Neuro-Oncology 15(2):189–197, 2013.
doi:10.1093/neuonc/nos305
Advance Access publication December 21, 2012

NEURO-ONCOLOGY

Comparing routes of delivery for nanoliposomal irinotecan shows superior anti-tumor activity of local administration in treating intracranial glioblastoma xenografts

Pin-Yuan Chen†, Tomoko Ozawa†, Daryl C. Drummond, Ashish Kalra, Jonathan B. Fitzgerald, Dmitri B. Kirpotin, Kuo-Chen Wei, Nicholas Butowski, Michael D. Prados, Mitchel S. Berger, John R. Forsayeth, Krystof Bankiewicz, and C. David James

Department of Neurological Surgery, University of California, San Francisco, California (P.-Y.C., T.O., N.B., M.D.P., M.S.B., J.R.F., K.B., C.D.J.); Department of Neurosurgery, Chang-Gung University and Memorial Hospital, Taoyuan, Taiwan (P.-Y.C., K.-C.W.); Graduate Institute of Clinical Medical Sciences, Chang-Gung University, Taoyuan, Taiwan (P.-Y.C.); Merrimack Pharmaceuticals, Cambridge, Massachusetts (D.D., A.K., J.B.F., D.B.K.)

Background. Liposomal drug packaging is well established as an effective means for increasing drug half-life, sustaining drug activity, and increasing drug efficacy, whether administered locally or distally to the site of disease. However, information regarding the relative effectiveness of peripheral (distal) versus local administration of liposomal therapeutics is limited. This issue is of importance with respect to the treatment of central nervous system cancer, for which the blood-brain barrier presents a significant challenge in achieving sufficient drug concentration in tumors to provide treatment benefit for patients.

Methods. We compared the anti-tumor activity and efficacy of a nanoliposomal formulation of irinotecan when delivered peripherally by vascular route with intratumoral administration by convection-enhanced delivery (CED) for treating intracranial glioblastoma xenografts in athymic mice.

Results. Our results show significantly greater anti-tumor activity and survival benefit from CED of nanoliposomal irinotecan. In 2 of 3 efficacy experiments, there were animal subjects that experienced apparent cure of tumor from local administration of therapy, as indicated by a lack of detectable intracranial tumor through bioluminescence imaging and histopathologic analysis. Results from investigating the effectiveness of combination therapy with nanoliposomal irinotecan plus radiation revealed that CED administration of irinotecan plus radiation conferred greater survival benefit than did irinotecan or radiation monotherapy and also when compared with radiation plus vascularly administered irinotecan.

Conclusions. Our results indicate that liposomal formulation plus direct intratumoral administration of therapeutic are important for maximizing the anti-tumor effects of irinotecan and support clinical trial evaluation of this therapeutic plus route of administration combination.

Keywords: convection-enhanced delivery, glioblastoma, irinotecan, liposome, xenograft.

Received September 7, 2012; accepted October 30, 2012.

†Authors contributed equally to this work.
Corresponding author: C. David James, PhD, Department of Neurological Surgery, University of California San Francisco, 1450 Third Street, Room HD-283, San Francisco, CA, 94158 (david.james@ucsf.edu).

The benefits of liposomal drug packaging have been well documented and include improved drug half-life, sustained drug activity, and increased drug efficacy. We and others have shown that liposomal formulation increases the activity of cytotoxic drugs when used in treating intracranial xenografts established

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from human glioblastoma (GBM), the most common and malignant of primary brain tumors in adults, irrespective of whether the liposomal drug is administered directly into the tumor or peripherally by a vascular route.[1–8]

Prior studies, however, have not compared the relative efficacy of peripheral and local administration of liposomal therapeutics for treating brain tumors. Information from such comparisons would help increase understanding of the influence of the blood-brain barrier (BBB) in limiting drug access to tumors and the extent to which therapeutic benefit is affected by peripheral administration of liposomal drugs.[9,10] This understanding, in turn, could influence clinical trial design for testing novel therapeutics or therapeutic formulations in treating patients with brain tumors.

Although there is widespread understanding and appreciation of BBB-restricted access of therapy to intracranial tumors, this concern tends to be lessened with respect to the conduct of clinical trials by animal model studies, the results of which show activity of peripherally administered therapeutics against brain tumors. Such results, combined with the expectation of repeated administration of therapy through oral or vascular routes, to achieve comparable if not superior anti-tumor activity, relative to a single intratumoral administration of drug, have been instrumental in promoting the preferential use of peripheral administration of therapy for treating patients with brain tumor.

Convection-enhanced delivery (CED) for local administration of therapy is an alternative to vascular administration that bypasses the BBB and delivers therapeutic agents directly into the brain.[11] Previously, we have shown that the use of controlled pressure and a specially designed cannula are key to maximizing the distribution of CED-administered therapy in the brains of animal subjects,[12,13] which is a critically important issue for effective treatment of GBM, the majority of which grow in an infiltrative, disseminated manner.

Irinotecan has been extensively studied as a therapeutic agent for glioma (reviewed by Vredenburgh et al.[14]) based on promising in vitro and in vivo preclinical results, its BBB penetrance, and its distinct mechanism of action, as compared with other agents used in the treatment of these tumors. Clinical experience with irinotecan has been at least modestly promising, with this therapeutic showing activity both as a single agent and in combination with other modalities. However, the free drug is associated with complex pharmacologic interactions, less than ideal pharmacokinetic properties, and toxicity. Liposomal formulation improves on irinotecan's pharmacokinetics, reduces toxicity, and, if delivered locally, could be combined with other treatment approaches as an attractive addition to brain tumor therapy.[1,4]

In the current report, we present the results of a study in which we have conducted experiments for comparing peripheral intravascular and CED administration of nanoliposomal irinotecan for efficacy against 2 distinct orthotopic xenograft models of GBM. Our data reveal that a single administration of nanoliposomal irinotecan by CED is significantly more effective than multiple systemic intravascular administrations of the same therapeutic and is safe for animal subjects. These results provide strong support for CED administration of nanoliposomal irinotecan in the investigational treatment of GBM.

Materials and Methods

Investigational Agent

Nanoliposomal irinotecan (MM-398) is a highly stabilized liposomal formulation containing nano-sized irinotecan crystals complexed with sucrose octasulfate in the liposome interior[15] and was generously provided by Merrimack Pharmaceuticals (Cambridge, MA). The preparation of nanoliposomal irinotecan, used in the experiments that follow, had a particle size of 111 nm, as determined by dynamic light scattering, and a drug-to-phospholipid (PL) ratio of 488 g irinotecan/mol PL.

GBM Xenografts

Human GBM primary tissues, GBM43 and SF7796, are maintained as serially passaged subcutaneous xenografts in athymic mice.[16,17] Both GBM43 and SF7796 have been modified by lentiviral infection for stable expression of firefly luciferase to enable in vivo bioluminescence imaging, as previously described.[18]

To prepare tumor cells from subcutaneous xenografts for transfer to the intracranial compartment, excised subcutaneous tumors were placed in culture dishes and minced with a scalpel then mechanically dispersed by repetitive pipetting to create small cellular aggregates that were passed repeatedly through 40-micron nylon mesh filters to produce single-cell suspensions. Cell suspensions were centrifuged at a rate of 1000 rpm for 10 min at 4°C and supernatants aspirated before resuspending pellets in 1 mL of sterile DMEM media. Cells were counted and then diluted to 1×10^5 cells/μL for intracranial injection.[19]

Intracranial Tumor Establishment in Athymic Mice

Five-week-old female athymic mice (nu/nu, homozygous; Simonsen Laboratories, Gilroy, CA), housed under aseptic conditions, received intracranial tumor cell injection as previously described[17] and as approved by the University of California San Francisco Institutional Animal Care and Use Committee. In brief, mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and then were injected with 3 μL of tumor cell suspension (300 000 cells total) into the right caudate putamen.

Bioluminescence Monitoring of Intracranial Tumor Growth

In preparation for bioluminescence imaging (BLI), mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), then administered 150 mg/kg of luciferin (D-luciferin potassium salt; Gold Biotechnology, St. Louis,

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MO) via intraperitoneal injection. Ten minutes after luciferin injection, mice were examined for tumor bioluminescence with an IVIS Lumina imaging station (Caliper Life Sciences, Alameda, CA). Regions of interest, defined using Living Image software (Caliper Life Sciences, Alameda, CA), were recorded as photons per second per steradian per square centimeter.[19] Beginning at 1 week after intracranial tumor cell injection, mice were imaged twice weekly until deaths were observed in control (untreated) mice, with data from the last imaging session used to evaluate the effect of therapy on tumor growth.

Vascular and Intratumoral Administration of Nanoliposomal Irinotecan

For vascular administration of therapy,[20] mice were warmed for 5–10 min with either a heating pad or a heat lamp to dilate tail vasculature. Injection sites were then cleaned with an alcohol swab, after which a 28 g a insulin syringe was inserted, with 50 or 100 μL liposomal drug that had been diluted in 5 mM HEPES-buffered saline (pH, 6.5) to a concentration of 0.004 mg irinotecan per microliter, and injected with steady pressure over 5–10 s. For CED administration, our approach was similar to that previously described.[21] In brief, infusion cannula were made with silica tubing (Polymicro Technologies, Phoenix, AZ) fused to a 0.1 mL syringe (Plastic One, Roanoke, VA) with a 0.5-mm stepped-tip needle that protruded from the silica guide base. Syringes were loaded with liposomal drug (0.04 mg per microliter) and attached to a microinfusion pump (Bioanalytical Systems, Lafayette, IN). The syringe with silica cannula was mounted onto a stereotactic holder then lowered through a puncture hole made in the skull[19,20] and to the same region in the caudate putamen at which tumor cells had been previously injected. Nanoliposomal irinotecan was infused at a rate of 1 μL/min until a volume of 5 or 10 μL had been delivered. Cannulae were removed 2 min after completion of infusion.

Mouse Irradiation

Mice were anesthetized via inhalation of 2.5% isoflurane with 1 L of oxygen per minute for 5 min prior to being positioned on an irradiation platform located 16.3 cm from a cesium-137 source (J. L. Shepherd & Associates, San Fernando, CA). Their eyes, respiratory tracts, and bodies were protected with lead shielding. Mice received whole brain irradiation at a dose rate of 247 cGy/min[22] until 1.5 Gy radiation had been administered. After irradiation, animals were monitored until recovery. For the experiment involving the analysis of combination therapy efficacy, mice were irradiated once daily for 5 consecutive days, Monday through Friday, with the first radiation treatment on day 7 following tumor cell implantation.

Analysis of Irinotecan Content in Intracranial Tumors

For the experiment involving analysis of tumor irinotecan content, athymic mice with intracranial GBM43 were administrated 0.4 mg of irinotecan by tail vein or directly into the tumor mass on day 13 after implantation of tumor cells and 30 min after the fifth and final of radiation fractions that had been initiated on day 9. Mice were euthanized 24 h after nanoliposomal irinotecan administration, with brains immediately resected and tumor tissue dissected prior to snap-freezing by immersion in liquid nitrogen. Analysis of irinotecan levels in tumor tissues was as described previously.[1] In brief, water was added to tissues at a 20% (w/v) ratio, and tissues were then homogenized with a mechanical homogenizer in an ice bath. Homogenates were extracted for the lactone form of irinotecan with an acidic methanol solution by vortexing and centrifugation at 13 000 rpm for 10 min, with the supernatants then transferred to autosampler vials for Dionex high-pressure liquid chromatography (HPLC) analysis.

Immunohistochemistry

Resected mouse brains were fixed in 10% buffered formalin, then paraffin-embedded and sectioned for hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) analysis. To determine cleaved caspase-3 reactivity, unstained sections were processed with a Ventana BenchMark XT automated system and a protocol consisting of pretreatment with 3% ethanolic hydrogen peroxide for 32 min at room temperature, epitope retrieval in Tris buffer (pH 8) for 8 min at 90°C, and incubation with primary antibody to cleaved caspase-3 (#9661, Cell Signaling Tech., Danvers, MA) at 0.2 mg/mL for 1 h at 37°C. Total and activated caspase-3–positive cells were counted in 5 high-powered fields within the tumor for each stained tissue section, with percent positive cells averaged for all fields associated with a specific treatment and subjected to statistical analysis as described below.

Statistical Analysis

PRISM 5, version 5.03 (GraphPad Software), was used to conduct all statistical analyses. For survival analysis, significance was determined by the log-rank (Mantel- Cox) test. Animals without tumor burden that died accidentally during anesthesia were excluded from the survival analysis. For all other statistical analyses, either a 2-tailed unpaired *t* test or Tukey's multiple comparison test was applied.

Results

Comparison of Intravascular Versus CED Administration of Nanoliposomal Irinotecan for Anti-Tumor Activity

Our initial experiment for comparing intracranial GBM xenograft response to peripherally and CED administered nanoliposomal irinotecan used GBM43, which is maintained as a serially propagated subcutaneous

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xenograft[16,17] and has been previously classified as a proneural GBM.[23] GBM43 cells, harvested from a disaggregated subcutaneous xenograft, produce rapidly growing intracranial tumors that have been shown to be relatively resistant to radiation therapy.[24]

Our experimental design included 3 CED and 2 intravascular administration treatment groups. CED administration was either once at 0.4 mg irinotecan, or twice at either 0.2 or 0.4 mg irinotecan each time. Intravascular administrations were 4 times at either 0.2 or 0.4 mg irinotecan administered each time. Thus, total irinotecan administered by CED was either 0.4 or 0.8 mg irinotecan, whereas total irinotecan administered by intravascular injection was either 0.8 or 1.6 mg. CED administrations were on day 5 only or on days 5 and 8, and intravascular administrations were on days 5, 8, 12, and 15 after tumor cell implantation. BLI of luciferase-modified GBM43 tumors showed an anti-tumor effect from irinotecan administration, regardless of amount or route of administration (Fig. 1A) and significantly ($P < .05$) or near significantly greater anti-tumor effect of direct over intravascular administration, irrespective of the amounts of administered irinotecan being compared (P values for all 2-way comparisons shown in Table 1).

Survival analysis from these treatments showed that all irinotecan treatment groups experienced significant survival benefit relative to control (Fig. 1B). Of importance, comparisons of all CED and intravascular administration groups showed significantly greater benefit from direct administration of therapy, even when the total amount of irinotecan administered by the vascular route was 4 times greater than that delivered directly into the tumor. More striking was the difference in number of mice that experienced apparent cure of tumor from CED of nanoliposomal irinotecan (6 of 7 in the group receiving 0.8 mg irinotecan, and a total of 4 of 18 in the 2 groups receiving 0.4 mg irinotecan), as indicated by a lack of detectable bioluminescence signal in mice at time of euthanasia. Serial sectioning of entire brains from 2 of the mice receiving CED administration of nanoliposomal irinotecan and showing lack of detectable bioluminescence at time of euthanasia revealed no detectable tumor on histopathologic analysis of H&E-stained tissues. None of the mice receiving intravascular administration of nanoliposomal irinotecan experienced cure of tumor.

Body weight monitoring of mice receiving vascular and CED of nanoliposomal irinotecan showed similar patterns of weight loss, irrespective of route of delivery (Fig. 1), with no animal losing >13% initial weight from treatment. All animals receiving liposomal therapy rapidly gained weight on completion of treatment and achieved weights comparable to or exceeding that of untreated control group mice prior to onset of symptoms indicative of tumor burden in control group mice, suggesting that administration of nanoliposomal irinotecan by either route of administration is without extended adverse effect and is well tolerated.

To investigate whether these results might prove to be generalizable to additional subtypes of GBM, we performed a second experiment with SF7796, which was

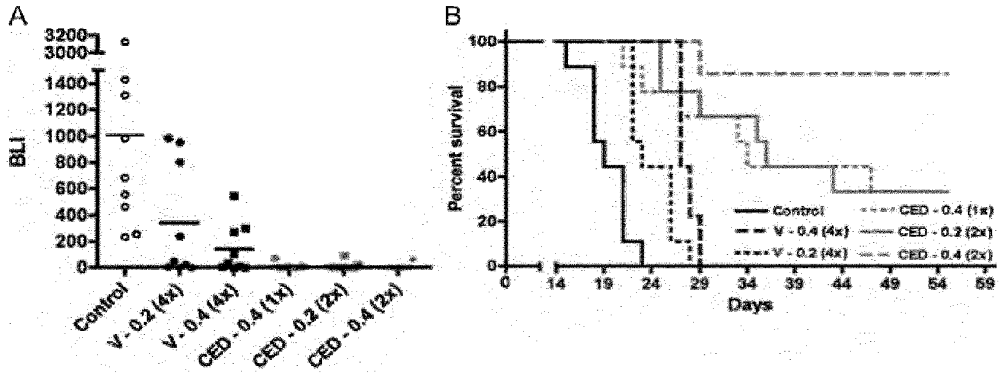


Fig. 1. Comparison of intravascular vs CED administration of nanoliposomal irinotecan for anti-tumor activity against intracranial GBM43 and corresponding survival benefit for animal subjects. (A) Treatment group day 15 normalized bioluminescence[18] (BLI) distributions (last imaging day in which all control group mice were alive). Route of administration identifiers: V, vascular administration; CED, convection enhanced delivery. Numbers following route of administration identifiers represent mg quantity of irinotecan administered with each dose; numbers in parentheses represent the number of administrations. Direct administrations were on day 5 or on days 5 and 8, and vascular administrations were on days 5, 8, 12, and 15. Student's t-test values for all 2-way comparisons are listed in Table 1s. (B) Corresponding survival plots for each treatment group. Log rank P values for all 2-way comparisons are listed in Table 2 and show that the survival benefit for mice receiving 2 CED administrations of 0.4 mg irinotecan was significantly greater ($P < .05$) than for any other treatment. Mice surviving at day 60, all of which had no detectable tumor by bioluminescence imaging, were euthanized, with analysis of serial H&E-stained sections of entire brains from 2 of these mice showing no detectable tumor. Control group mice in this experiment were untreated, which were established as valid for comparison by determining, in a separate experiment, that CED of vehicle caused no adverse or beneficial effect on animal survival relative to no treatment (Supplementary Figure 2). Number of mice included in the survival analysis for each treatment group (see Materials and Methods): Control = 9; CED 0.2 (2x) = 9; CED 0.4 (1x) = 9; CED 0.4 (2x) = 7; V 0.2 (4x) = 9; and V 0.4 (4x) = 9.

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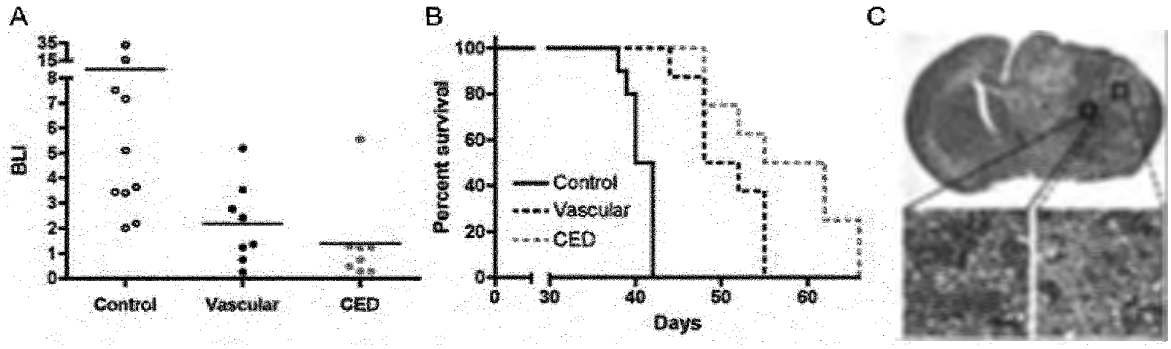


Fig. 2. Comparison of intravascular with CED administration of nanoliposomal irinotecan for anti-tumor activity and survival benefit for mice with intracranial SF7796. (**A**) Treatment group day 35 bioluminescence distributions (last imaging day for which all control group mice were alive). Mice receiving vascular administration received 0.4 mg doses of irinotecan on days 20 and 24 (0.8 mg total dose), whereas mice receiving CED administration were treated just once with 0.4 mg irinotecan on day 20 after tumor cell implantation. Student's *t*-test values for 2-way comparisons: 0.092 for control vs vascular; 0.061 for control vs CED; and 0.359 for vascular vs CED. (**B**) Corresponding survival plots for each treatment group. Log rank *P* values for 2-way comparisons: <0.001 for control vs vascular; <0.001 for control vs CED; and 0.048 for vascular vs CED. Number of mice included in the survival analysis for each treatment group: Control = 10; Vascular = 8; CED = 8. (**C**) 1.25x (upper) and 40x (lower) magnifications of an H&E-stained coronal section from the brain of a control group mouse that was euthanized on day 39 because of symptoms from increasing tumor burden. Lower left panel: high cellularity at the tumor core. Lower right panel: disseminated tumor cells at the tumor periphery.

established and has been maintained as a subcutaneous xenograft after initial implantation of a surgical specimen from a patient whose tumor had regrown after standard-of-care therapy (radiation + temozolomide[25]), which was followed by treatment of the recurrent tumor with bevacizumab[26] prior to second surgery. This GBM xenograft has been classified as mesenchymal using the classification scheme described by Verhaak et al.[23] For assessing SF7796 response to nanoliposomal therapy, we compared the anti-tumor effect and survival benefit of 0.4 mg irinotecan administered once by CED (day 20 after implantation) with 0.8 mg administered by intravascular route (0.4 mg administered twice: days 20 and 24). As for the previous experiment with GBM43, the results for SF7796 showed irinotecan anti-tumor activity by BLI (Fig. 2A) and significant survival benefit from treatment, irrespective of whether administered directly or intravascularly (Fig. 2B). SF7796 tumor cells produce a diffusely infiltrative intracranial tumor (Fig. 2C), and it is likely that this diffusely infiltrative nature, combined with the later time of treatment initiation for this experiment (day 20 vs day 5 for the initial experiment with GBM43), resulted in none of the mice with intracranial SF7796 experiencing cure of tumor from treatment. However, as before with the GBM43 model (Fig. 1), the survival benefit from one CED treatment was significantly greater than that resulting from multiple (2) intravascular administrations of liposomal irinotecan ($P = .048$).

Effect of Radiation when Combined with Intravascular or Intratumoral Nanoliposomal Irinotecan Therapy

The design for the prior 2 experiments (Figs. 1 and 2) is consistent with that which might be used in a clinical study for treating recurrent GBM and for which investigational therapies are often evaluated in isolation from co-treatment with other therapeutics and/or treatment modalities. To evaluate the activity of nanoliposomal irinotecan in a context consistent with a clinical trial for treating newly diagnosed GBM, we compared the anti-tumor activity of CED with intravascular administration of nanoliposomal irinotecan when used in combination with radiation therapy (RT). For this experiment, radiation was administered at 1.5 Gy/day × 5 (7.5 Gy total) beginning day 7 after implantation of GBM43 cells, with irinotecan administered once by CED (0.4 mg on day 7) or twice by the vascular route (0.4 mg on days 7 and 11). As with the previous GBM43 experiment (Fig. 1), CED of irinotecan outperformed intravascular administration, even though twice as much irinotecan was administered by the vascular route (Figs. 3A and B; $P = .035$ for survival benefit comparison). RT, which as a monotherapy, provided modest, albeit statistically significant, survival benefit to mice with intracranial GBM43 (Fig. 3B; $P = .011$ for RT vs control), further increased the anti-tumor effect and survival benefit from nanoliposomal irinotecan, with direct administration of irinotecan + RT providing the most extensive survival benefit of any treatment (Fig. 3B) and resulting in half (5 of 10) of the treatment group mice experiencing apparent cure of tumor. As for previous experiments, therapeutic regimens were well tolerated, with no animal subject experiencing >10% loss of pretreatment body weight at completion of therapy (data not shown).

For this latter experiment, untreated mice were included for euthanasia at day 7 to obtain samples providing indication of extent of intracranial tumor at time of treatment initiation (Fig. 4A) and at day 12 for all treatment groups (Figs. 4B–F) to allow qualitative

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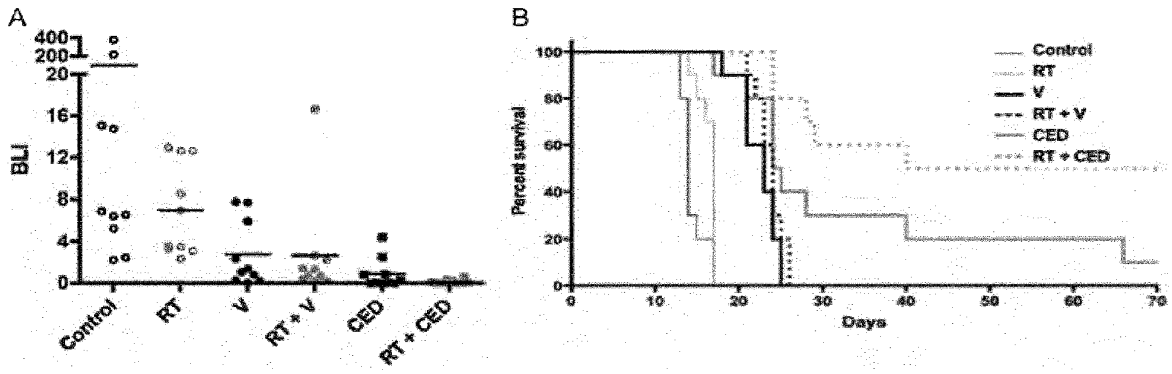


Fig. 3. Effect of radiation when combined with intravascular or CED administration of nanoliposomal irinotecan therapy. Radiation treatment for this experiment was 1.5 Gy/day x 5, beginning day 7 and ending day 11, for a total radiation dose of 7.5 Gy. Nanoliposomal irinotecan administration was 0.4 mg on day 7 for CED administration and was 0.4 mg on days 7 and 11 for intravascular administration. (A) Treatment group day 10 bioluminescence distributions (last imaging day at which all control group mice were alive). See Table 3 for all 2-way comparisons using the Student's *t*-test. (B) Corresponding survival plots for all treatment groups. Log rank *P* values for all 2-way comparisons are listed in Table 4 and show that CED administration of irinotecan as a monotherapy was significantly better than intravascular and RT monotherapies and that RT + CED of irinotecan was significantly better than all other therapies, including irinotecan monotherapy via CED. The experiment was terminated at 70 days, at which time there was no detectable bioluminescence signal in surviving mice. Ten mice included in the survival analysis for all treatment groups.

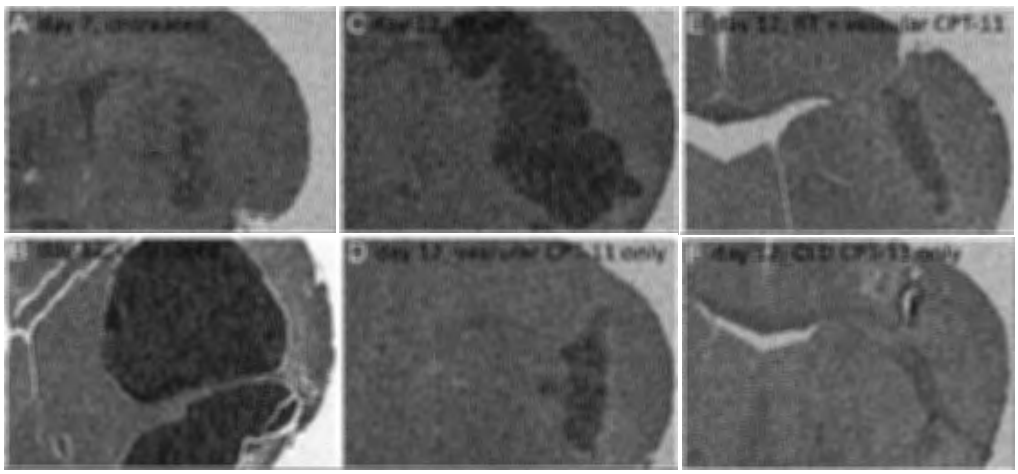


Fig. 4. H&E-stained coronal sections (2.5× magnification) from the brain of an untreated mouse that was euthanized on the first day of therapy (A) and from the brains of mice that were euthanized from each treatment group one day after the last day of therapy (day 12: panels B-F). H&E-stained sections from the brain of a mouse receiving RT + CED administration of nanoliposomal irinotecan (not shown) appeared to be similar to those of the mouse receiving local administration of liposomal therapy only (F) and showed a lack of identifiable tumor from combination therapy at day 12 after tumor cell implantation.

comparison of relative tumor size among treatment groups and quantitative IHC analysis of activated caspase 3 staining for assessing the pro-apoptotic effect of different treatments at 1 day after completion of therapy (Fig. 5). Inspection of H&E-stained tissues for sections with the largest tumor areas showed reasonable consistency between treatment effect on amount of H&E-stained tumor at time of completing therapy (Figs. 4B–F) and eventual treatment group survival (Fig. 3B). This was also the case for the activated caspase 3 IHC analysis, which showed the most extensive apoptotic response in mice receiving direct administration of nanoliposomal irinotecan + radiation therapy (Fig. 5; $P < .05$ for CED + RT vs any other treatment group; Student's *t* test results for all Fig. 5G; 2-way comparisons are shown in supplementary Table 5).

Analysis of Irinotecan Content in Intracranial Xenografts

To obtain information addressing whether radiation alters access of peripherally administered nanoliposomal irinotecan to intracranial tumor or, alternatively, alters retention of irinotecan when therapy is administered locally, additional mice received intracranial

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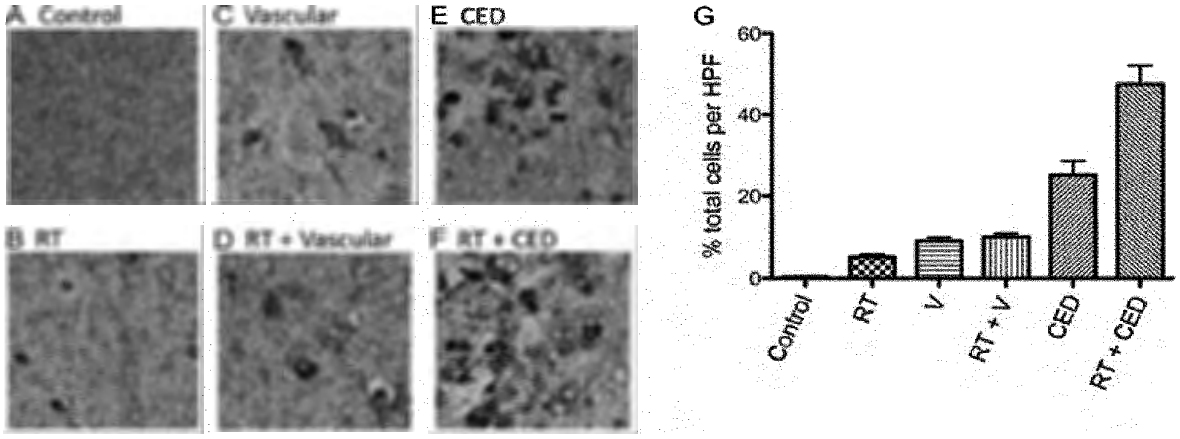


Fig. 5. (A–F) Examples of activated caspase 3 staining of tumors in the brains of mice from each of the treatment groups described in Fig. 3. Specimens were obtained from mice that were euthanized on the day after final treatments (day 12). (G) Histogram plot showing average values for percent positive cells from 5 high-powered fields examined in the brains of each of 3 mice from each treatment group: results are therefore based on a total of 15 high-powered fields per treatment group. Student's t test comparison of these values showed that radiation + CED administration of nanoliposomal irinotecan was significantly more effective in inducing apoptotic response, relative to all other treatment groups ($P < .05$).

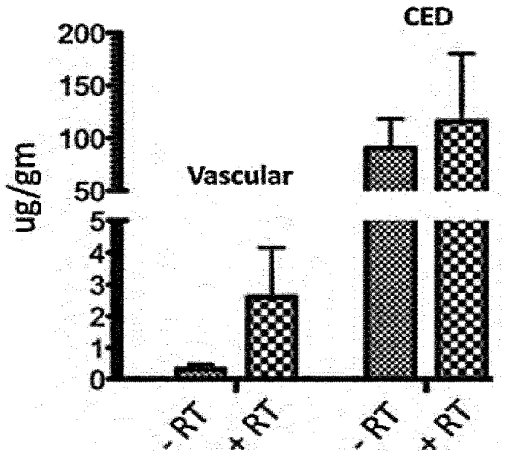


Fig. 6. Analysis of irinotecan content in intracranial GBM43 xenograft tumors 24 h after either vascular or CED administration of 0.4 mg nanoliposomal irinotecan and either in the presence or absence of radiation, with the last of 5 radiation treatments administered 30 min prior to the single irinotecan administration at day 13 subsequent to tumor cell implantation. The results show that RT does not cause a significant difference in tumor irinotecan, whether administered directly or by vascular route, but do show a significantly higher amount of irinotecan content in tumors receiving direct administration of therapy ($P < .05$). Analyzable samples were 2–4 for each treatment group.

implantation of GBM43, with tumors allowed to grow until day 9, at which time mice began receiving daily radiation for 5 consecutive days (1.5 Gy/day, 7.5 Gy total), with 0.4 mg nanoliposomal irinotecan administered either by CED or by a vascular route on day 13, the last day of radiation treatment. Twenty-four hours later, the mice were euthanized and tumor was dissected from surrounding normal brain of the euthanized mice, with dissected tumors subsequently examined for irinotecan content. The results of this analysis showed no significant difference in irinotecan content in tumors as a result of mice receiving pretreatment with radiation, although for mice receiving vascular administration of therapy, the mean value for tumor irinotecan was substantially higher in the group receiving radiation (Fig. 6). Not surprisingly, intratumoral irinotecan was substantially and significantly higher in mice receiving CED administration of liposomal therapy, irrespective of radiation pretreatment: 302-fold greater when comparing direct vs vascular administration in nonirradiated mice and 45-fold greater when comparing irinotecan content in mice from irradiated groups.

Discussion

In the current report, we have presented results addressing the relative activity and efficacy of intravascular and CED administration of nanoliposomal irinotecan in treating mice with orthotopic GBM xenografts. To the best of our knowledge, there are no previously published reports involving a route of administration comparison for liposomal therapy in treating an experimental animal model of GBM. Although our study is not exhaustive with respect to potential experimental variations, we feel that there is sufficient consistency of results for the variables tested in 2 distinct intracranial xenograft models to support the interpretation of CED administration of irinotecan nanoliposomes as being the more effective administration for maximizing anti-tumor effect of this therapy. Our cumulative experience in this area of research indicates that it is the combined effects of nanoliposomal packaging for extending the biologic half-life of active drug[1–5] and

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bypassing the limiting influence of the BBB through direct intratumoral administration of therapy[1–4] that are important for maximizing the anti-tumor effect of cytotoxic chemotherapy.

Our interpretation of these results is not at odds with clinical trial designs that use intravascular administration of liposomal therapy to treat GBM, which is an approach that, according to our results, could well provide benefit to patients with brain tumors. Our results do, however, support a clinical trial design in which nanoliposomal irinotecan is administered locally.

The advantage of CED is multifactorial. The combination of cannulae that minimize reflux[21] and the liposomal formulation of irinotecan allows for more robust and uniform distribution of the therapeutic. Catheter placement into an intact tumor, confirmed by neuronavigational methods with direct imaging assessment of catheter position and subsequent convective infusion of the liposome using real-time imaging,[13] would be the optimal strategy for initial clinical studies of this agent. This clinical setting would eliminate the risk of drug reflux back into a surgical cavity seen when using CED strategies at the time of surgical resection and minimize the risk of improper placement of catheters, often seen after expected changes in the geometry of the cavity hours to days after resection.

The increased efficacy of CED administration of therapy is consistent with the substantial disparity in irinotecan content of xenograft tumors removed 24 h after treatment of animal subjects with equivalent vascular and intratumoral amounts of liposomal drug (Fig. 6). In a previous study, we showed that CED of nanoliposomal irinotecan sustains higher intracranial levels of irinotecan, relative to intracranial administration of free irinotecan, and that CED administration of nanoliposomal irinotecan outperforms direct intratumoral administration of equivalent-free irinotecan.[1] Thus, liposomal formulation is important to maximizing anti-tumor activity and, potentially, clinical benefit from CED administration of irinotecan.

For the present study, it is noteworthy that the nanoliposomal irinotecan preparation used for direct intracranial administration was the same as that used for intravascular administration, and this formulation was developed for intravascular administration of therapy. Thus, it is conceivable that alternative nanoliposomal irinotecan formulations may further improve on the intracranial distribution and efficacy of CED administration of nanoliposomal irinotecan. Despite the use of vascular-optimized nanoliposomal irinotecan, our results suggest the diffusion of locally administered liposomal therapy to an extent that is effective in eradicating, at least in some instances, tumor that occupies a substantial portion of total brain (Figs. 4A and F). Because mice experiencing apparent cure of intracranial tumor (Figs. 1 and 3) were treated with 5–10 μL of liposomal therapy, our results support the concept that this therapeutic approach involving local administration of therapy is sufficiently scalable to anticipate efficacy against brain tumor in patients with GBM.

In addition to the superior efficacy of local administration of nanoliposomal irinotecan, it is important to emphasize the apparent safety of direct intracranial administration of therapy, as indicated by body weight monitoring of treated mice (Fig. 1), and lack of an indication of neurologic deficit (e.g., ambulation, activity, and seizure) from CED of liposomal therapy. Future preclinical studies will focus on dose escalation experiments to identify maximum tolerable amounts of nanoliposomal irinotecan that can be administered directly into brain and the optimization of convection-enhanced delivery approaches for sustained CED administration of nanoliposomal irinotecan.

Finally, our results show that CED administration of nanoliposomal irinotecan can be used with radiation therapy for further improvement of anti-tumor effect and survival benefit, relative to either monotherapy, and thereby support the potential use of direct administration of liposomal therapy with subsequent standard-of-care therapy for treating GBM: RT + temozolomide.[25] With irinotecan being administered locally, one would anticipate a lack of adverse effects associated with the peripheral administration of 2 cytotoxic therapies and that local administration of nanoliposomal irinotecan could be used safely with conventional treatment for newly diagnosed GBM. Thus, our results support the clinical trial evaluation of direct intratumoral administration of nanoliposomal irinotecan, both as a single agent in the treatment of recurrent GBM and as part of a combination therapy for patients with newly diagnosed GBM.

Supplementary Material

Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

Funding

This study was supported by National Cancer Institute (grant CA097257 to T. O., M. D. P., M. S. B., K. B., C. D. J.).

Acknowledgments

We thank Raquel Santos, Edgar Lopez-Lepe, Jacqueline De La Torre, and Christina Ng for their expert technical assistance.

Conflict of interest statement. D. C. D., A. K., J. B. F., and D. B. K. are employees of Merrimack Pharmaceuticals, the supplier of the therapeutic investigated in the study reported herein. All other authors declare no conflict of interest.

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GSPC Exhibit 1122
Page 9 of 375

Electronic Patent Application Fee Transmittal

Application Number:	15809815
Filing Date:	10-Nov-2017
Title of Invention:	Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin
First Named Inventor/Applicant Name:	Eliel Bayever
Filer:	Mary Rucker Henninger/Dawn MacPherson
Attorney Docket Number:	01208-0007-01US

Filed as Large Entity

Filing Fees for Utility under 35 USC 111(a)

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
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Pages:				
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Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
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		Total in USD ($)		**260**

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EFS ID:	45192010
Application Number:	15809815
International Application Number:	
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/Dawn MacPherson
Filer Authorized By:	Mary Rucker Henninger
Attorney Docket Number:	01208-0007-01US
Receipt Date:	10-MAR-2022
Filing Date:	10-NOV-2017
Time Stamp:	18:33:16
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
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Payment was successfully received in RAM	$260
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	Group Art Unit: 1612
Eliel BAYEVER et al.	
Application No.: 15/809,815	Examiner: Gollamudi S. Kishore
Filed: November 10, 2017	Confirmation No.: 5137
For: Methods for Treating Metastatic Pancreatic Cancer Using Combination Therapies Comprising Liposomal Irinotecan and Oxaliplatin	

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Respectfully submitted,

McNeill Baur PLLC

Dated: March 10, 2022

By: /Mary R. Henninger/
Mary R. Henninger
Reg. No. 56,992
404-891-1400

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(Not for submission under 37 CFR 1.99)

Application Number	15809815
Filing Date	2017-11-10
First Named Inventor	Eliel Bayever
Art Unit	1612
Examiner Name	Gollamundi S. KISHORE
Attorney Docket Number	01208-0007-01US

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15809815
	Filing Date	2017-11-10
	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Gollamundi S. KISHORE
	Attorney Docket Number	01208-0007-01US

	1	EP3337467: Sandoz AG Opposition dated September 9, 2021, 24 pages.
	2	EP3337467: Sandoz AG Opposition dated September 9, 2021, D1 (LORUSSO P, et al., "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," J Clin Oncol. 29(15) Suppl:3000 (2011), 2 pages).
	3	EP3337467: Sandoz AG Opposition dated September 9, 2021, D1a (LORUSSO P, et al., "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," Presentation presented at American Society of Clinical Oncology 2011 Meeting, 37 pages).
	4	EP3337467: Sandoz AG Opposition dated September 9, 2021, D2 (BERLIN J, et al., "A Phase 1 Dose-Escalation Study of Veliparib with Bimonthly FOLFIRI in Patients with Advanced Solid Tumors," J Clin Oncol. 32(15) Suppl:2574 (2014), 4 pages).
	5	EP3337467: Sandoz AG Opposition dated September 9, 2021, D3 (TAHARA M, et al., "The Use of Olaparib (AZD2281) Potentiates SN-38 Cytotoxicity in Colon Cancer Cells by Indirect Inhibition of Rad51-Mediated Repair of DNA Double-Strand Breaks," Mol Cancer Ther. 13(5):1170-80 (2014)).
	6	EP3337467: Sandoz AG Opposition dated September 9, 2021, D4 (NEIJZEN R, et. al., "Irinophore C™, a Lipid Nanoparticle Formulation of Irinotecan, Improves Vascular Function, Increases the Delivery of Sequentially Administered 5-FU in HT-29 Tumors, and Controls Tumor Growth in Patient Derived Xenografts of Colon Cancer," J Control Release. 199:72-83 (2015), Epub 2014).
	7	EP3337467: Sandoz AG Opposition dated September 9, 2021, D5 (Clinical Trials Identifier NCT01770353: 2015-05-05 update submitted, "A Pilot Study in Patients Treated with MM-398 to Determine Tumor Drug Levels and to Evaluate the Feasibility of Ferumoxytol Magnetic Resonance Imaging to Measure Tumor Associated Macrophages." 5 pages).
	8	EP3337467: Sandoz AG Opposition dated September 9, 2021, D6 (SHAH M, et al., "The Relevance of Drug Sequence in Combination Chemotherapy," Drug Resist Updat. 3(6):335-356 (2000)).
	9	EP3337467: Sandoz AG Opposition dated September 9, 2021, D7 (O'SULLIVAN C, et al., "Beyond Breast and Ovarian Cancers: PARP Inhibitors for BRCA Mutation-Associated and BRCA-Like Solid Tumors," Front Oncol. 4:42 doi: 10.3389/fonc.2014.00042 (2014), 13 pages).
	10	EP3337467: Sandoz AG Opposition dated September 9, 2021, D8 (ONIVYDE package insert, revision October 22, 2015, 18 pages).
	11	EP3337467: Sandoz AG Opposition dated September 9, 2021, D9 (CARNEVALE J and KO A, "MM-398 (Nanoliposomal Irinotecan): Emergence of a Novel Therapy for the Treatment of Advanced Pancreatic Cancer," Future Oncol. 12(4):453-64 (2016). Epub 2015).

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
	Application Number	15809815
	Filing Date	2017-11-10
	First Named Inventor	Eliel Bayever
	Art Unit	1612
	Examiner Name	Gollamundi S. KISHORE
	Attorney Docket Number	01208-0007-01US

	12	EP3337467: Sandoz AG Opposition dated September 9, 2021, D10 (Clinical Trials Identifier NCT02631733: 2015-12-15 submitted, "A Phase I Study of a Combination of MM-398 and Veliparib in Solid Tumors." 7 pages).
	13	EP3337467: Sandoz AG Opposition dated September 9, 2021, D11 (KOSHKARYEV A, et al., "Differential Tissue Clearance Results in Improved Therapeutic Index for Irinotecan Liposome Injection (ONIVYDE) When Combined with the PARP Inhibitor Veliparib in Preclinical Cervical Tumors," In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; Cancer Res. 76(14 Suppl):Abstract nr 2075 (2016), 2 pages).
	14	EP3337467: Sandoz AG Opposition dated September 9, 2021, D12 (LIVRAGHI L, et al., "PARP Inhibitors in the Management of Breast Cancer: Current Data and Future Prospects," BMC Med. 13:188; doi: 10.1186/ s12916-015-0425-1 (2015), 16 pages)).
	15	EP3337467: Proprietor's Submission in Response to Oppositions, dated February 3, 2022, including main request and auxiliary requests 1-23, 140 pages.
	16	EP3337467: Proprietor's Submission in Response to Oppositions, dated February 3, 2022, D13 (Written transcript of the presentation associated with D1a: LORUSSO P, et al., "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," American Society of Clinical Oncology 2011 Meeting), 7 pages).
	17	EP3337467: Proprietor's Submission in Response to Oppositions, dated February 3, 2022, D14 (SHAH M, et al., "A Phase I Clinical Trial of the Sequential Combination of Irinotecan Followed by Flavopiridol," Clin Cancer Res. 11 (10):3836-45 (2005)).
	18	EP3337467: Proprietor's Submission in Response to Oppositions, dated February 3, 2022, D15 (SADETZKI S, et al., "Childhood Exposure to External Ionising Radiation and Solid Cancer Risk," Br J Cancer. 100(7):1021-25 (2009)).
	19	EP3337467: Proprietor's Submission in Response to Oppositions, dated February 3, 2022, D16 (Practical Medical Oncology Textbook, Eds. Russio A, et al., Springer Nature Switzerland AG, Table of Contents, pp I-XI (2021)).
	20	EP3337467: Proprietor's Submission in Response to Oppositions, dated February 3, 2022, D17 (CAMPTOSAR package insert, 2014, 39 pages).

If you wish to add additional non-patent literature document citation information please click the Add button Add

EXAMINER SIGNATURE

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Attorney Docket Number	01208-0007-01US

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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	Examiner Name	Gollamundi S. KISHORE
	Attorney Docket Number	01208-0007-01US

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Mary R. Henninger/	Date (YYYY-MM-DD)	2022-03-10
Name/Print	Mary R. Henninger	Registration Number	56992

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

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LEDERER & KELLER

Patentanwälte Partnerschaft mbB
European Patent Attorneys

DR. FRANZ LEDERER
(1963 – 2012)

DR. GÜNTER KELLER
Dipl.-Biol.

DR. MICHAEL BEST
Dipl.-Chem.

DR. STEPHAN TEIPEL
Dipl.-Chem.

DR. GEORG KALHAMMER
Dipl.-Biochem.

DR. JÖRG-THOMAS BINDER
Dipl.-Chem.

DOTT. MARCO FACHINI
Laurea in Chimica

80538 MÜNCHEN
Unsöldstraße 2
Telefon +49 89 21 23 99 0
Telefax +49 89 21 23 99 22
E-Mail info@lederer-keller.de

European Patent Office

80298 Munich

Your reference	Our reference	09 September 2021
EP 3 337 467	ESP00521LED	MF

Re.: European Patent No. 3 337 467
European Patent Application No. 16757498.7
Patentee: Ipsen Biopharm Ltd.
Opponent: Sandoz AG

On behalf of

Sandoz AG
Lichtstrasse 35
4056 Basel
Switzerland

OPPOSITION

is lodged according to Article 99 EPC against the above-referenced patent of Ipsen Biopharm Ltd. entitled

"Combination therapy using liposomal irinotecan and a PARP inhibitor for cancer treatment".

The opposition fee amounting to EUR 815.-- is paid via online fee payment.

The opponent has appointed us as their representatives, and it is respectfully requested to effect all notifications to our address.

The European patent EP 3 337 467 (hereinafter referred to as the "contested patent") is opposed in its full extent (claims 1 to 14).

The opposition is based on the grounds of Article 100(a) EPC. In particular, it is submitted that the subject matter of the contested patent does not involve an inventive step.

It is requested to revoke the European patent in its entirety. Oral proceedings in accordance with Article 116 EPC are requested in the event that the Opposition Division does not reach the decision to revoke the patent on the basis of the written submissions of the opponent.

Detailed statement of the grounds for opposition

1. Bibliographic information

The contested patent is based on European application no. 16757498.7, which is the European phase of international application PCT/US2016/047814, published as WO 2017/031442 A1. Said application was filed on August 19, 2016, claiming the following priorities:

1. August 20, 2015 (2 documents),
2. December 18, 2015 (2 documents),
3. March 16, 2016 and
4. April 15, 2016.

2. Cited documents

2.1 Reference will be made to the following documents:

D1: LoRusso, P. *et. al, J. Clin. Oncol.* **2011**, 29(15S), abstract 3000 presented at the 2011 ASCO Annual Meeting;

D1a: LoRusso, P. *et. al*, slides presented at the 2011 ASCO Annual Meeting;

D1b: LoRusso, P. *et. al*, video of the presentation at the 2011 ASCO Annual Meeting;[1]

[1] A copy of the video has been made available under the following link: https://1drv.ms/u/s!ArKmWxrClBzZjw1QFTeNT2X62s51?e=xickoc. Please be so kind as to confirm that you downloaded the video. The video can also be downloaded from the website of the conference at https://meetinglibrary.asco.org/record/60307/video.

D2: Berlin, J. *et al.*, *J. Clin. Onc.* **2014**, 32(15S), abstract 2574;

D3: Tahara, M. *et al.*, *Mol. Cancer Ther.* **2014**, 13(5), 1170–1180;

D4: Neijzen, R. *et al.*, *J. Control. Release* **2015**, 199, 72–83;

D5: Protocol of clinical study NCT01770353 entitled *"Pilot Study to Determine Biodistribution of MM-398 and Feasibility of Ferumoxytol as a Tumor Imaging Agent"*, version 5 of May 5, 2015;

D6: Shah, M.A. *et al.*, *Drug Resist. Updat.* **2000**, 3, 335–356;

D7: O'Sullivan, C. *et al.*, *Front. Oncol.* **2014**, 4(42), 1-13;

D8: Onivyde prescribing information, October **2015**;

D9: J. Carnevale *et al.*, *Fut. Oncol.* **2016**, 12(4), 453-464;

D10: Protocol of clinical study NCT02631733 entitled *"Liposomal Irinotecan and Veliparib in Treating Patients With Solid Tumors That Are Metastatic or Cannot Be Removed by Surgery"*, version 1 of December 15, 2015;

D11: Koshkaryev, A. *et al.*, *Cancer Res.* **2016**, 76(14 Supp), abstract 2075 presented at the AACR 107th Annual Meeting, April 16-20, 2016,[2] New Orleans, LA;

D12: Livraghi, L. *et al.*, *BMC Med.* **2015**, 13:188.

2.2 Documents D1 to D7 and D12 were published prior to the earliest priority date of the contested patent and are thus full prior art for all claims.

2.3 Documents D8 and D10 were published between the 1st and the 2nd priority dates, document D9 between the 2nd and the 3rd priority dates and document D11 between the 3rd and the 4th priority dates of the contested patent.[2]

As we will show in the following, none of the claims of the contested patent are entitled to any of the claimed priorities. Hence, documents D8 to D11 are prior art according to Article 54(2) EPC for all claims.

2.4 Documents D6, D7, D9 and D12 are review articles. These documents are thus, by definition, an account of the common general knowledge and the state of the art prior to their own publication date (see e.g. T 777/08, r. 5.2, or T 1641/11). Document D8 is the prescribing

[2] The abstract book of the AACR 107th Annual Meeting was made available to the public no later than April 7, 2016, as it can be seen from https://web.archive.org/web/20160407082343/http://files.abstractsonline.com/SUPT/101/4017/AACR16_InstructionPage.html. The full text of the abstracts was posted at 4:30 p.m. ET on Wednesday, March 16, see http://web.archive.org/web/20160314031020if_/http://www.aacr.org/Meetings/Pages/MeetingDetail.aspx?EventItemID=63&DetailItemID=363#.VuYrq8Rxc2w.

information of an authorised drug and thus represents the general knowledge of the skilled person (T 734/12, r. 21).

2.5 Irrespective of the finding of the Opposition Division in relation to the priority entitlement, document D9 is cited as evidence of the common general knowledge prior to the effective date of the contested patent.

3. The subject matter of the contested patent

3.1 Technical field and background

3.1.1 The contested patent relates to liposomal irinotecan for use in an antineoplastic therapy in combination with a PARP inhibitor (contested patent, paragraph [0001]).

3.1.2 The contested patent explains in the background art section that nanoliposomal irinotecan (nal-IRI), a topoisomerase I (Top1) inhibitor, is a highly stabilized liposomal formulation of irinotecan that provides for sustained exposure of irinotecan and its active metabolite SN-38 (contested patent, paragraph [0003]).

Nal-IRI has shown promising preclinical and clinical activity in a range of cancer types, and has been approved in the United States in combination with 5-fluorouracyl / leucovorin (5-FU/LV) for patients with metastatic adenocarcinoma of the pancreas (contested patent, paragraph [0003]). Said liposomal irinotecan for injection, marketed as ONIVYDE®, was previously designated with the lab-code MM-398 (contested patent, paragraphs [0010] and [0047]).

3.1.3 Poly(ADP-ribose) polymerase (PARP) inhibitors are a new class of chemotherapeutic agents in development for the treatment of various cancer types (contested patent, paragraph [0004]).

3.1.4 The contested patent further explains in the background art section that combinations of PARP and Top1 inhibitors have shown to be synergistic in *in vitro* assays. However, the clinical development of PARP inhibitor and Top1 inhibitor combinations has allegedly been limited due to increased toxicities and resultant dose reductions (contested patent, paragraph [0005]).

3.1.5 The object of the contested patent has been defined as the provision of methods to safely and effectively combine a PARP inhibitor with a Top1 inhibitor to treat cancer (contested patent, paragraph [0006]).

3.2 The common general knowledge

3.2.1 Review D6 explains the relevance of drug sequence in combination chemotherapy (D6 title). D6 teaches that the sequence of drug administration can significantly enhance the therapeutic effect of chemotherapy. These sequence-dependent effects can be explained by chemotherapy-induced cell cycle perturbations, or by pharmacodynamic interactions between the agents in combination (D6, abstract).

3.2.2 D6 further teaches that the utilization of combination chemotherapy has become a standard practice in medical oncology. However, the combination of different chemotherapeutic agents to maximize tumour cell kill often requires dose reductions or delays in order to allow the simultaneous administration of all the drugs. The end result is to decrease the dose intensity and lessen the therapeutic benefit (D6, page 337, left-hand column, second paragraph).

3.2.3 In order to overcome this problem, the concept of *"dose density"* was introduced by Norton and Simon already in the '70s of the last century. This foresees a frequent, but staggered administration of the different chemotherapy agents that ultimately results in increased efficacy (D6, page 337, paragraph bridging the two columns). D6 then reports several examples of staggered administration of drug combinations, including combinations of irinotecan (D6, pages 345-346). In particular, D6 teaches that the combination of flavopiridol and irinotecan demonstrates sequence-dependent anti-tumour activity, with best results when SN-38 (irinotecan's active metabolite) was followed by flavopiridol (D6, pages 345, right-hand column, second paragraph).

3.2.4 Document D7 reviews the preclinical data and clinical development of PARP inhibitors and discuss their future development in solid tumours. D7 explains that PARP inhibitors have shown clinical activity in breast and ovarian cancers, but accumulating evidence suggests that they may have a wider application, such as in prostate, lung, endometrial, and pancreatic cancers. Several PARP inhibitors are in clinical investigation as single-agents and/or combination therapy in these solid tumours (D7, abstract). More particularly, D7 explains that veliparib is a sensitizer to irinotecan in colorectal cancer cells (D7, page 8, right-hand column, third paragraph). D7 concludes stating that it is anticipated that novel clinical trial design

strategies will help minimize toxicity and maximize therapeutic efficacy (D7, sentence bridging pages 9 and 10).

3.2.5 A similar teaching can be found in review article D12, published a week before the earliest priority date of the contested patent and 18 months after D7. In particular, D12 lists in tables 2 and 3 a large number of clinical trials of PARP inhibitors in patients with breast cancer and highlights *"the remarkable activity of olaparib and veliparib in multiple phase 2 trials"* (D12, page 8 of 16, left-hand column, last paragraph).

3.2.6 Document D8 is the US prescribing information of Onivyde (irinotecan liposome injection for intravenous use) and thus represents the general knowledge of the skilled person (see T 734/12, item 21). Onivyde is indicated, in combination with fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas. The recommended dosage regimen of Onivyde is 70 mg/m^2 per intravenous infusion every two weeks (D8, page 1, left-hand column, *"Indications and usage"*). Onivyde is formulated with irinotecan hydrochloride trihydrate into a liposomal dispersion for intravenous use (D8, page 10, last paragraph).

3.2.7 Document D9 is a review article on Onivyde (D9, title). D9 highlights in its abstract the favourable safety and tolerability profile of liposomal irinotecan compared to standard irinotecan (i.e., not encapsulated in liposomes). D9 also explains that Onivyde was previously called MM-398 and received its FDA approval in October 2015 (D9, page 462, left-hand column, second paragraph).

3.2.8 In conclusion, the skilled person was well aware at the relevant date that the sequence of drug administration can significantly enhance the therapeutic effect of chemotherapy. He or she was also familiar with PARP inhibitors and with the new liposomal formulation of irinotecan, which was known to have favourable safety and tolerability profile compared to standard irinotecan.

3.3 The claims of the contested patent

3.3.1 The contested patent comprises 14 claims. Claims 1 and 2 represent independent claims and claims 3 to 14 refer directly or indirectly back to claims 1 and/or 2.

3.3.2 Independent claim 1 relates to:

1. *Liposomal irinotecan*
for use in an antineoplastic therapy for the treatment of a solid tumor in a patient,
wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor,
wherein the liposomal irinotecan is repeatedly administered, and
wherein the treatment comprises:
i) administering to the patient liposomal irinotecan once every two weeks; and
ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan,
wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and
at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.

3.3.3 Independent claim 2 relates to:

2. *Liposomal irinotecan*
for use in an antineoplastic therapy for the treatment of a solid tumor in a patient,
wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor,
the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
ii) administering the PARP inhibitor on one or more days starting at least 3 days after the liposomal irinotecan and
ending at least 1 day prior to administration of additional liposomal irinotecan.

3.3.4 Claim 3 specifies that the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan. Claim 4 specifies that the PARP inhibitor is administered on one or more of days 5 to 12 or on one or more of days 3 to 12 of the antineoplastic therapy treatment cycle. Claim 5 specifies that the PARP inhibitor is administered on one or more of days 19 to 25 or on one or more of days 17 to 25 of the antineoplastic therapy treatment cycle. Claim 6 specifies that the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.

3.3.4 Claim 7 specifies that the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

3.3.5 Claim 8 specifies that each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base). Claim 9 specifies that each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

3.3.6 Claim 10 specifies that the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

3.3.7 Claim 11 specifies that the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer. Claim 12 specifies that the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib. Claim 13 specifies that the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.

3.3.8 Claim 14 specifies that the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.

3.4 The examples of the contested patent

The contested patent contains 10 examples.

3.4.1 Example 1 (described in paragraphs [0077-89] of the contested patent) illustrates the background of the invention.

It discloses a phase I study of a combination of irinotecan (as part of the so-called FOLFIRI regimen with 5-fluorouracyl) and veliparib in solid tumours, which corresponds to prior art document D2. It also provides a tabular overview of the prior art combination studies with a Top1 inhibitor (irinotecan or topotecan) and the PARP inhibitor veliparib and the references in which said studies were published (including prior art documents D1 and D2).

The example further illustrates the known mechanism of action of MM-398 and a clinical study with MM-398 in combination with the contrast agent ferumoxytol. The protocol of said study is prior art document D5. It further discloses the protocol of a dose-escalation study with MM-398 and veliparib.

3.4.2 Reference example 2 (described in paragraphs [0090-97] of the contested patent) discloses *in vitro* studies testing combinations of various PARP inhibitors and the topoisomerase inhibitor SN-38, which is the active metabolite of irinotecan (see contested patent, paragraph [0003]).

3.4.3 Example 3 (described in paragraphs [0098-105] of the contested patent) discloses pre-clinical dose tolerability studies in mice to evaluate if delayed dosing of veliparib relative to liposomal irinotecan can alleviate systemic toxicity. The results of said studies were disclosed in prior art document D11 (see in particular the third paragraph, which literally corresponds to paragraph [0102] of the contested patent).

3.4.4 Reference example 4 (described in paragraphs [0106-107] of the contested patent) discloses the pre-clinical efficacy of liposomal irinotecan in mice xenograft models.

3.4.5 Reference example 5 (described in paragraphs [0108-112] of the contested patent) discloses the pre-clinical activity of liposomal irinotecan and PARP inhibitors in mice xenograft models.

3.4.6 Example 6 (described in paragraphs [0113-121] of the contested patent) discloses the protocol a phase I dose escalation study to characterize the safety, tolerability, maximum tolerated dose and pharmacokinetic of the liposomal irinotecan MM-398 in combination with veliparib. Said protocol corresponds to prior art document D10. The example also discloses a copy of the same protocol for the combination of MM-398 and olaparib.

3.4.7 Reference example 7 (described in paragraph [0122] of the contested patent) discloses how to measure phosphorylated H2AX in tumour biopsies.

3.4.8 Reference example 8 (described in paragraphs [0123-129] of the contested patent) discloses a protocol making use of ferumoxytol and magnetic resonance to predict deposition of the topoisomerase inhibitor from liposomal irinotecan.

3.4.9 Reference example 9 (described in paragraph [0130] of the contested patent) explains that the clinical efficacy of MM-398 has been demonstrated in the prior art in the phase 2 study NAPOLI-1 (see also D9, abstract).

3.4.10 Reference example 10 (described in paragraphs [0131-132] of the contested patent) discloses the cell survival for various triple-negative breast cancer (TNBC) cell lines following SN-38 and PARP inhibitor combination treatment.

3.4.11 In conclusion, the examples of the contested patent confirm the known synergy of irinotecan and PARP inhibitors *in vitro*. They also show that a staggered administration of irinotecan and PARP inhibitors in mice xenograft models leads to increased tolerability compared to concomitant administration. The examples however provide no evidence of the efficacy of the claimed dosage regimen, neither in animals, nor in human patients.

4. Lack of priority

4.1 Liposomal irinotecan

4.1.1 The first priority application US 201562207709 filed on August 20, 2015 discloses in claims 1 and 2 the following subject matter:

1. *A method of treating a patient with cancer and having a tumor, the method comprising*
   i. intravenously administering to the patient an effective amount of an irinotecan liposomal formulation having a terminal elimination half-life of about 26.8 hours and a maximal irinotecan plasma concentration of about 38.0 micrograms/ml; and
   ii. administering to the patient an effective amount of a PARP inhibitor wherein the PARP inhibitor is administered after an effective irinotecan plasma clearing interval.

2. *A method of treating a patient with cancer and having a tumor, the method comprising*
   i. intravenously administering to the patient an effective amount of MM-398; and
   ii. administering to the patient and effective amount of a PARP inhibitor wherein the PARP inhibitor is administered after an effective irinotecan plasma clearing interval.

In paragraph [0037] it is specified that the effective plasma clearing interval is 24 hours or 2, 3, 4 or 5 days.

4.1.2 Hence, independent claim 1 of the first priority application is directed to a specific irinotecan liposomal formulation *"having a terminal elimination half-life of about 26.8 hours and a maximal irinotecan plasma concentration of about 38.0 micrograms/ml"*. As a matter of fact, the indicated terminal elimination half-life and maximal irinotecan plasma concentration correspond to those of the specific product MM-398 (see contested patent, paragraphs [0051-52]). The same teaching can be found in paragraph [0028] of the disclosure of the first priority document.

4.1.3 Granted claim 1, instead, does not contain said limitation and thus encompasses any liposomal irinotecan, irrespective of its terminal elimination half-life and a maximal irinotecan plasma concentration, such as the product Irinophore C disclosed in document D4. The specific product of the priority application is now claimed in claim 7 of the contested patent, thus confirming that this is a particular embodiment of granted claim 1.

4.1.4 Also independent claim 2 of the first priority application is directed to the specific irinotecan liposomal formulation MM-398. The contested patent teaches in paragraphs [0010] and [0047] that the lab-code MM-398 refers to the product ONIVYDE® (irinotecan liposome injection), which was approved by the FDA on 15 October 2015. Its US prescribing information is document D8, which reads:

> *"ONIVYDE is a sterile, white to slightly yellow opaque isotonic liposomal dispersion. Each 10 mL single-dose vial contains 43 mg irinotecan free base at a concentration of 4.3 mg/mL. The liposome is a unilamellar lipid bilayer vesicle, approximately 110 nm in diameter, which encapsulates an aqueous space containing irinotecan in a gelated or precipitated state as the sucrose octasulfate salt. The vesicle is composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) 6.81 mg/mL, cholesterol 2.22 mg/mL, and methoxy-terminated polyethylene glycol (MW 2000)-distearoylphosphatidyl ethanolamine (MPEG-2000-DSPE) 0.12 mg/mL. Each mL also contains 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) as a buffer 4.05 mg/mL and sodium chloride as an isotonicity reagent 8.42 mg/mL."*
> (D8, page, 11, first paragraph)

4.1.5 It is thus clear that MM-398 designates a very specific liposomal formulation of irinotecan. Such a formulation contains 43 mg of irinotecan free base encapsulated with

sucrose octasulfate, as drug entrapment agent, in a unilamellar bilayer vesicle of a certain size and with a very specific composition. Moreover, the MM-398 formulation contains HEPES and sodium chloride as further excipients. Also the general disclosure of the first priority application always refers to MM-398 and not to liposomal irinotecan in general.

4.1.6 However, claim 2 of the contested patent is not limited to the specific irinotecan liposomal formulation MM-398, but encompasses any liposomal irinotecan, such as the product Irinophore C disclosed in document D4.

4.1.7 The skilled person would have considered the teaching of the first priority document about the claimed dosage regimen to be applicable only to an irinotecan liposomal formulation having the specified terminal elimination half-life and maximal irinotecan plasma concentration, i.e. the specific product MM-398. The skilled person would have understood that said teaching cannot be generalised to any liposomal irinotecan.

4.1.8 Indeed, the terminal elimination half-life and maximal irinotecan plasma concentration of a liposomal irinotecan product influence its plasma clearing interval and thus the appropriate dosage regimen. The same is true for the choices of liposome type, composition, size, content of active ingredient, as well as the presence of further excipients in MM-398: these parameters are all critical to the stability of the liposomes and thus influence the plasma clearing interval of the active substance.

4.1.9 It is thus evident that the teaching of the first priority application in relation to a specific liposomal irinotecan formulation has been generalised in the international application to encompass any liposomal irinotecan, leading to a loss of priority.

4.2 The next administration of irinotecan

4.2.1 Granted claim 1 foresees that the liposomal irinotecan is administered repeatedly, i.e., more than once, and that the PARP inhibitor is administered at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan. Claim 2 refers to a 28-day antineoplastic therapy treatment cycle and foresees that the PARP inhibitor is administered at least 1 day prior to administration of additional liposomal irinotecan.

4.2.2 On the other hand, the original claims make no reference to a repeated administration of irinotecan, nor to a 28-day antineoplastic therapy treatment cycle. Moreover, they do not

specify a minimum time between the last administration of the PARP inhibitor and the next administration of irinotecan. The description does not disclose the missing features either.

4.2.3 The first priority document only specifies an interval between the administration of irinotecan and the one of the PARP inhibitor, but it is completely silent on when the latter administration should cease and on whether irinotecan should be administered again. It is therefore evident that only a part of the claimed dosage regimen is disclosed in the first priority application.

4.2.4 The full dosage regimen claimed in the contested patent cannot be directly and unambiguously derived from the first priority application. Therefore, none of the granted claims can validly claim said priority.

4.3 Solid tumour

4.3.1 Granted claims 1 and 2 are directed to liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumour in a patient. The first priority application, however, discloses the treatment of a tumour in general, i.e., thus encompassing also haematological malignancies.

4.3.2 In the first priority application references to solid tumours can be found only in the context of some examples: these references are thus inextricably linked to the other features disclosed in said examples. Moreover, none the examples of the first priority document disclose all the remaining features of the claimed dosage regimen.

4.3.3 Therefore, the reference to solid tumours in the granted claims finds no basis in the first priority application and the priority claim is invalid also for this reason.

4.4 Intravenous administration

4.4.1 The first priority application makes clear, both in its claims and in the general description, that liposomal irinotecan should be administered intravenously. References to other routes of administration cannot be found anywhere in said document.

4.4.2 However, the reference to the intravenous administration route is entirely missing in the granted claims, which thus encompass other routes of administration, such as the subcutaneous and the oral routes.

4.4.3 It is thus evident that the teaching of the first priority application in relation to a specific route of administration has been generalised in the international application to encompass any route of administration. The priority claim is therefore invalid.

4.5 The other priority documents

4.5.1 The reasonings illustrated above equally apply to the second to sixth priority documents. Indeed, the six priority documents have identical claims with the only exception of US 201662308924 filed on March 16, 2016, which contains 14 additional claims. These additional claims, however, are not directed to the same invention of the granted patent: on the contrary, they specify the liposomal irinotecan, its dosage and the dosage regimen further.

4.5.2 Moreover, the general disclosures of the six priority documents are very similar and cannot serve as suitable basis for the priority claims either.

4.5.3 Hence, the claims of the contested patent are entitled only to their filing dates of August 19, 2016 and documents D8 to D11 are full prior art under Article 52(2) EPC for all claims.

5. Lack of inventive step

5.1 The closest prior art

5.1.1 Document D10 discloses the protocol of a phase I study of the combination of liposomal irinotecan MM-398 and veliparib in solid tumours (D10, title and page 3, *"Study identification"*, *"Official title"*). Veliparib is a well-known PARP inhibitor (see D7 and D12).

5.1.2 D10 thus discloses the same therapeutic indication and the same active ingredients of claim 1 of the contested patent. Said protocol corresponds to the disclosure of example 6 of the contested patent.

5.1.3 D10 further discloses the dosage regimen of the two drugs:

> *"Patients receive liposomal irinotecan intravenously (IV) over 90 minutes on days 1 and 15 and veliparib orally (PO) on days 5-12 and 19-25 or 3-12 and 17-*

25. Courses repeat every 28 days in the absence of disease progression or unacceptable toxicity"
(D10, page 4, *"Study description"*).

5.1.4 Therefore, liposomal irinotecan is administered repeatedly every two weeks, on days 1 and 15 of the 28-day treatment cycle. The dosage regimen of liposomal irinotecan of claims 1 and 2 of the contested patent is thus disclosed in D10.

5.1.5 As far as veliparib is concerned, two dosage regimen are disclosed. In the first option, veliparib is administered on days 5-12 and 19-25, i.e. for a first period of 8 days and a second period of 7 days. Said administration starts each time 4 days after the administration of liposomal irinotecan; it then ceases 3 days prior to the next administration of the liposomal irinotecan in the first period and 4 days prior to the next administration of the liposomal irinotecan in the second period.

5.1.6 In the second option, veliparib is administered on days 3-12 and 17-25, i.e., for a first period of 10 days and a second period of 9 days. Said administration starts each time 2 days after the administration of liposomal irinotecan; it then ceases 3 days prior to the next administration of the liposomal irinotecan in the first period and 4 days prior to the next administration of the liposomal irinotecan in the second period.

5.1.7 The first option discloses all integers of claims 1 and 2 relating to the administration of the PARP inhibitor, while the second one at least those of claim 1. The dosage regimen of the PARP inhibitor of claims 1 and 2 of the contested patent is thus disclosed in D10.

5.1.8 D10 has thus the same purpose (the treatment of solid tumours) and the most technical features in common with claim 1 and 2 of the contested patent. D10 thus qualifies as the closest prior art.

5.2 The objective technical problem

5.2.1 D10 does not explicitly disclose the efficacy of the dosage regimen described therein.

5.2.2 The technical effect of this difference is the confirmation of the efficacy of said treatment.

5.2.3 Therefore, the objective technical problem to be solved starting from D10 can be formulated as the provision of a dosage regimen of liposomal irinotecan and a PARP inhibitor that effectively treats a solid tumour.

5.2.4 However, neither the contested patent, nor its file history provide any evidence that such a problem has been solved.

5.2.5 The objective technical problem must thus be reformulated in a less ambitious manner as the provision of an alternative dosage regimen of liposomal irinotecan and a PARP inhibitor, without any reference to its efficacy.

5.3 Obviousness

5.3.1 The solution to such a problem is obvious taking into consideration the general knowledge of the skilled person. Liposomal irinotecan and PARP inhibitors, as well as their combinations were known treatments for solid tumours at the relevant date (see section 3.2 above on the common general knowledge and documents D1, D2 and D11). It would have been straightforward for the skilled person to design an arbitrary dosage regimen including the two medicaments. As shown in the previous paragraph, no particular requisite of safety or efficacy must be fulfilled by said regimen, as a more ambitious formulation of the technical problem is not justified by the available data. The subject matter of claims 1 and 2 is thus obvious based on D10 alone.

5.3.2 In the following we will assume that the patentee is able to prove with further experimental data, such as the results of the trial of D10, that the claimed dosage regimen is effective in the treatment of solid tumours. In such a case, the objective technical problem would be the provision of a dosage regimen of liposomal irinotecan and a PARP inhibitor that effectively treats a solid tumour.

5.3.3 The Boards of Appeal have consistently ruled that the mere fact that a certain treatment is explored in a clinical trial gives the skilled person a reasonable expectation of success. It thus worthwhile to review the relevant case law and its applicability to the present case.

5.3.4 Decision T 2506/12 of Board 3.3.07 deals, like the present case, with a combination treatment of cancer. The two drugs are ET-743 (Yondelis) and liposomal doxorubicin (PLD, Doxil). Prior-art citations disclosed that a clinical phase I trial of Yondelis in combination with Doxil was in progress. The Board ruled that the information that a phase I clinical trial of the

claimed combination was in progress gave the skilled person a reasonable expectation of success. Moreover, the performance of an already designed clinical trial amounts to a routine exercise:

> *"Thus, at the publication date of D2, the information was available that the envisaged combination treatment was considered by pharmaceutical researchers with an expectation of success sufficient to justify a clinical phase I trial. In this context it is pointed out that drug compounds to be used in a clinical trial with human subjects are not selected based on a general "try-and-see" attitude, but based on existing favourable scientific data, for both ethical and economical reasons. Thus a clinical trial is not a mere screening exercise."*
> (T 2506/12, r. 3.10, emphasis added)

> *"The reason why clinical studies are carried out at all is that they have uncertain outcomes. But they are routine tests and the fact that their outcome is uncertain does not in itself turn their results into an invention."*
> (T 2506/12, r. 3.12.2, emphasis added)

5.3.5 Decision T 239/16 of Board 3.3.01 deals with zoledronic acid for the treatment of osteoporosis by intravenous once-a-year administration. There was no disclosure anywhere in the prior art that the active ingredient was efficacious in any dosage for the claimed indication (T 239/16, IX, page 8, first paragraph and r. 5.2, page 29, third paragraph). Nevertheless, the Board concluded

> *"The board considers that the mere fact that an active agent selected from the group of bisphosphonates is being tested in a clinical study for the treatment of osteoporosis (as disclosed in document (55)) leads to an expectation of success, due to the fact that clinical studies are based on data obtained by preclinical testing both in vitro and in animals and require authority approval which takes ethical considerations into account. This means in the present case that the skilled person would expect all study arms to treat osteoporosis effectively, unless he was dissuaded from this by the prior art."*
> (T 239/16, r. 6.5, second paragraph)

> *"Clinical trials in humans are planned scientific investigations. They require authority approval, which is only given after a risk/benefit evaluation. For ethical (but also economic) reasons it has to be ensured that research risks are*

minimised and are reasonable in relation to any potential benefits. Ethical and economical considerations require that the "benefit" will arise with reasonable certainty and will not only "be hoped for"."
(T 239/16, r. 6.6, fifth paragraph)

5.3.6 Coming back to the contested patent, liposomal irinotecan was a commercial product at the effective date of the contested patent, as the product Onivyde was authorised by the FDA already on October 15, 2015 (see label D8 and review D9, page 462, left-hand column, second paragraph). The efficacy of liposomal irinotecan on many solid tumours (see, e.g., D9, page 459, table 2 and right-hand column, second paragraph) and in particular metastatic adenocarcinoma of the pancreas was thus part of the common general knowledge at the relevant date. The prior art situation in relation to Onivyde is thus very similar to that of Doxil in T 2506/12.

5.3.7 Although veliparib was not yet a commercial product at the relevant date, its remarkable activity in patients with breast cancer, a solid tumour, had been demonstrated in multiple phase 2 trials (D12, page 8 of 16, left-hand column, last paragraph).

5.3.8 Moreover, the efficacy of the combination of irinotecan and veliparib had been confirmed in clinical trials before the priority date of the contested patent, although using the standard formulation of irinotecan (i.e., not encapsulated in liposomes). E.g., document D1 discloses that *"A clinical benefit (CB) rate of 61% (5 PR, 2 MR, 10 SD) was observed"* (D1, *"Results"*). Document D2 discloses that *"Ovarian and breast cancer pts experienced the greatest anti-tumor activity"* (D2, *"Conclusions"*).

5.3.9 These prior art studies also disclose the safety of the combination. D2 discloses that the combination *"was generally well tolerated without drug-drug interactions"* (D2, *"Conclusions"*). D1 discloses that the maximum tolerated dose of the combination was determined (D1, *"Conclusions"*).

5.3.10 Compared to the standard irinotecan formulation, it belonged to the common general knowledge that liposomal irinotecan had a favourable safety and tolerability profile (see paragraph 3.2.7 above). For these reason, the skilled person would have had no concerns that the combination of liposomal irinotecan and veliparib disclosed in D10 might exhibit unacceptable toxicities to render the treatment ineffective.

5.3.11 In conclusion, in the present case, unlike the one of T 2506/12 (see r. 2.12) the safety of the claimed combination therapy was already known in the art. Therefore, this knowledge in the prior art even increases the expectations of success in the present case compared to the case of T 2506/12.

5.3.12 As D10 already discloses the protocol of the trial, no scientific input is needed by the skilled person to design the trial. Although the disclosure of D10 cannot confer absolute certainly of success, a reasonable expectation of success is sufficient to lead to a finding of lack of inventive step.

5.3.13 Hence, the person skilled in the art would have performed the clinical trial whose protocol is already disclosed in D10 and confirmed with a reasonable expectation of success the efficacy of the claimed dosage regimen of liposomal irinotecan and a PARP inhibitor in solid tumours. The claimed subject matter is thus obvious over D10.

5.4 Obviousness in the light of D11

5.4.1 The skilled person would have considered also document D11, which discloses the improved therapeutic index for irinotecan liposome injection (ONIVYDE) when combined with the PARP inhibitor veliparib in preclinical cervical tumours (D11, title).

5.4.2 D11 discloses a pre-clinical dose tolerability study in mice, showing that the combination resulted in tumour growth inhibition and that toxicity could be alleviated by delaying the start of veliparib dosing (D11, third paragraph), like in the dosage regimen of D10. D11 concludes stating that said promising preclinical regimen will be examined further in a clinical phase I study to be initiated in 2016, i.e., in the very study of D10. The skilled person would have thus found in D11 the rationale on which the study of D10 is based, therefore reinforcing his or her expectation of success.

5.4.3 Moreover, it belonged to the common general knowledge that the liposomal irinotecan tested in D10 had a favourable safety and tolerability profile compared to the standard irinotecan formulation (see paragraph 3.2.7 above). For this reason, the skilled person would have expected the combination of liposomal irinotecan and veliparib disclosed in D10 to be well-tolerated.

5.4.4 Therefore, claims 1 and 2 of the contested patent do not involve an inventive step over document D10 in in the light of the general knowledge of the person skilled in the art, or

additionally in combination with document D11, and thus do not fulfil the requirements of article 56 EPC.

5.5 Lack of inventive step of the remaining claims

5.5.1 Claim 3 specifies that the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan. D10 discloses that the administration of veliparib ceases 3 days prior to the next administration of liposomal irinotecan in the first period and 4 days prior to the next administration of liposomal irinotecan in the second period, i.e., at least 3 days prior to said administration (D10, page 4, *"Study description"*).

5.5.2 Claim 4 specifies that the PARP inhibitor is administered on one or more of days 5 to 12 or on one or more of days 3 to 12 of the antineoplastic therapy treatment cycle. Claim 5 specifies that the PARP inhibitor is administered on one or more of days 19 to 25 or on one or more of days 17 to 25 of the antineoplastic therapy treatment cycle. Claim 6 specifies that the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered. D10 discloses that veliparib is administered on each of said days (D10, page 4, *"Study description"*).

5.5.3 Claim 7 specifies that the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml. The claimed terminal elimination half-life and maximal irinotecan plasma concentration correspond to those of the specific product MM-398 disclosed in D10 (see contested patent, paragraphs [0051-52]).

5.5.4 Claim 8 specifies that each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base). This is the standard dosage of the marketed product MM-398 (Onivyde) used in D10 (D8, page 14, last paragraph).

5.5.5 Claim 9 specifies that each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day. Claim 9 encompasses the standard dosage regimens of PARP inhibitors (see e.g. Table 3 of D7 and Tables 2 and 3 of D12). Moreover, it was known from D1 and D2, in particular their conclusions, that veliparib was administered in combination with irinotecan at a dose of 40 mg bid or 200 mg bid, respectively.

5.5.6 Claim 10 specifies that the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib. D10 already discloses veliparib.

5.5.7 Claim 11 specifies that the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer. Claim 12 specifies that the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib. Claim 13 specifies that the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib. D10 already discloses veliparib and cervical cancer (as well as several other solid tumours, see page 5, *"Conditions"*). Moreover, olaparib is a standard alternative to veliparib (see D7 and D12), which was already on the market at the priority date of the contested patent (see paragraph [0061] and end of paragraph [0120].

5.5.8 Claim 14 specifies that the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use. The sucrose octasulfate salt is contained in the marketed product MM-398 (Onivyde) disclosed in D10 (see D8, page 11, first paragraph).

5.5.9 In conclusion, the additional features recited in claims 3-14 are either already disclosed in the closest prior art document D10 or belong to the common general knowledge. Hence, the dependent claims are obvious for the reasons discussed in connection with claims 1 and 2.

5.6 Lack of inventive step if priority is claims considered valid

5.6.1 Purely as precautionary measure, the following arguments are presented in the unlikely case of finding that the granted claims are entitled to priority. In such a case, documents D8, D10 and D11 would not belong to the state of the art.

5.6.2 Conference abstract D1 would then constitute a suitable starting point for the inventive step reasoning, as it discloses the results of a phase I study of veliparib in combination with irinotecan in patients with advanced solid tumours (D1, title). Indeed, D1 has the same purpose (the treatment of solid tumours) and the key features in common with claim 1 of the contested patent (the two active ingredients).

The disclosure of D1 should be considered in combination with that of the PowerPoint slides presented at the conference (see D1a), as well as the video of the presentation (see D1b). For the present discussion it will be however sufficient to refer to abstract D1 only.

5.6.3 D1 discloses that cycles were of 21 days. Irinotecan was given intravenously on days 1 and 8. Twice daily oral dosing of veliparib occurred on days 3-14 (cycle 1) and days 1-14 (subsequent cycles) followed by a 6-day rest (D1, *"Background"*). Co-administration of irinotecan did not affect the pharmacokinetics of veliparib. A clinical benefit rate of 61% was observed (D1, *"Results"*), i.e., the efficacy of the treatment is disclosed in D1.

5.6.4 D1 does not disclose that liposomal irinotecan is used: the standard formulation of irinotecan is used instead. Moreover, the dosage regimen employed in D1 is different from the claimed dosage regimen.

5.6.5 The effect of these differences is not apparent from the contested patent, which contains no clinical data, let alone a comparison between the dosage regimen of D1 and the claimed one.

5.6.6 The objective technical problem to be solved starting from D1 is thus the provision of an alternative treatment of solid tumours with irinotecan and veliparib.

5.6.7 The solution to said problem is the use of liposomal irinotecan and veliparib according to the claimed dosage regimen. However, such a solution would have been obvious for the skilled person, who was well aware of the favourable safety and tolerability profile of liposomal irinotecan compared to standard irinotecan (D9, abstract; see also D4, page 73, third paragraph). The skilled person would have thus considered liposomal irinotecan as a good and even better alternative to standard irinotecan.

5.6.8 As far as the dosage regimen is concerned, claims 1 and 2 encompass several of the many dosage regimens that can be envisaged for the administration of a PARP inhibitor and irinotecan. The skilled person was well aware that the staggered administration of different chemotherapy agents ultimately results in increased efficacy (D6, page 337, paragraph bridging the two columns). D6, which reflects the common general knowledge in this respect, reports several examples of staggered administration of drug combinations, including combinations of irinotecan (D6, pages 345-346). In particular, D6 teaches that the combination of flavopiridol and irinotecan demonstrates sequence-dependent anti-tumour activity, with best results when SN-38 (irinotecan's active metabolite) was followed by flavopiridol (D6, pages 345, right-hand column, second paragraph). The common general knowledge thus clearly suggests to administer the second drug (veliparib in the case of the closest prior art D1) after irinotecan.

5.6.9 A similar teaching can be found also in secondary document D4, which the skilled person would have certainly consulted. D4 discloses in its title that Irinophore C, a liposomal formulation of irinotecan, increases the delivery of sequentially administered 5-fluorouracil (5-FU) in solid tumours (HT-29 is a human colon cancer cell line). D4 concludes that the treatment effects of sequential 5-FU dosing following liposomal irinotecan are additive with no additional toxicity. In contrast, in previous studies concurrent 5-FU and liposomal irinotecan treatment exacerbated 5-FU toxicity (D4, abstract, second-last sentence).

5.6.10 The skilled person, starting from D1 and looking for an alternative treatment of solid tumours with irinotecan and veliparib, would have considered the teaching of D4 and thus administered veliparib after liposomal irinotecan (liposomal irinotecan is also taught in D4) in a staggered fashion. The common general knowledge would have also reassured him or her that such a dosage regimen results in increased efficacy (see D6).

5.6.11 The additional features recited in claims 1 and 2, such as the number of days of veliparib treatment or the number of days after and prior to the irinotecan treatment, are arbitrary and cover many possible obvious variations of the dosage regimen: the contested patent does not contain any evidence that the recited intervals are connected with any technical effect, let alone a surprising one.

5.6.12 In conclusion, the skilled person would have modified the dosage regimen of D1 in the light of D4 to include liposomal irinotecan and a staggered administration of veliparib to provide an alternative treatment of solid tumours in an obvious manner. Moreover, he or she would have had a reasonable expectation that the modified dosage regimen resulted in increased efficacy, thus rendering even a more ambitious formulation of the objective technical problem obvious.

5.6.13 As shown in section 5.5, the dependent claims do not contain any features that could change such a finding.

5.6.14 Therefore, claims 1 to 14 of the contested patent do not involve an inventive step over document D1 in the light of the general knowledge of the person skilled in the art, or additionally in combination with document D4, and thus do not fulfil the requirements of article 56 EPC.

6. Conclusion

It has been shown above that the contested patent is not in accordance with the requirements of Article 56 EPC. Therefore, the request to revoke EP 3 337 467 in its entirety is fully justified.

