{nal-IRI}$	3.181e-3		Tissue-capillary partition coefficient of nal-IRI	Estimated
σ_{CPT-11}	5.24e-1		Tissue-capillary partition coefficient of CPT-11	Estimated
σ_{SN-38}	2.109e-1		Tissue-capillary partition coefficient of SN-38	Estimated
$k_{Release,t}$	1.681e-4	1/min	Rate coefficient for CPT-11 release from nal-IRI in tumor tissue compartment	Estimated
$V_{max,CES,t}$	2.17e-2	nmol/min	Maximum rate coefficient for CES enzyme in tumor tissue compartment	Estimated
$K_{m,CES,t}$	2.3e-6	nmol/L	Michaelis-Menten constant for CES enzyme in tumor tissue compartment	Estimated
V_{cap}	7e-7	L	Volume of tumor capillary compartment	(14)
V_t	1e-5	L	Volume of tumor tissue compartment	Fixed

Data. The models were built and implemented using Simbiology toolbox in MATLAB 8.2 (The MathWorks).

Results

nal-IRI displays a prolonged exposure in both plasma and tumor compared with free irinotecan

The pharmacokinetic profiles of the prodrug CPT-11 and its active metabolite SN-38 were measured in plasma and tumors following administration of either free irinotecan or nal-IRI (Fig. 1A). At similar doses of both free irinotecan and nal-IRI, the CPT-11 and SN-38 plasma levels cleared rapidly from circulation within 8 hours after free irinotecan injection,

whereas the levels of CPT-11 and SN-38 following nal-IRI administration were persistent and remained in circulation for over 50 hours. An approximately 10-fold higher plasma CPT-11 peak level was observed with nal-IRI as compared with free irinotecan. However, the plasma peak level of SN-38 achieved with nal-IRI was 10-fold lower compared with free irinotecan, probably due to the ability of the lipid bilayer to protect the conversion of prodrug CPT-11 to SN-38 by the systemic CES enzyme present in mouse models (17). Administration of free irinotecan resulted in the clearance of greater than 90% of CPT-11 from tumors within 24 hours; however, following nal-IRI administration, CPT-11 levels persisted above

10,000 nmol/L levels for 168 hours. Similar peak levels of SN-38 were achieved with both free irinotecan and nal-IRI in HT-29 tumors, though a prolonged SN-38 exposure for up to 168 hours (measured as the AUC from 0 to 168 hours) was achieved with nal-IRI as compared with less than 48 hours tumor exposure with free irinotecan. In summary, CPT-11 and SN-38 were still present in tumors at 168 hours following nal-IRI administration, though both CPT-11 and SN-38 had cleared from plasma.

Tumor SN-38 duration drives *in vivo* activity

We developed a mechanistic PK model to identify the determinants that may differentiate the plasma and tumor PK profiles between free irinotecan and nal-IRI (Fig. 1B). The experimental PK data were used to estimate the optimal model

parameters (Table 1) fitting the model simulations within the standard deviations of *in vivo* PK profiles of both CPT-11 and SN-38 (Fig. 1A). As the *in vitro* cytotoxic effects of irinotecan on tumor cells is dependent on the concentration and the time of exposure of cells to active metabolite SN-38 (7, 8), we sought to understand if the overall plasma and tumor SN-38 exposure predicts the *in vivo* activity of both nal-IRI and free irinotecan. The trained model determined that a 5-fold higher dose of free irinotecan (50 mg/kg) was required to achieve similar SN-38 exposure in both plasma and tumor as compared with nal-IRI (10 mg/kg; Fig. 2A). The TGI of HT-29 xenograft model at these equal exposure doses, was significantly greater with nal-IRI (~110%) treatment as compared with free irinotecan (~40%), despite the 5-fold lower total dose administered (*, $P < 0.05$,

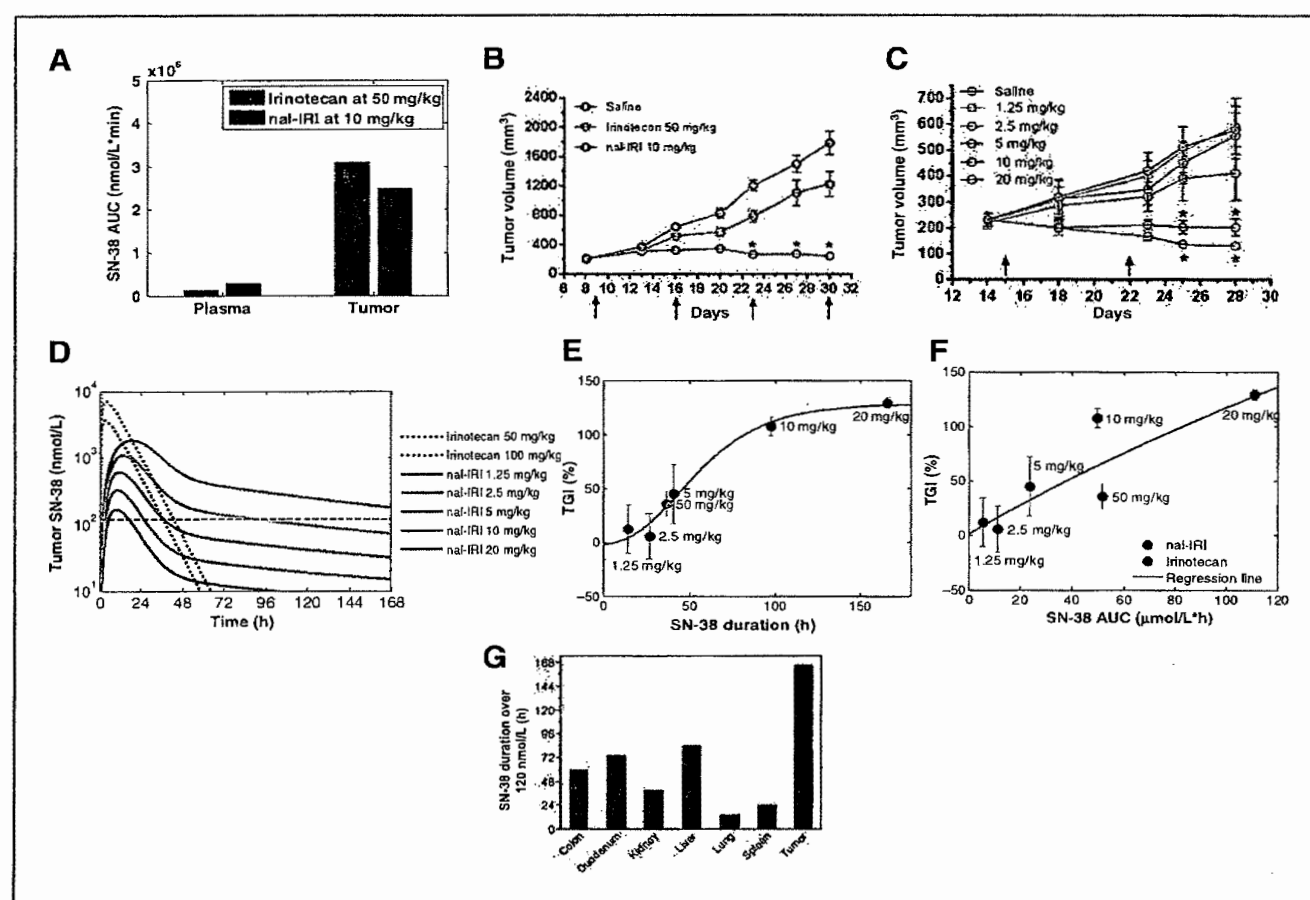


Figure 2. Relation of nal-IRI *in vivo* activity to tumor SN-38 duration. A, model predictions for similar SN-38 AUC in plasma and tumor following free irinotecan (50 mg/kg) and nal-IRI (10 mg/kg) administration. B, tumor response observed in HT-29 xenograft following weekly administration (arrows) of 50 mg/kg free irinotecan and 10 mg/kg nal-IRI ($n = 8$ /group). The tumor volumes for nal-IRI (10 mg/kg) were significantly lower (*, $P < 0.05$) compared with saline and irinotecan groups (one-way ANOVA test). C, tumor response in HT-29 xenografts following weekly administration (arrows) of various nal-IRI doses ($n = 5$ /group). The tumor volumes for nal-IRI (10 mg/kg) and nal-IRI (20 mg/kg) groups were significantly lower (*, $P < 0.05$) compared with saline tumors on day 25 and day 28 (one-way ANOVA test). D, model simulations were used to compare tumor SN-38 concentration following the administration of varying doses of free irinotecan or nal-IRI. Black dashed line represents threshold concentration of 120 nmol/L to determine tumor SN-38 duration. E and F, TGI(%) achieved by nal-IRI and free irinotecan treatment in HT-29 xenografts were compared with the tumor SN-38 duration above 120 nmol/L (E) and SN-38 AUC (F) at varying doses of nal-IRI or free irinotecan. Solid lines represent nonlinear regression lines based on five parameter logistic curve fitting. G, the SN-38 duration over a threshold of 120 nmol/L was computed from the pharmacokinetic profiles of SN-38 in tumor and normal tissues following 20 mg/kg of nal-IRI.

one-way ANOVA test; Fig. 2B). In addition, other studies have shown no additional HT-29 TGI at the maximum tolerated dose of free irinotecan (100 mg/kg; ref. 18). To identify a dose level of nal-IRI that gave comparable *in vivo* activity to 50 mg/kg free irinotecan, we performed a dose escalation study in the HT-29 xenograft model (Fig. 2C). nal-IRI at 5 mg/kg showed partial inhibition of tumor growth (~40% TGI) that was comparable with 50 mg/kg free irinotecan, whereas 10 mg/kg and 20 mg/kg nal-IRI showed significant (*, $P < 0.05$, one-way ANOVA test) TGI compared with saline (~110%–130% TGI). Furthermore, we have previously tested control liposomes (that have comparable composition with nal-IRI except for the absence of irinotecan, the active pharmaceutical ingredient) and did not observe any TGI (data not shown).

The intratumor SN-38 concentrations achieved from 50 to 100 mg/kg doses of free irinotecan and 1.25, 2.5, 5, 10, and 20 mg/kg doses of nal-IRI were then simulated using the trained mechanistic PK model (Fig. 2D). Although a nal-IRI dose of 5 mg/kg achieved similar TGI as 50 mg/kg free irinotecan, the tumor SN-38 AUC and peak levels were approximately 2-fold and 6-fold lower respectively for nal-IRI as compared with free irinotecan. Furthermore, we noted at these doses both drugs were able to maintain the tumor SN-38 concentration above 120 nmol/L for the same duration of approximately 40 hours. To determine if the tumor SN-38 concentration impacts *in vivo* activity, we used the tumor SN-38 concentration of 120 nmol/L as a threshold. We also determined the duration for which the various doses of nal-IRI or free irinotecan could maintain the tumor SN-38 concentration above 120 nmol/L, hereon referred to as "tumor SN-38 duration." A sigmoidal relationship between TGI (%) and tumor SN-38 duration (Fig. 2E) was observed for both nal-IRI and free irinotecan ($R^2 = 0.62$). However, when comparing TGI (%) with tumor SN-38 AUC (Fig. 2F) the relationship was

less significant ($R^2 = 0.45$), due to the lower TGI (%) achieved by 50 mg/kg free irinotecan compared with 10 mg/kg nal-IRI. We also observed longer SN-38 duration in tumors (>100 hours) compared with normal tissues (<72 hours; Fig. 2G and Supplementary Fig. S1).

Identification of liposome tumor permeability and local tumor activation as critical determinants for tumor SN-38 duration

A local sensitivity analysis on the model parameters was performed to identify processes impacting the tumor SN-38 duration (Supplementary Data). In response to the administration of free irinotecan (50 mg/kg), the tumor SN-38 duration was relatively insensitive to most model parameters (Fig. 3A), suggesting the inability of free irinotecan to modulate it. In contrast, several model parameters were found to significantly impact tumor SN-38 duration following the administration of nal-IRI (10 mg/kg; Fig. 3B). The sensitive parameters for nal-IRI can be classified into three different categories: (i) PK, rate of breakdown of liposomes in blood (Release rate in blood, $V_{\max, \text{Release}, p}$), (ii) activation of prodrug CPT-11 to SN-38 by CES enzyme (CES activity in tumor; $V_{\max, \text{CES}, t}$ and blood; $V_{\max, \text{CES}, p}$), and (iii) liposome uptake within tumors, that is, nal-IRI tumor deposition (nal-IRI tumor permeability, $PS_{\text{nal-IRI}}$). Among these parameters, the release rate in plasma negatively affected tumor SN-38 duration due to a decrease in the overall systemic exposure of nal-IRI. CES enzyme activity, particularly from tumor CES (local tumor activation of irinotecan) and nal-IRI permeability (tumor deposition), positively affected the tumor SN-38 duration. To assess the identifiability of parameter estimates, log likelihood profiling was performed for the sensitive parameters, $V_{\max, \text{CES}, t}$ and $PS_{\text{nal-IRI}}$ (19). The confidence intervals suggested that both parameters were precisely estimated (Supplementary Fig. S2).

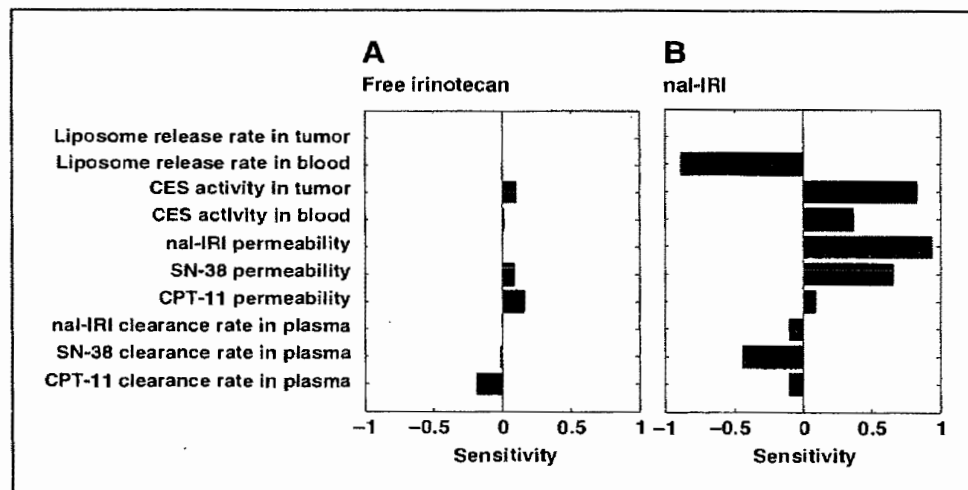


Figure 3. Model parameters impacting tumor SN-38 duration. Sensitivity analyses for free irinotecan (A) and nal-IRI (B) were performed on key model parameters that are responsible for plasma clearance, tissue deposition and metabolic reactions. Parameters whose values were not estimated in this study, including compartment volumes and tumor blood flow, were excluded from the analysis. The doses of free irinotecan (50 mg/kg) and nal-IRI (10 mg/kg) that achieved similar SN-38 plasma and tumor exposure were used for sensitivity analysis. The model parameters were modulated by 10% and their effect on tumor SN-38 duration was determined as a sensitivity index (Supplementary Equation S6).

Biologic variability and simulated perturbation of nal-IRI tumor deposition and local activation

To determine the biologic relevance of these sensitive parameters toward driving tumor SN-38 duration (namely nal-IRI tumor deposition and local tumor activation of irinotecan), the parameters were measured in a panel of 13 xenograft models. We used the total CPT-11 concentrations in tumors as a surrogate for nal-IRI tumor deposition as model simulations based on nal-IRI pharmacokinetics showed that majority of CPT-11 in plasma and tumor was encapsulated and protected within the liposomes and less than 10% was available as free CPT-11 (Supplementary Fig. S3). The intratumor con-

centrations of CPT-11 varied substantially across the tumor panel (Fig. 4A). The tumor models from cell-lines displayed overall higher levels of prodrug CPT-11 deposition (from 5,000–15,000 ng/g) as compared with patient-derived tumor models (1,000–2,000 ng/g). In addition, a high degree of variability was observed between individual tumors within the same xenograft model (66% average coefficient of variation). Model simulations were used to test the effect of altering nal-IRI tumor deposition on tumor SN-38 duration (Fig. 4B). By decreasing the nal-IRI permeability parameter to zero, which simulates an impermeable tumor microenvironment, the tumor SN-38 duration of approximately 100 hours achieved

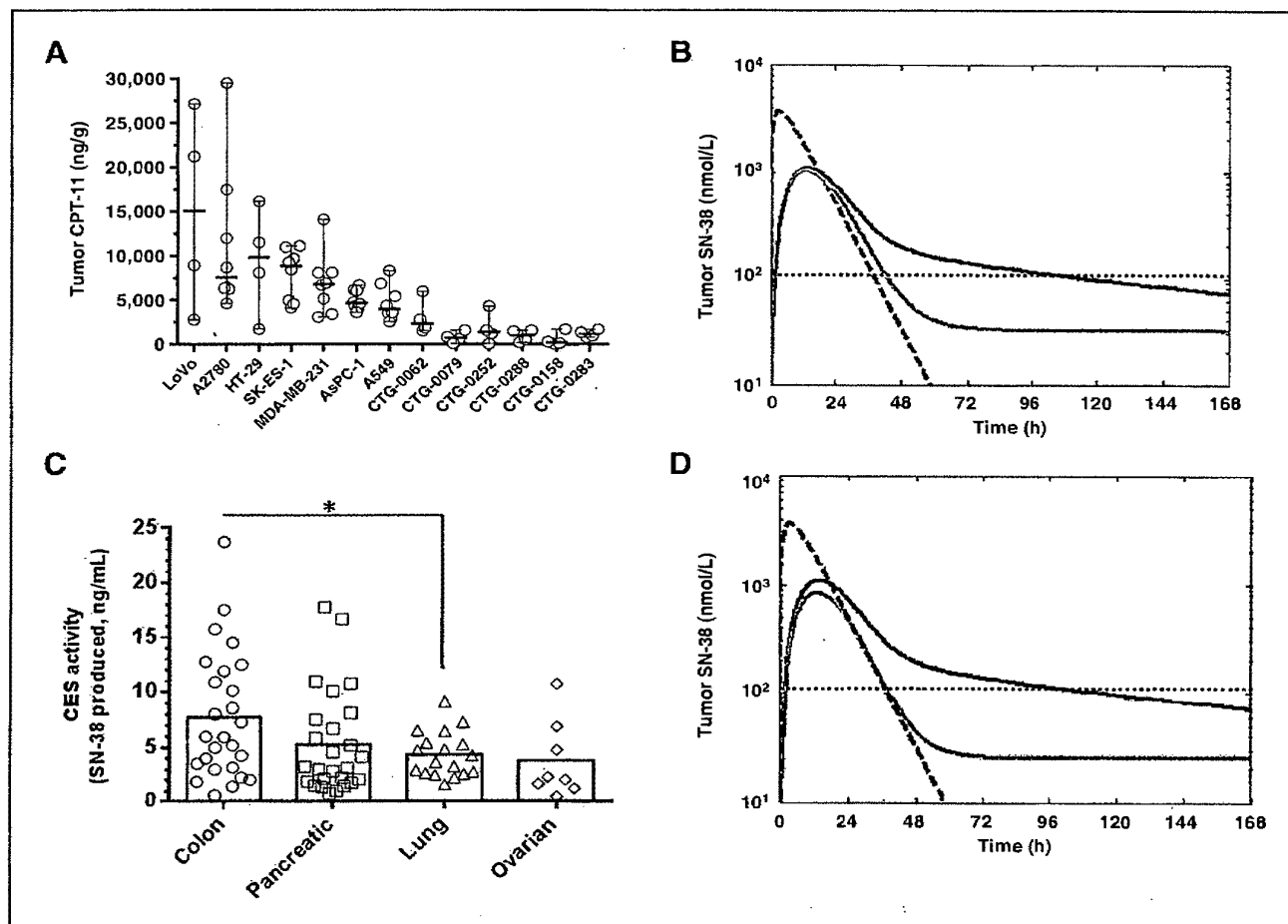


Figure 4. *In vivo* variability in nal-IRI tumor deposition and local activation. A, intratumor CPT-11 concentrations were measured across cell-line-derived (HT-29, SK-ES-1, A549, MDA-MB-231, LoVo, AsPC-1, and A2780) and patient-derived (CTG-0062, CTG-0079, CTG-0252, CTG-0288, CTG-0158, and CTG-0283) tumor models. Tumor-bearing mice were administered a single i.v. dose of 10 mg/kg nal-IRI and tumors excised 24 hours later. CPT-11 concentrations were determined in the tumor lysates using HPLC analysis as described in Materials and Methods ($n = 4-8$ tumors/model). B, the effect of nal-IRI permeability on tumor SN-38 concentrations was simulated by reducing the nal-IRI permeability parameter $PS_{nal-IRI}$ to zero. The simulated tumor SN-38 concentrations were compared at the equal exposure doses of 10 mg/kg nal-IRI and 50 mg/kg free irinotecan. Black solid line, nal-IRI (10 mg/kg) with base $PS_{nal-IRI}$. Gray solid line, nal-IRI (10 mg/kg) with zero $PS_{nal-IRI}$. Black dashed line, free irinotecan (50 mg/kg). Dotted line, threshold concentration of 120 nmol/L CES activity (C) for 80 patient-derived xenograft tumors across different indications was determined using *ex vivo* irinotecan activation assay. Tumor lysates (250 μ g of protein) from untreated mice was incubated with free irinotecan (5 μ mol/L) for 24 hours at 37°C and the amount of SN-38 produced was measured with HPLC analysis (*, $P < 0.05$; t test). D, the effect of knocking out tumor CES activity on tumor SN-38 duration was simulated by reducing the tumor CES parameter $V_{max,CES,t}$ to zero. The simulated tumor SN-38 concentrations were compared at the equal exposure doses of 10 mg/kg nal-IRI and 50 mg/kg free irinotecan. Black solid line, nal-IRI 10 mg/kg with base $V_{max,CES,t}$. Gray solid lines, nal-IRI 10 mg/kg with zero $V_{max,CES,t}$. Black dashed line, free irinotecan 50 mg/kg with base $V_{max,CES,t}$. Dotted line, threshold concentration of 120 nmol/L.

with 10 mg/kg nal-IRI was substantially reduced to approximately 50 hours and approached the levels observed with 50 mg/kg free irinotecan. Taken together, these results suggest that the tumor deposition of nal-IRI is highly tumor specific and will dramatically impact tumor SN-38 duration.

To determine the degree to which local tumor activation of irinotecan varied in human tumors, we measured CES activity using an *ex vivo* assay in a panel of 80 patient-derived tumors. The tumor lysates varied in their ability to activate prodrug irinotecan and produce SN-38 (1–25 ng/mL SN-38 produced), suggesting a high degree of variability in local tumor activation of irinotecan across indications. A significant difference in local tumor activation of irinotecan was observed between colon and lung tumors ($P < 0.05$). However, there was no significant difference between other indications, which may be due to high variability observed within each indication (Fig. 4C). The impact of varying the tumor CES activity on tumor SN-38 duration was evaluated by simulating a knockout of tumor CES enzyme (Fig. 4D). In the absence of local tumor activation, tumor SN-38 duration with nal-IRI (10 mg/kg) decreased from approximately 100 to 40 hours, similar to that achieved by free irinotecan (50 mg/kg).

nal-IRI tumor deposition and local activation collectively predict tumor SN-38 duration

The relative contribution of nal-IRI tumor deposition and local tumor activation on tumor SN-38 duration was evaluated using model simulations. On the basis of the findings from the sensitivity analysis (Fig. 3B), nal-IRI permeability ($PS_{\text{nal-IRI}}$) and tumor CES activity ($V_{\text{max,CES,t}}$) values were used to create a map relating these parameters to tumor SN-38 duration following nal-IRI administration (Fig. 5A). Model simulations predicted a concave relationship, where the tumor SN-38 duration is dependent upon both the tumor permeability and the tumor CES activity. The tumor SN-38 duration could be increased by either increasing the $PS_{\text{nal-IRI}}$ or $V_{\text{max,CES,t}}$ (white arrows) and the maximum tumor SN-38 duration of 168 hours was only

reached with CES activity at 0.025 nmol/min and tumor permeability at $1.5E-4$ L/min/kg.

To experimentally test the model predictions, we used the same panel of 13 xenograft models to measure the tumor concentrations of CPT-11 (as a surrogate for tumor deposition, Supplementary Fig. S4A); tumor SN-38 concentrations (as a surrogate for tumor SN-38 duration; Supplementary Fig. S4B) and CES activity (for local tumor activation of irinotecan). The experimental data supported the model simulations, confirming that the SN-38 concentration within tumors was dependent on both the tumor CPT-11 concentration and tumor CES activity (Fig. 5B). All tumor models with high CPT-11 concentration $>2,000$ ng/mL or high CES activity >5 ng/mL displayed high tumor SN-38 concentrations ("red") ranging from 25 to 125 ng/mL (Supplementary Table S2). In certain tumor models, one of the parameters contributed predominantly toward higher SN-38 concentrations (black arrows). A2780 and SK-ES-1 tumors displayed high tumor SN-38 concentrations of 97 ng/mL and 127 ng/mL respectively (Supplementary Table S2), which was mainly due to high CPT-11 concentrations ($>2,000$ ng/mL), whereas in other tumor models (CTG-0062 and AsPC-1) the CES activity (>5 ng/mL) was the dominant factor contributing toward high tumor SN-38 concentrations. Further tumor models with the lowest tumor SN-38 concentrations ranging from 5 to 12 ng/mL ("blue"), including several patient-derived tumor models (boxed area) also displayed lower tumor CPT-11 concentrations ($<2,000$ ng/mL) and CES enzyme activity (<5 ng/mL).

Tumor SN-38 duration correlates with nal-IRI *in vivo* activity

In vivo tumor response studies were performed in three tumor models in which different tumor SN-38 durations had been observed (as indicated by tumor SN-38 concentration at 72 hours) to determine the impact of tumor SN-38 duration on *in vivo* activity of nal-IRI. The tumor volumes observed for both HT-29 (Fig. 6A) and SK-ES-1 (Fig. 6B) models were significantly

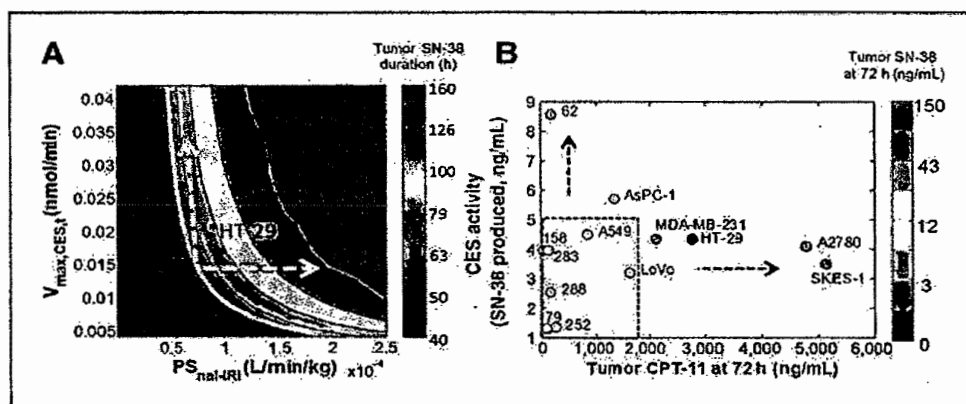


Figure 5. nal-IRI tumor deposition and local activation impacts tumor SN-38 duration. A, the effect of changing tumor CES activity and nal-IRI permeability parameters (arrows) on tumor SN-38 duration (color-coded in hours) in tumors was simulated. The optimal parameter values for HT-29 were marked with the symbol **. B, experimental data in tumor xenograft models showing the impact of tumor CPT-11 and CES activity on tumor SN-38 concentrations. Tumor CES activity (as surrogate for local tumor activation of irinotecan) and tumor CPT-11 concentration at 72 hours (as surrogate for tumor deposition) for different xenograft models were plotted and color-coded on the basis of their SN-38 concentrations in the tumor 72 hours after nal-IRI (each data point represents median of $n = 4-8$ tumors). Dotted arrows, dependence of tumor SN-38 concentrations on tumor CPT-11 concentration and CES activity.

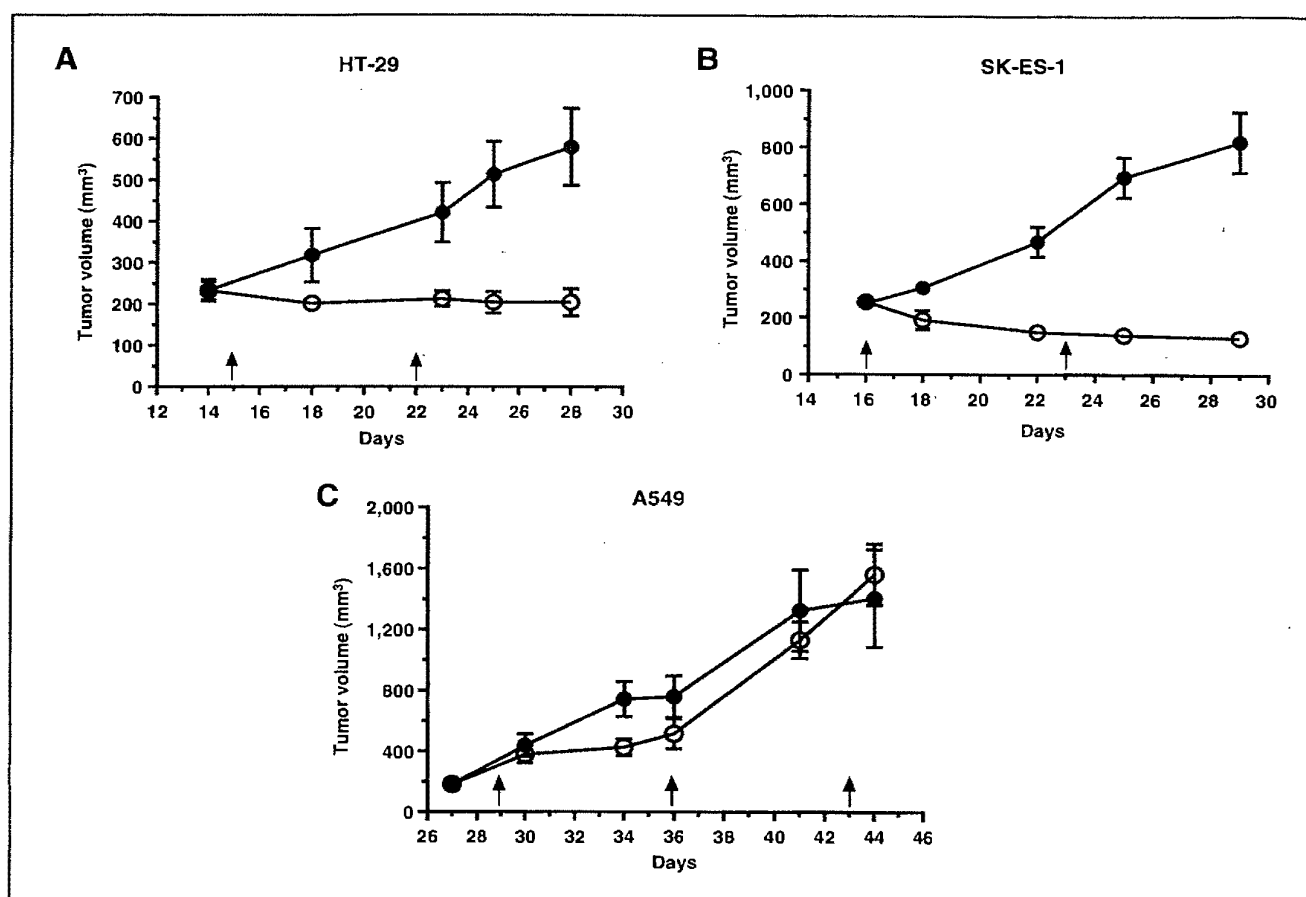


Figure 6. *In vivo* tumor response for nal-IRI. NOD/SCID mice were inoculated with HT-29; colon (A), SK-ES-1; Ewing's (B) and A549; lung (C) cell lines. Tumor-bearing mice were randomized when the tumor volume was approximately 200 mm³. Each group received weekly i.v. dose (arrows) of either saline (●) or 10 mg/kg dose of nal-IRI (○). Tumor volumes were measured twice per week ($n = 5$ –10 animals/group).

lower ($P < 0.05$) following 10 mg/kg nal-IRI as compared with untreated tumors. In both these models, tumor regression was observed immediately after the first dose and was sustained through the course of the study. A549 tumors achieved lower SN-38 tumor levels (Fig. 4A) and did not respond to nal-IRI treatment (Fig. 6C). Interestingly, both A549 and HT-29 cells displayed similar *in vitro* sensitivity to SN-38 with IC₅₀ values of 53 and 44 nmol/L, respectively (20). In summary, nal-IRI induced stronger responses (~100% TGI) in tumor models that had higher tumor SN-38 duration (>~100 hours).

Discussion

The nal-IRI formulation dramatically alters the pharmacologic properties of irinotecan as well as its active metabolite, SN-38 (9). In this study, we identified a pharmacologic parameter—namely, tumor SN-38 duration—as a driver of irinotecan-based *in vivo* activity and propose biomarkers that can impact tumor SN-38 duration achieved by nal-IRI. Our study indicates that nal-IRI can completely inhibit tumor growth compared with free irinotecan, despite administering doses that achieve similar SN-38 exposure (measured as the AUC). Instead, the duration of prolonged exposure of SN-38 within tumors

achieved by nal-IRI was shown to be a major pharmacologic determinant for *in vivo* activity in mice.

Several studies have shown improved *in vitro* cytotoxic activity of SN-38 when cells are exposed to drug for longer duration (21). The *in vitro* cell doubling time for HT-29 cells is approximately 20 hours (21), whereas *in vivo* the tumor volume doubles (Fig. 2B) at a slower rate (~8–9 days). In addition, at a given time only 35 to 50% of cells are in the S-phase of cell cycle wherein the maximum cytotoxicity of free irinotecan has been observed (21). Thus, to exert maximum cytotoxic effects across different cell-cycle phases, the cells have to be exposed to free irinotecan across multiple cell cycles. Our *in vivo* study confirms these findings as the free irinotecan is rapidly cleared from plasma and tumor tissue (tumor SN-38 duration of approximately 40 hours), thereby not allowing sufficient time for tumor cells to be exposed to SN-38 (for only 2 cell-cycle doubling time) as compared with more than 5 cell-cycle doubling times with nal-IRI (tumor SN-38 duration for >100 hours). Thus the extended exposure of tumor cells to SN-38, which is achieved by nal-IRI, can contribute toward the enhanced cytotoxicity as compared with free irinotecan.

We observed higher tumor concentrations of CPT-11 and SN-38 at 168 hours following administration of nal-IRI. In contrast, the peak plasma concentrations of SN-38 was lower with nal-IRI as compared with free irinotecan, suggesting that most of the CPT-11 from nal-IRI remains inside the liposomes and is protected from systemic conversion as described with free irinotecan (17). In addition, prolonged SN-38 duration from nal-IRI administration was observed only in tumors and much less in normal tissues, suggesting that toxicity might not be exacerbated by nal-IRI treatment. The preferential accumulation of nal-IRI in tumors as compared with normal tissues can be attributed to the EPR effect, where the leaky vasculature in tumor facilitates the extravasation of liposomal nanoparticles and the defective lymphatic drainage helps increase the retention within tumor (1, 2). Thus, with the EPR effect, nal-IRI creates a large depot of CPT-11 only in tumors thereby prolonging tumor SN-38 duration. In contrast, free irinotecan can easily be transported in and out of the tissues with a short plasma half-life, resulting in minimal SN-38 duration in tumors.

The enhanced *in vivo* activity of nal-IRI as compared with free irinotecan was attributed to the ability of nal-IRI to extend the tumor SN-38 duration. Sensitivity analysis identified two key determinants that impact the ability of nal-IRI to extend tumor SN-38 duration—(i) nal-IRI tumor deposition, as measured by the extent of prodrug CPT-11 deposition within tumors and (ii) nal-IRI local activation, from prodrug CPT-11 to SN-38 facilitated by the local tumor CES enzyme. The experimental data, in this study supported the importance of each of these determinants. We observed high degree of variability in the overall nal-IRI tumor deposition across the 13 xenograft models that were tested. Several studies have highlighted a role for tumor permeability, tumor perfusion, and stromal matrix in limiting the delivery of therapeutic agents into tumors (22). In our model simulations, when the nal-IRI tumor permeability was decreased to zero, the benefit of higher tumor SN-38 duration with nal-IRI was negatively impacted and reduced to levels simulated for free irinotecan. We also observed that the tumors with lower nal-IRI deposition had considerable lower SN-38 tumor levels. These data are consistent with other findings suggesting that a dense tumor stroma can impede drug permeability and limit drug delivery within tumors (23, 24).

Use of tumor CES activity as a cellular parameter for predicting free irinotecan response had limited success both in preclinical (25, 26) and clinical studies (27). Through the sensitivity analysis performed in this study, we identified CES activity as a critical parameter for nal-IRI activity. Tumor models that displayed high ability to activate CPT 11, achieved high tumor SN-38 concentrations despite limited deposition of CPT-11, thus suggesting the importance of local tumor CES enzyme expression in facilitating longer SN-38 exposure following nal-IRI administration. In fact, others have shown that *in vitro* and *in vivo* activity of free irinotecan can be enhanced by overexpressing of CES enzyme in tumor cells (28, 29). In addition to tumor cells expressing CES enzyme (30), other components of the extracellular matrix such as tumor-associated macrophages (TAMs) express CES1 enzyme and play a role in CPT-11 activation (31). In fact, we performed *in vitro*

studies that confirmed the ability of TAMs to hydrolyze CPT-11 to SN-38 (Supplementary Fig. S5). Thus our data suggests the extended tumor PK achieved by nal-IRI provides high local depot of prodrug CPT-11 for prolonged time, thus allowing for activation by tumor CES enzymes. Collectively our data provides rational for investigating tumor CES enzyme activity as a potential marker for nal-IRI activity.

Pharmacogenetic and pharmacodynamic markers such as TOP1 have shown limited correlations with free irinotecan response (6, 32–34). In addition to the intrinsic sensitivity of tumor cells to SN-38, our data indicate that the duration for which tumor cells are exposed to SN-38 (tumor SN-38 duration) also plays a critical role in driving treatment response to irinotecan. Tumor models with extended SN-38 duration (HT-29, SK-ES-1) showed robust *in vivo* response to nal-IRI, whereas A549 with shorter tumor SN-38 duration did not respond to therapy. The fact that *in vitro* sensitivity of both HT-29 and A549 to SN-38 is very similar (20) corroborates the finding that the duration of SN-38 is driving the tumor response.

In conclusion, our data demonstrate that nal-IRI enhances the pharmacokinetic profile of tumor SN-38, prolonging tumor exposure to SN-38 compared with free irinotecan, and therefore has the potential for therapeutic effect in human cancers. Liposome permeability and CES activity were the critical factors that emerged from model simulation of tumor SN-38 duration, which were experimentally shown to vary across and within tumor indications. Thus, translational research exploring the utility of tumor liposome permeability and local activation of irinotecan as biomarkers for nal-IRI clinical activity is warranted.

Disclosure of Potential Conflicts of Interest

D.C. Drummond and J.B. Fitzgerald have ownership interest (including patents) in Merrimack Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: A.V. Kalra, J. Kim, S.G. Klinz, D.C. Drummond, U.B. Nielsen, J.B. Fitzgerald

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.V. Kalra, N. Paz, J. Cain

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.V. Kalra, J. Kim, D.C. Drummond, J.B. Fitzgerald

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Kim, N. Paz

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Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

Preclinical Activity of Nanoliposomal Irinotecan Is Governed by Tumor Deposition and Intratumor Prodrug Conversion

Ashish V. Kalra, Jaeyeon Kim, Stephan G. Klinz, et al.

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Magnetic resonance imaging with an iron oxide nanoparticle demonstrates preclinically the feasibility of predicting intratumoral uptake and activity of MM-398, a nanoliposomal irinotecan (nal-IRI)

Ashish Kalra¹, Joseph Sperry², Jaeyeon Kim¹, Walid Kamoun¹, Arnold Sengooba¹, Stephan Klinz¹, Nancy Paz¹, Jason Cain¹, Ninfa Straubinger³, Yang Qu³, Sheryl Trueman³, Eliel Bayever¹, Ulrik Nielsen¹, Daryl Drummond¹, Jonathan Fitzgerald¹, Robert M. Straubinger³ ¹Merrimack Pharmaceuticals, Cambridge, MA; ²Roswell Park Cancer Institute, Buffalo, NY; ³University at Buffalo, SUNY, Buffalo, NY

Abstract #2065

Abstract

Sustained intratumoral delivery of cytotoxic agents is a major challenge for effective cancer treatment, and motivated the development of MM-398, nanoliposomal irinotecan (nal-IRI) that has been shown to have an extended plasma half-life and greater tumor deposition than free irinotecan in preclinical models. We have previously shown through a systems pharmacology approach that tumor deposition of nal-IRI and the subsequent carboxylesterase conversion of irinotecan to the active metabolite, SN-38, are determinants for nal-IRI activity *in vivo*.

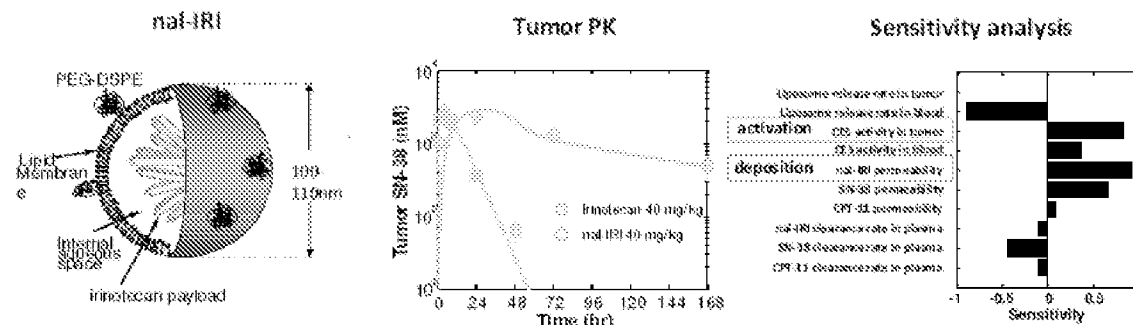
Ferumoxytol (FMX) is a 30nm iron-oxide, super-paramagnetic nanoparticle, known to be taken up by macrophages (as is nal-IRI), and exhibits magnetic resonance imaging properties. Since the size of a nanoparticle affects the rate of transcapillary transport significantly, we hypothesized that nal-IRI tumor biodistribution may be predicted by FMX-based MRI (Fe-MRI).

Biodistribution and imaging studies were performed in mice bearing cell-line derived (A2780, HT-29, A549) and patient-derived (pancreatic adenocarcinoma) tumor xenografts. The protocol consisted of a baseline MRI scan, an i.v. injection of FMX (20 mg/kg), followed by an i.v. injection of fluorescently labeled nal-IRI (10 mg/kg) 24hr later. Mice were sacrificed 24hr and 72hr after nal-IRI injection, and irinotecan and SN-38 concentrations were determined in plasma, tumor, and tissues by HPLC analysis.

The presence of FMX did not interfere with nal-IRI PK or biodistribution. Cellular distribution of liposomes within tumors was also not affected by FMX at up to 50 mg/kg as measured by flow cytometry. Furthermore, immunohistochemistry showed that both liposomes and FMX were co-localized with tumor-associated macrophages. The drug metabolite measurements from tissue samples showed that the xenograft tumor models display wide ranges of nal-IRI deposition capacity (irinotecan concentrations at 24hr: 2,104-26,096 ng/g). A2780 tumors displayed the highest concentration of both iron (3.92 mg/ml) and irinotecan (9,466 ng/g) at 72hr after nal-IRI injection, whereas A549 tumors displayed the lowest levels of both iron (0.23 mg/ml) and irinotecan (436 ng/g). We observed a correlation between the tumor Fe-MRI signal and intratumoral levels of irinotecan 72hr after nal-IRI injection ($R^2=0.9$, $p<0.001$). Furthermore, *in vivo* activity studies confirmed that xenograft models with higher intratumoral levels of irinotecan and SN-38 at 72hr showed greater tumor growth inhibition.

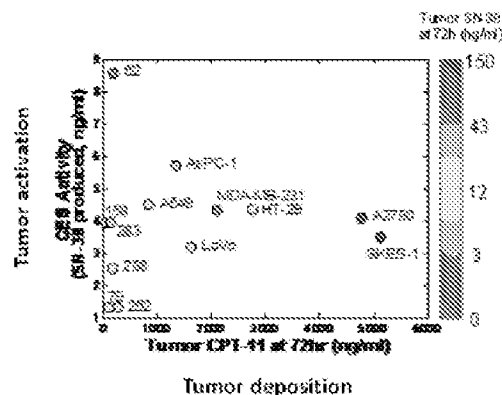
In summary, preclinical studies demonstrate the potential of utilizing Fe-MRI as a potential diagnostic tool to identify patients with higher tumor permeability. Based on encouraging preclinical data, a pilot study in patients with advanced solid tumors with extensive Fe-MRI scanning and paired tumor biopsies (NCT # 01770353) is being conducted.

nal-IRI and ferumoxytol (FMX) background



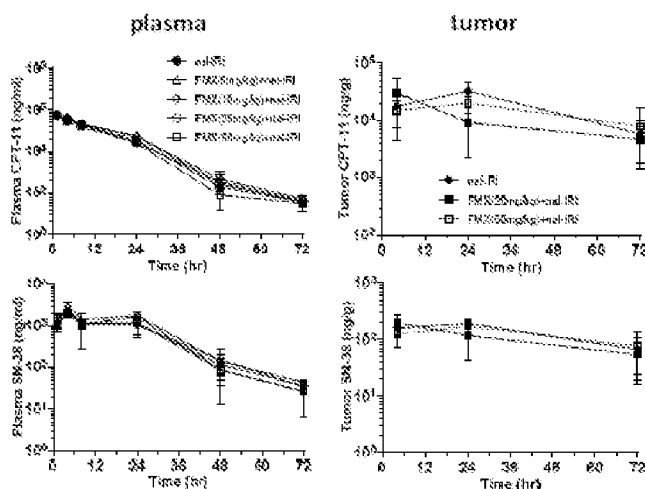
1. nal-IRI is a 100nm nanoliposomal formulation of irinotecan that displays higher plasma and tumor exposure compared to free irinotecan in preclinical models
2. Sensitivity analysis highlights two parameters, (1) tumor drug uptake: "tumor deposition" and (2) ability of tumor carboxylesterase enzyme to activate pro-drug irinotecan to SN-38: "tumor activation" as critical factors driving nal-IRI *in vivo* activity
3. Experimental data in a panel of xenograft models confirm the critical role of tumor drug deposition and activation.
4. Ferumoxytol (FMX) is a 30nm iron-oxide, super-paramagnetic nanoparticle, known to be taken up by macrophages
5. We are currently evaluating the feasibility of correlating FMX-based MRI acquisition to estimate tumor drug delivery in a pilot clinical study (NCT01770353)

Experimental validation

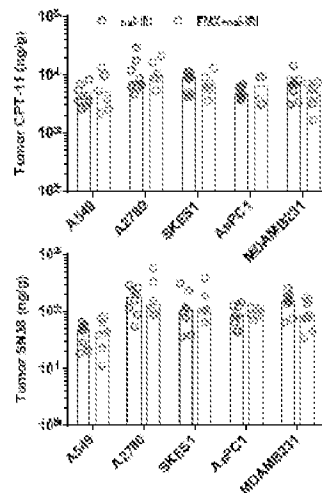


nal-IRI PK and intratumor distribution remains unaltered by ferumoxytol

A. Effect of FMX administration on nal-IRI PK



B. nal-IRI uptake in a panel of xenografts following FMX



C. Ex vivo FACS analysis to determine impact of FMX administration on liposome uptake by tumor cells (EPCAM+) and macrophages (CD11b+)

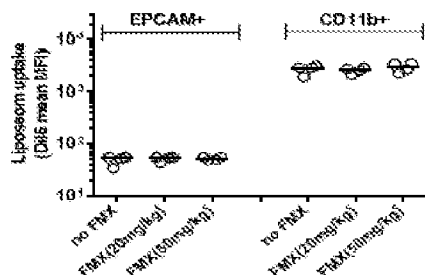
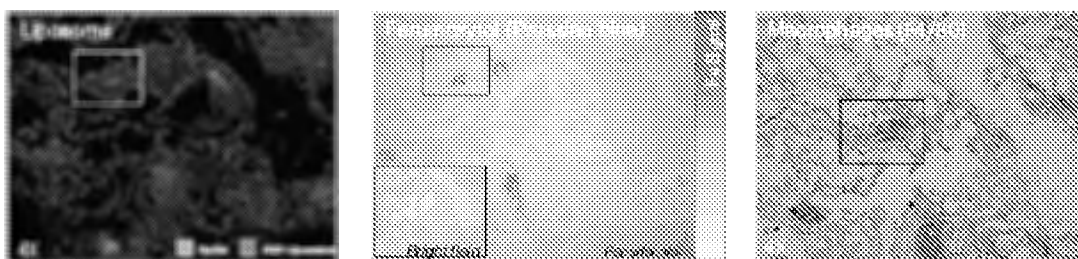


Figure 1. (A) HT-29 colon tumor bearing NOD SCID mice were injected (i.v.) with FMX followed (24h later) with nal-IRI (20 mg/kg). (B) Tumor bearing NOD SCID mice were injected with FMX (20 mg/kg) followed (24h later) with nal-IRI (10 mg/kg). Plasma and tumor samples were analyzed by HPLC to determine CPT-11 and SN-38 levels. (C) HT-29 colon tumor bearing NOD SCID mice were treated with FMX (20 mg/kg) followed by injection of DiI5-liposomes.

Ferumoxytol co-localizes with labeled liposomes and TAMs

A. IHC analysis to determine the intratumor distribution of liposomes, ferumoxytol and tumor associated macrophages in a pancreatic PDX tumor



B. Correlating FMX uptake to liposome uptake in 18254 (pancreatic PDX) and HT-29 (colon) xenograft

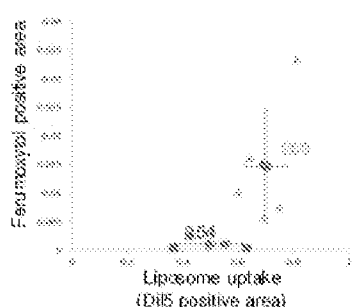
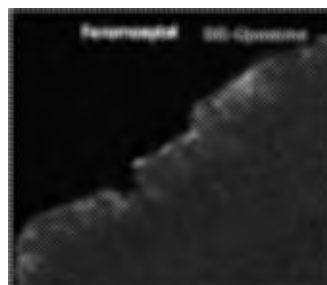
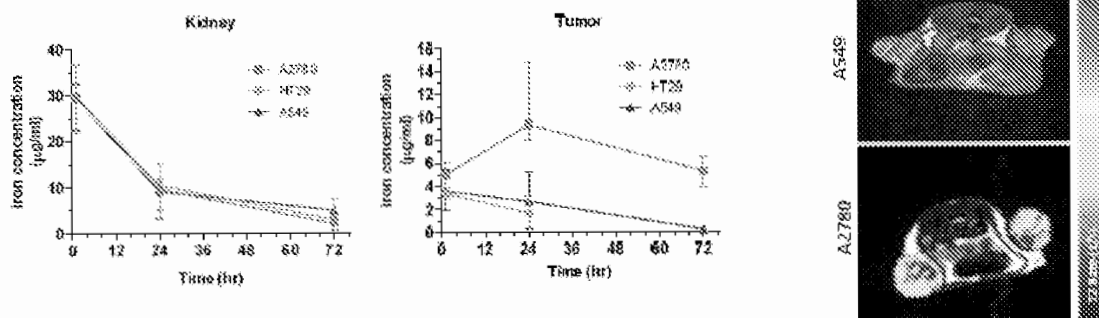


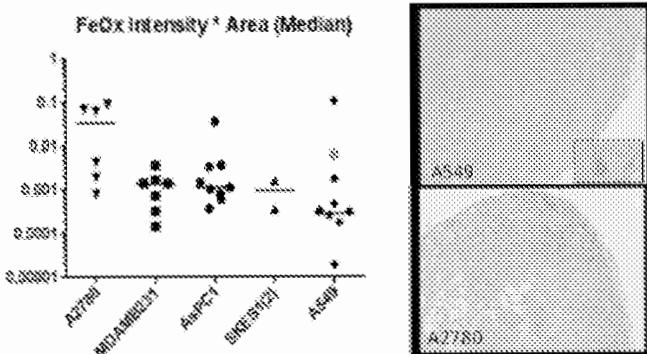
Figure 2. Pancreatic patient-derived xenograft models 18254 and 18269 injected with FMX (20 mg/kg) followed by DiI5-labeled liposomes. (A) IHC analysis of 18254 model shows co-localization of liposomes, FMX and macrophages. (B) Correlating liposome uptake to FMX levels within tumors (left panel). Comparing co-localization of FMX and DiI5 liposomes in HT-29 xenograft (right panel).

Tumor models with high ferumoxytol concentrations showed correlation with high intratumor irinotecan pro-drug levels

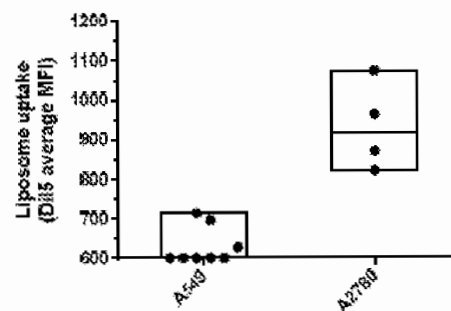
A. Iron concentration was estimated from MRI acquisition (T2 relaxation rates) across different xenograft models



B. IHC analysis to measure intratumor FMX uptake (Prussian blue staining)



C. Quantifying tumor uptake of labeled (Dil5) liposomes



D. Tumor irinotecan (CPT-11) and SN-38 concentrations correlate with the intratumor concentrations of FMX

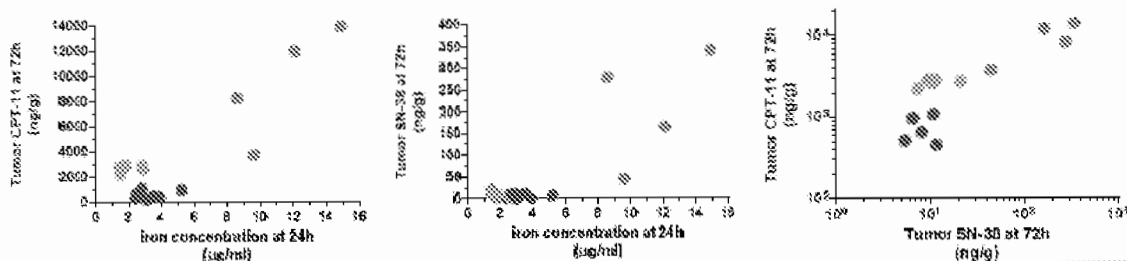


Figure 3. (A) C57BL tumor bearing mice were injected with FMX (20 mg/kg). Contrast enhanced MRI was performed using 4.7T CSI magnet incorporating Bruker AVANCE electronics and the ParaVision 3.0.2 imaging. Iron concentrations were extrapolated from the T2 relaxation rates using a phantom FMX standard curve. A2780 tumors displayed higher FMX (B) and Dil5 liposome (C) uptake compared to A549 tumors. (D) FMX-MRI confirms high FMX uptake in A2780 tumors which correlates with irinotecan (CPT-11) and SN-38 tumor levels.

Summary

- In preclinical studies ferumoxytol is co-localized with liposomes as well as tumor associated macrophages within the tumor microenvironment and does not alter the pharmacokinetic properties of nai-IRI.
- Contrast-enhanced MRI using ferumoxytol shows correlation between the FMX MRI signal and tumor drug uptake particularly in tumors with high liposomal drug delivery.
- A clinical study to assess the feasibility of ferumoxytol as tumor imaging agent prior to investigational nai-IRI treatment is ongoing (NCT01770353).

CONCERN

CONCLUSIONS

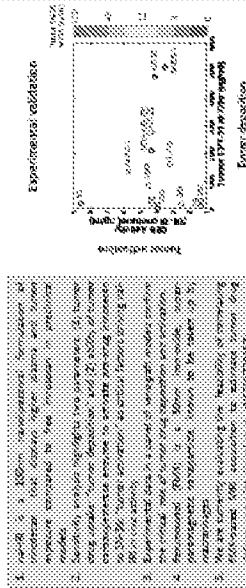
Ninfa Straubinger², Yang Qu³, Sheryl Trueman³, Eliel Bayewer¹, Ulrik Nielsen¹, Daryl Drummond⁶, Jonathan Fitzgerald¹, Robert M. Straubinger¹,
¹ Merrimack Pharmaceuticals, Cambridge, MA; ² Roswell Park Cancer Institute, Buffalo, NY; ³ University at Buffalo, SUNY Buffalo, NY

5902# 43214545

Abstract

biochemical and genetic studies were performed using breeding pairs of females (J77-30, J77-35, J50-6) and sperm-derived transgenic after-transfected female progenies. The protocol consisted of a breeding 1800-day, an oviposition of 250–260 mg/egg, followed by a 1% injection of fluorescent isothiocyanate (FITC) (200 mg/egg) and a second injection of 250–260 mg/egg. The other male, injection, and transgene and 20–25 concentrations were determined in several experiments.

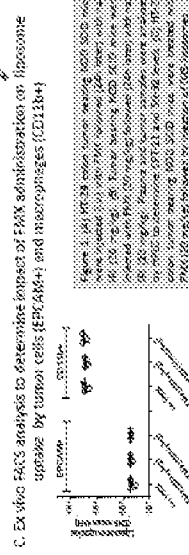
rad-RT and ferromagnet (FMX) background



transmission, or contact with labeled footsoles and socks.



nal-81 PK and intratumor distribution remains unaltered by peritoneal lavage



Tumor models with high serum/urine concentrations showed correlation with high intratumor iminodiacetic acid levels

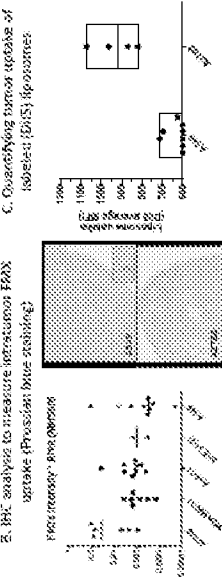


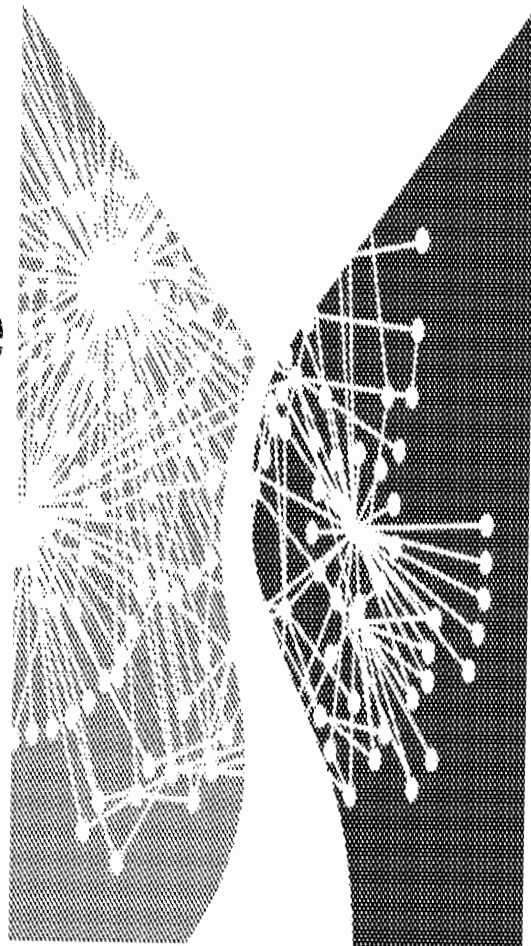
Figure 1 consists of three scatter plots arranged horizontally, each showing the relationship between the number of seeds per fruit (y-axis, 0 to 1000) and the number of fruits per plant (x-axis, 0 to 100). The plots are labeled 'Tricardium 2N', 'Tricardium 4N', and 'Tricardium 8N' from left to right. In the 'Tricardium 2N' plot, data points are scattered with a positive linear regression line drawn through them. In the 'Tricardium 4N' and 'Tricardium 8N' plots, data points are scattered with no regression line shown, indicating no significant correlation.

References

3. **Preclinical studies:** Preclinical is conducted with lymphomas as well as tumor-associated macrophages within the tumor microenvironment and does not alter the pharmacokinetic properties of T-CE.
4. **Controlled-randomized trial using biomarkers:** Shows correlation between the PD-L1 expression and tumor control outcome parameters in tumors with high expression drug delivery.
5. **Clinical study:** to assess the feasibility of immunotherapy as tumor imaging agent prior to immunotherapy. PD-L1 expression is assessed using PET/CT/768/33.

Magnetic resonance imaging (MRI) to predict tumor drug delivery and response to nanoliposomal therapy

Ashish Kalra, Ph.D.
Merrimack Pharmaceuticals



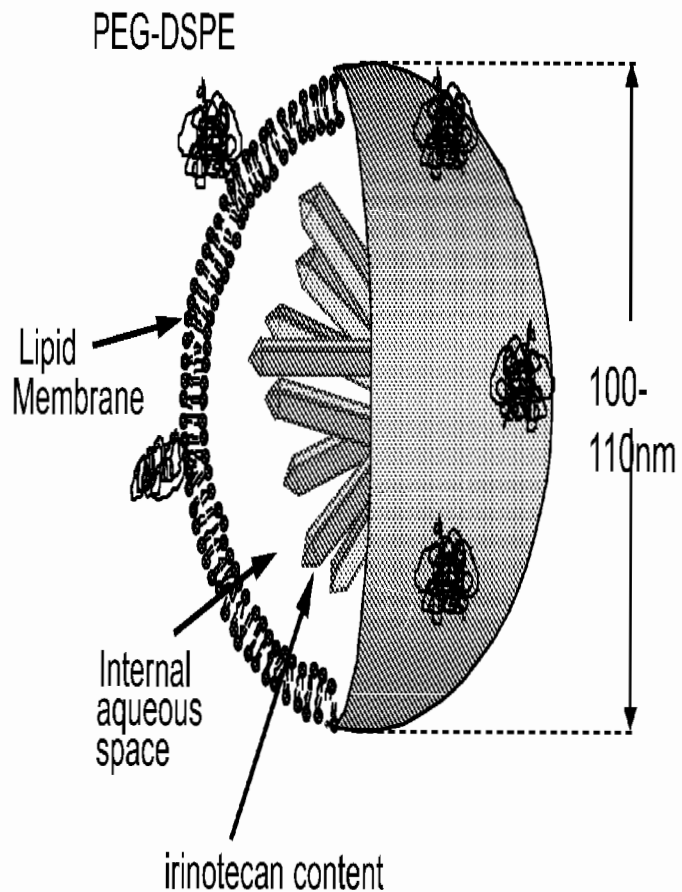
Tumor Models
Boston 2014

- MM-398 mechanism of action
 - Factors influencing MM-398 activity
- Diagnostic strategy to predict the tumor delivery of MM-398
 - Ferumoxytol based contrast enhanced MRI in preclinical tumor models
- Translating into clinical setting
 - Preliminary data from Phase 1 feasibility study

MM-398 nanoliposomal irinotecan

Extended circulation

- Low Pegylation & size sustain circulation half-life
- Liposomal encapsulation reduces systemic conversion of prodrug irinotecan
- Effective packaging of irinotecan with ~70,000 molecules/liposome

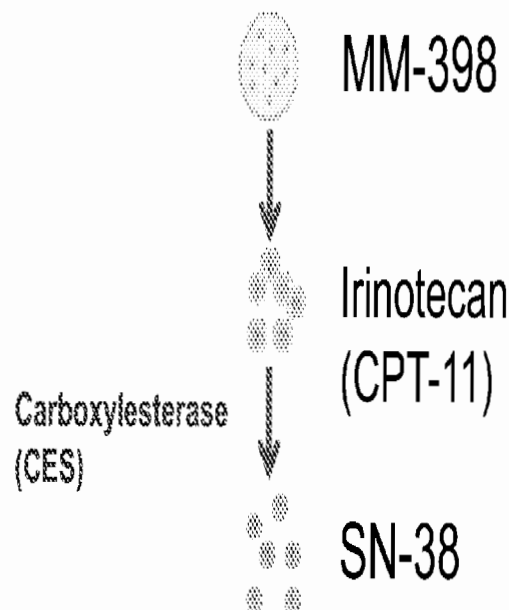


Enhanced tumor accumulation

- Passive accumulation in tumor due to EPR effect to achieve high drug levels

Local cytotoxic activation

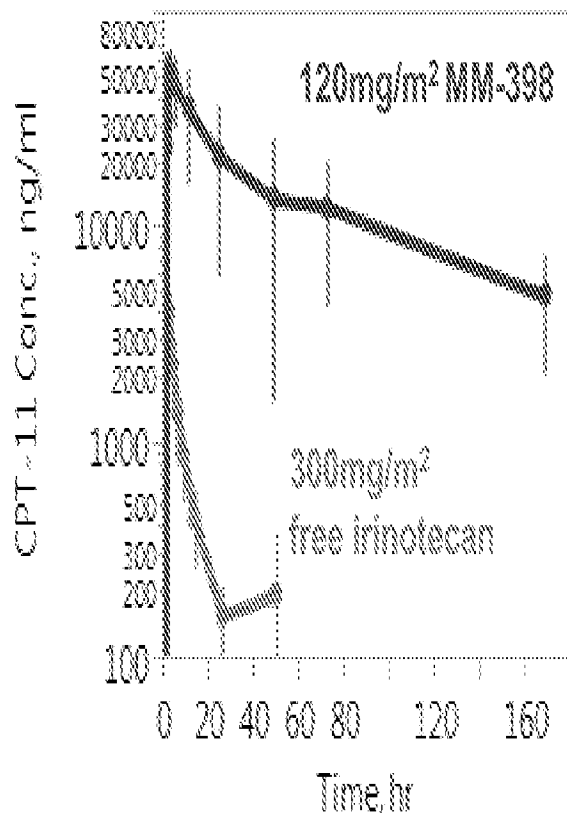
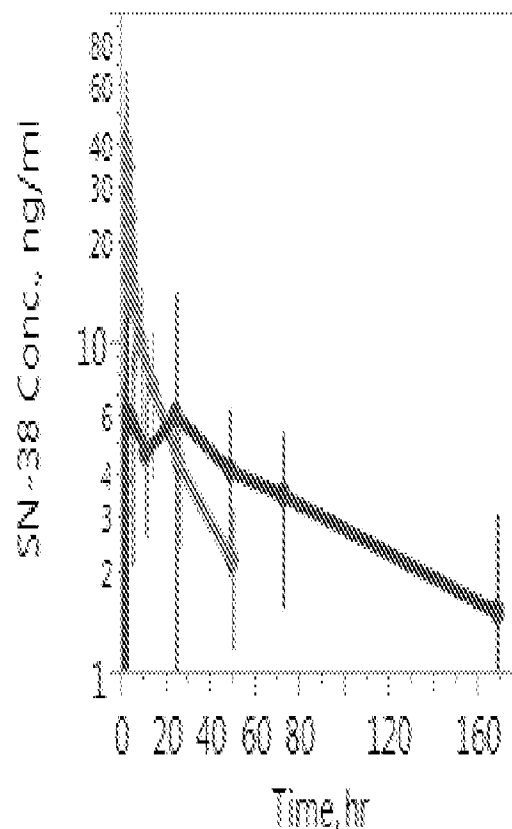
- After conversion to SN-38 binds to TOP1 and induces DNA damage



Sustained circulation of MM-398 in patients compared to irinotecan, with lower peak SN-38 levels

Irinotecan (CPT-11)

SN-38



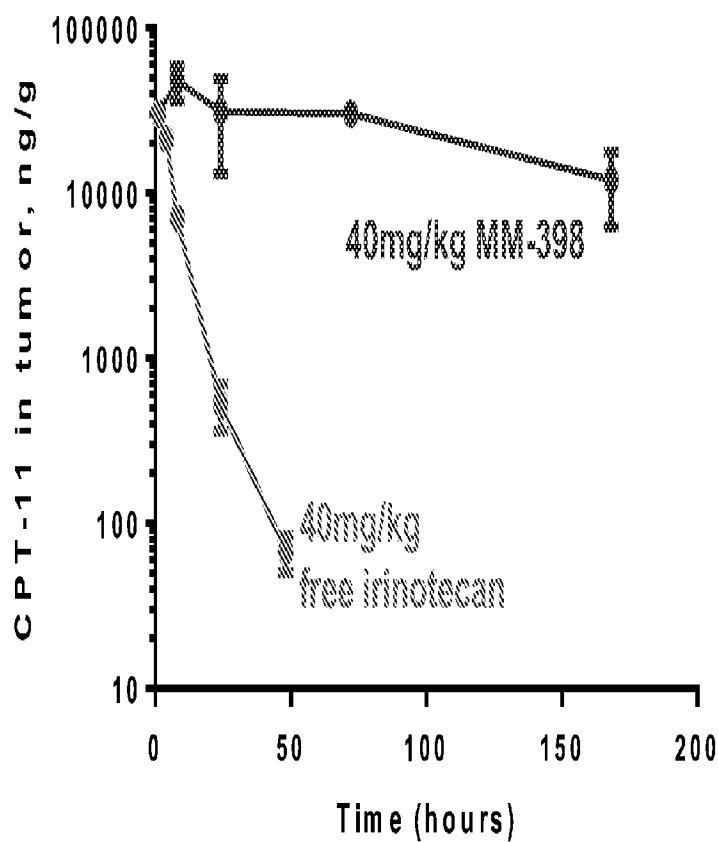
70-fold higher AUC of CPT-11 in the blood vs irinotecan

PK analysis of patients show extended circulation

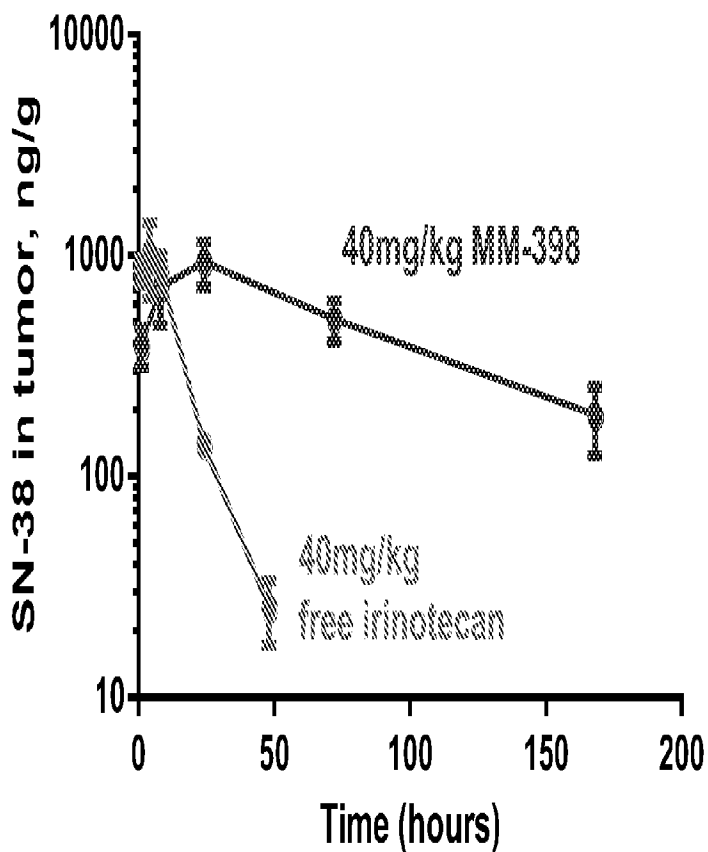
- Pharmacokinetic data is from a secondline study in gastric cancer patients
- 88 patients were dosed Q3W with MM-398 or free irinotecan

MM-398 increases & extends tumor exposure

HT-29 CRC tumor levels
of irinotecan (CPT-11)

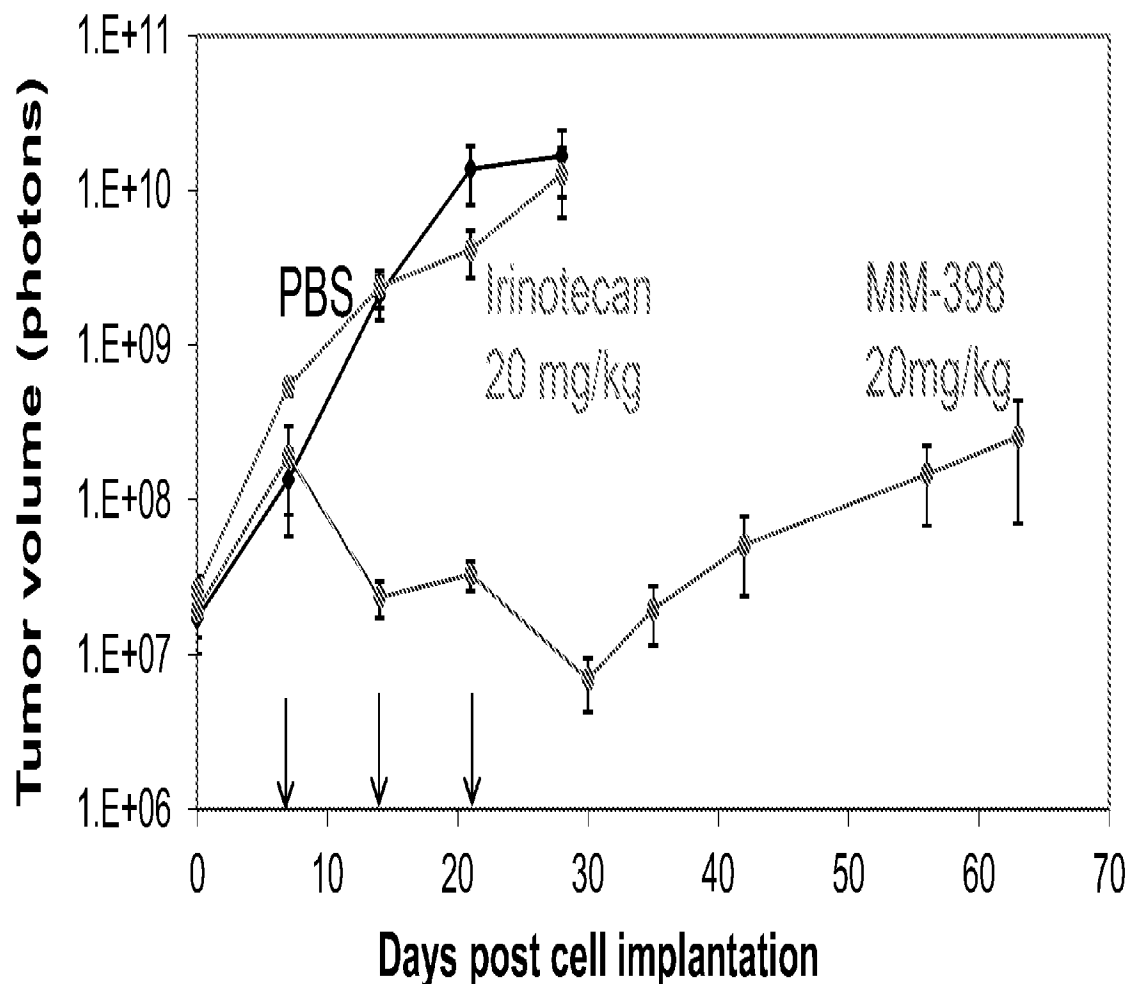


HT-29 CRC tumor levels
of SN-38



Superior activity and drug activation in pancreatic cancer xenografts

Orthotopic, hypoxic, L3.6pl pancreatic xenograft



	Irinotecan	SN-38
	(µg/g)	(µg/g)
irinotecan-24h	0.043	0.019
MM-398 - 24h	6.1	0.17
MM-398 - 72h	1.6	0.09

9X

Mechanistic PK model predicts factors influencing SN-38 tumor exposure (duration)

PK model trained on

- plasma and tumor samples
- multiple dose levels
- multiple time points

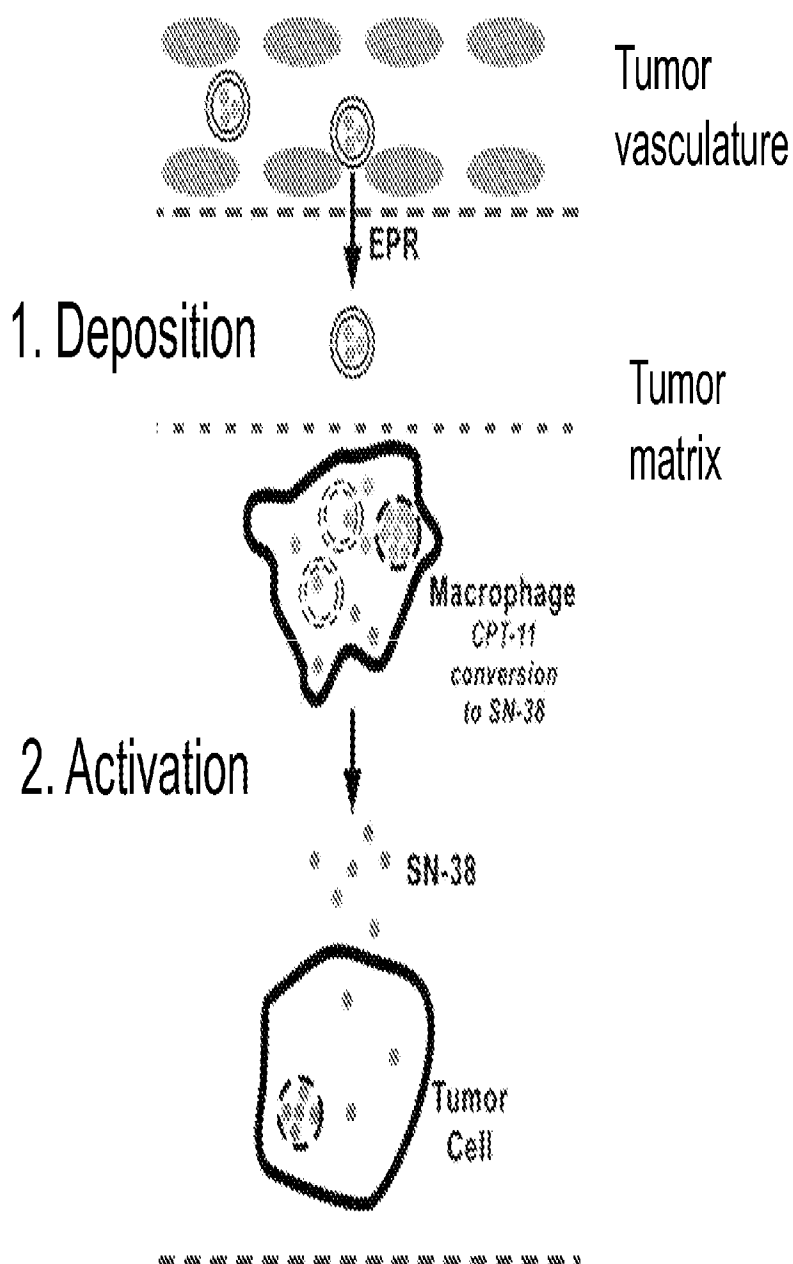
"SN-38 duration in tumor"

duration for which active drug SN-38 was present in the tumor

Two factors influence "SN-38 duration in tumor"

1. MM-398 deposition
2. MM-398 activation

Measure in preclinical xenograft models



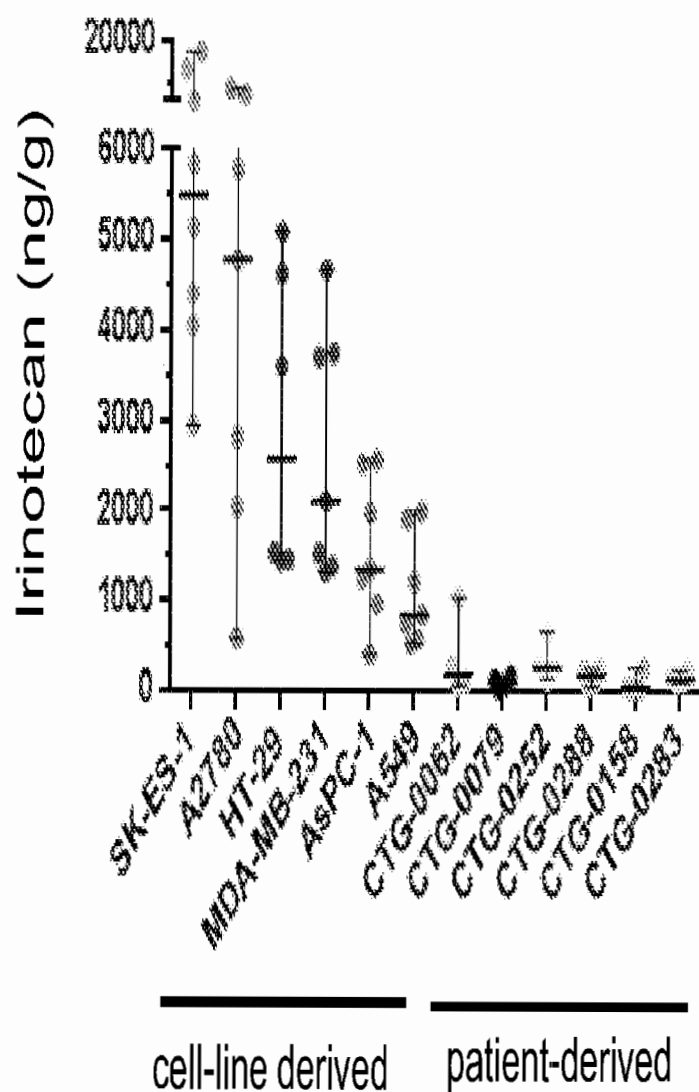
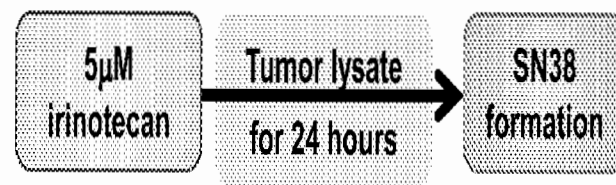
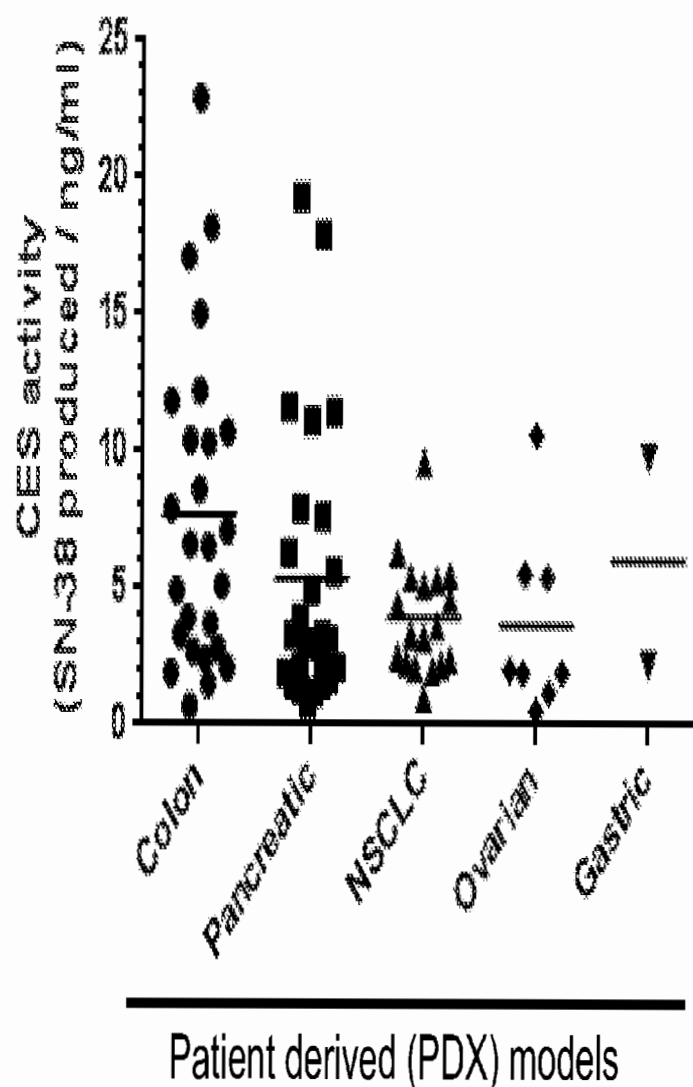
Factors affecting delivery of SN-38 vary considerably in preclinical tumor models

Deposition

Activation

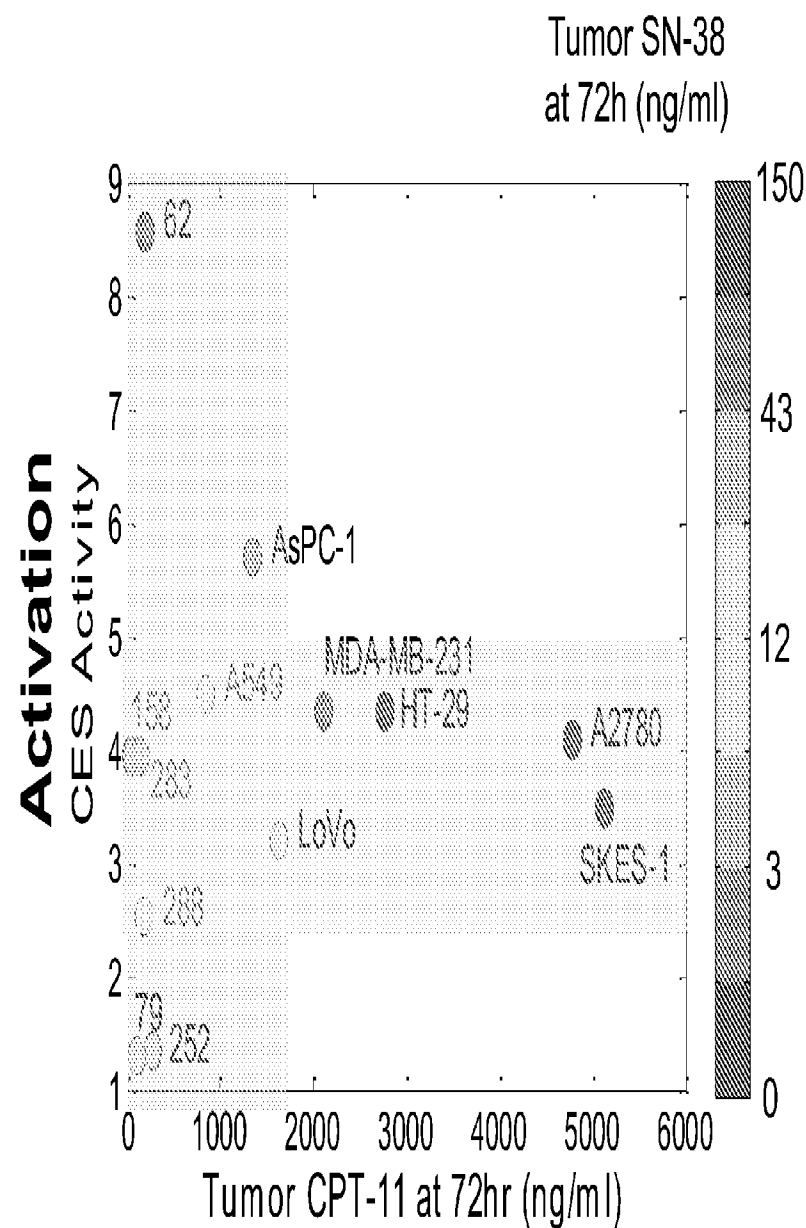
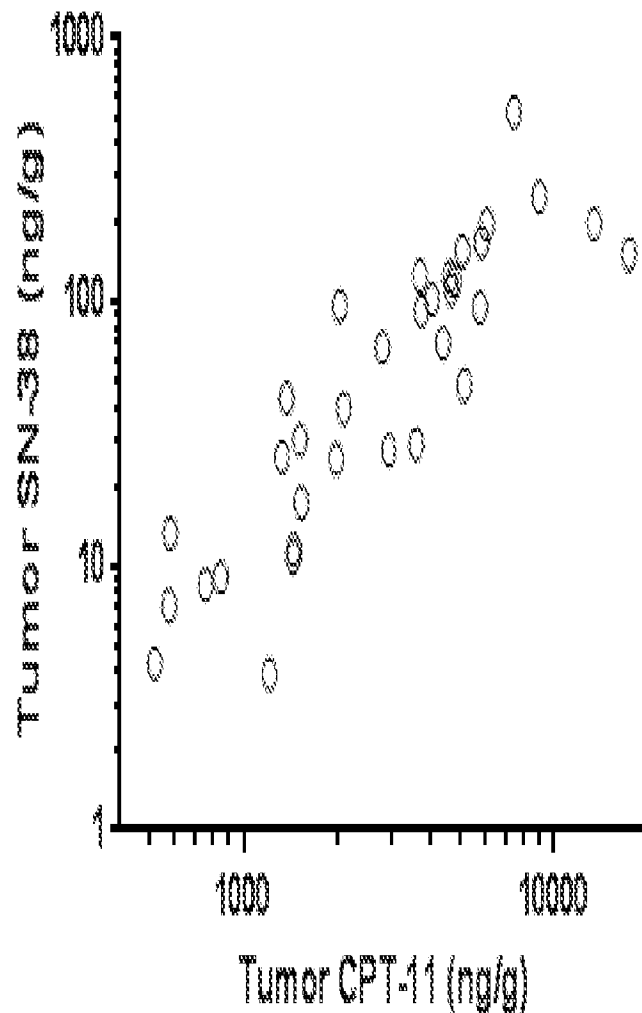
as measured by irinotecan level (>90% encapsulated)

Irinotecan activation assay



Experimental data confirms importance of tumor deposition and activation

A surrogate to measure variability in tumor drug deposition?



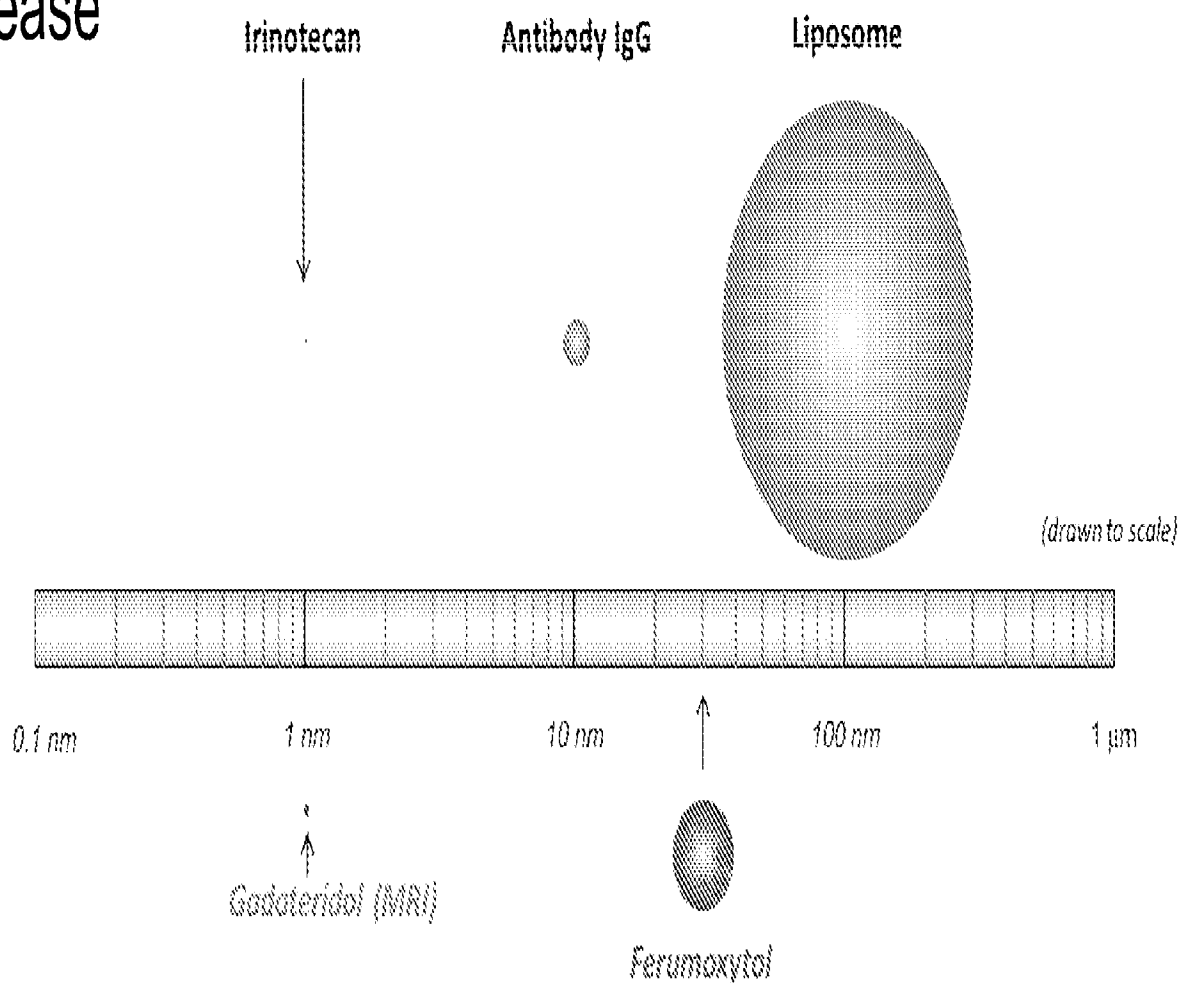
(median, n=4-8)

Deposition

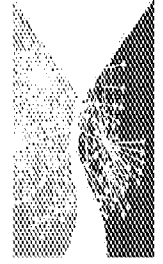
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Ferumoxytol (FMX) compared to therapeutic agents

- Superparamagnetic iron oxide nanoparticle coated with polyglucose sorbitol carboxymethylether
- FDA approved: iron supplement in patients with chronic kidney disease



- Semisynthetic coating and 30 nm size - longer plasma half life
- Taken up by phagocytic cells
- Recent off label use in MRI – CNS lesions and brain aneurysm

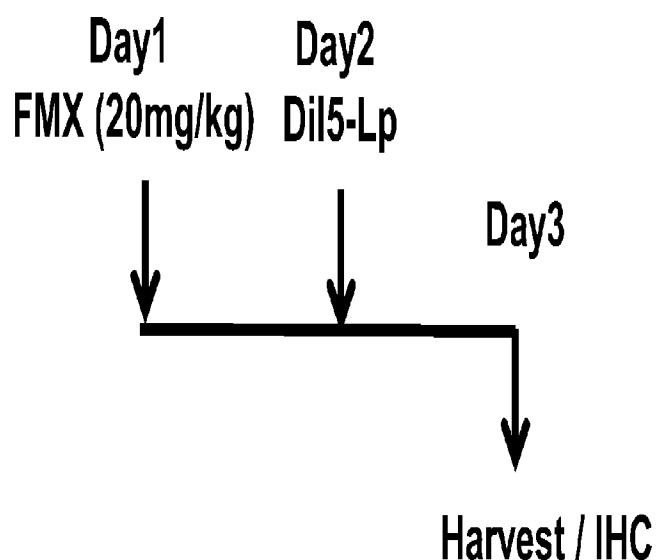


FMX based MRI to predict MM-398 deposition

1. Does FMX show similar intra tumor distribution as liposomes (co-localize with liposomes)?
2. Can we observe extended intra tumor accumulation of FMX?
3. Can we quantify tumor FMX signal as a surrogate for MM-398 tumor deposition?

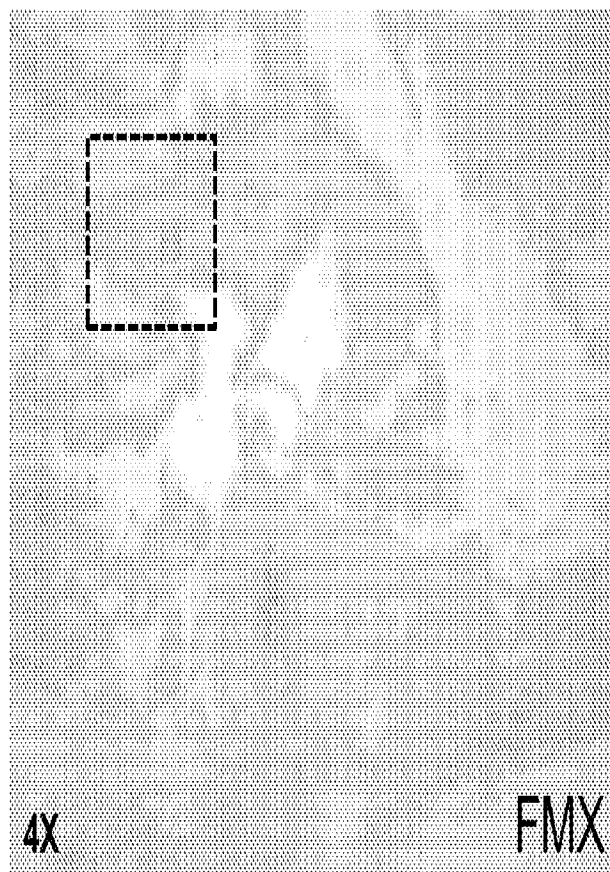
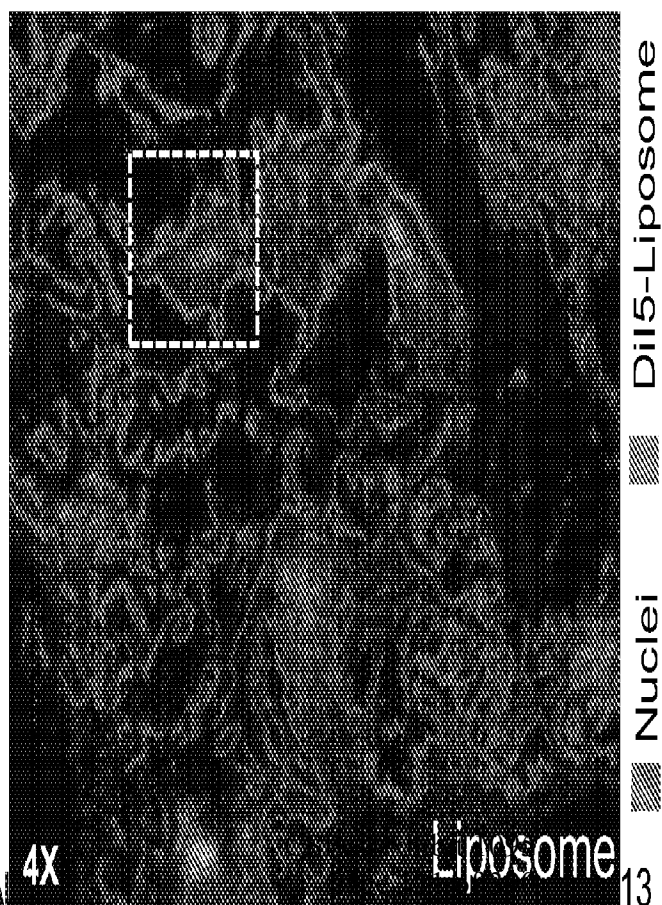
FMX staining co-localize with labeled liposomes

Pancreatic patient-derived xenografts
18254 and 18269



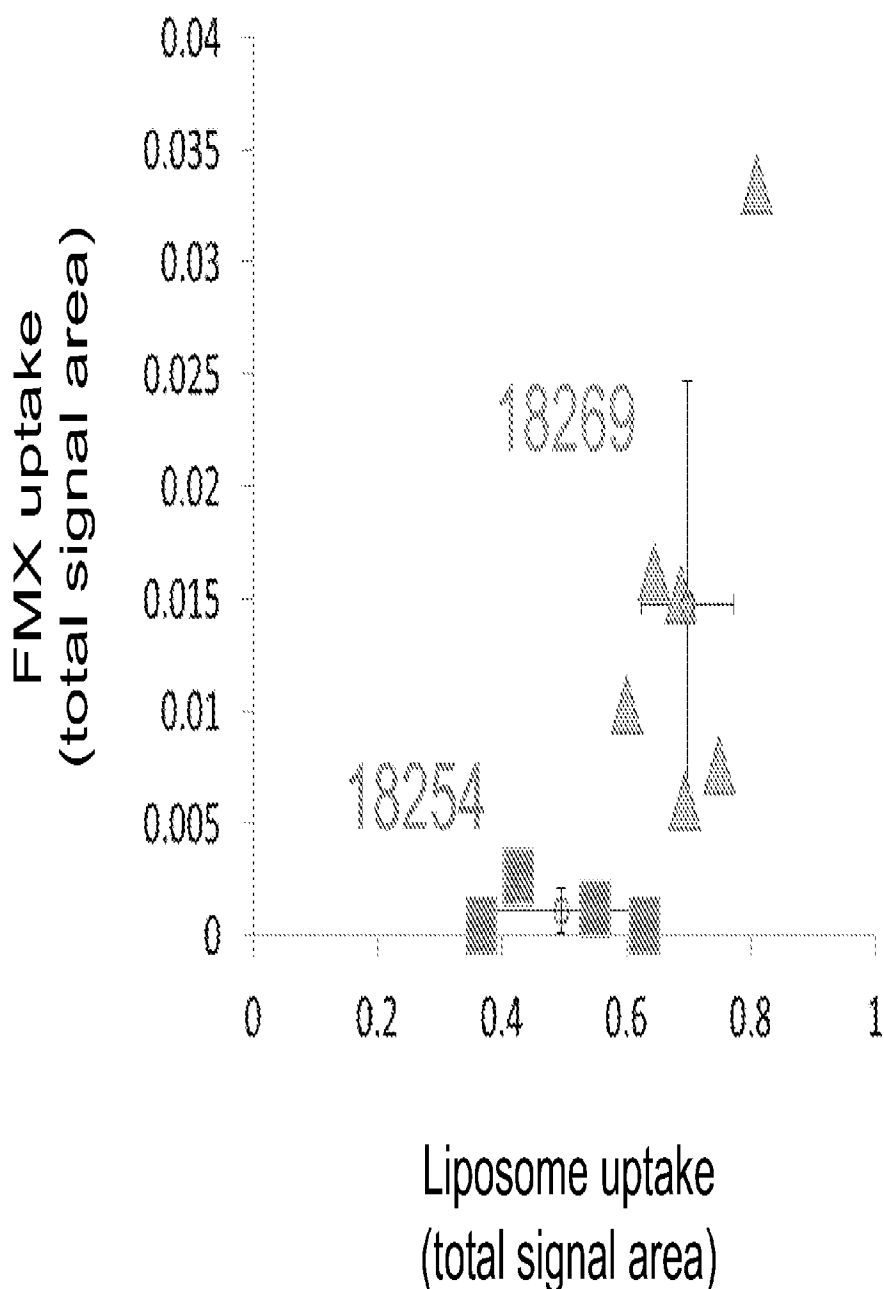
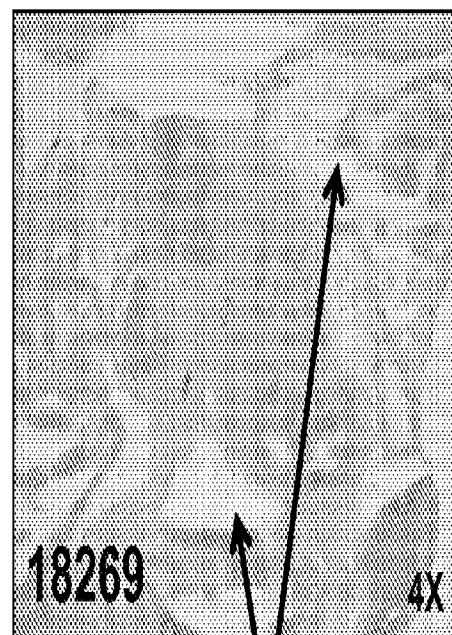
FMX – prussian blue stain

Liposomes – fluorescently (Dil5) labeled



FMX signal can differentiate between tumor model with varying liposome uptake (deposition)

IHC based quantification of FMX and liposome uptake in PDX tumor models

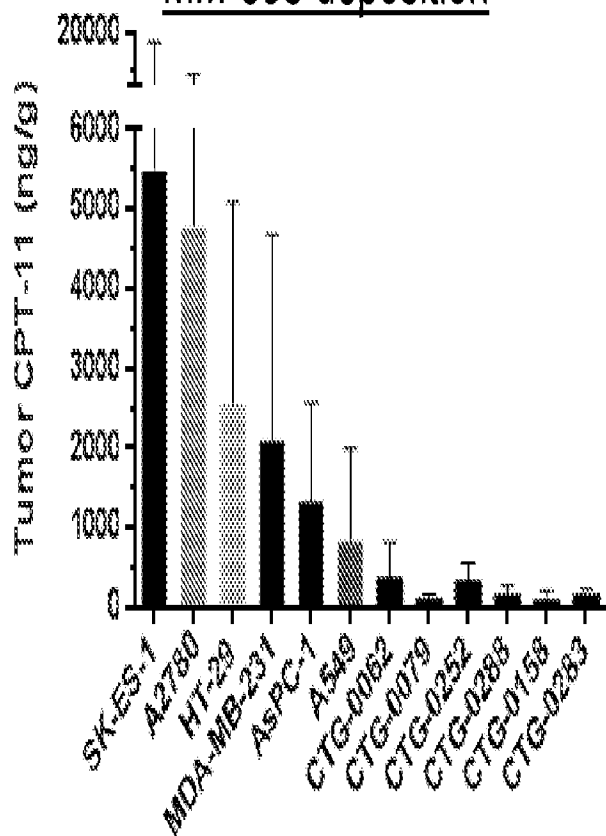


mucinous glands

FMX tumor accumulation: quantifying MRI signal

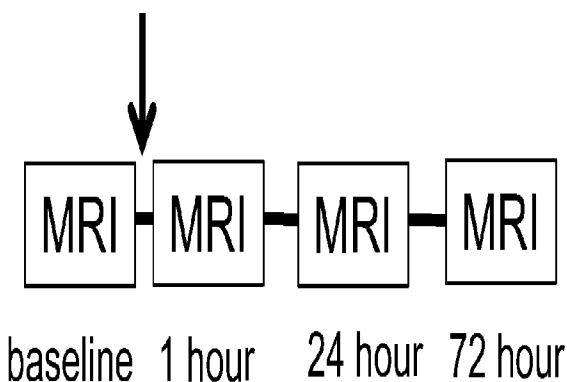
Selected models with varying

MM-398 deposition



Outline

FMX (20mg/kg)



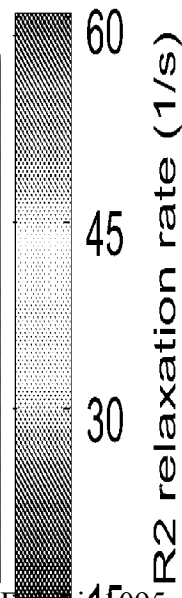
Majority of FMX cleared from plasma in 8 hours

R2 map images

Baseline

1 hour

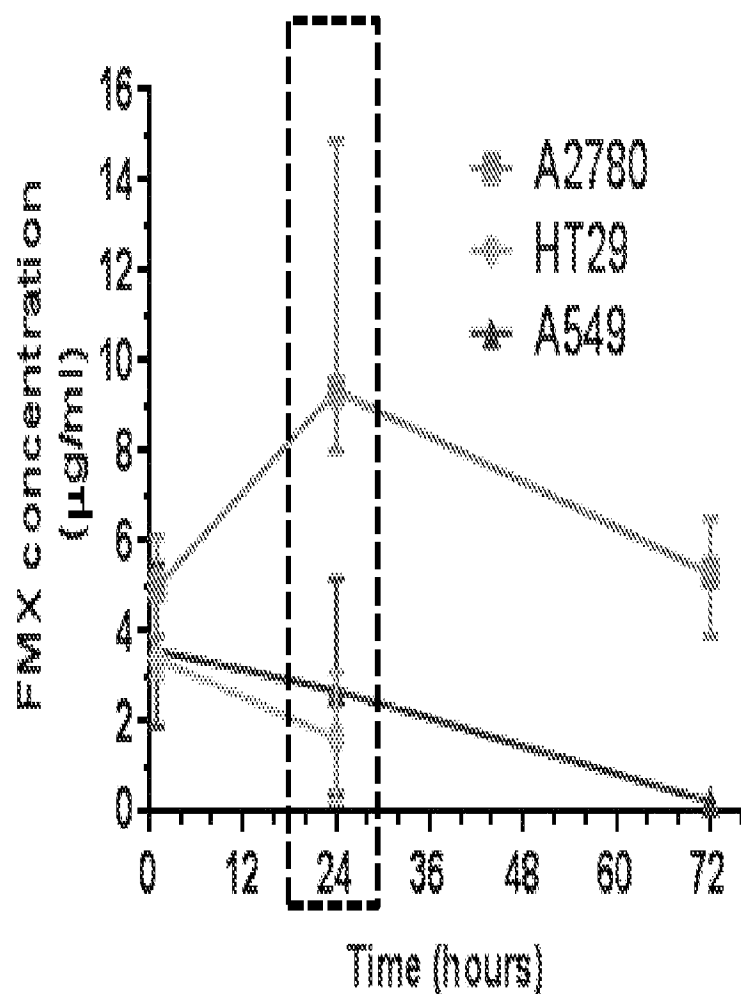
24 hour



Blood volume contribution

Tumor accumulation

Extended FMX accumulation observed in cell-line derived tumors



- Similar FMX levels at 1h
- Tumor accumulation of FMX observed at 24h and 72h in A2780
- Compare FMX signal at 24h to MM-398 deposition

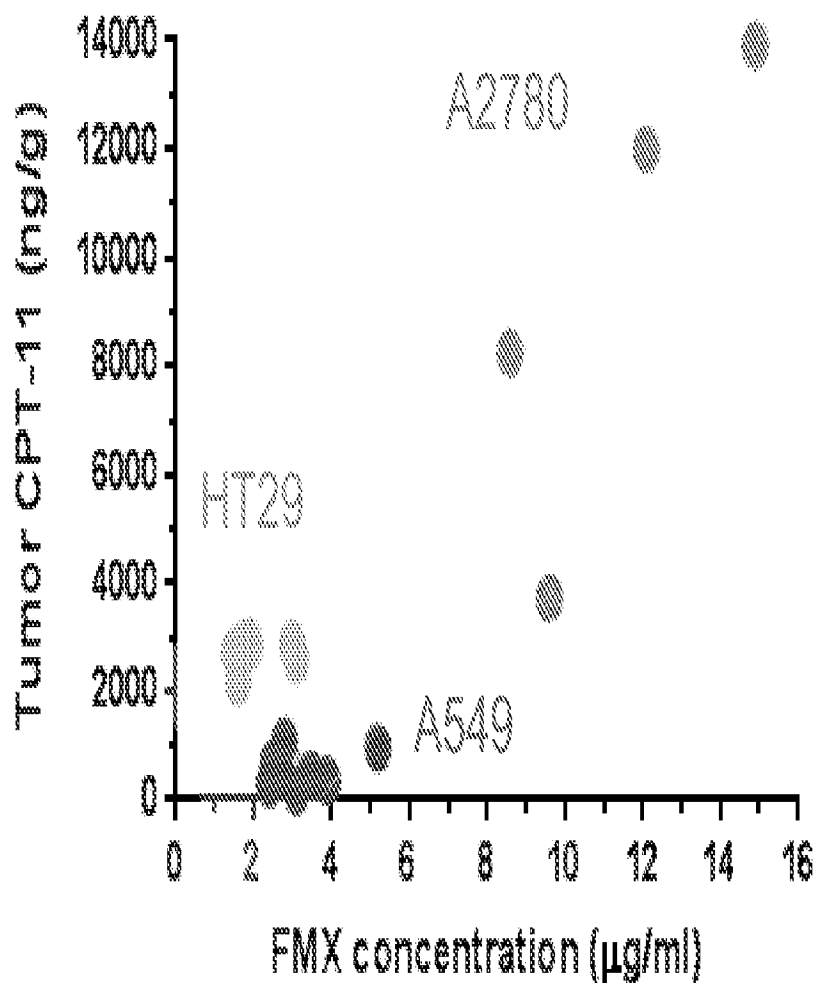
A2780

A549



T_2 relaxation Rate (1/s)

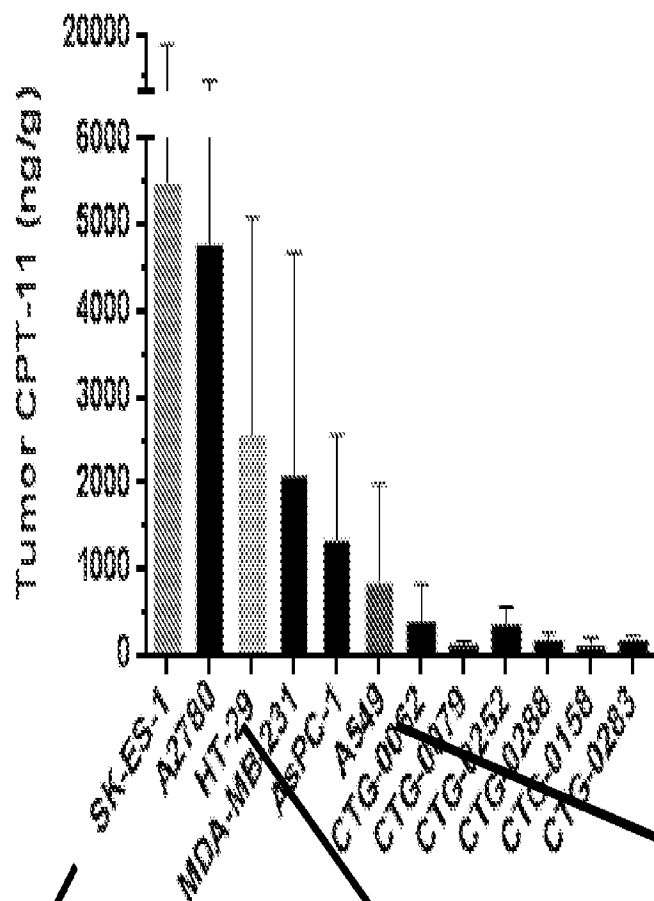
FMX-MRI can be used as a surrogate to determine MM-398 deposition



- MRI signal can differentiate between tumor models with high drug uptake and low drug uptake
- Studies to determine sensitivity of MRI signal ongoing

In vivo activity of MM-398 influenced by tumor deposition

Tumor deposition



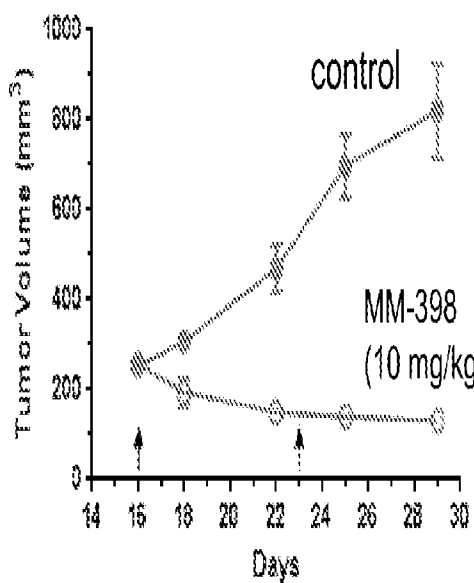
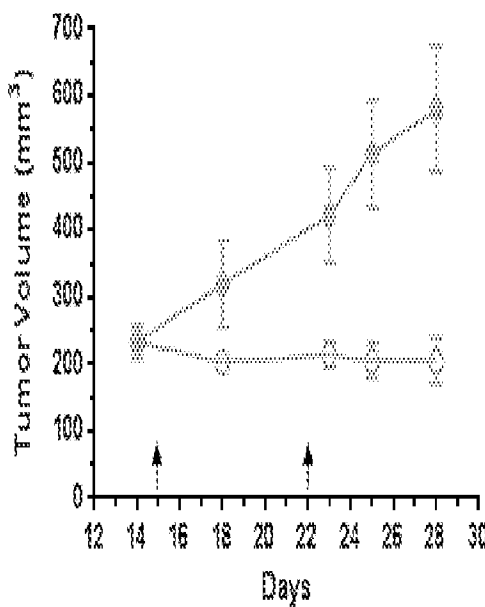
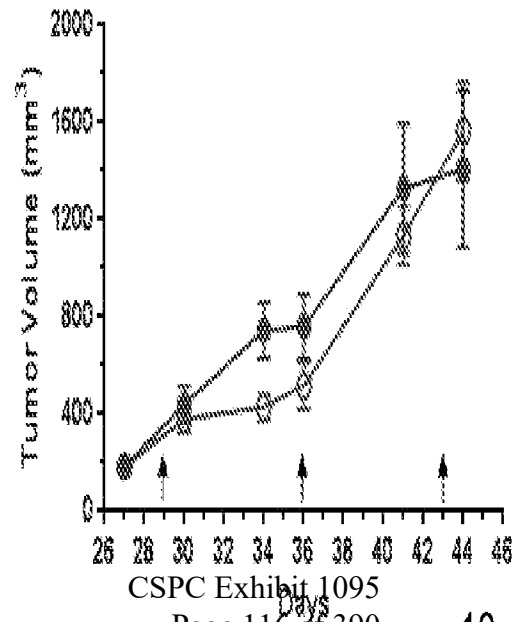
Similar in vitro IC_{50}
 HT-29 = 53nM
 A549 = 44nM

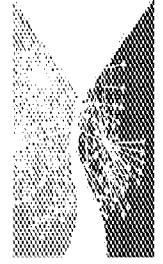
Tumor response

SK-ES-1

HT-29

A549

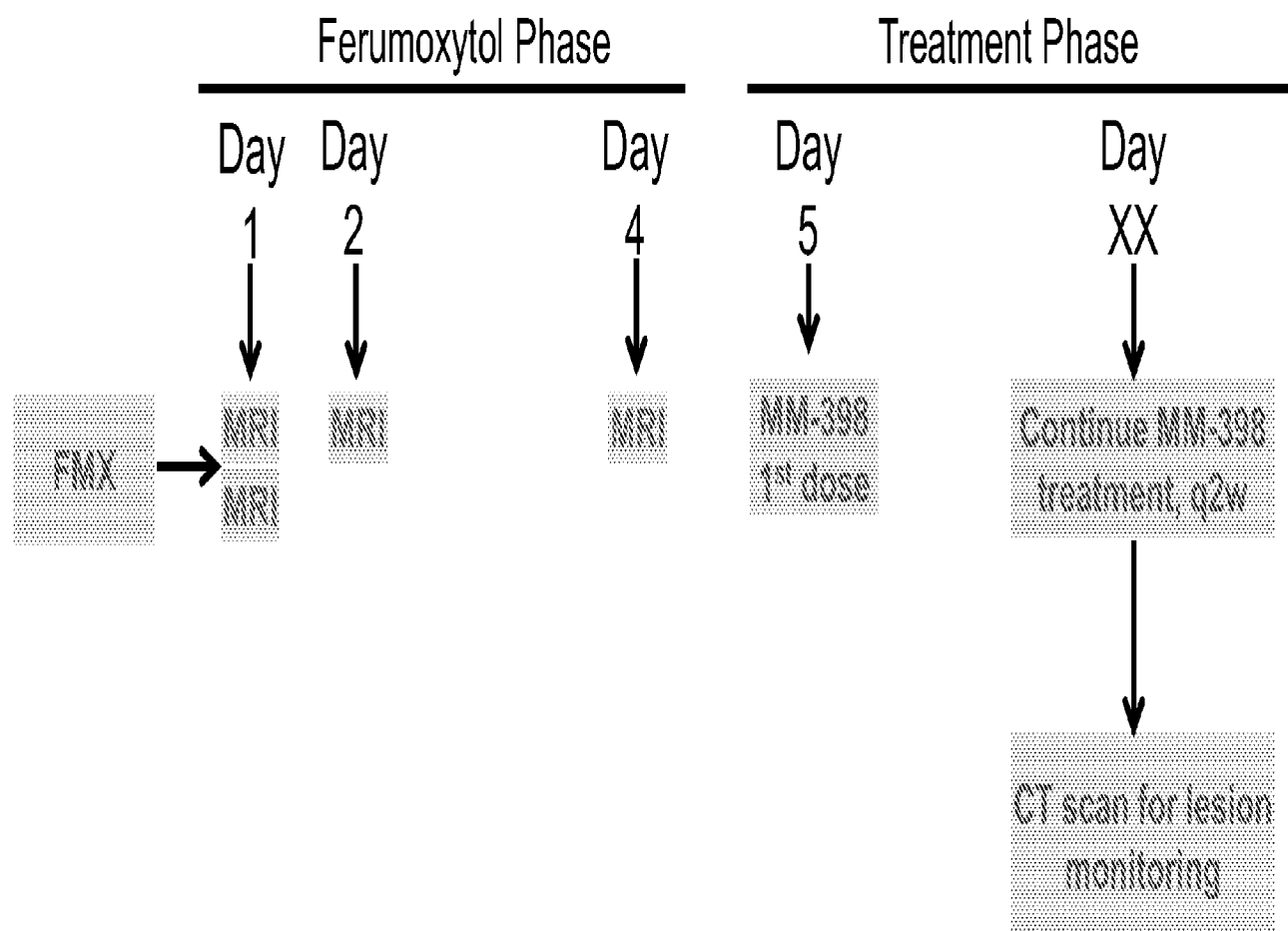
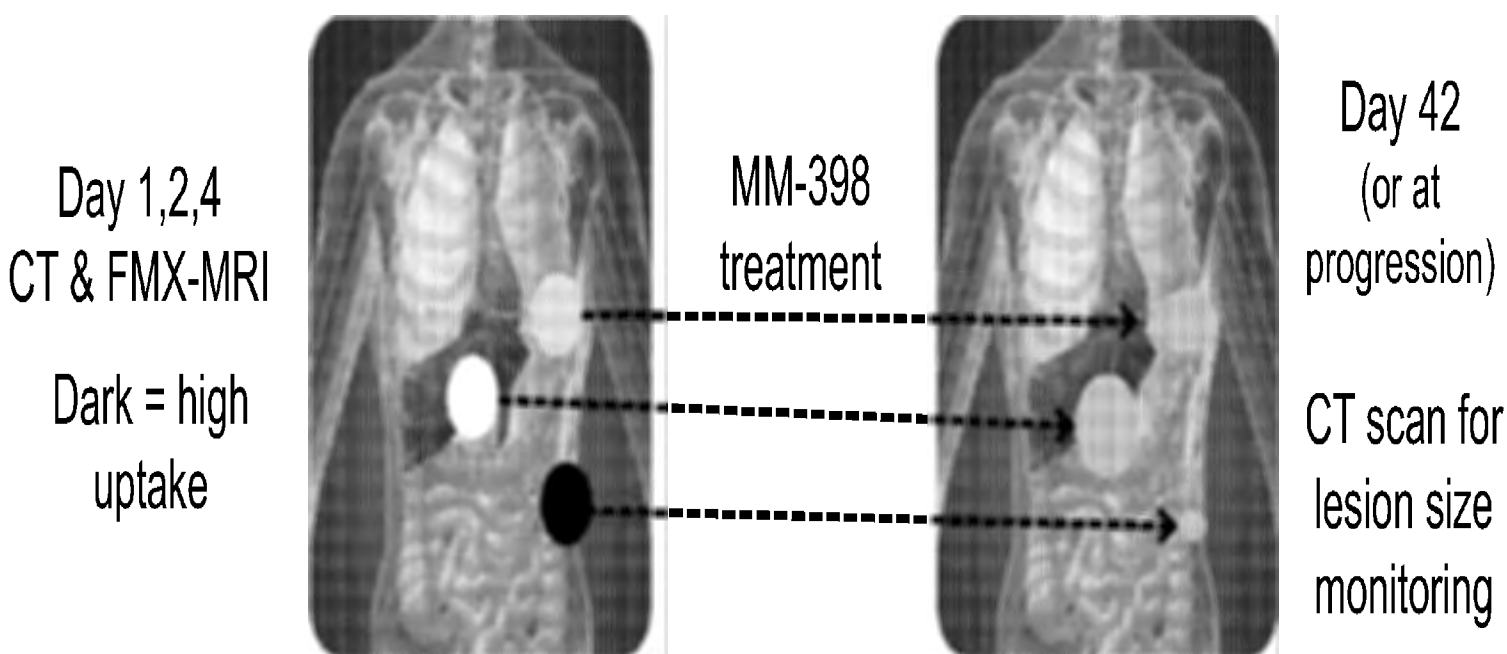




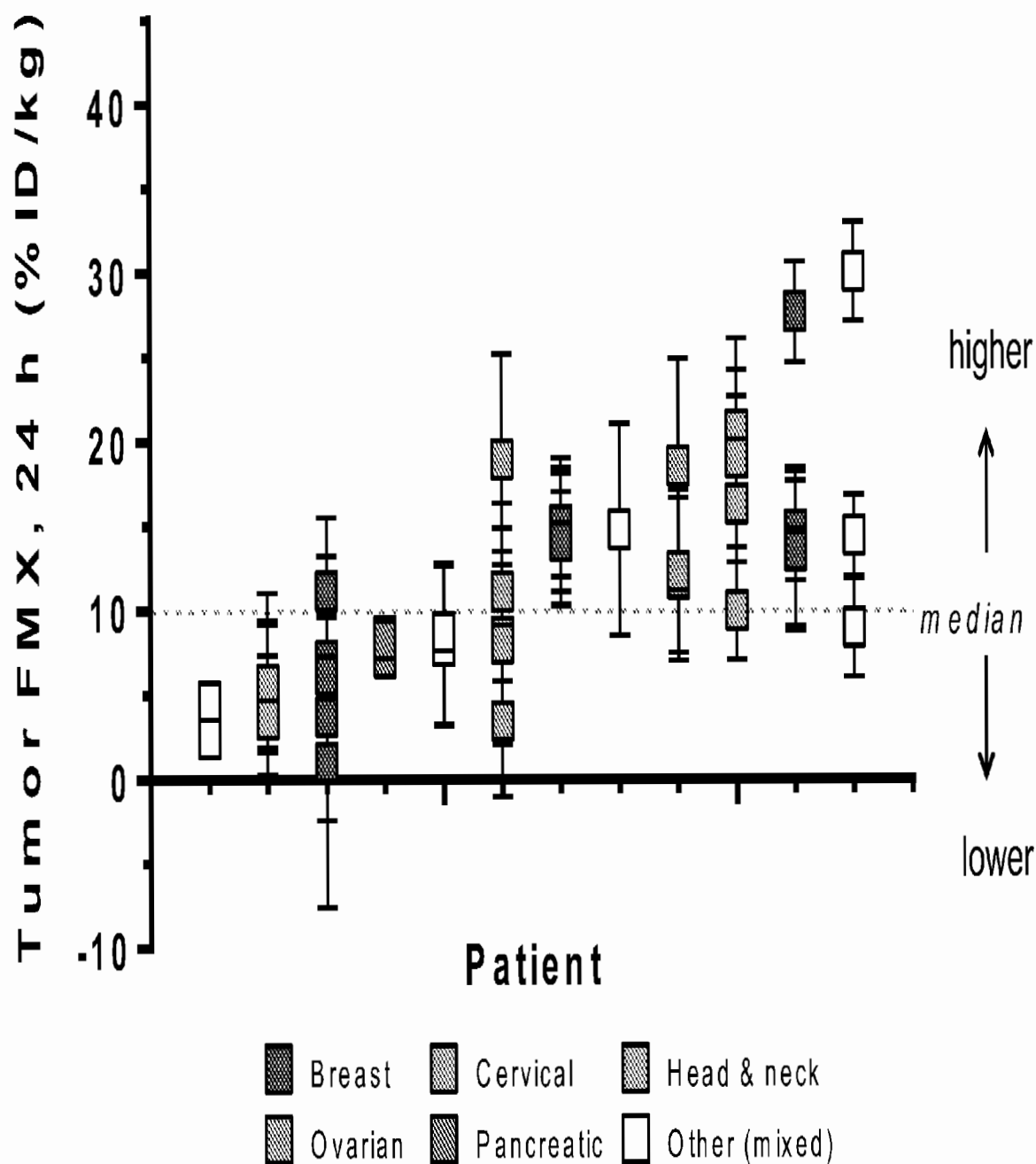
Outline

- MM-398 mechanism of action
 - Factors influencing MM-398 activity
- Diagnostic strategy to predict tumor delivery of MM-398
 - Ferumoxytol based contrast enhanced MRI in preclinical tumor models
- Translating into clinical setting
 - Preliminary data from Phase 1 feasibility study

Completed Phase 1 feasibility study quantifying ferumoxytol MRI

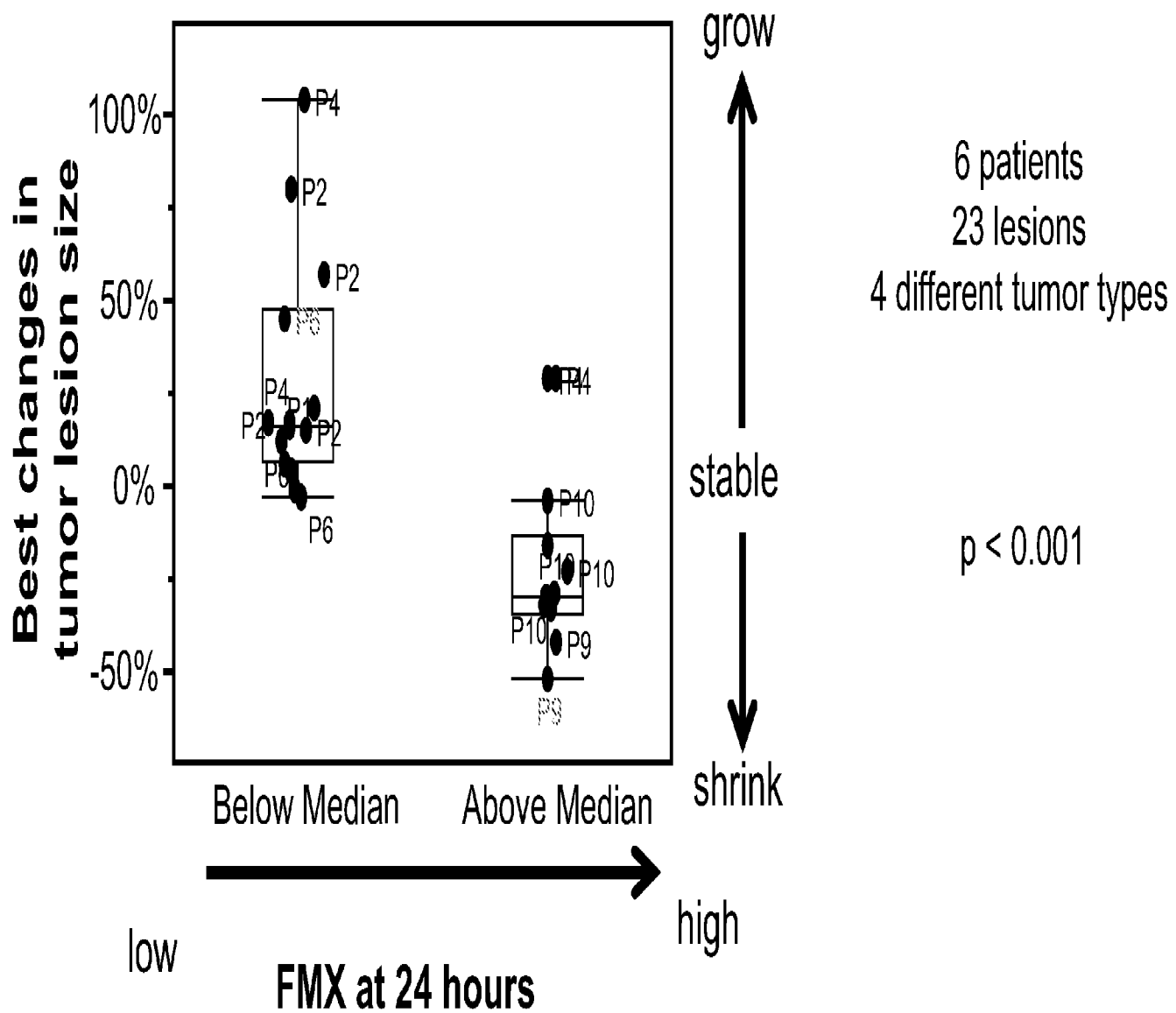


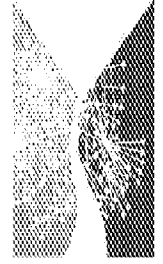
Quantification of FMX tumor delivery with MRI



- Deposition is variable across lesions and across patients
- Deposition is a continuous variable (ie. not bimodal)

FMX signal associated with lesion size shrinkage





Key messages

- Tumor deposition and activation are critical factors to determine MM-398 *in vivo* activity.
- Preclinical tumor models support the utility of FMX based MRI as a surrogate for MM-398 deposition.
- Potential use as an imaging diagnostic agent to predict tumor response to liposomal therapies.