Marco Fachini

Enc.
Documents D1-D12

Journal of Clinical Oncology

Issue: Volume 29(15) Supplement, 20 May 2011, p 3000-3000
Copyright: (C) 2011 American Society of Clinical Oncology
Publication Type: [Developmental Therapeutics - Experimental Therapeutics: 2011 ASCO Annual Meeting Abstracts]
DOI: 10.1200/jco.2011.29.15_suppl.3000
ISSN: 0732-183X
Accession: 00005083-201129151-00587

[Developmental Therapeutics - Experimental Therapeutics: 2011 ASCO Annual Meeting Abstracts]

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.: 3000

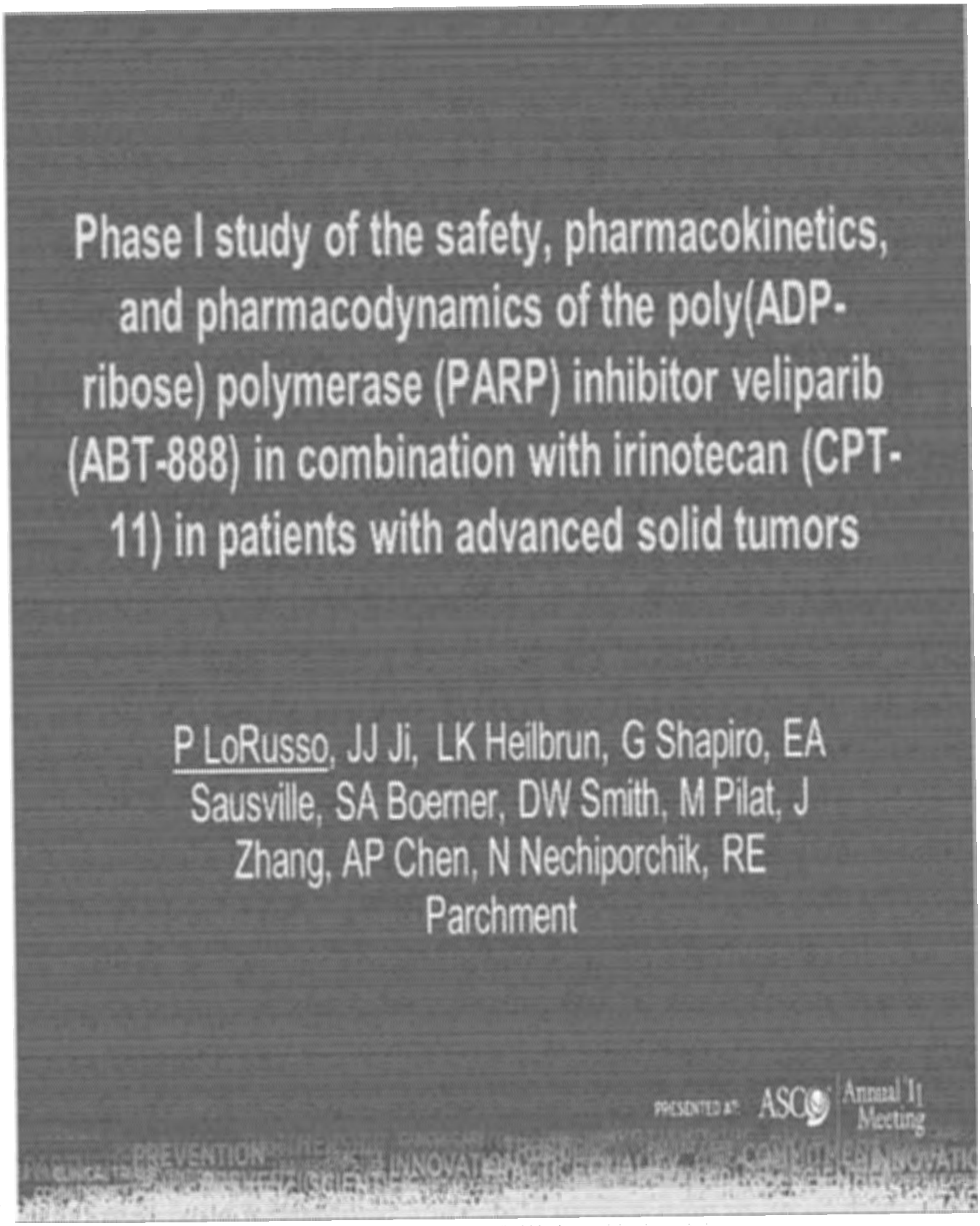
LoRusso, P.[1]; Ji, J. J.[1]; Li, J.[1]; Heilbrun, L. K.[1]; Shapiro, G.[1]; Sausville, E. A.[1]; Boerner, S. A.[1]; Smith, D. W.[1]; Pilat, M. J.[1]; Zhang, J.[1]; Chen, A. P.[1]; Nechiporchik, N.[1]; Parchment, R. E.[1]

Author Information

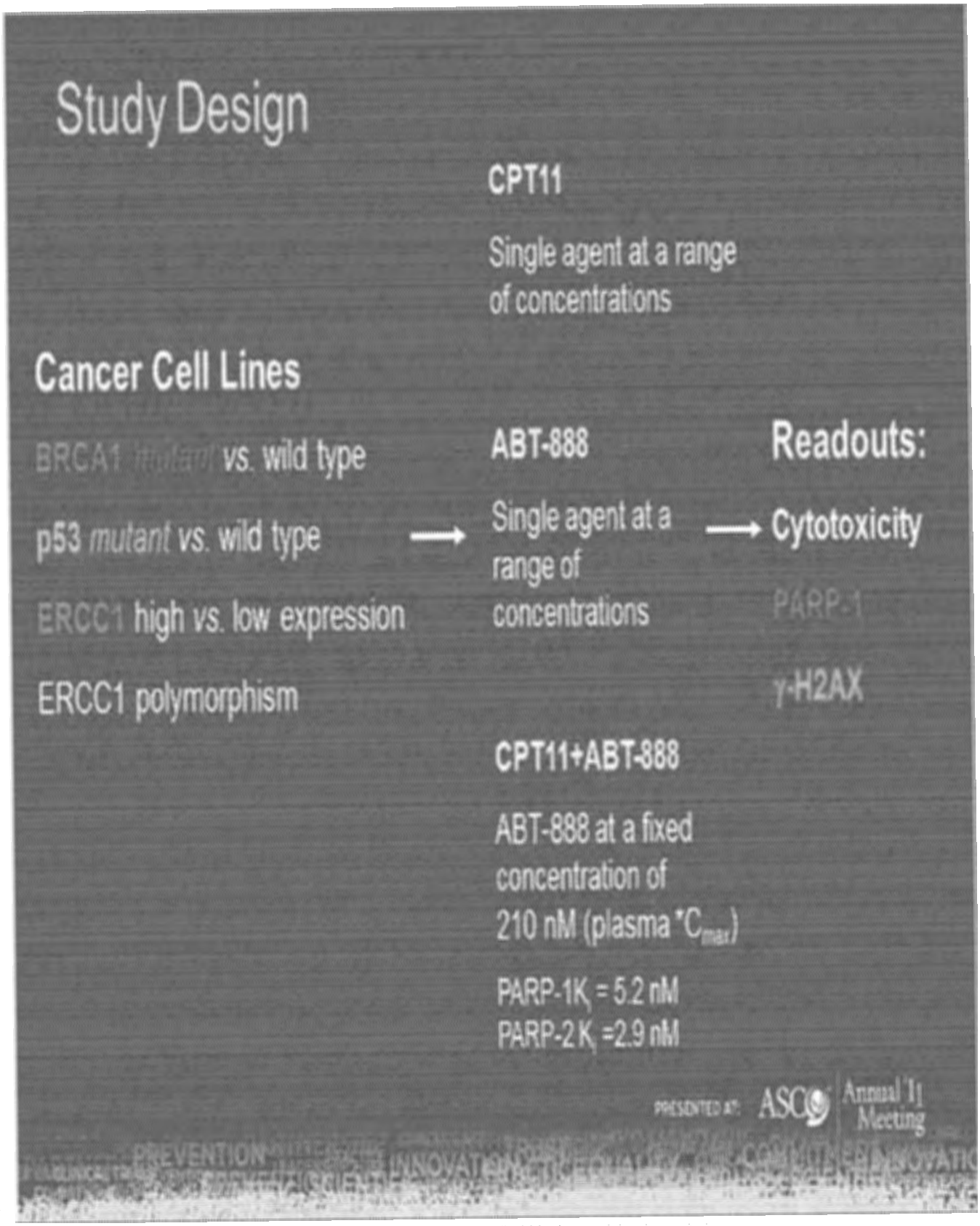
[1] Karmanos Cancer Institute, Detroit, MI; SAIC-Frederick, Bethesda, MD; Karmanos Cancer Institute, Wayne State University, Detroit, MI; Dana-Farber Cancer Institute, Boston, MA; University of Maryland School of Medicine and University of Maryland Greenebaum Cancer Center, Baltimore, MD; National Cancer Institute, Bethesda, MD
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; >= age 18; adequate bone marrow, hepatic and renal function. Cycles were 21 days. Ir was given i.v. 100 mg/m^2 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, [gamma]-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%), leucopenia (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/m^2 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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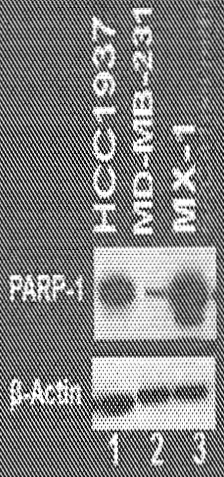


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ABT-888/CPT11 Single Agents vs Combination Effects

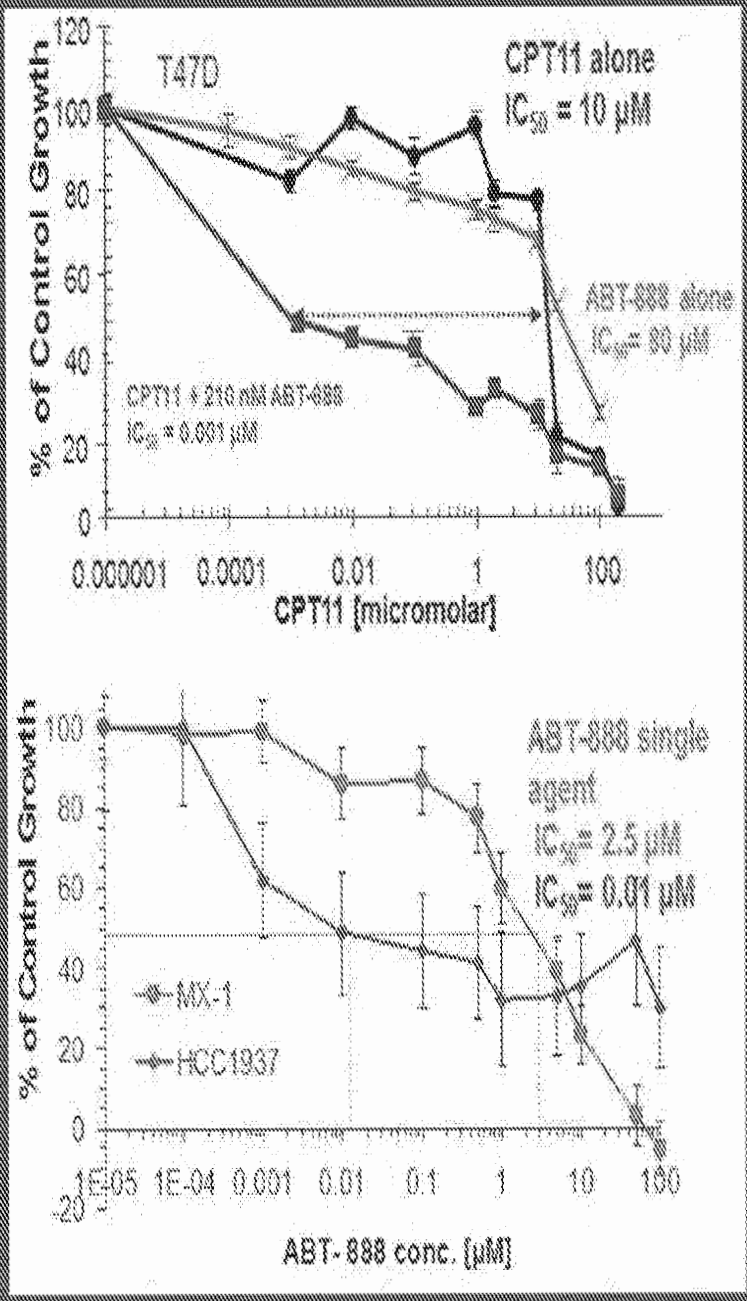
ERCC1 (+)

Mt p53



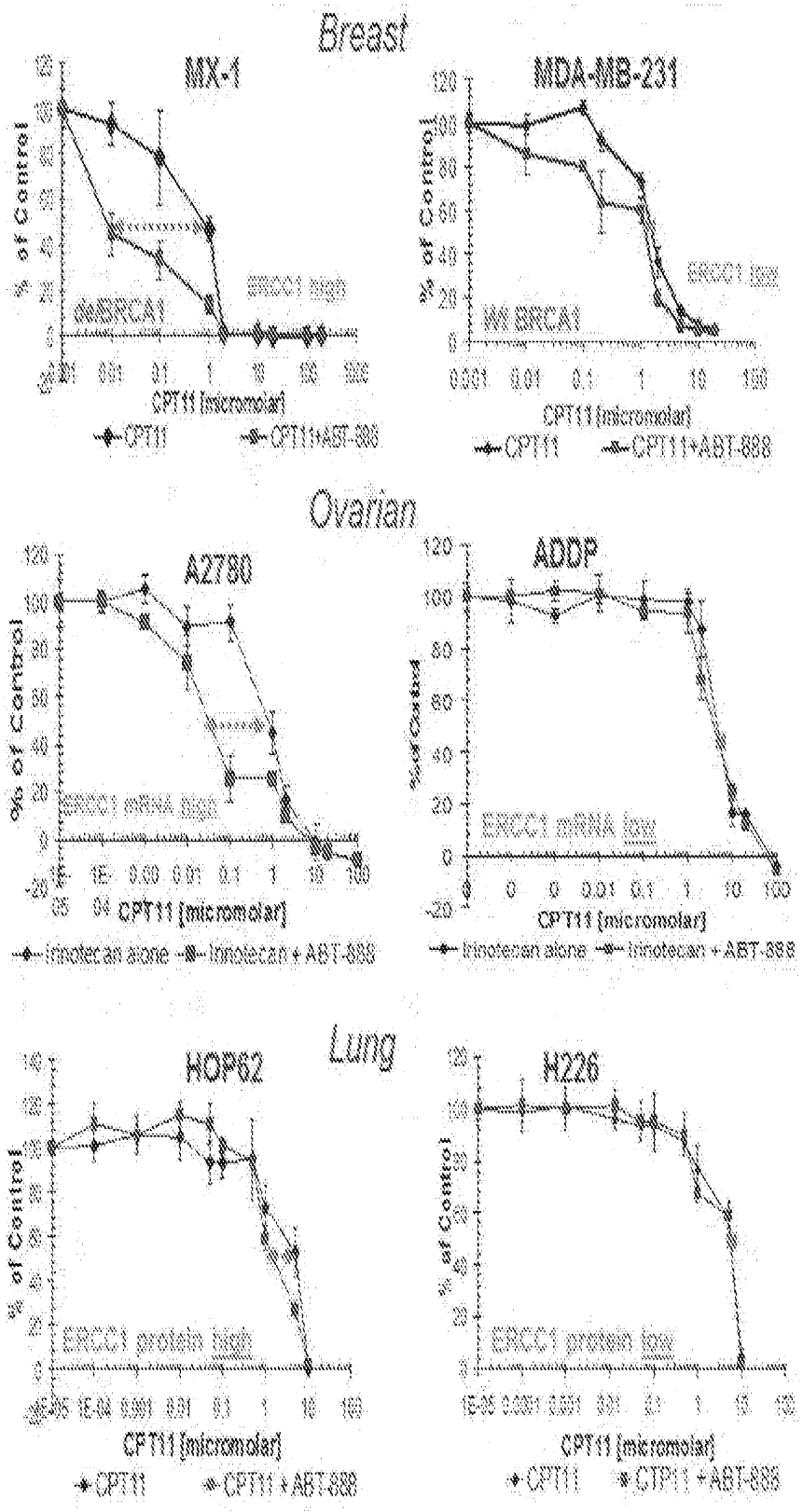
ERCC1 (-)

Mt p53

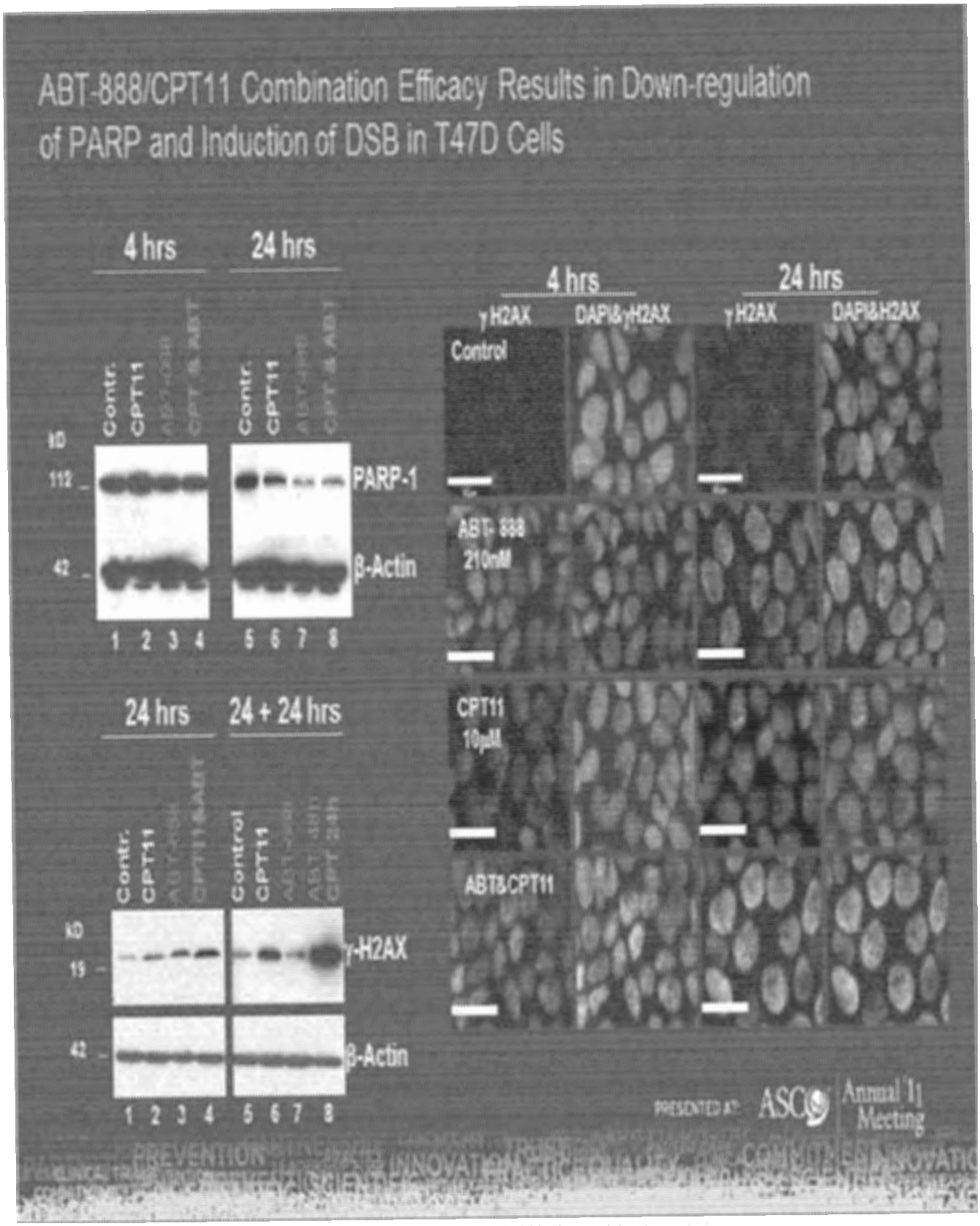


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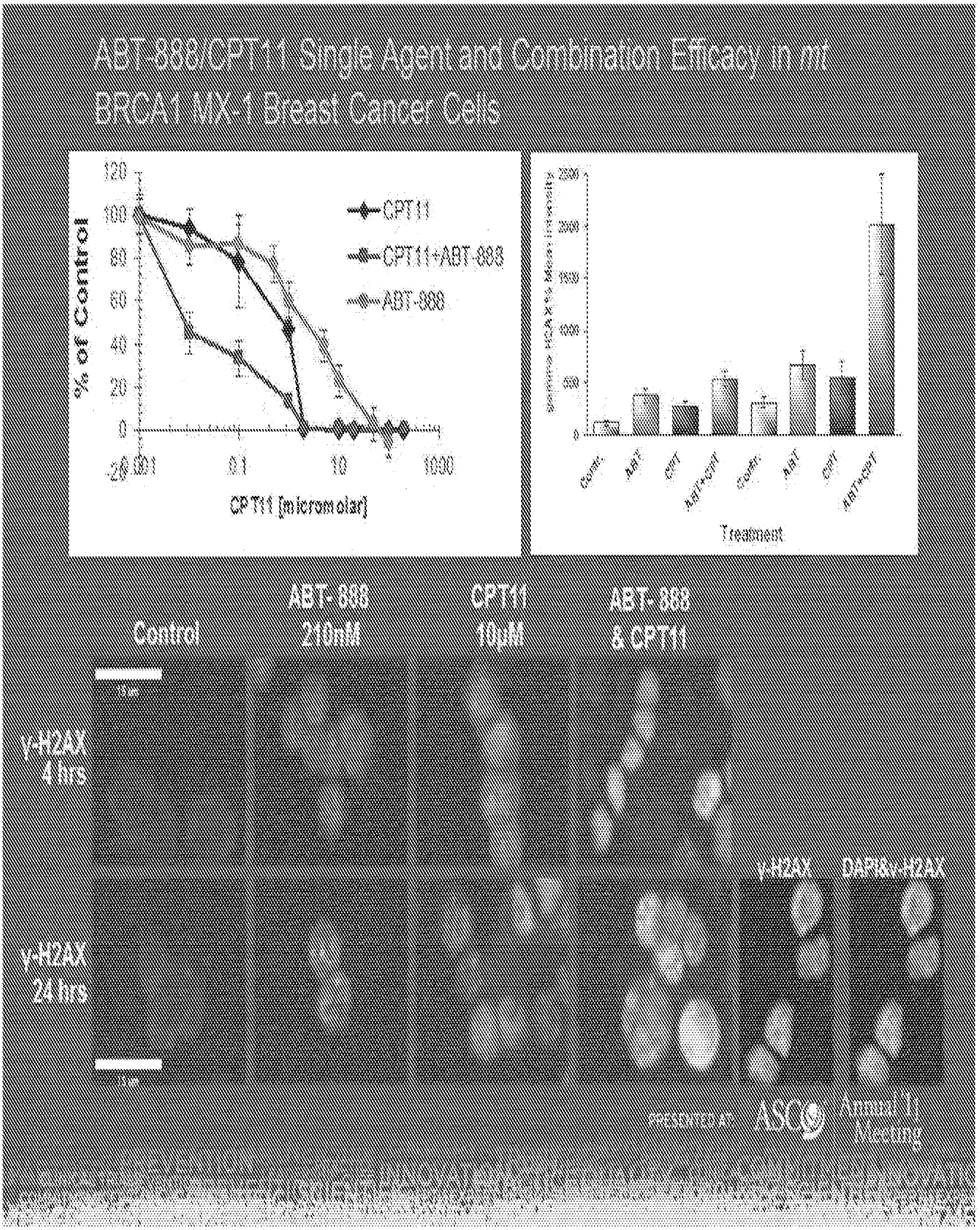
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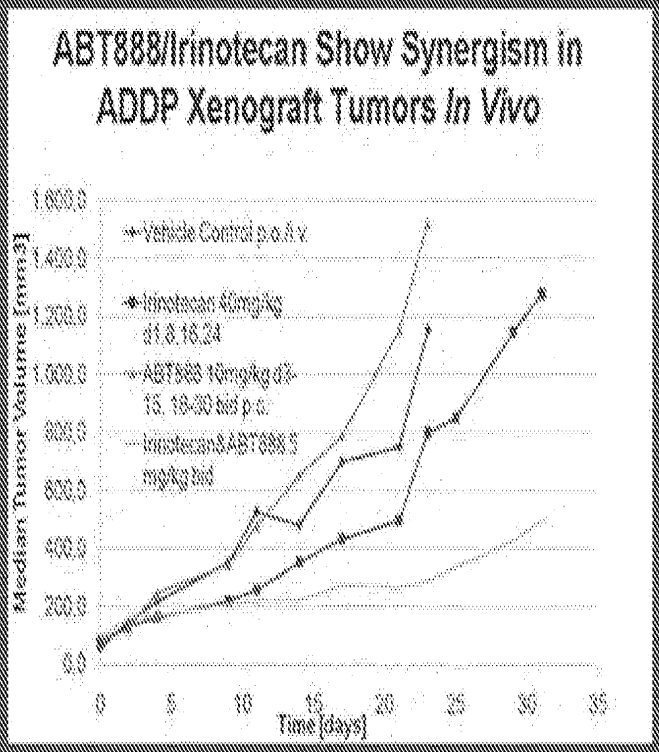


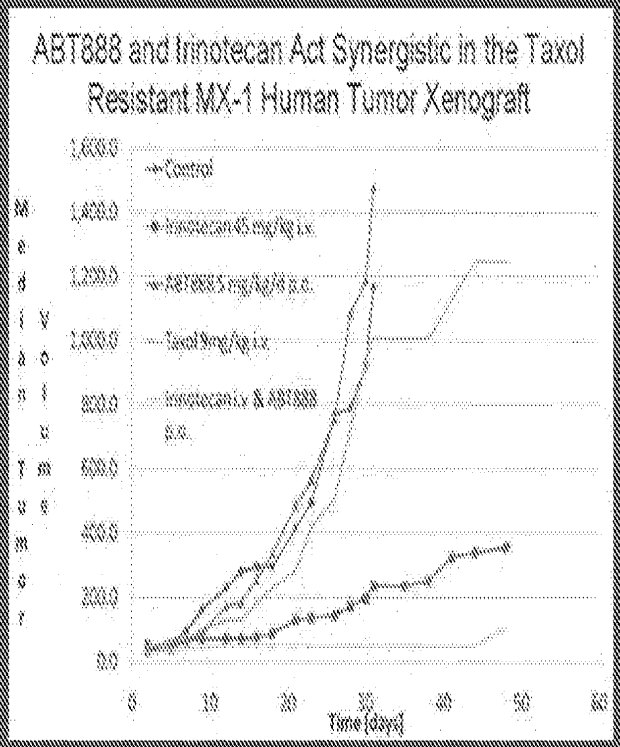
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Combination Irinotecan + ABT-888 *In Vivo*





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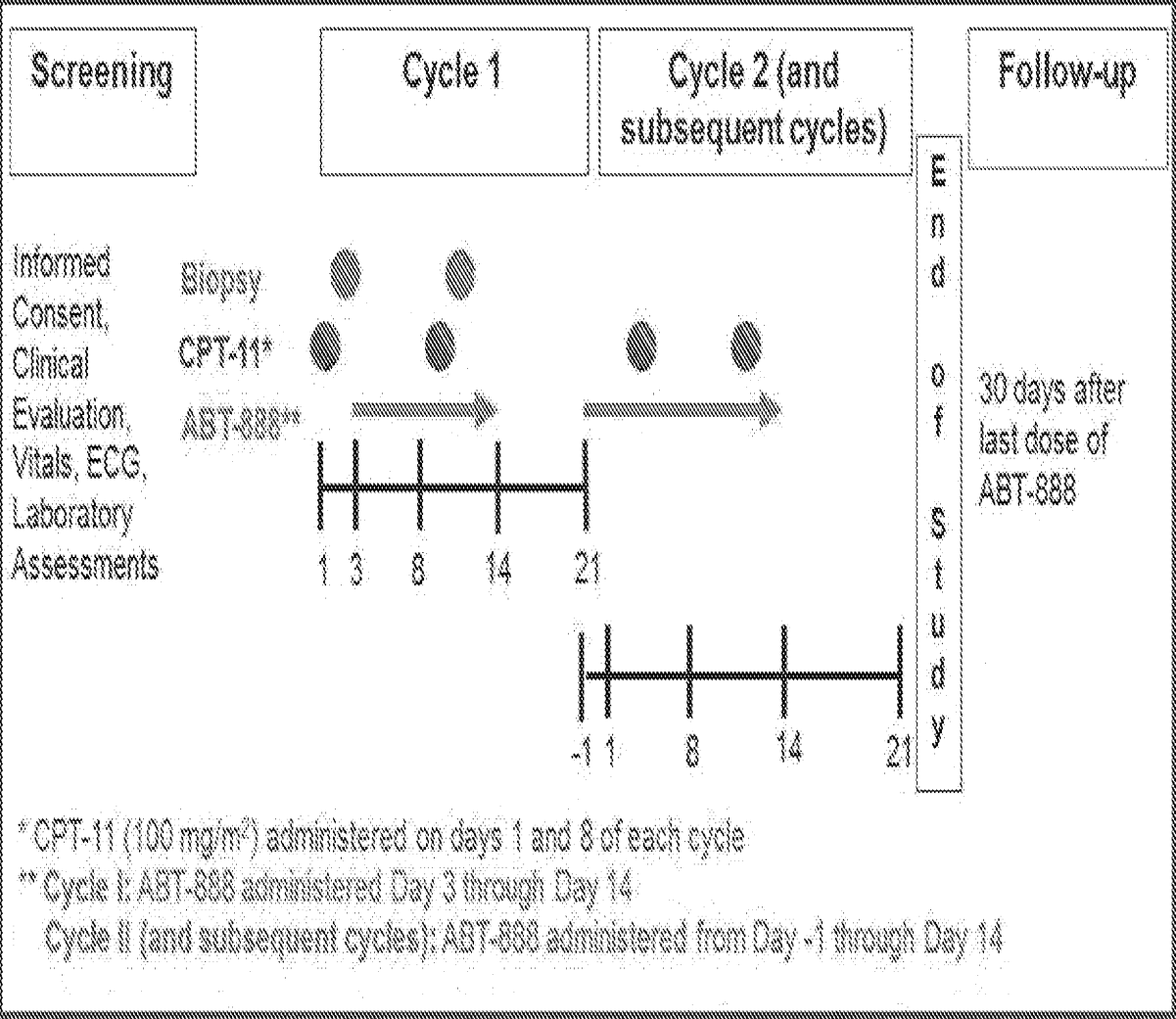
NCI #7977: Objectives

- 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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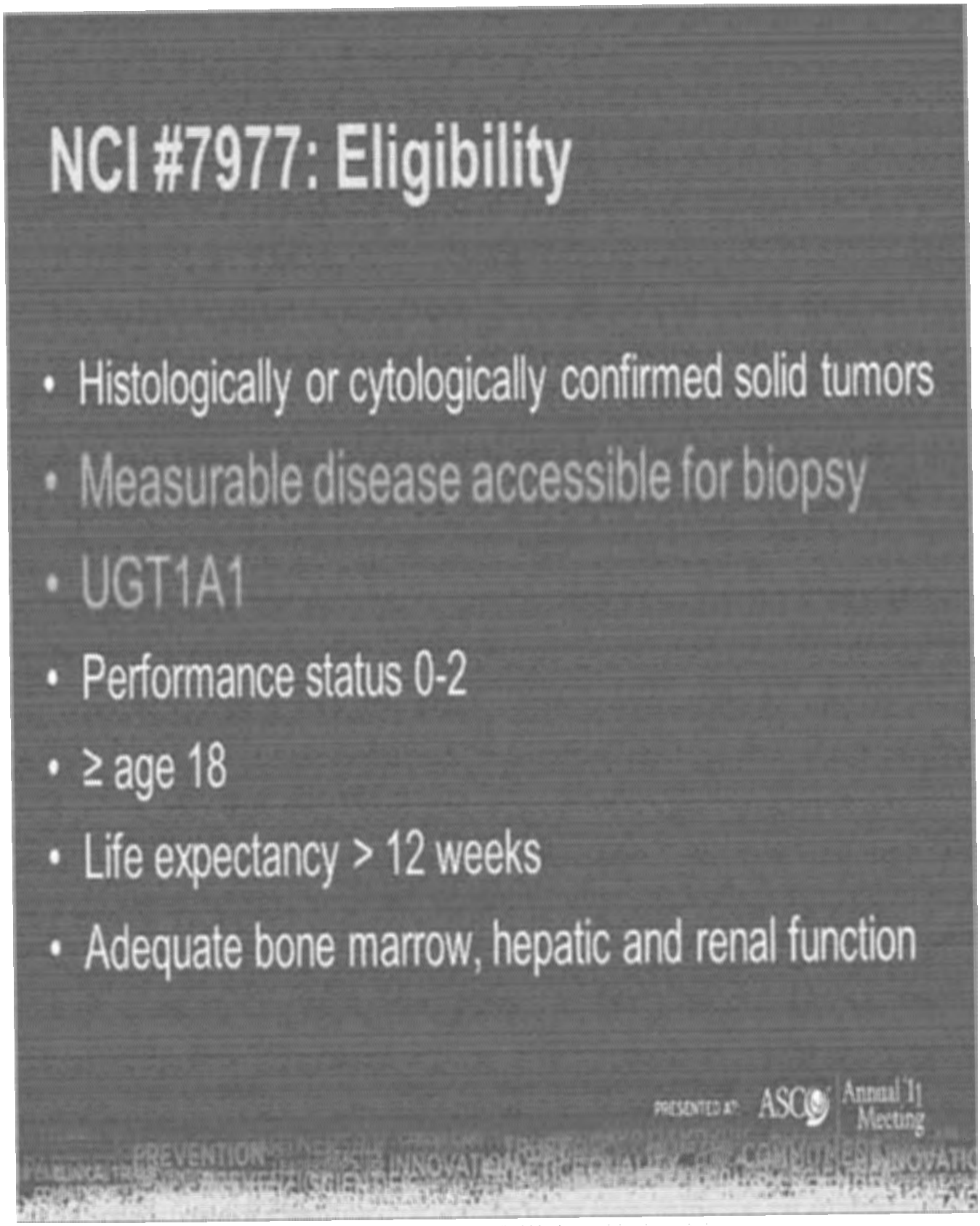
NCI #7977: Trial Schema



Blood Collections: D1: 0, 3.5, 5.5, 8.5, 23, 48 h
D8: 0, 3.5, 5.5, 8.5, 23, 48 h

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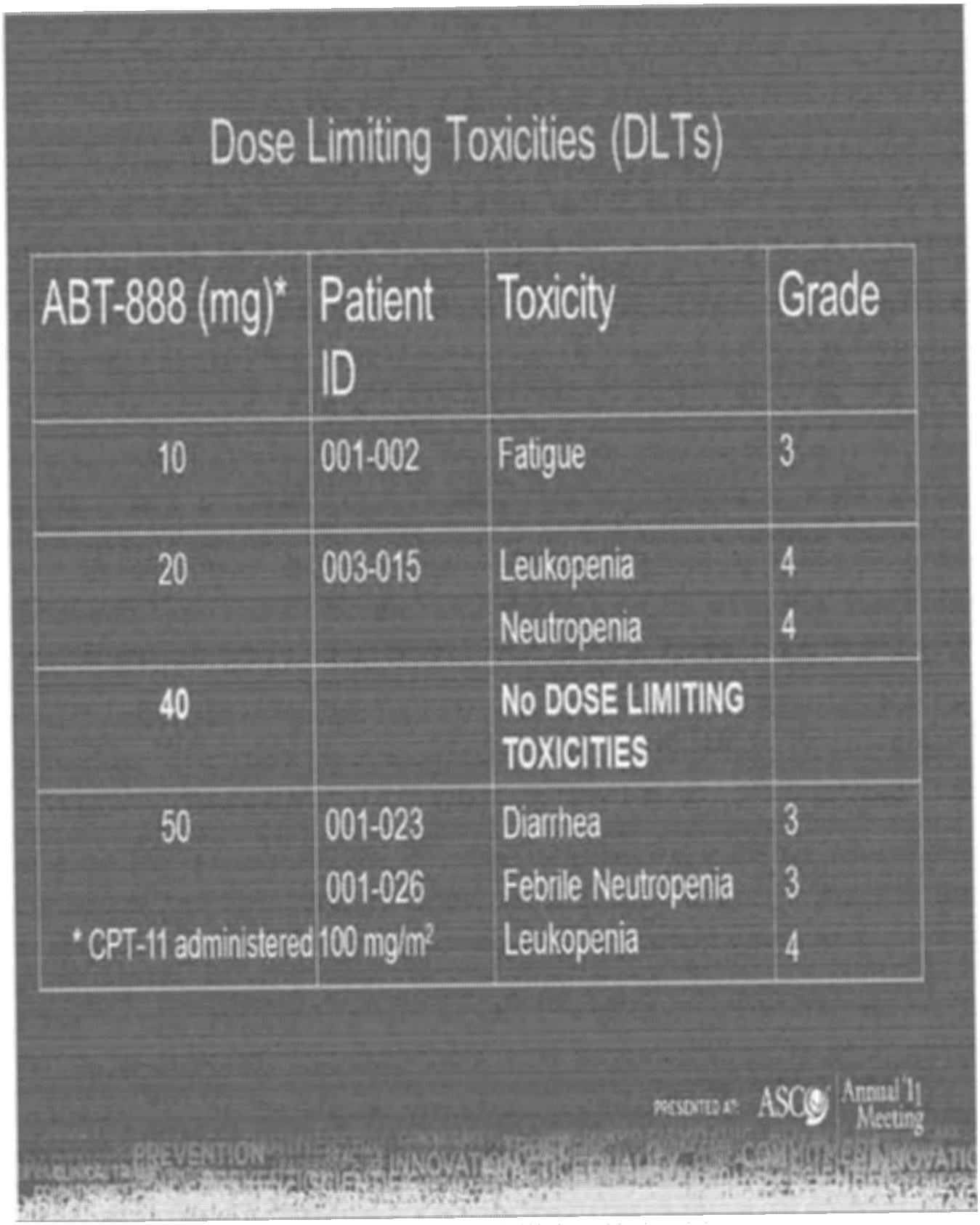


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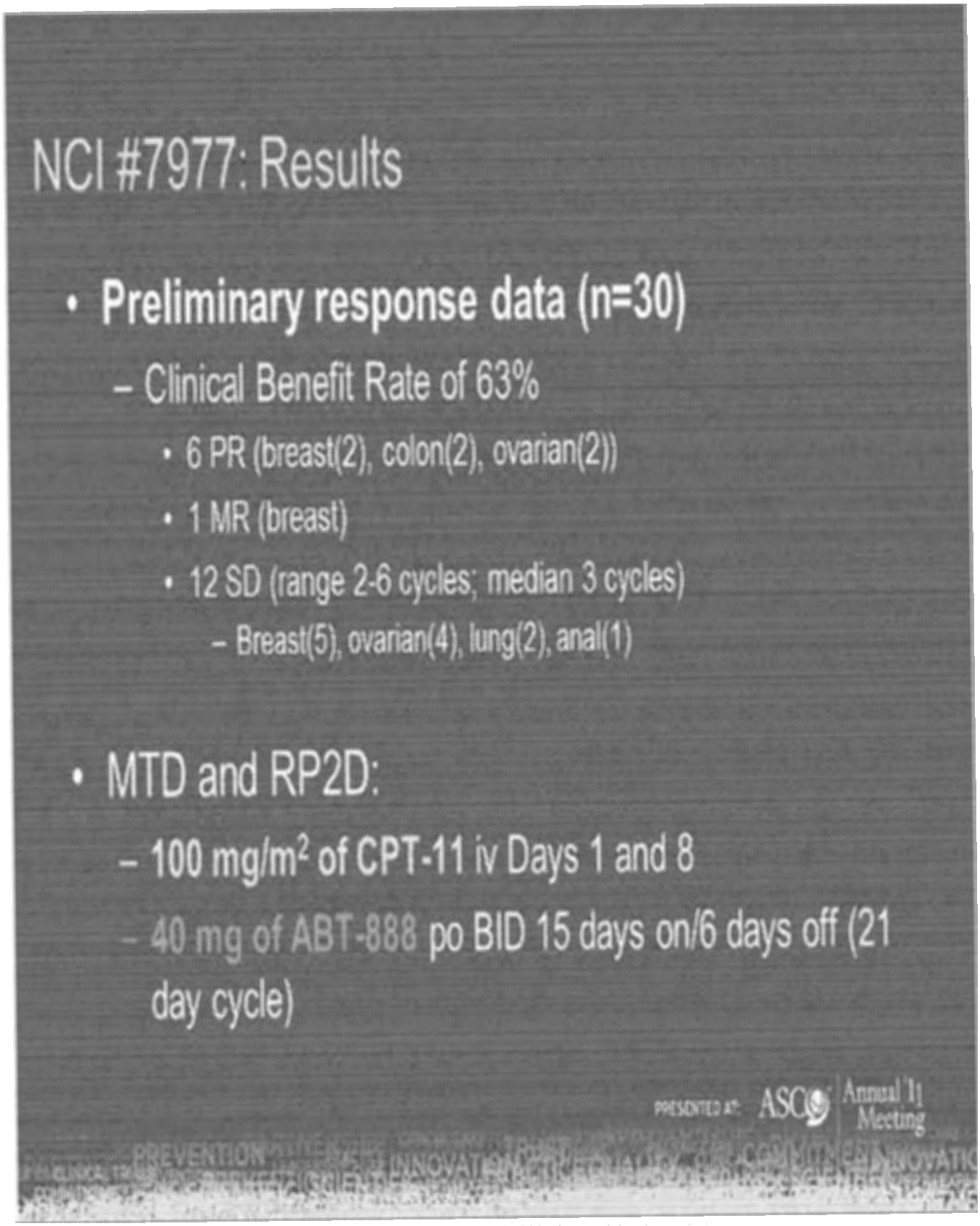
Most Common Toxicities (>10%)

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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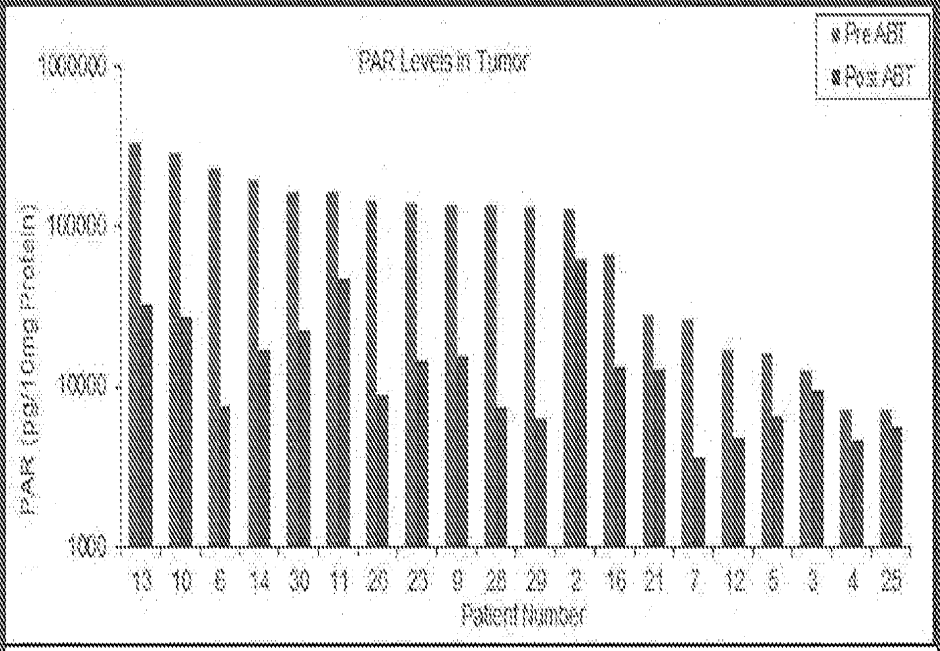


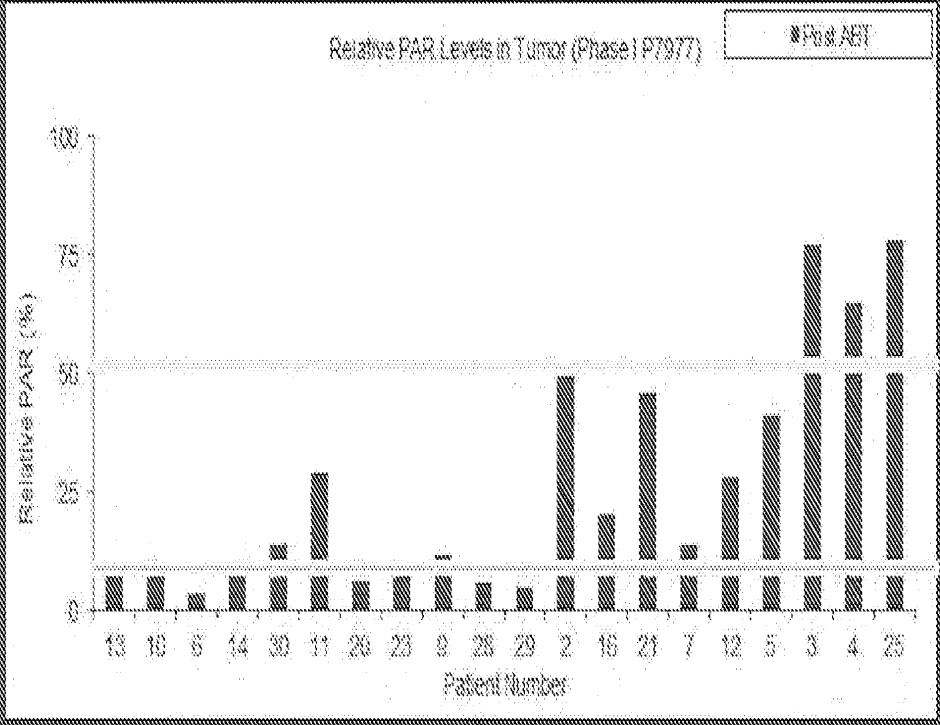
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PAR Levels in Tumors of Predose vs. Postdose

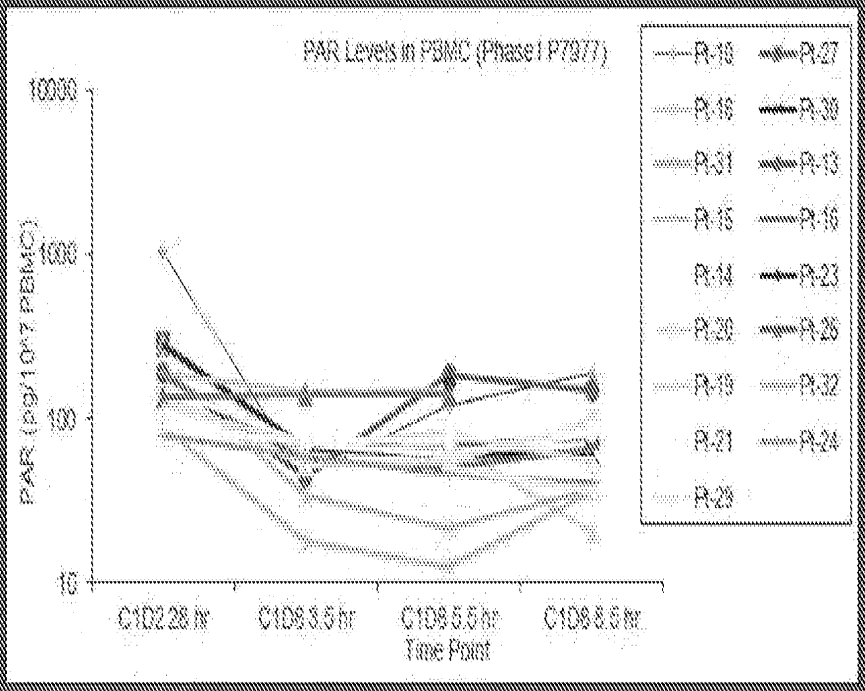


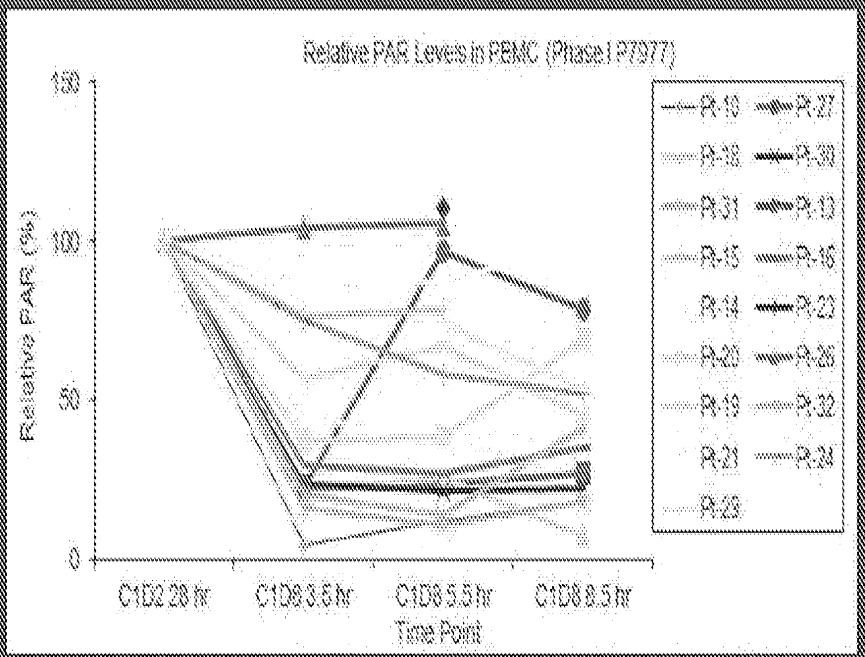


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PAR Levels in PBMCs

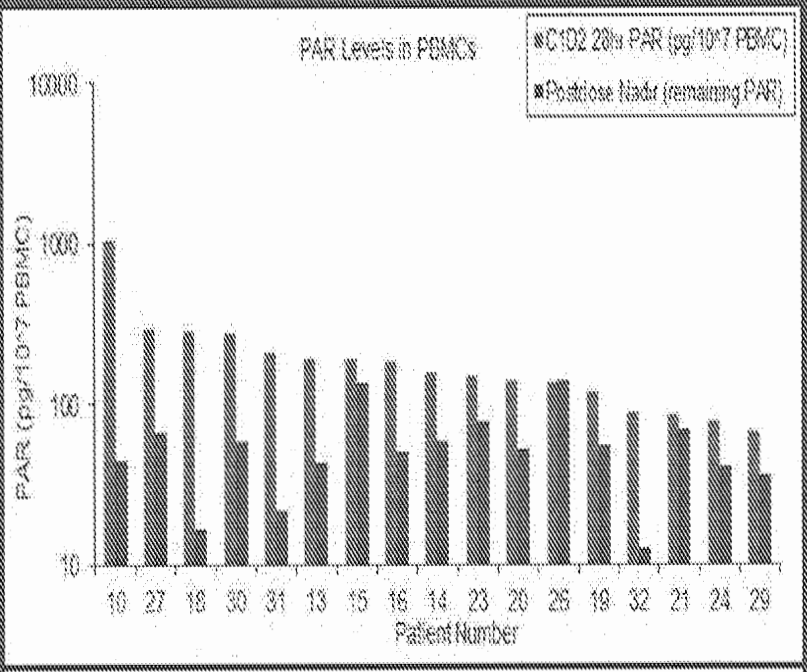


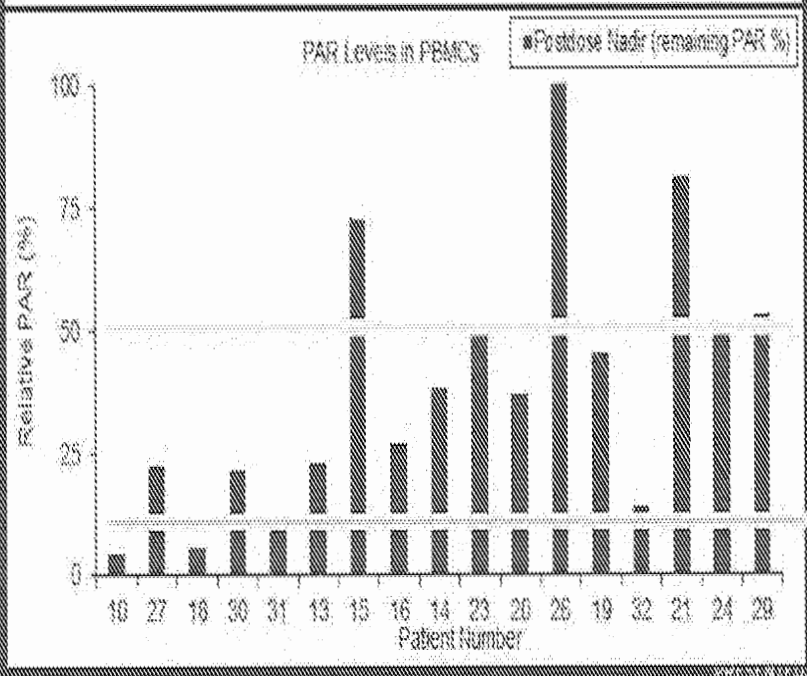


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PAR Levels in PBMCs of Predose vs. Postdose

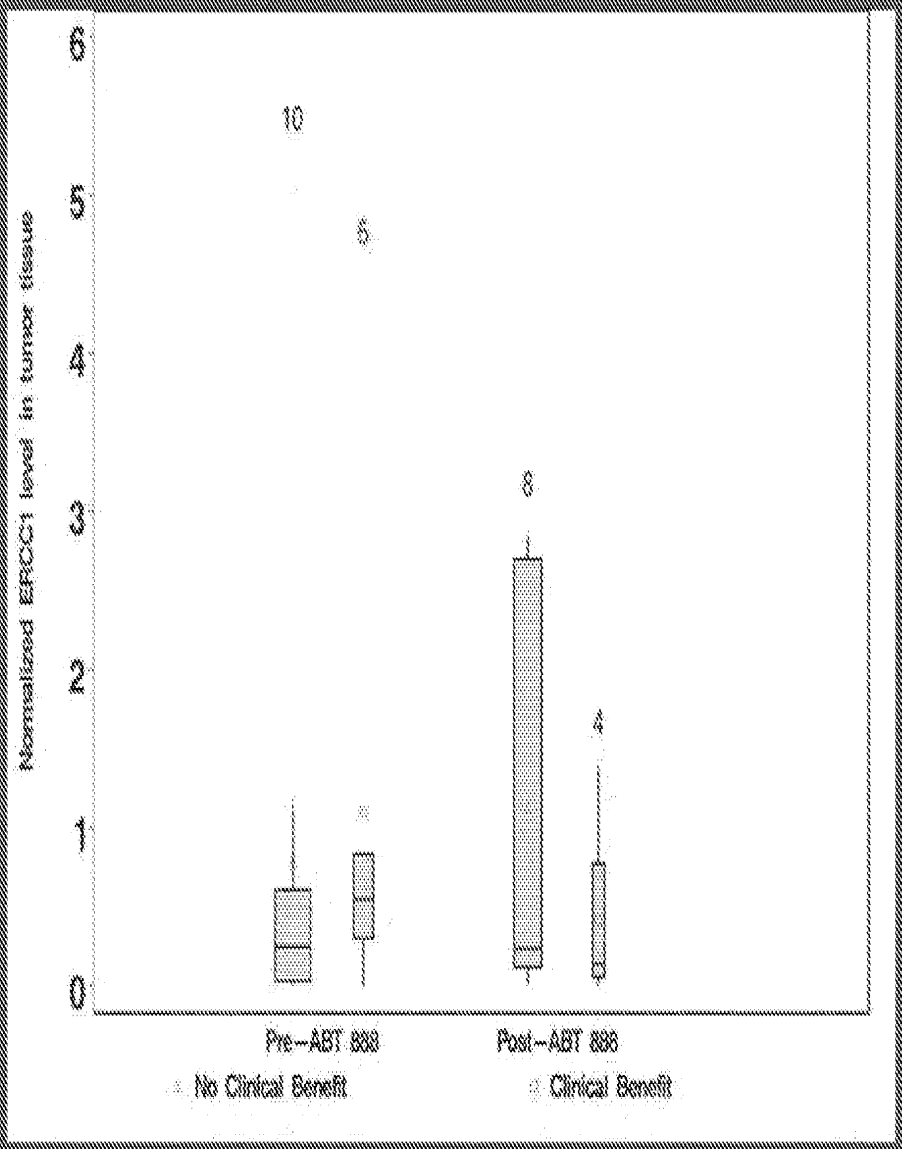




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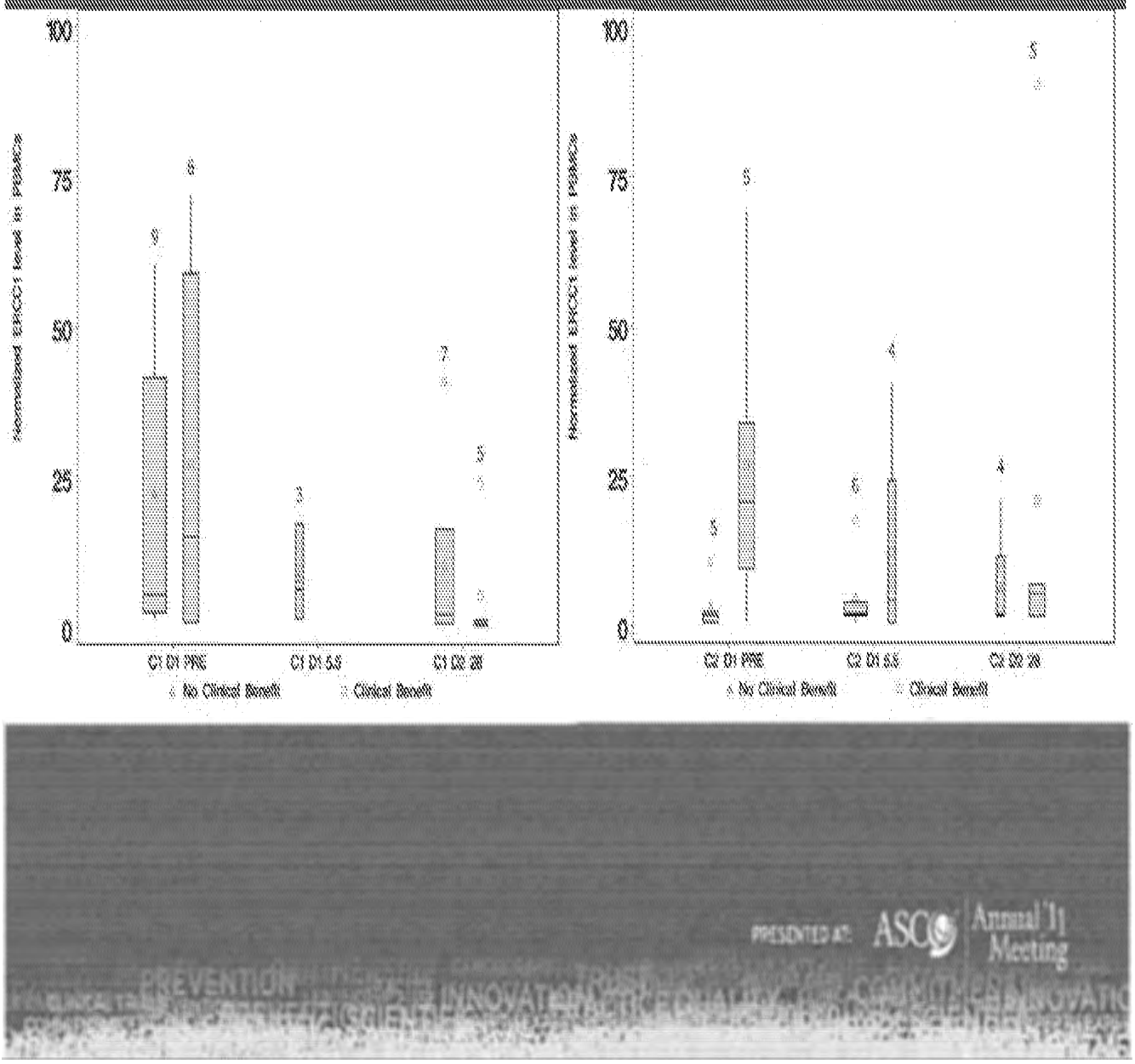
ERCC1 Levels in Tissue



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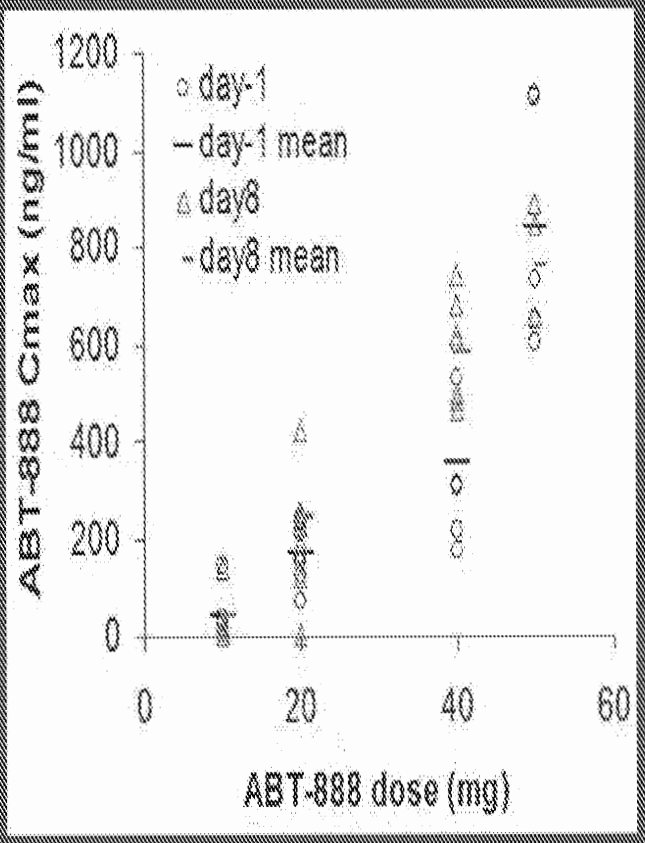
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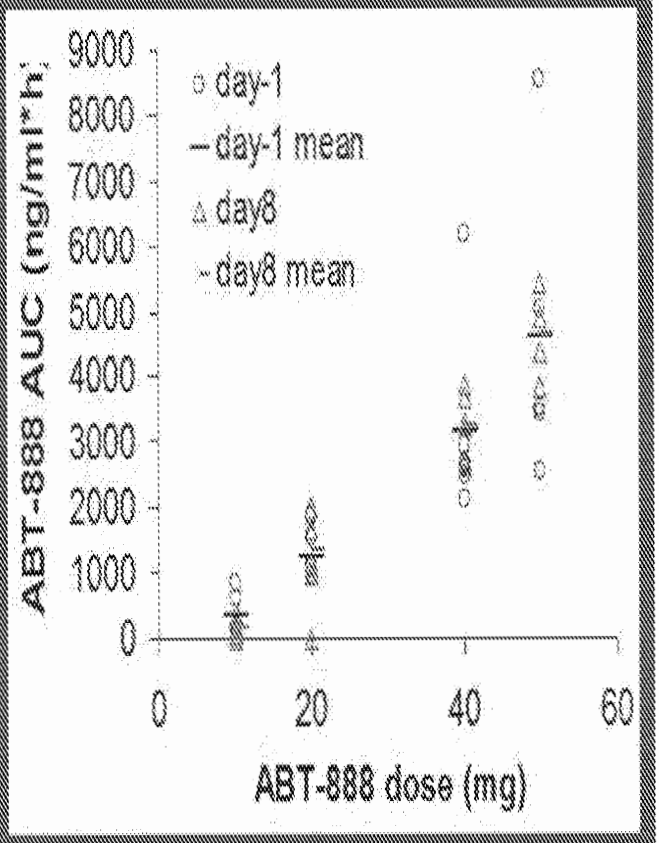
ERCC1 Levels in PBMCs



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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) ↑d with ↑g dose
- ABT-888 exhibited linear PK from 10 to 60 mg BID

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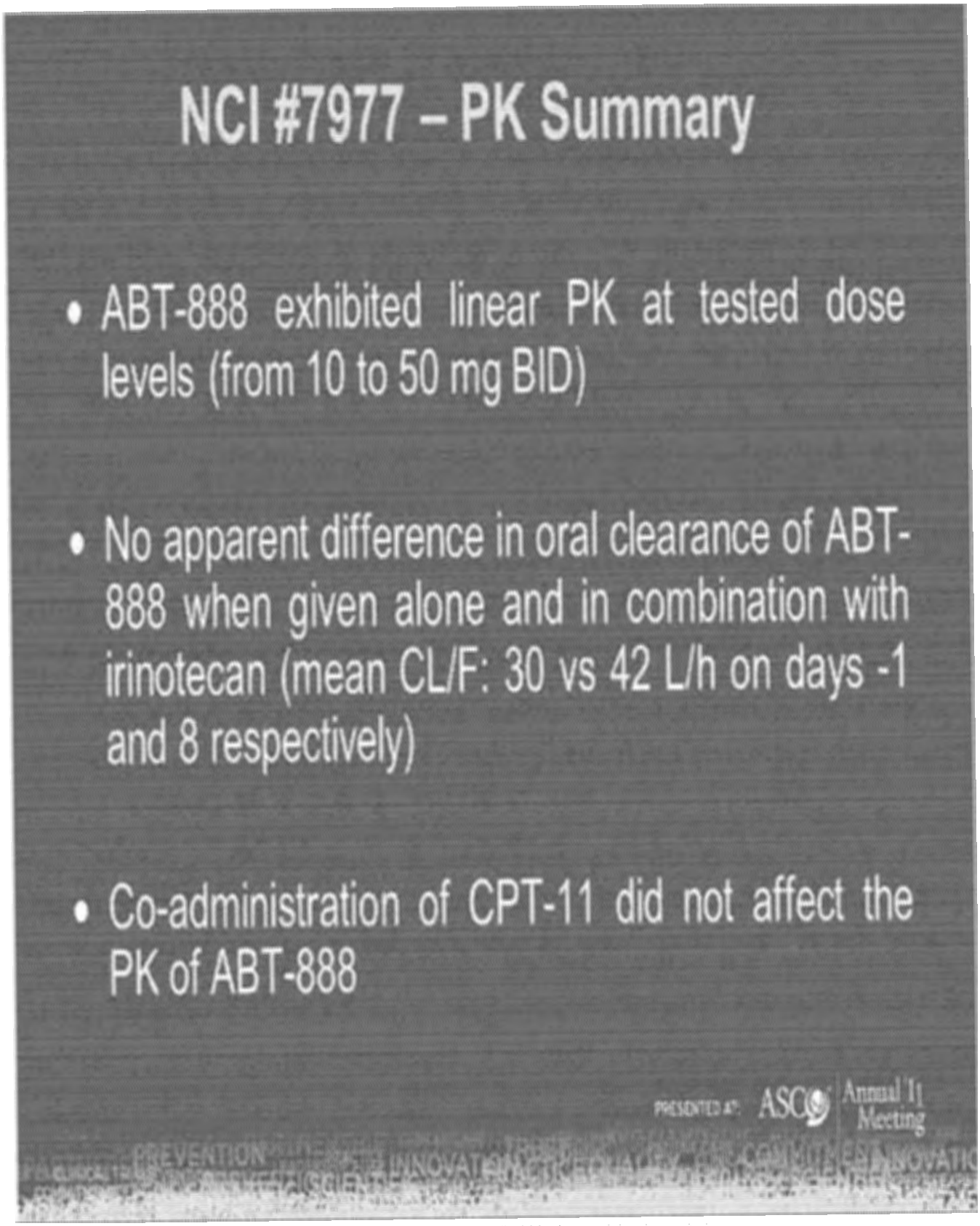
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PK parameters of CPT-11, alone (cycle 1) and in combination with ABT-888 (cycle 2)

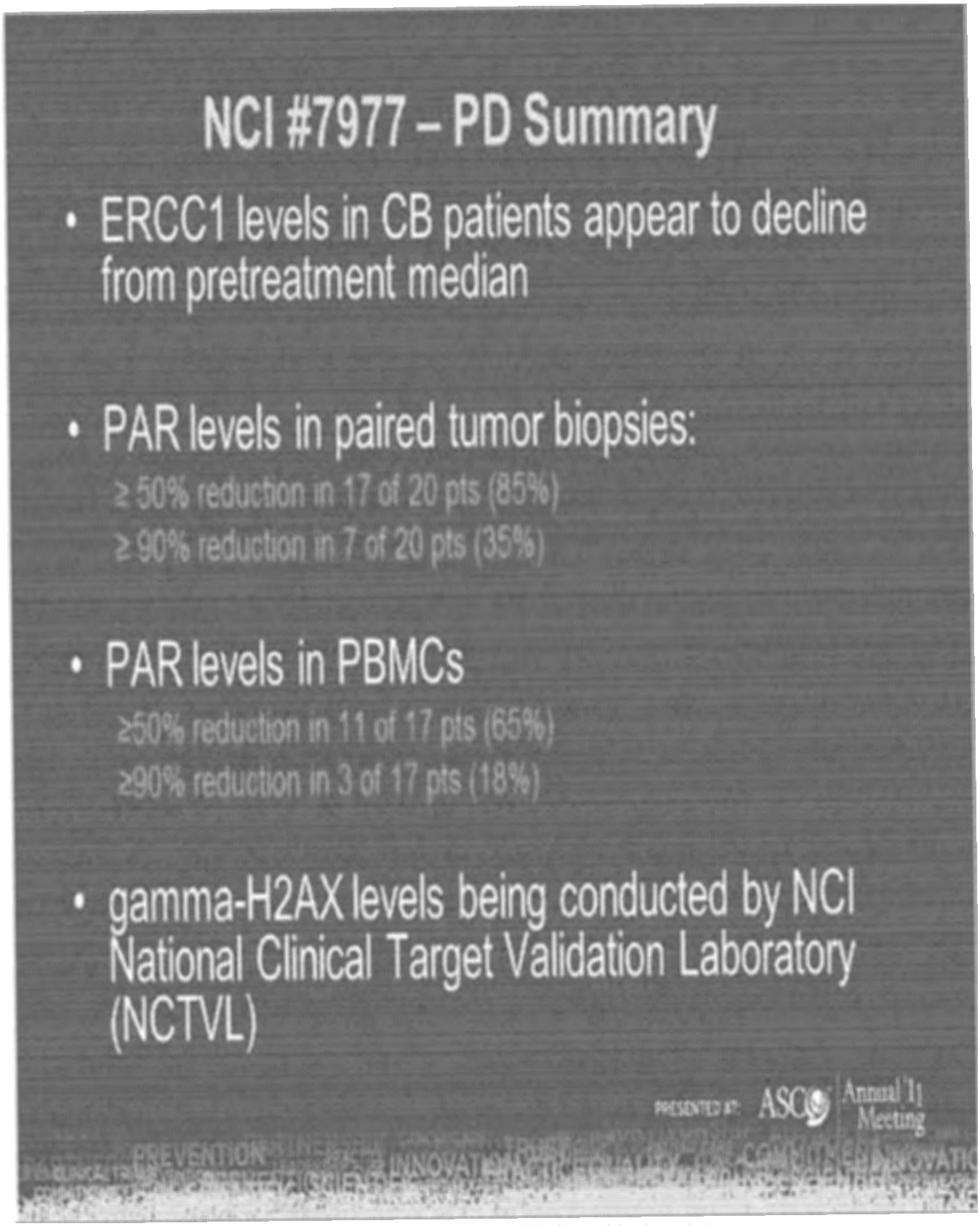
Cycle	T1/2 (h)	Tmax (h)	Cmax (ng/ml)	AUCINF (ng/ml*h)	Cl (L/h/m2)	Vz (L/m2)	Vss (L/m2)
CPT-11							
1	7.8±1.1	1.5±0.1	1060±341	5278±1900	21±5	230±70	151±38
2	7.6±1.1	1.5±0.2	818±230	4149±1036	25±6	270±63	178±55
SN-38							
1	14.0±10.6	2.0±0.9	11±6	117±61			
2	13.6±12.3	1.9±1.0	10±4	98±55			
SN-38G							
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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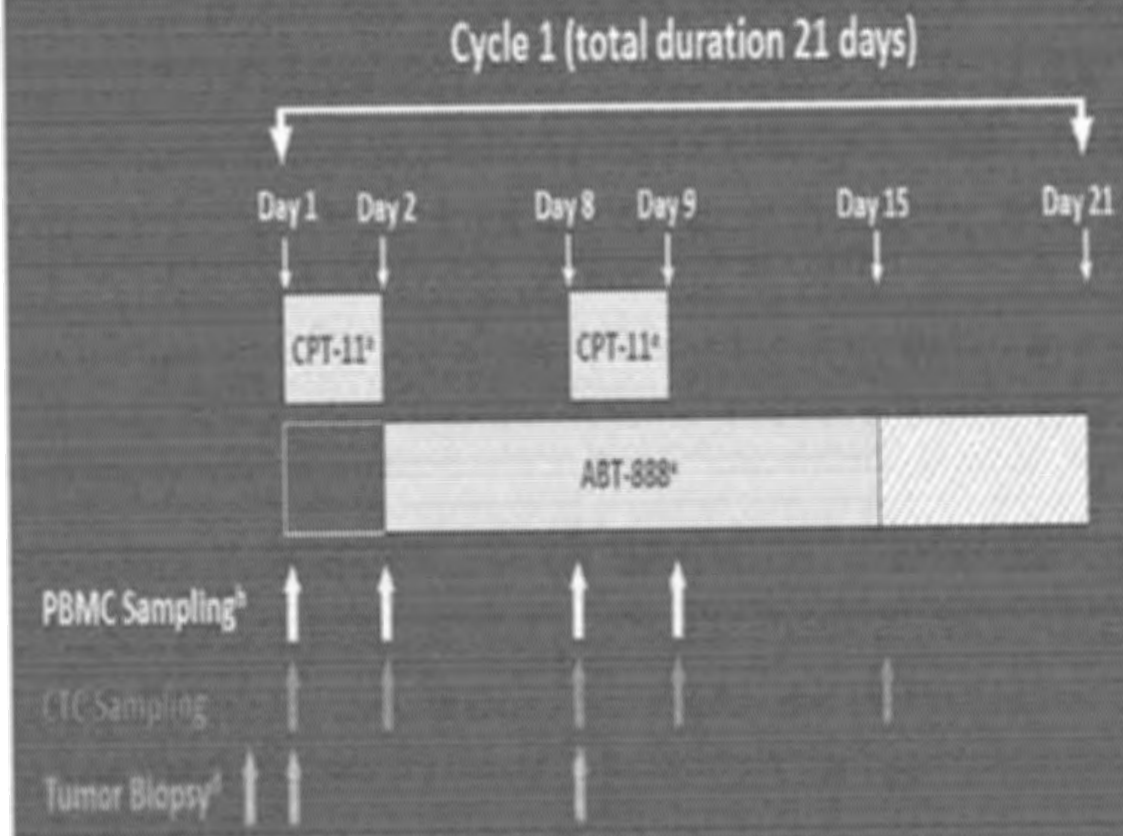
Proposed Expansion

- CTCs
  - +γH2Ax response in tumor @ 6-8 hrs vs CTCs at 12-24 hrs
  - Does PARPi ↑↑γH2Ax in CTCs by 6-8 hrs post CPT-11?
  - When does γH2Ax 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

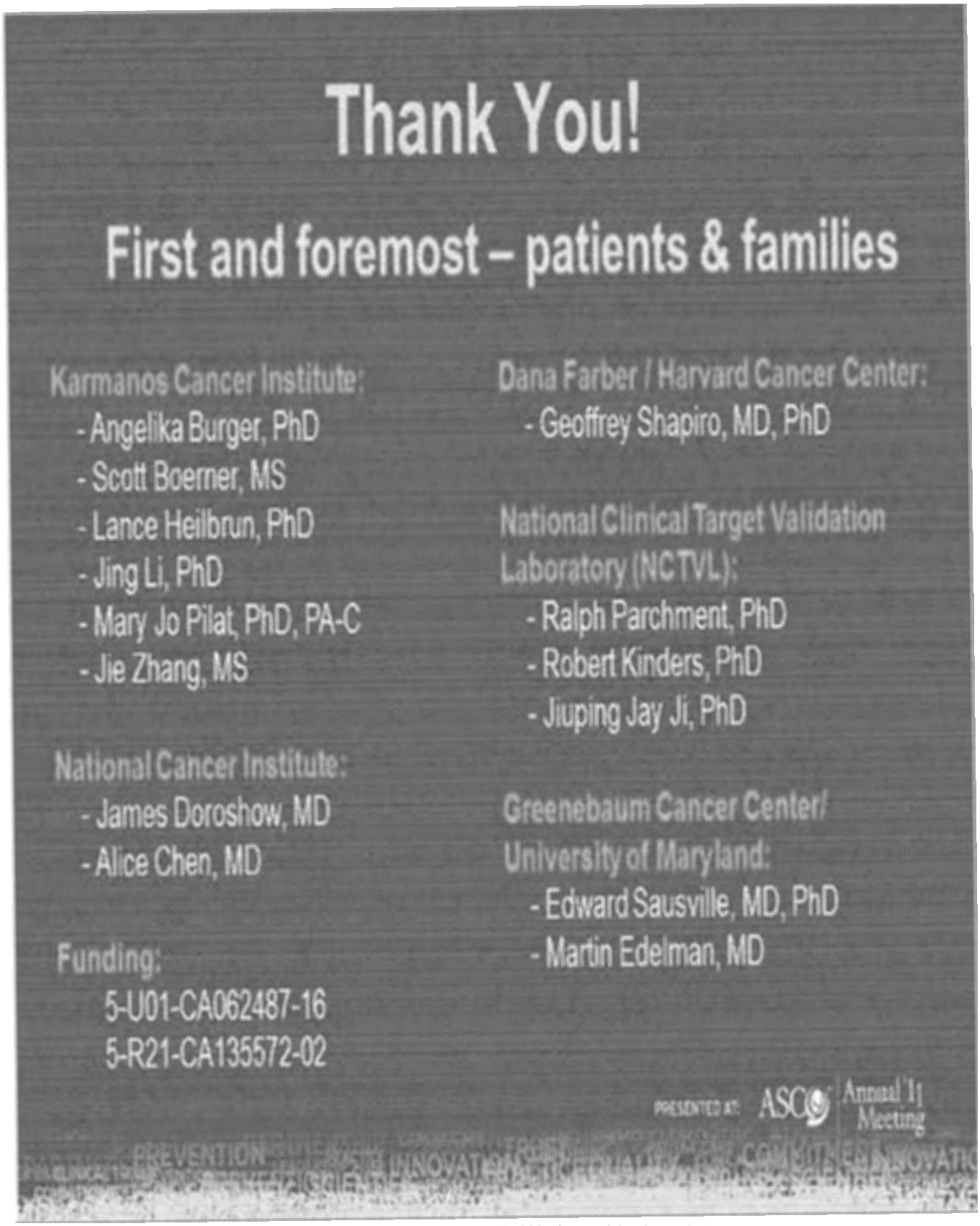


[a] CPT-11 (irinotecan) IV 100 mg/m² of CPT-11 given i.v. Days 1 and 8 administered for 90 min on days 1 and 8 of each cycle. ABT-888 40 mg po bid on days 2-15 in cycle 1; **Decision to administrate ABT-888 on a continuous schedule predicated on results from first 6 patients**

[b] PBMC samples will be obtained at baseline, 6-8, 12, 22, and 24 h after CPT alone on day 1 and after the combination dose on day 8 in cycle 1

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"My work is done."
Albert Einstein

The Lord is my Shepard. He makes me lie down in green pastures.
He leads me beside still waters. He restores my soul. *Psalm 23*



Prof. Dr. Dr. Angelika Burger

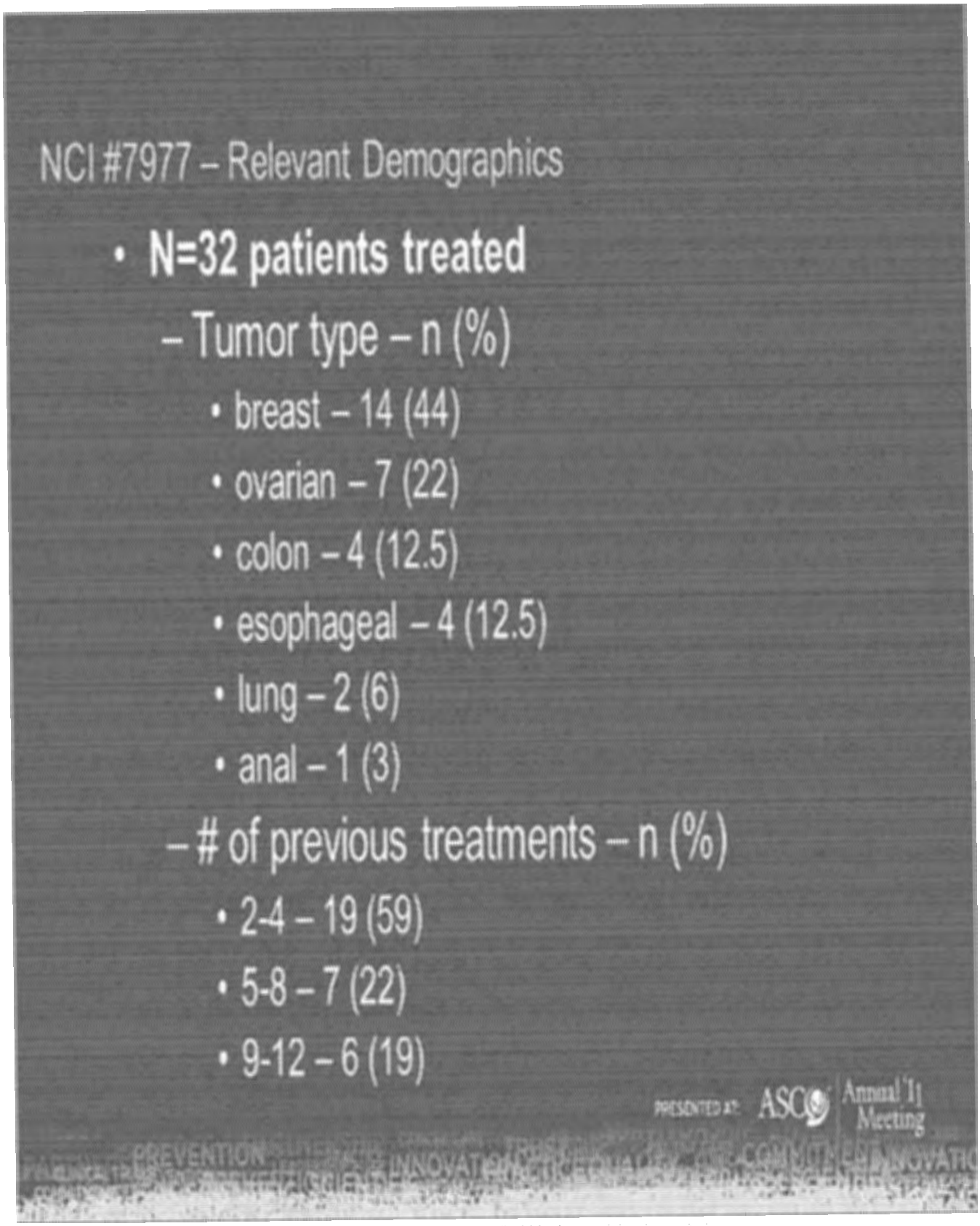
* 08.02.1965 † 04.05.2011

Our beloved daughter, sister, sister in law and nice 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

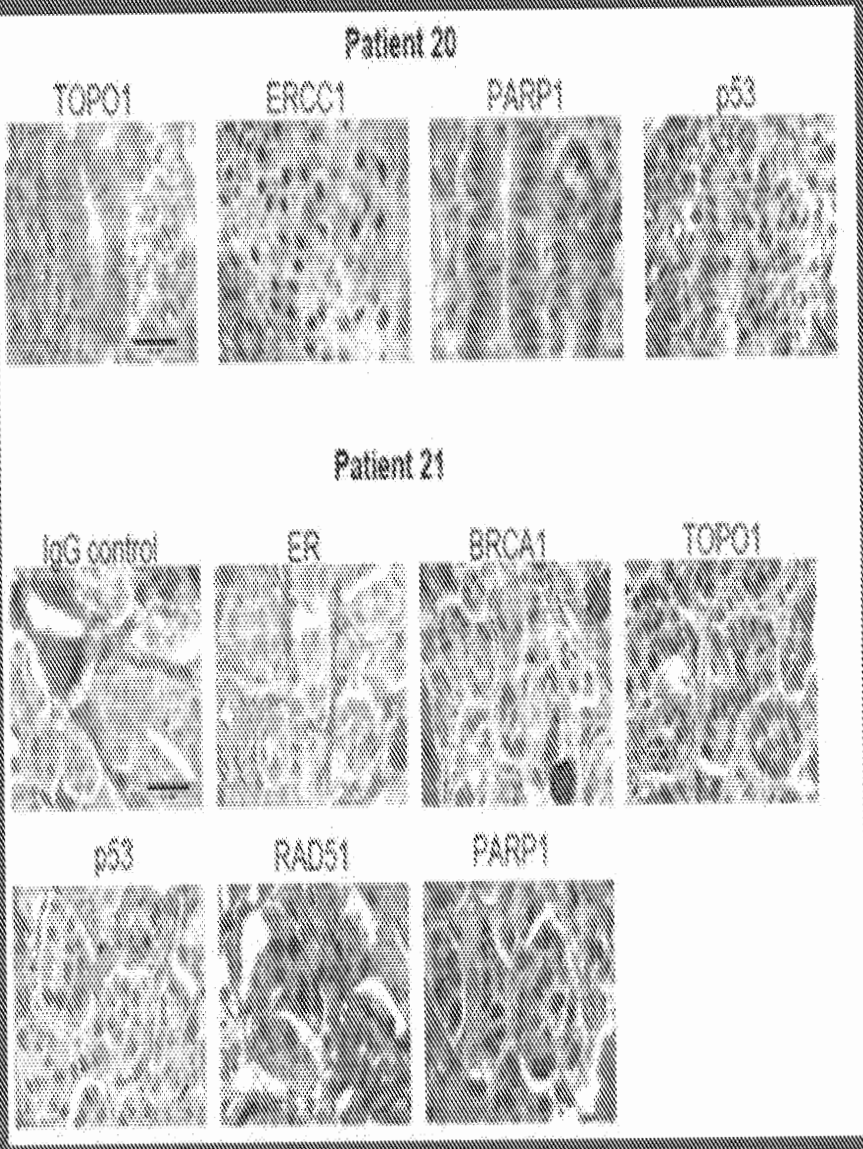
Neupotz/Germany, Detroit/USA, May 4th 2011

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/DonationFeb4.pp)*



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Immunohistochemistry (IHC) staining of archival tissue



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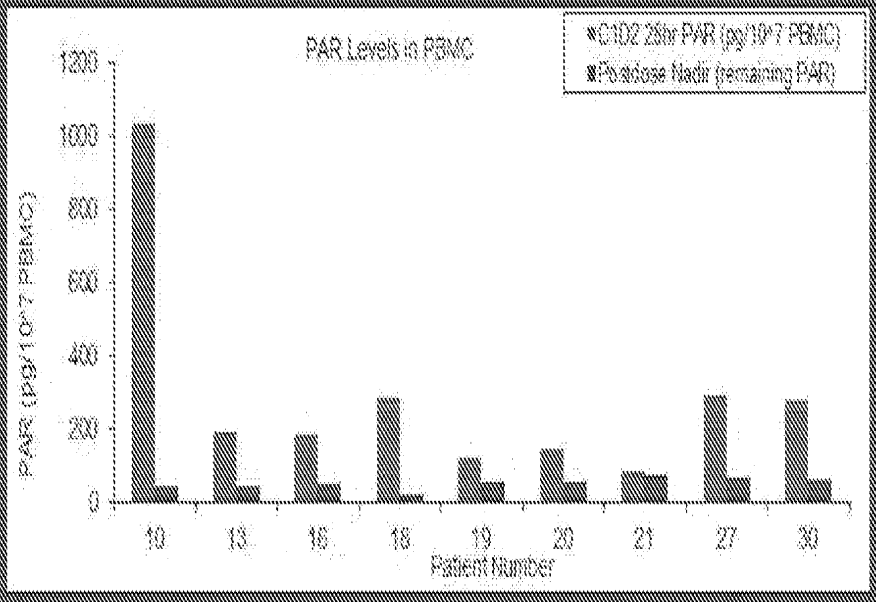
Results of Initial Immunohistochemistry staining

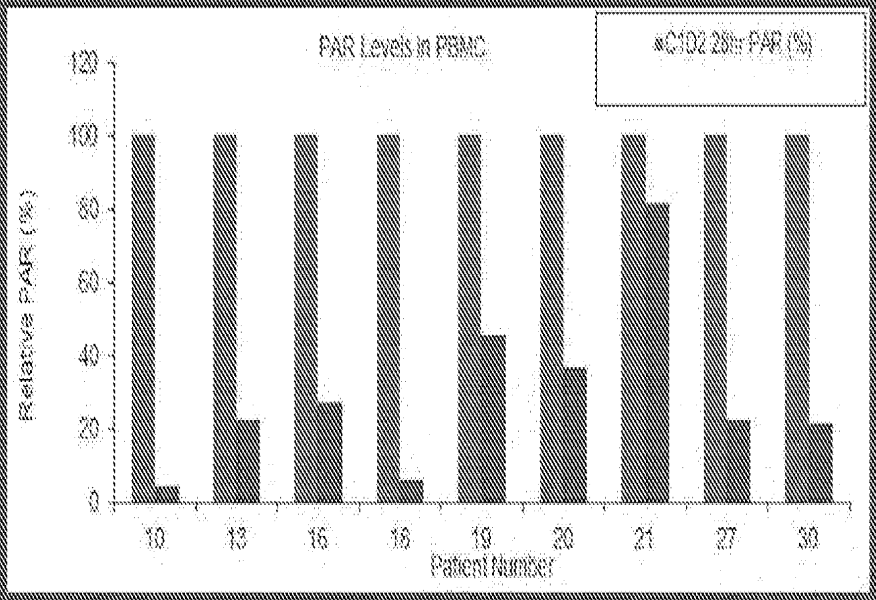
Antibody	Pt 6	Pt 13	Pt 14	Pt 18	Pt 20	Pt 21	Pt 23	Pt 24	Pt 26
ER	negative	negative	negative	positive	negative	negative	positive 3+	positive 1.5+	negative
p53	wild type	negative	not done	mutant	mutant	wild type	mutant	mutant	mutant
BRCA1	cytoplasm	negative	not done	cytoplasm	negative	nuclear	nuclear	nuclear	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+
TOPO1	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

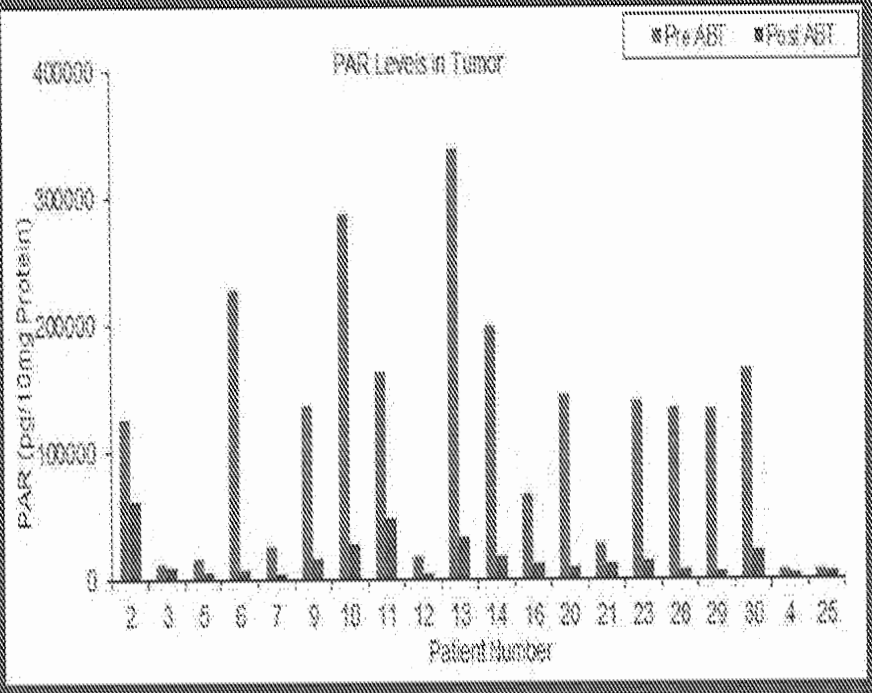


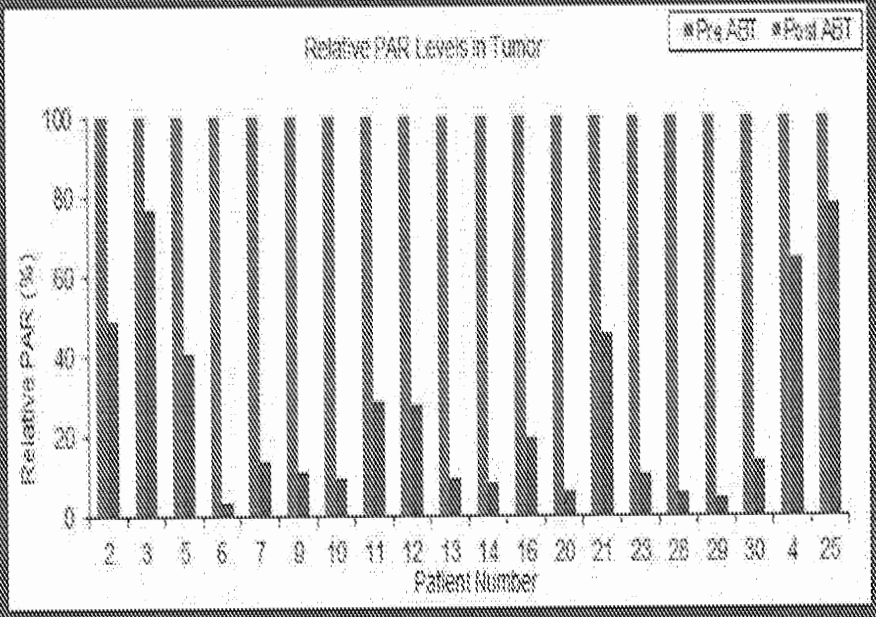


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PAR Levels in Tumors: Predose vs. Postdose

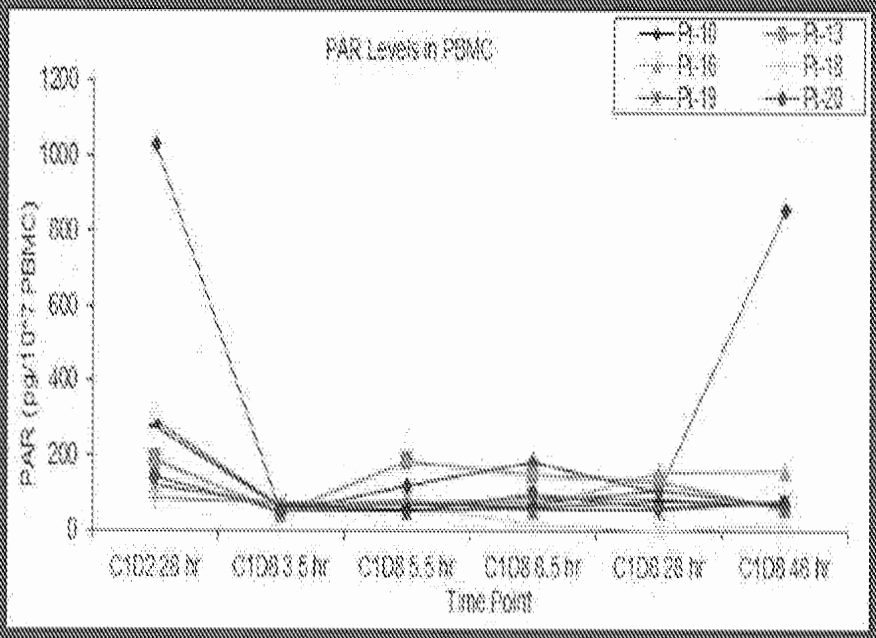


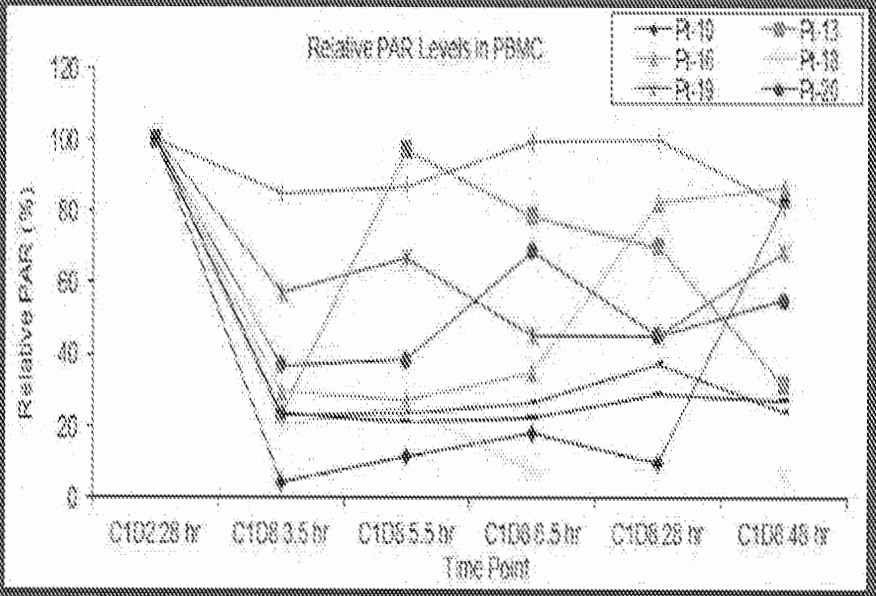


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PAR Levels in PBMCs

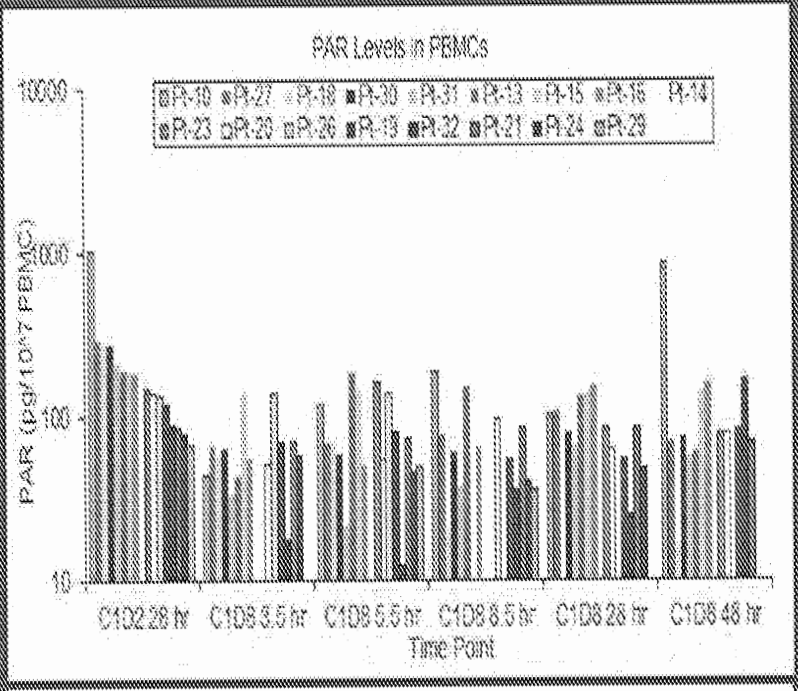


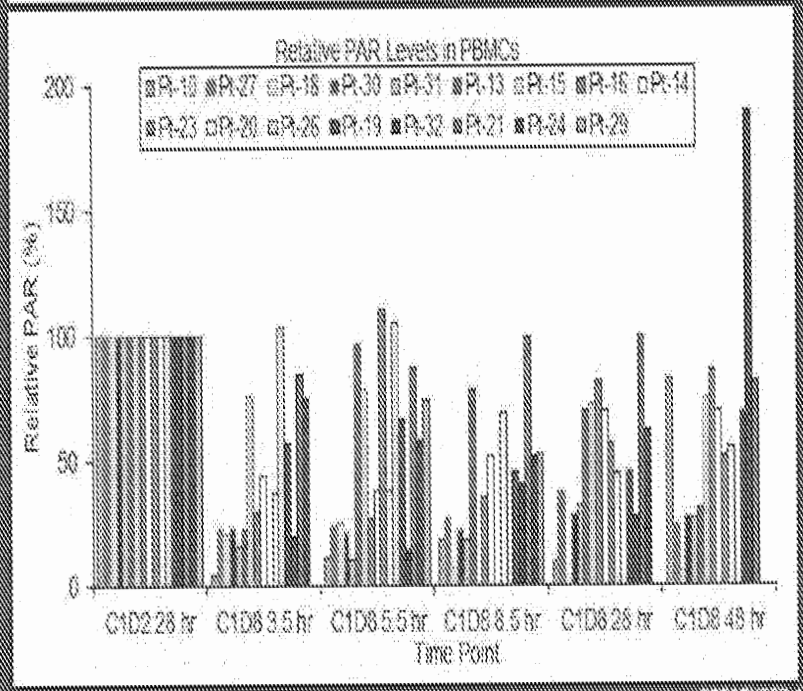


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PAR Levels in PBMCs





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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

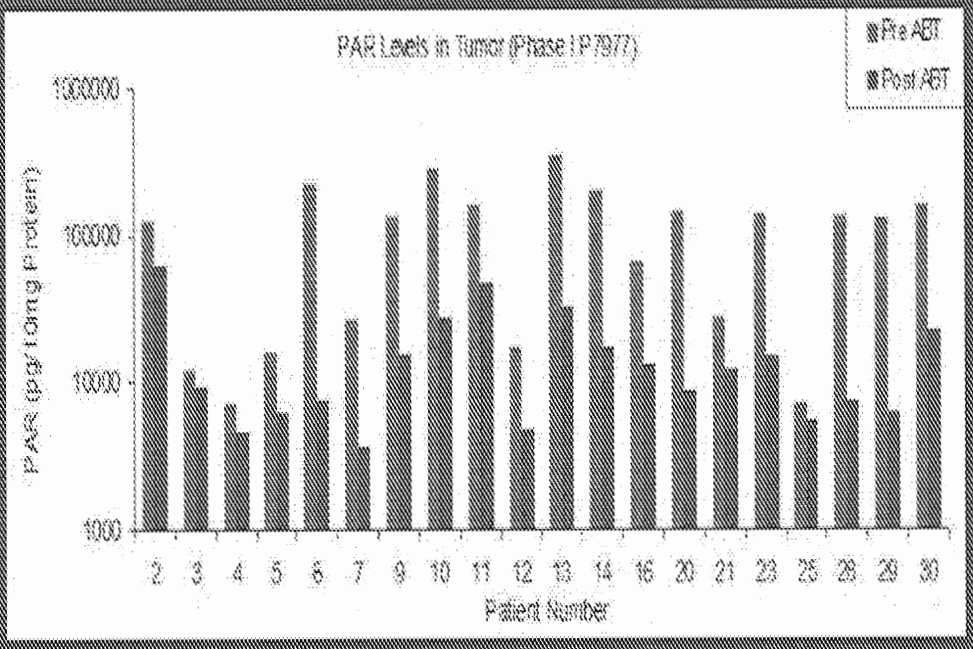
- There was no apparent difference in the apparent clearance (CL/F) of ABT-888 when given alone and in combination with irinotecan (mean, 23 vs. 25 L/h, $P = 0.79$), suggesting co-administration of irinotecan did not influence ABT-888 PK.