Acknowledgements MM-398

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CSPC Exhibit 1095



Backup slides

Emerging utility of ferumoxytol-enhanced MRI

Recent investigational clinical applications

- Imaging intracranial aneurysm
- Imaging CNS inflammatory lesions
- Magnetic resonance angiography

Features	Ferumoxytol (FMX)	Gd-DTPA
Molecular composition	Ironoxide coated with semisynthetic carbohydrate	Gadolinium(III) chelated with DTPA
Particle size	~ 30nm	0.375nm
Plasma half life	14h	1.6h
Peak enhancement in brain tumors	24h	3.5-25min
Distribution	Initially estimates blood pool and later inflammatory cells	Extracellular space
Clinical significance of imaging	abnormalities in BBB (by imaging inflammatory cells)	Imaging abnormal vascularity in BBB
Clearance	Stored as iron reserve	renal

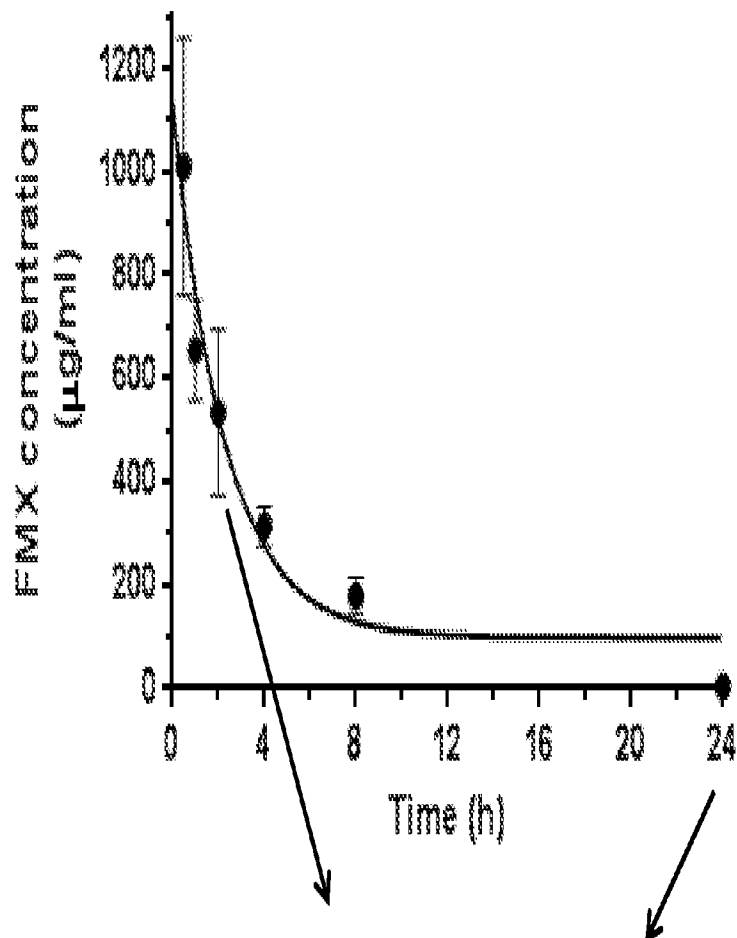
Kidney Int. 2009 March;75(5): 465-474

CSPC Exhibit 1095

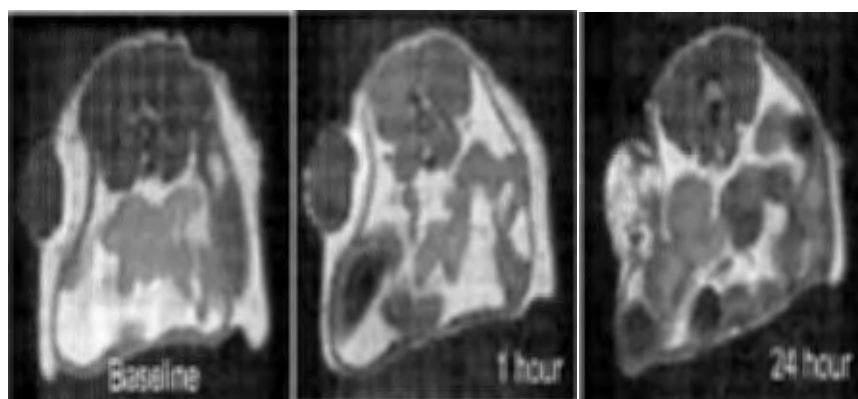
Page 124 of 390

FMX plasma PK and expected tumor accumulation: experimental outline

Plasma PK with FMX (20 mg/kg)



- Cleared from plasma within 8 hours
- Longer plasma circulation compared to other imaging agents (Gd) or small molecule drugs (irinotecan)
- Expect enhanced tumor accumulation (as MM-398)
- Tested in tumor models with varying deposition (A2780, HT29, A549)



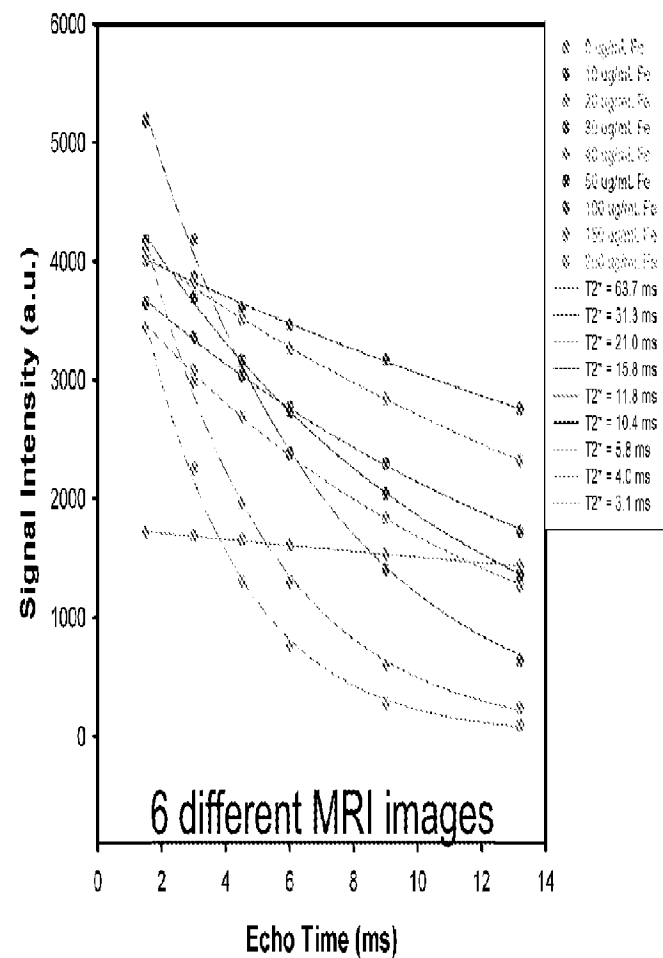
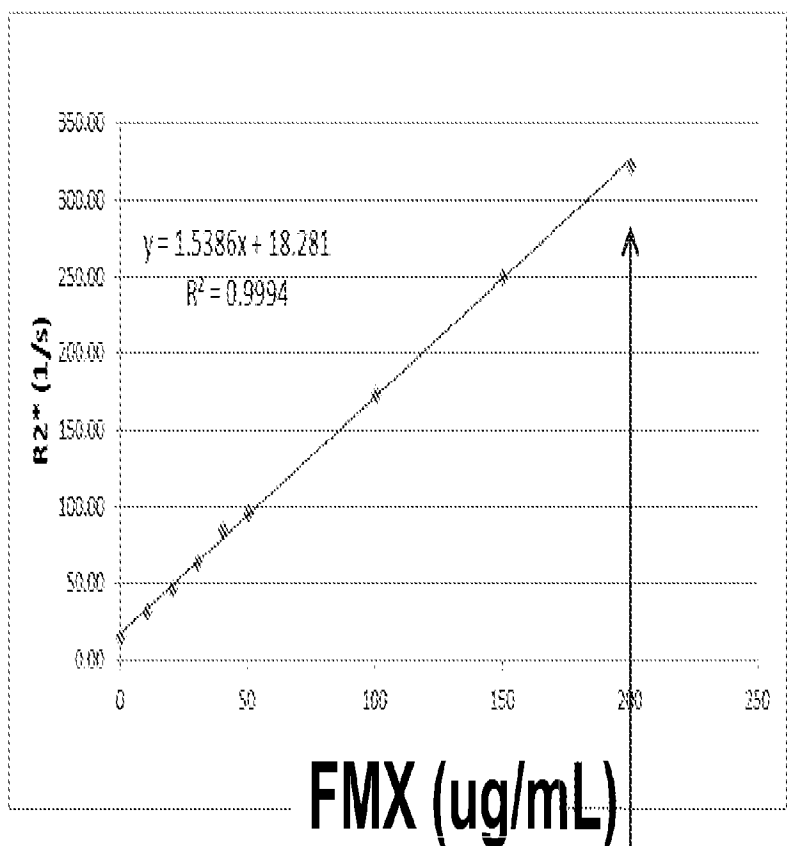
Blood volume
contribution

Tumor accumulation

R2 relaxation rate (1/s)

Estimating Relaxivity

Ferumoxytol Phantom, ~37°C, 1.5 Tesla



- Fit signal intensity vs. echo time with decaying exponential to determine relaxivity (R_{2*}).
- Slope is the relaxivity constant for ferumoxytol.

$$R_{2*} = \frac{1}{T_{2*}} = (R_{2*})_0 + (r_{2*}) \cdot [FMX]$$

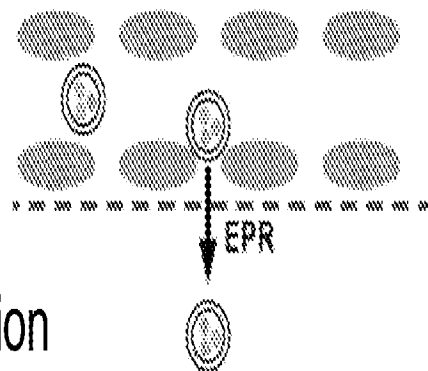
28

Mechanistic PK model predicts factors influencing SN-38 tumor exposure (duration)

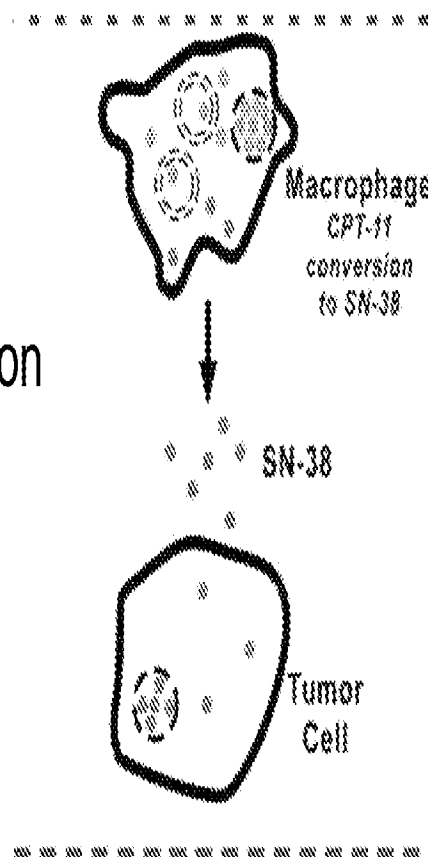
PK model trained on:

- multiple dose levels
- multiple time points
- plasma and tumor samples

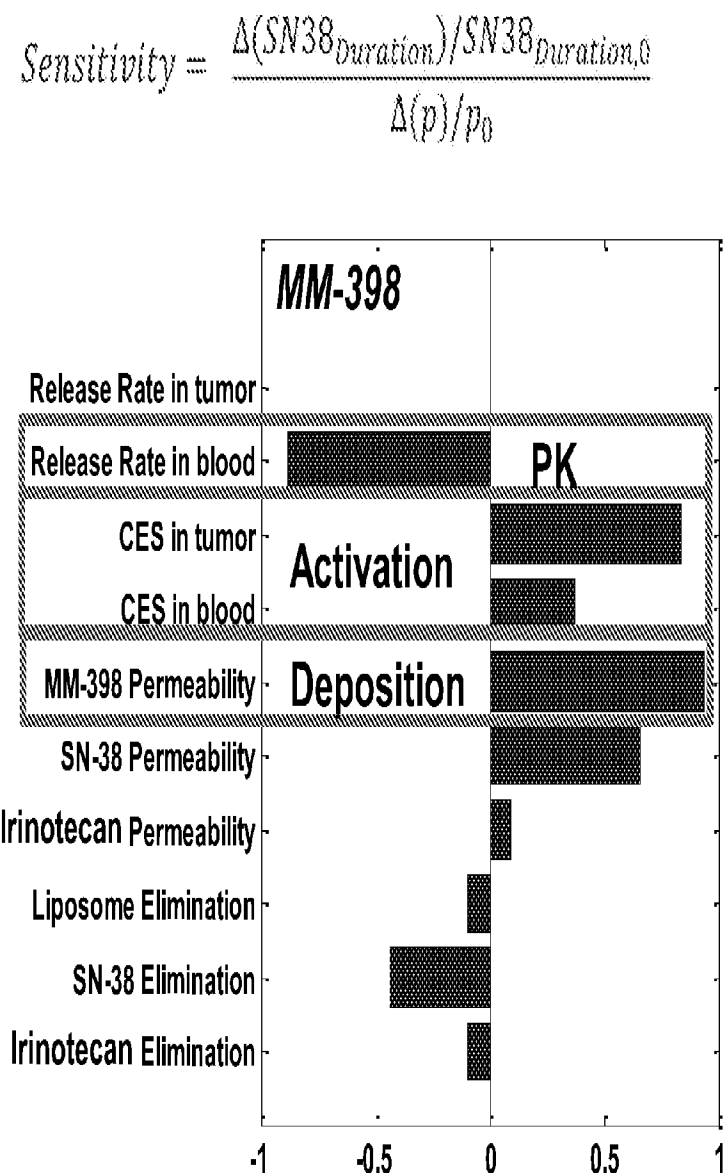
Measure in preclinical
xenograft models



1. Deposition



2. Activation



FMX quantification by MRI

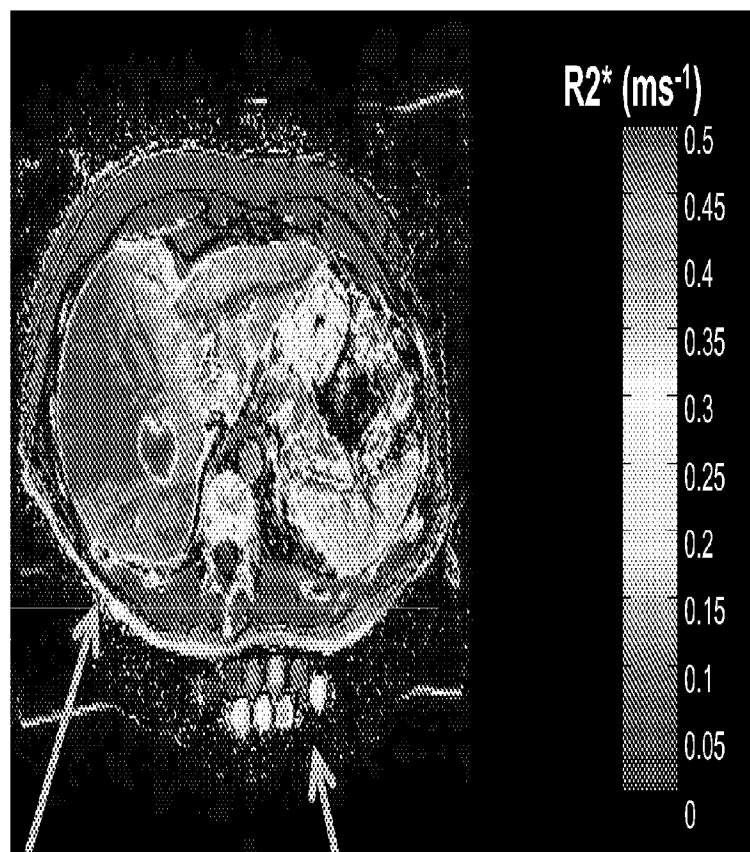
$$R_{2^*} = \frac{1}{T_{2^*}} = (R_{2^*})_0 + (r_{2^*}) \cdot [FMX]$$

Relaxivity Relaxation time Baseline relaxivity Ferumoxytol relaxivity constant Ferumoxytol concentration

If we measure these, we can determine this.

The diagram illustrates the equation for R_{2^*} and its components. The equation is enclosed in a dashed box. Arrows point from each term in the equation to a label below it: R_{2^*} to 'Relaxivity', T_{2^*} to 'Relaxation time', $(R_{2^*})_0$ to 'Baseline relaxivity', (r_{2^*}) to 'Ferumoxytol relaxivity constant', and $[FMX]$ to 'Ferumoxytol concentration'. A bracket under the first four terms points to the text 'If we measure these, we can determine this.', which then has an arrow pointing to the $[FMX]$ term.

Quantitative Ferumoxytol MRI



Patient
Ferumoxytol
Standard
Curve

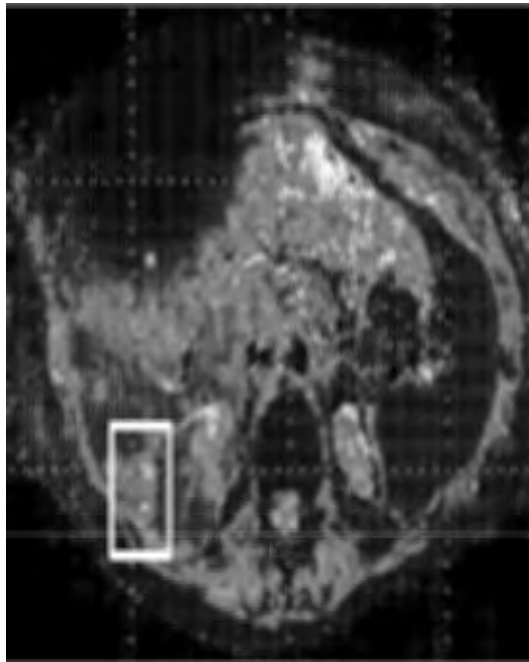
$$R_{2*} = \frac{1}{T_{2*}} = (R_{2*})_0 + (r_{2*}) \cdot [FMX]$$

Relaxivity Baseline relaxivity Ferumoxytol concentration

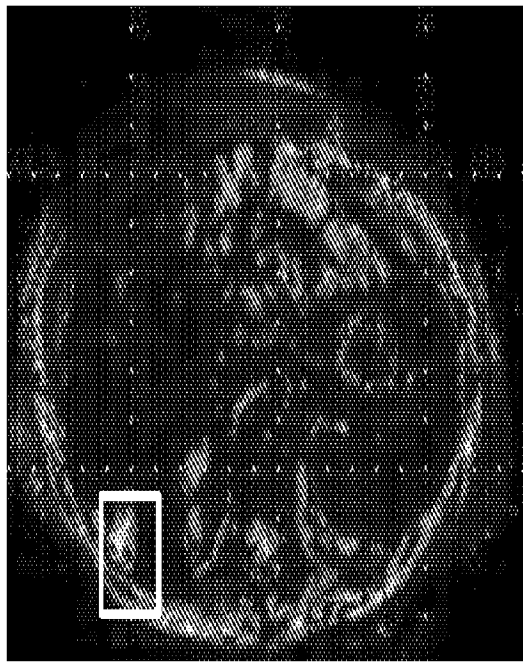
Relaxation time Ferumoxytol relaxivity constant

Ferumoxytol delivery is quantified by scanning the patient with a standard curve and subtracting off baseline tissue enhancement.

Anecdotal comparison of FMX signal from two patients and outcome



Pre-FMX

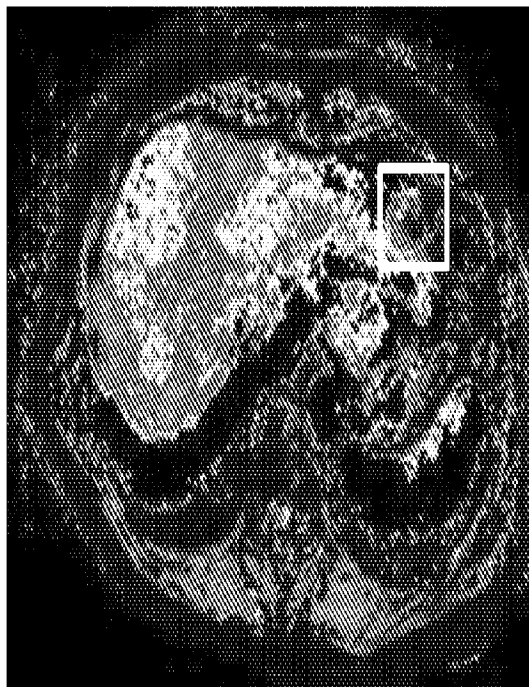
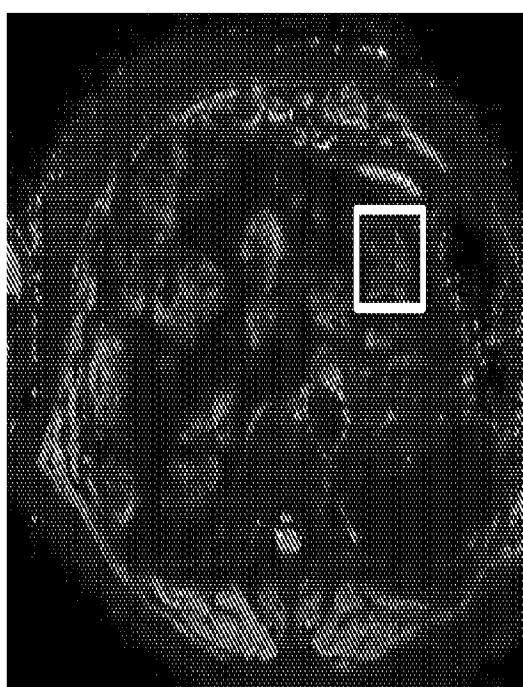


24 hours post FMX

ER+ BrCa patient

[FMX] = low

Lesion size $\uparrow$ 45%



ER+ BrCa patient
with partial response

[FMX] = high

Lesion size $\downarrow$ 49%

----- T2* weighted images -----

(darker = more FMX)

Activity of MM-398, Nanoliposomal Irinotecan (nal-IRI), in Ewing's Family Tumor Xenografts Is Associated with High Exposure of Tumor to Drug and High *SLFN11* Expression

Min H. Kang^{1,2}, Jing Wang^{1,2}, Monish R. Makena^{1,2}, Joo-Sang Lee^{1,2}, Nancy Paz³, Connor P. Hall^{1,2}, Michael M. Song^{1,2}, Ruben I. Calderon^{1,2}, Riza E. Cruz^{1,2}, Ashly Hindle^{1,2}, Winford Ko^{1,2}, Jonathan B. Fitzgerald³, Daryl C. Drummond³, Timothy J. Triche⁴, and C. Patrick Reynolds^{1,2}

Abstract

Purpose: To determine the pharmacokinetics and the antitumor activity in pediatric cancer models of MM-398, a nanoliposomal irinotecan (nal-IRI).

Experimental Design: Mouse plasma and tissue pharmacokinetics of nal-IRI and the current clinical formulation of irinotecan were characterized. *In vivo* activity of irinotecan and nal-IRI was compared in xenograft models (3 each in nu/nu mice) of Ewing's sarcoma family of tumors (EFT), neuroblastoma (NB), and rhabdomyosarcoma (RMS). *SLFN11* expression was assessed by Affymetrix Hux arrays, Taqman RT-PCR, and immunoblotting.

Results: Plasma and tumor concentrations of irinotecan and SN-38 (active metabolite) were approximately 10-fold higher for nal-IRI than for irinotecan. Two doses of NAL-IRI (10 mg/kg/dose) achieved complete responses maintained for >100 days in 24 of 27 EFT-xenografted mice. Event-free survival for mice with

RMS and NB was significantly shorter than for EFT. High *SLFN11* expression has been reported to correlate with sensitivity to DNA damaging agents; median *SLFN11* mRNA expression was >100-fold greater in both EFT cell lines and primary tumors compared with NB or RMS cell lines or primary tumors. Cytotoxicity of SN-38 inversely correlated with *SLFN11* mRNA expression in 20 EFT cell lines.

Conclusions: In pediatric solid tumor xenografts, nal-IRI demonstrated higher systemic and tumor exposures to SN-38 and improved antitumor activity compared with the current clinical formulation of irinotecan. Clinical studies of nal-IRI in pediatric solid tumors (especially EFT) and correlative studies to determine if *SLFN11* expression can serve as a biomarker to predict nal-IRI clinical activity are warranted. *Clin Cancer Res* 21(5): 1139–50. ©2015 AACR.

Introduction

Irinotecan hydrochloride, a water-soluble analogue of the natural alkaloid camptothecin (CPT; ref. 1), is first-line therapy in combination with 5-fluorouracil (5-FU) and leucovorin for metastatic colorectal cancer (2, 3). Carboxylesterase catalyzes irinotecan into SN-38, which is >100-fold more cytotoxic than irinotecan against tumor cells *in vitro* (4, 5). However, the complicated pharmacology of irinotecan has likely limited its clinical

activity. An active lactone form spontaneously hydrolyzes into the inactive carboxylate form in normal physiologic pH until the carboxylate form reaches equilibrium (~30% of total irinotecan; refs. 6, 7). Other pharmacologic disadvantages of irinotecan include fast elimination of the drug (8, 9), and diarrhea, due to β -glucuronidases produced by intestinal microflora that mediate hydrolysis of SN-38 glucuronide (inactive phase II metabolite of SN-38) to form the active metabolite SN-38 (10).

Liposomal formulations of irinotecan have been introduced (11, 12), which slow elimination and minimize exposure of the drug to serum (which at neutral to basic pH promotes hydrolysis of irinotecan), and thus stabilize the active lactone form versus the inactive carboxylate form (13). A nanoliposomal formulation of irinotecan, nal-IRI (also known as MM-398 or PEP02), employs a modified gradient loading method with sucrose octasulfate (13). Macrophage-mediated disruption of the nal-IRI, drug release, and conversion to SN-38 for *in vivo* require >24 hours, and preclinical studies demonstrated activity against colorectal and breast cancer subcutaneous xenografts (13), as well as intracranial glioblastoma tumor xenografts (14–16). A three-arm phase II study of nal-IRI versus irinotecan or docetaxel as a second-line therapy in patients with locally advanced or metastatic gastric or gastro-esophageal junction adenocarcinoma demonstrated an objective response rate comparable with docetaxel (17). A nal-IRI phase II study in

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-14-1882

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Translational Relevance

Despite high *in vitro* activity of the irinotecan active metabolite (SN-38), chemical and pharmacokinetic properties of irinotecan have potentially limited clinical activity. A nanoliposomal formulation of irinotecan (nal-IRI) with improved pharmacokinetic properties relative to irinotecan was developed, and is being tested in clinical trials. We demonstrated for nal-IRI compared with irinotecan advantageous pharmacokinetic properties and significantly greater antitumor activity in mouse xenograft models of pediatric solid tumors, especially in Ewing's sarcoma family of tumors (EFT). Expression of *SLFN11*, a putative DNA/RNA helicase, correlated with the *in vitro* activity of SN-38. In a panel of cell lines and in primary tumors, *SLFN11* mRNA expression was significantly higher for EFT relative to neuroblastoma or rhabdomyosarcomas. Our data confirm that nal-IRI improves systemic and tumor exposure to irinotecan and its active metabolite in murine xenograft models of childhood solid tumors, leading to enhanced antitumor activity. The high expression of *SLFN11* in EFT suggests a molecular mechanism for the striking activity of nal-IRI in EFT xenografts and provides a potential marker for drug sensitivity in clinical trials including an on-going phase I clinical trial of nal-IRI (SPOC-2012-001; NCT 02013336).

patients with metastatic, gemcitabine-refractory pancreatic cancer achieved a one-year survival rate of 25% (18). Nal-IRI combined with 5-FU and leucovorin demonstrated significantly improved overall survival for gemcitabine-refractory pancreatic cancer patients compared with 5-FU/leucovorin alone in a recently completed phase III pivotal trial (NAPOLI-1; ref. 19).

Here, we report the comparative pharmacokinetic profiles and antitumor activity in mouse xenograft models of pediatric solid tumors of nal-IRI and irinotecan (Camptosar). High expression of *SLFN11* has been reported for Ewing's family of tumors (20), and *SLFN11* expression has been associated with sensitivity to cytotoxic agents (21). Therefore, we also examined the correlation between *in vitro* activity of SN-38 and *SLFN11* expression in panels of pediatric cancer cell lines, the relative expression of *SLFN11* in pediatric primary tumors, and the correlation of *SLFN11* expression with the Ewing's-specific aberrant transcription factor EWS/FLI (22).

Materials and Methods

Drugs and chemicals

Merrimack Pharmaceuticals provided nal-IRI (nanoliposomal irinotecan-HCl, 5.0 mg/mL). Irinotecan (Camptosar, 20 mg/mL, free drug formulation) was from Baxter Healthcare Corporation. 4-HC was purchased from Niomech (Germany), and cyclophosphamide for *in vivo* studies was from Baxter. Topotecan, pure irinotecan-HCl, SN-38 (active metabolite of irinotecan), camptothecin (internal standard: IS), and all other chemicals were LC-MS grade and were purchased from Sigma-Aldrich Chemical Company. Methanol and acetonitrile were from Fisher Scientific. Normal mouse plasma was from Lampire Biological Laboratories and filtered by 6 mL charcoal packing Resprep SPE cartridges (Restek). Drug-free human plasma was obtained from the TTUHSC Blood Bank.

Cell lines

For these studies, we employed 20 Ewing's sarcoma (EFT), 20 neuroblastoma (NB), and 12 rhabdomyosarcoma (RMS) cell lines that were established in our laboratory or obtained from the originator of the line (Supplementary Table S1). All cell lines were grown in antibiotic-free RPMI-1640 supplemented with 10% heat-inactivated FBS or Iscoves Modified Dulbeccos Medium (IMDM) supplemented with 3 mmol/L L-glutamine, 5 µg/mL insulin, and 20% heat-inactivated FBS, were mycoplasma free, and were cultured and treated with drugs in a 37°C incubator with 5% CO₂. Cell line identity was confirmed at the time of experimentation by short tandem repeat (STR) genotyping (23) and compared with the Children's Oncology Group STR database (www.COGcell.org).

Plasma, tissue, and tumor pharmacokinetics

All animal studies were approved by the Animal Care and Use Committee at TTUHSC. Female BALB/c mice (6–8 weeks, 16–18 g) and nu/nu mice (6–8 weeks, 24–28 g) were from Charles River Laboratories. Irinotecan or nal-IRI was injected intravenously at 5, 10, or 20 mg/kg into the lateral tail vein of BALB/c mice once. Blood was collected by cardiac puncture at various time points under isoflurane anesthesia and centrifuged immediately at 15,700 × g for 5 minutes at 4°C to isolate the plasma. Organs were harvested, minced, mixed with 3 volumes of PBS, and homogenized on ice using a Tissue Tearor (BioSpec Products). Protein content in tissues was determined as previously described (24). Plasma and tissue homogenates were stored at –80°C until analyses.

For tumor pharmacokinetics, the human CHLA-136 neuroblastoma cell line (1.5×10^6 cells/100 µL) was injected subcutaneously into nu/nu mice and after 42 days irinotecan or nal-IRI was injected intravenously (20 mg/kg). Blood, organs, and tumors were collected and processed as described above 4, 24, and 48 hours after injection.

Pharmacokinetic analyses

The concentrations of irinotecan and SN-38 in mouse plasma and tissues were determined by an Agilent 1200 HPLC system (Agilent Technologies) and a Applied Biosystems 4000QTRAP LC-MS/MS system (Applied Biosystems) with data analysis software Analyst 1.42 (Applied Biosystems). Isocratic separation was achieved using 50% of methanol (0.1% formic acid) and 50% of 2 mmol/L ammonium formate in water (0.1% formic acid) on a 2.1 × 50 mm Waters XBridge C18 Column (3.5 µm; Waters) with a 2.0 × 4 mm Phenomenex Security Guard C18 cartridge (Phenomenex) at a flow rate of 0.3 mL/min. The retention times for irinotecan, SN-38, and internal standard (I.S.) were 1.20, 2.02, and 2.55 minutes, and the total run time was 6 minutes.

The column effluent was connected to an electrospray ionization interface operated in the positive mode (600°C and 3,000 V ionization voltage); nebulizer gas = 40 psi and turbo gas = 50 psi; collision-activated dissociation gas was set at medium and the curtain gas at 15 psi. The collision energies for irinotecan, SN-38, and camptothecin were 59, 37, and 33 eV, and the collision exit potential for the analytes were 12, 20, and 16 eV, respectively. The detection of the analytes was performed using multiple reaction monitoring. The transitions were *m/z* 588.4 → *m/z* 167.2 for irinotecan, *m/z* 393.2 → *m/z* 349.1 for SN-38, and *m/z* 348.8 → *m/z* 305.0 for camptothecin.

Plasma calibration standards of irinotecan were prepared at 1.0 to 500 ng/mL, and for SN-38 at 0.5 to 250 ng/mL in purified mouse plasma. Tissue calibrations of irinotecan and SN-38 were performed in blank mouse liver homogenate (1:3 dilution with PBS). Method validation was per FDA guidelines (<http://www.fda.gov/CDER/Cuidance/4252fnl.html>). The regression parameters of slope, intercept, and correlation coefficient were calculated by least-squares linear-regression analysis using a weight factor of $1/x^2$. For noncompartmental analysis, the area under the concentration *versus* time curve (AUC_{0-inf}) was calculated by using the trapezoidal rule extrapolated to infinity. Calculations were as follows: elimination half-life: $T_{1/2} = 0.693/ke$, systemic clearance: $CL_{tot} = \text{dose}/AUC$; and apparent volume of distribution: $V_d = \text{Dose}/(AUC_{0-inf} \times ke)$.

Conversion of irinotecan to SN-38 in mouse and human plasma

Irinotecan was spiked into blank human and mouse plasma to achieve final concentrations of 0, 10, 20, 50, 100, 250, 500, 750, and 1,000 ng/mL, incubated at 37°C for 24 hours, and reaction terminated by adding 600 μ L of ice-cold acetonitrile-methanol (1:1, 0.1% formic acid) and 2 μ L of internal standard solution (camptothecin 5 μ g/mL). The concentrations of SN-38 in plasma were determined by LC-MS/MS. V_{max} and K_m values were calculated using GraphPad Prism software.

Antitumor activity of nal-IRI in murine xenografts

Subcutaneous xenografts with human cell lines from EFT (CHLA-258, COG-E-352, CHLA-32, SK-N-MC, and CHLA-25; refs. 25), RMS [CB-NJR (26), SMS-CTR (27), and Rh30 (direct xenografts; ref. 28)], and neuroblastoma (CHLA-136, CHLA-79, and CHLA-119; ref. 29) were established in nu/nu mice. All cell lines were grown before xenograft implantation in antibiotic-free medium and verified to be free of mycoplasma (MycopAlert kit; Lonza) and cell line identity confirmed by STR at the time of experimentation (23). For each 6- to 8-week-old female athymic nu/nu mouse, 2.5 to 10 million tumor cells were mixed with RPMI-1640:Matrigel (1:1) and injected subcutaneously between the shoulder blades. Tumor volume was measured twice weekly (28, 30). Drug treatment (irinotecan or nal-IRI, 10 mg/kg/d, Monday and Thursday, a total of 2 doses were given) began when progressively growing tumors reached a mean of 150 mm³. Mice were sacrificed when the tumor volume exceeded 1,500 mm³ or serious morbidity was observed. To assess combination activity of nal-IRI + cyclophosphamide given in multiple doses, mice were treated for 4 weeks with intravenous nal-IRI 10 mg/kg/d, each Monday and Thursday, and intraperitoneal cyclophosphamide 25 mg/kg (every Monday).

Quantifying macrophage content in tumors

Chromogenic immunohistochemistry was conducted on 5- μ m-thick tumor frozen sections on fresh cut slides. Slides were baked for 30 minutes at 65°C, soaked in xylene, rehydrated through 100% and 80% graded alcohols, and pretreated with proteinase K (Invitrogen) for 15 minutes at room temperature. All further steps were done at room temperature in a hydrated chamber. Slides were blocked using Peroxidized1 (Biocare) and serum-free protein block (Dako) to quench endogenous peroxidase activity, and nonspecific binding, respectively, stained overnight with the F4/80 antibody (Serotec, MCA497C, 1.0 mg/mL) incubated at a 1:1,000 dilution, and was developed using the rat

on mouse HRP polymer kit (Biocare) as per the manufacturer and counterstained with hematoxylin. Slides were imaged on an Aperio Brightfield microscope at a 20 $\times$ magnification. The percentage of macrophage-positive area was quantified as a ratio of total viable tumor tissue area was carried out using Definiens Tissue Studio XD (Definiens).

SLFN11 expression in association with sensitivity to SN-38

We assessed cytotoxicity of SN-38 using the DIMSCAN cytotoxicity assay as previously described (31, 32) and *SLFN11* expression by Taqman real-time reverse transcription-PCR (RT-PCR) as previously described (33) using primers and a probe from Applied Biosystems in 20 EFT, 20 NB, and 12 RMS cell lines (Supplementary Table S1). Relative IC₅₀ (rIC₅₀) was calculated based on NCI Pediatric Preclinical Testing Program (PPTP) guidelines (32). *SLFN11* expression was also measured by Human Exon (HuEx) 1.0 ST Array (Affymetrix) in 17 EFT cell lines and expression measured by HuEx arrays correlated with expression by RT-PCR ($r = 0.62$, $P = 0.007$). *SLFN11* protein expression was assessed by immunoblotting (antibody from Santa Cruz) as previously described (33).

SLFN11 expression in primary tumors

Transcriptional profiling was performed on 150 cases of childhood tumors, 50 each of EFT, NB, and RMS (both embryonal and alveolar, in equal numbers) using Affymetrix Human Exon (HuEx) arrays. Data were imported and normalized using PLIER and quantile normalization. Mean expression values were set to 80 per the manufacturer's recommendation. The expression levels for both Schafen family member 11 (*SLFN11*) and Friend leukemia integration site 1 (*FLI1*) were analyzed for each tumor in each category and compared individually and categorically. Results were expressed as the log to base 10 in each case. For comparison of *SLFN11* versus *FLI1*, a scatter analysis with equal x and y axes (log₁₀) was employed. The R^2 value was calculated using a simple linear regression model to determine the correlation coefficient.

Statistical analysis

Mouse event-free survival (EFS) was graphically represented by the Kaplan-Meier analysis, and EFS between groups was analyzed by the log-rank test using GraphPad Prism (GraphPad Software). EFS was defined as the time from initiating treatment until death, tumor size greater than or equal to 1,500 mm³, or treatment-related toxicity. For pharmacokinetic experiments, the statistical differences were evaluated by the Student t test. Correlation between rIC₅₀ and *SLFN11* expression was determined by Pearson r correlation. The differences in macrophage content in tumors were assessed by the one-way ANOVA. $P < 0.05$ was considered significant.

Results

Antitumor activity of irinotecan or nal-IRI in xenograft models of pediatric cancers

We compared the antitumor activity of nal-IRI (10 mg/kg/dose, total 2 doses: Monday and Thursday) to irinotecan (10 mg/kg/dose, total 2 doses: Monday and Thursday) in xenograft models (three each) of EFT, RMS, and NB. Treatment with nal-IRI significantly extended EFS compared with control in seven of nine xenograft models and compared with irinotecan in six of nine models (Fig. 1, P values are included in Supplementary Table S2).

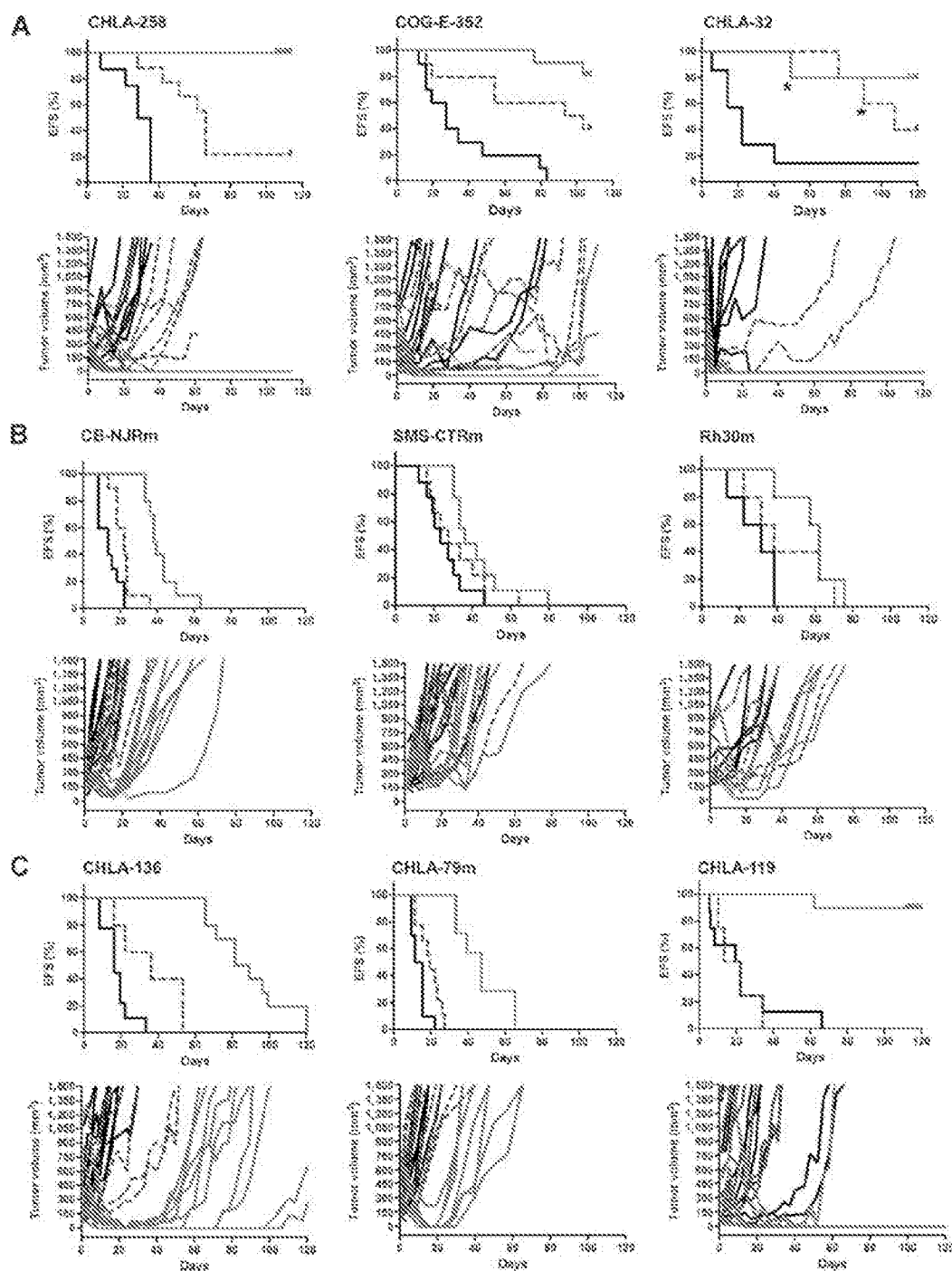


Figure 1.

EFS of tumor-bearing mice. Athymic (nu/nu) mice were engrafted subcutaneously with cell lines from (A) EFT (CHLA-258, COG-E-352, and CHLA-32), (B) RMS (CB-NJRM, SMS-CTRM, and Rh30m), or (C) NB (CHLA-136, CHLA-79m, and CHLA-119). When the average tumor size reached 150 mm³, the mice were treated with vehicle control (solid black line), 10 mg/kg/d of irinotecan (dotted blue line), or 10 mg/kg/d of nal-IRI (solid red line) in a single injection on day 1 and day 5. Then, the mice were observed for weight changes, diarrhea, or any signs of distress. Mouse EFS (top plot) was quantified as the time taken from the initiation of treatment until the tumor size reached 1,500 mm³, or for mice to be killed due to treatment-related toxicity. Tumor volume (bottom plot) of individual mice is presented for control (solid black line) versus irinotecan (dotted blue line) nal-IRI (solid red line). Each line represents the percentage of mice remaining event free over time. The number of mice per group, the median EFS, and the *P* values are listed in Supplementary Table S2. *, mice found dead, unknown cause not by tumor progression.

Maintained complete responses were observed over 110 days in the majority of mice treated with nal-IRI, but not free irinotecan, in all EFT xenograft models (CHLA-258, COC-E-352, and CHLA-32). The number of mice per group, the median EFS, and the *P* values are listed in Supplementary Table S2. In one of three RMS mouse models (CB-NJRm), nal-IRI-treated mice showed significantly greater EFS (median survival, 38.5 days) compared with irinotecan (22 days, $P < 0.0001$) or control mice (13 days, $P < 0.0001$). In the three neuroblastoma xenograft models (CHLA-136, CHLA-79m, and CHLA-119), EFS with nal-IRI was greater than with irinotecan ($P < 0.0004$) or vehicle control ($P < 0.0001$), but duration of responses in two of the three NB models was <100 days in the majority of mice treated with nal-IRI. Dosing of 10 mg/kg/d for both nal-IRI and irinotecan was well tolerated in xenografted mice; body weight during the course of therapy is shown for two representative models in Supplementary Fig. S1A. Overall, EFT xenograft mice treated with nal-IRI showed significantly longer EFS relative to RMS or NB (Supplementary Fig. S1B).

We also evaluated nal-IRI alone and when combined with cyclophosphamide when given weekly for 4 weeks in five xenograft models of EFT, including xenografts from cell lines established from progressive disease after myeloablative therapy (CHLA-258 and COC-N-352), compared with three RMS xenograft models. Cyclophosphamide was chosen for the combination studies due to its common use in pediatric oncology, and our data showing that the active metabolite of cyclophosphamide (4-HC) showed the best synergy with topoisomerase I inhibitors among tested agents (34). EFS for all 5 EFT xenograft models treated with nal-IRI + cyclophosphamide was 92% at 150 days, but only 13% at 110 days for the 3 RMS xenografts (Supplementary Fig. S1C). EFS for nal-IRI + cyclophosphamide in RMS xenografts at 80 days was 27% compared with 0% for nal-IRI as a single agent ($P = 0.0014$). However, due to the high activity against EFT of nal-IRI, the increase in EFS for nal-IRI + cyclophosphamide of 92% compared with 73% for nal-IRI alone was not significant ($P = 0.08$).

Plasma and tissue pharmacokinetics in nontumor-bearing mice

Plasma and tissue pharmacokinetic profiles for irinotecan and SN-38 following the administration of free irinotecan or nal-IRI at 5, 10, and 20 mg/kg in nontumor-bearing BALB/c mice are shown in Fig. 2A. After a 20 mg/kg dose of free irinotecan, the observed maximum plasma concentration of irinotecan was 2.8 ± 0.3 $\mu\text{g/mL}$ at 5 minutes after injection, whereas nal-IRI at the same dose achieved 197.0 ± 10.3 $\mu\text{g/mL}$ at 1 hour ($P < 0.01$). At 8 hours after injection with free irinotecan, the irinotecan concentration was <0.01 $\mu\text{g/mL}$, whereas it was 98.0 ± 12.7 $\mu\text{g/mL}$ at 8 hours after nal-IRI injection ($P < 0.01$). The rapid clearance of SN-38 from mouse blood following free irinotecan administration was consistent with the observed irinotecan clearance. The plasma concentration of SN-38 after injection of nal-IRI showed an increase up to 12 hours after injection followed by a gradual decrease over time. The pharmacokinetic parameters in mice for both formulations are summarized in Supplementary Table S3. The half-life of irinotecan (0.8–1.1 hours) in plasma was significantly shorter than that of nal-IRI (2.6–4.4 hours, $P = 0.001$), and the clearance was greater with irinotecan (138–300 mL/h) compared with nal-IRI (0.2–0.4 mL/h, $P = 0.024$).

After the administration of irinotecan we observed a concentration drop to 1/20–1/300 of the maximum concentrations over 8 hours in all tissues (Fig. 2B). Irinotecan and SN-38 levels were higher in liver, spleen, and kidney relative to the lung and brain. Irinotecan and SN-38 concentrations achieved by nal-IRI were comparable with free irinotecan soon after intravenous injection, but elimination of both irinotecan and SN-38 from tissues was substantially slower for nal-IRI than for free irinotecan (Fig. 2C).

Biodistribution in tumor-bearing mice

In nu/nu mice bearing human NB xenografts (CHLA-136), intravenous injection of 20 mg/kg free irinotecan achieved a irinotecan plasma level of 0.06 ± 0.01 $\mu\text{g/mL}$ at 4 hours after injection, barely detectable above the limit of quantitation at 24 hours (Fig. 3A, left). At the same dose, nal-IRI showed plasma irinotecan concentrations at 4 hours (131.4 ± 35.8 $\mu\text{g/mL}$) 1,000-fold higher than achieved with free irinotecan. Irinotecan was above the detection limit (0.014 ± 0.005 $\mu\text{g/mL}$) 48 hours after nal-IRI injection. Plasma SN-38 concentrations were >100-fold higher with nal-IRI relative to irinotecan (Fig. 3A, right).

Elimination of irinotecan and SN-38 for most tissues was more rapid with free irinotecan relative to nal-IRI in tumor-bearing nu/nu mice (Fig. 3B–F), similar to what was observed in nontumor-bearing mice. At 48 hours after injecting free irinotecan, the irinotecan levels in tissues and tumor were decreased by 2 orders of magnitude compared with those at 4 hours, and the concentration in the brain was below the detection limit (Fig. 3B). Elimination of nal-IRI in tissues and tumor was considerably slower than observed for irinotecan (Fig. 3B–F). After 4 hours of nal-IRI injection, the tissue levels of irinotecan and SN-38 in the liver reached 548 ± 396 $\mu\text{g/g}$ protein (irinotecan) and 15.1 ± 8.6 $\mu\text{g/g}$ protein (SN-38), which are the highest in all tissues measured (Fig. 3E). The levels of SN-38 in tumor at 48 hours with nal-IRI were almost 10-fold higher than we observed with irinotecan (Fig. 3F).

In vitro conversion of irinotecan to SN-38

As liposomal stabilization of irinotecan should minimize release of the drug before it reaches tumor sites, we did not expect to see >100-fold higher plasma SN-38 levels in nal-IRI-injected mice compared with free irinotecan-injected mice (Fig. 3A). As nal-IRI-injected mice did not experience greater systemic toxicities relative to irinotecan-injected mice, it was speculated that higher SN-38 concentrations might have resulted after samples were collected. Thus, we assessed two methods of blood sample collection, direct extraction without freezing plasma samples versus freezing samples for future analyses. Freezing samples for future analyses resulted in approximately 2-fold higher SN-38 concentrations up to 20 hours after the injection of nal-IRI (Supplementary Fig. S2A and S2B), indicating that irinotecan is further converted to SN-38 during the freeze-thaw process by carboxylesterase in mouse plasma.

A plasma carboxylesterase content is higher in mice than in humans (35), and postcollection conversion of irinotecan to SN-38 may be less of an issue in humans. Indeed, *in vitro* enzyme kinetic experiments demonstrated more limited conversion of irinotecan to SN-38 for human versus mouse plasma at 24 hours (37°C). The Michaelis–Menten kinetic parameters of V_{max} (maximum velocity) and K_m (substrate concentration that yield a half-maximal velocity) were 0.3 pmol/hr/mg protein and 12,471 ng/mL (31.8 $\mu\text{mol/L}$) in human plasma, and 1.3 pmol/hr/mg

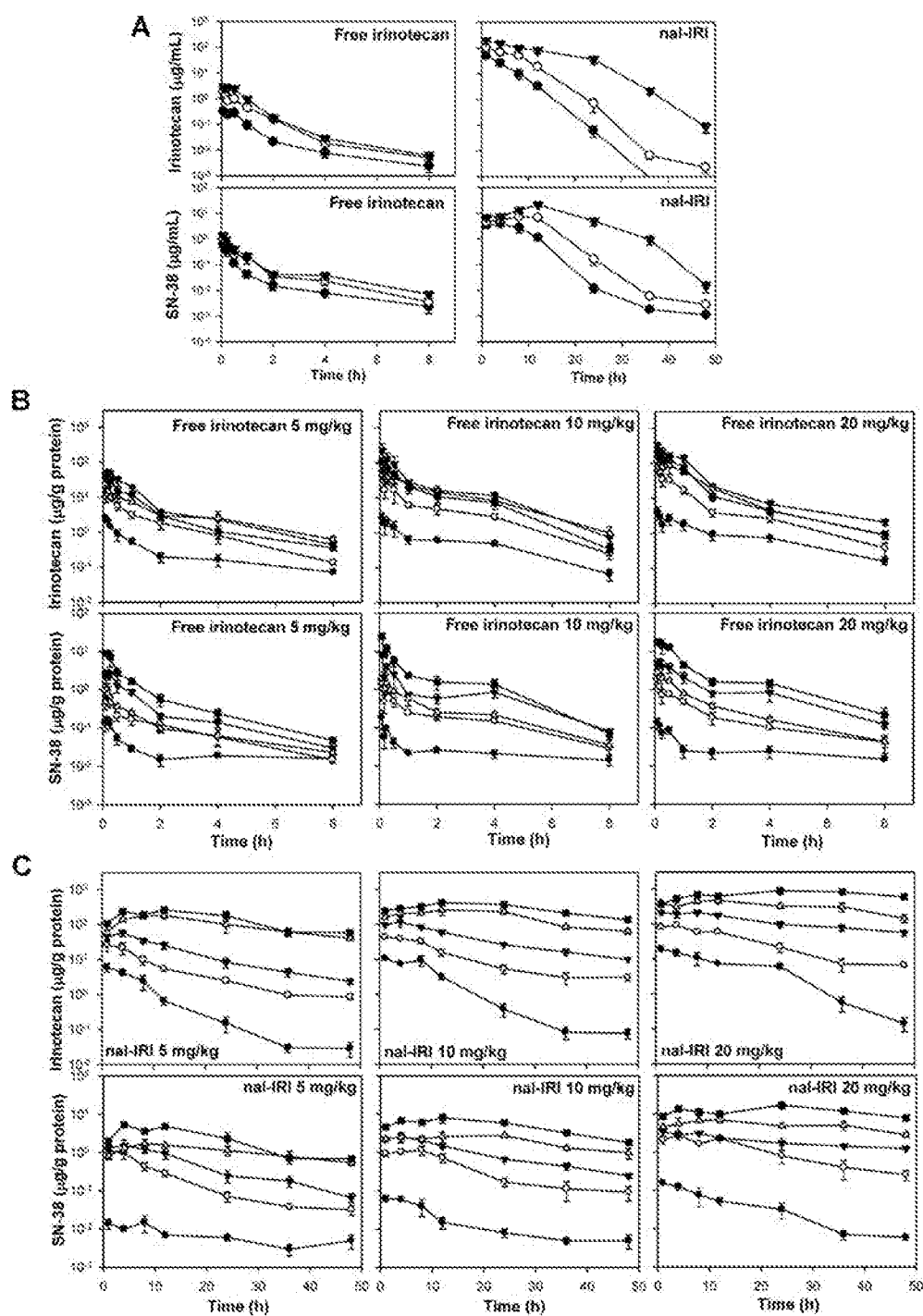


Figure 2.

Plasma and tissue pharmacokinetic profiles for irinotecan and SN-38 following single doses of irinotecan or nal-IRI. A, concentration of irinotecan (top plot) and SN-38 (bottom plot) as a function of time after irinotecan (left) or nal-IRI (right) injection. Female BALB/c mice were injected intravenously with a single dose of 5 mg/kg (●), 10 mg/kg (○), or 20 mg/kg (■) of drug solution. Blood samples were collected by cardiac puncture at various time points: 5, 10, 15, and 30 minutes, 1, 2, 4, and 8 hours for irinotecan; 1, 4, 8, 12, 24, 36, and 48 hours for nal-IRI. Points, mean; bars, $\pm$ SE ($n = 3$). B and C, tissue elimination profiles for irinotecan and SN-38 following three single doses of irinotecan (B) or nal-IRI (C). Female BALB/c mice were injected intravenously with 5, 10, or 20 mg/kg dose of drug solution. Mouse tissues include brain (●), lung (○), kidney (▼), spleen (△), and liver (■). Concentrations detected below the lower limit of quantification were not shown. Points, mean; bars, $\pm$ SE ($n = 3$).

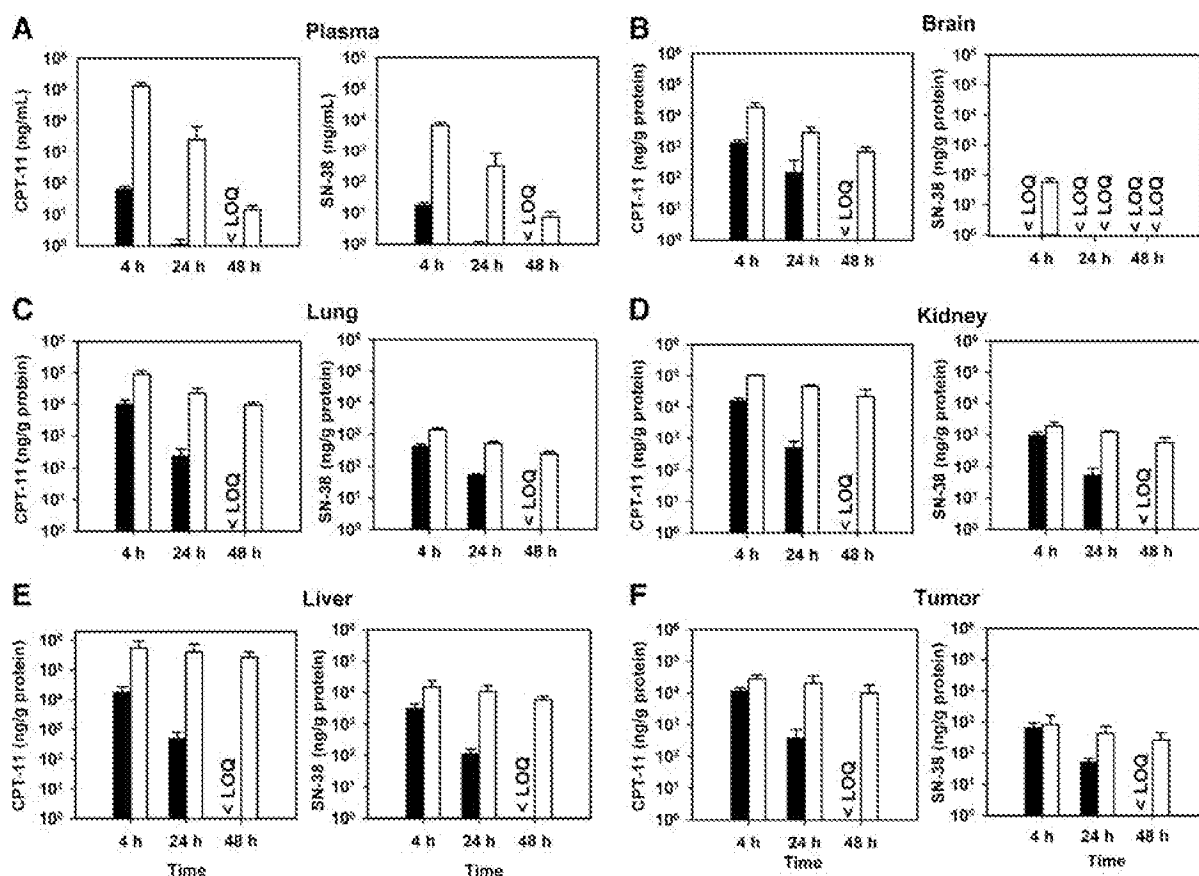


Figure 3.

Plasma and tissue/tumor concentrations for irinotecan and SN-38 following a single dose of irinotecan or *nal-IRI* in *nu/nu* mice. Female *nu/nu* mice bearing subcutaneous human neuroblastoma xenografts (CHLA-136) were injected intravenously with a single dose of 20 mg/kg of drug solution. A, the plasma concentrations of irinotecan (left) and SN-38 (right) were measured at 4, 24, and 48 hours after a single intravenous injection of irinotecan or *nal-IRI*. B–E, the tissue concentrations of irinotecan (left) and SN-38 (right) were measured at 4, 24, and 48 hours after a single intravenous injection of irinotecan or *nal-IRI*. F, the intratumoral concentrations of irinotecan and SN-38 were assessed at 4, 24, and 48 hours after a single intravenous injection of irinotecan or *nal-IRI*. Points, mean; bars, $\pm$ SE ($n = 3$). Black bars, irinotecan; white bars, *nal-IRI*. <LOQ, the detected concentration was below the lower limit of quantification.

protein and 393 ng/mL (1.0 μ mol/L) in mouse plasma (Supplementary Fig. S2C).

Macrophage infiltration and tumor pharmacokinetics (PK) after *nal-IRI* treatment

Responses to *nal-IRI* in xenograft models varied widely, with activity in EFT being consistently greater than what was observed for RMS or NB xenografts (Fig. 1 and Supplementary Fig. S1B). Tumor-associated macrophages could potentially concentrate *nal-IRI* and facilitate release of the more potent SN-38 into tumors. However, we observed no significant differences in macrophage infiltration among NB, RMS, or EFT tumor xenografts at 0, 48, or 168 hours following *nal-IRI* treatment (Fig. 4A, $P = 0.31$, representative pictures in Supplementary Fig. S3). Treatment with *nal-IRI* significantly increased macrophage infiltration in all three models ($P < 0.005$), but no greater infiltration was seen in any specific tumor type relative to other models at 168 hours after the treatment (Fig. 4A, $P = 0.37$).

The tumor concentrations of irinotecan (but not of SN-38) in EFT or RMS xenografts were significantly higher than NB

xenografts (Fig. 4B, $P < 0.05$). Plasma concentrations of irinotecan and SN-38 at 48 hours after injection in RMS xenograft mice were apparently lower compared with NB or EFT xenograft mice, although the differences were not statistically significant ($P = 0.07$). However, SN-38 plasma concentrations were above the detection limit (2.5 ng/mL) at 168 hours in the RMS model, whereas NB or EFT models were below the limit of detection. Given that no differences in macrophage infiltration were seen for the 3 tumor types, and that the tumor concentrations of the drug and the metabolite were not higher in EFT models (which showed durable complete responses in all five models tested, Fig. 1 and Supplementary Fig. S1B and S1C), macrophage infiltration or tumor concentrations of irinotecan or SN-38 were excluded as the major mechanism for the remarkably high *nal-IRI* activity observed in EFT relative to NB or RMS xenografts.

SLFN11 expression in association with sensitivity to SN-38

High expression of the putative DNA/RNA helicase *SLFN11* has been recently reported to correlate with sensitivity to DNA

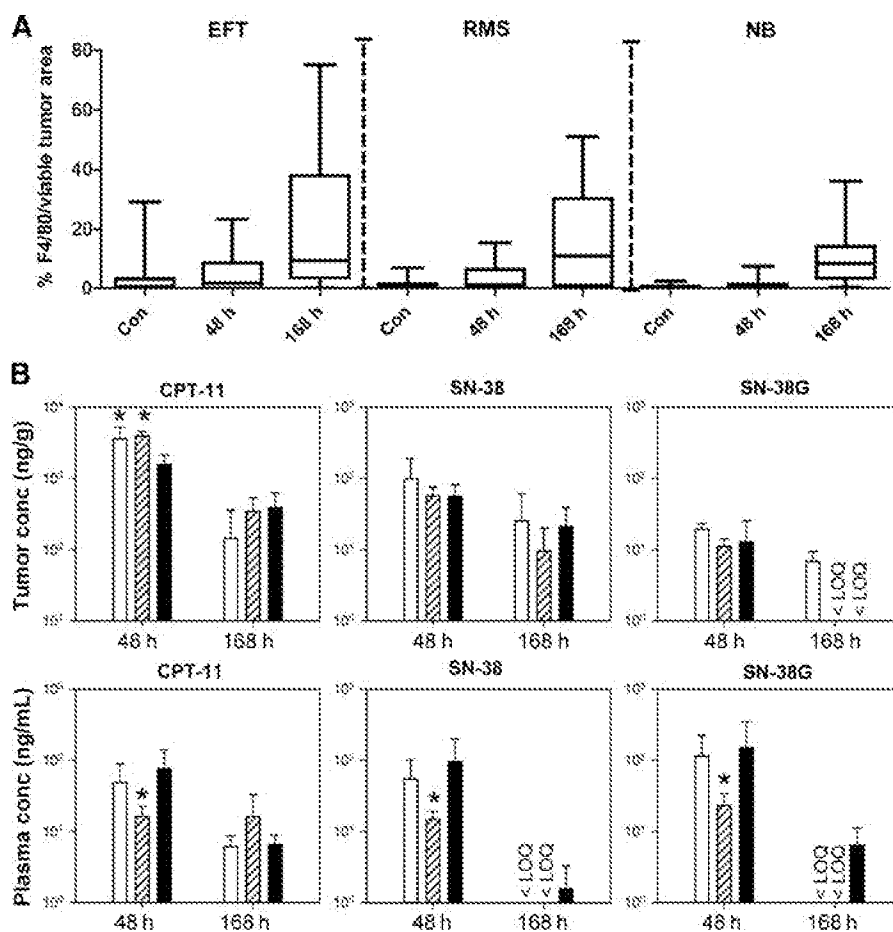


Figure 4.

Macrophage infiltration in pediatric tumors after nal-IRI treatment. Tumors from mice xenografted with EFT (CHLA-258), RMS (CB-NJRm), or NB (CHLA-79) were collected 48 and 168 hours after treatment with 10 mg/kg/d of nal-IRI. The tumors were snap-frozen for macrophage infiltration assays (A) and for PK analyses (B). A, macrophage infiltration was assessed as described in Materials and Methods; data are the % F4/80 staining cells per viable tumor area. A minimum of 5 fields were evaluated for macrophage content, and each time point has 3 or more mice. Differences in macrophage content by tumor type were not significant ($P = 0.37$ at 168 hours), but for all 3 tumor types macrophage infiltration was significantly ($P < 0.005$) increased by nal-IRI treatment. B, blood was collected 48 and 168 hours after treatment by cardiac puncture. Plasma samples were stored in -80°C until analyses. White bar, EFT; hatched bar, RMS; solid bar, NB. SN-38G, SN-38 glucuronide. *, statistically significant difference ($P < 0.05$). <LOQ, below the limit of detection (2.5 ng/mL).

damaging agents (20, 21). We observed that sensitivity to DNA damaging agents (4-HC, active metabolite of cyclophosphamide, topotecan, SN-38), as determined by the DIMSCAN cytotoxicity assay, showed a significant correlation with *SLFN11* expression assessed by the Affymetrix Human Exon Array in 17 EFT cell lines [Supplementary Fig. S4, $P < 0.005$ for all three drugs, $r = -0.95$ (4-HC), -0.76 (topotecan), -0.84 (SN-38)]. Using quantitative RT-PCR for 20 EFT, 20 NB, and 12 RMS cell lines, we observed that *SLFN11* expression was >100-fold higher in EFT relative to NB cell lines, whereas the expression varied widely in RMS (Fig. 5A). *SLFN11* protein and mRNA expression were consistent, showing higher expression in the majority of EFT cell lines relative to NB or RMS (Fig. 5B). We observed an inverse correlation between *SLFN11* mRNA expression measured by RT-PCR and the relative inhibitory/cytotoxic concentrations (rIC_{50}) concentrations (measured by DIMSCAN) of SN-38 for 20 EFT cell lines ($P = 0.004$, $r = -0.63$, Fig. 5C). However, no significant correlation between rIC_{50} and *SLFN11* mRNA expression was observed in RMS or NB cell lines. ($P = 0.26$ and $P = 0.21$; Fig. 5D and E).

As *SLFN11* mRNA expression assessed by RT-PCR and Affymetrix HuEx microarray correlated well in cell lines ($r = 0.62$, $P = 0.007$), we examined *SLFN11* mRNA expression data from HuEx microarrays for primary tumors (50 each for EFT, RMS, and NB).

Of the 50 EFT samples, 90% had the EWS-FLI1 translocation and 10% had EWS-ERG. Rhabdomyosarcomas were 1/3rd alveolar and 2/3rds embryonal and neuroblastomas were 1/3rd favorable biology, 1/3rd *MYCN*-amplified, and 1/3rd *MYCN*-nonamplified with unfavorable histopathology. Consistent with the expression in the cell line panels, median *SLFN11* mRNA expression was approximately 10-fold higher in EFT primary tumors compared with NB or RMS primary tumors ($P < 0.0001$; Fig. 6A). FLI1 is not normally expressed, and thus using the 3'-end of the gene provides a surrogate for the chimeric EWS-FLI1 gene. Using this approach, coexpression of FLI1 and *SLFN11* was evaluated using the same microarray data employed for Fig. 6A, comparing an exon of *SLFN11* (3753518) with the 8th exon of *FLI1* (3355784), which is expressed at high levels only when translocated, as part of EWS-FLI1 ($r = 0.51$, $P < 0.001$; Fig. 6B).

Discussion

Although diarrhea is the dose-limiting toxicity of irinotecan, it can be managed to some degree by employing antidiarrhea medications at the early stage of treatment (14). Other limitations of irinotecan for its clinical application include (i) poor pharmacokinetic properties only allowing short tumor exposure to the

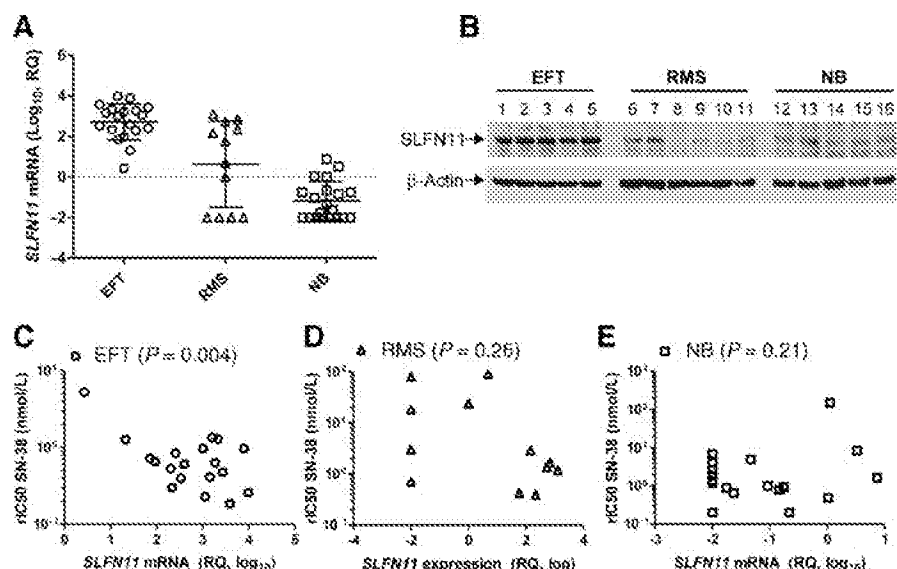


Figure 5.

Correlation between *SLFN11* expression and sensitivity to SN-38 in pediatric solid tumor cell lines. Cytotoxicity of SN-38 was evaluated, and mRNA was isolated from 20 NB, 12 RMS, and 20 EFT cell lines (see Supplementary Table S1 for cell line characteristics). A, *SLFN11* mRNA expression in panels of NB, RMS, and EFT cell lines. *SLFN11* mRNA expression was compared among the three histotypes of pediatric cancers using quantitative RT-PCR. The data were expressed in relative quantitation (RQ) using the BIRCH cell line (RMS) as the standard (marked as 0 on the Y axis). Each data point represents an average of 3 biologic replicates measured in three technical replicates. B, *SLFN11* protein expression in NB, RMS, and EFT cell lines assessed by immunoblotting. Lanes 1–5 (EFT), from left, CHLA-32, CHLA-258, TC-32, TC-72, CHLA-9; Lanes 6–11 (RMS), from left, TTC-442, Rh30, Rh18, RD, BIRCH, Rh41; Lanes 12–16 (NB), from left, CHLA-144, SK-N-BE(1), CHLA-90, CHLA-136, SMS-KCN. EFT cell lines were used in both experiments to compare the protein expression levels in NB and RMS relative to EFT cell lines. C–E, correlation between *SLFN11* mRNA expression and SN-38 cytotoxicity in EFT (C), RMS (D), and NB (E) cell lines. The rC₅₀ of SN-38 was correlated with *SLFN11* expression in EFT ($r = -0.63$), NB ($r = 0.31$), and RMS ($r = -0.31$) cell lines by Pearson r correlation. rC₅₀ was determined using GraphPad Prism from survival fractions of cells after treating them with 9 different concentrations of SN-38 in 3-fold increments. Each concentration had 6 replicates.

drug and its active metabolite (SN-38) and (ii) hydrolysis of active lactone rings in irinotecan and SN-38 to inactive carboxylate forms in neutral to basic pH (36). Stable liposomal encapsulation of irinotecan may minimize the transformation of the irinotecan into its inactive carboxylate form by reducing the direct exposure of the drug to physiologic pH and prolong the residence time of active drug and metabolites in the system. Because only the lactone form of camptothecin analogues is able to transverse cellular membranes (37), decreasing hydrolysis of irinotecan by encapsulating in liposomes has the potential to improve clinical activity.

Recent advances in intraliposomal encapsulation technology showed that irinotecan formulated as a nanoliposomal formulation (nal-IRI = MM-398) achieved high *in vivo* chemical stability, liposome retention, and improved residence time of irinotecan in rats (13). We observed that levels of SN-38 in xenografted tumors were significantly higher with nal-IRI than with irinotecan. The increased tumor exposure to irinotecan and SN-38 achieved with nal-IRI relative to irinotecan was consistent with the higher antitumor activity and superior EPS seen in the mouse xenograft models of pediatric solid tumors treated with nal-IRI compared with irinotecan in the majority of xenograft models tested.

The accumulation of liposomal drugs in tumors is suggested to occur via two pathways. One is via the enhanced permeability and retention (EPR) effect (11, 38). Liposomes do not readily diffuse out of the blood vessels in healthy tissues (39), but the leaky vasculature and impaired lymphatic drainage in tumor can facil-

itate the spontaneous accumulation of liposomes in these areas (40). The other pathway is through the localization of liposomes to mononuclear phagocytes such as macrophages (38). Although the number of macrophages varied greatly among different tumors, the percentage of tumor-associated macrophages is usually maintained at a stable level for a particular tumor type (41). In addition, macrophage carboxylesterases in tumor is a presumed mechanism for the conversion of irinotecan to SN-38 in human tumors (14, 42, 43), and the macrophage levels in nu/nu mice are comparable with levels observed in immunocompetent mice (44). However, we observed that neither macrophage infiltration nor intratumoral concentrations of irinotecan or SN-38 were significantly higher in EFT models in which durable responses to nal-IRI were seen relative to xenografts of NB and RMS that were less responsive to nal-IRI. Our data showed that macrophage infiltration into tumors or tumor pharmacokinetics does not vary among xenografts of the 3 histotypes of pediatric solid tumors that we tested, and thus is unlikely to be the reason for the consistently high responsiveness of EFT xenografts to nal-IRI relative to other histotypes.

The mRNA expression of *SLFN11*, a putative RNA/DNA helicase, has been reported to correlate with the sensitivity to DNA damaging agents in the NCI60 panel of cell lines (21) or with the sensitivity to irinotecan in three EFT cell lines (20). Consistent with the previous report by Barretina and colleagues showing highest expression of *SLFN11* in EFT primary samples among various cancer types (20), we found that *SLFN11* mRNA expression was significantly higher (>100-fold) in EFT cell lines relative

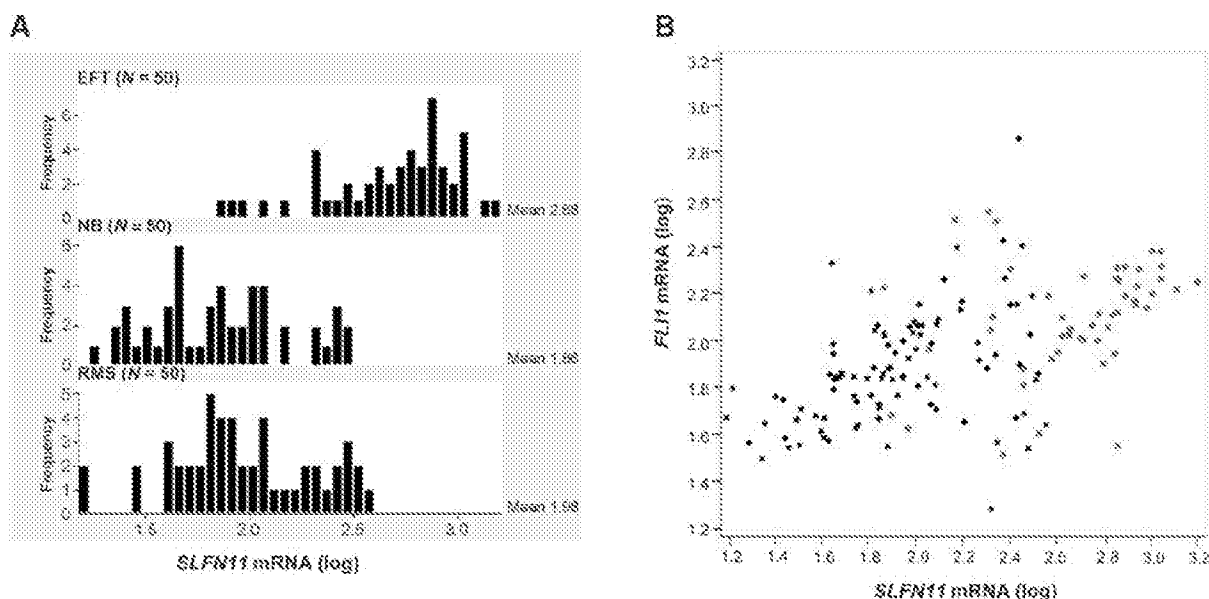


Figure 6.

SLFN11 expression in primary patient samples. A, histograms of *SLFN11* expression (measured using Affymetrix Exon arrays) for 50 primary tumors each of EFT, RMS, and NB. Data are displayed on a log₁₀ scale, with mean log expression values for each. B, using the same samples as shown in Fig. 6A, we observed a significant correlation ($P < 0.001$) between *SLFN11* expression and *FLI1* expression (measured using Affymetrix Exon arrays) in EFT, RMS, and NB tumors ($r = 0.51$). As shown in Fig. 6A, *SLFN11* is highly expressed in EFT; virtually all the high expressors for both *SLFN11* and *FLI1* are EFT, suggesting a functional link between expression of the two genes. EFT samples are shown as red dots.

to NB and RMS cell lines (Fig. 5). Although NB cell lines exhibit much lower *SLFN11* expression, the sensitivity to SN-38 in NB cell lines was similar to that in EFT cell lines, suggesting that *SLFN11* expression is not the primary driver of SN-38 sensitivity across all histotypes. For example, CHLA-119 (established at time of relapse after intensive consolidation chemotherapy; CCG-3891) that included topoisomerase II but not a topoisomerase I inhibitor is one of four NB cell lines with the lowest *SLFN11* expression, but nine of the ten xenograft mice survived >120 days after two doses of nal-IRI (Fig. 1).

In 20 EFT cell lines, we observed a strong correlation between *SLFN11* expression and SN-38 sensitivity that was confirmed by both microarray and quantitative RT-PCR experiments. The high sensitivity of EFT cell lines to SN-38 *in vitro* was consistent with *in vivo* EFT tumor xenograft responses to nal-IRI. Thus, the high activity seen for nal-IRI in EFT xenografts is likely due to the improved pharmacokinetic properties of nal-IRI enhancing exposure to SN-38 in the tumor combined with the high sensitivity to SN-38 associated with high *SLFN11* expression.

We observed significantly higher expression of *SLFN11* mRNA in primary tumors from EFT relative to RMS and NB, which is consistent with our cell line data and with the xenograft response data, suggesting that clinically EFT may be particularly sensitive to nal-IRI. *EWS/FLI1* is thought to be an aberrant transcription factor that is specifically expressed in EFT (22). *FLI1* is normally not expressed in EFT or other childhood tumors, other than as a partner of *EWS* (which is ubiquitously expressed in most tissues) to form the *EWS-FLI1* chimeric gene found in 85% to 95% of Ewing's sarcomas. Thus, we examined the correlation between expression of *FLI1* (which on Affymetrix microarrays provides a surrogate for *EWS/FLI1* expression)

and *SLFN11*. We observed a strong correlation between *SLFN11* and *FLI1* mRNA expression, and *SLFN11* may be a direct target of *EWSR1-FLI1* as evidenced by ChIPSeq analysis data from Kauer and colleagues (45). The relationship of *SLFN11* expression to sensitivity of tumors to nal-IRI will ultimately need to be addressed in clinical trials, and the mechanism by which *SLFN11* expression confers sensitivity to SN-38 will need to be explored in further laboratory studies.

Clinical trials of nal-IRI in patients with metastatic colorectal cancer and pancreatic cancer are ongoing (ref. 46; clinicaltrials.gov ID: NCT01494506, NCT01375816), with positive results being recently reported for nal-IRI in a pivotal phase III study of pancreatic cancer (19). Based on published data and data from the present study, evaluations of *SLFN11* as a potential biomarker for nal-IRI activity are planned. Our study is the first to show that nal-IRI has substantially improved antitumor activity over irinotecan in xenograft models of pediatric cancers. The striking antitumor activity in EFT xenografts seen with nal-IRI was likely due to the combination of the advantageous pharmacokinetic properties of nal-IRI combined with the high sensitivity to SN-38 of EFT, which was significantly correlated with *SLFN11* expression. Based on the preclinical studies reported here, a phase I study of nal-IRI combined with cyclophosphamide in pediatric solid tumors (SPOC 2012-001; NCT 02013336) is under way in the South Plains Oncology Consortium (www.SPONC.org).

Disclosure of Potential Conflicts of Interest

J.B. Fitzgerald has ownership interest (including patents) in Merrimack Pharmaceuticals. D.C. Drummond is employed by and has ownership interest (including patents) in Merrimack Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.H. Kang, J. Wang, M.R. Makena, C.P. Hall, W. Ko, J.B. Fitzgerald, T.J. Triche, C.P. Reynolds

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Study supervision: M.H. Kang, D.C. Drummond, C.P. Reynolds

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Clinical Cancer Research

Activity of MM-398, Nanoliposomal Irinotecan (nal-IRI), in Ewing's Family Tumor Xenografts Is Associated with High Exposure of Tumor to Drug and High *SLFN11* Expression

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Systems Pharmacology Based Biomarker Potentially Predicts Clinical Anti-Cancer Activity of MM-398, nanoliposomal irinotecan, nal-IRI

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ABSTRACT

Objectives:

With a systems pharmacology approach we have identified tumor permeability to nal-IRI and ability of tumor carboxylesterase to activate irinotecan as critical factors for in vivo activity. In order to test the importance of these parameters for anti-cancer activity of nal-IRI in patients we have conducted a clinical study to measure and quantify them by using tissue- and imaging-based methods as well as mechanistic PK model.

Methods:

Eligible patients (n=12) with refractory solid tumors were treated with nal-IRI (80mg/m² q2w). Plasma PK was measured at multiple time points, and tissue biopsies were collected 72h post-treatment, with drug metabolite levels measured by mass spectrometry. Prior to nal-IRI treatment patients underwent ferumoxytol-MRI to test the feasibility to non-invasively measure nanoparticle permeability in tumors. A mechanistic tumor PK model for ferumoxytol was developed to estimate the permeability of ferumoxytol in tumor.

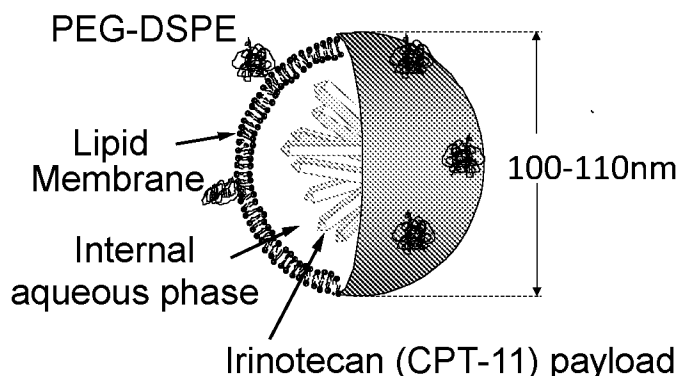
Results:

Patient-derived data showed that SN-38 concentrations in tumor were 5-fold higher than in plasma 72h post-treatment in agreement with our simulations incorporating the enhanced permeability and retention effect for tumor deposition of liposomes. The ferumoxytol tumor PK model was able to describe both plasma and tumor ferumoxytol-MRI data ($R^2 > 0.9$, n=9). Analyses indicated that tumor permeability to ferumoxytol contributed to MRI signals at 24h, while tissue retention capacity of ferumoxytol via binding contributed at 72h. Ferumoxytol levels above the median were significantly associated with better lesion responses as measured by change in lesion size ($p < 0.001$ at 1h; $p < 0.003$ at 24h) resulting in the receiver operating characteristics $AUC > 0.8$ for lesion classification. However, no significant relationship was observed at 72h.

Conclusions:

Systems pharmacology approaches can be used to identify parameters of clinical relevance for biomarker development.
promising biomarker strategy for nal-IRI.

Low-pegylated non-targeted
liposomal irinotecan



Systems pharmacology approach to identify MOA for nal-IRI

Figure 1. Mechanistic tumor PK model was developed and trained with plasma and tumor PK data in mice bearing HT-29 xenografts.

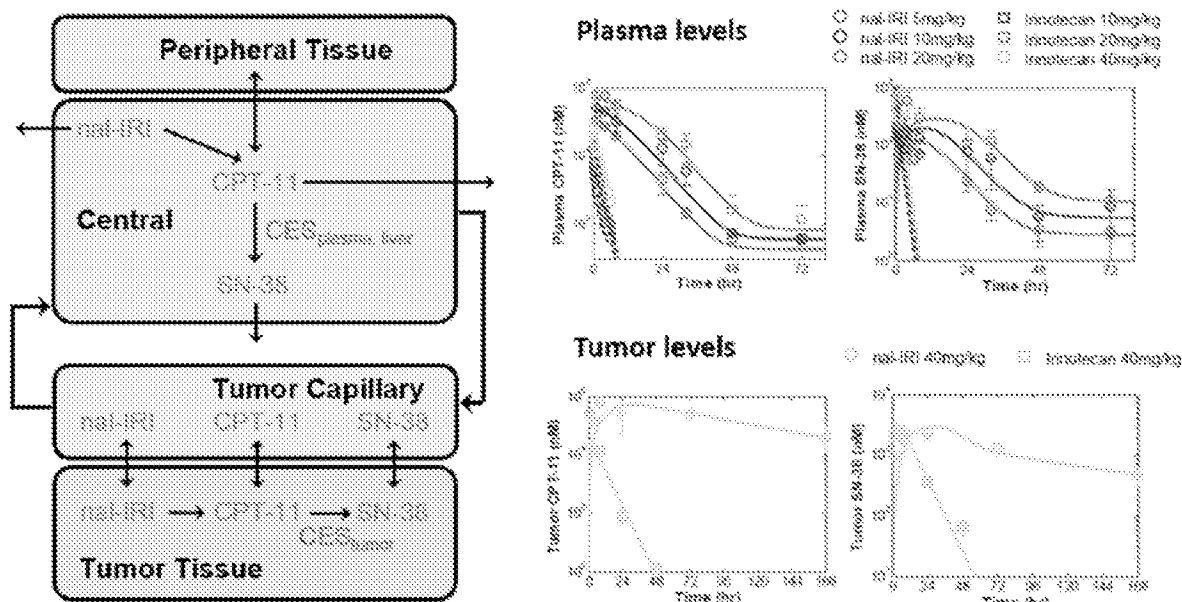
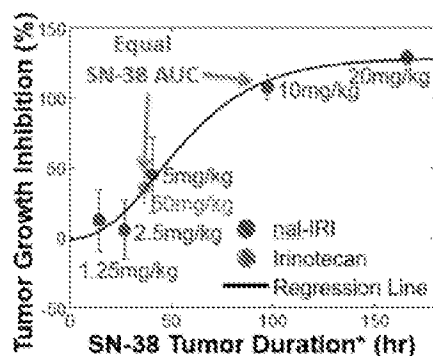
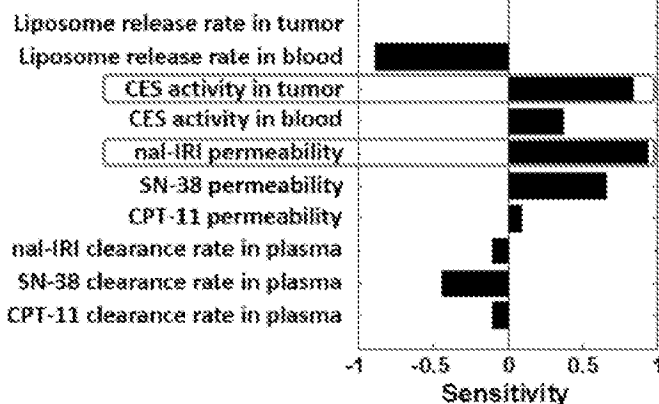


Figure 2. Mechanistic PK model identified that longer duration of SN-38 in tumor differentiates nal-IRI from free irinotecan for *in vivo* activity. Sensitivity analysis on the model parameters predicted critical processes to influence SN-38 tumor duration.



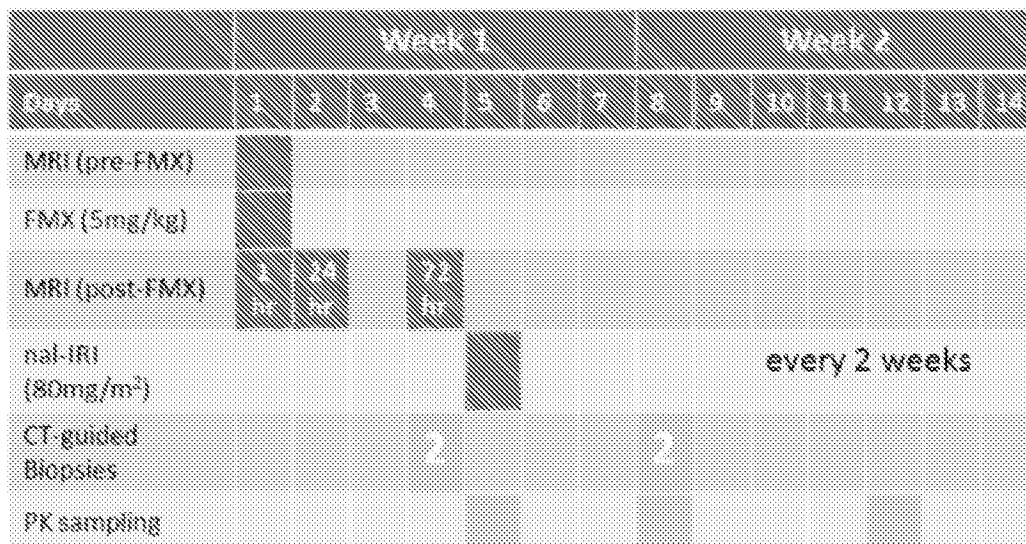
*Tumor duration is defined as the length of time the SN-38 tumor concentration is above 120nM

$$Sensitivity = \frac{\Delta(SN38_{Duration})/SN38_{Duration,0}}{\Delta(p)/p_0}$$



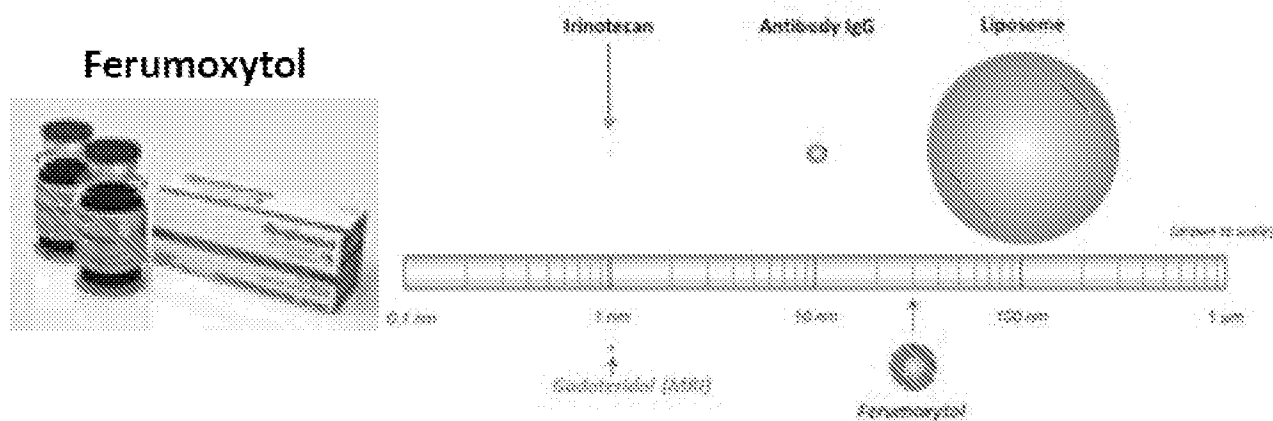
Design of Clinical Translational Study

Figure 3. Eligible patients with refractory solid tumors were recruited. PK samples for FMX were collected at 0.5h, 2h, 24h and 72h. PK samples for nal-IRI were collected at 1.5h, 3.5h, 72h and 168h. RECIST v1.1 evaluation was done every 8 weeks.



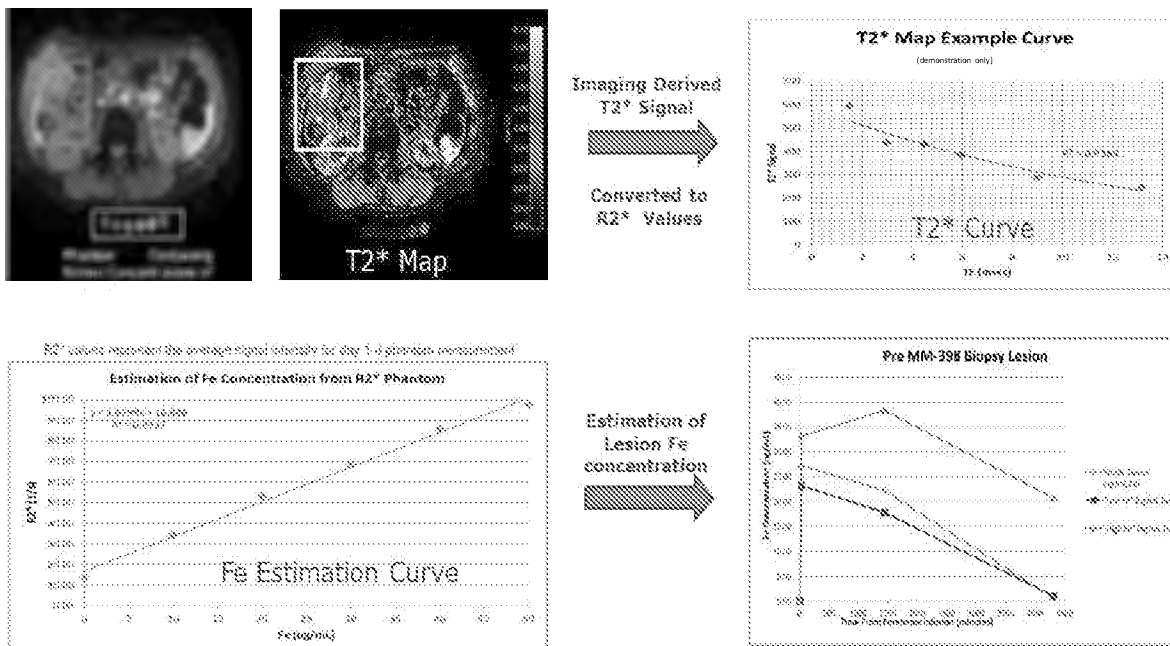
Ferumoxytol

Figure 4. Ferumoxytol (FMX) is a 30 nm size superparamagnetic iron oxide nanoparticle coated with polyglucose sorbitol carboxymethylether. FMX is approved for iron supplement in patients with chronic kidney disease and recently has been used as MRI contrast agent (off-label).



Ferumoxytol Imaging and Quantitation

Figure 5. MR images were acquired on a GE 1.5T MRI instrument with a T1 FSPGR series with echo delay times from 1.5 – 13.2ms. Slice thickness and spacing was 6mmx1mm using a 256x256 matrix. T2* values were extrapolated from each image series to construct a T2* map. Phantom tubes containing known FMX concentrations from 10 – 200mg/ml was included during each MRI session and demonstrated a linear relationship between $R2^*=1/T2^*$ and FMX levels.



MRI FSPGR FatSat TE 13.2 ms Breathhold Images

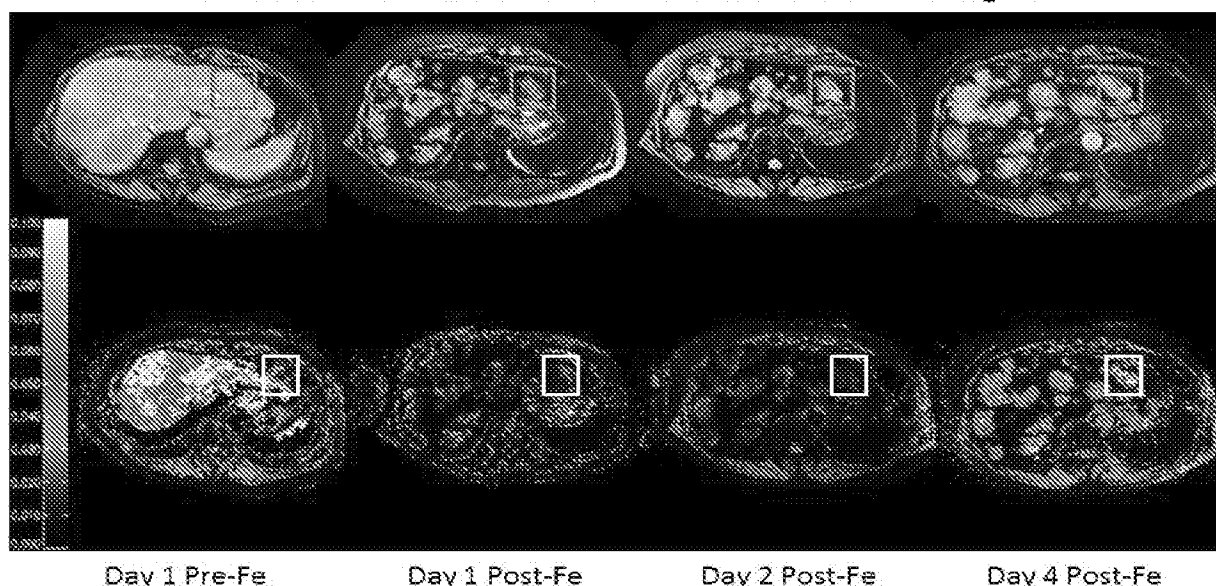
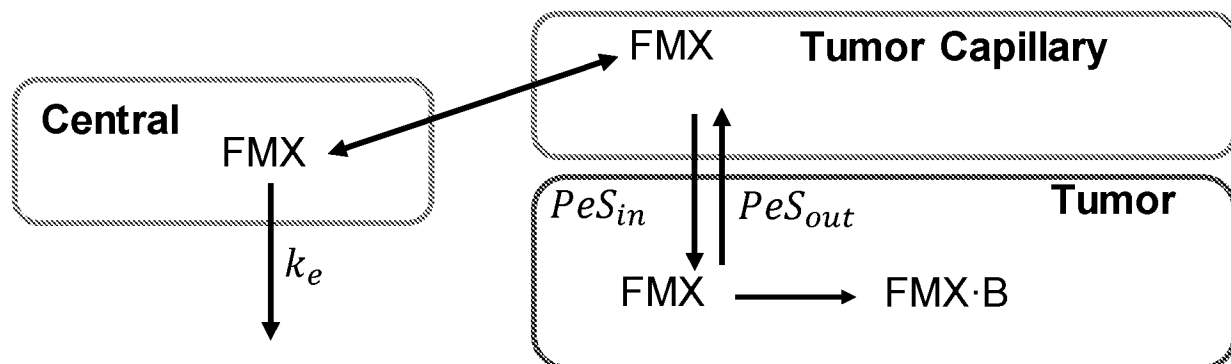


Figure 6. Plasma and tumor PK models were integrated to simulate FMX signals for each patient tumor lesion. FMX tumor PK model was developed by using SimBiology® toolbox in MATLAB®. Particle swarm optimization was used to estimate the model parameters.



PeS_{in} (Ktrans): Responsible for vascular wall permeability

v_e , Extravascular volume fraction, $v_e = PeS_{in} / PeS_{out}$: Responsible for permeability of extravascular tissue bed and distance that drug can diffuse out from the vascular wall

B: Number of tissue binding site (e.g., macrophages)

Figure 7. FMX tumor PK model could quantify the degree of tissue permeability and FMX binding activity across all tumor lesions

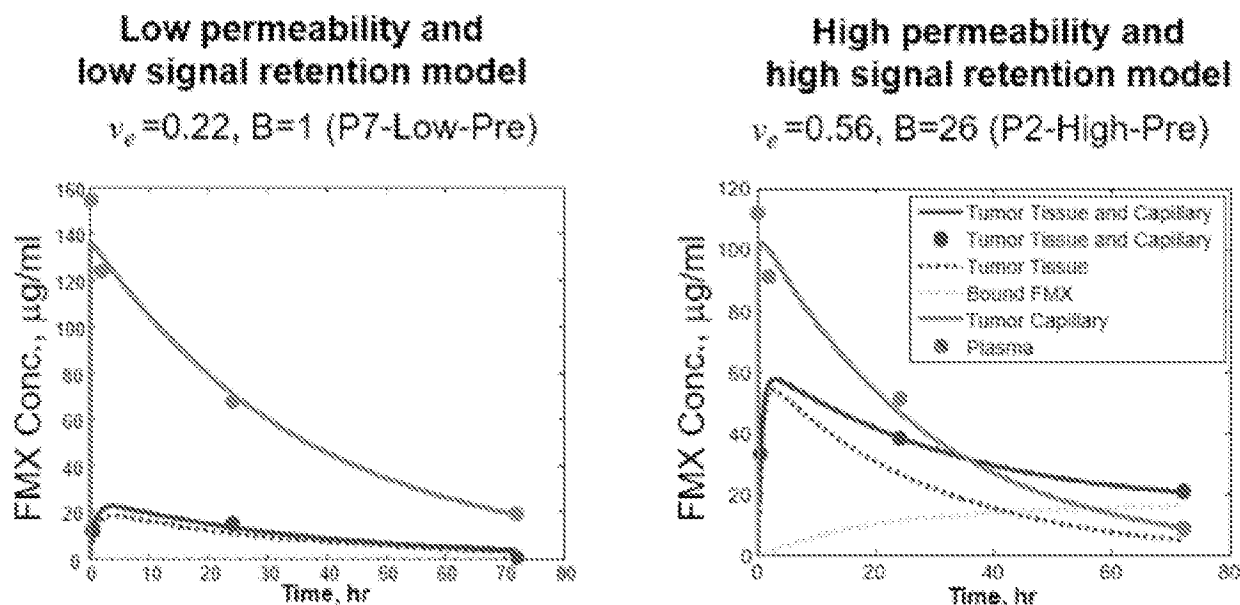
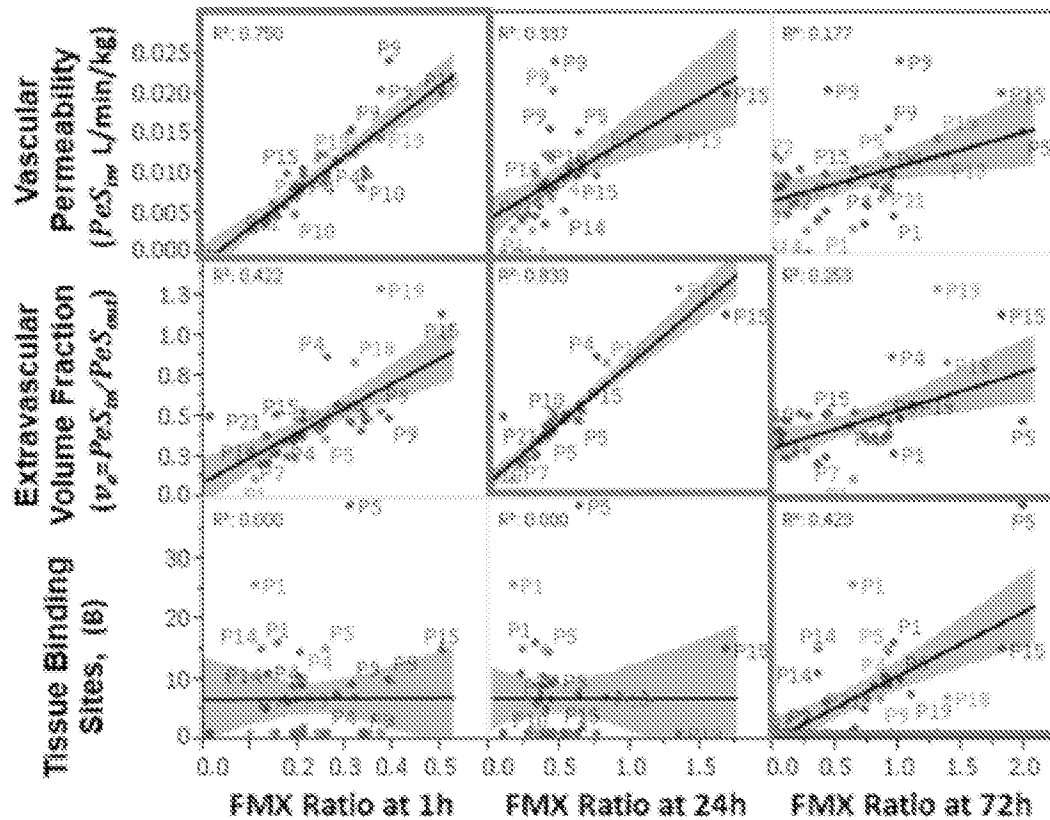


Figure 8. Earlier FMX signals (1h and 24h) were explained by the model parameters related to vascular permeability, whereas FMX signals at 72h were explained by the model parameter for FMX binding to tumor tissue.

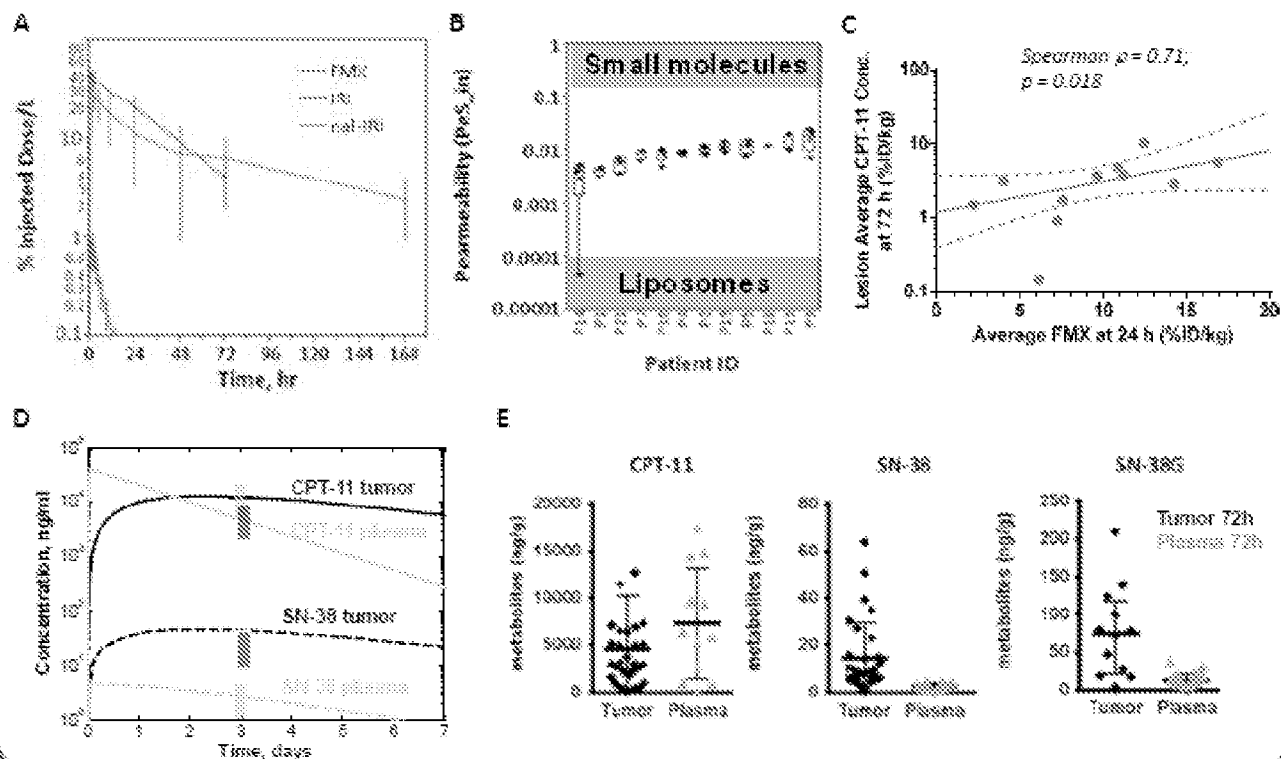


Conclusions

- This phase I study demonstrated the feasibility of incorporating FMX-MRI into a clinical workflow
- FMX tumor PK model identified that early FMX signals at 1h and 24h contributed to tumor permeability of FMX
- FMX-MRI correlated well with nal-IRI delivery to tumor lesions
- Significantly higher SN-38 levels in tumor suggested strong local conversion activity of nal-IRI
- Early FMX signals showed significant relationship with lesion size change response suggesting the potential use as a diagnostic tool

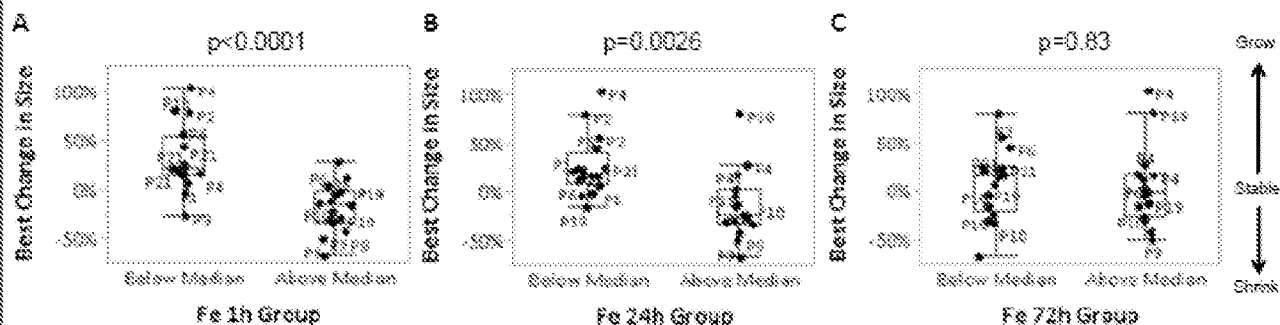
Plasma and Tumor PK of FMX and nai-IRI

Figure 9. FMX plasma half-life was similar to nai-IRI as compared to free IRI (A). Even though the estimated tissue permeability parameters for FMX were in between small molecules and liposomes (B), average FMX tumor levels correlated well with nai-IRI deposition to tumor in each patient (C). The mechanistic tumor PK model of nai-IRI predicted higher SN-38 levels in tumor suggesting strong local conversion activity of nai-IRI (D). The predictions were confirmed by the metabolite data from tumor biopsy samples in patients (D, E).



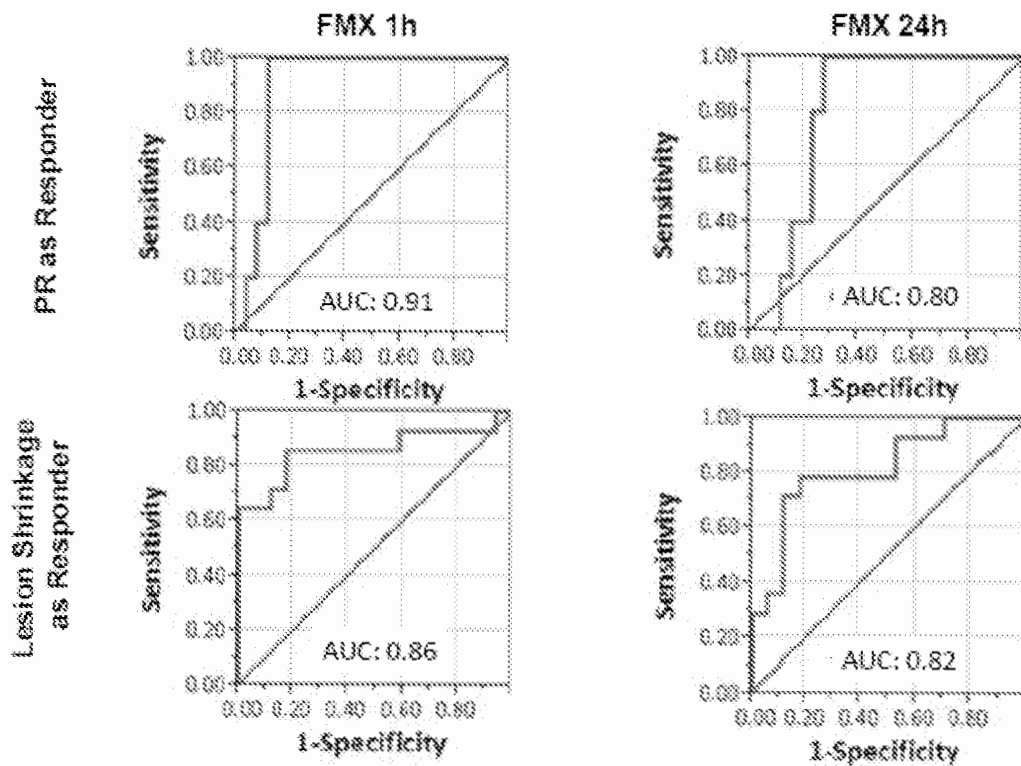
FMX Signal and Lesion Response Relationship

Figure 10. Lesions with FMX levels above the population median showed statistically significant shrinkage in individual lesion size*. Earlier FMX signals (1h and 24h) showed significant lesion response relationship (A,B), whereas no significant relationship was observed at 72h (C).

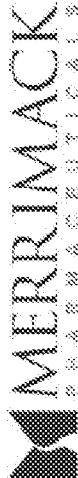


*Lesions from one patient were treated as independent samples

Figure 11. FMX signals at 1h and 24h were used to explore the utility of FMX-MRI as a diagnostic test for nal-IRI *in vivo* activity in humans. Receiver operating characteristic (ROC) curves were calculated by using two different definitions for responders; 1. Partial Response (PR) in lesion size change (Size Change < -30%) and 2. Decrease in lesion size change (Size Change < 0%). Area under curves (AUC) for ROC curves at both time points (1h and 24h) were >0.8 suggesting the potential usefulness of FMX-MRI as a diagnostic tool for nal-IRI *in vivo* activity



Systems Pharmacology Based Biomarker Potentially Predicts Clinical Anti-Cancer Activity of MM-398, nanoliposomal irinotecan, nal-IRI



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Design of Clinical Translational Study

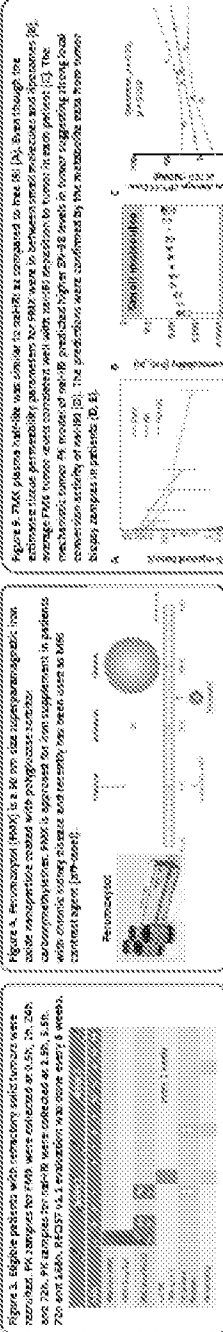


Figure 1. Design of Clinical Translational Study. Phase I: Single patients with refractory solid tumors. Phase II: 20 patients with refractory solid tumors. Phase III: 20 patients with refractory solid tumors. Pre-treatment: Baseline, Post-treatment. Immunogenicity: Anti-drug antibody, Anti-tumor antibody. Toxicity: Grade 3-4 toxicity, Grade 1-2 toxicity.

Figure 1. Single patients with refractory solid tumors were enrolled in Phase I. Phase II patients were enrolled in Phase II. Phase III patients were enrolled in Phase III. Pre-treatment: Baseline, Post-treatment. Immunogenicity: Anti-drug antibody, Anti-tumor antibody. Toxicity: Grade 3-4 toxicity, Grade 1-2 toxicity.



Figure 2. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

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Figure 3. Mechanistic PK model. The mechanistic PK model was used to estimate the PK parameters of MM-398. The mechanistic PK model was used to estimate the PK parameters of MM-398.

Figure 3. Mechanistic PK model. The mechanistic PK model was used to estimate the PK parameters of MM-398. The mechanistic PK model was used to estimate the PK parameters of MM-398.



Figure 4. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 4. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

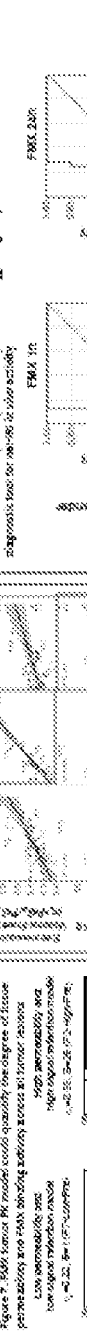


Figure 5. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

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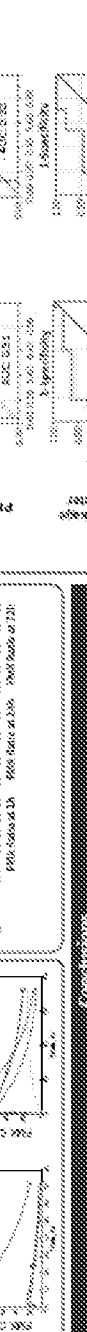


Figure 6. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

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Figure 7. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 7. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Systems Pharmacology Approach

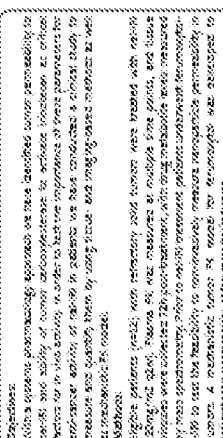


Figure 8. Systems Pharmacology Approach. The systems pharmacology approach was used to estimate the PK parameters of MM-398. The systems pharmacology approach was used to estimate the PK parameters of MM-398.

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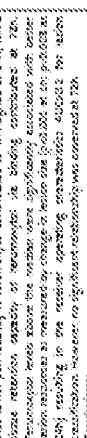


Figure 9. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 9. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

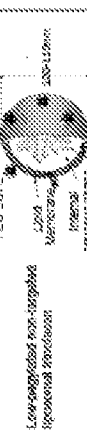


Figure 10. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 10. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

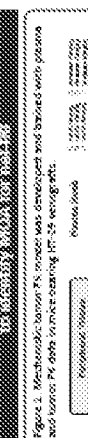


Figure 11. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 11. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.



Figure 12. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 12. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

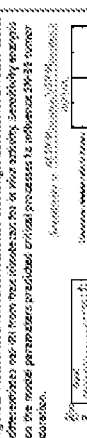


Figure 13. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 13. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

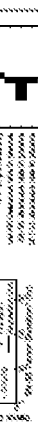


Figure 14. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Figure 14. PK model. The PK model was used to estimate the PK parameters of MM-398. The PK model was used to estimate the PK parameters of MM-398.

Antibody Targeting of Long-Circulating Lipidic Nanoparticles Does Not Increase Tumor Localization but Does Increase Internalization in Animal Models

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Abstract

We describe evidence for a novel mechanism of monoclonal antibody (MAb)-directed nanoparticle (immunoliposome) targeting to solid tumors *in vivo*. Long-circulating immunoliposomes targeted to HER2 (ErbB2, Neu) were prepared by the conjugation of anti-HER2 MAb fragments (Fab' or single chain Fv) to liposome-grafted polyethylene glycol chains. MAb fragment conjugation did not affect the biodistribution or long-circulating properties of i.v.-administered liposomes. However, antibody-directed targeting also did *not* increase the tumor localization of immunoliposomes, as both targeted and nontargeted liposomes achieved similarly high levels (7-8% injected dose/g tumor tissue) of tumor tissue accumulation in HER2-overexpressing breast cancer xenografts (BT-474). Studies using colloidal gold-labeled liposomes showed the accumulation of anti-HER2 immunoliposomes within cancer cells, whereas matched nontargeted liposomes were located predominantly in extracellular stroma or within macrophages. A similar pattern of stromal accumulation without cancer cell internalization was observed for anti-HER2 immunoliposomes in non-HER2-overexpressing breast cancer xenografts (MCF-7). Flow cytometry of disaggregated tumors posttreatment with either liposomes or immunoliposomes showed up to 6-fold greater intracellular uptake in cancer cells due to targeting. Thus, in contrast to nontargeted liposomes, anti-HER2 immunoliposomes achieved intracellular drug delivery via MAb-mediated endocytosis, and this, rather than increased uptake in tumor tissue, was correlated with superior antitumor activity. Immunoliposomes capable of selective internalization in cancer cells *in vivo* may provide new opportunities for drug delivery. (Cancer Res 2006; 66(13): 6732-40)

Introduction

Targeted delivery of anticancer drugs to tumors has been long sought to improve the therapeutic index of cancer treatment. Liposomes represent a now well-established strategy for drug delivery, and have entered clinical use after years of optimization. Although the first generation of liposomes was limited by modest drug encapsulation efficiencies and rapid clearance by the reticuloendothelial system, progressive optimization has resulted

in more stable and long-circulating liposomes with increased deposition in tumors (1, 2), which provide potential clinical benefits in certain cases. The next generation of nanoparticle-based drug delivery systems includes immunoliposomes, in which antibody fragments are conjugated to long-circulating liposomes for targeted drug delivery (for review, see ref. 3).

We have previously described anti-HER2 immunoliposomes, in which sterically stabilized liposomes containing polyethylene glycol (PEG) were conjugated to monoclonal antibody (MAb) fragments specific for the HER2 (ErbB2, Neu) oncoprotein (4, 5). Anti-HER2 immunoliposomes selectively bind to and internalize in HER2-overexpressing cancer cells *in vitro* via receptor-mediated endocytosis, thus potentially providing a vehicle for intracellular drug delivery (5, 6). Doxorubicin-loaded anti-HER2 immunoliposomes produced marked therapeutic results in HER2-overexpressing xenograft models, and were significantly superior to all other treatment conditions tested, including free doxorubicin, nontargeted liposomal doxorubicin, recombinant anti-HER2 MAb trastuzumab, and combinations of these other agents (4).

Here, we provide experimental evidence for the mechanisms underlying this increased antitumor efficacy, and show that it does not involve enhanced accumulation of anti-HER2 immunoliposomes in tumor tissue due to antigen binding. Rather, both liposomes and immunoliposomes localized at high levels in tumor tissue, but revealed a marked difference in pharmacodynamics with respect to tumor cells *in vivo*: immunoliposomes, but not liposomes, mediated intracellular drug delivery to HER2-overexpressing cancer cells in animal models.

Materials and Methods

Liposome and immunoliposome preparations. The liposome matrix was phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), cholesterol (Calbiochem, San Diego, CA), and *N*-methoxy-PEG (molecular weight, 1,900)-oxycarbonyl-distearoylphosphatidylethanolamine (DSPE; Genzyme, Cambridge, MA) as previously described (4). MAb fragments consisted of either Fab' fragments, rhuMAbHER2-Fab (7), from recombinant humanized anti-HER2 MAb trastuzumab (Genentech, Inc., South San Francisco, CA), or human anti-HER2 single chain Fv (scFv) F5 with COOH-terminal cysteine (8-11). The linker was *N*-[α -(2-[*N*'-maleimido]propionylamido)-PEG- ω -oxycarbonyl]-DSPE (6, 10, 11). PEG chains had a molecular weight of 2,000. For biodistribution and tissue microdistribution studies, immunoliposomes were prepared as previously described (6), including a linker at 2 mol% of liposomal phospholipid, and conjugation to recombinant anti-HER2 Fab' derived from trastuzumab; Fab' density was ~33 Fab'/immunoliposome. For flow cytometry studies, immunoliposomes were prepared by micellar incorporation of anti-HER2 scFv F5 as previously described (10, 11). The final linker concentration, including F5-conjugated and residual unconjugated (cysteine-quenched) linker was ~0.2 mol% of the liposomal phospholipid; scFv density was ~20 scFv/immunoliposome. Unused linker

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was cysteine-quenched in all liposomes where present. Liposome/immunoliposome size was 90 to 110 nm by dynamic light scattering.

For radiolabeling, ^{67}Ga (1.0–1.5 $\mu\text{Ci}/\mu\text{mol}$ phospholipid) was loaded into liposomes and immunoliposomes containing 95 mmol/L of sodium diethylenetriamine pentaacetate (DTPA; pH 5.5) using USP diagnostic grade (^{67}Ga)-gallium citrate (12). Encapsulation of colloidal gold was done as described (13); to facilitate the entrapment of gold chloride solution, liposomes were prepared with egg phosphatidylcholine and reverse-phase evaporation (14), and MAb fragments were conjugated following gold encapsulation. Liposomes containing water-soluble fluorescent marker, ADS645WS (American Dye Source, Inc., Quebec, Canada), were prepared with hydration of the neat lipid matrix of DSPC, cholesterol, and PEG-DSPE (3:1:1 by weight) in 5 mmol/L dye solution in normal saline, followed by sequential membrane extrusion and Sepharose CL-4B (GE Healthcare, Piscataway, NJ) chromatography to remove the unencapsulated dye. The mean nanoparticle size was 86 nm. Immunoliposomes were prepared from ADS645WS-loaded liposomes by coinubation with a micellar solution of F5-maleimide-PEG-DSPE conjugate at a ratio of 10 μg protein/ μmol liposomal phospholipid (9–11). ADS645WS-loaded nanoparticles were concentrated in a stirred ultrafiltration cell on an Amicon YM100 membrane (Millipore, Billerica, MA) to reach a concentration of 40 to 44 mmol/L of phospholipid. Liposomal phospholipid concentration was determined by phosphate assay (15). Conjugated protein was assayed by the dye-binding method (Bio-Rad Laboratories, Hercules, CA) and SDS-PAGE with Coomassie blue staining and spectrophotometry of the 49 kDa band at 595 nm. The HER2-specific reactivity of radiolabeled immunoliposomes was confirmed in cultured SK-Br-3 cells (0.2 mmol/L liposome phospholipid after 4 hours of incubation at 37°C).

Tumor and tissue uptake of lipidic nanoparticles in mice. Tumor cells (2×10^7 ; BT-474 or MCF-7) were inoculated s.c. in the upper back of 4- to 6-week-old female NCR homozygous nude mice, with sustained-release 17 β -estradiol pellets (Innovative Research of America, Sarasota, FL) implanted in the lower back, and allowed to grow for 10 to 14 days postinoculation. Studies included 54 animals with tumors ranging from 220 to 1,200 mm³ (median, 450 mm³). For each agent (HER2-targeted or nontargeted), animals were randomly assigned to nine groups of three animals each, corresponding to time points of 4, 8, 12, 24, 48, 72, 99, 123, and 168 hours postinjection. Liposomes with encapsulated ^{67}Ga -DTPA were injected via tail vein at 40 μmol phospholipid/kg of body weight. At the indicated times, the animals were anesthetized, exsanguinated via open heart puncture, and perfused through the left ventricle. Blood, tumor, and other tissues were collected and weighed; liposome levels were determined by radioactivity counting, and corrected for radioactive decay. The total injected dose was calculated from the injection volume and specific activity of the liposome/immunoliposome preparations measured simultaneously with tissue samples. Linear regression analysis did not show significant correlation between liposome uptake (expressed as the percentage of injected dose per gram of tissue) versus tumor size, which was consistent with previous observations (16).

Microdistribution of lipidic nanoparticles within tumor tissue. Colloidal gold-labeled liposomes/immunoliposomes were injected i.v. into tumor-bearing mice at a dose of 0.4 mmol phospholipid/kg. Results represent six separate experiments in BT-474 or MCF-7 models and a total of 24 animals. Tumor volumes ranged from 100 to 700 mm³. Forty-eight hours later, animals were sacrificed; tumors were fixed overnight with 4% formaldehyde in PBS, embedded in glycol methacrylate, and cut into 3- μm sections. Sections were made serially through multiple regions of each tumor (4, 5), excluding grossly necrotic areas, followed by silver enhancement using the Aurion R-Gent kit (Electron Microscopy Sciences, Fort Washington, PA) and light staining with H&E. To avoid artifacts, the silver-enhancement time was adjusted to prevent the appearance of silver grains in the tissue-free section area and on the concurrently enhanced sections of blank (liposome-free) tumors. Sections were examined using bright-field and dark-field microscopy. At low power magnifications, light-reflective silver staining was visualized by side illumination using the Darklite fiber optic illuminator (Micro Video Instruments, Avon, MA). Observations from tumor-to-tumor and section-to-section varied somewhat

in terms of total liposome/immunoliposome accumulation, but were consistent as to tumor cell versus stromal partitioning. Representative micrographs are shown in Results.

Quantitative analysis of immunoliposome/liposome uptake in tumor cells *in vivo*. ADS645WS-loaded nontargeted liposomes or F5-conjugated immunoliposomes were injected i.v. into mice bearing BT-474 s.c. tumors (360–600 mm³; four animals per group) at 0.4 mmol phospholipid/kg. Nontreated animals provided tumors that served as controls. Forty-four hours later, animals were sacrificed by cervical dislocation, and tumors were excised, mechanically fragmented, and treated sequentially with two portions of disaggregation solution [0.1% collagenase type IV (Sigma Chemical Co., St. Louis, MO) and 0.003% DNase I in Hank's buffered salt solution] for 20 minutes at 37°C with slow agitation. Following centrifugation at 4°C, cell pellets were gently resuspended in cold PBS containing 0.1% bovine serum albumin (BSA; type V, Sigma) and 0.02% sodium azide (BSA-PBS-azide). To differentiate cancer cells from cells of the mouse host, the cell suspension was stained with FITC-conjugated mouse MAb against human epithelial antigen (HEA125; Miltenyi Biotec, Auburn, CA) on ice for 15 minutes. The cells were diluted with cold BSA-PBS-azide, centrifuged and resuspended in BSA-PBS-azide, and centrifuged again. After three washes, cells were resuspended in PBS, fixed with paraformaldehyde (0.9% final concentration), and strained through a 53- μm nylon mesh. Flow cytometry was done (FACSCalibur, BD Biosciences, San Jose, CA) using the fluorescein (FL1) channel for HEA-FITC and Cy5 (FL-4) channel for liposomal ADS645WS. Cultured BT-474 cells served as a positive control for HEA staining. Ten thousand scatter-gated signals were collected per sample.

To verify intracellular localization of particles, cell suspensions were examined with a Nikon T2000 inverted confocal microscope employing FITC and Cy5 detection laser-filter sets for visualization of HEA and liposomes, respectively.

Cytochemical identification of tissue macrophages. Glycol methacrylate-embedded tissue sections $\pm$ silver enhancement were stained for nonspecific (α -naphthyl acetate) esterase using a commercial kit (91-A, Sigma), except that Fast Violet Red LB was substituted for Fast Blue BB to afford nonoverlapping colors between the esterase reaction (violet) and black silver grains signifying liposome localization. Sections were counterstained in Gill's hematoxylin no. 3, mounted in crystal/mount (Biomed, Foster City, CA), and examined by microscopy. There was no difference in pigment localization in sections stained before or after the silver enhancement step.

Results

Design of anti-HER2 immunoliposomes. Anti-HER2 immunoliposomes were produced by covalent conjugation of recombinant MAb fragments to sterically stabilized/pegylated liposomes. Liposomes consisted of small (80–100 nm) unilamellar vesicles containing phosphatidylcholine, cholesterol, PEG (molecular weight, 2,000)–modified PEG-DSPE, and maleimide-terminated PEG-DSPE to afford covalent conjugation of MAb fragments to the termini of surface-grafted PEG chains (“PEG-MAB linkage”; ref. 6). Conjugation was very efficient (>90% of added MAB fragment) and yielded high ligand density (>20 MAB fragment per immunoliposome). As controls, nontargeted pegylated liposomes were prepared identically except for the omission of MAB fragment conjugation.

Anti-HER2 MAB fragments included either Fab' derived from recombinant humanized MAB trastuzumab (17) or scFv F5, isolated via phage antibody screening for internalization in target cells (8). Immunoliposomes containing these or other MAB fragments have not shown any significant differences in pharmacokinetic properties (ref. 4, 18; data not shown). Whether constructed with trastuzumab-derived Fab' (4) or scFv F5 (9), anti-HER2 immunoliposomal doxorubicin showed superior antitumor efficacy over nontargeted liposomal doxorubicin in HER2-overexpressing tumor models *in vivo*.

Table 1. Biodistribution of radiolabeled HER2-targeted and nontargeted sterically stabilized liposomes in nontumor tissues 24 hours post-i.v. injection

Tissue	Liposome uptake, injected dose/g tissue (%)	
	Nontargeted sterically stabilized liposomes	Anti-HER2 immunoliposomes
Blood	7.98 ± 0.85	7.04 ± 0.82
Liver	14.2 ± 1.9	15.6 ± 3.8
Spleen	33.5 ± 5.5	41.6 ± 4.7
Kidneys	3.72 ± 0.24	3.21 ± 0.24
Lungs	0.61 ± 0.10	0.42 ± 0.1
Heart	0.68 ± 0.16	0.58 ± 0.05
Skin	3.97 ± 0.72	4.71 ± 0.86
Muscle	0.74 ± 0.20	0.37 ± 0.12
Bone	3.50 ± 0.82	2.06 ± 0.35

NOTE: Mice with 200 to 300 mm³ s.c. tumor xenografts received a single i.v. injection of liposomes or anti-HER2-Fab'-conjugated immunoliposomes containing encapsulated ⁶⁷Ga-DTPA (4 μmol liposomal phospholipid/kg). Twenty-four hours later, animals were sacrificed and tissue radioactivity was determined (Materials and Methods). Total recovery of label >80% of calculated. Data represent mean ± SD (five animals/group).

Biodistribution of anti-HER2 immunoliposomes. Radiolabeled liposomes and anti-HER2 immunoliposomes were prepared by the encapsulation of ⁶⁷Ga-DTPA chelate, which is essentially membrane-impermeable and released only on breakdown of liposomes in tissues; once released, ⁶⁷Ga-DTPA is then rapidly excreted (12). We have reported that liposomes containing encapsulated ⁶⁷Ga-DTPA display essentially identical pharmacokinetics to cognate liposomes containing doxorubicin (4). This pharmacokinetic profile differs markedly from that of free Gd chelates, including Ga-DTPA, and rules out any significant Ga or Ga-DTPA release from liposomes during circulation. Furthermore, even if any released Ga were subject to transchelation such as to transferrin, the pharmacokinetics of liposome-encapsulated ⁶⁷Ga-DTPA corresponds to that of the liposomes themselves ($t_{1/2}$, 12-16 hours or more; ref. 4) and not to Ga-transferrin complexes ($t_{1/2}$, ~140 minutes; refs. 15, 19). Thus, liposome-encapsulated ⁶⁷Ga-DTPA provides an ideal *in vivo* marker for liposomal drug. A dose of 40 μmol phospholipid/kg was used in the biodistribution studies as it is equivalent to the therapeutic dose used in antitumor efficacy studies of liposomal versus immunoliposomal doxorubicin, which showed increased efficacy for immunoliposome treatment (4).

Twenty-four hours after i.v. injection, both anti-HER2 immunoliposomes and nontargeted liposomes distributed to comparable levels in all of the sampled normal tissues (Table 1). Immunoliposomes displayed a biodistribution pattern characteristic of long-circulating liposomes (20), including reduced reticuloendothelial uptake and high blood concentrations. It is notable that the presence of MAb fragments on immunoliposomes did not result in any apparent alteration of liposome biodistribution or pharmacokinetics. These results are consistent with the plasma pharmacokinetics previously observed for anti-HER2 immunoliposomes in normal adult rats, in which immunoliposomes and sterically stabilized liposomes displayed equally long circulation times (4).

Tumor localization of anti-HER2 immunoliposomes versus nontargeted liposomes. Increased tumor localization of MABs and immunoconjugates has long been considered a paradigm for antibody-based cancer treatments. Because both MABs and liposomes accumulate in tumors, we carefully evaluated the tumor localization of anti-HER2 immunoliposomes, including direct comparisons with nontargeted liposomes in HER2-overexpressing tumors following i.v. administration. Importantly, the anti-HER2 MAb-mediated targeting of immunoliposomes did not increase tumor accumulation over that of nontargeted sterically stabilized liposomes (Table 2). Both anti-HER2 immunoliposomes and nontargeted liposomes showed efficient localization in breast tumor xenografts: liposome levels reached 7% to 8% injected dose/g tumor tissue whether MAB fragments were present or not, and whether the tumor was HER2-overexpressing (BT-474) or not (MCF-7).

Some MAB/ligand-directed liposomes have been reported to provide prolonged retention in tumor tissue (21), suggesting increased tumor exposure to liposomal drug, and therefore increased efficacy. We evaluated the kinetics of liposome and anti-HER2 immunoliposome accumulation in tumors for 7 days postinjection (Fig. 1A). The time course of tumor concentration was largely identical for nontargeted liposomes and immunoliposomes. In both cases, tumor levels increased during the initial 24 hours postinjection, remained at high levels until 72 hours, and then slowly declined thereafter, with >1% injected dose/g of tumor tissue still remaining after 7 days. Although tumor levels of immunoliposomes were somewhat higher than those of nontargeted liposomes at certain time points, these differences were not significantly different ($P > 0.05$, two-tailed unpaired Student's *t* test) with the exception of a single time point (120 hours). We did not observe any significant label washout in these studies. As noted, the pharmacokinetic profile of liposome-encapsulated ⁶⁷Ga-DTPA seen here corresponds closely to that of liposomal and immunoliposomal doxorubicin in our previous studies (4), indicating highly stable encapsulation of marker or drug while circulating. The cellular metabolism of Gd-DTPA was not examined.

Table 2. Uptake of radiolabeled nontargeted liposomes and anti-HER2 immunoliposomes in breast tumor xenografts 24 hours post-i.v. injection

Tumor	Liposome uptake, injected dose/g tissue (%)		<i>P</i>
	Nontargeted sterically stabilized liposomes	Anti-HER2 immunoliposomes	
BT-474	7.32 ± 1.05	8.34 ± 1.54	0.256
MCF-7	8.59 ± 2.32	7.18 ± 1.04	0.376
BT-474 tumor/muscle	9.9	22.5	
MCF-7 tumor/muscle	11.6	19.4	

NOTE: For details, see Table 1. *P*, probability of the null hypothesis by the independent two-tailed *t* test, targeted versus nontargeted liposomes.

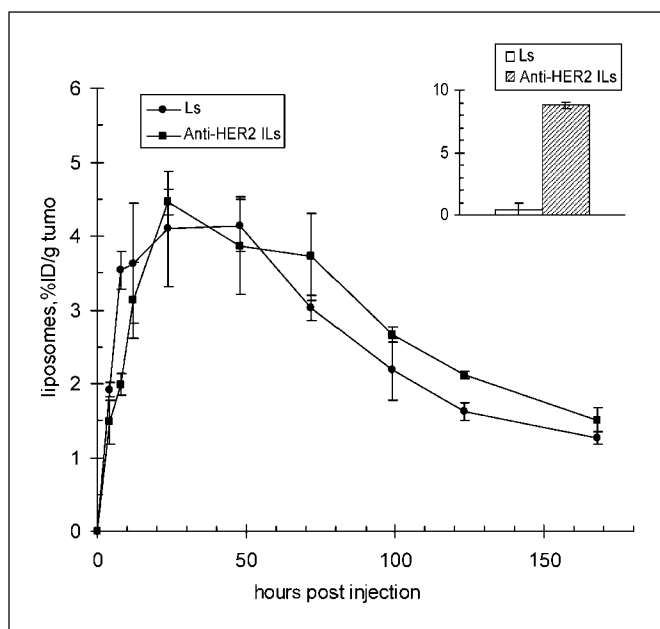


Figure 1. Tumor pharmacokinetics of ^{67}Ga -labeled anti-HER2 immunoliposomes (Fab' targeted) versus control pegylated liposomes (Ls) in s.c. BT-474 breast cancer xenografts in nude mice. Liposomes/immunoliposomes were administered i.v. at 40 μmol phospholipid/kg. Points, mean; bars, $\pm$ SD; three animals per group. *Inset*, uptake of anti-HER2 immunoliposomes (cross-hatched column) versus control liposomes (solid column) in HER2-overexpressing breast cancer cells (SK-BR-3) *in vitro*. Uptake units, nmol liposome phospholipid/ 10^6 cells (mean $\pm$ SD; $n = 4$ experiments).

As a positive control for the targeting effect *in vitro*, the same preparation of anti-HER2 immunoliposomes showed markedly greater uptake in cultured HER2-overexpressing breast cancer cells than nontargeted liposomes (Fig. 1, *inset*).

Doxorubicin-loaded anti-HER2 immunoliposomes showed significantly greater antitumor efficacy than nontargeted liposomal doxorubicin in this same BT-474 tumor model (4, 9), as well as in other HER2-overexpressing models (4). Yet, anti-HER2 immunoliposomes at equivalent doses failed to show increased accumulation or retention in tumor tissue compared with their nontargeted counterparts, thus ruling out increased tumor localization as the mechanism responsible for the therapeutic advantage of immunoliposome delivery.

Intratumoral distribution of anti-HER2 immunoliposomes versus nontargeted liposomes. This paradox was further investigated in detailed analyses of liposomes and immunoliposomes within tumor tissue, which revealed fundamental differences in microdistribution and cellular localization for anti-HER2 immunoliposomes in HER2-overexpressing tumors versus their nontargeted counterparts. As previously reported, anti-HER2 immunoliposomes undergo receptor-mediated endocytosis in target cells *in vitro*, resulting in intracellular delivery of encapsulated agents, whereas nontargeted liposomes do not bind or internalize in these cells (5, 6). To evaluate the distribution of these particles within tumor tissue following i.v. administration, anti-HER2 immunoliposomes or matching nontargeted liposomes labeled with encapsulated colloidal gold were administered i.v. in nude mice bearing BT-474 or MCF-7 tumors. Microdistribution studies using gold or fluorescently labeled liposomes/immunoliposomes employed a dose of 0.4 mmol/kg, which was higher than that used in bio-distribution and therapy studies in order to achieve greater signal.

Forty-eight hours postinjection, tumors were harvested and immunoliposomes/liposomes were visualized in tumor sections by light microscopy following silver enhancement (13).

Low-power magnification microscopy of BT-474 tumors showed that both nontargeted liposomes and anti-HER2 immunoliposomes had extravasated in large amounts from tumor vessels (Fig. 2A and B), consistent with the high tumor levels previously measured. Both nanoparticles were observed in areas of apparently viable tumor tissue, in necrotic regions, and particularly at the interface between these regions—a zone often associated with high local concentrations of angiogenic and permeability factors and consequent neovascularization (22, 23). Tumor penetration of liposomes and immunoliposomes clearly exceeded the predominantly perivascular deposition often reported with liposomes in other studies (24, 25); furthermore, a “binding site barrier” phenomenon described for some tumor cell-specific antibodies (28) was not observed for anti-HER2 immunoliposomes. At intermediate magnification of BT-474 tumor tissue, immunoliposome localization seemed to be qualitatively different from that of liposomes. Whereas immunoliposomes were distributed throughout tumor tissue in close association with tumor cells (Fig. 2C and D), nontargeted liposomes showed a more uneven or patchy distribution, with high focal accumulation within tumor stroma and less association with tumor cells (Fig. 2E and F).

At high-power magnification, anti-HER2 immunoliposomes were predominantly detected within tumor cells, as evidenced by silver grains in tumor cell cytoplasm (Fig. 3A). Internalized immunoliposomes were often concentrated in the perinuclear region of the cytoplasm, consistent with our previous observations in HER2-overexpressing cells *in vitro* (5, 6). In contrast, nontargeted liposomes accumulated predominantly within tumor stroma, either in the extracellular space or within tumor-resident macrophages (Fig. 3B). This extracellular and macrophage disposition is consistent with previous reports of the microdistribution of long-circulating liposomes within tumors (24). In marked contrast with anti-HER2 immunoliposomes, intracellular localization of nontargeted liposomes in cancer cells was a rare event. The accumulation of nontargeted liposomes in macrophages, however, was extremely high, with the amount of silver grains per cell too numerous to count.

As a further control, gold-labeled immunoliposomes or nontargeted liposomes were administered i.v. in the MCF-7 breast cancer xenograft model, which lacks HER2 gene amplification and expresses HER2 at low or basal levels ($\sim 10^4$ HER2 receptors/cell; immunohistochemistry score “1+”; ref. 26). The microdistribution of immunoliposomes in MCF-7 xenograft tissue was identical to that of nontargeted liposomes, showing only stromal and macrophage accumulation and virtually no tumor cell internalization (Fig. 3C). The mononuclear cells accumulating both HER2-targeted and nontargeted liposomes were confirmed to be macrophages by cytochemical staining for the monocyte marker, nonspecific esterase (Fig. 3D). In contrast, anti-HER2 immunoliposomes in BT-474 tumors were observed within tumor cells lacking nonspecific esterase staining (Fig. 3E). Thus, in the absence of tumor targeting, either due to the absence of MAb fragments (nontargeted liposomes) or due to insufficient antigen expression (anti-HER2 immunoliposomes in MCF-7 tumors), liposomes could accumulate to high levels in tumor tissue but fail to associate directly with cancer cells.

Cellular analysis of systemically treated tumors. To provide quantitative assessment of this targeting effect, we did two-color

ex vivo flow cytometry to measure the uptake of liposomes/immunoliposomes by tumor or stromal cells in systemically treated tumors. Following i.v. treatment, tumors were disaggregated and suspended, and populations of human carcinoma cells were distinguished from host/murine stromal cells via cytometric detection of HEA (EpCAM; ref. 27; Fig. 4). The extent of *in vivo* uptake of liposomes or immunoliposomes in these respective cell populations was then determined by flow cytometric quantitation of fluorochrome ADS645WS stably encapsulated in liposomes/immunoliposomes.

This assay clearly delineated two distinct cell populations in BT-474 xenografts: EpCAM(+) carcinoma cells and EpCAM(−) stromal cells (Fig. 4A). Forty-four hours after i.v. injection of fluorescently labeled liposomes or immunoliposomes, the EpCAM(+) tumor cell population showed a noticeable shift in fluorescence in the case of immunoliposomes, but showed a minimal shift with nontargeted liposomes. In the EpCAM(−) cell population, fluorescence uptake was comparably low in both liposome- and immunoliposome-treated animals (Fig. 4B and C). Observations of the event density (frequency) profiles of EpCAM and liposome signal intensity on two-dimensional graphs (Fig. 4D-F) indeed showed two distinct populations with high or low EpCAM staining. In animals receiving i.v. nontargeted liposomes, the liposome signal was distributed evenly between these two populations (Fig. 4D). In contrast, in animals receiving i.v. immunoliposomes, there was a pronounced

shift of the EpCAM(+) population into higher immunoliposome signal (Fig. 4E).

To distinguish intracellular versus surface uptake in tumor cells, confocal microscopy was done on the same samples (Fig. 4G and H). This analysis demonstrated that in cells showing positive staining with anti-EpCAM-FITC (i.e., in cancer cells), immunoliposomes were indeed internalized, whereas nontargeted liposomes showed minimal internalization. EpCAM-negative cells containing immunoliposomes or liposomes in high amounts were also observed, consistent with liposome-laden macrophages seen in histologic studies (Fig. 3).

Finally, the flow cytometric assay enabled quantitative analysis of the differential uptake of liposomes versus immunoliposomes in tumor cells versus host/murine cells following i.v. treatment of BT-474 tumor xenografts (Table 3). Immunoliposome delivery achieved true tumor cell targeting; mean immunoliposome-associated fluorescence in tumor cells was greater than in host stromal cells by a factor of 4-fold ($P = 0.005$). With nontargeted liposome treatment, liposome-associated fluorescence was comparable in cancer cells and host cells (cancer/host ratio of 1.5-fold; $P = 0.18$), indicating no selectivity for tumor cells. Comparison of tumor cell internalization following treatment with immunoliposomes or liposomes showed an almost 6-fold greater targeting of cancer cells by immunoliposomes, whereas the uptake of immunoliposomes by host cells increased only ~2-fold compared

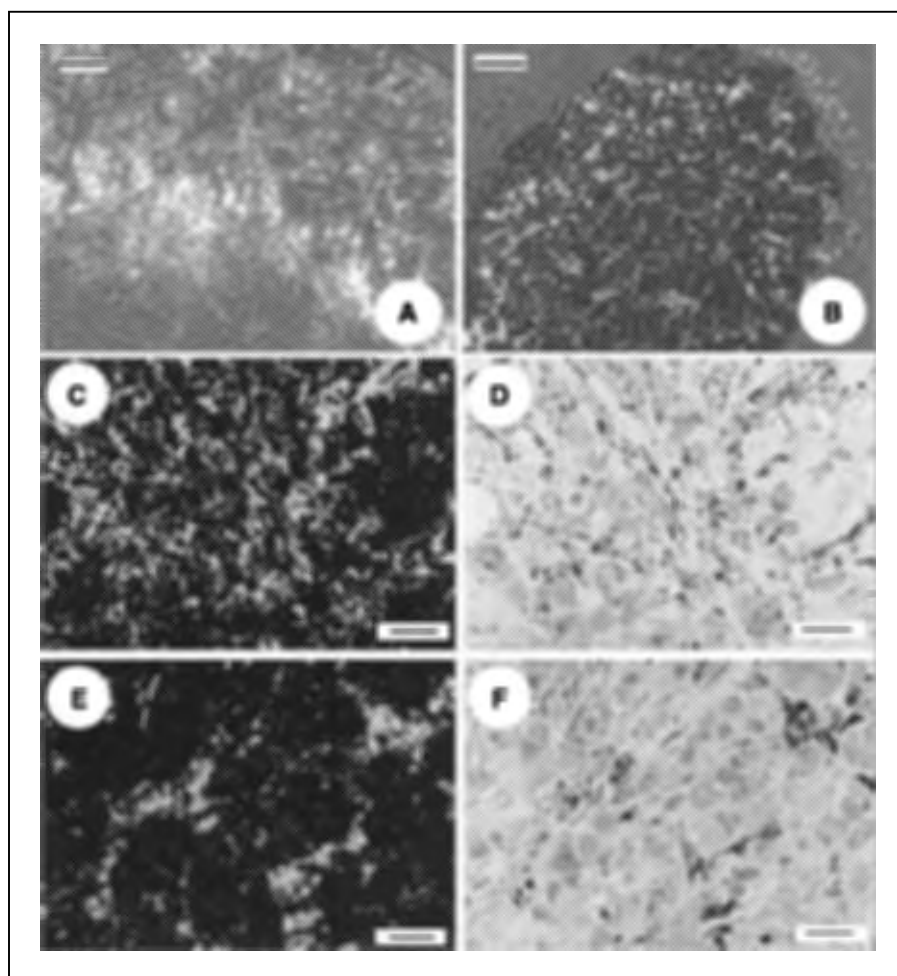


Figure 2. Microdistribution of anti-HER2 immunoliposomes (Fab' targeted) versus control pegylated liposomes in BT-474 breast cancer xenografts in nude mice. Forty-eight hours following i.v. injection, liposomes/immunoliposomes were visualized in tumor sections by silver enhancement of liposome-encapsulated colloidal gold, and appeared as bright dots on dark-field illumination microscopy (A, B, C, and E) or as black grains on bright-field microscopy (D and F). A and B, localization of anti-HER2 immunoliposomes (A) or control liposomes (B) in BT-474 tumors at low-power magnification ($\times 4$ objective; bar, 250 μm /L). Note the somewhat higher concentrations of liposomes at the interface between the tumor core and periphery. C-F, intermediate magnification ($\times 40$ objective; bar, 25 μm /L) of immunoliposome and liposome localization in BT-474 tumors. Anti-HER2 immunoliposomes showed extensive distribution within tumors (C, dark field; D, bright field). Control liposomes showed high accumulation but patchy distribution in these tumors (E, dark field; F, bright field).

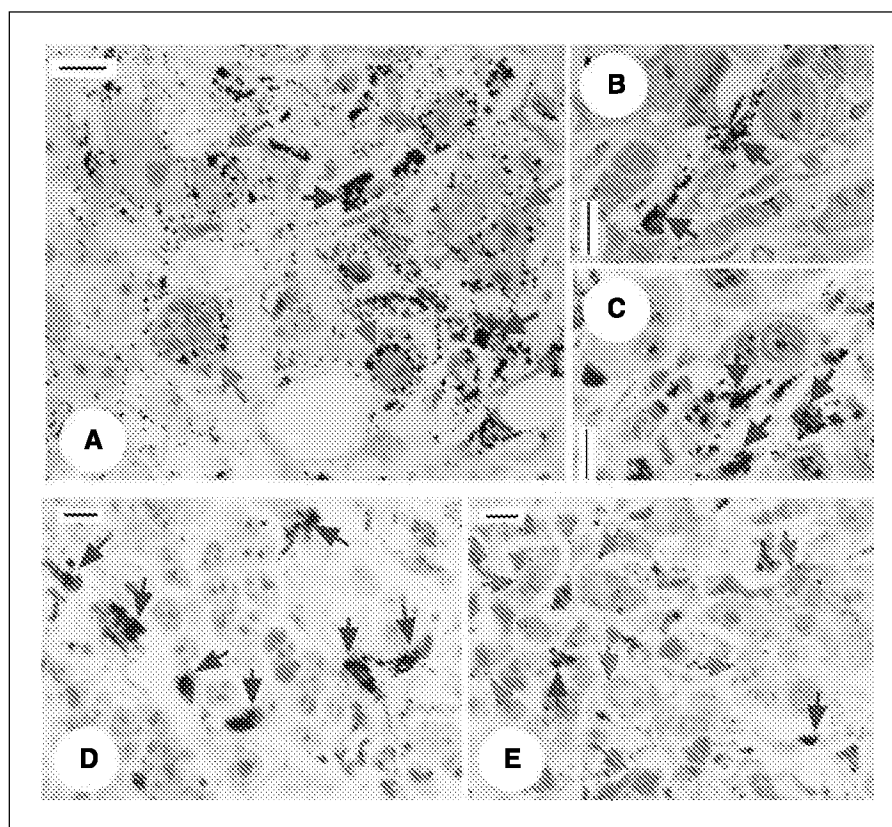


Figure 3. Detailed histologic analysis of the cellular internalization of anti-HER immunoliposomes (Fab' targeted) versus control pegylated liposomes in breast cancer xenografts in nude mice. Colloidal gold-encapsulating immunoliposomes/liposomes were administered i.v. in nude mice bearing either HER2-overexpressing BT-474 tumors (A, B, and E) or nonoverexpressing MCF-7 tumors (C and D). Forty-eight hours postinjection, tumors were excised and colloidal gold was visualized by silver enhancement. At high-power magnification ($\times 100$ objective; bar, 10 μm /L), cancer cells could be identified as large cells, often in clusters, with large nuclei and prominent nucleoli (magenta arrows). Macrophages could be identified as smaller, elongated, or pyramidal cells, typically within stromal gaps, with small densely stained nuclei (blue arrows). A-C, high-power magnification of immunoliposome and liposome uptake in cells within BT-474 or MCF-7 tumors. A, anti-HER2 immunoliposomes showed marked internalization in BT-474 tumor cells and in macrophages. B, nontargeted liposomes showed internalization only in macrophages within BT-474 tumor tissue, with minimal tumor cell uptake. C, anti-HER2 immunoliposomes showed internalization only in macrophages within MCF-7 tumor tissue, with minimal tumor cell uptake in this HER2-low tumor. D-E, high-power magnification with cytochemical staining of macrophages in MCF-7 or BT-474 tumor tissue from animals injected with anti-HER2 immunoliposomes. Macrophages were stained for nonspecific esterase activity (red) and tissue was counterstained with Gill's hematoxylin. Immunoliposomes were confirmed to be restricted to macrophage uptake in HER2-low MCF-7 tumors (D), but in contrast, had predominantly internalized within tumor cells in HER2-overexpressing BT-474 tumors (E).

with nontargeted liposomes. Because these measurements were obtained at a single time point (44 hours posttreatment) following one injection, this 6-fold increase in tumor cell uptake of immunoliposomes represents a robust therapeutic advantage in terms of total intracellular drug exposure. We cannot exclude that the higher doses used in the microdistribution studies may have altered the precise ratio of internalized versus noninternalized liposomes/immunoliposomes as compared with the therapy studies, perhaps decreasing this difference at higher doses due to greater nonspecific uptake.

Discussion

Anti-HER2 immunoliposomes were designed to achieve targeted intracellular delivery to HER2-overexpressing tumor cells. This design has several key features. First, immunoliposomes were rendered long-circulating by modifications including steric stabilization with a PEG-lipid conjugate. Second, the conjugation of multiple antibody fragments was achieved without compromising the pharmacokinetics or biodistribution of long-circulating liposomes. Antibody fragments Fab' and scFv, rather than intact

immunoglobulins, were used to minimize immunogenicity and to circumvent clearance via Fc receptor-mediated mechanisms. Finally, immunoliposomes were optimized for internalization in target cells, including the use of Fab' fragments from trastuzumab (28) or scFv F5 independently selected based on internalization ability in HER2-overexpressing cells (8). MAb fragments were also positioned distally from the liposome-grafted PEG layer to allow unhindered interaction with the target (6, 29).

We have previously reported that targeted delivery of doxorubicin via anti-HER2 immunoliposomes produced significantly enhanced antitumor efficacy in multiple HER2-overexpressing breast cancer xenograft models as compared with all other treatments tested, including liposomal doxorubicin lacking MAb fragments and the commercial preparation of pegylated liposomal doxorubicin (Doxil; ref. 4). Thus, targeting conferred substantial therapeutic advantage over nontargeted liposomal drug delivery. The present study now provides direct experimental evidence for a distinct mechanism for this enhanced efficacy, i.e., specific association of anti-HER2 immunoliposomes within HER2-overexpressing cancer cells rather than extracellular accumulation within tumor tissue.

Antibody-based agents for cancer therapy, either MABs themselves ("naked MABs") or immunoconjugates, have been proposed to provide selective, increased tumor localization due to antibody binding. Contrary to this paradigm, tumor tissue accumulation of immunoliposomes was not significantly increased

by MAB targeting, even though immunoliposomes treatment was associated with superior antitumor efficacy over nontargeted liposomes. This result is consistent with the following model for macromolecular agents such as liposomes or other nanoparticles: the rate-limiting step for their tumor localization is extravasation

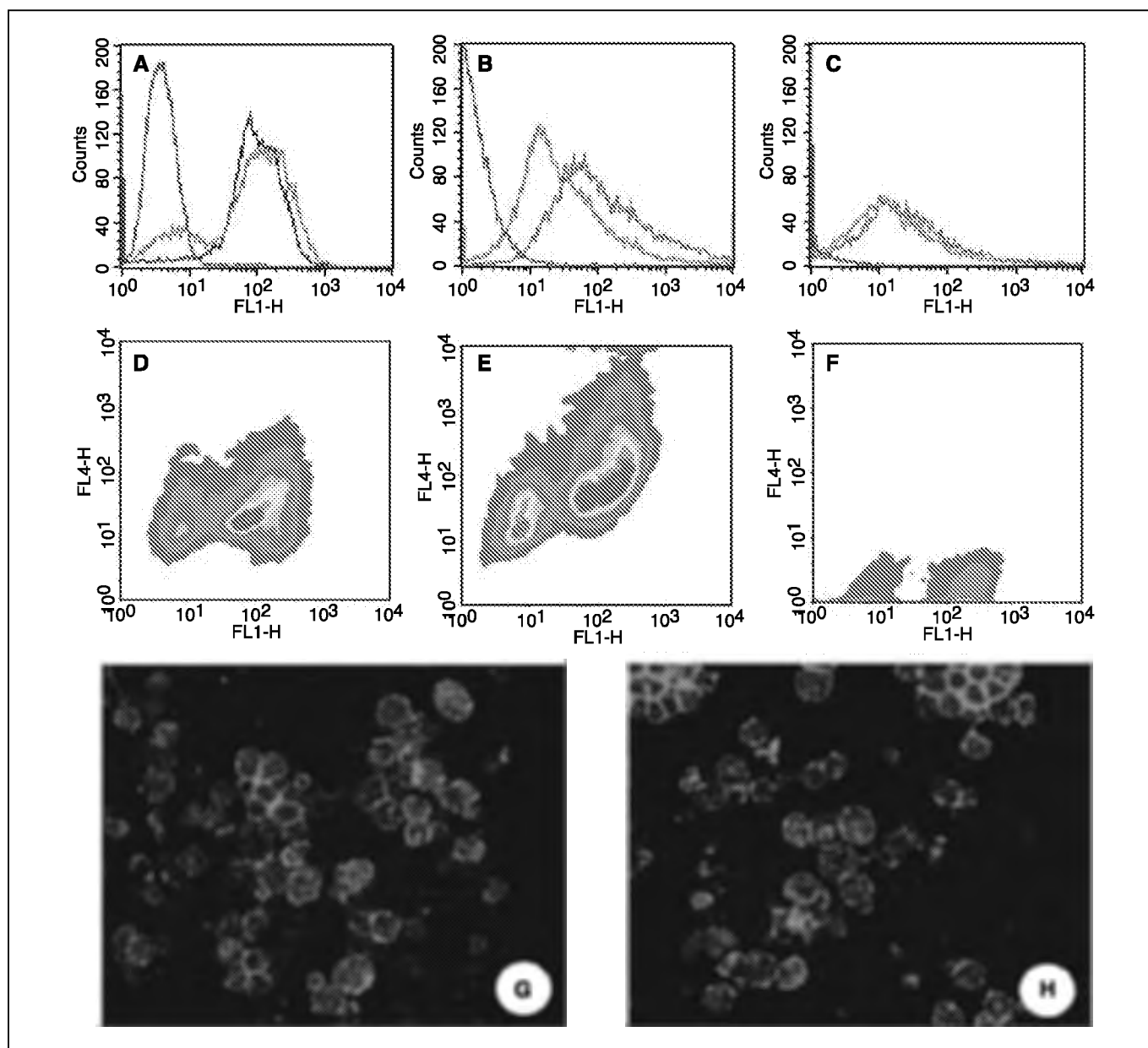


Figure 4. Flow cytometric and confocal fluorescence microscopic analyses of cells obtained from disaggregated s.c. BT-474 xenografts following i.v. injection of liposomes or anti-HER2 immunoliposomes (scFv F5-targeted). **A**, disaggregated BT-474 xenografts were stained for HEA (EpCAM) expression using anti-EpCAM MAb-FITC conjugate, demonstrating two distinct peaks (magenta line): EpCAM(+) carcinoma cells and EpCAM(-) host (murine) cells. As a control, BT-474 cells grown in culture were similarly stained, and appeared as a single peak (black line) corresponding with the EpCAM(+) cells harvested from disaggregated tumors. Blue line, unstained cells (autofluorescence control). **B** and **C**, liposomes or immunoliposomes containing encapsulated fluorescent marker, ADS645WS, were administered i.v. in BT-474 xenograft-bearing nude mice; 44 hours postinjection, tumors were disaggregated and analyzed by flow cytometry for anti-EpCAM MAb-FITC (to distinguish tumor versus host cells) and ADS645WS. Gating on the EpCAM(+) cell population (**B**) showed a clear shift indicating higher uptake of immunoliposomes (red line) as compared with nontargeted liposomes (green line). In contrast, gating on the EpCAM(-) cell population (**C**) showed equivalent and low uptake for both liposomes and immunoliposomes. Blue lines, tumor samples obtained from animals which received saline only (autofluorescence controls). **D-F**, two-dimensional event density plots of disaggregated tumor cell suspensions from animals injected with ADS645WS-labeled anti-HER2 immunoliposomes (**E**), control liposomes (**D**), or saline (**F**). Liposome label fluorescence was recorded in channel FL4-H and anti-EpCAM MAb-FITC fluorescence in channel FL1-H. Relative event density is color-coded as follows: blue (1-5%), light blue (5-15%), green (15-30%), yellow-green (30-45%), yellow (45-60%), orange (60-75%), red (75-90%), and magenta (90-100%). Note the shift to higher FL4 values in the FL1-gated cell population in animals injected with immunoliposomes as opposed to liposomes, in which there is no such shift. **G** and **H**, confocal microscopy of tumor cell suspensions obtained from mice injected with anti-HER2 immunoliposomes (**G**) or control liposomes (**H**). Liposomes/immunoliposomes were labeled with encapsulated ADS645WS (blue); tumor cells were counterstained with anti-EpCAM MAb-FITC conjugate (green). Magnification, $\times 400$.

Table 3. Flow cytometric analysis of uptake of immunoliposomes versus liposomes in cancer cells (EpCAM-positive) versus mouse host cells (EpCAM-negative) recovered from HER2-overexpressing BT-474 tumor xenografts in nude mice 44 hours after i.v. injection

Injected formulation	Cancer cells (EpCAM+)	Host/mouse cells (EpCAM-)	Ratio, cancer/host	P, tumor vs. host
Liposomes	74.1 ± 25.0	49.4 ± 4.1	1.5	0.1838
Anti-HER2 immunoliposomes	392.3 ± 84.4	98.3 ± 11.6	4.1	0.00843
Control (no treatment)	9.1 ± 4.7	4.9 ± 0.9	not done	0.3275
Ratio, immunoliposomes/liposomes	5.9	2.1		
P, immunoliposomes vs. liposomes	0.00503	0.00298		

NOTE: Data represent mean fluorescence ± SD of the indicated treatment groups ($n = 4$ per group). *P* values were calculated using nonpaired two-tailed Student's *t* test.

from tumor vasculature, and this is driven by factors such as circulation time and particle size. MAb-antigen interactions do not necessarily facilitate this process. Rather, both anti-HER2 immunoliposomes and nontargeted liposomes equally benefited from the “enhanced permeability and retention” (EPR) effect, in which long-circulating agents undergo preferential extravasation at sites of abnormal tumor vessel microanatomy associated with tumor angiogenesis, and combined with deficient tumor lymphatics leads to “passive targeting” or accumulation in tumors (30). Preclinical (31, 32) and clinical studies (33) previously showed that long-circulating liposomes do in fact preferentially accumulate in tumors as predicted by the EPR effect. Indeed, the efficiency of tumor localization observed for both targeted and nontargeted liposomes in this study (7-8% injected dose/g at 24 hours) was superior to that of free anti-HER2 MAb in similar models (34). The lack of increased tumor accumulation of alternatively designed anti-HER2 immunoliposomes as compared with nontargeted sterically stabilized liposomes has been previously reported (35). However, in these studies, antibody-directed targeting also did not result in therapeutic gain, possibly because of insufficient immunoliposome internalization.

The mechanism of anti-HER2 immunoliposome-mediated drug delivery is novel and distinct from that associated with drug delivery by nontargeted liposomes. Nontargeted sterically stabilized liposomes have been shown to accumulate in perivascular areas and adjacent extracellular space; eventually, liposome breakdown or phagocytic uptake could occur, resulting in the release of encapsulated drug for subsequent diffusion to nearby cancer cells (31). The studies in this report confirmed that nontargeted liposomes act by efficient tumor extravasation followed by accumulation in tumor stroma, including the extracellular space and within tumor-associated macrophages. In contrast, anti-HER2 immunoliposomes, after equally efficient extravasation, predominantly accumulated within the cytoplasm of HER2-overexpressing tumor cells, indicating that binding and internalization of immunoliposomes in tumor cells occurs *in vivo* as well as *in vitro*. The internalization of anti-HER2 immunoliposomes was previously shown to be mediated by the MAb fragment, as immunoliposomes bearing irrelevant MAb fragments failed to bind or internalize in HER2-overexpressing cancer cells *in vitro* (5). To our knowledge, this is the first direct demonstration of immunoliposome-mediated intracellular drug delivery in tumor cells *in vivo*.

How does the internalization of drug-carrying immunoliposomes in tumor cells enhance therapeutic efficacy against solid tumors? One explanation is that internalization leads to the intracellular release of bioactive drug near its subcellular site(s) of action. In the case of doxorubicin, acidification of the immunoliposome milieu upon receptor-mediated endocytosis would lead to drug release into the endolysosomal compartment, followed by rapid permeation of doxorubicin into the cytosol and nucleus. Redistribution of doxorubicin and other weak lipophilic bases across lipid membranes towards acidic pH is well-established; for example, the efflux of doxorubicin from liposomes into acidified medium has been directly shown (36). Another possibility is that MAb-targeting of immunoliposomes may facilitate better tumor penetration; this is supported by the more extensive distribution of immunoliposomes as compared with liposomes when visualized in tumor tissue. Although uptake by tumor-resident macrophages has been proposed as a mechanism for drug release from sterically stabilized liposomes (37), this mechanism seems to be less direct and less efficient than intracellular release within tumor cells. Hence, although immunoliposome delivery did not quantitatively increase drug levels in tumor tissue, it qualitatively altered the mechanism of delivery to tumor cells for enhanced activity.

In conclusion, we have shown a distinct mechanism for immunoliposomal drug delivery to tumor cells *in vivo*. This mechanism does not involve increased tumor localization, which was due to the EPR effect, but rather exploits antibody-dependent binding and internalization in tumor cells. These results may have implications for other nanoparticle-based agents containing MAb fragments or other ligands, and suggest that such approaches should be engineered for efficient internalization. Anti-HER2 immunoliposomes capable of penetrating tumor tissue, internalizing specifically in HER2-overexpressing cancer cells and intracellularly releasing encapsulated drugs represent a potentially advantageous strategy for molecularly targeted drug delivery.

Acknowledgments

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Antibody Targeting of Long-Circulating Lipidic Nanoparticles Does Not Increase Tumor Localization but Does Increase Internalization in Animal Models

Dmitri B. Kirpotin, Daryl C. Drummond, Yi Shao, et al.

Cancer Res 2006;66:6732-6740.

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Molecular Cancer Therapeutics

Topoisomerase Inhibitors

Abstract C207: Identifying differential mechanisms of action for MM-398/PEP02, a novel nanotherapeutic encapsulation of irinotecan.

Stephan G. Klinz, Nancy Paz, Ashish Kalra, Jaeyeon Kim, Bart Hendriks, Daryl Drummond, Dmitri Kirpotin, Victor Moyo, C. Grace Yeh, Clet Niyikiza, and Jonathan Fitzgerald

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Article

Info & Metrics

Abstracts: AACR-NCI-EORTC International Conference: Molecular Targets and Cancer Therapeutics—Nov 12-16, 2011; San Francisco, CA

Abstract

MM-398 (aka PEP02) is a stable, nanotherapeutic encapsulation of the pro-drug CPT-11 (irinotecan) that is currently in clinical development. In preclinical experiments, treatment with MM-398 resulted in significantly higher intratumoral concentrations of both irinotecan (142-fold) and its major metabolite, SN-38 (9-fold) and exhibited superior anti-tumor activity compared to free irinotecan in multiple tumor xenografts. Subsequently, multiple phase 1 and 2 studies have established a pharmacokinetic and safety profile that supports continued clinical development, including in pancreatic, gastric, colorectal and potentially other cancers.

Because current evidence suggests that resistance to pancreatic cancer is driven largely by inadequate drug penetration into these often poorly vascularized, stromally dense and hypoxic tumors, we sought to better understand how this relatively large (100nm) liposomal nanotherapeutic could potentially result in increased efficacy in very advanced gemcitabine-resistant pancreatic cancer and other cancer types.

We developed a mechanism-based PK model of MM-398 and free irinotecan designed to predict intratumor SN-38 levels. Sensitivity analysis revealed that for MM-398 local activation of irinotecan to SN-38 was a far more important parameter than systemic activation. Through cellular uptake studies, we demonstrated in vitro that MM-398 was

preferentially internalized by phagocytic macrophage/monocyte cell lines and, to a far lesser extent, by tumor cell lines. Furthermore, tumor microdistribution studies by flow cytometry and IHC showed uptake of MM-398 liposomes in both tumor cells and tumor-associated macrophages with more liposomal material being present in the macrophages. This distribution also suggests that macrophages may contribute to the postulated rate limiting process of irinotecan activation.

The sensitivity analysis also suggested that tumor permeability and vascularization are important determinants of tumor-associated SN-38 levels for both free irinotecan and MM-398. To determine the effect of MM-398 on these parameters we treated mice bearing HT29 (colorectal cancer) xenografts with a single dose of MM-398 and measured hypoxic markers (CAIX) and microvessel density (CD31) by IHC. Tumors treated with MM-398 showed a greater degree of CD31 staining and lower CAIX staining, indicating that MM-398 may be able to affect tumor characteristics that traditionally have contributed to therapy resistance and limited the delivery of cancer therapeutics and resistance.

In summary, encapsulation of irinotecan alters rate-limiting processes that determine tumoral SN-38 levels. Delivery of MM-398 is believed to alter tumor microvessel density and decrease hypoxia. These intriguing mechanisms of action findings support the continued clinical development of MM-398 as a differentiated therapeutic for several cancer types.

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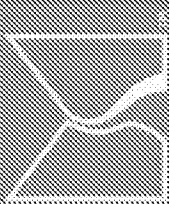
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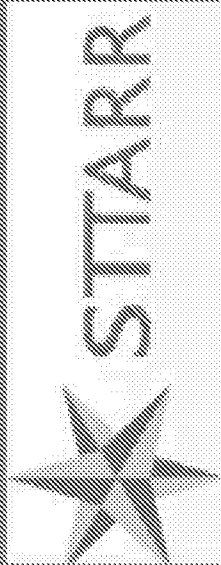
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Abstract #B47



Nanoliposomal irinotecan (nal-IRI) is an active treatment and reduces hypoxia as measured through longitudinal imaging using [¹⁸F]FAZA-PET in an orthotopic patient-derived tumorgraft model of pancreatic cancer

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Abstract

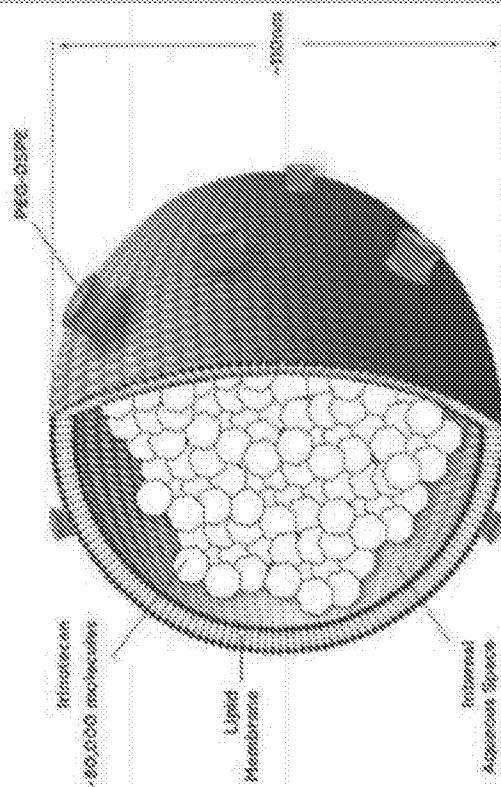
Introduction: Tumor hypoxia has been strongly linked to aggressive disease progression and resistance to therapy, especially in pancreatic cancer where the desmoplastic reaction is thought to also interfere with deposition of both small molecule drugs and nanotherapeutics. [⁶⁴Cu]fluorazomycin arabinoside (FAZA) is a radioactive tracer that allows for non-invasive quantification of tumor hypoxia during treatment by positron emission tomography (PET). We have previously shown that in the HT-29 cell-line derived xenograft model of colorectal cancer, nanoliposomal irinotecan (nal-IRI) achieves improved tumor growth control and is able to maintain a significantly lower level of tumor hypoxia as compared to non-liposomal irinotecan. Here, we evaluate the effects of nal-IRI on the kinetics and magnitude of hypoxia changes in an orthotopic patient-derived xenograft model of a pancreatic cancer (OCIP51) that is highly hypoxic.

Experimental Procedures: Tumor growth of orthotopically implanted OCP51 tumors was monitored using magnetic resonance imaging. Longitudinal FAZA-PET imaging of tumor hypoxia changes was performed over a 21-day period following weekly administration of nat-IRI at 20 mg/kg (n = 10) and compared to untreated controls (n = 5). Mean tumor FAZA uptake (%ID/g) and hypoxic fractions were calculated. In addition to PET, tumor cell proliferation was assessed using 3rd dosing cycle to assess tumor cell proliferation. Tumor growth was evaluated using an HPLC method in samples harvested 24 h after the last administration of a single dose of nat-IRI at 10 mg/kg. Nat-IRI induced DNA damage was assessed using γH2AX immunohistochemistry.

Results: nal-IRI treatment resulted in tumor growth inhibition of 71.6% compared to controls at study end. Tumor growth control was observable under Day 5 post treatment initiation. FAZA uptake in treated tumors decreased by 36% within the first treatment cycle, while average FAZA levels in control tumors remained unchanged during this period. Nal-IRI treatment resulted in statistically significant decreases in the FLU_{max} and FLU_{mean} values compared to pre-treatment values. 100% of nal-IRI treated mice survived to study end compared to only 40% of controls. Tumor weights at study end were almost 4 times smaller in nal-IRI-treated mice compared to the controls. Tumors from treated mice were fluid-filled and showed extensive blood pooling, while tumors from untreated mice appeared to be much less vascularized. Irinotecan levels detected in the OCIP51 tumors were 2 times lower at 72 h after nal-IRI administration, while SN-38 levels were ~28 times lower when compared to previous findings in

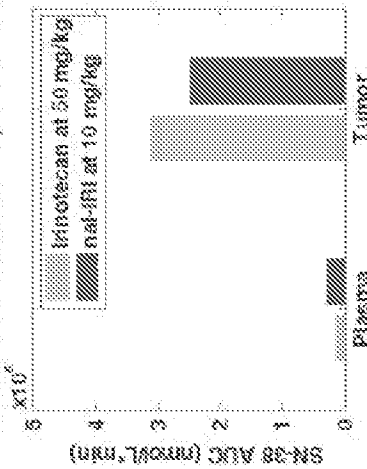
HT-29 tumors. Treatment with nal-IRI in the OCIP51 tumors significantly increased the frequency and intensity of γ H2AX staining across tumor cell areas compared to that observed in the untreated tumors, which were characterized by only a scattered and sporadic γ H2AX staining. Importantly, the stromal areas did not show γ H2AX staining in either the treated or the control group.

Conclusions: This study demonstrated the feasibility of performing longitudinal tumor hypoxia and proliferation assessments using FAZA- and FLT-PET imaging in a highly hypoxic orthotopic model of pancreatic cancer. Although this model showed reduced levels of liposomal drug deposition compared to cell-line derived xenograft models, treatment with nal-IRI led to effective tumor growth control, as well as significant changes in the tumor microenvironment as measured by reduced hypoxia levels compared to baseline and control tumors. Results from this study support the utility of FAZA-PET for evaluation of tumor hypoxia after anti-cancer therapy with nal-IRI as a means to provide early assessment of treatment activity.



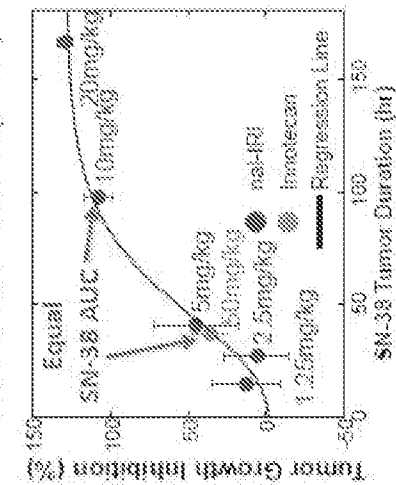
1 - Exposure-activity relationship for nal-IRI and irinotecan

A Dose levels of nal-IRI and irinotecan with similar SN-38 AUC (Simulation)



Yoon A et al., *Cancer Research* 2014 Oct;74(23):7603-13.

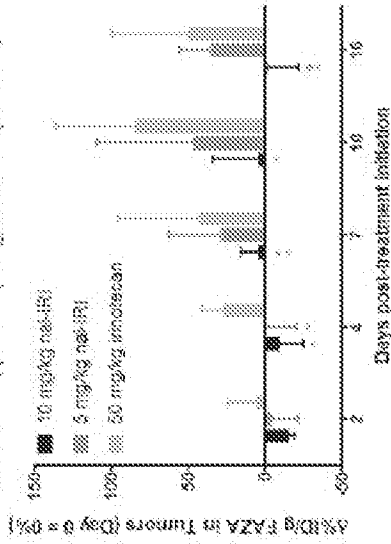
B Tumor growth inhibition is a function of SN-38 tumor duration (HT-29)



SN-38 duration is defined as the length of time the

SN-38 tumor concentration is above 120 nmol/L.

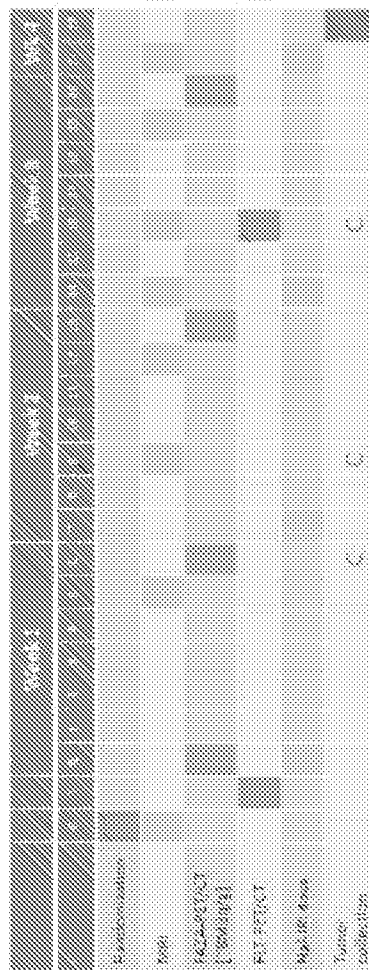
C Treatment with nal-IRI at 10 mg/kg inhibits hypoxia progression (HT-29)



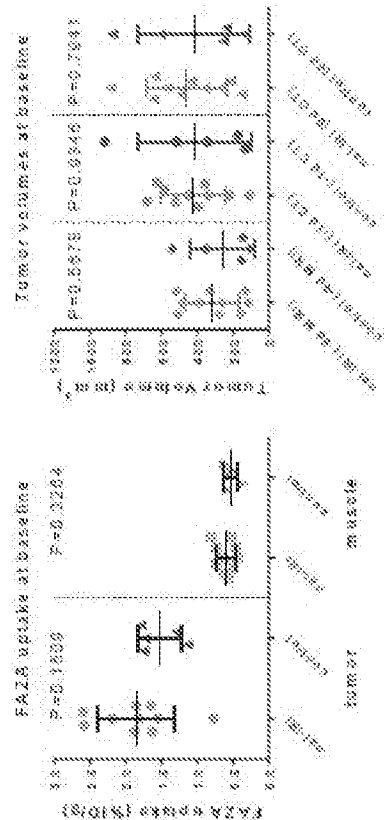
Zhang L et al., *BMC Cancer Research* 2015;5(1):52.

2 - Baseline characteristics for longitudinal FAZA-PET study

A Experimental schedule



8. FAZA uptake in tumor or muscle and tumor volumes are comparable between treatment cohorts at baseline.

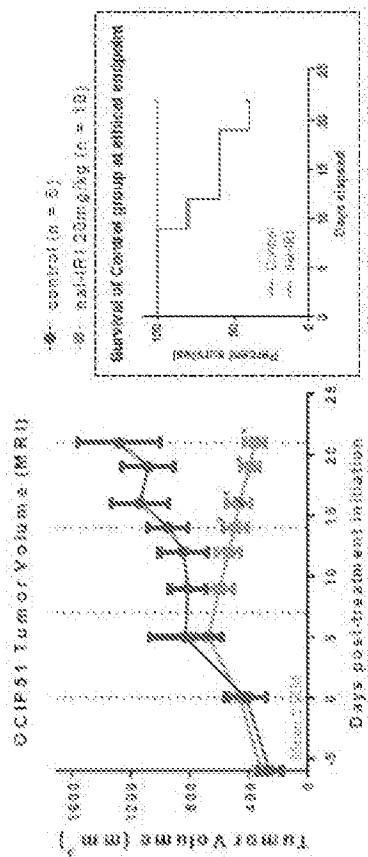


OCIP51 tumor fragments were implanted orthotopically into female NOD/SCID mice. Rindomycin treatment occurred 22 days post-implantation (day -8).

tumor CAZA uptake is higher compared to muscle ($p < 0.001$).

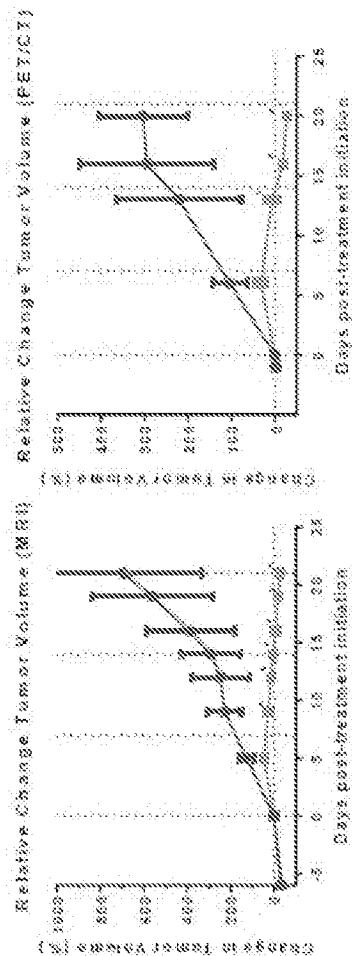
3 - nal-IRI inhibits OCIP51 growth in orthotopic setting

A OCIP51 tumor growth is well controlled by nal-IRI and extends animal survival



Nal-IRI is dosed weekly
p=0.0151 for day 14

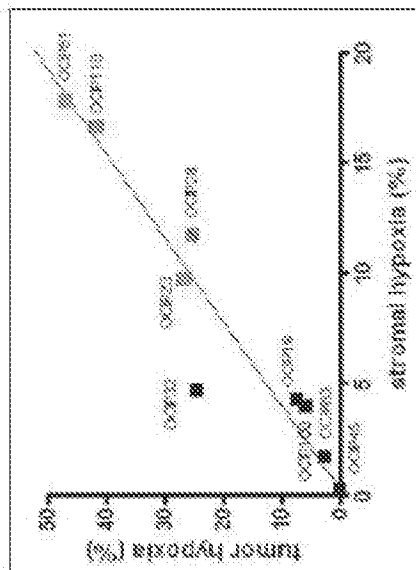
B Changes in tumor volume relative to pre-treatment sizes for MRI (left) and PET/CT (right) imaging



MRI: p=0.0011 for day 9; two-way ANOVA with multiple comparisons
PET/CT: p<0.0001 for days 9, 16, 20

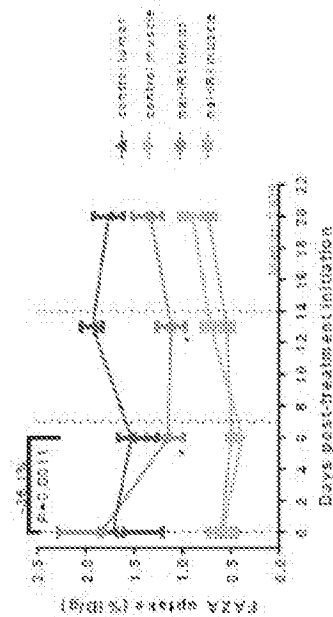
4 - nal-IRI reduces hypoxia in the OCIP51 model

A High levels of tumor and stromal hypoxia in OCIP51 PDAC tumor model



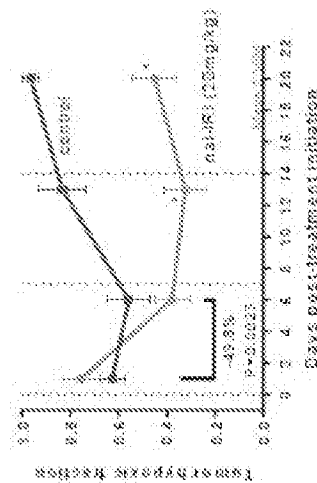
Lohse J et al. *Cancers (Basel)* 2012 Jan;6(1):459–71.

B Tumor FAZA uptake decreases after nal-IRI in OCIP51 tumors



Differences in FAZA uptake between treatment groups are significant at day 6 ($P=0.0496$), unpaired t test with Welch's correction) and day 13 ($P<0.0001$).

C Hypoxic fraction¹ in nal-IRI treated tumors is greatly reduced

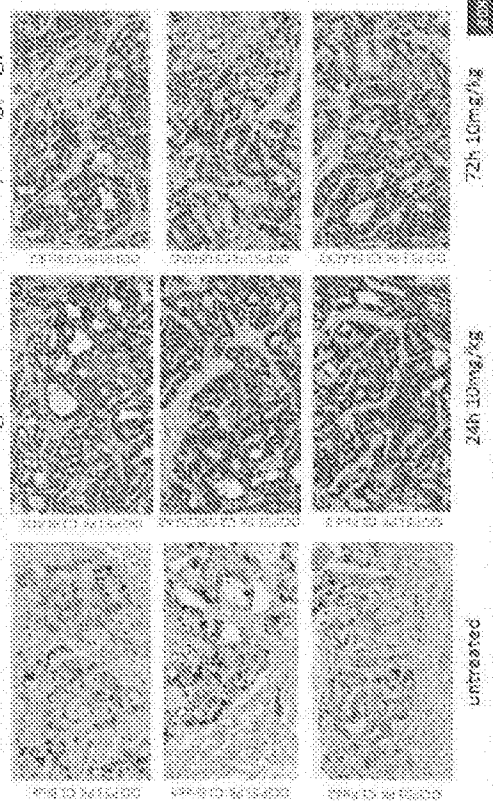


Differences in hypoxic fractions between treatment groups are significant at day 13 ($P=0.0035$) and day 20 ($P=0.0035$).

¹Mortensen IS et al. *Radiotherapy & Oncology* 2012;105(1):14–20.

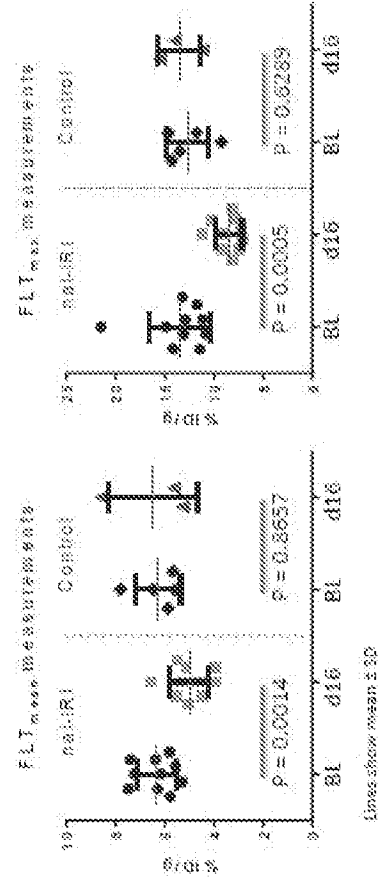
5 - nal-IRI induces γ H2AX and reduces FLT-PET signals in OCIP51

A γ H2AX expression is increased in tumor, but not stromal areas of OCIP51 after single dose of nal-IRI (10mg/kg)



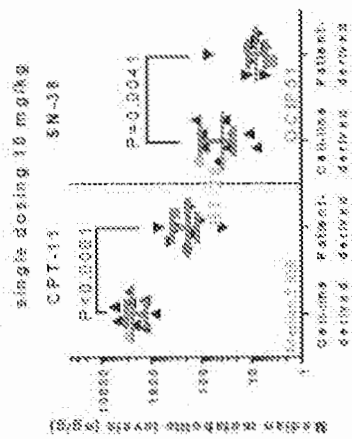
Anti- γ H2AX antibody clone 18W301 (MABipore)
Image acquisition on an Aperio® ScanScope® AT Turbo

B Mean and maximal FLT-PET signals are decreased by nal-IRI, but not in control tumors at day 16 of treatment



6 - Tumor metabolite drug levels and post-treatment weights

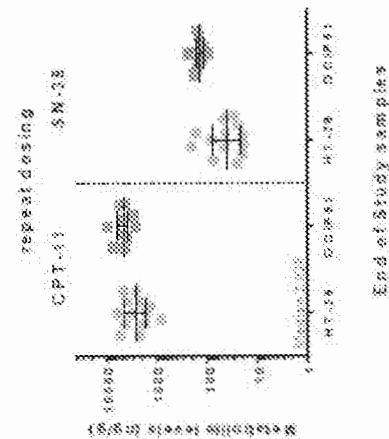
A Metabolite levels after single dose nat-IRI (72 h)



Medians with n=4-5 tumors/model are plotted.

Kara AV et al. Cancer Research 2014 Oct 74(25):7003-12

B Metabolite levels after repeat dose nat-IRI (day 22)

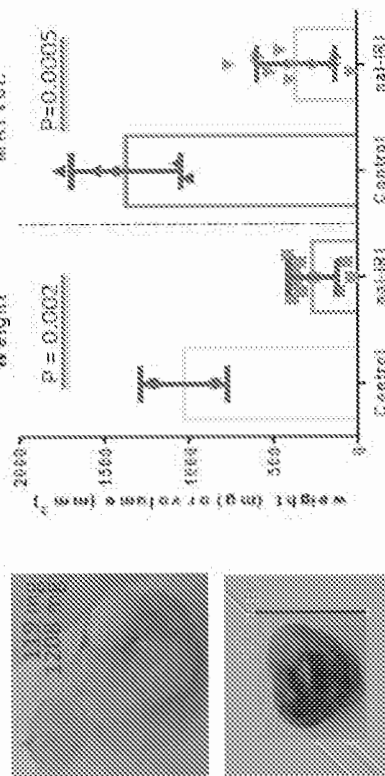


End of study samples

HT-29 data (10mg/kg) from:

Kara AV et al. Molecular Cancer Therapeutics 2013 12(11 Suppl):C293

C Tumor weights and MRI volumes at end of treatment



Tumor weights at study end (day 22) and MRI volumes for all lesions from last available time-point prior to sacrifice.

Summary

- The highly hypoxic orthotopic OCIP51 pancreatic patient-derived xenograft model is sensitive to nal-IRI treatment.
- Nal-IRI treatment reduced overall [^{18}F]FAZA uptake as well as hypoxic fraction compared to control tumors.
- Nal-IRI increased the expression of the DNA damage marker γH2AX at 24 – 72 h after dosing in tumor cells, but not stromal areas.
- [^{18}F]FLT- PET demonstrated decreased tumor proliferation as a function of thymidine salvage pathway utilization 48h after the 3rd dose of nal-IRI.
- Compared to cell-line derived tumor models, initial liposome deposition and SN-38 levels are reduced in the OCIP51 and other patient-derived tumorgraft models .

Despite greater hypoxia and lower initial deposition in pancreatic patient derived xenografts compared to cell-line derived xenografts, nal-IRI was effective at controlling tumor growth of OCIP51. Changes in hypoxia or tumor proliferation following treatment with nal-IRI may allow early assessment of treatment activity.

Irinotecan sucrosfate liposome injection, MM-398, demonstrates superior activity and control of hypoxia as measured through longitudinal imaging using [¹⁸F]FAZA PET compared to free irinotecan in a colon adenocarcinoma xenograft model

Stephan G. Klinz¹, Jinzi Zheng², Raquel De Souza², Michael Dunne², Jason Cain¹, Nancy Paz¹, Arnold Sengooba¹, Ashish Kalra¹, Jaeyeon Kim¹, David Jaffray², Jonathan Fitzgerald¹

¹Merrimack Pharmaceuticals, Cambridge, MA, USA;

²STARR Innovation Centre, Radiation Medicine Program, Princess Margaret Cancer Centre, Toronto, ON, Canada

Abstract #C293

Abstract

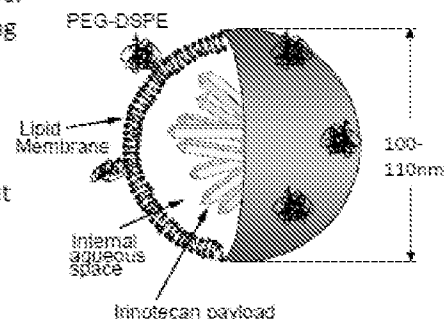
Tumor hypoxia is strongly linked to aggressive disease progression and resistance to therapy. Positron emission tomography (PET) imaging with hypoxia tracers such as [¹⁸F]fluoroazomycin arabinoside (FAZA) allows for non-invasive quantification of tumor hypoxia during treatment. We and others have previously shown by immunohistochemical methods that treatments with longer lasting camptothecin formulations reduce tumor hypoxia after either single or multiple treatment cycles. Here we evaluated the kinetics and magnitude of hypoxia changes in tumors after treatment with Irinotecan sucrosfate liposome injection (MM-398) which has shown an extended plasma half-life and higher intratumoral deposition in animal models relative to free pro-drug and compare it to the effects of free irinotecan at equivalent exposure levels.

FAZA-PET/CT was used for longitudinal monitoring of tumor hypoxia changes in the HT29 mouse xenograft model of colon cancer over a 21-day period following weekly chemotherapy administrations of either MM-398 (5 & 10mg/kg) or free irinotecan (50mg/kg). These dosages were predicted to result in comparable SN-38 exposure in either plasma or tumor based on a mechanistic pharmacokinetic model of MM-398 and free irinotecan that was developed using a systems pharmacology approach.

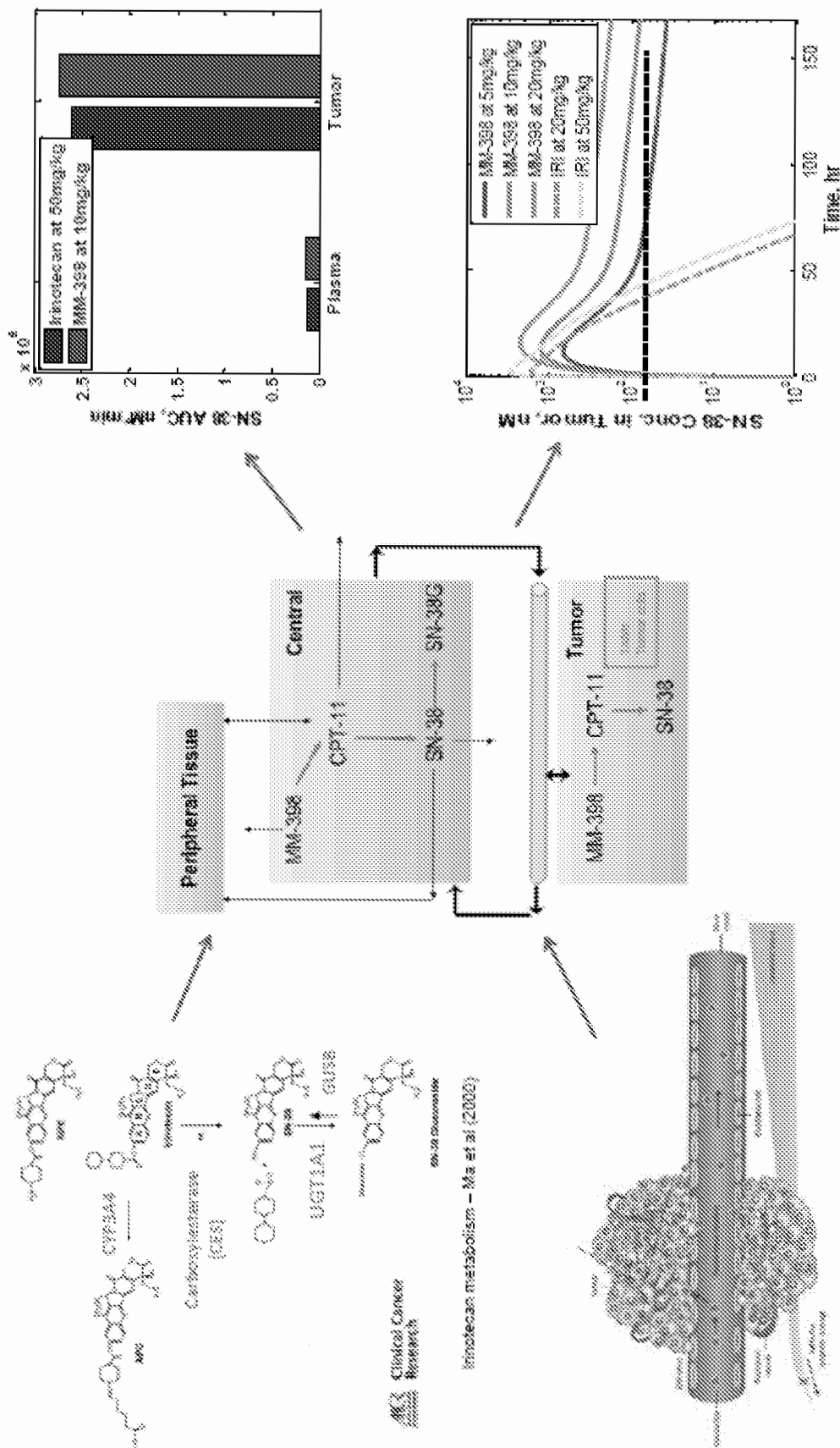
Baseline levels of FAZA uptake in tumors were similar across treatment groups. Significant differences in tumor FAZA uptake were observed between these groups as early as Day 7 post treatment initiation, with increased FAZA uptake seen in tumors treated with free irinotecan. In contrast, differences in tumor volume only became statistically significant on Day 16. MM-398 at 10mg/kg was the most active treatment for control of tumor volume and also minimized changes in FAZA uptake at all time-points. Background FAZA levels in the muscle were consistent over time across all treatment groups (0.78 ± 0.18 %ID/g, 0.81 ± 0.11 %ID/g and 0.71 ± 0.20 %ID/g). However, normalization with muscle signal did not improve quantification of FAZA uptake differences in tumors.

Tumor-specific hypoxia status at the study end point was confirmed by co-staining for CA9 and EF5 levels, which were, as expected, highly correlated. Average EF5 intensity/tumor area was lowest in the MM-398 (10mg/kg) treatment group, while being highest in the irinotecan (50mg/kg) treatment group.

This study demonstrated the feasibility of performing longitudinal and repeated tumor hypoxia assessment using FAZA-PET imaging. Treatment with MM-398, but not free irinotecan, led to significant changes in the tumor microenvironment as measured by reduced hypoxia levels that occurred far earlier than anatomical changes assessed by tumor volume. Imaging of hypoxia levels after anti-cancer therapy with MM-398 has the potential to allow early assessment of treatment activity.



MM-398 Background and pharmacokinetic modeling



The EPR effect – Peer D et al (2007)

Figure 1. Irinotecan succinate liposome injection (MM-398) is a nanopiposomal delivery system for the existing anticancer drug, irinotecan. MM-398 was designed to attempt to protect the drug from unwanted metabolism, extending the residence time in plasma and tissue, and increasing tumor accumulation via the enhanced permeability and retention (EPR) effect. A mechanistic tumor PK model was developed that identifies determinants that differentiate MM-398 from free irinotecan. Equal exposure levels of SN-38 in plasma and tumor can be achieved at either 10mg/kg MM-398 or 50mg/kg free irinotecan. However, only MM-398 treatment results in prolonged duration of potentially therapeutic SN-38 levels in the tumor.

Baseline characteristics for longitudinal FAZA-PET study

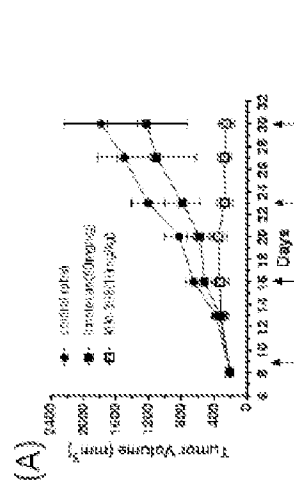
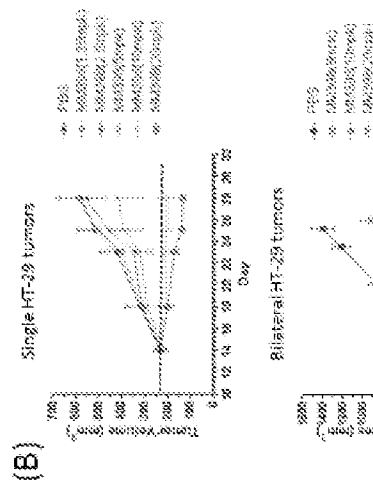
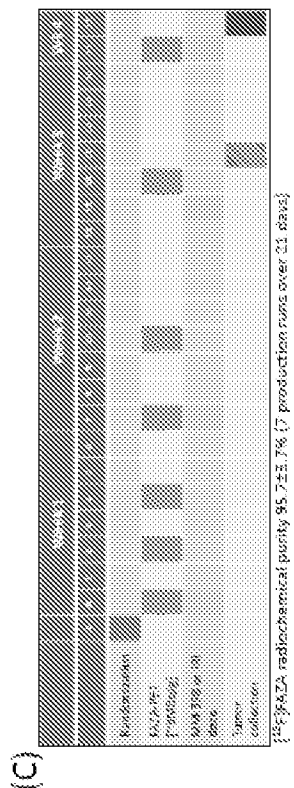
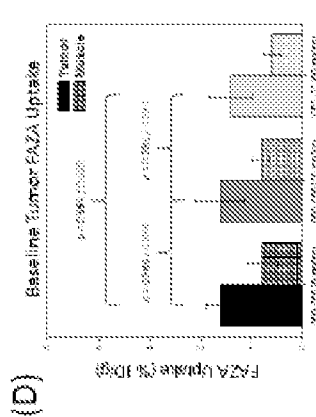
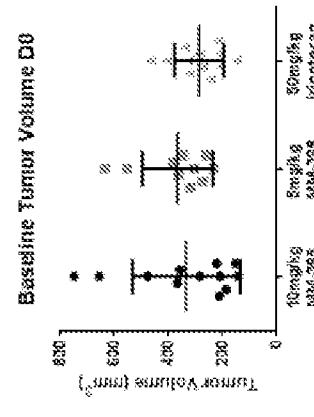
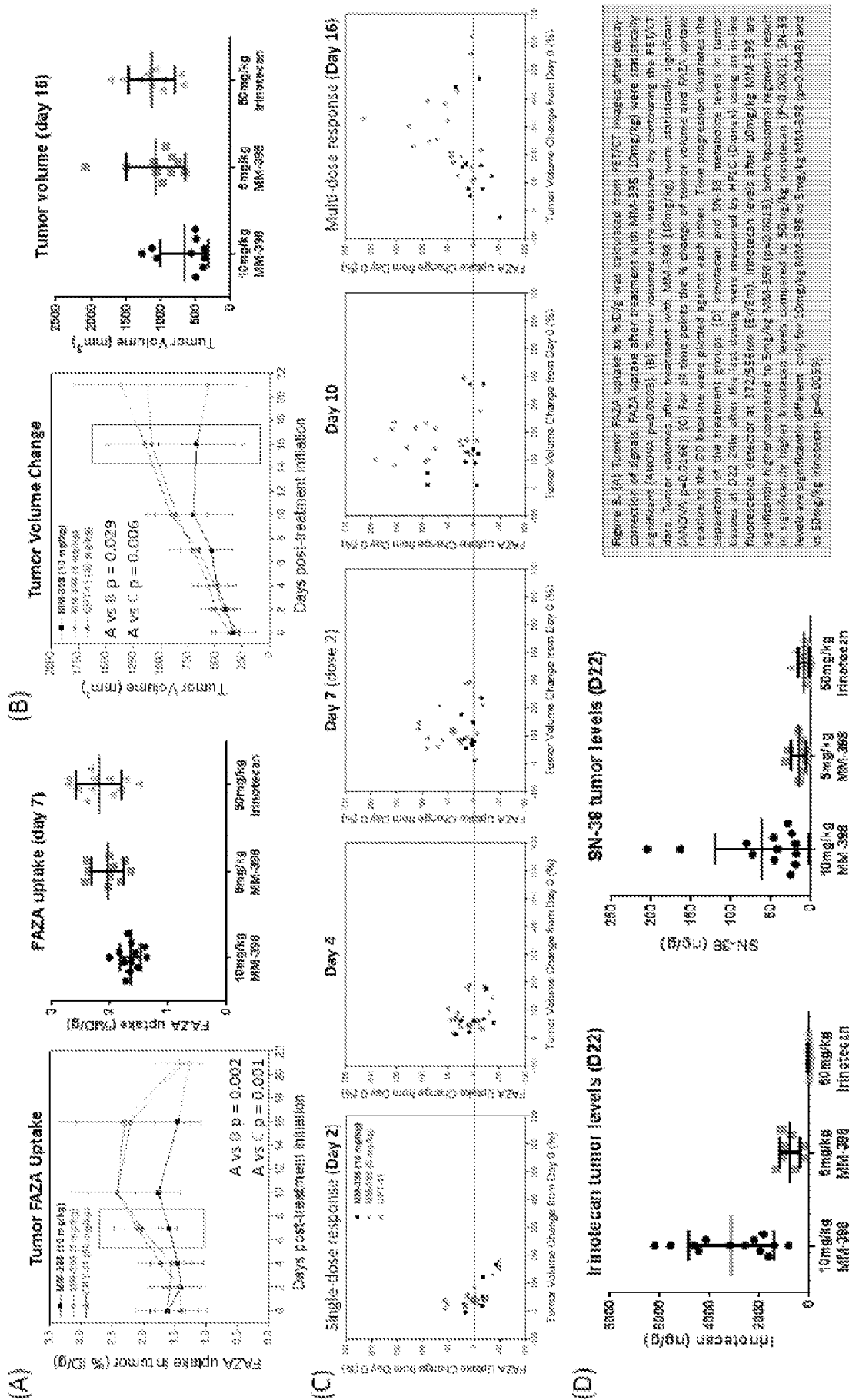


Figure 1. (A) Typical HT-29 tumor growth curves after treatment with irinotecan (50mg/kg or 100mg/kg) resulting in equal HT-29 tumor exposure. Growth inhibition with MM-398 was complete, while irinotecan (50mg/kg or 100mg/kg) showed moderate growth inhibition compared to single tumors when treated with equal amounts of MM-398. Dosing was qd. (C) Experimental schedule for FAZA-PET/CT imaging and drug dosing. (D) FAZA-PET/CT was injected 24h prior to imaging. Drug was injected after FAZA using the same iv line to preserve injection points on the tail. Then mice were imaged together using a Minneapoli Med system. Imaging was acquired on a microPET Focus 220 preclinical scanner (Siemens). Attenuation correction data was obtained via a rotating Co-57 point source. Anatomical CT scans were obtained on a Locus Ultra microCT preclinical scanner (GE Healthcare). PET/CT images were fused using the Invenia Research Workplace software (Siemens). (E) At study baseline FAZA uptake in tumor vs muscle was comparable across all three treatment groups (n=10). Tumor volumes were measured by contouring the PET/CT data and did not show obvious differences (ANOVA p=0.4403). No correlation between tumor FAZA uptake and tumor volume for individual tumors was observed at Day 0 (Pearson's r=-0.094).

Hypoxia within HT-29 tumors is stabilized by MM-398



Markers for Hypoxia and DNA damage are differentially modulated by Irinotecan and MM-398

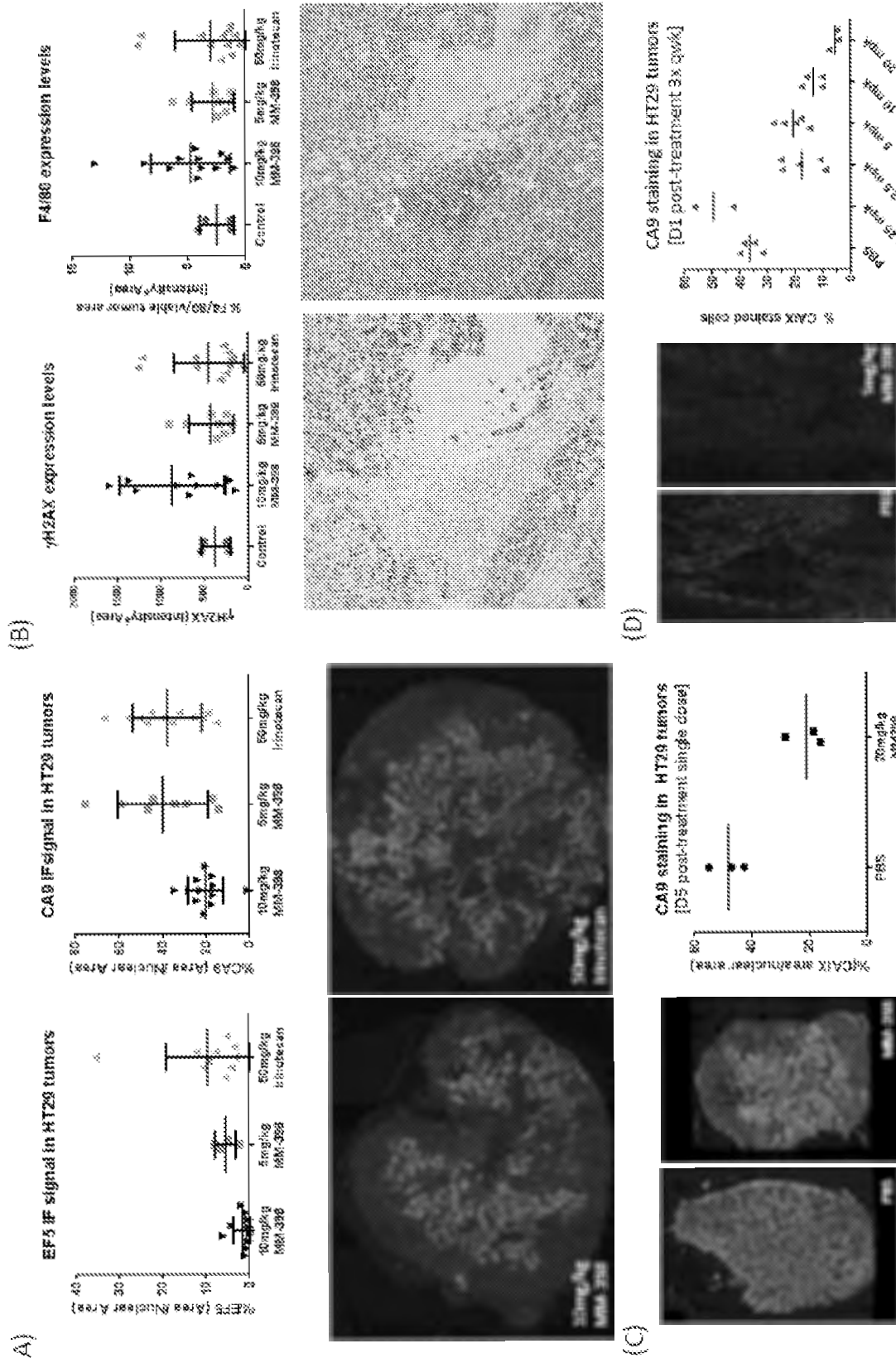


Figure 3. (A) EF5 and CA9 expression was evaluated in tumors harvested at end of study (D17-D22) by co-immunofluorescence. Fluorescence were Cx5 for EF5 and AF568 for CA9. Whole-tumor image acquisition was done on a Nikon TeComanager TissueScope scanner with optimized exposure conditions. Confocal TissueStudio software was used to quantify EF5 and CA9 marker area content normalized to total tissue nuclear area. MM-398 (10mg/kg, 50mg/kg) showed a statistically significant decrease in signal (look-way, 2400x, EF5 post-D22, CA9 post-D22) compared to the other groups. Fluorescent images show tumors (CA9 black, EF5 green) at tumors of comparable size harvested at D17 (B) γH2AX and F4/80 expression was evaluated in tumors harvested at end of study (D17-D22) by TissueStudio software. Whole-tumor image acquisition was done on an Aperio ScanScope 2.7. Marker quantification was done with a custom-built Matlab-based image analysis program. A representative section from tumor Fox Black EF (10mg/kg) for γH2AX (left) and F4/80 (right) is shown. (C) Single Rank HT-29 tumors were isolated 5 days after i.v. injection of MM-398 (20mg/kg) corresponding to the earliest time point growth reduction observed. Frozen sections were stained for CA9. Whole-tumor image acquisition was done on an Aperio ScanScope 2.7 with optimized exposure conditions. Confocal TissueStudio software was used to quantify CA9 marker area content normalized to total tissue nuclear area. MM-398-treated tumor showed a 50% reduction in CA9 staining (2400x) compared to PBS-treated controls. (D) Single Rank HT-29 tumors were dosed 3x wk with different concentrations of MM-398 (1.25mg/kg to 20 mg/kg). Shown here for 5mg/kg MM-398. The tumors were collected at the end of the study. The samples were stained with CA9 as a hypoxia marker and whole-tumor image acquisition was done on an Aperio ScanScope 2.7. Quantification with Defining TissueStudio software showed a significant dose-dependent reduction in CA9 staining following MM-398 treatment as compared to PBS controls.

Current Clinical Activities

- Ongoing Phase 3 study of MM-398 with or without 5-FU and leucovorin in metastatic pancreatic cancer patients
- Ongoing Phase 2 combination with 5-FU & Leucovorin in 2nd line colorectal cancer
- Ongoing cross-indication translational study to identify predictive biomarkers

Summary

- Demonstrated feasibility in performing longitudinal and repeated tumor hypoxia assessment with [¹⁸F]FAZA-PET
- Detected differences in tumor hypoxia levels within tumor-size matched groups and in response to treatment
- Confirmed hypoxia status for tumors at study endpoint and MM-398 treatment-dependent decrease of hypoxic fraction by EF5 and CA9 staining
- Hypoxia changes following treatment with MM-398 may allow early assessment of treatment activity
- Irinotecan sucrosfate liposome injection (MM-398) , but not free irinotecan, leads to early changes in the tumor microenvironment and demonstrates improved tumor growth control in a preclinical HT-29 colorectal cancer model

Cancer Research

cancerres.aacrjournals.org

doi: 10.1158/1538-7445.AM2011-3637

Cancer Res April 15, 2011 71; 3637

Abstract 3637: MM-302, a HER2-targeted liposomal doxorubicin, shows binding/uptake and efficacy in HER2 2+ cells and xenograft models

Stephan Klinz¹, Isabelle Eckelhofer¹, Daniel Gaddy¹, Elena Geretti¹, Bart Hendriks¹, Helen Lee¹, Shannon Leonard¹, Joe Reynolds¹, and Tom Wickham¹

Author Affiliations

Proceedings: AACR 102nd Annual Meeting 2011-- Apr 2-6, 2011; Orlando, FL

Abstract

Introduction: MM-302 is an ErbB2/HER2-targeted liposomal doxorubicin formulation designed to target doxorubicin to HER2-overexpressing cancer cells while limiting uptake into non-target cells. The characterization of the binding and uptake of MM-302 as a function of HER2 surface levels on various cell lines and in tumor models will help determine the minimum HER2 receptor levels required for MM-302 patient selection.

Methods: Cellular models included 16+ established human tumor cell lines as well as 4T1 murine breast cancer and HeLa human cervical cancer cells engineered to overexpress HER2 from 1E05-1E06 receptors/cell. Cells were incubated with MM-302, untargeted liposomes or free doxorubicin and binding/uptake was measured by HPLC or flow cytometry. In vivo efficacy was determined in select mouse xenograft models. Tumors were mechanically and enzymatically dissociated, followed by flow cytometry to quantify the uptake of targeted and untargeted liposomes into HER2-expressing tumor cells, macrophages and other cell types.

Results: Binding and uptake of MM-302 increased with rising HER2 surface levels in cells of common parental origin (4T1, HeLa), with significant uptake into cells with >2E05 HER2/cell. Further, total cell binding/uptake of MM-302 also increased with rising HER2 expression across a panel of 16+ cell lines. There was negligible uptake of untargeted liposomes under all conditions that showed no dependence on HER2 expression. Improved anti-tumor efficacy of MM-302 was observed in BT474-M3 and NCI-N87 mouse xenograft models relative to untargeted liposomal doxorubicin and free doxorubicin. Further, using disaggregated tumors, the relationship between HER2 expression and MM-302 uptake was confirmed in vivo in both models.

Conclusions: Our studies identified a HER2 threshold for uptake of MM-302 of approximately 2E05 receptors/cell, and suggest a role for MM-302 not only in HER2 3+ cancers, but also in HER2 2+ cancers as well. The ability to treat HER2 2+ tumors offers an exciting opportunity to serve a major unmet clinical need.

Citation Format: {Authors}. {Abstract title} [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, FL, Philadelphia (PA): AACR; Cancer Res 2011;71(8 Suppl):Abstract nr 3637. doi:10.1158/1538-7445.AM2011-3637

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Pancreatic Cancer 2014

February 21st- 22nd, 2014

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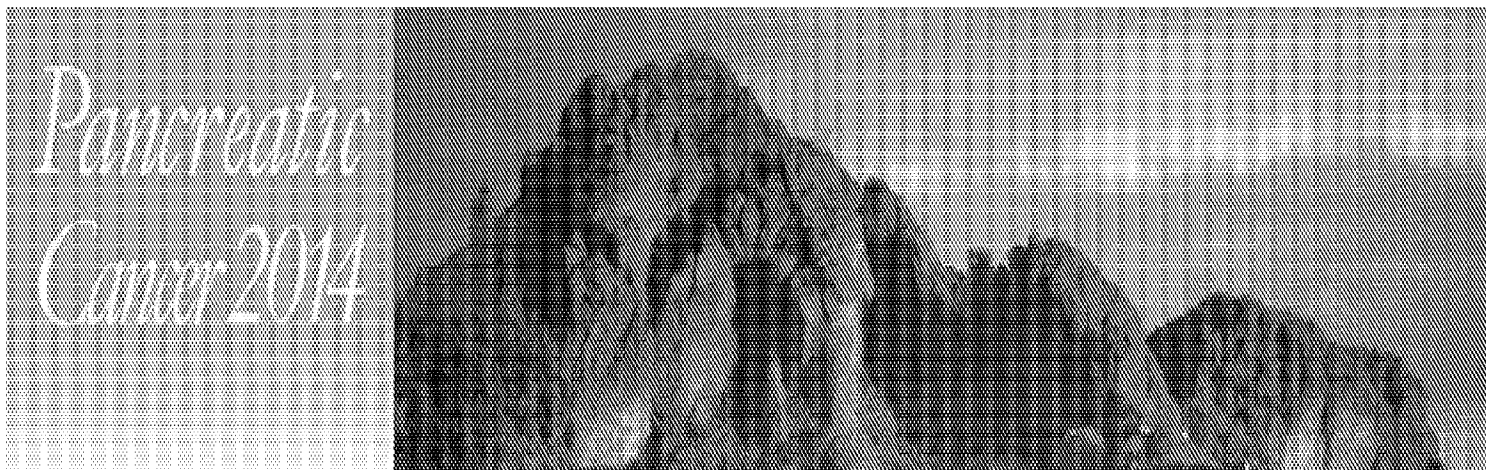
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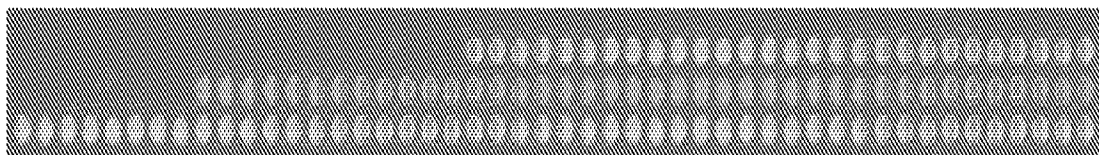
Advanced Imaging with Ferumoxytol MRI to Predict Drug Delivery

Ronald L Korn MD PhD

Chief Medical Officer, Imaging Endpoints Core Lab

Medical Director, Virginia G Piper Cancer Center Service Line

February 22, 2014



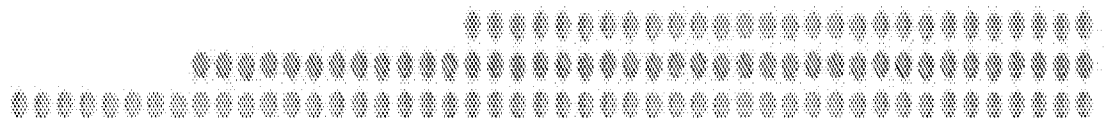
Ron Korn, MD, PhD

Disclosure Information

- I will discuss investigational and off-label use in my presentation.
- I have financial relationships to disclose:

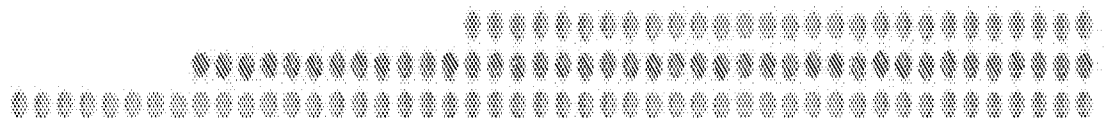
Shareholder, Founder and Chairman of Imaging Endpoints Research and Core Lab, Scottsdale, AZ

Grant/Research Support for: Merrimack Pharmaceuticals, Inc.

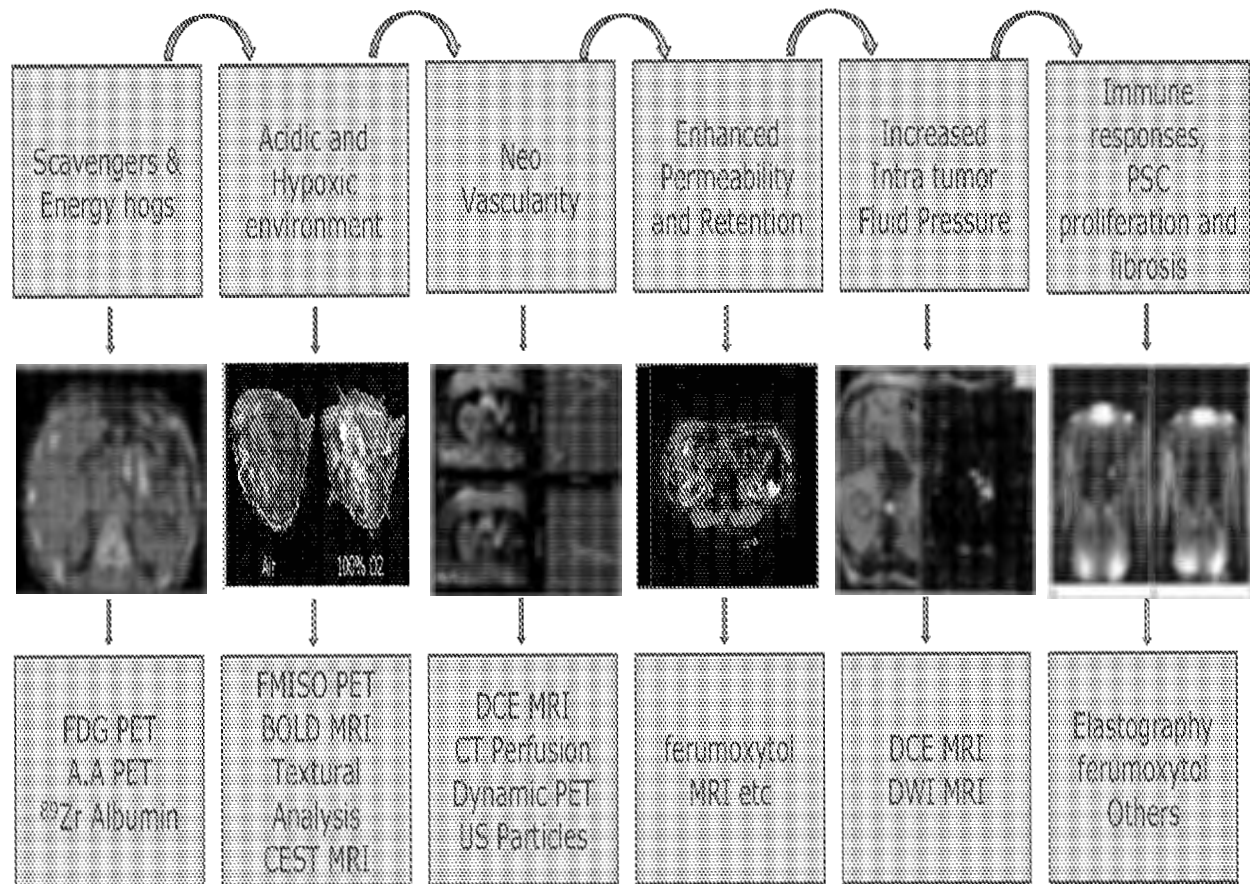


Objectives

- Discuss the use of imaging as a predictive biomarker of drug delivery
 - Emphasis on Enhanced Permeability and Retention (EPR)
 - Discuss potential association of Tumor Associated Macrophages (TAM) in drug delivery
 - Illustrate how ferumoxytol MRI might be used as a quantitative biomarker for liposomal drug delivery and TAM uptake

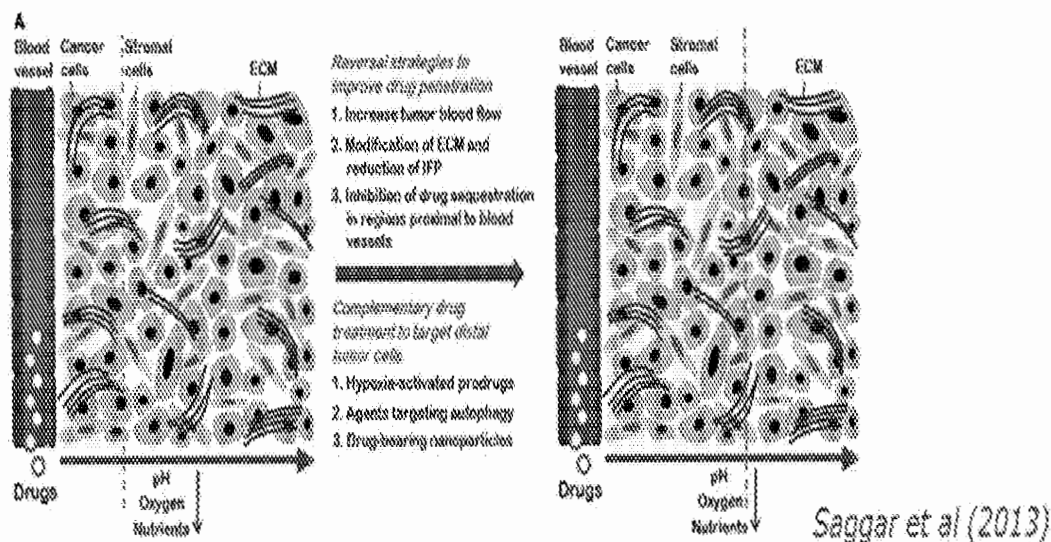
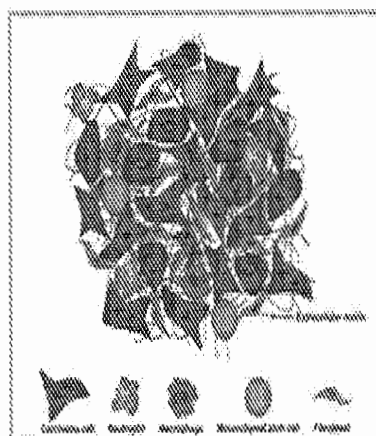


Imaging Can Probe the Hostile Environment In Pancreas Cancer



***Getting Drugs Into the Tumor Mass Can Be Very Difficult
Knowing That They Will Get There Is Critical***

Pancreas Cancer Develops A Hostile Environment

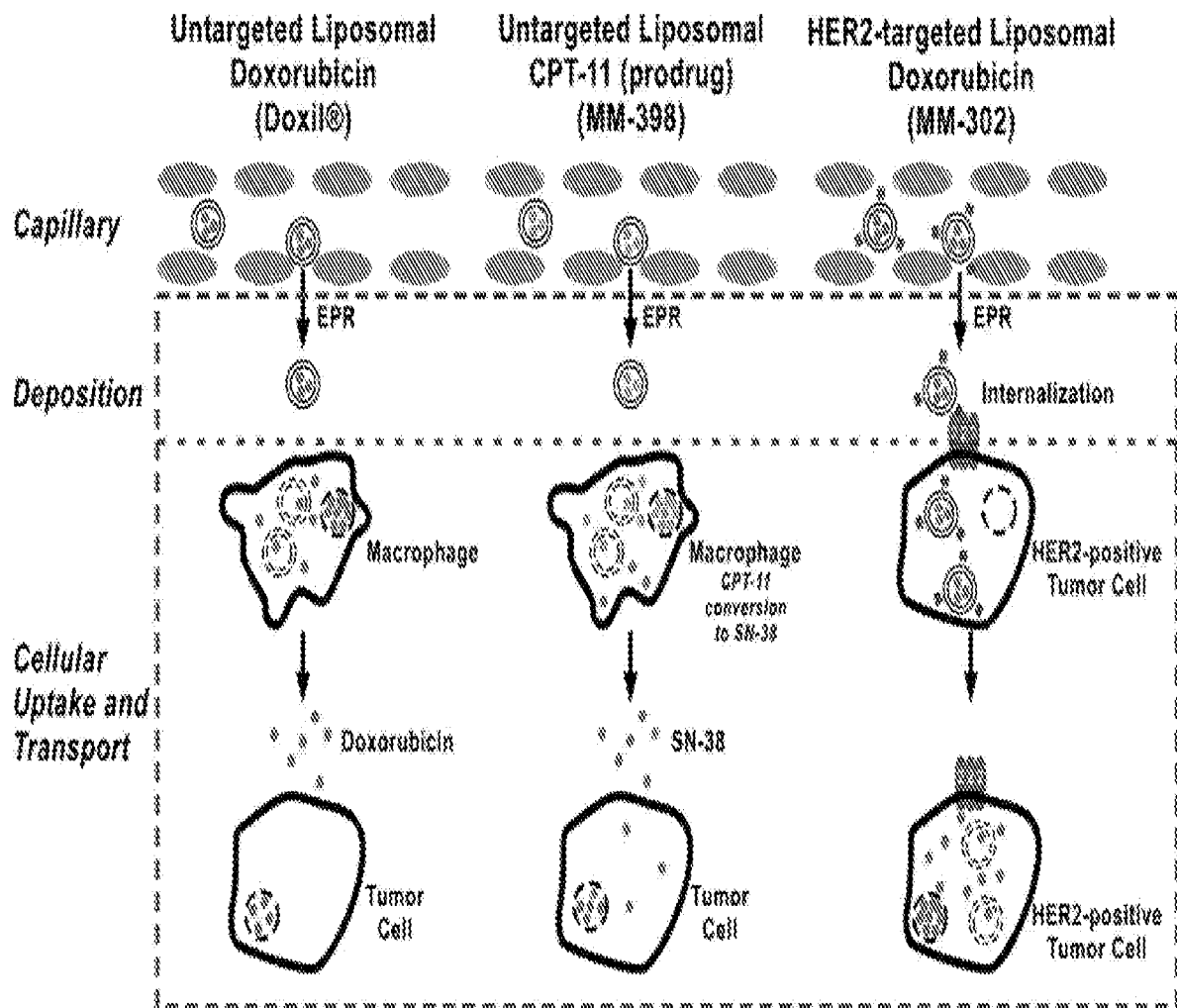


Choi et al (2013)

Contributes to Drug Resistance

Mechanisms of Liposomal Chemotherapeutics

All share same mechanism of deposition



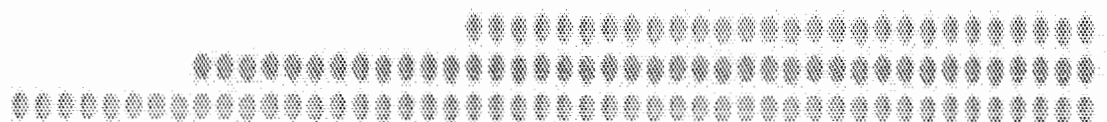
Properties of MRI Imaging Compared To PET *Superparamagnetic Iron Oxide (SPIO) Agents & other Nanoparticles*

MRI

- High spatial resolution
- FDA cleared contrast agent choices
- Quantitative
- Long physical half-lives
- Larger particle size
- Non Ionizing
- Low sensitivity
- Not all subjects are candidates for MRI imaging
- High cost-high availability

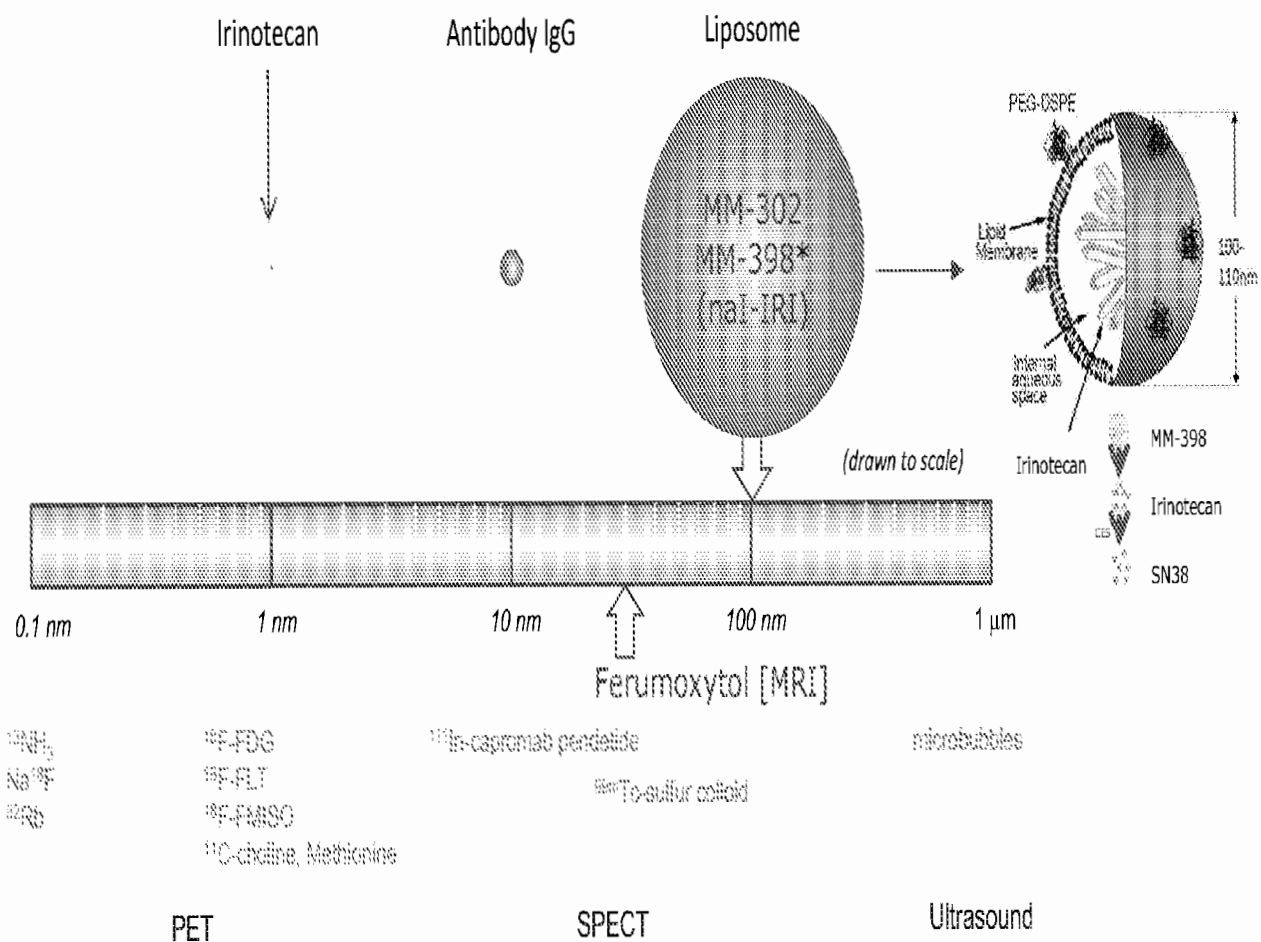
PET

- Low spatial resolution
- Many agents are not yet FDA cleared
- Quantitative
- Shorter physical half-lives
- Smaller particle size
- Ionizing radiation
- High sensitivity
- Most subjects are candidates for PET imaging
- High cost-low availability



How Can We Image Liposomal Deposition?

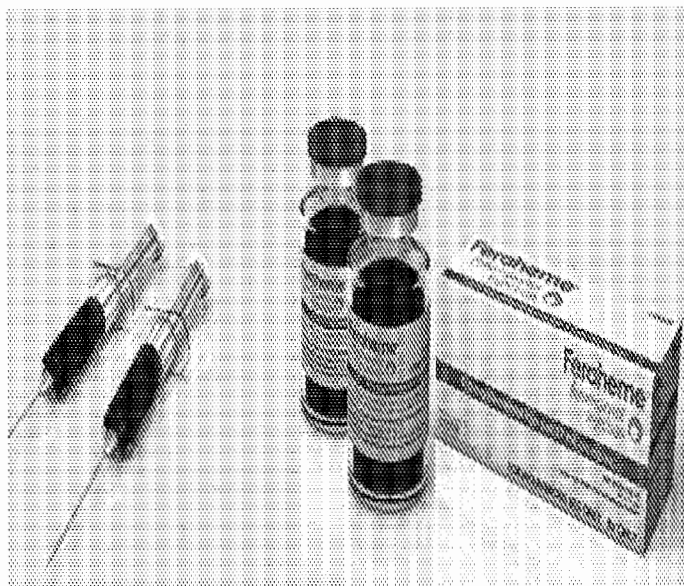
Relative Sizes of Approved Imaging Agents



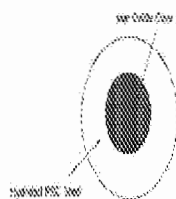
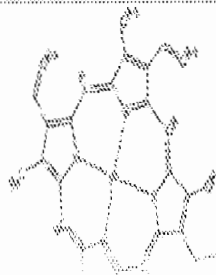
* Drug supplied by Merrimack Pharmaceuticals, Inc

9

Ferumoxytol-Feraheme®

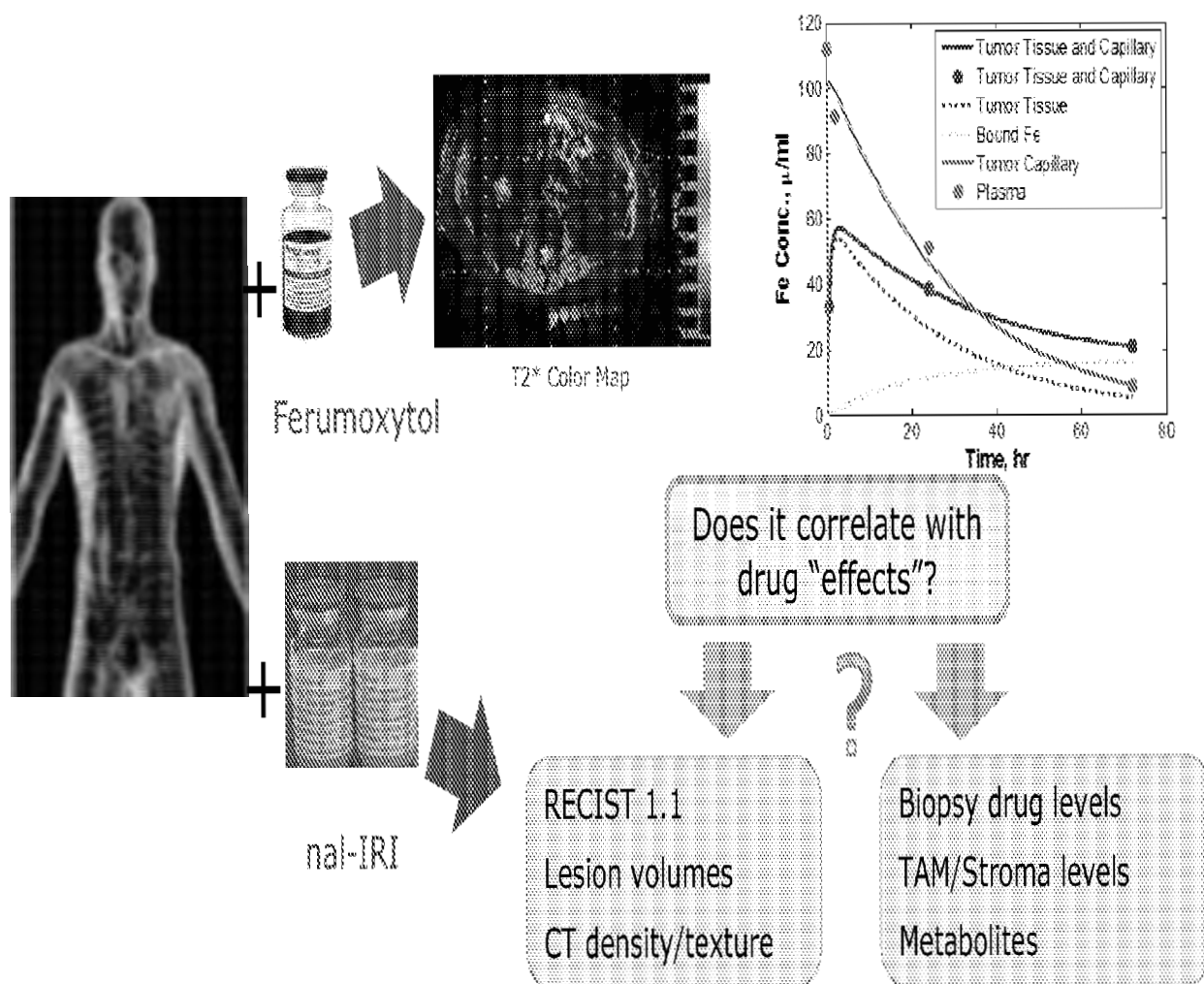


- 30 nm nanoparticle
- Fe(III) core surrounded by polyglucose sorbitol carboxymethylether shell
- FDA approved for treatment of iron deficiency anemia in chronic kidney disease
- Published reports have used this agent experimentally in neurological and oncology conditions
- Known to be taken up by macrophages
- Supraparamagnetic



The Core Question!

Can Nanoparticle MRI Be Used as an Imaging Diagnostic for Liposomal Agents?



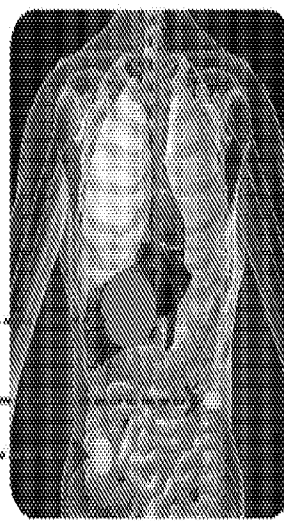
The Goal of the Feasibility Study

To evaluate the feasibility of delayed ferumoxytol MRI (Fe MRI) to identify tumor associated macrophages (TAM)

To measure tumor levels of irinotecan and SN-38

nal-IRI treatment

Fe-MRI
Dark = high
uptake



CT scan for
lesion size
monitoring

Day 1 or 3
CT & Fe-MRI

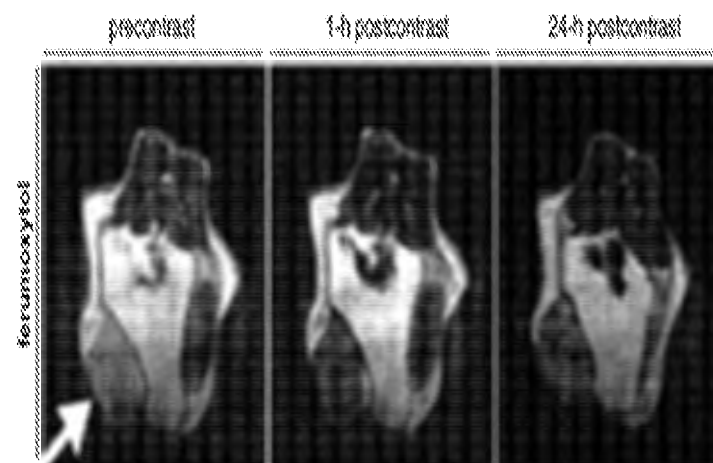
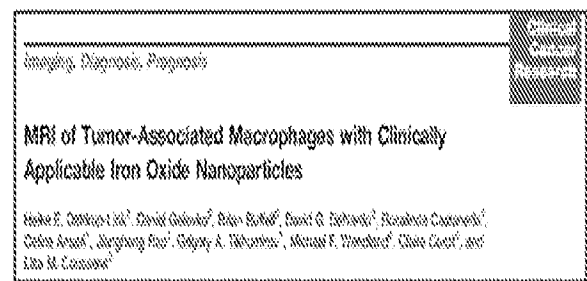
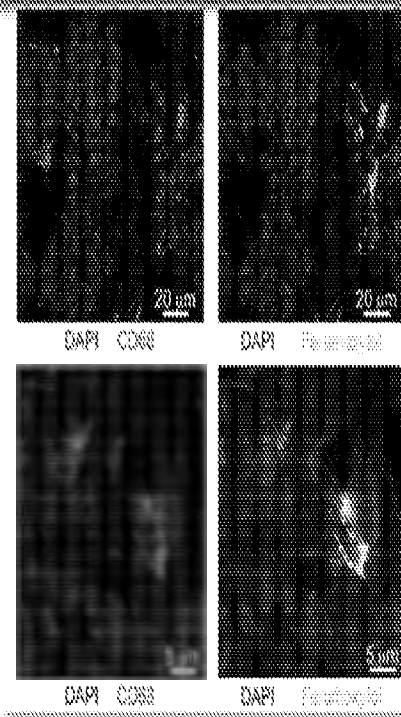
Single site

Hypothesis: Can ferumoxytol imaging be used to predict nal-IRI deposition, TAM-based activation or patient/lesion response?

Proof Of Concept

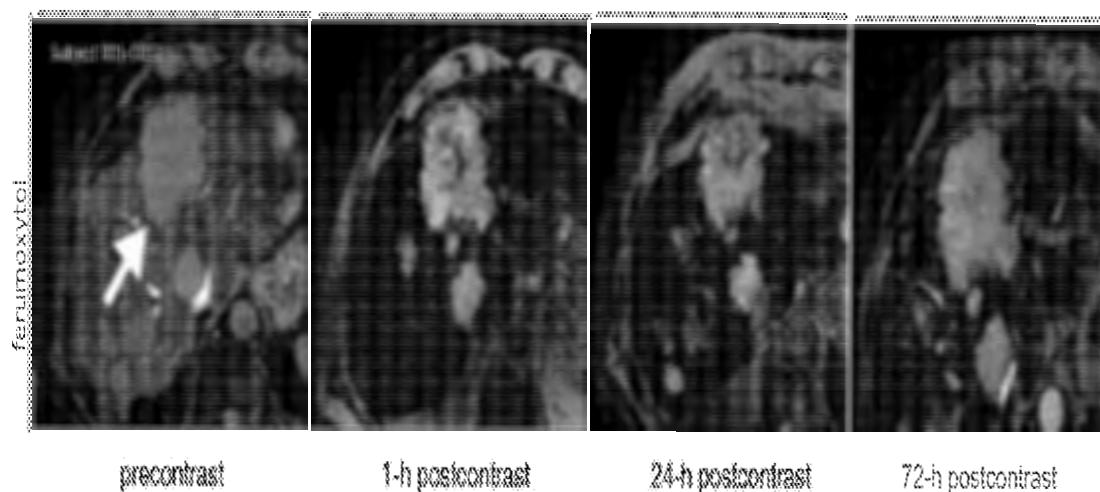
Does FMX Get Taken Up By Macrophages?

A In vivo MRI imaging

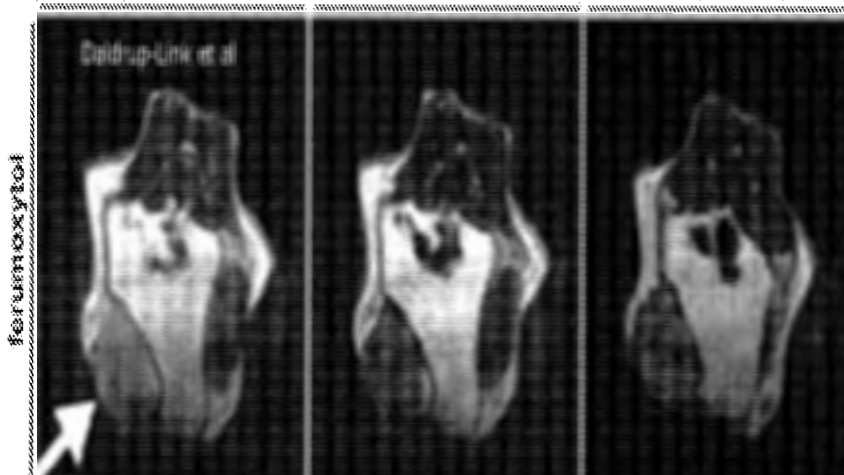


Comparison of Clinical Metastatic Disease to Preclinical Signal Using FMX

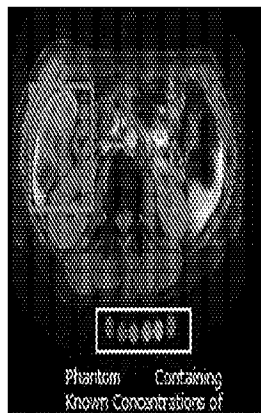
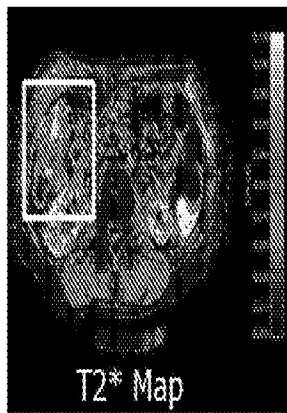
CITS Pilot Clinical Trial



Pre-Clinical



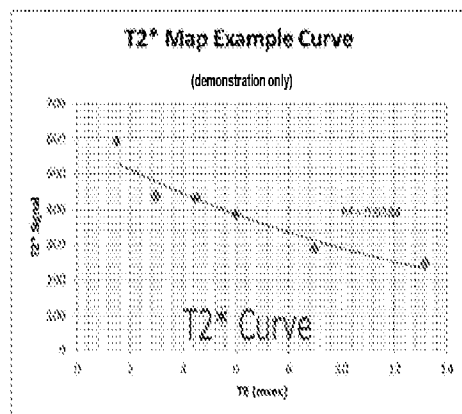
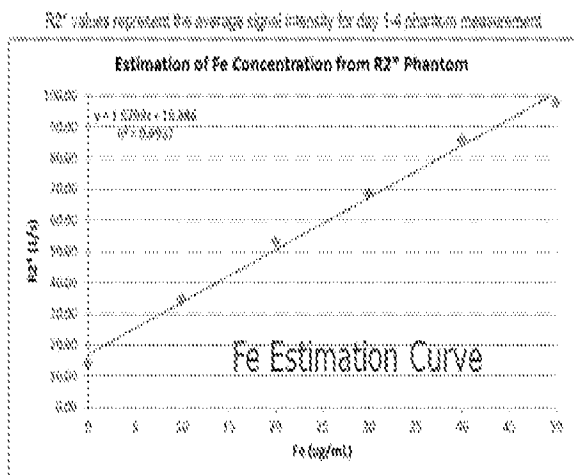
MRI Signal To Color Map To FMX Concentration



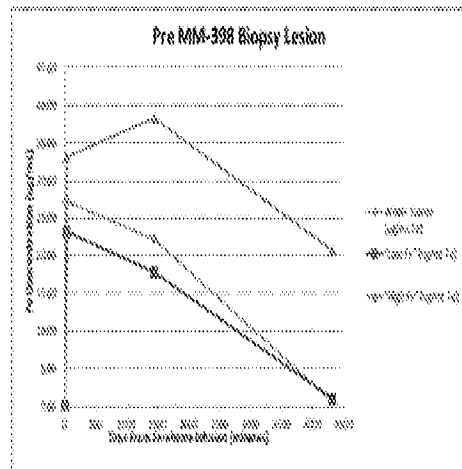
Imaging Derived
T2* Signal



Converted to
R2* Values

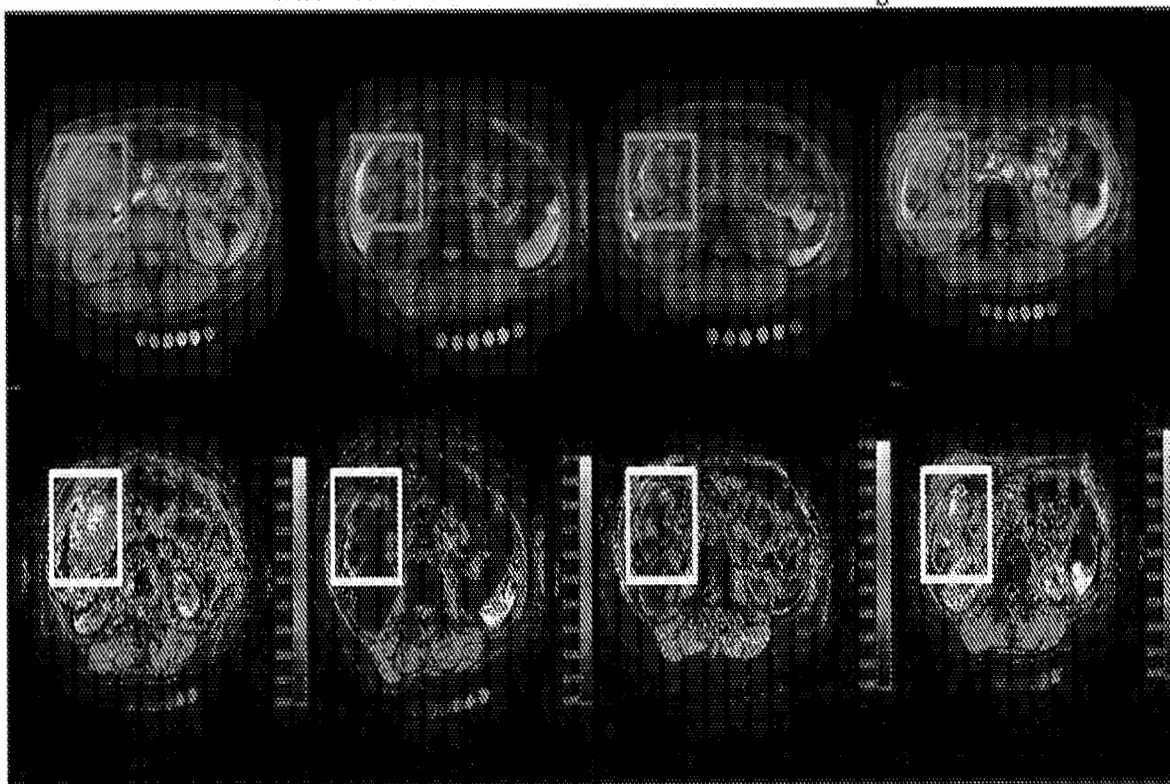


Estimation of
Lesion Fe
concentration



Example Images of Changes In MRI Signal Over Time Following FMX Infusion

MRI FSPGR FatSat TE 13.2 ms Breathhold Images



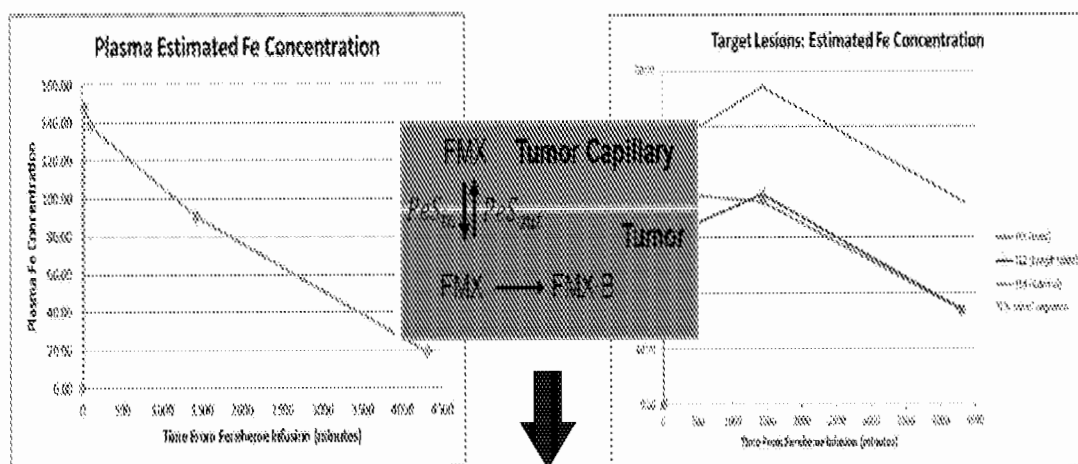
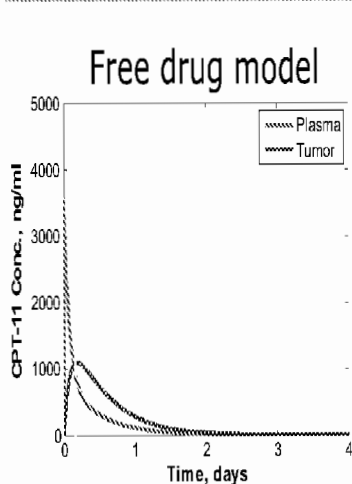
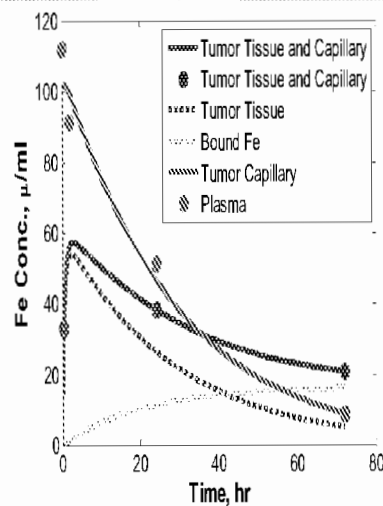
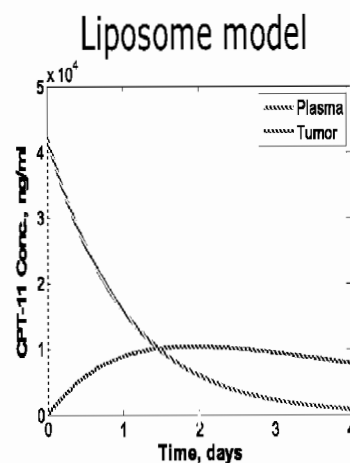
Day 1 Pre-Fe

Day 1 Post-Fe

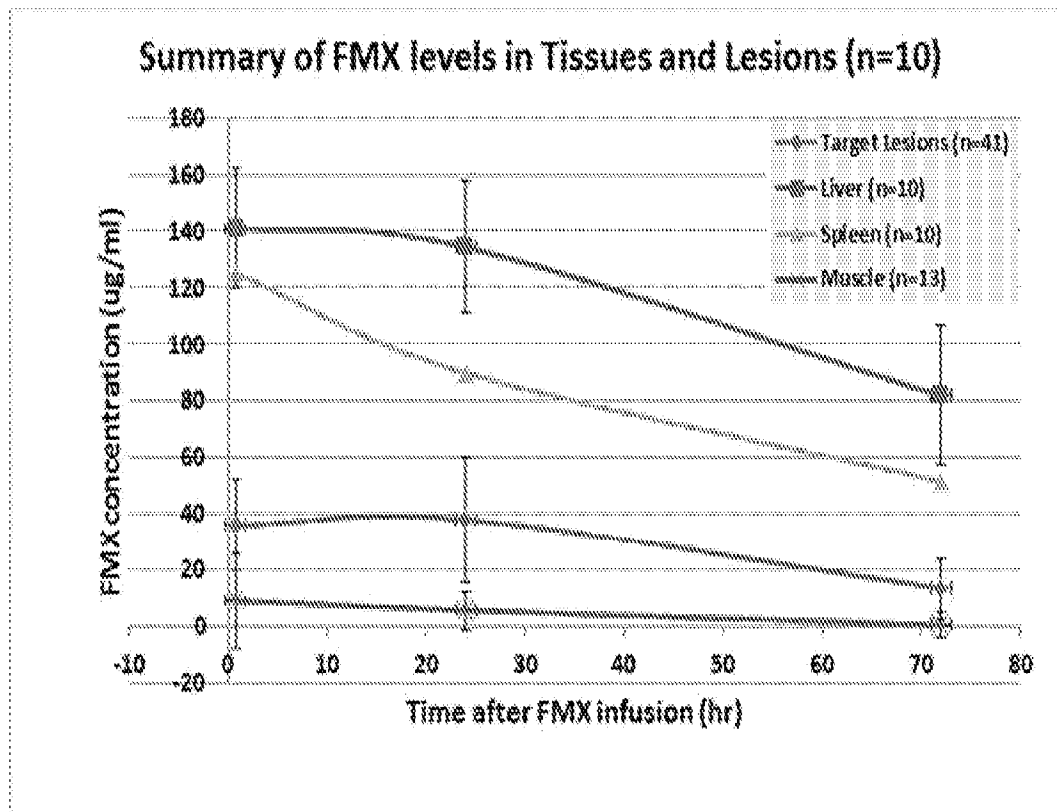
Day 2 Post-Fe

Day 4 Post-Fe

Compartmental Models of FMX Distribution



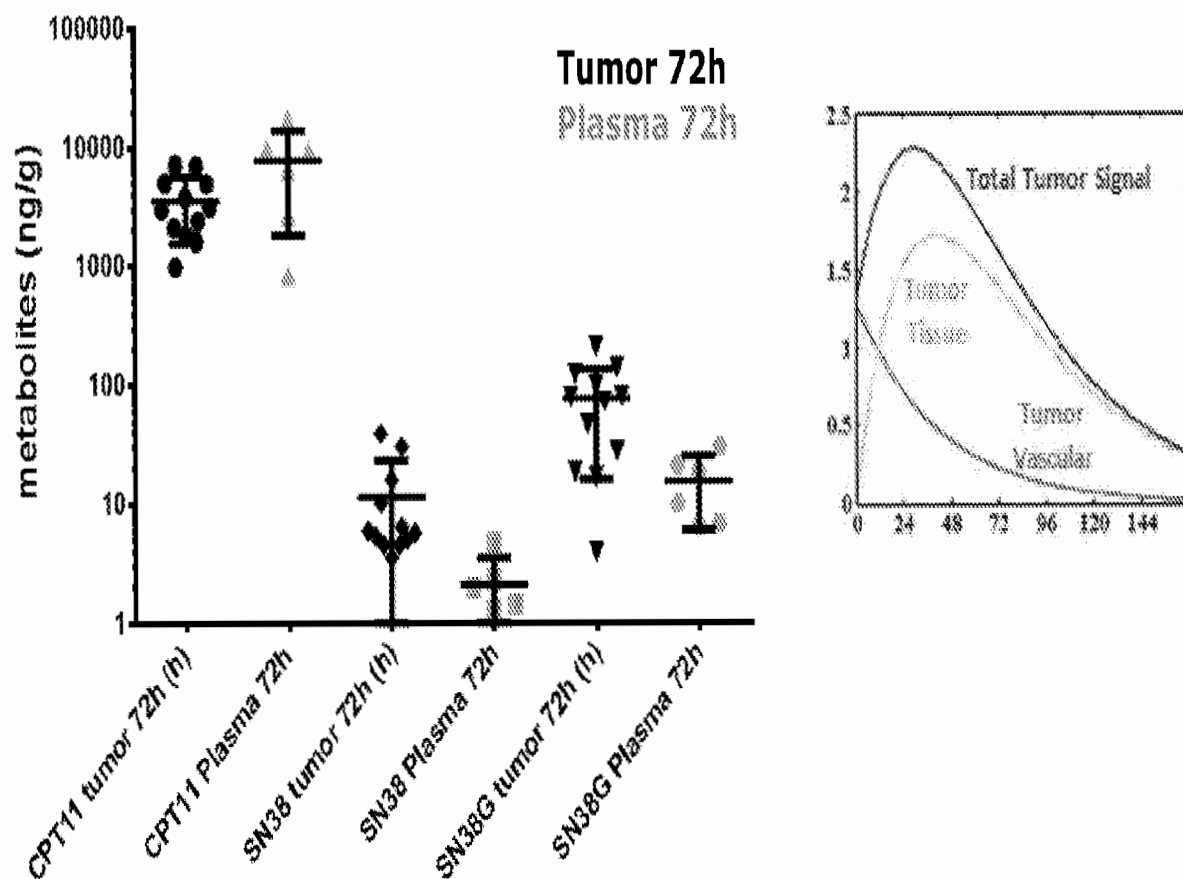
FMX Tissue Levels are Consistent With Particle Clearance and Permeability Differences



Tissues that are part of the RES clearance system retain FMX for a longer time

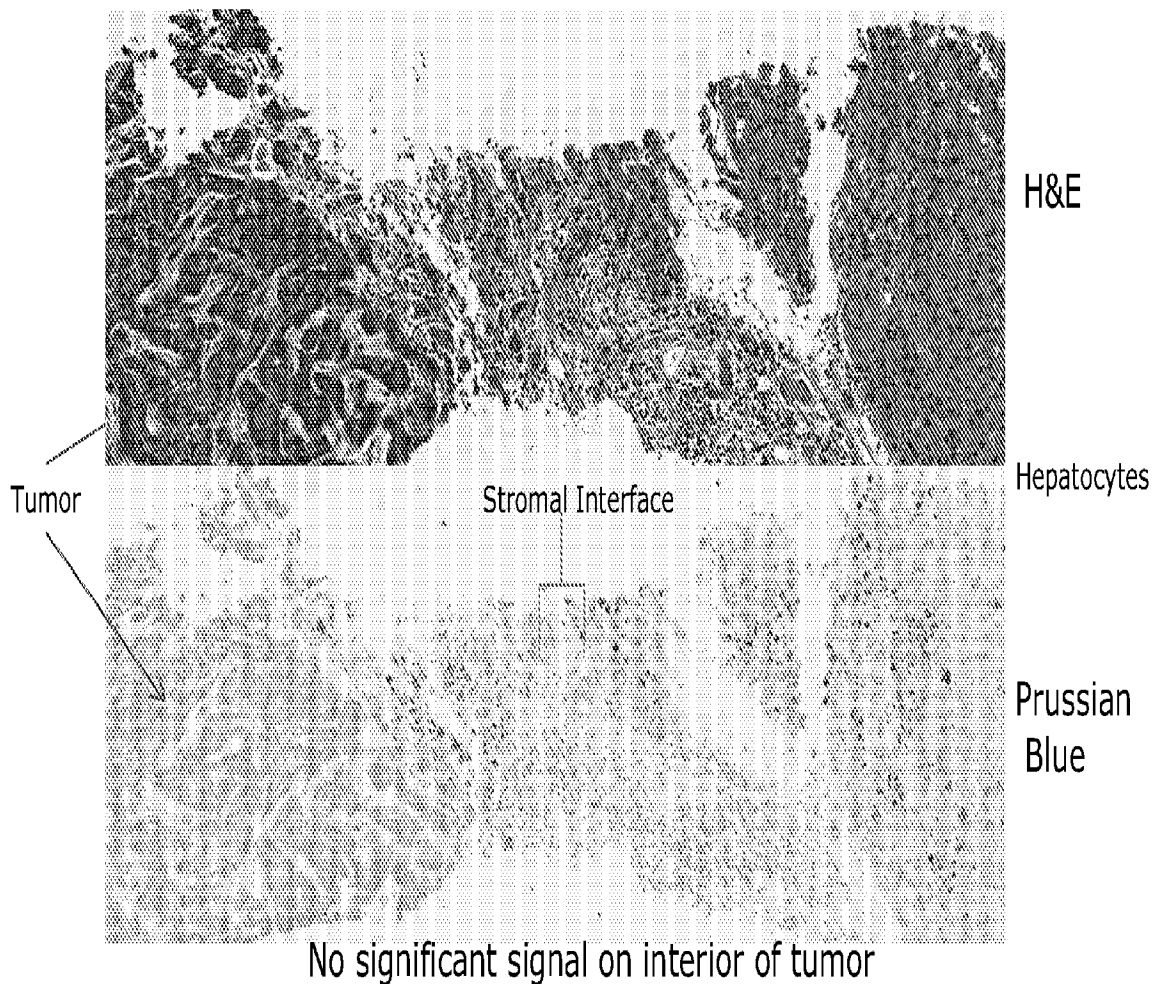
Muscle tissue has smaller endothelial fenestrations, and FMX deposition is minimized

Irinotecan Metabolite Measurements in Plasma and Tumor



*preliminary observations from the first 6 patients

Stromal Interface Exhibits Highest Fe Content in Macrophages



FeOx (Prussian Blue) Found in Primarily Stromal Macrophages



- High macrophage levels are observed in the tumor capsule and adjacent stroma.
- FMX deposition is observed in isolated hot-spots and overlaps with macrophage localization in adjacent slide sections

FMX MRI as an Imaging Diagnostic For Liposomal Therapy

- What we have learned to date:
 - ◆ Ferumoxytol MRI has been well tolerated with no serious adverse side effects
 - ◆ Phantom evaluation shows that accurate estimates of tumor/tissue Fe concentrations can be obtained with T2* MRI based sequences
 - ◆ The signal on MRI has allowed for modeling of ferumoxytol behavior and results seems to be consistent with naI-IRI models
 - ◆ Drug and metabolites are found in the tumor mass
 - ◆ Fe staining is predominately in the stroma-tumor interface and coincides with vascular associated macrophages
 - ◆ Awaiting correlation of FMX MRI tumor signal and response
 - ◆ If confirmatory, the FMX MRI may be a useful maging diagnostic for liposomal therapies

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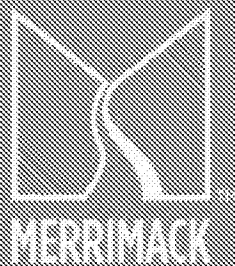
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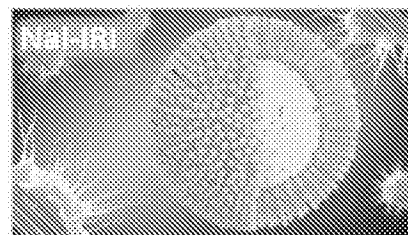
Differential Tissue Clearance Results in Improved Therapeutic Index for Nanoliposomal Irinotecan (nal-IRI; Onivyde) when Combined with the PARP inhibitor veliparib

Alexander Koshkaryev¹, Jonathan Fitzgerald¹, Jaeyeon Kim¹, Ashish Kalra¹, Walid Kamoun², Sarah Blanchette¹, Lia Luus¹, Stephan Klinz¹, Ozan Alkan³, Tad Kornaga¹, Susan Bates², Yves Pommier¹, and Daryl C Drummond¹

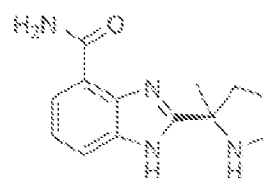
¹Merrimack, Cambridge, MA, USA; ²Columbia University, New York, NY, US; ³NCI Center for Cancer Research, Bethesda, MD, USA;

INTRODUCTION

- Nanoliposomal Irinotecan (nal-IRI) is a liposomal formulation of Irinotecan that provides for sustained exposure of Irinotecan and its active metabolite SN-38 in the tumor [1].
- Nal-IRI has shown promising preclinical and clinical activity in a range of cancer types, and was recently approved in the United States for the treatment of gemcitabine-refractory pancreatic cancer.
- PARPs are a family of enzymes involved in DNA repair that act via two mechanisms: Catalytic inhibition and trapping of PARP-DNA complexes, and inhibition of this repair pathway can result in cell death following DNA damage [2].
- It was hypothesized that combining PARP inhibitors (veliparib) with Top1 inhibitors will result in increased efficacy in the clinic compared to either agent alone, but promising preclinical activity has given rise to unacceptable toxicity in the clinic for these combinations [3].
- We hypothesized that delayed dosing of veliparib relative to nal-IRI will allow for the expected window of maximum Irinotecan-induced toxicity to pass in the absence of concurrent veliparib toxicity.