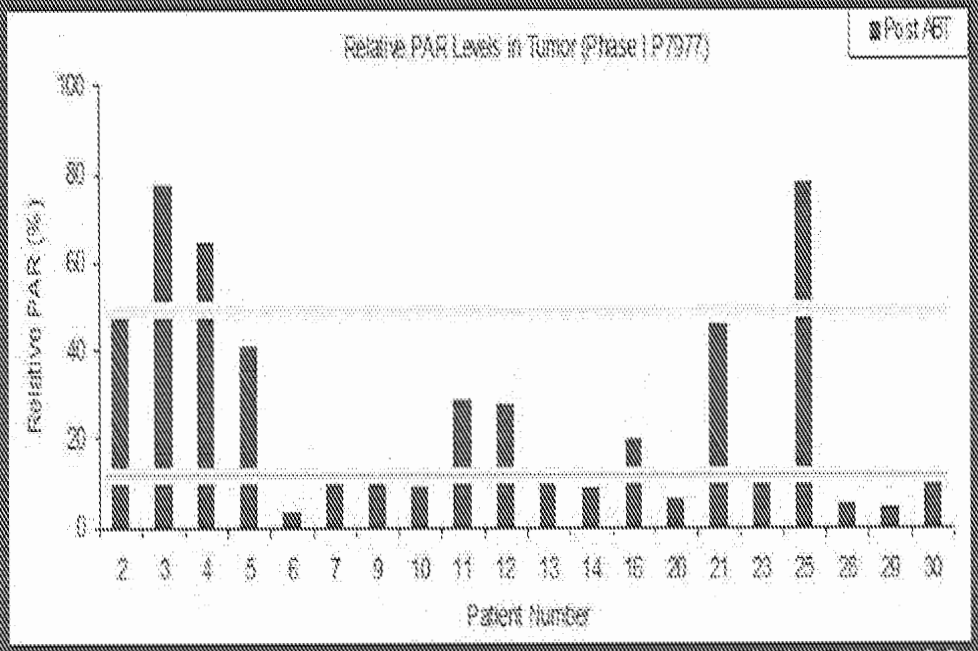
- The mean ratio of ABT-888 Cmax on day 8 relative to that on day -1 was 1.4, suggesting an average 1.4-fold drug accumulation at the steady state following twice daily dosing. This was consistent with the T1/2 of ABT-888 with a mean value of 7.3 h.

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PAR Levels in Tumors of Predose vs. Postdose

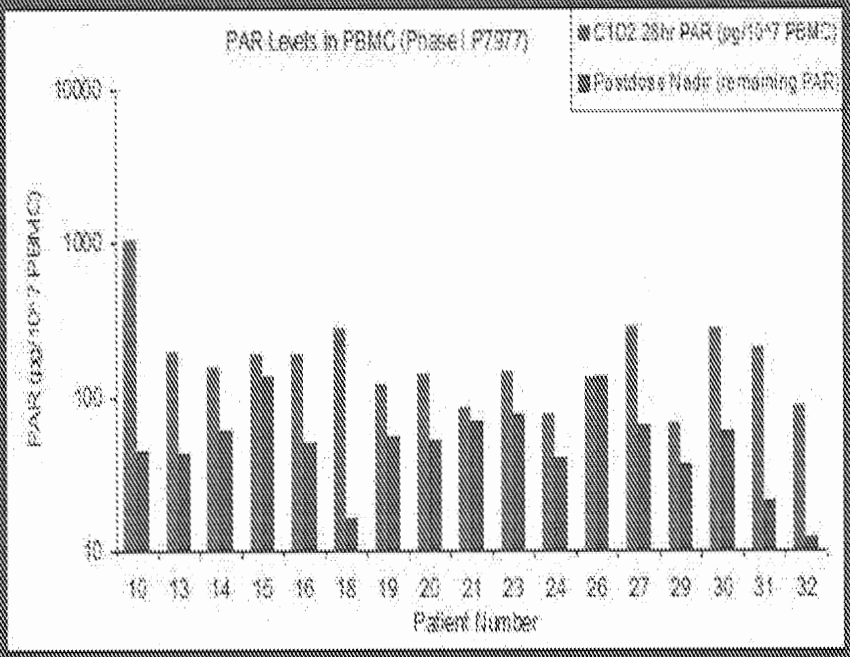


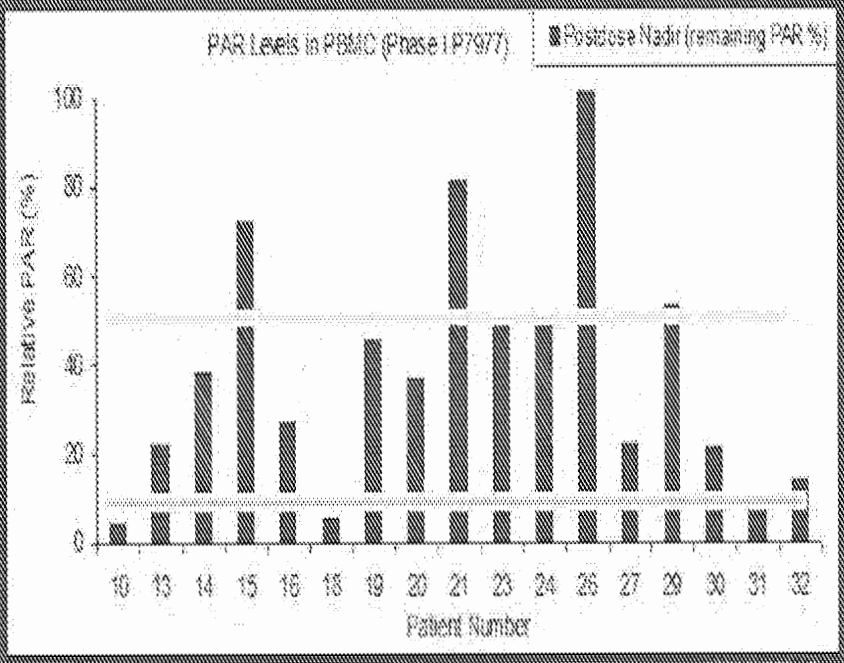


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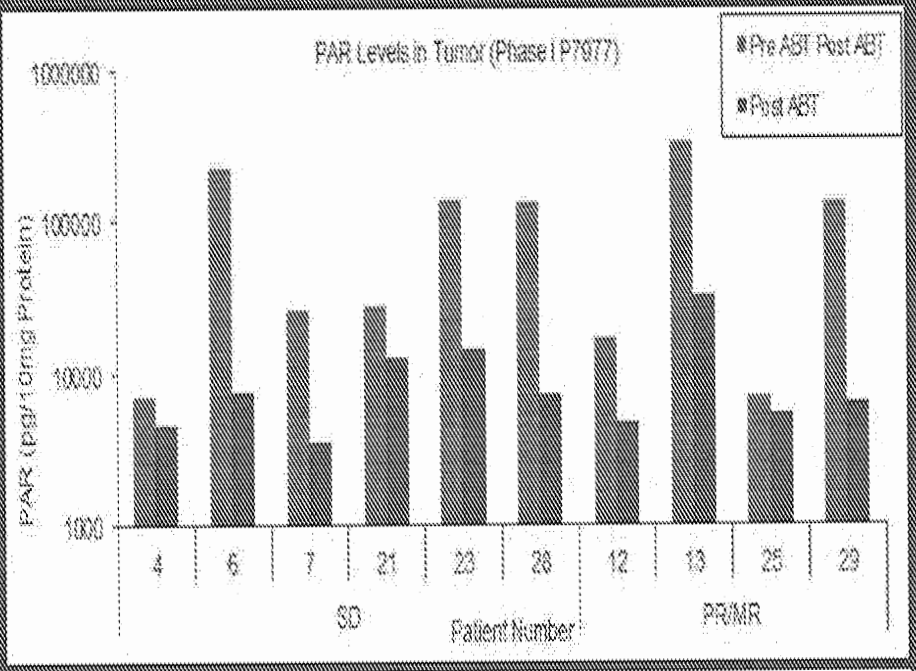


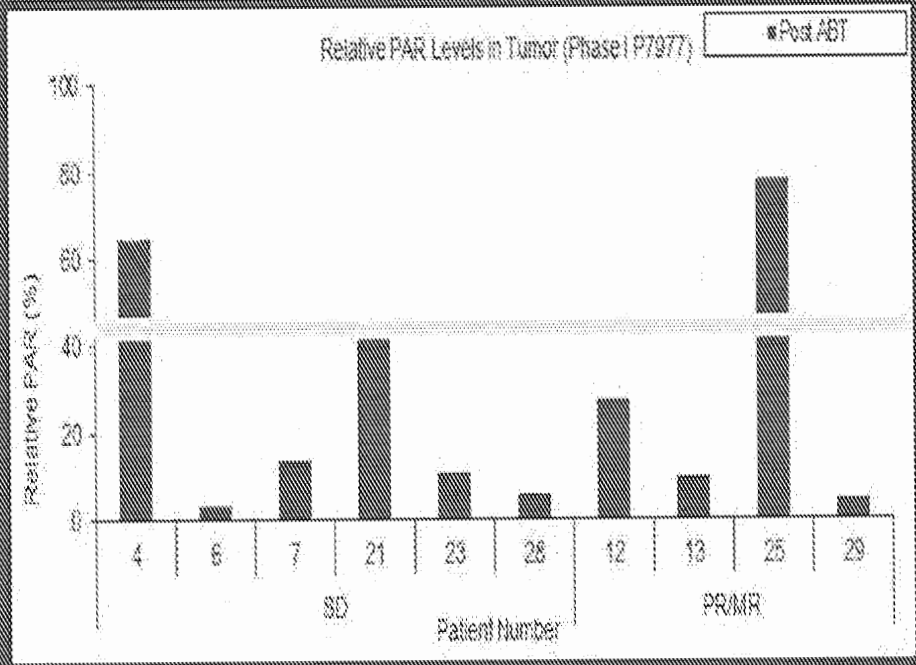


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PAR Levels in Tumors of Predose vs. Postdose





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DEVELOPMENTAL THERAPEUTICS—CLINICAL PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

A phase 1 dose-escalation study of veliparib with bimonthly FOLFIRI in patients with advanced solid tumors.

Jordan Berlin , Ramesh K. Ramanathan , John H. Strickler , Deepa Suresh Subramaniam , Herbert Hurwitz , Yoon-Koo KangTae-You Kim , Stacie Peacock Shepherd , Hao Xiong , Robert Hetman , Caroline Nickner , Matthew W. Dudley , Vincent L. Giranda , Heinz-Josef Lenz

Vanderbilt-Ingram Cancer Center, Nashville, TN; TGen - Virginia G. Piper Cancer Center at Scottsdale Healthcare, Scottsdale, AZ; Duke University Medical Center, Durham, NC; Lombardi Comprehensive Cancer Center, Georgetown University, Washington, DC; Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea; Department of Internal Medicine and Cancer Research Institute, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, South Korea; AbbVie, Inc., North Chicago, IL; AbbVie, Inc., Saint-Laurent, QC, Canada; Division of Medical Oncology, University of Southern California Norris Comprehensive Cancer Center, Los Angeles, CA

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DOI: 10.1200/jco.2014.32.15_suppl.2574
Journal of Clinical Oncology 32, no. 15_suppl (May 20, 2014) 2574-2574.

Published online May 20, 2014.

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WE RECOMMEND

A phase I clinical trial with weekly irinotecan (IRI) and capecitabine (CAP) in patients with advanced

Abstract

2574

Background: Veliparib (V; ABT-888), a potent, competitive poly (ADP-ribose) polymerase (PARP)-1 and -2 inhibitor enhances the activity of irrinotecan (IRI). This study seeks to establish the maximum tolerated dose (MTD) and recommended Phase 2 dose (RPTD) of V in combination with bimonthly FOLFIRI. **Methods:** Solid tumorpatients (pts; ≤3 prior DNA-damaging regimens) were administered V BID (days 1-5; 15–19) and FOLFIRI in three parts (P) : (P1) IRI 150 mg/m^2; (P2) IRI 180 mg/m^2 with 5-FU 400 mg/m^2 bolus; or (P3) IRI 180mg/m^2. All regimens included folinic acid 400 mg/m^2, immediately followed by 5-FU 46-hour continuous infusion. Dose escalation began with V 10 mg BID in P1 and 100 mg BID in P2 and P3. Two expansion cohorts were evaluated in gastric or colorectal cancer pts with no prior PARP inhibitor therapy. **Results:** 96 pts (median age 54.5 years; range, 24–77) were enrolled in cohorts from 10–300 mg BID V. Tumor types were: other (n=30); gastric (n=20); pancreatic (n=14); ovarian, breast, and colorectal (each, n=9). 88 pts discontinued: 83 due to progressive disease; 2 withdrew consent; and 3 other. Most common AEs (>40% pts) were diarrhea (61%), nausea (60%), neutropenia (59%), vomiting (48%), fatigue (47%), anemia and alopecia (each, 41%). Grade 3/4 AEs (>30 pts) were neutropenia (47%), nausea (38%), and diarrhea (34%). Four DLTs occurred: neutropenia (n=3; P1, 160 mg and 270 mg BID V; P2, 100 mg BID V); and gastritis and vomiting (P1, 270 mg BID V). 12 pts (gastric, n=3; ovarian, breast, pancreatic, and other, each n=2; colorectal, n=1) achieved a partial response and 1 (ovarian) a complete response. V exposure was approximately dose-proportional in combination with FOLFIRI. FOLFIRI PKs were comparable with or without V administration, indicating the lack of drug-drug interaction. **Conclusions:** V in combination with chemotherapy was generally well-tolerated without drug-drug interactions.

gastrointestinal and other solid malignancies
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PARP Inhibitors in Gynecologic Malignancies
Thao K. Huynh et al., US Pharmacist, 2020

AbbVie Initiates Veliparib Phase III Trial in BRCA-mutated Advanced Breast Cancer Patients
GenomeWeb, 2014

AbbVie's PARP Inhibitor Candidate Veliparib Plus Chemo Prolongs PFS in BRCA-Mutated Breast Cancer
Precision Oncology News, 2020

Ovarian and breast cancer pts experienced the greatest anti-tumor activity with an objective response rate of 33% and 22%, respectively. The RPTD was 200 mg BID V with bimonthly FOLFIRI (IRI 150 or 180 mg/m^2 without 5-FU bolus). This study supports further evaluation of V in combination with FOLFIRI in specific tumor types. Clinical trial information: NCT01123876.

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Dan Halbert, Iconix s New R&D Head, on the Firm s Toxicogenomics Goals
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Published OnlineFirst February 27, 2014; DOI: 10.1158/1535-7163.MCT-13-0683

Molecular Cancer Therapeutics

The Use of Olaparib (AZD2281) Potentiates SN-38 Cytotoxicity in Colon Cancer Cells by Indirect Inhibition of Rad51-Mediated Repair of DNA Double-Strand Breaks

Makiko Tahara[1,3], Takeshi Inoue[1], Futoshi Sato[1], Yasuyuki Miyakura[3], Hisanaga Horie[3], Yoshikazu Yasuda[3], Hirofumi Fujii[4], Kenjiro Kotake[2], and Kokichi Sugano[1]

Abstract

Potent application of topoisomerase I inhibitor plus PARP inhibitor has been suggested to be an effective strategy for cancer therapy. Reportedly, mismatch repair (MMR)–deficient colon cancer cells are sensitive to topoisomerase I inhibitor, presumably due to microsatellite instability (MSI) of the *MRE11* locus. We examined the synergy of SN-38, an active metabolite of irinotecan, in combination with the PARP inhibitor olaparib in colon cancer cells showing different MMR status, such as MSI or microsatellite stable (MSS) phenotype. Treatment with SN-38 and olaparib in combination almost halved the IC_{50} of SN-38 for a broad spectrum of colon cancer cells independent of the MMR status. Furthermore, olaparib potentiated S-phase–specific double-strand DNA breaks (DSB) induced by SN-38, which is followed by Rad51 recruitment. siRNA-mediated knockdown of Rad51, but not Mre11 or Rad50, increased the sensitivity to olaparib and/or SN-38 treatment in colon cancer cells. *In vivo* study using mouse xenograft demonstrated that olaparib was effective to potentiate the antitumor effect of irinotecan. In conclusion, olaparib shows a synergistic effect in colon cancer cells in combination with SN-38 or irinotecan, potentiated by the Rad51-mediated HR pathway, irrespective of the Mre11-mediated failure of the MRN complex. These results may contribute to future clinical trials using PARP inhibitor plus topoisomerase I inhibitor in combination. Furthermore, the synergistic effect comprising topoisomerase I-mediated DNA breakage–reunion reaction, PARP and Rad51-mediated HR pathway suggests the triple synthetic lethal pathways contribute to this event and are applicable as a potential target for future chemotherapy. *Mol Cancer Ther; 13(5); 1170–80. ©2014 AACR.*

Introduction

Colorectal cancer is the third most common cancer in men and the second in women worldwide, while almost 60% of the cases occur in developed countries. Colorectal cancer is estimated to be the cause of about 8% of all cancer-related deaths, making it the fourth most common cause (1).

The incidence of distant metastasis (stage IV) is reported to be 18% to 25%, with differences depending on ethnicity, and the 5-year relative survival rate for stage IV disease is as low as 12% (2), which shows the need for developing novel systemic chemotherapy. Conventionally, 5-fluorouracil (5-FU) and its analogs were employed as the first-line or adjuvant chemotherapy for colon cancer, while cases relapsing after 5-FU–based adjuvant chemotherapy were mostly resistant to 5-FU–based systemic chemotherapy. Irinotecan (CPT-11) was introduced as a drug effective for 5-FU–resistant colorectal cancer, the molecular target of which is type I topoisomerase. At present, either FOLFOX (5-FU + leucovorin + oxaliplatin) or FOLFIRI (5-FU + leucovorin + irinotecan) is recommended as the first-line regimen for stage IV colorectal cancer.

DNA repair systems have drawn attention as molecular targets of cancer therapy. Cancers with DNA repair deficiency could be more sensitive to certain therapeutic agents. One of the well-known syndromes is hereditary breast/ovarian cancer (HBOC) harboring a mutated *BRCA1* or *BRCA2* gene, which is involved in double-strand DNA break (DSB) repair. PARP inhibitors showed synthetic lethality to HBOC-related tumors (3, 4) and clinical trials on cases with HBOC have been ongoing worldwide. PARP inhibitors mainly target PARP-1 and PARP-2, which synthesize poly(ADP-ribose) (PAR) and are involved in DNA repair, cell differentiation, regulation of chromatin structure, and gene regulation (5–7).

Authors' Affiliations: [1]Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, [2]Department of Surgery, Tochigi Cancer Center, Utsunomiya; and [3]Department of Gastrointestinal Surgery and [4]Division of Clinical Oncology, Jichi Medical University, Shimotsuke, Tochigi, Japan

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org).

Corresponding Author: Kokichi Sugano, Oncogene Research Unit/Cancer Prevention Unit, Tochigi Cancer Center Research Institute, 4-9-13 Yohnan, Utsunomiya, Tochigi 320-0834, Japan. Phone: 812-8658-5151; Fax: 812-8684-5756; E-mail: ksugano@tcc.pref.tochigi.lg.jp

doi: 10.1158/1535-7163.MCT-13-0683

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PARP-1 has an important role in single-strand DNA break (SSB) repair and base excision repair (8). Recently, PARP has been reported to participate in the DSB repair pathway by nonhomologous end joining (NHEJ; refs. 9, 10) or homologous recombination (HR; ref. 11). The mechanism of PARP in DSB repair has been elucidated.

On the other hand, mismatch repair (MMR) deficiency showing microsatellite instability (MSI) due to aberration of mismatch repair genes such as *MSH2*, *MLH1*, *MSH6*, or *PMS2* could be a novel target for cancer treatment because such cases often show frameshift mutation in *MRE11* and/or *RAD50* genes, of which the gene products form a heterotrimer with Nbs1 and are associated with DSB repair (12–15). Reportedly, MMR deficiencies in colorectal cancer could be available as a surrogate marker for various chemotherapeutic agents. MMR-deficient cells were sensitive to topoisomerase I inhibitors (16–19), such as irinotecan and camptothecin, which form a covalent bond with topoisomerase I and inhibit the breakage–reunion reaction in DNA replication or translation; they are now available as the major chemotherapeutic drugs for colorectal cancer (20, 21). *In vivo*, irinotecan is converted to its active metabolite, SN-38, in the presence of carboxylesterase (22, 23).

The combinational treatment of PARP inhibitor and topoisomerase I inhibitor has been considered as a potent strategy for cancer therapeutics, especially for colorectal cancer cells (24–30). However, the mechanisms of inducing topoisomerase I inhibitor hypersensitivity using PARP inhibitor remain unclear and the correlation of the MMR status with the sensitivity to topoisomerase I inhibitor treatment in combination with PARP inhibitor has not been elucidated yet.

Olaparib (AZD2281) is a PARP inhibitor that has been used in clinical trials targeting breast or ovarian cancers. In this study, first, we investigated the sensitivity to SN-38 plus olaparib combinational treatment in colon cancer cell lines characterized by their MMR status, using the ^{3}H-thymidine incorporation assay, the cell counting assay and the clonogenic assay. Second, we investigated the intracellular response of treated cells by using cell diameter measurements as a surrogate marker of G_2–M arrest and apoptosis. Third, we examined the expression of DSB repair-related proteins and the incorporation of 5-bromo-2′-deoxyuridine (BrdUrd), that is, a S-phase–specific cell-cycle marker, using immunofluorescence microscopy to elucidate the mechanisms of enhanced chemosensitivity evoked by olaparib. Finally, we examined the antitumor effect of irinotecan plus olaparib in mouse xenograft models to confirm the results of *in vitro* study as the final stage of the preclinical setting.

Materials and Methods

Reagents

SN-38 and irinotecan (CPT-11) were purchased from Sigma-Aldrich and SANDOZ, respectively. Olaparib (AZD2281) was kindly provided by AstraZeneca K.K. SN-38 and olaparib were dissolved in dimethyl sulfoxide at a concentration of 10 mmol/L and stored at −20°C until the *in vitro* experiment. Irinotecan (20 mg/mL) was stored at room temperature for the *in vivo* experiment.

Cell lines and culturing

SW48, RKO, HCT116, and HT29 were purchased from American Type Culture Collection (ATCC; Summit Pharmaceutical International) from January 2010 through March 2011, which had been characterized using short tandem repeat polymorphism analysis by ATCC. LoVo was purchased from Health Science Research Resources Bank (HSRRB) in January 2011, which had been verified using isoenzyme analysis by HSRRB. These cell lines were passaged for less than 6 months after receipt. HCT-15 was kindly provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan) in January 2011. SW1116, C-1, and colo320HSR were provided by the National Cancer Center (Tokyo, Japan) in 1999. These four cell lines had not been authenticated in our laboratory.

All of these human colon cancer cell lines were cultured in RPMI-1640 medium (Life Technologies Japan) supplemented with 10% FBS (Thermo Fisher Scientific Japan) and 100 μg/mL kanamycin (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO_2.

SW48 and RKO lack *MLH1* expression due to promoter hypermethylation, causing MSI (31). HC116, LoVo, and HCT-15 have mutated MMR genes, *MLH1* (p.S252X), *MSH2* (p.R359fsX8), and *MSH6* (p.L290fsX1 and p.D1171fsX4), respectively (Supplementary Table S1).

Cell proliferation assay

The procedures for the ^{3}H-thymidine incorporation assay and the cell counting assay using the Scepter 2.0 cell counter were as described previously in detail (32) and are described in the Supplementary Methods.

Western blot analysis

The procedures for Western blot analysis were described in the Supplementary Methods. The primary antibodies used were as follows: anti-PAR (10H) mouse monoclonal antibody (1:500; GeneTex), anti–PARP-1 mouse monoclonal antibody (1:2,000, Santa Cruz Biotechnology), anti–PARP-2 mouse monoclonal antibody (1:1,000, Merck Millipore), anti-Mre11 rabbit polyclonal antibody (1:2,000, Cell Signaling Technology), anti-Rad50 mouse monoclonal antibody (1:1,000, GeneTex), anti-Nbs1 rabbit polyclonal antibody (1:1,000, Novus Biologicals), and anti-Rad51 rabbit polyclonal antibody (1:2,000, Santa Cruz). Anti–β-actin mouse monoclonal antibody (1:200, Santa Cruz Biotechnology) was used as an internal control.

Immunofluorescence microscopy

The detail of the procedures for the immunofluorescence staining is described in the Supplementary Methods. The primary antibodies were as follows: anti-53BP1 rabbit polyclonal antibody (1:500, Abcam), anti-Rad51 rabbit polyclonal antibody (1:50, Santa Cruz

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Biotechnology), anti-γ-H2AX mouse monoclonal antibody (1:500, Merck Millipore) and anti-BrdUrd mouse monoclonal antibody (1:500, Sigma-Aldrich). Fluorescence images were recorded with a BZ-9000 fluorescence microscope (KEYENCE) and analyzed using BZ-II Analyzer (KEYENCE). For quantification of 53BP1, Rad51, and γ-H2AX foci, haze-reduced images of about 100 to 200 nuclei from at least 4 fields were observed and the number of foci per nucleus was counted. For counting nuclei with BrdUrd incorporation, at least 6 fields (about 200–1,300 nuclei/field) were observed.

RNA interference

siRNAs directed to Mre11, Rad50, and Rad51 as well as a negative control were purchased from Life Technologies Japan. As commercially available negative control siRNA was cytotoxic for transfection with HCT116, we synthesized nontargeted siRNA using the Silencer siRNA Construction Kit (Life Technologies Japan). The nontarget siRNA sequences were designed as the scrambled sequences of the siRNA directed to RAD51: ACGTATGGATCTAAGATGCtt (sense) and CCATCTCGATCCATACGTat (antisense).

A total of 1–3 × 10^4 cells were plated in 24-well culture plates (500 μL/well). After 24 hours, the cells were transfected with 10 nmol/L siRNA using Lipofectamine RNAiMAX transfection reagent (Life Technologies Japan) in accordance with the manufacturer's recommended protocol. After 24 hours, the medium was replaced with fresh growth medium and exposed to SN-38 and/or olaparib for 48 hours, and then cells were harvested for the cell counting assay. For Western blot analysis, cells were plated in 100 mm dishes (10 mL/dish) and harvested 24 hours after transfection.

In vivo growth inhibitory assay

Five-week-old SCID hairless outbred (SHO) mice (Crl:SHO-Prkdc^{scid}Hr^{hr}) were purchased from Charles River Japan, Inc.. Xenograft models were established by injecting HT29 or SW1116 cells (5 × 10^6) subcutaneously into the left flank of mice. When the tumors reached a size of 100 mm^3, xenografted mice were divided into four groups as follows: control, olaparib alone, irinotecan alone, and olaparib plus irinotecan, and treatment was initiated (day 0). Olaparib (50 mg/kg) was dissolved in 9% β-cyclodextrin (33) and administered orally (*per os*) once a day for 5 days followed by an intermission of two days. In the no treatment group, 9% cyclodextrin alone was administered. Irinotecan (10 mg/kg) was administered intraperitoneally twice weekly. In the no treatment group, PBS alone was administered. In the combinational treatment group, olaparib was administered 1 hour before irinotecan treatment. The tumor diameters were measured with calipers and the tumor volumes were calculated using the following formula: length (mm) × width $(mm)^2/2$. The mice were killed on day 18 for HT29 and on day 30 for SW1116. The tumor sizes and body weights were measured three times weekly. In the study of SW1116 xenografted mice, blood sampling was carried out for analysis of hematologic toxicity and liver dysfunction. The blood samples were analyzed by SRL. Relative tumor volume was determined using the value relative to that on day 0. The data of the relative tumor volume are shown as mean ± SEMs.

Statistical analysis

The data of the *in vitro* study are presented as means ± SDs and that of the *in vivo* study are presented as mean ± SEMs. Statistical significance of *in vitro* study was either analyzed by the Student *t* test (between 2 groups) or one-way ANOVA with the Tukey test or Tukey–Kramer method (between 4 groups). In *in vivo* study, a linear trend test was performed as a utility of one-way ANOVA in each group, and statistical significance between four groups was analyzed by one-way ANOVA with the Tukey–Kramer method. All statistics were calculated using the GraphPad Prism V5.0 software (GraphPad Software, Inc.).

Results

Molecular profiles of the colon cancer cell lines

The molecular profiles of the cell lines used for the assay were examined and summarized as regards MSI status, genetic or epigenetic alterations of the MMR genes, and mutational status of *TP53*, *MRE11*, and *RAD50* (Supplementary Table S1). Of the 9 cell lines examined, 5 cell lines (SW48, RKO, HCT116, LoVo, and HCT-15) showed MSI, whereas 4 cell lines (C-1, colo320HSR, HT29, and SW1116) had a MSS phenotype. MMR-deficient cell lines also had MSI in the genes responsible for HR, such as *MRE11* and *RAD50*. SW48, RKO, HCT116, and LoVo had biallelic MSI in the poly(T)11 tract of *MRE11*, whereas HCT-15 had monoallelic MSI. As for *RAD50* poly(A)8 mutations, RKO, HCT116, and LoVo were heterozygous while SW48 and HCT-15 showed the wild-type phenotype. *TP53* gene mutations were detected in 1 MMR-deficient cell line (HCT-15) and 3 MMR-proficient cell lines (colo320HR, HT29, and SW1116).

Olaparib potentiates SN-38 sensitivity by inhibiting the synthesis of PAR polymer

To assess the effect of olaparib, we first evaluated the cell growth inhibitory effects of olaparib alone by using the ^{3}H-thymidine incorporation assay (Fig. 1A). Of all the cell lines examined, HCT116 was the most sensitive to olaparib, the IC_{50} of which was 2.5 μmol/L and SW1116 was the most resistant, with the IC_{50} of more than 100 μmol/L. We also evaluated the expression of PAR, PARP-1, and PARP-2 in HCT116 cells treated with various concentrations of olaparib. Poly (ADP-ribosyl)ation (PARylation) induced by 1 nmol/L SN-38 in HCT116 was inhibited by olaparib in a dose-dependent manner without affecting the expression levels of PARP-1 and PARP-2 (Fig. 1B).

Next, we evaluated the cell growth inhibitory effects of SN-38 with various concentrations of olaparib in HCT116 and HT29, which have different MMR status and sensitivities to SN-38 (17, 32, 34). In the presence of 0.5 nmol/L SN-38, cell growth was inhibited with 10 nmol/L ($P < 0.01$) or

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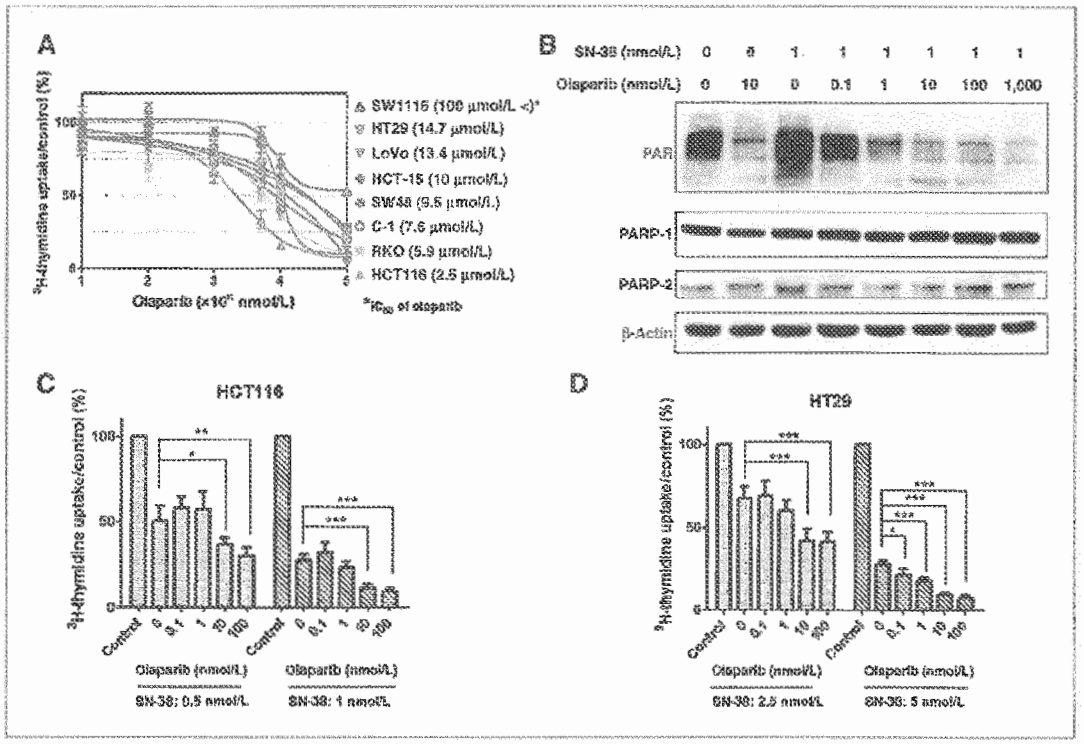


Figure 1. Effects of olaparib in the presence or absence of SN-38 on colon cancer cell lines. A, cell growth inhibition curves of colon cancer cell lines exposed to various concentrations of olaparib were determined using ^{3}H-thymidine incorporation assay. For each cell line, cell growth inhibition (%) was calculated as follows: (^{3}H-thymidine uptake of treated cells − background) × 100/(^{3}H-thymidine uptake of untreated cells − background). Error bars, SDs from replicate experiments ($n = 6$). Each number in parentheses shows the IC_{50} of olaparib analyzed using the cell growth inhibition curves. B, Western blot analysis of PAR, PARP-1, PARP-2, and β-actin (internal control) of HCT116 exposed to various concentrations of olaparib in the presence or absence of 1 nmol/L SN-38. C, cell growth inhibition (%) of HCT116 exposed to various concentrations of olaparib with SN-38 (0.5 or 1 nmol/L). D, cell growth inhibition (%) of HT29 exposed to various concentrations of olaparib with SN-38 (2.5 and 5 nmol/L). Each value is expressed as mean ± SD ($n = 6$). Control, nontreated cells. The Student t test was performed between cells exposed to SN-38 alone and cells exposed to SN-38 plus each concentration of olaparib in HCT116 and HT29 (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$).

100 nmol/L ($P < 0.001$) olaparib, and these effects were enhanced when cells were treated with 1 nmol/L SN-38 ($P < 0.0001$ for 10 nmol/L and $P < 0.0001$ for 100 nmol/L olaparib) in HCT116. Concentrations of olaparib less than 1 nmol/L had no inhibitory effect on cell growth in HCT116 treated with 0.5 nmol/L or 1 nmol/L SN-38 (Fig. 1C). Likewise, olaparib also potentiated SN-38 sensitivity at concentrations above 10 nmol/L in HT29 (Fig. 1D). Olaparib concentrations less than 1 μmol/L did not show cell cytotoxicity (Fig. 1A). Considering these results, we determined the concentration of olaparib as 10 nmol/L in the subsequent *in vitro* experiments.

Olaparib potentiates SN-38 cytotoxicity irrespective of MMR status

To assess whether the potentiating effects of olaparib on SN-38 sensitivity were correlated with MMR status or not, we evaluated the SN-38 sensitivity in the presence or absence of olaparib by a variety of assays using colon cancer cell lines showing either MMR-deficient or -proficient phenotypes.

First, we evaluated the IC_{50} of SN-38 in the presence or absence of olaparib by the ^{3}H-thymidine incorporation assay (Fig. 2). Each cell line was treated with SN-38 and/or olaparib for 48 hours. Olaparib significantly potentiated the sensitivity to SN-38 for all cell lines except HCT-15 (Fig. 2 and Supplementary Table S2). SN-38 in combination with olaparib almost halved the IC_{50} of SN-38 in each cell line.

In four cell lines (HCT116, RKO, HT29, and SW1116) showing different MMR status, the results of the ^{3}H-thymidine incorporation assay were reproducible with those measured by the Scepter 2.0 cell counter (Supplementary Fig. S1).

We previously reported that cell diameter changes measured by the Scepter 2.0 cell counter could be used as a marker of G_2–M arrest and apoptosis evoked by SN-38 (32). The increase of the mean cell diameter evoked by SN-

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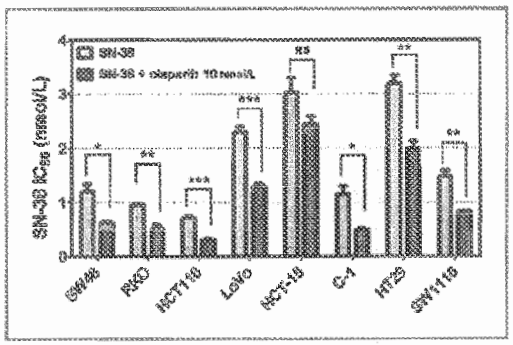


Figure 2. Comparison of SN-38 IC_{50} in the presence or absence of 10 nmol/L olaparib. ^{3}H-thymidine incorporation assay was carried out to calculate the IC_{50} values by cell growth inhibition curves. Each cell line was exposed to either SN-38 alone or SN-38 plus 10 nmol/L olaparib for 48 hours. Data of IC_{50}, mean ± SD of three independent experiments. In each cell line, the Student *t* test was performed between the IC_{50} of cells exposed to SN-38 alone and those exposed to SN-38 plus olaparib in combination (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant).

38 plus olaparib was greater than that of SN-38 alone in each cell line (Supplementary Table S3). These results indicate that olaparib enhanced G_2–M arrest induced by SN-38, together with the increase of the apoptotic cell fraction shown by the appearance of small-sized particles. These results show that olaparib potentiates SN-38 sensitivity in various colon cancer cell lines by inducing G_2–M arrest and apoptosis, irrespective of MMR status.

To assess the efficacy of the treatments with shorter duration of treatment, we performed robust colony assays. Two cell lines (HCT116 and HT29) were exposed to SN-38 and/or olaparib for 6, 12, 24, and 48 hours and subjected to colony formation. Olaparib potentiated the inhibitory effect of colony formation by SN-38, while these effects were not observed in the treatment using SN-38 alone after 12 hours of exposure in both cell lines (Supplementary Fig. S2). Colony formation was suppressed significantly after 24 hours in both cell lines treated with SN-38 alone or SN-38 plus olaparib. These results were well correlated with the data obtained by the ^{3}H-thymidine incorporation assay or the cell counting assay in which cells were treated for 48 hours as described above.

Olaparib increases DNA double-strand breaks induced by SN-38

Reportedly, SN-38 induces DSBs and PARP inhibitor is involved in the inhibition of SSB repair and PARylation of the proteins involved in DNA repair (5–8, 35). To elucidate whether olaparib increases DSBs induced by SN-38, we examined the formation of the 53BP1 nuclear foci, for example, a DSB marker in HCT116 and HT29 exposed to SN-38 and/or olaparib for 12 hours (Fig. 3).

The formation of 53BP1 nuclear foci did not increase by the treatment with olaparib alone, while they increased significantly in the groups treated with SN-38 or SN-38 plus olaparib (Fig. 3A and B). In analysis using one-way ANOVA, numbers of foci were significantly increased in the group treated with SN-38 plus olaparib as compared with SN-38 alone in both cell lines ($P < 0.001$; Fig. 3C and D).

We compared the results of the 53BP1 foci formation with those of ^{3}H-thymidine incorporation assay, using immunofluorescence double staining for BrdUrd and 53BP1 in HCT116 cells exposed to SN-38 and/or olaparib for different periods ranging from 6, 12, 24, and 48 hours (Supplementary Fig. S3). Exposed to SN-38 plus olaparib for 48 hours, BrdUrd incorporation decreased significantly as compared with SN-38 alone (Supplementary Fig. S3A). As BrdUrd incorporation is a surrogate marker of the S-phase, this was compatible with the data of ^{3}H-thymidine incorporation assay shown in Fig. 1C. When exposed to SN-38 plus olaparib for 6 hours, 53BP1 foci seemed to increase in the nuclei with BrdUrd incorporation (Supplementary Fig. S3B). This finding lasted until 12 hours of treatment, but BrdUrd-stained cell decreased markedly when they were exposed over 24 hours, leaving 53BP1 foci in the nuclei without BrdUrd incorporation (Supplementary Fig. S3B).

Olaparib increases Rad51 recruitment to the DSB sites induced by SN-38

As the major DSB repair pathway is mediated by HR (36–38), we examined formation of the Rad51 and γ-H2AX foci and their colocalization in HT29 cells. As antibodies directed against 53BP1 and Rad51 were both rabbit polyclonal antibodies and unsuitable for double staining, we used mouse anti-γ-H2AX monoclonal antibody as a DSB marker and examined its colocalization with Rad51 foci using double staining. Numbers of γ-H2AX foci and Rad51 foci showed a significant increase between SN-38 versus SN-38 plus olaparib and a part of γ-H2AX foci colocalized with Rad51 foci (Fig. 4). These data indicate that olaparib potentiates the recruitment of Rad51 to the DSB site in SN-38–treated cells.

Baseline levels of the DNA repair–related protein expression in cell lines were examined by Western blot analysis (Supplementary Fig. S4). It was found that the potentiating effects of olaparib on SN-38 sensitivity might not be correlated with the baseline levels of PARP-1, PARP-2, Mre11, Rad50, Nbs1, and Rad51 expressions. Furthermore, Rad51 expression by Western blot analysis did not differ between cells treated with SN-38 alone and SN-38 plus olaparib in RKO, HCT116, HT29, and SW1116 (data not shown).

Rad51 knockdown increases sensitivity to SN-38 and/or olaparib

To elucidate an effector molecule concerned with the potentiating effects of olaparib on SN-38 sensitivity, we performed a siRNA-mediated knockdown experiment of HR-related molecules, including Mre11, Rad50, and Rad51. SW1116 cells harboring wild-type *MRE11* and *RAD50* were used for the assay in that it is suitable for siRNA transfection. Drug sensitivities were evaluated by the cell counting assay. SW1116 cells transfected with

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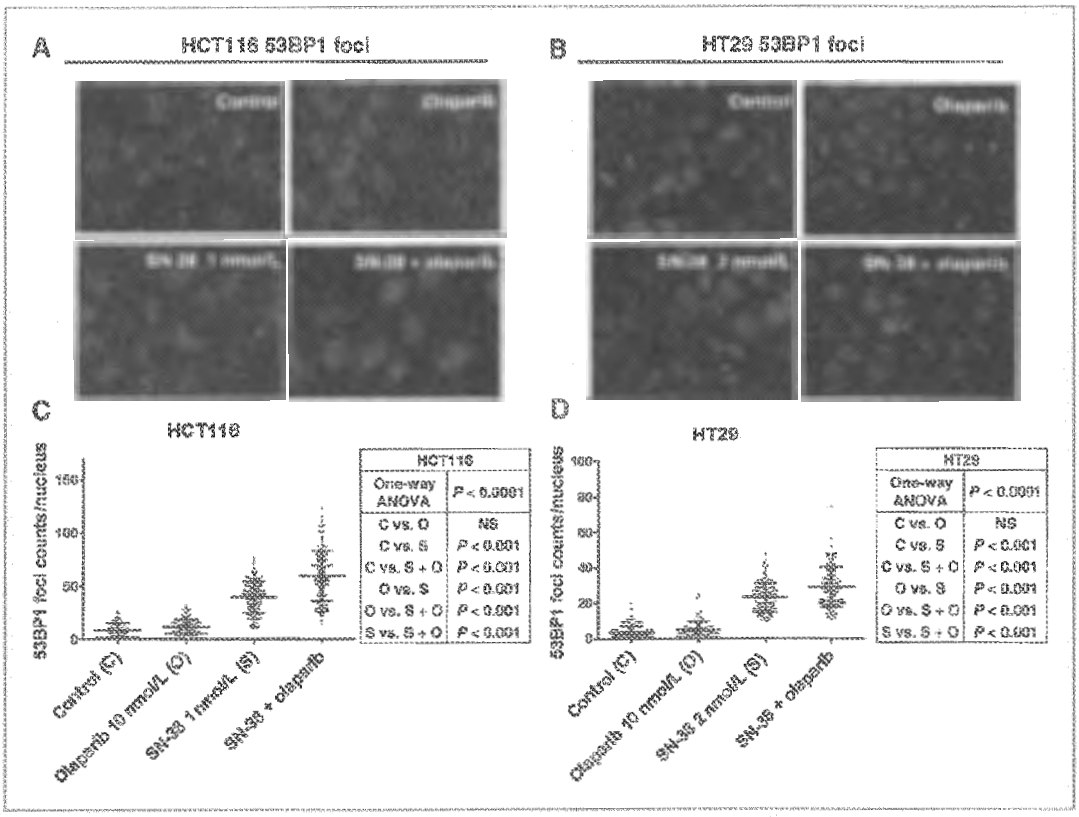


Figure 3. Olaparib potentiates DNA DSBs induced by SN-38. The formation of 53BP1 foci was examined by immunofluorescence microscopy upon counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI) in HCT116 (A) and HT29 (B) exposed to SN-38 (1 nmol/L for HCT116 and 2 nmol/L for HT29) in the presence or absence of 10 nmol/L olaparib for 12 hours. Counts of 53BP1 foci per nucleus were performed for HCT116 (C) and HT29 (D). Error bars, mean ± SD. The significance of differences between the four groups was analyzed using one-way ANOVA with the Tukey–Kramer method (NS, not significant).

Rad51 siRNA became significantly sensitive in all conditions, that is, with SN-38, SN-38 plus olaparib, and even with olaparib alone, as compared with the cells transfected with control siRNA (Fig. 5A, Supplementary Fig. S5). In cells transfected with Mre11 or Rad50 siRNA, there were no changes in the sensitivities to the olaparib or SN-38 treatments. Cell diameters of the transfectant with Rad51 siRNA increased according to the order of the treatments, namely, control < olaparib < SN-38 < SN-38 plus olaparib, indicating that G_2–M arrest occurred preferentially for SN-38 or SN-38 plus olaparib compared with olaparib alone (Fig. 5B and Supplementary Table S4). Furthermore, the increase of small particles indicated the increase of cell apoptosis in each treatment (Fig. 5B and Supplementary Table S4). Knockdown effects of these cells were confirmed by Western blot analysis, in which the protein expressions of Mre11, Rad50, and Rad51 were diminished 24 hours after transfection with each targeted siRNA (Fig. 5C–E). In addition, we observed the formation of Rad51 foci in cells transfected with Rad51 or control siRNA after SN-38 treatment by immunofluorescence microscopy and confirmed that the formation of Rad51 foci was inhibited in cells transfected with Rad51 siRNA compared with control siRNA (Fig. 5F).

We also evaluated the effects of Rad51 knockdown in HCT116 cells, which have biallelic *MRE11* mutations. HCT116 cells have endogenously weak expression of Mre11 and Rad50 (Supplementary Fig. S4). In cells transfected with Rad51 siRNA, the sensitivities to olaparib, SN-38, and combinational treatment increased significantly more than in cells transfected with control siRNA (Supplementary Fig. S6A). Moreover, the cell diameter changes indicated the increase of G_2–M arrest and apoptosis in cells transfected with Rad51 siRNA and subsequently treated with SN-38 and/or olaparib (Supplementary Fig. S6B and Supplementary Table S5). The expression of Rad51 protein was markedly diminished 24 hours after transfection with Rad51-targeted siRNA (Supplementary Fig. S6C). These results suggest that Rad51 may play an important role in DSB repair upon SN-38 and/or

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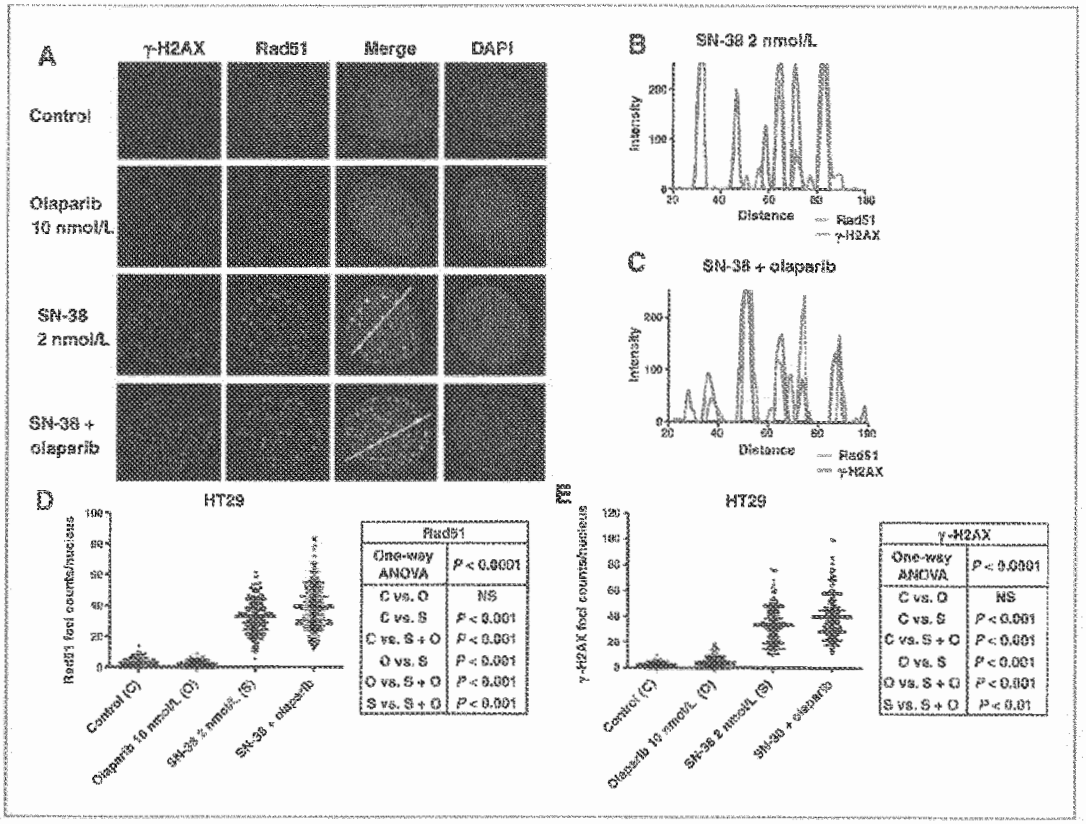


Figure 4. Olaparib potentiates Rad51 recruitment for double-strand breaksites induced by SN-38. A, the formation of Rad51 and γ-H2AX foci was examined by immunofluorescence microscopy in HT29 exposed to 2 nmol/L SN-38 and/or 10 nmol/L olaparib for 12 hours. For analysis of the colocalization of Rad51 and γ-H2AX, fluorescence intensity of Rad51 (green) and γ-H2AX (red) above the white line in the merged image was measured and we adopted two-color line plots to overlay the two-color histograms as shown in graphs B (SN-38–treated cell) and C (SN-38 plus olaparib–treated cell). Counts of Rad51 foci (D) and γ-H2AX foci (E) per nucleus in HT29 were carried out. Error bars, mean ± SD. The significance of differences between the four groups was analyzed using one-way ANOVA with the Tukey–Kramer method (NS, not significant).

olaparib treatment, irrespective of the Mre11 or Rad50 pathway.

Olaparib potentiates the antitumor effect of irinotecan in mouse xenograft models

To confirm whether the treatment with irinotecan plus olaparib in combination has an antitumor effect compared with irinotecan alone in *in vivo* experiment, we established two mouse xenograft models using HT29 and SW1116, of which the *in vitro* sensitivities to SN-38 were different. In HT29 xenografts, tumor growth in the irinotecan-treated group was not significantly suppressed compared with that in the control group. However, the group treated with irinotecan plus olaparib showed a significant decrease of the tumor volumes compared with the control group and the irinotecan-treated group ($P < 0.01$ for control vs. combination and olaparib vs. combination, $P < 0.05$ for irinotecan vs. combination; Fig. 6A). In SW1116 xenografts, tumor growth in the irinotecan-treated group was significantly suppressed compared with that in the control group. In the combinational treatment group, antitumor effects also increased significantly compared with those in the control group and the olaparib-treated group ($P < 0.001$ for control vs. irinotecan or combination and olaparib vs. irinotecan or combination; Fig. 6B). There was no significant change between irinotecan- and combinational-treated group, due to high sensitivity of SW1116 to irinotecan; however, the result of the trend test showed decrease of the slope in the irinotecan plus olaparib-treated group compared with the irinotecan alone (0.008 vs. 0.04; $P < 0.0001$; Fig. 6B). No significant body weight loss was observed in the treated groups compared with the control group (data not shown). In the experiment using SW1116 mouse xenograft, clinical blood chemistry was examined to analyze hematologic toxicity and liver dysfunction. In each treated group, neither

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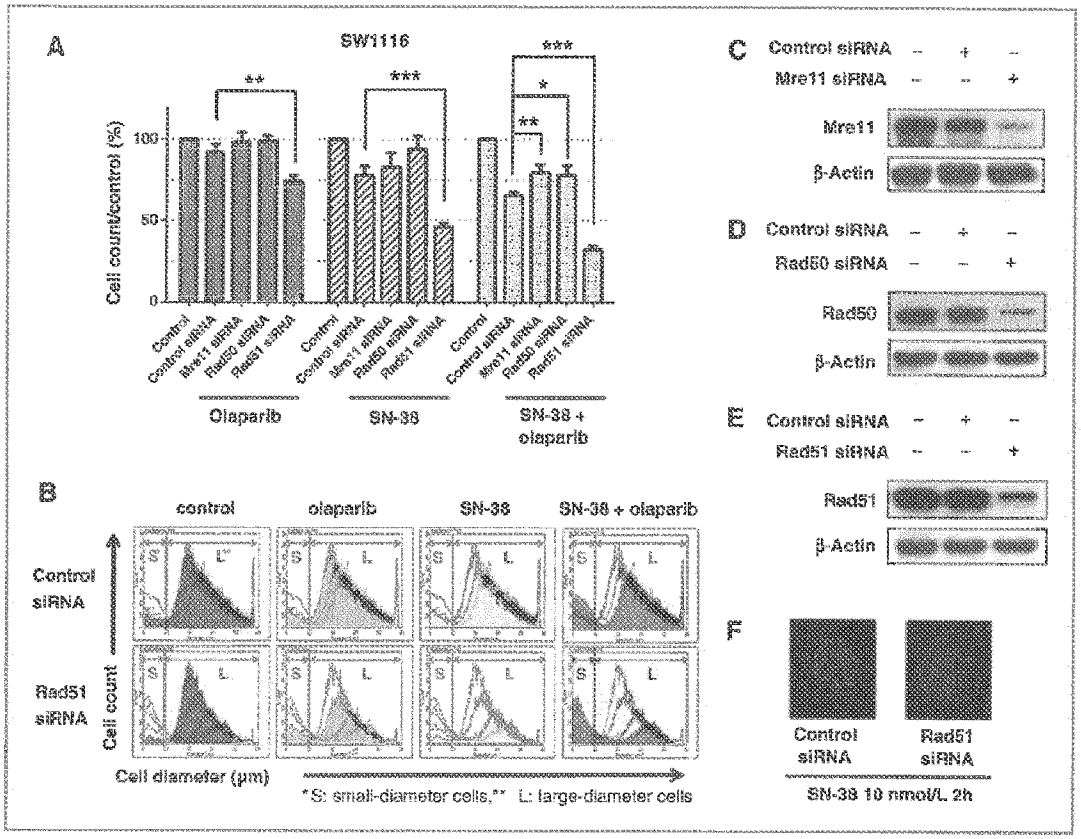


Figure 5. The siRNA-mediated knockdown targeting Rad51 potentiates the sensitivity to SN-38 and/or olaparib in SW1116. A, cell counting assay of SW1116 exposed to 1 nmol/L SN-38 and/or 10 nmol/L olaparib for 48 hours after transfection with control siRNA, Mre11 siRNA, Rad50 siRNA, or Rad51 siRNA. Data, mean ± SD of triplicate experiments. Control means nontreated cells. The Student *t* test was performed between cells transfected with control siRNA and those with the other siRNA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$). B, measurement of the cell diameter and cell count was performed using the Scepter 2.0 cell counter. The *x*-axis of each graph represents the cell diameter (μm) and the *y*-axis represents the cell count (demonstrated in the inset of Supplementary Fig. S5). Experiments were performed in triplicate in each condition and each graph of cells transfected with control siRNA and Rad51 siRNA represents the result of a single run in triplicate experiments. The mean ± SD of the cell diameters and the proportion of the cells with small diameter in each condition are shown in Supplementary Table S4. Graphs of cells transfected with Mre11 siRNA and RAD50 siRNA are shown in Supplementary Fig. S5. Effects of knockdown were confirmed by Western blot analysis 24 hours after siRNA-mediated transfection targeting Mre11 (C), Rac50 (D), or Rad51 (E). F, formation of the Rad51 foci 24 hours after transfection was examined by immunofluorescence microscopy. Cells were exposed to 10 nmol/L SN-38 for 2 hours before PFA fixation to induce double-strand DNA breaks.

hematocytopenia nor liver dysfunction was observed at the doses of irinotecan and olaparib used in this study, and the regimen was well tolerable in the mouse experiments (Supplementary Table S6).

Discussion

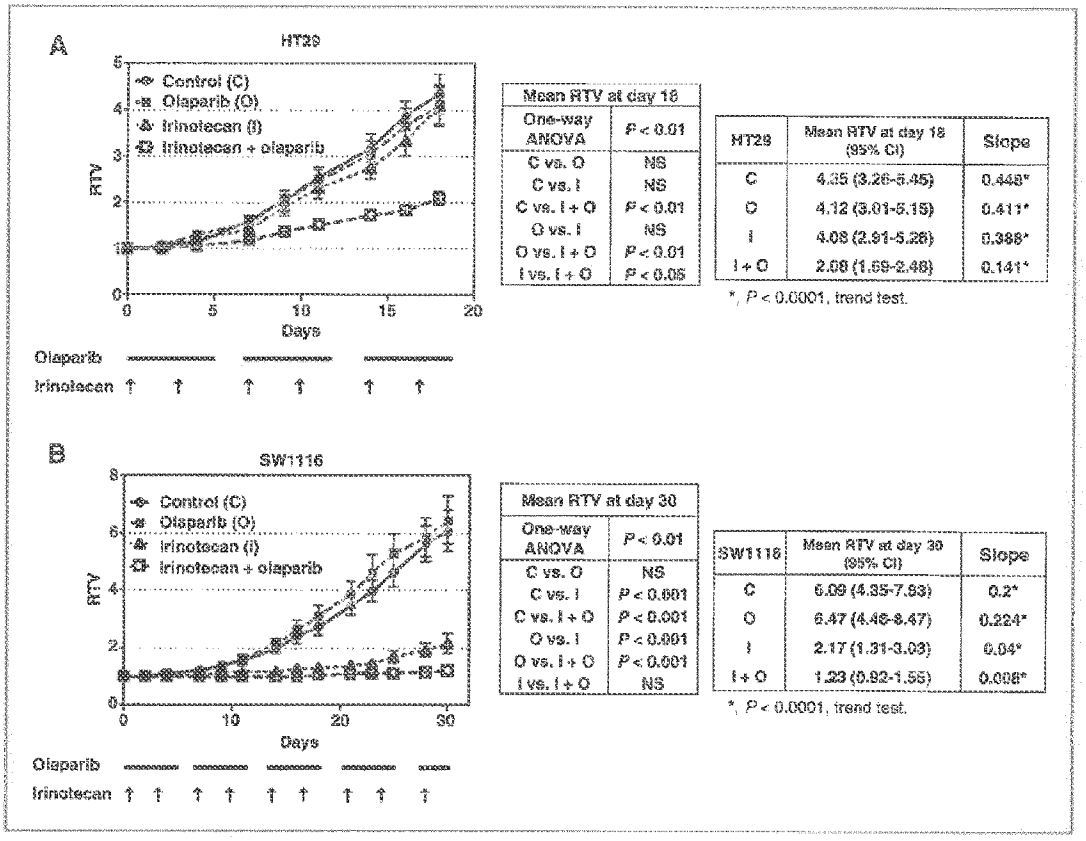
In this study, we assessed the effect of olaparib on potentiating the tumor cytotoxicity of SN-38 in colon cancer cell lines and whether the administration of olaparib in combination with irinotecan had suppressed the tumor growth in mouse xenograft models. As reported previously, the IC_{50}s of SN-38 were lower in MMR-deficient cells harboring homozygous mutations in *MRE11* and genetic or epigenetic alterations of *MLH1* as compared with the MMR-proficient cells, suggesting that Mre11 deficiency brought about the sensitization of the MMR-deficient cells to the topoisomerase I inhibitor (19, 34, 39). In analysis using ^{3}H-thymidine incorporation assay, olaparib almost halved the IC_{50} of SN-38 in each cell lines, irrespective of MMR status (Fig. 2 and Supplementary Table S2).

We counted the number of the 53BP1 foci formation in 2 cell lines, HCT116 and HT29 exposed to SN-38 or SN-38 plus olaparib for 12 hours: the former as a SN-38–sensitive cell line and the latter as a SN-38–resistant one. There was a significant increase in the number of 53BP1 foci in cells treated with SN-38 plus olaparib as compared with those treated with SN-38 alone, irrespective of the original sensitivity in each cell line (Fig. 3).

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Mean RTV at day 18	
One-way ANOVA	$P < 0.01$
C vs. O	NS
C vs. I	NS
C vs. I + O	$P < 0.01$
O vs. I	NS
O vs. I + O	$P < 0.01$
I vs. I + O	$P < 0.05$

HT29	Mean RTV at day 18 (95% CI)	Slope
C	4.35 (3.26–5.45)	0.448*
O	4.12 (3.01–5.15)	0.411*
I	4.08 (2.91–5.28)	0.388*
I + O	2.08 (1.59–2.48)	0.141*

*, $P < 0.0001$, trend test.

Mean RTV at day 30	
One-way ANOVA	$P < 0.01$
C vs. O	NS
C vs. I	$P < 0.001$
C vs. I + O	$P < 0.001$
O vs. I	$P < 0.001$
O vs. I + O	$P < 0.001$
I vs. I + O	NS

SW1116	Mean RTV at day 30 (95% CI)	Slope
C	5.09 (4.35–7.93)	0.2*
O	6.47 (4.46–8.47)	0.224*
I	2.17 (1.31–3.03)	0.04*
I + O	1.23 (0.92–1.55)	0.008*

*, $P < 0.0001$, trend test.

Figure 6. Olaparib increases the antitumor effects of irinotecan in mouse xenograft models. Tumor xenografts of HT29 and SW1116 were treated with olaparib (50 mg/kg, *per os*, every day consecutively for 5 days with intermission for 2 days per week) and/or irinotecan (10 mg/kg i.p., twice a week). A, relative tumor volumes (RTV) were measured in HT29 xenograft models (control, $n = 8$; olaparib, $n = 7$; irinotecan, $n = 5$; irinotecan plus olaparib, $n = 5$). B, relative tumor volumes were measured in SW1116 xenograft models (control, $n = 7$; olaparib, $n = 7$; irinotecan, $n = 6$; irinotecan plus olaparib, $n = 7$). Data, mean ± SEM. A linear trend test was performed in each group, and statistical significance between four groups was analyzed by one-way ANOVA with the Tukey–Kramer method (NS, not significant).

Results of the ^{3}H-thymidine incorporation assay and the correlation with the formation of 53BP1 foci were further analyzed by immunofluorescence double staining using antibodies directed to BrdUrd and 53BP1. Accumulation of nuclear BrdUrd incorporation persisted at 12 hours and decreased after 24 hours of exposure to SN-38 with or without olaparib (Supplementary Fig. S3A), while the formation of 53BP1 foci was consistent with positive BrdUrd nuclear staining in cells exposed to SN-38 or SN-38 plus olaparib for 12 hours, while in cells exposed for over 24 hours, 53BP1 foci was observed in the nuclei without BrdUrd incorporation (Supplementary Fig. S3B). Furthermore, results of the clonogenic assay showed that colony formation was suppressed significantly when cells were exposed to SN-38 or SN-38 plus olaparib over 24 hours as compared with the control or olaparib alone, whereas those exposed to SN-38 or SN-38 plus olaparib for less than 12 hours did not show reduced colony formation (Supplementary Fig. S2). Wu and colleagues reported that DSBs induced by SN-38 were biphasic events: an immediate phase was S-phase specific and inhibited by aphidicolin, a DNA polymerase inhibitor and a lagging phase, associated with apoptotic cell death (35). The data of BrdUrd and 53BP1 double staining indicated that DSBs at 12 hours after exposure to SN-38 or SN-38 plus olaparib seemed to be S-phase–specific immediate phase, and the cytotoxic effects observed at 48 hours exposure correspond to the lagging phase. The data of ^{3}H-thymidine incorporation assay were well compatible with those of the clonogenic assay (Fig. 2 and Supplementary Fig. S2).

IC_{50}s of olaparib were more than 2.5 μmol/L in all cell lines and 10 nmol/L olaparib used in *in vitro* experiments seemed to have the least cytotoxic effect (Fig. 1). At this concentration, olaparib alone did not increase DSBs and there were no differences in cell counts, cell cycle, and apoptotic change between control and olaparib-treated cells

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(Fig. 3 and Supplementary Fig. S1). Nonetheless, olaparib in combination with SN-38 increased DSBs and potentiated SN-38 sensitivity, leading to G_2–M arrest and apoptosis.

SN-38 plus olaparib treatment in HT29 cells increased the number of Rad51 foci colocalized with γ-H2AX, another marker of DSB, implying that DSBs induced by SN-38 would be repaired by the Rad51-mediated DNA repair pathway (Fig. 4). On the other hand, Mre11-Rad50-Nbs1 complex forms a MRN trimmer, which has 3′ to 5′ exonuclease activity and acts as a sensor molecule in the initiating process of DSB repair with the activation the checkpoint kinase ATM (40–45). If the hypersensitivity to SN-38 is caused by *MRE11* mutations resulting in disruption of the MRN complex, does it arise independent of the Rad51-mediated HR or not? To address this, we performed a knockdown experiment using Mre11, Rad50, and Rad51 siRNAs for SW1116 cells, a cell line with MMR-proficient phenotype without MSI in the *MRE11* locus. As a result, cell proliferation was suppressed significantly in the absence of Rad51, but not MRE11 nor RAD50 (Fig. 5).

A series of experiments denoted that single-strand DNA breaks caused by treating cells with SN-38 proceeds to S-phase–specific DSBs, which is repaired through HR-mediated DNA repair. The addition of olaparib in combination with SN-38 increased the number of Rad51 foci, which is colocalized with γ-H2AX foci, indicating the DSBs repaired by the HR-mediated DNA repair pathway (Figs. 3 and 4). Suppression of PARylation enhanced the sensitivity to SN-38, leading to G_2–M arrest and apoptosis in most of the colon cancer cell lines used in this study (Fig. 1 and Supplementary Fig. S1). The use of olaparib in combination with SN-38 may indirectly inhibit HR-mediated DNA repair through suppressing PARylation. This effect was also reproducible in the experiment using HCT116, an MMR-deficient cell line harboring biallelic *MRE11* mutations (Supplementary Fig. S6). SiRNA-mediated Rad51 knockdown increases the sensitivity of HCT116 cells to SN-38 and olaparib, indicating that PARP plays an indirect role for this synthetic lethality mediated by Rad51, irrespective of neither MMR deficiency nor *MRE11* inactivation.

In the experiment using the mouse xenograft models, combination of irinotecan and olaparib suppressed tumor growth significantly, without critical systemic or hematologic toxicity (Fig. 6 and Supplementary Table S6). The results of the *in vivo* study well confirmed the synergistic effect observed in the *in vitro* study. Dose of irinotecan employed in this study seems to be lower than LD_{50} reported in the nude mouse (177 mg/kg, i.p.), although LD_{50} of irinotecan in SHO mouse was not reported elsewhere (46). Tentori and colleagues reported that PARP inhibitor prevents irinotecan-induced intestinal damage in *in vivo* study using rat models (29). In the clinical settings, topoisomerase I inhibitor has adverse effects such as gastrointestinal symptoms, hematologic toxicity, and liver dysfunction, while olaparib was reported to be less toxic than irinotecan and its combined use may improve chemotherapeutic compliance by reducing the dose or frequency of irinotecan administration.

In summary, we found that SN-38 or irinotecan plus olaparib in combination could be applicable as a useful approach in a broad spectrum of colon cancer cells, regardless of the MMR status. *In vivo* study demonstrated that olaparib would be effective to potentiate the antitumor effect of irinotecan without serious adverse effects. Results of this preclinical study endorse further approaches to be pursued in clinical settings. Furthermore, triple synthetic lethality comprising topoisomerase I-mediated DNA breakage–reunion reaction, PARylation, and Rad51-mediated HR pathway may contribute as a potential target for future chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Tahara, T. Inoue, K. Sugano
Development of methodology: M. Tahara, T. Inoue
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Tahara, F. Sato, K. Kotake
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Tahara, K. Sugano
Writing, review, and/or revision of the manuscript: M. Tahara, Y. Miyakura, Y. Yasuda, K. Sugano
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Tahara, H. Horie, Y. Yasuda, H. Fujii, K. Sugano
Study supervision: Y. Miyakura, Y. Yasuda, K. Kotake, K. Sugano

Grant Support

This study was supported, in part, by Grants-in-Aid for Cancer Research and for the Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare, Japan, as well as by the National Cancer Center Research and Development Fund (21ban-shi-9-(3), 23-A-2 and 23-A-7)to K. Sugano).

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Received August 30, 2013; revised January 26, 2014; accepted February 17, 2014; published OnlineFirst February 27, 2014.

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Published OnlineFirst February 27, 2014; DOI: 10.1158/1535-7163.MCT-13-0683

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Molecular Cancer Therapeutics

The Use of Olaparib (AZD2281) Potentiates SN-38 Cytotoxicity in Colon Cancer Cells by Indirect Inhibition of Rad51-Mediated Repair of DNA Double-Strand Breaks

Makiko Tahara, Takeshi Inoue, Fuioshi Sato, et al.

Mol Cancer Ther 2014;13:1170-1180. Published OnlineFirst February 27, 2014.

Updated version	Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-13-0683
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Journal of Controlled Release 199 (2015) 72–83

Contents lists available at ScienceDirect

Journal of Controlled Release

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Irinophore C™, a lipid nanoparticulate formulation of irinotecan, improves vascular function, increases the delivery of sequentially administered 5-FU in HT-29 tumors, and controls tumor growth in patient derived xenografts of colon cancer

Robert Neijzen [a,b,*,1], May Q. Wong [b], Navdeep Gill [b], He Wang [b], Tamanna Karim [b], Malathi Anantha [b], Dita Strutt [b], Dawn Waterhouse [b], Marcel B. Bally [b,c,d], Isabella T. Tai [e,f], Sylvia S.W. Ng [b,g], Donald T. Yapp [b,g,**]

[a] Department of Pharmacy, University Medical Centre Utrecht, Postbus 85500, 3508 GA Utrecht, The Netherlands
[b] Experimental Therapeutics, The British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada
[c] The Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC V6T 2B5, Canada
[d] The Centre for Drug Research and Development, Vancouver, BC V6T 1Z4, Canada
[e] Michael Smith Genome Sciences Centre, The British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC, Canada
[f] Division of Gastroenterology, Department of Medicine, University of British Columbia, University of British Columbia, Vancouver, BC V6T 2B5, Canada
[g] The Faculty of Pharmaceutical Sciences, University of British Columbia, 2146 East Mall, Vancouver, BC V6T 1Z3, Canada

ARTICLE INFO

Article history:
Received 7 August 2014
Received in revised form 18 November 2014
Accepted 26 November 2014
Available online 8 December 2014

Keywords:
Colorectal cancer
Liposomes
Irinotecan
Sequential therapy
Patient derived xenografts
HT-29 tumors

ABSTRACT

Purpose: A liposomal formulation of irinotecan, Irinophore C™ (IrC™) is efficacious in a panel of tumor models, normalizes tumor vasculature, and increases the accumulation of a second drug in the same tumor. We now show that Irinophore C™ is also effective against patient derived xenografts (PDX) of colon cancer, and examine the kinetics of vascular normalization in the HT-29 tumor model and assess how these changes might be used with 5-FU sequentially.

Materials and methods: Rag2M mice bearing HT-29 tumors were treated with IrC™ (25 mg/kg; Q7D × 3) for up to three weeks. Groups of tumors were harvested for analysis at 7, 14 and 21 days after the start of treatment. Drug and lipid levels in the tumor were evaluated using HPLC and scintillation counts, respectively. Changes in tumor morphology (H&E), vasculature (CD31), perfusion (Hoechst 33342) and apoptosis (TUNEL) were quantified using microscopy. The accumulation of a second drug ([^{14}C]-5-FU, 40 mg/kg) given 3 h before sacrifice was determined using liquid scintillation. The efficacy of IrC™ (Q7D × 3) followed by 5-FU treatment (Q7D × 3) was assessed in mice bearing established HT-29 tumors. The efficacy of IrC™ was also evaluated in primary human colorectal tumors grown orthotopically in NOD-SCID mice.

Results: Following a single dose of IrC™ the active lactone forms of irinotecan and its metabolite SN-38 were measurable in HT-29 tumors after 7 days. The treatment reduced tumor cell density and increased apoptosis. Hoechst 33342 perfusion and accumulation of [^{14}C]-5-FU in the treated tumors increased significantly on days 7 and 14. This was accompanied by an increase in the number of endothelial cells relative to total nuclei in the tumor sections. Pre-treatment with IrC™ (Q7D × 3) followed by 5-FU (Q7D × 3) delayed the time taken for tumors to reach 1 cm^3 by 9 days ($p < 0.05$). IrC™ was just as effective as free irinotecan when used on patient derived xenografts of colorectal cancer.

Conclusions: Treatment with IrC™ reduces tumor cell viability and appears to normalize the vascular function of the tumor after a single treatment cycle. A concomitant increase in the accumulation of a second drug (5-FU) in the tumor was observed in tumors from IrC™ treated animals and this was correlated with changes in vascular structure consistent with normalization. The treatment effects of sequential 5-FU dosing following IrC™ are additive with no additional toxicity in contrast to previous studies where concurrent 5-FU and IrC™ treatment exacerbated 5-FU toxicity. The studies with PDX tumors also indicate that IrC™ is just as effective as free irinotecan on PDX tumors even though the delivered dose is halved.

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* Correspondence to: R. Neijzen, Department of Pharmacy, University, Medical Centre Utrecht, Postbus 85500, 3508 GA Utrecht, The Netherlands.
** Correspondence to: D.T. Yapp, Experimental Therapeutics, The British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver V5Z 1L3, BC, Canada.
E-mail addresses: robertneijzen@gmail.com (R. Neijzen), dyapp@bccrc.ca (D.T. Yapp).
[1] Formerly at the Experimental Therapeutics, The British Columbia Cancer Agency, 675 West 10th Avenue, Vancouver, BC V5Z 1L3, Canada.

http://dx.doi.org/10.1016/j.jconrel.2014.11.031
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1. Introduction

Treatment regimens for colorectal cancer are based on 5-fluorouracil (5-FU), which together with leucovorin, is used in combination with irinotecan or oxaliplatin [1–3]. These combinations have increased median overall survival to 20–24 months from 6 months with best supportive care. However, the consensus is that a treatment plateau has been reached with the 5-FU, leucovorin, oxaliplatin or irinotecan triad [1,4]. Inherent or acquired drug resistance and adverse drug effects are contributing factors to treatment failure, and adding another cytotoxic agent to this triad will likely exacerbate existing systemic toxicities. The addition of targeted therapeutics, such as agents targeting EGFR to these regimens has therapeutic value [5], but is limited to a subset of patients with KRAS wild-type tumors [6].

In humans, free irinotecan is metabolized by carboxylesterases into SN-38, a drug that is considered more potent than the parent compound [7,8]. Both compounds inhibit topoisomerase I and cause DNA strand breaks during replication which lead to cell death. The lactone forms of irinotecan and SN-38 are therapeutically active. However, under physiological conditions the lactone forms of irinotecan and SN-38 are hydrolyzed by esterases into their biologically inactive carboxylate forms [9,10] thereby reducing their biological availability. High levels of gastrointestinal carboxylesterases in organs such as the gut can result in toxic concentrations of SN-38 that severely damages the intestinal mucosa [11,12]. The resulting diarrhea can be so severe that it is life-threatening or dose-limiting [9,13,14]. The therapeutic efficacy of free irinotecan is thus unfortunately hampered by localized toxicity and limited biological availability.

Our group has shown that encapsulating irinotecan in liposomes with an unbuffered, acidic interior which contains copper (IrC™) helps to maintain the drug in its active lactone form in vivo [15]. This formulation makes more of the active lactone forms of irinotecan and SN-38 available over extended time periods in the blood, and the change in pharmacokinetics is associated with lower irinotecan toxicity and a significant increase in anti-tumor efficacy [16,17]. IrC™ is more efficacious than the free drug in several tumor models including colorectal cancer models [15,18–21]. Irinotecan associated toxicities are markedly reduced in mice; doses equivalent to 350 mg/kg irinotecan in the liposomal form can be administered to immune competent mice without side-effects in contrast to a maximum tolerated dose (MTD) of only 80 mg/kg for free irinotecan [18]. Pre-clinical studies carried out by our group show very specifically in a rat model of late onset diarrhea that IrC™ is less GI toxic then free irinotecan (Waterhouse, private communication). IrC™ therefore has immediate advantages over the free drug since the formulation reduces toxicity in the gut, while prolongation of the active lactone forms in liposomes helps increase exposure of the active drug to the tumor. Further studies in colorectal cancer and glioma tumor models further suggest that tumor perfusion and accumulation of a second drug is improved following treatment with IrC™ treatment [19–21]. Vascular changes in the glioma model also indicate that IrC™ treatment is associated with blood vessel normalization [21]. Vascular normalization has not been observed previously when using irinotecan and this may represent a unique mechanism of action for the IrC™ product.

Recognizing that IrC™, if approved for clinical use, will be used in combination with other drugs, our research team has examined the therapeutic effects when IrC™ and 5-FU are administered concurrently [22]. The data show that any therapeutic potential of the combination is compromised by an unexpected increase in toxicity [22]. The reasons for the enhanced toxicity are not well-understood although it may be due to IrC™ treatment prolonging the circulation lifetime of 5-FU [22]. The observed improvements in tumor perfusion following IrC™ treatment [20,21,23] suggest the possibility that combination treatments with IrC™ and 5-FU might be more efficacious if 5-FU was administered after IrC™ treatment in a sequential, rather than concurrent, manner. We hypothesize that vascular normalization engendered by IrC™ will improve tumor perfusion, increase the accumulation of 5-FU in the tumor and subsequently improve its therapeutic efficacy.

The data presented here show that a single dose of IrC™ is sufficient to improve vascular function in tumors, and that this correlates with enhanced 5-FU accumulation in tumors. Subsequent efficacy studies suggest that sequential administration of 5-FU after IrC™ treatment, in contrast to concurrent administration, is well tolerated, and resulted in at least additive therapeutic effects without additional toxic effects. We also show that doses of IrC™ (equivalent to 25 mg/kg of irinotecan) are as active as free irinotecan (given at 40 mg/kg) against patient derived xenografts (PDX) of colorectal cancer. The data also show that IrC™ is also active against a PDX tumor line that did not respond to free irinotecan.

2. Materials and methods

2.1. Cell culture

All cell lines were tested to verify the absence of mycoplasma before use. The HT-29 human colon adenocarcinoma cell line (ATCC, Manassas, VA, USA) cells were derived from an original stock that was expanded in antibiotic free media, frozen and stored in liquid nitrogen. The cells are maintained in culture for no more than 20 passages and at that time are replaced with frozen stock. The HT-29 cells were cultured in McCoy's 5A with 1% L-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (StemCell Technologies; Vancouver, CA). Plated flasks (BD Falcon, BD Sciences, Bedford, MA) were kept in a humidified incubator (5% CO_2, 37 °C) and stocks divided when ~80–90% confluent.