Veliparib



PROLONGED ACCUMULATION OF SN-38 IN TUMOR AFTER nal-IRI ADMINISTRATION COMPARED TO OTHER ORGANS

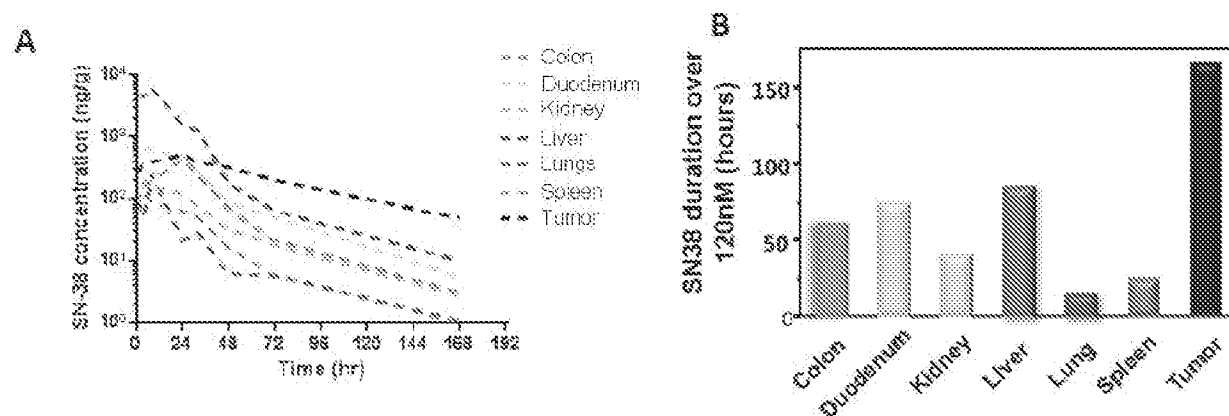


Figure 1. Biodistribution of liposomal SN-38 in mice [4]. A) HT-29 colorectal cancer tumor xenograft-bearing mice were injected intravenously with nal-IRI at a dose of 17 mg/kg and following a single injection, tissue samples were collected at various time points (1, 4, 8, 24, 48, 72, 168 hours). HPLC analysis was used to measure the levels of SN-38 in these samples. (B) Time of SN-38 duration over a threshold of 120 nmol/L was computed from the pharmacokinetic profiles of SN-38 in tumor and normal tissues following 17 mg/kg of nal-IRI.

IN VITRO ACTIVITY AND TUMOR ACCUMULATION OF SN-38 IN CERVICAL MODELS

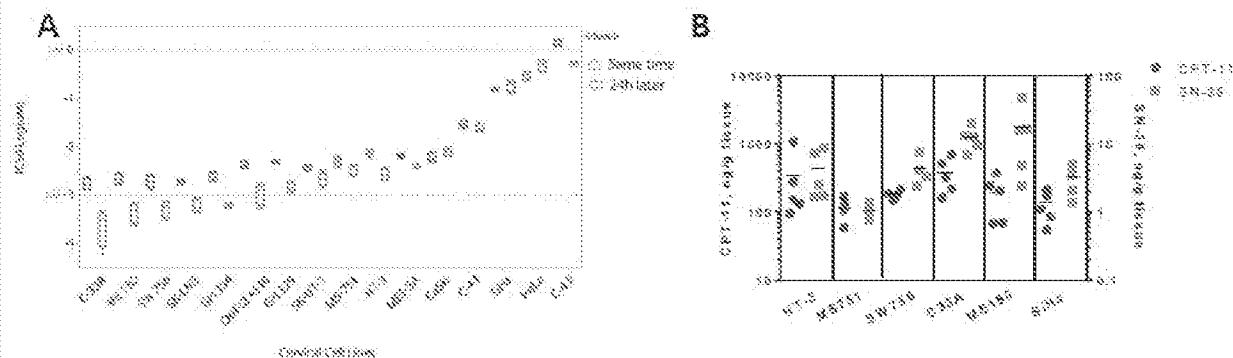


Figure 2. *In vitro* activity (A) and tumor content (B) of SN-38 in cervical models. (A) Cervical cells lines were treated with veliparib and SN-38 at either the same time or with scheduling with veliparib being added 24 h after SN-38, and cell viability was measured using CTG assay (B) Nude mice bearing cervical tumors were injected with a single dose of nal-IRI at 8.6 mg/kg and tumor content of CPT-11 and SN-38 were measured by LC-MS.

DELAYED DOSING OF VELIPARIB INCREASES TOLERABILITY OF COMBINATIONAL TREATMENT WITH NAL-IRI

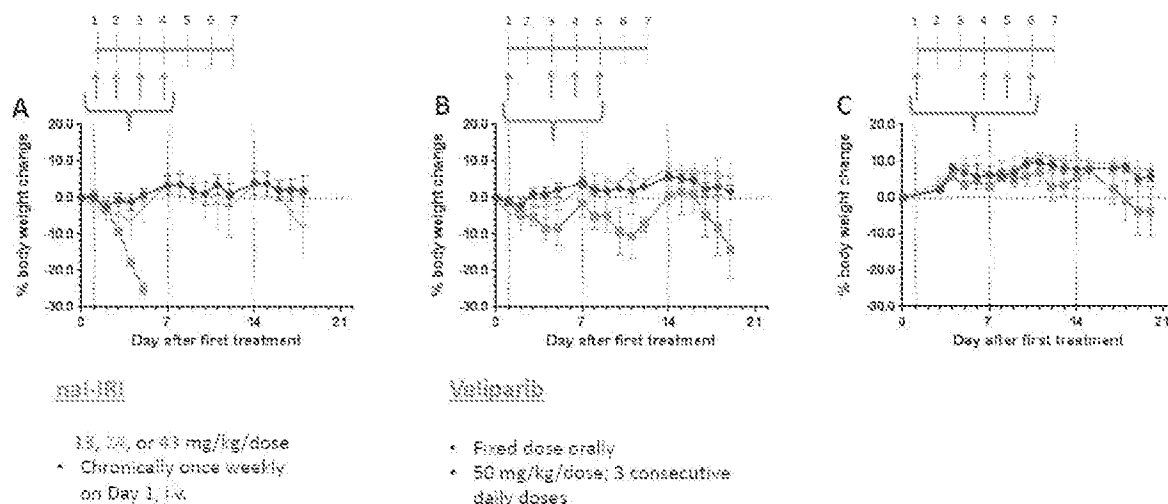


Figure 3. Tolerability of nal-IRI in combination with veliparib. Swiss-Webster mice were dosed chronically with nal-IRI once weekly at various doses on Day 1, while veliparib was dosed orally once daily at a fixed dose (50 mg/kg) for 3 consecutive days each week either on Days 2-4 (A), Days 3-5 (B), or Days 4-6 (C). Body weight was followed as a gross measure of toxicity.

VELIPARIB IMPROVES ANTI-TUMOR ACTIVITY OF NAL-IRI IN CERVICAL XENOGRAFT MODELS

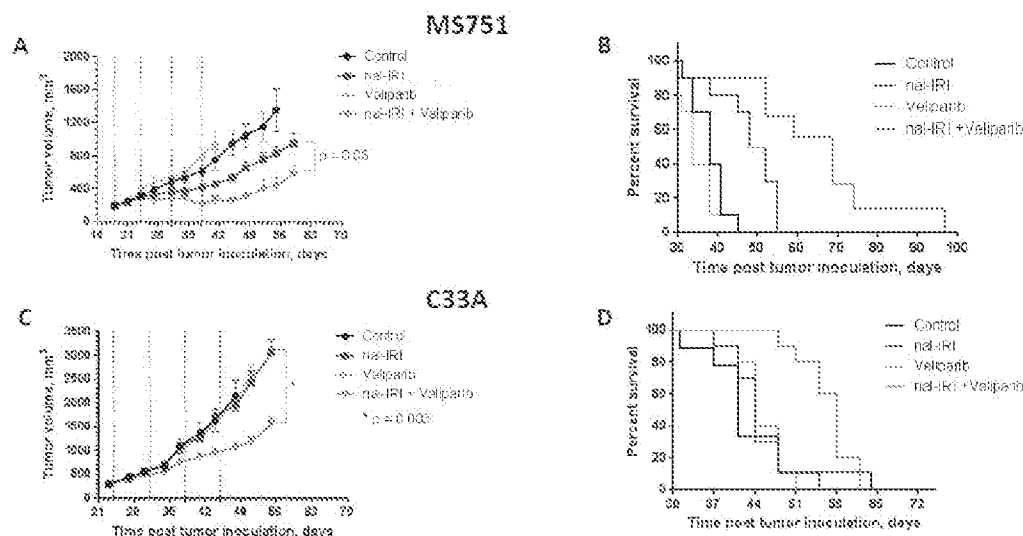
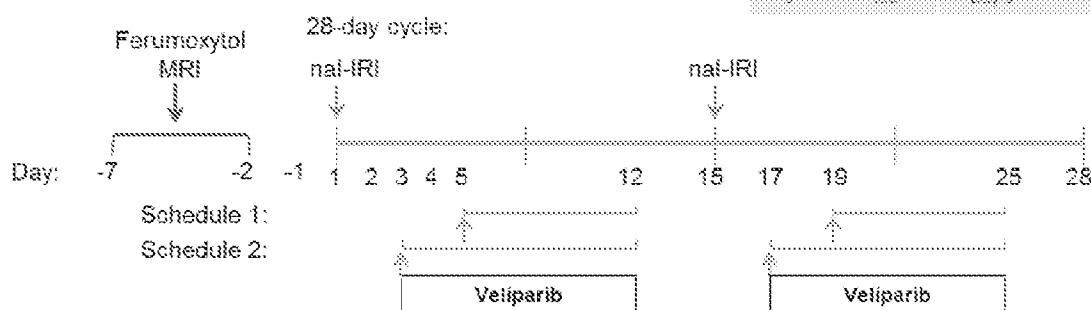


Figure 4. Anti-tumor activity of nal-IRI in combination with veliparib. (A, C) Nude mice bearing either MS751 (A-B) or C33A (C-D) cervical tumors were dosed i.v. with nal-IRI (1.7 mg/kg for C33A or 4.3 mg/kg for MS751) once weekly on Day 1, while veliparib was given orally once daily at dose 50 mg/kg for 3 consecutive days each week on Days 3-5. Tumor sizes were measured twice a week with a caliper. (B-D) Kaplan-Meier survival curves for individual animals were plotted when tumor's size achieved 1.5 cm³.

PROPOSED CTEP-SPONSORED PHASE I TRIAL DESIGN

- Primary endpoint
 - Identify MTD / RP2D
- Secondary endpoints
 - AE profile
 - PK parameters
 - Biomarker Analysis: Includes pre-treatment MRI to measure nanoparticle tumor delivery
 - Efficacy

Dose Escalation Cohorts			
Dose Level	Veliparib Dose (mg QW)	Veliparib Start Day	nal-IRI Dose (mg/m ² q2w)
1	100	Day 5	70
2	200	Day 5	70
3	200	Day 3	70
4	300	Day 3	70
5	400	Day 3	70



SUMMARY

- Differential tissue clearance is observed with nal-IRI, allowing drug to clear from the circulation and potential sites of toxicity, while being retained in the tumor.
- The veliparib + SN-38 combinations is significantly more active in cervical cancer cell lines when the exposure of both drugs is together.
- Delayed dosing of veliparib relative to nal-IRI substantially reduced toxicity.
- The combination of delay-dosed-veliparib and nal-IRI demonstrated more efficient tumor growth inhibition in cervical tumor xenografts comparing to the nal-IRI monotherapy.
- This preclinical study warrants further clinical investigation
- A CTEP-Sponsored Phase I trial is planned to be initiate in 2016, NCT02631733 (Clinicaltrials.gov).

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2. S. Kummar, A. Chen, R. E. Parchment, R. J. Kinders, J. Ji, J. E. Tomaszewski, and J. H. Doroshow, "Advances in using PARP inhibitors to treat cancer," *BMC Med.*, vol. 10, no. 1, p. 25, Jan. 2012.
3. J. Murai, S. N. Huang, B. B. Das, A. Renaud, Y. Zhang, J. H. Doroshow, J. Ji, S. Takeda, and Y. Pommier, "Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors," *Cancer Res.*, vol. 72, no. 21, pp. 5588–99, Nov. 2012.
4. A. V. Kaira, J. Kim, S. G. Kilinz, N. Paz, J. Cain, D. C. Drummond, U. B. Nielsen, and J. B. Fitzgerald, "Preclinical activity of nanoliposomal irinotecan is governed by tumor deposition and intratumor pro-drug conversion," *Cancer Res.*, vol. 74, no. 23, pp. 7003–13, Oct. 2014.

Differential Tissue Clearance Results in Improved Therapeutic Index for Nanoparticulate Irinotecan (nal-IRI, Onhydel) when Combined with the PARP inhibitor veliparib

Abstract

PROPOSED ONCOLOGY PHASE I TRIAL DESIGN

- Phase I design
- Study MTD - 500 mg
- Secondary endpoint
- AE profile
- PK parameters
- Biological activity: reduces expression of the tumor
- Biological activity: reduces expression of the tumor
- Biological activity: reduces expression of the tumor

Abstract

Abstract

Abstract

Abstract

Abstract

Abstract

Abstract

Abstract

Convection-enhanced delivery of nanoliposomal CPT-11 (irinotecan) and PEGylated liposomal doxorubicin (Doxil) in rodent intracranial brain tumor xenografts

Michal T. Krauze, Charles O. Noble, Tomohiro Kawaguchi, Daryl Drummond, Dmitri B. Kirpotin, Yoji Yamashita, Erika Kullberg, John Forsayeth, John W. Park, and Krystof S. Bankiewicz

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We have previously shown that convection-enhanced delivery (CED) of highly stable nanoparticle/liposome agents encapsulating chemotherapeutic drugs is effective against intracranial rodent brain tumor xenografts. In this study, we have evaluated the combination of a newly developed nanoparticle/liposome containing the topoisomerase I inhibitor CPT-11 (nanoliposomal CPT-11 [nLs-CPT-11]), and PEGylated liposomal doxorubicin (Doxil) containing the topoisomerase II inhibitor doxorubicin. Both drugs were detectable in the CNS for more than 36 days after a single CED application. Tissue half-life was 16.7 days for nLs-CPT-11 and 10.9 days for Doxil. The combination of the two agents produced synergistic cytotoxicity in vitro. In vivo in U251MG and U87MG intracranial rodent xenograft models, CED of the combination was also more efficacious than either agent used singly. Analysis of the parameters involved in this approach indicated that tissue pharmacokinetics, tumor microanatomy, and biochemical interactions of the drugs all contributed to the therapeutic efficacy

observed. These findings have implications for further clinical applications of CED-based treatment of brain tumors. *Neuro-Oncology* 9, 393–403, 2007 (Posted to *Neuro-Oncology* [serial online], Doc. D06-00150, July 24, 2007. URL <http://neuro-oncology.dukejournals.org>; DOI: 10.1215/15228517-2007-019)

Keywords: brain tumor, CED, CPT-11, Doxil, irinotecan, liposome, xenograft

Despite intensive multimodal treatment such as surgical resection, radiation therapy, and systemic chemotherapy, malignant brain tumors (e.g., glioblastoma multiforme) remain the most difficult neoplasms to treat. Poor penetration of the blood–brain barrier (BBB) by many anticancer drugs results in the need for high doses of systemic chemotherapeutics.^{1,2} Systemic side effects are therefore the limiting factor in chemotherapeutic protocols for brain tumor patients. Intratumoral chemotherapy has been proposed in order to overcome these difficulties and in recognition of clinical observations that 90% of malignant gliomas recur within 2 cm of an original resection site.³ However, many local drug delivery techniques face the persistent problem of poor distribution of infused drugs.⁴ Convection-enhanced delivery (CED) was introduced by Bobo et al.⁵ to overcome this difficulty. CED is a direct infusion technique that utilizes bulk flow to deliver molecules to a

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targeted site, offering an improved volume of distribution over that of simple diffusion. CED of therapeutic agents bypasses the BBB, delivers a high concentration of therapeutic agents to the infusion site, provides a wider distribution of therapeutic agents within the target site, and minimizes systemic exposure, resulting in fewer systemic toxicities.⁶⁻⁹ Promising results have been reported from clinical trials with CED of several therapeutic agents in malignant brain tumors.¹⁰⁻¹³ A major disadvantage of all these clinical trials is that delivery to the CNS cannot be monitored in real time. Therefore, side effects (e.g., chemical meningitis) can be explained only empirically.

In order to further improve the clinical prospects for CED-based therapy, we have introduced a number of innovations to CED for both current and future clinical applications. We have defined four steps essential for more efficient brain tumor therapies in the future: (1) visualized controlled local delivery of therapeutic agents to the CNS, (2) highly active therapeutic combinations that display relatively low toxicity in healthy brain, (3) optimized pharmacokinetic profile of the therapeutic agent that allows for a long half-life of the active agent in the brain in order to alleviate the need for frequent dosing and to introduce metronomic chemotherapy that targets dormant cancer cells as well as the supporting angiogenic blood vessels, and (4) establishment of therapeutic delivery parameters flexible enough to accommodate multiple tumor types.

Liposomes provide stable encapsulation for various anticancer drugs and have a number of advantages over the corresponding free drugs for the systemic treatment of cancer.^{14,15} Liposomal drugs are promising candidates for local delivery within the CNS, because they are inert until the drug is made bioavailable via release from the carrier. Liposomes now also play an important role in neuro-oncology not only because of their pharmacokinetic profile but also because their distribution in the CNS can be visualized by MRI.^{16,17} The introduction of a novel step-design delivery catheter in our CED studies provided for distribution of the therapeutic agent, free of detectable reflux at any infusion rate examined thus far, up to 50 $\mu\text{L}/\text{min}$.¹⁸ We have previously shown excellent distribution of liposomes throughout the primate brain with this catheter.¹⁷ Considerable progress has been reported in developing real-time imaging strategies with liposomal MRI contrast agents in primate CNS.^{16,17,19}

Drug delivery kinetics to the brain have been optimized by encapsulation of small-molecule drugs in liposomes, thereby markedly improving the pharmacokinetic profile of these drugs in the CNS.⁸ A unique intraliposomal stabilization technology has resulted in stable controlled-release formulations for a variety of difficult-to-encapsulate drugs, for example, irinotecan.^{20,21} We have previously evaluated in separate studies liposomes containing either the topoisomerase I (Topo I) inhibitor doxorubicin⁹ or the topoisomerase II (Topo II) inhibitors CPT-11 and topotecan, against human brain tumor xenografts.^{8,22} Results from these studies have been encouraging, in part because Topo I and Topo II inhibitors exert their principal effects on the two major classes of enzymes involved in regulating DNA topology. This

functional overlap suggested that a combination of both drugs could act synergistically against orthotopic brain tumor xenografts. Administration of a combination of nonliposomal Topo I and Topo II inhibitors to patients with advanced solid malignancies has been evaluated in numerous clinical studies.²³⁻²⁵

In this study, we evaluated PEGylated liposomal doxorubicin (Doxil) and nanoliposomal CPT-11 (nLs-CPT-11) *in vitro* and *in vivo* with respect to toxicity, tissue half-life, and efficacy in U87MG and U251MG xenografts. We demonstrated that only distribution within the entire tumor is able to prolong animal survival in the rodent brain tumor xenograft model.

Materials and Methods

Liposomal Therapeutics

Control "empty" liposomes, not loaded with drug, were composed of L- α -distearoylphosphatidylcholine (DSPC), choline (Chol), 1,1'-dioctadecyl-2,3,3',3'-tetramethylindocarbocyanine perchlorate [DiIC₁₈(3)], and polyethylene glycol-coupled 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (PEG-DSPE) in the molar ratio of 3:2:0.03:0.015. nLs-CPT-11 was composed of DSPC, Chol, and PEG-DSPE at a molar ratio of 3:2:0.015. Liposomes were prepared by dissolution of all lipids in chloroform/methanol (9:1, vol/vol) and subsequent removal of the solvent by rotary evaporation to form a dried lipid foam. The dried lipids were hydrated in 81 mM aqueous triethylammonium sucrose octasulfate solution (0.65 M triethylamine, pH 5.2) at 60°C, and the hydrated lipid suspension was subjected to eight cycles of freezing (-80°C) and thawing (60°C). After hydration, trace organic solvent was removed from the lipid suspension on a rotary evaporator. Unilamellar liposomes were formed by extrusion at 60°C in a pressurized, thermostat-controlled barrel extruder (Lipex Biomembranes, Vancouver, Canada) through double-stacked polycarbonate membranes (Whatman Nucleopore, Clifton, NJ, USA) of pore size 200 nm (6 times) and 100 nm (12 times), yielding a final liposomal diameter of 95–110 nm as determined by dynamic light scattering (N4Plus particle size analyzer; Beckman Coulter, Fullerton, LA, USA). Extraliposomal triethylammonium sucrose octasulfate was removed by size-exclusion chromatography on a Sepharose CL-4B column eluted with HEPES-buffered dextrose (5 mM HEPES, 5% dextrose, pH 6.5).

CPT-11 was loaded into the liposomes by addition of a 15 mg/ml solution of CPT-11-HCl to a final drug-to-lipid ratio of 750 g CPT-11-HCl per mole of phospholipid, and incubation of the drug/liposome mixture at 60°C for 45 min, followed by quenching on ice for 15 min. Unencapsulated CPT-11 was removed by chromatography on a Sephadex G-75 size-exclusion column that was eluted with HEPES-buffered saline (5 mM HEPES, 145 mM NaCl, pH 6.5). Drug-loaded liposomes were stored at 4°C until use. Drug-loading efficiencies of >95% were typically observed. Nanoliposomal CPT-11 (nLs-CPT-11)

was concentrated on a stirred cell concentrator (Millipore Corp., Billerica, MA, USA) containing a regenerated cellulose 1×10^5 nominal molecular-weight-limit membrane (Millipore) and sterilized by passage through a 0.2- μ m polyethersulfone syringe filter. The concentration of CPT-11 was determined by measuring the absorbance at 375 nm of a solubilized liposome sample. Briefly, 0.1 ml of an aqueous portion of the sample containing nLs-CPT-11 or standards was added to 0.9 ml of a solution containing 72 vol% methanol, 18 vol% 0.1 M phosphoric acid, and 10 vol% chloroform. Phospholipid was measured by a spectrophotometric assay.²⁶ All drug values for nLs-CPT-11 in this manuscript refer to equivalents of CPT-11-HCl.⁸

PEGylated liposomal doxorubicin was obtained as Doxil (Alza Pharmaceuticals, Mountain View, CA, USA). All drug values for Doxil refer to equivalents of doxorubicin-HCl.⁹

Tumor Cell Lines

Human glioblastoma multiforme cell lines U87MG and U251MG were obtained from the Brain Tumor Research Center Tissue Bank at the University of California, San Francisco (UCSF). Cells were maintained as monolayers in Eagle's minimal essential medium supplemented with 10% fetal calf serum, antibiotics, and nonessential amino acids. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell Cycle Analysis

One day prior to treatment, 2×10^5 cells were seeded into each well of a six-well plate (Corning Inc., Corning, NY, USA). Cells were exposed to drug-free DiIC₁₈(3)-DS liposomes (control liposomes), Doxil (0.2 μ g doxorubicin/ml), nLs-CPT-11 (1 μ g CPT-11/ml), or both, in complete medium. After 24 h, cells were fixed in 70% ethanol, washed, digested with RNase A (Sigma, St. Louis, MO, USA), stained with propidium iodide (Sigma), and subjected to flow cytometry in a FACScan (Becton-Dickinson, San Jose, CA, USA) with 10,000 events/determination. ModFit LT software (Verity Software House, Inc., Topsham, ME, USA) analyzed cell cycle distribution.

Cell Viability Assay

Cells were seeded at 1,000 cells/well in 96-well plates (Corning Inc.), allowed to attach for 24 h, and then exposed to drug-free DiIC₁₈(3)-DS liposomes (control liposomes), Doxil, nLs-CPT-11, or both, in complete medium. For combination studies, the ratio of Doxil to nLs-CPT-11 was kept constant at 1:5 (wt/wt of doxorubicin to CPT-11). MTT [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent was added 48 h after initiation of treatment, and plates were read at an absorbance of 490 nm 3 h later with a SpectraMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA). All treatments were performed in triplicate. The background absorbance was determined by incubating media

with substrate alone and subtracting the values from wells containing cells only.

Animals and Intracranial Xenograft Technique

Male Sprague-Dawley rats (250 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Congenitally athymic, male, homozygotic, nude rats (*rrnu/rrnu*; 150–200 g) were purchased from the National Cancer Institute Animal Production Program (Frederick, MD, USA) and were housed under aseptic conditions. All protocols were approved by the Institutional Animal Care and Use Committee at UCSF. For the intracranial xenograft tumor model, U87MG and U251MG cells were harvested by trypsinization, washed once with Hank's balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS), and resuspended in HBSS for implantation. A cell suspension of 5×10^5 cells/10 μ l HBSS was implanted into the striatal region of the athymic rat brains. Under isoflurane anesthesia, rats were placed in a small-animal stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A sagittal incision was made to expose the cranium, and a burr-hole was made in the skull 0.5 mm anterior and 3 mm lateral from the bregma. At a depth of 4.5 mm below the brain surface, 5 μ l of cell suspension was injected. Two minutes later, the needle tip was elevated 0.5 mm, and another 5 μ l was injected at a depth of 4 mm. After a further 2 min, the needle was removed and the wound sutured.

Convection-Enhanced Delivery

Throughout this study, drugs were delivered in a volume of 20 μ l by CED as described previously.^{6,27} Briefly, a fused-silica cannula was connected to a loading line (containing liposomes) and an oil-infusion line. A 1-ml syringe (filled with oil) was mounted onto a microinfusion pump (BecHive; Bioanalytical Systems, West Lafayette, IN, USA) that regulated the flow of fluid through the system. Based on tumor injection site coordinates, a reflux-free and backflow-free step-design cannula¹⁸ was mounted onto stereotactic holders and guided to the targeted region (4.5 mm depth) of the brain through burr holes made in the skull as described above. An infusion rate of 0.5 μ l/min over 40 min was applied to achieve a final infusion volume of 20 μ l.

Tissue Pharmacokinetics

Rats were given a single 20- μ l infusion by CED of Doxil (2 mg, 0.1 mg/ml) together with nLs-CPT-11 (0.8 mg, 40 mg/ml), and the animals were sacrificed at prescribed times. The brain hemisphere was perfused with phosphate-buffered saline, surgically removed, and frozen. Water was added to the tissue at a 50% wt/wt ratio, and the tissue was homogenized mechanically in an ice bath. The homogenates (0.1 ml) were extracted with 0.9 ml of chloroform/methanol (4:1) by vortexing for 15 s, and the organic phase was collected. This process was repeated, and the combined organic extracts were evaporated to dryness with a centrifugal concentrator. The residue was

dissolved in 0.5 ml methanol, and centrifuged at 10,000 rpm for 10 min. The supernatant solution was analyzed by high-performance liquid chromatography (HPLC). Standards were prepared by extraction of spiked blank tumor tissue. Analysis was conducted on a Dionex HPLC system with a C₁₈ reverse-phase silica column (Supelco C-18 column, 250 mm × 4 mm inner diameter, 5 mm particle size), preceded by a Supelco C₁₈ guard column. A sample injection volume of 50 µl was used, and the column was eluted at a flow rate of 1.0 ml/min with a mobile phase consisting of 0.21 M aqueous triethylammonium acetate (pH 5.5) and acetonitrile. A linear gradient elution was used with the acetonitrile content increasing from 27% to 45% over 10 min. Each drug was detected by a fluorescence detector (excitation, 485 nm; emission, 590 nm). Typical retention times for CPT-11 and doxorubicin were 5.4 min and 7.1 min, respectively.

Pharmacokinetic parameters that included the tissue half-lives of the drug ($t_{1/2}$), clearance, the mean residence time in the brain or brain/tumor, and the area under the concentration versus time curve were all determined by noncompartmental pharmacokinetic data analysis with PK Solutions 2.0 software (Summit Research Services, Montrose, CO, USA).

Evaluation of Toxicity

Three normal Sprague-Dawley rats were evaluated for potential local toxicity after CED-mediated co-infusion of Doxil and nLs-CPT-11. Rats were monitored daily for general health (alertness, grooming, feeding, excreta, skin, fur, mucous membranes, ambulation, breathing, and posture). Animal weights were reported weekly. Doxil was used at 50% of the maximum tolerated dose (MTD), determined previously.⁹ Little nLs-CPT-11 toxicity has been observed in previous studies, and the drug was used at a safe dose determined previously.⁸ Sixty days after CED of a 20-µl solution containing Doxil (2 mg, 0.1 mg/ml) and nLs-CPT-11 (0.8 mg, 40 mg/ml) into the striatum, rats were euthanized and their brains fixed in 4% formaldehyde. Fixed brain tissue was subjected to paraffin sectioning (5 µm), and sections were stained with hematoxylin and eosin (H&E).

Combination Therapy in the U87MG and U251MG Intracranial Xenograft Models

Forty-two rats were implanted with U251MG tumor cells as described above. Animals treated on day 7 after tumor implantation were randomly divided into four groups: (1) a control consisting of CED of DiIC₁₈(3)-DS fluorescent liposomes ($n = 6$), (2) CED of Doxil ($n = 6$), (3) CED of nLs-CPT-11 ($n = 6$), and (4) a combination treatment of Doxil together with nLs-CPT-11 ($n = 6$). In a second study, animals were randomly divided into two groups on day 14 after tumor implantation: a control consisting of CED of drug-free liposomes ($n = 9$) and a combination treatment of Doxil and nLs-CPT-11 ($n = 9$).

In a third study, 24 rats implanted with U87MG tumor cells were randomly divided into four groups on

day 10 after tumor implantation: (1) a control group consisting of drug-free liposomes ($n = 6$), (2) CED of Doxil ($n = 6$), (3) CED of nLs-CPT-11, and (4) a combination treatment of Doxil and nLs-CPT-11 ($n = 6$). CED of 20 µl of the specified drug was performed for each group in all survival studies. Rats were monitored daily for survival and general health (alertness, grooming, feeding, excreta, skin, fur, mucous membrane conditions, ambulation, breathing, and posture). Animal weights were reported weekly. The studies in U251MG were terminated 100 days after tumor implantation, and the U87MG survival study was terminated 70 days after tumor implantation. Surviving animals were euthanized, and their brains were sectioned and stained with H&E.

Distribution of Liposomes in U87MG and U251MG Brain Tumor Xenografts

Animals were implanted with U87MG ($n = 3$) and U251MG ($n = 3$) tumor cells. CED with 20 µl of DiIC₁₈(3)-DS fluorescent liposomes was performed on day 10 after implantation of U87MG and on day 30 after tumor implantation of U251MG. Animals were euthanized immediately after the infusion procedure. Brains were frozen and cut into 25-µm sections on a cryostat. Fluorescent images of liposome distribution were taken of each brain from rostral to caudal in 100-µm intervals. The fluorescent signal generated by DiIC₁₈(3) was visualized with a fluorescence microscope equipped with a 540/25 nm band-pass filter for excitation, together with a long-pass filter at 565 nm for emission. A charge-coupled device camera with a fixed aperture was used to capture the image.

Statistical Analysis

Results for the survival studies are expressed as a Kaplan-Meier curve. Survival between the treatment groups was compared with an unpaired Student's *t*-test and expressed as median survival (MS).

Results

Tissue Pharmacokinetics of Doxil and nLs-CPT-11 Coadministered by CED in Rat Brain

A mixture of liposomes containing 2 mg Doxil and 0.8 mg nLs-CPT-11 was infused by a single CED treatment into the brains of adult rats, and tissue levels were determined by HPLC at various times after infusion (Fig. 1). Both drugs were detectable for more than one month after a single CED treatment. The decline in the tissue concentration of nLs-CPT-11 was exponential ($R^2 = 0.9821$) with a half-life of 16.7 days (Table 1). Doxil, at a 400-fold lower dose, decreased linearly ($R^2 = 0.9975$) with a half-life of 10.9 days. The small differences may reflect differences in drug release from the carrier, or differences in clearance of highly PEGylated (Doxil) versus mildly PEGylated liposomes (nLs-CPT-11).

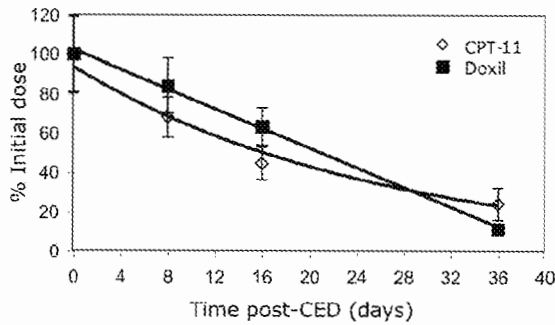


Fig. 1. Tissue pharmacokinetics of nanoliposomal CPT-11 and Doxil in the normal adult rat brain after single convection-enhanced delivery (CED) infusion. All values are percent injected dose versus time after CED of 20 μ l infusate. Drug concentrations were determined by high-performance liquid chromatography assay for CPT-11-HCl and doxorubicin-HCl. Values are means $\pm$ SD of four animals per time point.

Cell Cycle Distributions In Vitro

Twenty-four hours after treatment of U87MG and U251MG cells with drug-free liposomes (control), Doxil, nLs-CPT-11, or a mixture of the two therapeutic liposomes, changes in cell cycle were recorded (Figs. 2 and 3). U87MG cells exposed to Doxil, a G2/M-active antineoplastic drug, underwent G2 arrest, with the percentage of cells in G2/M increasing from 12% (control) to 46% (Doxil; Fig. 2). U87MG cells exposed to nLs-CPT-11 entered both S-phase, to a marked extent (19% with control liposomes vs. 39% with nLs-CPT-11), and also significant G2 arrest (from 12% with control liposomes to 55% with nLs-CPT-11). After treatment with the combination of Doxil and nLs-CPT-11, both S-phase accumulation and G2 arrest were observed in U87MG cells.

When the same drug concentrations used with U87MG cells were applied to U251MG cells, complete cell cycle arrest was observed (Fig. 3). Doxil, nLs-CPT-11, and the combination of both each caused a complete elimination of cells in S-phase, with virtually all cells in G2 arrest. This difference in tumor cell sensitivity to chemotherapeutics later translated to effects on animal survival (Fig. 4).

Table 1. Tissue pharmacokinetics of nLs-CPT-11 (0.8 mg) and Doxil (2 μ g)

	$t_{1/2}$ (d)	AUC (μ g·d/g)	CL (g/d)	MRT (d)
nLs-CPT-11	16.7	11,600	0.069	24.1
Doxil	10.9	47.2	0.042	15.8

Pharmacokinetic data from Fig. 1 were analyzed with respect to tissue half-life ($t_{1/2}$), area under the curve (AUC), rate of clearance of either drug from brain tissue (CL), and mean residence time (MRT).

Synergistic Cytotoxic Effects of Doxil and nLs-CPT-11 Liposomes In Vitro (U87MG and U251MG)

Synergy, determined by isobologram analysis,²⁸ was not observed between the two agents in U87MG cells (Fig. 5). EC_{50} values (median effect doses) calculated from this experiment were 1.18 μ g/ml for Doxil and 0.75 μ g/ml for nLs-CPT-11. In contrast, synergy was observed between the two agents in U251MG cells (Fig. 5B). EC_{50} values from this experiment were 1.29 μ g/ml for Doxil and 0.56 μ g/ml for nLs-CPT-11.

Combined Effect of Doxil and nLs-CPT-11 in U251MG and U87MG Brain Tumor Xenografts

First, we studied survival in rodents with intracranial U251MG brain tumor xenografts seven days after tumor cell implantation. Rats that received the control liposomes were all euthanized 36–44 days after tumor cell implantation due to neurological symptoms indicative of tumor progression (Fig. 4). MS for this group was 38.5 days. Three of six rats that received 2 mg Doxil survived until termination of the study. However, neurological symptoms due to large tumor formations were observed in the three remaining rats, which required euthanasia 52–71 days after tumor cell implantation. Significant improvement in survival was noted in this treatment group ($p = 0.0007$), with an MS of 85.5 days. Three of six rats that received treatment at 0.8 mg nLs-CPT-11 by CED survived until termination of the study. The three remaining rats in this group were euthanized 54–62 days after tumor cell implantation due to neurological symptoms indicating tumor progression. Small tumors in the striatum were still evident in two of the surviving rats (Fig. 6D). Nevertheless, a significant survival benefit for nLs-CPT-11 over the control group was found (MS = 81 days; $p = 0.0016$). All rats in the group that received the combination treatment of Doxil and nLs-CPT-11 survived until the end of the study (MS > 100 days; $p < 0.0001$).

Next, we studied survival in rodents with U251MG brain tumor xenografts treated 14 days after tumor cell implantation. Rats in the control group that received drug-free liposomes were euthanized 38–46 days after tumor cell implantation due to neurological symptoms indicative of tumor progression (Fig. 4). The MS for the control group was 41 days. All animals receiving combination treatment of 0.8 mg nLs-CPT-11 and 2 mg Doxil survived 63–78 days, with an MS of 68 days ($p < 0.0001$).

A separate group of U87MG xenografts treated on day 10 was also evaluated. Rats in the control group that received drug-free liposomes were euthanized 18–21 days after implantation due to neurological symptoms indicative of tumor progression (MS = 19 days; Fig. 4). Five of six animals that received 2 mg Doxil were euthanized due to tumor-related symptoms 17–30 days after implantation (MS = 24 days). No significant survival benefit was observed for this group ($p = 0.215$). All animals that received 0.8 mg nLs-CPT-11 were euthanized due to neurological symptoms indicative of tumor

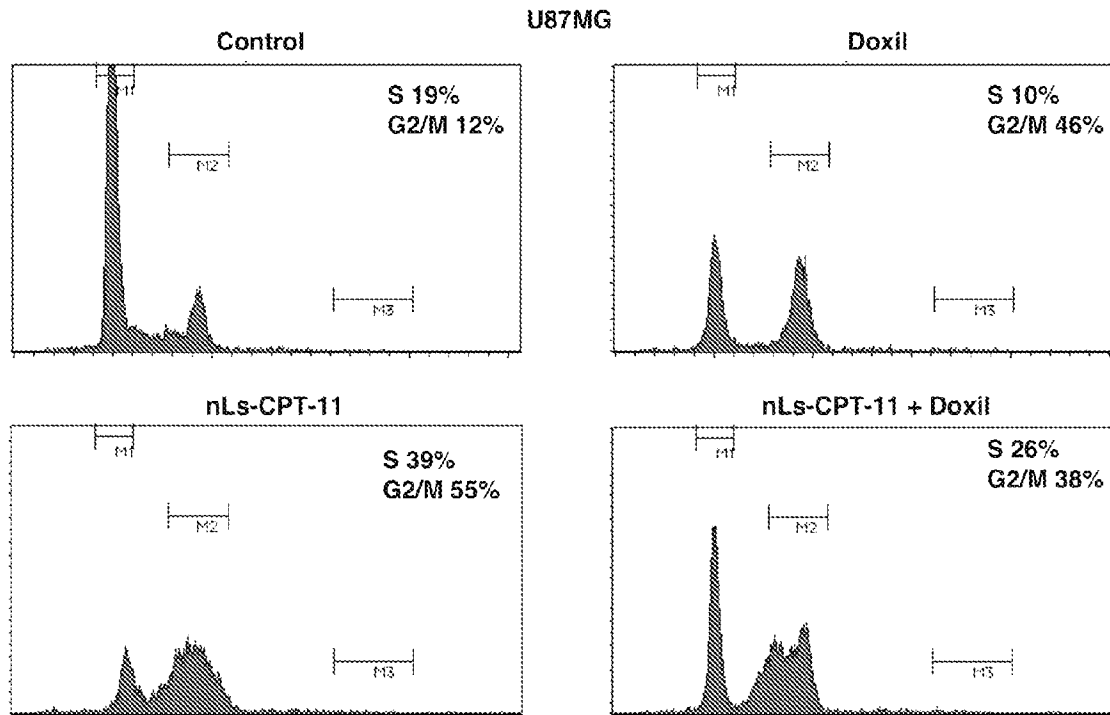


Fig. 2. Cell cycle profiles of U87MG cells examined by flow cytometry. U87MG cells were exposed to drug-free liposomes (control), Doxil (0.2 μ g/ml), CPT-11 nanoliposomes (nLs-CPT-11) (5 μ g/ml), or a combination of Doxil (0.2 μ g/ml) and nLs-CPT-11 (5 μ g/ml) for 24 h. Cells were harvested and analyzed by fluorescence-activated cell sorting as described in Materials and Methods.

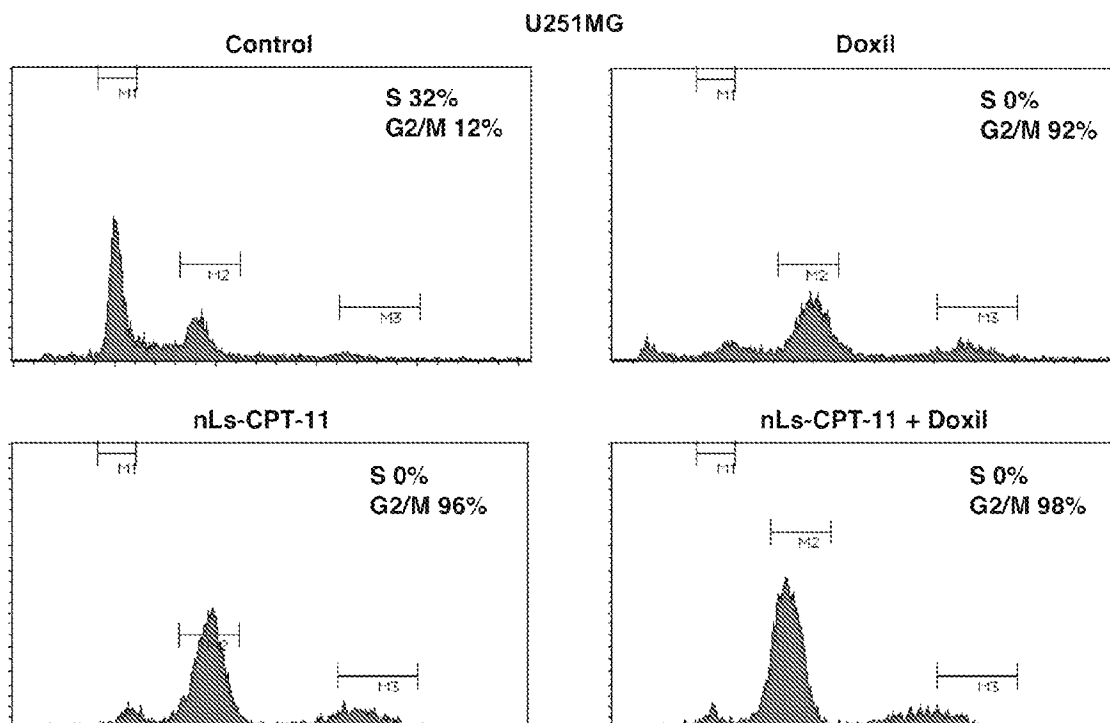


Fig. 3. Cell cycle profiles of U251MG cells examined by flow cytometry. U251MG cells were exposed to drug-free liposomes (control), Doxil (0.2 μ g/ml), CPT-11 nanoliposomes (nLs-CPT-11; 5 μ g/ml), or a combination of Doxil (0.2 μ g/ml) and nLs-CPT-11 (5 μ g/ml) for 24 h. Cells were harvested and analyzed by fluorescence-activated cell sorting as described in Materials and Methods.

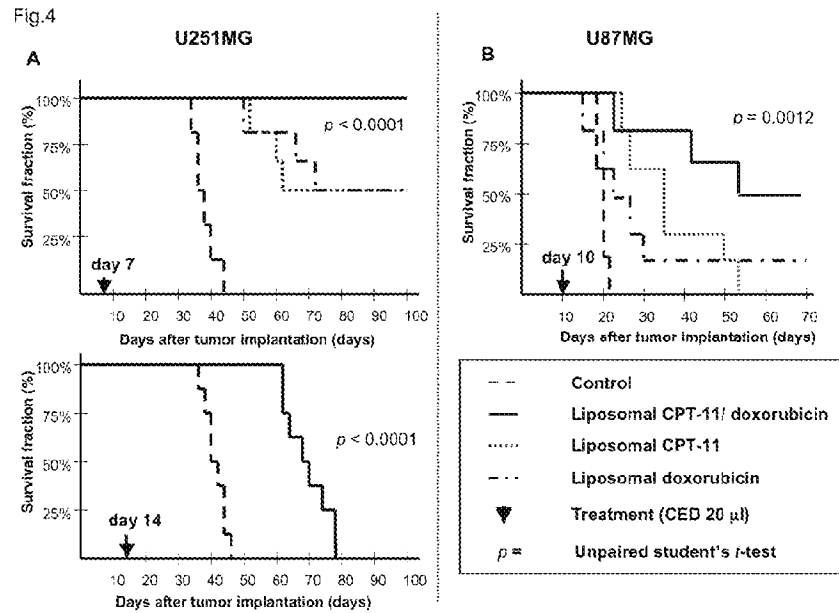


Fig. 4. Animals implanted with U251MG tumor cells. (A) Animals received liposome CED on day 7 and day 14 after tumor cell implantation. CED treatment on day 7 with nanoliposomal CPT-11 (nLS-CPT-11) and Doxil combination was able to eradicate all U251MG tumors in rodent striatum (see Fig. 5 caption). Each agent alone yielded only partial survival when delivered by CED on day 7. No animal in the day 14 combination therapy CED group survived longer than 78 days after tumor cell implantation. (B) Animals implanted with U87MG tumor cells received liposome CED on day 10 after tumor cell implantation. Three of six animals in the combination therapy group survived until termination of the study at day 70 after tumor cell implantation. Only one animal in the Doxil group survived until day 70. No animals in the nLS-CPT-11 group survived to the projected end of this survival study.

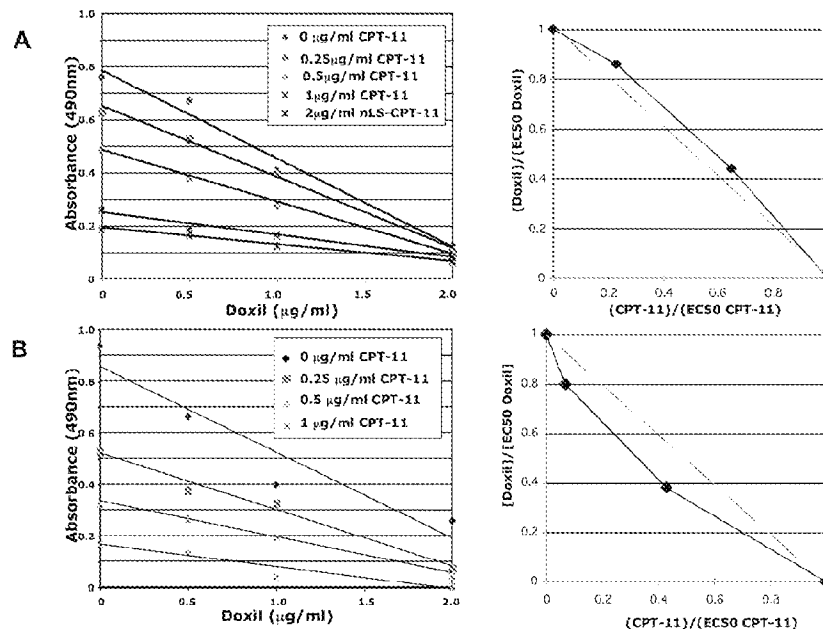


Fig. 5. Synergistic induction of cell death by Doxil and nanoliposomal CPT-11 (nLS-CPT-11) in U251MG glioma cells. (A) U87MG cells were treated for 24 h with increasing nLS-CPT-11 (0–2 μ g/ml) concentrations and 24 h with increasing Doxil (0–2 μ g/ml) concentrations. No synergy between the two agents was found by analysis of an isobologram (points above dotted line). (B) U251MG cells were treated identically to the U87MG cells with increasing Doxil and nLS-CPT-11 concentrations for 24 h. Synergy was determined between the two agents in the U251MG cell line as seen in the isobologram analysis (points below dotted line).

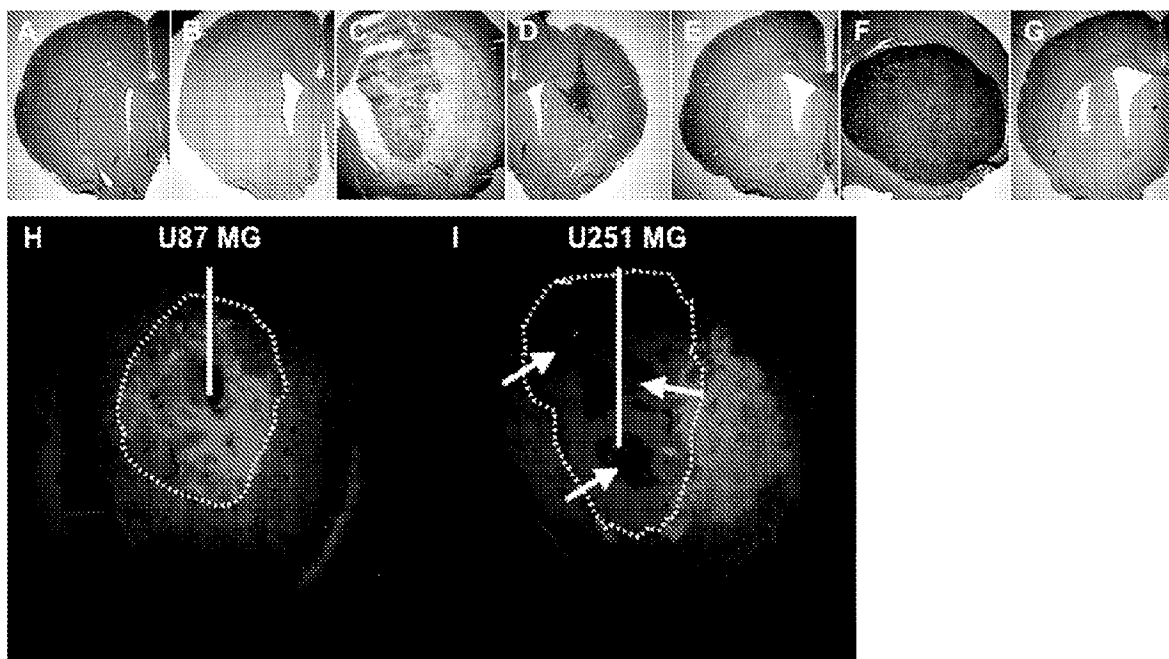


Fig. 6. Representative histology of animals used in this study. (A and B) Brain sections of animals used for toxicity study, euthanized 60 days after receiving the combination of Doxil (2.0 µg, 0.1 mg/ml) and nanoliposomal CPT-11 (nLs-CPT-11; 0.8 mg, 40 mg/ml). (C) Representative section of animals bearing U251MG xenografts. (D) Section from nLs-CPT-11 survivor of day 7 convection-enhanced delivery (CED) still bearing small U251MG tumor. (E) Survivor of day 7 CED combination therapy in U251MG at day 100. (F) Animal bearing U87MG xenografts. (G) Survivor of day 10 CED combination therapy in U87MG at day 70. (H and I) Representative sections showing DiIC₁₈(3) fluorescent liposome distribution in U87MG (H) and U251MG (I) intracranial xenografts after 20 µl CED. White line represents infusion catheter placement; dotted line delineates tumor margin on histology sections; white arrows show necrotic areas in U251MG brain tumor xenografts.

progression 24–53 days after tumor cell implantation (MS = 30 days). CED treatment with nLs-CPT-11 alone resulted in a significant survival benefit ($p = 0.0048$). Three of six animals receiving combination therapy of 0.8 mg nLs-CPT-11 and 2 mg Doxil were euthanized 23–54 days after tumor cell implantation. Three animals survived until termination of the study 70 days after tumor cell implantation. A three-fold increase in mean survival was observed for the combination therapy group (MS = 62 days), compared to the control ($p = 0.0012$).

Distribution of Liposomes in U87MG and U251MG Brain Tumor Xenografts

After CED of 20 µl, liposomal DiIC₁₈(3) distributed extensively throughout the U87MG brain tumor xenografts (Fig. 6H). However, the same amount of DiIC₁₈(3) liposomes distributed mainly outside U251MG brain tumor xenografts and within necrotic areas (white arrows; Fig. 6I). The observed tumor-specific distribution highlights one contribution to variability in therapeutic efficacy between tumor models.

Discussion

Further improvement of current CED-based drug delivery protocols is critical for future clinical application. Clinical trials in which CED is employed for drug delivery to the brain have indicated considerable potential for this approach in neurooncology and in neurodegenerative diseases.^{10,29} In this study, we have elucidated three important factors that affect overall survival: drug efficacy, drug tissue half-life, and drug distribution.

Liposomal encapsulation of chemotherapeutic drugs improves their pharmacokinetic properties.^{14,20,21,30} An ideal combination of two liposomal chemotherapeutics would have similar half-lives in the CNS and rates of drug release from the carrier in order to exert maximum combined toxicity on tumor cells over extended periods of time. In our study, although the dose of Doxil used was 400-fold lower than that of nLs-CPT-11, their tissue half-lives were similar. Liposomal encapsulation not only enhances drug half-life but also improves distribution of compounds with high tissue affinity (e.g., Doxil),⁹ as well as therapeutic index of the active agent (e.g., CPT-11).⁸

With respect to drug efficacy, we compared two brain tumor cell lines in our *in vitro* studies that are known to have different chemosensitivity *in vitro*.³¹ The same dose of the drug combination that produced an intermediate response in U87MG caused almost complete G2 arrest in U251MG. Our drug combination showed synergy in the more chemosensitive cell line, U251MG, whereas no synergy was found in U87MG. Various mechanisms are known by which tumor cells escape G2 cell cycle arrest,^{32,33} but different gene expression profiles, depending on whether cells are grown *in vitro* or *in vivo*, may be the key to the differences seen in this study.³⁴ Gene expression profiles in U87MG and U251MG, as determined by Camphausen et al.,³⁴ differ significantly *in vitro* but are similar when grown intracranially. We believe that contrast between the similar efficacy seen in the survival studies and the differences seen in our *in vitro* studies may be attributed to these effects. Moreover, the heterogeneity of malignant brain tumors warrants individual approaches to define the appropriate cancer treatment in each case.

We have shown previously that CED of liposomes easily covers an entire seven-day-old U251MG brain tumor xenograft (tumor diameter ≤ 0.5 mm).³⁵ The synergistic action of our liposomal therapeutic combination leads to complete eradication of U251MG xenografts, whereas Doxil or nLs-CPT-11 alone was only moderately efficacious. Interestingly, two of the three survivors in the nLs-CPT-11 group had relatively small tumor cell formations in the striatum at the termination of the study (day 100). Consistent with previous findings with nLs-CPT-11, we attribute this strong tumor growth inhibition *in vivo* to its central effect on the S-phase of the cell cycle.⁸ In contrast, a survival study with 14-day-old U251MG brain tumor xenografts (vs. the seven-day xenografts; tumor diameter up to 1 mm) showed significant prolongation of survival. However, none of the animals survived past day 78 with the combination treatment. In order to explain this discrepancy, we conducted a series of liposome distribution studies in U251MG brain tumor xenografts. Large necrotic areas in U251MG brain tumor xenografts that increase with growth of the tumor appeared to alter liposome distribution within the tumor. As shown in Fig. 6, a large fraction of infused liposomes accumulated outside of the tumor or within necrotic areas. We conclude that the slow clearance of liposomal therapeutics inhibits tumor growth at its margins, but after eventual clearance of the therapeutic agents, tumor in areas not subject to liposome accumulation can lead to continued tumor growth. In the U87MG survival study, a significant extension of animal survival was also achieved, but only 50% of animals survived until termination of the study. As in the U251MG experiments, we also studied liposome distribution in U87MG brain tumor xenografts. Due to its rather homogeneous growth pattern, good distribution of liposomes was observed in U87MG xenografts (tumor diameter ~ 1.5 mm on day 10).³⁵ However, despite the better distribution of liposomes in U87MG tumors, the fast growth of U87MG, the slow release of therapeutics from liposomes, and the lower sensitivity to

Doxil/nLs-CPT-11 combination *in vitro*, compared with U251MG, all contribute to reduced efficacy in U87MG versus U251MG tumors.

Several important conclusions can be drawn from this study. For patients with brain tumors, systemic delivery of therapeutics is usually associated with systemic side effects while achieving only marginal therapeutic concentrations in the CNS. This limits the effectiveness of systemic treatment. Thus, clinical trials to evaluate the combination of Topo I and II inhibitors via intravenous administration in patients with solid neoplasms demonstrated substantial toxicity.^{23,24} The defined MTD was frequently lower than the typical dose level for the respective individual agent. CED of nonencapsulated chemotherapeutic agents has produced better outcomes but was also highly toxic upon extensive distribution of free drug within the CNS.³⁶ CED of a mixture of Doxil and nLs-CPT-11 has shown excellent therapeutic potential without signs of toxicity to the CNS at the doses of each liposomal drug employed. Both therapeutics were used at doses previously shown to be individually non-toxic but to be substantially therapeutic in brain tumors when delivered by CED.^{8,9} The present drug combination was therefore capable of increasing therapeutic efficacy by synergistic action in U251MG without increase in CNS toxicity. Although synergy was not formally demonstrated via isobologram analysis in U87MG *in vitro*, the combination therapy *in vivo* nevertheless resulted in improved survival over each agent alone.

Another important aspect of this study is the prolonged release of the two drugs from liposomes.⁸ This slow-release phenomenon might benefit patients with brain tumors, since human tumors grow far more slowly than do animal tumor xenografts. As shown by our animal experiments, eradication of tumor is a realistic possibility only when complete coverage with a liposomal drug is achieved. This is why we have developed a real-time imaging method to visualize direct liposome delivery into the CNS.^{17,19} This real-time imaging technique, in combination with several infusion catheters, may permit complete liposomal coverage of tumors in human brain, because the drug may then be infused until optimal distribution is achieved while avoiding untoward leakage of the drug into healthy surrounding tissue. Currently, we are undertaking experiments in canine *de novo* brain tumors in order to validate the present rodent experiments in a larger mammalian brain and to strengthen the hypothesis that distribution of liposomes in tumors is greatly affected by tumor histology. Although all studies were conducted on a relatively small number of animals per group, our findings were consistent with previous rodent and primate studies. In the human setting, malignant tumors are usually more than 1 cm in diameter at time of diagnosis, and are located in various parts of the brain. Our previous studies on naive primate brains have clearly shown that our CED delivery technique, when combined with our step cannula design, allows liposome delivery into every CNS structure at any given depth.^{17,19} Application of this delivery technique will probably require multiple cannulas in order to cover the entire tumor mass. We

are aware that more work needs to be done in the field of distribution characterization as a function of tumor histology, but a more complete conceptual understanding of liposome therapeutics and distribution has now been established.

Acknowledgments

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Phase I Study of PARP Inhibitor ABT-888 in Combination with Topotecan in Adults with Refractory Solid Tumors and Lymphomas

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Abstract

A phase I trial of ABT-888 (veliparib), a PARP inhibitor, in combination with topotecan, a topoisomerase I-targeted agent, was carried out to determine maximum tolerated dose (MTD), safety, pharmacokinetics, and pharmacodynamics of the combination in patients with refractory solid tumors and lymphomas. Varying schedules and doses of intravenous topotecan in combination with ABT-888 (10 mg) administered orally twice a day (BID) were evaluated. Plasma and urine pharmacokinetics were assessed and levels of poly(ADP-ribose) (PAR) and the DNA damage marker γ H2AX were measured in tumor and peripheral blood mononuclear cells (PBMC). Twenty-four patients were enrolled. Significant myelosuppression limited the ability to coadminister ABT-888 with standard doses of topotecan, necessitating dose reductions. Preclinical studies using athymic mice carrying human tumor xenografts also informed schedule changes. The MTD was established as topotecan 0.6 mg/m²/d and ABT-888 10 mg BID on days one to five of 21-day cycles. Topotecan did not alter the pharmacokinetics of ABT-888. A more than 75% reduction in PAR levels was observed in 3 paired tumor biopsy samples; a greater than 50% reduction was observed in PBMCs from 19 of 23 patients with measurable levels. Increases in γ H2AX response in circulating tumor cells (CTC) and PBMCs were observed in patients receiving ABT-888 with topotecan. We show a mechanistic interaction of a PARP inhibitor, ABT-888, with a topoisomerase I inhibitor, topotecan, in PBMCs, tumor, and CTCs. Results of this trial reveal that PARP inhibition can modulate the capacity to repair topoisomerase I-mediated DNA damage in the clinic. *Cancer Res*; 71(17): 5626–34. ©2011 AACR.

Introduction

PARP enzymes are involved in the recognition of DNA damage and the facilitation of DNA repair. Inhibition of PARP activity is a promising approach for enhancing the DNA-damaging effects of chemotherapeutic agents (1, 2). PARP inhibitors in clinical development have generated considerable interest for patients whose tumors are driven by *BRCA1* or *BRCA2* mutations, based on reports of increased sensitivity of homozygous *BRCA*-deficient cells to PARP inhibition (3, 4).

ABT-888 (veliparib) is a potent, orally administered small molecule inhibitor of PARP-1 and PARP-2. In preclinical

models, ABT-888 potentiates the antitumor activity of multiple cytotoxic agents, including topoisomerase I-targeted inhibitors (5). PARP inhibition prevents efficient repair of DNA damage induced by camptothecins (6–10). Therefore, we hypothesized that coadministration of ABT-888 with topotecan would enhance the antitumor activity of this topoisomerase I inhibitor.

We conducted a phase I trial of ABT-888 combined with topotecan hydrochloride in patients with advanced, refractory solid tumors and lymphomas. The objectives were to establish the safety, tolerability, and maximum tolerated dose (MTD) of the combination of ABT-888 with topotecan; to evaluate the pharmacokinetics of each agent alone and in combination; and to determine the effects of the study treatment on the level of PARP activity and DNA damage in peripheral blood mononuclear cells (PBMC), circulating tumor cells (CTC), and tumor biopsy samples.

Patients and Methods

Eligibility

Patients eligible for this trial had histologically documented solid tumors and low-grade lymphoid malignancies that were refractory to standard therapy; an Eastern Cooperative

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Oncology Group performance status ≤ 2 ; and adequate organ and marrow function defined as leukocytes $\geq 3,000/\mu\text{L}$, absolute neutrophil count $\geq 1,500/\mu\text{L}$, platelets $\geq 100,000/\mu\text{L}$, total bilirubin $\leq 1.5 \times$ the upper limit of normal (ULN), aspartate aminotransferase and/or alanine aminotransferase $< 2.5 \times$ ULN, and creatinine $< 1.5 \times$ ULN.

Prior anticancer therapy must have been completed at least 4 weeks prior to enrollment. Prior therapy with a PARP inhibitor was allowed. Patients were excluded if they had an uncontrolled intercurrent illness or were pregnant or lactating.

This trial was conducted under a National Cancer Institute (NCI)-sponsored IND with Institutional Review Board approval. The protocol design and conduct complied with all applicable regulations, guidance, and local policies. ClinicalTrials.gov identifier: NCT00553189.

Trial design

This was an open-label, single-arm phase I study of the combination of ABT-888 administered orally with topotecan hydrochloride administered intravenously in patients with advanced malignancies. ABT-888 was supplied under a Collaborative Research and Development Agreement between the Division of Cancer Treatment and Diagnosis, NCI, and Abbott Laboratories, Inc. Topotecan was obtained from commercial sources.

To evaluate the pharmacokinetics and pharmacodynamics of each agent alone compared with the combination, we initially evaluated a schedule of topotecan administered alone on day -8, followed a week later by ABT-888 administered twice a day (BID) on days 1 to 7, and topotecan on days 2 to 5 for cycle 1. Starting on cycle 2, ABT-888 was administered on days 1 to 7 and topotecan on days 1 to 5 (21-day cycle; Fig. 1,

schedule A). The starting dose was topotecan $1.2 \text{ mg}/\text{m}^2/\text{d}$ and ABT-888 10 mg BID (dose level 1; Table 1). However, we observed grade 4 myelosuppression at this dose level, initially necessitating dose reduction of topotecan to $0.9 \text{ mg}/\text{m}^2/\text{d}$ with ABT-888 10 mg BID, followed by a revision of the drug administration schedule. We hypothesized that administering a dose of topotecan a week before giving 4 additional days of topotecan with 5 days of ABT-888 may be priming bone marrow progenitors, increasing the likelihood of myelosuppression (11). Therefore, we revised the schedule to administer topotecan on days 1 to 5 and ABT-888 on days 2 to 5 in cycle 1 and then gave both drugs together on days 1 to 5 in subsequent cycles (21-day cycles; Fig. 1, schedule B). The topotecan dose was reduced to $0.75 \text{ mg}/\text{m}^2/\text{d}$. However, we again observed grade 3 and 4 myelosuppression, resulting in reduction of the topotecan dose to $0.6 \text{ mg}/\text{m}^2/\text{d}$ given on days 1 to 5 (dose level -3). This dose level was well tolerated. Because of the clinical toxicities, human xenograft model studies were carried out indicating that delivering 1 day of ABT-888 with 5 days of topotecan produced the same antitumor effect as 5 days of both drugs (Fig. 2). Therefore, to safely escalate the dose of topotecan, we explored administering topotecan at $0.75 \text{ mg}/\text{m}^2/\text{d}$ given on days 1 to 5, with ABT-888 administration on day 1 only in each cycle (21-day cycles; Fig. 1, schedule C).

Adverse events were graded according to NCI Common Terminology Criteria version 3.0. Dose-limiting toxicity (DLT) was defined as an adverse event that occurred in the first 2 cycles (given that the schedules were different between cycles 1 and 2), was believed to be study drug related, and fulfilled one of the following criteria: grade 3 or higher nonhematologic toxicity (except for nausea/vomiting and diarrhea without maximal symptomatic/prophylactic treatment); grade 4

Figure 1. Days of administration of ABT-888 (ABT) and topotecan (TPT) during cycle 1 for schedules A, B, and C.

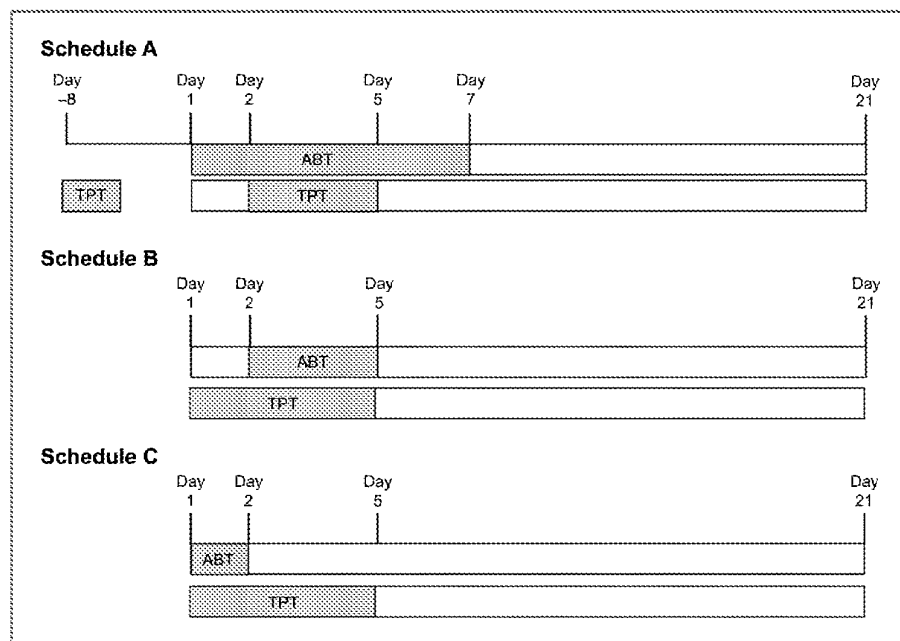


Table 1. Dose levels, number of patients enrolled, and dose-limiting toxicities in cycles 1 and 2

Dose level	Schedule	ABT-888		Topotecan		No. of patients	Dose-limiting toxicities (No. of patients)
		Dose (po BID)	Days	Dose (mg/m ² /d IV)	Days		
1	A	10 mg	1–7	1.2	C1: –8, 2–5	6	Neutropenia (2), thrombocytopenia (1), febrile neutropenia (1)
–1	A	10 mg	1–7	0.9	C2 onwards: 1–5 C1: –8, 2–5	3	Neutropenia (2)
–2	B	10 mg	C1: 2–5 C2 onwards: 1–5	0.75	1–5	3	Febrile neutropenia (1), neutropenia (1)
–3	B	10 mg	C1: 2–5 C2 onwards: 1–5	0.6	1–5	4 ^a + 3	None
1A	C	10 mg	1	0.75	1–5	5	Thrombocytopenia (2)

^aOne patient progressed before completing 2 cycles so was not evaluable for toxicity and was therefore replaced.

Abbreviation: C, cycle.

thrombocytopenia; grade 4 neutropenia lasting 5 days or more; or febrile neutropenia. Any degree of anemia, leukopenia in the absence of neutropenia as defined above, or lymphopenia was not considered dose limiting. Patients were

not allowed to receive prophylactic growth factors for the first 2 cycles.

Three patients were initially enrolled per dose level. Patient enrollment and dose escalation followed a traditional 3+3 design. The MTD was defined as the dose at which no more than 1 of 6 patients or 33% or less experienced a DLT (one dose level below the dose at which at least 2 of 3 to 6 patients experienced DLT).

Safety and efficacy evaluations

A complete patient history and physical examination were conducted at baseline and prior to each cycle. Complete blood counts with differential and serum chemistries were done at baseline, weekly for the first 2 cycles, and then prior to each cycle. More frequent blood counts were done for patients experiencing grade 3 and 4 myelosuppression to determine the duration of neutropenia and thrombocytopenia and to document recovery. Radiographic evaluation was done at baseline and every 2 cycles to assess for tumor response based on the Response Evaluation Criteria in Solid Tumors (RECIST 1.0; ref. 12).

Pharmacokinetics

Blood samples (7 mL) for pharmacokinetic analysis were collected during cycle 1 at baseline prior to drug administration and 0.5, 1, 2, 4, 7, 10, and 24 hours postdose on days –8, 1, and 2 for dose levels 1 and –1; days 1 and 2 for dose levels –2 and –3; and days 1 and 3 for dose level 1A.

A urine sample of 10 mL was collected before drug administration and at every void from 0 to 24 hours postdose on the same days that blood was collected for pharmacokinetic analysis.

Total topotecan plasma concentrations were measured by high-performance liquid chromatography (HPLC) with fluorescence detection as previously described (13). ABT-888

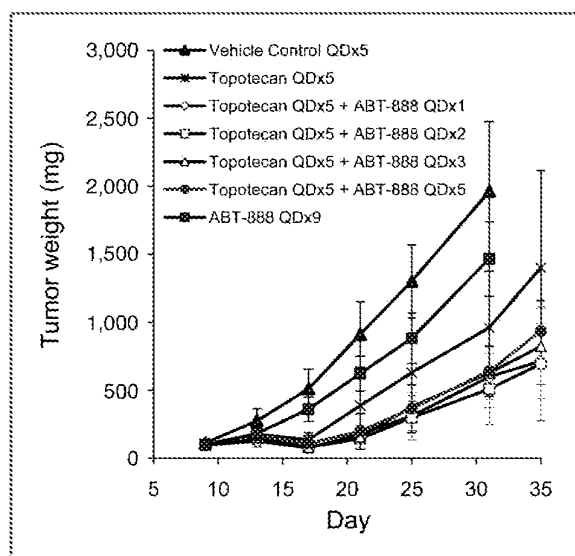


Figure 2. Tumor growth delay with revised ABT-888 and topotecan dosing schedules in mice bearing A375 human melanoma xenografts. Topotecan (1.5 mg/kg) was administered by intraperitoneal injection once a day for 5 consecutive days (QDx5) starting on study day 9. ABT-888 (3.13 mg/kg) was administered orally starting on study day 9 as follows, relative to topotecan: 1 hour before the first dose (QDx1), first and second doses (QDx2), first, second, and third doses (QDx3), and first, second, third, fourth, and fifth doses (QDx5). The antitumor efficacies of the revised dose schedules (1, 2, or 3 days of ABT-888 administration in combination with 5 days of topotecan), measured on day 31, were not significantly different from 5 days of ABT-888 administration. Animal weight loss did not exceed 10% in any cohort. Data presented as mean $\pm$ SD.

plasma concentrations were measured by an HPLC-based method (14) modified to include drug isolation by solid-phase extraction by using a Bond Elut Certify (Varian) column and fluorescence (excitation, 255 nm and emission, 390 nm) detection (R. Klecker, personal communication). Additional details are included in the Supplementary Appendix. Topotecan and ABT-888 plasma concentration–time data were analyzed by standard noncompartmental methods by using the program WinNonlin Pro (Pharsight Corp).

Pharmacodynamics

Blood samples for PBMCs were collected in 8-mL Cell Prep Tubes (Becton Dickinson) before drug treatment and 2, 4, 7, and 24 hours after drug administration on the first day of topotecan alone, ABT-888 alone, and the combination. PBMCs were isolated as previously described (15). A pretreatment biopsy was obtained just prior to drug administration on study, and a postdose biopsy was collected 3 to 7 hours after administration of the combination. Levels of poly(ADP-ribose) (PAR), a product of PARP, were measured by using a validated chemiluminescent PAR immunoassay as previously described (15, 16). Blood samples were also collected pre- and postdosing (day 2 or 5) to examine the effect of the combination on phosphorylated histone H2AX (γ H2AX), a marker for the formation of DNA double-strand breaks, in CTCs by using a quantitative, validated assay (17).

γ H2AX was also measured in tumor samples by using a quantitative immunofluorescence assay as previously described (18). γ H2AX in PBMCs was measured as described in the Supplementary Appendix.

Human tumor xenograft models

Xenograft models were used before and during the trial to help determine an optimum clinical dosing schedule. The effects of ABT-888, topotecan, or the combination on tumor growth were assessed in athymic nude (nu/nu NCr) mice bearing A375 (topotecan sensitive) human melanoma xenografts as described in the Supplementary Appendix. A375 cells were obtained from American Type Culture Collection, cultured for less than 6 months, and not reauthenticated.

Results

Patient demographics

A total of 24 patients were enrolled between November 2007 and June 2009 (Table 2). All patients had previously received prior standard therapy and had evidence of disease progression. In the initial cohorts, most patients had received more than 5 prior systemic therapies (range 2–14). In light of the degree of myelosuppression observed, we enrolled less heavily pretreated patients (3 or fewer prior systemic therapies) starting with patient 13.

Toxicity

We observed 3 DLTs in the first 6 patients enrolled on dose level 1 (ABT-888 10 mg BID on days 1 to 7; topotecan 1.2 mg/m²/d on day –8 and days 2 to 5 in cycle 1 and on days 1 to 5 in cycle 2 onward; schedule A, Fig. 1). One patient developed

Table 2. Patient characteristics

No. of patients	24
Male	13
Female	11
Age, y.	
Median	57
Range	25–77
Eastern Cooperative Oncology	
Group performance status	
0	1
1	23
Tumor types	
Colorectal	5
Ovarian	5
Melanoma	2
Pancreas	1
Endometrial cancer	2
Hurthle cell thyroid	1
Other ^a	8
No. of lines of prior systemic	
therapies (range 2–14) ^b	
2–3	14
>3	10

^aPleural mesothelioma, hepatocellular, non-Hodgkin's lymphoma (low grade), external ear adenocarcinoma, bile duct adenocarcinoma, small cell lung cancer, esophageal adenocarcinoma, and chondrosarcoma.

^bBecause of the observed myelosuppression, we enrolled less heavily pretreated patients (3 prior therapies or less) on Schedules B and C (patient 13 onwards).

grade 4 neutropenia and thrombocytopenia, one had grade 4 neutropenia lasting longer than 5 days, and one had febrile neutropenia. All 6 patients experienced grade 3 or 4 myelosuppression in the first 2 cycles, necessitating a topotecan dose reduction to dose level –1 (0.9 mg/m²/d). One patient developed grade 4 neutropenia for longer than 5 days, meeting the criteria for DLT. Because 2 other patients on this dose level also developed grade 4 neutropenia (even though it was less than 5 days in duration), the dose level was not considered tolerable. The drug administration schedule was revised to reduce the number of days of ABT-888 dosing, eliminate treatment on day –8, and further reduce the dose of topotecan (schedule B, Fig. 1). On dose level –2, topotecan (0.75 mg/m²/d) was administered on days 1 to 5 of every cycle, with ABT-888 (10 mg BID) administered on days 2 to 5 in cycle 1 and days 1 to 5 in cycle 2 onward. Three patients were enrolled and 2 DLTs were observed, febrile neutropenia and grade 4 neutropenia lasting longer than 5 days. This necessitated a reduction in topotecan dose to 0.6 mg/m²/d for dose level –3; 3 patients were treated at this dose level, and no DLTs were observed.

In view of the toxicities observed, we then employed xenograft models to explore alternative dosing schedules in

an attempt to escalate both agents. Results from these studies indicated that 1 day of ABT-888 administration (dose of 3.13 mg/kg) with 5 days of topotecan (1.5 mg/kg) produced a nearly equivalent antitumor effect as 5 days of both drugs (Fig. 2, Supplementary Table S1). On the basis of these pre-clinical data, we amended the trial to evaluate escalating the dose of topotecan while reducing the duration of ABT-888 administration to 1 day only (schedule C, Fig. 1). Five patients were enrolled on dose level 1A, and 2 developed grade 4 thrombocytopenia, meeting criteria for DLT. Three additional patients were enrolled on dose level -3; no DLTs were observed. Thus, the MTD was established as topotecan 0.6 mg/m²/d on days 1 to 5 and ABT-888 10 mg BID on days 1 to 5 in 21-day cycles.

Efficacy

Four of 6 patients on dose level 1 had stable disease after 2 cycles; however, these patients were taken off study because of toxicity. One patient with Hürthle cell thyroid cancer received a total of 33 cycles on dose level -3 with disease stabilization. One patient (dose level -3) with refractory melanoma received a total of 14 cycles with disease stabilization. One patient with colorectal cancer and one with bile duct adenocarcinoma (both on dose level 1A) received total of 6 and 4 cycles, respectively, with disease stabilization.

Pharmacokinetics

The mean ($\pm$ SD) half-life for topotecan administered alone, 1 week before beginning combination treatment (schedule A), was 3.6 ± 3.0 hours, with mean ($\pm$ SD) clearance and steady-state volume of distribution values of 9.2 ± 5.0 L/hr/m² and 35.1 ± 17.3 L/m², respectively. The mean ($\pm$ SD) half-life, clearance, and steady-state volume of distribution values for topotecan after administration of ABT-888 were 3.0 ± 1.4 hours, 9.8 ± 5.1 L/hr/m², and 35.7 ± 16.3 L/m², respectively. Topotecan half-life, clearance, and steady-state volume of distribution values were also not altered by concomitant administration with ABT-888 in schedules B and C (data not shown). Topotecan was predominantly excreted in the urine: 24 hours after intravenous administration, the cumulative urinary recovery of total topotecan (mean $\pm$ SD) ranged from $94.1\% \pm 29.5\%$ (day 1, $n = 11$) to $105.1\% \pm 16.7\%$ (day 2) of the administered doses. The mean values ($\pm$ SD, $n = 9$) for maximum plasma concentration, time to maximum plasma concentration, half-life, and area under the concentration-time curve extrapolated to infinity for ABT-888 (30 mg, BID) administered alone in schedule A were 262 ± 80 nmol/L, 1.6 ± 1.1 hours, 4.4 ± 1.4 hours, and $1,460 \pm 450$ nmol/L•h, respectively. When administered with topotecan in schedules B and C, the values were 255 ± 125 nmol/L, 1.8 ± 0.9 hours, 4.9 ± 1.8 hours, and $1,740 \pm 786$ nmol/L•h, respectively. Thus, there was no evidence of a significant pharmacokinetic interaction between the 2 drugs.

Pharmacodynamics

A summary of pharmacodynamic responses for patients in this trial is included in Supplementary Table S2. PARP was modulated by ABT-888 for each of the administration sche-

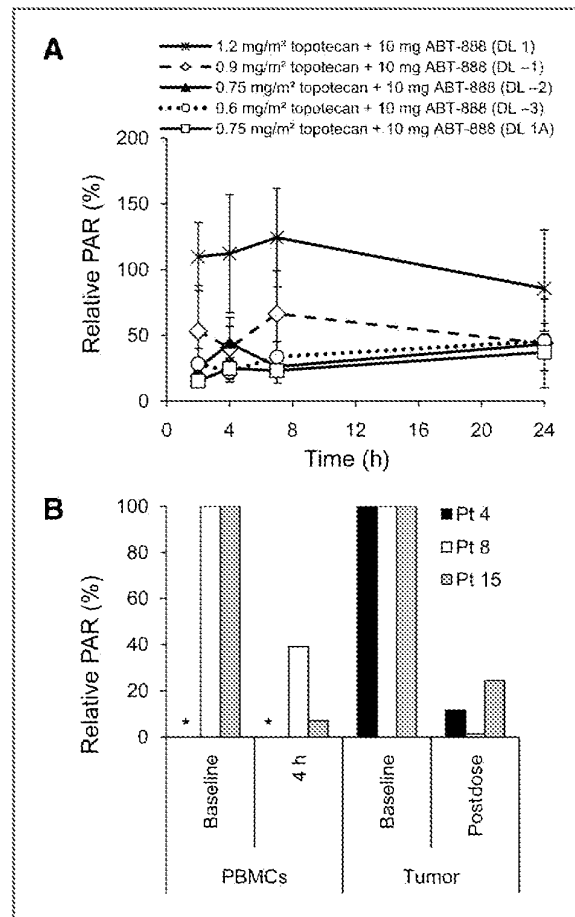
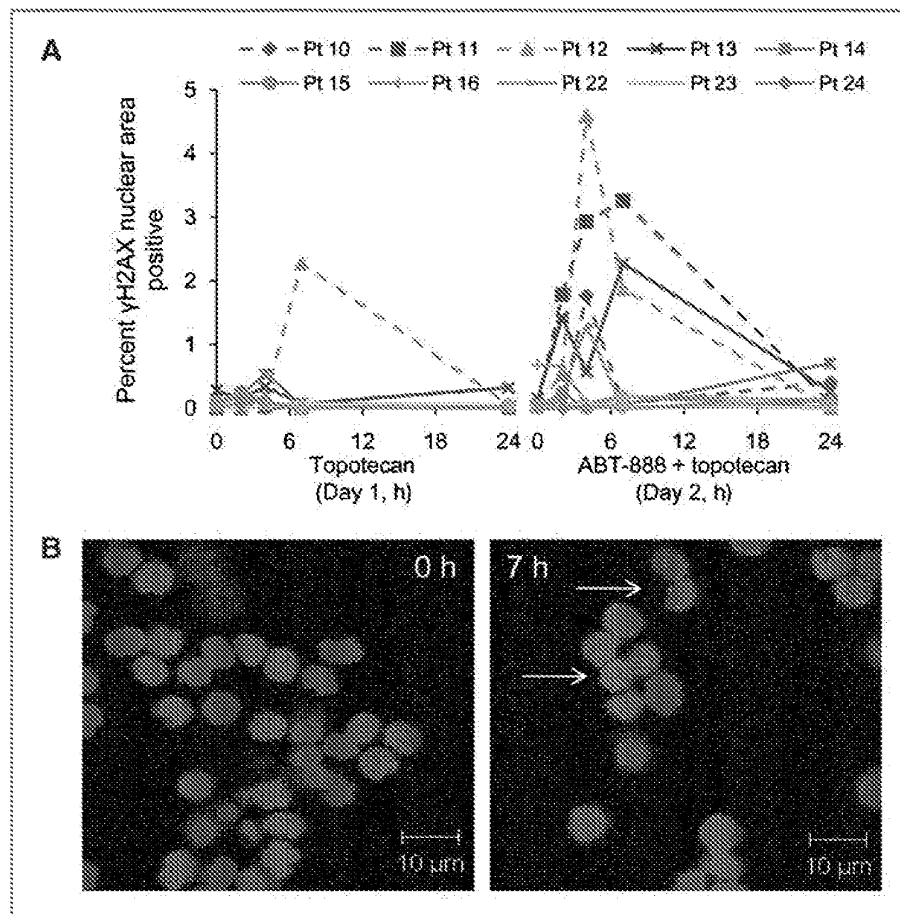


Figure 3. A, PAR levels relative to baseline (100%) in PBMCs collected at 2, 4, 7, and 24 hours after the combination of ABT-888 and topotecan. Data presented as mean $\pm$ SEM for dose level (DL) 1 ($n = 5$), DL -1 ($n = 3$), DL -2 ($n = 3$), DL -3 ($n = 7$), and DL 1A ($n = 5$). PAR levels in many individual samples decreased to values below the limit of detection of the assay. B, PAR levels relative to baseline (100%) in PBMCs and pre- and postdose tumor biopsies from patients 4 (DL 1), 8 (DL -1), and 15 (DL -3). Patients 4 and 8 were on schedule A and patient 15 was on schedule B. *The baseline PAR level in PBMCs for patient 4 was below the required minimum for analysis.

dules studied and in the majority of patients. Topotecan did not seem to alter the ability of ABT-888 to inhibit PARP. In PBMCs, a greater than 50% mean reduction in PAR was observed after administration of ABT-888 in all dose levels, except for the level with the highest dose of topotecan (1.2 mg/m²/d; Fig. 3A). Supplementary Figure S1 shows data by schedule for the 23 patients with evaluable PBMC PAR levels. A greater than 75% reduction in PAR levels in tumors was observed in each of the 3 patients for whom paired tumor biopsies were available (Fig. 3B); 2 of these patients also had a greater than 60% reduction in PAR levels in PBMCs. The baseline PAR level in PBMCs from the third patient was below the required minimum for analysis.

Levels of γ H2AX were measured in PBMCs after administration of topotecan alone and after the combination. One of 3

Figure 4. A, levels of γ H2AX measured as percent nuclear area positive (%NAP) in PBMCs from patients on dose level -2 (dashed lines) and -3 (solid lines). B, γ H2AX staining in PBMCs from the same patient at baseline (0 hour) and 7 hours after treatment with ABT-888 and topotecan on dose level -3. %NAP was increased after treatment. Arrows indicate γ H2AX signal (in red).



patients had elevated levels of γ H2AX in PBMCs after a dose of 0.75 mg/m²/d topotecan alone, but elevations were not observed with lower doses. However, after topotecan was given with ABT-888, PBMCs from 2 of 7 patients on dose level -3 (0.6 mg/m²/d topotecan) and all 3 patients on dose level -2 (0.75 mg/m²/d topotecan) showed increases in γ H2AX (Fig. 4A). The increases in γ H2AX were small but tended to be greater with the higher topotecan dose (dose level -2). By 24 hours after dosing, γ H2AX levels had returned to baseline levels. The two patients on dose level -3 who had increased γ H2AX levels in PBMCs after administration of ABT-888 with topotecan also had a reduction in PAR in PBMCs. These patients had a 100% PAR reduction in PBMCs at 2 hours postdose and 100% and 76.3% reductions at 4 hours postdose, compared with a 60.2% $\pm$ 29.6% PAR reduction (mean $\pm$ SD) at 2 hours postdose for the 5 patients on that dose level in whom a γ H2AX response was not observed (4 hours; 73.5% $\pm$ 22.1%). Paired tumor biopsies from 2 patients, one on dose level -1 and one on dose level -3, were analyzed for γ H2AX, but no effect of study treatment on γ H2AX levels was measured (data not shown).

Total CTCs and the number of γ H2AX-positive CTCs were measurable in samples from 4 patients at baseline and after combined ABT-888 and topotecan treatment (Fig. 5A and B).

The percentage of γ H2AX-positive CTCs increased in 3 of the 4 patients. The average increase for the 4 patients was 23% (95% CI: 5–40; the change is nonnegative with 0.06 significance, 1 sided, by the nonparametric sign test). Interestingly, whereas the patient with the highest total number of CTCs, patient 21, had a decrease in total CTCs after treatment, this patient had an increase in the percentage of γ H2AX-positive CTCs. Data from day 5 were available from one patient (patient 24). For this patient, the fraction of CTCs positive for γ H2AX increased from 16% at baseline to 100% by day 5, whereas the number of CTCs remained constant.

Discussion

This trial evaluated the toxicities, pharmacokinetics, and pharmacodynamics of ABT-888, a potent PARP inhibitor, in combination with a topoisomerase I inhibitor in clinical use, topotecan, in patients with advanced malignancies. The definition of DLT included drug-related adverse events that occurred in the first 2 cycles (given that the schedules were different between cycles 1 and 2). As 6 evaluable patients received at least 2 cycles without DLT at dose level -3, which included the regimen of a full 5 days of dosing of both ABT-888 and topotecan starting with cycle 2, this regimen

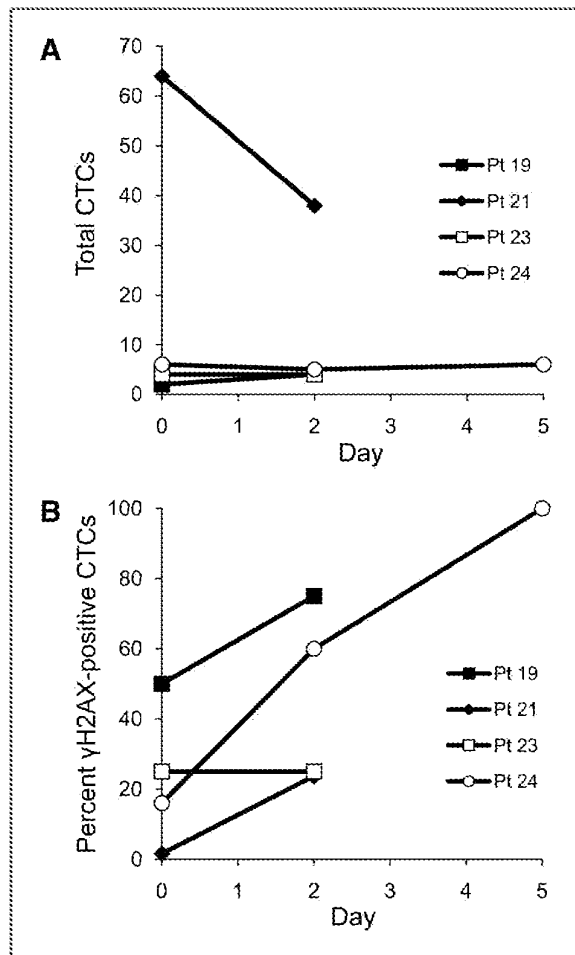


Figure 5. A, total CTCs isolated from patients on dose level -3 and 1A; data on day 5 was only available from patient 24. B, percentage of CTCs positive for γ H2AX from the same patient samples.

was established as the MTD (topotecan administered intravenously at 0.6 mg/m²/d on days 1 to 5 and ABT-888 given orally at 10 mg BID on days 1 to 5, in 21-day cycles). Myelosuppression was the principal toxicity on this trial, which is consistent with the severe neutropenia in 65% to 75% of courses and severe thrombocytopenia in 25% to 30% of courses when topotecan is administered to patients with lung or ovarian cancer at FDA-approved doses of 1.5 mg/m²/d for 5 days with 2 dose reductions allowed to 1 mg/m²/d (19, 20). Combination trials of topotecan with other cytotoxics such as cisplatin have required dose reduction of topotecan to 0.75 mg/m²/d or lower (21). Because of the degree of myelosuppression observed with topotecan alone, especially in heavily pretreated patients, it is difficult to draw conclusions about the contribution of ABT-888 to the toxicity of the combination regimen.

We used preclinical models during the conduct of the trial to evaluate our clinical observations and to revise the trial design. Although preclinical models indicated a better safety

profile for 1 day of ABT-888 in combination with 5 days of topotecan, we were unable to dose escalate topotecan in the clinic by reducing the number of days of ABT-888. It seems likely that a major reason for our difficulty in translating the results of our modeling of the ABT-888 plus topotecan combination in A375 melanoma xenografts to the clinic is the remarkable tolerance of mouse (versus human) bone marrow progenitors to topotecan (22).

One important question in developing PARP modulators is whether coadministration of a PARP inhibitor with reduced doses of topotecan results in superior benefit-to-risk profiles than full doses of topotecan alone. Enhanced normal tissue toxicity has been an important consideration in combination trials of PARP inhibitors with cytotoxic chemotherapies, requiring chemotherapeutic dose reductions (23, 24). As exemplified by our trial and trials with other PARP inhibitors in combination with chemotherapy, it has been difficult to define an optimum combination dose and schedule that would improve the therapeutic ratio. This could not be addressed in the current phase I dose escalation study because of a relatively small number of patients with a variety of refractory tumors. On the basis of our recent study in cancer cell lines with XPF-ERCC1 inactivation, it is possible that the potential benefit of using PARP inhibitors and topoisomerase I-targeted drugs will be seen in tumor cells with preexisting DNA repair deficiencies (10). To determine the effect of underlying genetic defects in the DNA repair pathways, such as ATM, ATR, BRCA, XRCC, XPF, and Fanconi anemia pathway, we are conducting this analysis in tumor samples from patients in our ongoing trial of the combination of ABT-888 with cyclophosphamide.

This is the first clinical study showing significant reduction in PAR levels (more than 75%) in all 3 paired tumor biopsies compared with baseline after administration of ABT-888 at 10 mg. Similar reductions were also observed in PBMCs. Whether this degree of PARP inhibition is sufficient (or a higher degree of inhibition is needed) to derive clinical benefit is currently unknown, making it difficult to define the "optimal biologic dose" of ABT-888 in combination with chemotherapy. Escalating the PARP inhibitor dose to the MTD may not be necessary to derive clinical benefit, especially given the narrow therapeutic index of certain combination regimens.

As previously reported in our phase 0 study of ABT-888 (15), tumor cells are more sensitive to PARP inhibition than PBMCs. Therefore, we evaluated the number of CTCs and the presence of γ H2AX in these tumor cells as a marker of drug effect on tumor. γ H2AX is a sensitive marker of DNA damage (18, 25). We hypothesized that increased DNA damage caused by the addition of an inhibitor of DNA repair, ABT-888, to topotecan could produce a measurable increase in γ H2AX levels in surrogate tissue and tumor (10). We observed an increase in γ H2AX in PBMCs after treatment with the combination of ABT-888 and topotecan when compared with topotecan alone. We did not observe a change in γ H2AX in the 2 paired tumor biopsies examined. The lack of increased γ H2AX in tumor biopsy samples may reflect an actual absence of effect on the level of DNA damage in tumor, given the low dose of topotecan administered, or the fact that tumor biopsy

timing was optimized to observe effects on PAR levels, and not γ H2AX levels, which are transient.

CTCs offer a relatively noninvasive way to repeatedly sample tumor cells over time, allowing assessment of dynamic markers. An increase in γ H2AX in CTCs following administration of topotecan has been previously reported by our group (17). In the current trial, we detected an increase in the fraction of CTCs positive for γ H2AX in 3 of 4 patients for whom CTC data were available after administration of the combination of topotecan with ABT-888 (Fig. 5). In the only patient in whom posttreatment samples were obtained at day 5 (following completion of all doses of study drugs), γ H2AX levels increased from 16% at baseline to 100%, suggesting an appreciable DNA damage response to drug treatment. This is the first clinical trial showing an increase in a marker of DNA damage in CTCs following administration of the combination of a PARP inhibitor with chemotherapy. The significance of these findings will be explored in further clinical trials. The actual number of CTCs collected (less than 10 at all time points in 3 of 4 patients) illustrates one of the major challenges for the wider application of CTCs to assess drug effect on target (Fig. 5B). Thus, newer technologies for CTC capture, which have been reported to substantially increase the number of CTCs harvested from a patient, could have a great impact on the future utility of CTCs as pharmacodynamic markers in clinical trials.

We investigated varying schedules of ABT-888 administration with topotecan in xenograft models after encountering toxicities with the initial schedule. Efficacy was equivalent whether ABT-888 was administered for 1 to 2 days or 5 days (with shorter treatment being better tolerated). This raises the question of the duration of exposure to PARP inhibitors necessary to derive clinical benefit. Ongoing clinical trials investigating combinations of PARP inhibitors with cytotoxic chemotherapies commonly evaluate multiple days of PARP inhibitor administration before, during, and after chemotherapy, in an attempt to increase DNA damage and delay DNA repair. The xenograft experiments carried out during this trial challenge the dogma that more is better. Issues such as chronic versus intermittent schedules and optimal sequencing

based on kinetics of DNA damage caused by the cytotoxic agent need to be explored further in clinical trials to maximize clinical benefit while ensuring patient safety. Further characterization of tumor-specific genetic defects in DNA repair and checkpoint pathways might also reveal the patients whose tumors will respond best to a combination of PARP- and topoisomerase I-targeted therapies.

Enhancing the therapeutic ratio between tumor cell killing and bone marrow toxicity for the combination of ABT-888 and topotecan will require evaluation of multiple, pharmacodynamically driven treatment schedules in the clinic. However, to our knowledge, this is the first clinical trial showing a mechanistic interaction between a PARP inhibitor and a topoisomerase I inhibitor in PBMCs, tumor, and CTCs. Results of this trial show that a PARP inhibitor can modulate the capacity to repair topoisomerase I-mediated DNA damage in the clinic.

Disclosure of Potential Conflicts of Interest

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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Phase I Study of PARP Inhibitor ABT-888 in Combination with Topotecan in Adults with Refractory Solid Tumors and Lymphomas

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Pharmacokinetic Study of Ferumoxytol: A New Iron Replacement Therapy in Normal Subjects and Hemodialysis Patients

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Key Words

Intravenous iron · Ferumoxytol · Iron pharmacokinetics · Renal anemia · Chronic kidney disease

Abstract

Background: Currently available intravenous iron preparations are not ideal, either because of safety concerns or dose limitations. We investigated the safety and pharmacokinetics of ferumoxytol, a new iron replacement therapy, in normal subjects and hemodialysis patients.

Methods: In a randomized, double-blind, ascending-dose study in normal volunteers ($n = 41$), 6 subjects received placebo, and 8 subjects each received ferumoxytol, at 1, 2 or 4 mg iron/kg, injected at 60 mg iron/min. The remaining subjects received 4 mg iron/kg at injection rates of 90 ($n = 3$), 180 ($n = 3$) or 1,800 mg iron/min ($n = 5$). In the second, open-label, ascending-dose study, 20 hemodialysis patients received 125 or 250 mg of iron over 5 min. **Results:** In normal subjects, the blood half-life of ferumoxytol increased with increasing dose from

9.3 to 14.5 h ($p < 0.05$) but not with increasing rate of injection. The drug half-life in hemodialysis patients was similar to normal subjects. Ferumoxytol was not removed with hemodialysis. Serum iron ($p < 0.001$), transferrin saturation ($p < 0.001$) and ferritin increased in both populations. No serious adverse events were attributable to ferumoxytol. **Conclusion:** Ferumoxytol was well tolerated in this study. Its pharmacokinetic properties and simplicity of administration suggest that it will be an attractive form of iron replacement therapy.

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Introduction

Normocytic, normochromic anemia is a common and treatable complication of chronic kidney disease (CKD) and end-stage renal disease (ESRD). According to the USRDS 2001 annual data report, there is a significant increase in the relative risk of cardiac, infectious, and all-cause mortality in ESRD patients with hematocrits $<33\%$ [1] and an even greater risk in patients with hematocrits $<30\%$. Although multiple factors contribute to the anemia of CKD, the primary cause is insufficient production of endogenous erythropoietin (EPO). Effective treatment

This work was performed at the University of Virginia Health System and at Jersey Shore Medical Center.

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of this anemia is associated with improved survival, decreased morbidity, and increased quality of life. Higher hematocrits have also been associated with marked improvements in various physiological parameters including oxygen utilization, muscle strength and function, cardiac function, possible regression of left ventricular hypertrophy, improvement in exercise tolerance and exercise-induced ST segment depression, cognitive and sexual function [2–7].

EPO use in hemodialysis patients has led to the successful treatment of anemia in most ESRD patients [8]. However, several factors can decrease the effectiveness of EPO including aluminum toxicity, hyperparathyroidism, infection, inflammatory conditions, malignancy, and, most importantly, iron deficiency [9]. Absolute iron deficiency in hemodialysis patients occurs for a variety of reasons, including blood retention in the dialysis membranes and connecting tubing system [9], occult gastrointestinal blood loss [10, 11], blood loss during surgery, frequent blood sampling [12], and increased erythropoiesis from the use of EPO [12]. Functional iron deficiency also occurs where adequate tissue iron stores are present but unavailable for erythropoiesis.

The estimated prevalence of iron deficiency in hemodialysis patients during EPO therapy is around 43–90% [9, 13, 14]. According to the National Kidney Foundation-Kidney Disease Outcomes Quality Initiative (KDO-QI) anemia guidelines [2], patients should have sufficient iron to achieve and maintain a hemoglobin of 11–12 g/dl and a hematocrit of 33–36%. To accomplish this, the guidelines suggest administering sufficient iron to a maintain transferrin saturation (TSAT) of $\geq 20\%$ and a serum ferritin ≥ 100 ng/ml [2]. Many studies have shown that oral iron is generally ineffective in maintaining adequate stores in most dialysis patients [3, 15, 16], as well as in patients not yet on dialysis [3, 16, 17]. Consequently, intravenous iron is commonly used to achieve and maintain adequate iron levels. Although intravenous iron replacement can be given over 10 min (125 mg iron gluconate) or 5 min (100 mg iron sucrose), it is generally given over 15–60 min, which requires significant expense for supplies such as tubing and infusion supplies, costly nursing time, multiple administrations, and may be inconvenient for the patient.

Until 1999, iron dextran was the only intravenous iron preparation available in the USA. Since then, iron gluconate and iron sucrose have become available for intravenous use. Although serious and life-threatening reactions occur most frequently with iron dextran [18], they do occur rarely with iron gluconate and iron sucrose [19, 20].

In addition, non-life-threatening reactions such as hypotension, back pain, vomiting, vertigo, and pruritus also occur with the gluconate and sucrose formulations and may be related to the rate of administration [20, 21]. These reactions, although not life-threatening, will sometimes preclude further dosing and therefore iron repletion [19, 22–24]. Finally, there is concern regarding the potential deleterious effects of parenteral iron in increasing oxidative stress and infection, and accelerating cardiovascular disease.

We examined the safety and pharmacokinetics of a new compound for iron replacement therapy, ferumoxytol, administered as an intravenous bolus, first in 41 healthy volunteers in a placebo-controlled, dose and injection rate escalation study, and then in 20 chronic hemodialysis patients receiving EPO therapy.

Materials and Methods

Ferumoxytol

The study drug, ferumoxytol (Advanced Magnetix, Inc., Cambridge, Mass., USA), is an ultrasmall superparamagnetic iron oxide (magnetite) nanoparticle coated with a semisynthetic carbohydrate designed to minimize immunological sensitivity. The drug has an average colloidal particle size of 30 nm by light scattering and a molecular weight of 750 kDa. Ferumoxytol is a sterile, isotonic, neutral pH liquid formulated to contain 30 mg/ml of elemental iron and 44 mg/ml of mannitol. Because of its magnetic properties, ferumoxytol can also be used as a magnetic resonance contrast agent.

Study Designs

Both trials were approved by Institutional Review Boards and conducted in accordance with the guidelines proposed in the Declaration of Helsinki, under an Investigational New Drug Exemption. All patients gave written informed consent to be in the study.

Normal Subjects. This phase I safety and pharmacokinetic study was a randomized, double-blind, placebo-controlled, ascending-dose study in adult male and female volunteers ($n = 41$). Subjects were 18–60 years old, not anemic, and in good general health. Eight subjects each received ferumoxytol at doses of 1, 2 and 4 mg iron/kg at injection rate of 60 mg iron/min (2 ml/min). Six subjects received saline as a placebo. The remaining 11 subjects received ferumoxytol at 4 mg iron/kg at an injection rate of 90 ($n = 3$), 180 ($n = 3$) or 1,800 mg iron/min ($n = 5$).

For pharmacokinetic analysis, serial blood samples were taken pre-dose, 5, 10, 15, 30 min, and 1, 4, 8, 12, 24, 48, 72 and 168 h post-dosing. For safety assessment, blood and urine samples were taken at screening, 48 and 24 h pre-dosing, at 8, 24, 48 and 72 h, and 7 days post-dosing. Evaluations included a complete blood count, biochemistry panel, iron metabolism panel (serum iron, percent TSAT, transferrin, and ferritin) and clotting function tests. Resting 12-lead electrocardiograms were obtained at screening, and 48 h pre-dosing, at 15 min, 1, 4, 8, and 24 h, and 7 days post-dosing.

ing. Vital signs were obtained at the same time points and additionally at 1, 5, 10, 30, 45 min, and 2, 4, 48 and 72 h.

Hemodialysis Patients. The study in hemodialysis patients was an open-label, dose escalation study to assess the safety and pharmacokinetics of two doses of ferumoxytol in non-anemic chronic hemodialysis patients ($n = 20$) receiving EPO therapy for anemia. Subjects were at least 18 years old, currently undergoing outpatient hemodialysis three times a week and receiving EPO therapy; hemoglobin ≥ 11 g/dl and/or hematocrit $\geq 33\%$ was required. Patients had not received oral or parenteral iron therapy for at least 2 weeks prior to entry; nor did they receive it for the duration of the study.

Two doses of ferumoxytol, containing 125 and 250 mg of elemental iron, were evaluated, given as an intravenous injection over 5 min, with the safety of the first dose established before proceeding to the higher dose. The first 10 patients were given a single dose of 4.2 ml of ferumoxytol (125 mg iron, 25 mg iron/min) and the second group of 10 patients was given a single dose of 8.4 ml of ferumoxytol (250 mg iron, 50 mg iron/min). Ferumoxytol was administered within 30 min of starting dialysis in all patients. No test dose was given.

Laboratory tests including complete blood count, electrolytes, comprehensive chemistry, hepatic function, iron metabolism panel and clotting function panel were drawn 1 week pre-dose, and at 48 h, 2 weeks, and 4 weeks following dosing. A panel consisting of complement levels (CH₅₀ and C3) and a complete blood count with differential was drawn immediately pre-dose and at 60 and 180 min post-dose to assess complement activation.

Blood clearance samples were drawn immediately pre-dose and post-dose at 5, 10, 15, 30, 60, 120, 180 min, 48 h, and 96 h. Patients were evaluated for evidence of adverse reactions during and after the drug administration and at 48 and 96 h following drug administration.

Drug Concentration Quantification

Because ferumoxytol has strong magnetic properties (T1 relaxivity of 38 mmol s⁻¹ and T2 relaxivity of 83 mmol s⁻¹) the intact, unmetabolized drug can be quantified by magnetic resonance spectrometer measurements [25], independent of serum iron, which has very low magnetic properties. The T1 spin-spin magnetic resonance relaxation time of the serum samples, standards, and controls were measured at 39.5°C using a Bruker PC-120 nuclear magnetic resonance spectrometer with an applied field of 0.47 T (20 MHz) using an inversion-recovery pulse sequence. The limit of detection of the assay was 3 µg/ml of ferumoxytol iron.

Safety Evaluation

Vital signs (blood pressure, heart rate, respiration rate and temperature) were evaluated at multiple time points before and after the drug administration in both studies. In addition, for the hemodialysis patients, the change in blood pressure recorded for each patient on the day of ferumoxytol injection was compared to the intradialytic blood pressure measurements recorded for that patient during the dialysis session 1 week prior to drug administration. Hypotension was considered an adverse event if the decrease in blood pressure was clinically significant based on the opinion of the investigator, regardless of the degree or absolute value.

Adverse events were identified by direct questioning and observation during drug administration, the post-administration observation period, and at later time points up to a week for the normal

subjects and a month for the hemodialysis patients. A serious adverse event was defined as one which constitutes a definite hazard and/or results in a handicap to the patient, including but not limited to: death; a life-threatening adverse drug experience; inpatient hospitalization or prolongation of existing hospitalization; a persistent or significant disability/incapacity; chest pain, dyspnea or other evidence of anaphylaxis.

Statistical Analysis

Data are presented as mean $\pm$ SD. Two-way analysis of variance (ANOVA) with between-groups repeated measures was used to evaluate differences in laboratory measures and pharmacokinetic parameters between groups. The pharmacokinetic parameters were calculated assuming a first order process in a single compartment model, with a first order elimination rate constant (K_{el}), area under the curve (AUC) calculated by trapezoidal rule (t_{0h} – t_{48h}), maximum concentration (C_{max}) estimated as the instantaneous concentration at $t = 0$ and clearance calculated as volume of distribution (V_d) $\times$ K_{el} . Parameters were calculated using PK Solutions 2.0 (Summit Research Services, Pharmacokinetics and Metabolism Software, Montrose, Colo., USA).

Other Analyses

Although these were not efficacy trials, and neither volunteers nor hemodialysis patients were anemic, changes in hematologic parameters including serum iron, serum ferritin, TSAT, hemoglobin, hematocrit, and reticulocyte count were evaluated for evidence of iron utilization.

Results

Demographics

Normal Subjects. The study population was comprised of comparable numbers of men and women, and was predominantly black (63%), with an average age of 33.5 $\pm$ 7.7 years (table 1). Seven of these patients had ferritin levels <12 ng/ml; none had hemoglobin values <10.8 g/dl. The four treatment groups did not differ significantly with respect to gender, race, age, height, and weight.

Hemodialysis Patients. The study population included 20 patients (age 34–77 years; 10 women, 10 men, 70% black, see table 1) currently undergoing hemodialysis three times weekly at an outpatient dialysis unit. The first 10 patients recruited were assigned to the low-dose group (125 mg iron). After this dose was found to be well tolerated in this patient population, 10 additional patients were recruited to the high-dose group (250 mg iron).

Pharmacokinetics

Normal Subjects. The pharmacokinetic summary data including V_d , K_{el} , AUC, C_{max} , half-life, and clearance for the dose escalation arm of the trial are shown in table 2. The AUC and C_{max} increased significantly with dose, as

Table 1. Subject characteristics

	Normal subjects	Hemodialysis patients
Number	41	20
Age	33.5 ± 7.7 (20–58)	60.7 ± 11.8 (34–77)
Gender, m/f	22/19	10/10
Race, B/W/H/O	26/8/7/0	14/5/0/1
Weight, kg	79.6 ± 15.1 (46.5–115.0)	84.2 ± 14.5 (55–107)
Height, cm	171.2 ± 9.8 (153.0–189.0)	168.4 ± 7.8 (157–183)
Hemoglobin, g/dl	13.0 ± 1.2 (10.8–16.0)	12.4 ± 1.0 (10.7–12.9)
Transferrin saturation, %	21.4 ± 7.6 (7.4–40.2)	29.6 ± 9.7 (14–47)
Serum iron, µg/ml	69.0 ± 20.7 (20–107)	58.8 ± 19.8 (32–99)
Ferritin, ng/ml	41.0 ± 32.5 (4–109)	259 ± 154 (86–677)

Table 2. Pharmacokinetic parameters at increasing dose, constant injection rate^a

Parameter	Dose group		
	1 mg Fe/kg	2 mg Fe/kg	4 mg Fe/kg
Rate, ml/min	2	2	2
Rate, mg iron/min	60	60	60
Number	8	8	8
Mean dose, mg	85 ± 17	152 ± 30	321 ± 71
K _{el} , h ^{-1b}	0.076 ± 0.010	0.069 ± 0.010	0.049 ± 0.007
Half-life, h ^b	9.3 ± 1.1	10.2 ± 1.5	14.5 ± 2.4
C _{max} , µg iron/ml ^b	26.3 ± 7.0	62.0 ± 11.6	126.0 ± 32.6
AUC, µg iron·h/ml ^b	396 ± 122	997 ± 320	2,771 ± 624
V _d , l	3.1 ± 0.8	2.4 ± 0.7	2.4 ± 0.6 ^d
V _d , ml/kg	36.3 ± 10.0	31.1 ± 7.4	31.6 ± 8.5
Cl, ml/h ^b	235.8 ± 91.8	162.9 ± 52.4	115.7 ± 15.1
Cl, ml/(h·kg) ^c	2.82 ± 1.21	2.17 ± 0.63	1.51 ± 0.33

^a Values are mean ± SD. K_{el} = First order rate constant; AUC = area under the curve; C_{max} = maximum plasma concentration of intact drug; half-life = elimination half-life; Cl = clearance; V_d = volume of distribution.

^b Significant by dose, *p* < 0.001.

^c Significant by dose, *p* < 0.02.

^d Significant by gender, *p* < 0.01 (see text).

did the half-life, while clearance decreased (*p* < 0.001). The elimination half-life increased from 9.3 to 14.5 h from 1 to 4 mg of iron/kg. The high-dose group showed a statistically significant difference in V_d by gender, also observed in all groups but not at statistically significant levels. This difference is attributable to a lower body weight in the females, since total blood volume is proportional to body weight. The V_d per kilogram was not significantly different.

The pharmacokinetic results from the injection rate escalation are shown in table 3. There were no significant differences in the pharmacokinetic parameters with increasing administration rates. Including all subjects who

received the dose of 4 mg iron/kg, a mean dose of 316 mg of iron in this study, the mean half-life is 14.7 h. The highest individual dose administered in this study was 420 mg of iron.

The concentration of ferumoxytol with time is shown in figure 1. The concentrations are dose-dependent (*p* < 0.001), and 90% of the highest dose is cleared from the blood by 48 h, approximately 3 half-lives.

Hemodialysis Patients. The pharmacokinetic summary data including V_d, K_{el}, AUC, C_{max}, half-life, and clearance are presented in table 4. The plasma pharmacokinetic parameters were similar to those observed in normal subjects. The half-life was different by dose (*p* < 0.01) as

Fig. 1. Serum concentration of ferumoxytol with time in normal subjects by dose. * = Placebo; ▲ = 1 mg/kg; ■ = 2 mg/kg; ● = 4 mg/kg. Concentration of ferumoxytol was dose-dependent ($p < 0.001$). The values are for intact drug and do not include serum iron.

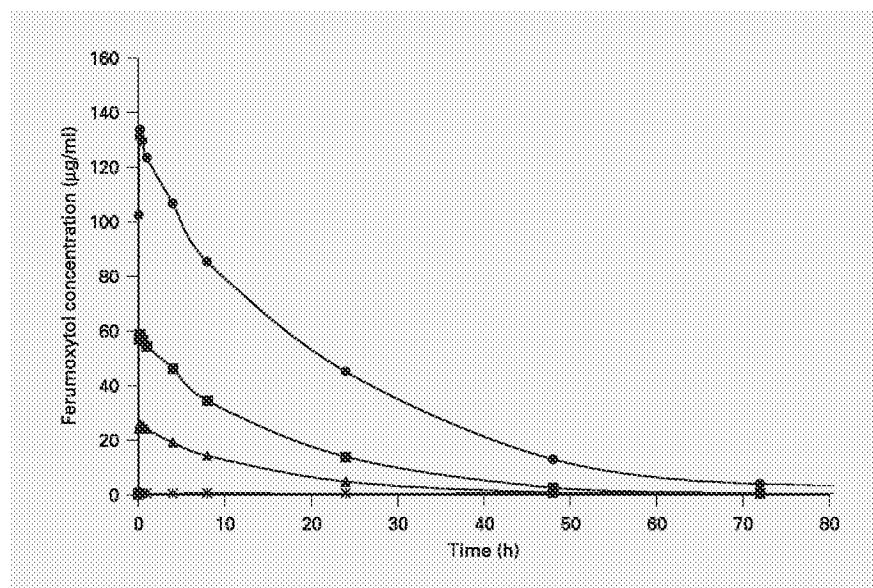


Table 3. Pharmacokinetic parameters at increasing injection rate, constant dose^a

Parameter	Administration rate group				
Dose, mg iron/kg	4 ^b	4	4	4	4
Rate, ml/min	2	3	6	60	—
Rate, mg iron/min	60	90	180	1,800	—
Number	8	3	3	3	17
Mean dose, mg Fe	321 ± 71	323 ± 69	337 ± 45	273 ± 81	316 ± 66
K_{el} , h ⁻¹	0.049 ± 0.007	0.049 ± 0.008	0.051 ± 0.005	0.043 ± 0.006	0.048 ± 0.007
Half-life, h	14.5 ± 2.4	14.5 ± 2.3	13.6 ± 1.4	16.2 ± 2.5	14.7 ± 2.2
C_{max} , µg iron/ml	126.0 ± 32.6	124.7 ± 31.1	141.4 ± 50.0	134.5 ± 30.3	130.0 ± 32.5
AUC, µg iron·h/ml	2,771 ± 624	2,857 ± 336	2,914 ± 984	3,343 ± 963	2,912 ± 683
V_d , l	2.4 ± 0.6	2.4 ± 0.9	2.4 ± 0.5	2.0 ± 0.4	2.3 ± 0.6
V_d , ml/kg	31.6 ± 8.5	29.6 ± 6.3	29.0 ± 9.3	29.1 ± 5.7	30.4 ± 7.3
Cl, ml/h	115.7 ± 15.1	116.1 ± 40.0	121.2 ± 27.1	83.2 ± 9.7	111.0 ± 24.1
Cl, ml/(h·kg)	1.51 ± 0.33	1.41 ± 0.18	1.47 ± 0.43	1.28 ± 0.43	1.44 ± 0.33

^a Values are mean ± SD. K_{el} = First order rate constant; AUC = area under the curve; C_{max} = maximum plasma concentration of intact drug; half-life = elimination half-life; Cl = clearance; V_d = volume of distribution.

^b Data in this group repeated from table 2, column 3, for comparison.

were C_{max} , AUC, and clearance ($p < 0.001$). The half-life was 10.7 h in the low-dose group and 16.2 h in the high-dose group, similar to that observed in normal subjects.

The concentration of ferumoxytol remained stable during the dialysis procedure, indicating that ferumoxytol is not removed with hemodialysis (fig. 2).

Laboratory Values

Normal Subjects. As expected, serum iron and TSAT increased in a dose-dependent manner, peaking at 1-day post-dose, whereas serum ferritin peaked at day 3; all returned toward baseline at 7 days. The changes in all were statistically significant ($p < 0.001$). The serum iron, TSAT, and serum ferritin are shown in figure 3.

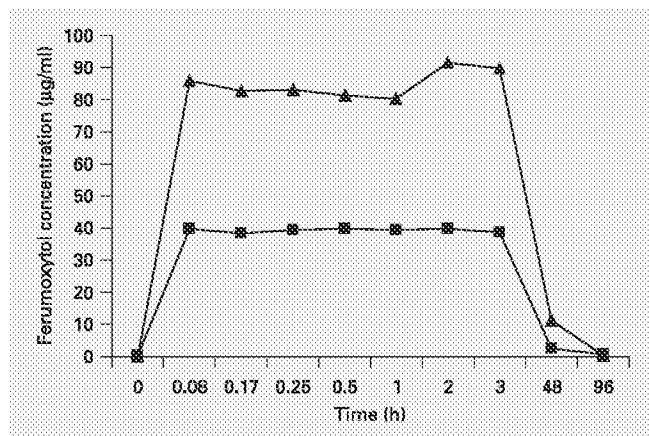


Fig. 2. Serum concentration of ferumoxytol with time in hemodialysis patients. ■ = 125 mg; ▲ = 250 mg. Concentration of ferumoxytol was dose-dependent ($p < 0.001$) and was constant during the hemodialysis session. The values are for intact drug and do not include serum iron.

The TSAT rose significantly ($p < 0.001$) at day 1, but did not exceed 100%. The rise in ferritin was delayed, relative to the TSAT and demonstrated that the drug was being metabolized and added to the normal body iron stores in these non-anemic subjects.

No changes were seen in hemoglobin or hematocrit, as expected. Reticulocytes increased from 1.1 to 1.3% by day 7 in the highest dose group but the changes did not reach statistical significance.

Hemodialysis Patients. No decrease in white blood cell count, platelet count, C3 or CH_{50} was observed following administration of ferumoxytol showing a lack of complement system activation (data not shown). No significant changes in the chemistry values were observed except for 1 patient who exhibited transient elevations of liver function tests.

There was a significant increase in serum iron ($p < 0.001$), TSAT ($p < 0.001$), and ferritin ($p < 0.05$ for the 250-mg dose group only) at 48 h in both groups which returned toward baseline levels by the end of the study, as shown in figure 4. There was no significant change in hemoglobin or hematocrit from baseline in either group over time.

Safety

Normal Subjects. No consistent, clinically relevant, or unexpected changes were observed. Only one treatment-associated adverse event (metallic taste) was considered possibly related to ferumoxytol administration.

Table 4. Pharmacokinetic parameters in hemodialysis patients^a

Parameter	Dose group	
	125 mg iron	250 mg iron
Rate, ml/min	24	48
Number	10	10
Mean dose, mg Fe/kg	1.5 ± 0.2	3.2 ± 0.6
K_{el} , h^{-1b}	0.073 ± 0.029	0.045 ± 0.008
Half-life, h^b	10.7 ± 3.6	16.2 ± 3.3
C_{max} , µg iron/ml ^c	44.3 ± 15.2	88.7 ± 26.1
AUC, µg iron·h/ml ^c	1,187 ± 484	2,831 ± 765
V_d , l	1.8 ± 0.8	2.3 ± 1.0
V_d , ml/kg	20.6 ± 7.5	27.2 ± 9.0
Cl, ml/h	121.4 ± 47.0	95.4 ± 30.2
Cl, ml/(h·kg)	1.43 ± 0.57	1.16 ± 0.24

^a Values are mean ± SD. K_{el} = First order rate constant; AUC = area under the curve; C_{max} = maximum plasma concentration of intact drug; half-life = elimination half-life; Cl = clearance; V_d = volume of distribution.

^b $p < 0.01$.

^c $p < 0.001$.

Hemodialysis Patients. There were no acute changes in blood pressure after infusion of ferumoxytol. There was, however, a statistically significant decrease in intradialytic systolic and diastolic blood pressures in both groups on the day of iron injection during the course of dialysis, $p < 0.05$. To confirm that the change in systolic and diastolic blood pressure over time was related to dialysis and ultrafiltration and not to administration of ferumoxytol, the change in systolic and diastolic blood pressure in this cohort during their dialysis treatments exactly 1 week prior to ferumoxytol administration was also evaluated. The results are shown in figure 5. The patients in both groups also experienced a significant decline in intradialytic blood pressure 1 week prior to iron administration. The rate of decline for systolic and diastolic blood pressure during dialysis on the day of injection was not significantly different from the rate of decline 1 week prior to injection.

There were no serious adverse events recorded in the hemodialysis patients, no episodes of hypersensitivity or anaphylaxis, and no episodes of hypotension caused by administration of the study drug. Adverse events considered remotely related to ferumoxytol administration included nausea and vomiting in 1 patient who reported nausea in the hours prior to starting dialysis and prior to receiving the study drug, and transient elevation of liver function tests in 1 patient.

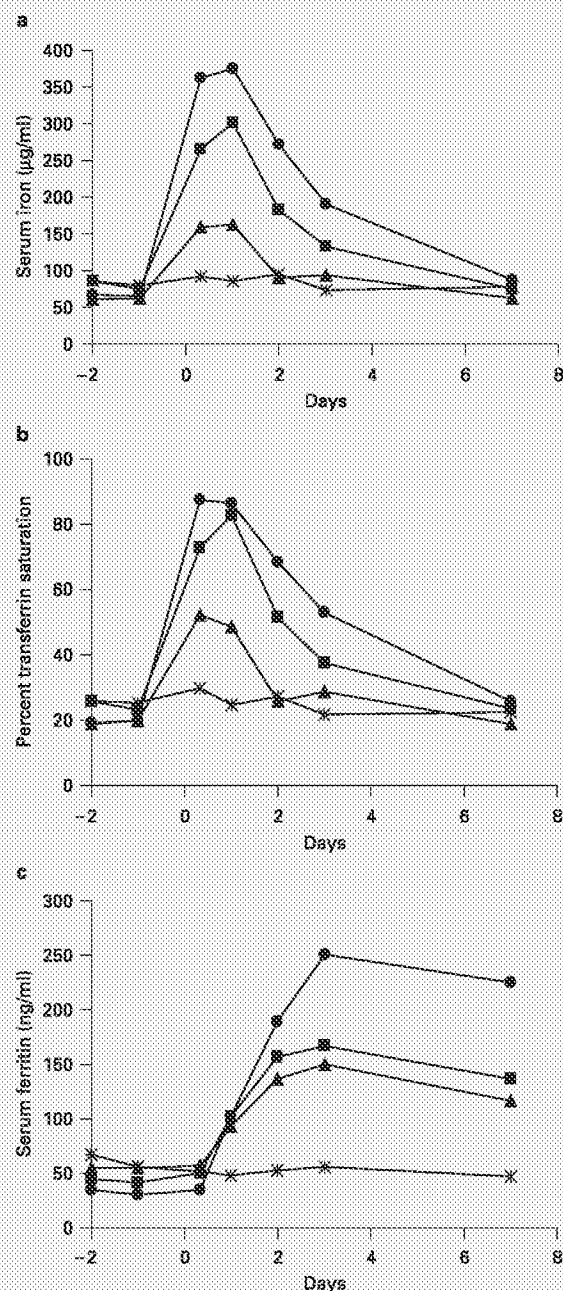


Fig. 3. Effect of ferumoxytol on serum iron (a), TSAT (b), and serum ferritin (c) in normal subjects. * = Placebo; ▲ = 1 mg/kg; ■ = 2 mg/kg; ● = 4 mg/kg. The changes were significant ($p < 0.001$) for all doses.