2.2. Irinophore C™

IrC™ was prepared as previously reported [16]. In brief, 1,2-distearoyl-*sn*-glycero-phosphocholine (DSPC; Avanti Polar Lipids, Alabaster, AL) and Cholesterol (Chol) were dissolved in chloroform with DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, Molecular Probes, Oregon). The mixture was spiked with ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE; Perkin Elmer Life Sciences; Boston, MA). The solvent was removed under vacuum to form a lipid film, which was rehydrated in 300 mM copper sulfate at 70 °C. The resulting multilamellar vesicles were then extruded 10× through polycarbonate membrane filters (100 and 80 nm pore size; Nuclepore, Pleasanton, CA) at 65 °C using a LIPEX™ extruder (Northern Lipids, Vancouver, BC, CA). The liposomes were subsequently incubated with A23187 (0.5 μg/mg lipid) followed by incubation with irinotecan (0.2 mol/mol lipid). Irinotecan concentration was determined by measuring UV–absorbance at 370 nm. The resulting product, IrC™, labeled with DiI and ^{3}H-CHE was used within 3 weeks. IrC™ is stable when maintained at 4 °C for >6 weeks where stability is assessed by no measureable change in product appearance, pH, liposome size, % encapsulated irinotecan, and irinotecan as well as DSPC and Chol content.

2.3. Animals

Studies were approved by the UBC's Institutional Animal Care Committee (IACC) which ensures that work conducted meets the standards defined by the Canadian Council for Animal Care. In the studies, animals were euthanized if tumors ulcerated or reached 1000 mm^3. Mice were also euthanized when their health status was compromised. A standard operating procedure, approved by the IACC, was used to provide comprehensive assessments of body weight, behavioral changes, appearance and level of activity. Mildly or markedly scruffed fur was scored 2 or 5, respectively. A hunched position was given a score of 2 or 5 depending on severity. Signs of lethargy were given a score of 2, 5 or 10 depending on severity. A 6–10% body weight loss (BWL) corresponded to a score of 2, 11–15% BWL corresponded to a score of 5 and 16 to 20%

BWL corresponded to a score of 10. A head tilt was given a score of 10. On the basis of this assessment methodology, animals would be euthanized when their health status score was 10 and the time of death was recorded as one day after the animals were terminated. In these studies, none of the animals was euthanized for humane reasons. When experimental endpoints were reached, all animals were terminated by CO_2 asphyxiation according to an IACC approved SOP.

2.4. Rag2M and HT-29 tumors

The shaved lower backs of 129S6/SvEvTac-*Rag2tm1Fwa* (Rag2M) mice were inoculated with HT-29 cells (5×10^6 cells, in 50 μL media). Mice were randomly assigned to treatment groups when tumors reached 150–200 mm^3 (Table 1). Tumor size and body weight were measured 3 times per week before and during treatments. Tumor volumes were calculated from two orthogonal caliper measurements ((length × width^2) / 2).

2.5. Patient derived xenografts (PDX)

Fresh samples of colon cancer tumors from consented patients undergoing surgical resection were initially implanted subcutaneously in NOD-SCID mice (Taconic). Tumors which grew were subsequently harvested, cut into smaller pieces (~2 × 2 × 2 mm) and implanted on the colon of new mice. Briefly, an incision was made on the abdomen of anesthetized mice, and the colon located. The tumor pieces were then sutured onto the surface of the colon, and the incision closed. Once established, the size of the implanted tumors was estimated by palpation and given a score between 1 and 5 by the same research technician. A tumor score of 1 is equal to ~200 mm^3 whereas a score of 5 is considered >800 mm^3 and defined as the experimental endpoint.

2.6. IrC™ treatments for assessment of early vascular changes in HT-29 tumors

Treatments are summarized in Table 1. Three groups of mice received IrC™ (25 mg/kg irinotecan) on day 0 and their tumors were harvested immediately, 1 and 7 days after treatment (labeled as groups IRC-0, IRC-1 and IRC-7, respectively). Mice in groups IRC-14 and IRC-21, received 2 (on days 0 and 7) and 3 IrC™ doses (on days 0, 7 and 14), respectively, and their tumors were harvested 7 days after the last treatment (i.e. on days 14 and 21, respectively). Mice in groups SAL-0, SAL-1, SAL-7, SAL-14 and SAL-21 were time-matched controls corresponding to those in groups IRC-0, IRC-1, IRC-7, IRC-14 and IRC-21 and received an i.v. injection of saline on the days as indicated.

2.7. Levels of irinotecan, SN-38, lipid (radiolabeled marker) and 5-FU (radiolabeled marker) in HT-29 tumors

On the day of harvest, IRC-7, IRC-14, IRC-21 and SAL-7, SAL-SAL-14 and SAL-21 received 5-FU (40 mg/kg + 1.5 μCi/mL ^{14}C-5-FU; Moravek Biochemicals, CA) and Hoechst 33342 (10 mg/kg; Sigma, MO) i.v. 3 h and 20 min, respectively, before euthanasia. Tumors were harvested from euthanized mice within 2 min and divided in half. One half was immediately frozen in tissue preservative (OCT). The other half was divided into two portions and each was weighed. One portion was prepared for measuring associated ^{14}C and ^{3}H as markers to quantify liposomal lipid and 5-FU levels; respectively. In brief, tumor samples were weighed and dissolved in 1 mL of Solvable (Sigma, St. Louis, MO) at 50 °C overnight. The next day, EDTA (200 mM, 50 μL), H_2O_2 and HCL were added with 4.5 mL of Pico-Fluor (Perkin Elmer Life Sciences, Mississauga, ON). The ^{14}C-5-FU present per gram tumor tissues was quantified by scintigraphy (Packard 1900TR Liquid Scintillation Analyzer; Perkin Elmer Life Sciences; Woodbridge, Ontario, Canada). ^{3}H/^{14}C quenched standard curves were used to quantify ^{14}C-FU and ^{3}H-lipid; DPM was used to estimate concentrations. The other portion was homogenized in ice cold water. Subsequently, MeOH:acetonitrile (50:50) was added and the supernatant was then analyzed by HPLC [18] to quantify the lactone to carboxy forms of irinotecan and SN-38 in the tumor. HPLC separation of irinotecan (CPT-11) and SN-38 (lactone and carboxylate forms) was performed by HPLC using a 250 × 4.6 mm C18 Symmetryshield column and C18 Symmetryshield guard column (Waters, Mississauga, ON). Gradient elution was used with mobile phase A composed of 75 mM ammonium acetate and 7.5 mM tetrabutylammonium bromide adjusted to pH = 6.4 with glacial acetic acid (Fisher Scientific, Nepean, ON) and mobile phase B was acetonitrile. Gradient profile was as follows: Time = 0 min: 78% A: 22% B, Time = 10 min: 64% A: 36% B, Time = 12 min: 78% A: 22% B, Time = 20 min: 78% A: 22% B. A 10 μL sample was injected onto the column (35 °C) and eluted at a flow rate of 1 mL/min. CPT-11 and SN-38 forms were detected using a Waters 2475 multi-wavelength fluorescence detector (Waters, Mississauga, ON) set with time program events of λex = 370 nm; λem = 425 nm between times 0 and 12.5 min for CPT-11 forms, and λex = 370 nm; λem = 535 nm between times 12.5 and 20 min for SN-38 forms. Prior to injection all samples were maintained at 4 °C to reduce conversion between lactone and carboxylate forms. Tumors were homogenized using ice cold water. 100 μL of homogenate was transferred to micro centrifuge tube and 900 μL of ice cold methanol:acetonitrile was added. The samples were than centrifuged to remove precipitated proteins. The supernatant was used for analysis. Dilutions of the supernatant were made in ice cold methanol:acetonitrile (50:50). Standard curves of CPT-11 and SN-38 lactone form were

Table 1
Treatment groups (n = 4). Mice in the IRC groups were treated with IrC™ (25 mg/kg irinotecan; i.v., once a week) on day 0 (start of treatment). Mice in groups IRC-1 and IRC-7 received one dose, and were euthanized for tumor harvest on days 1 and 7, respectively. Mice in groups IRC-14 and IRC-21 received 2 (days 0 and 7) and 3 (days 0, 7 and 14) doses of IrC™, respectively, and were euthanized for tumor harvest 7 days after the last dose (days 14 and 21, respectively). Mice in the SAL groups received saline in place of IrC™ and were euthanized on the same schedule as time-matched controls for analysis. 5-FU (spiked with ^{14}C-5-FU) and Hoechst 33342 were administered prior to harvest, 3 h and 20 min, respectively in all groups except in IRC-1 and SAL-1.

Irinophore C™-treated groups (25 mg/kg)					
Group	IRC-0	IRC-1	IRC-7	IRC-14	IRC-21
Treatment	1 dose, day 0			2 doses, days 0 & 7	3 doses, days 0, 7 & 14
Harvest day	Immediately	1	7	14	21
Hoechst 33342	n/a	20 min before harvest			
5-FU (40 mg/kg)	n/a		3 h before harvest		
Saline-treated groups					
Group	SAL-0	SAL-1	SAL-7	SAL-14	SAL-21
Treatment	1 dose, day 0			2 doses, days 0 & 7	3 doses, days 0, 7 & 14
Harvest day	Immediately	1	7	14	21
Hoechst 33342	n/a	20 min before harvest			
5-FU (40 mg/kg)	n/a		3 h before harvest		

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prepared by serial dilutions in a 2:1:1 sodium acetate (100 mM):MeOH: ACN pH = 4.0 buffer. For the carboxylate form of CPT-11 and SN-38, serial dilutions were prepared in a 2:1:1 sodium borate (100 mM):MeOH: ACN pH = 9.0 buffer.

2.8. Immunohistochemistry and quantitative microscopy

Frozen tumors were cryosectioned (10 μm), transferred to glass slides, air dried and stored at −20 °C. Entire tumor sections were scanned on an automated fluorescence microscope (Leica Microsystems). One set was imaged for Hoechst 33342 and DiI staining immediately; the same sections were then fixed, stained with an antibody specific for endothelial cells (CD31; rat anti-mouse; BD PharMingen) and visualized with a Cy5-goat anti-rat antibody (Invitrogen). Sections were also stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. A second set of adjacent sections was stained with hematoxylin and eosin to highlight tissue morphology and to identify necrotic areas in tissue sections which were excluded from the analysis. In a third set, apoptotic activity was assessed by TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining (Roche Pharmaceuticals). Positive staining for each fluorophore was determined empirically using Image J (Ver. 1.39). Initially, threshold values for staining were measured using a set of test samples. The average value (AV) and standard deviation (SD) for multiple measurements of each fluorophore were calculated, and a value of AV + 3× SD used as the cut-off for positive pixels in subsequent analysis. Necrotic areas in the section were cropped out of images, and the number of positive pixels normalized to the corresponding nuclei present (DAPI).

2.9. Therapeutic studies with 5-FU given after IrC™ administration

Mice with HT-29 tumors (150–200 mm^3) were treated with saline, 5-FU (40 mg/kg; Q7D × 3), IrC™ (25 mg/kg; Q7D × 3) or IrC™ (25 mg/kg; Q7D × 3) followed by 5FU (40 mg/kg; Q7D × 3) one week after the last IrC™ injection. Mice in the 5-FU alone group were treated when tumors were the same size as those treated with IrC™ for 3 weeks (approximately 150–200 mm^3). Tumor volumes were measured three times per week using the methods described above.

2.10. Treating patient derived xenografts (PDX) of colon cancer with IrC™

Three PDX tumors (CRC 02, 07 and CRC08) were treated with IrC™ (25 mg/kg irinotecan), saline or free irinotecan (40 mg/kg) when tumors reached a score of 2 (~250–300 mm^3). The various treatments were given once a week for six weeks. One week after the last dose of saline, IrC™ or irinotecan (i.e. on day 42 after the first dose), the mice were euthanized, the tumor harvested and its volume measured ex vivo to examine the effects of IrC™ and irinotecan on primary tumors of human colon cancer.

2.11. Statistics

Graphs were generated using GraphPad Prism 5.00 software. One-way ANOVA analysis was used to determine whether differences in one parameter (e.g. tumor volume, positive pixel staining in immunohistochemical experiments) between control and treated groups were statistically significant ($\alpha = 0.05$).

3. Results

The data summarized in Fig. 1a demonstrate that the biologically active lactone forms of irinotecan and SN-38 are still present in the tumor 7 days (IrC-7) after the first dose of IrC™. Similar drug levels were also found in tumors of mice that had received 2 and 3 doses of IrC™ (IrC-14 and IrC-21). Interestingly, the levels measured at days 7, 14, and 21 were not significantly different than those measured 24 h after drug administration (IrC-1). The data demonstrate that the use of IrC™ exposes the tumor to the parent drug and its more active metabolite for up to a week after administration. Moreover, both irinotecan and its active metabolite SN-38 are maintained in the ring closed active form. Liposomal lipid, as measured using DiI as a fluorescence marker was observed in the tumor (Fig. 1b) 24 h after administration of IrC™ and this marker appeared to be closely associated with Hoechst 33342 staining, which was used as a vascular marker (Fig. 1b). The fluorescent images were determined 1 day after administration; DiI is known as a non-exchangeable or metabolized fluorescent lipid over a 24 h time period (unpublished observations Kalra et al., 2013).

Fig. 2a shows H&E staining of tumors obtained from untreated mice consist of tightly-packed, continuous sheets of cancer cells (blue stain for nuclei) interspersed with mouse stroma (pink stain). The older and larger untreated tumors often had large areas of necrosis where the cell nuclei are disorganized, and often fragmented (arrows) whereas in the tumor rim there is healthy tissue with cell nuclei of similar size and shape that are tightly packed together consistent with cell growth being prevalent in the tumor rim. In contrast, the cell nuclei in tumors from mice treated with IrC™ were less densely packed by day 7 and stromal elements were more prominent (Fig. 2b). After two cycles of IrC™ the differences became even more obvious, with more open spaces throughout the section (Fig. 2b). Almost no necrosis was observed in the tumors obtained from treated animals and the number of nuclei (DAPI stained) per unit area of viable tissue was consistently lower compared to tumors from untreated animals (Fig. 2c). It is possible that the differences noted are underestimated as the size of the nuclei is larger in the tumors from mice treated with IrC™ (Fig. 2d). Increases in nuclei size are consistent with the action of agents that arrest cells in S-phase [24]. As shown in Fig. 2d the nuclei with tumors from untreated animals are tightly packed and regularly shaped. In contrast, nuclei in tumors from animals treated for 3 weeks with IrC™ are more variable in size with the majority being larger than normal and irregularly shaped. TUNEL staining was used to assess apoptosis and these data (Fig. 2e) show that apoptotic activity increases in the tumors from animals treated with IrC™ when compared to tumors from control mice. These data were obtained from stained sections and representative images are provided (Fig. 2f). The images show that apoptotic cells against DAPI-stained nuclei in control tumors are clustered around the edges of necrotic areas, but that when animals are treated with IrC™ (day 21) the cells within isolated tumors are distributed throughout the section.

The ratio of endothelial cells to cell nuclei was determined in HT-29 tumors and the results (Fig. 3a) indicate that within 24 h of drug administration there is a significant ($p < 0.05$) reduction in the % of CD31 cells within the tumor. However, over time and following multiple treatments with IrC™ a significant ($p < 0.05$) increase in the % of CD31 cells within the tumor is observed (IrC-21; Fig. 3a). Representative images of tumor sections obtained from saline and IrC™ treated animals are shown in Fig. 3b. Sections were stained for CD31 (red) and for nuclei (DAPI, blue) to show the presence of blood vessels (CD31 positive pixels) with respect to the cell nuclei (DAPI positive pixels) within viable tumor tissue. The density of positive CD31 cells in tumors treated with IrC™ (day 21) appears to be higher, and individual blood vessels appear thicker. In addition, Hoechst 33342 labeling of functional blood vessels was determined (Fig. 3c and d) and these data indicate that there are significant increases ($p < 0.05$) in the number of functional blood vessels in the tumors obtained from mice treated with IrC™. The Hoechst distribution is more homogenous across the tissue in sections from tumors obtained from treated animals (day 21).

5-FU delivery to tumors from control and IrC™ treated mice was measured following administration of a 5-FU (40 mg/kg) labeled with tracer levels of ^{14}C-5-FU. These results, summarized in Fig. 3e, are consistent with the Hoechst data and indicate that 5-FU accumulation increases after each IrC™ treatment cycle. For the IrC-21 group the amount of 5-FU accumulated within the tumors of treated animals

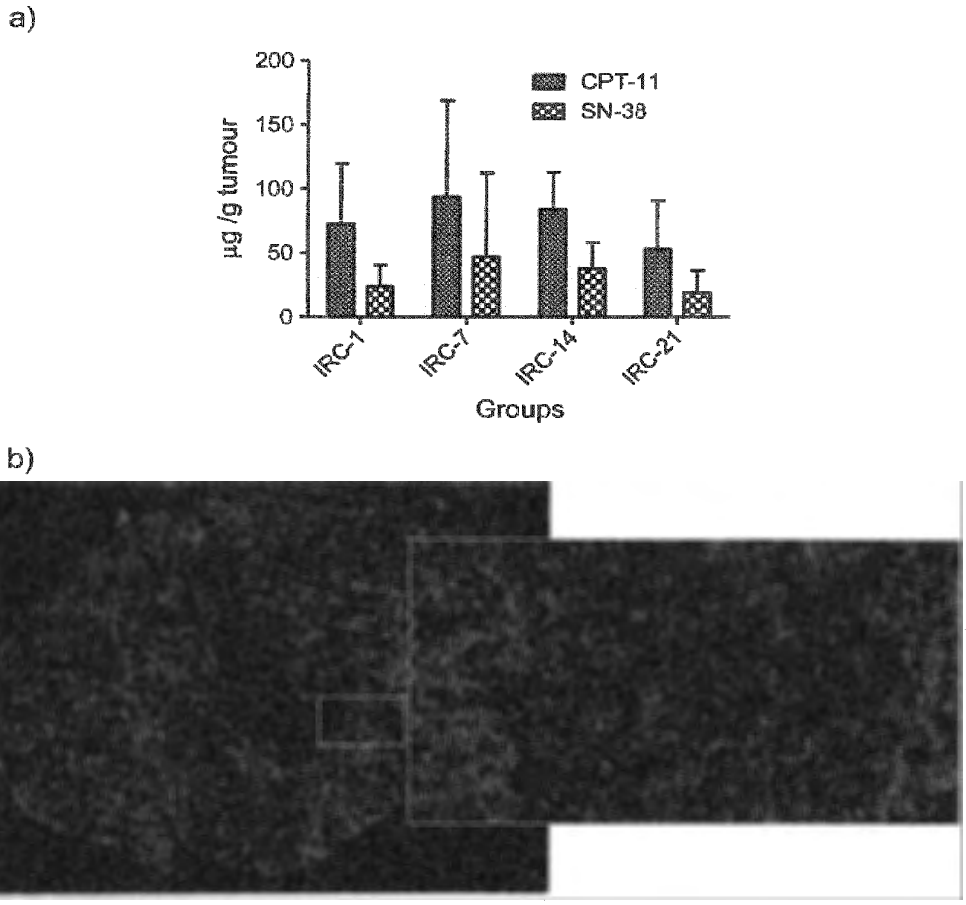


Fig. 1. a: Irinotecan and SN-38 are present for 7 days in HT-29 tumors. The levels of irinotecan and SN-38 present in HT-29 tumors after IrC™ treatment at selected time points. The drug levels are normalized to tumor weight and injected dose. Both drugs were present as the biologically active lactone form (>97%). Time points at days 7, 14 and 21, show drug levels 7 days after 1, 2 and 3 doses of IrC™, respectively. Error bars represent standard deviation. b: IrC™ is associated with tumor vasculature. The distribution of IrC™ in a representative tumor section from IRC-1 parallels the network of blood vessels. The cell nuclei are stained blue with DAPI, blood vessels in red (CD31) and the fluorescent dye, DiI, attached to the liposomal lipids are pink.

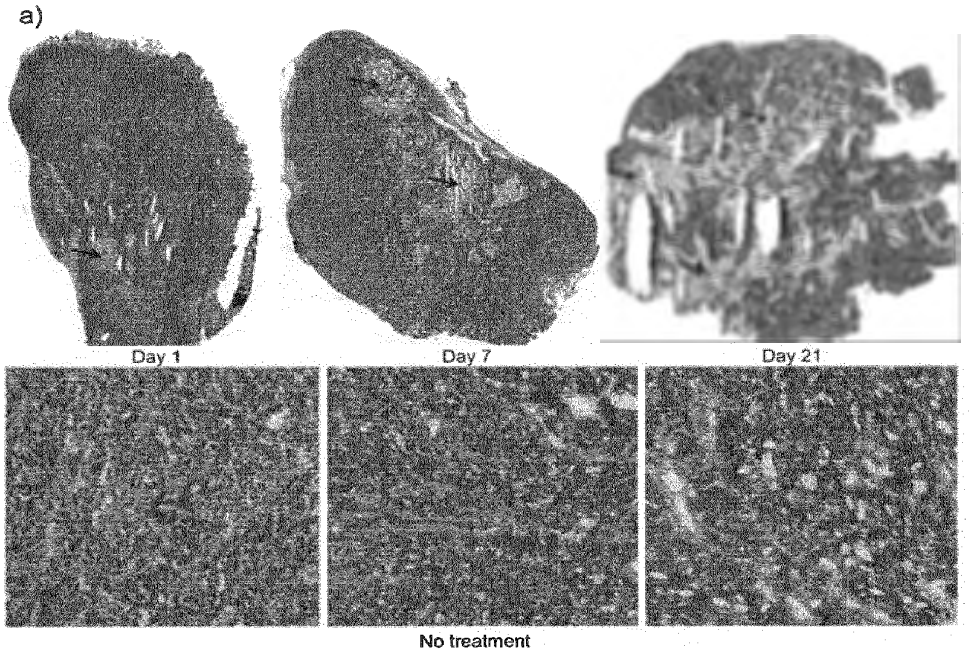
was almost 3-fold greater than that observed in tumors obtained from control mice.

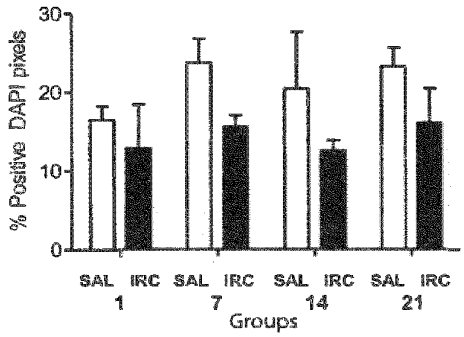
Previous studies showed that co-administration of IrC™ and 5-FU is effective in treating tumor bearing mice albeit with an increase in toxicity [22]. The observed increase in toxicity was attributed to IrC™ mediated changes in 5-FU pharmacokinetics and biodistribution. The data would suggest that dose sequencing could be important if 5-FU and IrC™ are to be used in the same treatment regimen. Sequential dosing would avoid the toxicity associated with concurrent administration of both drugs and, perhaps more importantly, could take advantage of IrC™ mediated increases in tumor perfusion and the associated enhancement in 5-FU delivery. To assess this hypothesis mice bearing HT-29 tumors were treated with IrC™ (Q7D × 3), and subsequently treated with 5-FU (40 mg/kg; Q7D × 3). Sequential treatment with IrC™ followed by 5-FU resulted in a significant growth delay compared to control. The time for untreated HT-29 tumors to reach 1000 mm^3 was 16 days. When treated with 5-FU alone (40 mg/kg; Q7D × 3), the time required to reach 1000 mm^3 was 24 days; a tumor growth delay of 8 days. Treatment with IrC™ (25 mg/kg; Q7D × 3) increases the time required for tumors to reach 1000 mm^3 to 40 days. When IrC™ treated animals were subsequently treated with 5-FU, the time required to reach 1000 mm^3 was 50 days; a tumor growth delay of 34 days. The delay in tumor growth attributed to 5-FU when used following IrC™ was 10 days; a delay that was comparable to the 8 day tumor growth delay observed with 5-FU alone. These results indicate that pretreatment with IrC™ did not enhance the therapeutic effects of 5-FU substantially; but the improved treatment outcomes that were observed were achieved with no increases in toxicity.

Three PDX tumor lines established from colon cancer tumors obtained from patients were treated with saline, irinotecan and IrC™. The effects of IrC™ on the tissue morphology of the three PDX lines resemble those seen in the HT-29 tumors (Figs. 5 and 2, respectively). In particular, the overall tissue structure in CRC 07 and 08 tumors treated IrC™ is destroyed, the cancer cells have undergone catastrophic cell death and what remains is empty space and a few surviving cancer cells with distorted and enlarged nuclei. The tumor volume data (Fig. 5b) shows that each tumor line responds to irinotecan and IrC™ differently compared to saline treated controls. Irinotecan and IrC™ reduce tumor volumes in CRC 02 albeit not significantly; however, both the free drug and liposomal form are effective in CRC 07. Interestingly, CRC 08 appears to be resistant to free irinotecan (40 mg/kg), but IrC™ (given at dose equivalent to 25 mg/kg irinotecan) reduces tumor volumes significantly.

4. Discussion

IrC™ was designed to improve the therapeutic efficacy of free irinotecan by reducing the drug's systemic toxicity while still maintaining


a)
Day 1
Day 7
Day 21
No treatment
b)
Day 1
Day 7
Day 21
Irinophore C™ treated


c)
% Positive DAPI pixels
30
20
10
0
SAL IRC SAL IRC SAL IRC SAL IRC
1 7 14 21
Groups

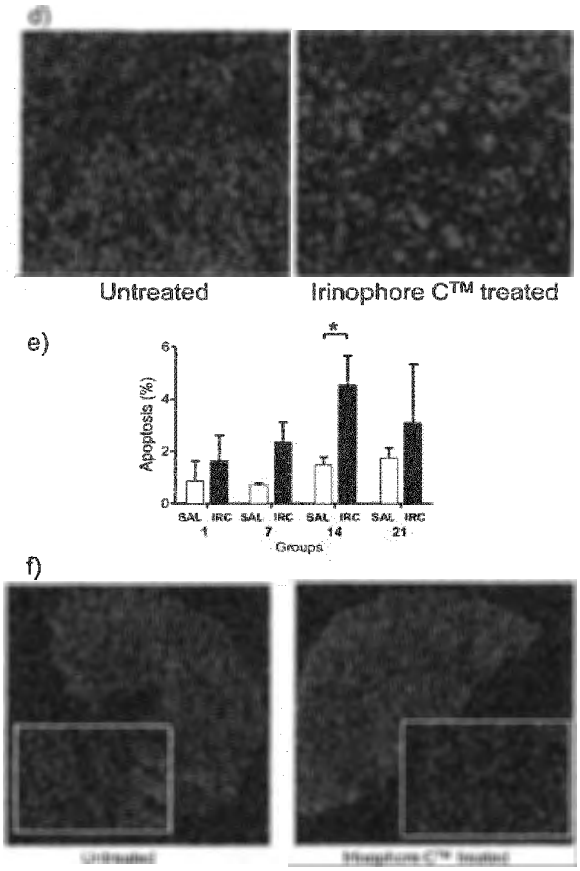


Fig. 2. a and b: Morphological changes in HT-29 tumors due to IrC™ treatment. H&E sections (20×) of representative control (a) and treated tumors (b) are shown in the figure from days 1, 7 and 21. Cancer cells are colored purple, the interspersing stromal elements are colored pink. c–d: Quantification of nuclear density in HT-29 tumors. Saline and IrC™ treated tumors are indicated by open (□) and filled bars (■), respectively. Cell nuclei were stained with DAPI, and positive pixels normalized to viable areas of the tumor section. The analysis counts all positive pixels and not objects. The results indicate that IrC™ treated tumors have lower numbers of DAPI positive pixels per unit area implying that there are fewer nuclei present. Moreover, irinotecan causes cell-cycle arrest in S-phase which increases the size of cell nuclei (see panel d) and subsequently the number of pixels counted so the differences are likely greater than that observed. (d) Fluorescence images (400×) of DAPI-stained sections. e–f: IrC™ increases apoptotic activity in HT-29 sections. Saline and IrC™ treated tumors are indicated by open (□) and filled bars (■), respectively. Treatment with IrC™ increases the percentage of apoptotic cells in HT-29 tumors significantly by day 14. Asterisks (*) indicate significant differences from controls ($p < 0.05$). Error bars represent standard deviation. (f) Fluorescence images of the tumor sections (20×). Apoptotic cells (green) are stained against DAPI-stained nuclei (blue).

its cytotoxic effects. Encapsulating irinotecan in liposomes prevents lactone ring hydrolysis while extending the circulation lifetime of the parent drug. Studies from our group subsequently showed that extended retention of irinotecan in liposomes improves the drug's therapeutic activity in multiple models of cancer, a result attributed to a substantial increase in systemic exposure to SN-38, the active metabolite of irinotecan. IrC™ is more potent than irinotecan even though the dose of irinotecan delivered by IrC™ is 3–5 fold lower than the maximum tolerated dose of irinotecan [17,18]. While IrC™ can be administered using a Q7D × 3 schedule at doses as high as 120 mg/kg; significant therapeutic effects are achieved at a dose as low as 25 mg/kg, as shown in this report. Reports from our group also show that IrC™ also improves the vascular function in several tumor types [19,21]. More recently, however, our group noted that concurrent administration of IrC™ and 5-FU compromised any therapeutic benefit due to exacerbated toxicity when the two drugs were given at the same time. Thus the objectives of the present studies were to determine when changes in the vasculature of HT-29 tumors occur with IrC™ treatment, and whether the therapeutic effects of 5-FU, given in a sequential approach to take advantage of IrC™ mediated improvements in the vascular function of HT-29 tumors, are improved.

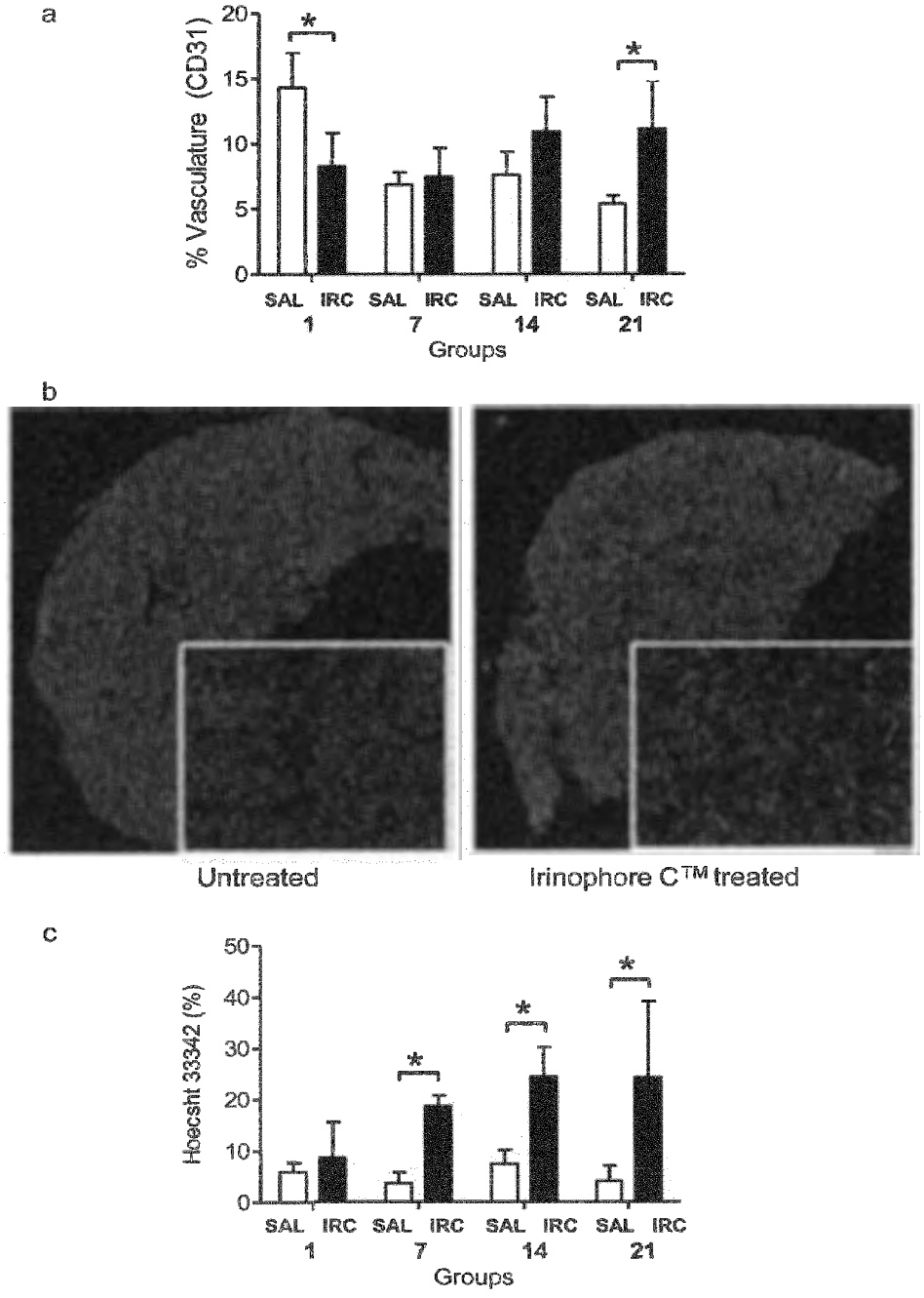
Our results show that the active lactone forms of irinotecan and SN-38 are present in the tumor 24 h after administration, and indicate for the first time that the level of tumor associated drug is maintained for at least 7 days after a single intravenous injection of IrC™ (Fig. 1a). The tumor levels of irinotecan and SN-38 (active lactone form) do not increase with multiple doses of IrC™, but it is clear that the tumor cells are exposed to the active lactone forms for extended time periods following IrC™ treatment. In contrast, it is known that free irinotecan is rapidly metabolized to its inactive carboxy form and quickly eliminated from the animal [9]. Although the free drug is converted to its more potent SN-38 derivative, this derivative is also rapidly hydrolyzed to the inactive carboxy form. Thus tumors treated with free irinotecan on a Q7D regimen would be exposed to doses of the lactone forms of irinotecan and SN-38 for short periods of time every 7 days followed by a drug-free period.

The continuous presence of drug in the tumor tissue following IrC™ treatment is associated with dramatic changes in tissue morphology. IrC™ causes profound damage to the tumor tissue and the overall result is a tumor that is much less dense, with cell nuclei that are enlarged and poorly defined, a higher apoptotic cell number, and a higher ratio of endothelial cells to cancer cells. Cell nuclei were stained with DAPI to estimate the overall cell density in the tumors. These data are a semi-quantitative measure of tumor cell density since the software measures the total number of DAPI positive pixels and not discrete objects such as nuclei (Fig. 2c). The results showed that tumors from IrC™ treated animals consistently had lower numbers of DAPI positive pixels per unit area (and thus lower nuclear density) than tumors from untreated time matched controls. The differences between treated and control tumors were not significant, but this may be because treatment causes cell-cycle arrest that associated with increases in the size of nuclei [21]. This would artificially increase the number of positive DAPI pixels detected. Indeed, the nuclei of the IrC™ treated cells were clearly enlarged and more variable in shape (see Fig. 2d). Of more relevance, however, the cell nuclei at these time points are poorly defined with evidence of condensed DNA; typical of dying cells; cell-cycle arrest and apoptosis. Interestingly, the levels of apoptosis seem to plateau or even drop by day 21; possibly indicating that cells that were not killed by exposure to the irinotecan or SN-38 entered cell-cycle arrest and became quiescent.

Our group has previously shown that IrC™, at the doses used here, can exert a cytostatic effect on tumor growth and is associated with normalization of tumor associated blood vasculature in three different tumor models [15,19–21]. The mechanism leading to vascular normalization remains unclear. The fluorescence DiI marker for liposomal lipid would suggest that the drug loaded liposomes become closely associated with blood vessels within the tumor of mice treated with IrC™ within 24 h (Fig. 1b). This could imply that the drug may target tumor vasculature initially by virtue of propinquity. Presumably, drug released from the localized liposomes will diffuse away from the blood vessel; however drug released from liposomes circulating in the blood system would expose endothelial cells to active forms of irinotecan and SN-38 for extended time frames [20,21]. IrC™ treatment likely targets proliferating endothelial cells in the tumor first and this would help explain the drop in the ratio of endothelial cells to cancer cells noted at 24 h (see Fig. 3a; SAL-1 vs. IRC-1). Cancer cells immediately adjacent to these vessels would subsequently die from lack of vascular support secondary to endothelial cell death. It is less likely that the active forms of irinotecan and SN-38 affect mature, non-proliferating endothelial cells [21,25]. Thus over a 3 week treatment period, mature blood vessels would be left behind while the population of cancer cells is reduced

resulting in an increase in the ratio of endothelial to cancer cells seen on days 14 and 21 (Fig. 3a). Regardless of mechanism, tumors from animals treated with IrC™ show a measurable improvement in tumor perfusion. We believe that this is a unique mechanism of irinotecan activity thus far only noted with use of IrC™. More importantly the changes in tumor associated blood vessels may provide a potential therapeutic opportunity that takes advantage of improved perfusion to deliver more of the same or a second drug [26,27].

Quantification of positive Hoechst pixels in tumor sections obtained from animals injected with this perfusion marker show that significantly more dye is present in tumors treated with IrC™. This result indicates that the tumor vascular function improves over the treatment period. These results are in keeping with the idea that sustained release of irinotecan from IrC™ may mimic metronomic-like dosing schedules. It is known that metronomic dosing can prune neovasculature; leaving behind mature, more functional blood vessels [19]. One would anticipate that the presence of more functional blood vessels would enhance the delivery of an agent known to cross the endothelial barrier, such as 5-FU. The results presented here show that 5-FU levels in the tumors increased when the mice were treated first with IrC™. This phenomenon was reported with doxorubicin and 5-FU in tumors treated with IrC™ for 6 weeks [19], and was suggested by the results in a



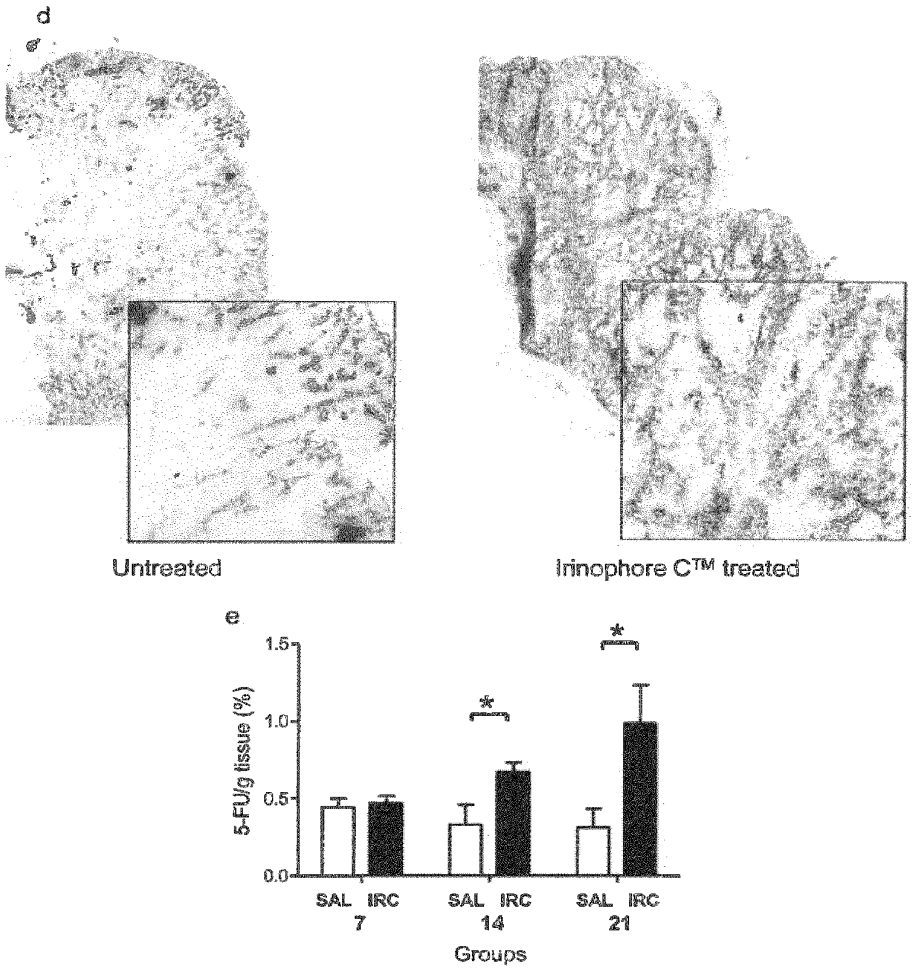


Fig. 3. a–b: The vascular density of HT-29 tumors increases IrC™ treatment. Saline and IrC™ treated tumors are indicated by open (□) and filled bars (■), respectively. The percentage of endothelial cells in HT-29 tumors decreases significantly within 1 day of the first dose of IrC™. However, with continuous treatment, the percentage increases by day 21. Asterisks (*) indicate significant differences from controls ($p < 0.05$). Error bars represent standard deviation. (b) Tumor sections (20×) were stained for CD31 (red) against DAPI-stained nuclei (blue). c–d: Tumor perfusion increases with IrC™ treatment in HT-29 tumors. Saline and IrC™ treated tumors are indicated by open (□) and filled bars (■), respectively. Accumulation of the perfusion marker Hoechst 33342, relative to tumor cell density (DAPI), increases after treatment with IrC™. Asterisks (*) indicate significant differences from controls ($p < 0.05$). Error bars represent standard deviation. (c) Representative images (20×) from control and treated tumors show the distribution of Hoechst 33342 (blue). e: Accumulation of 5-FU in HT-29 tumors increases after IrC™ treatment. Saline and IrC™ treated tumors are indicated by open (□) and filled bars (■), respectively. Concomitant with the increase in tumor perfusion, the accumulation of 5-FU delivered after treatment with IrC™ increases significantly compared to control tumors. Asterisks (*) indicate significant differences from controls ($p < 0.05$). Error bars represent standard deviation.

glioblastoma model also treated with IrC™ (Verreault, unpublished results), but this is the first indication that Irinophore C™ has measurable effects on tumor perfusion within one week of dose initiation, and that the effect increased over time (Fig. 3e). In these studies 5-FU was administered as a single bolus dose 7 days after the last dose of IrC™ in mice from groups IRC-7, IRC-14 and IRC-21, therefore the pharmacokinetic interactions noted with concurrent IrC™/5-FU administration were avoided and, in turn, there was no measurable effect on toxicity when the drugs were given sequentially.

It was important to establish whether increased accumulation of 5-FU in tumors post IrC™ treatment has therapeutic effects equal to or greater than 5-FU dosing alone (Fig. 4). The sequential combination of these two drugs at the doses used here resulted in at least additive therapeutic effects with no increase in toxicity, which was observed when these two drugs were given concurrently [22]. Other groups have examined treatment regimens where irinotecan treatment was followed by 5-FU; a treatment regimen that based on irinotecan treatment increasing the proportion of tumor cells in S-phase, which are

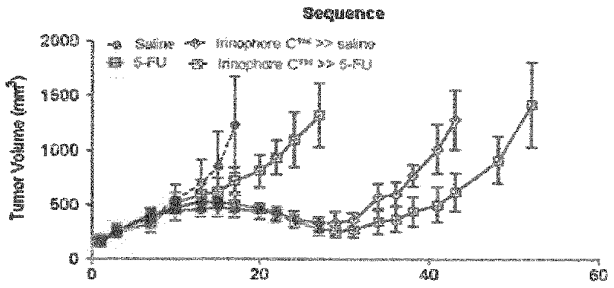


Fig. 4. Combining 5-FU with IrC™ sequentially has additive effects with no toxicity. Tumor growth delay in HT-29 tumors treated with 5-FU (~9 days compared to untreated controls) does not change significantly when tumors are pre-treated with IrC™ before 5-FU is given (~10 days compared to tumors treated only with IrC™ followed by saline). Treatment was initiated when average tumor sizes were 150–200 mm^3. Error bars represent standard deviation.

more susceptible to 5-FU [28,29]. If this is true than 5-FU administration after irinotecan dosing would result in synergistic efficacy in some tumor models [28–31]. However, these previous studies showed no improvements in efficacy in HT-29 tumors when the mice were treated with 5-FU following irinotecan [28]. In the clinic, 5-FU is given as an intravenous infusion to maximize exposure of the drug which results in a better response rate [32]. Extended exposure to 5-FU using intravenous infusion following IrC™ treatment in our studies may have improved treatment outcomes in the HT-29 model. If this is the case then the use of 5-FU in a drug carrier formulation could also result in significant improvements in therapeutic activity when combined with IrC™. Treatment with IrC™ may also have upregulated drug detoxification pathways which would subsequently adversely affect 5-FU activity and reduce the drug's therapeutic efficacy. The HT-29 study highlights the

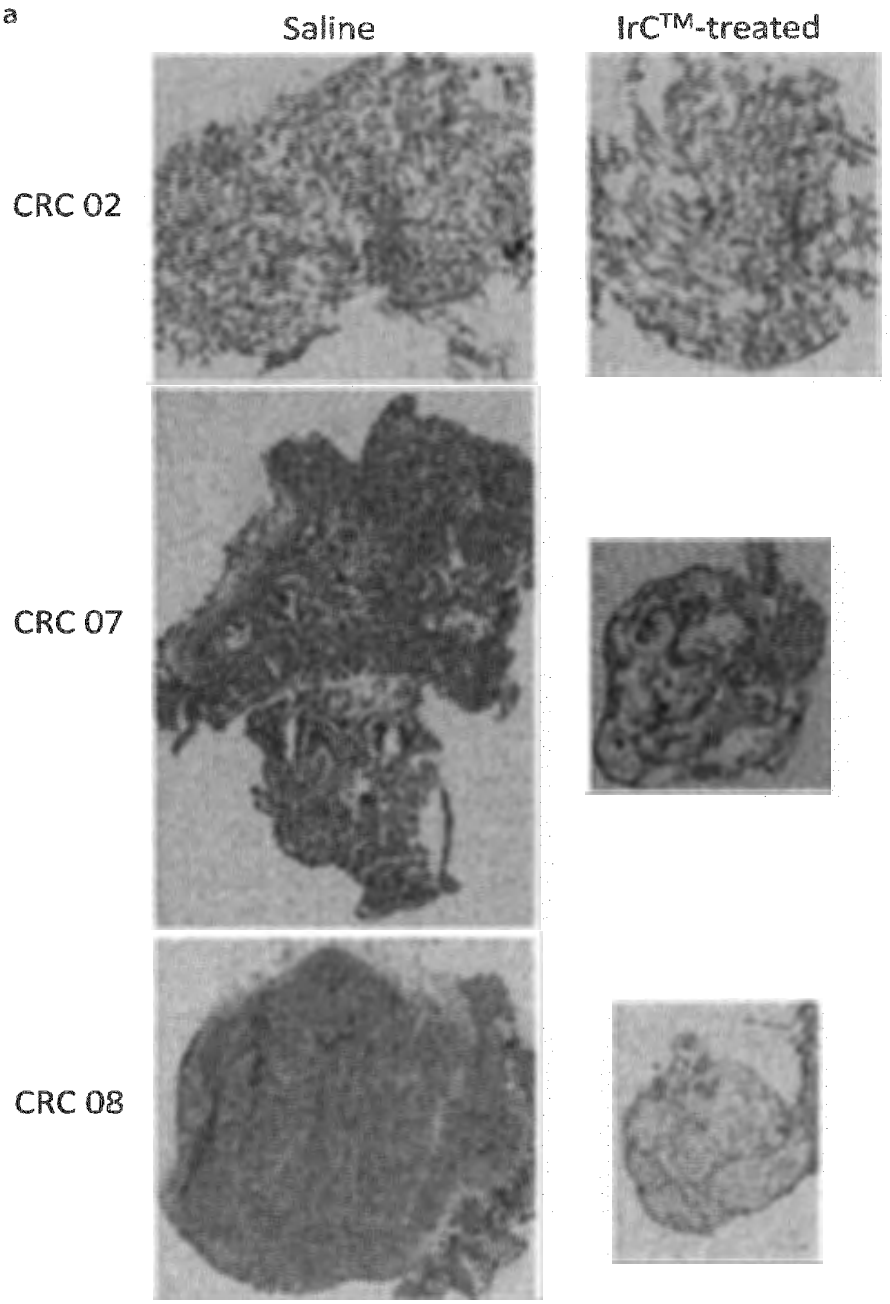


Fig. 5. a and b: The effects of IrC™ on patient derived xenografts of colon cancer are equivalent to or better than those of irinotecan. H&E stained tumor sections of CRC 02, 07 and 08 treated with IrC™ are shown (compared to saline treatment). Cancer cells in CRC 07 and 08 tumors are removed following treatment with IrC™, leaving behind empty spaces in the tissue. The effects are less obvious in CRC 02 because the mucinous nature of the tumor creates similar empty spaces. (b) The tumor volume data indicate that IrC™ has similar effects to free irinotecan in CRC 02 and 07 albeit at lower doses, but is more efficacious than free irinotecan in reducing tumor volumes in CRC 08.

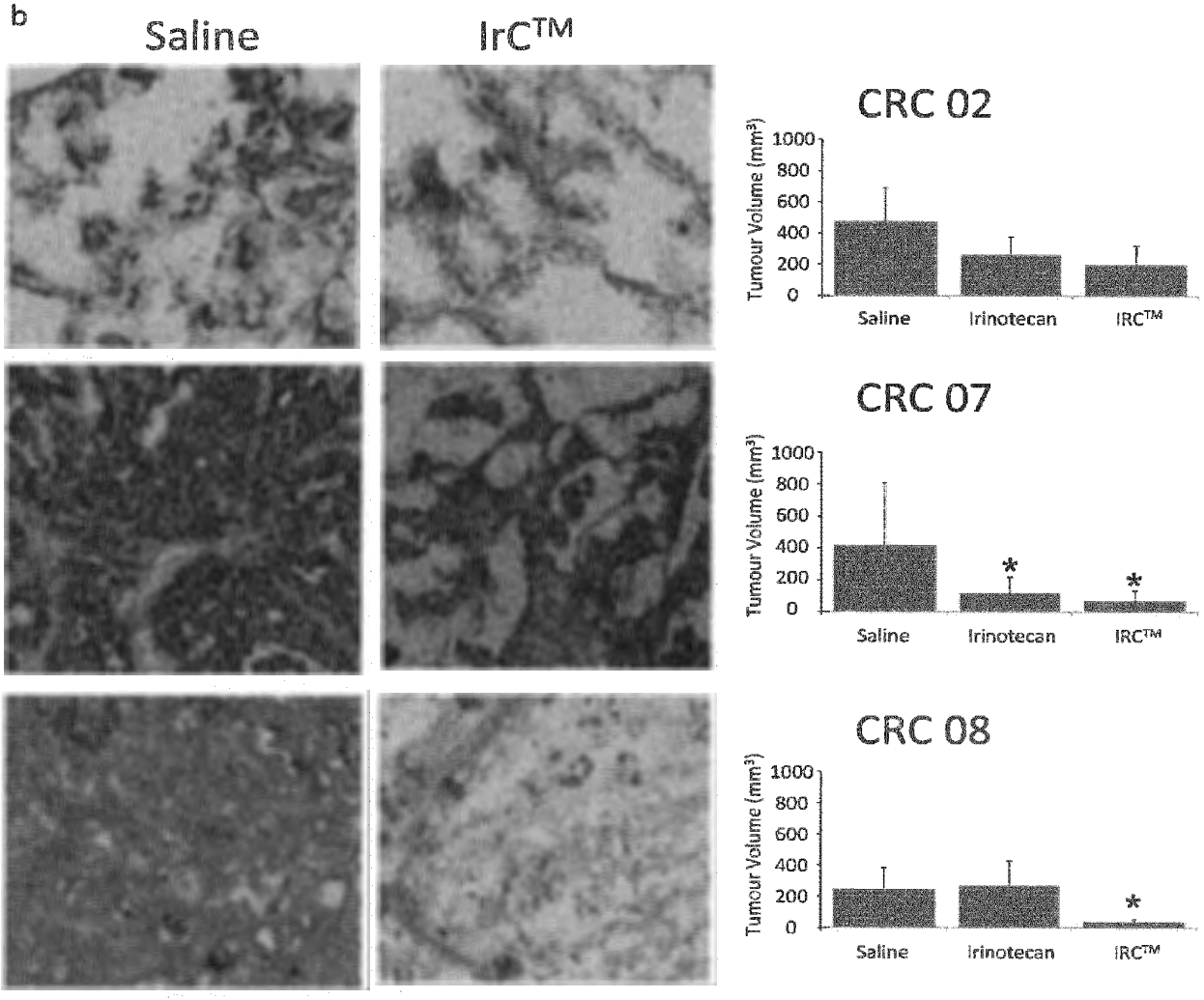


Fig. 5 (*continued*).

complexity of targeting cancer cell populations that are in a state of flux, whether due to disease progression, or as a result of prior drug treatment. The results confirm that if IrC™ is combined with 5-FU, sequential dosing of the two drugs is more efficacious than concurrent dosing, and we are now assessing other drug combinations and temporal sequences to better understand how best to take therapeutic advantage of changes in tumor physiology caused by IrC™.

We also report the effect of IrC™ on three patient derived colorectal tumors grown orthotopically in mice, also known as patient derived xenografts (PDXs). The response of PDXs to treatment is as individual as the patient tumors they are derived from. PDXs are known to maintain the original tissue structure and characteristics of the original sample and are thought to more closely replicate the response of tumors in the clinic. It is not surprising then that the PDX lines described here have different morphologies and therapeutic responses to irinotecan. For example, CRC 02 produces high levels of mucin which is reflected in the empty spaces seen in the H and E sections of untreated tumors (Fig. 5). In this PDX line, the cancer cells and vasculature are found within the linear streams of fibrotic tissue enclosing the mucinous areas. In contrast, CRC 07 and 08 are much less mucinous and consist of densely packed cancer cells, some of which are only partially dysregulated and still form pseudo glandular structures. In contrast, HT-29 tumors consist of homogenous sheets of cancer cells interspersed with a well distributed vascular system.

Each PDX tumor line responds differently to irinotecan. Compared to saline-treated tumors, CRC02 tumors respond somewhat to free irinotecan, CRC07 responds well, whereas CRC08 does not appear to respond to the drug. Not surprisingly, CRC 02 and 07 tumors respond in a similar fashion to IrC™, although it should be noted that the dose of irinotecan delivered by IrC™ is almost half that of free irinotecan. Interestingly, CRC 08 tumors, which do not respond to free irinotecan (40 mg/kg), respond extremely well to IrC™ (equivalent to 25 mg/kg irinotecan). The reasons for this are not immediately apparent and are the subject of on-going studies. The direct cytotoxic effects of irinotecan likely play the main role in controlling tumor growth, but how the drug is delivered to the tumor temporally may also be important. Regardless of its mechanism of action, the results reported here indicate clearly that Irinophore C™ has added advantages over free irinotecan since it has therapeutic effects that are as good, or superior to free irinotecan at a lower dose and fewer toxic effects. The effect of long-term exposure to the active forms of irinotecan and SN-38 may also be more effective against cancer cells that are inherently resistant to free irinotecan.

Acknowledgments

Funding for the study was provided by the The Cancer Research Society Inc., (DY), Canadian Cancer Society Research Institute (DY,SN,IT) (019488), Rethink Breast Cancer (DY), The Canadian Institutes of Health and Research (MBB) (MOP-102611), and Terry Fox Research Institute (MBB/DW). The authors would also like to acknowledge the Dr. Saal van Zwanenbergstichting and the KWF Kankerbestrijding for scholarships to RN for this study.

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History of Changes for Study: NCT01770353

Pilot Study to Determine Biodistribution of MM-398 and Feasibility of Ferumoxytol as a Tumor Imaging Agent

Latest version (submitted November 15, 2019) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare. One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of the study.
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in red.
- Study additions are displayed in green.

Study Record Versions

Version	A	B	Submitted Date	Changes
1	○	○	January 15, 2013	None (earliest Version on record)
2	○	○	January 16, 2013	Outcome Measures and Study Status
3	○	○	August 8, 2013	Conditions, Study Status, Eligibility and Outcome Measures
4	○	○	April 24, 2015	Arms and Interventions, Study Status, Study Design, Eligibility and Study Description
5	◉	◉	May 5, 2015	Arms and Interventions, Conditions, Study Status and Study Description
6	○	○	March 22, 2016	Contacts/Locations, Study Status, Arms and Interventions, Conditions, Study Description and Study Identification
7	○	○	July 7, 2016	Contacts/Locations and Study Status
8	○	○	April 4, 2017	Study Status, Sponsor/Collaborators, Study Identification and Study Design
9	○	○	April 18, 2017	Contacts/Locations and Study Status
10	○	○	September 29, 2017	Study Status, Study Identification, Contacts/Locations, Eligibility, Study Description and Oversight
11	○	○	December 22, 2017	Study Status
12	○	○	January 25, 2018	Recruitment Status, Study Status, Contacts/Locations, Eligibility, Study Design and Study Description
13	○	○	August 23, 2018	Arms and Interventions, Study Status, Contacts/Locations, Outcome Measures and Study Description
14	○	○	October 31, 2018	Recruitment Status and Study Status
15	○	○	December 20, 2018	Study Status and Contacts/Locations
16	○	○	October 2, 2019	Outcome Measures, Study Status, Arms and Interventions, Documents, Results and Study Description
17	○	○	November 15, 2019	Outcome Measures, Study Status, More Information, Reported Adverse Events, Participant Flow

Compare Comparison Format: ◉ Merged ○ Side-by-Side

Scroll up to access the controls

Study NCT01770353
Submitted Date: May 5, 2015 (v5)

Study Identification

Unique Protocol ID:	MM-398-01-01-02
Brief Title:	Pilot Study to Determine Biodistribution of MM-398 and Feasibility of Ferumoxytol as a Tumor Imaging Agent
Official Title:	A Pilot Study in Patients Treated With MM-398 to Determine Tumor Drug Levels and to Evaluate the Feasibility of Ferumoxytol Magnetic Resonance Imaging to Measure Tumor Associated Macrophages
Secondary IDs:	

Study Status

Record Verification:	May 2015
Overall Status:	Recruiting
Study Start:	November 2012
Primary Completion:	January 2016 [Anticipated]
Study Completion:	March 2016 [Anticipated]
First Submitted:	December 19, 2012
First Submitted that Met QC Criteria:	January 15, 2013
First Posted:	January 17, 2013 [Estimate]
Last Update Submitted that Met QC Criteria:	May 5, 2015
Last Update Posted:	May 7, 2015 [Estimate]

Sponsor/Collaborators

Sponsor:	Merrimack Pharmaceuticals
Responsible Party:	Sponsor
Collaborators:	

Oversight

U.S. FDA-regulated Drug:	
U.S. FDA-regulated Device:	
Data Monitoring:	No

Study Description

Brief Summary:	This is a Phase I Pilot study to understand the biodistribution of MM-398 and to determine the feasibility of using Ferumoxytol as a tumor imaging agent.
Detailed Description:	This study is conducted over two phases. The pilot phase of this trial is closed. The expansion phase of this trial is currently open to enrollment. Pilot Phase: This study will enroll approximately 12 patients, up to 20 in total in the Pilot Phase and 30 patients in the Expansion Phase. The first three patients that are enrolled can have any solid tumor type; however subsequent patients must have NSCLC, CRC, TNBC, ER/PR positive breast cancer, pancreatic cancer, ovarian cancer, gastric cancer, gastro-oesophageal junction adenocarcinoma or head and neck cancer. No more than three patients with ER/PR positive breast cancer can be enrolled in the Pilot Phase and similar restrictions may be placed on other tumor types to ensure a heterogeneous population. Expansion Phase: The expansion will enroll cohorts of single indications of patients with metastatic breast cancer in 3 cohorts: Cohort 1: ER and/or PR-positive breast cancer Cohort 2: TNBC Cohort 3: BC with active brain metastasis

Conditions

Conditions:	Solid Tumors Triple Negative Breast Cancer ER/PR Positive Breast Cancer Metastatic Breast Cancer With Active Brain Metastasis
Keywords:	solid tumors Triple Negative Breast Cancer Colorectal Cancer MM-398 nanoliposomal irinotecan Ferumoxytol ER/PR positive Breast Cancer Pancreatic Cancer Ovarian Cancer Gastric Cancer Gastro-Esophageal Junction Adenocarcinoma Head and Neck Cancer

Study Design

Study Type:	Interventional
Primary Purpose:	Other
Study Phase:	Phase 1
Interventional Study Model:	Single Group Assignment
Number of Arms:	2
Masking:	None (Open Label)
Allocation:	Non-Randomized
Enrollment:	45 [Anticipated]

Arms and Interventions

Arms	Assigned Interventions
Experimental: Pilot Phase: Ferumoxytol followed by MM-398 (CLOSED) Ferumoxytol 5 mg/kg IV at the rate of 1 mL/sec, given once on Day 1. MM-398 80 mg/m2 IV over 90 min on Days 1 and 15 of every 4 weekly cycle	Drug: Ferumoxytol followed by MM-398 Ferumoxytol 5 mg/kg IV at 1mL/sec, given once. MM-398 80 mg/m2 IV over 90 min every 2 weeks, until progressive disease or intolerable toxicity
Experimental: Expansion Phase: Ferumoxytol followed by MM-398 Ferumoxytol 5 mg/kg IV at the rate of 1 mL/sec, given once on Day 1. MM-398 80 mg/m2 IV over 90 min on Days 1 and 15 of every 4 weekly cycle Cohort 1: ER and/or PR-positive breast cancer Cohort 2: TNBC Cohort 3: BC with active brain metastasis	Drug: Ferumoxytol followed by MM-398 Ferumoxytol 5 mg/kg IV at 1mL/sec, given once. MM-398 80 mg/m2 IV over 90 min every 2 weeks, until progressive disease or intolerable toxicity

Outcome Measures

Primary Outcome Measures:

1. Measure tumor levels of irinotecan and SN-38 (in ng/mL)

   [Time Frame: 12 months]

Secondary Outcome Measures:

2. Safety profile of MM-398 in the presence of ferumoxytol (number and type of Adverse Events compared with histological control)

   [Time Frame: 12 months]

3. Tumor response Rate measured by RECIST (Response Evaluation Criteria in Solid Tumors) 1.1 guidelines

   [Time Frame: 12 months]

4. Half-life of the drug (t 1/2) in number of hours
   50% of total time of drug in plasma

   [Time Frame: 12 months]

5. Maximum concentration of drug in plasma (Cmax) in ng/mL

   [Time Frame: 12 months]

6. Measure of drug availability in the plasma (AUC) in ng/mL x hours
   Area under the plasma drug concentration versus time curve

   [Time Frame: 12 months]

Eligibility

Minimum Age:	18 Years
Maximum Age:	
Sex:	All
Gender Based:	
Accepts Healthy Volunteers:	No

Criteria: Inclusion Criteria:

- Pathologically confirmed diagnosis of solid tumors, CRC, TNBC, ER/PR Breast Cancer, NSCLC, Pancreatic Cancer, Ovarian Cancer, Gastric Cancer, GEJ adenocarcinoma, Head and Neck Cancer
- Metastatic disease
- ECOG Performance Status 0 to 2
- Adequate bone marrow, hepatic and renal function
- Normal ECG
- 18 years of age or above
- Able to understand and sign informed consent

Expansion Phase Additional Criteria:

The following invasive breast cancer tumor sub-types are required:

- Cohort 1: hormone receptor positive breast cancer patients with ER-positive and/or PR-positive tumors defined as ≥1% of tumor nuclei that are immunoreactive for ER and/or PR and HER2 negative
- Cohort 2: triple negative breast cancer (TNBC) patients with ER-negative, PR-negative tumors defined as < 1% of tumor nuclei that are immunoreactive for ER and PR and HER2 negative
- Cohort 3: Any sub-type of metastatic breast cancer and active brain metastases
- Documented metastatic disease with at least two radiologically measurable lesions as defined by RECIST v1.1 (except Cohort 3, see inclusion criterion o below)
- Received at least one cytotoxic therapy in the metastatic setting, with exception of TNBC patients who progressed within 12 months of adjuvant therapy
- Received ≤ 3 prior lines of chemotherapy in the metastatic setting (no limit to prior lines of hormonal therapy in Cohort 1)
- At least one lesion amenable to multiple pass core biopsy (exception: Cohort 3 patients)

Expansion Phase Cohort 3 additional inclusion criteria:

- Radiographic evidence of new or progressive brain metastases after prior radiation therapy with at least one brain metastasis measuring ≥ 1 cm in longest diameter on gadolinium-enhanced MRI (note: progressive brain lesions are not required to meet RECIST criteria in order to be eligible; extra-cranial metastatic disease is also allowed)

Exclusion Criteria:

- Active CNS metastasis (applies to pilot phase and expansion phase cohort 1 and 2 only)
- Clinically significant GI disorders
- Prior irinotecan or bevacizumab therapy within last 6 months
- Known hypersensitivity to MM-398 or ferumoxytol
- Inability to undergo MRI
- Active infection
- Pregnant or breast feeding

Contacts/Locations

Central Contact Person: Eliel Bayever, MD
Telephone: 617-441-1000
Email: ebayever@merrimackpharma.com

Study Officials: Eliel Bayever, MD
Study Director
Merrimack Pharmaceuticals

Locations: United States, Arizona

[Recruiting]
Scottsdale, Arizona, United States

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The relevance of drug sequence in combination chemotherapy

Manish A. Shah,[1,3] Gary K. Schwartz[1,2,3]

[1]Department of Medicine, [2]Division of Solid Tumor Oncology, [3]Laboratory of New Drug Development, Memorial Sloan-Kettering Cancer Center, New York, USA

Abstract The concept of combining chemotherapeutic agents to increase the cytotoxic efficacy has evolved greatly over the past several years. In the past, the rationale for combination chemotherapy centered on attacking different biochemical targets, overcoming drug resistance in heterogenous tumors, and increasing the dose-density of combination chemotherapy to take advantage of tumor growth kinetics. The overall goal was to improve clinical efficacy with acceptable clinical toxicity. It is now apparent that the sequence of drug administration can significantly enhance the therapeutic effect of chemotherapy. These sequence-dependent effects can be explained by chemotherapy-induced cell cycle perturbations, or by pharmacodynamic interactions between the agents in combination. In this review, we focus on drug combinations with taxanes and camptothecins, which we believe best illustrate the importance of the cell cycle and pharmacologic interactions in the sequential administration of chemotherapy. As our understanding of the cell cycle grows, our ability to appropriately sequence chemotherapy can have a great impact on the treatment of human cancers. © 2000 Harcourt Publishers Ltd

INTRODUCTION

The first attempt at systemic treatment of cancer was the use of alkylating agents for hematopoietic neoplasms in 1943.[1] Since then, there has been a tremendous growth in the development of chemotherapeutic agents, each with different mechanisms of action, toxicity and activity. However, except for a few select cancers (i.e. Burkitt's lymphoma and choriocarcinoma), individual chemotherapeutic agents for the majority of tumors have not increased cure rates in the treatment of cancer. The concept of combination chemotherapy to treat metastatic disease was an important early breakthrough in medical oncology, resulting from a natural evolution from single agent therapy having marginal individual success.[2] Early successes were demonstrated in Hodgkin's lymphoma,[3–5] childhood lymphocytic leukemia,[6–8] and advanced breast cancer.[2,9] A refinement in the delivery of combination chemotherapy, as proposed by Norton and Simon, was prompted by the Gompertzian mathematical model of tumor growth promoting the concept of dose intensification by increasing the dose density of therapy.[10] The successful test of this concept is best exemplified in breast cancer.[11–13] Presently, with advances in our understanding of the basic mechanisms of oncogenesis, cell cycle, and apoptosis, we are also gaining a better understanding of the effects of chemotherapy on normal and cancerous cells. With this knowledge, it is becoming increasingly apparent that the sequence of drugs used in combination chemotherapy is vitally important to their antitumor efficacy. In this review, we address the importance of sequence in the administration of combination chemotherapy, in particular as it relates to the cell cycle and apoptosis.

The cell cycle

The cell cycle is the sequence of events in eukaryotic cells by which cells grow and divide, and is typically divided into four phases (Fig. 1). The periods associated with DNA synthesis (S phase) and mitosis (M phase) are separated by gaps of varying length called G1 and G2. G0 is used to describe cells that have exited the cell cycle and are not actively replicating. Over the past decade, many of the mechanisms by which the cell enters and navigates this cycle have been elucidated.[14] In particular, several checkpoints within the cell cycle have been identified, which result in cell cycle arrest, followed by either DNA repair and continued survival or programmed cell death.[14,15] Additionally, it has become increasingly clear that alterations in the cell cycle may result in programmed cell death, or apoptosis. As our understanding of the cellular basis of cancer improves, we have gained a critical understanding of how chemotherapy may induce tumor cell death. Here, we propose an increased sophistication in the design of polychemotherapeutic regimens to treat cancer based on adjusting the sequence of drugs according to cell cycle perturbations resulting from each chemotherapeutic agent alone.

COMBINATION CHEMOTHERAPY

Historical rationale for its use

The utilization of simultaneous combination chemotherapy arose naturally from the inability to produce satisfactory results with single agents in the treatment of patients with advanced cancers (Table 1).[2] Several important concepts of systemic anti-tumor therapy form the foundation for this combination chemotherapy.[2,16]

First, using a leukemia L1210 mouse model, Skipper and Schabel introduced the concept of fractional or log-kill.[17,18] They found that the survival of animals bearing transplanted tumors is inversely related to the number of tumor cells inoculated or surviving after treatment. Specifically, the injection of a single malignant cell into a mouse could lead to death; the larger the number of cells in the inoculum, the shorter the interval to death; and that this interval can be predicted by the number of cells injected. Furthermore, they quantified the cytocidal effects of treatment by extrapolating back from the prolongation in host life span resulting from the treatment. For example, they reasoned that the increase in life span of 2 days would result from a 90% cell kill, or a reduction of 10^6 leukemic cells to 10^5 leukemic cells (1 log kill).[17] By this methodology, they determined that cell destruction followed first-order kinetics; a given dose of drug destroyed a constant fraction of cells, not a fixed number.[17] By increasing

CSPC Exhibit 1122
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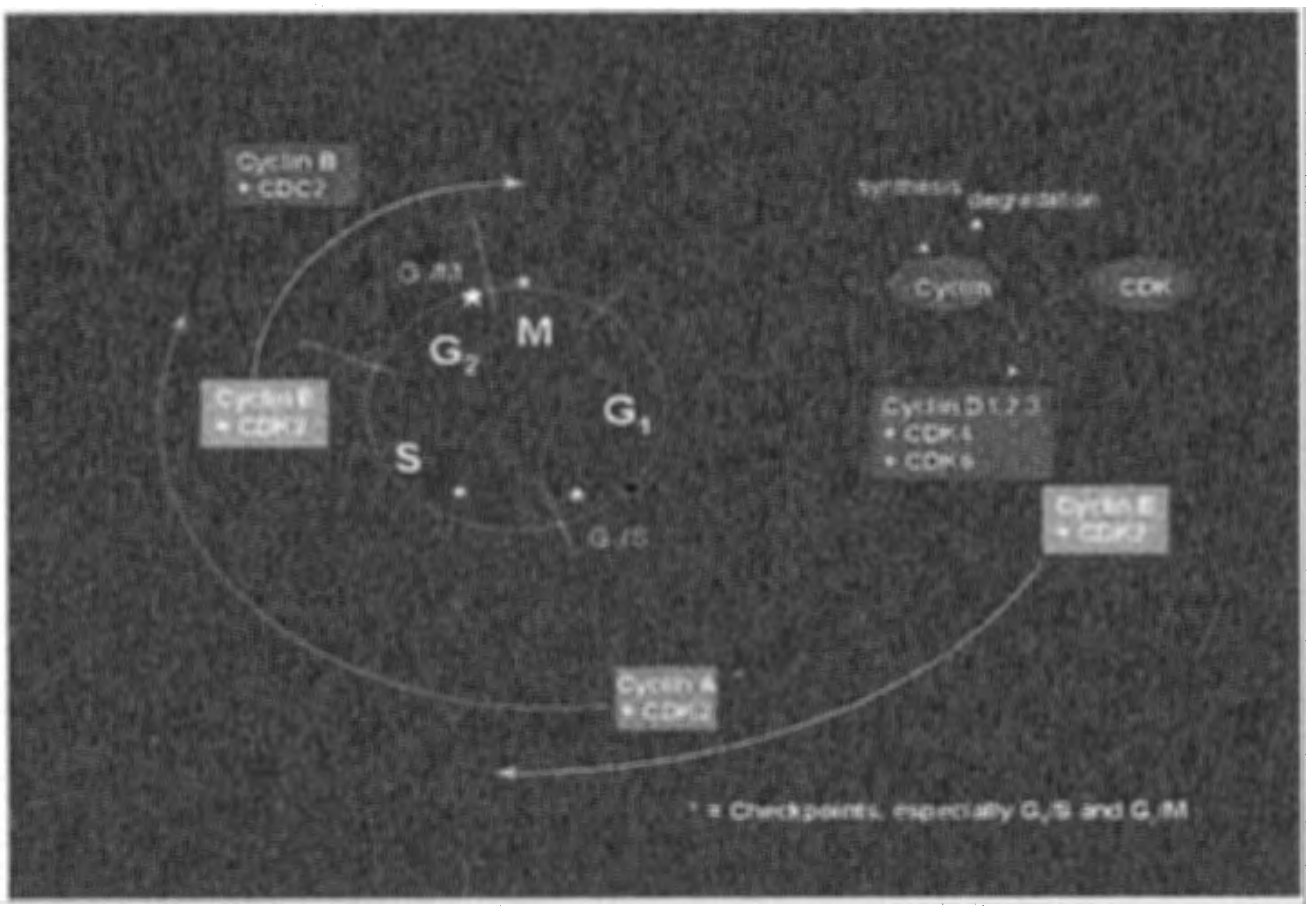


Fig. 1 The cell cycle.

the dose of chemotherapy, the log-kill could be increased, but at the expense of increased host toxicity. However, the addition of drugs would each individually add their own log-kill, resulting in improved tumor responses in the host. This naturally led to the use of drug combinations to improve anti-tumor efficacy.[2,16]

Circumvention of drug resistance is another rationale for the utilization of combination chemotherapy.[1,2,16] This concept is in large part based on the work of Luria and Delbruck, who found that bacteria spontaneously developed mutations that made them resistant to bacteriophage.[1,19] Unlike bacteria and many animal tumor models, human malignancies have a lower growth fraction, i.e. that portion of the population actively replicating,[2,20,21] and therefore require multiple repeated drug treatments over a prolonged period of time. The resultant repetitive exposure to chemicals can lead to the development of resistance in tumor cells.[2] Applying these concepts to cancer, Goldie and Coldman proposed that the probability that a given tumor will contain resistant clones at the time of diagnosis would be a function of the mutation rate of that cancer and the size of the tumor at diagnosis.[22,23] However, even with low mutation rates of 1 in 10^6 mitoses, it would be virtually certain that drug resistant mutants would populate the cells in a clinically detectable 1 cm^3 tumor deposit (10^9 tumor cells).[1,22,23] Therefore, they suggest that drug resistance would be a problem even with small tumor burdens, and that the maximal chance of cure occurs when all available effective drugs are used in combination, simultaneously if possible, or in an alternating schedule otherwise.[1,22,23] This was in large part the reasoning for the ABVD/MOPP alternating regimen for the treatment of Hodgkin's lymphoma.[24,25]

Biochemical synergy is a third historical rationale for combination chemotherapy. Synergy is defined as a greater than expected additive effect of individual drugs when combined together; antagonism is defined as less than expected additive effect. By choosing chemotherapeutic agents with different mechanisms of action, multiple sites in biosynthetic pathways can be attacked and/or several processes involved in the maintenance and function of essential macromolecules may be inhibited.[2] Conceptually, the mechanisms of drug interaction involve either the binding of each drug to different targets or to the same target,[26] with resultant additive, synergistic, or antagonistic interactions. Multidrug resistance is an important example of a combination of drugs working at different targets to have a greater than additive, or synergistic, anti-tumor effect. Multidrug resistant cells avoid drug cytotoxicity by maintaining the intracellular drug concentration at an extremely low level, associated with an increase in the transporter protein P-glycoprotein.[27]

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Table I **History of combination chemotherapy**

	Model	Concept
Skipper and Schabel[17,18]	Leukemia L1210 mouse model	Fractional or log-kill
Luria and Delbruck[19]	Bacterial mutations altered virus sensitivity to virus resistance	Tumor resistance
Goldie and Coldman[22,23]	Mathematical model applying a spontaneous mutation rate	Individual drug resistant mutant clones all ready exist
DeVita[2,5] and others[25,26]	Various: multidrug resistance;[27] thymidylate synthase[30]	Biochemical synergy
Norton and Simon[10,21]	Gompertzian mathematical model of tumor growth kinetics	Dose-density
Shah and Schwartz and others	Cell physiology and cell cycle	Cell cycle mediated drug resistance

Verapamil is a calcium channel antagonist that reverses the multidrug resistant phenotype in vitro by interacting with P-glycoprotein and increasing intracellular chemotherapeutic drug levels, thereby increasing their effectiveness at their own intracellular targets.[28,29] The combination of leucovorin and 5-fluorouracil provide an example of two drugs acting at the same target with resultant synergistic anti-tumor effects. In the presence of the reduced folate cofactor, 5,10-methylenetetrahydrofolate, the active 5-fluorouracil metabolite (FdUMP) forms a stable complex with thymidylate synthase.[30] Leucovorin expands the intracellular pools of 5,10-methylenetetrahydrofolate, thereby increasing the extent and duration of inhibition of thymidylate synthase, and enhancing the cytotoxicity of 5-fluorouracil.[31,32] Indeed this combination of drugs has demonstrated improved clinical efficacy, demonstrating an increased response rate when 5-fluoruracil is used with leucovorin (23%) vs. 5-fluorouracil alone (11%, $P = 10^{-7}$).[33,34] However, this improved response rate has not translated to improved overall survival.[33]

Gompertzian model of tumor growth and dose density

With benefit of combination chemotherapy first being demonstrated in the 1960s and 1970s,[2–9] the utilization of combination chemotherapy has become a standard practice in medical oncology. The primary goal of combination chemotherapy is to provide a broader range of anti-tumor activity, so as to improve the killing of resistant tumor cell lines in a heterogeneous tumor, and to circumvent or slow the development of drug resistance.[1] However, the combination of different chemotherapeutic agents to maximize tumor cell kill often requires dose reductions or delays in order to allow the simultaneous administration of all the drugs. The end result is to decrease the dose intensity and lessen the therapeutic benefit. This realization led Norton and Simon to the proposal of 'dose density' for combination chemotherapy,[10,16] a concept based on the model that tumor cells grow in accordance to the Gompertzian exponential growth curve. The underlying principle is that the doubling time of tumor cells is not constant, but rather increases with increasing tumor size.[21] This mathematical model predicts that, if the scheduled administration of chemotherapeutic agents is delayed because of toxicities, the delay in treatment will ultimately allow for resistant clones to develop and expand. It then follows that decreasing the interval between treatments would result in more cytotoxic drug being given over time and provide an increased 'dose density'. This shorter time interval would leave less time for tumor cells to grow and less time for resistant clones to develop;[16] i.e. if each drug represents a letter, then ABABCDCD is better than ABCDABCD. This concept is best illustrated in a randomized trial of patients with high risk primary breast cancer.[13] In this trial, patients were randomized to receive doxorubicin alternating with CMF vs. doxorubicin followed by CMF in the adjuvant setting. In the sequential arm, the doxorubicin was 'condensed' into the first 12 weeks of therapy followed by 24 weeks of CMF, vs. 36 weeks total to complete both doxorubicin and CMF in an alternating schedule. In this trial, at 10 years of follow up, those patients who received sequential, dose dense therapy received the most benefit (total survival 58% vs. 44%, $P = 0.002$).[13] In accordance with the concept of dose density, those cells sensitive to doxorubicin alone would have less time to regrow with the sequential therapy, and similarly those cells sensitive to CMF would have less time to regrow when CMF is given alone without interruption with doxorubicin.[16]

SEQUENCE DEPENDENCE IN COMBINATION CHEMOTHERAPY

In addition to the concepts of avoiding drug resistance, biochemical synergy, and dose-density to improve anti-tumor

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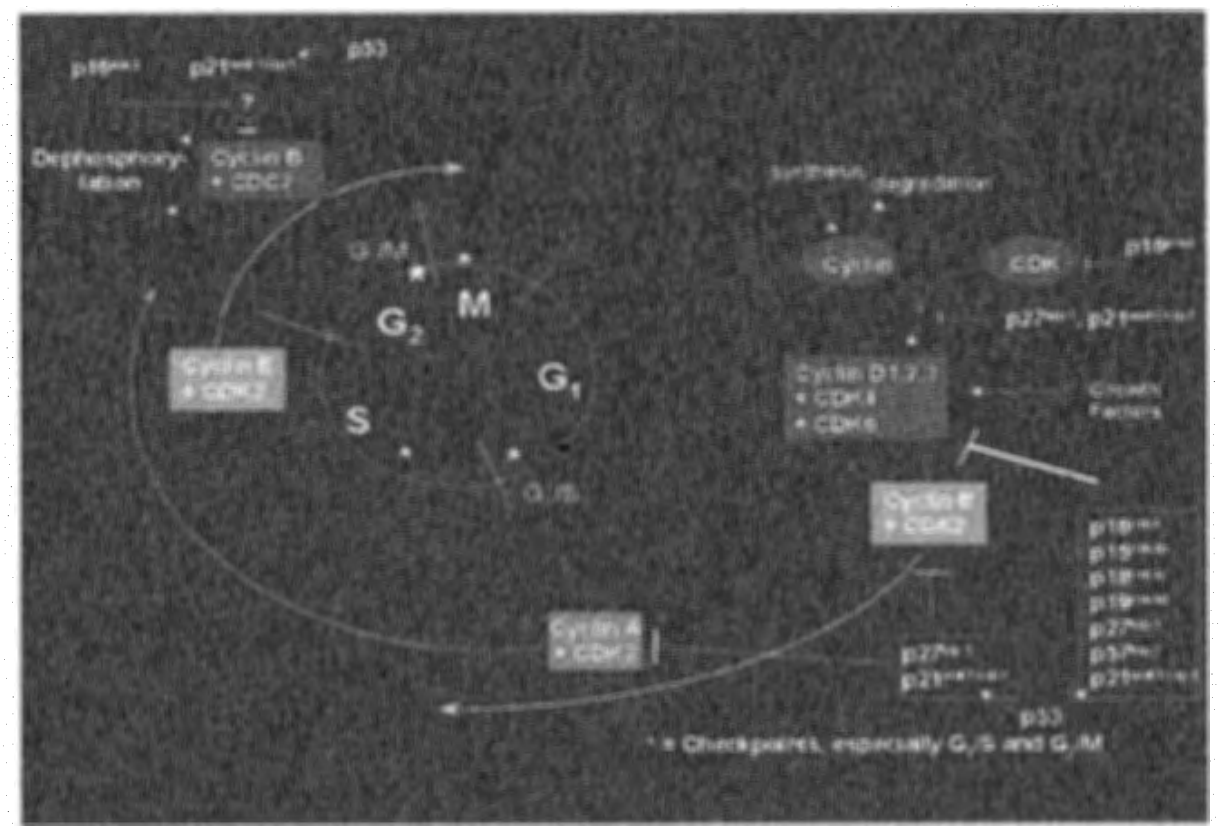


Fig. 2 Regulation of the cell cycle through cyclin dependent kinases and their inhibitors.

efficacy discussed above, we believe the sequence of administration of combination chemotherapy can be important in anti-cancer efficacy and chemotherapy-related toxicity. This sequence dependence is a direct extension of the physiological effects of chemotherapeutic agents on cancer cells, and depends on two processes: the direct cell-cycle interaction of individual drugs, and the pharmacodynamic interaction of the drugs when given in combination. The application of cell cycle influences has resulted in improved cancer killing in vitro by induction of apoptosis, and may translate into improved anti-tumor efficacy in the clinical arena. In this review, we will describe the regulation of the cell cycle, and our ability to influence this replicative cycle with a new class of anti-cancer agents in clinical development. We will then present pre-clinical to clinical data for and against combination chemotherapy with particular focus on sequence dependence, both from a cell cycle and pharmacodynamic point of view.

Prior to proceeding with a detailed description of the pre-clinical data describing the importance of sequence, one must first understand the methods used in the laboratory for determining synergy and antagonism, and their potential limitations. Synergy is defined as observing a greater than the expected additive effect when using drugs in combination; conversely, antagonism is demonstrated by a less than the expected additive effect. The quantitation of synergism and antagonism in experimental systems has evolved over the past several decades; however a uniformly accepted methodology remains elusive. Methodologies utilized include: isobologram method,[35] isobologram method of Steel and Pecham,[36] fractional product method of Webb,[37] combination index method of Chou and Talalay,[38] and more or less mathematical methods like surviving fraction,[39] percentage response to GM-CFU compared to controls,[40] and others.[41,42] For induction of apoptosis with drug combinations, there has been less reliance on these classical methods for testing for synergy and antagonism. Instead, investigators have utilized quantitative fluorescent microscopy to detect morphologic features of apoptosis, DNA fragmentation, poly-ADP-ribose polymerase (PARP) cleavage, cytochrome C release, and activation of caspases (cysteine proteases that are the common executioners of the apoptotic cascade). Still, varying methods may have disparate results, even within the same system. Furthermore, even for identical drug schedules in different experiments, the dosing and duration of exposure to drugs varied from experiment to experiment. Finally, in different cell culture systems, combination chemotherapy may have significantly varied effects. With these caveats in mind, we will summarize the existing data, from pre-clinical to clinical, for and against combination chemotherapy with particular focus on sequence dependence and its relationship to our understanding of the cell cycle.

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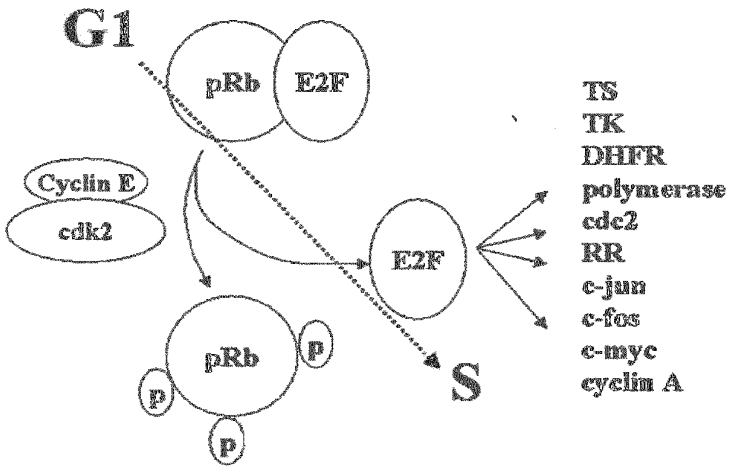


Fig. 3 The regulation of the G1/S transition.

Regulation of the cell cycle

A cell's progression through the cell cycle is promoted by a number of cyclin-dependent kinases (CDKs) which, when complexed with specific regulatory proteins called cyclins, drive the cell forward through the cell cycle (Fig. 2). There exist corresponding cell cycle inhibitory proteins (cyclin-dependent kinase inhibitors, CDKIs), that serve as negative regulators of the cell cycle, and can stop the cell from proceeding to the next phase of the cell cycle. The pattern of cyclin expression varies with a cell's progression through the cell cycle, and the specific cyclin expression patterns define the relative position of a cell in the cell cycle.[14,15] At least nine structurally related cdks (cdk1-cdk9) have been identified, though not all have clearly defined cell cycle regulatory roles. A considerable number of cyclins have been identified to date (cyclin A-cyclin T). Cdk/cyclin complexes themselves become activated by phosphorylation at specific sites on the cdk by cdk7/cyclin H, also referred to as cdk-activating kinase (CAK).[43] Cyclin D isoforms (cyclin D1-D3) interact with cdk 2,4, and 6 and drive a cell's progression through G1. The association of cyclin E with cdk2 is active at the G1/S transition and directs entry into S-phase progression is directed by the cyclin A/cdk2 complex, and the complex of cyclin A with cdk1 (also known as cdc2) is important in G2. Cdk1/cyclin B is necessary for mitosis to occur.

G1/S transition is closely controlled by the activity of the Retinoblastoma tumor suppressor gene product (pRb) (Fig. 3). In its active state pRb is hypophosphorylated, and forms a complex with a group of transcription factors known as E2F (E2F-1, -2, and -3). When pRb is inactivated by cdk2-mediated phosphorylation, E2F transcription factors are released resulting in transcriptional activation of numerous S- phase proteins, such as thymidylate synthase (TS) and dihydrofolate reductase (DHFR), to mention a few.

The progression-driving activity of CDKs is balanced by a number of cellular cyclin-dependent kinase inhibitors (CKIs). CKIs function to counter-balance the cell-cycle-accelerating effect of cdks. The INK4 (for inhibitor of cdk4) class of CKIs, notably p16Ink4a, p15Ink4b, p18Ink4c, and p19Ink4d, bind and inhibit cyclin D associated kinases (cdk2, 4, and 6). The KIP (kinase inhibitor protein) group of cdk inhibitors, p21waf1, p27kipl, and p57kip2, negatively regulates cyclin E/cdk2 and cyclin A/cdk2 complexes.[44]

TAXANE COMBINATIONS

The relevance of the cell cycle to the sequence of drug administration is best illustrated by combination chemotherapy involving taxanes, the prototypical class of cell-cycle specific chemotherapeutic agents. The taxanes act by stabilizing microtubules, thereby causing a G2/M arrest followed by apoptosis. Unlike other known mitotic spindle inhibitors (vinca alkaloids, colchicine and podophyllotoxin), which inhibit tubulin polymerization, taxanes markedly enhance microtubule assembly and disrupt a cell's transition through mitosis. The two primary drugs in clinical use today include paclitaxel (Taxol®) and docetaxel (Taxotere®). However significantly more pre-clinical and clinical information has been presented with paclitaxel.