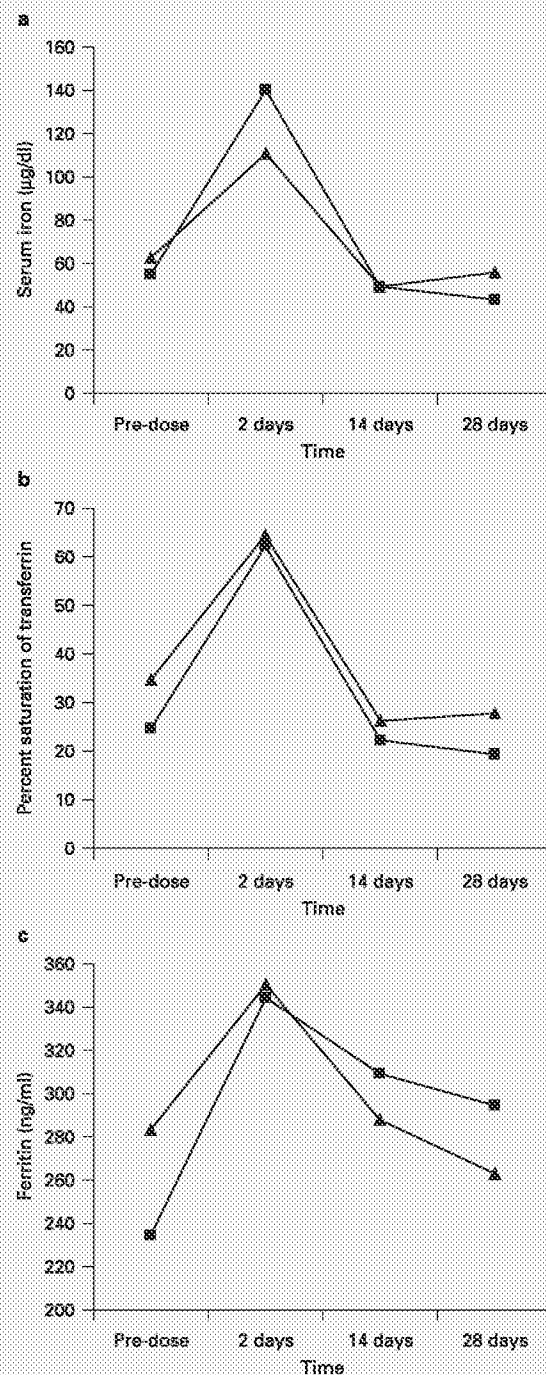
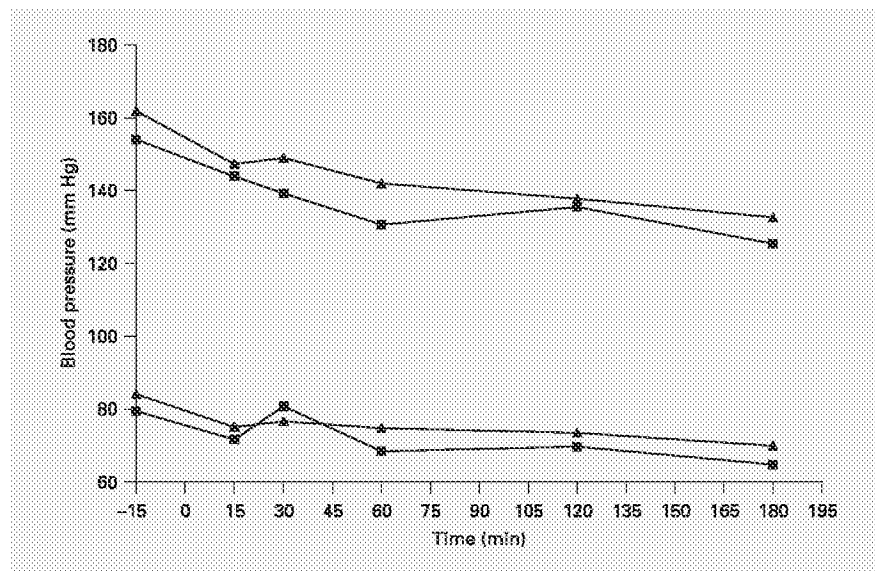


Fig. 4. Effect of ferumoxytol on serum iron (a), TSAT (b), and serum ferritin (c) in hemodialysis patients. ▲ = 125 mg; ■ = 250 mg. There was an acute increase in serum iron ($p < 0.001$), TSAT ($p < 0.001$), and ferritin ($p < 0.05$ for the 250-mg dose only) at 48 h after infusion of either dose of ferumoxytol which returned to or below baseline by the end of the observation period.

Fig. 5. Systolic and diastolic blood pressures in patients receiving ferumoxytol at both low and high doses decreased during dialysis ($p < 0.05$) but there were no acute decrements in blood pressure and the decrease during dialysis was comparable to that observed in a routine dialysis session the previous week. ▲ = Drug administration session; ■ = pre-administration session.



Discussion

In this study, we demonstrated that the new intravenous iron drug ferumoxytol can safely be administered at rates as high as 1,800 mg of iron/min. The highest dose administered in these trials was 420 mg of iron to a normal subject; in the high-dose group of normal volunteers, the mean dose was 316 mg. A total of 27 patients and subjects received a dose of at least 250 mg iron. The pharmacokinetic parameters were equivalent in normal subjects and in non-anemic hemodialysis patients. The drug was not removed by dialysis.

Hemoglobin and hematocrit did not change after the administration of single doses of ferumoxytol in this study. Because these were not efficacy trials, we enrolled normal subjects and patients who were at hemoglobin target by K/DOQI guidelines [2]. We did not expect an increase in hemoglobin with the regimen used in this study. The observed reticulocytosis in the high-dose normal group suggests that an increase in erythropoiesis did occur because of iron administration. In addition, a significant increase in serum iron, TSAT, and serum ferritin in all groups was observed after administration of one dose of ferumoxytol, confirming the bioavailability of the iron in ferumoxytol.

Kidney disease is a substantial public health problem. The prevalence of decreased glomerular filtration rate in patients not on dialysis is estimated at approximately 20 million with another 20 million at risk for CKD [26].

Iron deficiency is present in 43–90% of hemodialysis patients receiving EPO [5, 9, 14]. Many patients with decreased glomerular filtration rate and those at risk for CKD will also require iron therapy to maintain an acceptable hematocrit.

A safe, effective, and cost-effective agent to replete iron is imperative in this population given the large number of individuals who will ultimately require it. Currently available parenteral iron drugs, while effective at repleting iron stores, have risks associated with their use. Until recently, iron dextran was the only intravenous iron preparation available in the USA. Reactions have been reported in 4.7–43% of patients administered intravenous iron dextran [23, 27, 28]. Most of these reactions are mild, with 38% of patients given a total dose infusion [28] exhibiting delayed reactions of arthralgia, myalgia, and fever, but some patients experience anaphylactoid-type reactions (defined as any dyspnea, wheezing, chest pain, hypotension, urticaria, or angioedema) which can be serious and life-threatening.

The mechanism of allergic and anaphylactic reactions due to iron dextran is still poorly understood but is thought to be due to the dextran portion of the molecule which is known to be immunogenic [29]. Therefore, iron compounds such as sodium ferric gluconate complex in sucrose (Ferrlecit®) or iron sucrose (Venofer®) which do not contain dextran might be preferable. However, serious reactions also occur with these iron preparations.

Faich and Strobos [30] found the rate of allergic and anaphylactic reactions for ferric gluconate was 3.3 per million doses compared to 8.7 per million doses for iron dextran. Pascual et al. [31] reported that 3 (5%) of 60 hemodialysis patients treated with ferric gluconate (125 mg) as a 'slow injection' had reactions severe enough to stop further treatment. Nissenson et al. [32] reported that 3 of 88 (3.4%) patients given ferric gluconate had reactions severe enough to prevent further dosing. According to the drug package insert, 12 patients (0.8%) from a total of 1,498 sodium ferric gluconate-treated patients experienced serious reactions which precluded further therapy with sodium ferric gluconate [19]. True allergic and/or anaphylactoid reactions with ferric gluconate, however, are exceedingly rare [33].

Iron sucrose (Venofer[®]) has also been approved for use in the USA recently. In clinical trials of iron sucrose prior to FDA approval [24, 34], no patients were discontinued due to adverse drug reactions and no anaphylactoid reactions were reported. In addition, only 27 anaphylactoid reactions were reported among an estimated 450,000 exposed patients between 1992 and 1999 from the spontaneous reporting system [20]. However, adverse reactions including hypotension, back pain, vomiting, or vertigo have been reported in 0.9% of patients receiving 100-mg doses of iron sucrose and in 5.9% of patients receiving 200-mg doses (non-approved dose) of iron sucrose [21]. In a study including even higher doses of iron sucrose, 36% of the 22 patients given 500 mg of iron sucrose diluted in 250 ml of saline as a 2-hour infusion had hypotension, 2 requiring hospitalization [35]. These reactions, especially hypotension, may be due to rate of administration and total dose administered [20, 21]. These rate- and dose-related adverse reactions cannot be overlooked despite the excellent safety record of iron sucrose and low incidence of serious reactions at the labeled dose of 100 mg of iron [36].

The acute side effects noted above, related to excess intravenous iron administered too rapidly, are quite similar to the effects of free/labile iron reactions [37]. Indeed, increased oxidant stress, heightened risk of infection and increased cardiovascular risk have all also been postulated to result from free/labile iron in parenteral preparations of iron [38, 39]. The cytotoxic effects of parenteral iron preparations have been shown to parallel cellular iron uptake [38], which in turn may relate to structural differences of various iron preparations [40]. Of note, ferumoxytol contains much less ultrafiltrable iron than ferric gluconate or iron sucrose [41], and generates significantly less catalytic free iron than iron sucrose and

iron gluconate, with sixfold lower level than the latter two preparations after parenteral administration [42]. Further, since the acute adverse effects of parenteral iron carbohydrates seem related to labile/free-iron-like reactions, which in turn are inversely related to the molecular weight of the iron preparation [37], ferumoxytol (750 kDa) might be anticipated to be less likely to cause adverse effects than other parenteral iron carbohydrates (73–265 kDa), iron sucrose (43 kDa) or ferric gluconate (38 kDa) [40].

Recommendations for administration of Ferrlecit are 125 mg elemental iron diluted in 100 ml normal saline given intravenously over 1 h. It may be given as slow intravenous injection over 10 min (≤ 12.5 mg/min) and most patients require a minimum cumulative dose of 1.0 g administered over eight hemodialysis sessions [43, p. 3320]. Recommendations for administration of Venofer are 100 mg of elemental iron intravenously over 5 min by direct infusion (20 mg/min) or after dilution in 100 ml normal saline with slow infusion over at least 15 min. Most patients will require a minimum cumulative dose of 1,000 mg iron given over 10 dialysis sessions [43, p. 570]. Thus both Ferrlecit and Venofer require significant resources in terms of preparation, administration, oversight, supplies, and nursing time, regardless of whether infusion over 5–10 min or slower intravenous infusions are employed to achieve the required cumulative dose. On the other hand, ferumoxytol can be given by direct intravenous push over 1–5 min in doses up to 1,800 mg elemental iron/min, as shown here, without deleterious side effects, with total doses of iron up to 420 mg/injection.

The ability to give a medication such as intravenous iron as a rapid injection instead of a slow infusion has been evaluated and shown to be associated with significant potential cost savings. One study showed that administration of intravenous iron as a bolus compared to an infusion could have led to an annual cost savings of USD 223,529 (USD 19.22 per dose) in the representative dialysis program studied [44]. Additionally, the ability to administer a repletion course of intravenous iron in fewer doses has significant cost and convenience benefits in hemodialysis patients, and especially in patients not yet on dialysis or those on peritoneal dialysis. Dahdah et al. [45] reported 22 patients not yet on dialysis receiving iron dextran as either 500 mg administered twice or 200 mg administered five times. The group that received two divided doses showed a 35% cost savings (USD 965 vs. 1,490), in addition to three trips less to the clinic, fewer hours lost from work and personal time, and less travel

expense. Despite apparent safety benefits over iron dextran, iron gluconate and iron sucrose preparations are limited by rate of administration and total dose-related reactions that may limit or preclude use of such convenient and cost-saving dosing regimens [21, 44, 45].

In our study, we have shown that ferumoxytol is safe in normal subjects and in hemodialysis patients, and that it is safe when given as a bolus. We observed no allergic or hypotensive reactions. The ability to administer iron safely as a bolus in fewer doses as opposed to multiple infusions as described above will have considerable cost savings by eliminating the need for intravenous tubing, additional bags of saline, and nursing time [46]. This would free up nurses for other activities, as well. While the individual time savings per patient may be modest, the cumulative aggregate savings could be substantial [46]. These are attractive features for intravenous iron replacement in hemodialysis patients. Further study of

ferumoxytol in the broader CKD population is warranted. We would anticipate that the use of ferumoxytol as iron replacement in the CKD population should lead to significant cost savings, improved patient convenience, reduce hours lost from work and personal time, and less travel expense to an even greater degree than heretofore shown [44, 45].

Acknowledgements

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Cancer Research

Poster Session Abstracts

Abstract P4-02-05: A novel ^{64}Cu -liposomal PET agent (MM-DX-929) predicts response to liposomal chemotherapeutics in preclinical breast cancer models

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Article

Info & Metrics

Abstracts: Thirty-Fifth Annual CTRC-AACR San Antonio Breast Cancer Symposium-- Dec 4-8, 2012; San Antonio, TX

Abstract

Background: Liposomal anthracyclines (such as pegylated liposomal doxorubicin (PLD) or HER2-targeted liposomal doxorubicin (MM-302)) are being used and/or evaluated for the clinical management of breast cancer, but responses vary from patient to patient. It is hypothesized that variability in the deposition of liposomal therapeutics within tumors leads to differences in drug exposure, thereby directly influencing tumor response. We have developed MM-DX-929, a novel ^{64}Cu -liposomal PET imaging agent, as a clinically-implementable tool to investigate whether image-based quantification of liposome deposition in tumors can predict treatment response to liposomal chemotherapeutics, including PLD, MM-302 and/or liposomal irinotecan (MM-398).

Objectives: Our primary objective is to demonstrate that the extent of tumor uptake of MM-DX-929 is predictive of tumor response to MM-302 in preclinical breast cancer xenograft models. A secondary objective is to enable clinical translation of MM-DX-929 to enable incorporation into existing therapeutic trials.

Methods: Mice bearing BT474-M3 mammary and subcutaneous tumors were injected intravenously with MM-DX-929 prior to dosing with MM-302. PET/CT imaging was performed at 16h post MM-DX-929 injection, and tumor uptake was determined. Response to treatment was quantified as tumor volume changes measured over a 2-month period by MRI.

Results: Tumor deposition of MM-DX-929 administration correlated well with treatment response to MM-302 (Spearman correlation coefficient of -0.891 and a p-value of 0.0004). MM-DX-929 accumulation in tumors prior to the start of the MM-302 treatment successfully predicted improved tumor growth inhibition following MM-302 treatment.

Conclusion: These findings support trials of MM-DX-929 as a predictive imaging agent to select patients who are most likely to respond to liposomal therapies. The clinical development of MM-DX-929 for identification of breast cancer patients likely to respond to liposomal therapeutics is currently being pursued.

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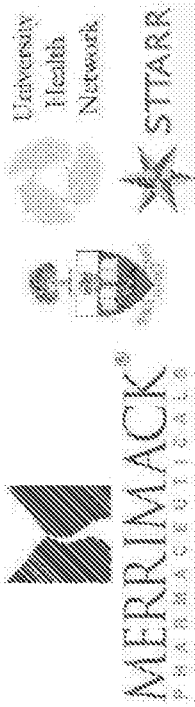
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Advertisement



A Novel ⁶⁴Cu-Liposome PET Agent (MM-DX-929) Predicts Response to Liposomal Chemotherapeutics in Preclinical Breast Cancer Models

Helen Lee¹, Jinzi Zheng², Daniel Gaddy¹, Dmitri Kirpotin¹, Michael Dunne², Daryl Drummond¹, Christine Allen^{2,3}, David Jaffray², Bart Hendriks¹, Thomas Wickham¹

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² STTARR Innovation Center, Radiation Medicine Program, Princess Margaret Hospital, University Health Network, Toronto, ON, Canada.

³ Leslie Dan Faculty of Pharmacy, University of Toronto, ON, Canada

ABSTRACT

Background: Liposomal anthracyclines (such as pegylated liposomal doxorubicin (PLD) or HER2-targeted liposomal doxorubicin (MM-302)) are being used and/or evaluated for the clinical management of breast cancer, but responses vary from patient to patient. It is hypothesized that variability in the deposition of liposomal therapeutics within tumors leads to differences in drug exposure, thereby directly influencing tumor response. We have developed MM-DX-929, a novel ⁶⁴Cu-liposomal PET imaging agent, as a clinically implementable tool to investigate whether image-based quantification of liposome deposition in tumors can predict treatment response to liposomal chemotherapeutics, including PLD, MM-302 and/or liposomal irinotecan (MM-398).

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Conclusion: These findings support trials of MM-DX-929 as a predictive imaging agent to select patients who are most likely to respond to liposomal therapies. The clinical development of MM-DX-929 for identification of breast cancer patients likely to respond to liposomal therapeutics is currently being pursued.

TUMOR DEPOSITION AS A PREDICTIVE MARKER

Tumor Deposition via EPR: Key to Delivering Liposomal Drugs

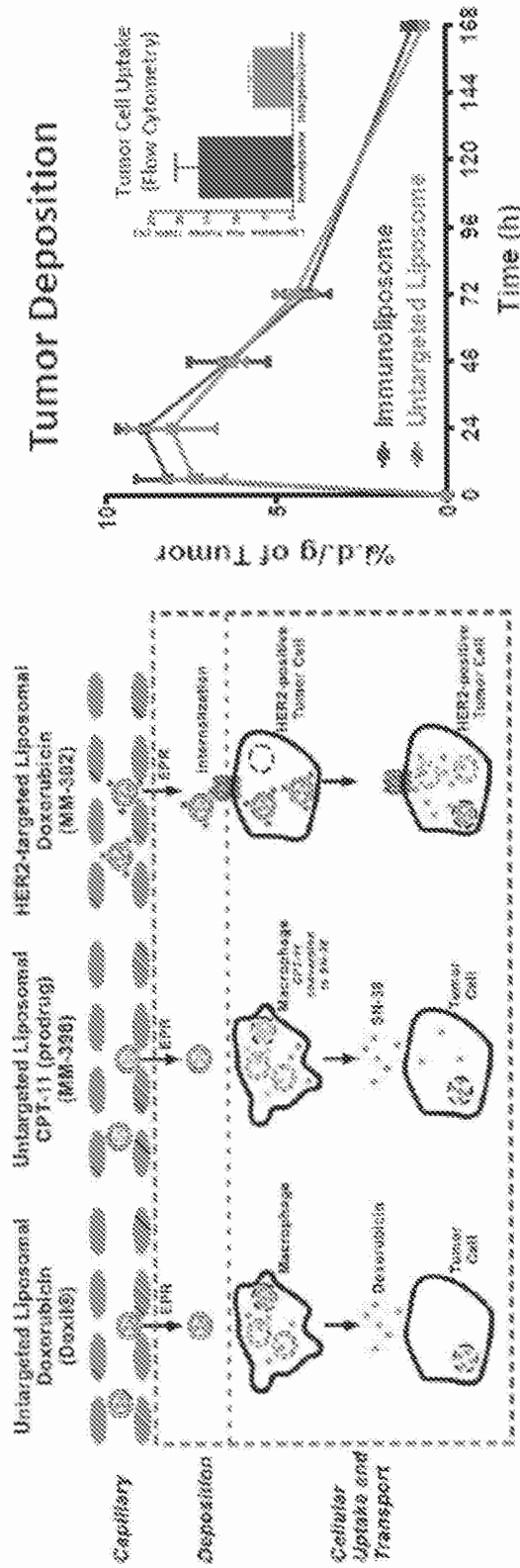
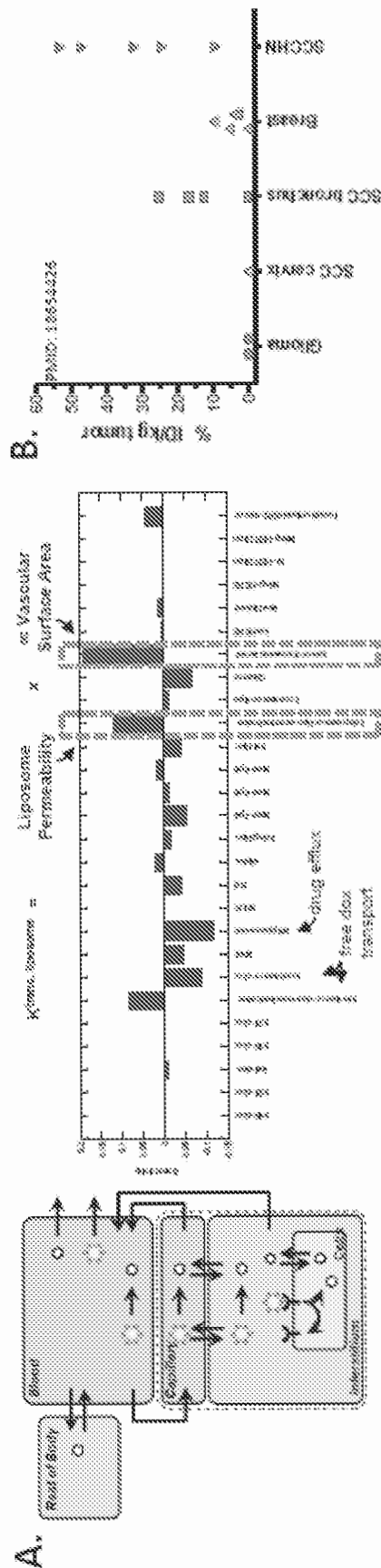


Figure 1. (Left) Schematic depicting key mechanism in which liposomal chemotherapeutics deliver their payloads to tumor cells, illustrating that tumor deposition is the rate-limiting step to effective liposomal drug delivery. This is supported by the preclinical data provided on the right panel, where tumor deposition of immunoliposomes and untargeted liposomes were similar. At the cellular level, tumor cell uptake of immunoliposomes in the tumor was significantly increased compared to untargeted liposomes (insert).

Tumor Deposition of Liposome is Highly Variable: Clinical Evidence



THE LIPOSOMES: MM-DX-929 AND MM-302

MM-DX-929: The Imaging Agent

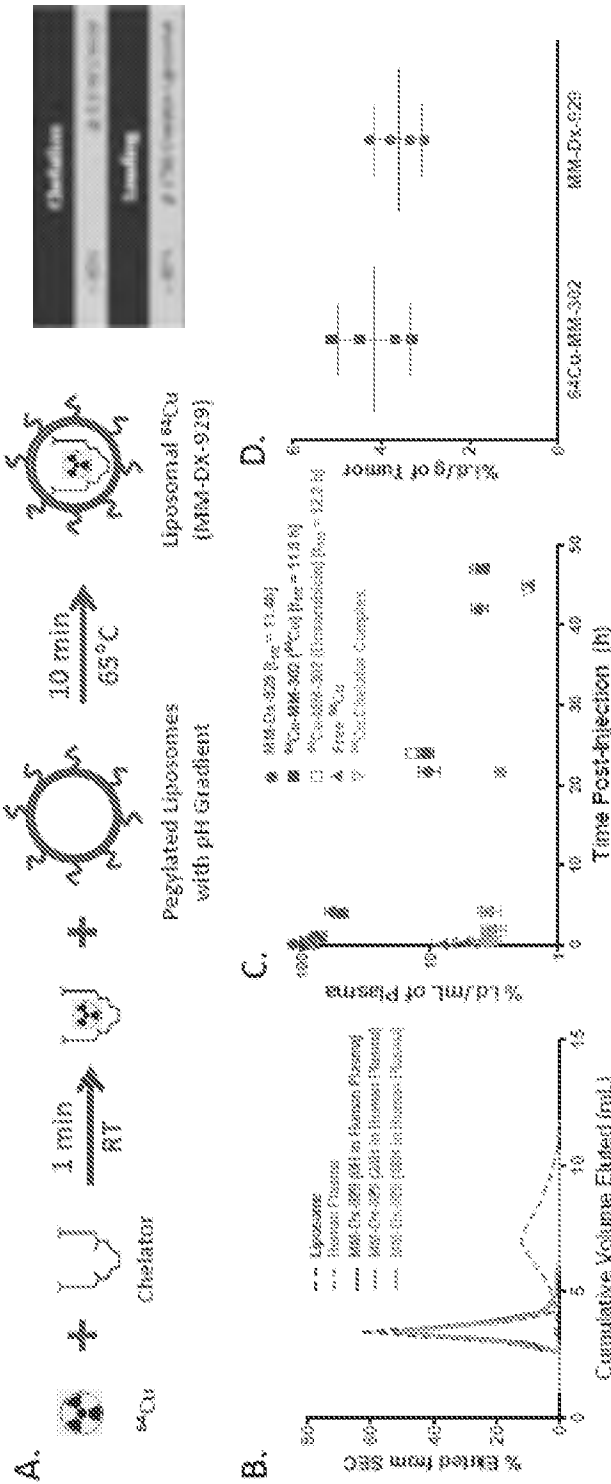
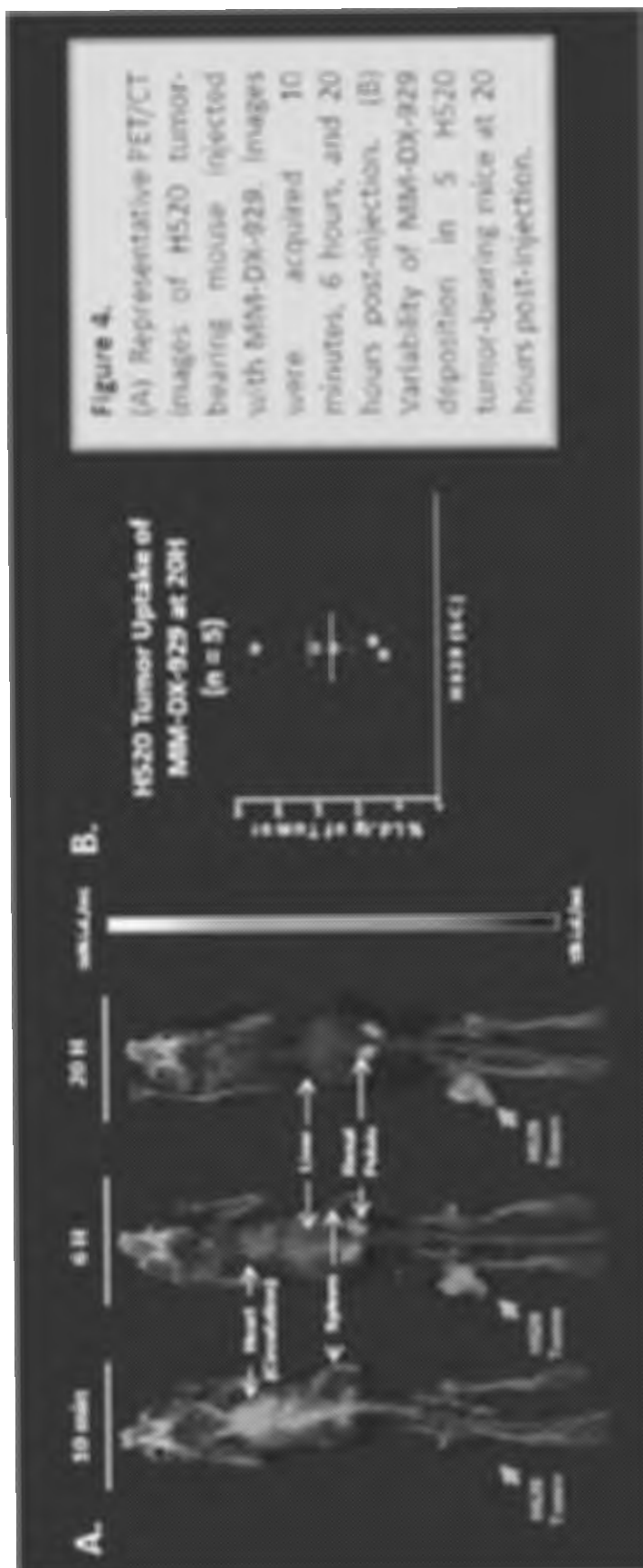
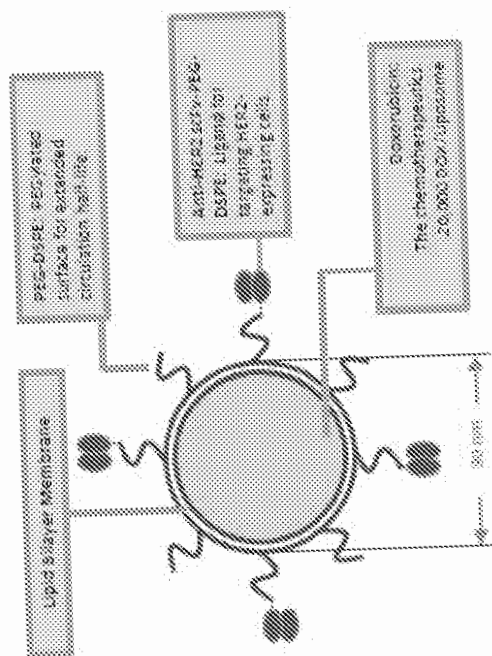
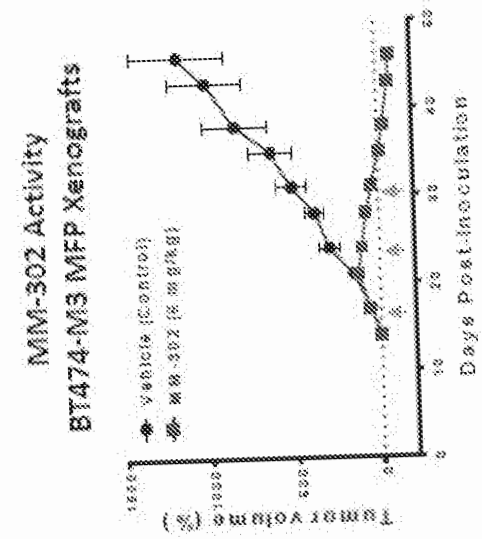
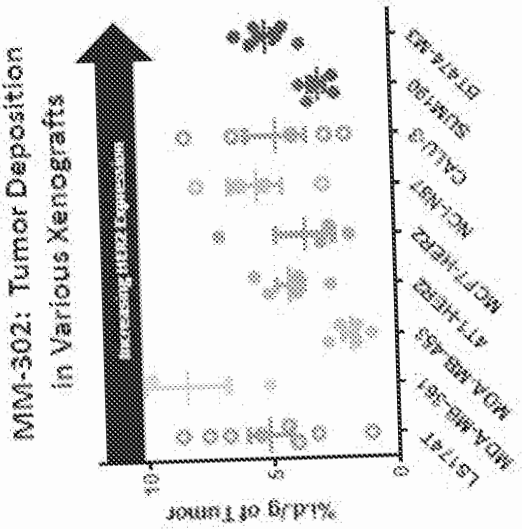


Figure 3. (A) Schematic describing the radiolabeling procedure of MM-DX-929. (B) *In vitro* stability of MM-DX-929. MM-DX-929 was incubated in human plasma at 37°C; at designated time points an aliquot of MM-DX-929 was assayed to separate liposomal-, protein-bound, or released- ^{64}Cu . Over a 48-hour incubation period, >96% of ^{64}Cu was still associated with the liposome. (C) Pharmacokinetics of MM-DX-929, ^{64}Cu -labeled-MM-302 (determined by ^{64}Cu and drug contents), free ^{64}Cu , and ^{64}Cu -chelator complex. (D) Tumor uptake of ^{64}Cu -MM-302 and MM-DX-929 in BT474-M3 (HER2 3+) mammary fat pad tumors.

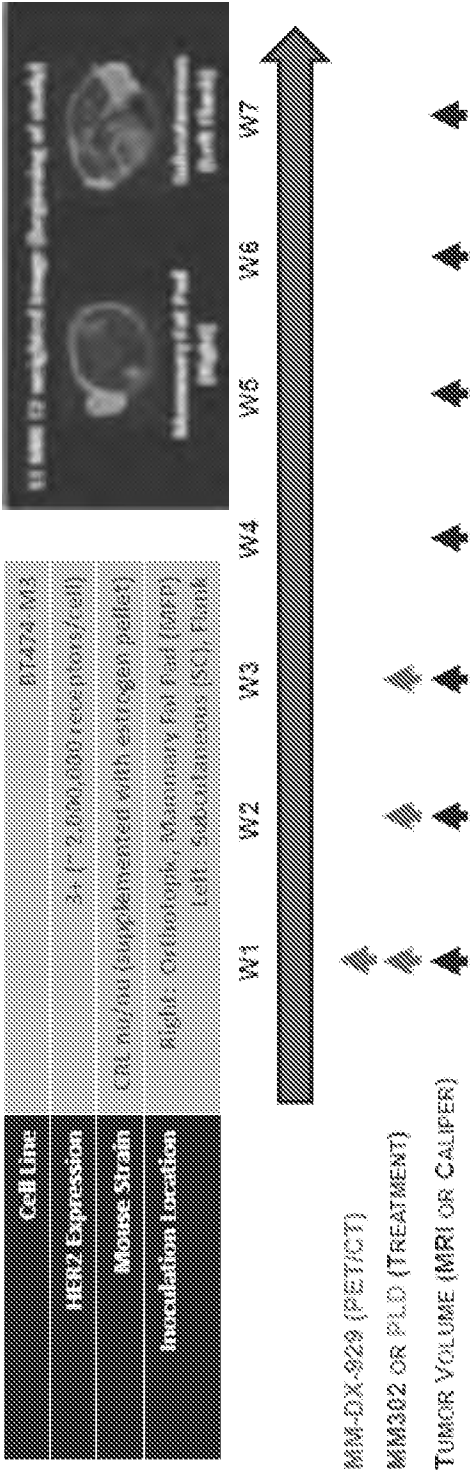


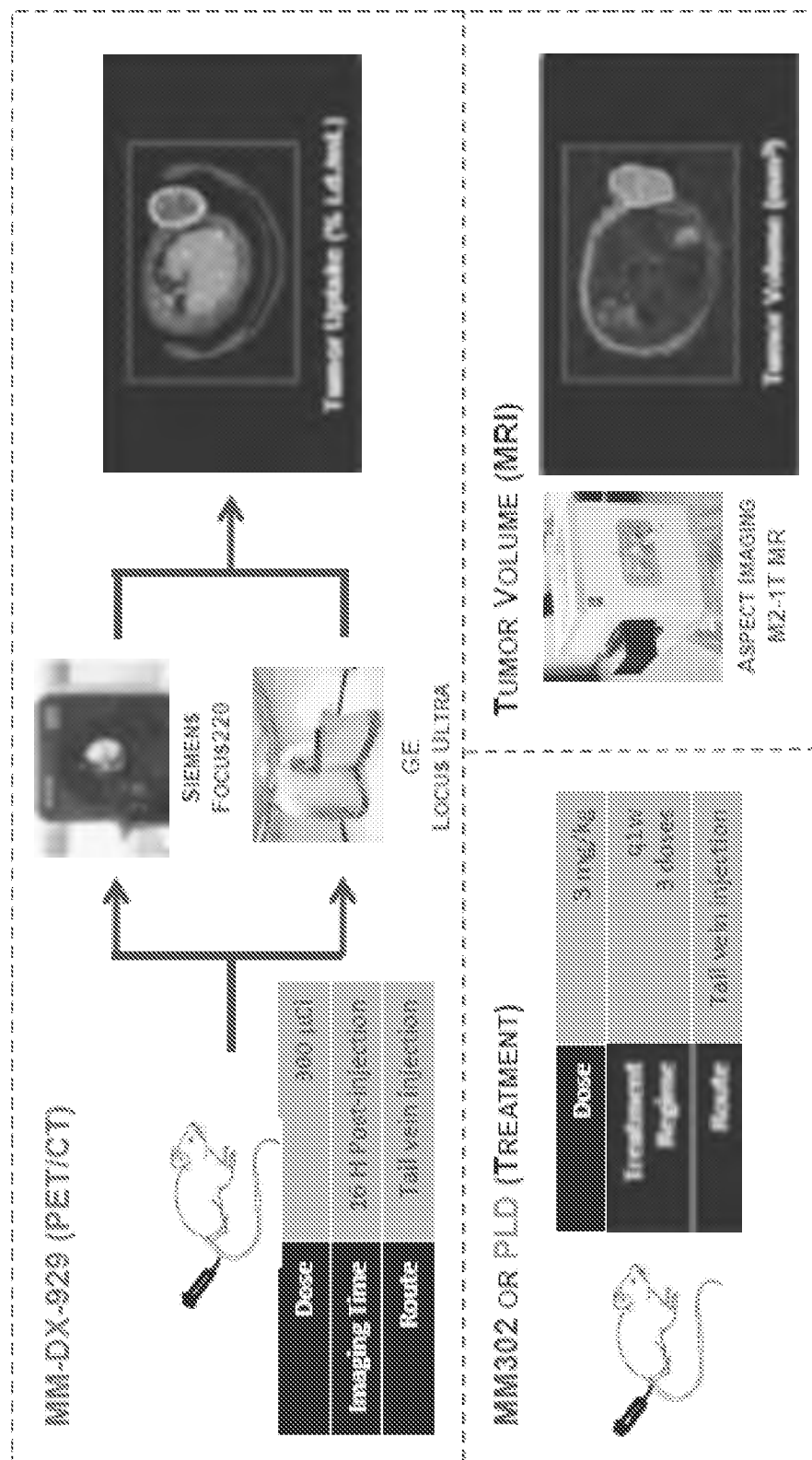
MM-302: The Drug



TUMOR DEPOSITION OF MM-DX-929 PREDICTS RESPONSE TO LIPOSOMAL CHEMOTHERAPEUTICS

Experimental Design





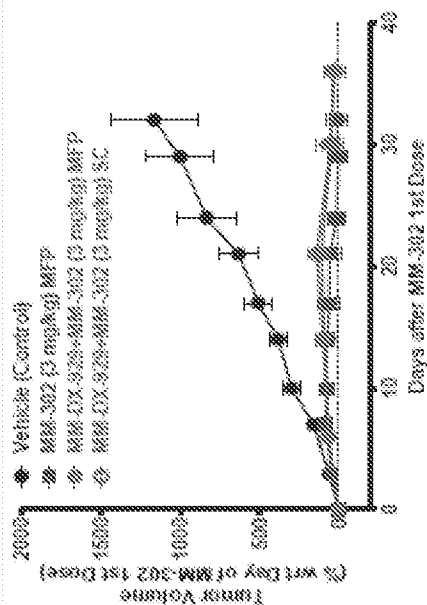
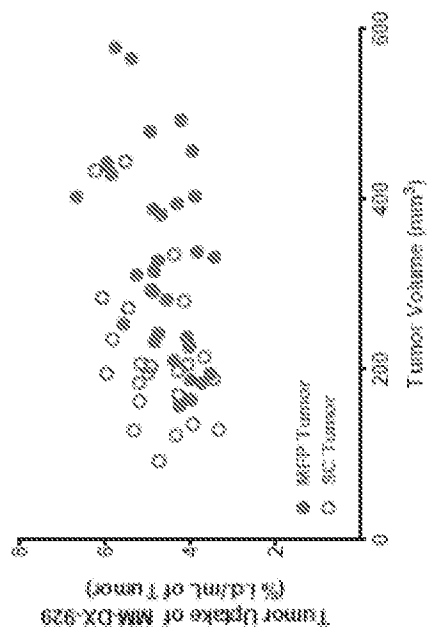


Figure 5. (Left) Anti-tumor activity of MM-302, MM-DX-929 + MM-302 in BT474-M3 tumor model. Administration of MM-DX-929 as a diagnostic agent does not interfere with the therapeutic activity of MM-302.



Tumor Deposition of MM-DX-929 Correlates with Liposomal Chemotherapeutics Activity

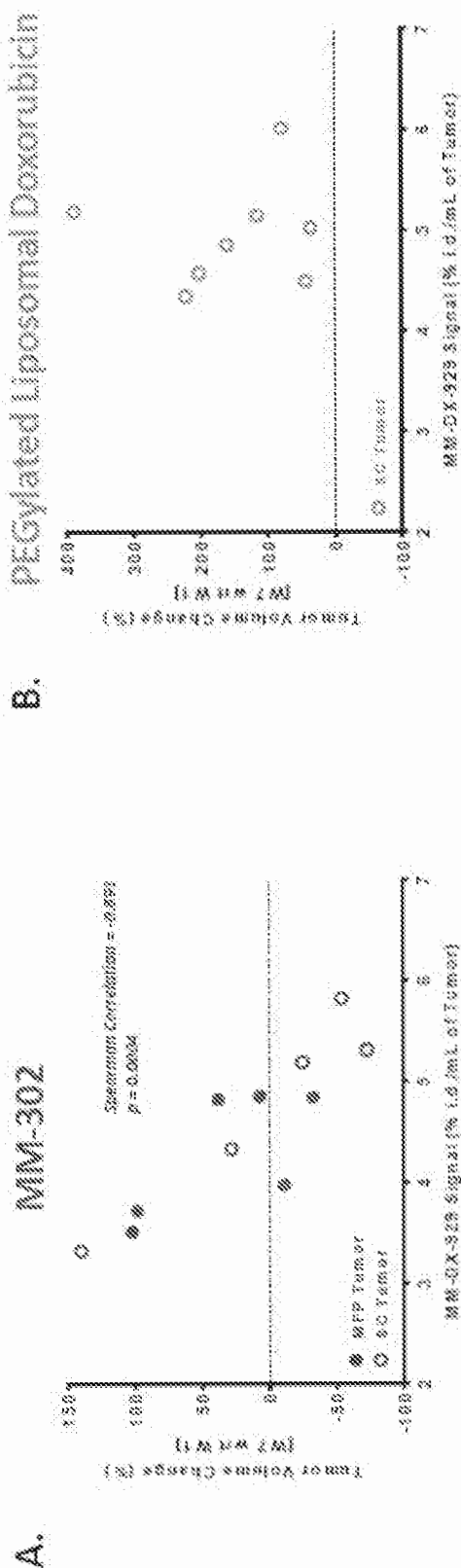


Figure 6. (A) A significant correlation was detected between tumor uptake of MM-DX-929 and therapeutic activity of an immunoliposome, MM-302, in a HER2-3+ xenograft breast cancer model. These results support the hypothesis in which tumor deposition is a key parameter in determining the activity of liposomal drugs, regardless of the presence of a targeting moiety. (B) Tumor deposition of MM-DX-929 is shown to have a similar trend when mice were treated with a non-targeted agent, PEGylated liposomal doxorubicin (PLD).

STUDY DESIGN FOR PROOF-OF-CONCEPT IN THE CLINIC WITH ⁶⁴CU-MM-302 AS A TRANSLATIONAL TOOL

⁶⁴Cu-MM-302: Translational Tool for Tracking Deposition of MM-302 in Patients

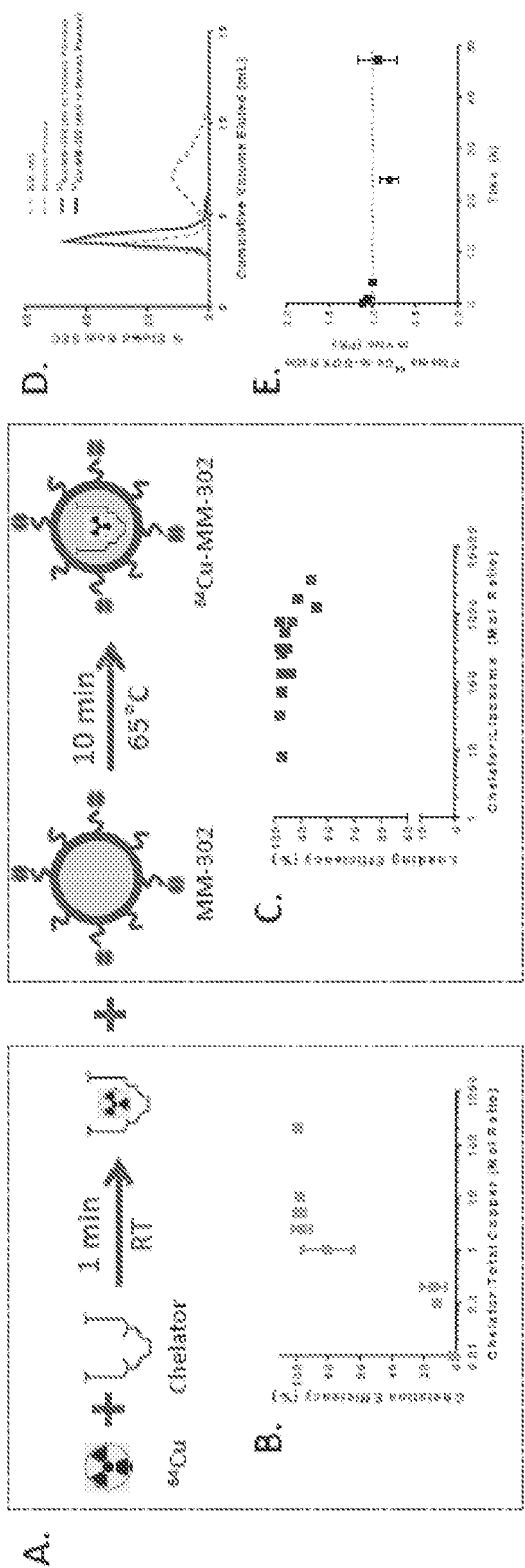
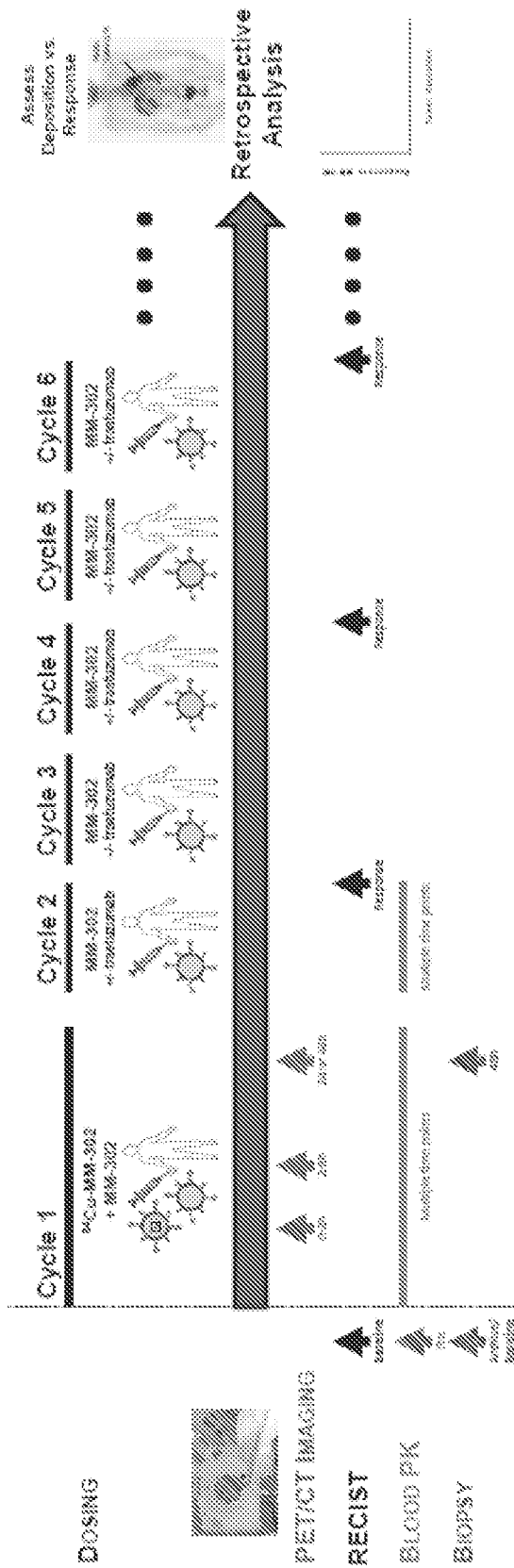


Figure 7. (A) Schematic describing the radiolabeling procedure of ⁶⁴Cu-MM-302. (B) & (C) Optimization of clinical kit chelation and radiolabeling efficiencies by varying chelator:copper and chelator:liposome ratios. (D) *In vitro* stability of ⁶⁴Cu-MM-302 in human plasma at 37°C; at designated time points, ⁶⁴Cu-MM-302 was assayed to separate liposomal-, protein-bound, or released- ⁶⁴Cu. Over a 48-hour incubation period, >98% of ⁶⁴Cu was still associated with the liposome. (E) *In vivo* plasma ratios of ⁶⁴Cu and MM-302 drug contents (doxorubicin) in mice administered with ⁶⁴Cu-MM-302 (derived from pharmacokinetics data presented in Figure 3C).

Proposed Clinical Study Design: ^{64}Cu -MM-302 Deposition vs. MM-302 Response



SUMMARY

MM-DX-929: A ^{64}Cu -liposome was developed as a tool for accurately measuring tumor deposition *in vivo*. This radiolabeling method has also been shown to successfully label drug-loaded liposomes (MM-302) with excellent stability.

Tumor Deposition is Variable: Tumor deposition of liposomes can be measured via administration of MM-DX-929/ ^{64}Cu -MM-302 and PET imaging, and was demonstrated to be variable across and within tumor models. Most importantly, this study has shown that variability in tumor deposition is a key mechanism for immunoliposome anti-tumor activity.

Tumor Deposition as a Predictive Marker for Liposomal Chemotherapeutics: This proof-of-principle study demonstrated the potential use of MM-DX-929 as a predictive imaging agent to select patients that are most likely to respond to liposomal therapies. ^{64}Cu -MM-302 may be used as a translational tool for a first step clinical proof-of-principle study in assessing the relationship between tumor deposition and activity of MM-302.



Delivery and Anti-Tumor Activity of Nanoliposomal Irinotecan (nal-IRI, MM-398) in Metastatic Xenograft Models of Triple Negative Breast Cancer

Helen Lee¹, Manuela Ventura², Nicholas Bernards², Afroz S. Mohammad³, Warren Foltz², Jonathan B. Fitzgerald¹, David Jaffray^{2,4,5}, Paul R. Lockman², Jinzi Zheng^{2,5}, Bart S. Hendriks¹

¹Merrimack Pharmaceuticals, Inc., Cambridge, MA; ²TECHNA Institute for the Advancement of Technology for Health, University Health Network, Toronto, ON, Canada; ³West Virginia University HSC, School of Pharmacy, Morgantown, WV; ⁴Department of Radiation Oncology, University of Toronto, Toronto, ON, Canada; ⁵Institute of Biomaterial and Biomedical Engineering, University of Toronto, Toronto, ON, Canada.

P6-12-16

Abstract

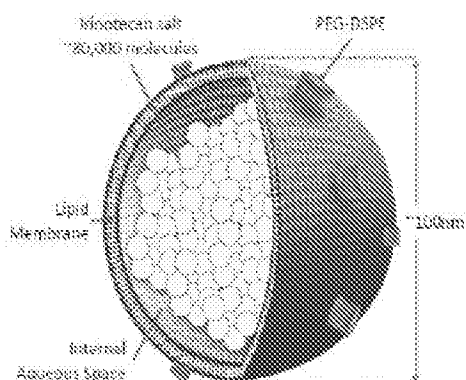
Introduction: nal-IRI (MM-398, nanoliposomal Irinotecan), is designed for extended circulation relative to nonliposomal Irinotecan and to exploit leaky tumor vasculature for enhanced drug delivery to tumors. nal-IRI (ONIVYDE®), in combination with 5-fluorouracil and leucovorin, was recently approved for the treatment of patients with advanced pancreatic cancer who have been previously treated with gemcitabine. A pilot clinical study established ferumoxytol (FMX) measured by magnetic resonance imaging (MRI) as a potential surrogate for assessing nanoparticle delivery to tumors. Lesions with FMX uptake above the median were associated with greater reductions in tumor size following treatment with nal-IRI. nal-IRI with FMX MRI is currently being evaluated in patients with metastatic breast cancer (NCT01770353). Here we present preclinical data demonstrating the activity of nal-IRI monotherapy in multiple models of triple negative breast cancer (TNBC).

Methods: Imaging and treatment efficacy studies were carried out in the MDA-MB-231 derived mouse models of TNBC (SCID mice), including the LM2-4 variant with spontaneous metastases. Delivery of nal-IRI to brain metastases was assessed in MDA-MB-231-Br-Luc model (intracardiac implantation) using fluorescently labeled nal-IRI. Kinetics of FMX tumor uptake were evaluated with 7T MRI. Total tumor Irinotecan and the active metabolite SN-38 were quantified by high performance liquid chromatography.

Results: nal-IRI treatment was well-tolerated based on body weight monitoring. In the LM2-4 model, nal-IRI (10 mg/kg salt) was more effective in suppressing primary tumor regrowth (median tumor volume of 155 mm³ vs. 946 mm³ at day 14), reducing metastatic burden (median bioluminescence flux of 0.4x10⁹ vs. 2.1x10⁹ at day 12), and prolonging overall survival (median survival of 66 days vs. 14 days), compared to nonliposomal Irinotecan (50 mg/kg salt). Similarly, nal-IRI demonstrated benefits in reducing brain metastatic burden and extended survival compared to untreated control in the MDA-MB-231 brain metastasis model. Fluorescence microscopy revealed that nal-IRI primarily localized in the metastatic lesions, with undetectable signal in normal brain tissue. At 24 h post FMX-injection, FMX uptake correlated positively with tumor SN-38 levels at 24 h following treatment with nal-IRI ($p = 0.0222$, Spearman correlation), supporting that nanoparticle imaging may be useful as a surrogate measure of nal-IRI tumor delivery. Furthermore, higher tumor FMX deposition was associated with increased tumor growth inhibition with nal-IRI, corroborating observations from the pilot Phase 1 clinical study.

Conclusions: These studies support the evaluation of nal-IRI in patients with TNBC, as well as further evaluation of nanoparticle imaging as a potential biomarker for identifying lesions likely to respond.

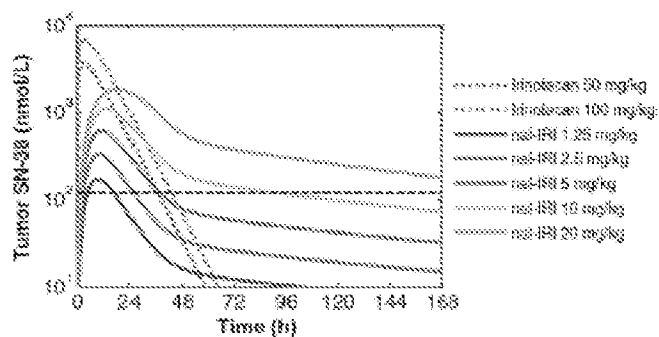
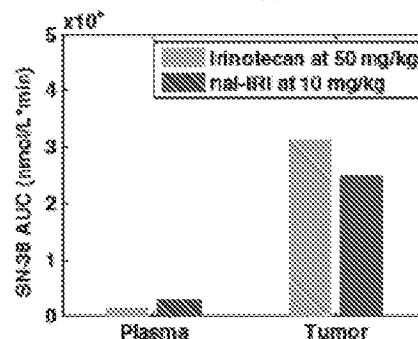
Nanoliposomal Irinotecan (MM-398, nal-IRI)



Key Attributes:

- Long circulation and sustained release
- Tumor accumulation via the enhanced permeability and retention (EPR) effect
- Improved treatment efficacy and reduced toxicity compared to non-encapsulated irinotecan when administered at comparable SN-38 AUC in plasma and tumor in preclinical models

nal-IRI extended tumor SN-38 duration at equivalent exposure as Irinotecan

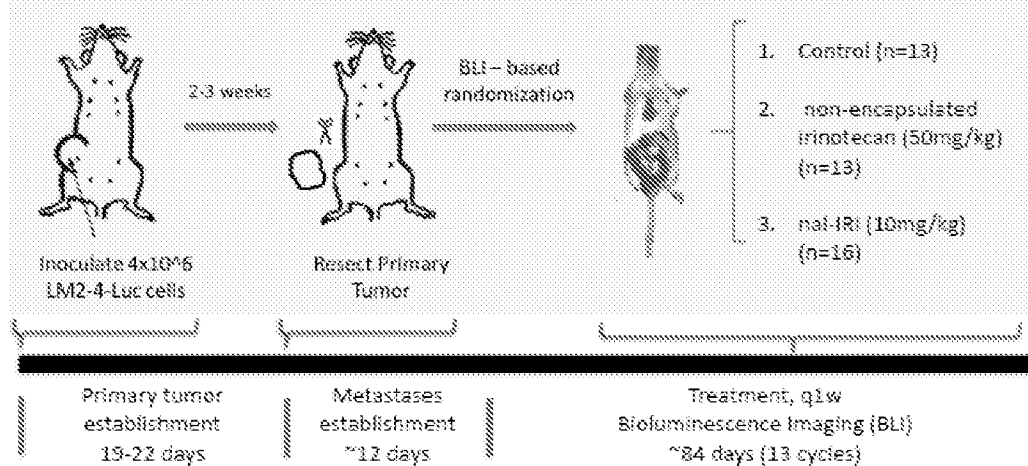


Note: nal-IRI and IRI concentrations in this poster are specified in salt concentrations.
nal-IRI 10 mg/kg (salt) is approximate to 9 mg/kg (free base).

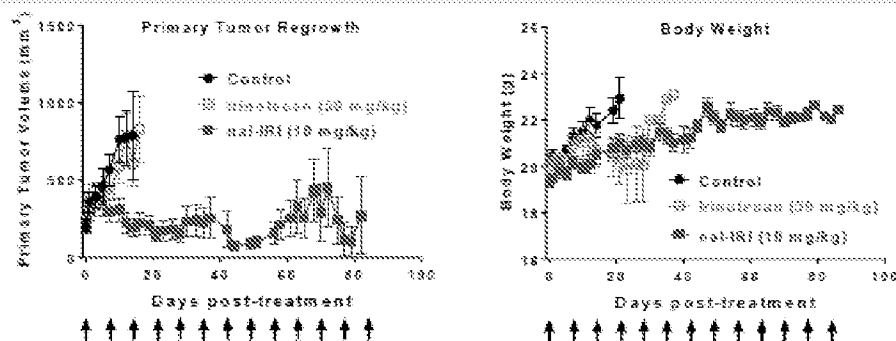
Kalra (2014)
Cancer Research

nal-IRI Reduces Metastatic Burden and Significantly Extended Overall Survival in LM2-4 Metastatic Model

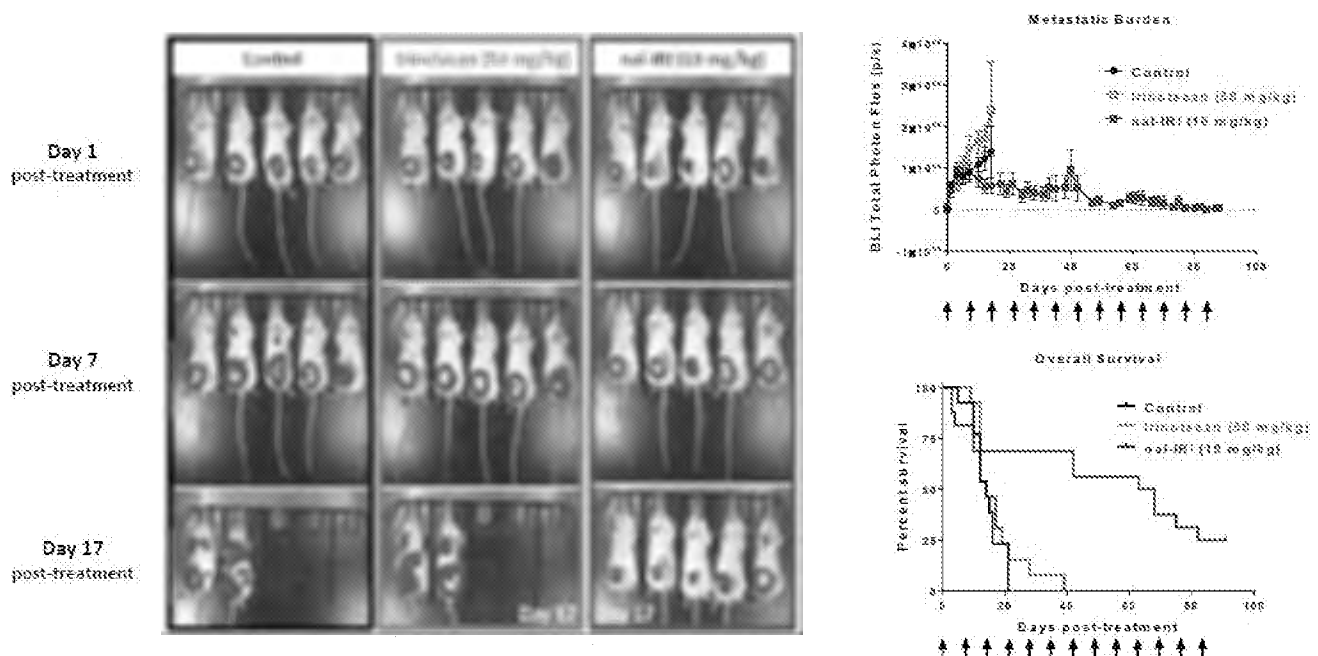
Experimental Design



nal-IRI is more effective at suppressing primary tumor regrowth than nonliposomal irinotecan with comparable tolerability

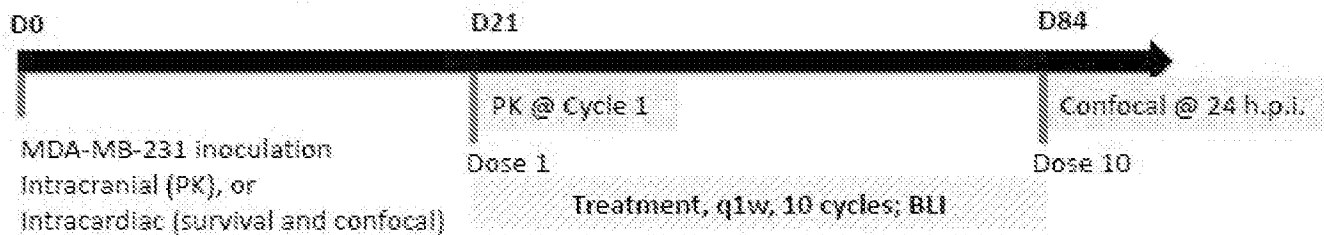


nal-IRI treated animals have significantly lower metastatic burden as detected by BLI and extended overall survival

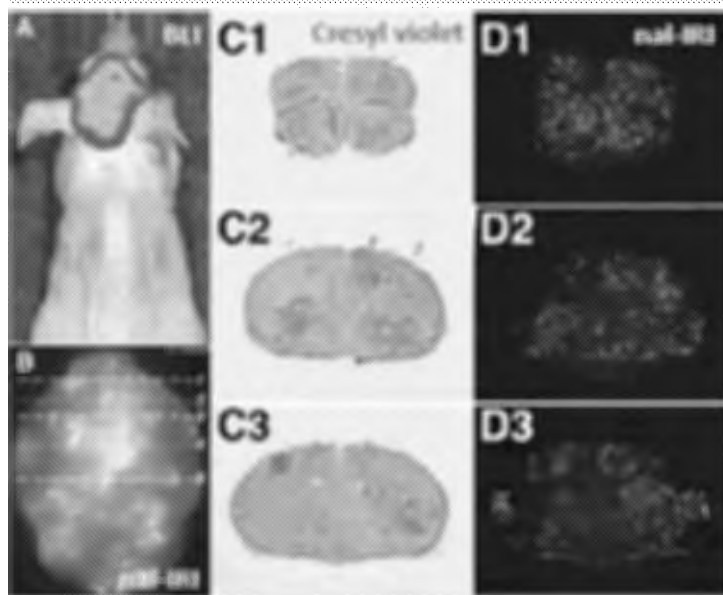


nal-IRI Improves Delivery of Irinotecan and SN-38 to TNBC Brain Tumors and Improved Survival

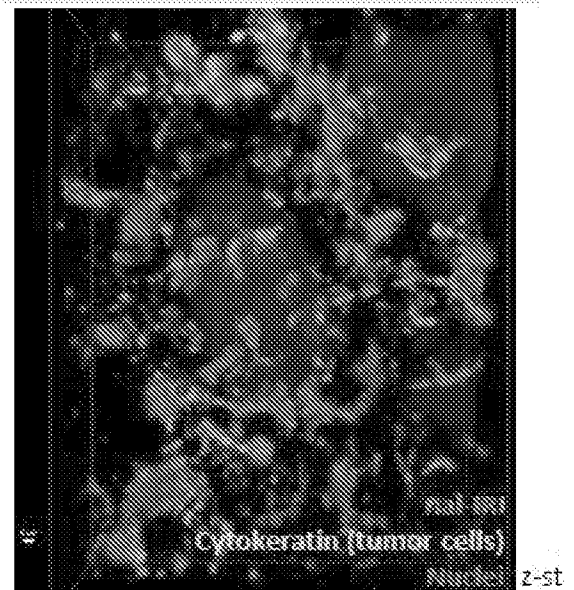
Experimental Design



nal-IRI preferentially accumulates at brain tumors with minimal uptake in normal brain tissue

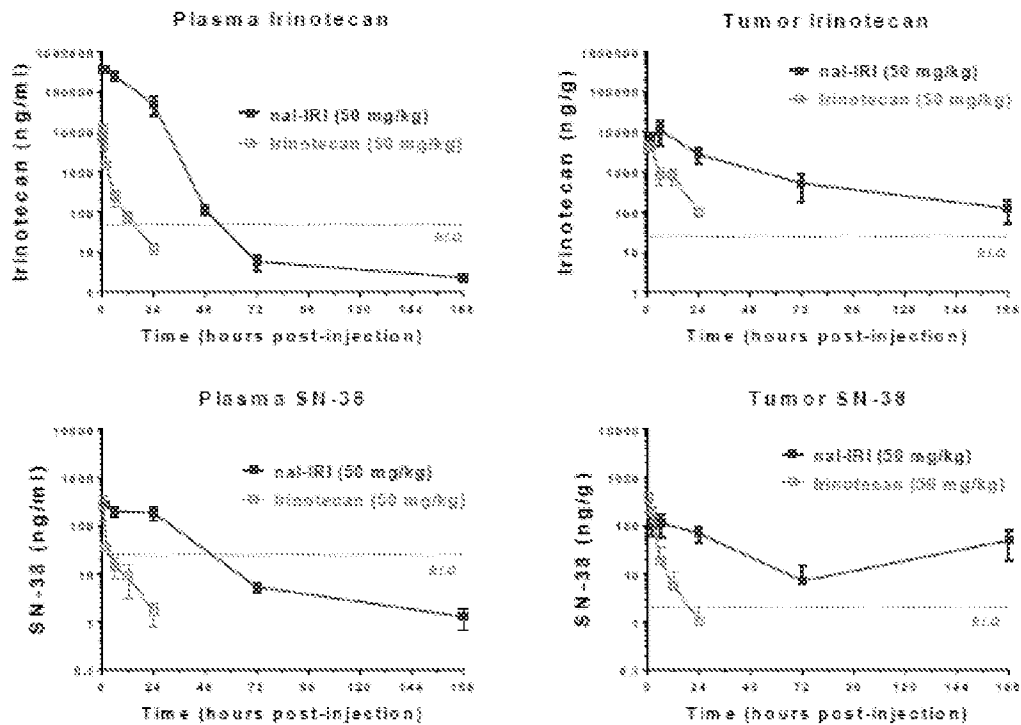


nal-IRI was detected inside brain tumor cells at 24 hours post-injection

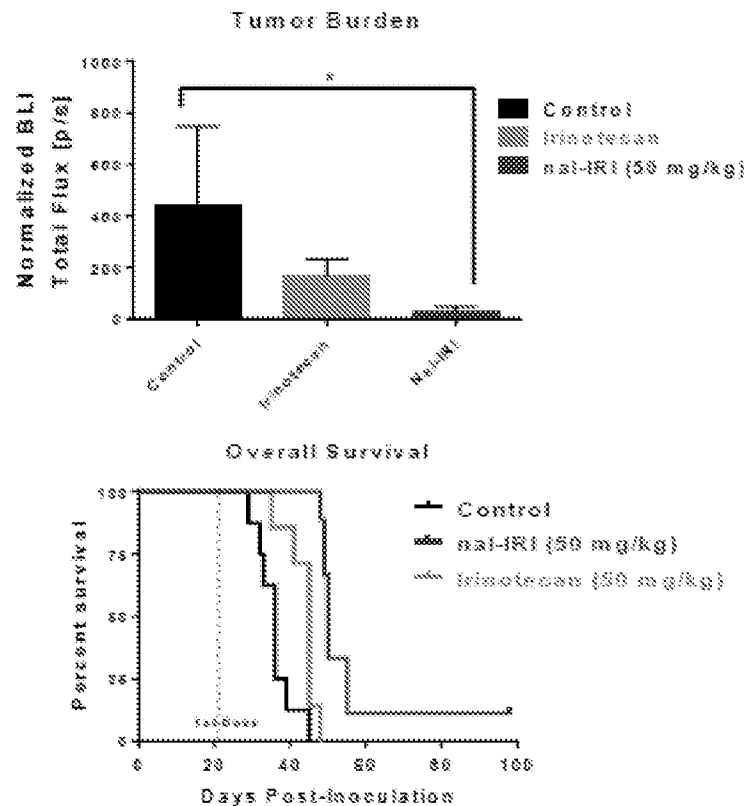


z-stack image by Nikon N-storm microscope

nal-IRI extends circulation of irinotecan and SN-38, and improves delivery to brain tumors

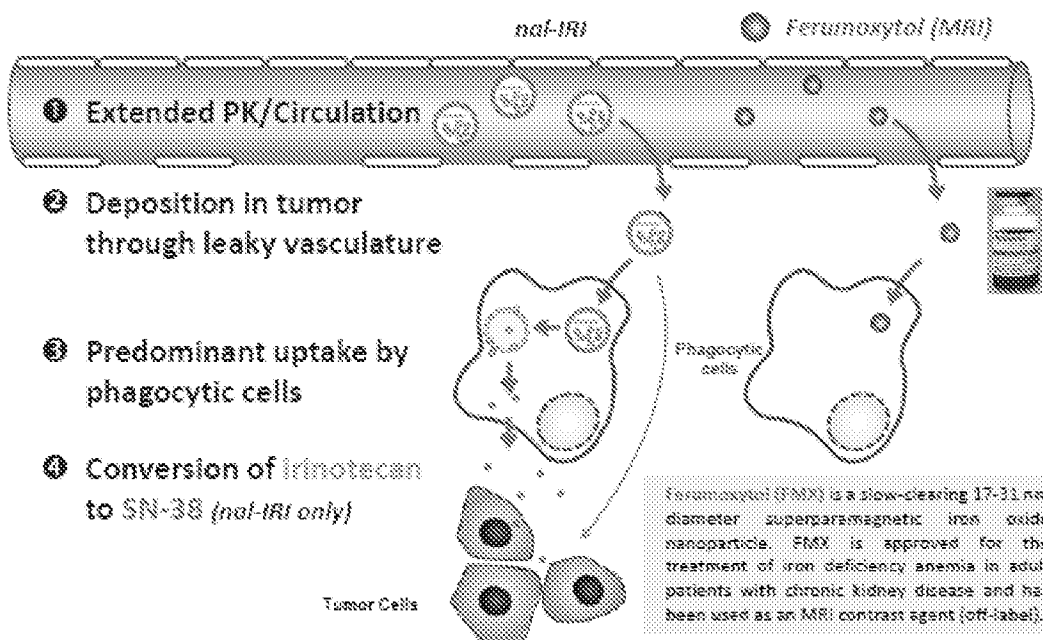


Mice treated with nal-IRI have fewer brain and peripheral metastases than irinotecan

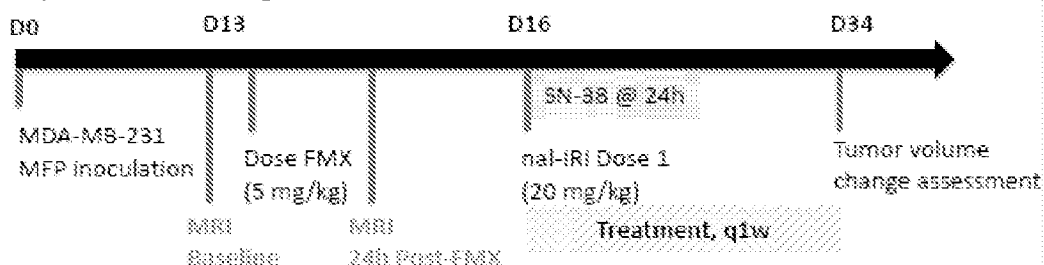


FMX-MRI as a Surrogate for nal-IRI Delivery and Response

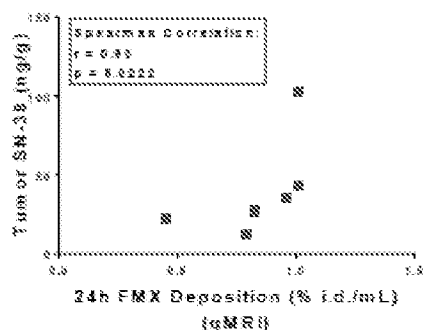
- We hypothesize that local tumor delivery of nal-IRI to solid tumors is potentially rate-limiting for effective delivery and treatment response
- FMX is delivered to tumors in a similar mechanism as nal-IRI
- FMX tumor uptake quantified by MRI may serve as a surrogate for nal-IRI delivery, and may be predictive of nal-IRI treatment response



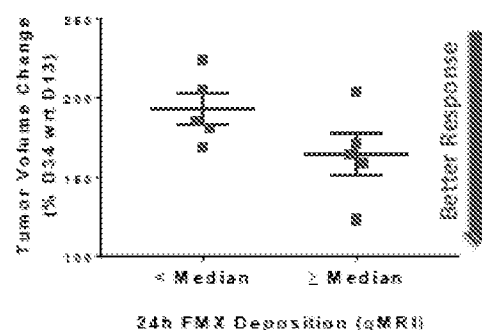
Experimental Design



Tumor SN-38 delivered by nal-IRI is correlated with FMX tumor deposition



High FMX tumor deposition is associated with better response to nal-IRI



Summary

- nal-IRI increases exposure and sustains delivery of irinotecan and SN-38 (active metabolite) to brain tumors, compared to nonliposomal irinotecan.
- nal-IRI preferentially accumulates in brain tumors with minimal uptake in adjacent normal brain tissues.
- We have demonstrated, in two preclinical TNBC brain metastatic models, that nal-IRI is more effective in reducing primary tumor regrowth, peripheral and brain metastatic burden, as well as prolonging overall survival, compared to nonliposomal irinotecan.
- FMX tumor uptake as quantified by MRI predicts nal-IRI delivery and treatment response in an orthotopic TNBC mouse model.

Abstract

Delivery and Anti-Tumor Activity of Nanodiposol Nanotubes (nan-DB) (M02-358) in Metastatic Xenograft Models of Triple Negative Breast Cancer

Abstract: Metastatic breast cancer is the leading cause of cancer death in women. Triple negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer that lacks expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Nanodiposol nanotubes (nan-DB) are a novel nanotechnology platform for the delivery of chemotherapeutic drugs. In this study, we evaluated the anti-tumor activity of nan-DB in metastatic xenograft models of TNBC. The results show that nan-DB significantly reduces tumor growth and increases survival in mice. The mechanism of action involves the disruption of the tumor vasculature and the induction of apoptosis. These findings suggest that nan-DB is a promising platform for the delivery of chemotherapeutic drugs in the treatment of TNBC.

nan-DB Reduces Metastatic Burden and Significantly Extended Overall Survival in L1210-6 Metastatic Model

Figure 1: nan-DB treatment significantly reduced the metastatic burden in L1210-6 mice. The graph shows the number of metastatic nodules per lung for control (n=10) and nan-DB treated (n=10) groups. The nan-DB group showed a significant reduction in metastatic burden (p < 0.05).

nan-DB Improves Delivery of Irinotecan and 5-Fluorouracil to Tumor and Improves Survival

Figure 2: nan-DB significantly improved the delivery of irinotecan and 5-fluorouracil to the tumor. The graph shows the tumor-to-plasma ratio of the drugs for control (n=10) and nan-DB treated (n=10) groups. The nan-DB group showed a significant increase in the tumor-to-plasma ratio (p < 0.05).

nan-DB Reduces Tumor Growth and Increases Survival in Mice

Figure 3: nan-DB treatment significantly reduced tumor growth and increased survival in mice. The graph shows the tumor volume (mm³) and survival percentage for control (n=10) and nan-DB treated (n=10) groups. The nan-DB group showed a significant reduction in tumor volume and a significant increase in survival (p < 0.05).

nan-DB Reduces Tumor Growth and Increases Survival in Mice

Figure 4: nan-DB treatment significantly reduced tumor growth and increased survival in mice. The graph shows the tumor volume (mm³) and survival percentage for control (n=10) and nan-DB treated (n=10) groups. The nan-DB group showed a significant reduction in tumor volume and a significant increase in survival (p < 0.05).

Extended topoisomerase 1 inhibition through liposomal irinotecan results in improved efficacy over topotecan and irinotecan in models of small-cell lung cancer

Shannon C. Leonard^a, Helen Lee^a, Daniel F. Gaddy^a, Stephan G. Klinz^a, Nancy Paz^a, Ashish V. Kalra^a, Daryl C. Drummond^a, Daniel C. Chan^b, Paul A. Bunn^b, Jonathan B. Fitzgerald^a and Bart S. Hendriks^a

Liposomal irinotecan (irinotecan liposome injection, nal-IRI), a liposomal formulation of irinotecan, is designed for extended circulation relative to irinotecan and for exploiting discontinuous tumor vasculature for enhanced drug delivery to tumors. Following tumor deposition, nal-IRI is taken up by phagocytic cells followed by irinotecan release and conversion to its active metabolite, SN-38. Sustained inhibition of topoisomerase 1 by extended SN-38 exposure as a result of delivery by nal-IRI is hypothesized to enable superior antitumor activity compared with traditional topoisomerase 1 inhibitors such as conventional irinotecan and topotecan. We evaluated the antitumor activity of nal-IRI compared with irinotecan and topotecan in preclinical models of small-cell lung cancer (SCLC) including in a model pretreated with carboplatin and etoposide, a first-line regimen used in SCLC. Nal-IRI demonstrated antitumor activity in xenograft models of SCLC at clinically relevant dose levels, and resulted in complete or partial responses in DMS-53, DMS-114, and NCI-H1048 cell line-derived models as well as in three patient-derived xenograft models. The antitumor activity of nal-IRI was superior to that of topotecan in all models tested, which generally exhibited limited control of tumor growth and was superior to irinotecan in four out of five models. Further, nal-IRI demonstrated antitumor activity in tumors that progressed following treatment with topotecan or irinotecan, and

demonstrated significantly greater antitumor activity than both topotecan and irinotecan in NCI-H1048 tumors that had progressed on previous carboplatin plus etoposide treatment. These results support the clinical development of nal-IRI in patients with SCLC. *Anti-Cancer Drugs* 28:1086–1096 Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc.

Anti-Cancer Drugs 2017, 28:1086–1096

Keywords: in-vivo pharmacology, irinotecan, small-cell lung cancer, topotecan

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Received 27 March 2017 Revised form accepted 24 June 2017

Introduction

Lung cancer is the leading cause of cancer-related death worldwide. Small-cell lung cancer (SCLC) accounts for about 15% of lung cancers overall and is a particularly aggressive neoplasia with a 5-year survival rate of less than 10%. Little progress has been made in improving the outcome of this malignancy over the last 30 years. Approximately 30% of patients with SCLC are diagnosed with limited-stage disease, and are typically treated with a combination of radiation and chemotherapy [1]. With current treatments, only ~20% of patients with limited-

stage disease maintain the potential to be cured [2]. Extensive-stage disease remains incurable with an expected median survival of 7–9 months [1].

Extensive-stage disease is typically treated in the front-line setting with a combination of platinum (carboplatin or cisplatin) plus the topoisomerase 2 inhibitor etoposide in the USA and European Union [3,4]. About 50–90% of patients with extensive-stage disease respond to initial treatment [1]; however, patients typically relapse rapidly and develop resistance. Irinotecan, a prodrug for its active metabolite SN-38, has shown activity in front-line settings in patients with SCLC, and its combination with cisplatin is commonly used in Japan [5] and is included in the NCCN and ESMO guidelines [3,4]. A number of trials have compared the combination of irinotecan plus cisplatin or carboplatin with etoposide plus cisplatin or carboplatin.

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Several of these trials demonstrated a significant advantage for the irinotecan combination, and meta-analyses of all trials showed significantly longer overall survival in the irinotecan combination relative to etoposide plus platinum combinations [6–9]. Nevertheless, etoposide plus platinum combinations are used most frequently in North America and Europe. In the second-line setting, single-agent irinotecan has been evaluated in a few small studies (overall response rate, time to treatment progression, and overall survival in the range of 0–47%, 1.7–2.6 months, and 4.6–13.3 months, respectively) [10–12] with similar outcomes to that of topotecan [13]. On the basis of these and other studies, irinotecan monotherapy is included in major treatment guidelines for the treatment of patients with relapsed SCLC [3,4].

Topotecan is the only agent approved for second-line therapy in the USA and Europe. The approved dose of intravenous topotecan is 1.5 mg/m² given on days 1–5 every 3 weeks. Clinically, the administration of topotecan is often limited by its tolerability, and in practice many oncologists reduce both the starting dose and number of days when the drug is administered [14]. Topotecan, like irinotecan, is a camptothecin derivative and topoisomerase 1 (TOP1) inhibitor that stabilizes the typically transient TOP1–DNA cleavable complex. This results in the accumulation of single-stranded breaks in DNA followed by conversion to replication-mediated DNA double-strand breaks and cell death.

There is evidence that the pharmacokinetics and tolerability of topotecan create an exposure problem that limits its activity. Topotecan is water soluble, contributing to rapid clearance from the bloodstream. Following administration, plasma levels peak at about 70 nmol/l and are maintained above a clinically relevant threshold of 10 nmol/l for about 2.6 h/daily infusion [10]. However, in-vitro studies using cell lines of SCLC origin have reported that the inhibitory concentrations required to achieve 50% of maximal activity range from single-digit nanomolar up to micromolar concentration [15,16]. In addition, the complex ionization chemistry of topotecan contributes to a relatively low cellular permeability [10,17]. In a translational study, tumor TOP1 inhibition was assessed by measuring TOP1 protein levels in biopsies at day 4 or 5 from patients with gastric and esophageal cancers treated with 1.5 or 2.0 mg/m² topotecan [18]. At the 1.5 mg/m² dose level, the degree of TOP1 inhibition measured 30 min after drug administration was only ~75%, indicating that topotecan is not able to fully inhibit its target. Thus, these data suggest that clinical administration of topotecan likely results in suboptimal peak levels and time of exposure in patients.

Multiple studies have demonstrated schedule-dependent efficacy of TOP1 inhibitors in preclinical models [19–21] in which increased antitumor activity is generally observed with prolonged exposure directed at the tumor.

Indeed, for this drug class it is well understood that liposomal formulations can improve the therapeutic activity through a slow and controlled release of the payload and by providing sustained duration of tumor cell exposure. Stable and long-circulating liposomal formulations for irinotecan and topotecan with potent in-vivo efficacy against cancer xenograft models have been described [17,22]. In particular, liposomal irinotecan (irinotecan liposome injection, nal-IRI) is designed for extended circulation relative to irinotecan and for exploiting leaky tumor vasculature for enhanced drug delivery to tumors [22]. Liposomal encapsulation of irinotecan resulted in detectable levels of irinotecan and SN-38 in the plasma for over 50 h after administration in mice. By contrast, irinotecan and its metabolite SN-38 were cleared from circulation in under 8 h when dosed at similar levels [23]. Nal-IRI has been demonstrated to mediate prolonged SN-38 exposure, subsequent DNA damage, and tumor cell death in models of colorectal and pancreatic cancer, as well as in other cancer models [23,24]. On the basis of a demonstrated improvement in overall survival, nal-IRI, in combination with 5-fluorouracil and leucovorin, has recently been approved in the USA and European Union for the treatment of patients with metastatic pancreatic adenocarcinoma after disease progression following gemcitabine-based therapy [25].

It is hypothesized that nal-IRI may have utility in additional indications including SCLC. Greater than 80% of SCLC tumor lesions express detectable levels of vascular endothelial growth factor [2], which is involved in angiogenesis and vessel permeability and may indicate the ability to achieve effective liposome delivery into SCLC tumors. Following tumor deposition, nal-IRI is taken up by phagocytic cells followed by irinotecan release and conversion to SN-38 [23]. Macrophages are the primary phagocytic cell type that is believed to take up nal-IRI within the tumor tissue and express high levels of carboxylesterase (CES) 1 [24,26], one of the enzymes needed to convert irinotecan to SN-38. CES in SCLC cell lines has been described by van Ark-Otte *et al.* [27] who found that CES activity was significantly higher when compared with non-SCLC cell lines while showing similar sensitivity to SN-38. Further, staining of samples from patients with SCLC has indicated consistent macrophage infiltration, which was found to increase with tumor stage [28,29].

For the reasons outlined above, it is hypothesized that nal-IRI has the potential to greatly extend TOP1 inhibition in SCLC tumors relative to topotecan and provide improved antitumor activity. Here, we evaluate and compare nal-IRI, irinotecan, and topotecan in xenograft models of SCLC.

Materials and methods

Materials

Nal-IRI was prepared as described previously [22]. Irinotecan and SN-38 were obtained from Areva

Pharmaceuticals (Elizabethtown, Kentucky, USA) and topotecan was obtained from Accord Healthcare (Durham, North Carolina, USA). SCLC cell lines (DMS-114, DMS-53, NCI-H1048, and NCI-H841) were obtained from ATCC and grown under recommended conditions. Solvents and reagents were obtained from Sigma Aldrich (St. Louis, Missouri, USA) and VWR (Radnor, Pennsylvania, USA). All irinotecan, topotecan, and nal-IRI concentrations and/or dose levels are described on the basis of their respective HCl salts.

Cell proliferation assay

SCLC cell lines were transfected with NucLight Red Lentivirus (Essen BioScience Ann Arbor, Michigan, USA) and selected with puromycin treatment following the manufacturer's instructions. For cell proliferation assay, NucLight Red-transfected cells were harvested using 0.25% trypsin and plated into 96-well plates at a density of 5000 cells/well. Cells were allowed to adhere overnight before addition of drugs (SN-38 or topotecan). Drug solutions were prepared in media containing no more than 1% of DMSO. Various concentrations (0.01–10 000 nmol/l) of drugs were added to each well. Plates were then transferred to the IncuCyte ZOOM (Essen Bioscience, USA) system (in a 37°C incubator, supplemented with 5% CO₂) for an incubation period of 88 h. Images were acquired in three fields of view per well every 4 h. Cell numbers were quantified using IncuCyte ZOOM (Essen Bioscience, USA) software by segmenting cell nuclei based on the NucLight Red fluorescence. The total number of cells was determined by summing the number of cells in the three fields of view for each well.

Animal studies

Cell line-derived xenograft models were developed in nonobese diabetic/severe combined immunodeficiency mice (5–7-week-old females) that were purchased from Charles River Laboratory (Wilmington, Massachusetts, USA), with the exception of NCI-H841 efficacy studies, which were performed in athymic nude mice, also from Charles River Laboratory. At the time of treatment, cell line-derived tumors averaged 200–300 mm³. Irinotecan and nal-IRI were administered once a week as an intravenous bolus injection. Topotecan was administered intraperitoneally once or twice weekly, as indicated. Body weight was recorded throughout the study and tumor volumes were measured using calipers. Before tumor collection, intracardial perfusion was performed to remove blood from the tumor tissue.

Patient-derived xenograft (PDX) models, used for assessment of drug delivery and CES activity, were established in nude mice (Harlan Laboratories, Washington, DC, USA) by Champions Oncology (Hackensack, New Jersey, USA) using their Champions Tumorgraft technology. PDX models of SCLC origin

used in antitumor activity studies were derived from donor mice and engrafted subcutaneously into the flanks of 6–8-week-old Balb/c nude mice (Vital River Laboratories, Beijing, China) and run by GenenDesign (Shanghai, China). At the time of treatment, patient-derived tumors averaged 250–300 mm³ and were at passage 6 or less. Irinotecan and nal-IRI were administered once a week as an intravenous bolus injection. Topotecan was administered intraperitoneally twice a week. All treatments continued for a total of 6 weeks. Body weight was recorded throughout each study and tumor volumes were measured with calipers twice a week until tumors reached 1500 mm³, animals were in poor general health, or until 8 weeks after the first dose.

The care and treatment of animals was performed in accordance with Institutional Animal Care and Use Committee guidelines. Institutional requirements for humane endpoints were used as surrogates for survival.

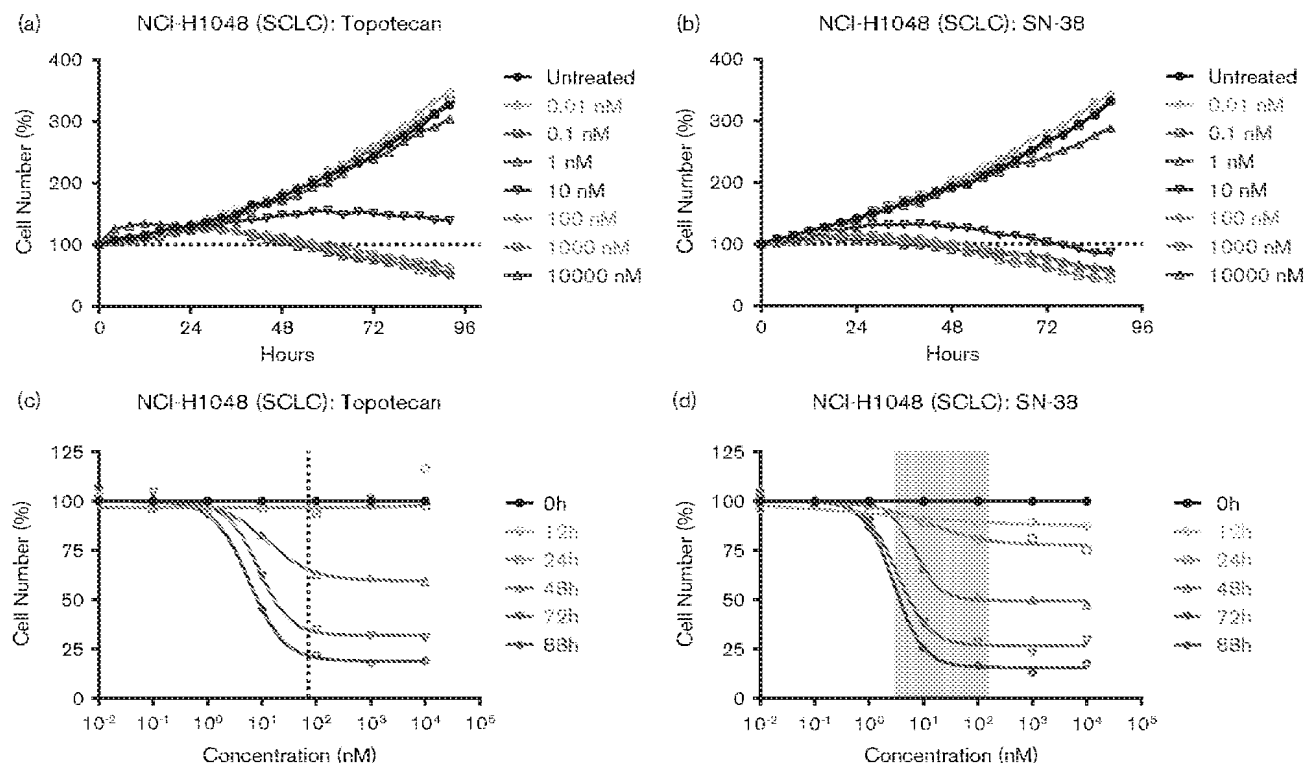
High-performance liquid chromatography quantification of irinotecan and SN-38

Tumor levels of irinotecan and SN-38 were quantified using high-performance liquid chromatography (HPLC) as previously described [23]. In brief, tissues were homogenized for 2 min in 20% w/v water using a Tissue-Lyser (Qiagen, Hilden, Germany), extracted by mixing 0.1 ml homogenate with 0.9 ml 1% acetic acid/methanol, vortexed for 10 s, and placed at –80°C for 1 h. Samples were centrifuged at 10 000 rpm for 10 min at room temperature and supernatants were collected. Samples and standards were analyzed using HPLC (Dionex; Thermo Fisher, Sunnyvale, California, USA) with a C18 reverse-phase column [Synergi Polar-RP 80A 250 × 4.60 mm² 4-μm column from Phenomenex (Torrance, California, USA)]. Irinotecan and SN-38 were eluted using a gradient of 30% acetonitrile, 70% of 0.1% trifluoroacetic acid/H₂O to 68% acetonitrile, and 32% of 0.1% trifluoroacetic acid/H₂O over 13 min at 0.1 ml/min. Irinotecan and SN-38 peaks eluted at ~7.7 and 8.4 min, respectively, and were detected using an in-line fluorescence detector excited at 372 nm and emitting at 556 nm.

Irinotecan activation assay

Irinotecan activation in tissues was measured as previously described [23]. Tumor tissues were homogenized in 6% w/v 0.1 mol/l Tris-HCl/1% Triton X-100 solution (pH 7.5) using a Tissue-Lyser for 2–4 min. A measure of 250 μg of protein lysate was mixed with an equal volume of 10 μmol/l irinotecan and incubated at 37°C for 24 h. Reactions were terminated by addition of an equal volume of 1% acetic acid/methanol. Samples were centrifuged at 10 000 rpm for 15 min and processed using HPLC as described above.

Fig. 1



Cytotoxicity of TOP1 inhibitors increases with exposure. Kinetic growth curves of the NCI-H1048 SCLC cell line were acquired on an Incucyte ZOOM instrument over 88 h at various concentrations of (a) topotecan or (b) SN-38, as indicated. Concentration-dependent growth inhibition at selected time points is shown for (c) topotecan and (d) SN-38. Vertical line, human plasma C_{max} for 1.5 mg/m² topotecan [14]. Shaded area, range of SN-38 measured in post-treatment biopsies at 72 h after 70 mg/m² liposomal irinotecan [31]. SCLC, small-cell lung cancer; TOP1, topoisomerase 1.

Statistical analysis

Data were analyzed using GraphPad Prism, version 7.0 (GraphPad Software, La Jolla, California, USA). Results were analyzed using a nonparametric unpaired two-tailed Student's *t*-test (Mann–Whitney), unless otherwise indicated.

Results

Cell growth inhibition of TOP1 inhibitors increases with exposure

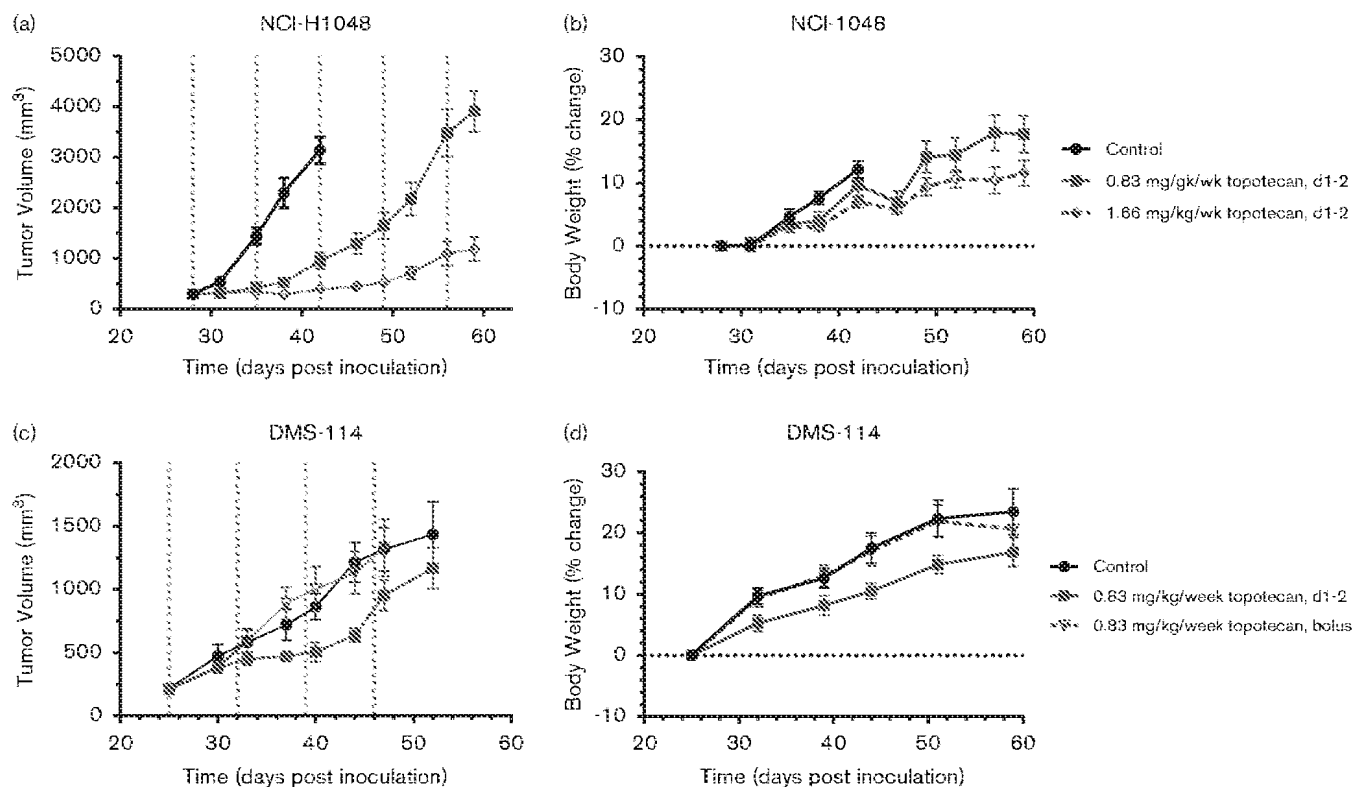
Although the mechanism of TOP1 inhibition has been extensively studied [30], we sought to specifically investigate the effect of dose and duration of inhibition. The activity of two TOP1 inhibitors, topotecan and SN-38 (the active metabolite of irinotecan), was tested in SCLC cell lines in in-vitro growth and viability assays. Cell growth inhibition kinetics of topotecan and SN-38 in the NCI-H1048 SCLC cell line over a time course of 88 h are shown in Fig. 1a and b. Effective cell growth inhibition was observed at ~10 nmol/l for both topotecan and SN-38, whereas cell killing was observed after prolonged incubation times at concentrations greater than or equal to 10 nmol/l. Concentration-dependent growth inhibition at selected time points is shown in Fig. 1c and d. Treatment with both topotecan and SN-38 decreased

cell viability by greater than 90%. The IC₅₀ values were variable and spanned several orders of magnitude depending on time of incubation. The human plasma C_{max} for 1.5 mg/m² topotecan is indicated in Fig. 1c and falls within the concentration required for cytotoxicity; however, the C_{max} is observed shortly after administration and concentrations decline rapidly on a time scale of hours [14]. The concentration range of SN-38 at which cell killing begins to occur coincides with the amount of SN-38 measured in tumor biopsies taken from patients with various solid tumors 72 h after administration of nal-IRI (range: 3–163 nmol/l [31]), which is depicted in Fig. 1d as the gray shaded area. Similar results were observed in the DMS-114 cell line (Lee H, unpublished data). These experiments demonstrate that for both SN-38 and topotecan the concentration and time of exposure are important determinants of cytotoxicity.

Dose and exposure are potentially limiting for topotecan

The antitumor activity of topotecan was evaluated for different doses and schedules in xenograft models. The dose used for mouse studies was based on published clinical dosing of topotecan in second-line treatment of SCLC [32] and calculated using standard surface area to weight ratios conversion per NCI guidelines. Using this

Fig. 2



Antitumor activity of topotecan in models of SCLC. NOD/SCID mice with NCI-H1048 SCLC xenograft tumors were treated with intraperitoneal (i.p.) topotecan 0.83 mg/kg/week split over days 1 and 2 (squares), i.p. topotecan 1.66 mg/kg/week split over days 1 and 2 (diamonds), or vehicle control (circles). Tumor growth curves (a) and body weight (b) are shown. NOD/SCID mice with DMS-114 SCLC xenograft tumors were treated with i.p. topotecan 0.83 mg/kg/week administered as a bolus (triangles), i.p. topotecan 0.83 mg/kg/week split over days 1 and 2 (squares), or vehicle control (circles). Tumor growth curves (c) and body weight (d) are shown. Vertical dotted lines indicate start of weekly dosing and error bars indicate SEM ($n = 10$ for all groups). NOD/SCID, nonobese diabetic/severe combined immunodeficiency; SCLC, small-cell lung cancer.

method, the equivalent mouse dose corresponding to the clinical dose of 1.5 mg/m^2 (days 1–5, every 3 weeks) is $\sim 0.83 \text{ mg/kg/week}$ and should be divided between days 1 and 2, repeating every 7 days to result in a similar exposure time as the clinical regimen (29%/cycle for preclinical vs. 24%/cycle for clinical).

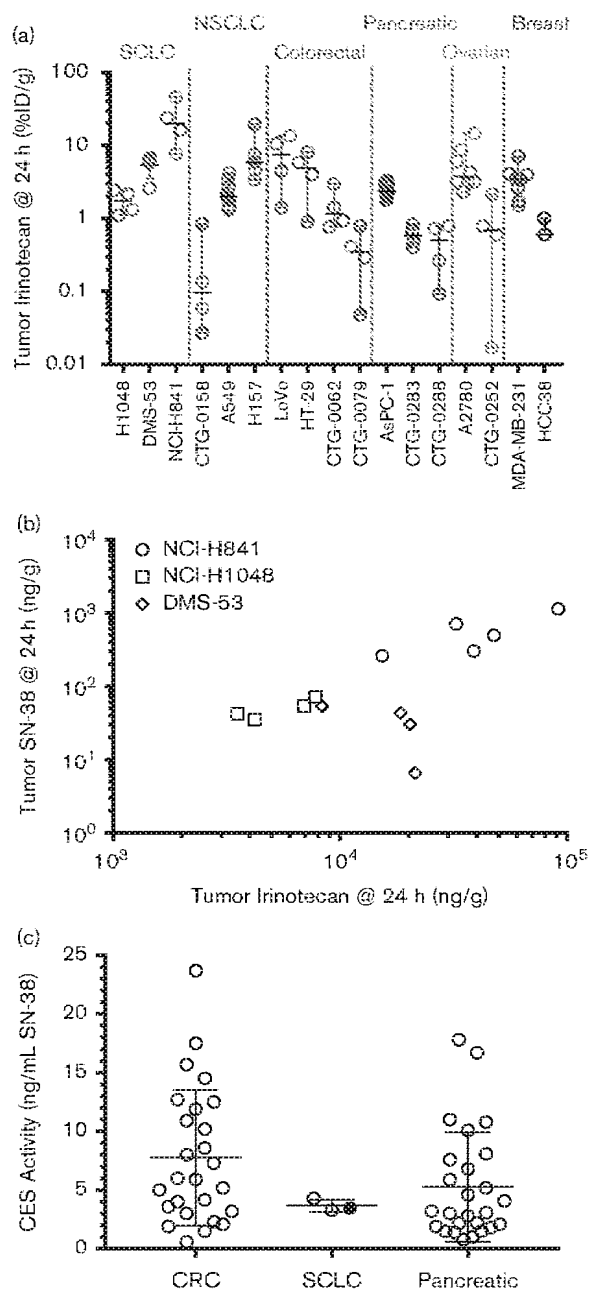
To explore the effect of dose, a higher dose (1.66 mg/kg/week divided over days 1 and 2) was also evaluated in the NCI-1048 model in addition to the clinically relevant dose. As seen in Fig. 2a and b, topotecan administered at 0.83 mg/kg/week split over days 1 and 2 was well tolerated and had modest antitumor activity, eliciting some growth delay but failing to control tumor growth. Doubling the dose, using the same schedule, improved antitumor activity and resulted in longer growth delay, but ultimately failed to control tumor growth. To test the effect of schedule on antitumor activity, the same total dose delivered as a bolus on day 1 was also evaluated in the DMS-114 model. As seen in Fig. 2c and d, tumor growth curves for mice treated with topotecan at 0.83 mg/kg/week delivered as a bolus were indistinguishable from those of the control mice; however,

a slight relative reduction in body weight was observed. Dividing the same total dose over 2 days demonstrated a trend toward improved antitumor activity and tolerability although the differences were not statistically significant and provided minimal delay in tumor growth.

Nal-IRI effectively delivers irinotecan and SN-38 to SCLC tumors

The ability of nal-IRI to deposit in tumors through leaky vasculature and deliver irinotecan and SN-38 was evaluated in SCLC cell line-derived xenograft models (NCI-H1048, DMS-114, NCI-H841) relative to other cell line-derived and PDX models of other tumor types. Nal-IRI was administered intravenously to mice bearing xenograft tumors. At 24 h after administration, mice were sacrificed and tumors were harvested. Irinotecan and SN-38 in tumors were measured using HPLC. Figure 3a demonstrates that tumors derived from SCLC cell lines have similar or higher levels of nal-IRI deposition, as assessed by irinotecan content, compared with other tumor types. Further, analysis of SN-38 levels indicates that increased irinotecan delivery was associated with increased levels of SN-38 (Pearson

Fig. 3



Delivery of irinotecan and SN-38 to SCLC xenograft tumors. (a) H841, NCI-H1048, and DMS-53 mouse xenograft tumors were excised 24 h after administration of nal-IRI and were processed into tumor lysates, followed by measurement of irinotecan levels using HPLC. Data were normalized to injected dose per tumor weight. Horizontal lines and associated error bars indicate the mean and SD. (b) Tumor irinotecan and tumor SN-38 levels, shown as ng of SN-38 per tumor weight, were measured using HPLC at 24 h after administration in three SCLC xenograft models [NCI-H841 (squares), NCI-H1048 (circles), DMS-53 (diamonds)]. (c) CES activity of patient-derived tumor xenografts of colorectal (CRC), SCLC, and of pancreatic origin and the H841 cell line-derived xenograft (filled circle) were evaluated by spiking irinotecan into tumor lysates and measuring the production of SN-38 by HPLC after incubation at 37°C for 24 h. CES, carboxylesterase; HPLC, high-performance liquid chromatography; SCLC, small-cell lung cancer; NSCLC, non-small-cell lung cancer.

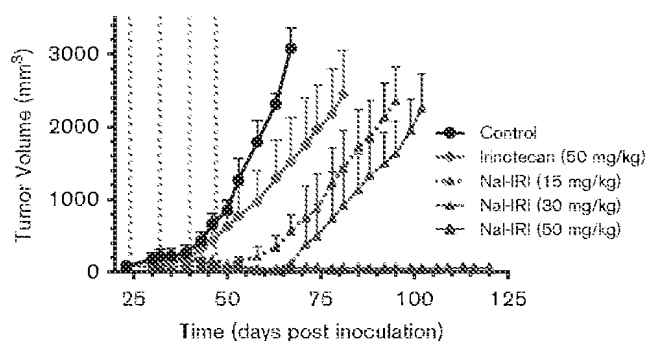
correlation = 0.62, $P = 0.02$; Fig. 3b). These findings are consistent with a proposed mechanism of liposome deposition and local conversion of irinotecan to SN-38 within the tumor [23].

Irinotecan is converted by CES to its active metabolite, SN-38, which is 100–1000× more active than its parent compound [21]. CES activity was measured by incubating tumor extracts with irinotecan and measuring SN-38 levels after 24 h using HPLC. Tumor grafts corresponding to the SCLC indication were extracted from the lung data set previously published [23] and are compared with colorectal and pancreatic tumor samples. As shown in Fig. 3c, SCLC tumors have CES activity comparable to that in gastrointestinal indications such as colorectal and pancreatic cancer, in which irinotecan has demonstrated clinical activity.

Nal-IRI has dose-dependent anti-tumor activity

The activity of nal-IRI as a single agent was investigated in H841 SCLC subcutaneous flank xenografts in female nude mice. Dosing started at day 24 after inoculation. Irinotecan, dosed at 50 mg/kg, yielded a tumor growth delay (defined as the time for tumor volumes to reach 1000 mm³) of about 8 days compared with controls, whereas nal-IRI, dosed at 15 mg/kg, yielded a tumor growth delay of ~25 days compared with controls. After dosing nal-IRI at 30 or 50 mg/kg, the greatest reductions in tumor volume were reached at 16 days after the last dose of nal-IRI. Tumors in mice dosed with 30 mg/kg nal-IRI steadily increased in size after the nadir, whereas tumors in mice dosed with 50 mg/kg nal-IRI maintained a small residual volume that was sustained for the duration

Fig. 4



Dose-dependent antitumor activity of nal-IRI. (a) Athymic nude mice bearing subcutaneous NCI-H841 tumor xenografts were treated with nal-IRI IV (squares) at 15 (dotted line), 30 (dashed line), or 50 (solid line) mg/kg starting on day 25 and given weekly at 4 doses, or irinotecan at 25 mg/kg at the same schedule (diamonds), or left untreated as controls (circles). Vertical dotted lines indicate days of dosing and error bars indicate SEM ($n = 5$ for all groups). Only upper error bars are drawn for clarity of presentation. Nal-IRI, liposomal irinotecan; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; SCLC, small-cell lung cancer.

of the study up to 120 days (Fig. 4). No obvious toxicities or weight loss was noted in the nal-IRI-treated groups.

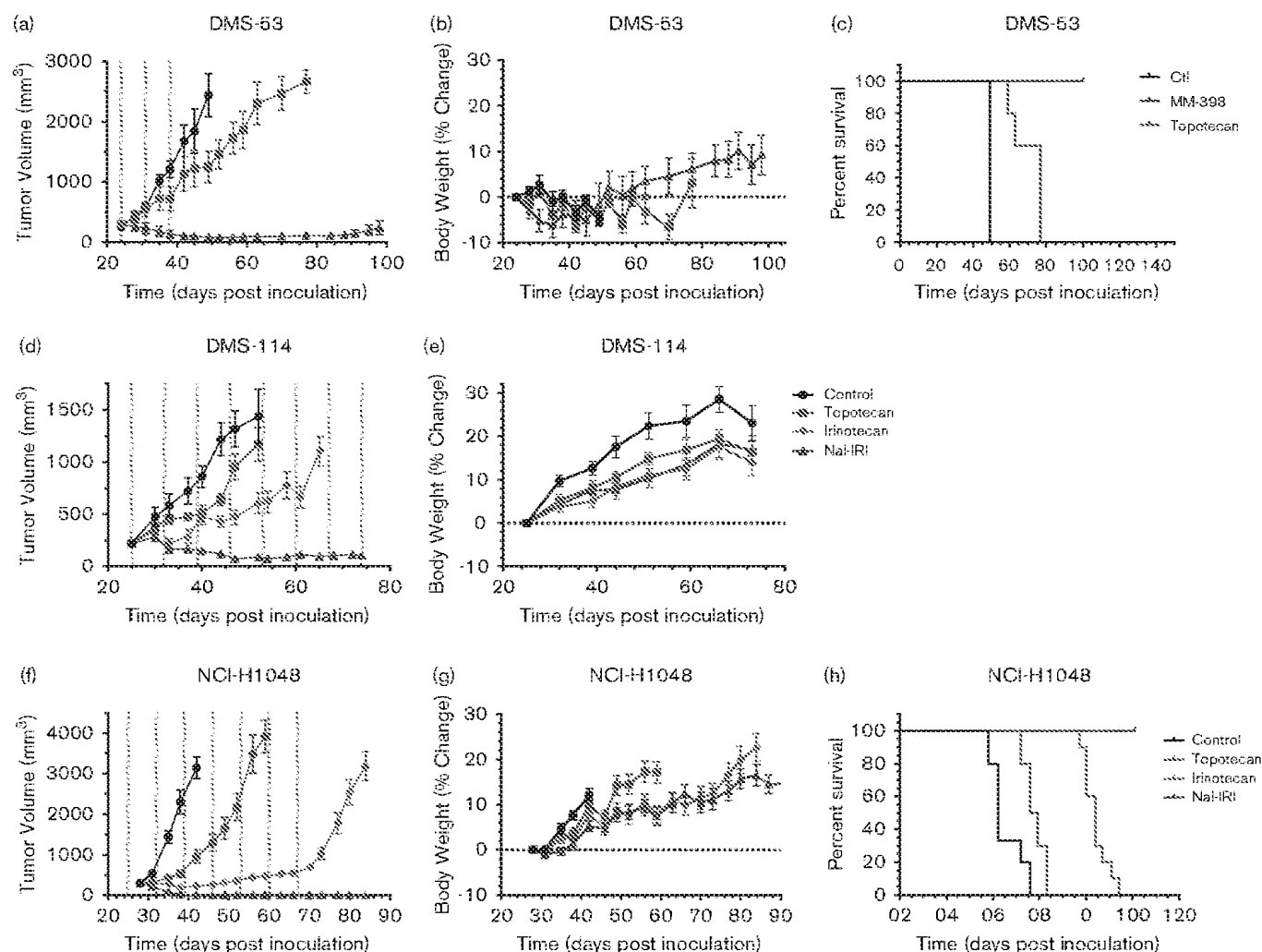
Nal-IRI improves antitumor activity compared with irinotecan and topotecan

The activity of nal-IRI, irinotecan, and topotecan were directly compared at clinically relevant doses in three cell line-derived models and three PDX models. Using the standard surface area to weight ratio conversion method as described above, the proposed clinical dose of nal-IRI (90 mg/m^2 free base, every 2 weeks) converted for mouse is 16 mg/kg salt every week. For irinotecan at a clinical dose of 300 mg/m^2 every 3 weeks, the equivalent mouse dose is $\sim 33 \text{ mg/kg/week}$. As discussed previously, for

topotecan at a clinical dose of 1.5 mg/m^2 (days 1–5, every 3 weeks), the equivalent mouse dose is $\sim 0.83 \text{ mg/kg/week}$, divided between days 1 and 2, repeating every 7 days.

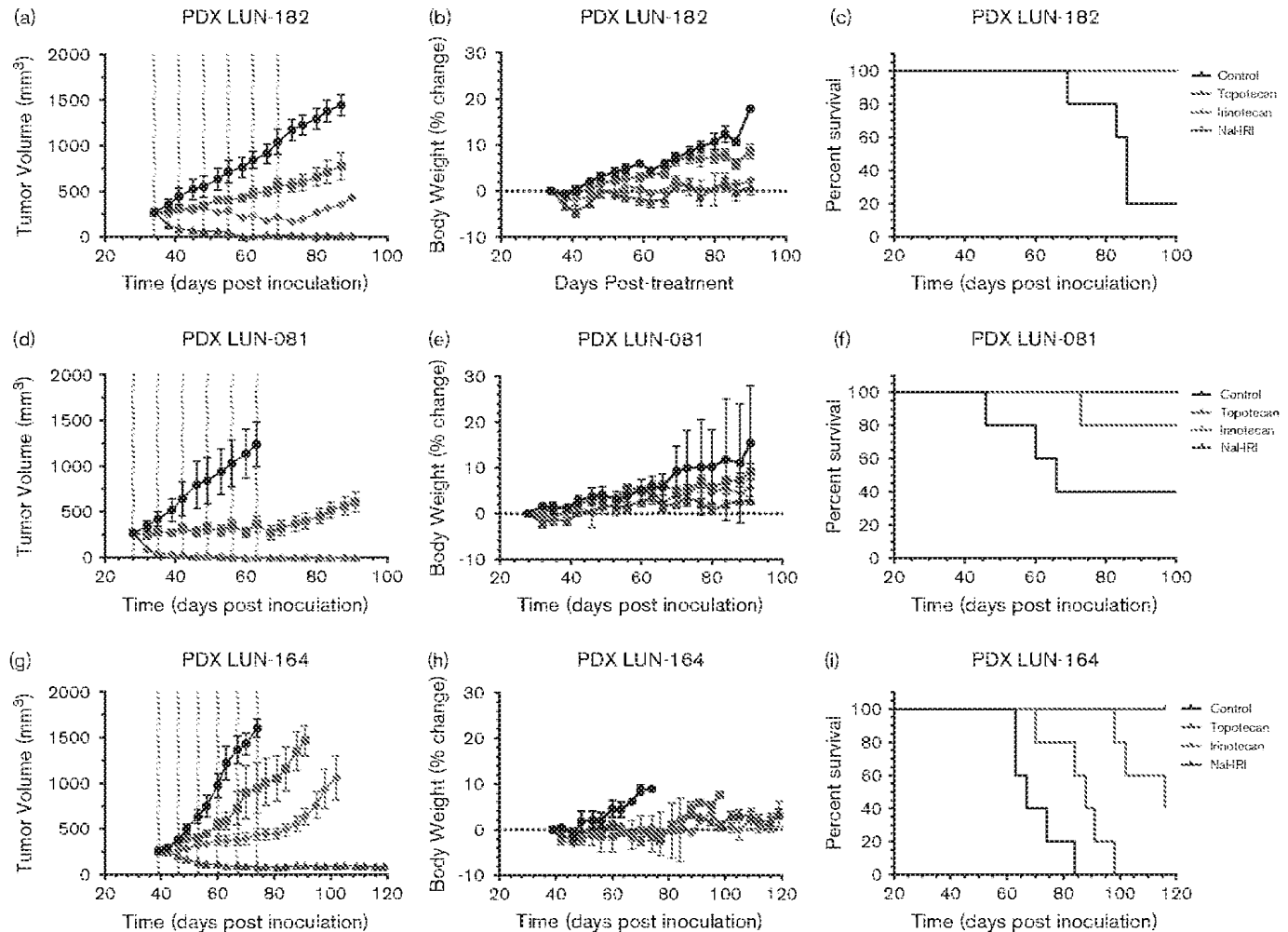
Figures 5 and 6 present tumor growth kinetics, body weight, and survival of mice bearing SCLC xenograft tumors treated weekly with nal-IRI, irinotecan, or topotecan at clinically relevant dose levels, described previously. In the DMS-53, DMS-114, and NCI-H1048 cell line-derived models, nal-IRI displayed significantly greater antitumor activity than topotecan (Fig. 5). In addition, comparison with irinotecan was also tested in DMS-114 and NCI-H1048, in which nal-IRI demonstrated significantly greater antitumor activity than

Fig. 5



Nal-IRI has greater antitumor activity than irinotecan and topotecan. NOD/SCID mice with subcutaneous DMS-53, DMS-114, or NCI-H1048 SCLC xenograft tumors were treated with intravenous (i.v.) nal-IRI (16 mg/kg ; triangles), i.v. irinotecan (33 mg/kg ; diamonds), intraperitoneal (i.p.) topotecan (0.83 mg/kg/week days 1–2; squares), or vehicle control (circles). Tumor growth curves, body weight, and survival are shown for DMS-53 (a–c), tumor growth curves and body weight for DMS-114 (d, e), and tumor growth curves, body weight, and survival are shown for NCI-H1048 (f–h). For DMS-114 and NCI-H1048, all groups have $n = 10$; for DMS-53 $n = 4, 5$, and 5 for control, topotecan, and nal-IRI, respectively. Balb/c nude mice bearing subcutaneous patient-derived xenografts. Nal-IRI, liposomal irinotecan; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; SCLC, small-cell lung cancer.

Fig. 6



Nal-IRI has greater antitumor activity than irinotecan and topotecan. Patient-derived xenografts LUN-182 (a-c), LUN-081 (d-f), and LUN-164 (g-i) were treated with intravenous (i.v.) nal-IRI (16 mg/kg; triangles), i.v. irinotecan (33 mg/kg; diamonds), intraperitoneal topotecan (0.83 mg/kg/week days 1-2; squares), or vehicle control (circles). Tumor growth curves (a, d, g), body weight (b, e, h), and survival (c, f, i) are shown. For all PDX models $n = 5$ for all groups. Vertical dotted lines indicate the start of weekly dosing and error bars indicate the SEM. Nal-IRI, liposomal irinotecan; PDX, patient-derived xenograft.

irinotecan ($P < 0.0001$ for DMS-114 on day 52 and $P < 0.0001$ for NCI-H1048 on day 59; nonparametric t -test) and topotecan ($P < 0.0001$ for DMS-114 on day 65 and $P < 0.0001$ for NCI-H1048 on day 84; nonparametric t -test). Furthermore, 10 out of 10 NCI-H1048-bearing mice treated with nal-IRI experienced complete regressions of their tumors compared with 0 out of 10 mice treated with topotecan. All treatments were well tolerated in all models and survival data reflected observed differences in tumor growth curves in the DMS-53 and NCI-H1048 models. Nal-IRI, irinotecan, and topotecan were also evaluated in three PDX models with a similar experimental design (Fig. 6). In all three models, nal-IRI showed significantly greater antitumor activity than did topotecan ($P < 0.0001$ for LUN-182 on day 87, $P < 0.0001$ for LUN-081 on day 91, and $P < 0.0001$ for LUN-164 on day 91; nonparametric t -test), with nal-IRI resulting in

tumor regression in all models, whereas treatment with topotecan resulted in tumor stasis or modest growth delay. In two out of the three models, nal-IRI showed greater antitumor activity than irinotecan and resulted in tumor shrinkage and sustained tumor control past the final week of dosing ($P < 0.0001$ for LUN-182 on day 90 and $P < 0.0001$ for LUN-164 on day 102; nonparametric t -test). By contrast, treatment with irinotecan resulted in tumor stasis during treatment, and then tumor growth following the end of treatment. In the LUN-081 PDX model (Fig. 6d), nal-IRI and irinotecan had comparable antitumor activity, resulting in durable tumor regressions that persisted beyond the final week of treatment. In this model, topotecan also demonstrated the greatest control of tumor growth during the treatment period, suggesting that LUN-081 may be inherently more sensitive to TOP1 inhibitors. This was further reflected in the

corresponding survival data in Fig. 6f. All treatments were well tolerated by the mice, as reflected in the body weight data. Survival data reflected observed differences in tumor growth. The LUN-164 model demonstrated the fastest tumor growth rates in the absence of any treatment and was also the least responsive to either topotecan or irinotecan; this was the only tumor model for which survival curves based on humane endpoints were able to differentiate treatments over the time scale of the experiment, whereas insufficient events were observed in the LUN-182 and LUN-081 models.

Nal-IRI has activity after failure of other TOP1 inhibitors

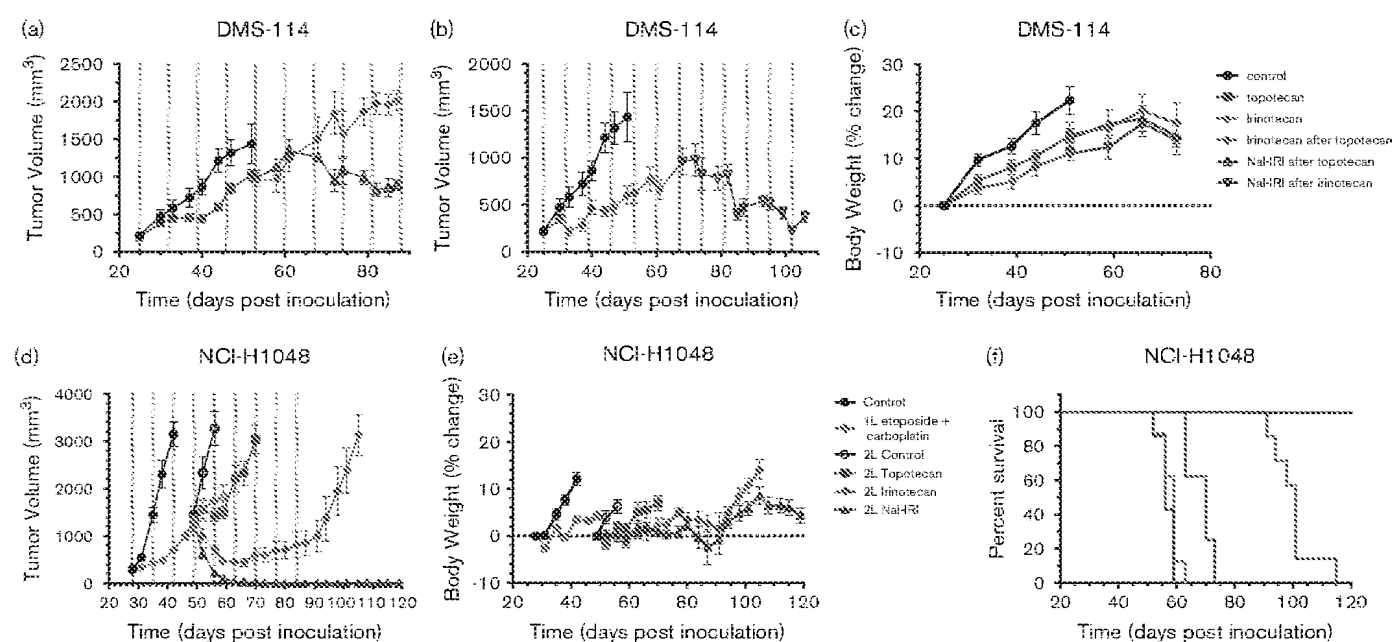
We next sought to further evaluate the hypothesis that the antitumor activity of topotecan and irinotecan are limited by their ability to provide sustained inhibition of TOP1. Mice bearing DMS-114 tumor xenografts that had been treated with topotecan (0.83 mg/kg/week split over days 1–2 for 4-weekly cycles) and failed to respond to treatment were randomized to treatment with either irinotecan or nal-IRI. Despite not responding to TOP1 inhibition by topotecan and having a comparatively large average tumor size of $\sim 1000 \text{ mm}^3$, mice receiving nal-IRI showed significant tumor shrinkage whereas mice receiving irinotecan failed

to respond (Fig. 7a). These data are consistent with the hypothesis that the antitumor activity of topotecan may be limited by insufficient delivery rather than by resistance to TOP1 inhibition. Similarly, nal-IRI was also evaluated in mice bearing DMS-114 tumor xenografts that had been first treated with irinotecan. As with topotecan, the tumors failed to respond to 4-weekly treatments with irinotecan, but showed significant tumor shrinkage following the switch to nal-IRI (Fig. 7b). These results are further consistent with the schedule-dependent efficacy of TOP1 inhibitors and support the reasoning that sustained delivery of SN-38 through nal-IRI may result in increased antitumor activity.

Nal-IRI has activity in the second-line setting

Finally, the activities of nal-IRI, irinotecan, and topotecan were directly compared in a second-line setting of SCLC. Mice bearing NCI-H1048 SCLC tumors were treated with carboplatin plus etoposide, a first-line regimen in SCLC. Once the tumors escaped growth control by carboplatin plus etoposide (tumor average 1320 mm^3), mice were randomized to either continue treatment with carboplatin plus etoposide or switched to second-line treatment with either nal-IRI, irinotecan, or topotecan. All treatment sequences

Fig. 7



Nal-IRI has activity in the second-line setting. NOD/SCID mice bearing subcutaneous DMS-114 xenograft tumors were treated with topotecan (0.83 mg/kg/week days 1–2; $n=9$; squares), irinotecan ($n=10$; diamonds), or left untreated (control; $n=10$; circles). Upon failure of topotecan (a; day 53) mice were randomized to receive irinotecan (33 mg/kg; $n=5$; diamonds) or nal-IRI (16 mg/kg; $n=4$; triangles). Upon failure of irinotecan (b; day 67), mice were switched to nal-IRI (16 mg/kg; $n=10$; triangles). (c) Body weight is shown for animals corresponding to (a) and (b). NOD/SCID mice with NCI-H1048 SCLC xenograft tumors were treated weekly with the combination of 30 mg/kg carboplatin plus 25 mg/kg etoposide (green diamonds), or with vehicle control (filled circles). When tumors reached $\sim 1320 \text{ mm}^3$, mice were randomized to receive weekly treatment with topotecan (1.66 mg/kg/week administered intraperitoneally in equal fractions on days 1 and 2; squares), irinotecan [33 mg/kg/week administered intravenous (i.v.) on day 1; red-filled diamonds], nal-IRI (16 mg/kg/week administered i.v. on day 1; triangles), continued treatment with carboplatin plus etoposide (open diamonds), or vehicle control (open circles). Tumor growth curves (d), body weight (e), and survival data (f) are shown. Vertical dotted lines indicate the start of weekly dosing and error bars indicate the SEM. Nal-IRI, liposomal irinotecan; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; SCLC, small-cell lung cancer.

were well tolerated as reflected by body weight, as shown in Fig. 7b. As shown in Fig. 7d, nal-IRI displayed significant antitumor activity compared with topotecan and irinotecan after tumors progressed on first-line treatment with carboplatin plus etoposide ($P=0.0002$ on day 70 and $P=0.0002$ on day 84 for topotecan and irinotecan, respectively). All treatments were well tolerated as reflected by body weight (Fig. 7e). The median survival from the initiation of second-line treatment was 7 days with vehicle control, 10 days with continued etoposide + carboplatin, 21 days with topotecan, and 59 days with irinotecan. All tumors treated with second-line nal-IRI completely regressed with a median time to complete regression of 24 days. Improved antitumor activity of nal-IRI versus irinotecan or topotecan also resulted in increased survival of mice [$P<0.0001$ and <0.0001 for comparison with irinotecan and topotecan, respectively; log-rank test (Mantel–Cox)], as shown in Fig. 7f.

Discussion

In this study, we provide further evidence of the schedule-dependent efficacy of the TOP1 inhibitors topotecan and irinotecan with its active metabolite SN-38 in SCLC. The in-vitro data suggest that time of inhibition is as important as concentration in determining the extent of cytotoxicity. Moreover, we demonstrate that the in-vivo activity of topotecan appears to be limited by the dose level and pharmacokinetics that result in its inability to provide sustained inhibition of TOP1. By contrast, we hypothesize that nal-IRI is able to provide sustained TOP1 inhibition and subsequent significant antitumor activity through the extended circulation afforded by liposomal encapsulation, specific accumulation in tumors, and the local conversion of irinotecan to SN-38. We provide data demonstrating effective liposomal delivery of irinotecan to tumors of SCLC origin and subsequent conversion of irinotecan to SN-38. In direct head-to-head comparisons, taking careful consideration of scaling of doses from standard clinical doses, nal-IRI displayed significantly greater antitumor activity than both irinotecan and topotecan in cell line-derived and patient-derived xenograft models. Further, complete regressions and/or sustained antitumor activity of nal-IRI for multiple weeks beyond the last dose were observed in multiple cell line-derived and patient-derived xenograft models. The activity of nal-IRI after failure of traditional TOP1 inhibitors irinotecan and topotecan is consistent with the premise that the activity of these agents is, in part, limited by their ability to provide sustained inhibition of TOP1.

The dose level of topotecan used in this study was specifically chosen to mimic the clinically approved dosing and regimen of 1.5 mg/m^2 administered on days 1–5, every 3 weeks. Given the shorter cycle length of 1 week used in mouse studies, the 0.83 mg/kg dose was divided over 2 days to provide more sustained exposure. The importance of dosing on multiple days has been observed by

others [33] and is emphasized by the improved growth inhibition of DMS-114 tumors by 2-day versus single-day dosing Fig. 2. This is in contrast with other studies in which higher single-day doses were used ($5\text{--}10 \text{ mg/kg}$) [34–36]. Limits of detection of the analytical methods may further provide impetus for use of dose levels higher than those that are clinically relevant. Assuming a typical mouse blood volume of 1 ml, the single-day dose used in our studies has a theoretical C_{max} of nearly $20 \mu\text{mol/l}$ and is still well above the 70 nmol/l C_{max} observed in patients [14].

Human plasma C_{max} values for topotecan [14] do not exceed the IC_{50} values for many cell lines [15,16]. Coupled with rapid clearance, this suggests that many patient tumors would be unlikely to receive saturating doses of topotecan, which would thus result in conditions permissive for continued cell growth. Consequently, tumor relapse following treatment with topotecan may be a result of insufficient inhibition, in addition to, and potentially contributing to, possible evolution of resistance. The ability to achieve higher local concentrations of TOP1 inhibition by liposomal delivery and local conversion of irinotecan to SN-38 may enable killing of a wide spectrum of tumor cells, including those with greater intrinsic levels of drug resistance. This is supported by the observations of complete regressions and sustained antitumor activity after the cessation of dosing in multiple efficacy studies (Fig. 5). Additional work to explore the capacity of nal-IRI in killing specific cell subpopulations, including cancer stem cells, and study of resistance mechanisms are areas of future interest.

There is comparatively little clinical study of liposomal agents in patients with lung cancer. A small phase 2 study of pegylated liposomal doxorubicin (PLD) in patients with recurrent SCLC concluded that PLD had limited activity [37] and the combination of PLD with vincristine and cyclophosphamide had modest activity in a subsequent phase 2 study as well [38]. Previous studies have suggested that doxorubicin-based combinations are rarely active after failure of platinum plus etoposide failure [37]. These studies, however, leave unanswered the question of the potential contribution of liposomal delivery of a TOP1 inhibitor, such as irinotecan.

The preclinical findings presented here support the potential for effective liposomal drug delivery to human tumors *in vivo*. Clinical reports of macrophage infiltration in tumors from patients with SCLC [28,29] fit well with the proposed predominant mechanism of action of nal-IRI involving cellular uptake and drug conversion. Preclinical data demonstrating SN-38 levels in xenograft tumors of SCLC origin, presumably mediated by CES activity in macrophages, suggest sufficient capacity of SCLC tumors for liposome uptake and local production of SN-38. Collectively, these data provide mechanistic rationale and support for a clinical study of nal-IRI versus topotecan in patients with previously treated SCLC. Evaluation of a

combination of nal-IRI with platinum agents for the treatment of patients with SCLC in the first-line setting is also warranted. Nal-IRI is not labeled for use in SCLC and the product is still investigational in that setting.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

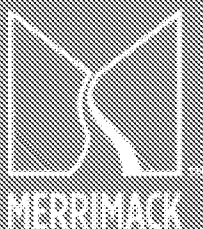
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Irinotecan Liposome Injection has Greater Anti-Tumor Activity than Topotecan and Irinotecan in Mouse Models of Small Cell Lung Cancer

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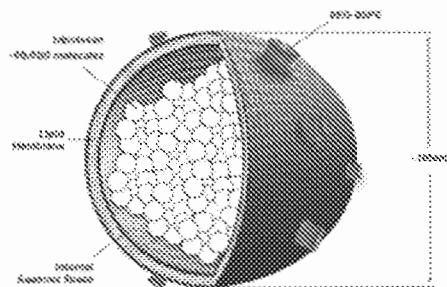
Abstract

Irinotecan Liposome Injection (nal-IRI, MM-398), a liposomal formulation of irinotecan, is designed for extended circulation relative to non-liposomal irinotecan and to exploit leaky tumor vasculature for enhanced drug delivery to tumors. Following tumor deposition, nal-IRI is taken up by phagocytic cells and subsequently irinotecan is released from liposomal particles and converted to its active metabolite, SN-38. Sustained inhibition of topoisomerase 1 (TOP1) by extended SN-38 exposure is hypothesized to enable superior anti-tumor activity compared to traditional TOP1 inhibitors. Topotecan, another TOP1 inhibitor, is an approved second-line treatment option for small cell lung cancer (SCLC). Here, we evaluate the anti-tumor activity of nal-IRI compared to irinotecan and topotecan in preclinical models of SCLC including those that have been pre-treated with therapeutics used clinically to treat SCLC.

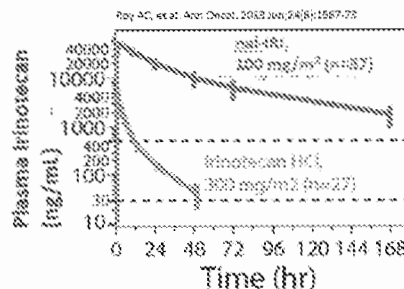
Anti-tumor activity of nal-IRI, irinotecan, or topotecan was evaluated based on tumor volume assessments in DMS-53, DMS-114 and NCI-H1048 subcutaneous xenograft models in NOD-SCID mice, as well as in a patient-derived xenograft (PDX) model in nu/nu mice. To approximate clinical dosing: Nal-IRI was dosed at 16 mg/kg (irinotecan HCl basis), q1w, equivalent to a proposed clinical dose of 90 mg/m² free base, q2w; irinotecan was dosed at 33 mg/kg q1w, equivalent to a clinical dose of 180 mg/m² q2w; and topotecan was dosed at 0.83 mg/kg/week, day 1-2 every 7 days, which approximates a clinical dose intensity of 1.5 mg/m² (days 1-5 every 21 days). Additionally, the activity of these agents was evaluated after tumors progressed following prior treatment with either topotecan, irinotecan or the combination of weekly carboplatin (30 mg/kg) plus etoposide (25 mg/kg), a standard first line regimen.

Nal-IRI demonstrated anti-tumor activity in xenograft models of SCLC at clinically relevant dose levels, and resulted in complete or partial responses in DMS-53, DMS-114, NCI-H1048 and a PDX model in comparison with irinotecan or topotecan, which each had limited tumor growth control. Furthermore, Nal-IRI demonstrated anti-tumor activity in tumors that progressed following treatment with topotecan, and demonstrated significantly greater anti-tumor activity than both topotecan ($p < 0.0001$) and irinotecan ($p < 0.0001$) in NCI-H1048 tumors (8/8 complete responses) that had progressed on prior carboplatin plus etoposide treatment. These results support the further clinical development of nal-IRI versus IV topotecan in patients with SCLC that progressed on or after prior platinum containing therapy.

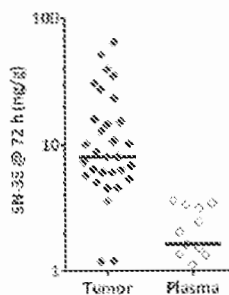
Irinotecan Liposome Injection (nal-IRI, MM-398)



Nal-IRI comprises irinotecan encapsulated in liposomal particles.



The half-life of total irinotecan and total SN-38 following administration of nal-IRI, 70 mg/m² free base (equivalent to 80 mg/m² of irinotecan as the hydrochloride trihydrate) was 25.8 and 67.8 hours, respectively in patients with gastric cancer.



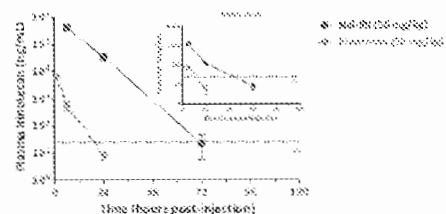
Preliminary data from a pilot study across different cancer types included metabolite profiling of tumor biopsies 72 h post nal-IRI administration indicated 5-fold higher SN-38 levels in tumor than plasma. (Ramangtham, et al. EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics 2014 #261)

Proposed Mechanism of Action

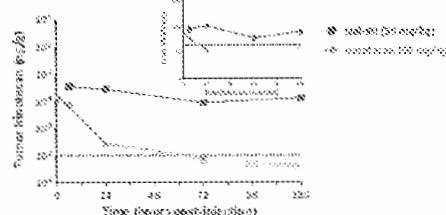
- Extended PK
- Deposition in tumor via leaky vasculature
- Uptake by phagocytic cells and tumor cells
- Conversion of irinotecan to SN-38
- Topoisomerase 1 inhibition in tumor cells leading to cell death



Plasma



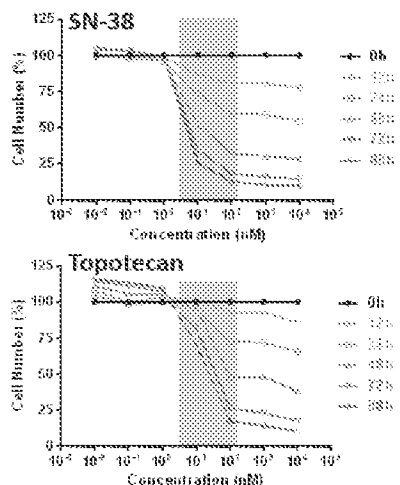
Tumor



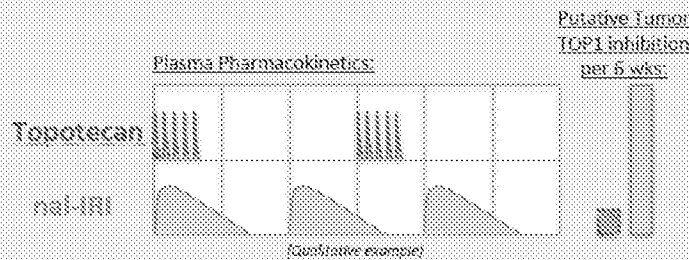
nal-IRI increases exposure and sustains delivery of irinotecan and SN-38 (active metabolite) in HsPC-3 mouse xenograft tumors.

Pharmacology of TOP1 Inhibition

Cytotoxicity of TOP1 Inhibitors Increases with Exposure



Pharmacology of Topotecan and nal-IRI

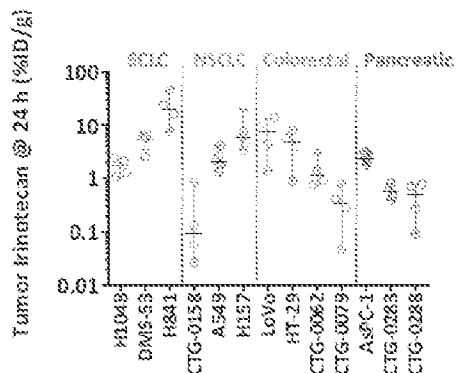


Topotecan is dosed on days 1-5 on an every 3 week schedule and nal-IRI is dosed on day 1 every 2 weeks. Shown here are approximate plasma pharmacokinetics and the subsequent TOP1 inhibition expected in the tumor.

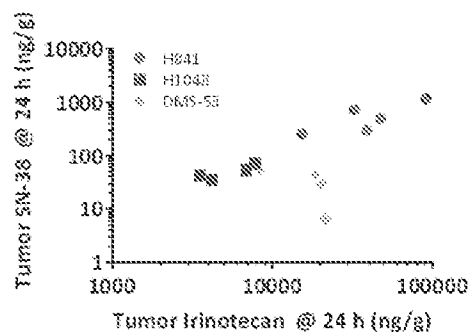
In vitro cell death of DdS-114 cells increased with concentration and time following treatment with SN-38, the active metabolite of irinotecan or topotecan. The range of SN-38 detected in tumor biopsies (grey box) from a previous clinical study (Ramanathan, 2014) in patients with various solid tumors is overlaid on the time-dependent SN-38 growth inhibition curves.

nal-IRI Mediated Tumor Delivery of SN-38

nal-IRI effectively delivers irinotecan to tumors in mouse models of SCLC



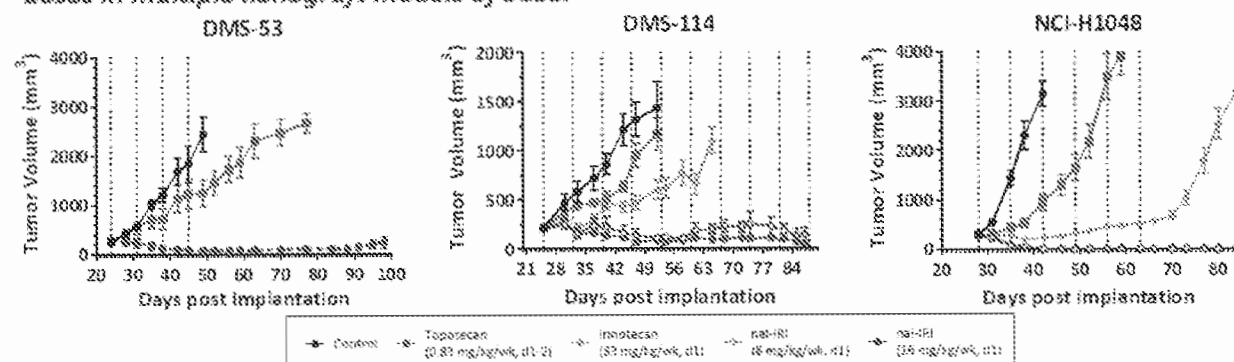
Increased tumor SN-38 levels were associated with increased tumor deposition of nal-IRI



Mice bearing tumor xenografts, as indicated, were treated with nal-IRI and tumors harvested 24 h post administration. Tumor levels of irinotecan and SN-38 were measured by HPLC.

nal-IRI Anti-Tumor Activity in Preclinical Models of SCLC

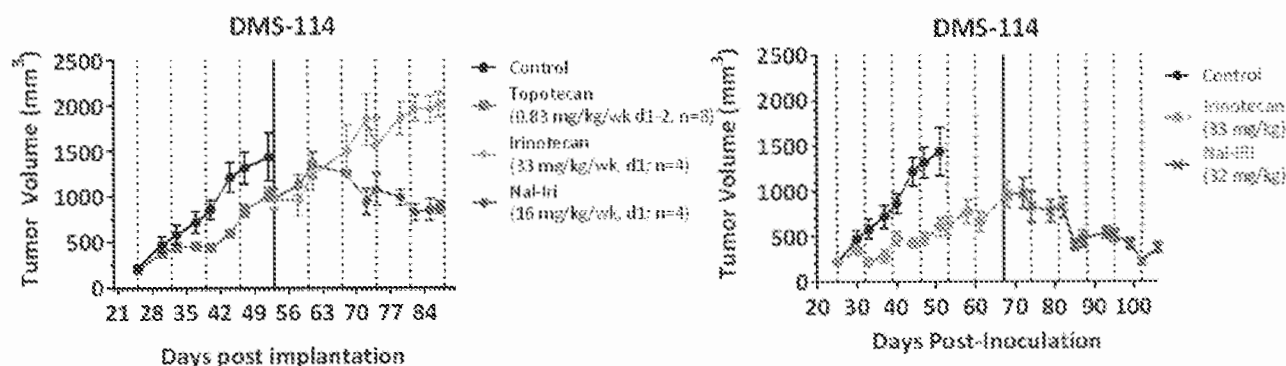
nal-IRI has greater anti-tumor activity than irinotecan and topotecan at clinically relevant doses in multiple xenograft models of SCLC.



NOD/SCID mice bearing subcutaneous xenografts, as indicated, were used to evaluate the anti-tumor activity of topotecan, irinotecan and nal-IRI. Clinically relevant doses were calculated by using standard surface area to weight ratio conversion per NCI guidelines. Using this method, the proposed clinical dose of nal-IRI (80 mg/m² free base, q2w) converted for mouse is 16 mg/kg sub q1w. For non-ileosarcoma irinotecan at a clinical dose of 120 mg/m² q2w, the equivalent mouse dose is approximately 33 mg/kg/week, for topotecan at a clinical dose of 1.5 mg/m² (D1-5, q2w), the equivalent mouse dose is approximately 0.83 mg/kg/week; the preclinical dosing schedule was divided between days 1 and 2 repeating every 7 days which result in a similar exposure time as the clinical regimen (25% per cycle for preclinical vs. 24% per cycle for clinical). Vertical lines indicate start of weekly dosing. Error bars indicate standard error of the mean. For all groups, n=10, except DMS-53 (n=4, 5 and 5 for control, topotecan and nal-IRI, respectively).

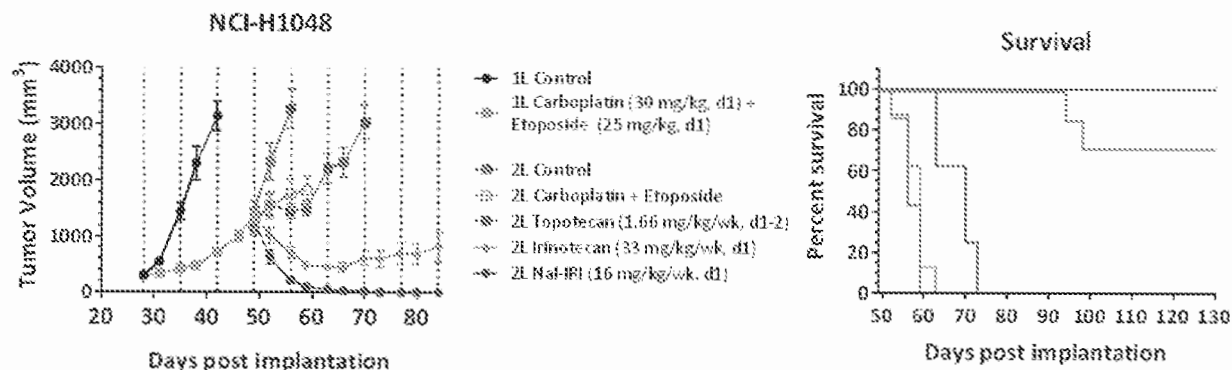
nal-IRI Anti-Tumor Activity in Preclinical Models of SCLC (cont.)

nal-IRI has anti-tumor activity after failure of topotecan or irinotecan.



NOD/SCID mice bearing subcutaneous DMS-114 xenograft tumors were treated with topotecan (n=9), irinotecan (n=10) or untreated (control; n=10). Upon failure of topotecan (left; day 53) mice were randomized to receive irinotecan (n=5) or nal-IRI (n=4). Upon failure of irinotecan (right; day 57), mice were switched to nal-IRI (n=10). Vertical lines indicate start of weekly dosing. Error bars indicate standard error of the mean.

nal-IRI has greater anti-tumor activity and survival than irinotecan and topotecan after failure of 1st line carboplatin + etoposide.



NOD/SCID mice bearing subcutaneous NCI-H1048 xenograft tumors were treated with carboplatin (30 mg/kg) + etoposide (25 mg/kg) (n=40) or untreated (1L control; n=10). Upon failure of carboplatin + etoposide (day 43), mice were randomized to receive no treatment (2L control; n=8), carboplatin + etoposide (n=8), topotecan (n=8), irinotecan (n=7) or nal-IRI (n=2). Vertical lines indicate start of weekly dosing. Error bars indicate standard error of the mean.

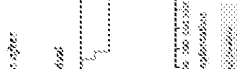
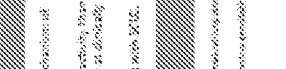
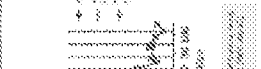
Summary

- The biology of SCLC shares features that are well-matched with the proposed mechanism of action for nal-IRI although several features merit further investigation in patients.
- nal-IRI has anti-tumor activity in multiple models of SCLC and greater anti-tumor activity than both irinotecan and topotecan in a preclinical models of SCLC when administered at clinically relevant doses.
- These preclinical studies support the rationale for evaluation of nal-IRI in patients with SCLC.

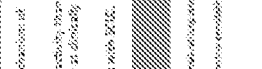
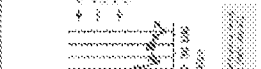
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- Ramanathan, et al. EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics 2014 #261

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P1.07-006

Preclinical Support for Evaluation of Irinotecan Liposome Injection (nal-IRI, MM-398) in Small Cell Lung Cancer



Topic: Drug Treatment Alone and in Combination with Radiotherapy

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Merrimack Pharmaceuticals, Inc., Cambridge/MA/United States of America

Background: Nal-IRI (irinotecan liposome injection, MM-398, ONIVYDE®), in combination with 5-fluorouracil and leucovorin, is currently approved by the FDA for treatment of patients with advanced pancreatic cancer who have progressed on gemcitabine-based therapies. Nal-IRI is designed for extended circulation relative to non-liposomal irinotecan and to exploit leaky tumor vasculature for enhanced drug delivery to tumors. Following tumor deposition, nal-IRI is taken up by phagocytic cells followed by irinotecan release and conversion to its active metabolite SN-38 in the tumors. Sustained inhibition of topoisomerase 1 (TOP1) by extended SN-38 delivery is hypothesized to enable superior anti-tumor activity compared to traditional TOP1 inhibitors. Topotecan, a TOP1 inhibitor, is currently a standard of care for second-line treatment of small cell lung cancer (SCLC). Here, we evaluate nal-IRI compared to topotecan in preclinical models of SCLC.

Methods: Anti-tumor activity of nal-IRI as a monotherapy was evaluated in DMS-53 and NCI-H1048 xenograft models. Cells were implanted subcutaneously into right flanks of NOD-SCID mice; treatments were initiated when tumors had reached approximately 280 mm³. Nal-IRI was dosed at 16 mg/kg salt, q1w, which is equivalent to a proposed clinical dose of 90 mg/m² free base, q2w. Topotecan was dosed at 0.83 mg/kg/week, Day 1-2 every 7 days, which approximates a clinical dose intensity of 1.5 mg/m² (Day 1-5 every 21 days). Tumor metabolite levels were compared in mice treated with nal-IRI or non-liposomal irinotecan at 24 hours post-injection, using previously established high performance liquid chromatography methods.

Results: Carboxylesterase activity and sensitivity to SN-38 in mouse SCLC models were comparable to those measured in tumor types where either nal-IRI or irinotecan HCl has proven to be efficacious clinically (e.g. pancreatic cancer, colorectal cancer). Nal-IRI delivered irinotecan to SCLC tumors to a similar or greater extent than other tumor types including pancreatic tumors. The tumor irinotecan and SN-38 levels of nal-IRI (16 mg/kg

salt) were 12 to 57-fold and 5 to 20-fold higher than non-liposomal irinotecan (30 mg/kg salt), respectively. Nal-IRI demonstrated anti-tumor activity in both xenograft models of SCLC at clinically relevant dose levels, and resulted in complete or partial responses after 4 cycles in NCI-H1048 or DMS-53 models, respectively, compared with topotecan which had limited tumor growth control.

Conclusion: This study demonstrated that nal-IRI is more active than topotecan at clinically relevant doses in SCLC preclinical models, and thus support further clinical development of nal-IRI versus topotecan in patients with SCLC that have progressed on prior platinum-based therapy.

Keywords: small cell lung cancer treatment, irinotecan liposome, MM-398, topoisomerase 1 inhibitor

P1.07-007

Clinical Outcomes of Patients with LS-SCLC Treated with Chemoradiotherapy. Can We Find Candidates for Salvage Surgery?



Topic: Drug Treatment Alone and in Combination with Radiotherapy

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¹Thoracic Oncology, Aichi Cancer Center Hospital, Nagoya/Japan, ²Thoracic Surgery, Aichi Cancer Center Hospital, Nagoya/Japan, ³Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Nagoya/Japan

Background: Although small cell lung cancer (SCLC) is generally considered a systemic disease even in patients with limited stage (LS). Selected recurrent LS-SCLC patients after chemoradiation treatment have been reported long survival with receiving salvage surgery. Purpose of this study was to find candidates for salvage surgery.

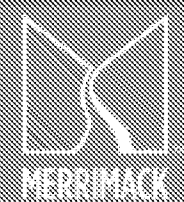
Methods: We retrospectively reviewed the charts of 43 consecutive patients who were treated with chemoradiotherapy for LS-SCLC at our hospital from January 2011 to December 2015 to search for the patients with locoregional progression without mediastinal lymph node involvement.

Results: Of the 43 patients, the median age was 69 (38-83), 91% were male and all of them had ECOG PS 0 or 1. Clinical stage: IIA (12%), IIIA (53%), IIIB (35%). 35 (81%) received hyperfractionated RT (45Gy/30fr/3w).

Preclinical Support for Evaluation of Irinotecan Liposome Injection (nal-IRI, MM-398) in Small Cell Lung Cancer

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¹Merrimack Pharmaceuticals, Inc., Cambridge, MA, USA, ²Division of Medical Oncology, University of Colorado, Denver, CO, USA

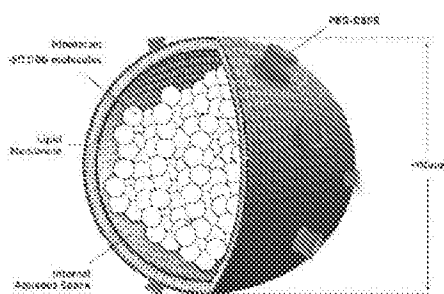


Introduction

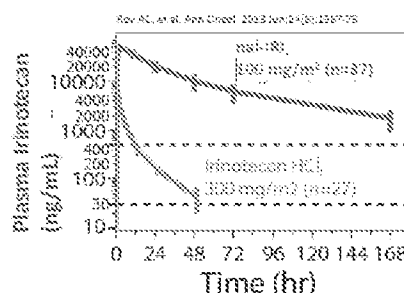
Background: Nal-IRI (irinotecan liposome injection, MM-398, ONIVYDE®), in combination with 5-fluorouracil and leucovorin, is currently approved by the FDA for treatment of patients with advanced pancreatic cancer who have progressed on gemcitabine-based therapies. Nal-IRI is designed for extended circulation relative to non-liposomal irinotecan and to exploit leaky tumor vasculature for enhanced drug delivery to tumors. Following tumor deposition, nal-IRI is taken up by phagocytic cells followed by irinotecan release and conversion to its active metabolite SN-38 in the tumors. Sustained inhibition of topoisomerase 1 (TOP1) by extended SN-38 delivery is hypothesized to enable superior anti-tumor activity compared to traditional TOP1 inhibitors. Topotecan, a TOP1 inhibitor, is currently a standard of care for second-line treatment of small cell lung cancer (SCLC). Here, we evaluate nal-IRI compared to topotecan in preclinical models of SCLC.

Objectives: The objectives of this study were to evaluate the ability of nal-IRI to deliver irinotecan and SN-38 to tumors and evaluate the subsequent anti-tumor activity of nal-IRI in comparison to irinotecan and topotecan at clinically relevant dose levels in mouse xenograft models of SCLC.

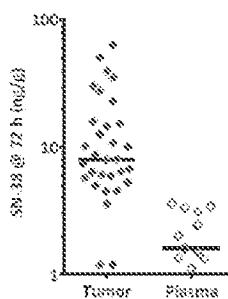
Nanoliposomal irinotecan (nal-IRI, MM-398)



Nal-IRI comprises irinotecan free base encapsulated in liposomal particles.



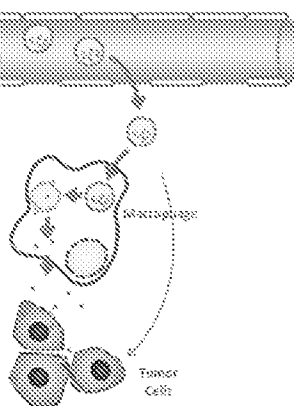
The half-life of total irinotecan and total SN-38 following administration of nal-IRI, 70 mg/m² free base (equivalent to 80 mg/m² of irinotecan as the hydrochloride trihydrate) was 25.8 and 67.8 hours, respectively.



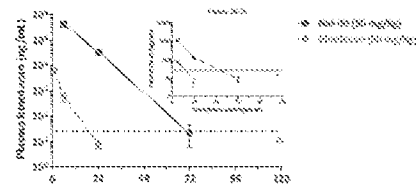
Metabolite profiling of tumor biopsies 72 h post nal-IRI administration indicated 5-fold higher SN-38 levels in tumor than plasma. (Ramanathan, et al. EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics 2014 #261)

Proposed Mechanism of Action

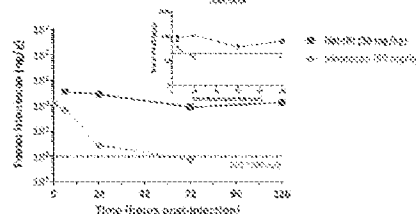
- ① Extended PK
- ② Deposition in tumor via leaky vasculature
- ③ Uptake by phagocytic cells and tumor cells
- ④ Conversion of irinotecan to SN-38
- ⑤ Topoisomerase 1 inhibition in tumor cells leading to cell death



Plasma



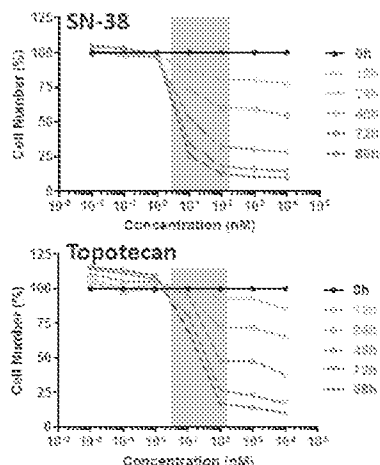
Tumor



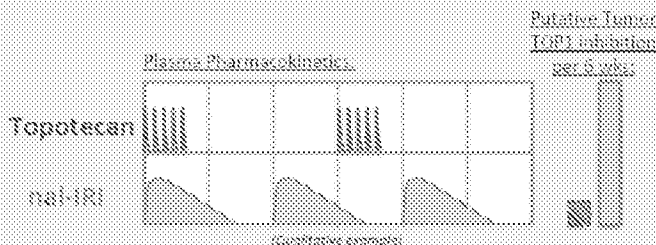
nal-IRI increases exposure and sustains delivery of irinotecan and SN-38 (active metabolite) in ExPC 2 mouse xenograft tumors.

Pharmacology of TOP1 Inhibition

Cytotoxicity of TOP1 Inhibitors Increases with Exposure



Pharmacology of Topotecan and Nal-IRI

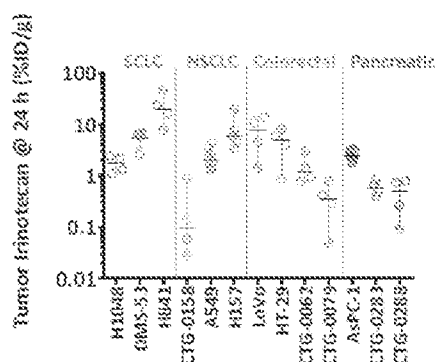


Topotecan is dosed on days 1-5 on an every 3 week schedule and nal-IRI is dosed on day 1 every 3 weeks. Shown here are approximate plasma pharmacokinetics and the subsequent TOP1 inhibition expected in the tumor.

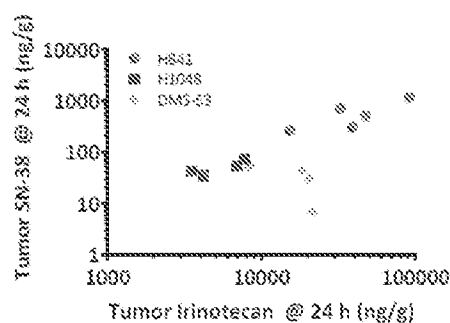
In vitro cell death of DMS-114 cells increased with concentration and time following treatment with SN-38, the active metabolite of irinotecan or topotecan. The range of SN-38 detected in tumor biopsies (gray box) from a previous clinical study (Iliyanathan, 2014) in patients with various solid tumors is overlaid on the time-dependent SN-38 growth inhibition curves.

nal-IRI Mediated Tumor Delivery of SN-38

nal-IRI effectively delivers irinotecan to tumors in mouse models of SCLC



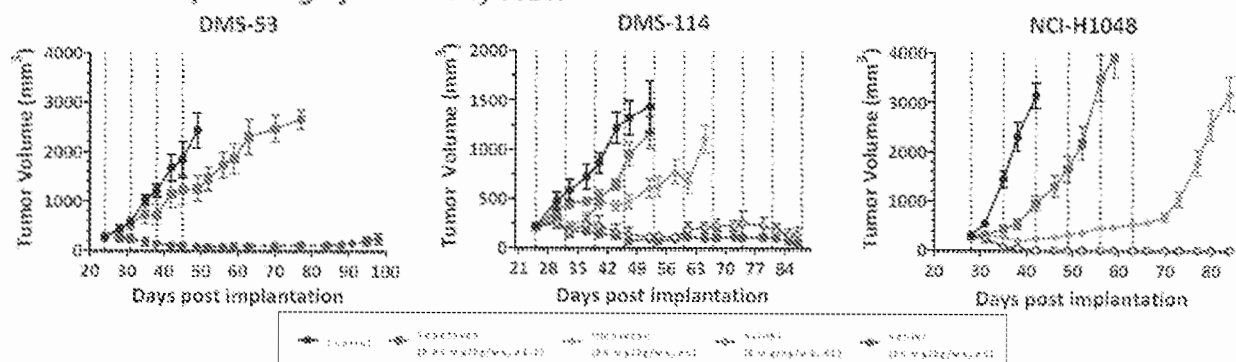
Increased tumor SN-38 levels were associated with increased tumor deposition of nal-IRI



After bearing tumor xenografts, as indicated, were treated with nal-IRI and tumors harvested 24 h post administration. Tumor levels of irinotecan and SN-38 were measured by HPLC.

nal-IRI Anti-Tumor Activity in Preclinical Models of SCLC

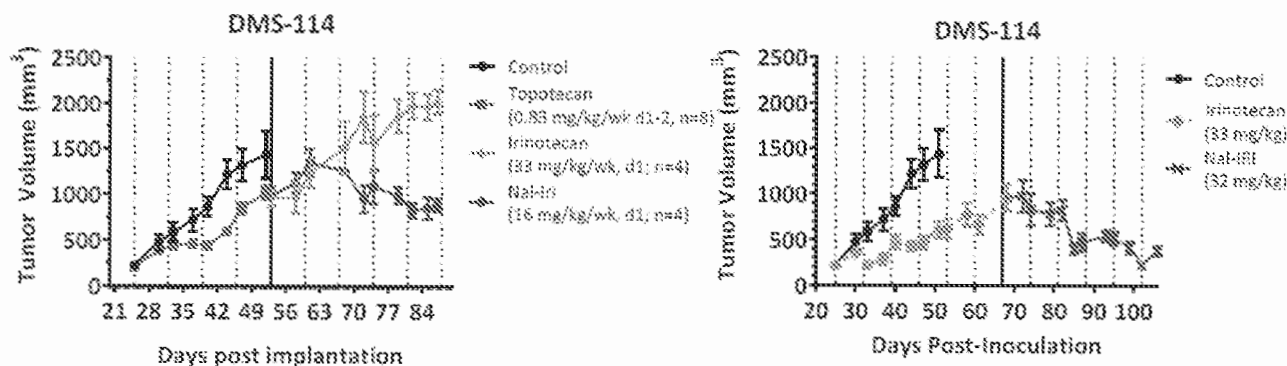
nal-IRI has greater anti-tumor activity than irinotecan and topotecan at clinically relevant doses in multiple xenograft models of SCLC.



NOD/SCID mice bearing subcutaneous xenografts, as indicated, were used to evaluate the anti-tumor activity of topotecan, irinotecan and nal-IRI. Clinically relevant doses were calculated by using standard surface area to weight ratio conversion per NCI guidelines. Using this method, the proposed clinical dose of nal-IRI (86 mg/m² per dose, q2w) converted for mouse is 26 mg/kg, q2w. For non-topotecan irinotecan at a clinical dose of 1.83 mg/m² q2w, the equivalent mouse dose is approximately 33 mg/kg/week. For topotecan at a clinical dose of 1.5 mg/m² (D1-5, q2w), the equivalent mouse dose is approximately 0.83 mg/kg/week. The preclinical dosing schedule was divided between days 1 and 2 repeating every 7 days which result in a similar exposure time as the clinical regimen (20% per cycle for preclinical vs. 24% per cycle for clinical). Vertical lines indicate start of weekly dosing. Error bars indicate standard error of the mean. For all groups, n=10 except DMS-53 (n=4, 3 and 5 for control, topotecan and nal-IRI, respectively).

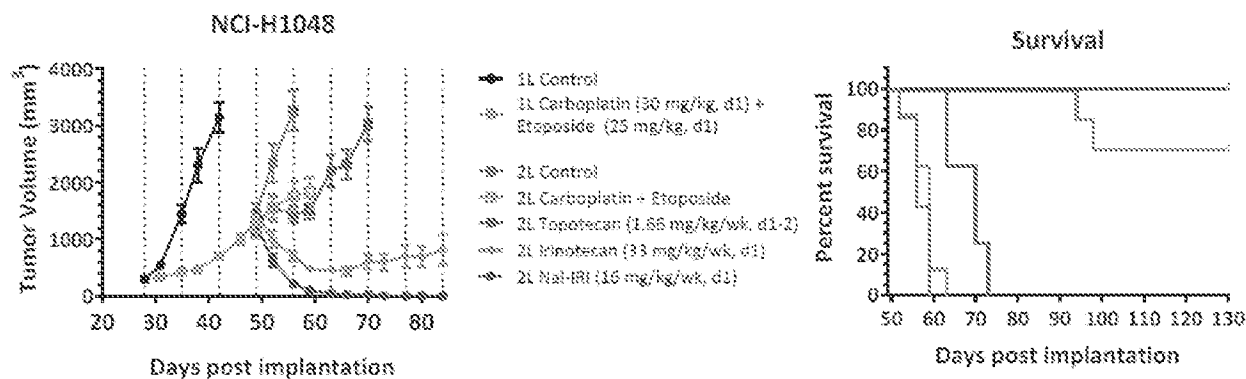
nal-IRI Anti-Tumor Activity in Preclinical Models of SCLC (cont.)

nal-IRI has anti-tumor activity after failure of topotecan or irinotecan.



NOD/SCID mice bearing subcutaneous DMS-114 xenograft tumors were treated with topotecan (n=8), irinotecan (n=10) or untreated (control; n=10). Upon failure of topotecan (left; day 53) mice were randomized to receive irinotecan (n=5) or nal-IRI (n=4). Upon failure of irinotecan (right; day 67), mice were switched to nal-IRI (n=10). Vertical lines indicate start of weekly dosing. Error bars indicate standard error of the mean.

nal-IRI has greater anti-tumor activity and survival than irinotecan and topotecan after failure of 1st line carboplatin + etoposide.



NOD/SCID mice bearing subcutaneous NCI-H1048 xenograft tumors were treated with carboplatin (30 mg/kg) + etoposide (25 mg/kg) (n=10) or untreated (1L control; n=10). Upon failure of carboplatin + etoposide (day 48), mice were randomized to receive no treatment (2L control; n=8), carboplatin + etoposide (n=8), topotecan (n=8), irinotecan (n=7) or nal-IRI (n=8). Vertical lines indicate start of weekly dosing. Error bars indicate standard error of the mean.

Summary

- The biology of SCLC shares features that are well-matched with the proposed mechanism of action for nal-IRI although several features merit further investigation in patients.
- nal-IRI has anti-tumor activity in multiple models of SCLC and greater anti-tumor activity than both irinotecan and topotecan in a preclinical models of SCLC when administered at clinically relevant doses.
- Preclinical studies support the rationale for evaluation of nal-IRI in patients with SCLC.
- A Phase 3 study of nal-IRI versus IV topotecan in patients with SCLC progressing on or after prior platinum-containing therapy is planned.

References

- von Pawel T, et al. Randomized phase III trial of irinotecan versus topotecan as second-line treatment for patients with small-cell lung cancer. *J Clin Oncol*. 2014 Dec 10;32(35):4012-9.
- Wang-Gillam A, et al. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemtabine-based therapy (NAPOLI-1): a global, randomized, open-label, phase 3 trial. *Lancet*. 2016 Feb 6;387(10016):545-57.
- Ramanathan, et al. EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics 2014 #261

Figure 1. The effect of the concentration of the *Agrobacterium* strain on the transformation efficiency of *Agrobacterium* strain 101. The concentration of the *Agrobacterium* strain 101 was varied from 10⁶ to 10⁹ cells/ml. The transformation efficiency was determined by the number of transformants per 10⁶ cells of the *Agrobacterium* strain 101. The data are the mean $\pm$ SD of three independent experiments.

THEY WERE NOT THE ONLY PEOPLE WHO WERE NOT

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Figure 1 consists of three schematic diagrams labeled (a), (b), and (c), illustrating the progression of a tumor.

- (a) Extension of the primary tumor:** This diagram shows a cross-section of a tumor (shaded area) growing from a basement membrane (dashed line) into the underlying stroma. The tumor is labeled "Tumor" and the stroma is labeled "Stroma".
- (b) Deposition of tumor cells into lymphovascular spaces:** This diagram shows the tumor cells (shaded area) breaking through the basement membrane and entering a lymphovascular space (dashed line). The tumor is labeled "Tumor" and the lymphovascular space is labeled "Lymphovascular space".
- (c) Deposition of tumor cells into the peritoneal cavity:** This diagram shows the tumor cells (shaded area) breaking through the basement membrane and entering the peritoneal cavity (dashed line). The tumor is labeled "Tumor" and the peritoneal cavity is labeled "Peritoneal cavity".

- ▶ The biology of SCDL breast tumours that are not associated with the proposed mechanism of action for metformin should be studied to further identify novel therapeutic targets in patients with metformin-resistant advanced breast cancer
- ▶ A phase 3 study of metformin versus IV trastuzumab in patients with SCDL progressing on or after prior trastuzumab-containing therapy is planned

Instituto de Física de la Universidad Nacional Autónoma de México, Ciudad de México, México

Phase I Safety, Pharmacokinetic, and Pharmacodynamic Study of the Poly(ADP-ribose) Polymerase (PARP) Inhibitor Veliparib (ABT-888) in Combination with Irinotecan in Patients with Advanced Solid Tumors

Patricia M. LoRusso¹, Jing Li¹, Angelika Burger¹, Lance K. Heilbrun¹, Edward A. Sausville², Scott A. Boerner¹, Daryn Smith¹, Mary Jo Pilat^{1,3}, Jie Zhang¹, Sara M. Tolaney⁴, James M. Cleary⁴, Alice P. Chen⁵, Lawrence Rubinstein⁵, Julie L. Boerner¹, Adam Bowditch¹, Dongpo Cai⁴, Tracy Bell⁴, Andrew Wolanski⁴, Allison M. Marrero⁶, Yiping Zhang⁶, Jiuping Ji⁶, Katherine Ferry-Galow⁶, Robert J. Kinders⁶, Ralph E. Parchment⁶, and Geoffrey I. Shapiro⁴

Abstract

Purpose: PARP is essential for recognition and repair of DNA damage. In preclinical models, PARP inhibitors modulate topoisomerase I inhibitor-mediated DNA damage. This phase I study determined the MTD, dose-limiting toxicities (DLT), pharmacokinetics (PK), and pharmacodynamics (PD) of veliparib, an orally bioavailable PARP1/2 inhibitor, in combination with irinotecan.

Experimental Design: Patients with advanced solid tumors were treated with 100 mg/m² irinotecan on days 1 and 8 of a 21-day cycle. Twice-daily oral dosing of veliparib (10–50 mg) occurred on days 3 to 14 (cycle 1) and days –1 to 14 (subsequent cycles) followed by a 6-day rest. PK studies were conducted with both agents alone and in combination. Paired tumor biopsies were obtained after irinotecan alone and veliparib/irinotecan to evaluate PARP1/2 inhibition and explore DNA damage signals (nuclear γ -H2AX and pNBS1).

Results: Thirty-five patients were treated. DLTs included fatigue, diarrhea, febrile neutropenia, and neutropenia. The MTD was 100 mg/m² irinotecan (days 1 and 8) combined with veliparib 40 mg twice daily (days –1–14) on a 21-day cycle. Of 31 response-evaluable patients, there were six (19%) partial responses. Veliparib exhibited linear PK, and there were no apparent PK interactions between veliparib and irinotecan. At all dose levels, veliparib reduced tumor poly(ADP-ribose) (PAR) content in the presence of irinotecan. Several samples showed increases in γ -H2AX and pNBS1 after veliparib/irinotecan compared with irinotecan alone.

Conclusions: Veliparib can be safely combined with irinotecan at doses that inhibit PARP catalytic activity. Preliminary antitumor activity justifies further evaluation of the combination. *Clin Cancer Res* 22(13): 3227–37. ©2016 AACR.

Introduction

Poly (adenosine diphosphate-ribose) (PAR) polymerases 1 and 2 (PARP1 and PARP2) are members of an essential nuclear protein superfamily that play a role in recognition of DNA damage and facilitation of DNA repair. PARP inhibition has

emerged as a promising strategy as monotherapy for cancers defective in homologous recombination (HR) repair, such as those arising in *BReast CAncer susceptibility gene* (BRCA) carriers (1–4), and as chemopotentialization for a variety of DNA-damaging agents, including topoisomerase I poisons, alkylators, platinum-based agents, and γ -irradiation (5–12). Recently, it has

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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Prior presentation: Presented in part at the 2011 Annual Meeting of the American Society of Clinical Oncology, Chicago, Illinois.

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Translational Relevance

In preclinical models, PARP1/2 inhibitors enhance the activity of topoisomerase I inhibitors. This phase I trial established the tolerability of the PARP inhibitor veliparib in combination with irinotecan. The MTD of veliparib was lower than that required for monotherapy cytotoxicity, but consistent with catalytic inhibition of PARP, evidenced by paired tumor biopsies obtained after irinotecan alone and after veliparib/irinotecan, demonstrating veliparib-mediated reduction in PAR content. In several samples, expression of γ -H2AX and pNBS1 was increased following veliparib/irinotecan compared with irinotecan, with the greatest increase in pNBS1 noted in 1 patient who achieved partial response. In other patients, similarly increased γ -H2AX and pNBS1 levels were not observed. Administration schedules accommodating higher veliparib doses or alternative tumor sampling time points may be required to reproducibly demonstrate modulation by veliparib of the extent and repair of irinotecan-mediated DNA damage. Nonetheless, the observed clinical responses suggest that the combination merits further evaluation.

been shown that PARP inhibitors possess two activities: (i) inhibition of NAD^+ -competitive catalytic activity of PARP and (ii) ability to trap PARP-DNA complexes. Cytotoxicity of monotherapy in preclinical models has been correlated with the trapping of PARP-DNA complexes (13).

Several orally bioavailable small-molecule PARP inhibitors are under active clinical development, including veliparib (ABT-888; refs. 14, 15). Importantly, PARP-DNA complex trapping is drug-specific, with talazoparib (BMN673) and olaparib demonstrating a greater ability than veliparib, whereas all of the compounds are potent catalytic PARP inhibitors (13, 16). These findings may explain why inhibition of the catalytic activity of PARP, assayed by tumor content of PAR, was demonstrated at a dose of 25 mg veliparib in a phase 0 study (17), whereas doses of 300 mg and above were required for tumor responses in a monotherapy phase I study of veliparib in BRCA-deficient cancers (18).

Preclinical synergism between PARP inhibition and topoisomerase I poisons has been firmly established (19–25). The precise mechanism of synergy remains under intense investigation. *In vitro* studies combining a PARP inhibitor with camptothecin or the camptothecin derivative irinotecan have demonstrated variable effects on the onset and magnitude of DNA damage, the persistence of DNA damage, and the time required for cells to accomplish repair (20, 22). In addition, whether inhibition of PARP catalytic activity is sufficient or whether PARP-DNA trapping is required for potentiation of topoisomerase I inhibitor-mediated DNA damage remains controversial (26, 27). Nonetheless, *in vivo*, the addition of a PARP inhibitor has resulted in substantial potentiation of the antitumor efficacy of irinotecan and other topoisomerase I poisons against a variety of human tumor xenografts (19, 21, 22).

Based on these preclinical data, we conducted a clinical trial of veliparib combined with irinotecan in patients with advanced solid tumors. The primary objective was to determine the

recommended phase II dose (RP2D) by evaluating the feasibility, safety, adverse events (AE), dose-limiting toxicities (DLT), and the MTD. Secondary objectives were to characterize the pharmacokinetics (PK) of veliparib and irinotecan, alone and in combination, and to assess preliminary antitumor activity. Paired tumor biopsies obtained post-irinotecan and post-veliparib/irinotecan at time points established for quantifying veliparib-mediated reductions in PAR levels were used to confirm inhibition of PARP catalytic activity (17). In addition, we studied modulation of irinotecan-induced DNA damage and repair by veliparib at these time points by measuring two key proteins of the DNA damage response machinery: nuclear phosphorylated histone H2AX (γ -H2AX; ref. 28) and phosphorylated Nijmegen breakage syndrome 1 (pNBS1).

Materials and Methods

Patient selection

Eligible patients had histologically or cytologically confirmed metastatic or unresectable malignancy for which standard curative or palliative measures were nonexistent or ineffective or for which irinotecan was a viable treatment regimen; Eastern Cooperative Oncology Group (ECOG) performance status ≤ 2 ; measurable disease (per RECIST) accessible for biopsy; and adequate organ and marrow function defined as absolute neutrophil count $\geq 1,500/\mu\text{L}$, platelets $\geq 100,000/\mu\text{L}$, aspartate aminotransferase and/or alanine aminotransferase $\leq 2.5 \times$ upper limit of normal (ULN) or $\leq 5 \times$ ULN if liver metastases present; bilirubin $\leq 1.5 \times$ ULN; and creatinine $\leq 1.5 \times$ ULN or calculated or measured creatinine clearance $\geq 60 \text{ mL/min/1.73 m}^2$. Patients homozygous for the HCT1A1*28 allele (A(TA)_nTAA) were ineligible due to the potential increased risk of toxicity associated with irinotecan (29, 30).

Prior chemotherapy, experimental therapy, or radiotherapy to $>5\%$ of bone marrow must have been completed at least 4 weeks prior to treatment initiation. Patients who received prior radiation to $\geq 50\%$ of their total marrow volume were excluded. CYP3A4 isoform-inducing drugs had to be discontinued at least 2 weeks prior to the first administration of irinotecan. Patients were also excluded if they had an uncontrolled intercurrent illness, prior history of seizures (based on the ability of veliparib to lower the seizure threshold), known active brain metastases, or a requirement for chronic maintenance of growth factor support.

Study treatment and design

Supplementary Figure S1 shows the study design and sample collection time points. Treatment cycles were 21 days. Irinotecan was administered intravenously at 100 mg/m^2 over 90 minutes on days 1 and 8, a dose and schedule that is tolerable in heavily pretreated patients. Blood samples for single-agent irinotecan PK were collected on cycle 1, days 1 to 3 following administration of the first dose. Twice-daily oral administration of veliparib (Abbvie, Inc.) began on day 3 of cycle 1 and continued until day 14, followed by 6 days of no treatment (days 15–20). Four dose levels of veliparib were tested: 10, 20, 40, and 50 mg twice daily. A single dose of veliparib was administered on day 21 (cycle 2, day –1; i.e., 1 day prior to cycle 2 irinotecan), for evaluation of single-agent veliparib PK. Twice-daily veliparib continued in cycle 2 through day 14, followed by a 6-day rest. Serial blood samples were obtained

on cycle 2, days 8 to 10 for evaluation of veliparib and irinotecan PK when coadministered. For subsequent cycles, veliparib was administered twice daily from day -1 through day 14, followed by a 6-day rest (15 days on treatment/6 days off).

At least 3 patients received study treatment on each cohort in standard 3 + 3 fashion. DLTs were based on toxicities observed during the first cycle. Toxicities were graded using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 until July 31, 2010, after which CTCAE version 4.0 was utilized. DLTs were defined as any grade ≥ 3 nonhematologic toxicity; grade ≥ 3 nausea, vomiting, or diarrhea uncontrolled by aggressive treatment; grade 4 granulocytopenia lasting ≥ 5 days without hematopoietic growth factor support; grade 4 thrombocytopenia or febrile neutropenia; inability to begin cycle 2 of treatment (at full dose) within 2 weeks of the scheduled date of administration due to unresolved toxicity; grade ≥ 2 nonhematologic toxicity persisting beyond the first 42 days or the occurrence of grade 2 toxicities that, in the judgment of the PI, were dose-limiting. For grade ≥ 3 electrolyte imbalance secondary to another toxicity, grading of the precipitating toxicity was used for DLT definition.

A new cycle of therapy did not begin until any toxicity recovered to $\leq$ grade 1, with no more than a 2-week delay permitted. Patients were also discontinued if there was a >2 -week delay in reinstitution of veliparib due to drug-related toxicity during a cycle. For a ≤ 2 -week delay or a grade ≥ 3 toxicity related to veliparib, treatment proceeded with a one dose level reduction. Dose modifications for irinotecan followed those recommended in the package insert (recommended dose modifications for single-agent schedules; ref.31). Dose reductions could occur multiple times to the lowest protocol-specified dose level as long as there was clinical benefit. Dose re-escalation was not allowed.

Written informed consent was obtained from enrolled patients. Institutional Review Board approvals of the protocol and consent form were obtained at all sites. Protocol design and conduct complied with all applicable regulations, guidance, and local policies. The study was conducted in accordance with the Declaration of Helsinki. The trial (ClinicalTrials.gov identifier NCT00576654) was conducted under an NCI-sponsored Investigational New Drug application. Veliparib was supplied under a Collaborative Research and Development Agreement between Abbvie, Inc., and the Division of Cancer Treatment and Diagnosis, NCI. Irinotecan was obtained commercially.

PK evaluation

Plasma PK of veliparib and irinotecan was assessed for each agent alone and in combination. For single-agent irinotecan, blood samples were collected at predose and at 0.5, 1.5 (immediately at the end of infusion), 2, 3.5, 5.5, 8.5, 28, and 48 hours following the start of infusion on cycle 1, day 1. For single-agent veliparib, blood samples were collected at predose and at 0.5, 1, 1.5, 3.5, 5.5, 8.5, 10, and 28 hours after oral administration of a single dose on cycle 2, day -1. The PK of veliparib and irinotecan in combination was evaluated on cycle 2, day 8. Serial blood samples were collected at predose and at 0.5, 1, 1.5, 2, 3.5, 5.5, 8.5, 10 (prior to the administration of the afternoon dose of veliparib), 28, and 48 hours following the administration of both drugs on day 8 (prior to veliparib dosing days 9 and 10).

Following isolation of plasma by centrifugation, samples were stored at -80°C .

The plasma concentrations of veliparib and its main metabolite (A-925088) were determined using a validated liquid chromatography with tandem mass spectrometry (LC-MS/MS) method (32). The plasma concentrations of irinotecan and its major active metabolite SN-38 were determined using a validated high-performance liquid chromatographic method with fluorescence detection (33) with modifications described in the Supplementary Methods. The PK parameters of veliparib, irinotecan, and their metabolites were estimated using non-compartmental analysis with WinNonlin software (Pharsight).

Analysis of pharmacodynamic biomarkers

Tumor sampling was designed to compare the levels of PAR as a pharmacodynamic (PD) biomarker of PARP1/2 catalytic activity after irinotecan alone with those after the veliparib/irinotecan combination. Patients underwent tumor biopsies on cycle 1 of day 2, approximately 28 hours after the start of irinotecan and again on cycle 1 day 9, approximately 28 hours after the second dose of irinotecan, and approximately 4 hours after the morning dose of veliparib, a time point informed by preclinical studies and confirmed by the prior phase 0 trial (17, 34).

Tumor PAR content was quantified using a validated, fit-for-purpose sandwich immunoassay (IA) of denatured tumor extracts (17, 34) and was performed according to NCI standard-operating procedures (SOPs) detailed in Supplementary Methods.

Whenever feasible, each biopsy procured a second-pass specimen for surveying two nuclear PD biomarkers of the DNA Damage Response (DDR): γ -H2AX and pNBS1. Nuclear γ -H2AX was quantified using a validated single-plex immunofluorescence microscopy assay (IFA) according to NCI-DCTD SOPs detailed in the Supplementary Methods. This assay employs ImagePro-based image analysis of all viable cell types in the specified number of objectively selected fields, excluding necrotic areas, and reports the percentage of their total nuclear area that is covered by the nuclear γ -H2AX staining pattern (%NAP, nuclear area positive). A preclinical fit-for-purpose study demonstrated the utility of this assay for quantifying the repair response to DNA damage caused by topoisomerase-1 inhibitors, including camptothecins (35).

The timing of the DDR response relative to reduction in tumor PAR levels in cancer patients is unknown; therefore, an exploratory multiplex IFA was developed to evaluate a second PD biomarker, pNBS1, in concert with γ -H2AX. After a modified antigen retrieval step replacing citrate with EDTA in the SOP for the validated γ -H2AX IFA (35), both γ -H2AX and pNBS1 biomarkers were evaluated in the same cut paraffin section after sequential staining on a Bond-max Autostainer with antibodies conjugated to different fluorochromes. Details of this exploratory IFA are given in the Supplementary Methods. Following image capture, Tissue Studio software (Definiens) was used to quantify %NAP of γ -H2AX only in fields with a high content of viable tumor cells. Assessment of nuclear pNBS1 in paired biopsies was qualitative.

Statistical methods

Descriptive statistics were used to summarize the baseline patient characteristics, treatment-related adverse events, and PK parameters. Confidence interval (CI) estimates of grade ≥ 3 toxicity rates and response rates were calculated using the Wilson

Table 1. Patient characteristics

Characteristic	N = 35 (%)
Gender	
Male	7 (20)
Female	28 (80)
Median (range) age in years	54 (31–73)
ECOG performance status	
0	9 (26)
1	24 (68)
2	2 (6)
Tumor type	
Breast	15 (43)
Ovary	9 (26)
Colon	4 (11)
Esophagus	4 (11)
Lung	2 (6)
Anus	1 (3)
Received prior systemic therapy	35 (100)
Median number of prior therapies (range)	4 (1–12)
Median number of veliparib cycles/patient (range)	3 (1–28)

method. The comparison of PK parameters of veliparib or irinotecan between single-agent and combination treatments was performed using a paired, two-sided Student *t* test. Associations between changes in PD markers, PK parameters, and measures of clinical outcome were also assessed. We hypothesized a positive association between increasing veliparib dose and the percent reduction of PAR. This hypothetical gradient in PAR reduction was investigated with the exact version of the nonparametric Jonckheere–Terpstra test for ordered alternatives (1-sided), given the small sample sizes per dose level.

Results

Patient demographics

Thirty-five consented patients (Table 1) were enrolled between March 2008 and June 2011. Whenever possible, germline *BRCA* status documentation (performed by Myriad Genetics, Inc.) was obtained. All patients received at least one dose of study drug, with a median of three (range of 1–28) cycles of veliparib administered. Patients had ≥ 1 line of prior systemic therapy and had evidence of disease progression at enrollment. Four patients did not complete a full cycle of treatment and were not evaluable for response or MTD determination due to financial reasons (1), rapid clinical deterioration (1), or disease progression (2).

Dose escalation and determination of MTD and RP2D

During dose escalation, 4 DLTs were observed. The first grade 3 toxicity, fatigue, occurred at the initial dose level (veliparib 10 mg twice daily). A second DLT of grade 4 neutropenia was observed at 20 mg twice daily. Two additional DLTs, one each of grade 3 diarrhea and febrile neutropenia, occurred at 50 mg twice daily, resulting in expansion of the 40 mg veliparib twice-daily cohort with 8 additional patients. No DLTs were observed in the additional patients; therefore, 40 mg veliparib twice daily administered on a 15 days on/6 days off schedule combined with 100 mg/m² irinotecan on days 1 and 8 of a 21-day cycle was established as the MTD and RP2D.

Toxicity

The combination of veliparib and irinotecan was generally well tolerated (Table 2). The most common toxicities among the 35 patients treated across all dose levels included diarrhea (63%; 95% CI, 46%–77%), nausea (60%; 95% CI, 44%–74%), fatigue (60%; 95% CI, 44%–74%), neutropenia (49%; 95% CI, 33%–64%), and leukopenia (49%; 95% CI, 33%–64%). Beyond standard loperamide recommended during irinotecan treatment, 13 patients across dose levels required additional medications, including diphenoxylate and atropine, tincture of opium, and in one case, octreotide injections surrounding irinotecan doses. In 4 patients who achieved a partial or mixed response, pegfilgrastim was used after the first cycle in order to maintain the initial dose of 20 or 40 mg veliparib twice daily with irinotecan. Thirteen patients required dose reductions during their treatment course, for diarrhea (6), dehydration (3), neutropenia (5), fatigue (2), nausea (3), or a combination of these toxicities. Eleven patients required dose interruption, most commonly for recovery of neutropenia. One ovarian cancer patient on the 20 mg twice-daily veliparib dose experienced intolerable diarrhea, nausea, and fatigue with irinotecan, even after two dose reductions within the first 6 cycles. She was permitted to remain on veliparib alone and received an additional 11 cycles before progressive disease was documented.

Pharmacokinetics

PK samples for veliparib and irinotecan were obtained from 26 and 34 patients, respectively. The PK parameter estimates for veliparib following oral administration (Supplementary Table S1) were consistent with those reported previously (5, 6, 17). The systemic exposure (i.e., C_{max} and AUC_{last}) to veliparib or A-925088 increased with dose (Supplementary

Table 2. Most common ($\geq 10\%$) treatment-related adverse events (maximum grade, all cycles)

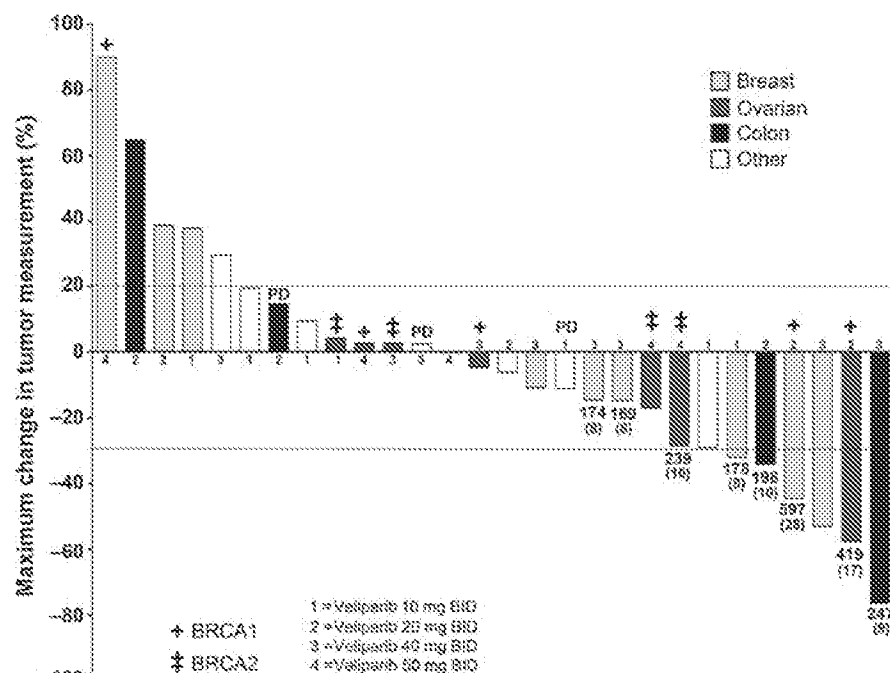
Adverse event	Total number of patients with event N = 35 (%)	Veliparib dose level							
		10 mg N = 7		20 mg N = 9		40 mg N = 12		50 mg N = 7	
		All (%)	Gr ≥ 3 (%)	All (%)	Gr ≥ 3 (%)	All (%)	Gr ≥ 3 (%)	All (%)	Gr ≥ 3 (%)
Diarrhea	22 (63)	5 (71)	1 (14)	5 (56)	1 (11)	8 (67)	—	4 (57)	1 (14)
Fatigue	21 (60)	5 (71)	1 (14)	5 (56)	2 (22)	6 (50)	—	5 (71)	—
Nausea	21 (60)	4 (57)	—	7 (78)	—	6 (50)	—	4 (57)	—
Leukopenia	17 (49)	4 (57)	3 (43)	4 (44)	1 (11)	4 (33)	2 (17)	5 (71)	2 (29)
Neutropenia	17 (49)	4 (57)	2 (29)	4 (44)	2 (22)	5 (42)	2 (17)	4 (57)	3 (43)
Anemia	13 (37)	4 (57)	1 (14)	1 (11)	—	5 (42)	—	3 (43)	—
Anorexia	11 (31)	3 (43)	—	1 (11)	—	4 (33)	—	3 (43)	—
Vomiting	9 (26)	2 (29)	—	3 (33)	—	3 (25)	—	1 (14)	—
Alopecia	7 (20)	1 (14)	—	1 (11)	—	4 (33)	—	1 (14)	—
Lymphopenia	6 (17)	2 (29)	2 (29)	1 (11)	1 (11)	3 (25)	—	—	—
Dehydration	5 (14)	1 (14)	1 (14)	2 (22)	—	1 (8)	—	1 (14)	—
Hypalbuminemia	5 (14)	1 (14)	—	1 (11)	—	3 (25)	—	—	—
Abdominal pain	4 (11)	1 (14)	—	1 (11)	—	1 (8)	—	1 (14)	—

Efficacy

Of the 9 patients with ovarian cancer enrolled to the study, all were BRCA1 or BRCA2 carriers. The sixth PR occurred in a BRCA1

One patient with advanced breast cancer on the 40 mg twice-daily dose level had a mixed response and received 8 cycles. Thirteen patients met the criteria of SD after two cycles of treatment (median, 2 cycles; range, 2-10 cycles) for an SD rate of $13/31 = 42\%$; 95% CI, 26%-59%. There were 9 patients progression-free at 4 months (29%; 95% CI, 16%-47%), and 5 patients progression-free at 6 months after start of treatment (16%; 95% CI, 7%-33%).

Paired biopsies were collected from 26 of the 35 patients; of these 26 pairs, 19 were fully evaluable for quantifying the primary PD endpoint of the PARP1/2 response to veliparib in the presence of irinotecan, including all 8 patients enrolled in the MTD cohort at veliparib 40 mg twice daily (Fig. 2 and Table 3). The reasons behind the attrition of seven biopsy pairs were (i) quality control failure in assay performance (5 pairs) with a root cause traced to a change in the supplier's method for producing the commercial antibody that was addressed prior to patient 13 by modifying the assay SOP (see Supplementary Methods), (ii) one pair with



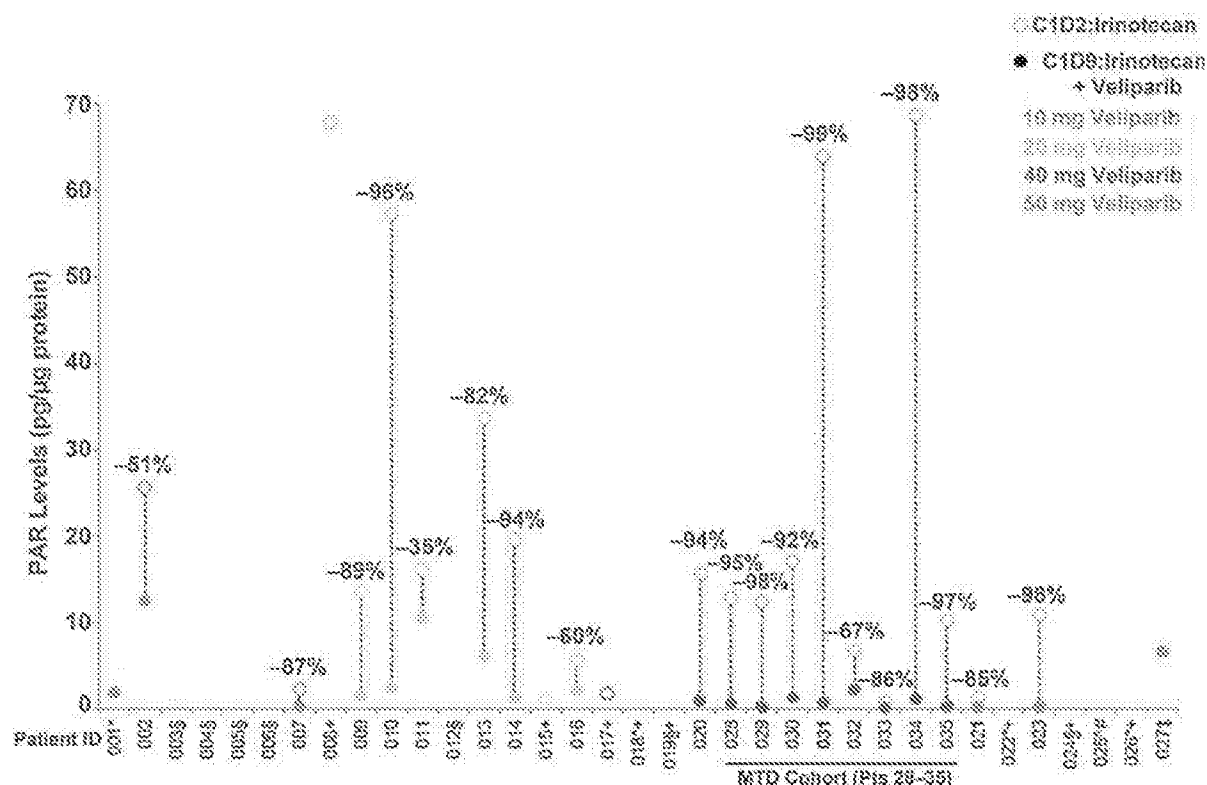


Figure 2.

PAR content in paired tumor biopsies after irinotecan alone and after combined veliparib/irinotecan. PAR levels in tumor obtained from biopsies performed on cycle 1, day 2, 28 hours after the first dose of irinotecan (open circles) and cycle 1, day 9, 28 hours after combined veliparib/irinotecan (closed circles), demonstrating substantial reductions in PAR after veliparib exposure across dose levels. Of 26 patients from whom paired biopsies were procured, 19 patients were evaluable for a quantitative change in PAR tumor content, including all 8 patients enrolled in the MTD cohort at 40-mg twice-daily veliparib. *, no day 2 sample; †, day 2 sample, insufficient quality; Δ, day 2 sample, < LLQ; +, no day 9 sample; #, day 9 sample, < LLQ; §, assay failed GC.

insufficient quality in the first biopsy, and (iii) one pair in which both biopsies contained PAR levels below the assay lower limit of quantitation (LLQ).

After 13 twice-daily doses, veliparib reduced PAR tumor levels in all PD-evaluable patients by 35% to 99%. The median PAR reduction across the veliparib dose levels of 10, 20, 40, and 50 mg was 69% (range, 51%–87%), 86% (range, 35%–96%), 95% (range, 67%–99%), and 92% (range, 85%–98%), respectively. This gradient in PAR reduction was statistically significant ($P = 0.0184$, 1-sided). However, there was no analogous-positive association between the degree of PARP inhibition and best response; several patients with progressive disease as the best response exhibited >90% reduction in tumor PAR content after veliparib (see Table 3). Nonetheless, the results demonstrate the effectiveness of veliparib at inhibiting PARP1/2 catalytic activity within 4 hours of its administration 1 day after irinotecan treatment.

DNA damage and repair pharmacodynamics

Second-pass core specimens from the paired biopsies of 11 patients yielded sufficient tissue for evaluating two nuclear PD biomarkers of the DDR response: γ -H2AX and pNBS1. Modulation of nuclear γ -H2AX was initially evaluated using

the validated single-plex IFA (35), and the response of this PD biomarker was low and highly variable (Table 3). In three sample pairs, the γ -H2AX NAP score (%NAP) increased above basal levels (i.e., >5% NAP) after veliparib/irinotecan compared with irinotecan alone (patients 2, 13, and 34). These patients had increases in NAP scores from <5% after irinotecan alone to 9.5%, 5.7%, and 9.9% after veliparib/irinotecan, with PD, PR, and PD as the best response, respectively. These %NAP values are considerably lower than those obtained in human tumor xenograft models that respond to topoisomerase 1 inhibitors, which reach 25% at 4 hours after dosing (35). Furthermore, these small changes in %NAP values were not confirmed in the exploratory multiplex assay, which uses an image analysis algorithm that reports %NAP only in fields predominately composed of viable tumor cells (Table 3 and Fig. 3). In the remaining patients evaluable for DDR biomarkers, the addition of veliparib to irinotecan treatment failed to increase nuclear γ -H2AX at the time of demonstrated decreases in tumor content of PAR (Table 3 and Supplementary Fig. S3).

Nuclear pNBS1 staining was assessed qualitatively in seven sample pairs (Table 3). In two cases, nuclear pNBS1 staining was increased after veliparib/irinotecan compared with irinotecan alone (Table 3 and Fig. 3). This included a robust biomarker

Table 3. Analysis of PAR levels and PD biomarkers of the DNA damage response in paired tumor biopsy specimens

Patient number	Dose level A81-888 (mg)	Best response	Validated PAR-1A		Decline in tumor PAR from days 2–9 (%)	Validated nuclear γ -H2AX-qIF ^a for 2008 in pRES without background		Exploratory nuclear γ -H2AX-qIF ^a (only fields with high content of viable tumor cells)		Exploratory nuclear pRES-qIF ^a (only fields with high content of viable tumor cells)	
			Day 2 tumor PAR content	Day 9 tumor PAR content		Day 2 nuclear γ -H2AX	Day 9 nuclear γ -H2AX	Day 2 nuclear γ -H2AX	Day 9 nuclear γ -H2AX	Day 2 nuclear pRES1	Day 9 nuclear pRES1
			28-hr of CPT-11 w/o A81-888 (pg/mg protein)	28-hr of CPT-11 4-hr after a.m. A81-888 dose (pg/mg protein)		28-hr of CPT-11 w/o A81-888 (% MAP)	28-hr of CPT-11 after a.m. A81-888 dose (% MAP)	28-hr of CPT-11 w/o A81-888 (% MAP)	28-hr of CPT-11 after a.m. A81-888 dose (% MAP)	28-hr of CPT-11 w/o A81-888 (% MAP)	28-hr of CPT-11 4-hr after a.m. A81-888 dose (% MAP)
002	10	PD	25.6	12.5	-51	<5.0	9.5	<5.0	<5.0	Background	Background
010	20	PD	57.3	2.4	-96	7.3	7.0	<5.0	<5.0	Background	Background
014	20	PD	19.6	1.2	-94	9.8	7.1	6.4	n/a	++	n/a
016	20	PD	5.7	2.2	-60	<5.0	<5.0	n/a	n/a	n/a	n/a
024	40	PD	60.8	1.2	-98	<5.0	9.9	6.7	<5.0	Detected	Background
006	10	SD	n/a	n/a	n/a	<5.0	<5.0	<5.0	<5.0	Background	Background
007	10	SD	2.3	0.5	-87	<5.0	<5.0	<5.0	<5.0	Detected	Detected
028	40	SD	12.8	0.5	-95	<5.0	<5.0	<5.0	<5.0	Detected	+
009	20	PR	13.6	1.8	-88	<5.0	<5.0	n/a	n/a	n/a	n/a
012	20	PR	n/a	n/a	n/a	6.0	<5.0	n/a	n/a	n/a	n/a
013	20	PR	33.7	6.1	-82	<5.0	5.7	<5.0	<5.0	++	+++

NOTE: Paired biopsies for evaluation of PAR levels in first-pass core specimens also yielded second-pass core specimens in some patients that were sufficient for analysis of nuclear PD biomarkers of the DNA damage response (DDR). Note that nuclear pRES1 was the only PD biomarker of the DDR that exhibited a signal at sampling times selected for evaluating PAR levels. Samples demonstrating evidence of increased DNA damage or persistent DNA repair after combined irinotecan/veliparib compared with irinotecan alone are bolded.

Abbreviations: hr, hour; n/a, not assessable (insufficient cells for analysis).

^aThe validated single-plex IFA for nuclear γ -H2AX employs ImagePro-based image analysis and reports %MAP for all viable cells in non-necrotic fields. The exploratory multi-plex IFA for nuclear γ -H2AX employs a Defensio-based image analysis algorithm that reports %MAP only in fields predominantly composed of viable tumor cells. Background %MAP values of nuclear γ -H2AX in untreated tumor specimens are <5%, so %MAP values must exceed this threshold to demonstrate an increase in DDR.

^bThe term "background" means <1% positive nuclei in all fields. "Detectable" means a few positive nuclei in only some fields, and "++" and "+++" indicate progressively higher numbers of strongly positive nuclei in every field.

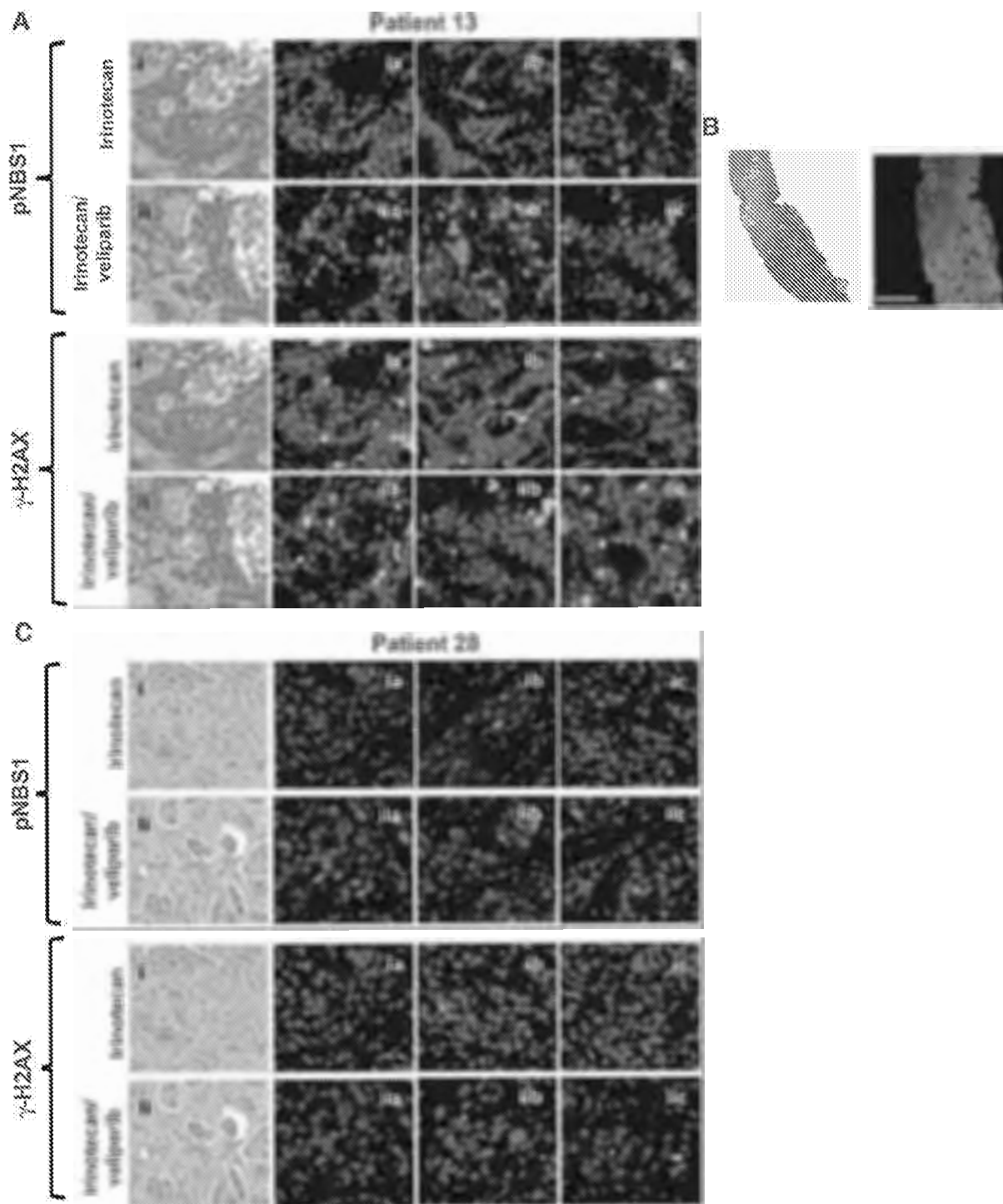


Figure 3.

Assessment of γ -H2AX and pNBS in the exploratory multiplex immunofluorescence assay. Multiplex immunofluorescence microscopy with Definiens image analysis was used to assess expression of γ -H2AX (green signal) and pNBS (red signal) after irinotecan alone and after combined veliparib/irinotecan. Hematoxylin and eosin plots and the "a" plots demonstrate cytology and pharmacodynamic response of adjacent sections. The "b" and "c" plots demonstrate typical staining patterns of other representative fields in the series of sections prepared from each biopsy specimen. A, sections from biopsies from patient 13 (colon cancer; best response PP). B, liver biopsy core after combination treatment from patient 13 demonstrating that pNBS staining is confined to tumor cells in the biopsy. Bar, 300 μ m. C, sections from biopsies from patient 28 (TNBC; best response SD). Qualitatively, all sections reveal a slight increase in nuclear pNBS staining after combination treatment (Table 3).

response in a patient with colorectal cancer treated at the 20 mg dose level, who achieved a PR (patient 13). Importantly, pNBS1 staining was limited to tumor cells, as demonstrated by a liver biopsy in which both tumor and surrounding normal liver were analyzed (Fig. 3 and Supplementary Fig. S3). A second patient with triple-negative breast cancer treated at the 40 mg dose level, who was not a BRCA carrier and who achieved SD for 5.3 months, also had a slight increase in pNBS1 staining after combination treatment (patient 28). The other five paired biopsies did not demonstrate increased nuclear pNBS1 after the addition of veliparib (Table 3 and Supplementary Fig. S4).

Discussion

This trial defined the MTD of day 1, 8 irinotecan given in combination with 15 days of veliparib on a 21-day cycle. Although veliparib dosing was attenuated during the first cycle to accommodate PK and PD analyses, it is unlikely that defining DLT in the first cycle led to an underestimation of toxicities; of the 10 evaluable patients treated at 40 mg veliparib twice daily, only 2 received dose reductions in the second and fourth cycles, respectively, indicating that the protocol-defined MTD was tolerable in the majority of patients.

Topoisomerase I poisons cause formation of a cleavable complex in which topoisomerase I is covalently attached to the DNA 3' phosphate. PARP1 is activated by topoisomerase I-associated single-strand breaks, and via recruitment of X-ray repair cross-complementing protein 1 (XRCC1) promotes both removal of the cleavable complex by tyrosyl-DNA phosphodiesterase-1 (TDP1) and subsequent completion of DNA repair (37–40). PARP inhibition therefore potentiates camptothecin-induced DNA damage by disabling the PARP1-XRCC1-TDP1 repair pathway (20, 37).

When PARP is inhibited, 3' endonucleases may participate in the removal of the topoisomerase I cleavable complex. During S phase, camptothecin-stabilized Topo I-DNA complexes are converted to collapsed replication forks and double-strand breaks. Removal of the cleavable complex by the MRE11/RAD50/NBS1 (MRN) complex results in a lesion processed by HR (37). However, in the presence of a PARP inhibitor, there is inhibition of replication fork restart (41) and an increase in collapsed replication forks, so that PARP inhibition preferentially sensitizes S phase cells to topoisomerase I-mediated cytotoxicity (25). The cleavable complex may also be removed by XPF/ERCC1 during the G₂ phase (25, 42), so that cells with low ERCC1 levels are more sensitive to combined camptothecin/veliparib (42).

In this study, we assayed levels of nuclear γ -H2AX and pNBS1 in tumor after veliparib/irinotecan compared with irinotecan alone. γ -H2AX scores the presence of DNA double-strand breaks (28); phosphorylation of NBS1 (part of the MRN complex) indicates that DNA damage has been detected and suggests that the DNA repair process has been initiated. Increases in these biomarkers were sought to provide evidence either for (i) increased irinotecan-mediated DNA damage in the presence of veliparib, or (ii) persistence of irinotecan-mediated DNA damage and repair in the presence of veliparib, resulting from a longer period of time required for DNA repair. The latter outcome is supported by one *in vitro* study, in which the increased detection of double-strand breaks after combination treatment was attributed to a significantly slowed repair process (20). Therefore, in samples demon-

strating increased γ -H2AX or pNBS1 after veliparib/irinotecan compared with irinotecan alone, it is possible that DNA damage was nearly resolved at the 28-hour time point after irinotecan alone, but still ongoing at that time point after veliparib/irinotecan, consistent with veliparib-mediated retardation of repair. Among four biopsy pairs from patients who achieved SD or PR for which both of these markers were assessable, two demonstrated an increase in nuclear pNBS1 after combination treatment compared with irinotecan alone.

In the colorectal cancer case, adjacent normal liver was not similarly affected, likely because irinotecan-mediated cytotoxicity and its potentiation by PARP inhibition occur primarily in cells with S phase DNA content (25), absent in noncycling hepatocytes. A small amount of DNA damage during G₁ may occur in response to irinotecan (25); if this occurred, the absence of PD effects in normal liver suggests proficient repair despite addition of veliparib. In either case, the lack of detectable DNA damage and repair markers in normal liver suggests a favorable therapeutic index for the irinotecan/veliparib combination.

However, increases in γ -H2AX foci after combination treatment measured using a validated single-plex assay did not correlate with response and were not confirmed with an exploratory multiplex assay. This discrepancy is likely related to the enhanced image analysis of the multiplex assay that is capable of excluding nonmalignant cells from the biomarker evaluation, and points to the confounding influence of DDR responses by nonmalignant yet cycling cells. In addition to technical issues, lack of corroboration of preclinical results may also be related to the timing of tumor sampling, designed in this trial to detect reduction of tumor PAR content after veliparib, and not optimized for detection of modulation of the DDR in malignant cells. Finally, underlying biology not predicted by preclinical models may be contributing, including the possibility that γ -H2AX focus formation may be dispensable for initial recruitment of other DNA repair factors in some instances (43).

Because a pNBS1 signal was detectable in samples negative for γ -H2AX response, pNBS1 may be useful for assessing DDR endpoints in future trials. A larger sample size will be required to determine if increased pNBS1 after combination treatment correlates with clinical outcome. Use of pNBS1 will also require determination of the optimal sampling window after topoisomerase I inhibition to measure this biomarker. In fact, a later time point might have demonstrated completion of repair (absent pNBS1) after irinotecan alone that was still ongoing (persistent pNBS1) after veliparib/irinotecan in a larger number of samples. It is also possible that NBS1 is involved in the removal of topoisomerase I cleavable complexes to a greater extent in some tumor cells than others. Assessment of XRCC1 or TDP1 recruitment into nuclear foci might have been a more direct measure of the action of veliparib in the presence of irinotecan, but quantitative IFAs for XRCC1 and TDP1 are not yet developed.

The MTD of veliparib when combined with irinotecan of 40 mg twice daily is comparable with doses used in the phase 0 study (17). Consistent with the inhibition of PARP catalytic activity, our PD data demonstrated reduction of tumor PAR content during veliparib exposure. Although the timing of biopsies has not allowed us to document persistent reduction in PAR over the 12-hour dosing interval, in the phase 0 trial, a single dose of 50 mg veliparib did reduce PAR levels measured at 24 hours by 76% and 97% in 2 patients, respectively (17).

In contrast with the documented effects on PARP catalytic activity, it is unlikely that PARP–DNA trapping has been achieved because monotherapy cytotoxicity, linked to PARP–DNA trapping, requires veliparib doses >300 mg twice daily, with 400 mg twice daily as the monotherapy MTD (18). The compromise in PARP inhibitor dose compared with the monotherapy MTD is similar to the doses of veliparib given in combination with other DNA-damaging agents (5, 44); the need for dose reduction has also been observed when other PARP inhibitors are combined with chemotherapy (10, 45, 46).

The requirement for PARP–DNA trapping in the potentiation of topoisomerase I poisoning by PARP inhibition is unclear. *PARP1*^{−/−} cells are hypersensitive to camptothecin, and the addition of a PARP inhibitor does not further sensitize these cells, suggesting that the absence of PARP catalytic activity is sufficient for sensitization (26). However, in other studies, parental and *PARP1*^{−/−} mouse embryonic fibroblasts have indistinguishable camptothecin sensitivities; transfection with catalytically inactive PARP1 or its isolated DNA-binding domain sensitizes to camptothecin, consistent with a model in which small-molecule inhibitors convert PARP1 into a protein that potentiates topoisomerase I poisons by binding to damaged DNA and preventing repair (27). If trapping of PARP–DNA complexes is a component of topoisomerase I inhibitor–mediated sensitization, alternative schedules using higher veliparib doses may be required.

In this study, PRs were seen in *BRCA* carriers and WT patients. HR is required to faithfully process double-strand breaks arising during S phase following combined topoisomerase I and PARP inhibition, so that HR-deficient tumors may be overall more vulnerable. For this reason, the irinotecan/veliparib combination is being studied in two triple-negative breast cancer populations, including those with and without *BRCA* germline mutations.

In summary, we have demonstrated the ability to safely inhibit PARP1/2 catalytic activity in combination with topoisomerase I inhibition. Further studies are necessary to reproducibly demonstrate modulation of the DDR in the context of loss of PARP1/2 function. Ultimately, randomized trials will be required to definitively demonstrate increased efficacy afforded by PARP inhibition in concert with DNA damage.

Disclosure of Potential Conflicts of Interest

P.M. LoRusso is a consultant/advisory board member for Akebia, Genentech, Novartis, and Pfizer. S. Tolane reports receiving commercial research grants from Genentech. No potential conflicts of interest were disclosed by the other authors.

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Clinical Cancer Research

Phase I Safety, Pharmacokinetic, and Pharmacodynamic Study of the Poly(ADP-ribose) Polymerase (PARP) Inhibitor Veliparib (ABT-888) in Combination with Irinotecan in Patients with Advanced Solid Tumors

Patricia M. LoRusso, Jing Li, Angelika Burger, et al.

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Phase I study of the safety, pharmacokinetics (PK), and pharmacodynamics (PD) of the poly(ADP-ribose) polymerase (PARP) inhibitor veliparib (ABT-888; V) in combination with irinotecan (CPT-11; Ir) in patients (pts) with advanced solid tumors.

Presented Sunday, June 5, 2011

Authors:

[Print](#)

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Abstract Disclosures

Background:

The nuclear enzyme PARP is essential in recognition and repair of DNA damage. In preclinical models, PARP inhibitors work as sensitizing agents for DNA-damaging agents such as Ir. V is an orally bioavailable PARP 1 and 2 inhibitor. This phase I study determined the maximum tolerated dose (MTD), dose-limiting toxicities (DLTs), PK and PD of V together with Ir.

Methods:

Eligibility included patients with performance status 0-2; $\geq$ age 18; adequate bone marrow, hepatic and renal function. Cycles were 21 days. Ir was given i.v. 100 mg/m² over 90 min on Days 1 and 8. Twice daily (BID) oral dosing of V (10-50mg) occurred Days 3-14 (Cycle 1) and Days -1-14 (subsequent cycles) followed by a 6-day rest. PK studies were conducted with both agents alone and in combination. Tumor biopsies and matched peripheral blood mononuclear cells (PBMCs) were collected for PD evaluation of PAR, γ -H2AX, PARP1, TOPO1, and ERCC1 after Ir alone and after the combination.

Results:

32 pts were treated (2 lung, 14 breast, 4 esophageal, 7 ovarian, 4 colon, 1 anal). Median age was 53 (range 31-73). Most frequent drug-related toxicities included: diarrhea (59%), nausea (56%), leukopenia (50%), fatigue (47%), neutropenia (47%), anemia (34%), and vomiting (31%). DLTs included fatigue, diarrhea, febrile neutropenia (gr 3), leukopenia and, neutropenia (gr 4). Response has been determined for 28 pts. A clinical benefit (CB) rate of 61% (5 PR, 2 MR, 10 SD) was observed. V exhibited linear PK over the dose range of 10 to 50 mg BID; co-administration of Ir did not affect PK of V. Exploratory analysis of pre/post-V treatment changes indicated median ERCC1 levels in tumor tissue and PBMCs differed somewhat by CB status. PAR levels in paired tumor biopsies showed > 50% reduction in 17 of 19 pts and > 90% reduction in 10 of 19 pts.

Conclusions:

The MTD and recommended phase II dose was established as 100 mg/m² of Ir given i.v. Days 1 and 8 combined with 40 mg of V given BID 15 days on/6 days off (21 day cycle). Exploratory analysis demonstrated that V reduced PAR levels in tumor. Support: NCI U01-CA062487 and NCI R21-CA135572.

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Phase I study of the safety, pharmacokinetics,
and pharmacodynamics of the poly(ADP-
ribose) polymerase (PARP) inhibitor veliparib
(ABT-888) in combination with irinotecan (CPT-
11) in patients with advanced solid tumors

P LoRusso, JJ Ji, LK Heilbrun, G Shapiro, EA
Sausville, SA Boerner, DW Smith, M Pilat, J
Zhang, AP Chen, N Nechiporchik, RE
Parchment

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Study Design

CPT11

Single agent at a range of concentrations

Cancer Cell Lines

BRCA1 *mutant* vs. wild type

p53 *mutant* vs. wild type

ERCC1 high vs. low expression

ERCC1 polymorphism



ABT-888

Single agent at a range of concentrations



Readouts:

Cytotoxicity

PARP-1

γ -H2AX

CPT11+ABT-888

ABT-888 at a fixed concentration of 210 nM (plasma $\times C_{min}$)

PARP-1K_i = 5.2 nM

PARP-2K_i = 2.9 nM

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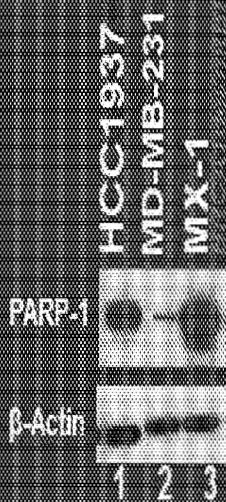
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ABT-888/CPT11 Single Agents vs Combination Effects

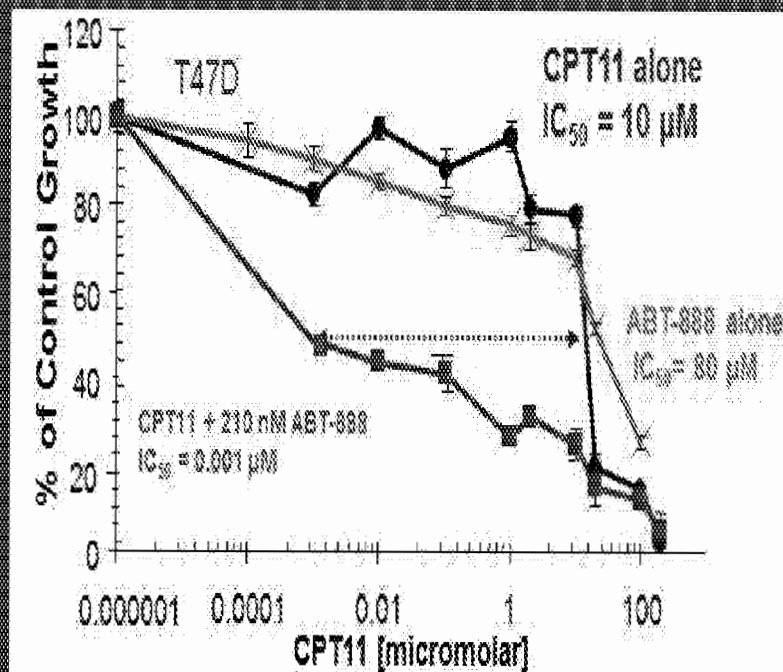
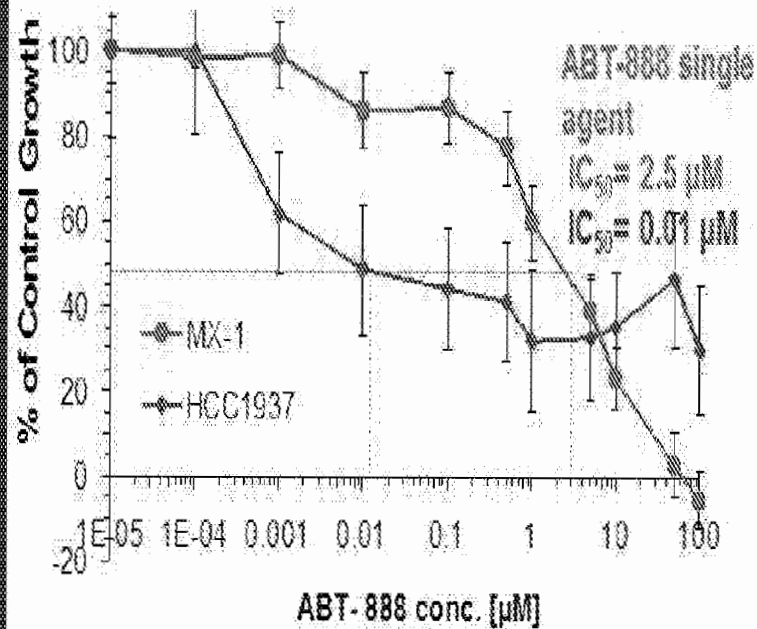
ERCC1 high

Mt p53



ERCC1 high

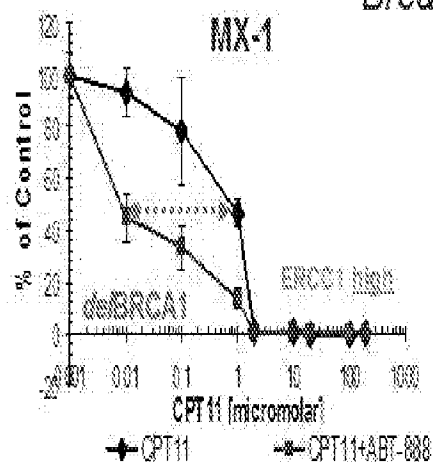
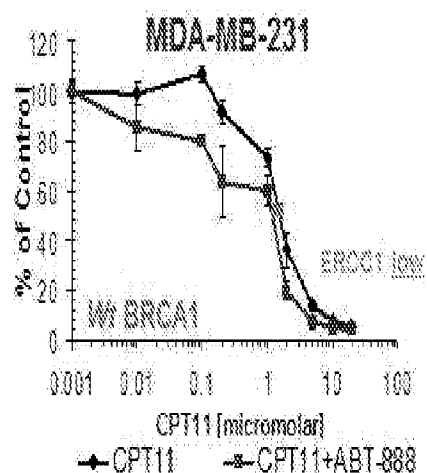
Mt p53



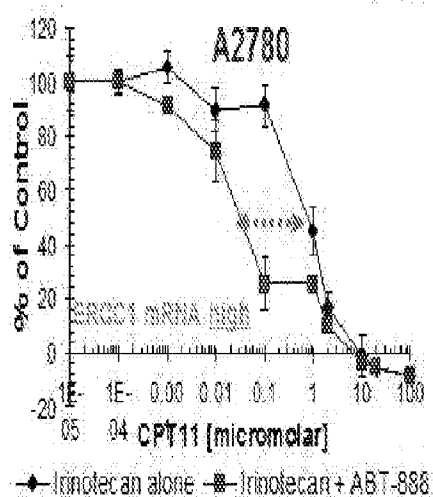
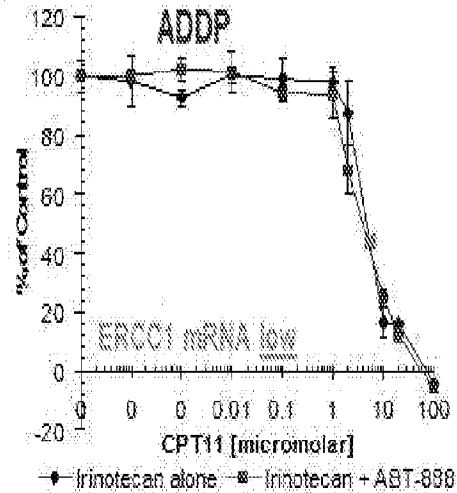
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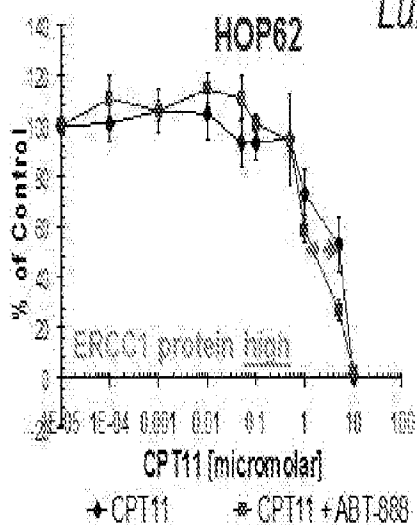
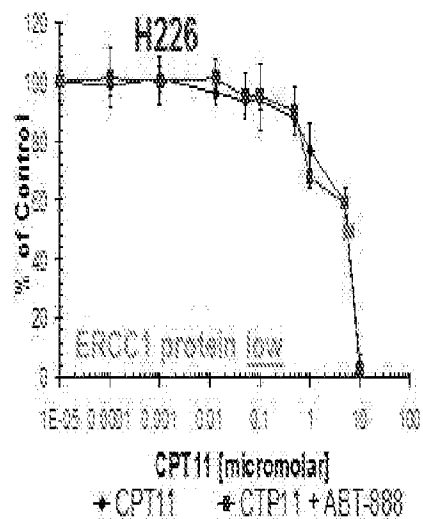
Breast



Ovarian

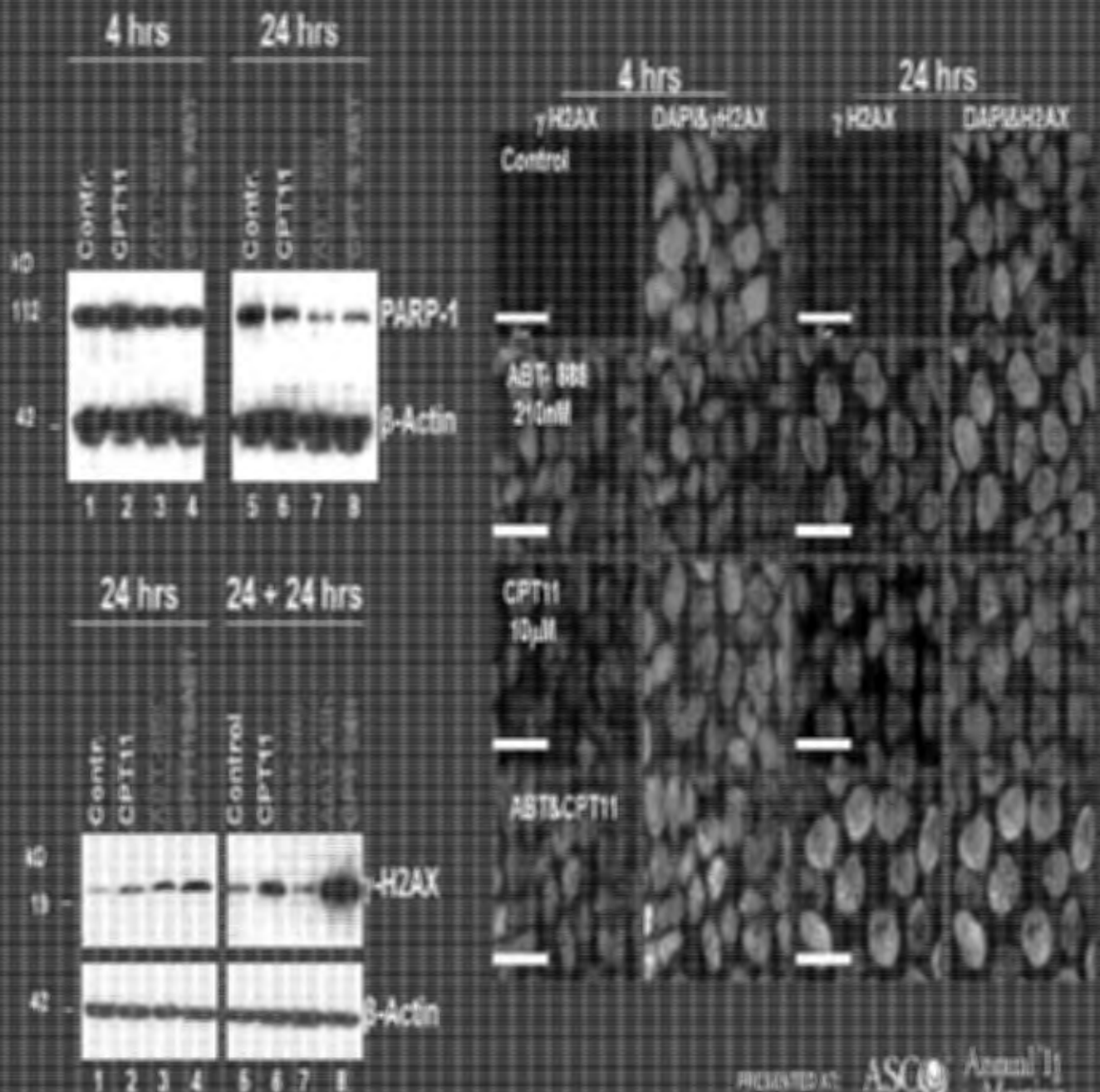


Lung



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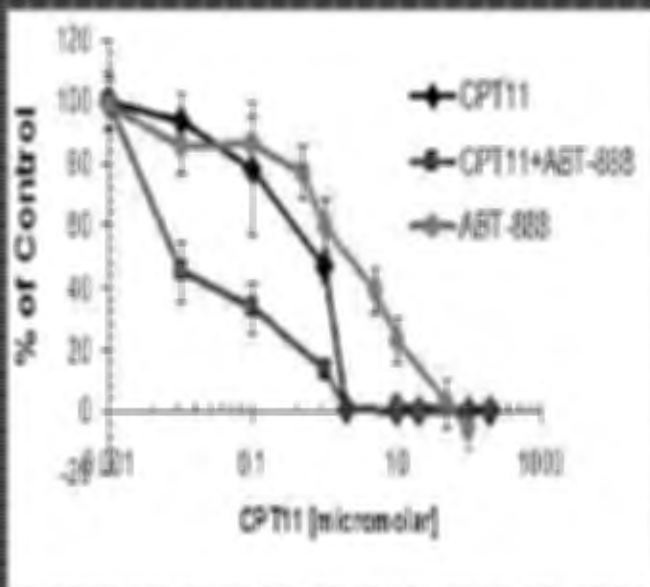
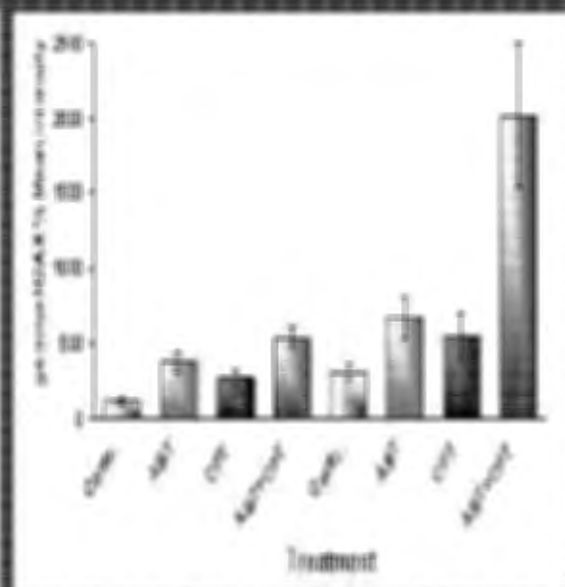
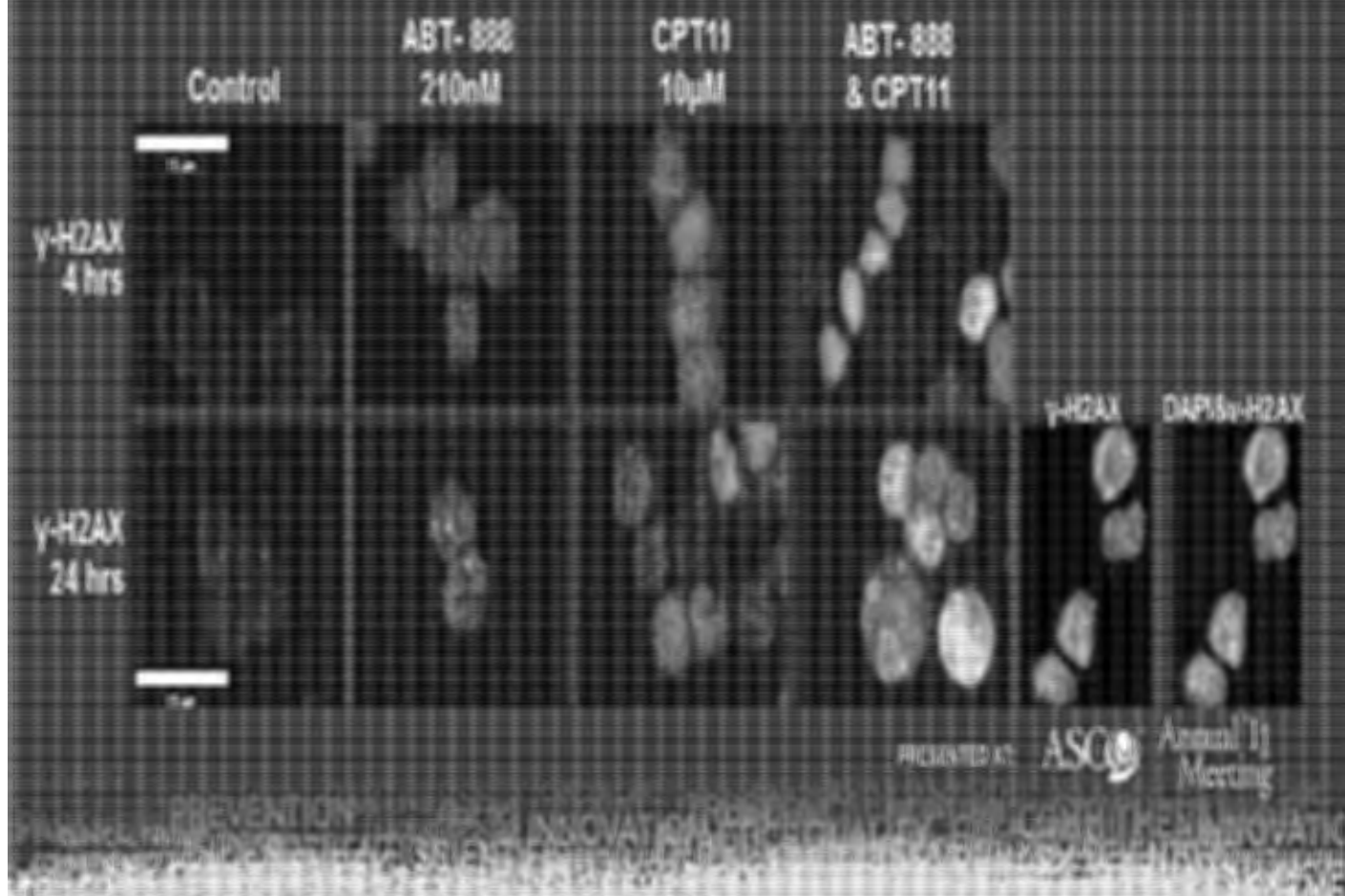
ABT-888/CPT11 Combination Efficacy Results in Down-regulation of PARP and Induction of DSB in T47D Cells



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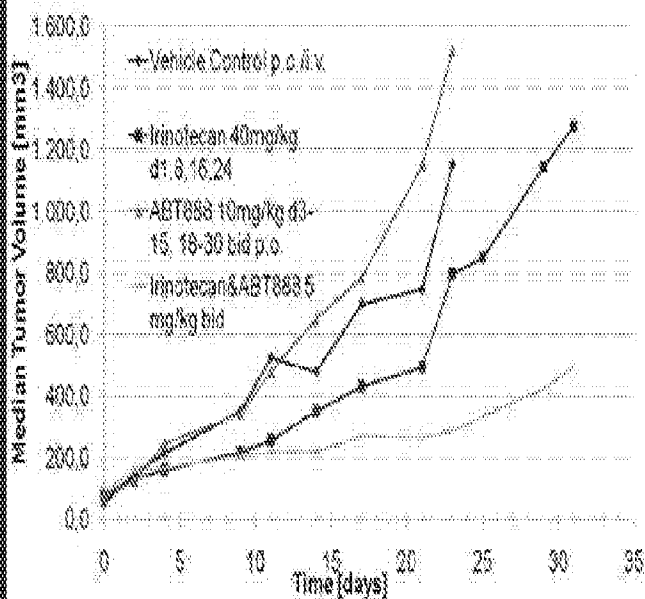
ABT-888/CPT11 Single Agent and Combination Efficacy in *mt* BRCA1 MX-1 Breast Cancer Cells



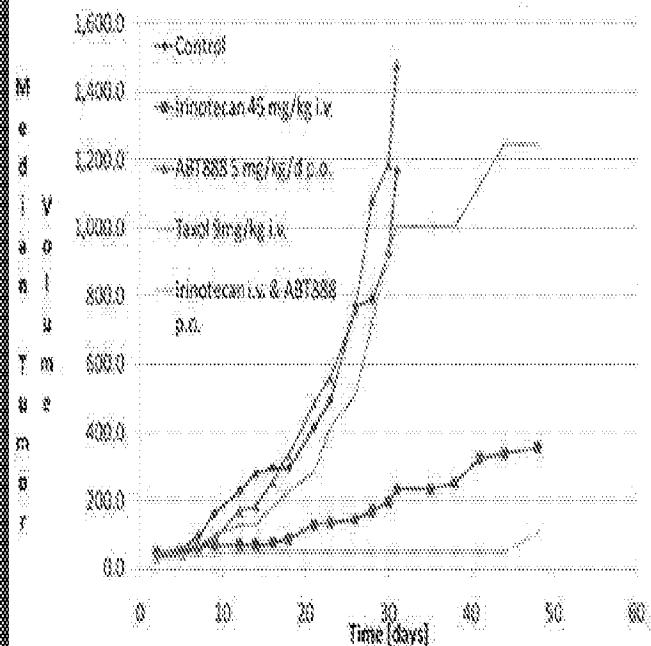
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Combination Irinotecan + ABT-888 *In Vivo*

ABT888/Irinotecan Show Synergism in ADDP Xenograft Tumors *In Vivo*



ABT888 and Irinotecan Act Synergistic in the Taxol Resistant MX-1 Human Tumor Xenograft



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NCI #7977: Objectives

- Primary Objectives:

- To determine the RP2D for PARP inhibition for CPT11/ABT888
- To determine the safety profile of CPT11/ABT888

- Secondary Objectives:

- To determine the PK profile of CPT11/ABT888, both as single agents and in combination
- To determine the tumor response as assessed by RECIST

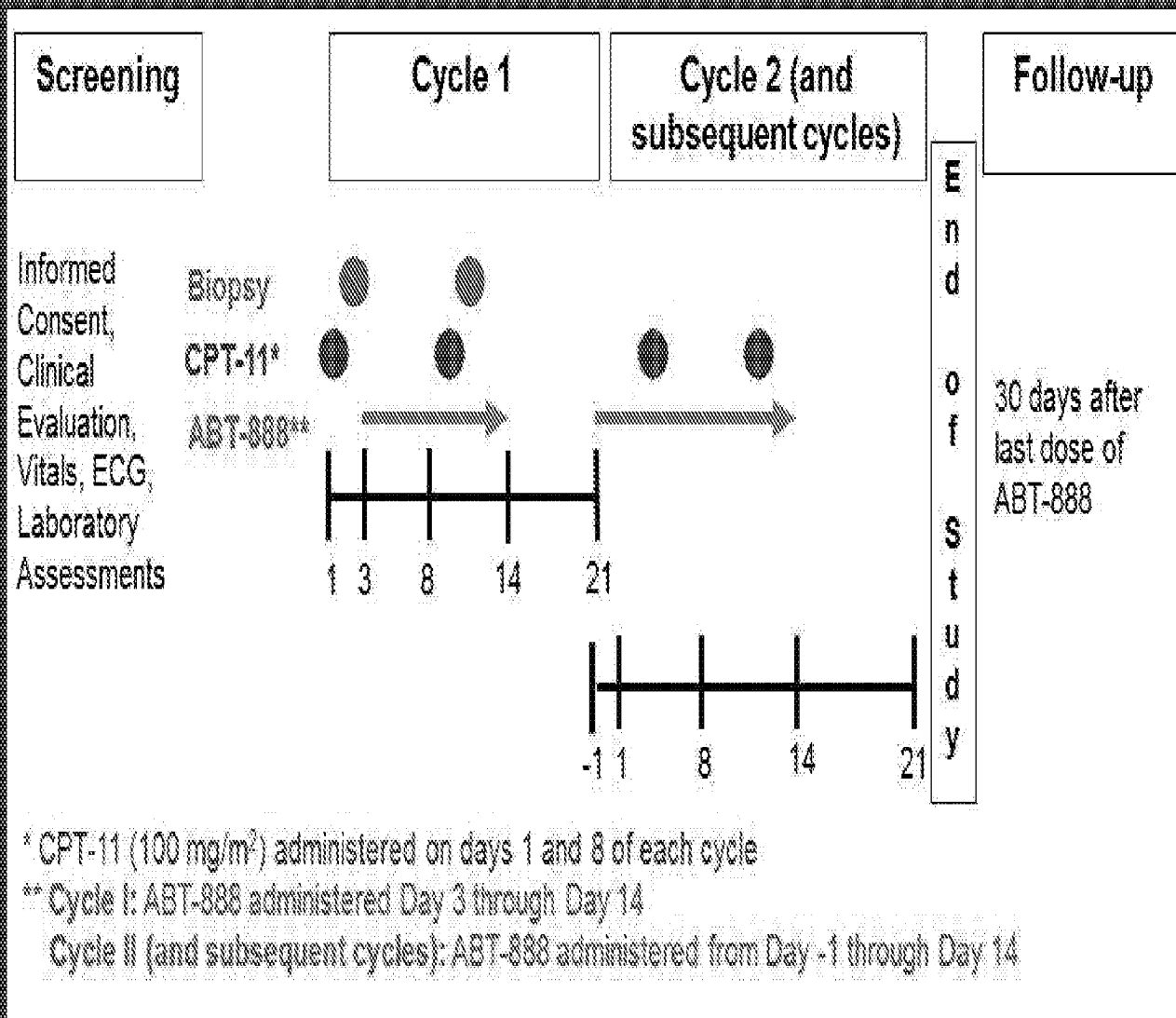
- Exploratory Objectives:

- PD response: PARP inhibition in PBMC and tissue (PAR levels)
- DNA damaging effects of CPT11 and CPT11/ABT888
ABT-888 by measuring levels of markers of DNA damage and repair (DDR)

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NCI #7977: Trial Schema



Tumor Collections: D2: 28h D8: 28h

Blood Collections: D1: 0, 3.5, 5.5, 8.5, 28, 48 h

D8: 0, 3.5, 5.5, 8.5, 28, 48 h

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NCI #7977: Eligibility

- Histologically or cytologically confirmed solid tumors
- Measurable disease accessible for biopsy
- UGT1A1
- Performance status 0-2
- $\geq$ age 18
- Life expectancy > 12 weeks
- Adequate bone marrow, hepatic and renal function

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Most Common Toxicities (>10%)

Toxicity	Grade of Adverse Event, n				Total, n (%)
	1	2	3	4	
Diarrhea	9	8	3	-	20 (59)
Nausea	15	4	-	-	19 (56)
Fatigue	9	5	2	-	16 (47)
Leukopenia	3	6	4	3	16 (47)
Neutropenia	1	6	2	6	15 (44)
Anemia	4	6	1	-	11 (32)
Vomiting	6	4	-	-	10 (29)
Alopecia	6	1	-	-	7 (21)
Lymphopenia	1	2	2	1	6 (18)
Anorexia	4	-	-	-	4 (12)
Dehydration	1	2	1	-	4 (12)
Hemoglobin	1	2	1	-	4 (12)
Hypoalbuminemia	2	2	-	-	4 (12)

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Dose Limiting Toxicities (DLTs)

ABT-888 (mg)*	Patient ID	Toxicity	Grade
10	001-002	Fatigue	3
20	003-015	Leukopenia	4
		Neutropenia	4
40		No DOSE LIMITING TOXICITIES	
50	001-023	Diarrhea	3
	001-026	Febrile Neutropenia	3
* CPT-11 administered	100 mg/m ²	Leukopenia	4

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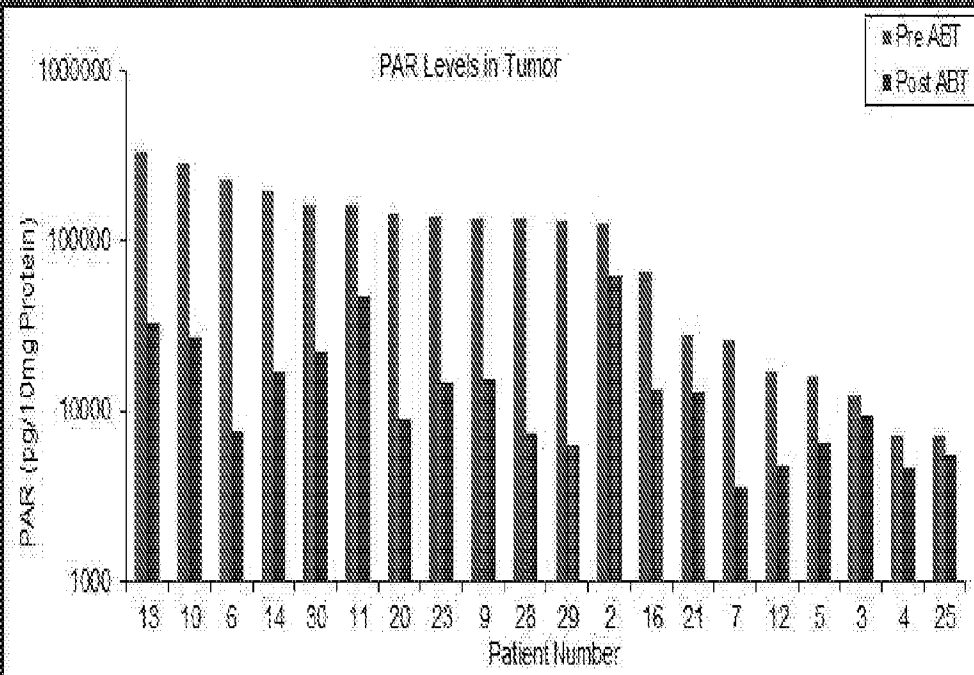
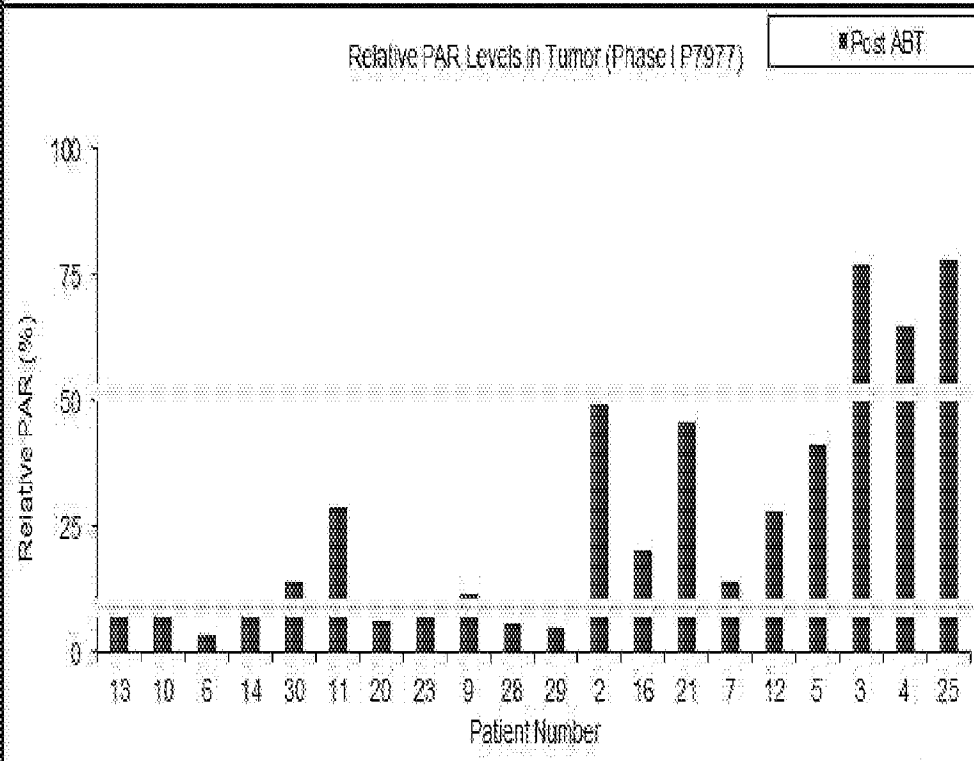
NCI #7977: Results

- Preliminary response data (n=30)
 - Clinical Benefit Rate of 63%
 - 6 PR (breast(2), colon(2), ovarian(2))
 - 1 MR (breast)
 - 12 SD (range 2-6 cycles; median 3 cycles)
 - Breast(5), ovarian(4), lung(2), anal(1)
- MTD and RP2D:
 - 100 mg/m² of CPT-11 iv Days 1 and 8
 - 40 mg of ABT-888 po BID 15 days on/6 days off (21 day cycle)

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PAR Levels in Tumors of Predose vs. Postdose



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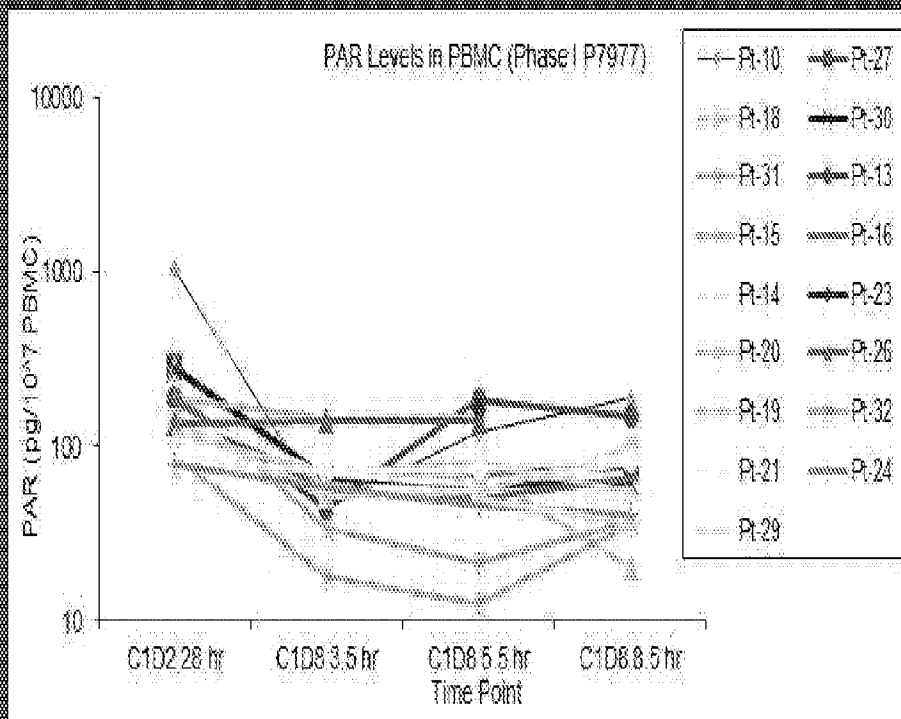
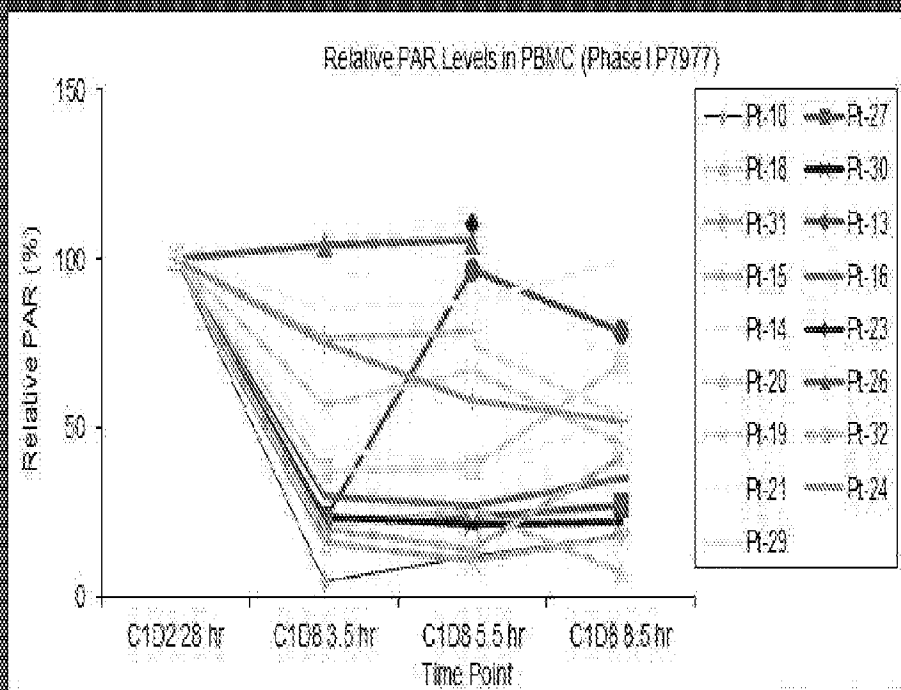
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CSPC Exhibit 1095

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PAR Levels in PBMCs



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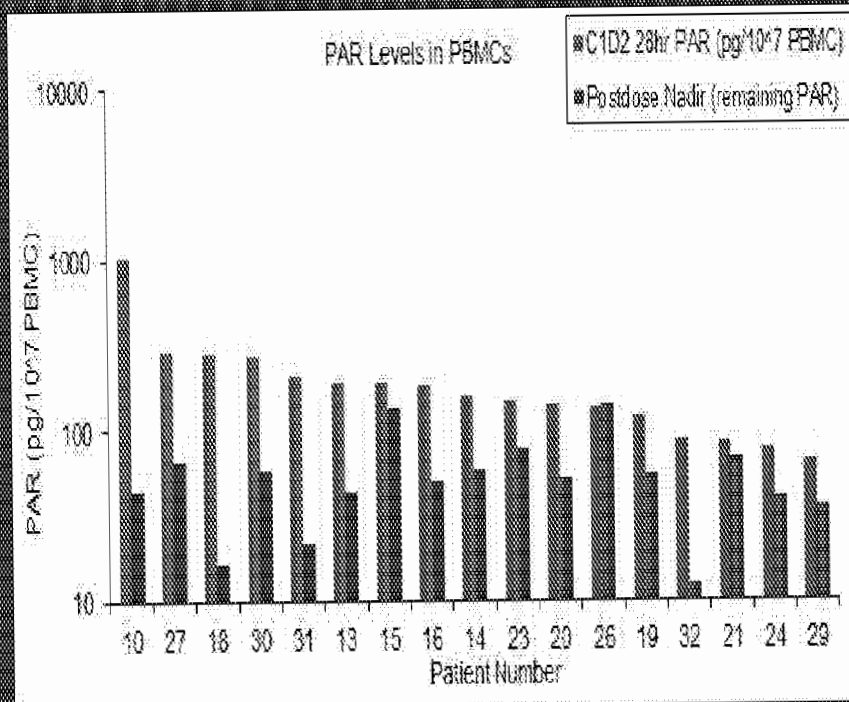
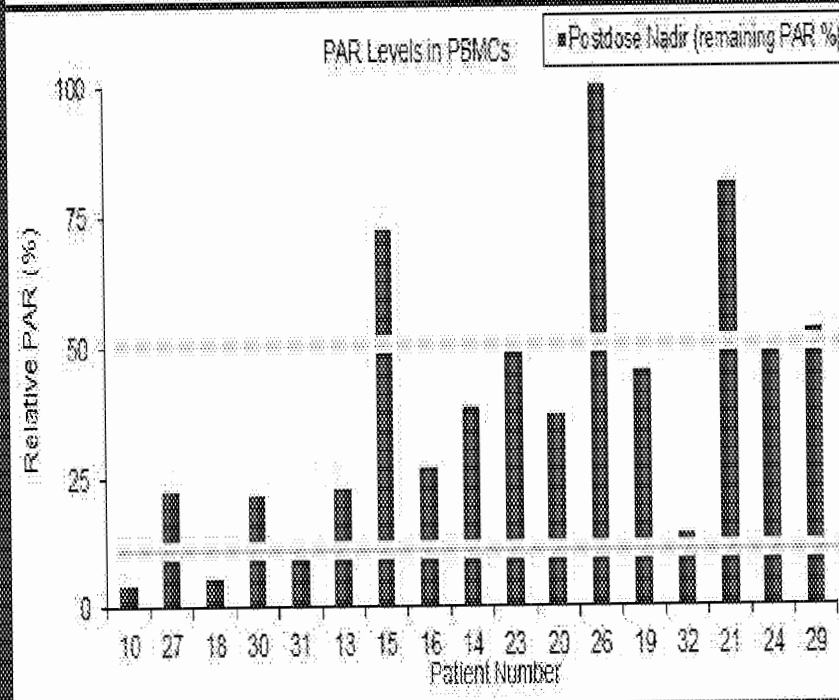
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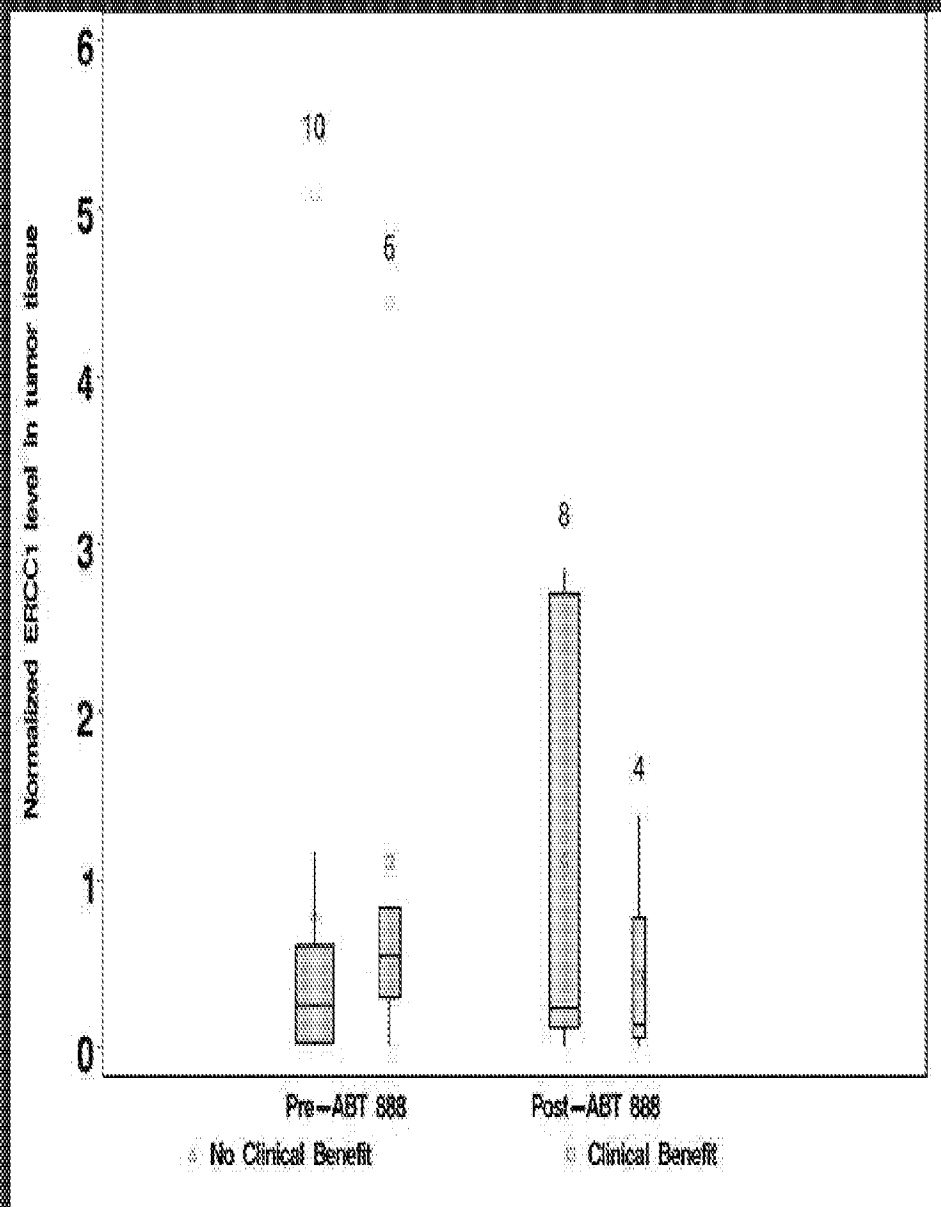
PAR Levels in PBMCs of Predose vs. Postdose



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ERCC1 Levels in Tissue



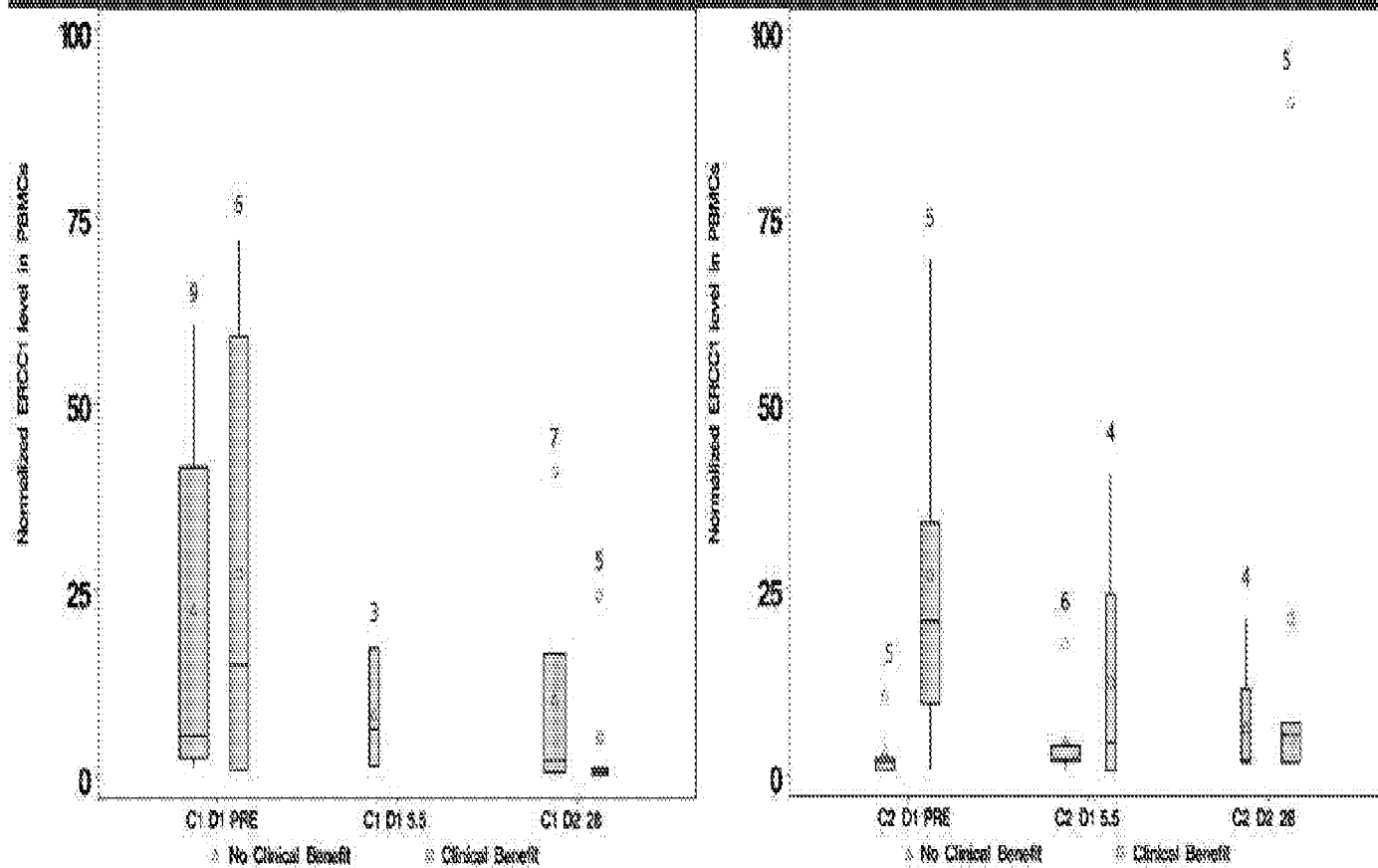
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ERCC1 Levels in PBMCs

CYCLE1

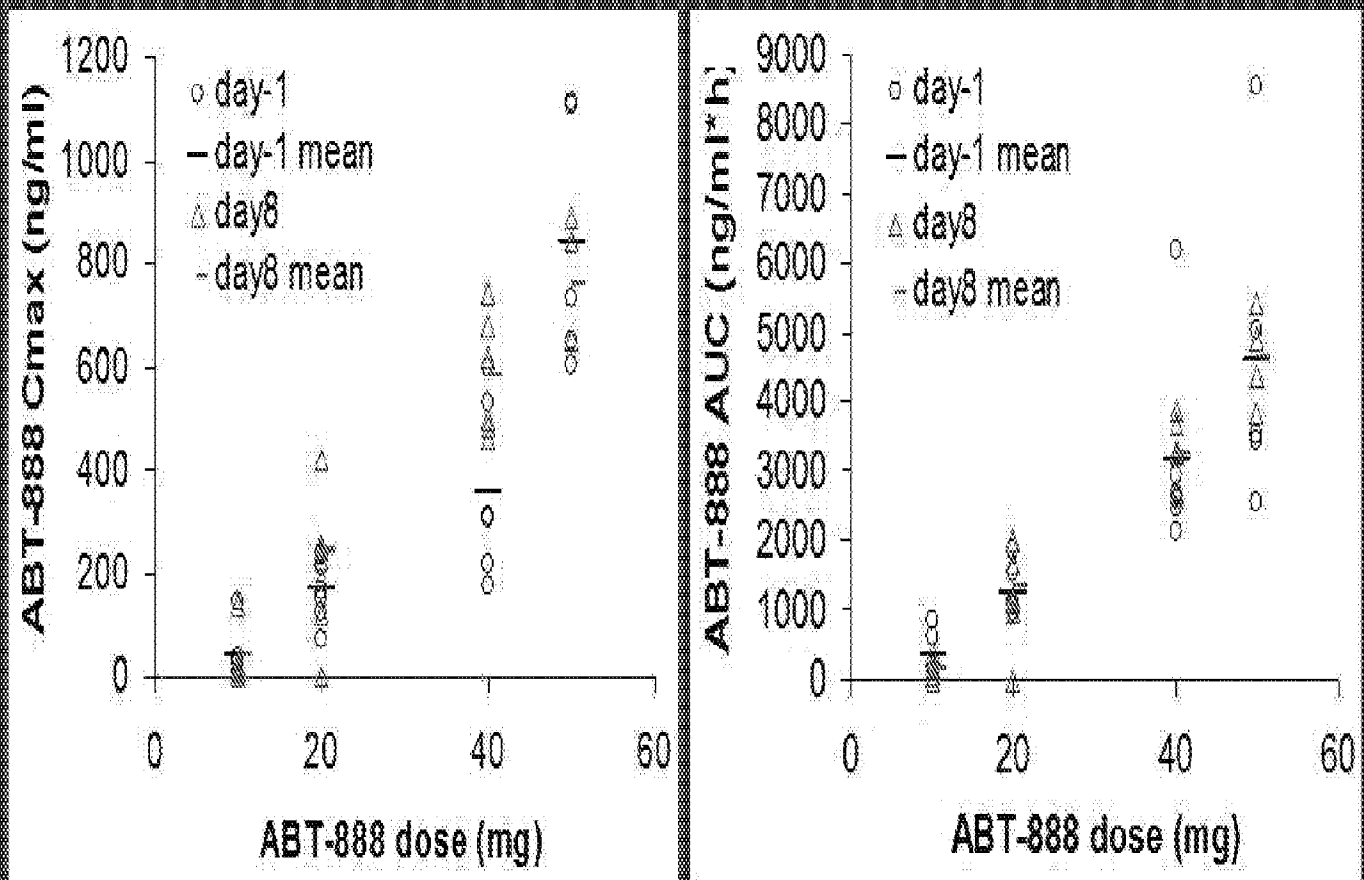
CYCLE2



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Systemic exposure to ABT-888, when given alone (on day 1) and in combination with irinotecan (on day 8)



- Systemic exposure to ABT-888 (i.e., Cmax and AUC) 1d with 1g dose
- ABT-888 exhibited linear PK from 10 to 50 mg BID

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PK parameters of CPT-11, alone (cycle 1) and in combination with ABT-888 (cycle 2)

Cycle	T1/2 (h)	Tmax (h)	Cmax (ng/ml)	AUCINF (ng/ml*h)	Cl (L/h/m2)	Vz (L/m2)	Vss (L/m2)
CPT-11							
1	7.8±1.1	1.5±0.1	1060±341	5278±1900	21±5	230±70	151±38
2	7.6±1.1	1.5±0.2	818±230	4149±1036	25±6	270±63	178±55
SN-38							
1	14.0±10.6	2.0±0.9	11±6	117±61			
2	13.6±12.3	1.9±1.0	10±4	98±55			
SN-38G							
1	11.3±5.1	2.5±0.9	21±16	240±173			
2	11.0±5.6	2.8±1.2	21±15	223±162			

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NCI #7977 – PK Summary

- ABT-888 exhibited linear PK at tested dose levels (from 10 to 50 mg BID)
- No apparent difference in oral clearance of ABT-888 when given alone and in combination with irinotecan (mean CL/F: 30 vs 42 L/h on days -1 and 8 respectively)
- Co-administration of CPT-11 did not affect the PK of ABT-888

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NCI #7977 – PD Summary

- ERCC1 levels in CB patients appear to decline from pretreatment median
- PAR levels in paired tumor biopsies:
 - ≥ 50% reduction in 17 of 20 pts (85%)
 - ≥ 90% reduction in 7 of 20 pts (35%)
- PAR levels in PBMCs
 - ≥ 50% reduction in 11 of 17 pts (65%)
 - ≥ 90% reduction in 3 of 17 pts (18%)
- gamma-H2AX levels being conducted by NCI National Clinical Target Validation Laboratory (NCTVL)

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Proposed Expansion

- CTCs
 - + γ H2Ax response in tumor @ 6-8 hrs vs CTCs at 12-24 hrs
 - Does PARPi $\uparrow\uparrow$ γ H2Ax in CTCs by 6-8 hrs post CPT-11?
 - When does γ H2Ax response peak in CTCs (correlation with tumor response)?
 - Is there a tumor switch between PARP and ERCC1 mediated repair in the presence of PARPi, (i.e., confirm initial PBMC and tumor findings)
 - Day 15 CTCs analysis for ABT-888 scheduling decision making (15 vs 21 days)
- Genomic profiling of TNBC or both expansion arms

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Expansion – TNBC and mtBRCA

Cycle 1 (total duration 21 days)



* CPT-11 (irinotecan) IV 100 mg/m² of CPT-11 given on Days 1 and 8 administered for 90 min on days 1 and 8 of each cycle. APT-888 40mg po bid on days 2-15 in cycle 1; Decision to administer APT-888 on a continuous schedule predicated on results from first 6 patients

* PBMC samples will be obtained at baseline, 5-8, 12, 22, and 24h after CPT alone on day 1 and after the combination dose on day 8 in cycle 1.