Paclitaxel is an anticancer agent with a broad spectrum of activity, currently being used in patients with ovarian, breast, lung, head and neck, bladder, and esophageal cancers.[45] Paclitaxel promotes microtubule assembly and stabilizes tubulin polymer formation,[46] thereby interrupting the dynamic cellular reorganization necessary for mitosis,[47] and resulting in a G2-M arrest.[48] Paclitaxel is also associated with down-regulation of CDK4[49] with concomitant G1/S arrest (Table 2). The primary effect of paclitaxel is to interfere with the assembly of the mitotic spindle, resulting in the failure of chromosomes to segregate.[50] As a microtubule promotor, paclitaxel shifts the equilibrium in favor of the microtubule and thus decreases the concentration of tubulin necessary for subsequent assembly.[51] Mitosis is initiated by the activation of the cyclin B1-CDK1 complex (also called cyclin B1-cdc2 kinase), and as mitosis progresses, cyclin B is destroyed by ubiquitin-mediated proteolysis. Cyclin B and Cdc2 kinase activity are closely related to paclitaxel function. Expression of cyclin B and the activation of CDK1 occur coincidentally

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Table 2 **Cell cycle events with chemotherapeutic agents**

	Cell cycle events	*Etiology*
Taxanes	G2/M arrest	• Promotes microtubule assembly and stabilizes tubulin polymer formation[44] • destruction of cyclin B1[55]
	G1 arrest	• CDK4[49]
	Apoptosis	• Bcl-2 hyper-phosphorylation[57,58] • c-Raf-1 phosphorylation[57,58]
Platinum	Sensitive in G1 and early S	[56]
	Duration of S phase and arrests cells in G2	[75]
Methotrexate	S phase activity	Inhibits DHFR
Vinca Alkaloids	G2/M phase activity	Microtubule disassembly[105,106]
	Apoptosis	Bcl-2 phosphorylation and bcl-2/bax heterodimers[108]
Anthracyclines	S phase activity	Covalent topoisomerase II-DNA complex formation[126]
	G2 arrest	[128]
Etoposide	Metaphase	[137–139]
Camptothecin	S phase active	Topoisomerase I inhibitor[148,167]
	G1 and G2 arrest	[153,157]
	Bax from prolonged exposure	[154]
	Unscheduled cyclinB1/cdc2 activity	[155]
Fluorouracil	S phase active	TS activity,[30] RNA incorporation [197]
	p53 and p21	[198]
	Cells accumulate in early S phase	Cyclin A, cyclin A/cdk2 activity[199]

with paclitaxel-induced apoptosis,[52,53] and destruction of cyclin B1 can be inhibited by paclitaxel.[54] Furthermore, a dominant negative mutant of p34cdc2 blocks paclitaxel-induced apoptosis.[55]

Although cytotoxicity is maximal at G2-M and minimal at G1-S,[56] paclitaxel may induce apoptosis by other mechanisms as well. In particular, paclitaxel exposure is also asso ciated with hyperphosphorylation of bcl-2 and phosphorylation of c-Raf-1,[15,57,58] steps perhaps necessary for apoptosis. However, the exact mechanism of programmed cell death induced by paclitaxel has been confounded by seemingly contradictory laboratory observations.[59] These contradictions occur in vitro due to cell type specificity,[60] as well as concentration and duration of exposure related effects.[59,61] Nanomolar concentrations appear to be sufficient to polymerize tubulin, and micromolar concentrations have demonstrated tubulin-independent effects, and may in fact be clinically irrelevant. With these caveats, we will review the sequence dependent data of combination chemotherapy with taxanes.

In view of the fact that paclitaxel is an M-phase-specific drug, one would hypothesize that agents that arrest cells in G1 before they enter M would antagonize paclitaxel effects. This issue is of clinical importance as cell cycle inhibitors enter clinical trials in combination with chemotherapy, in particular with paclitaxel. Flavopiridol is a novel anti-neoplastic agent that originally was noted for its ability to inhibit the activity of a number of protein kinases. It is a synthetic flavone with a novel structure compared with that of polyhydroxylated flavones including quercetin and genistein, and is identical to a compound obtained by derivation from a natural product obtained from *Dysoxylum benectariferum*, a plant indigenous to India.[62] Flavopiridol is now best classified as a cyclin-dependent kinase inhibitor (CDKI) because of its considerable affinity for CDKs and its ability to induce cell cycle arrest in a number of cell lines.[63–66] It has been shown to bind to and directly inhibit CDK1 (cyclin B1-cdc2 kinase), CDK2, CDK4 and CDK6.

Flavopiridol was combined in various sequences with paclitaxel in the MKN-74 human gastric cancer cell line as well as the human breast cancer cell line MCF-7, which are both heterozygous for p53.[67,68] Using clinically achievable doses of flavopiridol, paclitaxel for 24 h followed by flavopiridol for 24 h was most effective in inducing apoptosis, as evidenced by caspase activation and PARP degradation.[67] This result was sequence dependent, as apoptosis was only modestly increased when the two drugs were given concurrently for 24 h, and when the reverse sequence of

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flavopiridol followed by paclitaxel was examined, apoptosis was markedly suppressed. Similar sequence dependent antagonism and synergy were seen in A549 human non-small cell lung cancer cells in vitro.[69] The significant reduction in apoptosis demonstrated in our experiments when flavopiridol preceded paclitaxel may be explained by cell cycle effects. Flavopiridol inhibits cyclin B1-cdc2 kinase,[68] an event that is critical for the paclitaxel effect.[55] Additionally, flavopiridol induces a G1/S cell cycle arrest, thus preventing cells from entering M-phase, the phase during which paclitaxel is active by stabilizing microtubules. Therefore, when flavopiridol is given first, it antagonizes the action of paclitaxel by not permitting cells to enter M-phase. Conversely, when flavopiridol follows paclitaxel, it accelerates the exit of cells from mitosis. These events are believed to be critical for the sequence dependent enhancement of paclitaxel induced apoptosis.

Similar sequence-dependent effects have been demonstrated with bryostatin-1.[70,71] Bryostatin-1 is an activator of protein kinase C (PKC) with a short duration of action by translocating it from the cell membrane, cytoskeleton or nucleus. Over time, it leads to an overall decrease in activity thought to be secondary to down regulation of PKC.[72] Additionally, bryostatin-1 is an inhibitor of CDK2.[73] In a mouse mammary tumor xenograft system, paclitaxel prevents the physiological destruction of cyclin B1-cdc2 kinase activity, with consequent G2-M arrest followed by apoptosis and ultimately tumor regression. However, when the mice were first treated with bryostatin-1, cyclin B1 was suppressed, thereby decreasing cyclin B1-cdc2 kinase activity, and decreasing paclitaxel sensitivity. There were significantly fewer cells in mitosis with bryostatin-1 pretreatment. This corresponded to a significantly shortened tumor doubling time compared with paclitaxel alone (9.3 ± 1.9 days vs. 22.7 ± 2.5 days, $P < 0.001$). In contrast, the mean tumor doubling time increased significantly when bryostatin-1 followed paclitaxel, compared with paclitaxel alone (30.0 ± 0.9 days vs. 22.7 ± 2.5 days, $P < 0.01$),[70,71] demonstrating a sequence dependent enhancing effect. Thus, it is evident by these studies that it is critical to understand the cell cycle effects of each drug before they are given in combination. The issue is critical with the combination of cell cycle specific drugs, especially when one of these cell cycle drugs is a chemotherapeutic agent.

We have further examined the sequential combination of paclitaxel followed by flavopiridol in a phase I study (NCI T96-0091).[74] We were able to escalate the dose of paclitaxel to 175 mg/m^2 over 3 h on day 1 followed by a 24 h infusion of flavopiridol at a dose of 80 mg/m^2 on day 2, repeated on a 21-day schedule. The clinical results were remarkable for major responses in patients with chemotherapy refractory malignancies (i.e. prostate and esophagus), including patients who have received prior paclitaxel therapy. In particular, five of seven patients with esophageal cancer responded to the combination treatment, and three of them received prior paclitaxel therapy. These are patients who would not have been expected to respond to further paclitaxel therapy. These responses therefore suggest that flavopiridol, when given in a sequence-dependent manner may render paclitaxel-resistant cancers paclitaxel-sensitive. We have also seen stable disease in patients with cancer of the prostate (4), pancreas (3), sarcoma (3), neuroendocrine (1), and colon (2).[74] In this clinical trial, there was no effect of flavopiridol on paclitaxel pharmacokinetics.

We have also translated the pre-clinical studies demonstrating a sequence dependent synergy between paclitaxel and bryostatin-1 to a phase I clinical trial (NCI T97-0118). Patients were treated with a weekly dose of paclitaxel 80 mg/m^2 over followed 24 h later with increasing doses bryostatin-1. Two partial responses were demonstrated, with 9/27 patients demonstrating stable disease, including a patient with metastatic pancreatic carcinoma whose disease remained radiographically stable for 15 months. Again, we found no pharmacokinetic effects on paclitaxel by bryostatin-1.[70,71]

Taxanes and cisplatin (analogues)

Cisplatin belongs to the alkylating class of anticancer agents. It binds to DNA base pairs, creating adducts, crosslinks, and strand breaks that inhibit DNA replication. As such, cisplatin is not cell cycle specific, though cells appear to be maximally sensitive to cisplatin in G1, just prior to the onset of DNA synthesis, and minimally sensitive in peak DNA synthesis, with entry into S phase resulting in a 2-fold decrease in sensitivity.[56] Cells that remained arrested at the G1/S phase boundary during cisplatin treatment remain maximally sensitive after release.[56] Detection of damaged DNA leads to the activation of cyclin-dependent kinase inhibitors such as p21 and wee1/mik1, which subsequently arrest cells in either G1 or G2. Cisplatin exposure increases the duration of S phase and arrests cells in G2 in a dose-dependent manner.[75] This arrest is accompanied by the accumulation of inactive, phosphorylated p34cdc2 protein. Following a protracted delay, the p34cdc2 protein is dephosphorylated and an aberrant mitosis occurs. In fact, a number of agents that abrogate the G2 cell cycle checkpoint and induce premature mitosis have demonstrated enhancement of cisplatin-induced cytotoxicity.[76–78] Resistance to cisplatin has been associated with increased glutathione levels, increased metallothioneins, decreased drug uptake, increased DNA repair, and the tolerance of the formation of platinum-DNA adducts.[79]

Though the precise mechanism by which platinum-DNA damage results in cell death remains unknown, unrepairable DNA damage often results in activation of the apoptotic pathway.[80,81] P53 plays a significant role in DNA repair, proliferative arrest, and apoptosis[82] and has led to a correlation between p53 and cisplatin sensitivity.[83–85] Apoptosis has been associated with an unscheduled activation of cdc2 kinase in cisplatin-resistant cells, and with the p53/p21 Waf1 pathway in cisplatin sensitive cells.[86]

Combination studies with paclitaxel and cisplatin have been pursued in the hopes of increased anti-tumor effects due to each individual drug's broad range of clinical activity and different mechanisms of action. These pre-clinical studies have demonstrated a synergistic or additive interaction when paclitaxel precedes cisplatin,[81,86–90,92,93] and an antagonistic or additive relationship when paclitaxel follows cisplatin.[81,86–90,92,93] The reasons for this sequence-dependent synergy and antagonism may be multifactorial: paclitaxel given prior to cisplatin may increase intracellular uptake of

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cisplatin[94] and inhibit repair of cisplatin-induced DNA damage,[92] whereas cisplatin given prior to paclitaxel may block cells in G2,[75,81,86] interfere with tubulin or tubulin-associated proteins.[95] In addition, cisplatin may hasten the exit from mitosis, thereby increasing cytotoxicity, if given following paclitaxel.[93] These findings appear to be cell line and schedule dependent. For example, in a cisplatin-sensitive ovarian tumor cell line, Zaffaroni et al. demonstrated that cisplatin followed by paclitaxel antagonism was associated with a transient arrest in G2, increase in p53 and the CDKI p21cip and in cyclin B1.[86] In cisplatin-resistant ovarian cancer cells, cisplatin followed by paclitaxel was synergistic and was associated with premature activation of cyclin B1-Cdc2 kinase during S phase and loss of p21cip.[86] In both an in vitro cell culture[91] and mouse ovarian tumor model,[93] the duration of time prior to administration of the second drug was important to anti-tumor efficacy. Judson et al. found antagonism of paclitaxel effects when there was a 3 h time interval with either sequence.[81] Though cisplatin-resistant ovarian carcinoma cell lines retain sensitivity to paclitaxel, they found that concomitant exposure blocked paclitaxel-induced apoptosis, but did not inhibit paclitaxel-induced stabilization of microtubules or Bcl-2 degradation.[81] This is consistent with the mouse mammary tumor pre-clinical experiments that demonstrated that the maximal anti-tumor effect occurred when cisplatin followed paclitaxel exposure by 48 h.[93] Earlier treatment with cisplatin (as well as when cisplatin preceded paclitaxel) demonstrated a suppression of cells arrested in G2-M, thereby reducing paclitaxel activity.[93]

Clinical trials have further demonstrated a sequence-dependent effect, such that if cisplatin is given prior to paclitaxel, there is a 33% reduction in paclitaxel clearance with resulting increased myelotoxicity without improvement in anti-tumor efficacy.[90,96,97] However, this sequence dependence may be unique to paclitaxel and cisplatin. Other drugs within the same class of chemotherapeutic agents have failed to demonstrate a pharmacokinetic effect with either carboplatin → paclitaxel or the reverse sequence of paclitaxel → carboplatin.[98,99] These studies demonstrated a dose-response anti-tumor effect with no pharmacokinetic interactions, and neutropenia to be the dose-limiting toxicity.[98,99] Both sequences were also associated with a protective effect on thrombocytopenia.[98] Similarly, no pharmacokinetic interactions were seen with docetaxel (1 h infusion) followed by cisplatin (3 h infusion) or reverse sequences.[100] Again, docetaxel dose-dependent myelotoxicity was the dose-limiting toxicity, with responses seen with both sequences.[100]

Taxanes and methotrexate

Methotrexate is an antifolate and is one of the oldest and still most commonly used anticancer agents. Methotrexate inhibits dihydrofolate reductase, which converts dihydrofolate to tetrahydrofolate and is ultimately responsible for the maintenance of intracellular folate pools. Tetrahydrofolate is required for the de novo synthesis of thymidylate, purines, and methionine. Methotrexate is most toxic during S phase. The wide range of antitumor activity of paclitaxel and methotrexate, their different cytotoxic mechanisms, and the absence of cross-resistance provide the rationale for the combination of these agents.

Using the isobologram method of Steel and Peckham,[36] combinations of paclitaxel and methotrexate were evaluated for additive or antagonistic effects in four human cell cancer lines (non-small cell lung cancer A549, breast cancer MCF-7, ovarian cancer PA-1, and colon cancer WiDr).[101] Concurrent administration of methotrexate and paclitaxel was additive at low doses of paclitaxel in A549 and PA-1 cell lines, and antagonistic at higher levels of paclitaxel. For MCF-7 and WiDr both, the concurrent administration was antagonistic.[101] Similarly, Yeh et al. demonstrated antagonistic effects for combination therapy for 3 or 12 days in MDA-MB 435 human breast cancer cells.[102] The cell cycle effects induced by paclitaxel may offer an explanation: given concurrently, cells may be arrested in G2/M, during which they are not sensitive to methotrexate (an S-phase active drug).

This hypothesis would therefore predict increased sensitivity to methotrexate if given several hours following paclitaxel, when cells that do not undergo apoptosis exit from their paclitaxel-induced G2/M arrest and proceed onto G1 and S phases in a synchronized manner. When methotrexate followed a 24 h exposure of paclitaxel, additive and synergistic anti-tumor effects were seen in the four cell lines, A549, MCF-7, PA-1, and WiDr.[101] However, when 6 h paclitaxel exposure preceded treatment with methotrexate for 3 days or 12 days in MDA-MB-435 human breast cancer cells, there was a combined antagonistic effect,[102] consistent with the time-sensitive nature of this particular schedule. When methotrexate preceded paclitaxel in sequential administration in vitro, both studies demonstrated synergy.[101,102] Kano et al. noted that methotrexate exposure for 24 h synchronized most the cells into S phase. This would increase the number of cells that would be sensitive to subsequent exposure to paclitaxel,[101] as cells in S phase are also sensitive to paclitaxel exposure.[103]

This most promising schedule of methotrexate followed by paclitaxel was tested in a Phase I clinical study, and demonstrated no change in paclitaxel pharmacokinetics, but did demonstrate significant neutropenia, complicated by neutropenic fevers despite the addition of granulocyte-colony stimulating factor.[104] Here, we highlight the point that the synergistic effects seen in an in vitro system may not lead to a therapeutic advantage clinically; the drug combination may have an equally synergistic relationship for critical normal tissues, resulting in unacceptable toxicity.

Taxanes and vinca alkaloids

The vinca alkaloids (vincristine, vinblastine, and vinorelbine) represent a group of antimicrotubule agents that induce microtubule disassembly, particularly mictrotubules that compose the mitotic spindle.[105,106] They bind to sites on tubulin that are distinct from the binding sites of the taxanes, and result in the disruption of the microtubule process and a net disassembly of the microtubules. Their cytotoxic effects are most clearly related to their propensity to induce metaphase arrest by altering these microtubule dynamics at the ends of the mitotic spindle. This disruption occurs at the lowest effective drug concentration with minimal effects on microtubule depolymerization or disorganization.[107] The vinca alkaloids also phosphorylate bcl-2 and decrease the

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formation of anti-apoptotic bcl-2/bax heterodimers.[108] Sensitivity to vinca alkaloids and taxanes appear to be closely related to p53-dependent expression of microtubule-associated protein 4 (MAP4).[109] When p53 is transcriptionally inactive, MAP4 expression increases, in turn increasing microtubule polymerization and paclitaxel binding, with resultant increased sensitivity to paclitaxel. Concomitantly, increased microtubule polymerization is also associated with decreased vinca alkaloid binding and decreased sensitivity to vinca alkaloids.[109] Thus, although both taxanes and vinca alkaloids owe their cytotoxicity to microtubules, with maximal cytotoxicity at G2/M, they act by different mechanisms (for a review, see ref. 110). This multifocal attack at the same target may lead to increased anti-tumor efficacy when the drugs are used in combination, and therefore have been tested together for potential advantageous interactions. As paclitaxel and first generation vinca alkaloids have similar spectra of toxicity (including neurotoxicity), their clinical application of this combination has been limited. However, with the advent of vinorelbine, a second-generation vinca alkaloid with reduced potential for neurotoxicity, there has been renewed interest in this combination chemotherapy.

Taxanes and vinca alkaloids are predominantly additive or synergistic when given simultaneously.[111–116] These experiments demonstrated synergy in a wide array of cell lines including human lung cancer, melanoma, breast, colon, and ovarian cancer cells. Synergy was maximal at low concentrations of both agents. In fact, in a murine leukemia model, cures were demonstrated when the two drugs were given less than 6 apart.[113] However, a few experiments demonstrate antagonism with simultaneous exposure.[117,118] These discordant results may be due to concentration related effects on tubulin polymerization.[118] These researchers demonstrated a diminution of paclitaxel-induced tubulin polymerization with increasing doses of vinorelbine in human epidermoid carcinoma KB and breast carcinoma MCF-7 cells. This corresponded to reduced combination cytotoxicity, with antagonism for simultaneous administration of vinorelbine and paclitaxel.[118]

Similar concentration related effects affect sequential exposure of vinorelbine and paclitaxel.[114,116] Carles et al. has demonstrated that sequential exposure (vinorelbine → paclitaxel, and paclitaxel → vinorelbine) with low drug concentrations is synergistic for both sequences in human colon cancer cells, HT-29. However they did demonstrate increased cytotoxicity with the sequence vinorelbine → paclitaxel. For this sequence, as vinorelbine concentrations were increased, synergism turned to additivity, and finally to antagonism. When vinorelbine followed paclitaxel, a similar reduction in synergy was demonstrated with increasing paclitaxel concentrations. These results are likely due to cell cycle arrest of cells in G2/M, as the concentration of the first drug in sequence is increased.[114] If cells are arrested in G2/M by the first drug, they become increasingly less sensitive to the second drug in combination. Kano et al. similarly demonstrated antagonistic effects in MCF-7 breast cancer cells at mitotic arrest doses of paclitaxel when preceded by vinorelbine.[116]

There have been several clinical studies in non-small cell lung cancer and in breast cancer that have examined the simultaneous exposure of paclitaxel and vinorelbine, with varying schedules of delivery, including a 96 h simultaneous continuous infusion of vinorelbine,[119] bolus schedules every other week,[120] every 3 weeks,[121] and 6 weeks on and 2 off.[122] Each schedule has had clinical responses. Several phase II studies of different schedules have been reviewed by Krug et al.[123] There have been two clinical studies examining sequential administration of paclitaxel and vinorelbine.[124,125] Both studies examined the sequence of vinorelbine → paclitaxel. Budman et al. used a schedule of vinorelbine daily for 3 days followed by paclitaxel on day 3 in patients with metastatic breast cancer,[125] and Retsas et al. used vinorelbine on day 1 and paclitaxel on day 2 in patients with disseminated malignant melanoma.[124] Retsas et al. also reversed the sequence (paclitaxel → vinorelbine) in the same population.[124] All combinations demonstrated clinical activity, and pharmacokinetic effects were not reported.

Taxanes and topoisomerase II inhibitors

Anthracyclines

Anthracyclines have been in clinical practice since the 1960s and represent one of the most commonly used classes of anticancer drugs.[126] Anthracyclines induce the formation of covalent topoisomerase II-DNA complexes, thereby preventing the enzyme from completing the religation portion of the ligation-religation reaction. Anthracyclines also insert part of their planar structures between two adjacent base pairs of DNA, causing single-stranded and double-stranded breaks. Doxorubicin, the prototypical anthracycline, is a widely used anticancer agent, with significant activity against breast cancer. As a single agent in breast cancer, doxorubicin has a response rate of 30% to 50% in treated and previously untreated breast cancer patients, respectively. Paclitaxel is also highly active against breast cancer with a 60% response rate in previously untreated patients, and a 50% response rate in doxorubicin-refractory breast cancer patients.[127] For this reason, combinations of taxanes and anthracyclines have been pursued.

There is in vitro synergism when doxorubicin (4 h exposure) precedes paclitaxel (24 h exposure) in human breast cancer cell culture.[39] The sequence doxorubicin (4 h) → paclitaxel (24 h) is additive for the estrogen receptor-positive (ER+) MCF-7 cell line, and is synergistic for the estrogen receptor-negative (ER–) BRC-230 cell line, using a sulforhodamine B (SRB) chemosensitivity survival assay. However, in colon cancer cells (HCT-116), researchers demonstrated an antagonistic relationship when cytostatic doses of doxorubicin exposure preceded paclitaxel,[128] whereas in HCT-116 p21-/-cells, exposure to doxorubicin → paclitaxel was additive. This disparate result is associated with a doxorubicin-induced G2 arrest in wild-type HCT-116 cells, in part due to induction of p21. In the p21-negative HCT-116 cell line, cells continued to M phase with paclitaxel treatment, resulting in a G2/M arrest followed by apoptosis.[128] However, the researchers note that p21 induction alone (by transfection of the p21 expression vector Ad-p21) was only partially protective of paclitaxel effect and was associated with a G1 arrest (not G2).[128]

The reverse sequence of paclitaxel → doxorubicin was antagonistic in both ER+ (MCF-7) and ER-(BRC-230) cell lines.[39] This antagonism appears to be closely related to the

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timing of the anthracycline, when following paclitaxel. In a separate mouse mammary carcinoma xenograft system, efficacy of paclitaxel → epirubicin increased as the interval between the two agents increased.[129] This time-sensitive antagonism may be explained by paclitaxel-induced cell cycle changes. Paclitaxel exposure is associated with a G2/M arrest. Both studies demonstrated that, following paclitaxel exposure, there was a G2/M arrest, followed by tumor regression and by a coordinated portion of cells proceeding to G1 and S phase.[39,129] The researchers hypothesize that, since the anthracycline is more active in S phase (where it has a higher probability to stabilize the topoisomerase II-DNA cleavable complex), the treatment delay following paclitaxel would then allow more exited cells to enter S phase, thereby subjecting them to a greater anti-tumor effect of doxorubicin. Indeed, cells synchronized in S and G2/M phase were 3-fold more sensitive to doxorubicin than cells in G1.[130] The sequence of paclitaxel followed by doxorubicin does appear to be more toxic on hematopoetic stem cells, however.[40] In this system, the effect of each sequence of chemotherapeutic drugs was assayed by quantifying the reduction in response to granulocyte macrophage colony forming units (GM-CSF). Increased cytotoxicity was seen when paclitaxel preceded doxorubicin versus the reverse order (13 ± 2% vs. 26 ± 3% stem cell growth, $P < 0.01$),[40] indicating that this sequence of administration may not only be less cytotoxic in preclinical tumor models, but also more myelotoxic in cell culture.

There also appear to be sequence-dependent pharmacokinetic interactions that are independent of the cell cycle effects with the combination of paclitaxel and anthracyclines. When paclitaxel (24 h infusion) precedes doxorubicin, there is a 32% reduction in doxorubicin clearance.[107,131] This was believed to be the etiology of the increased myelotoxicity and severity of mucositis.[131] When paclitaxel (3 h) infusion) and doxorubicin (15 min infusion) were given over a shorter duration, Gianni et al. reported increased peak levels of doxorubicin and its primary metabolite doxorubicinol [132] This sequence was also associated with a 20% incidence of congestive heart failure, thought to be directly associated with the increased serum levels of the anthracycline.[133] Similarly, in a recent study evaluating the sequence-dependent pharmacodynamics of paclitaxel and epirubicin, patients who received the sequence of paclitaxel followed by epirubicin experienced a significant (37%) increase in epirubicin AUC, and a 25% reduction in total clearance of epirubicin. This was also associated with increased myelotoxicity (26% grade 4 leukopenia vs. 10%).[134] Thus, both the pre-clinical and clinical evaluations of the sequence dependence of taxanes and anthracyclines in combination have demonstrated a significant sequence-dependent effect, such that anthracycline → taxane is less toxic in the clinic and perhaps more efficacious in vitro.

Zoli et al. has added gemcitabine in a sequence-dependent manner[39] to the sequence-specific combination of anthracycline → paclitaxel. Gemcitabine is a new pyrimidine antimetabolite, active against a number of cancers, including lung, pancreas, and lymphoma.[135] Using the most effective combination (4 h doxorubicin → 24 h paclitaxel), Zoli et al. observed antagonism in vitro when gemcitabine immediately followed paclitaxel, or followed paclitaxel by 24 h of drug-free medium. However, when gemcitabine followed paclitaxel by 48 h of drug-free medium, there was marked synergy for both ER + and ER – cell lines. This sequence and schedule dependency can again be explained by paclitaxel-induced cell cycle effects. Forty-eight hours following paclitaxel exposure, the surviving cells are coordinated with regard to their position in the cell cycle. Specifically, shortly following paclitaxel exposure, cells were predominately arrested in G2/M with very few cells in G0–G1 or in S phase. When assayed at 24 and 48 h following doxorubicin → paclitaxel treatment, the cells have exited out of G2/M and entered in a synchronized fashion into S phase. At this time, administration of gemcitabine, which acts by incorporation into DNA (during S phase), allows gemcitabine to induce its maximal cytocidal effect in a cell-cycle coordinated manner.[39] Similar findings were reported for docetaxel and gemcitabine combinations as well.[136] This sequence- and schedule-sensitive combination has not yet been evaluated in a clinical setting. However the pre-clinical work does suggest that a series of chemotherapeutic agents can be given sequentially, according to their cell cycle effects, to maximize efficacy.

Etoposide

Etoposide is a semisynthetic epipodophyllotoxin derived from the plant *Podophyllum peltatum*. It is a spindle-damaging agent causing a phase-specific, schedule-dependent metaphase arrest.[137–139] Its primary cytotoxic effect is to inhibit cells from entering mitosis through the production of DNA strand breaks by inhibiting topoisomerase II.[140] The simultaneous combination of paclitaxel and etoposide is antagonistic,[141] perhaps because concurrent administration could be associated with a mitotic block (by paclitaxel) that would not allow for etoposide's activity in interfering with DNA repair mechanisms. Paclitaxel → etoposide combination chemotherapy was found to produce conflicting results; both synergistic,[141,142] and antagonistic.[40,140] Hahn suggested that cell cycle redistribution and cell delay at G2/M may explain the decreased efficacy[140]; however he used a one hour etoposide exposure duration which may be reversible. Perez demonstrated synergy in two breast cancer cell lines and a single lung cancer cell line with a 24 h exposure of etoposide which causes irreversible cellular damage.[141] Furthermore, the synergy was demonstrated at drug concentrations greater than IC_{90} for all cell lines.[141]

For the reverse sequence (etoposide → paclitaxel), confounding results were again noted. Synergy was found at drug doses higher than IC_{90} in human breast and lung cancer cells,[141] whereas antagonism or less than additive effects were noted in ovarian cancer cells,[140,142] human myeloid cells,[40] and lung and breast cancer cells.[140] For human ovarian cancer cells, the mechanism for antagonism was thought to be decreased cellular retention of paclitaxel when preceded by etoposide.[142] Perez et al. found that the efficacy of the combination improved with increasing the drug free interval between the two drugs.[141] A drug-free period between the two agents could allow for partial cell repair of the cytotoxic activity, followed by a continuation of the cell

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cycle and thereby allowing the cells to be sensitive to the other agent.[141]

Clinical studies with paclitaxel and etoposide have also been somewhat confounding. In the first study evaluating the sequence dependence of paclitaxel and etoposide, a 100 mg fixed dose of oral etoposide was given daily on days 1 to 5 and increasing doses of paclitaxel was given as a 3 h infusion either on day 1 (prior to etoposide) or day 5 (following etoposide).[143] When paclitaxel followed etoposide, the dose-limiting toxicity of myelosuppression was reached with 160 mg/m^2 paclitaxel, whereas the dose-limiting toxicity (non-hematologic toxicity) was reached with 220 mg/m^2 paclitaxel in the reverse sequence. Tumor regression was seen in both arms of the study.[143] The next clinical trials evaluating this sequence comprised two phase II studies; etoposide given as an intravenous infusion on days 1 to 3, and 175 mg/m^2 paclitaxel was given as a 3 h infusion either on day 1 (before etoposide) or day 3 (following etoposide).[144,145] These studies demonstrated increased myelotoxicity when paclitaxel was given on day 1 (70% grade 3–4 neutropenia) vs. day 3 (37% grade 3–4 neutropenia, $P = 0.04$). Despite the increased myelotoxicity, there were no responses demonstrated with this sequence (14/20 patients with stable disease as best response), whereas when paclitaxel followed etoposide, there were two complete responses and seven partial responses (RR 37.5%, 95% CI: 21–58%). Despite the variable in vitro results, there does appear to be clinical sequence-dependent activity with improved anti-tumor efficacy when paclitaxel is given following etoposide.[144,145]

TOPOISOMERASE I INHIBITORS: CAMPTOTHECINS

Having discussed the issues regarding the importance of sequence dependence with taxanes, a class of chemotherapeutic agents that have their peak activity during G2/M, we will now focus on camptothecins, another class of drugs that induce their primary cytotoxicity during the period of DNA synthesis. These alkaloids, derived from the Chinese tree *Camptotheca acuminata*,[146] are potent inhibitors of the nuclear enzyme topoisomerase I,[147] an enzyme that functions primarily in the S-phase of the cell cycle. In fact, cells in S phase are 100 to 1000 times more sensitive to camptothecin than cells in G1 or G2[148] Topoisomerase I induces transient single-stranded breaks of DNA, relieving torsional strain and permitting DNA unwinding ahead of the replication fork during S-phase. Camptothecins stabilize the 'cleavable complex' between topoisomerase I and DNA. When the cleavable complex collides with the moving DNA replication fork, double-stranded DNA breaks occur leading to cell death.[149–151] This apoptotic cell death is mediated by caspase activation, and inhibition of caspase activation shifts cells from apoptosis to transient G1 arrest followed by cell necrosis.[152] Camptothecin treatment is associated with a G2 cell cycle arrest.[153] Apoptosis from short bolus exposure to camptothecin is not associated with changes in bcl-2, bax, p53, or p21 acutely, however prolonged exposure (greater than 72 h) is associated with increased expression of bax.[154] Finally, camptothecin treatment is associated with the transient and unscheduled stimulation of cyclin B1-cdc2 kinase activity prior to apoptosis in HL60 cells.[155] Clinically important members of the camptothecin class include irinotecan and topotecan, and have been approved for clinical use in Japan for small cell lung cancer, non-small cell lung cancer, uterine cancer, ovarian cancer, stomach cancer, colorectal cancer, breast cancer, skin cancer, and non-Hodgkin's lymphoma.[156]

The combination of flavopiridol and irinotecan again demonstrates sequence-dependent anti-tumor activity. In a human colon cancer cell line (HCT-116), maximal apoptosis was demonstrated by PARP cleavage and caspase-3 activation when SN-38 (irinotecan's active metabolite) was followed by flavopiridol. Improved PARP cleavage and caspase-3 activation correlated with DNA laddering and QFM data for apoptosis which revealed that both SN-38 followed by flavopiridol and SN-38 combined simultaneously with flavopiridol induced apoptosis in 44±2% and 30±2% of the treated cells, respectively. SN-38 and flavopiridol as single agents respectively induced apoptosis in only 1±1% and 7±1% of the HCT-116 cells. Exposure to flavopiridol followed by SN-38 did not result in a significant apoptosis (15% ± 2%), whereas exposure to SN-38 followed by flavopiridol resulted in significant apoptosis (44% ± 1%), thereby again demonstrating sequence dependence.[157]

In this system, we examined the role of specific cell cycle checkpoints in relation to the induction of apoptosis and sequence dependence. The p21 gene is transcriptionally activated by p53 and is responsible for the p53-dependent checkpoint that results in G1 arrest following DNA damage. Tumor cells deficient in p21 display a different response to DNA damaging agents than cells with intact p21 checkpoint.[158] HCT-116 cells rendered deficient in p21 (or p53, its inducer) have previously been shown to be highly sensitive to apoptosis induction by γ-irradiation, the DNA damaging drug doxorubicin, the topoisomerase II inhibitor etoposide, as well as cisplatin and nitrogen mustard.[159–162] In our laboratory HCT-116 p21−/− cells are induced to undergo apoptosis with SN-38, alone. HCT-116 p21+/+ cells are resistant to SN-38, however sequence specific flavopiridol exposure decreased p21 expression and resulted in sensitization to SN-38, with induction of caspases and PARP cleavage. How flavopiridol suppresses p21 is currently under active investigation. We are now actively pursuing the combination of irinotecan followed by flavopiridol in a phase I clinical trial.

Camptothecins and taxanes

The rationale for the combination of paclitaxel with camptothecins is based on their broad anti-tumor activity, different cytotoxic mechanisms, and absence of cross-resistance.[163] Kano used the isobologram method of Steel and Pechham[36] to determine the effects of combining irinotecan with paclitaxel on four different human carcinoma cell lines (non-small cell lung cancer, A549, breast, MCF-7, ovarian, PA-1, and colon cancer, WiDr).[164] He found that concurrent administration of paclitaxel and SN-38 (active metabolite of irinotecan) was sub-additive or protective for MCF-7, A549, and WiDr, and uncertain to additive for PA-1. These results are consistent with data from Kaufman, who reported that simultaneous exposure of paclitaxel and topotecan for 7–8 days produced antagonistic effects in human lung cancer A549.[165] However, Chou et al. reported a synergistic effect when 4 day

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exposure of paclitaxel and topotecan was tested against human teratocarcinoma cell lines.[166]

When paclitaxel preceded SN-38, or when SN-38 preceded paclitaxel, the combined effects were primarily additive with no supra-additive or synergistic effects seen.[164] Of note, both paclitaxel and camptothecins induce cell cycle-dependent effects and cause a delay in cell-cycle transit at specific phases. Paclitaxel induces a G2-M arrest, and camptothecin has its major cytotoxic effects on cells in the S phase.[148,167] Thus, for simultaneous administration, one agent may limit the efficacy of the other due to a cell-cycle arrest. However, for sequential administration, the cell cycle arrest may result in cell synchronization, leading to greater drug sensitivity. For example, paclitaxel will induce the majority of cells to arrest in M. When drug is then withdrawn, cells which do not undergo apoptosis exit from M phase in synchrony and move onto S. Since the majority of cells are now in S, subsequent irinotecan exposure should theoretically result in a greater degree of cell kill. Madden et al. demonstrated that exposure of MDAH and MCF-7 breast cancer cells to sub-lethal concentrations of docetaxel or paclitaxel for 90 min prior to exposure to topotecan decreased the IC_{50} for topotecan by 10-fold.[168] Conversely, irinotecan (SN-38) induces accumulation of cells in G2. Thus, pre-treatment of cells with SN-38 should induce a synchronization of cells that results in a coordinated entry of cells into M phase, at which point, they are more susceptible to taxane exposure. However, contrary to Kano et al.'s results, Madden et al. demonstrated to additive effect when the camptothecin preceded the taxane.[168]

There have been several clinical trials combining these two classes of drugs in the past several years, varying both the schedule and sequence of the combination.[169–175] There are two studies that have altered the sequence of administration of taxane and camptothecin.[169,175] The first examined paclitaxel 24 h continuous infusion either on day 1 or day 5, with topotecan administered as a 30 min infusion daily on days 1 to 5. This study found responses in both arms, and no sequence-dependent toxicity or pharmacokinetic effects.[169] The next study examined docetaxel either before or after a 72 h continuous infusion of topotecan. Although responses have been seen, this study has been presented only in abstract form.[175]

Paclitaxel as a 3 h infusion followed by topotecan as a 30 min infusion daily for 5 days has been examined in a phase I[170] and phase II[171] study. The dose-limiting toxicity was neutropenia and although 3 partial responses were seen[170] in the phase I study, only one partial response was seen out of 17 evaluable patients with metastatic breast cancer in the phase II study,[171] thereby limiting the clinical development of this schedule. Couteau et al. administered docetaxel 1 h infusion followed by irinotecan 90 min infusion every 3 weeks, and found no pharmacokinetic interactions between these to agents in this combination.[174]

The reverse sequence (camptothecin → taxane) has been examined as well.[172,173] Adjei et al. examined the combination of irinotecan followed by docetaxel every 3 weeks.[172] They found neutropenia to be the dose-limiting toxicity, with diarrhea as a prevalent toxicity as well, and noted five partial responses out of 16 evaluable patients. They also found a mild significant increase in docetaxel clearance (12.5%) with this sequence of irinotecan followed by docetaxel, but no relationship between docetaxel clearance and dose of irinotecan.[172] The authors note that both irinotecan and docetaxel are hepatically metabolized via the cytochrome P450 3A system, and may explain the altered clearance of docetaxel; although other factors may also play a role, such as differences in liver function, and the use of dexamethasone premedication (a potent inducer of cytochrome P450).[172] Kudoh et al. administered irinotecan over 90 minutes on days 1, 8, and 15 with docetaxel on day 2 in a phase I/II trial in patients with non-small cell lung cancer. They found considerable responses (8/32 partial responses – 32%), however this study too has been presented only in abstract form.[173]

Camptothecin and topoisomerase II inhibitors

Topoisomerase I and II inhibitors demonstrate no cross-resistance in vitro,[176,177] thereby providing a rationale for their combination clinically. Camptothecin and its derivatives are topoisomerase I poisons that do not affect topoisomerase II,[150] and etoposide and doxorubicin are topoisomerase II poisons that do not interact with topoisomerase I.[178] Resistance to both classes of topoisomerase inhibitors has been associated with a decrease in their respective protein levels,[179,180] thus providing additional evidence for the possible improved antitumor efficacy of the combination of topoisomerase I and II inhibitors. Nevertheless, in pre-clinical studies, combined treatment with topoisomerase I and II have yielded mixed results. Additive effects were noted when irinotecan was simultaneously combined with either doxorubicin or etoposide in a human T-cell leukemia cell line.[181] However, in separate experiments, antagonism was demonstrated in a human leukemia cell line,[182,183] and in the HT-29 human colon cancer cell line.[183,184] Bertrand et al. further demonstrated that this antagonism could be overcome by the sequential administration of camptothecin and etoposide separated by 6–8 hours.[184] This antagonism demonstrated by simultaneous exposure was correlated to reduction in nucleic acid synthesis, which is required to convert topoisomerase II-DNA adducts to cytotoxic lesions.[182,184,185] We propose that the reduced nucleic acid synthesis may be a direct result of G1 arrest induced by irinotecan, as has been demonstrated in HCT-116 cells with wild type p21.[157] Importantly, though topoisomerase I is present throughout the cell cycle,[186] topoisomerase II is absent in G0 and G1, only increasing at the onset of S and during G2.[187]

When administered in sequence, the combination has demonstrated additive and synergistic antitumor effects in mouse xenograft experiments.[188,189] Kim et al. demonstrated that doxorubicin cytotoxicity was potentiated significantly by irinotecan pretreatment in two esophogeal, gastric, and a single colon mouse xenograft. Simultaneous exposure abolished sensitivity to irinotecan and doxorubicin in all cell lines [189] Similarly, Eder et al. examined the combination of irinotecan and etoposide or doxorubicin in the EMT-6 mammary carcinoma murine system.[188] They found that simultaneous exposure of irinotecan and etoposide was synergistic, whereas simultaneous exposure with doxorubicin was

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antagonistic. For both combinations, however, there were reduced levels of both topoisomerase I and II RNA and protein. They also examined sequential treatment, giving intraperitoneal injections of the first drug for 5 days, followed by the second agent for the next 5 days. They found that irinotecan followed by etoposide and doxorubicin followed by irinotecan both demonstrated synergy, and the effect on RNA and protein levels of the topoisomerases reflected the last agent in sequence.[188] This data may be reconciled by cell cycle perturbations with each agent, and its consequences. Both irinotecan and doxorubicin cause DNA damage with resultant p21 induction and cell cycle arrest.[157,160,162] Expression of p21 protects cells form the cytotoxic effects of etoposide,[190] perhaps due to decreased nucleic acid synthesis.[182,184,185] Thus, pre-treatment with irinotecan may arrest cells in G2. Upon exiting, cells that are not destined for apoptosis proceed in synchrony through G1 and into S phase, where they are sensitive to etoposide.

Initial clinical combination studies of a camptothecin and topoisomerase II inhibitor used a simultaneous schedule of administration.[191–193] These studies demonstrated that this was a feasible schedule, however responses were not increased beyond that of irinotecan alone.[191] This is consistent with the predominant pre-clinical studies, as described above. Sequential studies of a camptothecin followed by etoposide have only completed phase I trials.[194,195] These studies both demonstrate neutropenia and thrombocytopenia to be the dose-limiting toxicities, and have seen responses. Hammond et al. additionally biopsied patients before and after each drug in sequence, attempting to verify the hypothesis that initial exposure to a topoisomerase I inhibitor was associated with a reduction topoisomerase I and a compensatory increase in topoisomerase II.[194] Contrary to this hypothesis however, they demonstrated that a continuous infusion of topotecan was associated with an increase in topoisomerase I levels. Ando et al. is the only study published that examined both sequential administrations of irinotecan and etoposide.[196] They administered irinotecan on days 1 to 3 followed by etoposide on days 4 to 6, or vice versa. Both arms demonstrated responses, with no differences in toxicity (major dose limiting toxicity of both arms was neutropenia and diarrhea). However, when etoposide preceded irinotecan, there was a significant reduction in irinotecan clearance.[196]

Camptothecin and fluorouracil

Fluorouracil is an anti-metabolite with broad activity in epithelial tumors arising in the breast, head and neck, gastrointestinal, and ovarian cancers, with single-agent response rates ranging from 10 to 30%.[197] Upon cell entry, fluorouracil is converted to floxuridine (FUdR) by thymidine phosphorylase, which is then again phosphorylated by thymidine kinase to its active form, 5-fluoro-2'-deoxyuridine monophosphate (FdUMP).[197] In the presence of a reduced folate cofactor, this active metabolite forms a stable complex with thymidylate synthase thereby limiting its ability to continually synthesize thymidine 5'-monophosphase,[30] thereby inhibiting DNA synthesis. Fluorouracil also is extensively incorporated into both nuclear and cytoplasmic RNA species, interfering with normal RNA processing (reviewed in ref. 197). It is purely an S-phase-active chemotherapeutic agent, with no activity when cells are in G0 or G1. Twenty-four hour exposure to fluorouracil is associated with an accumulation of cells in S phase, as well as a transient induction of p53 and p21.[198] The accumulation of cells in early S phase is associated with expression of cyclin A, and an increase in cyclin A/cdk2 kinase activity.[199]

The rationale for the combination of fluorouracil with camptothecins lies in their different mechanisms of action and different mechanisms of resistance. Several pre-clinical studies have demonstrated a sequence dependence with this combination.[200–203] Guichard et al. examined various combinations of irinotecan and fluorouracil in HT-29 colon cancer cells, using the median effect principle to determine synergy vs. antagonism. They found that the simultaneous combination of fluorouracil and irinotecan was antagonistic at low concentrations, and mildly additive at higher concentrations.

However, they demonstrated synergy for either sequence of fluorouracil followed by irinotecan or prior to irinotecan. This was confirmed in a mouse xenograft model with the same cell line, with a treatment delay of 6 h between the two drugs.[201] They proceeded to demonstrate that fluorouracil, when given first, induces an increase in irinotecan and SN-38 cellular uptake, with a concomitant increase in topoisomerase I-DNA complexes. Furthermore, irinotecan treatment prior to fluorouracil was associated with persistent inhibition of thymidylate synthase, perhaps due to a G2 cell cycle arrest.[153,204] Mullany et al. confirmed that SN-38 sequentially induces diminished DNA synthesis, elevated dTTP pools (which may inhibit thymidylate synthase), but also demonstrated that SN-38 is associated with an S phase delay.[202] The sequential combination of the two agents appears to be sensitive to the schedule of administration. In the same HT-29 cell line, Mans et al. demonstrated antagonism when irinotecan and fluorouracil were combined together or sequentially with fluorouracil for 24 h followed immediately by irinotecan for 24 h. However, irinotecan followed by fluorouracil continued to demonstrate synergy.[203] This may be explained by accumulation of cells in early S phase induced by fluorouracil,[199] making immediate irinotecan therapy ineffective. With a delay in irinotecan therapy, cells may be synchronized in S phase, with perhaps an increased cytotoxicity, as demonstrated by Guichard et al.[201,204] In separate experiments using a chemically induced Ward colorectal carcinoma murine model, irinotecan followed by fluorouracil was the most active regimen, demonstrating significantly higher complete responses than the other combination regimens.[200] Furthermore, this sequence also demonstrated significant potentiation, as synergy was seen with irinotecan and fluorouracil doses at a fraction of the maximum tolerated dose individually. Of note, there was again a 24 h delay between the two chemotherapeutic agents in these experiments.[200]

Though this pre-clinical data is quite compelling, and although there have been several clinical studies examining the combination of irinotecan and fluorouracil, none of these studies have examined the combination of irinotecan and fluorouracil in a sequence-specific manner.[205–210] Irinotecan administered concurrently with fluorouracil has demonstrated increased responses compared with fluorouracil alone [206,210] even conferring a survival advantage

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with the combination therapy.[210,211] No pharmacokinetic interactions have been demonstrated with the simultaneous combination.[206] One study did alternate irinotecan with fluorouracil/leucovorin administered daily for 5 days, however irinotecan was administered on day 1 and fluorouracil/leucovorin on days 22–26 repeated every 6 weeks.[207] This lengthy interval between alternating agents is likely to negate any potentiation by sequential administration of the two agents.

FLUOROURACIL AND THYMIDYLATE SYNTHASE MODULATORS

Raltitrexed (Tomudex®)

Although fluorouracil has multiple intracellular effects, inhibition of thymidylate synthase (TS) is likely the primary mechanism by which fluorouracil and its metabolites are cytotoxic. In fact, TS activity was the best predictor for sensitivity to fluorouracil and fluorouracil/leucovorin in a heterogenous non-selected panel of human colon cancer cell lines.[212] This provides the rationale for the combination of fluorouracil with other TS modulators. Raltitrexed (Tomudex®) is another inhibitor of TS, through polyglutamation within target cells,[213] working through a different mechanism of action and with different binding sites.[214] The polyglutamated formes of raltitrexed are retained withing the cells, thereby accounting for its slow release from tissues and enabling its infrequent every 3 week scheduling.[215] Of note, raltitrexed and leucovorin share the same transporter for cell entry, and compete for polyglutamation, thereby reducing raltitrexed's antitumor activity.[215]

Combination studies have demonstrated that raltitrexed followed by fluorouracil is additive or synergistic, with increased synergy demonstrated with shorter exposures (1 h or 4 h) to fluorouracil versus longer (24 h) exposure.[216,217] This combination demonstrated sequence dependence, as the reverse sequence never demonstrated synergy in six different colon cancer cell lines, demonstrating additivity or antagonism instead;[216,218] additionally, the simultaneous combination of raltitrexed and fluorouracil largely demonstrated additive effects.[218] Longo et al. found further that each drug individually inhibited thymidylate synthase by 70–90%, whereas the combination in sequence (either raltitrexed → fluorouracil or reverse) increased the inhibition of thymidylate synthase, demonstrating that the synergy found when raltitrexed precedes fluorouracil is not due to enhanced blockade of TS.[216] These researchers did show that raltitrexed pretreatment was associated with a 30% increase in intracellular fluorouracil nucleotides, with concomitant increases in fluorouracil incorporation into RNA. This may explain the sequence dependence demonstrated in vitro.[216]

Raltitrexed and fluorouracil have been combined clinically in two phase I studies,[219,220] and reviewed by Schwartz et al.[214] One study administered raltitrexed on day 1, followed by fluorouracil on day 2 as a bolus infusion;[220] the other administered fluorouracil as a 24 h infusion weekly with raltitrexed given just prior to fluorouracil on days 8 and 29.[219] Both studies demonstrated an increase in fluorouracil concentrations with raltitrexed pretreatment, with responses and stabilization of disease seen, even in fluorouracil refractory patients.[214] These studies have not yet entered phase II investigation, but do appear to confirm the synergistic sequence specific relation between raltitrexed and fluorouracil.

UCN-01 (7-hydroxystaurosporine)

UCN-01 (7-hydroxystaurosporine) is a staurosporine analogue isolated from the culture broth of *Streptomyces* species,[221,222] and is a selective inhibitor of protein kinase C.[222] UCN-01 is associated with a G1/S cell cycle arrest,[223–225] associated with the induction of p21CIP/Waf1, dephosphorylation of CDK2,[226] and with the resultant dephosphorylation of the retinoblastoma gene product (pRb).[225,226] Hypophosphorylated pRb remains tightly bound to E2F-1, thereby preventing cell cycle progression into S phase.[227] Both TS and dihydrofolate reductase (DHFR) gene expression increase substantially during G1/S phase boundary of cell cycle.[228,229] Since TS is the target for fluorouracil, it is likely that UCN-01's ability to prevent this part of cell cycle progression is relevant to its interaction with fluorouracil,[230] and could potentially increase antimetabolite chemosensitivity by down-regulating TS and DHFR gene expression.

We tested this hypothesis by examining the effects of UCN-01 on TS mRNA and protein expression levels in SK-GT5 cells, a human gastric adenocarcinoma cell line with a mutated p53 gene.[230] Exposure to UCN-01 resulted in a dose dependent decrease in TS protein expression, with complete suppression at 1 μM UCN-01,[229] a concentration which is clinically achievable.[231] In addition to the suppression of TS protein there was also a dose dependent decrease in TS mRNA. In contrast, a corresponding decrease in E2F-1 protein was seen but no change in E2F-1 mRNA, as determined by Northern blot.[230] This indicates that UCN-01 induces loss of E2F-1 protein, and a consequent lack of transcription of TS mRNA. Since UCN-01 did not significantly affect E2F-1 mRNA levels, the loss of E2F-1 must occur through a post-transcriptional event. In combination with fluorouracil, UCN-01 demonstrated a sequence dependent augmentation of fluorouracil induced cytotoxicity. When UCN-01 preceded fluorouracil, apoptosis as measured by QFM, was observed in 46% ± 1% of the cells. The reverse sequence demonstrated only 17 ± 1% apoptosis.[230] This preclinical data has led to our evaluation of this sequence dependent combination in a phase I clinical trial.

CONCLUSIONS

In this review, we propose a framework based on our current understanding of cellular physiology to better understand the potential importance of sequence of chemotherapeutic drug administration on antitumor efficacy. We have reviewed several classes of drugs, with particular focus on their cell-cycle effects, and have also summarized the pre-clinical and clinical data regarding their combination with other chemotherapeutic drug classes. There are several limitations to the pre-clinical studies that limit the direct translation of that work to the clinical arena. First, the additive effects of a particular drug combination seen in vitro do not always indicate a therapeutic advantage if the combination is synergistic for critical normal tissues in vivo. As an example to highlight

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this point, paclitaxel followed several hours later by methotrexate has demonstrated synergy and significant cytotoxic efficacy in vitro.[101,102] However, when examined in a phase I clinical trial, this sequence-specific combination demonstrated significant neutropenia and myelosuppression that could not be readily overcome by the growth factor support,[104] thereby demonstrating that the cytotoxicity of this combination was not specific for neoplastic cells and limiting its further clinical development. Second, the monolayer cell system cannot reproduce the cellular interactions occurring at a three-dimensional tumor level. Furthermore, these in vitro studies cannot take into account the pharmacokinetic behaviour or tissue distribution of the drugs involved, or their effect on these parameters in combination. In vivo studies are therefore vital to confirm preliminary evidence regarding potential synergistic benefits of particular combination chemotherapy. However, even with these caveats in mind, clinical evaluation will remain essential to the development of a particular drug combination, simply because the use of one or two monoclonal cell lines are not fully representative of the spectrum of malignant cells seen clinically in a single tumor type. The pre-clinical efforts can thus provide an essential guideline to refer to as we develop particular combinations of chemotherapy for clinical use.

In this review, we have demonstrated that the sequence of drug administration can be vitally important in cancer therapy. The underlying reason for the relevance of sequence is the cell cycle, and an understanding of the perturbations of the cell cycle caused by chemotherapeutic agents. As our understanding of cellular physiology grows, we may be able to use sequence-specific combination chemotherapy increasingly to our advantage.

Received 16 August 2000; Accepted 21 August 2000; Published online 9 November 2000

Correspondence to: Gary K. Schwartz, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York NY 10021, USA. Tel: +212 639 8324; Fax: +1212 717 3320; E-mail:schwartg@MSKCC.org

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frontiers in ONCOLOGY

REVIEW ARTICLE
published: 28 February 2014
doi: 10.3389/fonc.2014.00042

Beyond breast and ovarian cancers: PARP inhibitors for BRCA mutation-associated and BRCA-like solid tumors

Ciara C. O'Sullivan[1], Dominic H. Moon[1,2], Elise C. Kohn[1] and Jung-Min Lee[1]*

[1] Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA
[2] Medical Research Scholars Program, National Institutes of Health, Bethesda, MD, USA

Edited by:
Kristin Zorn, Magee-Womens Hospital of UPMC, USA

Reviewed by:
Silvia Soddu, Regina Elena Cancer Institute, Italy
Lin Liu, Nankai University, China

***Correspondence:**
Jung-Min Lee, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, 10 Center Dr. MSC 1906, Building 10, Room 12N/226, Bethesda, MD 20892, USA
e-mail: leej6@mail.nih.gov

Poly(ADP-ribose) polymerase inhibitors (PARPi) have shown clinical activity in patients with germline BRCA1/2 mutation (gBRCAm)-associated breast and ovarian cancers. Accumulating evidence suggests that PARPi may have a wider application in the treatment of cancers defective in DNA damage repair pathways, such as prostate, lung, endometrial, and pancreatic cancers. Several PARPi are currently in phase I/II clinical investigation, as single-agents and/or combination therapy in these solid tumors. Understanding more about the molecular abnormalities involved in BRCA-like phenotype in solid tumors beyond breast and ovarian cancers, exploring novel therapeutic trial strategies and drug combinations, and defining potential predictive biomarkers are critical to expanding the scope of PARPi therapy. This will improve clinical outcome in advanced solid tumors. Here, we briefly review the preclinical data and clinical development of PARPi, and discuss its future development in solid tumors beyond gBRCAm-associated breast and ovarian cancers.

Keywords: poly(ADP-ribose) polymerase inhibitors, solid tumors, BRCA mutation, BRCA-like, DNA damage repair pathway

INTRODUCTION

Increasing understanding of the cellular aberrations inherent to cancer cells has allowed the development of therapies targeting biological pathways. This approach has been an important step toward individualization of therapy for germline BRCA1/2 mutation (gBRCAm)-associated breast and ovarian cancers (1, 2). The clinical development of poly(ADP-ribose) polymerase inhibitors (PARPi), with their selective mechanisms of action involving the DNA damage repair pathways, is an example of this strategy. Early clinical trials have shown significant single-agent activity of PARPi in gBRCAm-associated breast and ovarian cancers (3–5). Response rates (RR) of 31–40% have been reported in gBRCAm ovarian cancer patients with measureable recurrent disease, and the RR and duration of response to PARPi monotherapy has been associated with platinum sensitivity (6, 7). Emerging evidence suggests that PARPi is an effective therapeutic strategy in subsets of other malignancies that have gBRCAm, such as melanoma, prostate, and pancreatic cancers. BRCA-like tumors have molecular and clinical characteristics in common with tumors occurring in patients with gBRCAm, which may have implications for PARPi-based therapy (8). Additionally, there is a potential therapeutic role for PARP inhibition in a wider subgroup of solid tumors that may have defective homologous recombination (HR) (9). Therefore, the utility of PARPi in other solid tumors is potentially greater than was previously envisioned (8).

PARPi have shown to enhance cytotoxicity in combination with DNA methylating agents (10, 11), topoisomerase inhibitors (12, 13), platinums (14, 15), alkylating agents (14), and radiation (16, 17) in numerous preclinical studies. These preclinical findings are being explored in clinical trials to elucidate the role of PARPi as chemo- and radiosensitizers in various tumor types (18). A large number of clinical trials are exploring the efficacy of combination strategies in malignancies such as non-small cell lung cancer (NSCLC), squamous cell cancer of the head and neck (HNSCC), esophageal, and colorectal cancers (CRCs) (**Tables 1** and **2**); the results of several phase I and II trials have already been reported (**Table 3**). These data suggest further clinical exploration of PARPi as monotherapy or combinations is warranted in patients not only with gBRCAm-associated breast or ovarian cancer, but also in solid tumors with HR dysfunction.

gBRCAm-associated and BRCA-like tumors are rare subsets of advanced solid tumors. Approximately 5–10% of breast (27) and 10–15% of ovarian cancers (28) occur in the setting of a hereditary cancer syndrome, the most common of which is a gBRCAm (29). This occurs less frequently in other solid tumors. Approximately 5% of cutaneous melanoma and gastric cancers are related to gBRCAm and 5–19% cases of familial pancreatic cancer are attributed to a gBRCAm (30, 31). Furthermore, gBRCAm are very rare events in patients with prostate cancer and NSCLC. gBRCAm are present in 0.44–1.2% of prostate cancer cases (32, 33). The overall incidence of gBRCAm in patients with NSCLC has not been reported from large trials; only 3 patients (2.7%) were noted to have a gBRCAm in a study of 110 Jewish men with epithelial growth factor receptor (EGFR) mutant-NSCLC (34). These subgroups of tumors with germline HR dysfunction constitute a rare population with recognized unmet therapeutic needs, and may be sensitive to treatment with PARPi. Additionally, there are significant unanswered questions of their use in solid tumors that have molecular and clinical characteristics in common with gBRCAm-associated tumors. Advances have been made in identifying new therapeutic targets and analyzing response to novel treatments

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Table 1 | PARPi in clinical development (excluding breast and ovarian cancer) (19).

Name	Treatment	Cancer types	Phase
Olaparib (AstraZeneca)	Monotherapy Combination with chemotherapy Combination with RT Combination with targeted therapies	GBM, prostate, ES, NSCLC, CRC, and gastric cancer Esophageal cancer and HNSCC	I/II
Rucaparib (Clovis)	Combination with chemotherapy	AST	I
Veliparib (Abbott)	Monotherapy Combination with chemotherapy Combination with RT Combination with targeted therapies	gBRCAm prostate cancer, HNSCC, NSCLC, SCLC, pancreatic cancer, biliary cancers, HCC, rectal cancer, cervical cancer, CRPC, and CNS malignancies	I/II
CEP-9722 (Cephalon)	Monotherapy Combination with chemotherapy	AST	I
E7016 (EISAI)	Combination with chemotherapy	Melanoma and AST	I/II
BMN-673 (BioMarin)	Monotherapy	AST	I

GBM, glioblastoma multiforme; ES, Ewing's sarcoma; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; CRC, colorectal cancer; AST, advanced solid tumors; HNSCC, head and neck squamous cell cancer; CRPC, castrate resistant prostate cancer; HCC, hepatocellular cancer; CNS, central nervous system; n/a, not applicable.

in these patient subgroups and this has led to an explosion of PARPi-based clinical trials extending the patient cohort to include BRCA-like tumors.

PARP FUNCTION AND INHIBITION IN DNA DAMAGE REPAIR PATHWAYS

DNA damage can occur through various mechanisms from environmental factors such as ultraviolet rays, ionizing radiation, and genotoxic chemicals, to endogenous processes including generation of reactive oxygen species and replication (35). Highly complex and intertwined repair pathways have evolved to provide broad and redundant mechanisms to address damaged DNA: mismatch repair (MMR), base excision repair (BER), and nucleotide excision repair (NER) for a low fidelity single strand DNA break (SSB) repair mechanism, and HR and non-homologous end-joining (NHEJ) for double-strand DNA breaks (DSBs) (36). The different repair mechanisms are orchestrated by numerous enzymes to ensure the integrity of DNA essential for cell survival.

PARP are a family of enzymes that catalyze nicotinamide adenine dinucleotide (NAD^+)-dependent ADP-ribosylation of DNA. PARP1 is the best characterized member of the PARP family, and PARP2 has a similar structure and function with varying affinity for substrates (37). PARP1 has been implicated in several DNA repair mechanisms including the repair of SSBs through the BER pathway. It recognizes and binds to DNA sites with SSB via its DNA binding domain, then subsequently synthesizes poly(ADP-ribose) (PAR) by transferring ADP-ribose molecules from NAD^+ to itself and other acceptor proteins (38). This activates the formation of a DNA repair complex consisting of multiple repair proteins, including DNA ligase III and X-ray repair cross-complementing 1 (XRCC1) (39). The PARylated PARP1 dissociates from DNA as the negative charge of PAR decreases its affinity for DNA, and poly(ADP-ribose) glycohydrolase then degrades the PAR on PARP1 (40). PARP has been shown to have a direct involvement in DSB repair in addition to its role in preventing DSB formation by promoting BER. In PARP1-deficient cells, ATM-kinase function is compromised leading to a reduction in DNA DSB in response to radiation, indicating a role of PARP1 in ATM activation and HR (38, 41). PARP1 has been shown to reduce DSB formation by sensing stalled replication forks and recruiting MRE11 for end processing to initiate HR (42). Increased PARP1 expression and/or activity in tumor cells have been demonstrated in many tumor types (43, 44). Thus, HR dysfunction sensitizes cells to PARP inhibition leading to further chromosomal instability, cell cycle arrest, and apoptosis (45, 46).

PARPi are a class of drugs designed to compete with NAD^+ for the substrate binding site of PARP, acting as an effective catalytic inhibitor (47). PARP inhibition has been shown to induce phosphorylation of DNA-dependent protein kinase (DNA-Pk), to further stimulate error-prone NHEJ in HR-deficient cells (44, 48, 49). More recently, another mechanism of action of PARPi involving PARP1-trapping has been proposed (50). PARPi have been shown to trap PARP1 and PARP2 while in complex with damaged DNA, resulting in cytotoxic consequences (51). Trapped PARP prevents its availability for repair function and secondarily causes replication and transcription fork blockade, and subsequent DNA breakage. This mechanism of action may be important to the clinical activity of the PARPi class. The potency in trapping PARP differs markedly among PARPi, with niraparib (MK-4827) and olaparib having greater potency than veliparib. This pattern is not correlated with the catalytic inhibitory properties of each drug. These findings suggest that PARPi have several mechanisms of action and multiple targets in the DNA repair pathway to potentially induce cancer cell death (**Figure 1**).

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Table 2 | Trials of PARPi in solid tumors (excluding breast and ovarian cancers).

Malignancy	PARPi	Combination agent(s)	Phase
GI			
Pancreatic	Olaparib Veliparib	Chemotherapy Cisplatin Gemcitabine Gemcitabine/IMRT Monotherapy (gBRCAm pancreatic cancer) Modified FOLFOX 6	I/II
Pancreatic, biliary, urothelial and NSCLC	Veliparib	Cisplatin and gemcitabine	I
Liver	Veliparib	Cisplatin and gemcitabine	I
Colorectal cancer	Veliparib Olaparib Veliparib	TMZ Irinotecan Capecitabine and RT	I/II
Colorectal cancer stratified by MSI	Olaparib	N/A	I/II
Esophageal cancer	Olaparib	RT	I
Gastric cancer	Veliparib Olaparib	FOLFIRI Paclitaxel	I/II
LUNG			
NSCLC (surgically unresectable)	Olaparib Veliparib	Concurrent RT ± cisplatin RT Carboplatin/paclitaxel Cisplatin/gemcitabine	I/II
EGFR mutation positive advanced NSCLC	Olaparib	Gefitinib ± olaparib	I/II
SCLC	Veliparib	Cisplatin/etoposide TMZ	I/II
GENITOURINARY			
CRPC	Veliparib	Abiraterone and prednisone TMZ	I/II
	Olaparib	N/A	II
GYNECOLOGIC			
Cervical cancer	Veliparib	Cisplatin and paclitaxel Topotecan Carboplatin and paclitaxel	I/II
Uterine carcinosarcoma	Veliparib	Carboplatin and paclitaxel	II
CENTRAL NERVOUS SYSTEM			
GBM	Olaparib Veliparib	TMZ TMZ	I I/II
Brain metastases	Veliparib	WBRT	I/II
DPG	Veliparib	RT TMZ	I/II
Refractory CNS tumors	Veliparib	TMZ	I
HEAD AND NECK			
HNSCC	Veliparib	RT Docetaxel 5-FU	I/II

(Continued)

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Table 2 | Continued

Malignancy	PARPi	Combination agent(s)	Phase
SARCOMA			
Ewing's sarcoma	Olaparib	N/A	II
SKIN CANCER			
Melanoma	Veliparib	TMZ	II
	E7016	TMZ	
ADVANCED SOLID TUMORS			
	Veliparib	Carboplatin and gemcitabine	I/II
		Gemcitabine	
		Carboplatin and paclitaxel	
		Mitomycin C	
		Capecitabine and oxaliplatin	
		Cyclophosphamide	
	Olaparib	Cisplatin/gemcitabine	
		PLD	
		Topotecan	
	Niraparib	Monotherapy	
	CEP-9722	Monotherapy	
	BMN-673	Monotherapy	

IMRT, intensity modulated radiotherapy; NSCLC, non-small cell lung cancer; RT, radiotherapy; MSI, microsatellite instability; CRPC, castrate resistant prostate cancer; SCLC, small cell lung cancer; GBM, glioblastoma multiforme; DPG, diffuse pontine glioma; HNSCC, squamous cell carcinoma of the head and neck; 5-FU, 5-fluorouracil; PLD, pegylated liposomal doxorubicin.

PARP INHIBITION IN gBRCAm AND BRCA-LIKE SOLID TUMORS

Understanding DNA repair biology has allowed the identification of patient subsets with high potential for response to PARPi treatment. The marked susceptibility of patients with gBRCAm has validated gBRCAm as a predictive biomarker for PARPi response in breast and ovarian cancer patients. In a series of pivotal preclinical studies, PARPi were noted to cause selective cytotoxicity for *in vitro* and *in vivo* models of BRCA-deficient cells (52, 53). Additionally, PARPi attenuates tumor formation in embryonic stem cell-derived teratocarcinoma xenograft models (46). These findings were translated into a phase I clinical trial of the PARPi, olaparib, in recurrent breast, ovarian, and prostate cancer patients with gBRCAm (4), initiating a new era of possibilities for the use of PARPi as single-agent therapy to treat gBRCAm-associated cancers.

The BRCA-like behavior has been described based on clinical and molecular features that parallel gBRCAm-associated cancers' characteristics. The major clinical BRCA-like behavior identified is susceptibility to platinums and other DNA-damaging agents (54–56). Some of the molecular events described in BRCA-like behavior include epigenetic silencing of BRCA1 through promoter methylation (57–59) and overexpression of EMSY, suppressing BRCA2 transcription (60). In addition, loss or disruption of proteins necessary for HR such as RAD51, ATM, ATR, CHK1, CHK2, FANCD2, and FANCA (53, 61–64) are observed in a variety of tumors (8, 65–71), and may confer sensitivity to PARPi (8, 53). Defects in translesion synthesis (TLS) also contribute to carcinogenesis but confer sensitivity to DNA-damaging agents (72, 73), requiring further investigation on sensitivity to PARPi. Homozygous mutation in the PTEN tumor suppressor gene may also lead to HR dysfunction (74). Increased PARPi sensitivity was shown in a series of cell lines with PTEN mutation or haploinsufficiency, and confirmed in xenograft models using olaparib (74). There is also clinical evidence that olaparib may have a therapeutic utility in PTEN-deficient endometrial cancer (75, 76). Further studies are needed to investigate whether PTEN loss can serve as a potential biomarker for PARPi sensitivity (77–79). Future studies should focus on DNA profiling and the use of predictive biomarkers to select those tumors which are more likely to respond to PARPi. Ongoing research suggests HR deficiency, rather than a specific mutation in the BRCA genes, may be the main driver of cytotoxicity of PARP inhibition (45).

TRIALS WITH PARPi IN gBRCAm AND/OR BRCA-LIKE ADVANCED SOLID TUMORS

MALIGNANT MELANOMA

Little is known about the underlying cause of hereditary cancer predisposition in melanoma and its impact on the prognosis and therapeutic decisions. Cutaneous melanoma has been associated with mutations in the BRCA2 gene although there are only a few cases reported for uveal melanoma in BRCA2 mutation carriers (80). In recent years, the advent of BRAF V600E inhibitors (e.g., vemurafenib) and anti-CTLA4 antibodies (e.g., ipilimumab) has significantly improved outcomes in patients with metastatic melanoma (81–83), with a median duration of response of 8 and 16 months, respectively (84, 85). However, most patients eventually progress and some do not tolerate therapy due to immune-related side effects, indicating the need to develop other therapeutic strategies.

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Table 3 | PARPi trials for which tumor response rates have been reported.

PARPi	Patient cohort	Combination	Drug and schedule	Toxicity	Response
Rucaparib (20) (phase I)	AST Melanoma (32 pts)	TMZ	D1: rucaparib 12 mg/m^2 IV D1–5: TMZ 200 mg/m^2 PO q 28 day cycle	No DLT Myelosuppression (13%) At MTD	CR: 1/32 pts (melanoma) PR: 2/32 pts (1 melanoma; 1 desmoid tumor) SD: 7/32 pts-6 mo or greater
Olaparib (19) (phase I)	Melanoma (40 pts)	Dacarbazine	D1–7: olaparib (20–200 mg) PO BID D1: (cycle 2 day 2): dacarbazine (600–800 mg/m^2 IV) q 21 day cycle MTD: 100 mg olaparib PO BID and decarbazine 600 mg/m^2 IV	Grade 3 hypophosphatemia-1 pt Grade 3 neutropenia-1 pt Grade 4 neutropenia-2 pt	CR: 0/40 pts PR: 2/40 pts SD: 8/40 pts
Olaparib (21) (phase I)	AST (19 pts)	Topotecan	D1–3: topotecan 0.5–1.0 mg/m^2 IV Olaparib (50–200 mg PO BID) q 21 day cycle	DLTs 16% Grade 3 thrombocytopenia-1 pt Grade 4 neutropenia-2 pts Treatment related death-1 pt (pneumonia)	CR: 0/19 pts PR: 1/19 pts SD:4/19 pts RECIST RR = 37%
Olaparib (22) (phase I)	AST (12 pts)	N/A	D1–28: olaparib (100–400 mg PO BID)	No DLTs Grade 3 toxicity in 16% Anemia-8% Elevated AST-8%	CR: 0/12 PR: 1/12 pts-13 mo SD: 4/12 > 8 weeks (unknown gBRCAm status)
Veliparib (23) (phase I)	AST (35 pts)	MCP	D1–21: cyclophosphamide 50 mg daily PO Olaparib (20 mg daily × 7 days >80 mg daily q 21 days cycle) MTD: veliparib 60 mg daily and cyclophosphamide 50 mg once daily	DLTs-6% Grade 3 ileus-1 pt Grade 4 respiratory Failure and death-1 pt Lymphopenia-84.3%	CR: 0/35 pts PR: 7/35 pts (gBRCAm) SD: 6/35 (3 BRCA+)
INO-1001 (phase Ib)	Melanoma (12 pts)	TMZ	D1–5: TMZ 200 mg/m^2 IV daily and INO-1001 (100–400 mg IV q 12 h) × 10 doses, q 28 day cycle MTD: INO-1001 = 400 mg	Anamia-17% Grade 4 hepatotoxicity-8% Grade 4 hematologic toxicity-58% Grade 3 myelosuppression	CR:0/12 pts PR:1/12 pts SD:4/12 pts RR = 4.2% CBR = 41.6%
Rucaparib (24) (phase II)	Melanoma (40 pts)	TMZ	D1–5: TMZ 200 mg/m^2 and rucaparib 12 mg/m^2 IV, q 28 day cycle	Grade 4 thrombocytopenia-12%	10% PR (4/40 pts) SD-4/40 pts
Veliparib (25) (phase I/II)	Pancreatic cancer (18/28 pts evaluable at time of reporting)	Modified FOLFOX6	Phase 1 dose-escalation: veliparib 40–100 mg BID D1–7, q 14 day cycle Phase II-two parallel groups; first line and untreated	Grade 3 neutropenia-1 pt Grade 5 neutropenia-1 pt Grade 3 lymphopenia-1 pt Grade 3 anemia-1 pt	11 pts (First line) RR-18% PFS-3.9 mo OS-7.4 mo

(Continued)

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Table 3 | Continued

PARPi	Patient cohort	Combination	Drug and schedule	Toxicity	Response
					Seven patients (pre-treated) RR-14% PFS-1.8 mo OS-5.4 mo CR-1/18 pts PR-1/18 pts
Veliparib (26) (phase II)	Colorectal cancer (47 pts)	TMZ	D1–5: TMZ 150 mg/m^2 PO daily D1–7: veliparib 40 mg BID q 28-day cycle	Grade 3 A/E in 5 pts (myelosuppression)	ORR (CR + PR)-5% 2/47 pts-PR, 0/47 pts = CR CR + PR + SD = 23% Median TTP = 11 weeks; 23 weeks for pts with controlled disease

DLT, dose limiting toxicity; MTD, maximum tolerated dose; CR, complete response; PR, partial response; SD, stable disease; IV, intravenously; PO, orally; D, days; mo, months; q, every; BID, twice a day; RECIST, response evaluation criteria in solid tumors; TMZ, temozolomide; AST, aspartate transaminase; pt(s), patient(s); MCP, metronomic cyclophosphamide; PFS, progression-free survival; OS, overall survival; ORR, overall response rate; TTP, time to progression.

PARPi have multiple targets in DNA repair pathways that can potentially promote cancer cell death. In the setting of melanoma, altered expression or new mutations in DNA MMR genes, MLH1 and MSH2, have been reported in brain metastases (86). A melanoma cell line (MZ7), derived from a patient who received dacarbazine therapy, exhibited a high level of resistance to temozolomide (TMZ) without expressing O(6)-methylguanine-DNA methyltransferase (MGMT), which was related to impaired expression of MSH2 and MSH6 (87). PARP inhibition with INO-1001 has been shown to restore sensitivity to TMZ in an MMR-deficient xenograft model of malignant melanoma (88), and another PARPi, GPI 15427, enhanced TMZ anti-tumor activity in various cancers, including metastatic melanoma in an orthotopic xenograft mouse model (24). These preclinical studies provide evidence that MMR loss of function is a potential predictive biomarker of PARPi responsiveness in patients with metastatic melanoma.

A number of clinical trials of PARPi in melanoma patients have been conducted or are ongoing although they have not specifically addressed the frequency of HR dysfunction/gBRCAm in their populations. Bedikian et al. reported the results of a phase IB study of intravenous INO-1001 and oral TMZ in unselected patients with unresectable stage III or IV melanoma (89). The dose limiting toxicities (DLTs) were elevation of liver transaminases and myelosuppression at the 400-mg dose of INO-1001. Of the 12 patients enrolled, 1 patient had a partial response (PR) and 4 patients had stable disease (SD). Several phase II studies using PARPi either as a single-agent or in combination with chemotherapy, radiotherapy, or targeted therapy are summarized in **Table 3**. A phase II trial sought to evaluate the combination of rucaparib and TMZ in patients with metastatic malignant melanoma (90). The disease-control rate was 40% (8/20), where four patients attained a PR and four others had prolonged SD. In total, 12 of the 40 patients required a dose reduction of TMZ secondary to myelosuppression (90). Another phase II study evaluated treatment with rucaparib 12 mg/m^2 and TMZ 200 mg/m^2 in patients with advanced melanoma. Myelosuppression was again noted, with 25 patients (54%) requiring a 25% dose reduction in TMZ. The RR was 17.4%, with median time to progression and OS of 3.5 and 9.9 months, respectively. This study demonstrated that TMZ could safely be given with a PARP-inhibitory dose (PID) of rucaparib (12 mg/m^2), based on 74–97% inhibition in PARP of peripheral blood mononuclear cells (PBMCs). This resulted in an increase in PFS compared with historical controls (91). Phase I and II trials evaluating E7016 in combination with TMZ in patients with advanced solid tumors and malignant melanoma are ongoing (92, 93). Eligibility criteria for the phase II study include BRAF wild-type status and no prior treatment with TMZ or PARPi. As substantial progress has been made in the management of malignant melanoma in recent years (94), it remains to be seen whether PARPi will be added to the treatment armamentarium.

PANCREATIC CANCER

Hereditary pancreatic cancer is rare and extremely heterogeneous, and it accounts for approximately 2% of all pancreatic cancer cases. The major component of hereditary pancreatic cancer is the familial pancreatic cancer syndrome. Although up to 20% of

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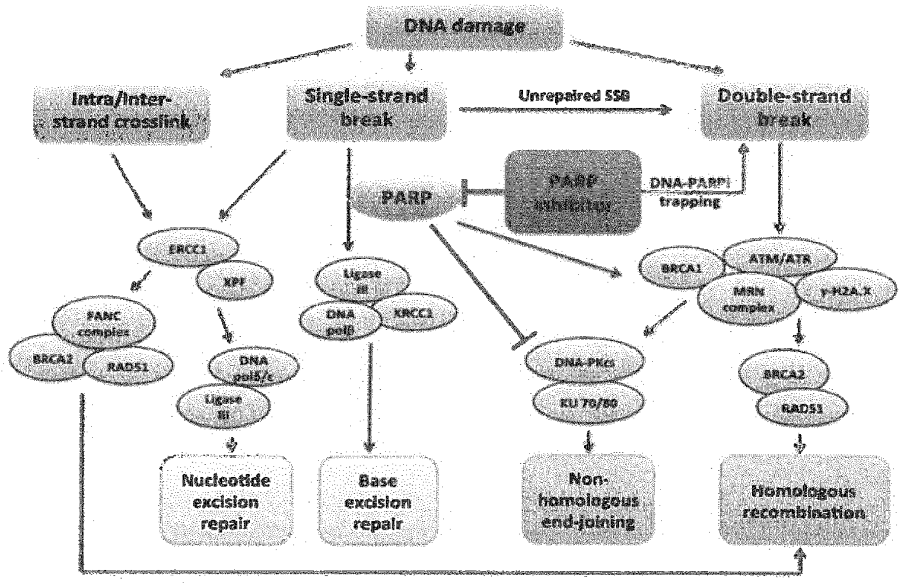


FIGURE 1 | PARP1 binds to DNA single strand break and catalyzes poly(ADP-ribosyl)ation of itself and acceptor proteins, which facilitates recruitment of DNA repair proteins. In addition to its reported role in base excision repair, PARP1 plays a role in activating ATM necessary for homologous recombination and inactivating DNA-dependent protein kinase, a key component of non-homologous end-joining. PARP inhibitors directly interfere with the above functions of PARP1. In addition, PARP inhibitors have been shown to trap PARP1 on damaged DNA, leading to replication and transcription fork blockage and subsequent double-strand DNA breakage. Repair of intra/interstrand crosslinks through nucleotide excision repair or homologous recombination are also important components of the DNA repair system, and whether defects in these repair pathways can confer sensitivity to PARPi are under investigation. PARP, poly(ADP-ribose) polymerase; PARPi, PARP inhibitor; DNA polβ/δ/ε, DNA polymerase beta/delta/epsilon; XRCC1, X-ray repair cross-complementing protein 1; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; KU 70/80, a.k.a XRCC6/5 (X-ray repair cross-complementing protein 6/5); ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; γ-H2A.X, gamma-histone H2A member X; RAD51, RAD51 homolog (*S. cerevisiae*); ERCC1, DNA excision repair protein ERCC1; XPF, DNA repair endonclease XPF (xeroderma pigmentosum group F-complementing protein); FANC, Fanconi anemia.

hereditary pancreatic cancer cases are associated with germline mutations in BRCA2, CDKN2A, PRSS1, STK11, or MMR genes, the major underlying gene defects are still unknown (95). BRCA2 mutation prevalence in familial pancreatic cancer patients varies between 5 and 19% (30), and a BRCA2 mutation increases the risk of developing pancreatic cancer by approximately 3.5-fold (96). The unique biology of cancer cells with BRCA mutations offers potential therapeutic advantages with agents such as platinums. However, one case series report patients with gBRCAm did not reveal a benefit to first line platinum chemotherapy in the treatment of advanced pancreatic cancer (97), although this needs to be further evaluated in a selected study for pancreatic cancer with gBRCAm. Preclinical studies have shown single-agent activity of PARPi (98), as well as radiosensitization in combination with chemoradiation in BRCA2-deficient pancreatic cells (25). Studies are ongoing to examine single-agent and combination PARPi therapy in BRCA2 mutant pancreatic cancers.

Interim results from an ongoing phase II study of olaparib monotherapy in gBRCAm-associated advanced solid cancers were recently reported (99). Nearly 8% of the patients (23/298) had advanced/recurrent pancreatic cancer. A RR of 5/23 (21.7%) was noted, with eight patients achieving SD. This yielded a clinical benefit rate of 57% in gBRCAm-associated pancreatic cancer patients. Pishvaian et al. reported a phase I study of veliparib with concurrent FOLFOX chemotherapy in patients with metastatic pancreatic cancer (100). Twenty-eight patients were enrolled in the trial and at the time of review, data were available for 18 patients. For the 11 patients who were treated in the first line setting, RR was 18%, with a PFS and OS of 3.9 and 7.4 months, respectively (**Table 3**). Therefore, the investigators concluded that the experimental combination regimen could be given safely, and was modestly active (100). These data support further evaluation of PARPi either as different combinations or more potent PARPi with chemotherapy and/or other targeted agents combination in this subgroup of pancreatic cancer patients.

PROSTATE CANCER

Germline BRCA2 mutation confers the highest genetic risk of prostate cancer known to date at 8.6-fold in men ≤65 years, whereas the effect of BRCA1 is more modest at 3.4-fold (32, 33, 101, 102). Prostate cancer in patients with gBRCAm tends to be more aggressive, with a higher likelihood of nodal involvement and distant metastasis with inferior survival outcomes (103). Trials analyzing the response of these patients to DNA-damaging agents, such as platinums, and identifying the therapeutic targets of this subgroup are urgently needed.

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Single-agent olaparib has demonstrated activity in patients with gBRCAm castration resistant prostate cancer (CRPC). A phase I olaparib study by Fong et al. reports one gBRCA2m patient treated with single-agent olaparib who sustained a CR lasting in excess of 2 years (4). Recently, Sandhu et al. presented clinical data on four patients with advanced gBRCAm CRPC, three of whom were treated with olaparib and one with niraparib (104). Two patients on olaparib showed prostate-specific antigen (PSA) and radiologic responses lasting 26 and 34 months, respectively, while the third patient had SD for 10 months. The patient on niraparib exhibited primary resistance with development of a new liver lesion and a rise in PSA of nearly threefold at the time of the first reassessment. Translational studies revealed positive ERG staining by immunohistochemistry, and ERG rearrangements by FISH, as well as either heterozygous or homozygous PTEN allelic loss in all four cases. Subsets of patients with CRPC are also known to manifest increased PARP activity (105). This potentially opens another avenue for therapy utilizing PARPi, although gBRCAm is a very rare event in prostate cancer.

Gene fusion between the ERG proto-oncogene and TMPRSS2 promoter is a major genomic alteration observed in approximately 50% of prostate cancers. Formation of the TMPRSS2-ERG fusion gene causes aberrant androgen-dependent ERG expression (106) and promotes tumorigenesis (107). Preclinical studies have shown that PARP1 directly interacts with ERG to inhibit ETS gene fusion protein activity. In turn, inhibition of PARP1 reduces ETS-positive, but not ETS-negative, prostate cancer xenograft growth (108). This may be a useful predictive biomarker for PARPi sensitivity.

Other preclinical studies include radiosensitization by rucaparib, most evident in PTEN-deficient prostate cancer cells containing the TMPRSS2-ERG fusion gene (109). However, no association was noted between loss of PTEN expression by immunohistochemistry and ETS rearrangements by FISH, with radiologic assessment of the anti-tumor activity of niraparib in 18 patients with prostate cancer (110). The HR/PARP synthetic lethality model may be more widely applicable in prostate cancer with germline or somatic inactivating mutations in the HR DNA repair genes, CHK2, BRIPI/FANCJ, NBS1, BRCA1, and ATM, collectively thought to occur in 20–25% of prostate cancer cases. Recently, a phase II study of olaparib in unselected patients with CRPC was initiated (111).

Veliparib has also been investigated and shown to enhance the anti-tumor activity of TMZ in prostate cancer xenografts, yielding tumor size reduction in TMZ-resistant PC3-Leu prostate cancer mice (112). This formed the rationale for testing the efficacy and safety of veliparib and TMZ in 26 patients with metastatic CRPC (113). Grade III/IV thrombocytopenia was noted in 15% of patients. Two patients had a confirmed PSA response and four patients had SD for at least 4 months. The median PFS and OS were 2.1 (95% CI: 1.8, 3.9) and 9.1 (95% CI: 5.5, 11.7) months, respectively. This study suggested veliparib and TMZ are tolerated well, but with limited clinical activity. Future trials will explore the use of different chemotherapy agents in combination with higher doses of veliparib. Overall, further evaluation of biochemical changes or predictive biomarkers in response to PARPi in advanced prostate cancer is needed.

COLON CANCER

Preclinical data suggest the utility of PARPi in tumors deficient in HR and displaying microsatellite instability (MSI) due to mutations in the coding microsatellites of the MRE11A and hRAD50 genes involved in DNA DSB repair (114). Preferential cytotoxicity to the PARP1 inhibitor ABT-888 was seen in MSI cell lines containing mutant copies of MRE11A, compared with wild-type or microsatellite stable (MSS) cells (115). In a recent study, the observed ability of MSH3 to protect against DSB was exploited by the combination of oxaliplatin and a PARPi, which produced a synergistic cytotoxic effect against CRC cells (116). Another study reporting high correlation between MRE11 mutations and MSI in CRC cell lines as well as primary tumors, found that PARPi preferentially kills MSI cell lines harboring MRE11 mutations (115). The data suggest a role for PARPi in MSI-CRC treatment, providing a rationale for clinical studies in this subset of patients.

Dozens of potential PARPi have been screened *in vitro* and *in vivo* to select candidates for clinical evaluation as a chemosensitizer in CRC (117). A phase II trial is currently evaluating the efficacy of olaparib in metastatic CRC (mCRC) stratified for MSI status (118). Twenty-two patients with MSI-negative tumors were enrolled and received a mean number of two cycles. Preliminary data indicate no single-agent activity of olaparib against non-MSI-high (MSI-H) mCRC. Accrual of MSI-H mCRC patients continues, along with active biomarker analysis. Other clinical trials of PARPi in MSI-CRC are in progress.

Studies have evaluated and validated veliparib as a sensitizer to irinotecan, oxaliplatin, and radiation therapy (RT) in CRC cells (26, 119). Several phase II studies are evaluating the role of PARPi as a chemosensitizer in patients with advanced and mCRC, irrespective of MSI status (**Table 2**). Pishvaian et al. (120) conducted a single arm, open label phase II study in patients with unresectable or mCRC. Patients were treated with TMZ (150 mg/m^2 orally daily) days 1–5, and veliparib (40 mg orally twice a day) days 1–7 of each 28-day cycle. Immunohistochemistry was performed on archived tumor samples to quantify MMR and PTEN protein expression. The combination of veliparib and TMZ was well tolerated in the 47 patients treated, with a disease-control rate of 23%. The results of immunohistochemistry for the MMR and PTEN proteins from 45 archived tumor samples are not yet reported. It was concluded that, in a heavily pre-treated population of patients with mCRC, the combination of veliparib and TMZ can be safely given, and displayed limited clinical activity.

LUNG CANCER

Reduced BRCA1 mRNA and protein expression levels have been observed in up to 44% of NSCLC, occurring through various mechanisms such as promoter hypermethylation (121). One study showed that BRCA1 silencing increased susceptibility to olaparib treatment in NSCLC cell lines (122), providing evidence for possible clinical application in this subset of NSCLCs. A future study will assess the utility of olaparib in delaying the time to disease progression in patients with advanced NSCLC who have responded to initial chemotherapy (123). The role of PTEN mutation and its effect on the susceptibility to PARPi is an area of continued research in lung and other malignancies. Up to 9% of NSCLCs

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have a somatic mutation in PTEN. Olaparib has yielded additive activity with cisplatin in homozygous deleted PTEN-deficient NSCLC cells and xenograft models (79). Another gene involved in DNA repair, excision repair cross-complementation group 1 (ERCC1), is a key component of NER and the main mechanism for removing platinum–DNA adducts (124). Preclinical studies have explored this repair pathway, demonstrating synergy of olaparib and veliparib with cisplatin in NSCLC cell lines with low ERCC1 expression levels (125, 126). PARPi have also been explored preclinically in combination with other DNA-damaging modalities such as RT (16).