† Tumor biopsy will be performed on day 1 and day 8 after the first dose of CPT-11 and APT-888.

‡ Tumor biopsy will be performed on day 1 and day 8 after the first dose of CPT-11 and APT-888.

§ Tumor biopsy will be performed on day 1 and day 8 after the first dose of CPT-11 and APT-888.

¶ Tumor biopsy will be performed on day 1 and day 8 after the first dose of CPT-11 and APT-888.

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¶ Tumor biopsy will be performed on day 1 and day 8 after the first dose of CPT-11 and APT-888.

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Thank You!

First and foremost – patients & families

Karmanos Cancer Institute:

- Angelika Burger, PhD
- Scott Boerner, MS
- Lance Heilbrun, PhD
- Jing Li, PhD
- Mary Jo Pilat, PhD, PA-C
- Jie Zhang, MS

National Cancer Institute:

- James Doroshow, MD
- Alice Chen, MD

Funding:

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Dana Farber/ Harvard Cancer Center:

- Geoffrey Shapiro, MD, PhD

National Clinical Target Validation Laboratory (NCTVL):

- Ralph Parchment, PhD
- Robert Kinders, PhD
- Jiuping Jay Ji, PhD

Greenebaum Cancer Center/ University of Maryland:

- Edward Sausville, MD, PhD
- Martin Edelman, MD

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"My work is done."

Albert Einstein

The Lord is my Shepherd. He makes me lie down in green pastures.
He leads me beside still waters. He restores my soul. *Psalms 23*



Prof. Dr. Dr. Angelika Burger

* 08.02.1965 † 04.05.2011

Our beloved daughter, sister, sister in law and niece passed away peacefully
at home with her family by her side, after a courageous battle against cancer

Herbert and Friedel Burger,
Dr. Joachim and Alexandra Burger,
Christine Burger and Peter Müller,
Günther Heid

Neupotz/Germany, Detroit/USA, May 4th 2011

The memorial service will be held on Friday May 6th, 5:00 pm in the Townhall in Neupotz, Germany.
The family suggests memorial donations to be made to the Barbara Ann Karmanos Cancer Institute,
Detroit, MI 48201 (<https://www.karmanos.org/DonationWebApp>)

NCI #7977 – Relevant Demographics

- **N=32 patients treated**

- Tumor type – n (%)

- breast – 14 (44)
 - ovarian – 7 (22)
 - colon – 4 (12.5)
 - esophageal – 4 (12.5)
 - lung – 2 (6)
 - anal – 1 (3)

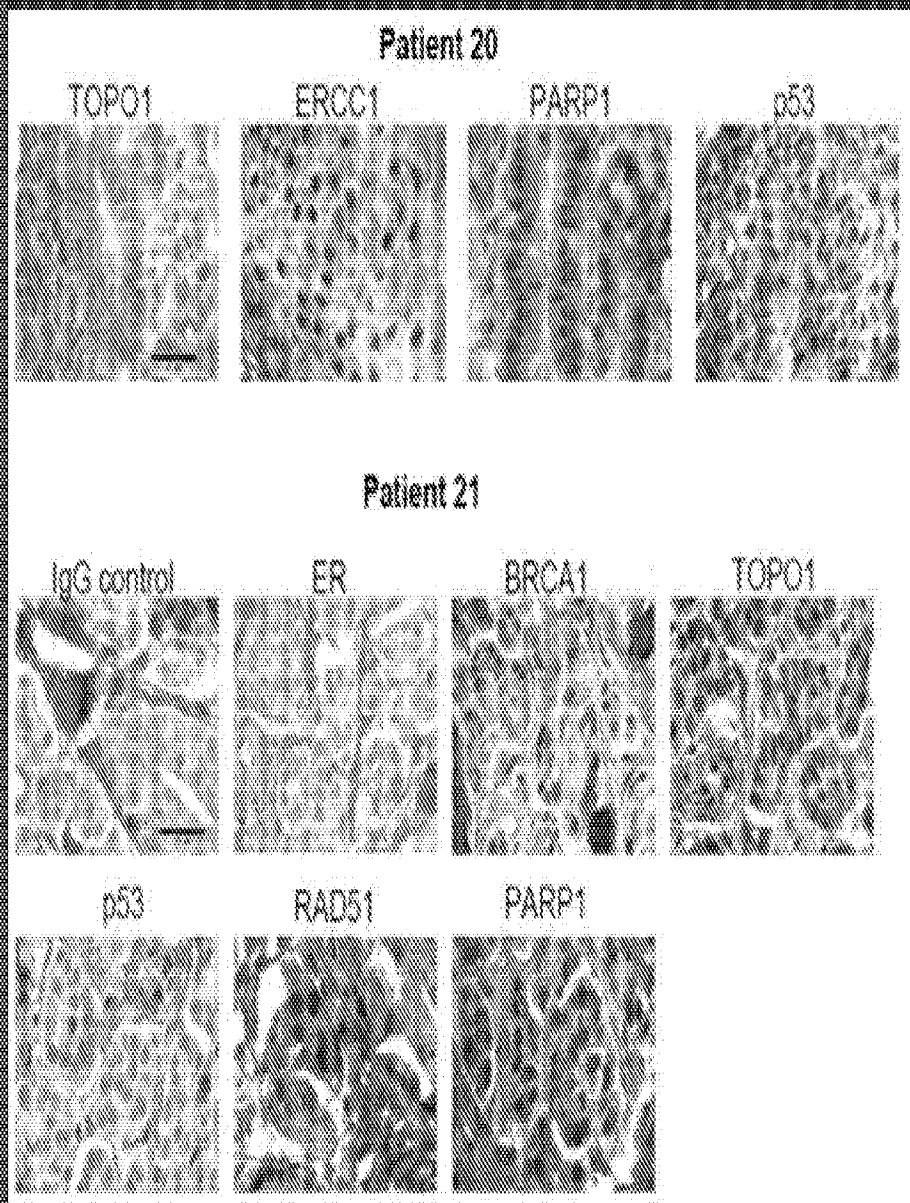
- # of previous treatments – n (%)

- 2-4 – 19 (59)
 - 5-8 – 7 (22)
 - 9-12 – 6 (19)

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Immunohistochemistry (IHC) staining of archival tissue



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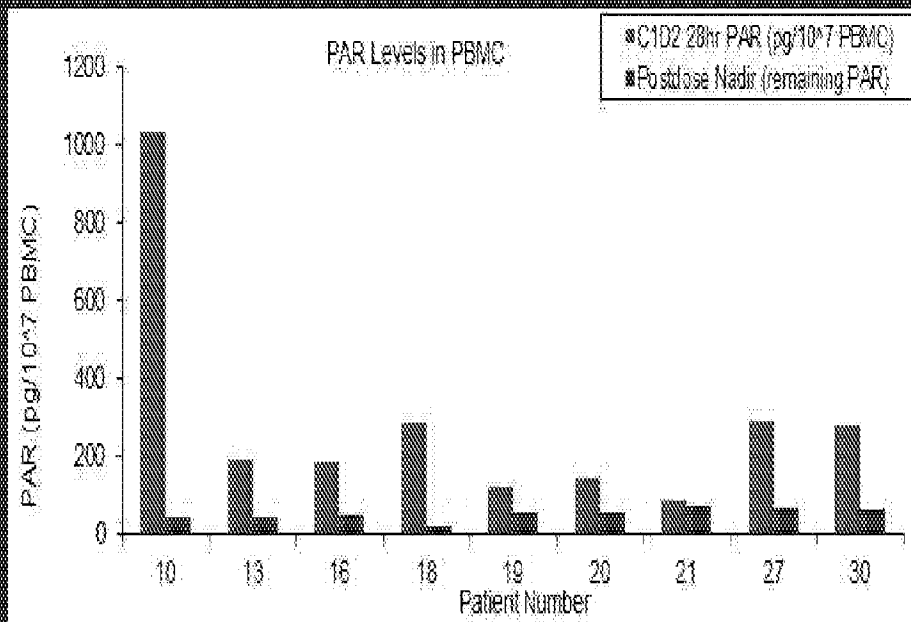
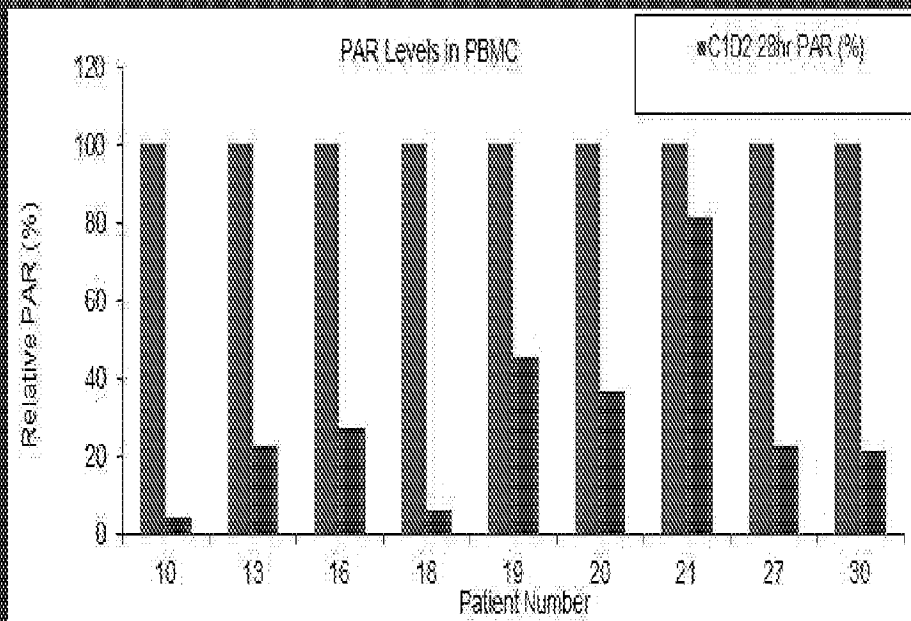
Results of Initial Immunohistochemistry staining

Antibody	Pt 8	Pt 13	Pt 14	Pt 18	Pt 20	Pt 21	Pt 23	Pt 24	Pt 25
ER	negative	negative	negative	positive	negative	negative	positive 3+	positive 1.5+	negative
p53	wild type	negative	not done	mutant	mutant	wild type	mutant	mutant	mutant
BRCA1	cytoplasm	negative	not done	cytoplasm	negative	nuclear	nuclear	nuclear	nuclear
BRCA2	cytoplasm	not done	not done	negative	not done	negative	nuclear	nuclear	negative
PARP1	positive 2+	not done	not done	positive 2+	positive 3+	positive 3+	positive 2+	positive 2+	positive 3+
TDP01	positive 1+	not done	not done	negative	negative	positive 2+	positive 2+	positive 1+	positive 2+
ERCC1	positive 1+	positive 2+	positive 3+	negative	positive 3+	weakly +	positive 3+	positive 2+	positive 3+
HER2	not done	not done	not done	not done	not done	not done	not done	not done	not done
Rad51	not eval.	not done	not done	not done	not done	positive 3+	positive 3+	positive 1.5+	negative

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PAR Levels in PBMCs: Predose vs. Postdose

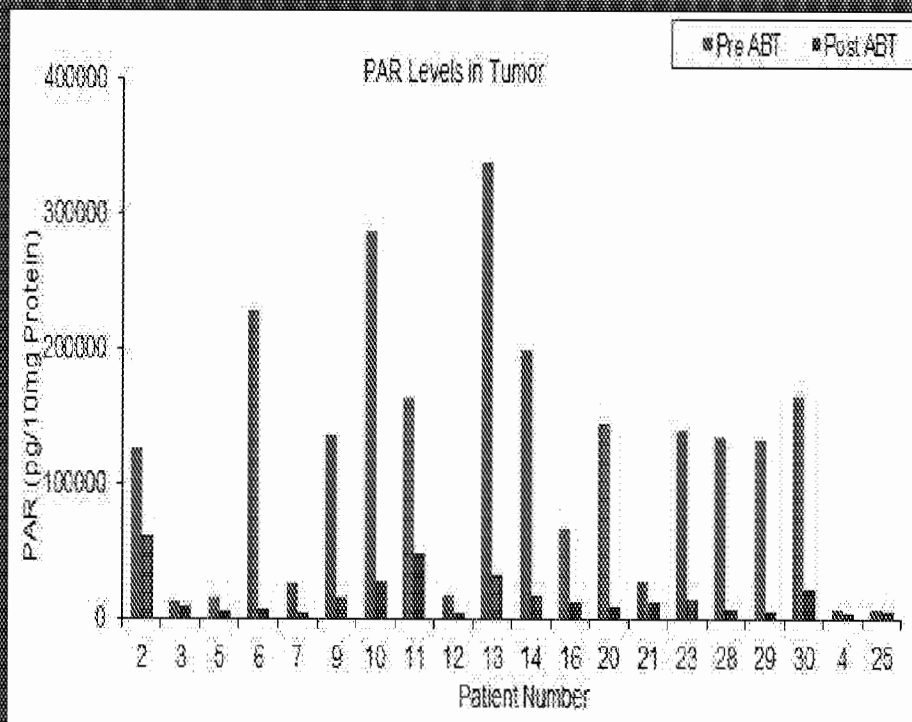
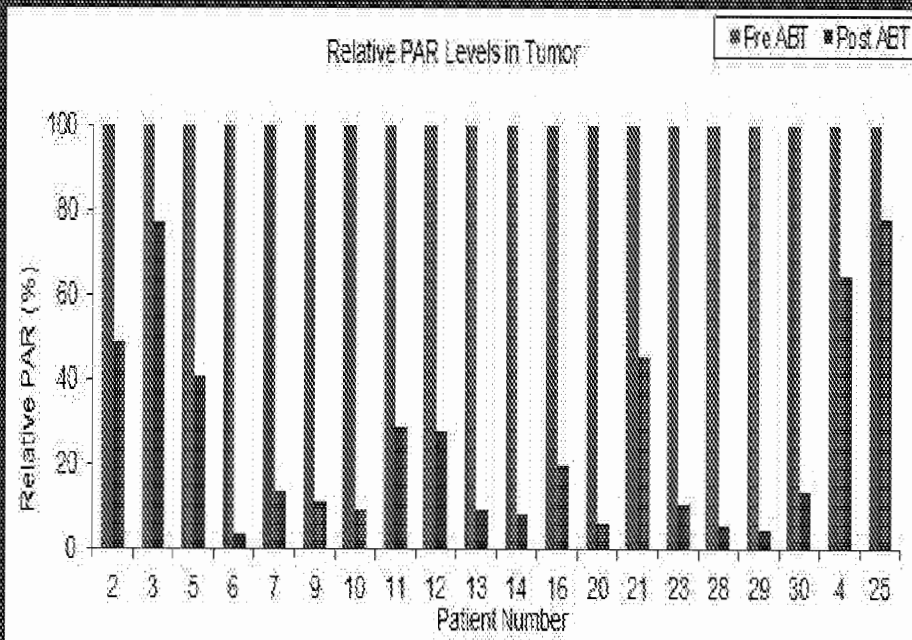


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PAR Levels in Tumors: Predose vs. Postdose

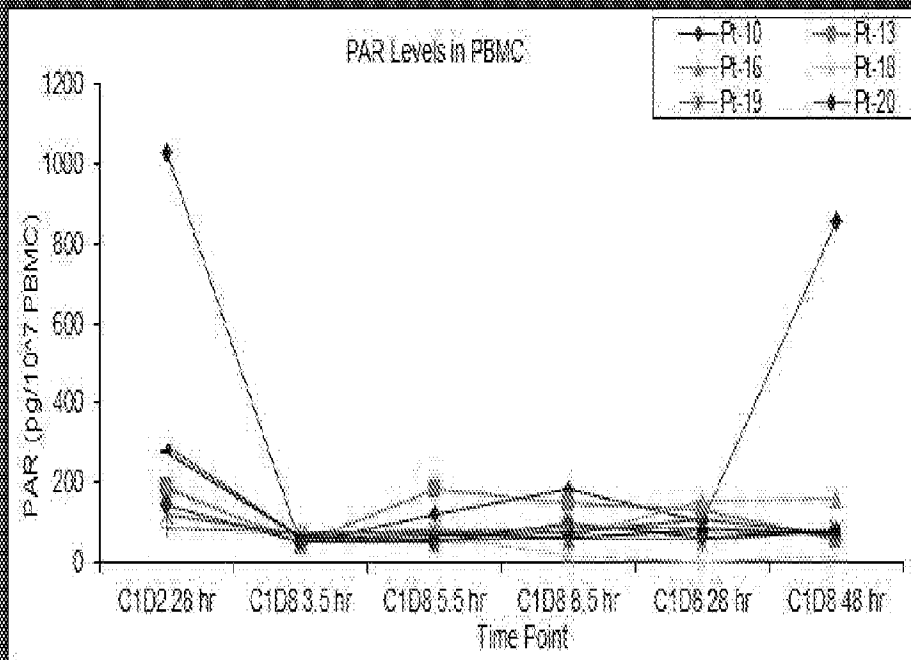
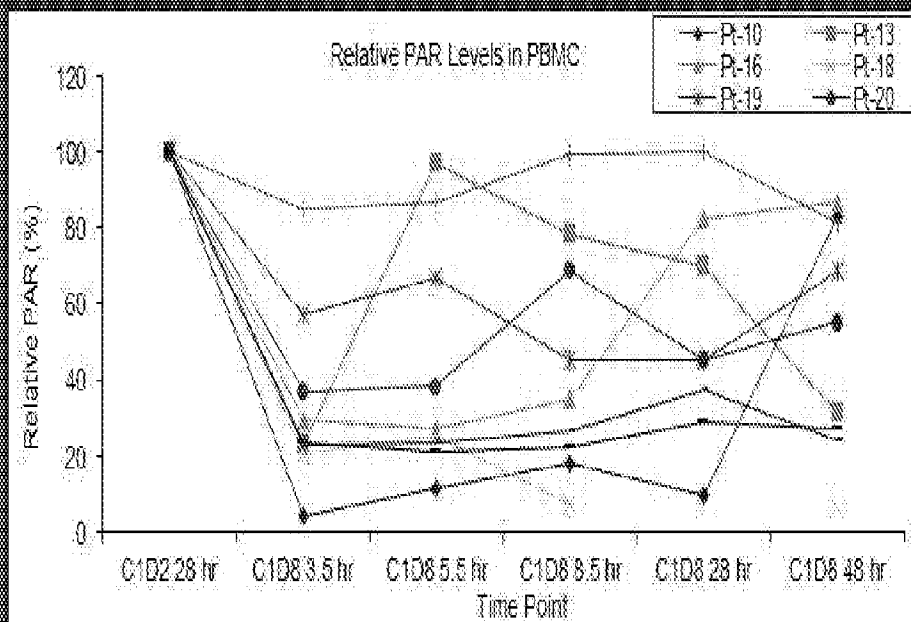


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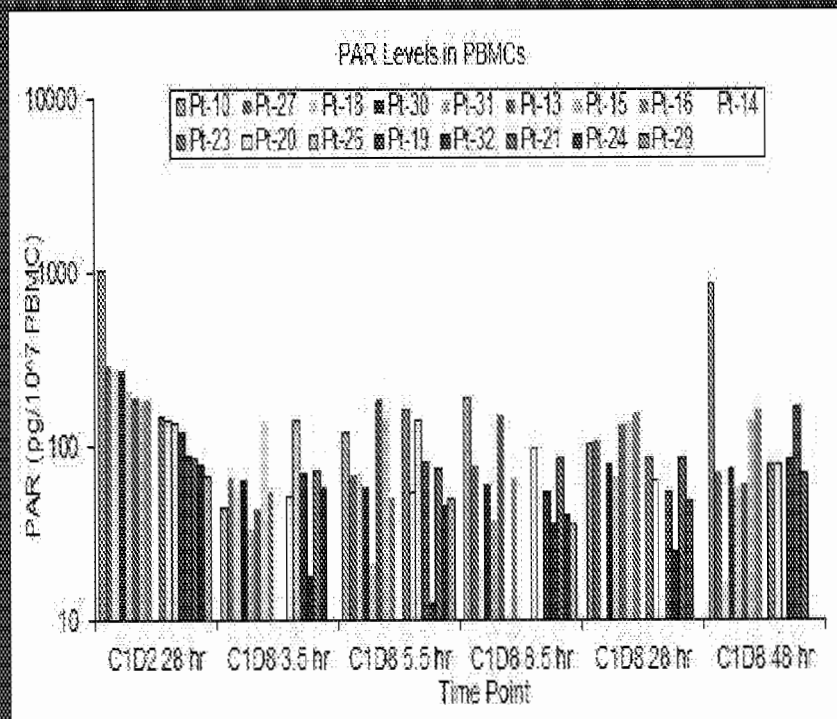
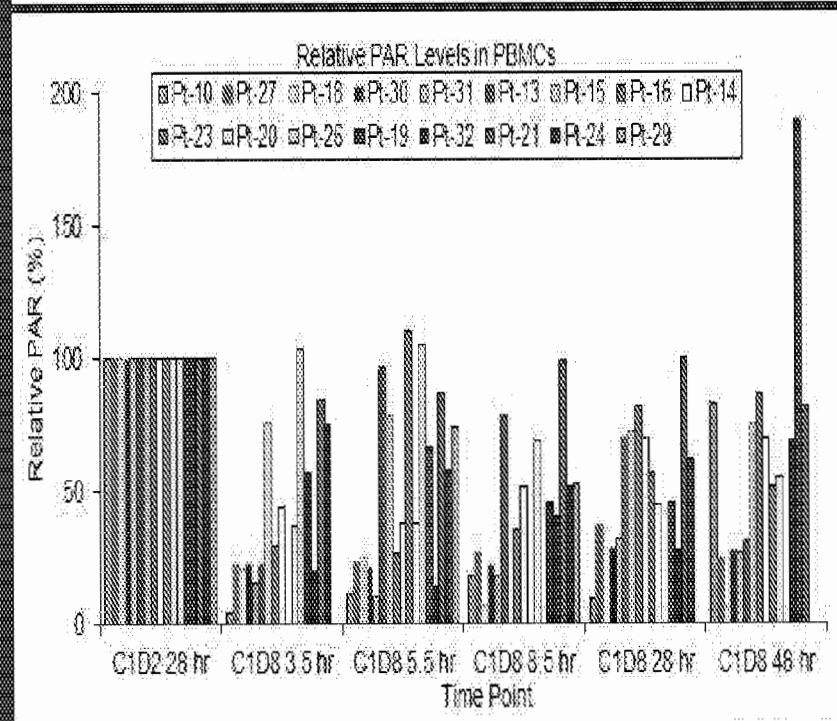
PAR Levels in PBMCs



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PAR Levels in PBMCs



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PK parameters of ABT-888, when given alone (on day 1) and in combination with irinotecan (on day 8)

Dose (mg)	N	Day	T1/2 (h)	Tmax (h)	Cmax (ng/ml)	AUC (ng/ml*h)	CL/F (L/h)
10	6	-1	8.0±7.8	2.1±1.2	42±51	336±331	60±40
	5	8		3.5±2.9	43±56	162±139	103±76
20	7	-1	6.1±1.1	2.4±1.9	170±62	1236±332	17±4
	7	8		2.4±1.3	244±101	1349±402	16±4
40	6	-1	8.7±5.9	1.9±1.3	357±170	3163±1499	14±4
	6	8		1.9±0.9	582±117	3239±500	13±2
50	5	-1	6.6±2.2	1.0±0.3	842±250	4611±2369	13±5
	4	8		2.8±0.9	760±124	4636±692	10±2
Overall	24	-1	7.3±4.8	1.9±1.7			23±24
	22	8	NE	2.6±1.7			25±29

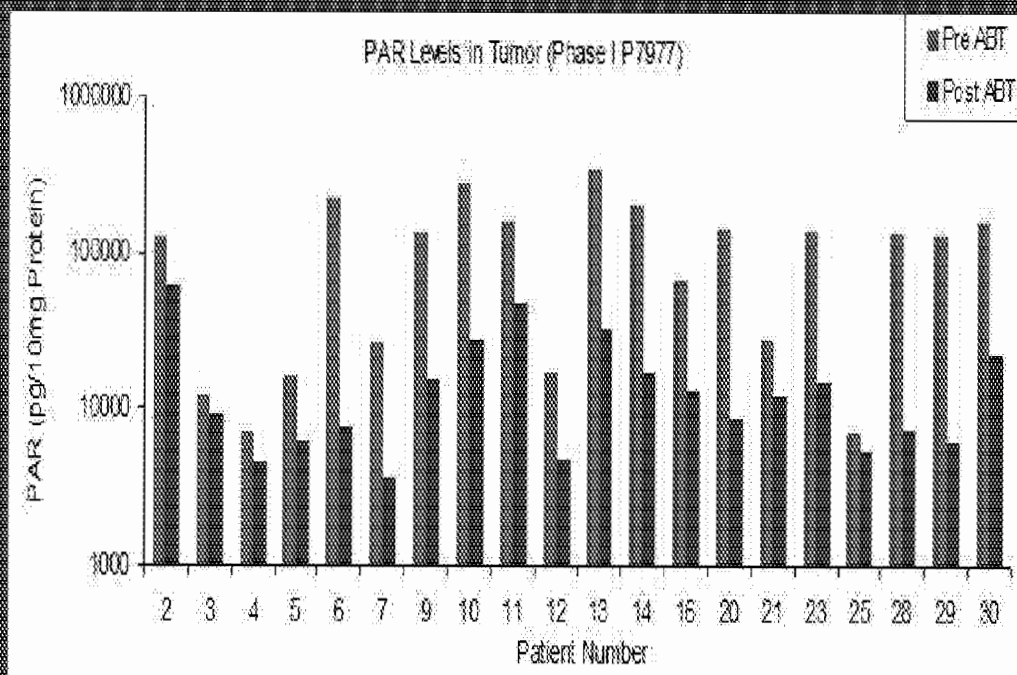
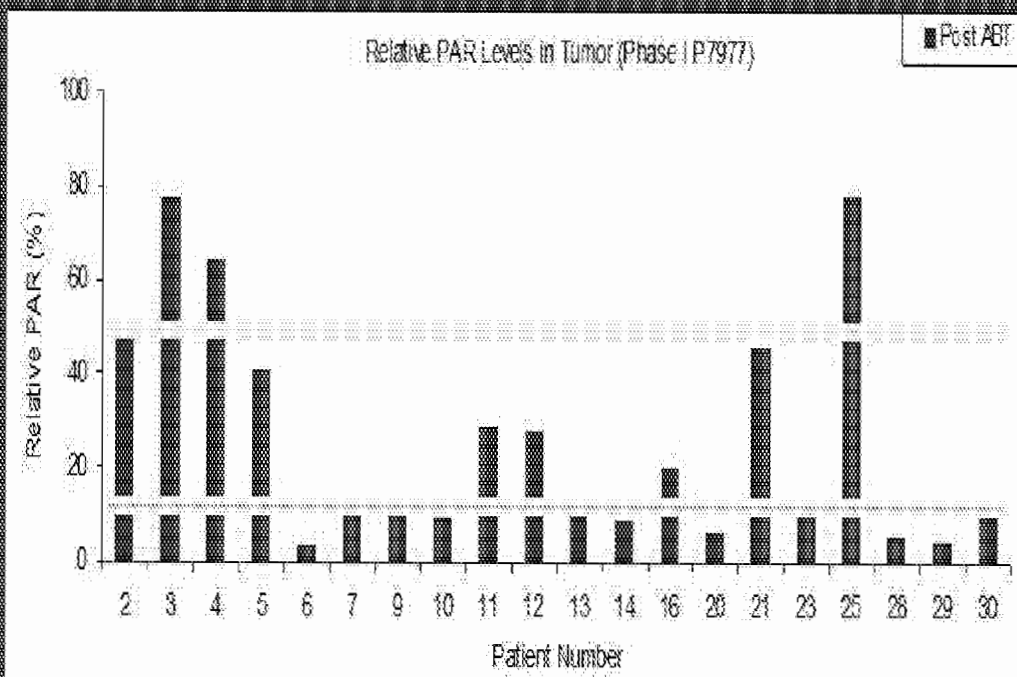
• There was no apparent difference in the apparent clearance (CL/F) of ABT-888 when given alone and in combination with irinotecan (mean, 23 vs. 25 L/h, $P = 0.79$), suggesting co-administration of irinotecan did not influence ABT-888 PK.

• The mean ratio of ABT-888 Cmax on day 8 relative to that on day -1 was 1.4, suggesting an average 1.4-fold drug accumulation at the steady state following twice daily dosing. This was consistent with the T1/2 of ABT-888 with a mean value of 7.3 h.

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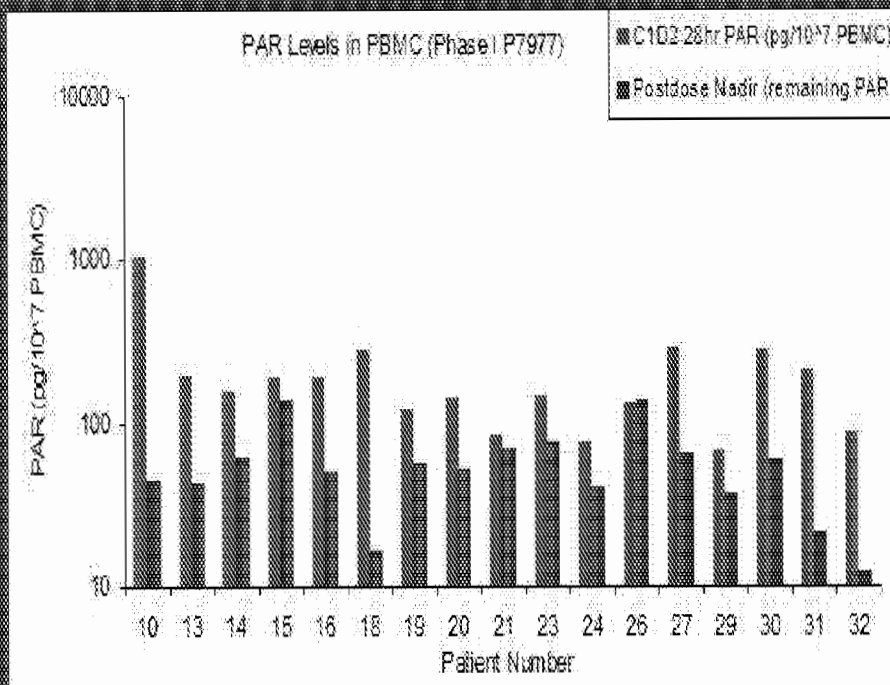
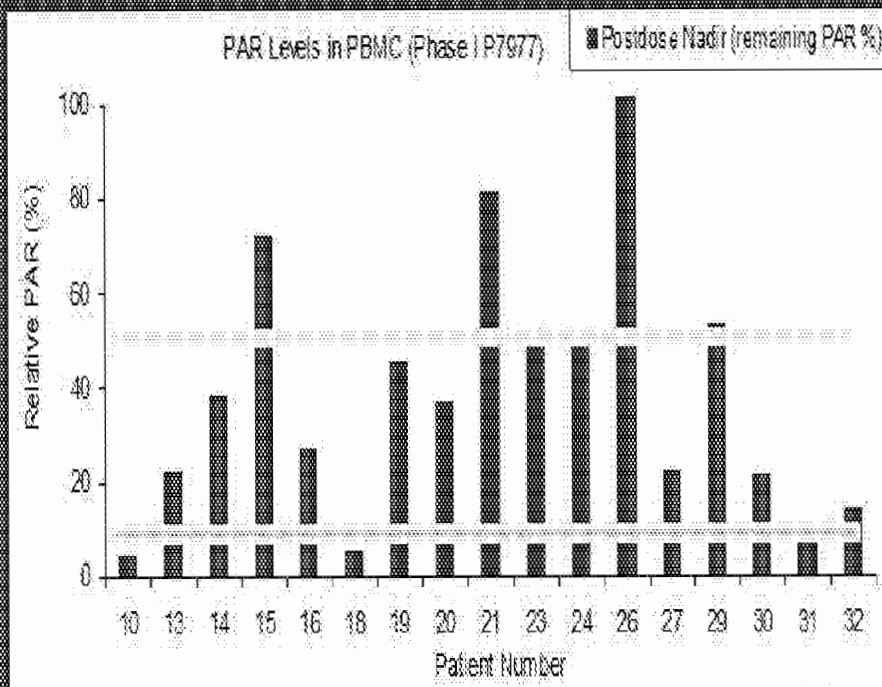
PAR Levels in Tumors of Predose vs. Postdose



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PAR Levels in PBMCs of Predose vs. Postdose

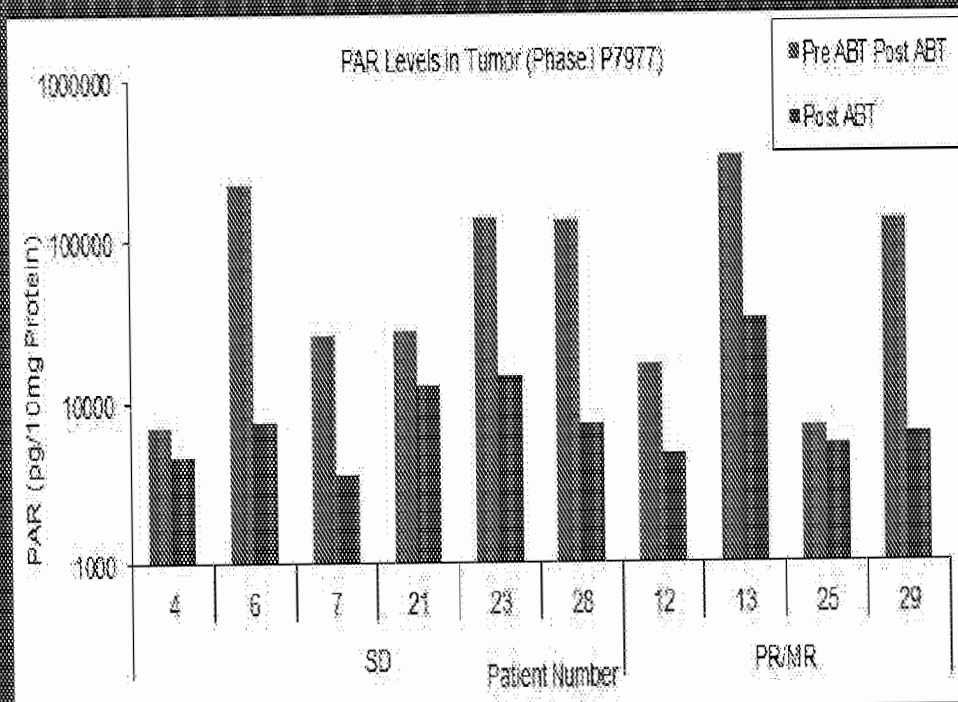
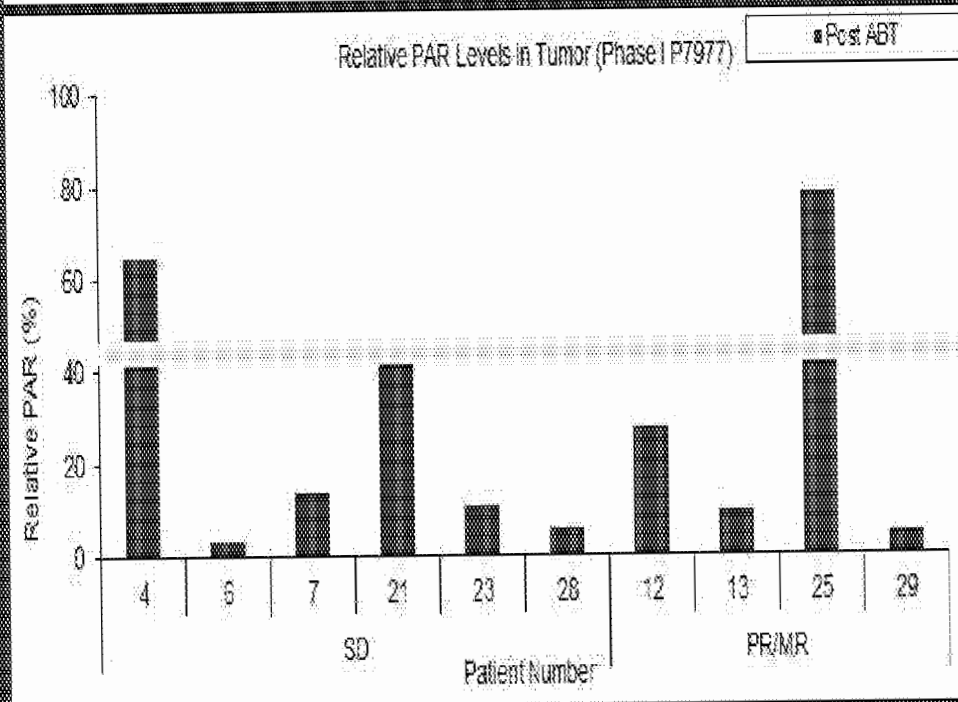


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PAR Levels in Tumors of Predose vs. Postdose



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HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use LYNPARZA safely and effectively. See full prescribing information for LYNPARZA.

LYNPARZA™ (olaparib) capsules, for oral use

Initial U.S. Approval: 2014

INDICATIONS AND USAGE

Lynparza is a poly (ADP-ribose) polymerase (PARP) inhibitor indicated as monotherapy in patients with deleterious or suspected deleterious germline *BRCA* mutated (as detected by an FDA-approved test) advanced ovarian cancer who have been treated with three or more prior lines of chemotherapy. (1.1)

The indication is approved under accelerated approval based on objective response rate and duration of response. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials. (1.1, 14)

DOSAGE AND ADMINISTRATION

- Recommended dose is 400 mg taken twice daily. (2.2)
- Continue treatment until disease progression or unacceptable toxicity. (2.2)
- For adverse reactions, consider dose interruption of treatment or dose reduction. (2.3)

DOSAGE FORMS AND STRENGTHS

Capsules: 50 mg (3)

CONTRAINDICATIONS

None

WARNINGS AND PRECAUTIONS

- Myelodysplastic syndrome/Acute Myeloid Leukemia: (MDS/AML) occurred in patients exposed to Lynparza, and some cases were fatal. Monitor patients for hematological toxicity at baseline and monthly thereafter. Discontinue if MDS/AML is confirmed. (5.1)

- Pneumonitis: occurred in patients exposed to Lynparza, and some cases were fatal. Interrupt treatment if pneumonitis is suspected. Discontinue if pneumonitis is confirmed. (5.2)
- Embryo-Fetal toxicity: Lynparza can cause fetal harm. Advise females of reproductive potential of the potential risk to a fetus and to avoid pregnancy. (5.3, 8.1)

ADVERSE REACTIONS

- Most common adverse reactions ($\geq 20\%$) in clinical trials were anemia, nausea, fatigue (including asthenia), vomiting, diarrhea, dysgeusia, dyspepsia, headache, decreased appetite, nasopharyngitis/pharyngitis/URI, cough, arthralgia/musculoskeletal pain, myalgia, back pain, dermatitis/rash and abdominal pain/discomfort. (6.1)
- Most common laboratory abnormalities ($\geq 25\%$) were increase in creatinine, mean corpuscular volume elevation, decrease in hemoglobin, decrease in lymphocytes, decrease in absolute neutrophil count, and decrease in platelets. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact AstraZeneca at 1-800-236-9933 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- CYP3A Inhibitors: Avoid concomitant use of strong and moderate CYP3A inhibitors. If the inhibitor cannot be avoided, reduce the dose. (2.3, 7.2)
- CYP3A Inducers: Avoid concomitant use of strong and moderate CYP3A inducers. If a moderate CYP3A inducer cannot be avoided, be aware of a potential for decreased efficacy. (7.3)

USE IN SPECIFIC POPULATIONS

- Nursing Mothers: Discontinue treatment or discontinue nursing. (8.3)

See 17 for PATIENT COUNSELING INFORMATION and MEDICATION GUIDE

Revised: 12/2014

FULL PRESCRIBING INFORMATION: CONTENTS***1 INDICATIONS AND USAGE**

- 1.1 Treatment of *gBRCA*-mutated advanced ovarian cancer

2 DOSAGE AND ADMINISTRATION

- 2.1 Patient Selection
- 2.2 Recommended Dosing
- 2.3 Dose Adjustments for Adverse Reactions
- 2.4 Dose Modifications for Use with CYP3A Inhibitors

3 DOSAGE FORMS AND STRENGTHS**4 CONTRAINDICATIONS****5 WARNINGS AND PRECAUTIONS**

- 5.1 Myelodysplastic syndrome/Acute Myeloid Leukemia
- 5.2 Pneumonitis
- 5.3 Embryo-Fetal Toxicity

6 ADVERSE REACTIONS

- 6.1 Clinical Trial Experience

7 DRUG INTERACTIONS

- 7.1 Anticancer Agents
- 7.2 Drugs that may Increase Olaparib Plasma Concentrations
- 7.3 Drugs that may Decrease Olaparib Plasma Concentrations

8 USE IN SPECIFIC POPULATIONS

- 8.1 Pregnancy
- Risk summary
- 8.3 Nursing Mothers
- 8.4 Pediatric Use
- 8.5 Geriatric Use
- 8.6 Females of Reproductive Potential
- 8.7 Hepatic Impairment
- 8.8 Renal Impairment

10 OVERDOSAGE**11 DESCRIPTION****12 CLINICAL PHARMACOLOGY**

- 12.1 Mechanism of Action
- 12.3 Pharmacokinetics

13 NONCLINICAL TOXICOLOGY

- 13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

14 CLINICAL STUDIES**16 HOW SUPPLIED/STORAGE AND HANDLING**

- 16.1 How Supplied
- 16.2 Storage

**17 PATIENT COUNSELING INFORMATION
MEDICATION GUIDE**

*Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION**1 INDICATIONS AND USAGE****1.1 Treatment of *gBRCA*-mutated advanced ovarian cancer**

Lynparza is indicated as monotherapy in patients with deleterious or suspected deleterious germline *BRCA* mutated (as detected by an FDA-approved test) advanced ovarian cancer who have been treated with three or more prior lines of chemotherapy.

The indication is approved under accelerated approval based on objective response rate and duration of response [see *Clinical Studies* (14)]. Continued approval for this indication may be contingent upon verification and description of clinical benefit in confirmatory trials.

2 DOSAGE AND ADMINISTRATION**2.1 Patient Selection**

Select patients for the treatment of advanced ovarian cancer with Lynparza based on the presence of deleterious or suspected deleterious germline *BRCA*-mutations [see *Indications and Usage* (1) and *Clinical Studies* (14)]. Information on FDA-approved test for the detection of *BRCA*-mutations is available at <http://www.fda.gov/companiendiagnosics>.

2.2 Recommended Dosing

The recommended dose of Lynparza is 400 mg (eight 50 mg capsules) taken twice daily, for a total daily dose of 800 mg. Continue treatment until disease progression or unacceptable toxicity.

If a patient misses a dose of Lynparza, instruct patients to take their next dose at its scheduled time.

Swallow capsule whole. Do not chew, dissolve, or open capsule. Do not take capsules which appear deformed or show evidence of leakage [see *How Supplied/Storage and Handling* (16.2)].

2.3 Dose Adjustments for Adverse Reactions

To manage adverse reactions, consider dose interruption of treatment or dose reduction. The recommended dose reduction is to 200 mg (four 50 mg capsules) taken twice daily, for a total daily dose of 400 mg.

If a further final dose reduction is required, then reduce to 100 mg (two 50 mg capsules) taken twice daily, for a total daily dose of 200 mg.

2.4 Dose Modifications for Use with CYP3A Inhibitors

Avoid concomitant use of strong and moderate CYP3A inhibitors and consider alternative agents with less CYP3A inhibition. If the inhibitor cannot be avoided, reduce the Lynparza dose to 150 mg (three 50 mg capsules) taken twice daily for a strong CYP3A inhibitor or 200 mg (four 50 mg capsules) taken twice daily for a moderate CYP3A inhibitor [see *Drug Interactions* (7.2)].

3 DOSAGE FORMS AND STRENGTHS

Lynparza (olaparib) is supplied as a white, opaque, hard capsule (50 mg), marked in black ink with "OLAPARIB 50 mg" on the cap and the AstraZeneca logo on the body.

4 CONTRAINDICATIONS

None

5 WARNINGS AND PRECAUTIONS**5.1 Myelodysplastic syndrome/Acute Myeloid Leukemia**

Myelodysplastic syndrome/Acute Myeloid Leukemia (MDS/AML) have been confirmed in 6 out of 298 (2%) patients enrolled in a single arm trial of Lynparza monotherapy, in patients with deleterious or suspected deleterious germline *BRCA*-mutated (*gBRCA*m) advanced cancers. In a randomized placebo controlled trial, MDS/AML occurred in 3 out of 136 (2%) patients with advanced ovarian cancer treated with Lynparza. Overall, MDS/AML were reported in 22 of 2,618 (<1%) patients treated with Lynparza. The majority of MDS/AML cases (17 of 22 cases) were fatal, and the duration of therapy with Lynparza in patients who developed secondary MDS/cancer-therapy related AML varied from <6 months to >2 years. All patients had previous chemotherapy with platinum agents and/or other DNA damaging agents.

Monitor complete blood count testing at baseline and monthly thereafter. Do not start Lynparza until patients have recovered from hematological toxicity caused by previous chemotherapy (≤CTCAE Grade 1). For prolonged hematological toxicities, interrupt Lynparza and monitor blood counts weekly until recovery. If the levels have not recovered to CTCAE Grade 1 or less after 4 weeks, refer the patient to a hematologist for further investigations, including bone marrow analysis and blood sample for cytogenetics. If MDS/AML is confirmed, discontinue Lynparza.

5.2 Pneumonitis

Pneumonitis, including fatal cases, occurred in <1% of patients treated with Lynparza. If patients present with new or worsening respiratory symptoms such as dyspnea, fever, cough, wheezing, or a radiological abnormality occurs, interrupt treatment with Lynparza and initiate prompt investigation. If pneumonitis is confirmed, discontinue Lynparza.

5.3 Embryo-Fetal Toxicity

Lynparza can cause fetal harm when administered to a pregnant woman based on its mechanism of action and findings in animals. Olaparib was teratogenic and caused embryo-fetal toxicity in rats at exposures below those in patients receiving the recommended human dose of 400 mg twice daily. If the patient becomes pregnant while taking this drug, apprise the patient of the potential hazard to a fetus [see *Use in Specific Populations* (8.1)].

Advise females of reproductive potential to avoid becoming pregnant while taking Lynparza. If contraceptive methods are being considered, use effective contraception during treatment and for at least one month after receiving the last dose of Lynparza [see *Use in Specific Populations* (8.6)].

6 ADVERSE REACTIONS

The following adverse reactions are discussed elsewhere in the labeling:

- Myelodysplastic syndrome/Acute Myeloid Leukemia [see *Warnings and Precautions* (5.1)]
- Pneumonitis [see *Warnings and Precautions* (5.2)]

6.1 Clinical Trial Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

Lynparza 400 mg twice daily as monotherapy, has been studied in 300 patients with *gBRCA*-mutated advanced ovarian cancer, and 223 of these patients had received 3 or more prior lines of chemotherapy.

In the 223 patients with *gBRCA*-mutated ovarian cancer who received 3 or more prior lines of chemotherapy (including 137 patients in Study 1 with measurable disease) [see *Clinical Studies* (14)] adverse reactions led to dose interruption in 40% of patients, dose reduction in 4%, and discontinuation in 7%. There were 8 (4%) patients with adverse reactions leading to death, two were attributed to acute leukemia, and one each was attributed to COPD, cerebrovascular accident, intestinal perforation, pulmonary embolism, sepsis, and suture rupture. Table 1 presents the frequency of adverse reactions reported in ≥20% of 223 patients (in 6 studies) with *gBRCA*-mutated advanced ovarian cancer who had received 3 or more prior lines of chemotherapy who were treated with Lynparza 400 mg twice daily. The median exposure to Lynparza in these patients was 158 days.

Table 1 Adverse Reactions Reported in ≥20% of Patients with *gBRCA*-Mutated Advanced Ovarian Cancer Receiving Lynparza

Adverse Reaction	3 or more lines of prior chemotherapy	
	Grades 1-4 N=223 %	Grades 3-4 N=223 %
Blood and Lymphatic disorders		
Anemia	34	18
Gastrointestinal disorders		
Abdominal pain/discomfort	43	8
Decreased appetite	22	1
Nausea	64	3
Vomiting	43	4
Diarrhea	31	1
Dyspepsia	25	0

Table 1 Adverse Reactions Reported in ≥20% of Patients with *gBRCA*-Mutated Advanced Ovarian Cancer Receiving Lynparza (continued)

Adverse Reaction	3 or more lines of prior chemotherapy	
	Grades 1-4 N=223 %	Grades 3-4 N=223 %
General disorders		
Fatigue/asthenia	66	8
Infections and infestations		
Nasopharyngitis/URI	26	0
Musculoskeletal and Connective Tissue disorders		
Arthralgia/musculoskeletal pain	21	0
Myalgia	22	0

Table 2 presents the frequency of abnormal laboratory findings in the 223 patients with *gBRCA*-mutated advanced ovarian cancer who had received three or more prior lines of chemotherapy receiving Lynparza 400 mg twice daily.

Table 2 Laboratory Abnormalities Reported in Patients with *gBRCA*-Mutated Advanced Ovarian Cancer Receiving Lynparza

Laboratory Parameter*	3 or more lines of prior chemotherapy	
	Grades 1-4 N=223 %	Grades 3-4 N=223 %
Decrease in hemoglobin (anemia)	90	15
Decrease in absolute neutrophil count (neutropenia)	25	7
Decrease in platelets (thrombocytopenia)	30	3
Decrease in lymphocytes (lymphopenia)	56	17
Mean corpuscular volume elevation	57	-
Increase in creatinine*	30	2

* Patients were allowed to enter clinical studies with laboratory values of CTCAE Grade 1.

The following adverse reactions and laboratory abnormalities have been identified in ≥10 to <20% of the 223 patients receiving Lynparza and not included in the table: cough, constipation, dysgeusia, peripheral edema, back pain, dizziness, headache, urinary tract infection, dyspnea, and rash. The following adverse reactions and laboratory abnormalities have been identified in ≥1 to <10% of the 223 patients receiving Lynparza and not included in the table: leukopenia, stomatitis, peripheral neuropathy, pyrexia, hypomagnesemia, hyperglycemia, anxiety, depression, insomnia, dysuria, urinary incontinence, vulvovaginal disorder, dry skin/eczema, pruritis, hypertension, venous thrombosis (including pulmonary embolism), and hot flush.

Table 3 presents adverse reactions reported in ≥20% of patients from a randomized trial of Lynparza 400 mg twice daily as maintenance monotherapy compared to placebo in patients with platinum sensitive, relapsed, high-grade serous ovarian cancer following treatment with 2 or more platinum-containing regimens. Table 4 presents the laboratory abnormalities in patients from this randomized trial. Of the 96 patients with *gBRCA*-mutation, 53 received Lynparza, and 43 received placebo. The median duration on treatment with Lynparza was 11.1 months for patients with a *gBRCA* mutation compared to 4.4 months for patients with *gBRCA* mutation on placebo.

Adverse reactions led to dose interruptions in 26% of those receiving Lynparza and 7% of those receiving placebo; dose reductions in 15% of Lynparza and 5% of placebo patients; and discontinuation in 9% of Lynparza and 0% in placebo patients. One (2%) patient on Lynparza died as a result of an adverse reaction.

Table 3 Adverse Reactions Reported in ≥20% of Patients with *gBRCA*-Mutated Ovarian Cancer in the Randomized Trial

Adverse Reactions	Lynparza N=53		Placebo N=43	
	Grades 1-4 %	Grades 3-4 %	Grades 1-4 %	Grades 3-4 %
Blood and Lymphatic disorders				
Anemia	25	4	7	2
Gastrointestinal disorders				
Abdominal pain/discomfort	47	0	58	2
Decreased appetite	25	0	14	0
Nausea	75	2	37	0
Vomiting	32	4	9	0
Diarrhea	28	4	21	2
Dyspepsia	25	0	14	0
Dysgeusia	21	0	9	0
General disorders				
Fatigue (including asthenia, lethargy)	68	6	53	2
Infections and infestations				
Nasopharyngitis/Pharyngitis/URI	43	0	16	0

Table 3 Adverse Reactions Reported in >20% of Patients with gBRCA-Mutated Ovarian Cancer in the Randomized Trial (continued)

Adverse Reactions	Lynparza N=53		Placebo N=43	
	Grades 1-4 %	Grades 3-4 %	Grades 1-4 %	Grades 3-4 %
Musculoskeletal and Connective tissue disorders				
Arthralgia/Musculoskeletal pain	32	4	21	0
Myalgia	25	2	12	0
Back pain	25	6	21	0
Nervous system disorder				
Headache	25	0	19	2
Respiratory, Thoracic, Mediastinal disorders				
Cough	21	0	14	0
Skin and Subcutaneous Tissue				
Dermatitis/Rash	25	0	14	0

Table 4 Laboratory Abnormalities in Patients with gBRCA-Mutated Ovarian Cancer in the Randomized Trial

Laboratory parameter*	Lynparza N=53		Placebo N=43	
	Grades 1-4 %	Grades 3-4 %	Grades 1-4 %	Grades 3-4 %
Decrease in hemoglobin	85	8	58	2
Decrease in absolute neutrophil count	32	8	23	0
Decrease in platelets	26	6	19	0
Mean corpuscular volume elevation	85	-	44	-
Increase in creatinine*	26	0	5	0

* Patients were allowed to enter clinical studies with laboratory values of CTCAE Grade 1.

7 DRUG INTERACTIONS

Olaparib is primarily metabolized by CYP3A.

7.1 Anticancer Agents

Clinical studies of Lynparza in combination with other myelosuppressive anticancer agents, including DNA damaging agents, indicate a potentiation and prolongation of myelosuppressive toxicity.

7.2 Drugs that may Increase Olaparib Plasma Concentrations

In patients (N=57), co-administration of itraconazole, a strong CYP3A inhibitor, increased AUC of olaparib by 2.7-fold. A moderate CYP3A inhibitor, fluconazole, is predicted to increase the AUC of olaparib by 2-fold.

Avoid concomitant use of strong CYP3A inhibitors (e.g., itraconazole, telithromycin, clarithromycin, ketoconazole, voriconazole, nefazodone, posaconazole, ritonavir, lopinavir/ritonavir, indinavir, saquinavir, boceprevir, telaprevir) and moderate CYP3A inhibitors (e.g., amprenavir, aprepitant, atazanavir, ciprofloxacin, crizotinib, darunavir/ritonavir, diltiazem, erythromycin, fluconazole, fosamprenavir, imatinib, verapamil). If the strong or moderate CYP3A inhibitors must be co-administered, reduce the dose of Lynparza [see *Dosage and Administration* (2.4)].

Avoid grapefruit and Seville oranges during Lynparza treatment [see *Dosage and Administration* (2.4) and *Clinical Pharmacology* (12.3)].

7.3 Drugs that may Decrease Olaparib Plasma Concentrations

In patients (N=22), co-administration of rifampicin, a strong CYP3A inducer, decreased AUC of olaparib by 87%. A moderate CYP3A inducer, efavirenz, is predicted to decrease the AUC of olaparib by 50-60%.

Avoid concomitant use of strong CYP3A inducers (e.g., phenytoin, rifampicin, carbamazepine, St. John's Wort) and moderate CYP3A4 inducers (e.g., bosentan, efavirenz, etravirine, modafinil, nafcillin). If a moderate CYP3A inducer cannot be avoided, be aware of a potential for decreased efficacy of Lynparza [see *Clinical Pharmacology* (12.3)].

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Pregnancy Category D [see *Warnings and Precautions* (5.3)]

Risk summary

Lynparza can cause fetal harm when administered to a pregnant woman based on its mechanism of action and findings in animals. Olaparib was teratogenic and caused embryo-fetal toxicity in rats at exposures below those in patients receiving the recommended human dose of 400 mg twice daily. If this drug is used during pregnancy, or if a patient becomes pregnant while taking this drug, apprise the patient of the potential hazard to the fetus and the potential risk for loss of the pregnancy.

Animal Data

In a fertility and early embryonic development study in female rats, olaparib was administered orally for 14 days before mating through to day 6 of pregnancy, which resulted in increased post-implantation loss at a dose level of 15 mg/kg/day (with maternal systemic exposures approximately 11% of the human exposure (AUC_{0-24h}) at the recommended dose).

In an embryo-fetal development study, pregnant rats received oral doses of 0.05 and 0.5 mg/kg/day olaparib during the period of organogenesis. A dose of 0.5 mg/kg/day (with maternal systemic exposures approximately 0.3% of human exposure (AUC_{0-24h}) at the recommended dose) caused embryo-fetal toxicities including increased post-implantation loss and major malformations of the eyes (anophthalmia, microphthalmia), vertebrae/ribs (extra rib or ossification center; fused or absent neural arches, ribs, and sternbrae), skull (fused exoccipital) and diaphragm (hernia). Additional abnormalities or variants included incomplete or absent ossification (vertebrae/sternbrae, ribs, limbs) and other findings in the vertebrae/sternbrae, pelvic girdle, lung, thymus, liver, ureter and umbilical artery. Some findings noted above in the eyes, ribs and ureter were observed at a dose of 0.05 mg/kg/day olaparib at lower incidence.

8.3 Nursing Mothers

It is not known whether olaparib is excreted in human milk. Because many drugs are excreted in human milk and because of the potential for serious adverse reactions in nursing infants from olaparib, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

8.4 Pediatric Use

The safety and efficacy of Lynparza has not been established in pediatric patients.

8.5 Geriatric Use

In clinical studies of Lynparza enrolling 735 patients with advanced solid tumors [the majority (69%) of whom had ovarian cancer] who received Lynparza 400 mg twice daily as monotherapy, 148 (20%) of patients were aged ≥65 years. The safety profile was similar irrespective of age with the exception of AEs of CTCAE ≥3 which were reported more frequently in patients aged ≥65 years (53.4%) than those <65 years (43.4%). No individual adverse event or System Organ Class accounted for this observed difference.

8.6 Females of Reproductive Potential

Lynparza can cause fetal harm when administered to a pregnant woman [see *Use in Specific Populations* (8.1)]. Advise female patients of reproductive potential to avoid pregnancy while taking Lynparza. If contraceptive methods are being considered, use highly effective contraception during treatment with Lynparza and for at least one month following the last dose of Lynparza. Instruct patients to contact their healthcare provider if they become pregnant, or if pregnancy is suspected, while taking Lynparza.

8.7 Hepatic Impairment

The effect of hepatic impairment on exposure to Lynparza has not been studied. Patients with bilirubin >1.5 X ULN and AST/ALT ≥2.5 X ULN (≥5 X ULN in the presence of liver metastases) were excluded from Lynparza clinical trials. There are no data in patients with baseline hepatic impairment (serum bilirubin >1.5 X ULN) [see *Clinical Pharmacology* (12.3)].

8.8 Renal Impairment

Based on preliminary data, a 1.5 fold increase in mean exposure (AUC) was observed in patients with mild renal impairment (CL_{CR} = 50-80 mL/min) compared to patients with normal renal function (CL_{CR} >80 mL/min). No dose adjustment to the starting dose is required in patients with CL_{CR} of 50 to 80 mL/min, but patients should be monitored closely for toxicity. There are no data in patients with moderate or severe renal impairment (CL_{CR} <50 mL/min) or patients on dialysis [see *Clinical Pharmacology* (12.3)].

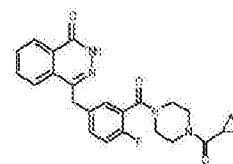
10 OVERDOSAGE

There is no specific treatment in the event of Lynparza overdose, and symptoms of overdose are not established. In the event of an overdose, physicians should follow general supportive measures and should treat symptomatically.

11 DESCRIPTION

Olaparib is an inhibitor of the mammalian polyadenosine 5'-diphosphoribose polymerase (PARP) enzyme.

The chemical name is 4-[(3-[[4-(cyclopropylcarbonyl)piperazin-1-yl]carbonyl]-4-fluorophenyl)methyl]phthalazin-1(2H)-one and it has the following chemical structure:



The empirical molecular formula for Lynparza is C₂₄H₂₃FN₄O₃ and the relative molecular mass is 434.46.

Olaparib is a crystalline solid, is non-chiral and shows pH-independent low solubility of approximately 0.1 mg/mL across the physiological pH range.

Lynparza is available in 50 mg capsules for oral administration. Each capsule contains olaparib as the active ingredient and the following inactive ingredients:

- **Capsule content:** lauroyl polyoxyglycerides
- **Capsule shell:** hypromellose, titanium dioxide, gellan gum, potassium acetate
- **Capsule printing ink:** shellac, ferrousferic oxide

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Lynparza is an inhibitor of poly (ADP-ribose) polymerase (PARP) enzymes, including PARP1, PARP2, and PARP3. PARP enzymes are involved in normal cellular homeostasis, such as DNA transcription, cell cycle regulation, and DNA repair. Olaparib has been shown to inhibit growth of select tumor cell lines *in vitro* and decrease tumor growth in mouse

xenograft models of human cancer both as monotherapy or following platinum-based chemotherapy. Increased cytotoxicity and anti-tumor activity following treatment with olaparib were noted in cell lines and mouse tumor models with deficiencies in *BRCA*. *In vitro* studies have shown that olaparib-induced cytotoxicity may involve inhibition of PARP enzymatic activity and increased formation of PARP-DNA complex, resulting in disruption of cellular homeostasis and cell death.

12.3 Pharmacokinetics

Absorption

Following oral administration of olaparib via the capsule formulation, absorption is rapid with peak plasma concentrations typically achieved between 1 to 3 hours after dosing. On multiple dosing there is no marked accumulation (accumulation ratio of 1.4 – 1.5 for twice daily dosing), with steady state exposures achieved within 3 to 4 days.

Limited data suggest that the systemic exposure (AUC) of olaparib increases less than proportionally with dose over the dose range of 100 to 400 mg, but the PK data were variable across trials.

Co-administration with a high fat meal slowed the rate (T_{max} delayed by 2 hours) of absorption, but did not significantly alter the extent of olaparib absorption (mean AUC increased by approximately 20%).

Distribution

Olaparib had a mean ($\pm$ standard deviation) apparent volume of distribution at steady state of 167 ± 196 L after a single 400 mg dose of olaparib. The *in vitro* protein binding of olaparib at plasma concentrations achieved following dosing at 400 mg twice daily is approximately 82%.

Metabolism

In vitro, CYP3A4 was shown to be the enzyme primarily responsible for the metabolism of olaparib.

Following oral dosing of ^{14} C-olaparib to female patients, unchanged olaparib accounted for the majority of the circulating radioactivity in plasma (70%). It was extensively metabolized with unchanged drug accounting for 15% and 6% of radioactivity in urine and feces, respectively. The majority of the metabolism is attributable to oxidation reactions with a number of the components produced undergoing subsequent glucuronide or sulfate conjugation.

Excretion

A mean ($\pm$ standard deviation) terminal plasma half-life of 11.9 ± 4.8 hours and apparent plasma clearance of 8.6 ± 7.1 L/h were observed after a single 400 mg dose of olaparib.

Following a single dose of ^{14} C-olaparib, 86% of the dosed radioactivity was recovered within a 7-day collection period, 44% via the urine and 42% via the feces. The majority of the material was excreted as metabolites.

Based on preliminary data from a dedicated renal impairment trial, the mean AUC and C_{max} of olaparib increased by 1.5- and 1.2-fold, respectively, when olaparib was dosed in patients with mild renal impairment ($CL_{cr} = 50$ -80 mL/min; $N=14$) compared to those with normal renal function ($CL_{cr} > 80$ mL/min; $N=8$). There are no data in patients with $CL_{cr} < 50$ mL/min or in patients on dialysis.

Drug Interactions

In vitro, olaparib was an inhibitor of CYP3A4 and an inducer of CYP2B6 at higher concentrations than are clinically achieved. Olaparib produced little/no inhibition of other CYP isozymes. *In vitro* studies have shown that olaparib is a substrate of CYP3A4.

Based on the data from a drug-interaction trial ($N=57$), the AUC and C_{max} of olaparib increased by 2.7- and 1.4-fold, respectively, when olaparib was administered in combination with itraconazole, a strong CYP3A inhibitor. Simulations using physiologically-based pharmacokinetic (PBPK) models suggested that a moderate CYP3A inhibitor (fluconazole) may increase the AUC and C_{max} of olaparib by 2- and 1.1-fold, respectively.

Based on the data from a drug-interaction trial ($N=22$), the AUC and C_{max} of olaparib decreased by 87% and 71%, respectively, when olaparib was administered in combination with rifampicin, a strong CYP3A inducer. Simulations using PBPK models suggested that a moderate CYP3A inducer (efavirenz) may decrease the AUC and C_{max} of olaparib by 50 - 60% and 20 - 30%, respectively.

In vitro studies have shown that olaparib is a substrate of P-gp and an inhibitor of BCRP, OATP1B1, OCT1, OCT2, OAT3, MATE1 and MATE2K. The clinical relevance of these findings is unknown.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenicity studies have not been conducted with olaparib.

Olaparib was clastogenic in an *in vitro* chromosomal aberration assay in mammalian CHO cells and in an *in vivo* rat bone marrow micronucleus assay. This clastogenicity is consistent with genomic instability resulting from the primary pharmacology of olaparib and indicates potential for genotoxicity in humans. Olaparib was not mutagenic in a bacterial reverse mutation (Ames) test.

In a fertility study, female rats received oral olaparib at doses of 0.05, 0.5, and 15 mg/kg/day for at least 14 days before mating through the first week of pregnancy. There were no adverse effects on mating and fertility rates at doses up to 15 mg/kg/day (maternal systemic exposures approximately 11% of the human exposure (AUC_{0-24h}) at the recommended dose).

In a male fertility study, olaparib had no effect on mating and fertility in rats at oral doses up to 40 mg/kg/day following at least 70 days of olaparib treatment (with systemic exposures of approximately 7% of the human exposure (AUC_{0-24h}) at the recommended dose).

14 CLINICAL STUDIES

The efficacy of Lynparza was investigated in a single-arm study in patients with deleterious or suspected deleterious germline *BRCA*-mutated (g*BRCA*m) advanced cancers (Study 1). A total of 137 patients with measurable, g*BRCA*m-associated ovarian cancer treated with three or more prior lines of chemotherapy were enrolled. All patients received Lynparza at a dose of 400 mg twice daily as monotherapy until disease progression or intolerable toxicity. Objective response rate (ORR) and duration of response (DOR) were assessed by the investigator according to RECIST v1.1.

The median age of the patients was 58 years, the majority were Caucasian (94%) and 93% had an ECOG PS of 0 or 1. Deleterious or suspected deleterious, germline *BRCA* mutation status was verified retrospectively in 97% (59/61) of the patients for whom blood samples were available by the companion diagnostic BRACAnalysis CDx™, which is FDA approved for selection of patients for Lynparza treatment.

Efficacy results from Study 1 are summarized in Table 5.

Table 5 Overall Response and Duration of Response in Patients with g*BRCA*-mutated Advanced Ovarian Cancer Who Received 3 or More Prior Lines of Chemotherapy in Study 1

	N=137
Objective Response Rate (95% CI)	34% (26, 42)
Complete Response	2%
Partial Response	32%
Median DOR in months (95% CI)	7.9 (5.6, 9.6)

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

Lynparza 50 mg is a white, opaque, hard capsule, marked in black ink with: "OLAPARIB 50 mg" on the cap and AstraZeneca logo on the body; available in:

Bottles of 112 capsules NDC 0310-0657-58

16.2 Storage

Store at 25°C (77°F), excursions permitted to 15-30°C (59-86°F) [see USP Controlled Room Temperature]

Lynparza should not be exposed to temperatures greater than 40°C or 104°F. Do not take Lynparza if it is suspected of having been exposed to temperatures greater than 40°C or 104°F.

17 PATIENT COUNSELING INFORMATION

SEE FDA-APPROVED PATIENT LABELING (MEDICATION GUIDE)

- Dosing Instructions:** Inform patients on how to take Lynparza [see *Dosage and Administration* (2.1)]. Lynparza should be taken twice daily. Instruct patients that if they miss a dose of Lynparza, not to take an extra dose to make up for the one that they missed. They should take their next normal dose at the usual time. Each capsule should be swallowed whole. Do not chew, dissolve, or open the capsule. Patient should not take Lynparza with grapefruit or Seville oranges.
- MDS/AML:** Advise patients to contact their healthcare provider if they experience weakness, feeling tired, fever, weight loss, frequent infections, bruising, bleeding easily, breathlessness, blood in urine or stool, and/or laboratory findings of low blood cell counts, or a need for blood transfusions. This may be a sign of hematological toxicity or a more serious uncommon bone marrow problem called 'myelodysplastic syndrome' (MDS) or 'acute myeloid leukemia' (AML) which have been reported in patients treated with Lynparza [see *Warnings and Precautions* (5.1)].
- Pneumonitis:** Advise patients to contact their healthcare provider if they experience any new or worsening respiratory symptoms including shortness of breath, fever, cough, or wheezing [see *Warnings and Precautions* (5.2)].
- Pregnancy and Females of Reproductive Potential:** Advise females to inform their healthcare provider if they are pregnant or become pregnant. Inform female patients of the risk to a fetus and potential loss of the pregnancy [see *Use in Specific Populations* (8.1)]. Advise females of reproductive potential to use effective contraception during treatment with Lynparza and for at least one month after receiving the last dose of Lynparza [see *Warnings and Precautions* (5.3) and *Use in Specific Populations* (8.1, 8.6)].
- Nursing Mothers:** Advise patients not to breastfeed while taking Lynparza [see *Use in Specific Populations* (8.3)].
- Nausea/vomiting:** Advise patients that mild or moderate nausea and/or vomiting is very common in patients receiving Lynparza and that they should contact their healthcare provider who will advise on available antiemetic treatment options.

3079900 12/14

December 2014

Medication Guide
Lynparza (Lin-par-zah)
(olaparib)
capsules

Read this Medication Guide before you start taking Lynparza and each time you get a refill. There may be new information. This Medication Guide does not take the place of talking to your healthcare provider about your medical condition or treatment.

What is the most important information I should know about Lynparza?

Lynparza may cause serious side effects that can lead to death, including:

Bone marrow problems called Myelodysplastic Syndrome (MDS) or Acute Myeloid Leukemia (AML). Some people who have ovarian cancer or who have received previous treatment with chemotherapy or certain other medicines for their cancer have developed MDS or AML during treatment with Lynparza. If you develop MDS or AML, your healthcare provider will stop treatment with Lynparza.

Symptoms of low blood cell counts are common during treatment with Lynparza, but can be a sign of serious bone marrow problems, including MDS or AML. Symptoms may include:

- weakness
- weight loss
- fever
- frequent infections
- blood in urine or stool
- shortness of breath
- feeling very tired
- bruising or bleeding more easily

Your healthcare provider will do blood tests to check your blood cell counts:

- before treatment with Lynparza
- every month during treatment with Lynparza
- weekly if you have low blood cell counts that last a long time. Your healthcare provider may stop treatment with Lynparza until your blood cell counts improve.

Lung problems (pneumonitis). Tell your healthcare provider if you have any new or worsening symptoms of lung problems, including shortness of breath, fever, cough, or wheezing. Your healthcare provider may do a chest x-ray if you have any of these symptoms. Your healthcare provider may temporarily stop treatment or completely stop treatment if you develop pneumonitis.

Tell your healthcare provider if you have any of the symptoms above during treatment with Lynparza.

What is Lynparza?

Lynparza is a prescription medicine used to treat women with advanced ovarian cancer who:

- have received previous treatment with 3 or more prior chemotherapy medicines or a combination of chemotherapy medicines for their cancer, **and**
- have a certain type of abnormal inherited BRCA gene.

Your healthcare provider will perform a test to make sure that Lynparza is right for you.

It is not known if Lynparza is safe and effective in children.

What should I tell my healthcare provider before taking Lynparza?

Before you take Lynparza, tell your healthcare provider if you:

- have lung or breathing problems
- have liver problems
- have kidney problems
- are pregnant or plan to become pregnant. **Lynparza can harm your unborn baby and may cause loss of pregnancy (miscarriage).** You should not become pregnant during treatment with Lynparza. Talk to your healthcare provider if you are pregnant or plan to become pregnant.
 - Females who are able to become pregnant should use effective birth control (contraception) during treatment with Lynparza and for at least 1 month after receiving the last dose of Lynparza.
 - Talk to your healthcare provider about birth control methods that may be right for you.
 - Tell your healthcare provider right away if you become pregnant or think you may be pregnant during treatment with Lynparza.
- are breastfeeding or plan to breastfeed. It is not known if Lynparza passes into your breast milk. You and your healthcare provider should decide if you will take Lynparza or breastfeed. You should not do both.

Tell your healthcare provider about all the medicines you take, including prescription and over-the-counter medicines, vitamins, and herbal supplements. Taking Lynparza and certain other medicines may affect how Lynparza works and may cause side effects.

Know the medicines you take. Keep a list of them to show your healthcare provider and pharmacist when you get a new medicine.

How should I take Lynparza?

- Take Lynparza exactly as your healthcare provider tells you.
- Your healthcare provider may temporarily stop treatment with Lynparza or change your dose of Lynparza if you have side effects.
- Take Lynparza 2 times a day.
- Swallow Lynparza capsules whole. Do not chew, dissolve, or open the capsules.
- Do not take Lynparza capsules if they look damaged or show signs of leakage.
- If you miss a dose of Lynparza, take your next dose at your usual scheduled time. Do not take an extra dose to make up for a missed dose.
- If you take too much Lynparza, call your healthcare provider or go to the nearest emergency room right away.

What should I avoid while taking Lynparza?

- Avoid grapefruit, grapefruit juice and Seville oranges during treatment with Lynparza. Grapefruit and Seville oranges may increase the level of Lynparza in your blood.

What are the possible side effects of Lynparza?

Lynparza may cause serious side effects.

See “What is the most important information I should know about Lynparza?”

The most common side effects of Lynparza are:

- nausea or vomiting. Tell your healthcare provider if you get nausea or vomiting. Your healthcare provider may prescribe medicines to treat these symptoms.
- tiredness or weakness
- diarrhea
- indigestion or heartburn
- headache
- loss of appetite
- changes in the way food tastes
- changes in kidney function blood test
- sore throat or runny nose
- upper respiratory infection
- cough
- pain in the joints, muscles, and back
- rash
- pain or discomfort in the stomach area

Tell your healthcare provider if you have any side effect that bothers you or that does not go away.

These are not all the possible side effects of Lynparza. For more information, ask your healthcare provider or pharmacist.

Call your doctor for medical advice about side effects. You may report side effects to FDA at 1-800-FDA-1088.

How should I store Lynparza?

- Store Lynparza at room temperature, between 68 to 77°F (20 to 25°C).
- Do not store Lynparza at temperatures greater than 104°F (40°C). Do not take Lynparza if you think it may have been stored at a temperature greater than 104°F (40°C).

Keep Lynparza and all medicines out of the reach of children.**General information about the safe and effective use of Lynparza**

Medicines are sometimes prescribed for purposes other than those listed in a Medication Guide. Do not use Lynparza for a condition for which it was not prescribed. Do not give Lynparza to other people, even if they have the same symptoms you have. It may harm them.

If you would like more information, talk with your healthcare provider. You can ask your healthcare provider or pharmacist for information about Lynparza that is written for health professionals.

For more information, call 1-800-236-9933
or go to www.Lynparza.com.

What are the ingredients in Lynparza?

Active ingredient: olaparib

Inactive ingredients:

Capsule contains: lauroyl polyoxyglycerides

Capsule shell contains: hypromellose, titanium dioxide, gellan gum, potassium acetate

Capsule printing ink contains: shellac, ferrosoferric oxide

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Epidermal Growth Factor Receptor–Targeted Immunoliposomes Significantly Enhance the Efficacy of Multiple Anticancer Drugs *In vivo*

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Abstract

We previously reported the development of epidermal growth factor receptor (EGFR)–targeted immunoliposomes that bind and internalize in tumor cells which overexpress EGFR and/or mutant EGFR variant III (EGFRvIII), enabling intracellular delivery of potent anticancer agents *in vitro*. We now describe *in vivo* proof-of-concept for this approach for the delivery of multiple anticancer drugs in EGFR-overexpressing tumor models. Anti-EGFR immunoliposomes were constructed modularly with Fab' fragments of cetuximab (IMC-C225), covalently linked to liposomes containing probes and/or anticancer drugs. Pharmacokinetic and biodistribution studies confirmed long circulation times ($t_{1/2}$ = 21 hours) and efficient accumulation in tumors (up to 15% ID/g) irrespective of the presence of the targeting ligand. Although total accumulations of anti-EGFR immunoliposomes and nontargeted liposomes in EGFR-overexpressing tumors were comparable, only immunoliposomes internalized extensively within tumor cells (92% of analyzed cells versus <5% for nontargeted liposomes), indicating different mechanisms of delivery at the cellular level. *In vivo* therapy studies in a series of xenograft models featuring overexpression of EGFR and/or EGFRvIII showed the superiority of immunoliposomal delivery of encapsulated drugs, which included doxorubicin, epirubicin, and vinorelbine. For each of these drugs, anti-EGFR immunoliposome delivery showed significant antitumor effects and was significantly superior to all other treatments, including the corresponding free or liposomal drug (P < 0.001-0.003). We conclude that anti-EGFR immunoliposomes provide efficient and targeted drug delivery of anticancer compounds and may represent a useful new treatment approach for tumors that overexpress the EGFR. (Cancer Res 2005; 65(24): 11631-8)

Introduction

Nanoparticles, including liposomes and other nanoscale constructs, can be used to deliver drugs to tumors. Certain nanoparticles, such as sterically stabilized liposomes, have already been optimized for prolonged circulation and redirection of drug, which can yield superior accumulation in tumors via a process often referred to as the "enhanced permeability and retention" effect

(for review, see refs. 1, 2). Notable examples are the liposomal anthracyclines pegylated liposomal doxorubicin (PLD; Doxil/Caelix) and liposomal daunorubicin (DaunoXome), both of which are currently approved for cancer treatment.

Immunoliposomes, in which monoclonal antibody (mAb) fragments are conjugated to liposomes, represent the next generation of molecularly targeted drug delivery systems. By combining the tumor targeting properties of mAbs with the pharmacokinetics and drug delivery advantages of liposomes, immunoliposomes offer the promise of selective drug delivery to tumor cells, including internalization and intracellular drug release within targeted cells (3). For example, we previously reported that immunoliposomes that bind to and internalize in HER2-overexpressing tumor cells (4, 5) show significantly increased therapeutic efficacy in delivering doxorubicin in preclinical tumor models (6) and can be manufactured from existing liposomal doxorubicin (7, 8).

We have also developed immunoliposomes that target epidermal growth factor receptor (EGFR; ErbB), which is involved in the pathogenesis of many tumors and is a proven target for cancer therapy (for review, see ref. 9). Anti-EGFR immunoliposomes were constructed using Fab' fragments of mAb C225 (cetuximab, Erbitux), a chimeric anti-EGFR mAb that is in clinical use for colorectal cancer treatment, as well as scFv C10, an independently derived phage antibody; these immunoliposomes mediated specific and efficient intracellular drug delivery to a variety of target cells *in vitro* (10). Here we describe *in vivo* properties of anti-EGFR immunoliposomes, including the therapeutic potential of this receptor-targeted vehicle for the delivery of various cytotoxic drugs in a series of EGFR-overexpressing tumor models.

Materials and Methods

Liposome preparation. Liposomes were prepared by a lipid film hydration-extrusion method using repeated freeze-thawing to hydrate the lipid films (11). Liposomes were composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol (molar ratio 3:2) with methoxy polyethylene glycol (mPEG)-1,2-distearoyl-3-*sn*-glycerophosphoethanolamine (DSPE; 0.5-5 mol% of phospholipid; Avanti Polar Lipids; Alabaster, AL). Following hydration, liposomes were extruded 10 times through polycarbonate filters (0.1 μ m pore size), followed by extrusion seven times through additional filters (0.08 μ m pore size). Liposome size was determined by dynamic light scattering (typically 80-100 nm). Phospholipid concentration was measured by phosphate assay (12).

For liposomes loaded with ADS645WS (American Dye Source, Quebec, Canada), the fluorescent dye (5 mmol/L) was dissolved in buffer for rehydration of the dried lipids. After passive loading, unencapsulated dye was removed using Sephadex G-75 chromatography.

For encapsulation of chemotherapeutic drugs doxorubicin (Bedford Laboratories, Bedford, OH) and epirubicin (Pharmacia, Kalamazoo, MI), a standard remote-loading method using ammonium sulfate was done

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(13, 14). For encapsulation of vinorelbine, liposomes were prepared as described following hydration in a solution of triethylammonium sucrose octasulfate (TEA₈SOS; 0.65 mol/L TEA, pH 5.2-5.5). Untrapped TEA₈SOS was removed on a Sepharose CL-4B size exclusion column. Vinorelbine was added at a drug-to-phospholipid ratio of 350 g drug/mol phospholipid and the pH adjusted to 6.5 with 1 N HCl before initiation of loading at 60°C for 30 minutes. The resulting liposomal vinorelbine was purified on a Sephadex G-75 column to remove unencapsulated drug.

Preparation of monoclonal antibody fragments and immunoliposomes. Intact C225 mAb (cetuximab, Erbitux; ImClone Systems, Inc., New York, NY) was cleaved and reduced as previously described (10). Fab' fragments were covalently conjugated to maleimide groups at the termini of PEG-DSPE chains (Mal-PEG-DSPE; Nektar, Huntsville, AL; ref. 8). Conjugation efficiencies were typically 30% to 50% for C225-Fab'. For incorporation into preformed liposomes or commercial PLD (Doxil; Alza Pharmaceuticals, Palo Alto, CA), mAb conjugates were incorporated into liposomes by coinubation at 55°C for 30 minutes at protein/liposome ratio of 30 µg Fab'/µmol phospholipid, resulting in incorporation efficiencies of 70% to 80% (10).

Pharmacokinetic studies. Healthy adult Sprague-Dawley rats (CD 1GS rats, Charles River Laboratories, Wilmington, MA) received single i.v. injections of liposomes or immunoliposomes loaded with doxorubicin (5 mg doxorubicin/kg). For two-component pharmacokinetic studies, serial blood samples were divided and analyzed for both doxorubicin and [³H]-cholesteryl hexadecyl ether [cholesteryl-1,2-³H(N); Perkin-Elmer, Boston, MA] as a liposome marker. Plasma concentrations of doxorubicin were determined by fluorescence and those of liposomes by radioactivity counting. Construct stability in circulation was evaluated by serial determinations of the doxorubicin-to-lipid ratio. Noncompartmental pharmacokinetic data analysis was done using PK Solutions 2.0 software (Summit Research Services, Montrose, CO).

Biodistribution studies. NCR nu/nu mice (5-6 weeks; Taconic, Germantown, NY) were injected s.c. with EGFR-overexpressing MDA-MB-468 tumor cells (2 × 10⁷ cells) in the dorsum of the animal. When tumor xenografts were fully established and had reached volumes of 400 to 800 mm³, animals were randomly assigned to different treatment groups: 3 mice per group × 2 groups (EGFR-targeted and nontargeted) × 2 time points (24 and 72 hours). Liposomes or anti-EGFR immunoliposomes, both loaded with doxorubicin and labeled with [³H]-cholesteryl hexadecyl ether, were injected i.v. via tail vein at a dose of 5 mg doxorubicin/kg. At 24 or 72 hours, animals were euthanized and tissues were collected following perfusion with PBS and analyzed for doxorubicin and lipid. Tissue samples were homogenized and drug (doxorubicin) and lipid (liposomes) concentrations were determined as described. The final distribution was expressed as percentage of injected dose for the concentration in circulation (blood) and as % of injected dose/g of tissue for all other samples. Analyses were done in triplicate; data indicate mean ± SD.

In vivo uptake studies. Nude mice (NCR nu/nu) were injected s.c. with U87 glioblastoma cells (1 × 10⁷) additionally transfected with EGFR variant III (EGFRvIII; ref. 15). Once tumors size reached 400 to 800 mm³, animals were randomly assigned to treatment group: 3 mice per group × 3 groups (saline, nontargeted, and EGFR-targeted liposomes). Liposomes and immunoliposomes loaded with the fluorophore ADS645WS were injected i.v. as a single dose via tail vein (total 10 µmol phospholipid/animal in 200 µL volume). After 24 hours, animals were euthanized and tumors were excised and disaggregated [1 hour at 37°C in 0.1% (w/v) collagenase IV (Sigma, St. Louis, MO) and 0.003% DNase I (Calbiochem, San Diego, CA)]. Cell suspensions were carefully washed, fixed [formaldehyde, 1% (v/v)], and analyzed by flow cytometry (FACSCalibur2, BD Biosciences, Inc., San Jose, CA) or confocal microscopy (LSM 510 meta, Carl Zeiss, Inc., Thornwood, NY).

Tumor xenograft models. Tumor cells (MDA-MB-468, U87, or U87vIII) were injected in the dorsum of nude mice as described. Once tumor xenografts had become established and 150 to 400 mm³ in volume, mice were randomly assigned to treatment groups (10-15 animals per group). Liposomes and immunoliposomes were administered via tail vein at approximate maximum tolerated dose (MTD) once weekly for 3 weeks.

Based on typical drug/lipid ratios of 150 µg drug/µmol phospholipid, the corresponding lipid dose was typically 0.8 to 1.3 µmol of phospholipid per injected dose and 2.4 to 3.9 µmol total. "Empty" anti-EGFR immunoliposomes were prepared identically, except for omission of cytotoxic drug, and were administered i.v. at the same lipid dose as anti-EGFR immunoliposomal drugs. Free drug and saline were injected on the same schedule as liposomes/immunoliposomes. Free mAb C225 was injected i.p. at 1 mg/dose twice a week for 3 weeks according to optimized regimens (16).

Tumor measurements were done [(length × width²) / 2] and mice were weighed and examined for toxicity thrice a week for 60 to 90 days post tumor implantation. Mice with complete clinical regressions were examined histopathologically and were classified as "cured" if no residual tumor was detected after histopathologic analysis.

Statistical analysis. Treatment effects were compared using Student's *t* test (two-sample individual *t* test) for tumor size at each time point. In addition, a multivariate (rank) test was done based on the sums of ranks for each mouse. Tumor sizes at each time point following final injection were ranked across all mice and the ranks were summed. The sum of the ranks was compared in each case for two treatments by a two-sample *t* test (17).

Results

Construction of immunoliposomal drugs targeted to EGFR. Immunoliposomes were constructed using a modular design that builds on sterically stabilized liposomes optimized for long circulation as drug-containing nanoparticles as previously described (3, 4, 10). Briefly, conjugation was done by covalent linkage of Fab' fragments derived from mAb C225 to modified termini of PEG chains on liposomes (5) or by covalent linkage to Mal-PEG-DSPE linker in solution, followed by incorporation of the resulting micellar conjugates into liposomes (18). The "micellar incorporation" method allowed stable addition of numerous mAb fragments to an existing drug-loaded liposome such as PLD (Doxil/Caelyx).

For delivery of other drugs, novel nanoparticle/liposome-based constructs were prepared using a new process for active drug loading and retention. Previous methods for liposome drug loading have been extremely variable, with excellent results with liposomal anthracyclines (14) but more problematic efficiency and/or stability with other drugs (19). In this new technique, liposomes were formed with an entrapped solution containing poorly permeable ionic species such as substituted ammonium salts of poly(phosphate) or sucrose octasulfate, and then transferred to a buffer solution containing the drug. The resulting transmembrane potential induced spontaneous accumulation of weakly basic drugs into the liposome interior, producing packing, gelation, or crystallization of drug within the liposome. This technique enabled highly robust encapsulation of a number of chemical classes into stabilized liposomes, including epirubicin, Vinca alkaloids, ellipticines, and camptothecins; the resulting constructs were notable for extremely efficient loading (~100% of added drug was encapsulated), unsurpassed drug yields (10⁴-10⁵ drugs per particle), and marked *in vivo* stability (20, 21). In this report, we converted new liposomal constructs of epirubicin and vinorelbine into immunoliposome versions targeted to EGFR.

Pharmacokinetics of anti-EGFR immunoliposome-doxorubicin. Pharmacokinetic studies of anti-EGFR immunoliposome-doxorubicin were done in healthy adult rats. Immunoliposomes or matched liposomes lacking mAb fragments were labeled with [³H]-cholesteryl hexadecyl ether and loaded with doxorubicin. After a single i.v. dose, anti-EGFR immunoliposome-doxorubicin showed prolonged circulation of both lipid and drug components, both of

which were still present at ~20% of 5-minute levels at 48 hours postinjection (Fig. 1A). In contrast, free doxorubicin at the same dose was already undetectable at 4 hours postinjection. These data confirmed that immunoliposome delivery greatly extended the circulation time of encapsulated doxorubicin and that carrier and drug circulated together without appreciable drug release. Furthermore, the pharmacokinetics of immunoliposome components were indistinguishable from those of sterically stabilized liposomal doxorubicin, indicating that mAb fragment conjugation did not compromise circulation time or stability. Plasma clearances for liposomal and immunoliposomal doxorubicin were equivalent

at 2.48 ± 0.21 and 2.74 ± 0.25 mL/h, as were the areas under the plasma concentration versus time curve at $2,025 \pm 170$ and $1,836 \pm 165$ $\mu\text{g h/mL}$, respectively (Table 1). Similarly, serial measurements of the doxorubicin-to-phospholipid ratio, indicative of drug retention, were equivalent at all time points. For example, the doxorubicin-to-phospholipid ratio at 48 hours was 89.14 ± 0.39 for nontargeted liposomal doxorubicin and 84.67 ± 1.92 for anti-EGFR immunoliposome-doxorubicin.

Biodistribution of anti-EGFR immunoliposomes versus nontargeted liposomes. Biodistribution and tumor tissue localization of liposomal and immunoliposomal doxorubicin were evaluated in nude mice bearing EGFR-overexpressing MDA-MB-468 breast tumor xenografts. Following a single i.v. injection of either liposomes or immunoliposomes, deliveries of liposomal lipid (^3H -cholesteryl hexadecyl ether) and encapsulated drug (doxorubicin) were separately assayed in various tissues at 24 or 72 hours (Fig. 1B and C).

At 24 hours, tissue levels for both anti-EGFR immunoliposomes and nontargeted liposomes were highest in blood, consistent with the long circulation times observed in rats. Blood levels were slightly lower in tumor-bearing nude mice than in normal rats: 18% ID to 20% ID for tumor-bearing mice versus >40% ID for rats at 24 hours postinjection. This differential may reflect species-specific differences in pharmacokinetics as well as an increase in clearance in tumor-bearing animals. These results again showed equivalent circulation times for immunoliposomes and liposomes, with no compromise in pharmacokinetics due to the presence of mAb fragments.

In addition to high blood levels, at 24 hours, both nanoparticles reached high levels in tumor, liver, spleen and skin. Nanoparticle accumulation in other organs, including heart, lung and kidney, was substantially lower. This biodistribution pattern is fully consistent with the well-established preclinical and clinical profile of sterically stabilized liposomes, such as PLD, which feature reduced but eventual clearance in reticuloendothelial system (RES) sites in liver and spleen (22).

At 72 hours, blood levels of both nanoparticles showed a decline whereas tumor levels increased and RES sites showed a more complex pattern. In liver and spleen, levels of cholesterol and doxorubicin, which were equivalent at 24, showed a relative decrease in doxorubicin as compared with cholesterol at 72 hours ($P < 0.05$). This result likely reflected metabolism and clearance of doxorubicin within the RES between 24 and 72 hours.

Importantly, tumor accumulation in EGFR-overexpressing MDA-MB-468 tumor cells was extremely high for nontargeted liposomes and was not further increased in the case of anti-EGFR immunoliposomes. At 24 hours, tumor levels for both nanoparticles reached 10% to 12% ID/g tissue, and continued to accumulate at 72 hours to 13% to 15% ID/g tissue. The observation that mAb-mediated targeting did not further increase accumulation of long circulating liposomes in tumors is consistent with previous results using anti-HER2 immunoliposomes in HER2-overexpressing tumor models (4, 23).

Internalization of anti-EGFR immunoliposomes in xenografted tumor cells *in vivo*. Although anti-EGFR immunotargeting did not confer additional tumor tissue localization, we evaluated whether the mAb component was capable of mediating binding and internalization of immunoliposomes in tumor cells *in vivo* as we reported previously in *in vitro* studies. Confocal microscopy was used to compare binding and uptake of EGFR-targeted immunoliposomes and nontargeted liposomes in the U87/EGFRvIII tumor model, which overexpresses both EGFR and

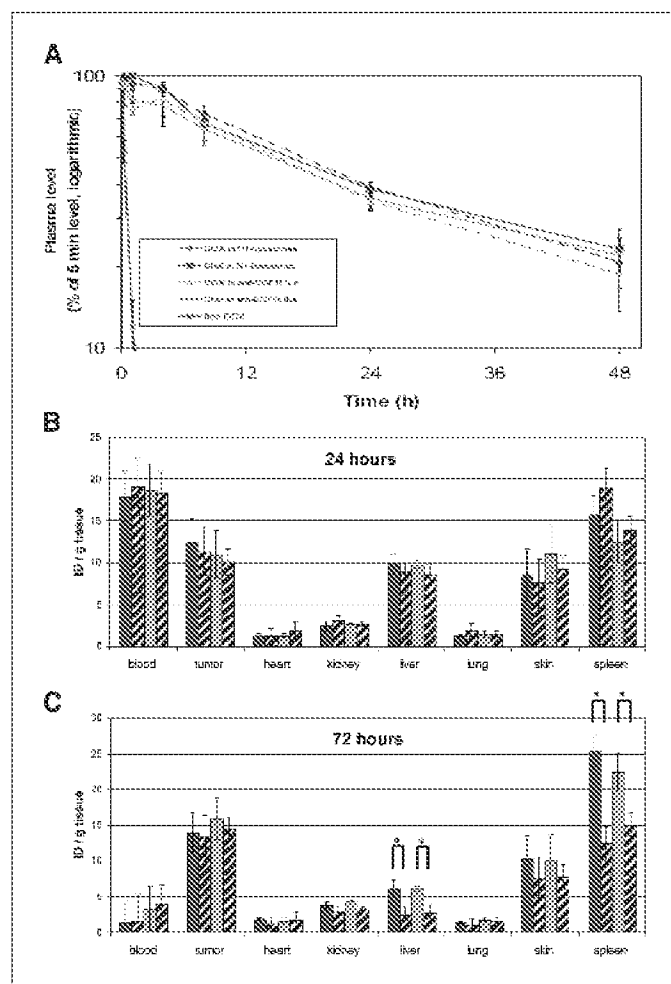


Figure 1. Pharmacokinetics and biodistribution of anti-EGFR immunoliposomes. Sterically stabilized liposomes were prepared with 10% PEG-DSPE and ^3H -Chol [cholesteryl-1,2- ^3H (N)], then loaded with doxorubicin and conjugated or not to C225-Fab'. A, pharmacokinetics in adult rats. Free doxorubicin (DOX), nontargeted liposomal doxorubicin, or anti-EGFR immunoliposomal doxorubicin was injected i.v. into normal healthy adult rats (three rats per group) at a dose of 5 mg doxorubicin/kg. Lipid [cholesteryl-1,2- ^3H (N); solid lines] and doxorubicin (dotted lines) were separately assayed at the indicated times. Injection of free doxorubicin resulted in no detectable drug after 4 hours (<2.5% ID). Points, mean; bars, SD. B and C, MDA-MB-468 xenograft model. EGFR-overexpressing MDA-MB-468 cells were implanted s.c. in nude mice. Either nontargeted liposomal doxorubicin (black columns) or anti-EGFR immunoliposomal doxorubicin (gray columns) was injected i.v. at a dose of 5 mg doxorubicin/kg. Lipid [cholesteryl-1,2- ^3H (N); solid columns] and doxorubicin (hatched columns) were separately assayed in the indicated tissues at 24 hours (B) or 72 hours (C) posttreatment ($n = 3$). Data for blood samples are expressed as % ID; for all other tissues, data are expressed as % ID/g tissue. *, $P < 0.05$; comparison of lipid versus doxorubicin levels showed significant differences in liver and spleen at 72 hours (two-sample individual t test), reflecting drug metabolism. Columns, mean; bars, SD.

Table 1. Plasma pharmacokinetics of free, liposomal, and anti-EGFR immunoliposomal doxorubicin in adult rats

Treatment*	$t_{1/2}$ (h)	AUC _∞ (μg h/mL)	CL (mL/h)	V _d (mL)	MRT (h)	DOX-to-PL [†] (%ID/%ID)
LS-DOX	21.5 ± 2.0	2,025 ± 170	2.48 ± 0.21	76.5 ± 0.7	31.0 ± 2.8	89.14 ± 0.39
Anti-EGFR ILs-DOX	20.7 ± 3.3	1,836 ± 165	2.74 ± 0.25	81.1 ± 5.9	29.9 ± 4.9	84.67 ± 1.92

Abbreviations: AUC_∞, area under the plasma concentration versus time curve based on the sum of exponential terms; MRT, mean residence time calculated from exponential terms; CL, clearance calculated from exponential terms; V_d, volume of distribution.

*Liposomal doxorubicin (LS-DOX) and anti-EGFR immunoliposome-doxorubicin (Anti-EGFR ILs-DOX) were prepared using DSPC/Chol/PEG-DSPE (3:2:0.3, mol/mol/mol) and loaded with doxorubicin at a ratio of 150 g doxorubicin/mol phospholipid using the ammonium sulfate gradient-loading method as described in Materials and Methods. Anti-EGFR immunoliposome-doxorubicin or nontargeted liposomes were administered i.v. at a single dose of 5 mg doxorubicin/kg; two animals per group.

[†]Ratio of doxorubicin-to-phospholipid at the 48-hour time point.

EGFRvIII. For these studies, liposomes were loaded with the water-soluble fluorescent dye ADS645WS, with or without mAb fragments, and administered in tumor-bearing mice as a single-dose i.v. injection. At 24 hours postinjection, tumors were removed, disaggregated, and extensively washed for analysis. Confocal microscopy of the treated and harvested tumor cells showed that anti-EGFR immunoliposomes had accumulated profusely throughout the cytoplasm in a pattern consistent with receptor-mediated endocytosis (Fig. 2A). In contrast, control liposomes lacking the mAb fragment showed only minimal binding or uptake (Fig. 2B), which did not seem to be significantly different than the background (Fig. 2C).

Quantitative analysis of cellular accumulation of immunoliposomes following i.v. administration was done using a flow cytometry assay. Twenty-four hours following i.v. injection of ADS645WS-loaded immunoliposomes or liposomes, disaggregated cells from U87 xenografts were gated for positive nuclear staining and evaluated for fluorescence uptake. Overall, anti-EGFR immunoliposomes achieved cellular uptake that was 6-fold greater than that of nontargeted liposomes (Fig. 2D). Tumors were also characterized in separate experiments by *ex vivo* analysis of the cell population. Tumors were excised from mice that did not receive i.v. treatment and processed as described; the resulting cell suspension was then incubated for 2 hours at 37°C with ADS645WS-loaded anti-EGFR immunoliposomes or nontargeted liposomes *ex vivo*. Immunoliposome uptake was observed in 92% of the population (Fig. 2E) whereas nontargeted liposomes showed minimal uptake in <5% of the population (Fig. 2F). These results confirmed that the preponderance of the analyzed cells in harvested tumor tissues consisted of tumor cells capable of taking up anti-EGFR immunoliposomes.

Taken together, these studies indicated that immunoliposomes and nontargeted liposomes were comparable in overall tumor tissue localization but, at the cellular level, displayed distinctly different mechanisms of delivery. Consistent with *in vitro* results, anti-EGFR immunoliposomes, but not liposomes, were capable of extensive cellular uptake and internalization in EGFR-overexpressing tumor cells *in vivo*.

Efficacy of anti-EGFR immunoliposomal doxorubicin in EGFR-overexpressing tumor xenograft models. The therapeutic efficacy of EGFR-targeted immunoliposomes containing doxorubicin (anti-EGFR immunoliposome-doxorubicin) was evaluated in two different EGFR-overexpressing tumor xenograft models: MDA-MB-468 human breast tumors ($\sim 5 \times 10^5$ receptors per cell) and

U87 human glioblastoma tumors ($\sim 1 \times 10^5$ – 2×10^5 receptors per cell). In both models, 1×10^7 to 2×10^7 tumor cells were implanted s.c. without matrigel in the flank of nude mice and allowed to grow until fully established and substantial in volume. To rigorously evaluate anticancer efficacy against advanced tumors, treatments were initiated when tumors were moderately large (mean volume, >150 mm³) or extremely large (mean volume, >400 mm³). We then evaluated the therapeutic effects of anti-EGFR immunoliposomal doxorubicin alongside various comparators.

In the MDA-MB-468 xenograft model with moderate-sized tumors (>150 mm³), anti-EGFR immunoliposome-doxorubicin was administered i.v. at a total dose of 15 mg doxorubicin/kg divided over three weekly doses of 5 mg/kg/wk (Fig. 3A). Other treatments included saline, free doxorubicin at its MTD of 7.5 mg/kg, nontargeted liposomal doxorubicin (commercial PLD) at the same dose and schedule as immunoliposomes, and irrelevant immunoliposomes containing a different mAb fragment not specific for EGFR, also at the same dose and schedule as anti-EGFR immunoliposomes.

As expected, free doxorubicin produced some tumor growth inhibition as compared with saline treatment. Both nontargeted liposome delivery via PLD and irrelevantly targeted immunoliposome-doxorubicin showed increased efficacy over free drug [$P < 0.0001$, multivariate (rank) test]; this is consistent with the established advantages of liposome delivery of anthracyclines in preclinical models (19). Treatment with anti-EGFR immunoliposome-doxorubicin produced substantial tumor regressions and was the most efficacious treatment. Because immunoliposomes were prepared by conjugation of Fab' to PLD itself, the superiority of anti-EGFR immunoliposome-doxorubicin ($P < 0.001$ versus PLD) was clearly due to targeting by the anti-EGFR mAb fragments. In addition, cured tumors, defined as complete eradication of tumor during treatment and confirmed by histopathology at sacrifice, occurred in 27% (3 of 11) of immunoliposome-treated mice versus 0% for all other treatments, including free doxorubicin (0 of 10), nontargeted PLD (0 of 11), and irrelevantly targeted PLD (0 of 10). This represented a statistically significant advantage for anti-EGFR immunoliposome-doxorubicin versus either nontargeted or irrelevantly targeted PLD ($P = 0.011$, individual two-sample *t* tests).

Anti-EGFR immunoliposome-doxorubicin was further evaluated in a different EGFR-overexpressing tumor model featuring U87 human glioblastoma xenografts (Fig. 3B). In this highly tumorigenic and rapidly growing model, treatment was initiated against very large U87 tumors (~ 450 mm³). Despite this, anti-EGFR

immunoliposome-doxorubicin produced substantial tumor growth inhibition and was clearly superior to all other treatments, including nontargeted PLD ($P = 0.002$), free doxorubicin ($P < 0.001$), and saline.

As an additional control, one group of mice received treatment with empty anti-EGFR immunoliposomes lacking encapsulated drug. Empty anti-EGFR immunoliposomes, administered i.v. at the same lipid dose and schedule as anti-EGFR immunoliposome-doxorubicin, produced no measurable effect on tumor growth. This result indicated that the potent activity of anti-EGFR immunoliposome-doxorubicin was due to targeted delivery of doxorubicin and not to the antiproliferative activity of C225-Fab'

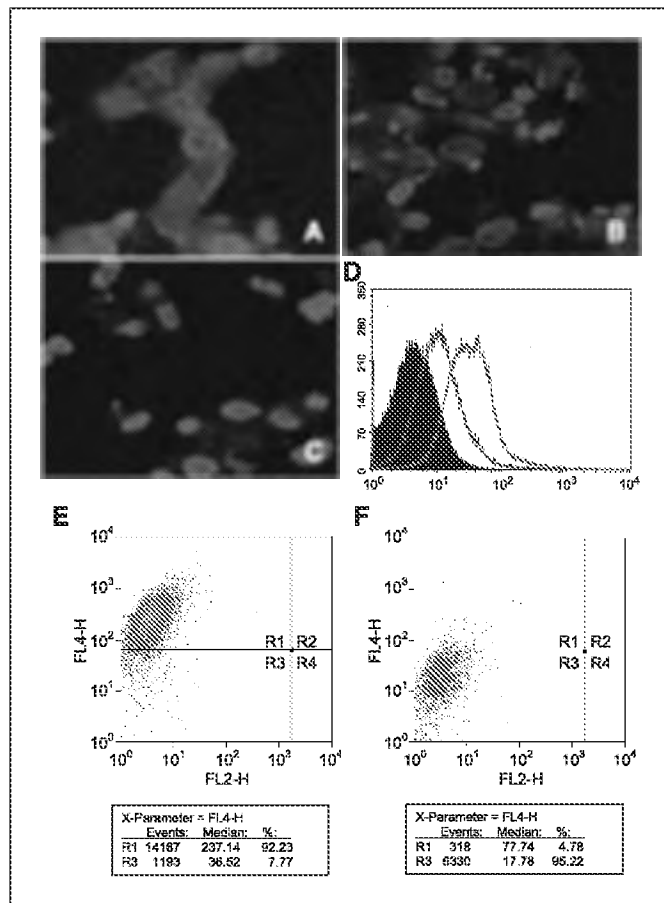


Figure 2. In vivo uptake of liposomes and immunoliposomes in the U87/EGFRvIII tumor xenograft model. U87/EGFRvIII tumor cells, overexpressing EGFR and EGFRvIII, were implanted s.c. in nude mice. After tumors had become fully established, mice received a single i.v. injection of saline, nontargeted liposomes, or anti-EGFR immunoliposomes. Liposomes and immunoliposomes were loaded with the fluorescent marker ADS645WS. At 24 hours, tumor cells were harvested by tissue disaggregation, extensive washing, and resuspension. Tumor cell uptake of liposomes or immunoliposomes was evaluated by confocal microscopy and flow cytometric analysis. **A**, tumor cells showing uptake of anti-EGFR immunoliposomes; **B**, tumor cells showing no apparent uptake of nontargeted liposomes; **C**, tumor cells treated with saline; **D**, flow cytometric analysis showing greater uptake of anti-EGFR immunoliposomes (red) as compared with nonspecific accumulation of nontargeted liposomes (green). Events were gated for positive nuclear staining. In separate control experiments, cell suspensions were derived from mice that did not receive i.v. treatment and processed as described, and then incubated ex vivo for 2 hours at 37°C with either ADS645WS-loaded anti-EGFR immunoliposomes (**E**) or nontargeted liposomes (**F**). Flow cytometry was done to evaluate immunoliposome/liposome uptake in the cell suspension and the proportion of cells positive or negative for uptake was calculated using the gates shown. Representative results from two therapy experiments (**A-D**; three animals per group) and four control experiments (**E-F**; one animal per study).

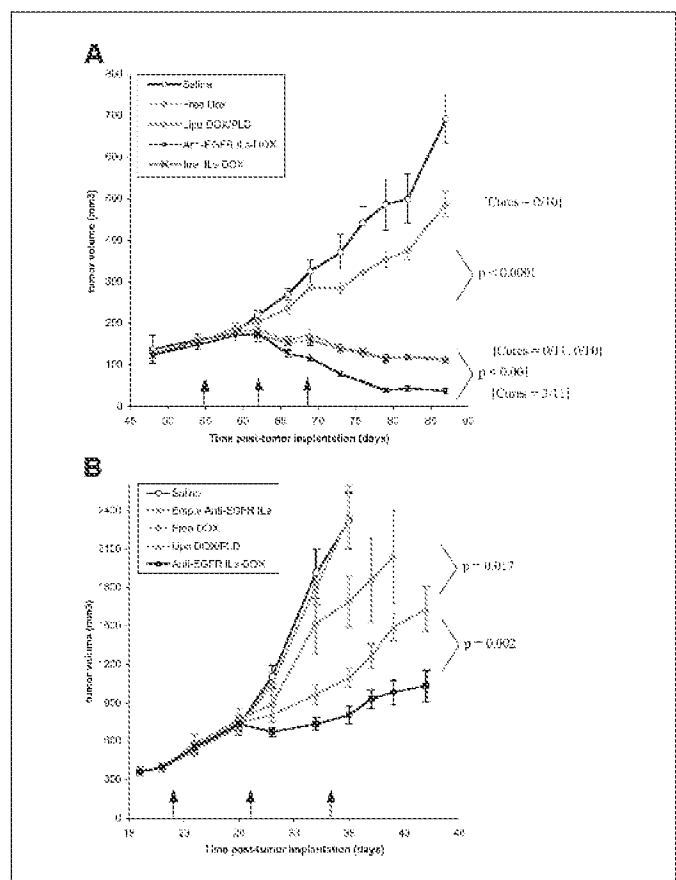


Figure 3. Therapeutic efficacy of anti-EGFR immunoliposome-doxorubicin in EGFR-overexpressing tumor models. **A**, MDA-MB-468 model. Anti-EGFR immunoliposomes containing doxorubicin (Anti-EGFR ILs-DOX; ■) were administered i.v. at a total dose of 15 mg doxorubicin/kg on the indicated days post tumor implantation (arrows). Other treatment groups included saline (○), free doxorubicin (◇) at its MTD of 7.5 mg/kg, PLD (Lipo Dox/PLD; ◆) at 15 mg/kg, and irrelevant immunoliposomes (Irrel ILs-DOX; ×) containing a mAb fragment not specific for EGFR. Ten to eleven mice in each group. Liposome delivery was significantly superior to free drug ($P < 0.0001$) whereas anti-EGFR immunoliposome-doxorubicin was in turn significantly superior to nontargeted liposomal doxorubicin ($P < 0.001$). **B**, U87 model. Anti-EGFR immunoliposome-doxorubicin (■) was administered i.v. at a total dose of 15 mg doxorubicin/kg over three weekly doses on the indicated days post tumor implantation (arrows). Other treatment groups included saline (○), free doxorubicin (◇) at its MTD of 7.5 mg/kg, PLD (Lipo Dox/PLD; ◆) at 15 mg/kg, and anti-EGFR immunoliposome-doxorubicin identically prepared but lacking doxorubicin (Empty Anti-EGFR ILs; ●). Ten to twelve mice in each group. Liposome delivery was significantly superior to free drug ($P = 0.017$) whereas anti-EGFR immunoliposome-doxorubicin was in turn significantly superior to nontargeted PLD ($P = 0.002$). Points, mean tumor volumes; bars, SE. P values were based on a multivariate (rank) test for the indicated treatment groups.

fragments present on the immunoliposomes. These results are also consistent with previous studies of anti-HER2 immunoliposomes containing trastuzumab-based Fab', which showed no antitumor efficacy when empty unless administered at much more frequent intervals to achieve steady-state concentrations of the mAb component (6).

Anti-EGFR immunoliposome-doxorubicin was well tolerated by the mice. Maximum weight loss was $6.3 \pm 3.5\%$ for anti-EGFR immunoliposome-doxorubicin and $4.6 \pm 3.9\%$ for Food and Drug Administration-approved PLD, which were not statistically different (individual two-sample t tests).

Efficacy of anti-EGFR immunoliposome-epirubicin in an EGFR/EGFRvIII-overexpressing tumor xenograft model. EGFRvIII (Δ EGFR), an in-frame deletion of the extracellular domain

associated with constitutive signaling, has been detected in many gliomas and other tumors (24–30); it seems to play a role in glioma tumorigenesis although its role in other tumor types is less clear (31).

Anti-EGFR immunoliposomes constructed from either C225-Fab' or scFv C10 can target EGFRvIII-expressing tumor cells as their epitopes are retained in the truncated receptor (10). Anti-EGFR immunoliposomes were evaluated in the U87/EGFRvIII tumor xenograft model in which U87 human glioma cells were stably transfected with mutant EGFRvIII (15). Coexpression at high levels of wild-type EGFR ($\sim 1 \times 10^5$ – 2×10^5 receptors per cell) and mutant EGFRvIII ($\sim 5 \times 10^5$ receptors per cell) in this model corresponds to a frequent phenotype in human gliomas and possibly other tumors (25).

For these studies, liposomes and anti-EGFR immunoliposomes were loaded with the anthracycline drug epirubicin. As a free drug, epirubicin has potential advantages over doxorubicin, including possibly reduced cardiotoxicity (32). Liposome delivery has also been shown to reduce this important limitation of doxorubicin (33, 34). Thus, liposome/immunoliposome delivery of epirubicin may provide potential clinical advantages.

In the U87/EGFRvIII model, free epirubicin at 24 mg/kg was associated with some antitumor efficacy, which was exceeded by that of the nontargeted liposomal version at equivalent drug dose (Fig. 4). The combination of liposomal epirubicin plus free mAb C225 showed enhanced efficacy as compared with liposomal epirubicin alone, consistent with preclinical and clinical results that C225 increases the efficacy of various chemotherapies (35). However, single-agent treatment with anti-EGFR immunoliposome-epirubicin at equivalent dose showed greater efficacy than all other treatments. Anti-EGFR immunoliposome-epirubicin was significantly superior to nontargeted liposomal epirubicin [$P = 0.009$, multivariate (rank) test], as well as to the combination of liposomal epirubicin plus free C225 ($P < 0.05$, individual two-sample t tests at three different time points).

Efficacy of anti-EGFR immunoliposomes containing alternative chemotherapy drugs. One of the theoretical advantages of immunoliposomal drug delivery versus other immunoconjugate approaches is the inherent versatility of the liposome component, which can be used to encapsulate various chemotherapeutic drugs or other compounds. To exploit this, we constructed anti-EGFR immunoliposomes containing either doxorubicin or vinorelbine; these were then directly compared for therapeutic effects *in vivo* in the EGFR-overexpressing U87 xenograft model (Fig. 5A).

For doxorubicin, anti-EGFR immunoliposome-doxorubicin was prepared from commercial PLD as described. Treatment with anti-EGFR immunoliposome-doxorubicin at a total drug dose of 15 mg/kg i.v. was associated with clear tumor regressions in this aggressive xenograft model and was significantly superior to treatment with nontargeted liposomal doxorubicin ($P = 0.002$; Fig. 5B).

For vinorelbine, a novel liposome construct was prepared by active loading across an induced transmembrane potential. Liposomal vinorelbine constructs achieved outstanding yields, with 26,000 drug molecules per nanoparticle, and stability, with prolonged drug retention during long circulation in rats ($t_{1/2} = 27.2$ hours). Treatments with free, liposomal, or immunoliposomal vinorelbine were all administered i.v. at a total drug dose of 15 mg vinorelbine/kg (Fig. 5C). Liposomal vinorelbine seemed to have greater efficacy than free vinorelbine, although this comparison was of borderline statistical significance ($P = 0.053$). Anti-EGFR

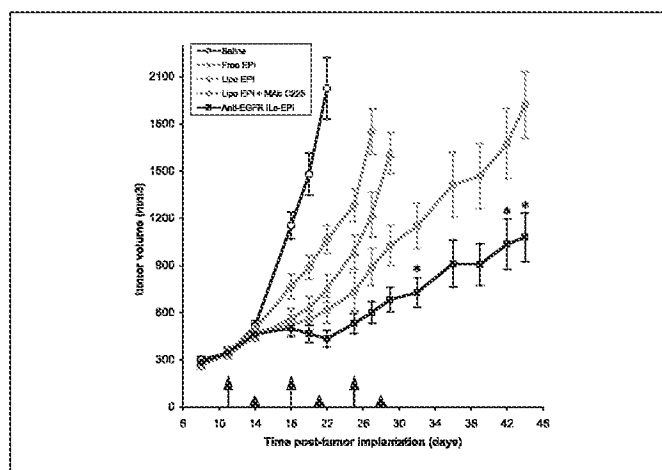


Figure 4. Therapeutic efficacy of anti-EGFR immunoliposomal epirubicin versus combination therapies in the U87/EGFRvIII tumor xenograft model. Anti-EGFR immunoliposomes containing epirubicin (Anti-EGFR ILs-EPI; ■) were administered i.v. once a week over 3 weeks at a total drug dose of 24 mg/kg on the indicated days post tumor implantation (arrows). Other treatment groups included saline (○), free epirubicin (Free EPI) at 24 mg/kg (◇), liposomal epirubicin at 24 mg/kg (Lipo EPI; ◇), and the combination of liposomal epirubicin at 24 mg/kg + free anti-EGFR mAb (Lipo EPI + MAb C225; ●) administered i.p. at 1 mg/dose twice a week over six doses (small arrows). Ten to twelve mice in each group. Anti-EGFR immunoliposome-epirubicin was significantly superior to liposomal epirubicin [$P = 0.009$, multivariate (rank) test] and was also superior to the combination of liposomal epirubicin + mAb C225 at the indicated time points (*, $P < 0.05$, individual two-sample t tests). Points, mean tumor volumes; bars, SE.

immunoliposome-vinorelbine showed a significant increase in efficacy over nontargeted liposomal vinorelbine ($P = 0.003$).

Comparison across the two drug classes showed that the efficacies of anti-EGFR immunoliposomes containing either doxorubicin or vinorelbine at 15 mg/kg of either drug were comparable (Fig. 5A). Similarly, the nontargeted liposomal versions of doxorubicin and vinorelbine, either commercial PLD or the new liposomal vinorelbine construct, were comparable to each other.

Discussion

Anti-EGFR immunoliposomes showed long circulation and stable drug retention, with no apparent compromise due to mAb fragment conjugation. Treatment studies with drug-loaded anti-EGFR immunoliposomes showed potent therapeutic effects in multiple tumor models, including tumor growth inhibition, regressions, and eradications. Anti-EGFR immunoliposomes loaded with doxorubicin, epirubicin, or vinorelbine were significantly superior to all other treatments, including the corresponding free drug, nontargeted liposomal drug, and other relevant comparators.

Of note, both nontargeted liposomes and anti-EGFR immunoliposomes showed largely comparable biodistributions at the tissue level. Both types of nanoparticles achieved extremely high tumor concentrations at 24 hours (10–12% ID/g tissue) and even higher levels at 72 hours (13–15% ID/g tissue). Hence, whereas drug-loaded immunoliposomes produced clearly greater antitumor efficacy than nontargeted versions, targeting was not associated with increased tumor tissue levels.

This apparent paradox, also observed with anti-HER2 immunoliposomes in HER2-overexpressing tumors (23), was clarified by further studies of the mechanism of delivery for liposomes and immunoliposomes at the cellular level. We previously

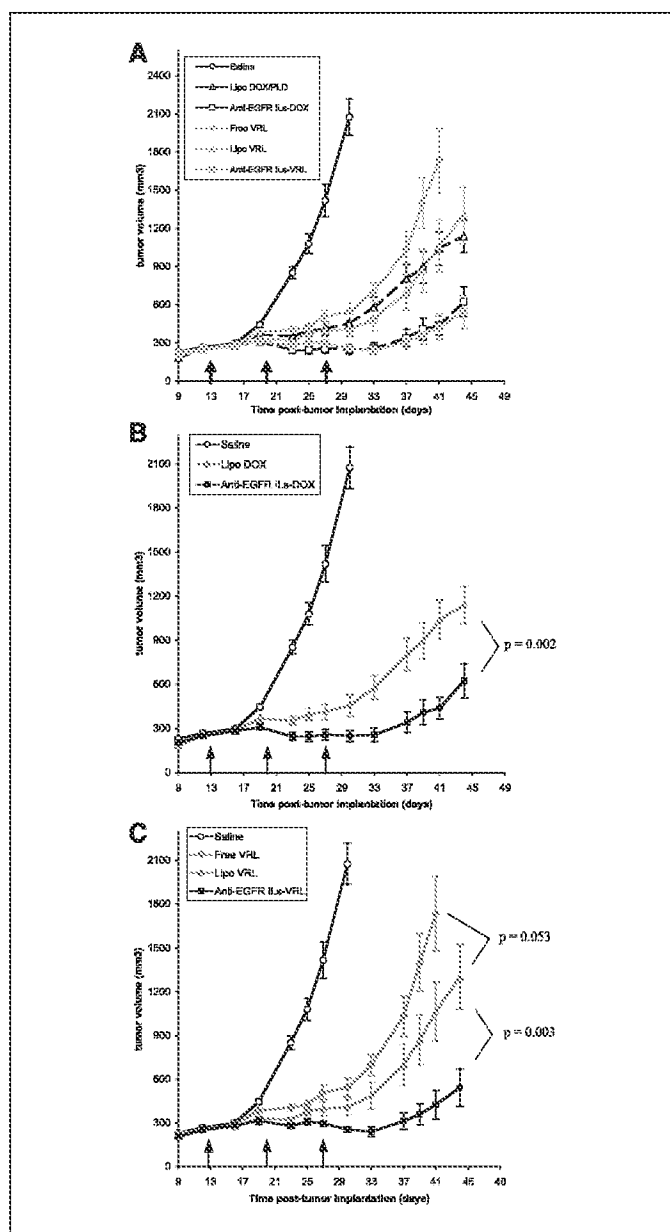


Figure 5. Efficacy of anti-EGFR immunoliposomes containing various drugs in the U87 tumor xenograft model. A, all treatments. Two cytotoxic drugs with different mechanisms of action were encapsulated into liposomes or anti-EGFR immunoliposomes: doxorubicin and vinorelbine (VRL). For each of these drug classes, nude mice with flank U87 tumor xenografts were treated with saline, free drug, nontargeted liposomal drug, or anti-EGFR immunoliposomal drug. Each group contained 10 to 12 tumor-bearing mice. Points, mean tumor volumes; bars, SE. Pairwise comparisons of treatments were done by multivariate (rank) test. For clarity, treatment results for each set of drugs are shown separately in (B and C). B, doxorubicin. Anti-EGFR immunoliposome-doxorubicin (Anti-EGFR ILs-DOX; ■) was prepared by micellar conjugation of Fab' to commercial PLD (Lipo DOX/PLD; ●), and both were administered i.v. at a total doxorubicin dose of 15 mg/kg over three doses (arrows). Anti-EGFR immunoliposome-doxorubicin was significantly superior to nontargeted PLD ($P = 0.002$). C, vinorelbine. Nanoparticle/liposome constructs encapsulating vinorelbine were prepared via induced transmembrane potential for active drug loading, resulting in highly concentrated drug packing of 26,000 drugs per particle. Anti-EGFR immunoliposomes containing vinorelbine (Anti-EGFR ILs-VRL; ◼) were administered i.v. at a total dose of 15 mg/kg on the indicated days post tumor implantation (arrows). Other treatment groups included saline (○), free vinorelbine (◇), and liposomal vinorelbine (Lipo VRL; ◆), both at 15 mg vinorelbine/kg. There was a trend towards superior efficacy for liposomal vinorelbine over free vinorelbine ($P = 0.053$) whereas anti-EGFR immunoliposome-vinorelbine was in turn clearly superior to nontargeted liposomal vinorelbine ($P = 0.003$).

reported that anti-EGFR immunoliposomes bind and internalize in EGFR-overexpressing tumor cells *in vitro*, thus enabling intracellular drug delivery (10). The present studies confirm that immunoliposomes internalize in target cells *in vivo*. Tumor cells analyzed after i.v. treatment showed internalization of immunoliposomes but not of nontargeted liposomes. These data are consistent with a mechanism for immunoliposome delivery involving two phases. In the first or tissue phase, nanoparticles with sufficient longevity in circulation (such as sterically stabilized liposomes or immunoliposomes) slowly accumulate in tumor tissue, ultimately reaching high tumor levels due to the enhanced permeability and retention effect. Molecular targeting is not required for this process and does not seem to facilitate it. In the second or cellular phase, nontargeted liposomes remain in the interstitial space and are subject to decomposition, degradation, or phagocytosis, with eventual release of drug. In contrast, immunoliposomes bind to and internalize in tumor cells via ligand-receptor interactions. Taken together, these results indicate that internalization of anti-EGFR immunoliposomes in tumor cells was a critical factor for the enhanced efficacy observed.

It was also clear that anti-EGFR immunoliposome efficacy was not attributable to signal inhibition mediated by the C225 component. Empty anti-EGFR immunoliposomes, administered at the same lipid dose and schedule as doxorubicin-loaded immunoliposomes, failed to show antitumor efficacy. This was in fact not surprising given the large difference in the dose requirements involved. Treatment with free mAb C225 required 1,000 μg per dose for six doses. Immunoliposome treatment, in contrast, required very small amounts of conjugated mAb fragments: $\sim 30 \mu\text{g}$ Fab' per dose for three doses, corresponding to more than 60-fold lower mAb content. It is also notable that anti-EGFR immunoliposomes showed greater efficacy than the combination of free C225 plus liposomal epirubicin.

Immunoliposomal drugs may provide a more potent strategy to treat EGFR-overexpressing tumors than current approaches. Strategies to inhibit EGFR signal transduction, including naked mAbs and small molecule inhibitors, have proved to be clinically useful (36–39); however, it remains unclear which subsets of patients are most likely to receive clinical benefit from these treatments (40, 41). Anti-EGFR immunoliposomes can target both wild-type EGFR and EGFRvIII and their efficacy is not dependent on signal transduction effects.

In conclusion, anti-EGFR immunoliposomes achieved favorable pharmacokinetics, biodistribution, and tumor localization due to its nanoparticle properties, followed by specific binding and internalization in tumor cells via mAb fragments. Targeting different drugs to tumor cells in this way produced potent anticancer activity in preclinical models, thus representing a potentially useful strategy for cancer treatment.

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Epidermal Growth Factor Receptor–Targeted Immunoliposomes Significantly Enhance the Efficacy of Multiple Anticancer Drugs *In vivo*

Christoph Mamot, Daryl C. Drummond, Charles O. Noble, et al.