The role of PARPi in patients with EGFR mutant NSCLC has been studied in a phase IB study of olaparib and the EGFR tyrosine kinase inhibitor (TKI) gefitinib (127). It was noted that high BRCA1 mRNA expression is associated with a shorter PFS in EGFR-mutated patients treated with erlotinib. To date, 18 patients have received treatment at four different dose levels of olaparib ranging 100–200 mg twice daily dose, and 200–250 mg three times daily dose. DLT was grade 3 anemia observed at dose level 4 (250 mg three times daily). Of the 17 patients in whom a disease response could be evaluated, 7 (41.1%) had a PR. All of the patients who responded were EGFR TKI naive. Another seven patients (41.1%), most of whom received prior treatment, had documented SD, and three patients (17.6%), all of whom had prior EGFR TKI treatment, progressed. The observed anti-tumor activity will be further evaluated in EGFR TKI treatment-naive patients with EGFR-mutated NSCLC; a phase II randomized trial comparing the efficacy of olaparib and gefitinib versus gefitinib alone was launched in July 2013.

Multiple studies are also exploring the role of PARPi in combination with chemotherapy and/or RT in NSCLC. A phase I dose-escalation trial of olaparib and concurrent RT, with or without cisplatin, is ongoing in patients with advanced NSCLC (128). SWOG 1206, a phase I/II trial, is evaluating the use of veliparib with or without RT and carboplatin/paclitaxel in patients with inoperable stage III NSCLC. Several similar studies involving other combinations of PARPi ± chemotherapy and/or RT are ongoing in patients with NSCLC (**Table 2**). Ultimately, combining PARPi with cisplatin or radiotherapy may prove to be a useful strategy in the treatment of NSCLC.

EWING'S SARCOMA

PARPi has preclinically shown anti-tumor activity in the treatment of Ewing's sarcoma. Gene fusions involving Ewing's sarcoma breakpoint region 1 (EWS) and ETS transcription factors have been implicated in abnormal proliferation, invasion, and tumorigenesis (129). PARP inhibition has been evaluated as an effective treatment option for Ewing's sarcoma with EWS–FLI1 or EWS–ERG genomic fusions in xenograft models (130), and olaparib has been shown to have potent activity in cell lines with a EWS/FLI1 translocation (131). Additionally, a study in preclinical models showed synergy between PARPi and TMZ (130) in the treatment of Ewing's sarcoma cell lines. Currently, a number of clinical trials investigating the utility of PARPi in Ewing's sarcoma are underway (132, 133).

CHALLENGES AND FUTURE DIRECTIONS FOR CLINICAL DEVELOPMENT IN CANCERS OTHER THAN BREAST AND OVARIAN

There is considerable interest in the clinical development of PARPi for use in solid tumors other than breast and ovarian cancers. The optimal dose, scheduling, and sequencing of PARPi, and combination with other cytotoxic or biologic agents need to be evaluated in carefully designed clinical trials. The incorporation of predictive biomarkers into studies of gBRCAm and BRCA-like cancers presents challenges. First is the development of a mechanism with which to identify patients who are most likely to benefit from PARPi therapy. Predictive biomarkers applied to readily available bioresources, such as archival tissue or non-tumor tissue, have been proposed. Changes in or baseline PAR incorporation into PBMC DNA was suggested and evaluated as a putative early on-treatment pharmacodynamic measure; while present, there was no relationship to clinical outcome (134). BRCA1/2 somatic mutation or promoter methylation, ATM mutation, MRE11-dominant negative mutations in MMR-deficient cancers, FANCF promotor methylation and PTEN deficiency are all potential biomarkers of sensitivity to PARPi (51). Importantly, not all patients with deficiencies in BRCA1 or 2 are responsive to PARP inhibition (135). Therefore, identification and validation of predictive biomarkers of those gBRCAm who will respond to PARPi is also an important area of ongoing research.

The second challenge is dissecting and defining mechanisms of development of resistance to PARPi, and whether they portend potential collateral resistance to other DNA-damaging agents. Acquisition of a secondary mutation in BRCA1/2 that allows BRCA1/2 gene read-through and yields a functional protein has been demonstrated in cell lines and some patients; this was correlated with loss of susceptibility to PARPi treatment (136). Other potential mechanisms of clinical resistance have been proposed based on preclinical models, including loss of 53BP1, or increased activity of RAD51 (137, 138). Whether these findings can be used as selective or predictive biomarker is yet to be determined. Ang et al. recently reported that gBRCAm-associated ovarian cancer patients retain the potential to respond to subsequent chemotherapy, including platinum-based agents, after progression on PARPi (139). This observation has implications for chemotherapy sequencing. Further studies are needed to evaluate outcomes to subsequent chemotherapies or another PARPi in other solid tumor patients who have a BRCA-like phenotype. Understanding the mechanism(s) of resistance to PARPi will lead to optimal application and sequencing of PARPi and other DNA-damaging agents.

CONCLUSION

PARPi are a class of agents with mechanisms of action beyond their documented role in BER pathway. They potentially have a broader application in the treatment of cancer patients, both within the confines of gBRCAm and BRCA-like disease, but also extending to a wide range of aberrations in DNA damage repair pathways. Ongoing research will aim to identify optimal predictive biomarkers in order to improve patient selection and thus, clinical responses to treatment. It is anticipated that novel clinical trial

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design strategies will help minimize toxicity and maximize therapeutic efficacy. Other pertinent questions relate to the duration of treatment and long-term effects of treatment, which need to be carefully investigated (20). Future directions for PARPi will include clinical trials directed at patient subsets that are most likely to respond to treatment, based on their molecular characteristics and predictive biomarkers. This may ultimately result in practice-changing treatments in malignancies such as pancreatic cancer, prostate cancer, and Ewing's sarcoma. The results of trials of PARPi, either as single-agents or in combination with chemotherapy, RT, or biological agents in other solid tumors are eagerly awaited.

AUTHOR CONTRIBUTIONS

All authors substantially contributed to the concept of the manuscript, drafted and revised, and approved final version.

ACKNOWLEDGMENTS

This work was supported by the Intramural Program of the Center for Cancer Research, National Cancer Institute. Dominic H. Moon was funded through the NIH Medical Research Scholars Program, a public–private partnership supported jointly by the NIH and through the Foundation for the NIH (details on program and donors: http://www.fnih.org/work/programs-development/medical-research-scholars-program).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 07 November 2013; accepted: 18 February 2014; published online: 28 February 2014.

Citation: O'Sullivan CC, Moon DH, Kohn EC and Lee J-M (2014) Beyond breast and ovarian cancers: PARP inhibitors for BRCA mutation-associated and BRCA-like solid tumors. Front. Oncol. 4:42. doi: 10.3389/fonc.2014.00042

This article was submitted to Cancer Molecular Targets and Therapeutics, a section of the journal Frontiers in Oncology.

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HIGHLIGHTS OF PRESCRIBING INFORMATION

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

ONIVYDE™ (irinotecan liposome injection), for intravenous use
Initial U.S. Approval: 1996

WARNING: SEVERE NEUTROPENIA and SEVERE DIARRHEA
See full prescribing information for complete boxed warning

- **Fatal neutropenic sepsis occurred in 0.8% of patients receiving ONIVYDE. Severe or life-threatening neutropenic fever or sepsis occurred in 3% and severe or life-threatening neutropenia occurred in 20% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Withhold ONIVYDE for absolute neutrophil count below 1500/mm^3 or neutropenic fever. Monitor blood cell counts periodically during treatment (2.2), (5.1).**
- **Severe diarrhea occurred in 13% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Do not administer ONIVYDE to patients with bowel obstruction. Withhold ONIVYDE for diarrhea of Grade 2-4 severity. Administer loperamide for late diarrhea of any severity. Administer atropine, if not contraindicated, for early diarrhea of any severity (2.2), (5.2).**

INDICATIONS AND USAGE

ONIVYDE is a topoisomerase inhibitor indicated, in combination with fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy. (1)

Limitation of Use: ONIVYDE is not indicated as a single agent for the treatment of patients with metastatic adenocarcinoma of the pancreas. (1)

DOSAGE AND ADMINISTRATION

- **Do not substitute ONIVYDE for other drugs containing irinotecan HCl. (2.1)**
- Recommended dose of ONIVYDE is 70 mg/m^2 intravenous infusion over 90 minutes every two weeks. (2.2)
- Recommended starting dose of ONIVYDE in patients homozygous for UGT1A1*28 is 50 mg/m^2 every two weeks. (2.2)
- There is no recommended dose of ONIVYDE for patients with serum bilirubin above the upper limit of normal. (2.2)
- Premedicate with a corticosteroid and an anti-emetic, 30 minutes prior to ONIVYDE. (2.2)

DOSAGE FORMS AND STRENGTHS

Injection: 43 mg/10 mL single dose vial (3)

CONTRAINDICATIONS

Severe hypersensitivity reaction to ONIVYDE or irinotecan HCl. (4, 5.4)

WARNINGS AND PRECAUTIONS

- Interstitial lung disease (ILD): Fatal ILD has occurred in patients receiving irinotecan HCl. Discontinue ONIVYDE if ILD is diagnosed. (5.3)
- Severe hypersensitivity reaction: Permanently discontinue ONIVYDE for severe hypersensitivity reactions. (5.4, 4)
- Embryo-fetal toxicity: Can cause fetal harm. Advise females of reproductive potential of the potential risk to a fetus and to use effective contraception. (5.5, 8.1, 8.3)

ADVERSE REACTIONS

The most common adverse reactions (≥ 20%) of ONIVYDE: diarrhea, fatigue/asthenia, vomiting, nausea, decreased appetite, stomatitis, and pyrexia. The most common laboratory abnormalities (≥ 10% Grade 3 or 4) were lymphopenia and neutropenia. (6)

To report SUSPECTED ADVERSE REACTIONS, contact Merrimack Pharmaceuticals, Inc. at 1-844-441-6225 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

DRUG INTERACTIONS

- Strong CYP3A4 Inducers: Avoid the use of strong CYP3A4 inducers if possible. Substitute non-enzyme inducing therapies at least 2 weeks prior to initiation of ONIVYDE. (7.1)
- Strong CYP3A4 Inhibitors: Avoid the use of strong CYP3A4 or UGT1A1 inhibitors, if possible, discontinue strong CYP3A4 inhibitors at least 1 week prior to starting therapy. (7.2)

USE IN SPECIFIC POPULATIONS

- Lactation: Do not breastfeed. (8.2)

See 17 for PATIENT COUNSELING INFORMATION.

Revised: 10/2015

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FULL PRESCRIBING INFORMATION

WARNING: SEVERE NEUTROPENIA and SEVERE DIARRHEA

Fatal neutropenic sepsis occurred in 0.8% of patients receiving ONIVYDE. Severe or life-threatening neutropenic fever or sepsis occurred in 3% and severe or life-threatening neutropenia occurred in 20% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Withhold ONIVYDE for absolute neutrophil count below 1500/mm^3 or neutropenic fever. Monitor blood cell counts periodically during treatment *[see Dosage and Administration (2.2) and Warnings and Precautions (5.1)]*.

Severe diarrhea occurred in 13% of patients receiving ONIVYDE in combination with fluorouracil and leucovorin. Do not administer ONIVYDE to patients with bowel obstruction. Withhold ONIVYDE for diarrhea of Grade 2-4 severity. Administer loperamide for late diarrhea of any severity. Administer atropine, if not contraindicated, for early diarrhea of any severity *[see Dosage and Administration (2.2) and Warnings and Precautions (5.2)]*.

1 INDICATIONS AND USAGE

ONIVYDE™ is indicated, in combination with fluorouracil and leucovorin, for the treatment of patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy.

Limitation of Use: ONIVYDE is not indicated as a single agent for the treatment of patients with metastatic adenocarcinoma of the pancreas *[see Clinical Studies (14)]*.

2 DOSAGE AND ADMINISTRATION

2.1 Important Use Information

DO NOT SUBSTITUTE ONIVYDE for other drugs containing irinotecan HCl.

2.2 Recommended Dose

Administer ONIVYDE prior to leucovorin and fluorouracil *[see Clinical Studies (14)]*.

- The recommended dose of ONIVYDE is 70 mg/m^2 administered by intravenous infusion over 90 minutes every 2 weeks.
- The recommended starting dose of ONIVYDE in patients known to be homozygous for the UGT1A1*28 allele is 50 mg/m^2 administered by intravenous infusion over 90 minutes. Increase the dose of ONIVYDE to 70 mg/m^2 as tolerated in subsequent cycles.
- There is no recommended dose of ONIVYDE for patients with serum bilirubin above the upper limit of normal *[see Adverse Reactions (6.1) and Clinical Studies (14)]*.

Premedication

Administer a corticosteroid and an anti-emetic 30 minutes prior to ONIVYDE infusion.

2.3 Dose Modifications for Adverse Reactions

Table 1: Recommended Dose Modifications for ONIVYDE

Toxicity NCI CTCAE v4.0[†]	Occurrence	ONIVYDE adjustment in patients receiving 70 mg/m^2	Patients homozygous for UGT1A1*28 without previous increase to 70 mg/m^2
Grade 3 or 4 adverse reactions	Withhold ONIVYDE. Initiate loperamide for late onset diarrhea of any severity. Administer intravenous or subcutaneous atropine 0.25 to 1 mg (unless clinically contraindicated) for early onset diarrhea of any severity. Upon recovery to ≤ Grade 1, resume ONIVYDE at:		
	First	50 mg/m^2	43 mg/m^2
	Second	43 mg/m^2	35 mg/m^2
	Third	Discontinue ONIVYDE	Discontinue ONIVYDE
Interstitial Lung Disease	First	Discontinue ONIVYDE	Discontinue ONIVYDE
Anaphylactic Reaction	First	Discontinue ONIVYDE	Discontinue ONIVYDE

[†] NCI CTCAE v 4.0=National Cancer Institute Common Toxicity Criteria for Adverse Events version 4.0

For recommended dose modifications of fluorouracil (5-FU) or leucovorin (LV), refer to the Full Prescribing Information; refer to Clinical Studies (14).

2.4 Preparation and Administration

ONIVYDE is a cytotoxic drug. Follow applicable special handling and disposal procedures.[1]

Preparation

- Withdraw the calculated volume of ONIVYDE from the vial. Dilute ONIVYDE in 500 mL 5% Dextrose Injection, USP or 0.9% Sodium Chloride Injection, USP and mix diluted solution by gentle inversion.
- Protect diluted solution from light.
- Administer diluted solution within 4 hours of preparation when stored at room temperature or within 24 hours of preparation when stored under refrigerated conditions [2°C to 8°C (36°F to 46°F)]. Allow diluted solution to come to room temperature prior to administration.
- Do NOT freeze.

Administration

- Infuse diluted solution intravenously over 90 minutes. Do not use in-line filters. Discard unused portion.

3 DOSAGE FORMS AND STRENGTHS

Injection: 43 mg/10 mL irinotecan free base as a white to slightly yellow, opaque, liposomal dispersion in a single-dose vial.

4 CONTRAINDICATIONS

ONIVYDE is contraindicated in patients who have experienced a severe hypersensitivity reaction to ONIVYDE or irinotecan HCl.

5 WARNINGS AND PRECAUTIONS

5.1 Severe Neutropenia

ONIVYDE can cause severe or life-threatening neutropenia and fatal neutropenic sepsis. In Study 1, the incidence of fatal neutropenic sepsis was 0.8% among patients receiving ONIVYDE, occurring in one of 117 patients in the ONIVYDE plus fluorouracil/leucovorin (ONIVYDE/5-FU/LV) arm and one of 147 patients receiving ONIVYDE as a single agent. Severe or life-threatening neutropenia occurred in 20% of patients receiving ONIVYDE/5-FU/LV compared to 2% of patients receiving fluorouracil/leucovorin alone (5-FU/LV). Grade 3 or 4 neutropenic fever/neutropenic sepsis occurred in 3% of patients receiving ONIVYDE/5-FU/LV, and did not occur in patients receiving 5-FU/LV.

In patients receiving ONIVYDE/5-FU/LV, the incidence of Grade 3 or 4 neutropenia was higher among Asian patients [18 of 33 (55%)] compared to White patients [13 of 73 (18%)]. Neutropenic fever/neutropenic sepsis was reported in 6% of Asian patients compared to 1% of White patients *[see Clinical Pharmacology (12.3)]*.

Monitor complete blood cell counts on Days 1 and 8 of every cycle and more frequently if clinically indicated. Withhold ONIVYDE if the absolute neutrophil count (ANC) is below $1500/mm^3$ or if neutropenic fever occurs. Resume ONIVYDE when the ANC is $1500/mm^3$ or above. Reduce ONIVYDE dose for Grade 3-4 neutropenia or neutropenic fever following recovery in subsequent cycles *[see Dosage and Administration (2.2)]*.

5.2 Severe Diarrhea

ONIVYDE can cause severe and life-threatening diarrhea. Do not administer ONIVYDE to patients with bowel obstruction.

Severe or life-threatening diarrhea followed one of two patterns: late onset diarrhea (onset more than 24 hours following chemotherapy) and early onset diarrhea (onset within 24 hours of

chemotherapy, sometimes occurring with other symptoms of cholinergic reaction) *[see Cholinergic Reactions (6.1)]*. An individual patient may experience both early and late-onset diarrhea.

In Study 1, Grade 3 or 4 diarrhea occurred in 13% receiving ONIVYDE/5-FU/LV compared to 4% receiving 5-FU/LV. The incidence of Grade 3 or 4 late onset diarrhea was 9% in patients receiving ONIVYDE/5-FU/LV, compared to 4% in patients receiving 5-FU/LV. The incidence of Grade 3 or 4 early onset diarrhea was 3% in patients receiving ONIVYDE/5-FU/LV, compared to no Grade 3 or 4 early onset diarrhea in patients receiving 5-FU/LV. Of patients receiving ONIVYDE/5-FU/LV in Study 1, 34% received loperamide for late-onset diarrhea and 26% received atropine for early-onset diarrhea. Withhold ONIVYDE for Grade 2-4 diarrhea. Initiate loperamide for late onset diarrhea of any severity. Administer intravenous or subcutaneous atropine 0.25 to 1 mg (unless clinically contraindicated) for early onset diarrhea of any severity. Following recovery to Grade 1 diarrhea, resume ONIVYDE at a reduced dose *[see Dosage and Administration (2.2)]*.

5.3 Interstitial Lung Disease

Irinotecan HCl can cause severe and fatal interstitial lung disease (ILD). Withhold ONIVYDE in patients with new or progressive dyspnea, cough, and fever, pending diagnostic evaluation. Discontinue ONIVYDE in patients with a confirmed diagnosis of ILD.

5.4 Severe Hypersensitivity Reaction

Irinotecan HCl can cause severe hypersensitivity reactions, including anaphylactic reactions. Permanently discontinue ONIVYDE in patients who experience a severe hypersensitivity reaction.

5.5 Embryo-Fetal Toxicity

Based on animal data with irinotecan HCl and the mechanism of action of ONIVYDE, ONIVYDE can cause fetal harm when administered to a pregnant woman. Embryotoxicity and teratogenicity were observed following treatment with irinotecan HCl, at doses resulting in irinotecan exposures lower than those achieved with ONIVYDE 70 mg/m^2 in humans, administered to pregnant rats and rabbits during organogenesis. Advise pregnant women of the potential risk to a fetus. Advise females of reproductive potential to use effective contraception during treatment with ONIVYDE and for one month following the final dose *[see Use in Specific Populations (8.1, 8.3), Clinical Pharmacology (12.1)]*.

6 ADVERSE REACTIONS

The following adverse drug reactions are discussed in greater detail in other sections of the label:

- Severe Neutropenia [see *Warnings and Precautions (5.1) and Boxed Warning]*
- Severe Diarrhea *[see Warnings and Precautions (5.2) and Boxed Warning]*
- Interstitial Lung Disease *[see Warnings and Precautions (5.3)]*

- Severe Hypersensitivity Reactions *[see Warnings and Precautions (5.4)]*

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in clinical trials of ONIVYDE cannot be directly compared to rates in clinical trials of other drugs and may not reflect the rates observed in practice.

The safety data described below are derived from patients with metastatic adenocarcinoma of the pancreas previously treated with gemcitabine-based therapy who received any part of protocol-specified therapy in Study 1, an international, randomized, active-controlled, open-label trial. Protocol-specified therapy consisted of ONIVYDE 70 mg/m^2 with leucovorin 400 mg/m^2 and fluorouracil 2400 mg/m^2 over 46 hours every 2 weeks (ONIVYDE/5-FU/LV; N=117), ONIVYDE 100 mg/m^2 every 3 weeks (N=147), or leucovorin 200 mg/m^2 and fluorouracil 2000 mg/m^2 over 24 hours weekly for 4 weeks followed by 2 week rest (5-FU/LV; N=134) *[see Clinical Studies (14)]*. Serum bilirubin within the institutional normal range, albumin ≥ 3 g/dL, and Karnofsky Performance Status (KPS) ≥ 70 were required for study entry. The median duration of exposure was 9 weeks in the ONIVYDE/5-FU/LV arm, 9 weeks in the ONIVYDE monotherapy arm, and 6 weeks in the 5-FU/LV arm.

The most common adverse reactions (≥ 20%) of ONIVYDE were diarrhea, fatigue/asthenia, vomiting, nausea, decreased appetite, stomatitis, and pyrexia. The most common, severe laboratory abnormalities (≥ 10% Grade 3 or 4) were lymphopenia and neutropenia. The most common serious adverse reactions (≥ 2%) of ONIVYDE were diarrhea, vomiting, neutropenic fever or neutropenic sepsis, nausea, pyrexia, sepsis, dehydration, septic shock, pneumonia, acute renal failure, and thrombocytopenia.

Adverse reactions led to permanent discontinuation of ONIVYDE in 11% of patients receiving ONIVYDE/5-FU/LV; the most frequent adverse reactions resulting in discontinuation of ONIVYDE were diarrhea, vomiting, and sepsis. Dose reductions of ONIVYDE for adverse reactions occurred in 33% of patients receiving ONIVYDE/5-FU/LV; the most frequent adverse reactions requiring dose reductions were neutropenia, diarrhea, nausea, and anemia. ONIVYDE was withheld or delayed for adverse reactions in 62% of patients receiving ONIVYDE/5-FU/LV; the most frequent adverse reactions requiring interruption or delays were neutropenia, diarrhea, fatigue, vomiting, and thrombocytopenia.

Table 2 provides the frequency and severity of adverse reactions in Study 1 that occurred with higher incidence (≥5% difference for Grades 1-4 or ≥2% difference for Grades 3-4) in patients who received ONIVYDE/5-FU/LV compared to patients who received 5-FU/LV.

Table 2: Adverse Reactions with Higher Incidence (≥5% Difference for Grades 1-4* or ≥2% Difference for Grades 3 and 4) in the ONIVYDE/5-FU/LV Arm

Adverse Reaction	ONIVYDE/5-FU/LV N=117		5-FU/LV N=134	
	Grades 1-4 (%)	Grades 3-4 (%)	Grades 1-4 (%)	Grades 3-4 (%)
Gastrointestinal disorders				
Diarrhea	59	13	26	4
Early diarrhea[†]	30	3	15	0
Late diarrhea[‡]	43	9	17	4
Vomiting	52	11	26	3
Nausea	51	8	34	4
Stomatitis[§]	32	4	12	1
Infections and infestations	38	17	15	10
Sepsis	4	3	2	1
Neutropenic fever/neutropenic sepsis[¶]	3	3	1	0
Gastroenteritis	3	3	0	0
Intravenous catheter-related infection	3	3	0	0
General disorders and administration site conditions				
Fatigue/asthenia	56	21	43	10
Pyrexia	23	2	11	1
Metabolism and nutrition disorders				
Decreased appetite	44	4	32	2
Weight loss	17	2	7	0
Dehydration	8	4	7	2
Skin and subcutaneous tissue disorders				
Alopecia	14	1	5	0

* NCI CTCAE v4.0

† Early diarrhea: onset within 24 hours of ONIVYDE administration

‡ Late diarrhea: onset > 1 day after ONIVYDE administration

§ Includes stomatitis, aphthous stomatitis, mouth ulceration, mucosal inflammation.

¶ Includes febrile neutropenia

Cholinergic Reactions: ONIVYDE can cause cholinergic reactions manifesting as rhinitis, increased salivation, flushing, bradycardia, miosis, lacrimation, diaphoresis, and intestinal hyperperistalsis with abdominal cramping and early onset diarrhea. In Study 1, Grade 1 or 2 cholinergic symptoms other than early diarrhea occurred in 12 (4.5%) ONIVYDE-treated patients. Six of these 12 patients received atropine and in 1 of the 6 patients, atropine was administered for cholinergic symptoms other than diarrhea.

Infusion Reactions: Infusion reactions, consisting of rash, urticaria, periorbital edema, or pruritus, occurring on the day of ONIVYDE administration were reported in 3% of patients receiving ONIVYDE or ONIVYDE/5-FU/LV.

Laboratory abnormalities that occurred with higher incidence in the ONIVYDE/5-FU/LV arm compared to the 5-FU/LV arm (≥5% difference) are summarized in the following table.

Table 3: Laboratory Abnormalities with Higher Incidence (≥5% Difference) in the ONIVYDE/5-FU/LV Arm*#

Laboratory abnormality	ONIVYDE/5-FU/LV N=117		5-FU/LV N=134	
	Grades 1-4 (%)	Grades 3-4 (%)	Grades 1-4 (%)	Grades 3-4 (%)
Hematology				
Anemia	97	6	86	5
Lymphopenia	81	27	75	17
Neutropenia	52	20	6	2
Thrombocytopenia	41	2	33	0
Hepatic				
Increased alanine aminotransferase (ALT)	51	6	37	1
Hypoalbuminemia	43	2	30	0
Metabolic				
Hypomagnesemia	35	0	21	0
Hypokalemia	32	2	19	2
Hypocalcemia	32	1	20	0
Hypophosphatemia	29	4	18	1
Hyponatremia	27	5	12	3
Renal				
Increased creatinine	18	0	13	0

*NCI CTCAE v4.0, worst grade shown.
Percent based on number of patients with a baseline and at least one post-baseline measurement.

7 DRUG INTERACTIONS

7.1 Strong CYP3A4 Inducers

Following administration of non-liposomal irinotecan (i.e., irinotecan HCl), exposure to irinotecan or its active metabolite, SN-38, is substantially reduced in adult and pediatric patients concomitantly receiving the CYP3A4 enzyme-inducing anticonvulsants phenytoin and strong CYP3A4 inducers. Avoid the use of strong CYP3A4 inducers (e.g., rifampin, phenytoin, carbamazepine, rifabutin, rifapentine, phenobarbital, St. John's wort) if possible. Substitute non-enzyme inducing therapies at least 2 weeks prior to initiation of ONIVYDE therapy *[see Clinical Pharmacology (12.3)]*.

7.2 Strong CYP3A4 or UGT1A1 Inhibitors

Following administration of non-liposomal irinotecan (i.e., irinotecan HCl), patients receiving concomitant ketoconazole, a CYP3A4 and UGT1A1 inhibitor, have increased exposure to irinotecan and its active metabolite SN-38. Co-administration of ONIVYDE with other inhibitors of CYP3A4 (e.g., clarithromycin, indinavir, itraconazole, lopinavir, nefazodone, nelfinavir, ritonavir, saquinavir, telaprevir, voriconazole) or UGT1A1 (e.g., atazanavir, gemfibrozil, indinavir) may increase systemic exposure to irinotecan or SN-38. Avoid the use of strong CYP3A4 or UGT1A1 inhibitors if possible. Discontinue strong CYP3A4 inhibitors at least 1 week prior to starting ONIVYDE therapy *[see Clinical Pharmacology (12.3)]*.

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8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

Based on animal data with irinotecan HCl and the mechanism of action of ONIVYDE, ONIVYDE can cause fetal harm when administered to a pregnant woman *[see Clinical Pharmacology (12.1)]*. There are no available data in pregnant women. Embryotoxicity and teratogenicity were observed following treatment with irinotecan HCl, at doses resulting in irinotecan exposures lower than those achieved with ONIVYDE 70 mg/m^2 in humans, administered to pregnant rats and rabbits during organogenesis *[see Data]*. Advise pregnant women of the potential risk to a fetus.

In the U.S. general population, the estimated background risk of major birth defects and miscarriage in clinically recognized pregnancies is 2-4% and 15-20%, respectively.

Data

Animal Data

No animal studies have been conducted to evaluate the effect of irinotecan liposome on reproduction and fetal development; however, studies have been conducted with irinotecan HCl. Irinotecan crosses the placenta of rats following intravenous administration. Intravenous administration of irinotecan at a dose of 6 mg/kg/day to rats and rabbits during the period of organogenesis resulted in increased post-implantation loss and decreased numbers of live fetuses. In separate studies in rats, this dose resulted in an irinotecan exposure of approximately 0.002 times the exposure of irinotecan based on area under the curve (AUC) in patients administered ONIVYDE at the 70 mg/m^2 dose. Administration of irinotecan HCl resulted in structural abnormalities and growth delays in rats at doses greater than 1.2 mg/kg/day (approximately 0.0002 times the clinical exposure to irinotecan in ONIVYDE based on AUC). Teratogenic effects included a variety of external, visceral, and skeletal abnormalities. Irinotecan HCl administered to rat dams for the period following organogenesis through weaning at doses of 6 mg/kg/day caused decreased learning ability and decreased female body weights in the offspring.

8.2 Lactation

Risk Summary

There is no information regarding the presence of irinotecan liposome, irinotecan, or SN-38 (an active metabolite of irinotecan) in human milk, or the effects on the breastfed infant or on milk production. Irinotecan is present in rat milk *[see Data]*.

Because of the potential for serious adverse reactions in breastfed infants from ONIVYDE, advise a nursing woman not to breastfeed during treatment with ONIVYDE and for one month after the final dose.

Data

Radioactivity appeared in rat milk within 5 minutes of intravenous administration of radiolabeled irinotecan HCl and was concentrated up to 65-fold at 4 hours after administration relative to plasma concentrations.

8.3 Females and Males of Reproductive Potential

Contraception

Females

ONIVYDE can cause fetal harm when administered to a pregnant woman *[see Use in Specific Populations (8.1)]*. Advise females of reproductive potential to use effective contraception during treatment with ONIVYDE and for one month after the final dose.

Males

Because of the potential for genotoxicity, advise males with female partners of reproductive potential to use condoms during treatment with ONIVYDE and for four months after the final dose *[see Nonclinical Toxicology (13.1)]*.

8.4 Pediatric Use

Safety and effectiveness of ONIVYDE have not been established in pediatric patients.

8.5 Geriatric Use

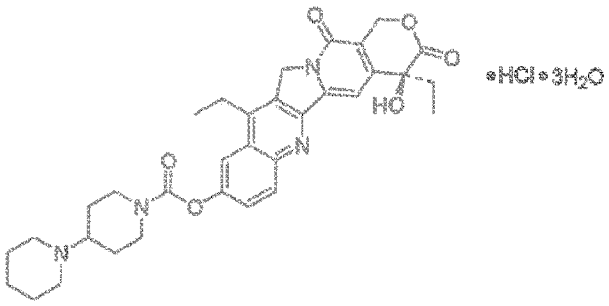
Of the 264 patients who received ONIVYDE as a single agent or in combination with 5-FU and leucovorin in Study 1, 49% were ≥ 65 years old and 13% were ≥ 75 years old. No overall differences in safety and effectiveness were observed between these patients and younger patients.

10 OVERDOSAGE

There are no treatment interventions known to be effective for management of overdosage of ONIVYDE.

11 DESCRIPTION

ONIVYDE is formulated with irinotecan hydrochloride trihydrate, a topoisomerase inhibitor, into a liposomal dispersion for intravenous use. The chemical name of irinotecan hydrochloride trihydrate is (S)-4,11-diethyl-3,4,12,14-tetrahydro-4-hydroxy-3,14-dioxo1H-pyrano[3',4':6,7]-indolizino[1,2-b]quinolin-9-yl-[1,4'bipiperidine]-1'-carboxylate, monohydrochloride, trihydrate. The empirical formula is $C_{33}H_{38}N_4O_6 \cdot HCl \cdot 3H_2O$ and the molecular weight is 677.19 g/mole. The molecular structure is:



ONIVYDE is a sterile, white to slightly yellow opaque isotonic liposomal dispersion. Each 10 mL single-dose vial contains 43 mg irinotecan free base at a concentration of 4.3 mg/mL. The liposome is a unilamellar lipid bilayer vesicle, approximately 110 nm in diameter, which encapsulates an aqueous space containing irinotecan in a gelated or precipitated state as the sucrose octasulfate salt. The vesicle is composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) 6.81 mg/mL, cholesterol 2.22 mg/mL, and methoxy-terminated polyethylene glycol (MW 2000)-distearoylphosphatidyl ethanolamine (MPEG-2000-DSPE) 0.12 mg/mL. Each mL also contains 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) as a buffer 4.05 mg/mL and sodium chloride as an isotonicity reagent 8.42 mg/mL.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

Irinotecan liposome injection is a topoisomerase 1 inhibitor encapsulated in a lipid bilayer vesicle or liposome. Topoisomerase 1 relieves torsional strain in DNA by inducing single-strand breaks. Irinotecan and its active metabolite SN-38 bind reversibly to the topoisomerase 1-DNA complex and prevent re-ligation of the single-strand breaks, leading to exposure time-dependent double-strand DNA damage and cell death. In mice bearing human tumor xenografts, irinotecan liposome administered at irinotecan HCl-equivalent doses 5-fold lower than irinotecan HCl achieved similar intratumoral exposure of SN-38.

12.3 Pharmacokinetics

The plasma pharmacokinetics of total irinotecan and total SN-38 were evaluated in patients with cancer who received ONIVYDE, as a single agent or as part of combination chemotherapy, at doses between 50 and 155 mg/m^2 and 353 patients with cancer using population pharmacokinetic analysis.

The pharmacokinetic parameters of total irinotecan and total SN-38 following the administration of ONIVYDE 70 mg/m^2 as a single agent or part of combination chemotherapy are presented in Table 4.

Table 4: Summary of Mean (±Standard Deviation) Total Irinotecan and Total SN-38

Dose (mg/m^2)	Total Irinotecan					Total SN-38		
	C_{max} [µg/mL] (n=25)	$AUC_{0-\infty}$ [h·µg/mL] (n=23)	$t_{1/2}$ [h] (n=23)	CL [L/h] (n=23)	V_d [L] (n=23)	C_{max} [ng/mL] (n=25)	$AUC_{0-\infty}$ [h·ng/mL] (n=13)	$t_{1/2}$ [h] (n=13)
70	37.2 (8.8)	1364 (1048)	25.8 (15.7)	0.20 (0.17)	4.1 (1.5)	5.4 (3.4)	620 (329)	67.8 (44.5)

C_{max}: Maximum plasma concentration
$AUC_{0-\infty}$: Area under the plasma concentration curve extrapolated to time infinity
$t_{1/2}$: Terminal elimination half-life
CL: Clearance
V_d: Volume of distribution

Over the dose range of 50 to 155 mg/m^2, the C_{max} and AUC of total irinotecan increases with dose. Additionally, the C_{max} of total SN-38 increases proportionally with dose; however, the AUC of total SN-38 increases less than proportionally with dose.

Distribution

Direct measurement of irinotecan liposome showed that 95% of irinotecan remains liposome-encapsulated, and the ratios between total and encapsulated forms did not change with time from 0 to 169.5 hours post-dose. The mean volume of distribution is summarized in Table 4.

Plasma protein binding is <0.44% of the total irinotecan in ONIVYDE.

Elimination

Metabolism

The metabolism of irinotecan liposome has not been evaluated. Irinotecan is subject to extensive metabolic conversion by various enzyme systems, including esterases to form the active metabolite SN-38, and UGT1A1 mediating glucuronidation of SN-38 to form the inactive glucuronide metabolite SN-38G. Irinotecan can also undergo CYP3A4-mediated oxidative metabolism to several inactive oxidation products, one of which can be hydrolyzed by carboxylesterase to release SN-38. In the population pharmacokinetic analysis using the results of a subset with UGT1A1*28 genotypic testing, in which the analysis adjusted for the lower dose administered to patients homozygous for the UGT1A1*28 allele, patients homozygous (N=14) and non-homozygous (N=244) for this allele had total SN-38 average steady-state concentrations of 1.06 and 0.95 ng/mL, respectively.

Excretion

The disposition of ONIVYDE has not been elucidated in humans. Following administration of irinotecan HCl , the urinary excretion of irinotecan is 11 to 20%; SN-38, <1%; and SN-38 glucuronide, 3%. The cumulative biliary and urinary excretion of irinotecan and its metabolites (SN-38 and SN-38 glucuronide), over a period of 48 hours following administration of irinotecan HCl in two patients, ranged from approximately 25% (100 mg/m^2) to 50% (300 mg/m^2).

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Specific Populations

Age, Gender, and Renal Impairment:

The population pharmacokinetic analysis suggests that age (28 to 87 years) had no clinically meaningful effect on the exposure of irinotecan and SN-38.

The population pharmacokinetic analysis suggests that gender (196 males and 157 females) had no clinically meaningful effect on the exposure of irinotecan and SN-38 after adjusting for body surface area (BSA).

In a population pharmacokinetic analysis, mild-to-moderate renal impairment had no effect on the exposure of total SN-38 after adjusting for BSA. The analysis included 68 patients with moderate (CLcr 30 - 59 mL/min) renal impairment, 147 patients with mild (CLcr 60 - 89 mL/min) renal impairment, and 135 patients with normal renal function (CLcr > 90 mL/min). There was insufficient data in patients with severe renal impairment (CLcr < 30 mL/min) to assess its effect on pharmacokinetics.

Ethnicity: The population pharmacokinetic analysis suggests that Asians (East Asians, N=150) have 56% lower total irinotecan average steady state concentration and 8% higher total SN-38 average steady state concentration than Whites (N=182).

Hepatic Impairment: The pharmacokinetics of irinotecan liposome have not been studied in patients with hepatic impairment. In a population pharmacokinetic analysis, patients with baseline bilirubin concentrations of 1-2 mg/dL (N=19) had average steady state concentrations for total SN-38 that were increased by 37% compared to patients with baseline bilirubin concentrations of <1 mg/dL (N=329); however, there was no effect of elevated ALT/AST concentrations on total SN-38 concentrations. No data are available in patients with bilirubin >2 mg/dL.

Drug Interactions

In a population pharmacokinetic analysis, the pharmacokinetics of total irinotecan and total SN-38 were not altered by the co-administration of fluorouracil/leucovorin.

Following administration of irinotecan HCl, dexamethasone, a moderate CYP3A4 inducer, does not alter the pharmacokinetics of irinotecan.

In vitro studies indicate that irinotecan, SN-38 and another metabolite, aminopentane carboxylic acid (APC), do not inhibit cytochrome P-450 isozymes.

12.5 Pharmacogenomics

Individuals who are homozygous for the UGT1A1*28 allele are at increased risk for neutropenia from irinotecan HCl. In Study 1, patients homozygous for the UGT1A1*28 allele (N=7) initiated ONIVYDE at a reduced dose of 50 mg/m^2 in combination with 5-FU/LV. The frequency of Grade 3 or 4 neutropenia in these patients [2 of 7 (28.6%)] was similar to the frequency in patients not homozygous for the UGT1A1*28 allele who received a starting dose of ONIVYDE of 70 mg/m^2 [30 of 110 (27.3%)].

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13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No studies have been performed to assess the potential of irinotecan liposome for carcinogenicity, genotoxicity or impairment of fertility. Intravenous administration of irinotecan hydrochloride to rats once weekly for 13 weeks followed by a 91-week recovery period resulted in a significant linear trend between irinotecan HCl dosage and the incidence of combined uterine horn endometrial stromal polyps and endometrial stromal sarcomas. Irinotecan HCl was clastogenic both in vitro (chromosome aberrations in Chinese hamster ovary cells) and in vivo (micronucleus test in mice). Neither irinotecan nor its active metabolite, SN-38, was mutagenic in the in vitro Ames assay.

Dedicated fertility studies have not been performed with irinotecan liposome injection. Atrophy of male and female reproductive organs was observed in dogs receiving irinotecan liposome injection every 3 weeks at doses equal to or greater than 15 mg/kg, (approximately 3 times the clinical exposure of irinotecan following administration to ONIVYDE dosed at 70 mg/m^2) for a total of 6 doses. No significant adverse effects on fertility and general reproductive performance were observed after intravenous administration of irinotecan HCl in doses of up to 6 mg/kg/day to rats; however, atrophy of male reproductive organs was observed after multiple daily irinotecan HCl doses both in rodents at 20 mg/kg (approximately 0.007 times the clinical irinotecan exposure following ONIVYDE administration at 70 mg/m^2) and in dogs at 0.4 mg/kg (0.0007 times the clinical exposure to irinotecan following administration of ONIVYDE).

14 CLINICAL STUDIES

The efficacy of ONIVYDE was evaluated in Study 1, a three-arm, randomized, open-label trial in patients with metastatic pancreatic adenocarcinoma with documented disease progression, after gemcitabine or gemcitabine-based therapy. Key eligibility criteria included Karnofsky Performance Status (KPS) ≥70, serum bilirubin within institution limits of normal, and albumin ≥3.0 g/dL. Patients were randomized to receive ONIVYDE plus fluorouracil/leucovorin (ONIVYDE/5-FU/LV), ONIVYDE, or fluorouracil/leucovorin (5-FU/LV). Randomization was stratified by ethnicity (White vs. East Asian vs. other), KPS (70-80 vs. 90-100), and baseline albumin level (≥ 4 g/dL vs. 3.0-3.9 g/dL). Patients randomized to ONIVYDE/5-FU/LV received ONIVYDE 70 mg/m^2 as an intravenous infusion over 90 minutes, followed by leucovorin 400 mg/m^2 intravenously over 30 minutes, followed by fluorouracil 2400 mg/m^2 intravenously over 46 hours, every 2 weeks. The ONIVYDE dose of 70 mg/m^2 is based on irinotecan free base (equivalent to 80 mg/m^2 of irinotecan as the hydrochloride trihydrate). Patients randomized to ONIVYDE as a single agent received ONIVYDE 100 mg/m^2 as an intravenous infusion over 90 minutes every 3 weeks. Patients randomized to 5-FU/LV received leucovorin 200 mg/m^2 intravenously over 30 minutes, followed by fluorouracil 2000 mg/m^2 intravenously over 24 hours, administered on Days 1, 8, 15 and 22 of a 6-week cycle. Patients homozygous for the UGT1A1*28 allele initiated ONIVYDE at a reduced dose (50 mg/m^2 ONIVYDE, if given with 5-FU/LV or 70 mg/m^2 ONIVYDE as a single agent). When ONIVYDE was withheld or discontinued for adverse reactions, 5-FU was also withheld or discontinued. When the dose of

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ONIVYDE was reduced for adverse reactions, the dose of 5-FU was reduced by 25%. Treatment continued until disease progression or unacceptable toxicity.

The major efficacy outcome measure was overall survival (OS) with two pair-wise comparisons: ONIVYDE versus 5-FU/LV and ONIVYDE/5-FU/LV versus 5-FU/LV. Additional efficacy outcome measures were progression-free survival (PFS) and objective response rate (ORR). Tumor status assessments were conducted at baseline and every 6 weeks thereafter. The trial was initiated as a two-arm study and amended after initiation to include a third arm (ONIVYDE/5-FU/LV). The comparisons between the ONIVYDE/5-FU/LV and the 5-FU/LV arms are limited to patients enrolled in the 5-FU/LV arm after this protocol amendment.

Four hundred seventeen patients were randomized to: ONIVYDE/5-FU/LV (N=117), ONIVYDE (N=151), or 5-FU/LV (N=149). Baseline demographics and tumor characteristics for the 236 patients randomized to ONIVYDE/5-FU/LV or 5-FU/LV (N=119) after the addition of the third arm to the study were a median age of 63 years (range 34-81 years) and with 41% ≥ 65 years of age; 58% were men; 63% were White, 30% were Asian, 3% were Black or African American, and 5% were other. Mean baseline albumin level was 3.97 g/dL, and baseline KPS was 90-100 in 53% of patients. Disease characteristics included liver metastasis (67%) and lung metastasis (31%). A total of 13% of patients received gemcitabine in the neoadjuvant/adjuvant setting only, 55% of patients had 1 prior line of therapy for metastatic disease, and 33% of patients had 2 or more prior lines of therapy for metastatic disease. All patients received prior gemcitabine (alone or in combination with another agent), 54% received prior gemcitabine in combination with another agent, and 13% received prior gemcitabine in combination with nab-paclitaxel.

Study 1 demonstrated a statistically significant improvement in overall survival for the ONIVYDE/5-FU/LV arm over the 5-FU/LV arm as summarized in Table 5 and Figure 1.

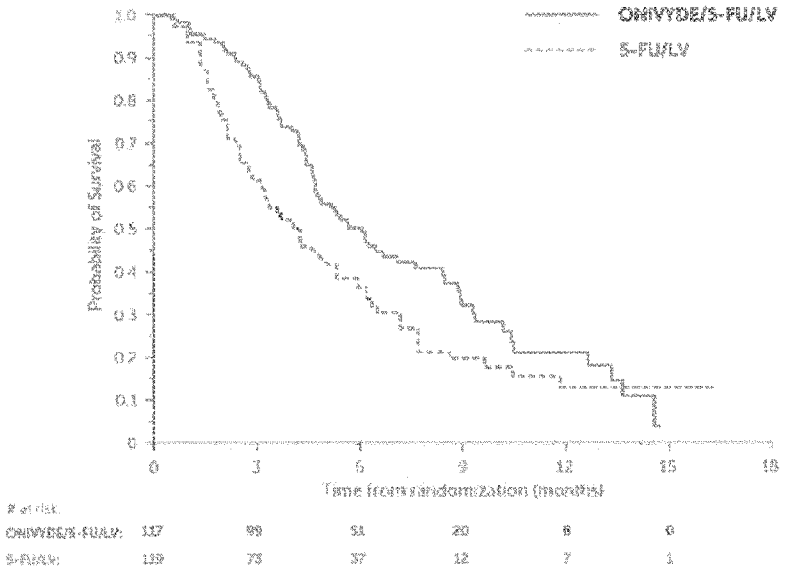
There was no improvement in overall survival for the ONIVYDE arm over the 5-FU/LV arm (hazard ratio=1.00, p-value=0.97 (two-sided log-rank test)).

Table 5: Efficacy Results from Study 1†

	ONIVYDE/5-FU/LV (N=117)	5-FU/LV (N=119)
Overall Survival		
Number of Deaths, n (%)	77 (66)	86 (72)
Median Overall Survival (months)	6.1	4.2
(95% CI)	(4.8, 8.5)	(3.3, 5.3)
Hazard Ratio (95% CI)	0.68 (0.50, 0.93)	
p-value (log-rank test)	0.014	
Progression-Free Survival		
Death or Progression, n (%)	83 (71)	94 (79)
Median Progression-Free Survival (months)	3.1	1.5
(95% CI)	(2.7, 4.2)	(1.4, 1.8)
Hazard Ratio (95% CI)	0.55 (0.41, 0.75)	
Objective Response Rate		
Confirmed Complete or Partial Response n (%)	9 (7.7%)	1 (0.8%)

† 5-FU/LV=5-fluorouracil/leucovorin; CI=confidence interval

Figure 1: Overall Survival



15 REFERENCES

1. OSHA Hazardous Drugs. *OSHA*. http://www.osha.gov/SLTC/hazardousdrugs/index.html

16 HOW SUPPLIED/STORAGE AND HANDLING

How Supplied

ONIVYDE is available in a single-dose vial containing 43 mg irinotecan free base at a concentration of 4.3 mg/mL

NDC: 69171-398-01

Storage and Handling

Store ONIVYDE at 2°C to 8°C (36°F to 46°F). Do NOT freeze. Protect from light.

ONIVYDE is a cytotoxic drug. Follow applicable special handling and disposal procedures.[1]

17 PATIENT COUNSELING INFORMATION

Advise patients of the following:

Severe Neutropenia
Advise patients of the risk of neutropenia leading to severe and life-threatening infections and the need for monitoring of blood counts. Instruct patients to contact their healthcare provider immediately if experiencing signs of infection, such as fever, chills, dizziness, or shortness of breath *[see Warnings and Precautions (5.1)]*.

Severe Diarrhea
Inform patients of the risk of severe diarrhea. Advise patients to contact their healthcare provider if they experience persistent vomiting or diarrhea; black or bloody stools; or symptoms of dehydration such as lightheadedness, dizziness, or faintness *[see Warnings and Precautions (5.2)]*.

Interstitial Lung Disease
Inform patients of the potential risk of ILD. Advise patients to contact their healthcare provider as soon as possible for new onset cough or dyspnea *[see Interstitial Lung Disease (5.3)]*.

Hypersensitivity to irinotecan HCl or ONIVYDE
Advise patients of the potential risk of severe hypersensitivity and that ONIVYDE is contraindicated in patients with a history of severe allergic reactions with irinotecan HCl or ONIVYDE. Instruct patients to seek immediate medical attention for signs of severe hypersensitivity reaction such as chest tightness; shortness of breath; wheezing; dizziness or faintness; or swelling of the face, eyelids, or lips *[see Contraindications (4) and Warnings and Precautions (5.4)]*.

Females and males of reproductive potential

Embryo-fetal toxicity: Inform females of reproductive potential of the potential risk to a fetus, to use effective contraception during treatment and for one month after the final dose, and to inform their healthcare provider of a known or suspected pregnancy *[see Warnings and Precautions (5.5), Use in Specific Populations (8.1, 8.3)]*.

Contraception: Advise male patients with female partners of reproductive potential to use condoms during treatment with ONIVYDE and for four months after the final dose *[see Females and Males of Reproductive Potential (8.3)]*.

Lactation

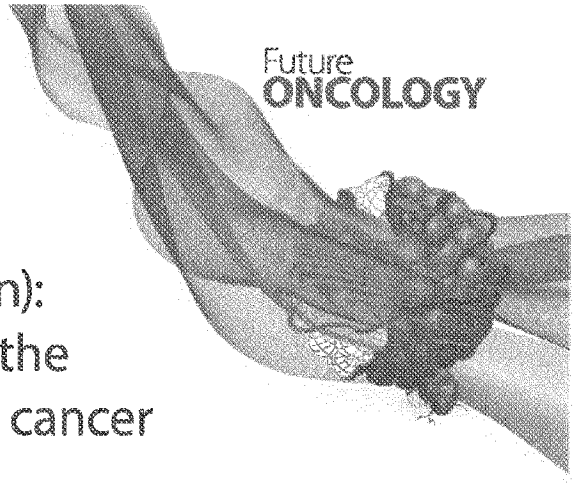
Advise women not to breastfeed during treatment with ONIVYDE and for one month after the final dose *[see Use in Special Populations (8.2)]*.

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MM-398 (nanoliposomal irinotecan): emergence of a novel therapy for the treatment of advanced pancreatic cancer

Julia Carnevale[1] & Andrew H Ko*[1]

While progress in the treatment of advanced pancreatic cancer has accelerated in recent years, this malignancy continues to have an exceedingly poor prognosis, with no standard of care options beyond front-line chemotherapy. Currently, there are a number of new therapeutic agents in varying stages of clinical development, including molecularly targeted agents, immunotherapies, and modified versions of cytotoxics. MM-398, a novel nanoliposomal formulation of irinotecan, was designed to maximize tumor exposure while minimizing systemic toxicity due to its favorable pharmacokinetic profile. Overall, across multiple clinical trials in multiple disease indications, MM-398 has been shown to have a favorable safety and tolerability profile compared with standard irinotecan. Recent results of the Phase III NAPOLI-1 trial in patients with metastatic pancreatic cancer refractory to gemcitabine-based chemotherapy have shown a significant improvement in overall survival of MM-398 when combined with 5-fluorouracil/leucovorin, compared with 5-fluorouracil/leucovorin alone. This review focuses on the development and pharmacokinetic properties of MM-398, followed by evaluation of its safety and efficacy with a primary emphasis on clinical trials in advanced pancreatic cancer.

First draft submitted: 15 October 2015; Accepted for publication: 26 November 2015; Published online: 21 December 2015

KEYWORDS
• irinotecan • MM-398
• nanoliposomal
• pancreatic cancer • PEP02

Notorious for its late presentation at diagnosis as well as its poor prognosis, pancreatic cancer continues to be one of the most deadly of all human diseases. The estimated incidence rate of pancreatic cancer in the USA is 48,960, with most patients expected to succumb to their disease, even those with early stage disease who are able to undergo resection [1]. Globally, pancreatic cancer is the eighth leading cause of cancer deaths in men (138,100 deaths annually) and the ninth in women (127,900 deaths annually) [2]. While progress in the therapeutic development of this disease has historically taken a slow and painstaking course, littered with numerous negative trial results, over the past 5 years several new treatment options have emerged and research is accelerating in this area. This review will focus on the development and clinical evaluation of MM-398, a novel nanoliposomal irinotecan, which, based on recent clinical results, is now being incorporated into standard treatment paradigms for patients with advanced pancreatic cancer.

Overview of the market: currently available therapeutics

Following the approval of gemcitabine for advanced pancreatic cancer in 1996, there followed well over a decade of numerous negative Phase III clinical trials, most of which compared gemcitabine monotherapy to gemcitabine combined with either a second cytotoxic or a molecularly targeted agent

[1]Division of Hematology & Oncology, University of California San Francisco, San Francisco, CA, USA
*Author for correspondence: andrewko@medicine.ucsf.edu

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in the front-line setting. During this period, only one major randomized study demonstrated an improvement in survival in this patient population [3]; while it did lead to the approval of the EGFR inhibitor erlotinib as an option to add to gemcitabine, the degree of clinical benefit, and hence its widespread usage for this indication, is relatively modest.

This stretch of failed or clinically inconsequential trials changed in 2010, when results of the PRODIGE 4/ACCORD 11 trial demonstrated that the combination of 5-fluorouracil (5-FU), leucovorin (LV), irinotecan and oxaliplatin (FOLFIRINOX) led to a significant improvement in median overall survival (OS; 11.1 vs 6.8 months; hazard ratio [HR]: 0.57; $p < 0.001$) when compared with gemcitabine for advanced pancreatic cancer patients with good performance status in the first-line setting [4]. Subsequently, in 2013, the combination of gemcitabine with nanoparticle albumin-bound paclitaxel (nab-paclitaxel; Abraxane, Celgene, NJ, USA) was also approved as an acceptable regimen for the first-line treatment of advanced pancreatic cancer patients. This approval was based on the results from the Phase III MPACT trial that showed an improvement in median OS of this doublet when compared with gemcitabine monotherapy (8.5 vs 6.7 months; HR: 0.72; $p < 0.001$) [5]. Therefore, for patients with advanced pancreatic cancer and good performance status, FOLFIRINOX and gemcitabine plus nab-paclitaxel both represent viable options in the first-line setting. These two regimens have not been directly compared head-to-head, and absent any predictive biomarker that allows for selection of one versus the other, the choice depends on clinical and practical factors such as age, medical co-morbidities and patient preference.

Upon progression following first-line treatment, the appropriate choice of therapy (for those who remain well enough to continue with some form of active treatment) becomes less clear. Before the introduction of FOLFIRINOX, most studies were designed to assess the efficacy of second-line therapy following progression on gemcitabine or a gemcitabine-based regimen. A prior systematic review of 34 clinical trials evaluating the efficacy of different second-line regimens after gemcitabine-based therapy concluded that there does appear to be a survival advantage of continuing with some form of therapy as opposed to best supportive care [6]. However, whether combination therapy is superior to monotherapy in this setting is uncertain. A randomized Phase III trial from Germany, CONKO-003, showed that oxaliplatin when combined with folinic acid and fluorouracil (OFF) significantly extended median OS (5.9 vs 3.3 months; HR: 0.66; log-rank $p = 0.010$) compared with folinic acid and fluorouracil (FF) in patients with gemcitabine-resistant pancreatic cancer [7]. Conversely, a similar trial conducted in Canada called PANCREOX did not find any benefit of FOLFOX over 5-FU/LV alone [8].

Now that both FOLFIRINOX and gemcitabine/nab-paclitaxel are being commonly used as first-line treatment, there is considerable interest in evaluating how well each of these regimens works in patients who have already received the other. Randomized clinical trial data sequencing these regimens one after the other are still lacking, although some small series (primarily retrospective) and case reports do indicate modest efficacy of each in the second-line setting [7,9–13]. Recognizing the limitations of available data, current consensus guidelines [14] recommend that patients who have progressed on first-line gemcitabine-based therapy could be offered a 5-FU-based regimen, albeit without offering clearer direction as to whether and when it should be offered as part of combination therapy. On the other hand, for those who have started with a 5-FU-based regimen such as FOLFIRINOX, a gemcitabine-based regimen would be the next logical step – although again, without certainty as to the magnitude of benefit of using such a sequencing approach.

Overview of the market: competitor compounds in the clinic/late development

Despite the significant clinical advances that have been made recently for patients with advanced pancreatic cancer, overall prognosis remains poor, with only around 2% of patients surviving 5 years [15]. Thankfully, a number of research efforts are ongoing evaluating new therapeutic agents for this disease, including some in later stages of clinical development. For organizational purposes, each of these drugs can be placed into one of four broad categories: cytotoxics, stromal targeting agents, targeted signaling inhibitors and immune modulators, although many may actually mediate effects across multiple of these categories [16].

The two main cytotoxic agents furthest along in clinical development include nanoliposomal irinotecan (MM-398, Merrimack

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Pharmaceuticals, MA, USA), the main focus of this article, and evofosfamide (TH-302, Merck/EMD Serono, Rockland, MA, USA). MM-398 is a novel nanoparticle/liposome construct containing irinotecan designed to improve drug delivery to the tumor while minimizing toxic effects to the rest of the body. By changing the pharmacokinetic properties of irinotecan, MM-398 enhances tumor retention and increases the therapeutic window [17]. As will be discussed in detail below, Phase III clinical data has led to the recent approval of this agent by the US FDA that should lead to incorporation of this agent into our treatment algorithms for pancreatic cancer patients. Evofosfamide, meanwhile, is a prodrug of the cytotoxic alkylating agent, bromo-isophosphoramide mustard. This drug is activated under hypoxic conditions, providing a strong rationale for its evaluation in pancreatic cancer, an exceedingly hypoxic tumor. A randomized Phase II study comparing gemcitabine versus gemcitabine plus evofosfamide in patients with advanced pancreatic cancer demonstrated an improvement in median progression-free survival (PFS) with the addition of evofosfamide (5.6 vs 3.6 months; HR: 0.63; p = 0.005) [18], prompting a Phase III study, the MAESTRO trial, for this same patient population, results of which are pending.

Due to the impressive desmoplastic stroma that characterizes most pancreatic cancers, there has been considerable interest in targeting the stroma to alter the biology and the therapeutic accessibility of the tumor. Most advanced in clinical development is an agent termed PEGPH20 (Halozyme, CA, USA), a pegylated form of recombinant human hyaluronidase, which acts by breaking down hyaluronic acid, a major stromal component. Interim analysis of the Phase II HALO-109-202 study, a study of gemcitabine/nab-paclitaxel with or without PEGPH20 in previously untreated stage IV pancreatic cancer patients, showed intriguing early results. In a subset of patients with high levels of hyaluronic acid in their tumor specimens, overall response rate (71 vs 29%) and median PFS (9.2 vs 4.3 months) were significantly improved in the PEGPH20-containing arm [19]. These results have prompted the development of a global Phase III randomized controlled trial of gemcitabine/nab-paclitaxel with or without PEGPH20 specifically in patients with high intratumoral levels of hyaluronic acid, scheduled to start in 2016. Other ongoing studies are evaluating this same agent in combination with different chemotherapy backbones, including FOLFIRINOX.

A number of agents targeting specific signaling pathways that drive the growth, proliferation and survival of pancreatic cancer are also fairly far along in clinical development for advanced pancreatic cancer. Ruxolitinib (Jakafi, InCyte Pharmaceuticals, NY, USA), a small molecule inhibitor of the JAK1 and JAK2 kinases, may act both through direct anti-oncogenic mechanisms as well as by reducing the cytokine burden that contributes to pancreatic cancer-related cachexia. This agent was evaluated in combination with capecitabine in patients who had failed gemcitabine-based chemotherapy in the Phase II RECAP trial. In a preplanned analysis of patients with elevated levels of C-reactive protein (CRP), reflecting high levels of systemic inflammation, median survival was significantly longer in those patients who received the ruxolitinib/capecitabine combination as opposed to capecitabine alone (83 vs 55 days; HR: 0.47; p = 0.01) [20]. Based on these encouraging results, two Phase III trials (JANUS 1 and JANUS 2) have been initiated to evaluate this agent specifically in pancreatic cancer patients with high CRP levels. Another recently opened randomized Phase II trial, this one in the frontline setting, is evaluating MM-141, a bispecific IGFR/HER3 monoclonal antibody, in combination with gemcitabine plus nab-paclitaxel for patients with metastatic pancreatic cancer who have high serum levels of free IGF-1. The use of IGF-1 as a selection biomarker was informed by results from a preceding Phase I trial that showed that patients with higher circulating levels of this growth factor were able to stay on MM-141 approximately twice as long [21].

Finally, while pancreatic cancer has historically been considered an immune-privileged tumor, there have been a number of attempts to stimulate an immune attack in this disease, with different immune modulatory agents currently in various stages of clinical testing. Vaccination-based strategies represent one such approach far along in clinical development. For example, CRS-207 (Aduro Biosciences, Berkeley, CA, USA) is a live-attenuated *Listeria monocytogenes* vaccine vector genetically engineered to express mesothelin, a tumor-associated antigen expressed in the majority of pancreatic cancers. CRS-207 was shown to prolong survival when combined with the cellular vaccine GVAX in a

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Phase II trial of patients with chemorefractory metastatic pancreatic cancer, as compared with GVAX alone (median OS: 6.0 vs 3.4 months; HR: 0.4477; p = 0.0057) [22]. These results have led to successor trials comparing this vaccine-based strategy to standard cytotoxic therapy, as well as evaluating it in combination with immune checkpoint blockade (nivolumab). Other immune modulatory agents currently under evaluation in pancreatic cancer include monoclonal antibodies directed against PD-1, PDL-1 and CTLA-4; IDO inhibitors; CD40 agonist antibodies; and Bruton tyrosine kinase inhibitors. One very novel approach still in pilot stages of clinical evaluation in pancreatic cancer (as well as other solid tumors) consists of adoptive T-cell transfer, using a patient's own (autologous) T cells that are genetically engineered to express chimeric antigen receptors (CAR) that then recognize tumor-specific proteins such as mesothelin.

Introduction to MM-398

MM-398 (also known as nal-IRI; previously referred to as PEP02) is a novel nanoparticle/liposome construct containing irinotecan, which has been engineered to optimize drug delivery and retention in the tumor while minimizing systemic toxicity [17]. First approved in 1996, irinotecan is a semisynthetic analog of the natural alkaloid camptothecin that is currently used widely for a variety of solid tumor indications. By stabilizing the complex between topoisomerase I and bound DNA, irinotecan induces stalling of replication forks which ultimately leads to DNA strand breaks and inhibits replication [23,24].

While it has been used in the treatment of many different malignancies, irinotecan has historically been most heavily used in the treatment of colon cancer, typically in combination with 5-FU and LV (FOLFIRI) [25]. Based on results of the PRODIGE 4/ACCORD 11 trial, irinotecan is also frequently used in combination with oxaliplatin, fluorouracil and LV (FOLFIRINOX) as first-line treatment in patients with pancreatic cancer and good performance status [4]. The primary dose limiting toxicities of irinotecan include diarrhea and myelosuppression. By improving accumulation and activation specifically within the tumor, MM-398 was designed to increase the therapeutic window of irinotecan to achieve maximum efficacy while minimizing these toxicities.

Chemistry

Liposomal-based systems have been used to improve the delivery of other cytotoxic agents, such as the example of PEGylated liposomal doxorubicin, which is presently used for the treatment of breast cancer, Kaposi sarcoma and other anthracycline-sensitive malignancies. However, development of liposomal constructs for other chemotherapeutics has proven more challenging. In 2006, Drummond and colleagues developed a new intraliposomal drug stabilization technique to successfully encapsulate irinotecan into liposomal nanoparticles [17]. To achieve this, they used highly charged anions of sucrose octasulfate to trap the irinotecan in the liposome (see Figure 1). The triethylammonium component of this salt compound is thought to provide a source of cations that exchange for the influx of the irinotecan molecules. The sucrose octasulfate forms a stable complex with the irinotecan and the triethylammonium crosses the lipid bilayer as triethylamine. This leads to an extremely efficient loading system, ultimately packaging 109,000 drug molecules per particle, which far exceeds liposomal formulations of other chemotherapies [17].

Pharmacokinetics & pharmacodynamics

Irinotecan is a prodrug that is converted by nonspecific carboxylesterases into its active metabolite, SN-38, which is about 100- to 1000-fold more potent [26]. In addition, irinotecan exists in an equilibrium between its active lactone form (acidic conditions) and an inactive carboxylate form (basic conditions). SN-38 is glucuronidated in the liver by UGT1A1 and then cleared by biliary excretion.

Genetic variants of UGT1A1 produce significant variability in the metabolism and excretion of irinotecan, which contributes substantially to interpatient differences in tolerability of this agent, particularly hematologic toxicity (see Pharmacogenomics section below). The liposomal carrier system of MM-398 offers a way to protect irinotecan from premature activation, allowing a longer duration in circulation and improved biodistribution. It is thought that these nanoliposomes passively accumulate preferentially in the tumor due in part to what is known as the enhanced permeability and retention (EPR) effect [27]. The EPR effect in tumors is attributed to a combination of irregular and permeable blood vessels along with an impaired lymphatic system that leads

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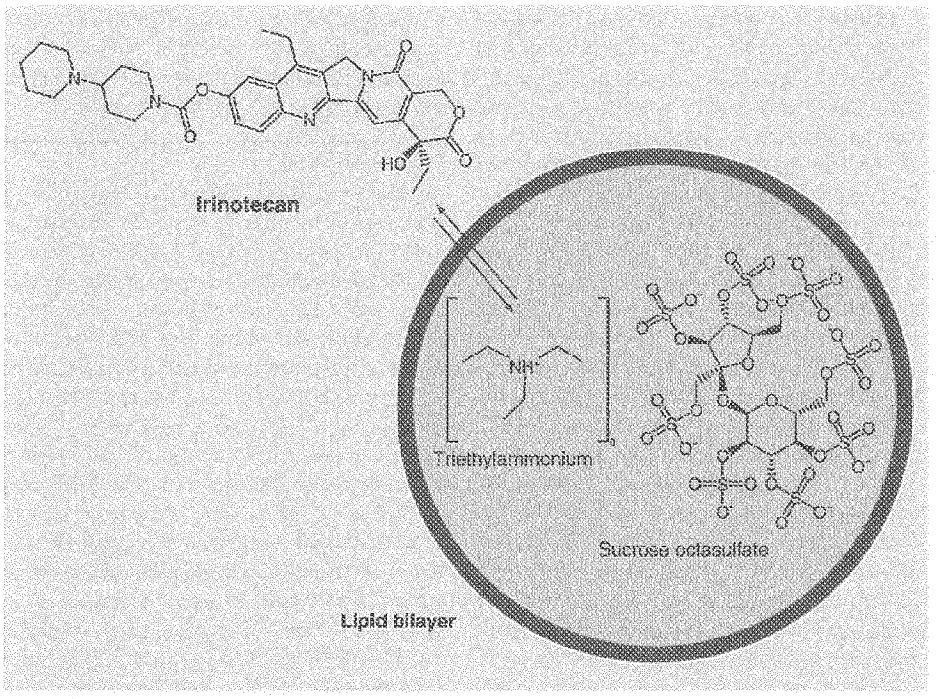


Figure 1. Depiction of exchange of triethylamine for irinotecan, which forms a stable complex with sucrose octasulfate inside the liposome.

to increased cumulative trapping of macromolecules in tumors relative to other tissues. Once deposited in the tumor, liposomes can be taken up by tumor-resident macrophages, which release the bound irinotecan and convert it to active SN-38 [17,28].

Preclinical pharmacokinetic (PK) analyses in rodents demonstrated a significant increase in the half-life of MM-398 in circulation compared with standard irinotecan, by a factor of 40. Standard irinotecan was rapidly cleared from the circulation, with only 2% of the injected dose remaining at 30 min and 35% present in the inactive carboxylate form. By comparison, 23.2% of MM-398 was detectable in circulation at 24 h, with no conversion of the irinotecan to either SN-38 or the carboxylate form in the blood [17]. These preclinical studies suggest that the nanoliposomal carrier could successfully provide protection from lactone hydrolysis or activation to the toxic SN-38 metabolite in circulation, thus increasing circulation time and tumor delivery. Additional studies of breast and colon cancer xenograft models in mice furthered the notion that this novel drug formulation can translate into improved antitumor activity. Specifically, when compared with standard irinotecan, nanoliposomal irinotecan led to greater inhibition of tumor growth and higher rates of complete tumor regressions, with less associated toxicity (transient weight loss representing the primary side effect) [17].

Two published studies in humans have reported on the phamacokinetics of MM-398. In a Phase I study of MM-398 in advanced solid tumor patients [29], the maximal tolerated dose (MTD) was determined to be 120 mg/m^2 every 3 weeks, with the primary toxicities including myelosuppression and diarrhea. In this study, the slow release of irinotecan from MM-398 resulted in a small volume of distribution and a prolonged terminal half-life of total irinotecan in circulation, while the active metabolite of irinotecan, SN-38, demonstrated a lower C_{max} and longer terminal half-life than respective values of these measures reported in the literature for standard irinotecan. Furthermore, the area under the curve (AUC) of SN-38 after 120 mg/m^2 MM-398 was in the same range as that achieved with 300–350 mg/m^2 of standard irinotecan described in the literature, suggesting that MM-398 confers an improved therapeutic window and may be a better choice for combination with other cytotoxic agents.

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Meanwhile, a randomized Phase II trial of MM-398 compared with either irinotecan or docetaxel as second-line treatment for patients with locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma [30] afforded the opportunity to directly compare the PK properties of MM-398 with those of standard irinotecan. PK values were analyzed for MM-398 dosed at 120 mg/m^2 and for irinotecan dosed at 300 mg/m^2. The dose-normalized PK results of these PK parameters evaluated are displayed in Table 1. Compared with standard irinotecan, MM-398 demonstrated a higher AUC, a lower clearance and smaller volume of distribution for total and encapsulated irinotecan. The active metabolite SN-38 showed a longer mean T_{max} (10.2 vs 2.1 h after infusion of 120 mg/m^2 MM-398 and 300 mg/m^2 irinotecan, respectively). After administration of MM-398, the dose-normalized C_{max} value for the formation of SN-38 was about 50% less than after infusion of standard irinotecan, indicating less premature activation of the prodrug irinotecan in the circulation in its encapsulated form. Additionally, the dose-normalized AUC_{0-t} and $AUC_{0-\infty}$ values of SN-38 following MM-398 administration were 3.3- and five-times higher, respectively, than those seen with standard irinotecan [30]. These favorable PK parameters confirmed that liposomally encapsulated irinotecan can stably circulate in plasma significantly longer than free irinotecan, enabling a slow release of irinotecan over time and increasing the likelihood of exposure of the tumor to the active SN-38 metabolite.

Pharmacogenetics

As discussed above, SN-38 is detoxified by *UGT1A1* to its inactive form SN-38 glucuronide (SN-38G), which is subsequently excreted via bile and urine. More than 60 genetic variants in the promoter region and exon 1 of *UGT1A1* have been identified, most of which are associated with reduced or absent enzymatic activity. This results in higher levels and prolonged exposure to SN-38, and consequently, a higher risk for irinotecan-associated toxicity, including neutropenia and diarrhea. The most well-studied among these *UGT1A1* genetic variants is the *28 polymorphism [31–33], which produces seven repeats of the two-base insertion TA in TATA box in the promoter region of the gene. Individuals who are homozygous for this *UGT1A1*28* allele (also known as *7/7*) are more than threefold likely to develop severe neutropenia compared with those with wild genotype (reviewed in [34]).

In the above referenced Phase I trial of MM-398, the study investigators were only able to analyze *UGT1A1* genetic polymorphisms in three study patients. Interestingly, the one

Table 1. Pharmacokinetic values from the Phase II trial by Roy *et al.*

Parameter	Unit	Drug given	Total irinotecan	MM-398/ irinotecan	SN-38	MM-398/ irinotecan
C_{max}	ng/ml	MM-398	60,842	36	9	0.498
		Irinotecan	4265		44	
T_{max}	h	MM-398	2.1	1.31	10.2	4.9
		Irinotecan	1.6		2.1	
AUC_{0-t}	h-ng/ml	MM-398	1,651,508	171	476	3.3
		Irinotecan	24,155		361	
$AUC_{0-\infty}$	h-ng/ml	MM-398	1,812,221	173	879	4.99
		Irinotecan	26,159		440	
Vss	ml/m^2	MM-398	2234	0.0227	NA	NA
		Irinotecan	98,527		NA	NA
CL	ml/h/m^2	MM-398	191	0.0148	NA	NA
		Irinotecan	12,886		NA	NA
$T_{½}$	h	MM-398	21.2	2.75	88.8	3.9
		Irinotecan	7.7		22.8	
$MRT_{0-\infty}$	h	MM-398	30.1	3.58	128.7	5
		Irinotecan	8.4		26	

AUC_{0-t}: Area under the curve at T_{max}; AUC0-∞: Area under the curve extrapolated to infinite time; Cl: Clearance; C_{max}: maximum concentration; MRT0-∞: Mean residence time; SN-38: Active metabolite of irinotecan; T½: Half-life; T_{max}: Time at which Cmax is reached; Vss: Volume of distribution at steady state.
Data taken from [30].

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Table 2. List of clinical trials evaluating MM-398

Year	Title	First author	Ref.
2010	Phase I study of liposome irinotecan (PEP02) in combination with weekly infusion of 5-FU/LV in advanced solid tumors	Chen *et al.*	[38]
2012	Phase I study of biweekly liposome irinotecan (PEP02, MM-398) in metastatic colorectal cancer failed on first-line oxaliplatin-based chemotherapy	Chen *et al.*	[39]
2013	A randomized Phase II study of PEP02 (MM-398), irinotecan or docetaxel as a second-line therapy in patients with locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma	Roy *et al.*	[30]
2013	A multinational Phase II study of nanoliposomal irinotecan sucrosofate (PEP02, MM-398) for patients with gemcitabine-refractory metastatic pancreatic cancer	Ko *et al.*	[35]
2015	Phase I study of nanoliposomal irinotecan (PEP02) in advanced solid tumor patients	Chang *et al.*	[29]
2015	PEPCOL: a randomized noncomparative Phase II study of PEP02 (MM-398) or irinotecan in combination with leucovorin and 5-fluorouracil as second-line therapy in patients with unresectable metastatic colorectal cancer – A GERCOR Study	Chibaudel *et al.*	[41]
2015	A Phase I trial of intravenous liposomal irinotecan in patients with recurrent high-grade gliomas	Clarke *et al.*	[42]
2015	Phase III study of MM-398 (nal-IRI), with or without 5-fluorouracil and leucovorin, versus 5-fluorouracil and leucovorin, in metastatic pancreatic cancer (mPAC) previously treated with gemcitabine-based therapy	Wang-Gillam *et al.*	[40]

patient experiencing treatment-related death due to myelosuppression on this study was heterozygous for both UGT1A1*6 and UGT1A1*28, and showed a higher $AUC_{0-\infty}$ and C_{max} of SN-38 (two- to three-fold higher than those of the other three patients treated at the same dose level) [29]. In the Phase II gastric cancer study, UGT1A1 variants were observed to correlate with toxicity; specifically, among patients treated with MM-398, a significantly higher frequency of grade 3–4 neutropenia was seen in patients heterozygous for UGT1A1*6 compared with wild-type patients [30]. A separate nonrandomized Phase II study of MM-398 monotherapy in gemcitabine-resistant metastatic pancreatic cancer patients included analysis of polymorphisms in both UGT1A1 and UGT1A9; however, no correlation was identified with either nonhematologic or hematologic toxicity [35].

It is worth noting a prior PK and dose-finding study performed by Innocenti and colleagues evaluating the MTD and dose-limiting toxicities in patients who were either homozygous or heterozygous carriers of the UGT1A1*28 variant. These results suggested that doses of irinotecan could be individualized based on *UGT1A1* genotype [36]. Despite these and other studies demonstrating differences in PK and toxicity based on *UGT1A1* genotype, there are currently not well-established guidelines on testing for *UGT1A1* polymorphisms in clinical practice. The package insert for irinotecan notes that a reduction in the starting dose of this drug should be considered for patients known to be homozygous for the UGT1A1*28 allele, although the appropriate dose reduction is not known [37]. It is reasonable to expect that similar fairly general recommendations will extend to MM-398 unless and until further larger-scale pharmacogenetic studies with this agent are conducted.

Clinical efficacy

A number of Phase I, II and III clinical trials of MM-398 have been conducted across many different solid tumors (listed in Table 2) [29,30,35,38–42]. As noted previously, Chang *et al.* [29] published a multicenter, first-in-human, open-label, Phase I, dose-escalation study of MM-398 in patients with advanced refractory solid tumors, including one patient with pancreatic cancer who had been refractory to gemcitabine and FOLFOX. Best response by RECIST criteria was partial response in 20% of the evaluable study cohort (including the patient with pancreatic cancer); with an overall disease control rate of 50%. The maximum tolerated dose was 120 mg/m^2.

Specific to pancreatic cancer, Ko *et al.* conducted a multinational Phase II study of MM-398 in patients with metastatic pancreatic cancer refractory to gemcitabine-based front-line chemotherapy [35]. Forty patients were enrolled and received MM-398 at a dose of 120 mg/m^2 every 3 weeks, with the option of dose-escalating to 150 mg/m^2 if the first cycle was tolerated well. The study met its primary end point with a 3-month OS rate of 75%, and median PFS and OS was 2.4 and 5.2 months, respectively. An objective response was noted in 7.5% of patients and disease control (partial response + stable disease) in 50%. Additionally, of the patients with an elevated CA19-9 level at baseline, 31.3% showed a decline in this tumor marker by greater

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than 50%.

Based on these results, an international randomized Phase III trial called NAPOLI-1 was developed to evaluate the efficacy of MM-398 for this same disease indication, with OS serving as the primary end point [40]. Patients were stratified according to albumin levels (less than or greater than 4.0 g/dl), Karnofsky Performance Status (70–80 vs 90–100) and ethnicity (Caucasian vs east Asian vs other). The study was originally designed with two arms only, comparing MM-398 (120 mg/m^2 every 3 weeks) to a control arm of 5-FU (administered as a weekly 24-h infusion at 2000 mg/m^2) plus LV (200 mg/m^2), for 4 out of 6 weeks. Subsequently, after the first 63 patients were enrolled, a third arm consisting of biweekly MM-398 (80 mg/m^2) plus 46-h infusion of 5-FU (2400 mg/m^2) and LV (400 mg/m^2) was added. The impetus for inclusion of this additional arm stemmed not only from the historical precedent of combining irinotecan with 5-FU in gastrointestinal malignancies, but also from preclinical evidence suggesting a biologic rationale to evaluate liposomal irinotecan in combination with other therapies. Specifically, studies of Irinophore-C (another liposomal irinotecan formulation) in subcutaneous and orthotopic mouse models showed that this agent increased microvessel density and thereby increased delivery of small molecule chemotherapies [43,44]. In a randomized Phase II trial of MM-398 in advanced colorectal cancer (PEPCOL), patients receiving MM-398 in combination with 5-FU and leucovorin demonstrated not only promising antitumor activity, but also an improved safety profile in comparison to subjects on the irinotecan + 5-FU/LV arm [41].

Overall, the three arms on NAPOLI-1 were well balanced according to age (median age 63 years), sex (54–59% male) and Karnofsky performance status (56% Karnofsky performance status 90–100). In the entire (intention to treat) cohort (n = 417), patients on the MM-398 + 5-FU/LV arm had an improved median survival compared with the 5-FU/LV alone arm (6.1 vs 4.2 months; HR: 0.67; p = 0.0122). Forest plot analyses showed that survival benefit for the MM-398-containing combination was retained across all subgroups, including according to Karnofsky performance status, age, ethnicity, baseline CA19-9 level and line of therapy (second-line or beyond). The combination arm also showed significant improvements in median PFS (3.1 vs 1.5 months; HR: 0.56, p = 0.0001), PFS at 12 weeks (57 vs 26%), objective response rate (16 vs 1%) and CA19-9 decline ≥50% (36 vs 12%). In a subsequent analysis of the per-protocol population (defined as those patients who received greater than 80% of their planned doses during the first 6 weeks of study treatment), improvement in median OS associated with MM-398 + 5-FU/LV was even more pronounced (8.9 vs 5.1 months; HR: 0.47; p = 0.0018). Notably, there was no statistically significant difference in median survival between patients receiving MM-398 monotherapy and those receiving 5-FU/LV alone (4.9 vs 4.2 months; HR: 0.99; p = 0.94). The main efficacy findings of NAPOLI-1 are summarized in Table 3.

Safety & tolerability

The primary adverse effects seen with MM-398, including bone marrow suppression and diarrhea, are similar to those typically associated with irinotecan. In the Phase II trial of MM-398 monotherapy in patients with gemcitabine-refractory metastatic pancreatic cancer, 15% of patients experienced grade 3 or 4 diarrhea and 30% developed grade 3–4 neutropenia (defined as an absolute neutrophil count less than 1000 cells/μl). Importantly, three of the 40 patients on this study had deaths attributable to treatment-related neutropenia [38]. Other notable grade 3–4 side

Table 3. Efficacy results of the Phase III trial NAPOLI-1 of MM-398 ± 5-fluorouracil/leucovorin compared with 5-fluorouracil/leucovorin alone.

Parameter	MM-398 + 5-FU/LV	5-FU/LV	MM-398
n	117	149	151
Median OS	6.1 months	4.2 months	4.9 months
Median PFS	3.1 months	1.5 months	2.7 months
PFS at 12 weeks	57%	28%	47%
ORR	16%	1%	6%
CA19-9 reduction ≥50%	36%	12%	31%

5-FU: 5-fluorouracil; LV: Leucovorin; ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival.

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Table 4. Adverse events ≥grade 3 in trials evaluating MM-398.

	Ko *et al.* (2013) [35]	Roy *et al.* (2013) [30]	Chang *et al.* (2015) [29]	Chibaudel *et al.* (2015) [41]	Wang-Gillam *et al.* (2015) [40]	
Study phase	II	II	I	II	III	III
Dosing regimen	MM-398 120 mg/m² q3 weekly	MM-398 120 mg/m² q3 weekly	MM-398 60–180 mg/m² q3 weekly	MM-398 80 mg/m² plus LV 400 mg/m² and 46-h infusion 5-FU 2400 mg/m² q2 weekly	MM-398 80 mg/m² plus LV 400 mg/m² and 46-h infusion 5-FU 2400 mg/m² q2 weekly	MM-398 120 mg/m² q3 weekly
Sample size	40	44	11	28	117	151
Nonhematologic toxicity (%)						
Diarrhea	15	27	33	33	13	21
Nausea	10	11	33	4	8	5
Vomiting	N/A	5	67	4	11	14
Fatigue	20	5	17	N/A	14	6
Hyponatremia	15	N/A	N/A	N/A	3	6
Anorexia	10	7	0	N/A	N/A	N/A
Hematologic toxicity (%)						
Neutropenia	30	7	17	11	20	16
Anemia	15	5	0	N/A	6	7
Thrombocytopenia	N/A	2	N/A	N/A	2	1

5-FU: 5-fluorouracil; LV: Leucovorin; N/A: Not applicable; q2 weekly: Once every 2 weeks; q3 weekly: Once every 3 weeks.

effects included fatigue (20%), anemia (15%), hyponatremia (15%), anorexia (10%) and nausea (10%). Meanwhile, in the Phase III NAPOLI-1 study, grade 3–4 diarrhea was reported in 13% of patients receiving the combination of MM-398 plus 5-FU/LV, and grade 3–4 neutropenia in an additional 20%. Other grade 3–4 toxicities associated with this combination included fatigue (14%), vomiting (11%), anemia (6%) and thrombocytopenia (2%).

In considering the toxicities of MM-398 in the future context in which it will be most commonly used in pancreatic cancer, perhaps the most appropriate comparison would be of (MM-398 + 5-FU/LV) to FOLFIRI. The previously discussed Phase II PEPCOL study, in which patients with advanced colorectal cancer received either MM-398 plus 5-FU/LV or FOLFIRI (including both FOLFIRI-1 and modified FOLFIRI-3 regimens), is instructive in that regard. This study utilized the same dose of 5-FU in all arms, and 80 mg/m² of MM-398 compared with 180 mg/m² total of irinotecan. In terms of grade 3–4 toxicity, lower levels of both neutropenia (10.7 vs 29.6%) and diarrhea (21.4 vs 33.3%) were observed in the MM-398 + 5-FU/LV arm compared with the FOLFIRI arm [41].

Taken together, the safety and toxicity profile of MM-398 appears to be comparable to, if not better than, that of irinotecan. Although administration of MM-398 as monotherapy is associated with substantial cytopenias, including several deaths associated with neutropenic complications in the Phase II pancreatic cancer trial, this does not appear to be as a major issue when using the biweekly dosing schedule of MM-398 with 5-FU/leuvocorin. Therefore, primary prophylaxis with granulocyte colony-stimulating factors is not necessarily indicated, except perhaps for patients with a prior history of neutropenia on prior antineoplastic agents. Table 4 provides a summary of the primary toxicity profile of MM-398 reported in various clinical studies, both as a single agent and in combination with 5-FU/LV.

Regulatory affairs

In 2011, MM-398 received orphan drug designation from both the US FDA and the EMA for the treatment of advanced pancreatic cancer. Based on the positive results of the NAPOLI-1 trial, in November 2014 the FDA granted MM-398 a Fast Track designation as second-line therapy, in combination with 5-FU and LV, for patients with metastatic pancreatic cancer who have progressed on a gemcitabine-based regimen. Merrimack Pharmaceuticals, in partnership with Baxter International, completed rolling submission of

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the New Drug Agreement (NDA) to the FDA in April 2015, where it was subsequently assigned priority review designation. This priority review assignment indicates that the FDA will take action on the marketing application within 6 months from the date of assignment (June 2015). In parallel, the EMA also accepted for review a Marketing Authorization Application (MAA) for the same indication.

In October 2015, MM-398 received official FDA approval, under the name Onivyde, for use in combination with fluorouracil and LV for the treatment of metastatic pancreatic cancer in patients who have previously received gemcitabine-based chemotherapy.

Conclusion

MM-398 is a novel nanoliposomal formulation of irinotecan designed to increase the drug payload to the tumor while minimizing systemic toxicity. There is now evidence from multiple clinical trials demonstrating improvement in the pharmacokinetic properties of MM-398 compared with standard irinotecan. Furthermore, at the maximum tolerated dose MM-398 appears to be as safe as, if not safer than, standard irinotecan. MM-398 is currently being tested across a range of different solid tumors, but has now completed Phase III evaluation in advanced pancreatic cancer. Based on results from the Phase III NAPOLI-1 trial of pancreatic cancer patients refractory to gemcitabine-based therapy, MM-398 monotherapy appears to perform similarly to 5-FU/LV, but significantly better in terms of OS and other clinical outcome measures when administered in combination with 5-FU/LV.

Future perspective

Based on the positive results of NAPOLI-1, MM-398 in combination with 5-FU/LV is the first therapeutic agent to gain approval specifically for second-line use in patients with metastatic pancreatic cancer. This regimen might now be expected to be used commonly in patients who have progressed on gemcitabine plus nab-paclitaxel, a first-line standard of care combination. It is also of interest whether the optimized PK and safety profile of MM-398 over standard irinotecan would make it an ideal substitute for irinotecan in the first-line FOLFIRINOX

EXECUTIVE SUMMARY

Mechanism of action

- MM-398 is a nanoliposomal formulation of irinotecan, designed to maximize tumor delivery and minimize systemic toxicity via optimized pharmacokinetic properties.

Pharmacokinetic properties

- Compared with standard irinotecan, MM-398 demonstrates a significantly increased area under the curve and half-life when both total irinotecan and SN-38 (the active metabolite) are measured in the blood.
- MM-398 also demonstrates a decreased clearance and decreased volume of distribution.
- Overall, this suggests a longer time in circulation and increased tumor exposure.

Clinical efficacy

- MM-398 has now been assessed in Phase I–III clinical trials in advanced pancreatic cancer patients.
- Based on results from the Phase III NAPOLI-1 trial of pancreatic cancer patients refractory to gemcitabine-based therapy, MM-398 significantly improves overall survival when combined with 5-fluorouracil/leucovorin compared with 5-fluorouracil/leucovorin alone.

Safety & tolerability

- The primary adverse effects of standard irinotecan are bone marrow suppression and diarrhea.
- Across studies, it appears that the safety and tolerability of MM-398 compares favorably to standard irinotecan.

Dosage & administration

- The maximum tolerated dose of MM-398 monotherapy has been determined to be 120 mg/m^2 every 3 weeks.
- When given in combination with 5-fluorouracil/leucovorin, MM-398 is dosed at 70 mg/m^2 every 2 weeks (this is the free base equivalent to the 80 mg/m^2 dose of the salt form used in the NAPOLI-1 trial), with 5-fluorouracil dosed at 2400 mg/m^2 (as a continuous infusion over 46 h) and leucovorin at 400 mg/m^2, every 2 weeks.

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regimen. This might represent a natural extension of MM-398's role in metastatic pancreatic cancer. Future clinical trials of MM-398, whether in the first-line setting or beyond, should also assess its combinability with other cytotoxics, including gemcitabine and nab-paclitaxel; as well as with novel therapeutics currently in development. It would also be instructive if such trials were to incorporate tumor biomarker evaluation as well as the pharmacodynamic effects of MM-398 on the tumor and its microenvironment. For instance, pre- and post-treatment evaluation of tumor stroma and microvasculature, macrophage composition and resultant small molecule penetrance, would be of great interest given the predicted modulation of MM-398 on these compartments based on preclinical studies.

Financial & competing interests disclosure

AH Ko has previously served as an advisory board member for Merrimack Pharmaceuticals, and currently receives funding support (paid to his institution) from Merrimack for the conduct of pancreatic cancer-specific clinical trials. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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U.S. National Library of Medicine
ClinicalTrials.gov archive

History of Changes for Study: NCT02631733

Liposomal Irinotecan and Veliparib in Treating Patients With Solid Tumors That Are Metastatic or Cannot Be Removed by Surgery

Latest version (submitted July 3, 2021) on ClinicalTrials.gov

- A study version is represented by a row in the table.
- Select two study versions to compare. One each from columns A and B.
- Choose either the "Merged" or "Side-by-Side" comparison format to specify how the two study versions are to be displayed. The Side-by-Side format only applies to the Protocol section of the study.
- Click "Compare" to do the comparison and show the differences.
- Select a version's Submitted Date link to see a rendering of the study for that version.
- The yellow A/B choices in the table indicate the study versions currently compared below. A yellow table row indicates the study version currently being viewed.
- Hover over the "Recruitment Status" to see how the study's recruitment status changed.
- Study edits or deletions are displayed in red.
- Study additions are displayed in green.

Study Record Versions

Version	A	B	Submitted Date	Changes
1	◉	◉	December 15, 2015	None (earliest version on record)
2	○	○	February 15, 2016	Arms and Interventions and Study Status
3	○	○	June 20, 2016	Study Status
4	○	○	June 21, 2016	Study Status
5	○	○	July 6, 2016	Study Status
6	○	○	July 11, 2016	Study Status
7	○	○	July 19, 2016	Recruitment Status, Study Status and Contacts/Locations
8	○	○	August 5, 2016	Contacts/Locations and Study Status
9	○	○	September 21, 2016	Recruitment Status, Study Status and Contacts/Locations
10	○	○	January 19, 2017	Study Status
11	○	○	January 20, 2017	Study Status
12	○	○	January 23, 2017	Study Status
13	○	○	January 24, 2017	Study Status
14	○	○	January 31, 2017	Study Status
15	○	○	March 1, 2017	Study Status
16	○	○	March 28, 2017	Study Status and Study Identification
17	○	○	May 8, 2017	Conditions and Study Status
18	○	○	May 19, 2017	Recruitment Status, Study Status, Outcome Measures, Contacts/Locations, Arms and Interventions, Study Description, Study Identification, Eligibility, Conditions and Oversight
19	○	○	May 23, 2017	Study Status
20	○	○	June 26, 2017	Study Status
21	○	○	June 30, 2017	Contacts/Locations and Study Status
22	○	○	July 7, 2017	Contacts/Locations and Study Status

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Version	A	B	Submitted Date	Changes
23	○	○	July 21, 2017	Contacts/Locations and Study Status
24	○	○	August 10, 2017	Study Status, Eligibility and Study Description
25	○	○	August 11, 2017	Contacts/Locations and Study Status
26	○	○	August 14, 2017	Contacts/Locations, Arms and Interventions and Study Status
27	○	○	August 19, 2017	Study Status
28	○	○	September 4, 2017	Arms and Interventions and Study Status
29	○	○	September 22, 2017	Contacts/Locations and Study Status
30	○	○	October 3, 2017	Contacts/Locations and Study Status
31	○	○	October 6, 2017	Contacts/Locations and Study Status
32	○	○	October 9, 2017	Contacts/Locations and Study Status
33	○	○	November 6, 2017	Study Status
34	○	○	December 11, 2017	Arms and Interventions and Study Status
35	○	○	January 12, 2018	Study Status, Eligibility and Outcome Measures
36	○	○	January 17, 2018	Contacts/Locations and Study Status
37	○	○	January 22, 2018	Study Status
38	○	○	March 2, 2018	Study Status
39	○	○	April 10, 2018	Contacts/Locations and Study Status
40	○	○	April 13, 2018	Contacts/Locations and Study Status
41	○	○	May 14, 2018	Outcome Measures, Contacts/Locations and Study Status
42	○	○	May 21, 2018	Study Status
43	○	○	June 25, 2018	Contacts/Locations and Study Status
44	○	○	July 9, 2018	Contacts/Locations and Study Status
45	○	○	July 11, 2018	Contacts/Locations and Study Status
46	○	○	July 16, 2018	Study Status
47	○	○	July 17, 2018	Recruitment Status, Study Status and Contacts/Locations
48	○	○	July 18, 2018	Study Status
49	○	○	August 28, 2018	Recruitment Status, Study Status, Contacts/Locations and Oversight
50	○	○	September 7, 2018	Study Status
51	○	○	September 13, 2018	Contacts/Locations and Study Status
52	○	○	October 9, 2018	Contacts/Locations and Study Status
53	○	○	October 31, 2018	Contacts/Locations and Study Status
54	○	○	December 14, 2018	Contacts/Locations and Study Status
55	○	○	January 17, 2019	Recruitment Status, Study Status and Contacts/Locations
56	○	○	February 14, 2019	Study Status
57	○	○	March 6, 2019	Contacts/Locations and Study Status
58	○	○	July 22, 2019	Study Status
59	○	○	August 12, 2019	Arms and Interventions and Study Status
60	○	○	November 14, 2019	Outcome Measures, Contacts/Locations, Eligibility, Arms and Interventions, Study Description, Oversight, Study Status and Study Identification

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Version	A	B	Submitted Date	Changes
61	○	○	November 16, 2019	Study Status
62	○	○	November 22, 2019	Study Status
63	○	○	December 9, 2019	Study Status
64	○	○	February 3, 2020	Study Status
65	○	○	March 2, 2020	Study Status
66	○	○	April 16, 2020	Study Status
67	○	○	May 2, 2020	Study Status
68	○	○	May 23, 2020	Study Status
69	○	○	June 18, 2020	Contacts/Locations and Study Status
70	○	○	June 24, 2020	Study Status
71	○	○	June 30, 2020	Study Status
72	○	○	July 2, 2020	Study Status
73	○	○	October 20, 2020	Arms and Interventions and Study Status
74	○	○	January 6, 2021	Study Status
75	○	○	January 7, 2021	Study Status
76	○	○	April 13, 2021	Arms and Interventions and Study Status
77	○	○	May 4, 2021	Arms and Interventions and Study Status
78	○	○	July 2, 2021	Study Status
79	○	○	July 3, 2021	Oversight and Study Status

Compare Comparison Format: ◉ Merged ○ Side-by-Side

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Study NCT02831733
Submitted Date: December 15, 2015 (v1)

Study Identification

Unique Protocol ID: NCI-2015-02125

Brief Title: Liposomal Irinotecan and Veliparib in Treating Patients With Solid Tumors That Are Metastatic or Cannot Be Removed by Surgery

Official Title: A Phase I Study of a Combination of MM-398 and Veliparib in Solid Tumors

Secondary IDs: NCI-2015-02125 [Registry Identifier: CTRP (Clinical Trial Reporting Program)]
9914 [National Cancer Institute LAO]
9914 [CTEP]

Study Status

Record Verification:	December 2015
Overall Status:	Not yet recruiting
Study Start:	May 2016
Primary Completion:	January 2018 [Anticipated]
Study Completion:	
First Submitted:	December 15, 2015
First Submitted that Met QC Criteria:	December 15, 2015
First Posted:	December 16, 2015 [Estimate]
Last Update Submitted that Met QC Criteria:	December 15, 2015
Last Update Posted:	December 16, 2015 [Estimate]

Sponsor/Collaborators

Sponsor:	National Cancer Institute (NCI)
Responsible Party:	Sponsor
Collaborators:	

Oversight

U.S. FDA-regulated Drug:	
U.S. FDA-regulated Device:	
Data Monitoring:	Yes

Study Description

Brief Summary: This phase I trial studies the side effects and best dose of veliparib when given together with liposomal irinotecan in treating patients with solid tumors that have spread to other parts of the body (metastatic) or cannot be removed by surgery. Liposomal irinotecan and veliparib may stop the growth of tumor cells by blocking some of the enzymes needed for cell growth.

Detailed Description: PRIMARY OBJECTIVES:

I. To evaluate the safety and tolerability of escalating doses of MM-398 (liposomal irinotecan) + veliparib combination.

II. To determine the maximum tolerated dose (MTD) and recommended phase 2 dose (RP2D) of the combination of MM-398 + veliparib.

SECONDARY OBJECTIVES:

I. To observe and record anti-tumor activity. II. To characterize the preliminary efficacy of the combination using key efficacy indicators, such as objective response rate, clinical benefit rate defined as complete response (CR), partial response (PR), or stable disease (SD) at 24 weeks, and progression free survival (PFS).

EXPLORATORY OBJECTIVES I. Imaging, tumor, and blood biomarkers to assess the sensitivity or resistance to each drug and correlation with clinical response.

OUTLINE: This is a dose-escalation of veliparib.

Patients receive liposomal irinotecan intravenously (IV) over 90 minutes on days 1 and 15 and veliparib orally (PO) on days 5-12 and 19-26 or 3-12 and 17-26. Courses repeat every 28 days in the absence of disease progression or unacceptable toxicity.

After completion of study treatment, patients are followed up for 4 weeks.

Conditions

Conditions: Estrogen Receptor Negative
HER2/Neu Negative
Neuroendocrine Neoplasm
Progesterone Receptor Negative
Stage IIB Cervical Cancer
Stage IIIA Cervical Cancer
Stage IIIB Cervical Cancer
Stage IIIB Non-Small Cell Lung Cancer
Stage IIIC Breast Cancer
Stage IV Breast Cancer
Stage IV Cervical Cancer
Stage IV Gastric Cancer
Stage IV Non-Small Cell Lung Cancer
Stage IV Ovarian Cancer
Stage IV Small Cell Lung Carcinoma
Triple-Negative Breast Carcinoma

Keywords:

Study Design

Study Type: Interventional

Primary Purpose: Treatment

Study Phase: Phase 1

Interventional Study Model: Single Group Assignment

Number of Arms: 1

Masking: None (Open Label)

Allocation: N/A

Enrollment: 48 [Anticipated]

Arms and Interventions

Arms	Assigned Interventions
Experimental: Treatment (liposomal irinotecan, veliparib) Patients receive liposomal irinotecan IV over 90 minutes on days 1 and 15 and veliparib PO on days 5-12 and 19-25 or 3-12 and 17-25. Courses repeat every 28 days in the absence of disease progression or unacceptable toxicity.	Drug: Ferumoxytol Non-Stoichiometric Magnetite Correlative studies Other Names: • Feraheme • Ferumoxytol Laboratory Biomarker Analysis Correlative studies Drug: Liposomal Irinotecan Given IV Other Names: • Irinotecan Liposome • Onivyde • PEP02 Procedure: Magnetic Resonance Imaging Correlative studies Other Names: • Magnetic Resonance Imaging Scan • Medical Imaging, Magnetic Resonance / Nuclear Magnetic Resonance • MRI • MRI Scan • NMR Imaging • NMRI • Nuclear Magnetic Resonance Imaging Drug: Veliparib Given PO Other Names: • ABT-888 • PARP-1 inhibitor ABT-888

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Outcome Measures

Primary Outcome Measures:

1. Incidence of adverse events, graded using the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0

   [Time Frame: Up to 4 weeks]

2. MTD and RP2D of liposomal irinotecan in combination with veliparib determined by incidence of dose limiting toxicities, graded using the NCI CTCAE version 4.0

   [Time Frame: 28 days]

Secondary Outcome Measures:

3. Clinical benefit rate defined as CR, PR, or SD assessed using RECIST version 1.1

   [Time Frame: At 24 weeks]

4. Objective response rate assessed using RECIST version 1.1

   [Time Frame: At 24 weeks]

5. PFS

   [Time Frame: Duration of time from start of treatment to time of progression or death, whichever occurs first, assessed up to 4 weeks after completion of study treatment]

6. Tumor response assessed using the revised Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1)

   [Time Frame: Up to 4 weeks after completion of study treatment]

Other Pre-specified Outcome Measures:

7. Biomarker levels
   Will be studied to assess the sensitivity or resistance to each drug and correlated with clinical response.

   [Time Frame: Up to 4 weeks after completion of study treatment]

Eligibility

Minimum Age: 18 Years

Maximum Age:

Sex: All

Gender Based:

Accepts Healthy Volunteers: No

Criteria: Inclusion Criteria:

- Patients must have pathologically confirmed diagnosis of one of the following solid tumors: cervical cancer, ovarian cancer, triple negative breast cancer (TNBC), non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), gastric cancer, and neuroendocrine tumors that is metastatic or unresectable and for which standard curative or palliative measures do not exist or are no longer effective
- Prior poly ADP ribose polymerase (PARP) inhibitor therapy is allowed
- Patients at the National Cancer Institute (NCI) site must be willing to undergo a pre-treatment ferumoxytol magnetic resonance imaging (MRI) (patients will be excluded from undergoing ferumoxytol MRI if they have evidence of iron overload, a known hypersensitivity to ferumoxytol or any other IV iron product, a documented history of multiple drug allergies, or those for whom MRI is otherwise contraindicated, including claustrophobia or anxiety related to undergoing MRI); this eligibility criterion applies only to patients enrolling at NCI
- Eastern Cooperative Oncology Group (ECOG) performance status =< 2 (Karnofsky >= 60%)
- Life expectancy of greater than 3 months
- Hemoglobin > 9 g/dL
- Leukocytes >= 3,000/mcL
- Absolute neutrophil count >= 1,500/mcL without the use of hematopoietic growth factors
- Platelets >= 100,000/mcL
- Total bilirubin within normal institutional limits
- Aspartate aminotransferase (AST)(serum glutamic oxaloacetic transaminase [SGOT])/alanine aminotransferase (ALT)(serum glutamate pyruvate transaminase [SGPT]) =< 2.5 x institutional upper limit of normal (=< 5 x upper limit of normal [ULN] is acceptable if liver metastases are present)
- Creatinine =< 1.5 x ULN OR creatinine clearance >= 60 mL/min/1.73 m^2 for patients with creatinine levels above institutional normal
- Women of child-bearing potential and men must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation; should a woman become pregnant or suspect she is pregnant while she or her partner is participating in this study

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she should inform her treating physician immediately; men and women treated or enrolled on this protocol must also agree to use adequate contraception prior to the study, for the duration of study participation, and 4 months after completion of veliparib, MM-398 and ferumoxytol MRI administration
- Ability to understand and the willingness to sign a written informed consent document
- IMAGING CORRELATIVE STUDY: Patients will be eligible to participate in the ferumoxytol (FMX) imaging study if they do not meet any of the following criteria
- Evidence of iron overload as determined by:
  - Fasting transferrin saturation of > 45% and/or
  - Serum ferritin levels > 1000 ng/ml
- A history of allergic reactions to any of the following:
  - Compounds similar to ferumoxytol or any of its components as described in full prescribing information for ferumoxytol injection
  - Any IV iron replacement product (e.g. parenteral iron, dextran, iron-dextran, or parenteral iron polysaccharide preparations)
  - Multiple drugs
- Unable to undergo MRI or for whom MRI is otherwise contraindicated (e.g. presence of errant metal, cardiac pacemakers, pain pumps or other MRI incompatible devices; or history claustrophobia or anxiety related to undergoing MRI)

Exclusion Criteria:

- Patients who have had chemotherapy or radiotherapy within 4 weeks (6 weeks for nitrosoureas or mitomycin C) prior to entering the study or those who have not recovered from adverse events due to agents administered more than 4 weeks earlier; patients must have completed prior biological therapies and/or targeted therapies >= 2 weeks prior to study enrollment; patients who have had radiation to the pelvis or other bone marrow-bearing sites will be considered on a case by case basis and may be excluded if the bone marrow reserve is not considered adequate (i.e. radiation to > 25% of bone marrow)
- Patients who are receiving any other investigational agents
- Patients with active brain metastases should be excluded from this clinical trial
- History of allergic reactions attributed to compounds of similar chemical or biologic composition to veliparib, MM-398 and ferumoxytol
- Clinically significant gastrointestinal (GI) disorders, including history of small bowel obstruction unless the obstruction was a surgically treated remote episode
- Active infection
- Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements
- Pregnant women are excluded from this study; breastfeeding should be discontinued if the mother is treated with any of these agents
- Human immunodeficiency virus (HIV)-positive patients on combination antiretroviral therapy are ineligible
- Patients who are taking medications which are strong inhibitors or inducers of cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4)

Contacts/Locations

Study Officials: Susan Bates
Principal Investigator
National Cancer Institute LAO

Locations: **United States, Maryland**

National Cancer Institute LAO
Bethesda, Maryland, United States, 20892
Contact: Contact: Susan E. Bates 301-402-1357 batess@helix.nih.gov
Contact: Principal Investigator: Susan E. Bates

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Abstract 2075: Differential tissue clearance results in improved therapeutic index for irinotecan liposome injection (ONIVYDE) when combined with the PARP inhibitor veliparib in preclinical cervical tumors

Alexander Koshkaryev, Jonathan Fitzgerald, Jaeyeon Kim, Ashish Kalra, Walid Kamoun, Sarah Blanchette, Li a Luus, Stephan Klinz, Ozan Alkan, Tad Kornaga, Susan Bates, Yves Pommier and Daryl C. Drummond

DOI: 10.1158/1538-7445.AM2016-2075 Published July 2016

Proceedings: AACR 107th Annual Meeting 2016; April 16-20, 2016; New Orleans, LA

Abstract

PARPs (poly ADP ribose polymerases) are a family of enzymes involved in DNA repair via two mechanisms: catalytic inhibition and trapping of PARP-DNA complexes. Inhibition of this repair pathway can result in cell death following DNA damage. Irinotecan liposome injection has shown promising preclinical and clinical activity in a range of cancer types, and was recently approved in the United States in combination with 5-FU/LV for patients with metastatic adenocarcinoma of the pancreas after disease progression following gemcitabine-based therapy. Irinotecan liposome injection is a highly stabilized liposomal formulation of irinotecan. It is hypothesized that combining PARP inhibitors with Top I inhibitors will result in increased efficacy compared to either agent alone. However, the preclinical promising activity has also given rise to unacceptable toxicity in the clinic for the combinations.

In preclinical study, compared with free irinotecan, irinotecan liposome injection has an extended PK profile with prolonged local tumor exposure of irinotecan and SN-38. Since SN-38 is cleared more quickly from normal tissues than from tumor, it is hypothesized that delayed dosing of the PARP inhibitor, veliparib, relative to irinotecan liposome injection will allow for the expected window of maximum irinotecan-induced toxicity to pass in the absence of concurrent veliparib toxicity. However, the tumor levels of SN-38 are predicted to be sustained by the time veliparib is given, therefore maintaining the ability of both drugs to act on tumor tissue simultaneously to maintain synergy.

To test the hypothesis that delayed dosing of veliparib relative to irinotecan liposome can alleviate systemic toxicity, a pre-clinical dose tolerability study was performed. Mice were dosed chronically with irinotecan liposome once weekly at various doses on Day 1, while veliparib was dosed once daily at a fixed dose for 3 consecutive days each week (either on Days 2-4, Days 3-5, or Days 4-6) and body weight was followed as a gross measure of toxicity. Toxicity of the combination was seen at the highest doses of irinotecan liposome when given in close proximity to the veliparib doses. However, this toxicity could be alleviated either by dose reducing irinotecan liposome or delaying the start of veliparib dosing. This dosing schedule was followed in studies in two cervical cancer tumor xenograft models, one in which veliparib alone was not efficacious, and a second in which neither irinotecan liposome or veliparib were efficacious as single agents, however the combination demonstrated tumor growth inhibition.

This promising preclinical regimen will be examined further in a clinical novel phase I study design led by the NCI/CTEP where both the dose and schedule will be optimized in parallel. The study is currently planned to be initiated in 2016.

Citation Format: Alexander Koshkaryev, Jonathan Fitzgerald, Jaeyeon Kim, Ashish Kalra, Walid Kamoun, Sarah Blanchette, Lia Luus, Stephan Klinz, Ozan Alkan, Tad Kornaga, Susan Bates, Yves Pommier, Daryl C. Drummond. Differential tissue clearance results in improved therapeutic index for irinotecan liposome injection (ONIVYDE) when combined with the PARP inhibitor veliparib in preclinical cervical tumors. [abstract]. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl):Abstract nr 2075.

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Livraghi and Garber *BMC Medicine* (2015) 13:188
DOI 10.1186/s12916-015-0425-1

BMC Medicine



Spotlight on breast cancer

REVIEW

Open Access



PARP inhibitors in the management of breast cancer: current data and future prospects

Luca Livraghi and Judy E. Garber*

Abstract

Poly(ADP-ribose) polymerases (PARP) are enzymes involved in DNA-damage repair. Inhibition of PARPs is a promising strategy for targeting cancers with defective DNA-damage repair, including *BRCA1* and *BRCA2* mutation-associated breast and ovarian cancers. Several PARP inhibitors are currently in trials in the adjuvant, neoadjuvant, and metastatic settings for the treatment of ovarian, *BRCA*-mutated breast, and other cancers. We herein review the development of PARP inhibitors and the basis for the excitement surrounding these agents, their use as single agents and in combinations, as well as their toxicities, mechanisms of acquired resistance, and companion diagnostics.

Keywords: BRCA, Breast cancer, PARP inhibitors, Poly(ADP-ribose) polymerases

Background

Modern strategies for the development of novel cancer therapies include agents targeting specific molecular defects that characterize certain cancer cells in order to increase treatment efficacy and reduce toxicities. In breast cancer, targeted therapies have long been effective, as agents targeting hormone receptors in tumors expressing them and as antibodies or tyrosine kinase inhibitors targeting overexpressed or amplified HER2 molecules. Breast tumors expressing none of these are called triple-negative breast cancers (TNBC), which comprise about 15 % of breast cancers overall, about 70 % of breast cancers in individuals harboring a germline *BRCA1* mutation, and 20 % in *BRCA2* mutation carriers [1–4]. The discovery of the family of nuclear enzymes poly(ADP-ribose) polymerases (PARPs) and their role in DNA-damage repair pathways opened the possibility of developing a new class of antineoplastic drugs with the ability to interfere with the DNA damage repair systems of cancer cells – PARP inhibitors (PARPi). One characteristic of *BRCA*-mutated cancers is defective function of one of the major DNA damage repair pathways, the homologous recombination (HR) pathway. The original concept of the activity of PARP inhibitors was that they acted through synthetic lethality by targeting the base excision repair pathway (BER); in tumor cells with defects in a different DNA repair mechanism, disruption of both pathways led to cell death. The preferential sensitivity of BRCA-associated breast and ovarian cancers was therefore predicted as the tumor cells are characterized by defective homologous recombination repair. Subsequently, PARPi have shown significant activity in BRCA-associated breast, ovarian, and other cancers [5, 6]. However, the activity in sporadic ovarian cancers suggests a more complex mechanism of action described below [7].

PARPs and DNA damage repair

PARPs are a family of enzymes involved in various activities in response to DNA damage [8]. Eighteen components of this family have been discovered; PARP-1 to -3 are so far the only members defined as DNA damage-dependent PARPs [9].

PARP activation, largely driven by DNA damage (other mechanisms may occur, as reviewed by Bürkle et al. [10]), determines post-transcriptional modification of nuclear proteins such as histones [9]. PARP-1 activation is one of the earliest responses to DNA damage in human cells [11, 12]. The ADP-ribosylation of histones and the recruitment of chromatin remodeling enzymes create a relaxed chromatin state that is appropriate for DNA repairing activities (Fig. 1a). The ADP-ribose polymer synthesized by PARP acts as a "flag" that drives the assembly of DNA-repair complex at sites of DNA damage, mainly promoting BER and single strand break repair (SSBR) pathways [9], while involvement of PARPs in

* Correspondence: judy_garber@dfci.harvard.edu
Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02215, USA

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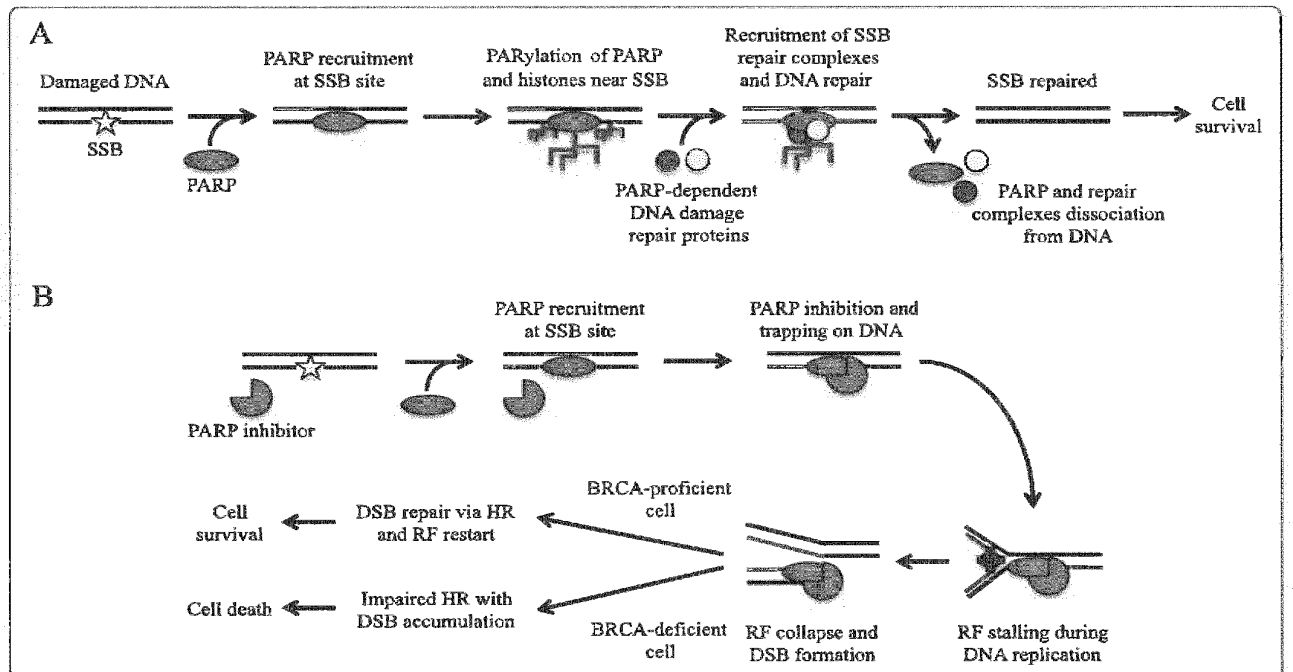


Fig. 1 Current model for PARP role in DNA damage repair and PARP inhibition – BRCA mutation synthetic lethality. **a** When single-strand break (SSB) is detected, PARP recruitment and activation leads to SSB repair through poly(ADP-ribosyl)ation (PARylation) of histones and chromatin remodeling enzymes, auto-PARylation of PARP, and recruitment of PARP-dependent DNA repair proteins. Repaired DNA can undergo replication determining cell survival. **b** In the presence of PARP inhibitors, PARPs recruited to DNA-damage sites are no longer able to activate PARP-dependent repair systems and to dissociate from DNA (due to catalytic activity inhibition and/or direct trapping), determining replication fork (RF) stalling during DNA replication. Stalled RF eventually collapse creating double strand break (DSB). DSB can be repaired by homologous recombination (HR) and replication may restart, leading to cell survival. In BRCA-deficient cells, HR is impaired, thus DSB cannot be efficiently repaired; in this context, DSB accumulate determining cell death

double strand break repair (DSB – an error-free DNA repair system) is likely limited [13].

Rationale for development of PARPi in breast cancer

Since cancer is a disease in which DNA replication is critical, replication errors are prominent, and deficiencies in DNA-repair pathways are common [14], the involvement of PARPs in DNA-repair pathways stimulated the development of agents capable of targeting PARP activity.

To maintain DNA integrity, HR-deficient cells rely on secondary DNA repair pathways, such as BER, SSBR, and non-homologous end joining. When PARP-dependent activation of BER/SSBR and non-homologous end joining is defective, cells rely on the HR pathway to restore DNA integrity. BRCA1 and BRCA2 proteins are key actors of the HR apparatus and deficiency of either (secondary to germline mutation in one copy and loss of heterozygosity inactivating or removing the other copy) results in inefficient activation of HR (Fig. 1b). Using *BRCA1*- and *BRCA2*-deficient cell lines and mouse xenografts, Bryant et al. [15] and Farmer et al. [16] demonstrated marked *in vitro* and *in vivo* cytotoxicity of PARPi monotherapy in tumor cells with intrinsic HR deficiency, with close to no effect on BRCA-proficient cells.

The model explaining this "synthetic lethality" effect of PARP inhibition in HR-deficient cells is comprehensively reviewed by Helleday [17]. Briefly, suppression of PARP catalytic activity blocks the formation of ADP-ribose polymers at site of SSB, hence PARP-dependent DNA-damage repair complexes cannot be efficiently recruited. Unrepaired SSB eventually lead to stalling of replication forks [17]. Stalled replication forks collapse into double strand breaks that are highly cytotoxic lesions if not repaired by HR [17], the repair mechanism inefficiently activated in *BRCA*-mutated cancers. Recent data suggest that another mechanism of action of PARPi, so-called "PARP trapping", is more important in determining PARPi cytotoxicity. Murai et al. [18] showed that PARPi prevent dissociation of recruited PARPs from DNA-damage sites: these stabilized PARP/DNA complexes determine stalling of the replication fork during DNA replication, with subsequent formation of double strand breaks.

The observation that *BRCA*-mutated breast cancers show an impairment in HR pathways [19], and that some sporadic TNBC are phenocopies of *BRCA1*-mutated cancers

(i.e. they display a phenotype resembling *BRCA1*-mutated cancers without harboring a *BRCA1* mutation, a feature also defined as "BRCAness", see below) [20, 21], led to exploration of the application of PARP inhibition to the treatment of breast cancer (BRCA-associated and TNBC).

Clinical application in breast cancer

Clinical development of PARPi started in 2003 and focused on two strategies: utilizing PARPi in combination with other drugs in a range of solid malignancies or using PARPi monotherapy in specific cancer types with features (like impairment of DNA-damage repair systems alternative to the PARP-dependent ones) that would be predicted to be highly sensitive to PARP inhibition. Testing of PARPi in combination with cytotoxic drugs showed the feasibility of this approach with overall good tolerability, but there was little evidence of activity in unselected patients [22]. In contrast, promising data emerged in the treatment of patients with breast and ovarian cancers [23, 24], the two malignancies most frequently associated with *BRCA* mutations.

Clinical testing of PARPi was initially slowed by negative results from a phase 3 trial of iniparib, a compound inaccurately classified as a PARPi [25]. Subsequently, it was shown that iniparib and its metabolites do not inhibit PARP in intact cells [26], and clinical development of genuine PARPi gained new vigor. Currently, five compounds with the ability to inhibit the activity of various PARPs are being investigated in clinical trials (Table 1). Below, we will present the most important findings from phase 1 and 2 clinical trials evaluating the efficacy of PARPi in the treatment of breast cancer. These data are also summarized in Tables 2 and 3.

Clinical trials in advanced disease

PARPi as single agent therapy

Following the demonstration by Bryant and Farmer [15, 16] of the cytotoxic effect of PARP inhibition in HR-deficient cells, there was interest in studying the activity of PARPi as monotherapy in solid tumors. In earlier studies, the population enrolled in these trials was not restricted to patients with known *BRCA* mutations, but encompassed also those whose cancer displayed a phenotype similar to *BRCA*-mutated cancers. Clinically, this group included triple-negative breast cancers and high-grade serous or poorly differentiated ovarian cancer. The term "BRCAness" was introduced to identify sporadic tumors that shared common phenotypic features with familial BRCA tumors [20]. Attempts to identify cancers with BRCAness included evaluation of epigenetic silencing of *BRCA* genes [27], measurement of levels of proteins involved in HR [28], and of foci of DNA-repair proteins like gammaH2AX [5, 29]. However, after preliminary data showing minimal efficacy of PARPi in sporadic breast cancers, some of the trials were amended to enrich the study cohorts for BRCA-associated tumors [5, 30].

Initial phase 1 testing of olaparib as monotherapy in BRCA-associated breast and ovarian cancers showed encouraging results: 47 % of patients with BRCA-associated breast, ovarian, or prostate cancers treated with olaparib achieved a partial response, and 63 % of them derived clinical benefit (tumor marker decrease or radiologic response or stable disease for 4 or more months) [5]. A phase 1 study of niraparib in patients with advanced solid tumors enriched for BRCA-associated cancers reported an overall response rate of 40 % (8 of 20) in patients with BRCA-associated ovarian cancer and 50 % (2 of 4) in patients with

Table 1 PARPi compounds in clinical development

Drug name	Pharmaceutical company	Current investigational phase in breast cancer
Olaparib (AZD2281)	AstraZeneca	Phase 3 studies in adjuvant and advanced settings in germline BRCAm breast cancer
Veliparib (ABT-888)	Abbvie	Phase 3 study in neoadjuvant setting in combination with carboplatin and standard therapy in triple-negative breast cancer
		Phase 2/3 studies in advanced setting as combination therapy in germline BRCAm breast cancer
Niraparib (formerly MK-4827)	Tesaro	Phase 3 study in advanced setting in germline BRCAm breast cancer
Talazoparib (BMN-673)	BioMarin Pharmaceuticals	Phase 3 study in advanced setting in germline BRCAm breast cancer
		Phase 2 studies in advanced setting in BRCAm breast cancer
		Phase 2 study in advanced setting in germline BRCA intact breast cancer
		Phase 2 study in neoadjuvant setting in BRCAm breast cancer
Rucaparib (formerly AG-14699)	Clovis Oncology	Phase 2 study in advanced setting in patients with known germline BRCAm solid tumors
		Phase 2 study in adjuvant setting in triple-negative breast cancer or germline BRCAm breast cancer
CEP-9722	Teva Pharmaceutical Industries	Phase 2 study in advanced setting in solid tumors

BRCAm, *BRCA1/2* mutation-associated

Table 2 Phase 1/2 studies of PARPi monotherapy in metastatic breast cancers, with spotlight on BRCA mutated patients

Study Name (NCT)	Ref.	Phase	Tumor type	No. of patients [total/BC (BRCAm BC)]	Investigation arm	Primary endpoint	Overall results	Results in BRCAm BC	Additional notes
Olaparib									
NCT00516373	[5]	1	Solid tumors	60/9 (3)	Olaparib (10–600 mg bid)	PK, PD, safety and tolerability	ORR: 15 %	ORR 33 %	One CR in BRCAm BC lasting more than 60 weeks
NCT00572364	[119]	1	Solid tumors	12/4 (NK)	Olaparib (100–400 mg bid)	Safety and tolerability	ORR: 8 %	–	One patient with BC and family history of BC had PR for 13 months
NCT00494234	[34]	2	BRCAm BC	54/54 (54)	C1: olaparib (400 mg bid)	ORR	–	ORR C1: 41 %	
					C2: olaparib (100 mg bid)			ORR C2: 22 %	
NCT00679783	[7]	2	TNBC or BRCAm BC, HGSOC or BRCAm OC	90/26 (10)	Olaparib (400 mg bid)	ORR	ORR in BC: 0 % ORR in OC: 29 %	ORR: 0 % 50 % of unconfirmed PR (by RECIST)	Evidence of activity in non-BRCAm OC and platinum-resistant OC
NCT01078662	[6]	2	BRCAm solid tumors	317/62 (62)	Olaparib (400 mg bid)	ORR	ORR: 26 %	ORR: 13 % DS ≥ 8 weeks: 47 %	Mean number of previous regimens for advanced disease: 4.6
Veliparib									
NCT00892736	[120]	1	TNBC, HGSOC and BRCAm BC and OC	98/35 (14)	Veliparib (50–500 mg)	Tolerability	ORR in BRCAm: 24 % ORR in BRCA wt: 4 %	ORR: 29 % CBR: 57 %	
Talazoparib									
NCT01286987	[32]	1	Solid tumors	39/8 (6)	Talazoparib (25–1100 μg)	PK, PD, safety and anti-tumor activity	ORR: 65 % in BRCAm OC	ORR: 33 %	
Niraparib									
NCT00749502	[31]	1	Solid tumors	100/12 (4)	Niraparib (30–400 mg)	Safety and tolerability	ORR: 18 % in overall population, 40 % in BRCAm OC	ORR: 50 %	
Rucaparib									
NCT01482715	[33, 121]	1–2	BRCAm BC and OC	56/27 (27)	Rucaparib (18 mg/m^2)	ORR	Data mixed between OC and BC, at RP2D ORR: 80 % (4/5)	–	

BC Breast cancer, *BID* bis in die, *BRCAm BRCA1/2* mutation-associated, *CBR* Clinical benefit rate; *CR* Complete response, *DS* Disease stabilization, *HGSOC* High-grade serous ovarian cancer, *NK* Not known, *OC* Ovarian cancer, *ORR* Objective response rate, *PD* Pharmacodynamics, *PK* Pharmacokinetics, *RP2D* Recommended phase 2 dose, *TNBC* Triple-negative breast cancer, *WT* Wild type
Clinical benefit: CR + PR + SD for ≥24 weeks

Table 3 Phase 1/2 studies of PARPi as combination therapy in metastatic breast cancers, with spotlight on BRCA mutated patients

Study name (NCT)	Ref.	Phase	Tumor type	No. of patients [total/BC (BRCAm BC)]	Investigation arm	Primary endpoint	Overall results	Results in BRCAm BC	Additional notes
Olaparib									
NCT00707707	[122]	1	TNBC	19/19 (NK)	Olaparib (200 mg bid) + PTX (90 mg/m^2)	Safety and tolerability	ORR: 37 %	–	First- or second-line treatment only
NCT00710268	[79]	1	Solid tumors	12/3 (NK)	Olaparib (100–400 mg bid) + BEV (10 mg/kg)	Safety and tolerability	No data on response reported	–	No grade 3 or 4 hematologic toxicities
NCT00782574	[23]	1	BC, OC, peritoneal cancer, pancreatic cancer	54/42 (17)	Olaparib (50–200 mg bid continuously vs. intermittent) + CDDP (75 mg/m^2)	Safety and tolerability	ORR: 41 %	ORR: 71 %	Continuous olaparib schedules not tolerable (hematologic toxicity)
NCT01116648	[45]	1	TNBC, OC	28/8 (3)	Olaparib (100–400 mg bid) + cediranib (20–30 mg)	Safety and tolerability	Overall ORR: 29 % BC ORR: 0 % BC CBR: 29 %	ORR: 0 %	
NCT01445418	[123]	1	BRCAm OC and BC	45/8 (8)	Olaparib (100–400 mg bid) + CBDCA (AUC 3–5)	Safety and tolerability	ORR: 52 %	ORR: 88 %	One CR in BRCAm BC for 3 months
Veliparib									
NCT00535119	[124]	1	Solid tumors	68/14 (NR)	Veliparib (20–120 mg) + CBDCA (AUC 5–6) + PTX (150–200 mg/m^2)	PK, safety and tolerability	ORR: 19 %	–	One CR in BC
NCT00740805	[125]	1	Solid and hematologic tumors	18/14 (5)	Veliparib (50–150 mg) + DOX (60 mg/m^2) + CYC (600 mg/m^2)	Tolerability	No overall results reported	ORR: 60 %	Expansion cohort study in BC ongoing
NCT01063816	[126]	1	Solid tumors	59/10 (NK)	Veliparib (250 mg bid) + CBDCA (AUC 4) + GEM (800 mg/m^2)	PK, safety and tolerability	ORR: 22 %	–	
NCT01104259	[53]	1	TNBC or BRCAm BC	45/45 (12)	Veliparib (20–300 mg) + CDDP (75 mg/m^2) + VNR (25 mg/m^2)	Tolerability	ORR: 55 %	ORR: 73 %	
NCT01251874	[127]	1	BC	44/44 (16)	Veliparib (50–200 mg) + CBDCA (AUC 5–6)	Safety and tolerability	ORR: 19 %	ORR: 25 %	
NCT01445522	[128]	1	Solid tumors and lymphomas	35/12 (NK)	Veliparib (20–80 mg) + CYC (50 mg)	Safety and tolerability	ORR: 20 %	–	
Not reported	[54]	1	BRCAm BC	26/26 (26)	Veliparib (50–200 mg) + CBDCA (AUC 5–6)	Safety and tolerability	–	ORR: 46 % CBF: 74 %	
NCT01281150	[55]	1	Solid tumors	30/24 (5)	Veliparib (50–200 mg) + PTX (80 mg/m^2) + CBDCA (AUC 5–6)	Tolerability	ORR: 48 %	ORR: 60 %	ORR in non-mutated BRCA BC: 67 %

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Table 3 Phase 1/2 studies of PARPi as combination therapy in metastatic breast cancers, with spotlight on BRCA mutated patients *(Continued)*

NCT01009788	[52]	2	BC	41/41 (8)	Veliparib (40 mg bid) + TMZ (150 mg/m^2)	ORR	ORR: 13 %	ORR: 50 % CBF: 63 %	Expansion cohort with additional 20 patients with *BRCA1/2* mutations: ORR 15 %, CBR: 45 %
Rucaparib									
NCT01009190	[129]	1	Solid tumors	23/5 (NK)	Rucaparib (80–360 mg) + CBDCA (AUC 3–5)	Safety and tolerability	DCR: 50 %	-	
CEP-9722									
NCT00920595	[130]	1	Solid tumors	26/7 (NK)	CEP-9722 (150–1000 mg) + TMZ (150 mg/m^2)	PK, PD, safety and anti-tumor activity	ORR: 5 %	-	

BC Breast cancer, *BEV* Bevacizumab, *BID* bis in die, *BRCAm BRCA1/2* mutation-associated, *CBDCA* Carboplatin, *CBR* Clinical benefit rate, *CDDP* Cisplatin, *CR* Complete response, *CYC* Cyclophosphamide, *DCR* Disease control rate, *DOX* Doxorubicin, *DS* Disease stabilization, *GEM* Gemcitabine, *HGSOC* High-grade serous ovarian cancer, *NK* Not known, *OC* Ovarian cancer, *ORR* Objective response rate, *PD* Pharmacodynamics, *PK* Pharmacokinetics, *PTX* Paclitaxel, *RP2D* Recommended phase 2 dose, *TMZ* Temozolomide, *TNBC* Triple-negative breast cancer, *VNR* Vinorelbine, *WT* Wild type
Clinical benefit: CR + PR + SD for ≥24 weeks
Disease control: CR + PR + SD for ≥12 weeks

BRCA-associated breast cancer [31]. Talazoparib monotherapy has shown antitumor activity in patients with *BRCA* mutations, with an objective response rate of 65 % in ovarian and peritoneal tumors and 33 % (2 of 6 patients) in breast cancers [32]. Data presented at ASCO 2014 on single agent rucaparib showed efficacy in BRCA-associated ovarian, breast, and pancreatic cancers [33].

These data from phase 1 trials guided the development of phase 2 studies in the population of patients with BRCA-associated cancers or with cancer usually associated with "BRCAness", namely triple-negative breast cancer and high-grade serous ovarian cancer (HGSOC).

Tutt et al. [34] reported efficacy of olaparib as monotherapy in 54 patients with advanced breast cancer and germline *BRCA1/2* mutations. At the maximum tolerated olaparib dose of 400 mg bid, a 41 % objective response rate was observed, with responses in both TNBC and hormone receptor-positive HER2-negative patients. Toxicities were generally manageable, with treatment-related adverse events reported in 81 % of patients, but grade 3 or 4 events occurred in only 24 % of patients. Efficacy data from this study compare favorably with response rates in studies of single agent cytotoxics (capecitabine [35], vinorelbine [36], eribulin [37], ixabepilone [38–40]) and of new anti-HER2 targeted therapies (pertuzumab [41] and T-DM1 [42]) in advanced breast cancer treatment. Similar results from a parallel phase 2 study of olaparib monotherapy in recurrent ovarian, fallopian tube, or peritoneal cancers were reported by Audeh et al. In germline *BRCA1/2* mutation-positive patients, the objective response rate was 33 % [43]. It should be noted that in both trials, for the first time, a documented germline *BRCA* mutation was an enrollment criterion [34, 43].

Gelmon et al. [7] assessed safety and efficacy of olaparib as a single agent in HGSOC and TNBC in an important trial that also demonstrated the feasibility of pre- and post-treatment biopsies. While sustained responses were documented in HGSOC, no confirmed objective response was shown in TNBC, regardless of *BRCA*-mutation status, although 50 % of *BRCA*-mutation carriers had a greater than 30 % reduction in the target lesion. The authors speculated that the lack of evidence of efficacy in BRCA-associated breast cancers in this trial could be due to chance because of small sample size or population characteristics (heavily pretreated patients) [7].

Kaufman et al. [6] reported data of a phase 2 study (NCT01078662) of olaparib monotherapy in 298 patients with diverse recurrent cancers (mostly ovarian, breast, pancreatic, and prostate) and confirmed *BRCA1/2* mutations (a study design called "basket trial"). Breast cancer tumor response rate was 12.9 % in 62 patients, and 47 % of patients had disease stabilization for ≥8 weeks. The lower objective response rate in this study compared with previous studies [5, 34] could be due to the fact that the study population was more heavily pretreated than in other trials (mean of 4.6 prior chemotherapy regimens in the metastatic setting vs. 3 in Tutt et al. [6]).

When tested in ovarian cancer, PARPi showed efficacy regardless of *BRCA* status. In the previously cited Gelmon et al. [7] study, olaparib induced sustained responses in non-*BRCA* mutant HGSOC. Responses to olaparib were also observed in ovarian cancer patients with wild type or unknown *BRCA* status in a study of maintenance therapy after platinum-based chemotherapy [44] and in a study of olaparib plus cediranib [45]. Molecular studies suggested that up to 20 % of HGSOC lose *BRCA1* or *BRCA2* function through epigenetic events [46], thus expressing an HR-deficient phenotype with sensitivity to PARPi even in the absence of somatic/germline *BRCA* mutation.

Studies of veliparib monotherapy in metastatic breast cancer are currently in progress [47, 48]; data on veliparib efficacy as single agent in gynecological cancers are already available. Coleman et al. [49] reported data from a multicenter phase 2 study in BRCA-associated persistent or recurrent ovarian, fallopian tube, or primary peritoneal cancer: objective response rate to single agent veliparib was 26 % and progression-free survival at 6 months was 54 %, without significant difference between platinum-sensitive or platinum-resistant tumors.

PARPi in combination therapy

PARPi have been tested in the treatment of metastatic breast cancer in combination with multiple compounds in phase 1 and 2 studies [22]. Preclinical data showed that veliparib exerts remarkable synergic activity with other cytotoxic compounds [50]: in particular, veliparib enhanced temozolomide's cytotoxic effect even in tumor types not typically responsive to temozolomide [51] with a good safety profile. Veliparib has been further clinically explored mainly as a part of combination therapy. In a phase 2 trial in BRCA-associated breast cancers, treatment with veliparib and temozolomide offered a response rate of 22 % and a clinical benefit rate of 50 % (defined as complete response, partial response, or stable disease) [52]. Efficacy was successively confirmed in a larger expansion cohort with patients previously treated with platinum compounds or PARPi [30].

Other combinations between PARPi and chemotherapy drugs have been proven effective in early clinical trials: the best results in terms of efficacy emerged from combination with cisplatin [23, 53] and carboplatin [54, 55], as well as topotecan [56], with response rates in BRCA-related breast cancers up to 73 % [23, 53]. Contrasting data about the safety of the combination therapy approach emerged from these studies. The combination topotecan-olaparib showed dose-limiting hematological adverse events at subtherapeutic doses of olaparib [57]; in contrast, veliparib combinations have been better tolerated overall.

It is still not clear which is the best chemotherapeutic companion for a PARPi, and studies show that different PARPi may combine more or less efficiently with cytotoxic drugs with different mechanisms of action [58, 59]. The differences in synergistic effect between cytotoxic drugs and PARPi may be explained by PARPi mechanisms of action. Indeed, some PARPi exert their cytotoxic effect mainly suppressing PARPs' catalytic activity (veliparib), while others more by trapping PARPs to DNA (olaparib, talazoparib, rucaparib, niraparib) [18]. It has been proposed that PARP trapping is synergistic with alkylating agents, while PARP catalytic inhibition synergizes with topoisomerase I inhibitors [58]. In preclinical models, proliferation of breast cancer cells is more potently suppressed when both mechanisms of PARP inhibition are present [18]. On the other hand, the higher toxicity of this class of PARPi may render them more toxic in combination with cytotoxic therapies.

Ongoing studies in the metastatic setting

Ongoing randomized phase 3 studies of PARPi in metastatic breast cancer are limited to patients with documented *BRCA1/2* mutations (Table 4). Three parallel study designs will test oral PARPi monotherapy vs. physician's choice single agent chemotherapy in breast cancer patients with PARPi-naive metastatic disease with germline *BRCA1/2* mutations: BRAVO (niraparib, NCT01905592 [60]), EMBRACA (talazoparib, NCT01945775 [61]), and OlympiAD (olaparib, NCT02000622 [62]). Finally, study NCT02163694 [63] will test the efficacy of veliparib versus placebo in combination with carboplatin and paclitaxel in HER2-negative metastatic or locally advanced, unresectable, BRCA-associated breast cancer.

Results from these studies are eagerly awaited and, if positive, will form the basis of applications for Food and Drugs Administration approval of PARPi for the treatment of metastatic BRCA-associated breast cancer. Approval will require an acceptable safety profile (see below) in a well-characterized and defined target population that currently lacks a specific targeted therapy. In 2014, both the European Medicines Agency and the Food and Drugs Administration [64, 65] granted accelerated approval to olaparib in high-grade serous ovarian, fallopian tube, and primary peritoneal cancer based on the results of two phase 2 trials [44, 66].

Going beyond the metastatic setting

Conventionally, new antineoplastic drugs are tested as adjuvant treatments for breast cancer after solid data from phase 3 trials in the metastatic setting become available. In the case of PARPi, the remarkable activity of olaparib and veliparib in multiple phase 2 trials and their manageable toxicity profiles have led to trials of several PARPi in the adjuvant and neoadjuvant settings (Table 4). The adjuvant trial OlympiA is evaluating 1 year of the PARPi olaparib [67]. Data for the acceptability of olaparib given for extended periods of time come from a phase 2 study of single agent olaparib as maintenance therapy in platinum-sensitive ovarian cancer – median duration of treatment 206 days – but some patients stayed on the medication for years [44].

The OlympiA trial (NCT02032823 [68]) will assess the efficacy and safety of up to 12 months of olaparib versus placebo as adjuvant treatment in patients with germline *BRCA1/2* mutations and high-risk hormone receptor-negative HER2-negative primary breast cancer who have completed definitive local treatment and neoadjuvant or adjuvant chemotherapy. Eligibility criteria have recently been expanded to allow for enrollment of high-risk hormone receptor-positive patients. Randomization will be stratified by prior neoadjuvant versus adjuvant chemotherapy, and according to the use of prior platinum-based chemotherapy for breast cancer. The post-neoadjuvant treatment group will comprise patients in whom pathologic complete response was not achieved following at least six cycles of neo-adjuvant chemotherapy. The primary end point will be interval disease-free survival, and the secondary end points will be overall survival, distant disease-free survival, and the development of new primary invasive cancers.

Rucaparib is being tested in a phase 2 trial as adjuvant treatment for TNBC or *BRCA*-mutated HER2-negative breast cancers with residual disease after preoperative chemotherapy (NCT01074970) [69]; preliminary data presented at ASCO 2014 showed no improvement in 1-year disease-free survival with rucaparib plus cisplatin versus cisplatin alone in the intent-to-treat population; rucaparib did not add substantial toxicity to the cisplatin treatment [70].

The I-SPY2 study assesses sequential novel agents in the neoadjuvant treatment of breast cancer. In the I-SPY-2 trial assessing the addition of veliparib and carboplatin to standard neoadjuvant therapy in TNBC, an estimated 52 % pathologic complete response rate was observed in the experimental arm versus 26 % in the standard treatment arm [71]. In the cooperative group neoadjuvant trials GeparSixto and Alliance 40603, the addition of carboplatin to standard neoadjuvant chemotherapy increased pathological complete response rates in TNBC from 42.7 % to 53.2 % and from 41 % to 54 %, respectively [72, 73]. In GeparSixto, this effect is most evident in patients with germline *BRCA1/2* or *RAD51* mutations (the pathological complete response rate with carboplatin was 66.7 % versus 43.5 % without carboplatin). Participants are currently being accrued to a randomized three arms phase 3 trial that will test the efficacy of the addition of carboplatin plus veliparib, carboplatin alone, or placebo to standard neoadjuvant chemotherapy (Brightness Study – NCT02032277) [74].

Table 4 Ongoing and recruiting phase 2/3 studies

Study name (NCT)	Ref.	Phase	Setting	Investigational arm(s)	Comparator arm(s)	Primary endpoint	Study status
Olaparib							
OlympiAD (NCT02000622)	[62]	3	ADV	Olaparib monotherapy	Physician's choice CT	PFS	R
OlympiA (NCT02032823)	[68]	3	ADJ	Olaparib monotherapy	Placebo	IDFS	R
Veliparib							
BROCADE (NCT01506609)	[96]	2	ADV	Veliparib + (Temozolomide) or (CBDCA + PTX)	Placebo + CBDCA + PTX	PFS	R
(NCT02163694)	[63]	3	ADV	Veliparib + CBDCA + PTX	Placebo + CBDCA + PTX	PFS	R
Brightness (NCT02032277)	[74]	3	NADJ	(Veliparib + CBDCA) or (Placebo + CBDCA) + Neoadjuvant CT	Placebo + Neoadjuvant CT	pCR rate	R
Talazoparib							
EMBRACA (NCT01945775)	[61]	3	ADV	Talazoparib monotherapy	Physician's choice CT	PFS	R
ABRAZO (NCT02034916)	[131]	2	ADV	Talazoparib monotherapy	Single arm study	ORR	R
(NCT02282345)	[75]	2	NADJ	Talazoparib monotherapy	Single arm study	Safety	R
Niraparib							
BRAVO (NCT01905592)	[60]	3	ADV	Niraparib monotherapy	Physician's choice CT	PFS	R
Rucaparib							
(NCT00664781)	[132]	2	ADV	Rucaparib monotherapy	Single arm study	ORR, safety	NR
(NCT01074970)	[133]	2	ADJ	Rucaparib + Cisplatin	Cisplatin	2y-DFS	NR

ADJ Adjuvant, *ADV* Advanced, *CBDCA* Carboplatin, *CT* Chemotherapy, *IDFS* Interval disease-free survival, *NADJ* Neoadjuvant, *NR* Not yet recruiting, *ORR* Objective response rate, *PFS* Progression-free survival, *PTX* Paclitaxel, *R* Recruiting, *2y-DFS* 2-year disease-free survival

A pilot phase 2 study of neoadjuvant talazoparib monotherapy in BRCA-associated breast cancer is ongoing at MD Anderson Cancer Center in Texas [75].

An interesting possibility for the future development of PARP inhibition in BRCA-related breast cancers has been raised by To et al. [76], who demonstrated a chemopreventive effect of veliparib and olaparib in delaying mammary tumor development in BRCA1-deficient mice. Data in this field are still too limited to speculate whether these findings could be translated to humans, but the concept of a chemopreventive drug active in a population at high risk of developing breast cancer is nonetheless intriguing. The future of PARPi in prevention is not clear at this time because of some chemotherapy-like toxic effects on bone marrow, in particular [22].

Safety of PARPi

Toxicities of PARPi monotherapy appear to be similar to cytotoxic chemotherapeutic agents. Data from prominent phase 1 and 2 studies are summarized in Table 5: the most frequently reported adverse events in published studies are grade 1–2 nausea, vomiting, diarrhea, fatigue, headache, and anemia. The most common grade 3–4 toxicities were nausea, vomiting, and hematological toxicity, with anemia, lymphopenia, and thrombocytopenia being the most common dose-limiting toxicities in dose-finding studies [5, 34].

Conversely, dose-limiting toxicities observed in trials of PARPi in combination with cytotoxic agents include primarily hematologic toxicities [77, 78]. These potentiated toxicities might restrict the future development of some olaparib-cytotoxic combinations [79]. However, using an intermittent schedule of PARPi administration instead of continuous dosing has proved effective in overcoming this limitation [23].

One major concern with drugs that inhibit DNA damage repair mechanisms is the risk of development of new primary malignancies. A small number of cases of

Table 5 Most common toxicities in studies of PARPi monotherapy in breast cancer patients

Compound	Adverse event (range of occurrence across studies)	
	Grade 1–2	Grade 3–4
Olaparib [5–7, 34, 134]	Nausea (32–58 %)	
	Fatigue (30–50 %)	
	Vomiting (11–34 %)	Nausea (4–15 %)
	Anorexia (12–27 %)	Fatigue (5–15 %)
	Anemia (5–25 %)	Anemia (11–15 %)
	Headache (22 %)	Vomiting (4–11 %)
	Diarrhea (11–18 %)	Thrombocytopenia (3 %)
	Taste alteration (13 %)	
Veliparib [135]	Dizziness (7 %)	–
	Nausea (7 %)	
	Dysgeusia (7 %)	
Talazoparib [32]	Fatigue (26 %)	
	Nausea (26 %)	
	Alopecia (grade 1 only, 26 %)	Neutropenia (8 %)
	Anemia (13 %)	Thrombocytopenia (8 %)
	Neutropenia (10 %)	Anemia (5 %)
	Flatulence (10 %)	
	Thrombocytopenia (3 %)	
Niraparib [31]	Anemia (48 %)	
	Nausea (42 %)	
	Thrombocytopenia (35 %)	Thrombocytopenia (15 %)
	Fatigue (34 %)	Anemia (10 %)
	Anorexia (25 %)	Fatigue (8 %)
	Neutropenia (24 %)	Neutropenia (4 %)
	Constipation (23 %)	
	Vomiting (19 %)	
	Insomnia (10 %)	
Rucaparib [33][a]	Fatigue (30–39 %)	
	Nausea (27–30 %)	
	Diarrhea (13–20 %)	
	Vomiting (23 %)	
	Dizziness (17 %)	
	Anorexia (11 %)	

Grading according to Common Toxicology Criteria for Adverse Event
[a]Grade of reported adverse events not specified

myelodysplastic syndrome and acute myeloid leukemia have been described in PARPi studies, with an incidence of <1 % [22]. It is noteworthy that most patients had already been treated with DNA-damaging classic chemotherapeutic drugs, which *per se*, represents a risk factor for development of new malignancies. Nonetheless, the increased concentration of gammaH2AX (a marker of DNA damage [80–82]) in tissues of patients treated with PARPi implies an accumulation of DSB in normal tissues and thus could lead to an increased risk of cancer secondary to DNA damage [22], warranting a high level of attention when developing PARPi therapy, especially in the adjuvant setting.

Resistance to PARP inhibition

As with most targeted therapies, cancers develop resistance to PARPi. All tumors that responded initially to treatment with PARPi have ultimately progressed. So far,

three mechanisms of resistance to PARPi have been demonstrated, while two others have been hypothesized [83–85] (Table 6). The first of the three established mechanisms is the development of secondary mutations that restore BRCA functionality. Preclinical and clinical evidence indicates that genomic instability promoted by PARPi in HR-deficient cells may result in secondary mutations in the mutated *BRCA1* or *BRCA2* gene with restoration of functional protein expression and induction of PARPi resistance [86–88]. The second mechanism involves increased drug efflux with consequent reduction of intracellular PARPi concentrations. PARP1 knock-out cells show dramatic overexpression of P-glycoprotein [89]; PARP inhibition induces up-regulation of P-glycoprotein expression in an *in vivo* mammary tumor model [59]. The third mechanism of PARPi resistance is based on loss of p53 binding protein 1 (53BP1). *In vitro* and *in vivo* experiments showed that mutations causing loss of 53BP1 are able to restore the HR in *BRCA1/2* mutated cells, at least partially [90–92]: this "DNA damage repair rewiring" ultimately leads to reduced sensitivity to PARPi [93].

Another hypothesized, but still unconfirmed, mechanism of resistance to PARPi at the time of this submission is the presence of *BRCA1/2* forms with low level of expression, but that can be enhanced in the presence of opportune stimuli (such as increase in DSB due to PARP inhibition) – so called hypomorphic *BRCA1/2* [84]. Furthermore, the hypomorphs may lead to the reduced formation of PARP-DNA complexes because of decreased PARP expression (for example, by epigenetic silencing of the gene or increased turnover of the protein) [85].

Some of the aforementioned mechanisms of resistance are shared between PARPi and platinum compounds [94], but the degree of overlap is not clear. For example, Audeh et al. [43] reported response to olaparib in ovarian cancer regardless of previous platinum sensitivity or resistance, while in the basket trial by Kaufman et al. [6], response rate to olaparib across breast cancer patients showed a trend in favor of patients without prior platinum exposure. However, platinum sensitivity can persist after resistance to PARPi develops [95]. It is notable that most of the ongoing studies of PARPi in advanced breast cancer exclude patients who had been previously treated with platinum compounds [61, 63, 96, 97] or who progressed on platinum-based chemotherapy regimens [60, 62].

The existence of resistance mechanisms can limit the clinical utility of PARPi; strategies to overcome acquired resistance are needed. For example, it has been shown that drugs able to block efflux pumps may revert their PARPi resistance [59]. Further, when PARPi resistance is due to restoration of BRCA-proficiency, induction of a BRCAness phenotype via CDK1 inhibition may render the tumor cells again susceptible to PARPi [98].

Predicting response to PARPi

No established biomarker of response to PARPi is currently available. A candidate biomarker is the homologous recombination deficiency (HRD) score, which combines three different DNA-based metrics of genomic instability that are highly associated with *BRCA1/2* mutational status or predictive of sensitivity to platinum chemotherapy [99]; Richardson et al. [100] demonstrated that the HRD score is able to identify patients with breast tumors with underlying HR deficiency (including *BRCA1/2* non-mutated tumors) that benefit from neoadjuvant platinum therapy. In PrECOG 0105, high HRD scores identified patients with a higher likelihood of achieving pathological complete response to platinum-based neoadjuvant chemotherapy [101]. However, data from the GeparSixto study showed a statistically significant increase in pathological complete response rates in patients with high HRD score; the benefit was observed irrespective of *BRCA1/2* status (mutated versus intact) [102]. These results could not be replicated in the advanced setting, although the fact that the HRD assay was performed on primary tumor specimens rather than metastatic samples may have limited its ability to predict responsiveness to carboplatin in metastatic breast cancers (TNT trial) [103]. The value of HRD score in predicting response to therapy is being prospectively tested in both neoadjuvant and advanced settings using platinum compounds [104] and PARPi [97, 105], respectively. Other promising biomarkers are the assessment of PARP activity through measurement of poly(ADP-ribose) levels [93, 106], the evaluation of HR proficiency through the formation of nuclear RAD51 foci [107, 108], the presence of miRNAs involved in the regulation of

Table 6 Mechanisms of resistance to PARP inhibitors

Mechanism of resistance	Proposed explanation	HR restoration
Restoration of BRCA functionality	Secondary mutation in *BRCA1/2*	Potentially complete
DNA damage repair rewiring	Mutations in p53 binding protein 1	Partial
Increased drug efflux	Overexpression of P-glycoprotein	None
Increased activity of BRCA1/2 proteins	Increased stimulation of hypomorphic BRCA1/2 protein expression	Partial
Decreased PARP expression	Epigenetic silencing or increased turnover	None

HR Homologous recombination. For further details refer to text and [83–85]

BRCA proteins (such as miR-182) [109], and the evaluation of levels of 53BP1 expression [17, 93].

Strategies to expand PARPi application to BRCA-proficient breast cancers

Theoretically, PARPi activity could be expanded to breast cancers without *BRCA1/2* mutations; several preclinical experiments support this possibility by focusing on the impairment of the HR pathway. PTEN [110] and ATM [111, 112] deficiencies correlate with sensitivity to PARPi both *in vitro* and *in vivo*; moreover, CDK1 inhibition [98] and histone deacetylase inhibition [113] have been shown to efficiently sensitize BRCA-proficient cells to PARPi *in vitro* and, in animal models, *in vivo*. A phase 1 study is ongoing in patients with solid tumors testing the association of veliparib, a selective CDK inhibitor (dinaciclib) and carboplatin: an expanded cohort of BRCA-proficient tumors is planned [114]. Unfortunately, no validated biomarker of HR dysfunction other than germline *BRCA1/2* mutations is currently available.

Alterations in the HR pathway different from *BRCA1/2* mutations may determine an HR-deficient phenotype similar to BRCA-deficient tumor (namely, BRCAness) [20]. Such alterations include *BRCA1/2* suppression (for example, by promoter methylation) or mutations in genes encoding other proteins involved in HR (such as *PTEN*, *FANCF*, *RAD51*, *ATM*, and *CDK1*) [20, 28, 110]. In line with this hypothesis, talazoparib will be tested in *BRCA1/2* wild type breast cancer with high HRD score or deleterious germline or somatic mutation implicated in the HR pathway [97].

Other options to exploit PARP inhibition in BRCA-proficient breast cancers currently under investigation (mainly in cell lines and animal models, but also in clinical trials) include PI3K inhibition [115, 116] and TGFβ activation [117]. Preliminary positive data of clinical efficacy of PARPi/PI3K inhibitors in *BRCA* wild type ovarian and breast cancer have been presented by Matulonis et al. [118] at the 2015 American Association for Cancer Research Annual Meeting.

Conclusions

PARP inhibition is a promising strategy for the treatment of breast cancer associated with germline *BRCA1/2* mutations and papillary serous ovarian cancers. Efficacy data from phase 1 and 2 studies showed encouraging objective response rates with acceptable toxicity profiles for PARPi monotherapy. The initial data are consistent with those of other targeted therapies in identifiable subsets of tumors. There is great excitement about the ongoing phase 3 trials in the metastatic, adjuvant, and neoadjuvant settings.

However, other questions apart from clinical efficacy need to be addressed before PARPi will become part of clinical practice. For example, the long-term effects of continuous administration of this class of drugs are not yet fully characterized: will prolonged exposure to PARPi confer increased risk of hematological toxicity or development of new primary malignancies? This is a concern of particular importance in the adjuvant setting. Increasing use of platinum in early triple-negative disease may influence the way PARPi are used given the overlapping mechanisms of action and resistance.

New strategies are being examined to expand the application of PARPi in BRCA-associated cancers beyond breast and ovarian, and in some sporadic tumors. PARPi should be more fully studied in ER-positive BRCA-associated tumors as well. PARPi appear likely to assume an important role in the management of patients with BRCA-associated tumors, and possibly in other carefully defined tumor subsets as well.

Abbreviations

53BP1: p53 binding protein 1; BER: Base excision repair pathway; HGSOC: High-grade serous ovarian cancer; HR: Homologous recombination; HRD: Homologous recombination deficiency; PARPi: PARP inhibitors; PARPs: Poly(ADP-ribose) polymerases; SSBR: Single strand break repair; TNBC: Triple-negative breast cancers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LL and JEG drafted and revised the manuscript. Both authors read and approved the final manuscript.

Acknowledgements

The authors thank Dr. Daniel Silver (Dana-Farber Cancer Institute, Boston, MA) for advising on the creation of figures for this review.

Received: 17 March 2015 Accepted: 17 July 2015
Published online: 13 August 2015

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CARPMAELS & RANSFORD

Chartered Patent Attorneys
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Solicitors

Carpmaels & Ransford LLP
One Southampton Row
London WC1B 5HA
United Kingdom
T +44 20 7242 8692
F +44 20 7405 4166
E email@carpmaels.com
www.carpmaels.com

European Patent Office
80298 Munich
Germany

Electronically submitted

Your Ref
Our Ref O008427EP:ECO/FJT/CEM
Date 3rd February 2022

Re: European Patent No. 3337467
In the name of IPSEN BIOPHARM LTD.

The proprietor's response to the opposition is enclosed.

As explained in the enclosed, the proprietor's Main Request is for the patent to be maintained on the basis of the enclosed set of claims labelled "Main Request". If the Main Request cannot be allowed, the proprietor requests that the patent be maintained on the basis of one of Auxiliary Requests 1-23, also enclosed.

Oral proceedings are requested should the Opposition Division be considering allowing anything other than the proprietor's Main Request.

Yours faithfully,

// ELECTRONICALLY SIGNED AND SUBMITTED //

OATES, Edward Christopher
Carpmaels & Ransford LLP Professional Association No. 182

Encl. Proprietor's response to opposition
Main Request (clean and marked-up)
Auxiliary Requests 1-23 (clean and marked up)
D13-D17

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PROPRIETOR'S RESPONSE TO OPPOSITION

EUROPEAN PATENT 3 337 467 B1

IN THE NAME OF IPSEN BIOPHARM LTD.

16757498.7 | O008427EP

1 INTRODUCTION AND REQUESTS

1.1 European patent EP 3 337 467 ("the patent") owned by Ipsen Biopharm Ltd. ("the proprietor") has been opposed by Sandoz AG ("the opponent"). This is the proprietor's response to the opposition.

1.2 The proprietor's Main Request ("MR") is that the patent be maintained on the basis of the enclosed claims entitled "Main Request".

1.3 In the MR, the granted claims have been amended in response to the inventive step attacks from the opponent by strengthening their priority entitlement. In particular, granted independent claim 1 has been deleted, and granted independent claim 2 has been renumbered as claim 1. New claim 1 (based on granted claim 2) has been amended to recite the list of specific cancers to be treated, the type of liposomal irinotecan and the dosing delay between irinotecan and the PARP inhibitor. Dependent claims 3, 6, 11, 13 and 14 have been deleted for consistency with new claim 1 and to strengthen the priority entitlement of the MR. The MR finds basis in PCT claims 3, 16 and paragraphs [0002], [0085] and [00141] of the application as filed.

1.4 If the MR cannot be allowed, the proprietor requests that the patent be maintained on the basis of one of the enclosed auxiliary requests 1-23, which are described in section 5 below.

1.5 The proprietor requests oral proceedings if the Opposition Division ("OD") is considering anything other than the MR.

1.6 The proprietor requests permission to make further amendments to the main and auxiliary requests, and/or to submit further auxiliary requests in the future in response to points raised by the opponents or the OD.

1.7 Should the OD deem amendment to the description necessary, the proprietor requests that any such amendment be deferred until final agreement on the claims has been reached.

1.8 For the avoidance of doubt, any unclaimed or deleted subject matter is not abandoned.

2 DOCUMENTS ENCLOSED

2.1 The proprietor files the following documents

- D13 – Written transcript of the presentation in the video of D1b
- D14 – Shah, M. A. et. al, Clin Cancer Res 2005;11(10) May 15, 2005
- D15 – Sadetzki, S. et al., British Journal of Cancer (2009) 100, 1021-1025

- D16 – Russo et al. Practical Medical Oncology Textbook (2021), pages 1-11
- D17 – Camptosar® label, December 2014 version

2.2 D14 is a paper describing the results of the clinical trial discussed on page 345 of D6. It provides details of the specific administration protocol used in the study referred to by the opponent in D6.

2.3 D15 and D16 are filed in response to the opponent's priority allegations, in support of the fact that treatment of solid tumors is disclosed in the priority documents.

2.4 D17 demonstrates that the non-liposomal irinotecan used in the clinical trial of D1 is non-liposomal irinotecan hydrochloride.

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3 PRIORITY ENTITLEMENT

3.1 The opponent has alleged that the claims are not entitled to any of the claimed priority dates. However, the first and second priority documents (P1: US 62/207,709 and P2: US 62/207,760), which were both filed on 20th August 2015, relate to the "same invention" as claim 1 of the MR. The MR is therefore entitled to the earliest priority date, contrary to the opponent's assertion.

3.2 It is noted that the opponent appears to have referred only to the claims of the earliest priority document when assessing whether each feature of the granted claims was entitled to priority. This is clearly an incorrect approach. Rather, in assessing whether the invention was disclosed in the priority application(s), the **entire disclosure** of the priority application(s) as filed must be taken into account[1]. The opponent has therefore failed to properly assess the entitlement of the claims to claim priority to P1 and P2.

3.3 When the full disclosures of P1 and P2 are considered, taking into account features implicit to the skilled person in what is expressly mentioned in P1 and P2 (i.e., the gold standard test, an assessment that is identical to the test required for compliance with Article 123(2) EPC)[2], it is clear that claim 1 of the MR is entitled to the earliest priority date. Therefore, D8, D9, D10 and D11, which were all published after the earliest priority date of 20th August 2015, do not form a part of the state of the art within the meaning of Article 54(2) EPC.

The subject matter of claim 1 has basis in P1 and P2

3.4 Claim 1 of the MR relates to subject matter which is directly and unambiguously derivable from P1 and P2. Therefore, claim 1 of the MR is entitled to the earliest priority date of 20th August 2015.

3.5 Claim 1 of the MR can be summarised as follows:

Drugs:	irinotecan sucrosofate salt encapsulated in liposomes for intravenous use

[1] Guidelines for Examination 2021, H-IV, 2.2.
[2] Guidelines for Examination 2021, F-VI-2.2, and G 2/98

Poly(ADP-ribose) Polymerase ("PARP") inhibitor

Condition treated: a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from a cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer

Dosage regimen: a 28-day antineoplastic therapy treatment cycle consisting of administering the liposomal irinotecan sucrosofate salt on days 1 and 15 of the treatment cycle and administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan sucrosofate salt and ending at least 1 day prior to administration of additional liposomal irinotecan sucrosofate salt

Claim 1 of the MR is derivable from P1 and P2

Drugs

3.6 P1 and P2 refer to "MM-398" throughout their disclosure. Paragraph [0031] of P1 and paragraph [0032] of P2, which are identical, state that "MM-398" includes "irinotecan sucrosofate salt encapsulated in liposomes for intravenous use":

> *The MM-398 irinotecan liposome injection) [sic] includes an irinotecan sucrosofate salt, encapsulated in liposomes for intravenous use.*

3.7 Therefore, any references throughout P1 and P2 to "MM-398" provide at least an implicit disclosure of "irinotecan sucrosofate salt encapsulated in liposomes for intravenous use", as recited in claim 1 of the MR.

3.8 Paragraph [0042] of P1 and paragraph [0043] of P2, which are identical, then disclose a treatment cycle wherein MM-398 (which includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use) and a PARP inhibitor are administered in at least one cycle. It is stated in these paragraphs that:

> *In certain embodiments, the MM-398 and the PARP inhibitor are administered in at least one cycle.*

3.9 Thus, paragraph [0042] of P1 and paragraph [0043] of P2 disclose the drugs from claim 1 of the MR in combination for use in a treatment cycle.

Condition treated

3.10 Claim 1 of the MR recites that the condition to be treated using the liposomal irinotecan sucrosofate salt is a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from various specific cancers. This condition to be treated is entitled to priority from both P1 and P2.

3.11 In particular, paragraph [0038] and claim 14 of P1 and paragraph [0039] and claim 14 of P2 each provide a list of cancers to be treated using the methods therein, which corresponds to the list of cancers recited in claim 1 of the MR. Paragraph [0058] of P2 then explicitly confirms

that each of these listed cancers is a "solid tumor" cancer, stating that the combination of liposomal irinotecan sucrosofate salt and a PARP inhibitor is used to (emphasis added):

> *...treat **solid tumors including the following tumor types**: cervical cancer, ovarian cancer, triple negative breast cancer (TNBC), non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), gastric cancer, pancreatic cancer, and neuroendocrine tumors*

3.12 Therefore, P1 and P2 disclose the specific cancers which appear in claim 1 of the MR. In addition, at least P2 discloses that these cancers are "solid tumor" cancers. Therefore, the condition treated in claim 1 is directly and unambiguously derivable from P1 and P2.

3.13 In addition, and for the avoidance of doubt, the fact that the listed cancers manifest as "solid tumors" in a patient is also explicitly confirmed in D10, where it is stated that patients involved in the clinical trial therein must have diagnosis of:

> *...one of the following **solid tumors**: cervical cancer, ovarian cancer, triple negative breast cancer (TNBC), non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), gastric cancer, and neuroendocrine tumors* [emphasis added]

3.14 The fact that the listed cancers invariably manifest as "solid tumors" is further supported by the disclosure of D15, which classifies "solid" cancers as all cancers forming tumours in solid organs, as opposed to cancers affecting the blood. In other words, cancers which affect any solid organ are "solid tumor" cancers[3]:

> *...solid cancer (malignancies that form tumours in solid organs, as opposed to leukaemia, which affects the blood)...*

D16 also confirms that the cancers listed in claim 1 manifest as solid tumors. This is clear from the contents page in D16, in particular on pages IX-XI. On these pages, the contents of a chapter entitled "*Clinical Oncology: Diagnosis and Treatment of Solid Tumors*" are listed. It should be noted that all of the cancers listed in claim 1 of the MR are mentioned in this chapter, thus indicating that these cancers are cancers which invariably manifest as solid tumors.

3.15 Thus, even if one hypothetically disregards the statement in paragraph [0058] of P2 discussed at paragraph 3.11, the skilled person (an oncologist) would appreciate that the treatment of the specific cancers listed in paragraph [0038] of P1, paragraph [0039] of P2, and in claim 1 of the MR would **inevitably result** in the treatment of a solid tumor. That is, the link between the specific cancers and solid tumors would not need to be stated *expressis verbis* because this link would already have been known by the skilled person.

3.16 Therefore, the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from those given in claim 1 of the MR, is directly and unambiguously derivable from P1 and P2.

[3] D15, page 1022, left-hand column, fifth paragraph.

Dosage regimen

3.17 In addition to specifying the claimed administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor in at least one cycle, paragraph [0042] of P1 and paragraph [0043] of P2 (discussed above at paragraphs 3.8 - 3.9) disclose a dosage regimen according to claim 1 of the MR.

3.18 In particular, the identical teaching in paragraph [0042] of P1 and paragraph [0043] of P2 is that the "cycle" of administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor can involve, in general terms, the administration of a first agent for a period of time followed by a second agent for a period of time, and the repetition of this sequential administration (emphasis added):

> *In certain embodiments, the MM-398 and the PARP inhibitor are administered in at least one cycle.* ***A cycle comprises the administration of a first agent*** *(e.g., a first prophylactic or therapeutic agents) for a period of time, followed by the* ***administration of a second agent*** *(e.g., a second prophylactic or therapeutic agents) for a period of time, optionally followed by the administration of a third agent (e.g., a third prophylactic or therapeutic agents) for a period of time and so forth, and* ***repeating this sequential administration, i.e., the cycle****.*

3.19 Paragraph [0042] of P1 and paragraph [0043] of P2 then specify that the administration cycle can be a 2 week administration cycle (e.g., a 14-day cycle):

> *In one embodiment the cycle is a 2 week cycle.*

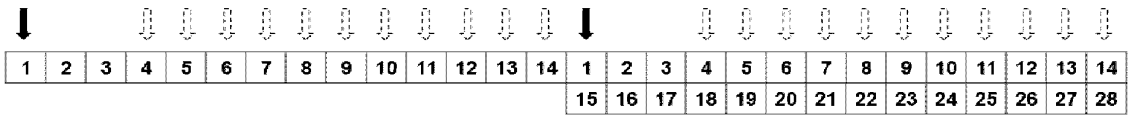
3.20 It would have been implicitly, but directly and unambiguously, apparent to the skilled person that this 2 week cycle could be a 14-day cycle, because the number of days in 2 weeks is 14. Thus, P1 and P2 both disclose, at least implicitly, a 14-day cycle.

3.21 It is then stated in paragraph [0042] of P1 and paragraph [0043] of P2 that in each cycle the liposomal irinotecan sucrosofate salt is administered at the beginning of the cycle (i.e. on the first day – "day 1"), and administration of a PARP inhibitor is delayed until at least about 12, 24, 48, 72, 96, or 120 hours after the administration of the liposomal irinotecan sucrosofate salt (i.e. delayed until at least about half a day, 1 day, 2 days, 3 days, 4 days or 5 days respectively, after administration of the liposomal irinotecan sucrosofate salt):

> *In one embodiment MM-398 is administered at the beginning of the cycle and administration of a PARP inhibitor ... is delayed until at least about 12, 24, 48, 72, 96, or 120 hours, after the administration of MM-398.*

3.22 Therefore, the above passages disclose to the skilled person a 14-day administration cycle wherein the liposomal irinotecan sucrosofate salt is administered on day 1 and the PARP inhibitor is administered for a period of time (i.e., one or more days) which is delayed until 72 hours (3 days) after the liposomal irinotecan sucrosofate salt administration. The preference for a delay of 3 days between administration of the liposomal irinotecan sucrosofate salt is indicated in P1 and P2, for example in paragraph [0029] of P2 where an interval of "3 days" is defined as the upper limit of time to be taken between administration of the liposomal irinotecan and the PARP inhibitor.

3.23 Given that P1 and P2 specify that the "cycle" described therein is **repeated**, as discussed above (see the final clause of the quote given in paragraph 3.18), the disclosure of the above-described 14-day administration cycle in P1 and P2 is a direct and unambiguous disclosure of the 28-day administration cycle of claim 1 of the MR. Specifically, if the 14-day administration cycle is repeated, following the disclosure discussed above, a 28-day cycle shown below would result. In this exemplary cycle, the numbers represent days in the cycle, solid arrows represent liposomal irinotecan sucrosofate salt administration, and dashed arrows represent PARP inhibitor administration.



3.24 As seen in the top row above, which shows a repetition of the 14-day cycle, each 14-day cycle involves the administration of liposomal irinotecan sucrosofate salt on day 1 (i.e., at the beginning of the cycle) and the administration of the PARP inhibitor for a period of time (i.e., one or more days) starting 3 days after administration of the liposomal irinotecan sucrosofate salt (i.e., day 4 onwards).

3.25 As shown in the bottom row, a repetition of this 14-day cycle equates exactly to a 28-day cycle wherein liposomal irinotecan sucrosofate salt is administered on days 1 and 15, and the PARP inhibitor is administered on one or more days starting 3 days after each administration of the liposomal irinotecan sucrosofate salt. In addition, the end of the first cycle is on day 14, and the start of the second cycle is on day 15. Therefore, given that each cycle is only 14 days in length, administration of the PARP inhibitor from the first 14-day cycle must end at least 1 day prior to the next administration of liposomal irinotecan sucrosofate salt.

3.26 The fact that paragraph [0042] of P1 and paragraph [0043] of P2 provide a direct and unambiguous disclosure of a 28-day cycle where liposomal irinotecan sucrosofate salt is administered on days 1 and 15 and administration of a PARP inhibitor is delayed for a period of days is supported by the cycles exemplified therein. Specifically, in the two treatment cycles exemplified in paragraph [0042] of P1 and paragraph [0043] of P2, MM-398 is administered as part of a 28-day cycle on days 1 and 15 and a PARP inhibitor is administered on either days 3-12 and days 17-25, or days 5-12 and days 19-25. Thus, the skilled person reading these paragraphs would be left in no doubt as to the treatment cycle defined therein.

3.27 As a result, paragraph [0042] of P1 and paragraph [0043] of P2 both disclose the dosage regimen of claim 1 of the MR.

3.28 Therefore, the invention of claim 1 of the MR is disclosed in P1 and P2 and claim 1 of the MR is entitled to the earliest priority date of 20th August 2015.

None of the opponent's arguments call compliance with Article 76(1) EPC into question

3.29 The opponent raised four specific points in its allegations that the granted claims were not entitled to priority. These points are either rendered moot in view of the amendments made in

the MR or are based on an incorrect interpretation of the priority documents. Therefore, they fail to advance the opponent's case.

"Liposomal irinotecan"

3.30 The opponent alleged that the priority documents are specific to MM-398, and do not provide a broader disclosure of liposomal irinotecan[4]. However, claim 1 of the MR specifies that the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use. As discussed at point 3.6 above, P1 and P2 state that MM-398 includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use. Thus, the claims of the MR are limited to irinotecan sucrosofate salt encapsulated in liposomes for intravenous use , which is disclosed in P1 and P2, and the opponent's objection on this point is no longer relevant.

"The next administration of irinotecan"

3.31 The opponent raised an objection that the priority documents did not disclose any treatment involving the repeated administration of irinotecan or involving a minimum time between administration of the PARP inhibitor and the next administration of irinotecan. However, as discussed in points 3.6-3.27 above, paragraph [0042] of P1 and paragraph [0043] of P2 each disclose that the sequential administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor described therein is performed as a "cycle", which is **repeated**. Therefore, both P1 and P2 disclose repeated administration of liposomal irinotecan sucrosofate salt.

3.32 Regarding the minimum time between administration of the PARP inhibitor and the next administration of irinotecan, as discussed above, paragraph [0042] of P1 and paragraph [0043] of P2 both disclose a 14-day cycle which, when repeated once in accordance with the disclosure of these paragraphs, corresponds exactly to the 28-day treatment cycle recited in claim 1 of the MR. As explained above, when the 14-day cycle is repeated (in accordance with the teaching of P1 and P2), there is a minimum delay of one day between administration of the PARP inhibitor on day 14 of the first cycle, and administration of the liposomal irinotecan sucrosofate salt on day 1 of the second cycle. Therefore, P1 and P2 both disclose a dosage regimen wherein administration of the PARP inhibitor ends at least 1 day prior to administration of additional liposomal irinotecan sucrosofate salt, as specified in claim 1 of the MR.

"Solid tumor"

3.33 The opponent raised an objection that the treatment of solid tumors is found in P1 "only in the context of some examples", and thus that there was no general disclosure of the treatment of a patient having a solid tumor in P1. However, claim 1 of the MR recites that the liposomal irinotecan sucrosofate salt is for use in treating a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from a list of cancers selected from the list in paragraph [0038] and claim 14 of P1 and paragraph [0039] and claim 14 of P2. Therefore, as discussed above, the treatment of a solid tumor in a patient having the list of cancers in claim 1 of the MR is disclosed in both P1 and P2.

[4] Notice of opposition, point 4.1.

"Intravenous administration"

3.34 The opponent raised an allegation that the priority documents only ever refer to the *intravenous* administration of irinotecan sucrosofate salt. However, this allegation is incorrect at least because several passages of P1 and P2 refer to the administration of irinotecan sucrosofate salt without requiring that the administration is intravenous. For example, paragraph [0042] of P1 and paragraph [0043] of P2 each disclose that liposomal irinotecan sucrosofate salt is "administered" in the treatment cycle, with no specific requirement as to which method of administration is used. Therefore, claim 1 of the MR, which simply recites administration of liposomal irinotecan sucrosofate salt, is entitled to priority.

3.35 As a result, none of the opponent's arguments are prejudicial to the priority entitlement of claim 1 of the MR, and 20th August 2015 is the effective date for the analysis of novelty and inventive step of claim 1. As a consequence, prior art documents D8, D9, D10 and D11, which were all published after this date, do not form a part of the state of the art for the consideration of inventive step.

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4 INVENTIVE STEP

Summary of the invention and introduction to inventive step

4.1 The invention relates to a combination therapy for treating a solid tumor in a patient involving treatment with liposomal irinotecan sucrosofate salt (a Topoisomerase I ("Top1") inhibitor) in combination with a PARP inhibitor. As discussed in paragraph [0004] of the patent, combinations of Top1 inhibitors and PARP inhibitors have previously been demonstrated to be synergistic as anticancer agents in *in vitro* assays. However, clinical development of combination therapies involving Top1 inhibitors and PARP inhibitors has been limited due to increased toxicities and resultant dose reductions, which have limited the clinical utility of the combination. Thus, there remains a need for a safe and effective combination therapy involving a Top1 inhibitor and a PARP inhibitor.

4.2 The present invention addresses this need by providing a combination therapy for a solid tumor cancer involving administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor, wherein the synergistic effect of the combination of the two drugs can be utilised. As demonstrated by the data in the patent (e.g., Figures 8A-8C, 11A and B), the present combination therapy harnesses the clinical potential of the synergistic effect of liposomal irinotecan sucrosofate salt and a PARP inhibitor without compromising on safety or requiring dose reductions of either drug.

4.3 The invention is defined in claim 1 of the MR, which recites a 28-day treatment cycle wherein irinotecan sucrosofate salt encapsulated in liposomes for intravenous use is administered on days 1 and 15 and a PARP inhibitor is administered on one or more days starting 3 days after each administration of the liposomal irinotecan sucrosofate salt and ending at least 1 day before the next administration of liposomal irinotecan sucrosofate salt.

4.4 In its opposition statement, the opponent presented two inventive step arguments, one of which assumed that the claims were not entitled to priority and used D10 as the closest prior art document, and the other of which assumed that the claims were entitled to the earliest priority date and used D1 as the closest prior art document.

4.5 As outlined above, the claims are entitled to the earliest priority date of 20th August 2015. Therefore, prior art documents D8, D9, D10 and D11, which were all published after this date, do not form a part of the state of the art for the consideration of inventive step.

4.6 For the below analysis, it will first be set out why the claims are inventive at the earliest priority date. However, should the OD disagree with the proprietor that the claims are entitled to the P1 and P2 priority dates, the proprietor has set out its arguments on why the claims are inventive in any case.

Inventive step at the P1 and P2 priority date

4.7 In its inventive step arguments assuming that the claims were entitled to the priority date of P1 and P2, the opponent took D1 to be the closest prior art document. D1 discloses some results of a clinical trial involving treatment of cancer with a combination of irinotecan and a PARP inhibitor.

4.8 D1 discloses a treatment cycle of 21 days in length, wherein non-liposomal irinotecan hydrochloride[5] is administered in a dose of 100 mg/m^2 on days 1 and 8 and veliparib is administered in a dose of 40 mg on days 3-14 in the first cycle and in a dose of 40 mg on days 1-14 in subsequent cycles[6]. The study in D1 is explicitly referred to in paragraph [0079] of the patent, where it is explained that dose-limiting toxicities included febrile neutropenia (grade 3), leukopenia and neutropenia (grade 4). As a result of these dose-limiting toxicities, a 10-fold lower dose of veliparib had to be used compared with the dose conventionally used in veliparib monotherapy[7].

Technical differences

4.9 As acknowledged by the opponent, there are several technical differences between the claims of MR and D1. In particular:

- The claims of the MR use **irinotecan sucrosofate salt encapsulated in liposomes for intravenous use**, whereas D1 uses **non-liposomal irinotecan**.
- The claims of the MR recite a **28-day treatment cycle** with administration of liposomal irinotecan sucrosofate salt on **days 1 and 15**, whereas D1 recites a **21-day treatment cycle** with administration of irinotecan on **days 1 and 8**.
- The claims of the MR specify that in **each treatment cycle**, the PARP inhibitor is administered on one or more days starting **3 days after** the liposomal irinotecan sucrosofate salt. In contrast, in the treatment cycle of D1, the PARP inhibitor is administered on **days 3 to 14 in the first cycle** and on **days 1-14 in subsequent cycles**, i.e., delayed by 2 days relative to the first irinotecan administration in the first cycle but then administered on the same day as irinotecan for the second irinotecan

[5] D1 does not state explicitly that irinotecan hydrochloride was used. However, at the priority date, irinotecan hydrochloride was the only form of irinotecan that had received regulatory approval (see D17, page 1, LH column under heading "*Dosage Forms and Strengths*"). Therefore, the clinical trial described in D1 must have used irinotecan hydrochloride.
[6] D1, "Conclusions"
[7] See Table 2B on page 15 of the patent, where it is stated that the conveniently "*Monotherapy dosing*" for veliparib is "*200-400 mg BID*" (i.e. 400-800 mg per day). This contrasts with the 40 mg BID dose mentioned in the "*Conclusions*" section of D1.

administration of the first cycle and each irinotecan administration in each subsequent cycle.