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Extensive distribution of liposomes in rodent brains and brain tumors following convection-enhanced delivery

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Key words: brain tumors, CNS, convection-enhanced delivery, glioblastoma, liposomes

Summary

Liposomes labeled with various markers were subjected to local–regional administration with either direct injection or convection-enhanced delivery (CED) into rodent brains and brain tumor models. Direct injection of liposomes containing attached or encapsulated fluorochromes and/or encapsulated gold particles indicated that tissue localization of liposomes could be sensitively and specifically detected in the central nervous system (CNS). When CED was applied, liposomes achieved extensive and efficient distribution within normal mouse brains. Co-infusion of mannitol further increased tissue penetration of liposomes. Liposomes were also loaded with gadodiamide to monitor their CNS distribution in rats by magnetic resonance imaging (MRI). CED-infused liposomes were readily seen on MRI scans as large regions of intense signal at 2 h, and more diffuse regions at 24 h. Finally, labeled liposomes were infused via CED into tumor tissue in glioma xenograft models in rodent hosts. In intracranial U-87 glioma xenografts, CED-infused liposomes had distributed throughout tumor tissue, including extension into surrounding normal tissue. Greater penetration was observed using 40 *versus* 90 nm liposomes, as well as with mannitol co-infusion. To our knowledge, this is the first report of CED infusion of liposomes into the CNS. We conclude that CED of liposomes in the CNS is a feasible approach, and offers a promising strategy for targeting therapeutic agents to brain tumors.

Introduction

Brain tumors remain among the most difficult neoplasms to treat, and a major barrier to progress is the relative inaccessibility of tumors in the central nervous system (CNS) to anticancer agents. Drug delivery approaches have begun to provide clinical benefits, such as the intracranial implantation of biodegradable polymer systems containing BCNU for higher local drug concentrations [1]. However, implantation or direct injection approaches ultimately rely upon diffusion, which is concentration-dependent, inversely related to the size of the agent, and usually very slow relative to tissue clearance; these limitations typically result in non-homogeneous distribution of

agents within very short distances from the source. In contrast to diffusion, convection-enhanced delivery (CED) uses a pressure gradient established at the tip of an infusion catheter to create bulk flow that ‘pushes’ the drug through the interstitial space [2]. CED can distribute large therapeutic molecules over considerable distances. While CED has been used in conjunction with small molecule drugs [3,4], antibody and other protein-based therapeutics [5], and viral vectors [6,7], we hypothesized that liposome-based agents could be infused via CED into the brain. Liposomes can provide stable encapsulation and delivery of a number of potent anticancer drugs [8]. While current liposomal drugs have been optimized for systemic administration, including stabilized versions with very

prolonged circulation time, liposomes can in principle be engineered to deliver drugs within the CNS. In conjunction with CED, such liposomes may penetrate significantly throughout brain tumor tissue; once the bulk flow is stopped, stabilized liposomes can reside in the interstitial space, followed by release of drug.

In this report, we evaluated the feasibility of combining CED with liposomal delivery, to achieve an efficient approach for targeting anticancer agents to brain tumors.

Materials and methods

Liposome preparation

1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine-5, 5'-disulfonic acid (DiIC₁₈(3)-DS) was obtained from Molecular Probes (Eugene, OR), 1-2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-(methoxy (polyethylene-glycol)-2000) (DSPC-PEG) from Avanti Polar Lipids (Alabaster, AL), and cholesterol (Chol) from Calbiochem (San Diego, CA). DOPC and Chol (molar ratio 3:2), PEG-DSPE (5 mol%) and DiIC₁₈(3)-DS (0.2 mol%) were mixed in chloroform, dried by rotary evaporation, rehydrated by six successive cycles of rapid freezing-thawing and subsequently extruded through polycarbonate filters with defined pore sizes (5×0.2 , $7 \times 0.05 \mu\text{m}$) [9], yielding liposomes of ~90 nm diameter as determined by dynamic light scattering. Further extrusion using $0.03 \mu\text{m}$ polycarbonate filters yielded liposomes of ~40 nm diameter. Liposome concentration was measured by standard phosphate analysis and adjusted to 20 mM phospholipid for all experiments.

For liposomes loaded with rhodamine B disulfonate (Sigma, St. Louis, MO), the fluorescence dye (5 mM) was dissolved in water for rehydration of the dried lipids. After passive loading, the unencapsulated dye was removed using a Sephadex G-75 size exclusion column. For magnetic resonance imaging (MRI) studies liposomes were loaded with gadodiamide (Omniscan®) accordingly after rehydration in 250 mM gadodiamide solution.

Liposomes loaded with gold particles composed of egg-PC/Chol/PEG-DSPE (molar ratio 3:2:0.3) with optional DiIC₁₈(3)-DS co-labeling were prepared by reverse-phase evaporation with gold chloride:citrate in the aqueous phase [10]. After gold particles were formed, unencapsulated free gold chloride and

excess citrate were removed by passing the liposome suspension through a Sephacryl S-400 column (Pharmacia, Piscataway, NJ), yielding liposomes of 120–150 nm.

Animal studies

Institutional approval for all animal studies with mice and rats were obtained at the University of California at San Francisco, and the requirements were followed strictly in each of the studies.

Injection of liposomes containing various labels in normal rat brain

Fifteen adult rats (three per group) received conventional intracranial injections of various probes into the striatum ($5 \mu\text{l}$ per site, $1.5 \mu\text{l}/\text{min}$), including liposomes containing (1) DiIC₁₈(3)-DS, (2) rhodamine B disulfonate, (3) a co-labeled moiety of colloidal gold and DiIC₁₈(3)-DS, or (4) 'free' DiIC₁₈(3)-DS and 'free' rhodamine B disulfonate, both dissolved in PBS but not incorporated into liposomes. All animals were euthanized immediately following liposome infusion (0 h); the brains were cut fresh frozen into $30 \mu\text{m}$ sections using a cryostat. The sections were analyzed by fluorescence microscopy (540/25 nm bandpass filter for excitation, 565 nm long pass filter for emission) for signal from the targeted area and volumes were calculated relatively at the center of injection in comparison to the distribution of DiIC₁₈(3)-DS-liposomes (=100%) using the software package NIH Image (Version 1.62, NIH). For the detection of liposomes loaded with colloidal gold and co-labeled with DiIC₁₈(3)-DS, the gold particles were visualized using a silver-enhancement kit (Amersham, Arlington Heights, IL) followed by direct comparison of fluorescence and phase contrast light microscopy of each slide. Importantly, for fluorescence analysis all camera settings, including exposure time and background correction, were set and kept unchanged to allow direct comparisons.

CED of liposomes in the rodent striatum

Six normal mice received intrastriatal liposome infusions using CED as previously described [11]. In all the animals, the left hemisphere received an acute infusion of very small DiIC₁₈(3)-DS labeled liposomes (40 nm) diluted with PBS (1:1; vol:vol) while the right

hemisphere received the same liposomes diluted with 25% mannitol (1 : 1; vol : vol) for direct comparison. A total of 10 μ l were infused using an ascending infusion rate as follows: 0.2 μ l/min for 10 min; 0.5 μ l/min for 8 min and 0.8 μ l/min for 5 min. Mice were sacrificed either immediately after infusion (0 h) or 48 h later. Tissue sections were cut and analyzed by fluorescence microscopy to evaluate liposome distribution as follows: every 10th section (40 μ m) was examined under fluorescent illumination using grid system. Regions of intense fluorescence were plotted over the grid and area of fluorescence was represented as summation of squares on each side of the brain (measurement units). PBS and mannitol-treated sides were compared for each group using student's *t*-test, $p < 0.005$.

CED of liposomes loaded with gadodiamide for MRI

For MRI studies, six male rats received slow infusions (CED) of liposomes loaded with gadodiamide and co-labeled with DiIC₁₈(3)-DS on the right and 'empty' liposomes on the left side. The coordinates for injection were: AP 0.5 mm, LAT +3 mm, depth 4.5 mm, and a total volume of 20 μ l (0.2 μ l/min for 15 min; 0.5 μ l/min for 10 min and 0.8 μ l/min for 15 min) were injected. Animals were sedated using sodium-pentobarbital (50 mg/kg, i.p.) and placed in a tray that held three animals at a time. Towels were placed around each animal to aid in the positioning and to maintain the body temperature of the animals during the 15 min of scanning. The two paddles of a pelvic phased array coil were placed on the underside and on top of the animal tray. Coronal T1-weighted spoiled-gradient echo (SPGR) images were captured with a Signa 1.5 Tesla machine (General Electric, Waukesha, Wis.). The imaging parameters for the SPGR images were: TR/TE/flip = 40/6 ms/30, FOV = 200 $\times$ 200 mm, matrix = 192 $\times$ 256, NEX = 3, slice thickness = 1.0 mm. The pixel size in the reconstructed images was 0.78 $\times$ 0.78 mm. Images were captured 2 and 24 h after liposome infusion.

CED of liposomes into flank and intracranial U-87 tumor xenograft models

Twenty athymic rats received intrastriatal transplantation of U-87 cells (5×10^6 cells/10 μ l) using the coordinates: AP 0 mm, LAT $\pm$ 3 mm and a depth +6 mm.

Tumor size reached ~ 100 mm³ on days 17–19 and animals were separated into two groups receiving either CED of 40 *versus* 90 nm liposomes. The groups were further divided into two subgroups ($\pm$ co-infusion of mannitol). The total liposome volume infused by CED intratumorally was 20 μ l (0.5 μ l/min for 16 min and 0.8 μ l/min for 15 min).

For studies in large flank tumors, 10 balb/c male nude mice received s.c. flank transplantation of U-87 cells (5×10^6 cells/100 μ l) and were followed for 3–4 weeks until the tumor size reached $\sim 1,000$ mm³. Animals were separated into two groups: liposomes/PBS *versus* liposomes/mannitol 25%. Intratumoral administration of DiIC₁₈(3)-DS labeled liposomes was performed using CED methods as described previously.

All animals were euthanized at 0 or 48 h post-CED and fresh frozen brains, respectively tumors were analyzed by fluorescence microscopy as previously described.

Results

Detection of liposomes containing various markers in the rat brain

To evaluate the distribution of liposomes following administration into the rodent CNS, liposomes containing a series of fluorescent or colorimetric markers were constructed. These studies were undertaken to establish the feasibility of liposome detection in the CNS, and to compare markers as to sensitivity and specificity of liposome localization. Small unilamellar liposomes were prepared and then either 'labeled', by incorporation of a marker as part of the liposomal membrane; or 'loaded', by encapsulation of the marker within the aqueous interior of liposomes, which is analogous to methods used in drug loading. Markers included: (1) liposomes labeled with DiIC₁₈(3)-DS, a fluorescent lipid that can be stably incorporated into liposomal membranes [12]; (2) liposomes loaded with water-soluble rhodamine B disulfonate; (3) liposomes labeled with DiIC₁₈(3)-DS and further loaded with gold particles, a histologic marker that can be visualized in tissue sections [10] (4) 'free' DiIC₁₈(3)-DS dissolved in PBS as a control and (5) 'free' rhodamine B disulfonate dissolved in PBS as a control.

Initial studies were performed to visualize the distribution of liposomes containing these markers following a single intracranial injection directly into the striatum of adult rats; identical detection settings

were used, including camera exposure time and background correction, allowing direct comparisons. Immediately following injection, liposomes labeled with DiIC₁₈(3)-DS were clearly visualized after having diffused around the needle track and into the corpus callosum (Figure 1A). Extent of distribution reached ~2 mm in diameter, and was inhomogeneous. As a control, injection of free DiIC₁₈(3)-DS was performed to exclude the possibility that the observed distribution was due to release of DiIC₁₈(3)-DS from liposomes or exchange with other membranes, rather than true liposomal distribution. Free DiIC₁₈(3)-DS, in fact, was distributed less extensively than liposomal DiIC₁₈(3)-DS (Figure 1B). Image analysis and quantitation of these tissue patterns indicated that the distribution of free DiIC₁₈(3)-DS was only $23 \pm 1.1\%$ that of liposomal DiIC₁₈(3)-DS. This may be due to tendency toward aggregation or membrane interactions of free DiIC₁₈(3)-DS that impeded tissue penetration, but which were ameliorated with liposome incorporation. Next, direct injection of liposomes containing encapsulated rhodamine B disulfonate showed comparable or slightly greater distribution than that of liposomal DiIC₁₈(3)-DS (Figure 1C). This differential was calculated to be $141.4 \pm 25.7\%$. The injection of free rhodamine B disulfonate as a positive control showed significantly greater distribution, and was $219.9 \pm 37.9\%$ that of liposomal DiIC₁₈(3)-DS. This distribution was expected based upon the facile diffusion of this small water-soluble compound. These results indicated that the distribution of liposomes injected into the CNS could be evaluated using labeling with DiIC₁₈(3)-DS or encapsulation of rhodamine B disulfonate, with roughly comparable results. The slightly greater distribution noted with rhodamine B disulfonate encapsulation than with DiIC₁₈(3)-DS-labeling was most likely due to the somewhat greater sensitivity of detection of the respective fluorochromes. Although a low level of leakage of the water-soluble marker out of liposomes cannot be ruled out, the sharp and discrete boundaries of liposomal rhodamine B distribution was readily distinguished from the more gradual gradient observed with injection of free marker.

Finally, liposomes were prepared with both DiIC₁₈(3)-DS-labeling and colloidal gold encapsulation, allowing a direct comparison of fluorescence and histologic markers in the same tissue sections. Injection of these liposomes resulted in comparable distribution patterns for the two markers (Figure 1E,F).

CED of liposomes into the mouse brain

In order to achieve superior tissue penetration of liposomes within the CNS, a CED technique was applied in conjunction with fluorescently labeled liposomes. In normal mouse brains, intrastriatal administration of very small, ~40 nm, liposomes via CED (10 μ l volume) produced an extensive and diffuse fluorescence distribution in the striatum, as well as in subcortical regions and the corpus callosum (Figure 2A). In contrast to direct injection, liposomes infused by CED covered a broad area with much more uniform accumulation. The area of distribution (A_d) was calculated to be 52.5 ± 2.1 measurement units immediately after CED (Figure 2B). At 48 h, A_d was 13.5 ± 3.2 units, indicating a substantial residual distribution of liposomes. To further optimize CED parameters for the administration of liposomes, mannitol was co-infused based upon previous observations that this hyperosmotic solute can enhance convection of other particles [13]. CED of liposomes with 25% mannitol co-infusion produced a significant enhancement in A_d within the brain compared to CED without mannitol. Enhanced distribution was observed immediately after infusion (78.5 ± 5.5 vs. 52.5 ± 2.1 units), and was even more pronounced after 48 h (34.8 ± 7.5 vs. 13.5 ± 3.2 units).

MRI of gadolinium (Gd)-loaded liposomes administered via CED into the rat brain

The distribution of liposomes in the CNS following CED administration was also evaluated by real-time MRI scanning, using liposomal constructs containing paramagnetic agents for MRI contrast effects. For these studies, the MRI contrast agent gadodiamide (Gd-DTPA-BMA) was stably encapsulated into liposomes. Gd-containing liposomes were infused via CED (20 μ l) into the rat striatum, and control liposomes lacking Gd were infused on the contralateral side. Animals were then subjected to MRI scanning, and images were acquired at various time points post-infusion. Consistent with the CNS distribution of liposomes as revealed by fluorescence and histologic markers, CED infusion had distributed Gd-loaded liposomes extensively away from the injection site (Figure 3). Two hours post-infusion, a discrete signal of high intensity was observed within the striatum due to contrast enhancement from the Gd-loaded liposomes. Liposomes had reached a significant cross-sectional area

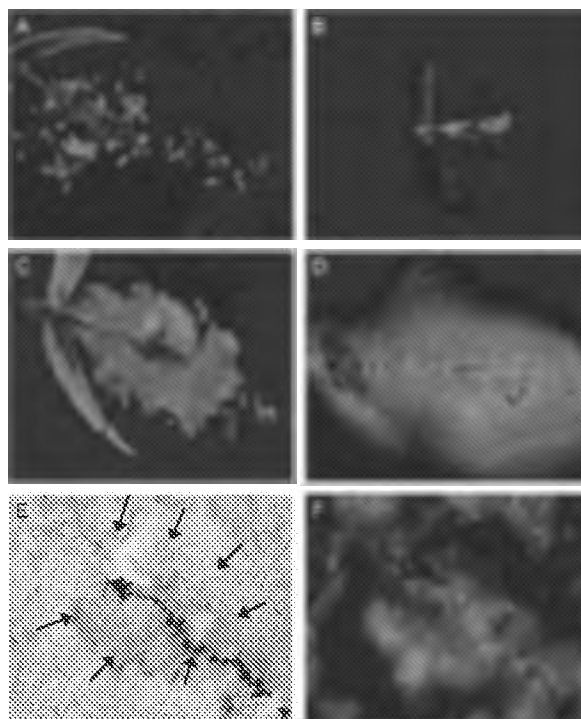


Figure 1. Direct injection of liposomes containing various markers into the rat brain. Fluorescent markers (i.e. lipid DiI₁₈(3)-DS and water-soluble rhodamine B, either dissolved in water or incorporated within liposomes) or liposomes loaded with colloidal gold particles and co-labeled with DiI₁₈(3)-DS were administered into the striatum of normal rats using conventional direct intraparenchymal injections. Fresh frozen brains were sectioned and analyzed by phase contrast and fluorescence microscopy immediately after the liposome administration. Identical detection settings were used, including camera exposure time and background correction. Panels A–D show representative tissue sections (dorsal surface to the left, ventral surface to the right) under fluorescence microscopy to detect the following agents: (A) DiI₁₈(3)-DS liposomes, (B) ‘free’ water-dissolved DiI₁₈(3)-DS, (C) rhodamine B disulfonate loaded liposomes and (D) ‘free’ water-dissolved rhodamine B disulfonate. (E) demonstrates colloidal gold loaded liposomes after silver-enhancement (using phase contrast light microscopy) and (F) shows the identical section under fluorescence microscopy, demonstrating comparable distribution of liposome membrane-bound fluorescent marker and liposomally entrapped gold particles.

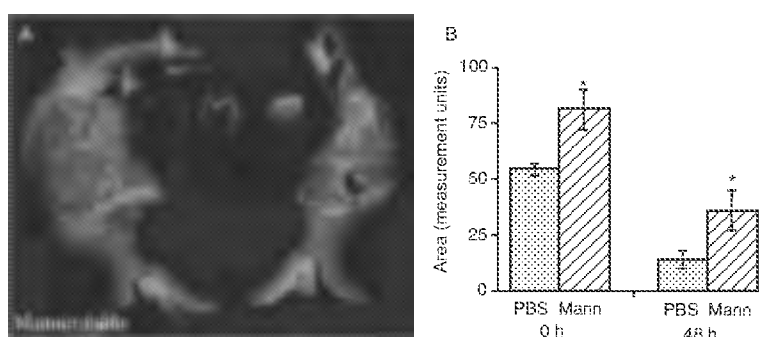


Figure 2. Convection-enhanced delivery of liposomes into the mouse brain. Normal mice ($n = 6$) were injected with fluorescence-labeled liposomes (40 nm) by CED into the striatum bilaterally $\pm$ mannitol and sacrificed at 0 or 48 h. (A) Fluorescence microscopy of liposomes in the infused striatum immediately after CED (0 h), shown as composite coronal sections. Extensive distribution of liposomes was observed in the striatum following CED alone (right), and distribution was further enhanced by co-infusion with 25% mannitol (left). (B) Quantitation of fluorescence distribution. Tissue sections were analyzed for fluorescence over regions of interest, and expressed as area in measurement units $\pm$ SE (* $P < 0.005$, student's t -test comparing PBS and mannitol (Mann) for each time point).



Figure 3. CED of liposomes loaded with gadodiamide (Gd) for MRI. Liposomes loaded with Gd were infused into the striatum of normal rats using CED. After 2 and 24 h, coronal images (1 mm cuts) were acquired by MRI. The scan on the left ('Control') shows a rat brain prior to any treatment. The scan in the center ('2 hours') shows a rat brain at that timepoint after infusion of Gd-encapsulated liposomes into the right striatum, and control liposomes lacking Gd into the contralateral or left striatum. Two hours post-infusion, a strong and discrete signal from Gd-loaded liposomes was observed. The scan on the right ('24 hours') shows a rat brain at that timepoint after infusion of Gd-encapsulated liposomes into the right striatum, and control liposomes lacking Gd into the left striatum. Twenty-four hours post-infusion, a diffuse signal encompassing a broader area was observed. At both timepoints (2 and 24 h) control liposomes lacking Gd showed minimal enhancement. Scale on the right represents size in centimeter.

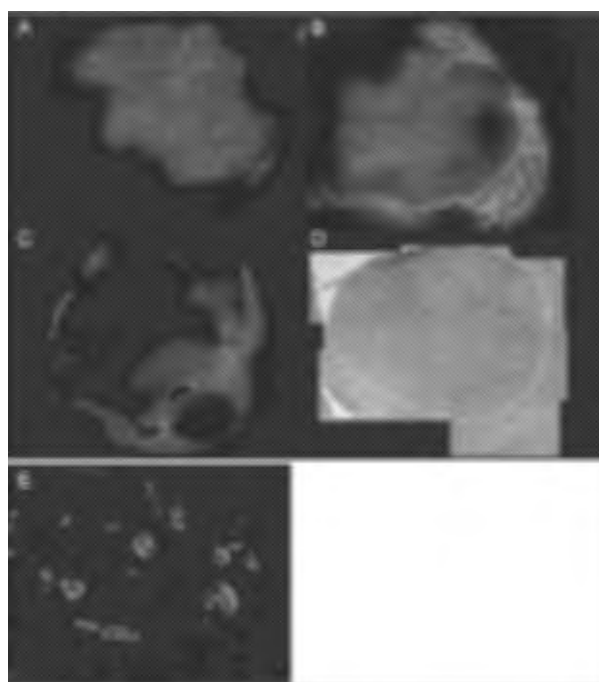


Figure 4. CED of liposomes into U-87 glioma xenografts in intracranial and flank tumor models. Nude rats bearing intracranial U-87 glioma xenografts were infused with fluorescence DiIC₁₈(3)-DS-labeled liposomes of either 40 or 90 nm in size via CED $\pm$ mannitol. Fluorescence micrographs indicated liposome distribution immediately after CED, shown as composite sections of tumor and surrounding tissue. (A) CED of 40 nm liposomes resulted in extensive distribution within the tumor. (B) CED plus mannitol significantly enhanced CNS distribution of 40 nm liposomes throughout tumor and beyond, including adjoining normal brain tissue. (C) CED of 90 nm liposomes with mannitol resulted in partial distribution within the tumor, >50% overall in multiple tumor sections. (D) Histological section of a representative U-87 glioma xenograft. Nude mice bearing U-87 flank tumors were also treated with DiIC₁₈(3)-DS-labeled liposomes (90 nm) infused via CED. (E) Fluorescence micrograph (high magnification) shows perivascular accumulation of liposomes after 48 h in the periphery of a large U-87 tumor.

at this early time point. The contralateral striatum showed no enhancement from the control liposomes lacking the MRI contrast agent, confirming that Gd-encapsulation was responsible for the contrast effect. Twenty-four hours post-infusion, Gd-loaded liposomes were observed to have diffusely distributed over the striatum, including penetration beyond external capsule and contralateral structures. DiIC₁₈(3)-DS-labeled liposomes administered under the same conditions showed a comparable distribution pattern in necropsied brains (data not shown). No toxic effects based on clinical and histopathological examination were observed in rats receiving Gd-loaded liposomes.

CED of liposomes into intracranial and flank U-87 tumor xenograft models

Liposomes were infused via CED into tumor tissue in two different glioma xenograft models in rodent hosts. Tumors consisted of U-87 glioma cells were implanted orthotopically in the brains of nude rats or s.c. into the flank of nude mice; in the intracranial xenograft model tumors were allowed to grow to $\sim 100 \text{ mm}^3$ (days 17–19) and in the flank xenograft model to $\sim 1,000 \text{ mm}^3$ (days 25–35). Liposomes of either ~ 90 or $\sim 40 \text{ nm}$ were labeled with DiIC₁₈(3)-DS, and infused into these relatively large tumors via CED $\pm$ mannitol. Both small (90 nm) and very small (40 nm) liposomes were selected since these represent the approximate sizes of the two liposomal anthracyclines that are currently approved for clinical use in the US, pegylated liposomal doxorubicin (Doxil[®]/Caelyx[®]) and liposomal daunorubicin (DaunoXome[®]). Again, identical detection settings were used, including camera exposure time and background correction, allowing direct comparisons.

In the orthotopic brain tumor model, the small 40 nm liposomes infused via CED without mannitol achieved distribution over nearly all the tumor tissue (Figure 4A). The addition of mannitol to CED further enhanced distribution, as liposomes had accumulated throughout all sections of tumor, and had further penetrated beyond the tumor boundary into adjacent normal brain tissue (Figure 4B). For the 90 nm liposomes, CED with mannitol resulted in excellent tumor penetration ($>50\%$ of total area evaluated), which also included extension into surrounding normal brain (Figure 4C). Distribution was somewhat less extensive, however, than with the 40 nm liposomes. Because this analysis of tissue fluorescence does not allow precise quantitation of liposome accumulation, it is not clear whether the

increased intensity in the periphery of tumors reflects greater liposome accumulation or a combination of accumulation and tissue effects; e.g., the peripheral zone features increased tissue density from compression of white matter tracts. In any case, these data indicate that CED produced excellent tumor penetration of liposomes that reached the outer periphery (or beyond) of large tumors. Such distribution is very promising for brain tumor treatment, since the interface frequently contains infiltrating tumor cells.

Convection-enhanced delivery infusion of liposomes into s.c. flank tumors also resulted in extensive tissue penetration. Interestingly, a prominent perivascular accumulation of liposomes was observed, which resulted in a less homogeneously diffuse pattern than with infusion into the orthotopic brain tumors (Figure 4E, higher magnification). In flank tumors, CED-infused liposomes had accumulated in areas that surrounding blood vessels, and especially in the outer periphery of large tumors. A possible explanation may include the ability of liposomes infused into tissue parenchyma to enter blood vessels, which may be facilitated by the abnormal structural features of tumor vessels during angiogenesis; liposomes may then undergo brief intravascular transit within the tumor, followed by extravasation, again facilitated by the hyperpermeability of tumor vessels. Liposome extravasation from tumor vessels, resulting in perivascular accumulation, often occurs following systemic administration [14,15]. On the other hand, minimal fluorescence was detected within blood vessels, suggesting that liposomes did not enter vessels to a significant degree. This makes it more likely that liposomes were preferentially channeled through the perivascular space by the process of CED. This is consistent with the perivascular space in tumors representing a path of low resistance to bulk flow produced by CED. It is not clear why this process was more operative in flank as opposed to brain tumors of U-87 glioma, however, greater tissue density of the flank tumors may have contributed to more efficient perivascular distribution of liposomes following CED.

Discussion

We have studied the CNS distribution produced by local-regional administration of liposomes containing various markers, including attached or encapsulated fluorochromes, encapsulated gold particles and encapsulated gadodiamide for MRI contrast. Results are presented using image analysis-based detection of

these markers. We did not attempt to quantify levels of fluorescence in tissues, which would require non-validated quantification procedures; instead, demarcations between fluorescence and non-infused tissue were used. These results indicate that application of CED to liposome agents achieved extensive and efficient distribution within normal rodent brains, s.c. flank tumor xenografts and intracranial tumor xenografts. To our knowledge, this is the first report of CED infusion of liposomes into the CNS.

Liposomal delivery systems offer a number of potential advantages for brain tumor treatment. Liposomes have been used as a systemic vehicle for a number of anticancer agents, and can provide improved pharmacologic properties including pharmacokinetics and biodistribution. Liposomes capable of long circulation can in fact accumulate preferentially in tumors due to enhanced extravasation from tumor vessels. The best-known examples are the highly stabilized liposomes developed for anthracycline delivery, which have entered standard clinical use in the treatment of Kaposi's sarcoma, ovarian cancer and other tumors (for review, see [8]). Systemic treatment with liposomal anthracyclines also appears capable of increased accumulation and efficacy in brain tumor models [16], and early clinical trials in glioblastoma and metastatic brain tumors have been performed [17]. Hence, liposomes containing potent anticancer drugs may be able to reach brain tumors via systemic administration, or based on this report can be distributed into brain tumors via CED.

Liposomes represent ideal agents for CED administration. First, stabilized liposomes composed of neutral phospholipids and other structural features display much reduced reactivity with proteins and membranes in their environment. CED infusion of such liposomes resulted in extensive penetration throughout normal brain and brain tumor tissue. In contrast, our previous studies have indicated that the distribution of certain proteins and viral vectors administered by CED was impeded by interactions with proteoglycans, which could be counteracted by co-infusion with heparin [7,18]. Second, the extensive distribution of infused liposomes within the CNS persisted for at least 48 h post-treatment. The very slow clearance of liposomes from tissues is favorable for sustained drug release from their sites of distribution. Third, the versatility of liposomes facilitated optimization for use with CED. Parameters such as liposome size and co-administration of mannitol were particularly evaluated, resulting in further increases in delivery efficiency. While both small (90 nm) and very small (40 nm)

liposomes achieved substantial penetration after CED, the pattern was more pronounced with the very small liposomes. Mannitol, which can act as a hyperosmotic agent to draw fluid from cells or the vasculature into the interstitial space [13], was able to increase the convective effect on liposomes. It is plausible that mannitol co-infusion increased the size of channels through which liposomes could transit, resulting in increased accessibility even in dense tissues.

Finally, liposomes can be used to deliver various agents, including those present in existing liposomal drug formulations, new liposome versions of chemotherapy drugs, and liposome-based constructs of novel agents. In the case of standard drugs, liposome delivery in conjunction with CED may significantly improve therapeutic index, either by increasing efficacy due to greater tumor exposure to drug, and/or by decreasing toxicity due to lesser systemic exposure. With novel agents, liposome formulations may enable clinical use of agents with otherwise limiting pharmacologic problems. Furthermore, we and others have developed immunoliposome agents, in which antibody-based tumor targeting is combined with liposomal drug delivery [19]. Immunoliposomes, such as those directed against glioma-associated antigens [20], may also be suitable for CED administration in brain tumor treatment, thereby combining anatomic and molecular targeting.

Another application of liposome technologies is delivery of probes or reporters as well as drugs. As described here, liposomes were loaded with gadodiamide for MRI detection following CED in rodent brains. This proved to be a useful non-invasive method to track infused liposomes in animal models. In principle, liposomal contrast agents can be developed for further uses, including real-time monitoring of liposome distribution in patients.

We conclude that CED of liposomes in the CNS is a feasible approach, and offers a promising strategy for targeting therapeutic agents to brain tumors. This approach may be useful to administer liposomes containing various anticancer drugs, liposomes containing probes or reporters for imaging, or immunoliposomes or other molecularly targeted liposome constructs.

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CPT-11: A New Derivative of Camptothecin for the Treatment of Refractory or Relapsed Small-Cell Lung Cancer

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Purpose: To evaluate the activity of CPT-11, which is a new derivative of camptothecin, against refractory or relapsed small-cell lung cancer (SCLC).

Patients and Methods: Sixteen patients with refractory or relapsed SCLC were entered onto a prospective, non-randomized, single-institution phase II trial. All 16 patients had been pretreated heavily with some form of cisplatin-based combination chemotherapy. Five patients had received previous chemotherapy with cisplatin, vincristine, doxorubicin, and etoposide (CODE) as an induction therapy. Six patients had been treated with concurrent cisplatin and etoposide plus chest x-ray. The median time off chemotherapy was 7.3 months (range, 1.9 to 15.1 months). Patients were treated with a CPT-11 starting dose of 100 mg/m² body surface given as a 90-minute

intravenous (IV) infusion every week with subsequent doses based on toxicity. Fifteen patients were assessable for toxicity, response, and survival.

Results: Seven patients (47%; 95% confidence limits for an overall response rate, 21.4% to 71.9%) responded to CPT-11 with a median duration of response of 58 days. The major toxicities were myelosuppression (predominantly leukopenia), diarrhea, and pulmonary toxicity.

Conclusion: CPT-11 is an active agent against refractory or relapsed SCLC and deserves to be studied more closely as both a single agent and in combination with other drugs to treat patients with SCLC.

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UNLIKE OTHER TYPES of lung cancer, small-cell lung cancer (SCLC) is sensitive to chemotherapy or radiation therapy.¹ Nonetheless, after apparently successful induction therapy, most patients show a relapse within 2 years because of the emergence of drug-resistant cancer cells during the induction therapy or the existence of such cells before chemotherapy. Therefore, cures are rare.²⁻⁷ Second-line chemotherapy in SCLC usually yields disappointing results.⁸ As a result, to achieve a more effective control of SCLC, we need new drugs that are effective for patients who have not responded to standard treatment, and who may have multidrug-resistant tumors.

Camptothecin, a plant alkaloid extract from *Camptotheca acuminata*,⁹ is a potent inhibitor of DNA topoisomerase I^{10,11} and has been shown to have a strong antitumor activity in vitro^{12,13} and in experimental animal tumor systems.^{9,14} However, it has not been used clinically because of its low response rates in clinical trials and its high toxicity.¹⁵⁻¹⁸ 7-ethyl-10-(4-[1-piperidino]-1-piperidino) carbonyloxycamptothecin (CPT-11) had been semisynthesized as a water-soluble derivative of camptothecin in an attempt to reduce its toxicity and to improve its therapeutic efficacy. CPT-11 exhibits a marked antitumor activity against not only a broad spectrum of experimental tumor models^{19,20} but against pleiotropically drug-resistant tumor-cell lines.²¹ An initial phase I study of CPT-11 by a single intravenous (IV) administration showed that the dose-limiting toxicity was severe myelosuppression.²² During this phase I study, objective responses were observed in previously treated patients with sarcoma of the stomach, mela-

noma, colon cancer, and non-SCLC. CPT-11 also has been found to be effective against refractory leukemia and lymphoma.²³ This unusual activity against several malignancies that generally were considered to be unresponsive to cytotoxic drugs was the basis for starting a phase II trial on refractory or relapsed SCLC.

PATIENTS AND METHODS

Patient Selection

Patients with refractory or relapsed SCLC who were referred to the Osaka Prefectural Habikino Hospital participated in the study of treatment with CPT-11. The criteria for study entry were (1) histologic or cytologic proof of SCLC with documented progression; (2) bidimensionally measurable disease; (3) no chemotherapy or radiotherapy within 4 weeks of study entry; (4) life expectancy at least 8 weeks; (5) performance status 2 or more by the Eastern Cooperative Oncology Group scale; (6) age less than 79 years; (7) adequate bone marrow function (leukocyte count $\geq 3,500/\mu\text{L}$, and platelet count $\geq 100,000/\mu\text{L}$), hepatic function (bilirubin level ≤ 1.5 mg/dL), transaminases ≤ 2 times the upper limit of normal), and renal function (creatinine level ≤ 1.4 mg/dL); (8) no medical problems that were sufficiently severe to prevent compli-

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ance with the study requirements, and no preexisting interstitial pulmonary disease; and (9) written informed consent of the patient. Patients were required to be free of any active concomitant malignant disease. Patients were not eligible if they showed allergic reactions to prick skin tests with CPT-11.

Between May 1990 and May 1991, 16 patients were entered onto the trial. One patient was ineligible for the study because of liver dysfunction. Fifteen patients were assessable for toxicity and survival. Fourteen patients were assessable for response. One patient was not assessed for response because he died on day 23 before an adequate response assessment could be performed. However, in this case, the treatment was considered to be a failure. He was not neutropenic at the time of his death.

The characteristics of the eligible patients treated during this trial are listed in Table 1. Three patients were women and 12 were men; their median age was 63 years (range, 38 to 76). Five (33%) patients exhibited limited disease (LD) and 10 (67%) exhibited extensive disease (ED) at CPT-11 treatment (at the time of relapse). The most common sites for metastases were the liver (four patients, 27%), adrenal gland (three patients, 20%), extrathoracic lymph node (three patients, 20%), and brain (three patients, 20%); other sites included the lungs (two patients, 13%), bones (one patient, 7%), and subcutaneous nodule (one patient, 7%). All 15 patients had been pretreated heavily with some form of cisplatin-based chemotherapeutic regimens; concurrent cisplatin and etoposide plus chest x-ray²⁷ had been used in six patients with LD, and cisplatin, vincristine, doxorubicin, and etoposide (CODE)²⁸ had been intended for five patients with ED. Ten of these patients had received thoracic irradiation after chemotherapy or simultaneously. Only one patient proved to be refractory to chemotherapy as defined by either tumor growth during chemotherapy or the recurrence of a tumor within 3 months after the completion of chemotherapy. Details of prior exposure to chemotherapy also are listed in Table 1.

Treatment Schedule

Based on our phase I data,²⁴ CPT-11 was dissolved in 500 mL of normal saline and was given at a dose of 100 mg/m² as a 90-minute IV infusion every week. CPT-11 was provided by Yakult Honsha Co, Ltd (Tokyo, Japan) and Daiichi Pharmaceutical Co, Ltd (Tokyo, Japan). If therapeutic effects were observed and the toxicities proved to be tolerable, treatment was continued. Premedication to prevent nausea and/or vomiting or diarrhea was not scheduled during this study. The dose of CPT-11 was adjusted based on the hematologic values that were obtained on the day when the dose was scheduled according to the following methods: (1) if the WBC count was $\geq 3,000/\mu\text{L}$, the treatment was continued with a full dose (100 mg/m²); (2) if the WBC count was 2,000/ μL to 3,000/ μL , the treatment was postponed until the leukopenia subsided, and then was restarted with a full dose; (3) if the WBC count was 1,000/ μL to 2,000/ μL , the treatment was postponed, and was restarted with 80 mg/m²; and (4) if the WBC count was less than 1,000/ μL or other grade 4 toxicities occurred, the treatment was discontinued.

Evaluation

Patients were evaluated to determine the stage of disease at the time of disease progression or at the time of relapse by a complete medical history and a physical examination, chest x-ray, bone scintiscan, bone marrow aspiration, computed tomography of the

Table 1. Characteristics of Eligible Patients Treated With CPT-11

Total no. of patients	15
Sex	
Male	12
Female	3
Age (years), median	63
Range	38-76
Performance status*	
0	1
1	5
2	9
Weight loss (%)	
≤ 10	15
> 10	0
Stage at initial diagnosis	
LD	8
ED	7
Stage at CPT-11 start (at relapse)	
LD	5
ED	10
Sites of metastases	
Liver	4
Brain	3
Lymph node	3
Adrenal gland	3
Lung	2
Bone	1
Subcutaneous nodule	1
Prior therapy	
Chemotherapy alone	5
Chemotherapy and chest irradiation	10
Induction chemotherapy	
PE + RT	6
CODE	5
PE	2
CAV/PE	1
Other	1
Response to prior chemotherapy	
CR	7
PR	7
NC	1
PD	0
Time from diagnosis (months)	
≤ 10	7
> 10	8
Chemotherapy-free interval (days)	
≤ 90	1
> 90	14

Abbreviations: PE, cisplatin and etoposide; RT, radiotherapy; CAV/PE, cyclophosphamide, doxorubicin, and vincristine alternating with cisplatin and etoposide; NC, no change; PD, progression of disease.

*Eastern Cooperative Oncology Group criteria.

head, chest, and abdomen, and fiberoptic bronchoscopy. LD was defined as disease confined to one hemithorax, which included bilateral mediastinal and bilateral supraclavicular nodes; any involvement beyond these confines was defined as ED. Before the first course, each patient was assessed by a complete blood cell (CBC) count, which included a differential count and a platelet count, and by serum chemistry for renal and hepatic functions,

electrolytes, and urinalysis. The assessments of CBC count, serum chemistry, electrolytes, urinalysis, and chest x-ray were repeated at least once a week after the initial assessment. Other appropriate investigations were repeated as necessary to evaluate the sites of marker lesions. The eligibility, assessability, and response of each patient were assessed by extramural reviewers. Tumor responses and drug toxicity were classified in accordance with the World Health Organization (WHO) criteria,²⁵ except that the duration of response was calculated from the day of the observed response until its progression. The duration of survival was determined as the number of days from the start of CPT-11 until October 10, 1991.

The Kaplan-Meier method was used to derive the survival curve.²⁶ The primary end point of this study was a response rate.

RESULTS

Response to Therapy and Survival

Although there were no complete responses (CR) in any of the 15 patients who were assessable, a partial response (PR) was obtained in seven (47%) patients (95% confidence intervals [CI] for an overall response rate, 21.4% to 71.9%), with a median duration of response of 58 days (range, 28 to 156); seven showed no change, and one patient exhibited disease progression. A PR was obtained in three (60%) of five patients with LD, and in four (40%) of 10 patients with ED. Responses were observed usually at a median of 23 days (range, 13 to 28) after the start of CPT-11 treatment and occurred at all sites, which included the brain (two of two).

By the time of the analysis, 11 (73%) of 15 patients that had been assessable previously had died. The median survival time from the start of CPT-11 therapy for the whole group of 15 eligible patients was 187 days.

Toxicity

A total of 102 cycles of weekly CPT-11 were administered (median, seven per patient; range, two to 13). Subjectively, the treatment was tolerated well. Table 2 lists the maximum toxicities that were experienced during treatment. The most frequent toxicity observed was myelosuppression that primarily affected leukocytes; 33% and 0% of patients had severe leukopenia and thrombocytopenia (WHO grade 3 or 4), respectively. Planned dosages remained unchanged in seven patients, were held for more than 14 days in seven patients, and were reduced and delayed in one patient at any time during the trial mostly because of marrow toxicity and diarrhea. No episode of fever during the period of leukopenia was observed. Transient eosinophilia in 10% or more of the leukocytes was observed in five (33%) patients at a median of 21 days (range, 20 to

Table 2. Worst Toxicity by 15 Patients on CPT-11

Toxicity	WHO Grade				No. of Patients With Grade $\geq$ 3	%
	1	2	3	4		
Leukopenia	4	5	4	1	5	33
Thrombocytopenia	0	1	0	0	0	0
Anemia	6	1	3	0	3	20
Nausea/vomiting	5	2	2	0	2	13
Diarrhea	7	3	1	0	1	7
Constipation	1	0	0	1	1	7
Alopecia*	2	2	0	0	0	0
Pulmonary toxicity	0	0	1	1	2	13
ECG abnormality	0	1	0	0	0	0
Fever	0	0	0	0	0	0
Elevation of serum creatinine	0	0	0	0	0	0
Elevation of transaminases	2	0	0	0	0	0

*N = 14.

21 days) after the start of CPT-11. Twenty percent had WHO grade 3 anemia.

Gastrointestinal toxicity was another prominent toxic effect of this treatment. A greater than grade 2 nausea and vomiting occurred in two (13%) patients. Diarrhea was observed in one (7%) patient, and was resolved 18 days after the end of CPT-11 treatment. Furthermore, one (7%) patient experienced grade 4 paralytic ileus, which was severe enough to stop CPT-11 administration after four weekly doses. Some instances of alopecia and transient atrial fibrillation were observed but never became a problem. No toxicity was observed in the urinary bladder, kidney, or liver, and no allergic reactions to CPT-11 administration were encountered.

Grade 3 or 4 pulmonary toxicity occurred after the administration of five and seven doses of CPT-11 in two (13%) patients. These two patients showed a diffuse reticulonodular pattern that was visible on the chest roentgenogram. At the same time, they experienced dyspnea on exertion and high fever ($> 38^{\circ}\text{C}$).

In one patient, pneumonitis responded well to steroid therapy. However, in another patient, pneumonitis did not subside with steroid therapy. A transbronchial biopsy was performed before treatment with steroids began in the patient. Forceps biopsy specimens showed interstitial edema associated with fibroblastic proliferation and lymphoid cell infiltration, and fibrinous exudate formed hyaline-like membrane in several air spaces. Most of the alveolar-lining cells were enlarged and atypically reacted without any evidence of a viral infection, such as an intranuclear or intracytoplasmic inclusion body. These histopathologic findings could have been indications of a drug-induced change. The patient subsequently died from progressive respiratory insufficiency and was considered to have had a treatment-related death.

DISCUSSION

This phase II study indicates that CPT-11 is an active single agent for treating refractory or relapsed SCLC as gauged by a response rate of 47% (95% CI, 21.4% to 71.9%) in 15 patients who were assessable. The extraordinary high response rate is remarkable. Because patients who were pretreated with aggressive chemotherapy have become resistant not only to those drugs but to a number of other drugs as well, a new drug tested in these patients would have to be more active than the first-line therapy to overcome drug resistance, or would have to be at least noncrossresistant to demonstrate activity. The response rate of 47% obtained with CPT-11 far exceeds the usual threshold of a 20% response rate for the determination of activity in previously untreated patients,^{29,30} and favorably compares with the 40% to 50% response rates that are reported for a combination of etoposide and cisplatin, which now is considered to be the most effective salvage chemotherapy.³¹⁻³⁴ Murray et al³⁵ reported that the potential of either cisplatin and etoposide or cyclophosphamide, doxorubicin, and vincristine regimens as salvage chemotherapy for patients with a relapse after intensive weekly CODE chemotherapy has been disappointing (only one PR in 10 attempts). However, a PR was documented in three of five patients who had been treated with CODE in this trial, which suggests that there is a lack of crossresistance between CPT-11 and the cisplatin-containing regimens that are presently available. CPT-11 is one of the potent inhibitors of topoisomerase I that is under extensive clinical evaluation, whereas cisplatin exerts its action through the involvement of the alkylation of DNA. Therefore, the difference in the mechanisms of action between CPT-11, cisplatin, and other drugs currently available may explain this partial noncrossresistance. Another inhibitor of topoisomerase I, topotecan, also is under current clinical investigation by the National Cancer Institute.

The toxicity profile noted in this trial is similar to that previously described in our phase I and phase II studies,^{24,36} with leukopenia and diarrhea as dose-limiting toxicities. All side effects except one case of pneumonitis

were manageable. The relatively low overall myelotoxicity with only one patient who experienced a WHO grade 4 toxicity and no episode of neutropenic fever observed in heavily pretreated patients (Table 2), mainly was a result of the decision to delay the CPT-11 doses when the WBC count was less than 3,000/ μ L. However, there was marked interpatient variability in the degree of these toxicities. Higher doses of CPT-11 seem to yield better response rates. Future trials with CPT-11 should consider the use of the recombinant human granulocyte colony-stimulating factor to determine whether this offers adequate protection against the neutropenic toxicity CPT-11 causes, as neutropenia is the major hematologic side effect of this drug. Although infrequent and sporadic in appearance, the severity of pulmonary toxicity in two (13%) patients suggests that this drug-induced pneumonitis may also be dose-limiting. Similar pulmonary toxicity on this schedule has been noted in 6% of all patients in a previous phase I study,²⁴ and in 8% of patients in a phase II study conducted on non-SCLC.³⁶

In conclusion, the weekly schedule of CPT-11 100 mg/m² is an active treatment for refractory or relapsed SCLC, although a more precise estimate of the degree of activity will require additional trials. The extraordinarily high response rate of 47% observed in heavily pretreated patients may be explained by the different CPT-11 mechanisms of action from presently available cytotoxic agents used in treating this disease. Given the greater activity in this schedule, further studies of this agent, both as a single agent and in combination with other drugs, in previously treated or untreated patients with SCLC, are warranted.

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December 19, 2012

Merrimack Pharmaceuticals Initiates Cross-Tumor Study to Investigate Potential Predictive Response Markers for a Developmental Nanotherapeutic Chemotherapy

Translational Study Examines Tissue and Imaging Based Biomarkers Across Indications

CAMBRIDGE, Mass., Dec. 19, 2012 (GLOBE NEWSWIRE) -- Merrimack Pharmaceuticals, Inc. (Nasdaq:MACK) today announced enrollment of its first patient in a cross indication translational study. The study is designed to identify predictive biomarkers associated with the novel compound MM-398 in advanced colorectal, lung and triple-negative breast cancers. Specifically, it aims to establish the feasibility of collecting specialized magnetic resonance-based images (MRI) and tissue-based biomarkers for the purpose of estimating drug delivery to the tumor and patient response to MM-398.

A stable, nanotherapeutic encapsulation of the chemotherapy irinotecan, MM-398 is designed to optimize the delivery of irinotecan by increasing levels of the drug deposited in the tumor, extending circulation and thereby enabling preferential activation of the therapy within the tumor. Preclinical research has shown that the length of time MM-398, specifically the active component, SN38, remains within tumors directly correlates with tumor response. Another key to MM-398's activity may be the number of macrophages within a tumor, which have been shown to preferentially internalize liposomes and release their contents.

"Our research indicates tumor micro-environments across these clinical indications allow for the deposition of large particles, such as nanotherapeutics. They also have high levels of tumor-associated macrophages, which means MM-398 can be broken down and release increased levels of chemotherapy to the adjacent cancer cells," said Ulrik Nielsen, Co-Founder and Chief Scientific Officer of Merrimack. "We believe this study is the first of its kind to combine the use of both imaging and biomarker diagnostic approaches and we hope that the insights we gain from this study will help to predict response to MM-398 in these hard-to-treat tumors."

The cross indication translational study will focus on patients that have progressed following standard of care therapy for colorectal, lung and triple-negative breast cancers. The study is taking place at Scottsdale Healthcare Medical Center in Arizona.

About MM-398

Merrimack initiated a pivotal Phase 3 clinical trial of MM-398 for the treatment of patients with metastatic pancreatic cancer who have previously failed treatment with gemcitabine. The trial, called NAPOLI-1 (NAnoliPOsomaL Irinotecan), is a global, randomized, open label Phase 3 study. NAPOLI-1 calls for a total enrollment of 405 patients equally randomized across the three arms: MM-398 as a monotherapy and MM-398 in combination with 5-FU/LV, compared with the shared control arm of 5-FU/LV. Merrimack believes that MM-398 has potential uses in a number of other indications, including colorectal cancer, lung cancer, gastric cancer and glioma. In addition to NAPOLI-1, there are ongoing Phase 1 and Phase 2 clinical trials of MM-398 in glioma and colorectal cancer. MM-398 has been tested in several clinical trials, including a Phase 2 single agent study of MM-398 in metastatic pancreatic cancer and an ongoing Phase 2 study of MM-398 in combination with 5-FU and leucovorin in patients with metastatic colorectal cancer.

In July 2011, the U.S. Food and Drug Administration (FDA) granted MM-398 orphan drug designation for the treatment of pancreatic cancer. Merrimack holds the development and commercialization rights to MM-398 worldwide, with the exception of Taiwan. Merrimack has licensed the Taiwanese commercialization rights to PharmaEngine, Inc. (Taipei), which conducted previous studies of MM-398 under the designation PEP02.

About Merrimack Pharmaceuticals, Inc.

Merrimack Pharmaceuticals is a biopharmaceutical company discovering, developing and preparing to commercialize innovative medicines paired with companion diagnostics for the treatment of serious diseases, with an initial focus on cancer. Merrimack applies Network Biology, its proprietary systems biology-based approach to biomedical research, throughout the research and development process. Merrimack currently has six targeted therapeutic oncology candidates in clinical development.

Forward-Looking Statement

To the extent that statements contained in this press release are not descriptions of historical facts, they are forward-looking

CSPE Exhibit 1095

statements reflecting the current beliefs and expectations of management made pursuant to the safe harbor provisions of the Private Securities Litigation Reform Act of 1995, as amended. Forward-looking statements include any statements about Merrimack's strategy, future operations, future financial position and future expectations and plans and prospects for Merrimack, and any other statements containing the words "anticipate," "believe," "estimate," "expect," "intend," "may," "plan," "predict," "project," "target," "potential," "will," "would," "could," "should," "continue," and similar expressions. In this press release, Merrimack's forward-looking statements include statements about the potential effectiveness of its drug candidates in new indications, the safety and tolerability of its drug candidates and its ability to translate clinical and pre-clinical data into future clinical success. Such forward-looking statements involve substantial risks and uncertainties that could cause Merrimack's clinical development programs, future results, performance or achievements to differ significantly from those expressed or implied by the forward-looking statements. Such risks and uncertainties include, among others, the uncertainties inherent in the initiation of future clinical trials, availability of data from ongoing clinical trials, expectations for regulatory approvals, development progress of Merrimack's companion diagnostics and other matters that could affect the availability or commercial potential of Merrimack's drug candidates or companion diagnostics. Merrimack undertakes no obligation to update or revise any forward-looking statements. Forward-looking statements should not be relied upon as representing Merrimack's views as of any date subsequent to the date hereof. For a further description of the risks and uncertainties that could cause actual results to differ from those expressed in these forward-looking statements, as well as risks relating to Merrimack's business in general, see the "Risk Factors" section of Merrimack's Quarterly Report on Form 10-Q filed with the Securities and Exchange Commission (SEC) on November 14, 2012 and other reports Merrimack files with the SEC.

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Source: Merrimack Pharmaceuticals

News Provided by Acquire Media

Liposomal Irinotecan: Formulation Development and Therapeutic Assessment in Murine Xenograft Models of Colorectal Cancer

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ABSTRACT

Purpose: The purpose is to demonstrate whether an appropriately designed liposomal formulation of irinotecan is effective in treating mice with liver-localized colorectal carcinomas.

Experimental Design: Irinotecan was encapsulated in 1,2-distearoyl-*sn*-glycero-3-phosphocholine/cholesterol (55:45 molar ratio) liposomes using an ionophore (A23187)-generated transmembrane proton gradient. This formulation was evaluated *in vivo* by measuring plasma elimination of liposomal lipid and drug after i.v. administration. Therapeutic activity was determined in SCID/Rag-2M mice bearing s.c. LS180 tumors or orthotopic LS174T colorectal metastases.

Results: Drug elimination from the plasma was significantly reduced when irinotecan was administered in the liposomal formulation. At 1 hour after i.v. administration, circulating levels of the liposomal drug were 100-fold greater than that of irinotecan given at the same dose. High-performance liquid chromatographic analysis of plasma samples indicated that liposomal irinotecan was protected from inactivating hydrolysis to the carboxylate form. This formulation exhibited substantially improved therapeutic effects. For the LS180 solid tumor model, it was shown that after a single injection of liposomal irinotecan at 50 mg/kg, the time to progress to a 400-mg tumor was 34 days (as compared with 22 days for animals treated with free drug at an equivalent dose). In the model of colorectal liver metastases (LS174T), a median survival time of 79 days

was observed after treatment with liposomal irinotecan (50 mg/kg, given every 4 days for a total of three doses). Saline and free drug treated mice survived for 34 and 53 days, respectively.

Conclusions: These results illustrate that liposomal encapsulation can substantially enhance the therapeutic activity of irinotecan and emphasize the potential for using liposomal irinotecan to treat liver metastases.

INTRODUCTION

The liver is the primary site of blood borne metastases from cancers of several different origins, including carcinomas of the lung and breast. However, the liver is especially susceptible to metastases derived from colorectal adenocarcinomas. These metastases extravasate from the primary tumor site in the colon and access the liver directly via the portal circulation. Consequently, unlike secondary sites originating from gastric and pancreatic neoplasms, the liver is often the sole site of metastases for colorectal carcinoma. As many as 70% of patients with colorectal cancer will present with (synchronous) liver metastases at the time of their primary diagnosis or develop liver metastases (metachronous) as their disease progresses (1).

The management of liver-localized tumors is challenging. Despite overall 5-year survival rates of only 25 to 40%, surgical resection remains the treatment of choice for appropriately selected patients (2, 3). The most common therapeutic modality used in patients presenting with colorectal hepatic metastases is systemic chemotherapy. This palliative approach almost exclusively uses 5-fluorouracil-based treatment regimes, which yield responses in the range of 20 to 30%. However, most of these patients later develop resistance to these cytotoxic agents.

The emergence of irinotecan has had a major impact on chemotherapeutic regimens used to treat colorectal cancer. Irinotecan has demonstrated considerable therapeutic activity even in refractory disease. It is now combined with 5-fluorouracil and leucovorin for the treatment of advanced colorectal carcinoma. Irinotecan is a water-soluble analogue of the natural alkaloid camptothecin. Camptothecins act during the S-phase of DNA replication by stabilizing the complex formed between topoisomerase I and DNA, eventually resulting in lethal DNA breaks (4, 5). Topoisomerase I is overexpressed in several tumor types, including breast, lung, and colorectal tumors (6).

Liposomal carriers have the potential to improve the therapeutic activity of anticancer drugs. Improvements manifested by encapsulation of the active agent can include reduced toxicity, increased drug stability, improved drug distribution measures, and most importantly, improved therapeutic effects (7–9). Resultantly, liposomal formulations of doxorubicin (10–13) and daunorubicin (14, 15) have gained regulatory approval for clinical use. In addition to the licensed anthracyclines, other liposome-encapsulated cytotoxic agents are undergoing clinical assessment. Examples include various liposomal camptothecins in

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early-stage clinical trials (16, 17) and liposomal vincristine, demonstrating positive results in refractory patient populations (18, 19).

The advent of clinically available liposomal formulations of anticancer drugs is encouraging. Nevertheless, these achievements should be considered in the context of the anticipated value of liposomes as drug carriers. Encapsulation of numerous distinct therapeutic agents has failed to improve their therapeutic profile sufficiently in model systems to warrant clinical development. Others that have progressed to clinical trials have demonstrated limited use (20, 21). These observations may reflect the inherent limitations of the encapsulated drug. To date, the focus has been on liposomal formulations of approved cytotoxic drugs. When used as single agents, these drugs are not anticipated to elicit cures in the majority of clinical cancers (22). Furthermore, disease localization has the potential to minimize the effectiveness of liposomal therapy. These limitations are illustrated by the minimal activity associated with the majority of liposomal drug formulations designed for the treatment of liver-localized disease (23). This is disappointing given the natural tendency for liposomes to accumulate in the liver.

Liposomal uptake by the liver is a consequence of kupffer cell activity, fenestrated vascular beds, and the distinctive nature of hepatic circulation. The resultant distribution of the liposomes is considered, in conjunction with the choice of drug and the rate of drug release from the carrier, as one of the critical parameters that dictate the success of liposome-mediated treatment of liver neoplasms (24). The pattern and degree of liver accumulation can, to some extent, be controlled by manipulation of liposome composition and size (25).

This study describes efforts to improve the treatment of liver carcinomas by encapsulating irinotecan in liposomes. The unencapsulated drug has demonstrated considerable activity in the clinic despite the dependence of camptothecin-mediated cytotoxicity on maintenance of the closed lactone ring form of the drug. At physiologic pH, the closed lactone ring undergoes rapid hydrolysis to an inactive carboxyl or ring-opened form (reviewed in ref. 26). The ability of liposomes to maintain internal aqueous environments of low pH is an attractive means to stabilize the active lactone form of water-soluble camptothecins (27, 28).

Irinotecan is shown to be an ideal candidate for liposome encapsulation. An ionophore-mediated proton gradient was used to efficiently trap irinotecan in the acidic interior of the liposomes at relatively high drug-to-lipid ratios. Liposome encapsulation of irinotecan significantly increased the circulation longevity of the drug as compared with the free agent while maintaining the agent in its active lactone form. In comparison to free irinotecan, the liposomal formulation was significantly more effective in the treatment of two models of colorectal cancer.

MATERIALS AND METHODS

Materials. Irinotecan hydrochloride (Camptosar; Pharmacia [Upjohn, Mississauga, ON, Canada]) was purchased from the British Columbia Cancer Agency Pharmacy. 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), the divalent cationic ionophore A23187, HEPES, and Sephadex

G-50 (medium) were obtained from the Sigma Chemical Company (St. Louis, MO). Tritiated cholesteryl hexadecyl ether (NEN, Boston, MA) was used as a liposome marker, and [^{14} C]methylamine hydrochloride, used in determining liposomal pH gradients, was obtained from Amersham Biosciences Corp. (Baie d'Urfe, Quebec, Canada). 1,1'-Diiododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), used as a fluorescent lipid probe, was purchased from Molecular Probes (distributed by Cedarlane Laboratories Limited, Hornby, Ontario, Canada). All other chemicals used in this study were analytical or high-performance liquid chromatography (HPLC) grade. The LS180 and LS174T tumor cell lines were originally purchased from the American Type Culture Collection (Manassas, VA) and were maintained in tissue culture. Cells were used for experiments when they were between passages 3 and 20. After passage 20, the cells were discarded, and the new cell lines were brought up from frozen stock. Male SCID/Rag-2M mice (8 to 10 weeks old) were bred at the British Columbia Cancer Agency Joint Animal Facility.

Preparation of Liposomes. Large unilamellar vesicles were prepared according to previously published procedures (29). Briefly, the appropriate mixture of lipids were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum. The dried lipid films were subsequently hydrated in 300 mmol/L manganese sulfate (pH 3.4) to a final lipid concentration of 50 or 100 mg/mL. Hydration was enhanced by multiple freeze-thaw cycles (30) before 10 high-pressure passes through two-stacked polycarbonate membrane filters (100/80 nm pore size; Nuclepore, Pleasanton, CA) using an extruder (Northern Lipids, Vancouver, British Columbia, Canada) at 65°C. The size of the large unilamellar vesicles was typically in the range of 115 ± 15 nm based on quasi-elastic light scattering (Nicom Particle Sizer model 270; NICOMP Particle Sizing Systems, Santa Barbara, CA). The external buffer was exchanged by running the sample down a Sephadex G-50 column equilibrated in 300 mmol/L sucrose, 20 mmol/L HEPES, and 15 mmol/L EDTA (SHE buffer) at pH 7.4. Liposomes composed of DSPC/Chol (55:45 mol%) or, if fluorescently labeled, DSPC/Chol/DiI (54.5:45:0.5 mol%). [^{3} H]CHE was incorporated into liposome formulations as non-exchangeable tracer (0.08 Ci/mol).

MnSO₄ pH Gradient Loading of Irinotecan. Irinotecan was actively loaded into DSPC/Chol (55:45 mol%) liposomes using an ionophore-mediated proton gradient (31). The divalent cation ionophore A23187 (0.5 μ g per 1 mg lipid) was incorporated into the liposomal bilayer after incubation at 37°C or 60°C for 10 minutes. Subsequently, the efficiency of irinotecan encapsulation by liposomes (drug-to-lipid weight ratio of 0.3:1) was determined at incubation temperatures of 37°C or 60°C over a 2-hour period. During this process, the mixture was kept in the dark because irinotecan has been shown to undergo degradation upon extended exposure to light (32). Encapsulated drug was separated from free drug using a Sephadex G-50 mini spin column (33). Irinotecan was quantified by measuring absorbance at 370 nm using a Beckman DU-64 Spectrophotometer (Beckman Coulter Canada, Inc., Mississauga, Ontario, Canada). Lipid concentration was determined by liquid scintillation counting of [^{3} H]CHE (Packard 1900 scintillation counter; Packard Instrument Co., Inc., Meriden, CT).

The drug release characteristics of this formulation were assessed by dialyzing (12,000 to 14,000 MWCO, Fisher Scientific, Nepean, ON) the irinotecan-loaded liposomes against 4 liters of SHE buffer (pH 7.4) for 72 hours at 37°C. At 1, 2, 4, 24, 48, and 72 h, 3 × 50 µL aliquots were removed from the dialysis cassette and analyzed for encapsulated drug and lipid concentrations as described above.

Irinotecan Encapsulation as a Function of Transmembrane pH Gradient. Irinotecan was encapsulated by liposomes at drug-to-lipid weight ratios of 0.1, 0.2, 0.3, 0.4, and 0.5 using the MnSO₄ loading procedure. The loading efficiency was determined as described previously. The magnitude of the transmembrane pH gradients in the absence of drug or, at the drug-to-lipid weight ratios above, was assessed by incubating the samples at 23°C for 1 hour in the presence of [¹⁴C]methylamine (34). Sephadex G-50 mini spin columns were used to separate encapsulated [¹⁴C]methylamine from unencapsulated [¹⁴C]methylamine. The eluant and the original sample were assayed for [³H] and [¹⁴C] using a dual label analysis method. With the assumption that the liposomes used have a trapped volume of 1.2 µL/µmol lipid, the distribution of radioactive methylamine was used to calculate the magnitude of the transmembrane proton gradient.

Animal Husbandry. Male SCID/Rag-2M mice (8 to 10 weeks) were purchased from the Joint Animal Facility at the British Columbia Cancer Research Center. Mice were housed under standard conditions and had access to food and water *ad libitum*. The Committee on Animal Care, Research Ethics Office, Research Services at the University of British Columbia approved all protocols in accordance with the Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care. All animals were observed at least once a day, more if deemed necessary, during the pretreatment and treatment periods for mortality and morbidity. In particular, signs of ill health were based on body weight loss, change in appetite, behavioral changes such as altered gait, lethargy, and gross manifestations of stress. Animals were terminated when signs of severe toxicity or tumor-related illness were seen (CO₂ asphyxiation), and a necropsy was performed to assess other signs of toxicity.

Plasma Elimination Studies. Free irinotecan (50 mg/kg) and liposomal irinotecan (50 mg/kg irinotecan, 167 mg/kg lipid) were injected via the lateral tail vein into male SCID/Rag-2M mice. At the indicated time points, mice were terminated by CO₂ asphyxiation, and whole blood was collected by cardiac puncture and transferred to EDTA-coated tubes (Microtainers; Becton Dickinson, Franklin Lakes, NJ). Plasma was isolated by centrifuging the whole blood at 2500 × g for 10 minutes. The liposomal carrier present in the plasma was quantified by scintillation counting of the nonexchangeable and nonmetabolizable lipid marker [³H]CHE. The concentration of total irinotecan and the relative percentages of the circulating drug existing as the lactone or carboxylate species were determined using HPLC analysis as described in ref. 35, with modifications. Briefly, irinotecan was extracted from plasma by diluting the sample in ice-cold methanol immediately before analysis. A standard curve for the lactone form of irinotecan was generated by dissolving the drug in DMSO. To generate an equivalent standard curve for the carboxylate species, irinotecan was processed in 50% MeCN: 50% 20 mmol/L borate buffer (pH 9). Separation

was achieved at 35°C using a Symmetry C₁₈ cartridge column (100Å, particle size, 5 µm; 250 × 4.6-mm inside diameter; Waters Corp., Milford, MA) with a Symmetry Sentry C₁₈ guard column (particle size, 5 µm; 20 × 3.9 inside diameter; Waters Corp.) with a run time of 20 minutes at a flow rate of 1.5 mL/minute. A two-solvent mobile phase consisted of mobile phase A (75 mmol/L ammonium acetate, 7.5 mmol/L tetra-butylammoniumbromide, pH 6.4 adjusted with glacial acetic acid) and mobile phase B (100% acetonitrile), with the elution gradient containing a mixture of 76% A:24% B. For sample analysis, 80 µL of each sample and standard were loaded into 1-mL HPLC sample vials (Waters Corp.) with 200 µL inserts (Chromatographic Specialties, Inc., Brockville, Ontario, Canada), and 10 µL were injected into the column. Irinotecan was quantified using a HPLC system equipped with a Waters Model 717 plus autosampler (set to 4°C), a Model 600E pump and controller, and a Model 470 Scanning Fluorescence Detector (Waters Corp.) set at an excitation wavelength of 362 nm and an emission wavelength of 425 nm. Data were acquired and processed with the Millennium32 chromatography manager (version 3.20; Waters Corp.).

Establishing a Maximum-tolerated Drug Dose. Limited dose ranging studies were used in an effort to determine the maximum-tolerated dose of free and liposomal irinotecan. Male SCID/Rag-2M mice in groups of two were given free or encapsulated drug by single or multiple [given every 4 days for a total of three doses (3 × q4d)] i.v. injections into the lateral tail vein. Qualified animal care technicians monitored the mice for weight loss and other signs of stress/toxicity for a period of 30 days. Individual animals that lost >30% of their original weight or those appearing severely stressed, as judged by appearance and/or behavior, were terminated. After 30 days, all remaining animals were terminated by CO₂ asphyxiation, and necropsies were conducted to identify any additional drug toxicities. Toxicity studies aimed at determining the exact LD₅₀ dose of a drug formulation are neither sanctioned by the Canadian Council on Animal Care nor by the Committee on Animal Care, Research Ethics Office, Research Services at the University of British Columbia.

Liposomal Irinotecan Antitumor Efficacy Using the Human LS180 Solid Tumor Model. Male SCID/Rag-2M mice (20 to 30 g, six per group) were inoculated with 1 × 10⁶ LS180 cells s.c. on the right posterior dorsal side. Once the tumor had established and reached a size accurately measurable with calipers (tumor volume, >0.04 cm³; day 11 postinoculation), free or encapsulated irinotecan dosing schedules of single or multiple (days 11, 15, or 19) injections of 50 or 100 mg/kg irinotecan via the lateral tail vein were initiated. Animals were monitored daily for any signs of stress or toxicity and were terminated when animals displayed increasing health deterioration including weight loss, lethargy, scruffy coats, or dehydration. Survival time for terminated animals was recorded as the following day. Animals were monitored for 60 days or until all animals had been terminated. Drug-mediated delays in tumor growth were estimated by extrapolation of a line tangential to exponential tumor growth down to the X axis, which indicated a time in days post cell inoculation.

Liposomal Irinotecan Antitumor Efficacy Using the Human LS174T Orthotopic Tumor Model. Male SCID/Rag-2M mice (20 to 30 g, six per group) were inoculated intrasplenically using the method developed by Kozlowski *et al.* (36–39). Briefly, a small incision was made in the skin lateral to the midline of the mouse. An additional incision (2 to 4 mm) in the abdominal wall allows exteriorization of the spleen. Fifty microliters (5×10^6 cells) of LS174T cell suspension were injected just under the capsule of the spleen. After inoculation, the spleen was returned to the abdominal cavity and the incision sutured. Wound clips were used to close the skin. Histologic assessment of the liver after intrasplenic inoculation indicated that at day 7, LS174T cells were well established in the liver. This reflects the previously reported observations (36). On day 7 after cell inoculation, animals began q4d dosing schedules for three injections (days 7, 11, or 15) of 50 mg/kg free or liposomal irinotecan given via the lateral tail vein. Animals were monitored daily and terminated upon signs of severe weight loss (>30%) and/or health deterioration as above. Survival time for terminated animals was recorded as the following day.

Liposome-mediated Drug Delivery to LS174T Liver Metastases. To illustrate liposome delivery to tumors in the liver, male SCID/Rag-2M mice (20 to 30 g, two per group) were inoculated intrasplenically as described in the previous section. Once the tumors were well established in the liver (day 19 after inoculation), animals were given a single dose of liposomal irinotecan (50 mg/kg) or mock-loaded DSPC/Chol/DiI (54.5:45:0.5 mol%) liposomes injected via the lateral tail vein. Twenty-four hours later, animals were terminated by CO₂ asphyxiation, whole blood was collected via cardiac puncture as outlined in the plasma elimination studies, and a portion of a lobe of the liver was harvested for sectioning and histochemistry. DiI inclusion in the liposomes enabled their visualization using fluorescent microscopy (λ_{excit} , 549 nm; λ_{emit} , 565 nm). Liver sections taken from untreated animals ($n = 2$) were H&E stained to reveal tumor localization.

Statistical Analysis. One-way ANOVA was performed on the tumor volume results obtained after the administration of free or liposomal irinotecan. Common time points were compared using the post hoc comparison of means (Scheffé test). Survival data were analyzed using a multiple-sample test in the Survival Analysis module of Statistica (Statsoft, Inc., Tulsa, OK). This test is an extension of Gehan's generalized Wilcoxon test, Peto and Peto's generalized Wilcoxon test, and the log-rank test. Differences were considered significant at $P < 0.05$.

RESULTS

Irinotecan Encapsulation by DSPC/Chol (55:45 mol%) Liposomes. The incorporation of an ionophore into the lipid bilayer can generate a stable transmembrane pH gradient. The ionophore exchanges cations present in the internal buffer of the liposomes for protons from the external buffer, thereby generating and maintaining a pH gradient. In this study, the divalent cation ionophore, A23187, facilitates the outward movement of Mn²⁺ ions across the bilayer in exchange for the inward movement of 2H⁺.

Fig. 1 illustrates the importance of the A23187 ionophore in mediating efficient encapsulation of irinotecan by liposomes

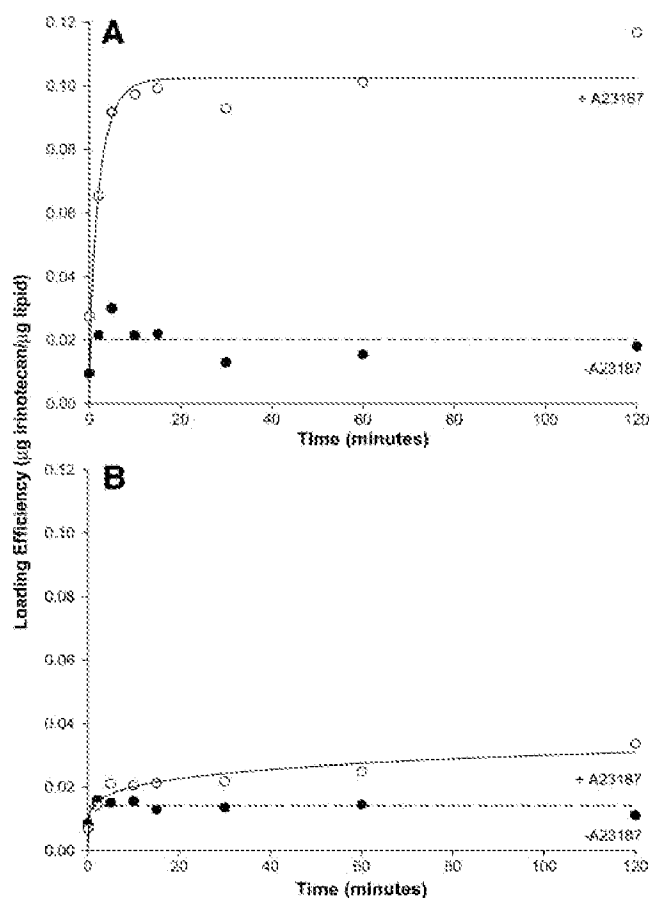


Fig. 1 Effect of the A23187 ionophore on pH gradient-mediated loading of irinotecan into DSPC/cholesterol (55:45 mol%) liposomes at 60°C (A) or 37°C (B). Loading was evaluated at two temperatures in the presence (○) or absence (●) of the A23187 ionophore at an initial drug-to-lipid ratio of 0.1:1 (wt:wt). Drug encapsulation was determined as described in Materials and Methods.

composed of DSPC and Chol (55:45mol%). In the absence of A23187, drug accumulation in the liposomes was <20%, irrespective of the incubation temperature used. In the presence of A23187, incubation at 60°C resulted in >98% of the added irinotecan being incorporated into the liposomes after 15 minutes (Fig. 1A). In contrast, <30% of the irinotecan was associated with the liposomes at the end of 2 hours of incubation at 37°C (Fig. 1B).

To determine the maximum level of irinotecan encapsulation achievable using this loading method, the starting drug-to-lipid weight ratio was increased incrementally from 0.1 to 0.5. The results, shown in Fig. 2A, indicate that the loading efficiency at 60°C decreases as the added drug-to-lipid weight ratio increases. Loading efficiencies of >80% were achieved for samples incubated for 1 hour at starting drug-to-lipid weight ratios of 0.1 to 0.4. When the starting drug-to-lipid weight ratio was 0.5, the loading efficiency was <70%. Because drug loading is anticipated to be dependent on the magnitude and stability of the ionophore-generated pH gradient, the transmembrane pH gradients of the samples were determined after 1 hour of incubation at 60°C (Fig. 2B). In the presence of A23187, but in the

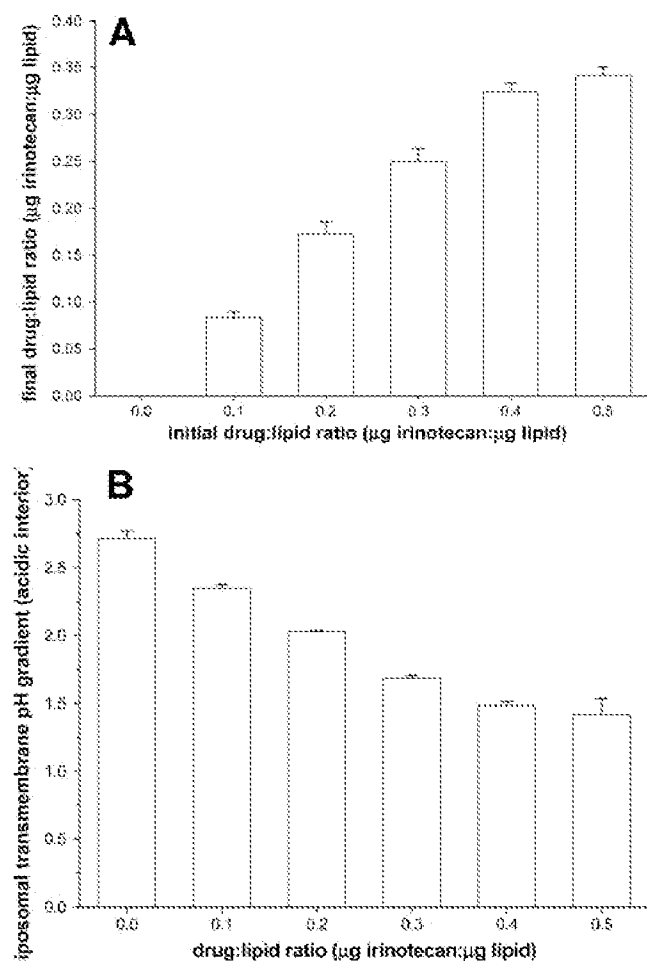


Fig. 2 The effect of the initial drug-to-lipid weight ratio on irinotecan loading (A) and transmembrane pH gradient before and after loading (B). A. DSPC/cholesterol liposomes (55:45 mol%) were incubated at 60°C for 1 hour at different drug-to-lipid weight ratios, and the extent of irinotecan encapsulation was determined (see Materials and Methods) and expressed as the average final drug-to-lipid weight ratio $\pm$ SD. B. Transmembrane pH gradients were determined as described in Materials and Methods using [^{14} C]methylamine as a probe. The [^{14} C]methylamine was added after 1 hour of incubation at 60°C in the absence of added drug or in the presence of the indicated irinotecan-to-lipid weight ratio.

absence of added drug, a transmembrane pH gradient of approximately three units was detected using the pH probe methylamine. The magnitude of this pH gradient decreased to 1.5 units when the initial drug-to-lipid weight ratio was ≥ 0.4 . Therefore, reduced loading efficiency could be attributable to lower transmembrane pH gradients, but regardless of the loading efficiency, all formulations exhibited a significant transmembrane pH gradient (inside acidic) after loading. The loading efficiency at all drug-to-lipid weight ratios tested was not improved by increasing the incubation time period beyond 1 hour. Indeed, the majority of irinotecan encapsulation occurred during the first 30 minutes of incubation (data not shown).

From the data presented thus far, it has been demonstrated that irinotecan can be efficiently loaded into DSPC/Chol liposomes at an initial drug-to-lipid weight ratio of 0.3. Moreover,

the resultant drug-loaded liposomes were able to retain a transmembrane pH gradient of >1.5 units. On the basis of these observations, the stability of this formulation was initially assessed by dialyzing the sample at 37°C against a large volume of buffer (pH 7.4). After 72 hours of incubation, $<2\%$ of the encapsulated irinotecan was released (data not shown). These results indicated that this formulation was worthy of additional evaluation.

***In vivo* Plasma Elimination of Free and Liposomal Irinotecan.** The plasma elimination profiles of both free irinotecan (50 mg/kg) and irinotecan encapsulated in DSPC/Chol liposomes (50 mg/kg drug, 167 mg/kg lipid) were determined after i.v. injection into SCID/Rag-2M mice. At 1, 4, and 24 hours after injection of the liposomal formulations or 5, 15, and 60 minutes after injection of free irinotecan, plasma was collected and analyzed for drug and/or liposomal lipid levels (Fig. 3).

DSPC/Chol liposomes exhibited an extended circulation life span and the elimination of irinotecan-loaded (Fig. 3A, filled symbols) and mock-loaded (Fig. 3A, open symbols) liposomes was not significantly different. Approximately 40% of the injected lipid dose could still be found in the plasma compartment 24 hours after i.v. injection. It should be noted that these circulation levels were achieved in the absence of surface grafted polyethylene glycol and are reasonably high because of the high lipid dose used (167 mg/kg).

Free irinotecan was eliminated rapidly from the plasma compartment (Fig. 3B; Table 1). In contrast, liposome-encapsulated irinotecan was maintained at higher irinotecan levels throughout the 24-hour time frame of the experiment. At 1 hour, the measured levels of liposomal irinotecan in the plasma were 100 times greater than those associated with the free drug (Fig. 3 and Table 1).

The therapeutic activity of irinotecan is dependent on maintenance of the drug in its closed lactone ring form. This is of particular importance when considered in the context of physiologic pH, which favors the existence of the inactive open-ring carboxyl form of irinotecan (40). Resultantly, the relative proportions of the lactone and carboxyl forms of irinotecan responsible for the plasma levels of total irinotecan presented in Fig. 3B were quantified by HPLC analysis. The results (summarized in Table 1) suggest that at 5 minutes after injection of the free drug, $\sim 28\%$ of the measured irinotecan was in the carboxyl form, and this increased close to 60% at the 1-hour time point. In contrast, $>95\%$ of the measured liposomal irinotecan was in the lactone form at 1 and 4 hours. This indicates that irinotecan remains encapsulated in the low pH environment of the liposomes, thereby maintaining its lactone configuration. At 24 hours after injection of the liposomal drug, the irinotecan concentration in plasma was 100.90 $\mu\text{g/mL}$ or $\sim 8\%$ of that measured at the 1-hour time point (1263.63 $\mu\text{g/mL}$; see Table 1). At this time point, $>80\%$ of the remaining drug exists in the lactone form and suggests that the vast majority of the detected drug is likely present in circulating liposomes that exhibit a low interior pH.

By calculating the ratio of irinotecan to liposomal lipid from the data shown in Fig. 3, A and B, and Table 1, it is possible to illustrate the loss of drug from liposomes after i.v. injection. This estimate assumes that 100% of the measured irinotecan in the plasma compartment is associated with circu-

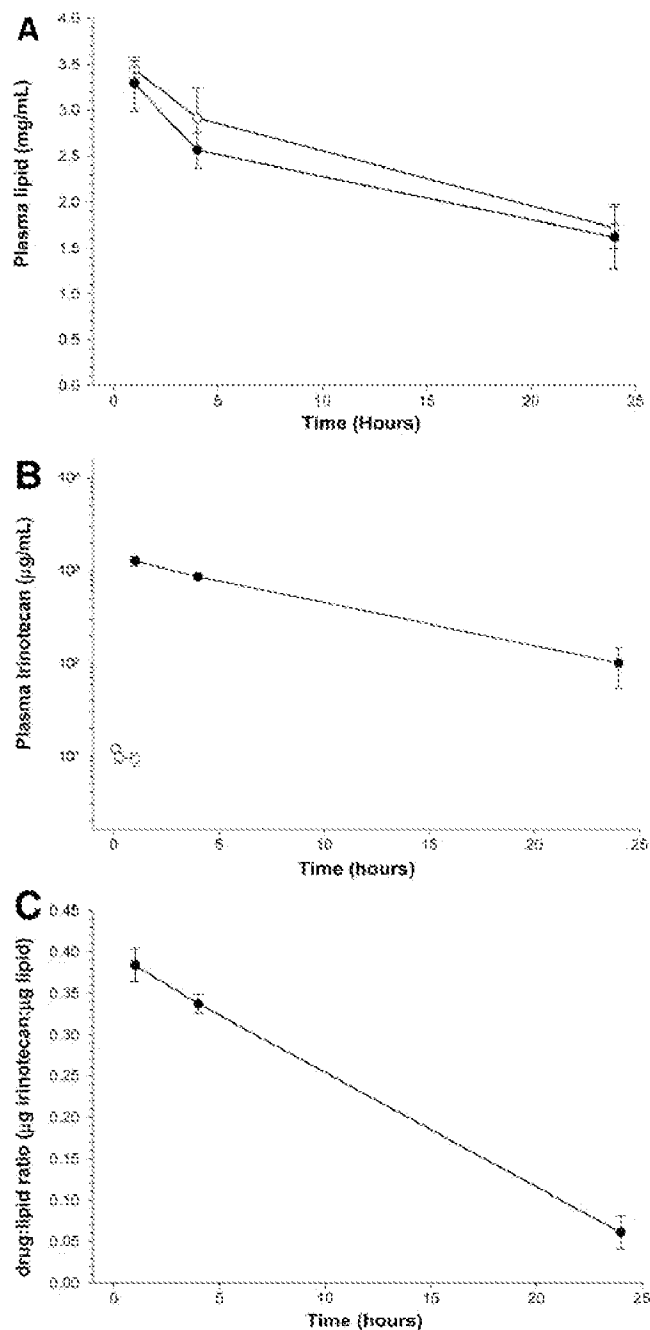


Fig. 3 Plasma levels of free and liposomal irinotecan determined at three time points after i.v. administration in SCID/Rag-2M mice. **A**. Mice received injections of liposomal irinotecan (drug-to-lipid weight ratio of 0.3) (●) or mock-loaded liposomes (○) at a lipid dose of 167 mg/kg and a drug dose of 50 mg/kg. At the indicated times, plasma was collected and the circulating liposomal lipid concentration was determined as described in Materials and Methods. **B**. Plasma levels of total irinotecan (carboxyl form + lactone form) after i.v. injection of the liposomal drug formulation (●) or free drug (○) at a dose of 50 mg/kg drug. **C**. Using the measured data shown in **A** and **B**, changes in drug-to-lipid weight ratio can be calculated at the indicated time points. Decreases in the drug-to-lipid ratio provide a measure of drug release from liposomes in the plasma compartment. All data points represent the mean values obtained from six mice per time point, and the error bars represent the SD.

lating liposomes. As shown in Fig. 3C, irinotecan is released at a steady rate from the liposomes over the 24-hour time course.

The Efficacy of Liposomal and Free Irinotecan in Treating an Established LS180 Human Xenograft Tumor Model. Efficacy studies were undertaken to determine whether liposome encapsulation engendered improvements in the activity of the irinotecan when compared with the free drug. The therapeutic benefits of the free and encapsulated drug were assessed in two advanced colorectal tumor models.

The Canadian Council of Animal Care does not authorize formal toxicity studies. Therefore, the maximum-tolerated dose of free and liposomal irinotecan was determined through limited dose range finding studies in tumor-free SCID/Rag-2M mice (two mice per dose). Unfortunately, due to the nature of commercially available irinotecan [20 mg/mL irinotecan dissolved in 45 mg/mL sorbitol, 0.9 mg/mL lactic acid (pH 5)], the dose escalation studies with free drug were inconclusive. A pronounced acute dose-limiting toxicity was observed when the drug was given at doses > 100 mg/kg. This toxicity was observed within 1 minute of injection, suggesting that it was a consequence of the formulation as opposed to drug-induced effects on normal cell populations typically observed as weight loss and/or myelosuppression. An evaluation of drug-induced weight loss during the dose escalation studies with DSPC/Chol irinotecan (data not shown) indicated that the drug was tolerated (on injection) at the 100 mg/kg dose but was toxic as judged by weight loss. Necropsies of the injected animals 30 days after drug administration revealed no gross abnormalities in any of the tissues examined. On the basis of these limited toxicity studies, efficacy experiments focused on drug doses of 50 and 100 mg/kg, given as a single injection or 3 × q4d.

Studies completed in LS180 tumor-bearing SCID/Rag-2M mice evaluated drug-induced weight loss as a measure of toxicity (Fig. 4) and drug-induced delays in tumor growth as a measure of efficacy (Fig. 5). Measurable LS180 solid tumors were obtained 11 days after s.c. inoculation of 10⁶ LS180 cells, and treatment was initiated at this time using a single dose or a 3 × q4d schedule.

Weight loss results clearly indicate that the liposomal irinotecan formulation was more toxic than the free drug when administered three times at 100 mg/kg on the q4d schedule (Fig. 4D). The nadir in weight loss after this treatment schedule occurred between 2 and 7 days after the final drug dose. At this time, animals treated with liposomal drug had lost >30% of their original body weight and had to be terminated. It should be noted that other indicators of toxicity were not evident, and the animals were terminated solely because of weight loss considerations at this time. In general, animal care protocols indicate that animals must be terminated when body weight loss is >20%; however, protocols used in these studies emphasized a number of different end points, and if animals were considered healthy as judged by other end points, then animals with >20% body weight loss were maintained on the study. Animals treated with 50 or 100 mg/kg free irinotecan or liposomal irinotecan at a dose of 50 mg/kg exhibited a weight loss in the range of 7 to 13%, suggesting that these drug doses were reasonably well tolerated (Fig. 4A–C).

Results obtained after treatment of mice bearing established LS180 tumors have been summarized in Fig. 5. These

Table 1 HPLC analysis of plasma irinotecan after i.v. administration of free or liposomal drug

Time	Plasma concentration ($\mu\text{g/mL}$)			Irinotecan (% of total)	
	Lactone	Carboxylate	Total	Lactone	Carboxylate
Free irinotecan					
5 minutes	8.72 ± 0.93	3.34 ± 0.58	12.06 ± 1.42	72.45 ± 2.38	27.55 ± 2.38
15 minutes	5.44 ± 0.79	4.35 ± 0.80	9.79 ± 1.43	55.67 ± 3.37	44.33 ± 3.37
1 hour	3.80 ± 0.61	5.46 ± 0.96	9.26 ± 1.51	44.12 ± 6.52	55.85 ± 6.52
Liposomal irinotecan					
1 hour	1236.66 ± 148.67	26.97 ± 5.84	1263.63 ± 151.43	97.87 ± 0.41	2.13 ± 0.41
4 hour	838.89 ± 66.41	25.46 ± 5.00	864.35 ± 66.35	97.02 ± 0.62	2.98 ± 0.62
24 hour	82.56 ± 46.10	18.34 ± 9.36	100.90 ± 47.47	79.32 ± 8.68	20.68 ± 8.68

NOTE. Plasma levels of irinotecan were determined as described in Materials and Methods. $n = 6$, values quoted are means $\pm$ SD.

data clearly establish that the DSPC/Chol irinotecan formulation was therapeutically more active than free drug. The tumors of untreated animals progressed to a size of 400 mg 19 days after inoculation (Fig. 5A). A single dose of 50 mg/kg free irinotecan delayed this progression to day 22. Increasing the dose of free irinotecan to 100 mg/kg slowed the rate of tumor growth further, such that a size of 400 mg was recorded on day 26 (Fig. 5A). The equivalent values observed for the liposomal formulations are shown in Fig. 5B. A single dose of 50 mg/kg liposomal irinotecan prevented the tumor mass reaching 400 mg until day

34. This growth retardation was extended to 39 days after one dose of 100 mg/kg liposomal irinotecan.

Additional gains in efficacy were observed for both free and liposomal irinotecan when the administration schedule was increased to three doses administered on days 11, 15, and 19 (Fig. 5, C and D). Tumors reached 400 mg in size on day 30 after three doses of 50 mg/kg free irinotecan (Fig. 5C). The tumor growth curve representing three doses of free 100 mg/kg irinotecan resembles that observed after a single dose of 100 mg/kg liposomal irinotecan, with tumors reaching 400 mg after 40 days

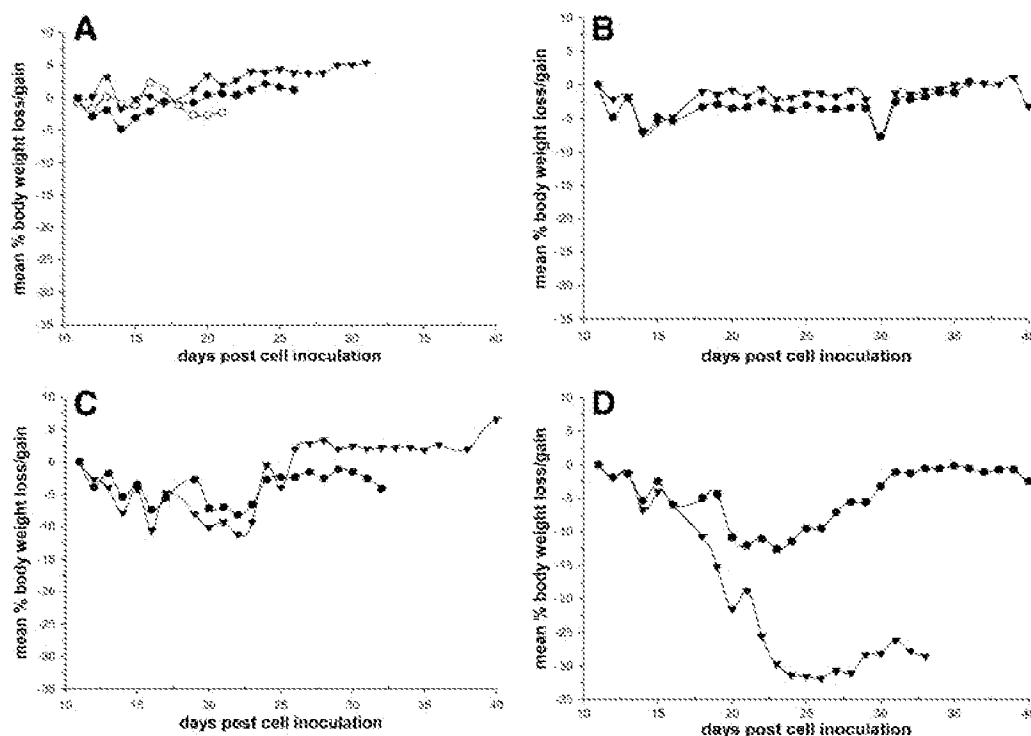
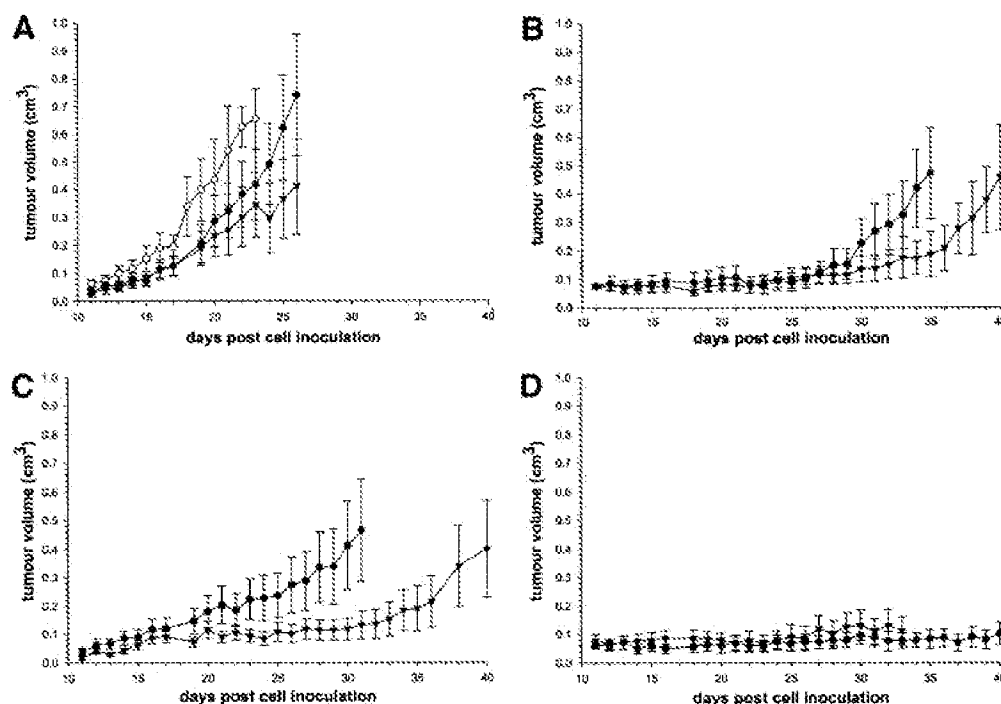


Fig. 4 Toxicity of free (A and C) and liposomal (B and D) irinotecan as assessed by weight loss in LS180 tumor-bearing mice. Single injections (A) free irinotecan or (B) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were administered i.v. on day 11 after cell inoculation (1×10^6 cells/50 μL), or three injections of (C) free irinotecan or (D) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were administered on day 11, 15, and 19 after cell inoculation. Changes in weight were obtained from saline-treated animals ($\circ$); animals given 50 mg/kg irinotecan/dose per injection ($\bullet$); and animals given a 100 mg/kg irinotecan/dose per injection ($\blacktriangledown$). For clarity purposes, only the mean change in weight (relative to the weights of animals determined on day 10) is shown on the indicated days. Saline-treated animals were terminated on day 23 because of progression and occasional ulceration of the solid tumors. Mice that received three 100 mg/kg injections of liposomal irinotecan were terminated on day 34 because of drug-induced toxicity manifested as severe weight loss. However, it should be noted that these animals appeared healthy by all other measurements of stress.

Fig. 5 Antitumor efficacy of free (A and C) and liposomal (B and D) irinotecan as determined in LS180 tumor-bearing animals. Drug efficacy was assessed by measuring tumor volume as a function of time after s.c. injection of LS180 tumor cells (1×10^6 cells/50 μ L, day 0). Single injections of (A) free irinotecan or (B) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were administered i.v. on day 11, or three injections of (C) free irinotecan or (D) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were given on days 11, 15, and 19. The data shown represents the mean and SE derived from a group size of six mice. These data were obtained from saline-treated animals ($\circ$); animals given a 50 mg/kg irinotecan/dose per injection ($\bullet$); and animals given a 100 mg/kg irinotecan/dose per injection ($\blacktriangledown$).



(compare Fig. 5C with Fig. 5B). Administration of three doses of 50 mg/kg or 100 mg/kg liposomal irinotecan mediated substantial delays in onset of tumor growth. There were no increases in tumor size recorded over the 40-day evaluation period (Fig. 5D).

Efficacy of Single and Multiple Dose Administration of Liposomal and Free Irinotecan in the Orthotopic LS174T Human Tumor Model. The results shown in Fig. 5 suggest that the liposomal formulation of irinotecan is therapeutically more active than free drug, but these results were achieved using a s.c. tumor model. The following studies were initiated to determine whether similar gains in therapeutic activity could be achieved using a murine LS174T model designed to mimic liver metastases arising from colorectal cancer (39). The LS174T is a relatively slow-growing model, and the effects of a large tumor burden do not become apparent until approximately day 30 after inoculation, after which, the host condition deteriorates quickly, and the animals must be terminated (Fig. 6). Thus, in the absence of treatment (saline control), the median survival time of inoculated animals was determined to be 34 days. Animals treated with free irinotecan ($3 \times$ q4d) exhibited a median survival of 53 days, whereas those treated using the same schedule with liposomal drug exhibited a median survival time of 79 days. This represented a 141% increase in life span, which was significantly greater ($P < 0.05$) than the 56% increase in life span achieved using an identical dose of free irinotecan. It should be noted that treatment was initiated 7 days after tumor inoculation. Studies with control mice terminated 7 days after LS174T cell injection indicated the presence of established disease in the liver (results not shown).

Liposome-mediated Delivery to Liver Metastases. To determine whether improved therapy was associated with liposome delivery to regions of LS174T tumor growth within the

liver, LS174T tumor-bearing animals were dosed with a liposomal irinotecan formulation prepared with 0.5 mol% DiI, a nonexchangeable fluorescent marker (41). Representative photomicrographs are shown in Fig. 7.

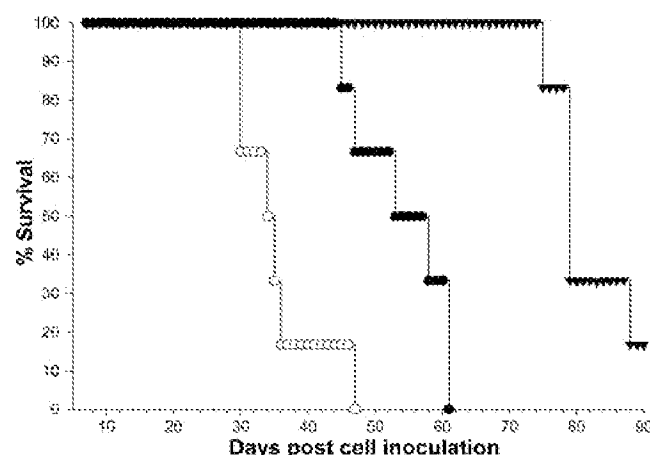


Fig. 6 Antitumor efficacy of free and liposomal irinotecan as determined in the orthotopic LS174T tumor model. Mouse survival was determined as a function of time after intrasplenic injection of LS174T tumor cells (5×10^6 cells/50 μ L, day 0) as described in Materials and Methods. Survival cannot be used as an experimental end point. Therefore, multiple indicators of animal stress were used to assess tumor progression. If an animal was terminated because of tumor progress, the day of death was recorded as the subsequent day. The technician assessing animal stress was blinded to the study groups. Three injections of 50 mg/kg free or liposomal drug (drug-to-lipid weight ratio 0.3) were administered i.v. on days 7, 11, and 15 after cell inoculation ($n = 6$). The data shown were obtained from saline-treated animals ($\circ$); animals given free irinotecan ($\bullet$); and animals given liposomal irinotecan ($\blacktriangledown$).

The tumor-bearing livers of untreated animals are shown in Fig. 7A. This image is characterized by the presence of large vacuole-like structures observed within regions of tumor growth. Because the LS174T tumors are derived from a mucin-producing adenocarcinoma cell line (42), it can be suggested that these structures may be mucin-containing vacuoles. Both empty (Fig. 7B) and irinotecan-loaded (Fig. 7C) liposomes were seen predominantly in regions of healthy liver tissue (thin arrows), as opposed to regions of tumor growth (large arrows). These images suggest that DSPC/Chol liposomes accumulate at the periphery of the tumor with no evidence of tumor localization 24 hours after i.v. administration.

DISCUSSION

DSPC/Chol (55:45 mol%) liposomes (~100 nm in diameter) proved to be an effective carrier for irinotecan. Encapsulation of irinotecan was mediated by the combination of 300 mmol/L MnSO_4 solution in the interior of the liposomes and A23187 incorporated into the liposomal bilayer. A23187 is capable of transferring two protons into the liposome for every one Mn^{2+} ion transported out. This creates a pH gradient across the liposomal membrane, which was crucial for efficient loading (>90%) of irinotecan (Fig. 1). Under these conditions, high drug-to-lipid weight ratios of 0.3 could be achieved (Fig. 2). The formulation proved to be stable after incubation in buffer at 37°C for 72 hours and therefore was considered a suitable candidate for further investigation.

The plasma elimination studies illustrated in Fig. 3, demonstrated that the encapsulated irinotecan was steadily released from the liposomes over a 24-hour period. Approximately 70% of the initial dose of liposomal irinotecan was present at 4 hours postinjection. This decreases to <10% after 24 hours. HPLC analysis revealed that the circulating drug associated with the liposomes was maintained in the active lactone form (Table 1). This is a consequence of irinotecan encapsulation within the favorable acidic environment of the liposomes. These observations are in contrast to the data recorded for free irinotecan. Very low concentrations of free drug were recorded in the plasma compartment 5 minutes postinjection. At the 15-minute and 1-hour time points, there was no marked change in total drug levels; however, there was a progression from the lactone form toward the inactive carboxyl form, as would be expected under physiologic conditions. The rapid disappearance of irinotecan from the circulation probably reflects the high concentrations of esterases present in mouse plasma. Resultantly, irinotecan is rapidly converted into its more active congener, SN-38 (43). SN-38 would not be detectable under the HPLC conditions used here.

Efficacy studies conducted in SCID/Rag-2M mice bearing established s.c. LS180 solid tumors indicated that free irinotecan was effective in slowing tumor growth. However, this effect was much more pronounced after administration of the liposomal drug, with tumor progression arrested over an extended time period (Fig. 5). These observations were extended to mice inoculated intrasplenically with LS174T cells. This orthotopic tumor model is designed to mimic liver metastases secondary to colorectal cancer (39). Liposomal irinotecan demonstrated sig-

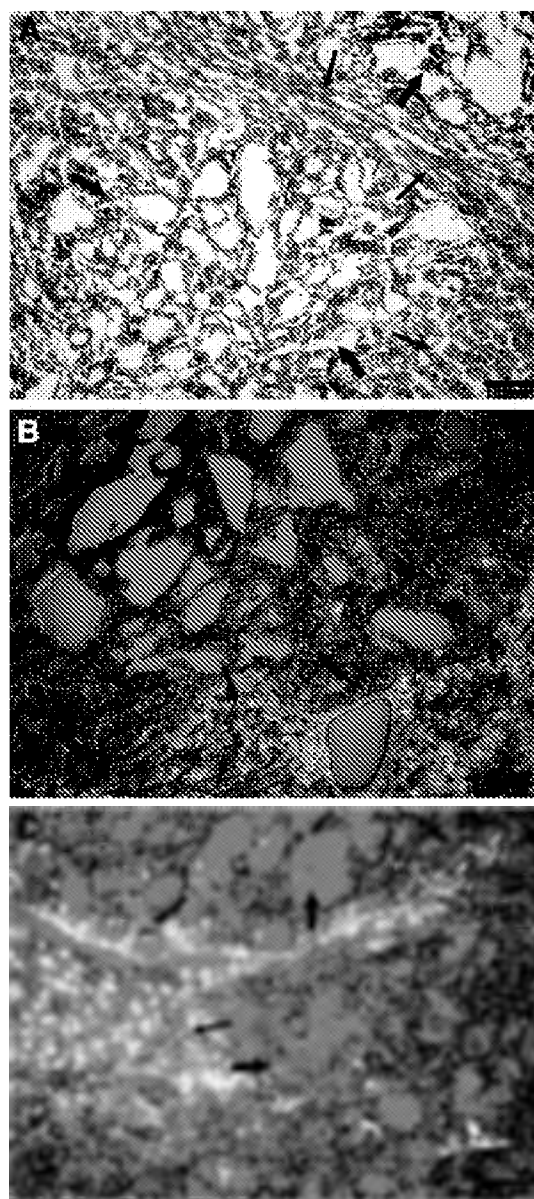


Fig. 7 Liposome-mediated drug delivery to livers of LS174T tumor-bearing mice. DiI-labeled DSPC/Chol liposomes, empty or irinotecan-loaded (drug-to-lipid weight ratio 0.3), were injected i.v. into mice (two per group) inoculated with LS174T tumor cells 19 days earlier as described in Materials and Methods (5×10^6 cells/50 μL). Twenty-four hours after injection of saline (A), mock-loaded liposomes (B) and irinotecan-loaded liposomes (C), the livers were harvested for histology as indicated in Materials and Methods. A, Representative H&E staining where the (thick arrows) indicate regions of tumor growth, whereas the (narrow arrows) indicate normal liver tissue; B, representative fluorescent micrograph of liver section derived from a mouse injected with a mock loaded liposome (no encapsulated drug); and C, representative fluorescent micrograph of liver section derived from a mouse injected with irinotecan-containing liposomes.

nificantly greater survival ($P < 0.05$) than free drug at equivalent doses in this model (Fig. 6).

The results described clearly demonstrate that liposomal encapsulation can potentiate the therapeutic activity of irinote-

can against models of human colorectal cancer and associated liver-localized metastases. This supports our belief that liposomal anticancer formulations encapsulating appropriately selected drugs can be of value in treating liver-localized neoplasia.

A notable advantage of encapsulating irinotecan using the methods described here is the maintenance of the drug as the active lactone form (see Table 1). Previous lipid-based techniques used to encapsulate camptothecins have exploited their ability to partition into the lipid bilayer. This has been shown to confer protection to the lactone species of irinotecan (44, 45). Therapeutically, this approach is limited by low drug loading efficiencies and the rapid exchange of membrane-localized drug from the liposomes to endogenous membranes after i.v. administration (46, 47). Tardi *et al.* (28) overcame this limitation by successfully encapsulating topotecan in the aqueous interior of liposomes using the ionophore-generated pH gradient described here. In both instances, encapsulation of topotecan and irinotecan was efficient (>90% loading). Furthermore, the internal acidic environment of the liposome limits hydrolysis of the lactone ring of camptothecins to the inactive carboxyl form. Consequently, liposomal topotecan and liposomal irinotecan are characterized by the maintenance of the lactone forms of the drugs in the circulation for extended periods after i.v. administration.

An additional facet of the liposomal irinotecan formulation described here is the steady release of drug over 24 hours. This is of particular interest in the context of improve therapeutic activity mediated by drug-carrier systems. Improvements manifested by drug carriers are often attributed to the ability of the carrier system to deliver the drug to the site of action. However, studies have demonstrated that carrier-mediated delivery of an effective agent to the tumor site is not in its self sufficient to achieve therapy. Furthermore, the concentration of drug achieved within the site of tumor growth does not necessarily predict therapeutic activity (48, 49). This apparent contradiction can be attributed to the properties of the liposomal carrier. Lipid compositions and/or encapsulation procedures selected to achieve improved drug delivery to the region of interest may hinder the release of the drug and consequently limit the amount of drug that is available to act on the target cell population.

Histologic evidence presented here (Fig. 7) indicates that this formulation accumulates in the liver, as would be expected based on experience with previous liposome preparations (50). However, 24 hours after i.v. administration, sites of liposomal localization were distinct from those of tumor growth. Moreover, the low concentrations of irinotecan associated with DSPC/Chol liposomes at this time (Table 1) suggest that the improved therapeutic activity observed does not appear to be a consequence of enhanced delivery of encapsulated irinotecan to the tumor metastases. This is supported by previous efficacy studies that demonstrated therapeutic improvements in the treatment of liver-localized disease mediated by liposomal drugs was dependent on release of the encapsulated drug from the liposomes at an appropriate rate after administration (24). It will be important to establish in future studies what release rates provide the most significant improvements in therapeutic activity.

The present study did not address how the liposome composition and irinotecan encapsulation procedure influences therapeutic activity. However, the results can be compared with

other studies using comparable lipid compositions (primarily DSPC and Chol) to encapsulate doxorubicin (51), vincristine (52), mitoxantrone (53), daunorubicin (54), idarubicin (55), topotecan (28), and cisplatin (56). Such a comparison clearly demonstrates that a composition that is useful for one anticancer drug may be not be of any therapeutic value for another.

When considering the importance of drug release from liposomes in the plasma compartment or within sites of liposome accumulation, it is reasonable to suggest that the liposomal formulation used here provides sustained drug exposure over an extended time period after administration. It can be suggested, therefore, that one of the benefits of using a liposomal carrier for irinotecan is to promote prolonged tumor cell exposure to this S-phase-specific drug. This, in turn, should increase tumor growth arrest, delays in tumor progression, and increases in median survival. As discussed previously, irinotecan cytotoxicity has the additional constraint of requiring carboxylesterase-mediated conversion to the vastly more potent SN-38. These enzymes are found in high concentrations in hepatic cells, as well as in serum and other tissues, including some tumor populations (57, 58). The environment responsible for liposomal irinotecan activation and the subsequent therapeutic activity observed against colorectal metastases is currently unclear. Initial animal studies evaluated the efficacy of liposomal irinotecan in the murine LS180 and LS174T models of metastatic colorectal cancer. Although the activity of camptothecins has been evaluated, to a limited extent, in these cell lines *in vitro* (59–61), no studies have assessed irinotecan activity *in vivo* against tumors derived from these cell lines. In both the LS180 and LS174T models, liposomal irinotecan was considerably more efficacious than free drug. As shown in Fig. 5, treatment with liposomal irinotecan did not result in a decrease in tumor size, rather tumor growth was prevented for extended time periods. It can be suggested that liposomal irinotecan provides adequate control of tumor expansion but alone it is not sufficient to eradicate the disease. This is not surprising given the fact that the benefits attributable to irinotecan use have often been shown when it is used in combination with second agents such as the fluorouracils (62) and platinum-based anticancer drugs (63). In fact, irinotecan is an approved agent for treatment of colorectal cancer in combination with 5-fluorouracil plus leucovorin and is undergoing advanced testing in combination with cisplatin for treatment of lung cancer (64). We are particularly interested in developing formulations that can mediate effective delivery of irinotecan in combination with other agents selected to achieve optimal therapeutic effects as judged by assays that measure the interactions between selected drug combinations.

In conclusion, liposome encapsulation of irinotecan results in a potent drug formulation for the treatment of models of colorectal cancer as a result of increased drug longevity, protection of the active lactone species, and rapid accumulation at the site of tumor development in the liver. The use of liposomal irinotecan for the treatment of liver metastases has tremendous potential, and continuing studies focusing on optimizing the lipid formulation and understanding the role of hepatic cells in processing these carriers, as well as studies with second agents that combine well with irinotecan, could result in a chemotherapeutic strategy that will improve survival for colorectal cancer patients.

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Combination Versus Sequential Single-Agent Therapy in Metastatic Breast Cancer

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Key Words. *Capecitabine · Taxanes · Breast cancer · Docetaxel · Sequential · Combination*

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Describe the current and evolving role of combination therapy in the management of the breast cancer patient with advanced (metastatic) disease.
2. Appreciate the principles of combination treatment design for breast cancer management.
3. Describe those combination treatment regimens, which are designed rationally.
4. Explain the current status of combination or sequential treatment in the management of advanced breast cancer and how clinicians might select options based on patient characteristics.

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ABSTRACT

The optimal treatment approach in metastatic breast cancer is controversial. Until recently, the arguments for administering antitumor agents sequentially in the metastatic setting have traditionally outweighed those for administering agents in combination. Older, often empirically designed drug combinations were no more effective and often more toxic than monotherapy, compromising quality of life for little or no clinical benefit. However, more recent studies show that combination and sequential therapy both have their place in the treatment of metastatic breast cancer, with the heterogeneity of breast cancer demanding flexibility in approach. Newer drug combinations, such as paclitaxel/trastuzumab or capecitabine/docetaxel, show

survival advantages over single-agent therapy and have manageable safety profiles. Such combination treatments may be preferable to sequential therapy for patients requiring urgent reduction in their tumor burden. Sequential therapy allows the optimal delivery of single-drug therapy and potentially reduces the risk of toxicity, which may improve quality of life. Sequential therapy may be especially appropriate in frail or elderly patients, who may be unable to tolerate the toxicity of combination therapy, or in patients with slowly growing tumors. This article expands on these issues by reviewing trials comparing combination regimens with sequential approaches. *The Oncologist* 2002;7(suppl 6):13-19

INTRODUCTION

The optimal treatment approach in metastatic breast cancer, with antitumor agents given sequentially or in

combination, remains controversial. It is doubtful that either strategy is appropriate for all patients. Breast cancer patients are most likely treated on an individual basis

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according to each patient's demographic and disease characteristics, based on clinical trial data and influenced by the personal experience of the physician. In the future, prospective evaluation of specific subpopulations may help identify optimal treatment approaches for patient groups. This article discusses the advantages and disadvantages of sequential and combination therapies in metastatic breast cancer, given the evidence currently available.

THE CURRENT STATUS OF COMBINATION THERAPIES IN BREAST CANCER

In the adjuvant setting, combination chemotherapy is used routinely due to widespread evidence that polychemotherapy offers a survival advantage compared with single-agent therapy [1]. A retrospective analysis of trials performed in 1992 by the Early Breast Cancer Trialists' Collaborative Group showed that adjuvant treatment with combination chemotherapy regimens was associated with a 12% reduction in the risk of death compared with no adjuvant treatment, translating into a 4.4% reduction at 5 years (unpublished data). The same group showed that anthracycline-containing regimens provided advantages in overall and recurrence-free survival rates over non-anthracycline-containing regimens [2]. Consequently, anthracycline-containing polychemotherapy became a standard therapy in the adjuvant setting.

In contrast, the use of combination treatments in the metastatic/advanced cancer setting is more controversial. A meta-analysis of trials in the metastatic setting published by *Fossati et al.* concluded that there was a survival benefit for polychemotherapy over single-agent therapy, the hazard ratio of 0.82 (0.75-0.90) translating into an 18% reduction in the risk of death [3]. However, most of the trials included were small (median number of patients <150), were sometimes poorly designed, and often featured old regimens. For example, the overall hazard ratio was biased by a 30% reduction in the risk of death in a subset of trials in which the combination treatment was based on cyclophosphamide and 5-fluorouracil (5-FU) [3]. The entire number of patients in the meta-analysis ($n < 1,000$) only modestly exceeds that in a single, recent, important randomized trial of sequential single-agent versus combination chemotherapy conducted by the Eastern Cooperative Oncology Group ($n = 739$) [4], as discussed later in this review.

An obvious important difference between the adjuvant and the metastatic disease settings is life expectancy. In the meta-analysis by *Fossati et al.*, anthracycline-containing combination regimens reduced the risk of death by 13% when compared with monotherapy [3], a reduction similar to that discussed above for polychemotherapy versus

single-agent therapy in the adjuvant setting. However, the life expectancy for patients receiving treatment in the metastatic setting is much shorter, typically months rather than years, than for those treated in the adjuvant setting. Furthermore, patients treated for metastatic disease typically receive several chemotherapy regimens, and there is general concern that any survival advantage may be counterbalanced by additive toxicity. Consequently, as treatment in the metastatic setting is palliative, tolerability and patients' quality of life are major factors guiding treatment decisions.

PRINCIPLES OF COMBINATION THERAPY DESIGN

Much of the concern about the toxicity and effectiveness of combination therapy has arisen from empirically chosen combination treatments. Ideally, a combination therapy should meet three criteria:

- Each component should have single-agent activity with no cross-resistance
- There should be preclinical evidence of synergy between the components
- The components should have nonoverlapping safety profiles

However, all three criteria are rarely met. Consequently, many combination therapies have failed to significantly improve outcomes compared with sequential administration of single agents because their components are administered at suboptimal doses due to dose-limiting toxicities.

COMBINATION THERAPIES THAT ARE NOT OPTIMALLY DESIGNED

An example of a combination therapy that does not meet all of the criteria described above, but which is frequently used in breast cancer, is the combination of a taxane and an anthracycline. There is little preclinical evidence of synergy, and myelosuppression is a major toxicity with both classes of agents [5]. The primary rationale for combining these agents is that each has high single-agent activity [6, 7]. Therefore, it is not surprising that the clinical impact of taxane/anthracycline combination regimens in metastatic breast cancer has been disappointing. Taxane/anthracycline combinations have been proven to be only marginally more effective and often more toxic treatments for metastatic breast cancer than anthracycline/cyclophosphamide combinations (Table 1) [8-13]. A difference in survival was reported for only one of five studies [9]. A superior response rate and superior time to disease progression were seen in two trials [9, 10], although the clinical relevance of a 5-week difference in time to disease progression at the

Table 1. Review of trials comparing anthracycline/taxane combinations with anthracycline/cyclophosphamide combinations

Reference	Regimen	Response rate (%)	Median time to disease progression (months)	Median overall survival (months)	Febrile neutropenia (%)
<i>Biganzoli et al.</i> , 2002 [8]	AP versus AC	58/54 NS	6.0/6.0 NS	20.6/20.5 NS	32/9 (<i>p</i> < 0.001)
<i>Jassem et al.</i> , 2001 [9]	AD versus FAC	68/55 (<i>p</i> = 0.032)	8.3/6.2 (<i>p</i> = 0.034)	23.3/18.3 (<i>p</i> = 0.013)	68/55 (<i>p</i> < 0.001)
<i>Nabholtz et al.</i> , 1999 [10]	AD versus AC	60/47 (<i>p</i> = 0.012)	7.3/8.5 (<i>p</i> = 0.01)	NR	33/10 (<i>p</i> < 0.001)
<i>Lück et al.</i> , 2000 [12]	EP versus EC	46/40 NS	9.0/7.4 NS	16.8/20.3 NS	2/2 NS
<i>Carmichael et al.</i> , 2001 [13]	EP versus EC	67/56 NS	6.5/6.7 (<i>p</i> = 0.072)	13.7/13.8 (<i>p</i> = 0.092)	NR

Abbreviations: A = doxorubicin; C = cyclophosphamide; D = docetaxel; E = epirubicin; F = 5-FU; P = paclitaxel; NR = not reported; NS = not significant.

Table 2. Doxorubicin plus vinorelbine combination therapy versus doxorubicin alone [14]

	Doxorubicin plus vinorelbine (<i>n</i> = 145)	Doxorubicin (<i>n</i> = 144)	<i>p</i> value
Overall response rate (%)	38.0	30.0	NS
Median duration of response (months)	7.2	6.8	NS
Median time to disease progression (months)	6.2	6.1	NS
Median overall survival (months)	13.8	14.4	NS

Abbreviation: NS = not significant.

median may be questionable [11]. In terms of safety, three trials reported markedly higher incidences of febrile neutropenia in the taxane/anthracycline arms than in the anthracycline/cyclophosphamide arms [8, 9, 11].

Another problematic drug combination according to the criteria above is doxorubicin/vinorelbine. Several trials have investigated anthracyclines with or without vinorelbine. In one of these, 303 patients with metastatic breast cancer were randomized to either doxorubicin, 50 mg/m² on day 1, plus vinorelbine, 25 mg/m² on days 1 and 8, or doxorubicin, 70 mg/m² alone on day 1 of a 21-day cycle [14]. As both components are associated with myelosuppression, toxicity was significant in the combination arm. After 16 of the first 59 patients developed febrile neutropenia, the regimens were changed to doxorubicin, 40 mg/m², plus vinorelbine, 20 mg/m², versus doxorubicin, 60 mg/m² alone. At the end of the trial, there was no significant difference between treatments for any of the efficacy parameters (Table 2). Moreover, toxicity was worse in the combination arm than in the monotherapy arm. There were significantly higher incidences of neurotoxicity/neuropathy, constipation, and venous reactions in the combination arm than in the monotherapy arm [14]. Doxorubicin/

vinorelbine, therefore, provides no advantage over doxorubicin monotherapy.

It has also been shown that CEF (cyclophosphamide/epirubicin/5-FU) does not offer superior efficacy to single-agent mitoxantrone [15]. In that study, 412 high-risk patients with metastatic breast cancer were randomized to either 500 mg/m² cyclophosphamide, 50 mg/m² epirubicin, and 500 mg/m² 5-FU, or to 12 mg/m² single-agent mitoxantrone, given every 3 weeks. There was no difference between the CEF arm and the mitoxantrone arm in terms of disease control (complete or partial response or stable disease: 66% versus 57% of patients, respectively, *p* = 0.182), time to disease progression (median 4.4 versus 6.2 months, respectively, *p* = 0.18), and overall survival (median 15.8 versus 14.1 months, respectively, *p* = 0.66).

In that study, quality-of-life-adjusted efficacy (modified Brunner score) was also calculated by adding scores assigned for time to disease progression, change in performance status, patient's subjective judgment of treatment utility, and toxicity. There were no significant differences between treatments for any of the parameters except toxicity (*p* = 0.0001). The overall quality-of-life-adjusted efficacy scores were -2.07 for the combination arm and 3.92

for the monotherapy arm, indicating that monotherapy was preferable to the combination therapy.

TRIALS COMPARING COMBINATION WITH SEQUENTIAL THERAPY REGIMENS

The trials described above and the meta-analysis by *Fossati et al.* have compared combination therapies with either other combination therapies or monotherapies [3]. However, perhaps a more pertinent question is how combination therapy compares with sequential chemotherapy. This question has largely gone unanswered, at least partly because the term “sequential chemotherapy” can be interpreted in several ways. It can mean sequential administration of chemotherapy, each new regimen introduced following disease progression, or it can mean a planned multicourse sequence of chemotherapy without a break between each different treatment. Furthermore, chemotherapy may be either monotherapy or polychemotherapy. Consequently, the design of trials comparing combination therapy with sequential therapy has been difficult, and only three trials have been reported examining this question to date.

The first trial to compare combination therapy with sequential therapy was a three-arm, phase III trial by *Sledge et al.* [4, 16]. A total of 739 chemotherapy-naïve patients were randomized to one of three treatment regimens: doxorubicin, 60 mg/m², followed at progression by paclitaxel, 175 mg/m²/day; paclitaxel, 175 mg/m²/day, followed at progression by doxorubicin, 60 mg/m²; or a combination

regimen of doxorubicin, 50 mg/m², plus paclitaxel, 150 mg/m²/day. The overlapping toxicity profiles of taxanes and anthracyclines and resulting risk of toxicity made it necessary to use lower doses of doxorubicin and paclitaxel in the combination arm than in the single-agent arms as well as G-CSF to ameliorate anticipated neutropenia.

The combination regimen resulted in a superior response rate and time to treatment failure compared with either of the sequential therapies (Table 3). However, combination therapy did not result in a survival benefit. There was no difference between the treatment arms in terms of safety or quality of life (functional assessment of cancer therapy-B scale).

Agents of the same class were used in a second, more recent trial comparing combination therapy with sequential therapy [17]. In this phase II trial, 193 patients were randomized to docetaxel, 100 mg/m², followed by doxorubicin, 75 mg/m², at progression (or after four cycles without progression), or to docetaxel plus doxorubicin combination therapy at one of two reduced dose levels: docetaxel, 75 or 60 mg/m², plus doxorubicin, 50 or 60 mg/m². Although there was no formal statistical analysis, there was no clinical difference among arms for overall survival, time to disease progression, response rate, or toxicity (Table 4). This trial, although smaller and less powerful, supports the notion that taxane/anthracycline combination therapy provides no advantage over sequential administration of the same agents.

Table 3. Doxorubicin and paclitaxel: combination therapy versus sequential therapy [16]

Regimen	A then P	P then A	A + P
Response rate (%)	34.0	33.0	46.0*
Median time to disease progression (months)	6.2	5.9	8.0*
Median overall survival (months)	20.1	22.2	22.4
Grade 3/4 adverse events (n of patients)	109.0	169.0	135.0
Treatment-related deaths (n of patients)	6.0	2.0	4.0

**p* < 0.01, A + P versus A then P and P then A

Abbreviations: A = doxorubicin, P = paclitaxel.

Table 4. Doxorubicin and docetaxel: combination therapy versus sequential therapy [17]

Regimen	T 75 mg + A 50 mg	T 60 mg + A 60 mg	T 100 mg then A 75 mg
Response rate (%)	49.0	12.0	21.0
Median time to disease progression (months)	6.7	8.3	6.9
Median overall survival (months)	11.9	14.5	13.8
Grade 3/4 neutropenia (%)	85.0	90.0	85.0

Abbreviations: A = doxorubicin; T = docetaxel.

The third trial to compare combination therapy with sequential therapy was conducted by *Joensuu et al.* [18]. This trial had a very relevant design in which 303 patients with metastatic breast cancer were initially randomized to either monotherapy or combination therapy as first- and second-line treatment. In the monotherapy arm, patients received epirubicin, 20 mg/m² every week until progression (or until the cumulative dose was 1,000 mg/m²), and then mitomycin C, 8 mg/m² every 28 days. In the combination therapy arm, patients received cyclophosphamide, 500 mg/m², epirubicin, 60 mg/m², and 5-FU, 500 mg/m² every 21 days until progression, followed by mitomycin C, 8 mg/m², plus vinblastine, 6 mg/m², every 28 days (*n* = 88). No significant difference in survival (*p* = 0.93) was found between the two arms, but quality of life and tolerability were more favorable in the sequential monotherapy arm.

The three trials described above comparing combination therapy with sequential monotherapy have not provided evidence of an advantage with the combination approach.

RATIONALLY DESIGNED COMBINATION THERAPIES

In contrast to the combinations described above, trastuzumab (a recombinant, humanized anti-HER2 antibody) plus a taxane represents a rationally designed combination fulfilling the criteria described earlier. Each component has single-agent activity with distinct mechanisms of action and without cross-resistance. There is also preclinical evidence of synergy: trastuzumab plus a taxane showed clearly enhanced antitumor activity in xenograft models [19]. Finally, the two components have nonoverlapping toxicities. It has now been established that trastuzumab plus paclitaxel offers a survival advantage of 25% over paclitaxel alone in women with metastatic breast cancer overexpressing HER2 [20].

Another rationally designed combination therapy is capecitabine plus docetaxel. Both agents have considerable single-agent activity and distinct mechanisms of action. There is preclinical evidence of synergy, as indicated in xenograft models [21]. Potential mechanisms underlying the observed synergy include thymidine phosphorylase (TP) upregulation and Bcl-2 downregulation, as shown in Figure 1 [21, 22]. Finally, the two agents have nonoverlapping key toxicities: the predominant toxicity with docetaxel is myelosuppression, but capecitabine monotherapy is associated with a particularly low incidence of myelosuppression.

Capecitabine/docetaxel combination therapy, compared with docetaxel monotherapy, significantly improves overall survival, time to disease progression, and response rates in women whose breast cancer has progressed during or following anthracycline treatment [23] (as described by *O'Shaughnessy et al.* pp. 4-12 [24]). Despite the lower dose

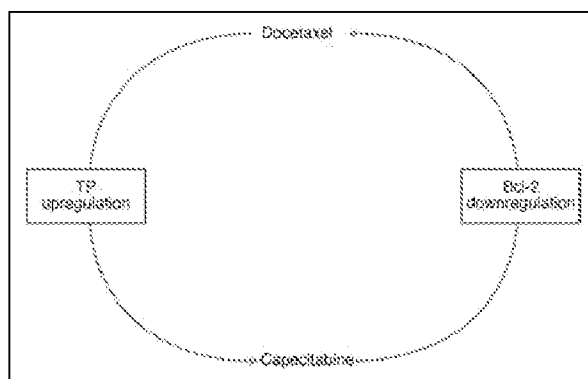


Figure 1. Mechanism of capecitabine/docetaxel synergy.

of docetaxel administered in the combination arm, the addition of capecitabine to docetaxel resulted in greater efficacy compared with the full dose of docetaxel monotherapy. The overall survival curves separated early and maintained a clear separation over time. This survival advantage, associated with the combination therapy, was maintained despite balanced numbers of patients receiving poststudy therapy [25]. In terms of safety, the incidence of grade 3/4 adverse events was higher in the combination arm than the docetaxel-only arm (71% versus 49%), due primarily to grade 3 hand-foot syndrome, which was managed by dose adjustment of capecitabine.

Of note, the survival benefit in the capecitabine/docetaxel combination arm was achieved without compromising quality of life. This is an important finding as the overall spectrum of toxicity was greater. Nevertheless, in that study, the impact of toxicity on perceived quality of life did not differ between treatment arms.

Figure 2 highlights an evolution of therapy in patients with anthracycline-pretreated breast cancer since *Nabholtz*

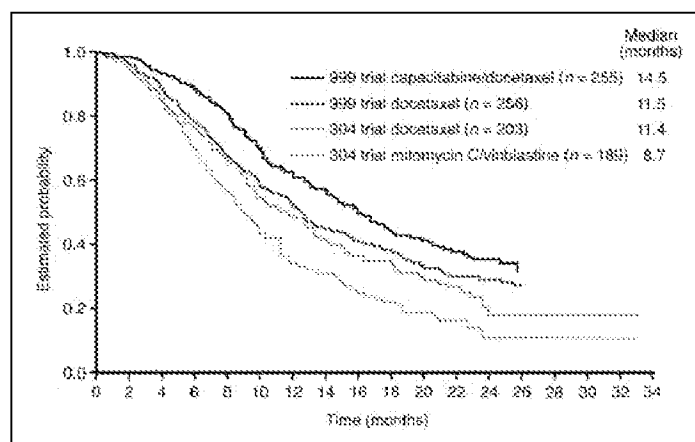


Figure 2. The evolution of therapy for anthracycline-pretreated metastatic breast cancer: probability of survival in two randomized trials.

et al. demonstrated that it was possible to extend the survival of such patients with docetaxel monotherapy, compared with mitomycin C plus vinblastine [26], to the current survival benefit offered by capecitabine plus docetaxel [23]. The combination of capecitabine plus docetaxel has not been evaluated against other combination regimens, but none of these have been shown to improve survival compared with docetaxel monotherapy.

However, it should be emphasized that neither the capecitabine/docetaxel trial [23] nor the trastuzumab/paclitaxel trial [20] has answered the question of whether combination therapy is preferable to sequential therapy because these combinations were compared only with monotherapy and a formal crossover was not part of the trial design. Indeed in the capecitabine/docetaxel trial, among the 164 patients initially randomized to docetaxel alone who received any kind of post-study chemotherapy, only one quarter (27%) subsequently received capecitabine monotherapy [23], and those patients appeared to have a survival advantage over those receiving other poststudy chemotherapies [25]. The survival advantage of combination over true sequential therapy (capecitabine then docetaxel or docetaxel then capecitabine) has not been demonstrated, and this question remains, as yet, unanswered.

THE CHOICE BETWEEN COMBINATION AND SEQUENTIAL THERAPY

Combination regimens appear to offer no advantage over sequential monotherapy in the metastatic setting, where palliation is traditionally the primary goal of treatment. However, newer, rationally designed combinations may offer clinically meaningful survival advantages over single monotherapy. Some cytotoxic combinations achieve this clinical benefit with a manageable safety profile, making them more attractive options in the metastatic setting. This was demonstrated in the capecitabine/docetaxel trial [23]. Furthermore, the early separation of the survival curves in the capecitabine/docetaxel trial shows that a high proportion of patients may benefit from combination treatment, whereas 30%–40% of patients initially randomized to docetaxel alone did not receive further therapy after disease progression. Combination therapy may be particularly useful in patients needing a prompt reduction in tumor burden

(e.g., patients with visceral disease and fast-growing tumors). Therefore, as in the adjuvant setting, survival data should play a more prominent role in motivating the choice of treatment.

However, as described above, the majority of combinations are more toxic than monotherapy alternatives, and the importance of maintaining quality of life cannot be underestimated in the metastatic setting. In the adjuvant setting, chemotherapy causes a temporary impairment in patients' quality of life. Nonetheless, most patients are willing to receive adjuvant chemotherapy with the potential for clinical benefit [27, 28]. In patients with metastatic disease, the survival benefit associated with treatment is generally modest at best, and therefore, maintenance of quality of life assumes greater importance, although patients may be unwilling to trade quantity for quality of life [29].

The newer drug combinations, however, do not entirely supplant sequential monotherapy in the metastatic setting. In multiple randomized trials designed to compare these strategies, no survival benefit has been seen with combination versus true sequential therapy. The combination of capecitabine/docetaxel has not yet been compared with true sequential therapy. Sequential monotherapy allows the optimal delivery of single-agent therapy, potentially reducing the risk of toxicity, and thereby improving quality of life. Sequential monotherapy may be especially appropriate in patients with slowly progressing tumors or in frail or elderly patients who may be unable to tolerate the toxicity of combination therapy.

It can be concluded that combination and sequential therapy may both have their place in the metastatic breast cancer setting, and the heterogeneity of breast cancer argues for flexibility in approach. For example, in patients for whom sequential monotherapy may not be optimal (e.g., those with more rapidly progressing disease and/or visceral metastases), capecitabine/docetaxel combination therapy could be considered optimal therapy. However, in some groups of patients, such as those with poor performance status or more indolent disease, sequential monotherapy may be more appropriate, and oral capecitabine, an active monotherapy for breast cancer, can be considered a valuable option.

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