Technical effect

4.10 The combination of these technical differences results in a technical effect, in that the claimed combination therapy harnesses the clinical potential of the synergistic effect of liposomal irinotecan sucrosofate salt and a PARP inhibitor without compromising on safety or requiring dose reductions of either drug. This technical effect is discussed in detail in the patent.

4.11 As discussed in paragraph [0099], the present inventors found that the use of MM-398 (which includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use) in combination with a PARP inhibitor (of which veliparib is one example) could provide a particularly effective therapy. In particular, they unexpectedly found that with an appropriate delay in administration between liposomal irinotecan sucrosofate salt and the PARP inhibitor in each treatment cycle, the synergistic anticancer effect of co-administration of the two drugs could be realised without the problematic toxicity usually associated with their co-administration (emphasis added):

> *The advantage of dosing with MM-398 compared to free irinotecan is the extended PK profile and prolonged local tumor exposure of MM-398. Since SN-38 is cleared more quickly from normal tissues than from tumor,* ***delayed dosing of veliparib (e.g. starting veliparib dosing a few days after MM-398 administration) allows for the window of maximum irinotecan-induced toxicity to pass in the absence of concurrent veliparib toxicity****. However, the tumor levels of SN-38 are sustained longer than in healthy tissue, such that* ***upon PARP inhibitor dosing subsequent to liposomal Top1 inhibitor (e.g., MM-398) administration, both drugs will act on tumor tissue simultaneously****.*

4.12 Therefore, the present inventors found that a repeated treatment cycle involving the initial administration of irinotecan sucrosofate salt encapsulated in liposomes for intravenous use, followed by administration of a PARP inhibitor 3 days later, was both safe and effective.

4.13 As discussed in paragraph [0100] of the patent, the inventors surprisingly discovered that the use of the treatment cycle of the invention meant that higher doses of liposomal irinotecan sucrosofate salt and PARP inhibitor could be used compared to when the two drugs were administered closer together in the treatment cycle, without compromising the safety of the treatment.

4.14 The ability to use higher doses of anticancer agents increases the efficacy of the treatment. This is discussed, for example, in D6, where it is stated that increasing the dose of chemotherapy increases the "log-kill" of a particular chemotherapy treatment (where the "log kill" is a measure of cell toxicity and 1 log kill is a 90% cell kill)[8]. As discussed in D6, higher chemotherapy doses are usually associated with increased host toxicity. Therefore, the fact that the present treatment cycle can safely deliver higher doses of irinotecan and PARP inhibitor than other treatment cycles represents an important advantage in the technical field.

[8] See, for example, the discussion in D6, page 335, right-hand column - page 336, left-hand column.

4.15 The higher dose tolerability achieved by the present treatment cycle is shown by data in the patent. In particular, paragraph [0100] describes the results of a dose tolerability study of irinotecan sucrosofate salt encapsulated in liposomes for intravenous use when administered in combination with a fixed dose of a PARP inhibitor (veliparib) in mice, the results of which are shown in Figures 8A-8C. It is concluded that the highest dose of liposomal irinotecan sucrosofate salt can only be successfully administered if the administration of the PARP inhibitor is delayed by 3 days, as required by claim 1 of the MR (emphasis added)[9]:

> *Toxicity of the combination was seen at the highest doses of MM-398 when given in close proximity to the veliparib doses (Figure 8A). However, this* ***toxicity could be alleviated either by dose reducing MM-398 or delaying the start of veliparib dosing, whereby the highest dose of MM-398 could be successfully dosed with veliparib if given on Days 4-6 following Day 1 dosing of MM-398*** *(Figures 8A-8C).*

4.16 Thus, the treatment cycle where administration of the PARP inhibitor was delayed by 3 days allowed higher doses of liposomal irinotecan sucrosofate salt to be delivered compared to the other tested treatment cycles. Further, contrary to the opponent's allegation at point 5.6.11 of the opposition statement, the data in the patent also confirms that the claimed treatment cycle provides effective treatment of a solid tumor. As described in paragraph [0100] of the patent and shown in Figures 11A and 11B (emphasis added):

> *The Day 4-6 veliparib dosing schedule (following day 1 dosing of MM398) was followed in subsequent efficacy studies which* ***demonstrated synergy of the combination in two cervical cancer tumor xenograft models****, in which veliparib alone was not efficacious (Figure 11A) and a second model in which neither MM-398 or veliparib were efficacious as single agents (Figure 11B), however the combination demonstrated tumor growth inhibition (Figure 11B).*

4.17 Paragraph [0100] thus provides evidence that the claimed combination therapy harnesses the clinical potential of the synergistic effect of liposomal irinotecan sucrosofate salt and a PARP inhibitor without compromising on safety or requiring dose reductions of either drug.

4.18 Similarly, paragraph [0101] describes the results of a dose tolerability study of a second PARP inhibitor (olaparib) when administered in combination with a fixed dose of irinotecan sucrosofate salt encapsulated in liposomes for intravenous use in mice, the results of which are shown in Figures 10A-10D. It is concluded that delayed administration of the PARP inhibitor reduces the toxicity observed with co-administration (emphasis added)[10]:

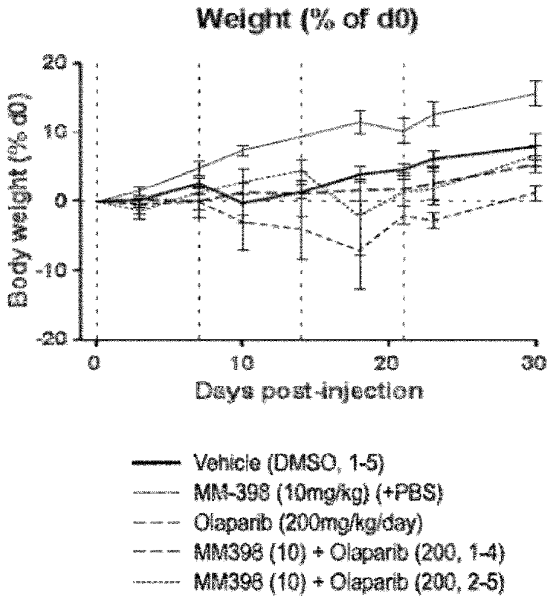
[9] As explained in paragraph [0019] of the patent, the three dosage regimens involved dosing with MM-398 on day 1, followed by 50mg/kg veliparib dosed daily on days 2-4 (regimen A, figure 8A); or 50mg/kg veliparib dosed daily on days 3-5 (regimen B, figure 8B) or 50mg/kg veliparib dosed daily on days 4-6 (regimen C, figure 8C).

[10] As explained in paragraph [0101] of the patent, the tested dosage regimens were as follows: Group 1: MM-398 alone IV (10mg/kg); Group 2: olaparib alone oral (200mg/kg); Group 3: MM-398 (d1) + olaparib (200mg/kg, d1-5); Group 4: MM-398 (d1) + olaparib (150mg/kg, d1-5); Group 5: MM-398 (d1) + olaparib (200mg/kg, d1-4); Group 6: MM-398 (d1) + olaparib (200mg/kg, d2-5); Group 7: MM-398 (d1) + olaparib (200mg/kg, d3-5).

Addition of olaparib seemed to be more toxic as compared to monotherapy, however delaying start of olaparib administration to d3 seemed to decrease olaparib specific toxicity as compared to concurrent therapy…

4.19 In addition, data from this study confirms that delayed dosing of the PARP inhibitor results in higher dose tolerability. For example, Figure 10A shows the toxicity of two different treatment cycles involving administration of 10 mg/kg of liposomal irinotecan sucrosofate salt (MM-398) in combination with 200 mg/kg/day of PARP inhibitor (olaparib). The first treatment cycle (labelled 1-4, shown as – – – in the figure) included co-administration of liposomal irinotecan sucrosofate salt and the PARP inhibitor on day 1, whereas in the second treatment cycle (labelled 2-5, shown as ——— in the figure) administration of the PARP inhibitor was delayed until one day after liposomal irinotecan sucrosofate salt administration.

4.20 It can be seen in Figure 10A that that when mice were administered the combination of liposomal irinotecan sucrosofate salt and the PARP inhibitor on the same day of each treatment cycle, a significant decrease in body weight was observed in the first 20 days post-administration. In contrast, when PARP inhibitor administration was delayed by one day in each treatment cycle, a net increase in body weight was observed. As stated in paragraph [0100] of the patent, bodyweight is a gross measure of toxicity, and weight loss indicates intolerability of the combination.



4.21 Therefore, Figure 10A shows that delayed administration of the PARP inhibitor relative to the liposomal irinotecan sucrosofate salt in each treatment cycle resulted in a notable reduction in toxicity of the combination.

4.22 Therefore, the patent provides data showing that the claimed dosage regimen for co-administration of irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and a PARP inhibitor provides a safe and effective treatment of a solid tumor. In particular, higher doses of both liposomal irinotecan sucrosofate salt and PARP inhibitor can be

administered to provide synergistic tumor treatment, without demonstrating the problematic toxicity usually associated with their co-administration.

Technical problem to be solved

4.23 The technical problem to be solved is to provide a treatment for a solid tumor using a combination of irinotecan and a PARP inhibitor which harnesses the synergistic effect of irinotecan and a PARP inhibitor without compromising the safety of the treatment.

Non-obviousness of the solution

4.24 When starting from D1 and faced with the present technical problem, the skilled person would not have arrived at the claimed treatment with a reasonable expectation of successfully solving the technical problem. In particular, none of the documents cited by the opponent would have led the skilled person to a 28-day treatment cycle involving administration of liposomal irinotecan sucrosofate salt on days 1 and 15 and administration of a PARP inhibitor on one or more days starting 3 days later.

4.25 D1, which is a conference abstract, (as well as D1a, the slides from the presentation given at the conference, and D1b, the video of the presentation given at the conference) describes the protocol and results of a phase I trial of combination therapy involving irinotecan and the PARP inhibitor veliparib. As laid out above, the protocol described in D1 differed from the claimed protocol both in that non-liposomal irinotecan was used, and in that it used a 21-day treatment cycle consisting of the administration of irinotecan on days 1 and 8 and the administration of veliparib on days 3-14 in the first treatment cycle and days 1-14 in subsequent cycles. There is no teaching or suggestion in D1 that anything other than non-liposomal irinotecan should be used. There is certainly nothing what would have led the skilled person to use liposomal irinotecan sucrosofate salt, as required by claim 1 of the MR.

4.26 Further, D1 would not have motivated the skilled person to modify the treatment cycle therein to introduce a delay of 3 days between the administration of the non-liposomal irinotecan and the PARP inhibitor. Although the protocol of D1 did delay dosing of veliparib to day 3 in the first treatment cycle, the reason for this was entirely unrelated to the safety of the treatment. This is explained in the presentation D1b, where it is stated that the delay of veliparib dosing to day 3 in the first treatment cycle was made so that the investigators had time to perform a biopsy on each of the patients in the study between irinotecan administration and veliparib administration[11]. After the first treatment cycle, when no more biopsies needed to be taken, the study investigators instead opted for concurrent administration of both drugs on day 1 of the cycle.

4.27 D1 thus provides no teaching that delayed dosing of the PARP inhibitor has any beneficial effects on treatment or safety, let alone that it could result in safe and effective treatment of a solid tumor. In fact, based on the results described in D1, the investigators proposed a phase II trial having a 21-day treatment cycle where in each treatment cycle, including the first

[11] D1b, 07m:14s – 08m:33s; D13, page 3, line 19-31: – it is explained by the presenter that irinotecan (CPT-11) was initially administered, then patients were biopsied before administration of veliparib (ABT-888) so that the investigators could determine whether PAR expression could be further inhibited by veliparib after administration of irinotecan. Thus, the delay between irinotecan and veliparib administration was due to the time taken to biopsy the patients, rather than being a deliberate dosing delay for, e.g., treatment efficacy or safety.

treatment cycle, irinotecan was administered on days 1 and 8 and veliparib was administered on days 1-15[12]. That is to say, on the basis of the results of the study described in D1, the investigators concluded that a dosage regimen in which irinotecan and a PARP inhibitor are **administered on the same day in each cycle** should be taken forward. Therefore, if anything, D1 would have led the skilled person toward the use of a dosage regimen where irinotecan and the PARP inhibitor were administered on the same day. There is nothing in D1 which would have led the skilled person to a completely different dosage regimen which, in each cycle, requires the administration of a PARP inhibitor a set number of days after the liposomal irinotecan sucrosofate salt with a reasonable expectation of success. There is certainly nothing that would have led the skilled person to a dosage regimen where, in each cycle, a PARP inhibitor is administered 3 days after the liposomal irinotecan sucrosofate salt with a reasonable expectation of providing a solution to the technical problem formulated at paragraph 4.23. The claims of the MR are thus inventive over D1 alone.

4.28 Of the other prior art documents cited by the opponent, only D2, D3, D4 and D6 relate to treatments for solid tumors involving the co-administration of irinotecan and a second anticancer agent. However, none of these documents would have led the skilled person to the claimed subject matter. For example, some of these documents do not disclose or suggest any kind of co-administration of irinotecan and a PARP inhibitor (e.g. D4), so these documents could not have led the skilled person to the claimed subject matter. In addition, some of the documents disclose dosage regimens which are totally different to that recited in claim 1. In particular, the dosage regimens taught in each of these documents fall into two distinct categories: either the second anticancer agent is only administered after irinotecan administration has finished completely or irinotecan and the anticancer agent are administered on the same day. That is, the dosage regimens disclosed in D2-D4 and D6 are fundamentally different to the claimed dosage regimen which requires, *inter alia*, that both the liposomal irinotecan sucrosofate salt and the PARP inhibitor be administered in each cycle, with administration of the PARP inhibitor occurring 3 days after the administration of irinotecan sucrosofate salt.

Administration of a second anticancer agent only after irinotecan treatment has ended (D4)

4.29 D4 relates to a treatment involving the repeated administration of irinotecan in liposomes with an unbuffered, acidic interior which contains copper (referred to therein as IrC™), followed by the subsequent administration of 5-fluorouracil (5-FU)[13]. The opponent alleged that D4 would have led the skilled person to provide a treatment cycle with "staggered" administration of irinotecan and a PARP inhibitor[14]. However, D4 is missing several of the key features of the claimed treatment cycle.

4.30 Most significantly, 5-fluorouracil is not a PARP inhibitor. Therefore, D4 is not related to treatment involving the co-administration of irinotecan and a PARP inhibitor **at all**, let alone to their co-administration in a treatment cycle where dosing of one is delayed relative to the other. In fact, D4 does not even mention PARP inhibitors. Thus, when faced with the present technical problem and starting from D1, the skilled person would have had no reason to look to D4, which provides no teaching regarding, for example, the synergy between irinotecan

[12] D1, "Conclusions".
[13] D4, page 73, Section 1: "Introduction".
[14] Notice of opposition, points 5.6.9-5.6.10.

and PARP inhibitors, let alone ways to harness this synergy without compromising treatment safety.

4.31 For completeness, even if the skilled person had for some reason looked to D4 and considered replacing 5-FU with a PARP inhibitor, which they would have had no reason to do, D4 would not have led the skilled person to use the treatment cycle recited in the claims of the MR.

4.32 Firstly, D4 discloses the use of irinotecan in the form of IrC™, which is irinotecan in liposomes with an unbuffered, acidic interior containing copper. D4 makes no mention of the possibility for using other forms of irinotecan, let alone specifically liposomal irinotecan sucrosofate salt. Therefore, D4 would not have led the skilled person to provide *any* treatment cycle involving the co-administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor, because it does not mention either of them.

4.33 Secondly, D4 does not disclose a treatment cycle wherein irinotecan and a PARP inhibitor are each administered and wherein there is a delay between administration of the irinotecan and the PARP inhibitor. Instead, D4 describes two possible treatment cycles – one of which involves "concurrent" administration of the two drugs, and the other of which is "sequential". The opponent alleged that the "sequential" administration referred to in D4 would have led the skilled person to administer irinotecan and a PARP inhibitor in a "staggered" fashion, such as the claimed treatment cycle[15]. However, the opponent's interpretation is incorrect, because the "sequential administration" described in D4 is entirely different from that required by the presently claimed dosage regimen. As discussed in D4, section 1, sequential administration in the context of D4 means administration of 5-FU **after treatment with irinotecan has completely ended**:

> *The observed improvements in tumor perfusion following IrC™ treatment suggest the possibility that combination treatments with IrC™ and 5-FU might be more efficacious if **5-FU was administered after IrC™ treatment** in a sequential, rather than concurrent, manner.*

4.34 This is confirmed in the detailed dosage regimen described in D4, which is summarised in section 2.9 as: "Therapeutic studies with 5-FU **given after** IrC™ administration". The dosage regimen therein is described as initial treatment with IrC™, followed by administration of 5-FU **one week after the last IrC™ injection**. Thus, D4 does not teach toward a "staggered" treatment in which irinotecan and a second anticancer agent are administered as part of a repeating cycle, as alleged by the opponent. On the contrary, D4 teaches the use of a dosage regimen wherein a second anticancer agent should be administered only after treatment with irinotecan has completely ended.

4.35 In fact, D4 actually teaches away from a treatment cycle involving concurrent use of irinotecan and a second anticancer agent in a cycle, such as the presently claimed dosage regimen, linking co-administration with problematic toxicity[16]:

> *...our research team has examined the therapeutic effects **when IrC™ and 5-FU are administered concurrently. The data show that any***

[15] Notice of opposition, point 5.6.10.
[16] D4, section 1: "Introduction".

> ***therapeutic potential of the combination is compromised by an unexpected increase in toxicity.*** *The reasons for the enhanced toxicity are not well-understood although it may be due to IrC™ treatment prolonging the circulation lifetime of 5-FU.*

4.36 Thus, when faced with the technical problem of providing a safe and effective treatment for a solid tumor using a combination of irinotecan and a PARP inhibitor, the skilled person would have had no reason to look to D4. However, even if they did, D4 would at most have taught toward a dosage regimen where the PARP inhibitor was administered after the end of irinotecan treatment, rather than concurrently with irinotecan treatment as part of a cycle.

Same-day administration of irinotecan and a second anticancer agent (D2, D3 and D6)

4.37 Each of the remaining prior art documents which describe protocols for combination therapy with irinotecan and a second anticancer agent (D2, D3 and D6) teach a dosage regimen wherein irinotecan and the second anticancer agent are both administered on the same day. Therefore, none of these documents would have led the skilled person to the claimed dosage regimen, which requires the administration of the PARP inhibitor to be delayed by 3 days after the administration of liposomal irinotecan sucrosofate salt.

4.38 Specifically, D2 provides the outline for a clinical trial wherein non-liposomal irinotecan (in the form of FOLFIRI, which is a combination including non-liposomal irinotecan, 5-fluorouracil, and leucovorin) is administered on days 1 and 15 of a 28 day cycle and the PARP inhibitor veliparib is administered on days 1-5 and 15-19[17]. In other words, in the protocol of D2, irinotecan and a PARP inhibitor are administered **on the same day of each cycle**. D2 makes no mention of the possibility for using liposomal irinotecan of any kind, let alone liposomal irinotecan sucrosofate salt, nor does it contemplate the possibility for any alternative treatment cycle other than the one used therein. Thus, D2 would not have led the skilled person to provide a treatment involving the use of liposomal irinotecan sucrosofate salt, nor would it have led them to provide a treatment cycle wherein administration of the PARP inhibitor was delayed relative to irinotecan in the treatment cycle.

4.39 D3 describes a study involving co-administration of non-liposomal irinotecan and olaparib (a PARP inhibitor), wherein when mice were treated with a combination of olaparib and irinotecan, "*olaparib was administered 1 hour before irinotecan treatment.*"[18]. D3 summarises that the treatment described therein was successful and synergistic[19], thus providing clear teaching that a treatment cycle involving **same-day administration of irinotecan and a PARP inhibitor** was advantageous. D3 makes no mention of the possibility for using liposomal irinotecan of any kind, let alone liposomal irinotecan sucrosofate salt, nor does it contemplate the possibility for any alternative treatment cycle other than the one used therein. If anything, D3 would have led the skilled person away from the subject matter of claim 1 towards a treatment cycle in which a PARP inhibitor (e.g. olaparib) is administered **before** irinotecan. Therefore, D3 would not have led the skilled person to provide a treatment involving the use of liposomal irinotecan sucrosofate salt, nor would it have led them to

[17] D2, page 2, "Background".
[18] D3, page 1172, "*In vivo* growth inhibitory assay".
[19] D3, "Abstract".

provide a treatment cycle wherein administration of the PARP inhibitor was delayed relative to irinotecan in the treatment cycle.

4.40 D6 describes a study involving the exposure of a human colon cancer cell line to SN-38 (the active metabolite of irinotecan) in combination with various other anticancer agents, such as flavopiridol and fluorouracil. The opponent has alleged that D6 would have taught the skilled person that they should administer a PARP inhibitor after irinotecan, such as in the presently claimed treatment cycle, particularly in view of its teaching that better *in vitro* anti-tumor activity was achieved when SN-38 was administered before flavopiridol compared to when the two drugs were administered the other way around[20]. However, D6 is missing several fundamental aspects of the claimed treatment and would not have led the skilled person to the treatment cycle recited in the MR.

4.41 Most significantly, flavopiridol is not a PARP inhibitor. Therefore, D6 is not related to treatment involving the co-administration of irinotecan and a PARP inhibitor **at all**, let alone to their co-administration in a treatment cycle where dosing of one is delayed relative to the other. In fact, D6 does not even mention PARP inhibitors. Thus, when faced with the present technical problem and starting from D1, the skilled person would have had no reason to look to D6, which provides no teaching regarding, for example, the synergy between irinotecan and PARP inhibitors, let alone ways to harness this synergy without compromising treatment safety.

4.42 For completeness, even if the skilled person had looked to D6 and considered replacing flavopiridol with a PARP inhibitor, which they would have had no reason to do, D4 would not have led the skilled person to use the treatment cycle recited in the claims of the MR.

4.43 Firstly, D6 discloses the use of irinotecan in the form of its active metabolite, SN-38[21]. D6 makes no mention of the possibility for using alternative forms of irinotecan, let alone liposomal irinotecan or specifically liposomal irinotecan sucrosofate salt. Therefore, D6 would not have led the skilled person to provide *any* treatment cycle involving the co-administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor, because it does not so much as mention either of them.

4.44 Secondly, in the "staggered" administration cycle referred to by the opponent in D6, although it is stated that flavopiridol was administered after SN-38, no specific administration times are given for either drug. However, the authors of D6 state that, on the basis of the finding that better *in vitro* anti-tumor activity was achieved when SN-38 was administered before flavopiridol, they are pursuing a phase I clinical trial based on this combination. D14 is a paper discussing the results of this clinical trial, which specifies that in the treatment cycle followed in the trial, irinotecan was initially administered and flavopiridol was administered 7 hours later – in other words, the two drugs were administered **on the same day**. Thus, the meaning of administration of one drug "after" another in D6 is on the order of hours, not days, and D6 does not teach a treatment cycle where the administration of one drug is delayed by as much as 3 days after the other.

[20] Notice of opposition, point 5.6.8; D6, page 345, right-hand column, second paragraph.
[21] D6, page 345, right-hand column, second paragraph.

4.45 Thus, not only does D6 fail to disclose combination therapy involving liposomal irinotecan sucrosofate salt and a PARP inhibitor, but it also does not disclose the use of a dosage regimen where administration of one drug is delayed by a number of days relative to the other. D6 therefore would not have led the skilled person to the claimed treatment.

Other cited prior art documents (D5, D7 and D12)

4.46 For completeness, none of the other prior art documents would have led the skilled person to the claimed treatment.

4.47 D5 is a clinical trial design for determining the biodistribution of irinotecan sucrosofate salt encapsulated in liposomes for intravenous use (referred to therein as MM-398). However, D5 makes no mention of any possible uses for the irinotecan sucrosofate salt encapsulated in liposomes for intravenous use in treatment[22]. In particular, D5 does not mention PARP inhibitors at all, let alone a treatment cycle involving the co-administration of irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and a PARP inhibitor. Therefore, D5 would not have led the skilled person to the claimed treatment cycle.

4.48 D7 relates in its entirety to the use of PARP inhibitors in treating breast and ovarian cancers[23], and does not disclose any treatment cycle involving the administration of irinotecan and a PARP inhibitor in combination. In fact, beyond making a statement on page 8 that veliparib has been proven to sensitise irinotecan therapy in CRC cells, D7 does not mention irinotecan at all. D7 thus fails to disclose a treatment cycle involving liposomal irinotecan sucrosofate salt, let alone a treatment cycle involving the PARP inhibitor administration delay recited in the MR. D7 therefore would not have led the skilled person towards the claimed subject matter.

4.49 D12 relates in its entirety to the use of PARP inhibitors in treating breast and ovarian cancers[24]. D12 makes no mention of irinotecan at all, let alone of irinotecan in the form of liposomal irinotecan sucrosofate salt. D12 thus clearly fails to disclose any treatment cycle involving the co-administration of PARP inhibitors and irinotecan. Therefore, D12 would not have led the skilled person towards the claimed subject matter.

4.50 It is noted that the opponent referred to D9 to allege that the skilled person would have been motivated to use liposomal irinotecan in view of its favourable pharmacokinetic profile. However, D9 was published on 21st December 2015, which is after the earliest priority date of 20th August 2015. The opponent has alleged that, because D9 is a review article, it represents the skilled person's common general knowledge, citing T 777/08 to support this allegation. However, unlike the situation in T 777/08, the opponent has cited no corroborating prior art documents to indicate that the specific information disclosed in D9 was part of the skilled person's common general knowledge at the earliest priority date. It therefore cannot be assumed that all of the information in D9 represents knowledge that the skilled person had at the priority date, and D9 should therefore be dismissed for the consideration of inventive step.

4.51 However, even if D9 is taken into consideration (which it should not be as it is not prior art), D9 does not disclose or suggest any kind of combination therapy involving the co-

[22] D5, "Study Identification".
[23] D7, page 1, "Abstract".
[24] D12, page 1, "Abstract".

administration of liposomal irinotecan and a PARP inhibitor. Therefore, D9 fails to advance the opponent's case.

4.52 Therefore, the skilled person starting from D1 and seeking to solve the objective technical problem would not have been led to the claimed subject matter with a reasonable expectation of success. If anything, D1 would have led the skilled person away from the claimed invention and towards a dosage regimen which required the administration of non-liposomal irinotecan and a PARP inhibitor on the same day. Moreover, the deficiencies in D1 are not remedied by the other prior art documents, and therefore claim 1 of the MR meets the requirements of inventive step. The dependent claims are inventive at least by virtue of their dependency on claim 1.

Inventive step at the PCT filing date

4.53 Should the OD come to the conclusion that the claims are not entitled to claim priority from P1 and P2 (which the proprietor contests for the reasons set out in section 3 above), the claims would, in any event, be inventive over the prior art available at the PCT filing date.

4.54 In its inventive step arguments assuming that the claims were not entitled to priority, the opponent took D10 to be the closest prior art document. D10 will therefore be taken as the closest prior art document for the below analysis.

Technical differences and technical effect

4.55 There are several technical differences between the claims of MR and D10. In particular:

- The claims of the MR specify that the claimed treatment involves the administration of **irinotecan sucrosofate salt encapsulated in liposomes for intravenous use**.
    - D10 simply refers to "liposomal irinotecan" and "MM-398" without giving further details.
- The claims of the MR specify that in **each treatment cycle**, the PARP inhibitor is administered on one or more days starting **3 days after** the liposomal irinotecan sucrosofate salt.
    - In contrast, in the 28-day treatment cycles contemplated in D10, MM-398 is administered on days 1 and 15 and the PARP inhibitor is either administered on **days 5-12 and 19-25** or **days 3-12 and 17-25** i.e., delayed by either 2 or 4 days relative to each irinotecan administration.
- The MR specifies that the treatment cycle **treats a solid tumor in a patient**, whereas D10 makes no mention of the efficacy of the regimen recited therein.

4.56 The combination of these technical differences results in a technical effect, in that the claimed combination therapy harnesses the clinical potential of the synergistic effect of liposomal irinotecan sucrosofate salt and a PARP inhibitor without compromising on safety or requiring dose reductions of either drug. This technical effect is discussed in detail under inventive step starting from D1 in sections 4.10-4.22 above. This discussion applies here *mutatis mutandis*.

Technical problem to be solved

4.57 The technical problem to be solved is to provide a treatment for a solid tumor using a combination of irinotecan and a PARP inhibitor which harnesses the clinical potential of the synergistic effect of irinotecan and a PARP inhibitor without compromising the safety of the treatment.

Non-obviousness of the solution

4.58 When starting from D10 and faced with the present technical problem, the skilled person would not have arrived at the claimed treatment cycle with a reasonable expectation of success.

4.59 D10 is the earliest version of a contemplated clinical trial protocol for a combination therapy involving MM-398 and veliparib (a PARP inhibitor). As laid out above, D10 does not disclose the efficacy of the protocol recited therein in treating patients with a solid tumor, let alone whether the recited protocol achieves treatment without compromised safety. In addition, while D10 discloses two possible treatment cycles, neither of these involves delaying the administration of a PARP inhibitor by 3 days after administration of liposomal irinotecan sucrosofate salt. Thus, D10 would have provided no motivation for the skilled person to modify these treatment cycles to instead include a PARP inhibitor dosing delay of 3 days.

4.60 If anything, based on the fact that the clinical trial protocol of D10 recited the exact dosing days for MM-398 and the PARP inhibitor, the skilled person would have been taught away from modifying the treatment cycle to change the dosing days. As discussed above in section 4.1, the skilled person in the technical field would have known that clinical development of combination therapies involving Top1 inhibitors (of which irinotecan is an example) and PARP inhibitors had been limited due to increased toxicities and resultant dose reductions limiting the clinical utility of the combination. Thus, based on the previous difficulties with combination therapies, the skilled person would have surmised that the treatment cycles of D10 had been suggested for a particular reason, possibly with a view to providing treatment without the toxicities associated with previous combination therapies. Thus, the skilled person would only have modified these treatment cycles if there were a strong motivation or suggestion to do so. There is no motivation or suggestion here (be it strong or otherwise), and therefore D10 would not have led the skilled person towards the claimed subject matter.

4.61 At this point, it should be noted that the opponent has cited two pieces of case law in an attempt to support its inventive step attack. In particular, it is noted that the opponent cited T2506/12, r.3.10, in its opposition statement:

> "*at the publication date* [of the clinical trial protocol]*, the information was available that the envisaged combination treatment was considered by pharmaceutical researchers with an expectation of success sufficient to justify a clinical phase I trial. In this context* ***it is pointed out that drug compounds to be used in a clinical trial with human subjects are not selected based on a general "try-and-see" attitude, but based on existing favourable scientific data****, for both ethical and economical reasons.*"

4.62 Another case cited by the opponent is T239/16, particularly r.6.6, fifth paragraph thereof:

> "*Clinical trials in humans are planned scientific investigations. They require authority approval, which is only given after a risk/benefit evaluation. For ethical (but also economic) reasons it has to be ensured that research risks are minimised and are reasonable in relation to any potential benefits. Ethical and economical considerations require that the "benefit" will arise with reasonable certainty and will not only "be hoped for".*"

4.63 Should the opponent wish to rely on T2506/12 and T239/16, and should the OD agree with the opponent's interpretation of these cases, several points should be noted. In particular, the opponent has argued that the skilled person would look to D10 and have a reasonable expectation that the dosage regimens described therein would have been successful. This can be seen from page 18 and 19 of the opposition statement, particularly paragraph 5.3.12. To arrive at the claimed subject matter starting from D10, the skilled person would have needed to modify the dosage regimens of D10 to include a 3 day delay in between administering the liposomal irinotecan sucrosofate salt and the PARP inhibitor. However, D10 provides no disclosure or suggestion which would have led the skilled person to make such a modification.

4.64 Thus, if the opponent wishes to rely on T2506/12 and T239/16, and if the OD agrees with the opponent's interpretation of these cases, the skilled person would have been in a situation in which a reasonable expectation of success existed for the dosage regimens described in D10. Faced with dosage regimens which they already reasonably expected to be successful, the skilled person would have had no *a priori* reason to make any modifications to these dosage regimens. In addition, there is nothing in any other passage of D10 which would have motivated the skilled person to make any sort of modification to the dosage regimens of D10, let alone the very specific modifications which would have been required to arrive at claim 1. Therefore, D10 would not have led the skilled person towards the claimed subject matter, and so the claims involve an inventive step in view of D10.

4.65 In addition, and as will be explained below, none of the additional cited prior art documents would have provided the skilled person with the motivation to modify the treatment cycles of D10.

Documents D8, D9 and D11 would not have led the skilled person to the invention

4.66 Of the cited prior art documents which are only relevant for inventive step if the claims are not entitled to priority (which the proprietor contests), only D11 discusses combination therapy involving a combination of irinotecan and a PARP inhibitor (veliparib). D11 describes the weekly dosing of mice with liposomal irinotecan sucrosofate salt and veliparib, wherein three different potential treatment cycles were tested (dosing on days 2-4, 3-5 or 4-6[25]). Crucially, D11 describes the protocol and results of a preclinical study – i.e., D11 is a study whose results influence the optimum protocol for a particular clinical trial. In other words, D11 could be thought of as being a study which produces the "*existing favourable scientific data*"

[25] D11, page 1, final paragraph.

referred to by the Board in T 2506/12 which is used to inform clinical trial design. In fact, D11 is **the preclinical study that led to the clinical trial of D10**[26].

4.67 Thus, when starting from D10 and faced with the technical problem of providing a treatment for a solid tumor using a combination of irinotecan and a PARP inhibitor which harnesses the clinical potential of the synergistic effect of irinotecan and a PARP inhibitor without compromising the safety of the treatment, D11 would not have assisted the skilled person, because the results of D11 had already been accounted for when the treatment cycle of D10 was designed. In particular, the preclinical results from the treatment cycles involving MM-398 dosing on day 1 and veliparib dosing on days 2-4 (a 1-day delay), days 3-5 (a 2-day delay) or days 4-6 (a 3-day delay) resulted in the study investigators proposing a clinical trial involving either a 2-day delay or a 4-day delay in PARP inhibitor dosing. That is, having studied a 1-day delay, a 2-day delay, and a 3-day delay, the 2-day delay was taken forward, but the 3-day delay was not taken forward and seemingly abandoned. Therefore, if anything, the preclinical study of D11 in combination with the contemplated clinical trial protocol of D10 actually would have taught the skilled person away from using a 3-day delay. This is because the skilled person would have noted that a dosage regimen using a 3-day delay had been tested in D11 and seemingly disregarded for the actual clinical trial of D10, possibly due to poor efficacy and/or safety results.

4.68 D9 is a drug evaluation paper discussing the properties and potential uses of MM-398, which is used in the trial of D10. D9 discusses various treatment cycles involving combination therapy with MM-398 and a second anticancer agent, focusing in particular on a phase III trial, NAPOLI-1, which involved the co-administration of MM-398 with 5-fluorouracil/leucovorin every two weeks[27]. However, D9 makes no mention of any combination therapy involving MM-398 and a PARP inhibitor, let alone the specific dosing days to be used in such a cycle. Therefore, D9 would not have led the skilled person to modify the clinical trial protocol of D10 to arrive at the claimed treatment cycle.

4.69 D8 is the label for ONIVYDE®, which is a form of liposomal irinotecan sucrosofate salt for injection that is indicated for treatment of patients with pancreatic cancer in combination with fluorouracil and leucovorin[28]. D8 discusses the approved treatment cycle for this combination of drugs, which is the same protocol as described in D9, i.e., a 2-week treatment cycle involving the administration of ONIVYDE® over 90 minutes, followed by the administration of leucovorin over 30 minutes, followed by the administration of fluorouracil over 46 hours[29]. Thus, not only does D8 make no mention of any treatment cycle involving the administration of liposomal irinotecan sucrosofate salt and a PARP inhibitor, but it also makes no mention of any treatment cycle wherein administration of a second anticancer agent is delayed by a number of days relative to liposomal irinotecan sucrosofate salt. Therefore, D8 would not have motivated the skilled person to modify the protocol of D10 to arrive at the claimed treatment cycle.

[26] See "Contacts/Locations" section of D10 (page 7), which lists the study official as Susan Bates, one of the authors of the preclinical study of D11, and the location as the National Cancer Institute, the institution by whom the phase I trial following on from the preclinical study of D11 is planned to be led.
[27] D9, page 462, "Executive summary; Dosage & administration".
[28] D8, page 1, left-hand column, "Indications and Usage".
[29] D8, page 14, "Clinical Studies".

Documents D1-D6, D7 or D12 would not have led the skilled person to the invention

4.70 None of the cited documents D1-D6, D7 or D12 would have led the skilled person to modify the protocol of D10 to include a delay of 3 days between administration of irinotecan and the PARP inhibitor for the reasons discussed in sections 4.25-4.49 above with respect to the protocol of D1.

4.71 In view of the above, when starting from D10 as closest prior art, which the proprietor refutes is the correct approach, the claims are still inventive. In particular, there is nothing that would have led the skilled person to make a very specific modification to the dosage regimens described in D10 with a reasonable expectation of success. Therefore, the requirements of Article 56 EPC are met.

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5 AUXILIARY REQUESTS

5.1 The proprietor considers the claims of the MR allowable for the reasons given above. However, should the OD unexpectedly disagree, the enclosed auxiliary requests are filed to address the opponents' various assertions of lack of priority entitlement, and lack of inventive step.

Auxiliary Request 1

5.2 Auxiliary Request 1 (AR1) is filed in response to the allegations of lack of priority entitlement, and lack of inventive step on this basis.

Article 123(2) EPC

5.3 The claims have been amended in the same way as for the MR (i.e., granted claim 2 has been renumbered as new claim 1 and amended to recite the list of cancers to be treated, the type of liposomal irinotecan and the dosing delay between irinotecan and the PARP inhibitor) and dependent claims 3, 6, 11, 13 and 14 have been deleted. However, the list of cancers in claim 1 of AR1 has been shortened with respect to the list of cancers in the MR. Basis for this amendment is found in paragraph [0010] of the application as filed.

Article 76(1) EPC

5.4 This amendment merely confirms priority entitlement to P1 and P2 and finds basis in paragraph [0038] of P1 and paragraph [0039] of P2.

Article 56 EPC

5.5 For the same reasons as the claims of the MR, the claims of AR1 are inventive.

Auxiliary Request 2

5.6 Auxiliary Request 2 (AR2) is filed in response to the allegations of lack of priority entitlement, and lack of inventive step on this basis.

Article 123(2) EPC

5.7 The claims have been amended in the same way as for the MR (i.e., granted claim 2 has been renumbered as new claim 1 and amended to recite the list of cancers to be treated, the type of liposomal irinotecan and the dosing delay between irinotecan and the PARP inhibitor)

and dependent claims 3, 6, 11, 13 and 14 have been deleted. However, AR2 has been amended to explicitly recite that the solid tumor is selected from the list of cancers in the MR. Basis for this amendment is found in paragraphs [0085] and [00155] of the application as filed.

Article 76(1) EPC

5.8 This amendment merely confirms priority entitlement to P1 and P2 and finds basis in paragraph [0038] of P1 and paragraphs [0039] and [0058] of P2.

Article 56 EPC

5.9 For the same reasons as the claims of the MR, the claims of AR2 are inventive.

Auxiliary Request 3

5.10 Auxiliary Request 3 (AR3) is filed in response to the allegations of lack of priority entitlement, and lack of inventive step on this basis.

Article 123(2) EPC

5.11 The claims have been amended in the same way as for the MR (i.e., granted claim 2 has been renumbered as new claim 1 and amended to recite the list of cancers to be treated, the type of liposomal irinotecan and the dosing delay between irinotecan and the PARP inhibitor) and dependent claims 3, 6, 11, 13 and 14 have been deleted. However, the claim has been amended to recite a dosing delay of 72 hours, with basis in paragraph [00108] of the application as filed.

Article 76(1) EPC

5.12 This amendment merely confirms priority entitlement to P1 and P2 and finds basis in paragraph [0042] of P1 and paragraph [0043] of P2.

Article 56 EPC

5.13 For the same reasons as the claims of the MR, the claims of AR3 are inventive.

Auxiliary Request 4

5.14 Auxiliary Request 4 (AR4) is filed in response to the allegations of lack of inventive step.

Article 123(2) EPC

5.15 The claims have been amended in the same way as for the MR (i.e., granted claim 2 has been renumbered as new claim 1 and amended to recite the list of cancers to be treated, the type of liposomal irinotecan and the dosing delay between irinotecan and the PARP inhibitor) and dependent claims 3, 6, 11, 13 and 14 have been deleted. However, the claim has been additionally amended to specify that the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, olaparib which is administered at a dose of 300mg BID, rucaparib which is administered at a dose of 240-600 mg BID, niraparib, which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD. This amendment finds basis in paragraph [00102] of the application as filed, which states that the PARP inhibitor may be administered at the dose selected for PARP inhibitor monotherapy and exemplifies monotherapy dosing of veliparib, in combination with Table 2B, which

provides monotherapy dosing for each of olaparib, rucaparib, niraparib and talazoparib. Dependent claims 9 and 10 have been deleted for consistency with this amendment.

Article 56 EPC

5.16 The claims of AR4 are inventive for the reasons laid out above for the main request. However, the claims of AR4 additionally recite that the PARP inhibitor is veliparib, olaparib, rucaparib, niraparib or talazoparib, each administered at their therapeutically effective dose as a monotherapy[30]. This amendment further strengthens the proprietor's inventive step position.

Inventive step at the priority date of P1 and P2

5.17 As discussed above in section 4.8, D1 discloses a study involving the coadministration of irinotecan and veliparib. Thus, AR4 has at least one additional technical difference over D1:

- The dose of PARP inhibitor administered is **a dose which can be used in PARP inhibitor monotherapy**, whereas in D1 the dose of PARP inhibitor administered is **significantly lower than the dose used in monotherapy** (e.g. for veliparib, the minimum dose administered in the claimed dosage regimen is 200mg/day, while in D1 the dose administered was 40mg/day – *five times lower than the minimum monotherapy dose*).

5.18 As discussed above in section 4.8, D1 discloses a study involving the coadministration of irinotecan and veliparib, where due to dose-limiting toxicities a **10-fold lower dose** of veliparib had to be used compared with the dose conventionally used in veliparib monotherapy (40 mg versus 400 mg). Thus, the claims of AR4 have been amended to more closely reflect the technical effect over D1 – i.e. as discussed above in sections 4.10-4.22, the inventors surprisingly found that the use of the treatment cycle of the invention means that higher doses of PARP inhibitor can be used compared to when the two drugs were administered closer together in the treatment cycle, without compromising the safety of the treatment. In view of the higher doses used, the efficacy (or "log kill") of the treatment is higher, without the expected increase in toxicity usually associated with the use of higher doses. AR4 specifies that the claimed treatment cycle uses a dose of PARP inhibitor which is equivalent to the therapeutically effective dose used in PARP inhibitor monotherapy. Thus, AR4 has an additional technical effect over D1 in that a higher effective dose of PARP inhibitor can be administered safely.

5.19 There was no teaching in any of the cited prior art documents to use a PARP inhibitor in combination therapy at its therapeutically effective dose in monotherapy. On the contrary, the teaching in D1 that dose limiting toxicities necessitated the use of a dose ten times lower than the monotherapy dose would have taught away from using veliparib, olaparib, rucaparib, niraparib or talazoparib at the doses recited in AR4. The claims are thus additionally inventive for this reason.

Inventive step at the PCT filing date

5.20 As discussed above in section 4.59, D10 is the earliest version of a contemplated clinical trial protocol for a combination therapy involving MM-398 and veliparib (a PARP inhibitor). D10

[30] See Table 2B on page 15 of the patent.

provides no information regarding the amounts of MM-398 or veliparib to be administered in the protocol therein. Thus, AR4 has at least one additional technical difference over D10:

- The dose of PARP inhibitor administered is **a dose which can be used in PARP inhibitor monotherapy**, whereas in D10, **no dose of PARP inhibitor is provided**.

5.21 As discussed above in sections 4.10-4.22, the inventors surprisingly discovered that the use of the treatment cycle of the invention means that higher doses of PARP inhibitor can be used compared to when the two drugs were administered closer together in the treatment cycle, without compromising the safety of the treatment. In view of the higher doses used, the efficacy (or "log kill") of the treatment is higher, without the expected increase in toxicity usually associated with the use of higher doses. AR4 specifies that the claimed treatment cycle uses a dose of PARP inhibitor which is equivalent to the therapeutically effective dose used in PARP inhibitor monotherapy. Thus, AR4 has an additional technical effect over D10 in that a high effective dose of PARP inhibitor can be administered safely.

5.22 There was no teaching in D10 to use a PARP inhibitor in combination therapy at its therapeutically effective dose in monotherapy – in fact, there was no teaching about dose at all in D10. However, if the skilled person had looked to the other cited prior art documents to determine what dose to use for the PARP inhibitor, they would have been taught away from using the same dose as in monotherapy. For example, as discussed above, D1 teaches that dose limiting toxicities in irinotecan-veliparib combination therapy necessitated the use of a dose **ten times lower than the monotherapy dose** for veliparib. Thus, when starting from D10 and solving the problem of providing a treatment for a solid tumor using a combination of irinotecan and a PARP inhibitor which harnesses the clinical potential of the synergistic effect of irinotecan and a PARP inhibitor without compromising the safety of the treatment, the skilled person would not have used a dose of veliparib, olaparib, rucaparib, niraparib or talazoparib as high as the one recited in AR4. The claims are thus additionally inventive for this reason.

Auxiliary Request 5

5.23 Auxiliary Request 5 (AR5) is filed in response to the allegations of lack of inventive step.

Article 123(2) EPC

5.24 The claims have been amended in the same way as for the MR (i.e., granted claim 2 has been renumbered as new claim 1 and amended to recite the list of cancers to be treated, the type of liposomal irinotecan and the dosing delay between irinotecan and the PARP inhibitor) and dependent claims 3, 6, 11, 13 and 14 have been deleted. However, the claim has been additionally amended to specify that the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID. This amendment finds basis in paragraph [00102] of the application as filed, which states that the PARP inhibitor may be administered at the dose selected for PARP inhibitor monotherapy, in combination with Table 2B, which provides monotherapy dosing for rucaparib. Dependent claims 9, 10 and 12 have been deleted for consistency with this amendment.

Article 56 EPC

5.25 The claims of AR5 are inventive for the reasons laid out above for AR4. However, the claims of AR5 additionally recite that the PARP inhibitor is **rucaparib** which is administered at a dose

of 240-600 mg BID (i.e., administered at its therapeutically effective dose in monotherapy[31]). This represents an additional technical difference over both D1 and D10, where the PARP inhibitor is **veliparib**. The claims are thus additionally inventive because the skilled person starting from D1 or D10 would have had no reason to change the PARP inhibitor to rucaparib, and to use it at the dose used in monotherapy.

5.26 In fact, as discussed above in paragraphs 5.19 and 5.22 with respect to AR4, the cited prior art documents would have led the skilled person to use a significantly lower dose than that used in monotherapy. In particular, as discussed above, D1 teaches that dose limiting toxicities necessitated the use of a dose ten times lower than the monotherapy dose of the PARP inhibitor, thus clearly teaching away from using rucaparib at the dose recited in AR5. The claims are thus additionally inventive for this reason.

Other auxiliary requests

5.27 AR6 is a combination of AR1 and AR2

5.28 AR7 is a combination of AR1 and AR3

5.29 AR8 is a combination of AR1 and AR4

5.30 AR9 is a combination of AR1 and AR5

5.31 AR10 is a combination of AR2 and AR3

5.32 AR11 is a combination of AR2 and AR4

5.33 AR12 is a combination of AR2 and AR5

5.34 AR13 is a combination of AR3 and AR4

5.35 AR14 is a combination of AR3 and AR5

5.36 AR15 is a combination of AR1, AR2, and AR3

5.37 AR16 is a combination of AR1, AR2 and AR4

5.38 AR17 is a combination of AR1, AR2 and AR5

5.39 AR18 is a combination of AR1, AR3 and AR4

5.40 AR19 is a combination of AR1, AR3 and AR5

5.41 AR20 is a combination of AR2, AR3 and AR4

5.42 AR21 is a combination of AR2, AR3 and AR5

5.43 AR22 is a combination of AR1, AR2, AR3 and AR4

5.44 AR23 is a combination of AR1, AR2, AR3 and AR5

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[31] See Table 2B on page 15 of the patent.

MAIN REQUEST

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800

mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

MAIN REQUEST

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~3~~6, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~4~~7, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~5~~8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~6~~9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

8~~12~~. The liposomal irinotecan for use of any one of claims 1-7~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 1

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 1

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~3~~6, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~4~~7, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~5~~8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~6~~9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

812. The liposomal irinotecan for use of any one of claims 1-~~7~~11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 2

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 2

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-3~~6~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-4~~7~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-5~~8~~, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7~~10~~. The liposomal irinotecan for use of any one of claims 1-6~~9~~, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

8~~12~~. The liposomal irinotecan for use of any one of claims 1-7~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 3

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800

mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 3

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~58~~, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~69~~, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

8~~12~~. The liposomal irinotecan for use of any one of claims 1-7~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 4

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 4

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

3~~5~~. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

4~~7~~. The liposomal irinotecan for use of any one of claims 1-3~~6~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5~~8~~. The liposomal irinotecan for use of any one of claims 1-4~~7~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative~~

~~breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 5

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 5

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 6

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 6

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~3~~6, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~4~~7, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~5~~8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~6~~9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

812. The liposomal irinotecan for use of any one of claims 1-~~7~~11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 7

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 7

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~3~~6, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~4~~7, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~5~~8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~6~~9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

812. The liposomal irinotecan for use of any one of claims 1-~~7~~11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 8

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 8

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 9

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 9

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 10

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 10

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~3~~6, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~4~~7, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~5~~8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~6~~9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

812. The liposomal irinotecan for use of any one of claims 1-~~7~~11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 11

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 11

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 12

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 12

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 13

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 13

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinoteca~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 14

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 14

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 15

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

7. The liposomal irinotecan for use of any one of claims 1-6, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

8. The liposomal irinotecan for use of any one of claims 1-7, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 15

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

2~~4~~. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~3~~6, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~4~~7, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

69. The liposomal irinotecan for use of any one of claims 1-~~5~~8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.

710. The liposomal irinotecan for use of any one of claims 1-~~6~~9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

812. The liposomal irinotecan for use of any one of claims 1-~~7~~11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 16

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 16

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 17

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 17

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least~~ 3 days after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 18

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 18

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

612. The liposomal irinotecan for use of any one of claims 1-511, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 19

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 19

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 20

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 20

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 21

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 21

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 22

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

6. The liposomal irinotecan for use of any one of claims 1-5, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

AUXILIARY REQUEST 22

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor , wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is veliparib which is administered at a dose of 200-800 mg/day, or olaparib which is administered at a dose of 300mg BID, or rucaparib which is administered at a dose of 240-600 mg BID, or niraparib which is administered at a dose of 300mg QD, or talazoparib which is administered at a dose of 1 mg QD, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

6~~12~~. The liposomal irinotecan for use of any one of claims 1-5~~11~~, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

AUXILIARY REQUEST 23

1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:
   i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and
   ii) administering the PARP inhibitor on one or more days starting 72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

2. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 of the antineoplastic therapy treatment cycle.

3. The liposomal irinotecan for use of any one of claims 1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 of the antineoplastic therapy treatment cycle.

4. The liposomal irinotecan for use of any one of claims 1-3, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

5. The liposomal irinotecan for use of any one of claims 1-4, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

AUXILIARY REQUEST 23

~~1. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor in a patient, wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the liposomal irinotecan is repeatedly administered, and wherein the treatment comprises:~~

~~i) administering to the patient liposomal irinotecan once every two weeks; and~~

~~ii) administering the PARP inhibitor daily for 3 to 10 days between consecutive administrations of the liposomal irinotecan, wherein the PARP inhibitor is administered at least 2, 3, 4 or 5 days after the administration of the liposomal irinotecan and at least 2, 3, 4 or 5 days prior to the next administration of the liposomal irinotecan.~~

1~~2~~. Liposomal irinotecan for use in an antineoplastic therapy for the treatment of a solid tumor selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer in a patient, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use and wherein the liposomal irinotecan is administered in combination with a Poly(ADP-ribose) Polymerase (PARP) inhibitor, wherein the PARP inhibitor is rucaparib which is administered at a dose of 240-600 mg BID, the treatment comprising a 28-day antineoplastic therapy treatment cycle consisting of:

i) administering the liposomal irinotecan on days 1 and 15 of the treatment cycle, and

ii) administering the PARP inhibitor on one or more days starting ~~at least 3 days~~72 hours after the liposomal irinotecan and ending at least 1 day prior to administration of additional liposomal irinotecan.

~~3. The liposomal irinotecan for use of claim 2, wherein the PARP inhibitor is not administered for at least 3 days prior to the next administration of liposomal irinotecan.~~

24. The liposomal irinotecan for use of ~~any one of claims 2-3~~claim 1, wherein the PARP inhibitor is administered on one or more of days 5 to 12 ~~or on one or more of days 3 to 12~~ of the antineoplastic therapy treatment cycle.

35. The liposomal irinotecan for use of any one of claims ~~2-4~~1-2, wherein the PARP inhibitor is administered on one or more of days 19 to 25 ~~or on one or more of days 17 to 25~~ of the antineoplastic therapy treatment cycle.

~~6. The liposomal irinotecan for use of claim 1, wherein the PARP inhibitor is administered on each of consecutive days 3 to 10 between the days when the liposomal irinotecan is administered.~~

47. The liposomal irinotecan for use of any one of claims 1-~~36~~, wherein the liposomal irinotecan has an irinotecan terminal elimination half-life of 26.8 hours and a maximal irinotecan plasma concentration of 38.0 micrograms/ml.

58. The liposomal irinotecan for use of any one of claims 1-~~47~~, wherein each administration of liposomal irinotecan is administered at a dose of 80 mg/m^2 (salt) or 70 mg/m^2 (free base).

~~9. The liposomal irinotecan for use of any one of claims 1-8, wherein each administration of the PARP inhibitor is administered at a dose of from about 20 mg/day to about 800 mg/day and/or wherein the PARP inhibitor is administered once or twice daily at a dose of from about 20 mg/day to about 400 mg/day.~~

~~10. The liposomal irinotecan for use of any one of claims 1-9, wherein the PARP inhibitor is selected from the group consisting of niraparib, olaparib, veliparib, rucaparib and talazoparib.~~

~~11. The liposomal irinotecan for use of any one of claims 1-10, wherein the patient with a solid tumor has a cancer selected from cervical cancer, ovarian cancer, triple negative breast cancer, non-small cell lung cancer, small cell lung cancer, gastrointestinal stromal tumors gastric cancer, pancreatic cancer, colorectal cancer, or a neuroendocrine cancer.~~

~~12. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is veliparib.~~

~~13. The liposomal irinotecan for use of any one of claims 1-11, wherein the patient with a solid tumor has cervical cancer and the PARP inhibitor is olaparib.~~

~~14. The liposomal irinotecan of any one of claims 1-13, wherein the liposomal irinotecan includes irinotecan sucrosofate salt encapsulated in liposomes for intravenous use.~~

Transcript of D1b

Thank you Dr Ivy and I'd like to thank the organising committee for allowing me to present to you today on this investigator-initiated study of

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the Phase 1 PARP inhibitor ABT-888 in combination with CPT-11 evaluating its safety of the combination, the pharmacokinetics as well as some pharmacodynamic endpoints in patients with advanced solid tumours, and I'd like to let you know that I'm presenting this on behalf of all of my co-investigators as you see here, both at the Karmanos cancer institute as well as the National Cancer Institute, most specifically JG, who did a lot of the pharmacokinetic work with us from the NCTLV lab, Dr. Geoff Shapiro from the Dana Farber cancer institute, and my colleagues as well from University of Maryland.

[Goes to slide 2 of D1a]

So this trial actually began with preclinical work that was done by one of my close collaborators at Karmanos cancer insistute, Dr Angelica Burge. And basically what we did is we initially began by doing a pre-clinical study design because we wanted to look at these two drugs in combination, the PARP inhibitor ABT-888 in combination with CPT-11, and so we chose several cancer cell lines that we would need to look at paired, to determine the exact....to help define the in vitro cytotoxicity differential potential looking at these cancer cell lines, and we looked at both BRCA mutant and wildtype, p53 mutant and wildtype, ERCC1 high vs low expression cell lines as well as cell lines that had ERCC1 polymorphism, and as we would develop a clinical trial we developed the pre-clinical trial looking at both monotherapy of CPT-11 and ABT-888 as well as the combination; and then we looked at specific readouts which included cytotoxicity as well as PARP-1 and gamma-H2AX levels.

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So looking specifically at a couple of these pre-clinical combinations, in the ERCC high mutant p53 T47D cell line what we identify is that with both CPT-11 at an IC_{50} of 10 as well as ABT-888 at 8 μM there, as you can see here, these monotherapy lines, and then when you gave these two drugs in combination you got significant enhanced increase in tumour growth inhibition or in vitro cytotoxicity. And then when you look specifically at two cell lines, both the MX-1, which is a BRCA1 deleted BRCA2 mutant line, as well as the HCC1937, another BRCA line, looking at ABT-888 as a monotherapy with much lower IC_{50}s of 2.5 and an IC_{50} of 0.01 μM, you see significant in vitro monotherapy cytotoxicity.

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So then what we also did is we looked at paired lines of deleted BRCA mutant vs wildtype BRCA mutant with the MX-1 vs the MDA-MB-231, demonstrating that in the BRCA1 mutant cell line, which is also ERCC1 high, there was enhanced cytotoxicity of the combination and we didn't really see that in the MDA-MB-231 cell line. We then went into the ovarian ERCC1 high A2780 vs the ADDP comparator ERCC1 low cell line and only in the ERCC1 high cell line did we see in vitro combination enhanced cytotoxicity which, as I said, was not demonstrated in the ERCC1 low line. And then we looked at two in vitro lung models, the HOP62 and H226, both lung, one ERCC1 high and one low, and we again really didn't demonstrate any significant enhanced in vitro cytotoxicity in either of the in vitro lines.

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So looking at the combination again, looking at its efficacy it resulted in downregulation of both PARP and induction of double strand breaks in the T47D cell line that I demonstrated previously; looking at...and we measured the differential in terms of gamma-H2AX expression at both 4 hours and 24 hours as well as looking at PARP inhibition and what we identified was that with either of the monotherapy arms there was essentially no alteration of PARP at either 4 or 24 hours but when you gave the two drugs in combination there was significant alteration in PARP expression and that was also manifest with significant increase in gamma-H2AX expression at 24 hours with the combination vs either monotherapy. You see some suggestion of that at 4 hours but it's really enhanced at 24 hours and again you see that as well here with the combination vs monotherapy at 24 hours.

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Again, we looked at it in a mutant BRCA MX-1 breast cancer model, again looking at the monotherapy vs the combination, significant increase in in vitro cytotoxicity and again looking at gamma-H2AX, again in the BRCA mutant model, we were able to again confirm the alteration in gamma-H2AX relative to the monotherapy arms, increasing at 4 hours but significantly increased at 24 hours in the combination relative to the monotherapy control arms.

[Goes to slide 7 of D1a]

And then obviously we went in vitro in several models...in vivo in several cell lines, looking at the combination vs monotherapy and this is just demonstrating two separate lines, again the ADDP ovarian model where we looked at CPT-11 vs ABT-888 monotherapy vs the combination, demonstrating an increase in efficacy, probably more synergism here, or additivism than synergism,

but when we went into the MX-1 taxol-resistant xenograft model, we saw significant alteration of efficacy of the combination relative to the monotherapy arms which were virtually devoid of any activity, especially the taxol arm in this taxol-resistant line.

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So then based on that we were then able to successfully compete for an LOI at the NCI, at CTAP, looking at the two drugs in combination, in a Phase-1 combination study, where our primary objective was obviously, as it is a Phase-1 trial, to determine what the recommended Phase-2 dose of the two drugs in combination are, as well as the primary objective of safety, followed by secondary objectives which included the pK profiling of each drug alone as well as in combination, to determine whether there or not there was any drug-drug interaction, and to secondarily also define any tumour efficacy by RECIST and we included several exploratory objectives – pharmacodynamics, looking at PAR inhibition, both in PBMCs and tumour tissue, as well as any DNA damaging effects with CPT-11 vs CPT-11 in combination with ABT-888, by measuring levels of DNA damage and repair.

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So it's a very complicated schema but I'll go through it. There were serial biopsies, we had 34 patients that were enrolled, were successfully able to get pre-biopsies on all patients, were successfully able to get a second hit on 30 of the 34 patients - because there were only 2 biopsies that we were doing - we were concerned that we wouldn't be able to successfully biopsy each patient 3 times - we did not get baseline levels so that we could determine what the maximum level of PAR expression would be after CPT-11, then after we gave the drug within 24 hours biopsied the patient, followed then by daily oral ABT-888 to obviously inhibit PAR and then biopsing them after the second dose of CPT-11 to determine whether or not, in the presence of a PAR inhibitor, could we inhibit PAR expression in the tumour despite giving a second dose of CPT-11. And then subsequent cycles just involved giving the ABT-88 first followed by CPT-11, the ABT given daily continuously for 15 out of 21 days, the CPT-11 given on both days 1 and 8. Beginning with ABT-88 in hopes that we could prevent the up-regulation of PAR when we gave the CPT-11, hopefully because we had PAR inhibitory agents on board.

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So the eligibility were pretty typical for a phase-1 trial with two exceptions – all patients had to have measurable disease that was accessible or evaluable disease that was accessible for biopsy, and because of the CPT-11 (and Dr Mark retains data with genomic profiling), and the fact that UGT1A1

homozygous patients can have enhanced ANT-toxicity, most notably amylosupression, all patients were pre-screened for UGT1A1 and were excluded if they met certain genomic parameters.

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In terms of the most common toxicities, they were exactly what you would expect with CPT-11 – diarrhoea, nausea being the most common toxicities, in excess of 50% of the patients, but only 3 patients having Grade 3 diarrhoea in this trial, followed by fatigue, leukopenia - very typical toxicities that you would expect with just CPT-11 alone. There were no really surprises here in terms of toxicity.

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We began with 10 mg BID of the ABT-11 in combination with 100 mg/m^2 of CPT-11. That dose was maintained at a constant 100 mg/m^2 throughout the dose escalation. We then escalated to 20 mg. We did get Grade 4 neutropenia and leukopenia. We expanded that cohort, went subsequently to 50 mg and because 2 out of the 6 patients demonstrated dose-limiting toxicities, in terms of diarrhoea, febrile neutropenia and leukopenia, we then de-escalated down to 40 mg of ABT-88 BID in combination with 100 mg/m2 day 1 in aid of CPT-11 which became the dose that we then continued in an expansion cohort for definition of safety in that two drug dose combination.

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Preliminary response data is available on 30 patients, there was a clinical benefit rate of 63% in those 30 patients. We had 6 partial responses, a triple negative breast cancer patient that remained on study for 28 cycles, another triple negative breast cancer patient that remained on cycle for approximately nine cycles, and actually her cycles were changed to Q4 weeks because of travel issues so she maintained response for approximately nine months. There were two colorectal cancer patients that obtained a PR1 that had had prior exposure to CPT-11 in a fall-fury regimen without response. And there were two BRCA mutant ovarian cancers that responded; both of those patients were at the Dana Farber. We had one MR, again in a triple negative breast cancer patient. And then there were 12 stable disease that ranged from 2 to actually 6+ cycles as we have two patients currently remaining on study, going into their 7th cycle pending their CT scan results.

The MTB and the recommended phase 2 dose (RP2D), as I said, was 100 mg/m^2 of CPT-11 on days 1 and 8 and 40 mg of ABT-888 twice daily, days 1-15, repeated every 21 days.

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So the beauty of serial tumour biopsies is that we were able to look at PAR levels, we looked at PAR levels instead of alteration in PARP because it was much easier to....and more consistent assay for evaluation of PAR levels within these tumours. As I said, Dr JG at the National Cancer Institute did all of our PD PAR evaluation. And as you can see the red are the baseline levels, the blue are those levels at day 9, so this is day 1 (24 hours after CPT-11) and then the blue is after the CPT-11/ABT-888. And as you can see, there was significant decline in several patients and if you look at relative PAR levels – day 1 vs day 9 – what you can see here is actually a more pronounced differential in the relative PAR, keep in mind that when we're looking at PAR levels in the tumour tissue, the vertical axis is...we see PAR levels about 2.5 logs here from...baseline here is recorded at 1000, 2 in excess of 100,000.

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So we also looked at PAR levels in PBMCs, to determine whether PBMCs could be used in the future as a surrogate. Obviously again on the vertical axis, those PAR...the vertical axis PAR measurement is significantly lower than in tumour, which makes sense because the tumour is where it's at its maximum. We did see alteration in PAR levels in PBMCs, but it did not look nearly as pronounced nor as reproducible as the PAR levels that we defined in the tumour biopsies. One would question whether or not in the tumour biopsies we were getting maximum PAR inhibition and one would also question why, many times, those PAR levels...never were the PAR levels dropped down to zero; but one must keep in mind that we were biopsing those patients at day 9, we don't know when the maximum inhibition of PAR would be, relative to the exposure of ABT-888 and CPT-11, and those studies will be ongoing in a subsequent amended trial that we will be talking about in the next few minutes.

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So one of the other things we did look at one was one of the downstream targets, ERCC1, and although we can't make concluding bio-statistically - relevant conclusions of our data, if you looked at the ERCC1 levels in tumour tissue - in the little red boxes are the medians and this is pre-ABT-888 and day 9 after several days exposure of ABT-888 – there's a significant decline in the median....there's a decline in the median ERCC1 levels which is highly suggestive that this could potentially be a marker. And the red boxes were those patients that had clinical benefit. So, we saw an alteration in ERCC1 in those patients with clinical benefit and that alteration was not manifest in patients that went on to have progressive disease.

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And we also looked at that, obviously, in PBMCs and although it wasn't as manifest and pronounced between pre- and after cycle 1 day 1 (C1 D1), what you can determine in cycle 1, if you look at the pre- in the patients with clinical benefit versus day 28, there's again significant decline.

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Pharmacokinetics: I'll just say because of time limitations – systemic exposure to ABT-888, when given alone or in combination with CPT-11, was not altered by various parameters such as Cmax and AUC;

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and again when we looked at CPT-11, either alone or in combination, there was no alteration of exposure or kinetics, whatsoever, based on the addition of ABT-888 to the CPT-11.

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So in summary, the kinetics demonstrated that ABT-888 exhibited linear kinetics at tested dose levels from 10-15 mg twice daily. There was no apparent difference in oral clearance of the compound when given either alone or in combination with irinotecan, and co-administration of CPT-11 did not at all affect the kinetics of ABT-888.

Regarding the pharmacodynamic summary, it appeared as if ERCC1 could potentially be a biomarker to define those patients for which clinical benefit may occur, as there was demonstrated a decline in those patients at day 8 vs baseline. Regarding PAR levels in paired tumour biopsies, there was at least a 50% reduction in 85% of the patients and a greater than 90% reduction in at least 35% of the patients. And if you looked at PAR levels in PBMCs, the differential was not as great, but it was still demonstrating a decline of at least 50 % in at least 65% of the patients. And the gamma-H2AX levels will be conducted by the NCI's national clinical trial validation laboratory as those sample will be submitted in the next few weeks to the NCI for analysis.

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We have a proposed expansion and that expansion will include two cohorts, one in triple negative breast cancer and one in BRCA mutant patients. We will be doing three serial biopsies in those patients at baseline relative to day 2 relative to day 9 and we will be looking at circulating tumour cell

(CTC) sampling in several time points because our hope is that CTCs in the future will help us define alteration and will be a more effective biomarker. And the CTC sample that I think is going to be most relevant is day 15 because if CTCs can be used as biomarker analysis, hopefully the day 15 sample will let us know whether or now we have achieved maximum PAR inhibition, based on relative comparison to day 9, and if we haven't then we will be altering our schedule of administration to conclude continuous administration of ABT-888.

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We are going to be again doing tumour biopsies and hopefully in addition to all of the CTC work that we're doing in this study, and tumour biopsy analysis for similar biomarkers that we did in the initial portion of the Phase 1 trial, we're hoping that we're going to be able to do deep-dive sequencing on the triple negative cancer patients for continuous evaluation of determination of whether or not the genomics can help us determine responders vs non-responders with this drug combination.

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And with that I'd like to first and foremost thank the patients and families, my colleagues at the various institutions, especially Geoff Shapiro, who's been a dynamite partner to work with, not only on this trial but multiple other trials, and Ralph and Jiu Ji at the NCI. And I'd like to also thank one of my colleagues, Dr Angelika Burger, who unfortunately died about a month ago, who was very instrumental in doing all of the PD biomarkers at our site, and helping to write the trial and do the pre-clinical work at our site. I recruited her from Baltimore about 3 years ago and unfortunately 6 months after her recruit she was diagnosed with metastatic disease and her life ended on May 4th of this year. Thank you very much.

A Phase I Clinical Trial of the Sequential Combination of Irinotecan Followed by Flavopiridol

Manish A. Shah,[1,5] Jeremy Kortmansky,[1,5] Monica Motwani,[5] Marija Drobnjak,[4] Mithat Gonen,[2] Sandy Yi,[1] Amanda Weyerbacher,[1] Carlos Cordon-Cardo,[4] Robert Lefkowitz,[3] Baruch Brenner,[1] Eileen O'Reilly,[1] Leonard Saltz,[1] William Tong,[1] David P. Kelsen,[1] and Gary K. Schwartz[1,5]

Abstract

Purpose: Flavopiridol potently enhances the effect of irinotecan with cures in colorectal cancer xenografts, and is associated with modulation of several molecular targets, including p21, Differentiation-related gene 1 (Drg1), and p53. We initiated a phase I trial of the sequential combination of irinotecan followed by flavopiridol to determine the maximal tolerated dose of this combination therapy.

Patients and Methods: Forty-five patients with advanced solid tumors were enrolled. Irinotecan was administered first (100 or 125 mg/m^2) followed 7 hours later by escalating flavopiridol (10-70 mg/m^2) given weekly over 1 hour for 4 of 6 weeks. At the maximal tolerated dose, the pharmacokinetic analysis was expanded and pre- and posttreatment tumor biopsies were done.

Results: At irinotecan 100 mg/m^2, dose-limiting diarrhea and myelosuppression were observed with flavopiridol 70 mg/m^2. At irinotecan 125 mg/m^2, we observed dose-limiting hyperbilirubinemia, fatigue, and myelosuppression at flavopiridol 60 mg/m^2. Peak flavopiridol concentrations of $\geq$2 μmol/L were achieved above flavopiridol 50 mg/m^2. No significant pharmacokinetic interactions with irinotecan were noted. Baseline serum bilirubin significantly predicted cycle 1 dose-limiting toxicity and neutropenia. We observed partial responses in three patients and prolonged stable disease (i.e., >6 months) in 36% of patients including adrenocortical cancer and hepatocellular cancer. Patients with wild-type p53 and either no change or low posttreatment biopsy p21 and a decrease in Drg1 expression showed stable or responsive disease to the combination therapy.

Conclusions: The recommended phase II dose with irinotecan 100 mg/m^2 is flavopiridol 60 mg/m^2 and with irinotecan 125 mg/m^2 is flavopiridol 50 mg/m^2. Toxicity can be predicted by baseline bilirubin. Clinical activity is encouraging and may correlate to changes in p21 and Drg1 levels in patients with wild type p53 tumors following therapy.

The rationale for targeting the cell cycle, and in particular cyclin-dependent kinases (CDK), in anticancer therapy lies in the high frequency of their perturbations in human malignancy and the observation that their inhibition, leading to cell cycle arrest, would induce apoptosis (1, 2). Targeting CDKs recapitulate aberrant cell cycle checkpoints, thereby limiting the ability of a tumor cell to cycle and thus facilitating the induction of apoptosis (3). This led to the development of CDK inhibitors as novel antitumor agents (4), with flavopiridol serving as an early model drug in this new class. Flavopiridol inhibits multiple CDKs (5–8), as well as attacks other molecular targets including inhibition of angiogenesis, survivin, IκB kinase and nuclear factor κB (5–11). Although single agent phase I studies (4, 12) showed safety and encouraging clinical activity, single agent phase II trials have shown disappointing activity (13–18), perhaps due to an inability to achieve adequate free drug concentrations due to high protein binding.

There is a growing body of data suggesting that flavopiridol may improve the efficacy of cytotoxic chemotherapy, including irinotecan (19–24). In our xenograft studies, maximal tumor regressions and pathologic cures were shown without significant toxicity in HCT-116 tumor–bearing mice treated with irinotecan followed by flavopiridol at 7 or 16 hours. We also noted the importance of p21 and Differentiation-related gene 1 (Drg1) in these experiments. Specifically, irinotecan administration was associated with the inhibition of cell proliferation, the induction of p53, p21, and Drg1, and a failure to undergo

Authors' Affiliations: [1]Department of Medicine, Division of Solid Tumor Oncology, Gastrointestinal Oncology Service, [2]Departments of Biostatistics, [3]Radiology, and [4]Pathology, and [5]The Laboratory of New Drug Development, Memorial Sloan Kettering Cancer Center, New York, New York

Received 12/22/04; revised 2/11/05; accepted 2/16/05.

Grant support: National Cancer Institute grant R01 CA 067819 (G.K. Schwartz), and American Society of Clinical Oncology Young Investigator Award 2001 and Cancer and Leukemia Group B Investigator Award 2001 (M.A. Shah).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Presented in part at the 39th Annual Meeting of the American Society of Clinical Oncology, May 31-June 3, 2003, Chicago, Illinois.

Requests for reprints: Gary K. Schwartz, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Phone: 212-639-8324; Fax: 212-717-3340; E-mail: schwartg@mskcc.org.

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apoptosis. However, the subsequent administration of flavopiridol was associated with decreases in p21 and Drg1, inhibition of X-linked inhibitor of apoptosis (an inhibitor of caspase 3), and an induction of apoptosis (21, 25).

In these xenograft studies, we administered flavopiridol as an intraperitoneal injection. The rapid i.p. absorption of this drug approximates a shortened i.v. infusion of flavopiridol. This implied that a prolonged continuous infusion of flavopiridol would not be necessary to potentiate the effect of irinotecan. Because prolonged infusions of flavopiridol had been associated with considerable clinical toxicity, we felt it would be possible to avoid these toxicities with the drug administered over a shorter time interval, thereby allowing for potentiation of chemotherapy. On the basis of these laboratory and preclinical studies, we initiated a phase I study of irinotecan at a fixed dose followed 7 hours later by escalating doses of flavopiridol given over 1 hour for 4 of 6 weeks.

Patients and Methods

Adult patients (≥18 years old) with a histologically confirmed solid tumor that was refractory to standard therapy (or for which there was no standard therapy) were eligible. Eligibility and exclusion criteria were as previously reported (26). Prior irinotecan therapy was allowed. The protocol was reviewed and approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center. Written informed consent was obtained from each patient.

Treatment plan. This was an open-label, nonrandomized dose-escalation study. Groups of three to six patients received sequentially a fixed dose of irinotecan i.v. over 30 minutes followed 7 hours later by increasing dosages of flavopiridol administered over 60 minutes on a schedule of 4 weeks of therapy followed by a 2-week break. At the initiation of this study, a weekly schedule of flavopiridol had never previously been tested. Therefore, we chose the starting dose for weekly flavopiridol to be 10 mg/m^2, representing a total dose of 40 mg/m^2 over the 6-week cycle, which is well below the safe dose of flavopiridol when administered on an every-21-day schedule. Irinotecan was first fixed at 100 mg/m^2 and flavopiridol was escalated in successive cohorts as follows: 10→30→50→60→70 mg/m^2. Once the maximal tolerated dose (MTD) of flavopiridol with irinotecan 100 mg/m^2 was defined, we then determined the MTD of flavopiridol with irinotecan 125 mg/m^2. Flavopiridol was decreased to one dose level below its MTD with irinotecan 100 mg/m^2, and then we continued flavopiridol dose escalation in successive cohorts with irinotecan 125 mg/m^2.

All treatments were administered in the outpatient setting, and once assigned to a dose level, dose escalation was not permitted. For the first two cycles, patients were evaluated by a physician weekly before chemotherapy administration and then at the initiation of every cycle thereafter. Treatment responses were evaluated after every two cycles. Response Evaluation Criteria in Solid Tumors were used for response assessment and done by an independent protocol radiologist.

Toxicity was graded in accordance with the Common Toxicity Criteria version 2.0. Dose-limiting toxicity (DLT) was defined in cycle 1 with any occurrence of grade 4 hematologic toxicity, grade 3 or 4 nonhematologic toxicity including diarrhea despite antidiarrheal prophylaxis. At the onset, we realized that, independent of the flavopiridol administration, some patients would be unable to tolerate 4 weekly treatments of irinotecan followed by a 2-week break. These patients often tolerate intermittent dosing, such as irinotecan for 2 weeks followed by a 1-week break, thereby still receiving 4 of 6 weeks of therapy. Therefore, DLT was also defined as any delay in treatment resulting in less than 4 treatments in a 6-week cycle. Patients were allowed to proceed with treatment at the same dose in the absence of DLT if, on the day of the scheduled treatment, the absolute neutrophil count (ANC) was ≥1,500/mm^3, and platelet count ≥100,000/mm^3. If counts were below these levels, then therapy was delayed until the blood counts recovered. Treatment was also held for incompletely resolved diarrhea or stomatitis. A minimum of three patients were followed for at least one complete cycle (6 weeks) before dose escalation continued. If one instance of DLT was observed, an additional three patients were treated at that dose level. Dose escalation proceeded if these additional patients did not experience DLT. The MTD was defined as the dose one level below the dose at which two or more patients experienced DLT.

At the final MTD, 10 additional patients were enrolled to better define the toxicity profile. We planned to choose the recommended phase II dose based on the cumulative experience of all patients treated at a particular dose level, including the patients in the expanded cohorts, with the use of a confidence interval for toxicity. If 12 patients were treated at a particular dose level, the occurrence of 4 DLTs would define an unacceptable dose for further study.

Drug supply. Flavopiridol (HMR 1275) was supplied by the National Cancer Institute (Bethesda, MD) in 10 and 50 mg sterile vials, as previously reported (26). Flavopiridol was reconstituted in 250 mL of 0.9% Sodium Chloride Injection, USP, or 5% Dextrose for Injection, USP, so that the final concentration did not exceed 0.5 mg/mL. Irinotecan (Camptosar, UpJohn/Pharmacia, New Brurswick, NJ) was commercially available in two forms: 2 mL vials containing 40 mg of drug and 5 mL vials containing 100 mg of drug. The appropriate dose was diluted in 250 mL of 5% dextrose (D_5W) solution.

Pharmacokinetics. We examined the plasma levels of flavopiridol and irinotecan and its metabolites, SN-38 and SN-38G, in all patients the first week of the first two cycles of chemotherapy. During week 1 of cycle 1, blood draws were done at the following time points, relative to flavopiridol administration: before flavopiridol (t = 0 hour), at flavopiridol completion (t = 1 hour), 2 hours, and 20 hours. During week 1 of cycle 2 only, the flavopiridol and irinotecan order was reversed so as to obtain pharmacologic data on flavopiridol without the influence of irinotecan. Flavopiridol was given alone on day 1 and irinotecan was given on day 2, 24 hours later. Relative to flavopiridol, blood draws during the first week of cycle 2 were done at the following time points: flavopiridol completion (t = 1 hour), 2, 3, and 24 hours (before irinotecan administration). For the next 3 weeks of cycle 2, and for subsequent cycles, irinotecan was given 7 hours before flavopiridol as usual.

At the expanded cohort, the blood draws were expanded to better characterize both irinotecan and flavopiridol pharmacology. During week 1 of cycle 1, blood draws occurred (relative to flavopiridol) at the following times: at irinotecan completion (t = −6.5 hours), before flavopiridol (t = 0 hour), 1, 1.5, 2, 2.5, 3, 17, and 20 hours. During cycle 2, week 1, blood draws occurred at the following times: flavopiridol completion (t = 1 hour), 1.5, 2, 2.5, 3, 7, 9, 10, 24 hours (before irinotecan), 24.5 hours (irinotecan completion), 25.5, 26, and 48 hours.

Irinotecan, SN-38, and SN-38G pharmacokinetics were done by high-performance liquid chromatography by published methods (27). Flavopiridol pharmacokinetics were done at Quintiles Laboratory (Kansas City, MO) by published methods (26).

Biological assays. Patients enrolled at the expanded cohort at the MTD underwent computed tomography–guided biopsies before initiation of protocol treatment and again after 2 weeks of treatment. All posttherapy computed tomography–guided biopsies occurred within 48 hours of the week 2 treatment. Eighteen-gauge core needle biopsies were done for liver or lymph node biopsies and fine needle aspirates for lung lesions to minimize complications. The biopsy specimen was split in half when possible, fixed in formalin, and embedded in paraffin. Five-micrometer thin sections were cut for H&E and immunohistochemistry staining. Monoclonal antibodies for p53 (PAb1801, Oncogene Calbiochem, Cambridge, MA) and p21 (Cip1/WAF1; Oncogene Calbiochem) were used at 0.2 and 1 μg/mL concentrations, respectively. Standard avidin-biotin peroxidase method and heat-induced epitope retrieval with 0.01 mol/L citric acid (pH 6) were applied.

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Drg1 rabbit polyclonal antibody (courtesy of Therese Commes, UPR Center National de la Recherche Scientifique, Universite Montpellier II, France) was used at 1:8,000 final dilution. Sections were immersed in 0.01 mol/L citric acid (pH 6) and boiled in a microwave oven for 15 minutes. After cooling to room temperature, sections were incubated with 10% normal goat blocking serum for 30 minutes, followed by the primary antibody and overnight incubation at 4°C. After washing, biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) was applied at 1:1,000 dilution for 30 minutes, followed by avidin-biotin complex (Vector Laboratories) at 1:25 dilution. Diaminobenzidine was used as the final chromogen and hematoxylin as the nuclear counterstain.

Both positive and negative controls were run at the time of each experiment. Nuclear staining was considered specific reactivity for both p53 and p21 antibodies and percent of positive tumor cells was estimated examining different fields throughout the entire tissue section. For Drg1, cytoplasmic/plasma membrane staining was considered specific staining and results were recorded as percent of tumor cells reactive with the antibody, as estimated from the examination of different fields throughout the entire tissue section. Data were recorded in a continuum as the percentage of tumor cells stained. The staining was reviewed by two pathologists (M.D. and C.C.) for a consensus assessment for each antigen. Mutant (or positive) p53 staining was considered if greater than 20% of the nuclei stained positive. A change in p21 staining was considered significant if it was greater than 30%. Because the SD of Drg1 expression in 131 patients used to characterize the antigen was ±20% (28), the posttreatment biopsy was considered significantly changed if it was at least 20% different, either increased or decreased, from the pretreatment biopsy for Drg1. This analysis was done without knowledge of patient outcomes.

Statistics. All statistical analyses were done with SAS version 9.0. The probability of DLT was estimated using the observed proportion and 95% confidence intervals were formed by inverting the exact binomial test. The difference between SN-38 levels before and after the administration of flavopiridol was compared using the signed ranks test (paired Wilcoxon test). The area under the curve (AUC) was determined by a one-compartment model for all patients. We noted that at equivalent flavopiridol dosing, the AUCs calculated in patients with expanded pharmacology blood draws were similar with the AUCs calculated with limited pharmacology blood draws, suggesting appropriate AUC estimates. The terminal half-life was determined in the expanded cohorts for which additional pharmacology blood draws were done using a two-compartmental method. Calculation of clearance requires the knowledge of the initial dose which is not available to us for irinotecan metabolites. For this reason, clearance of the metabolites is presented in terms of the formation fraction, which is computed using the irinotecan dose in the calculations (29). The association of AUC and ANC levels with cycle, cohort, and toxicity was assessed using an exact Wilcoxon test. Bilirubin levels, as dichotomized by the observed median of 0.7 mg/dL, were compared between those who had a DLT and those who did not using a Fisher's exact test.

Results

Patient characteristics. From May 30, 2001 to May 6, 2003, 51 patients with advanced solid tumors were enrolled on protocol. Six of these patients were inevaluable for the primary end point of toxicity assessment due to rapid progression of disease (four patients who received only one or two doses of therapy), medical noncompliance (one patient), and patient withdrawal (one patient). Table 1 provides the patient characteristics for the remaining 45 patients who were assessable for toxicity, of whom 43 were assessable for response to the combination therapy. The median age was 55 (range 35-80 years), and the median Karnofsky performance status was 90% (range 70-90%). There were 29 men and 16 women. The cancers treated and frequency included colorectal (28), pancreas and cholangiocarcinoma (three each), breast, hepatocellular, gastric, and adrenocorticoid (two each), esophageal, germ cell, and neuroendocrine (one each). Forty-three patients (96%) had received prior chemotherapy, and 35 (78%) had received prior irinotecan (33 patients) or the related camptothecin, DX-8951F (2 patients), with one patient having received both drugs before participation in this trial. The median number of prior regimens for metastatic disease was 2 (range 0 to 6), with 38 (84%) having received between 1 and 4 prior treatment regimens.

Table 1. Patient characteristics

Characteristic		No. patients
Total		45
Assessable for response		43
Male		29
Female		16
Age, y		
Median	55	
Range	35-80	
KPS, %		
Median	90	
Range	70-90	
Prior chemotherapy		43
Prior camptothecin		35
No. prior regimens		
Mean	2	
Range	0-6	
Primary sites of disease		
Colorectal		28
Pancreas		3
Cholangiocarcinoma		3
Breast		2
Hepatocellular		2
Gastric		2
Adrenocorticoid		2
Esophageal		1
Germ cell		1
Neuroendocrine		1

Toxicity. Table 2 provides common cycle 1 nonhematologic and hematologic grades 2 to 4 toxicity that was possibly, probably, or definitely attributable to chemotherapy. All toxicities shown were either DLTs or occurred more than once in each cohort. Patients enrolled in the first four cohorts completed cycle 1 without DLT. However, in cohort 5, we observed 3 DLTs. One patient developed grade 3 diarrhea and two other patients were unable to receive 4 weekly treatments within the 6-week cycle due to modest (grade 2) but sustained myelosuppression. As shown in Table 3, each of these patients had previously received and tolerated weekly doses of irinotecan at 125 mg/m^2, thereby suggesting the toxicity observed was due to the combination therapy.

We then continued enrollment by reducing flavopiridol by two dose levels (to 50 mg/m^2) and increasing irinotecan to 125 mg/m^2 in cohort 6. In this cohort, one of six patients developed dose-limiting grade 3 fatigue and diarrhea. In retrospect, this

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toxicity may have been predicted in this patient because he previously experienced similar toxicity with irinotecan, fluorouracil, and leucovorin (see Table 3). However, as this was the only patient with DLT at this dose level, dose escalation continued.

In the first three patients treated in cohort 7, one patient with metastatic cholangiocarcinoma developed dose-limiting hyperbilirubinemia and fatigue. After the first dose of irinotecan and flavopiridol, he presented with grade 2 fatigue and a bilirubin that had increased from 1.3 to 3.4 mg/dL. Week 2 was held, and the patient presented on week 3 with a bilirubin back to baseline, however, with grade 3 fatigue. A computed tomography scan at that point showed disease progression. Although this toxicity was unusual, we felt it was possibly attributed to the combination therapy, and the cohort was expanded to six patients. In the expanded cohort, another patient developed grade 3 fatigue. This patient was subsequently retreated at a reduced dose of irinotecan 100 mg/m^2 and did not develop subsequent significant toxicity.

Although we observed two of six patients with DLT, it was unclear whether we had exceeded our MTD because one of the patients also had evidence of disease progression. Therefore, to better evaluate the tolerability of this dose level, we subsequently expanded this dose level with six additional patients (cohort 7b) for a total of 12 treated at this dose level. We observed two additional patients developing DLT, both unable to receive 4 weeks of therapy within the 6-week cycle due to sustained modest myelosuppression. Thus, a total of 4 of 12 patients treated at this dose level developed DLT in cohort 7. Three of these patients had received prior weekly irinotecan-based therapy (Table 3), which they had tolerated without significant toxicity, thereby suggesting intolerance to the combination therapy rather than to irinotecan alone.

Thus, the MTD of flavopiridol with irinotecan 100 mg/m^2 is 60 mg/m^2, and with irinotecan 125 mg/m^2 is 50 mg/m^2. We next treated 10 patients in an expanded cohort at the MTD (cohort 8) to better establish the recommended phase II doses of the combination therapy. Based on the patient's number of prior regimens and the potential for increased myelosuppression, it was left to the discretion of the treating physician whether to treat with flavopiridol at full-dose irinotecan (125 mg/m^2) or a lower dose of irinotecan (100 mg/m^2). In the nine additional patients treated at irinotecan 100 mg/m^2 and flavopiridol 60 mg/m^2, we observed three patients with DLT; one patient was unable to receive 4 treatments in 6 weeks, and the other two with grade 4 hematologic toxicity. Two of these patients were previously heavily treated (four and six previous therapies, respectively), and the third DLT occurred in a patient who previously developed neutropenia with gemcitabine therapy alone. Of note, one patient in the expanded cohort was treated at irinotecan 125 mg/m^2 and flavopiridol 50 mg/m^2, and no DLT was observed.

Cumulative toxicity was not significantly different from cycle 1 toxicity (data not shown). The most common cumulative nonhematologic toxicities included grade 2 diarrhea (46%), fatigue (31%), nausea (20%), and vomiting (22%). Grade 3 diarrhea, nausea, and vomiting occurred in 5 (11%), 4 (9%), and 2 (4%) patients, respectively. Common cumulative hematologic toxicities included grade 2 leukopenia (42%), grade 2 neutropenia (36%), and grade 2 anemia (22%). Grades 3 to 4 neutropenia occurred in 12 patients (27%), and was complicated by fever and neutropenia in 4 patients (9%). There does not seem to be cumulative hematologic toxicity in these patients, as the mean hematologic values for leukocytes, neutrophils, platelets, and hemoglobin are similar for cycle 1 and for all cycles. There were no treatment-related deaths on this study.

Toxicity and its relationship with baseline bilirubin. In one patient, we observed an interesting relation between a fluctuating bilirubin and myelosuppression. This patient had a baseline bilirubin of 1.6 mg/dL and ranged from 0.9 to 2.4 mg/dL. This patient also developed grade III neutropenia

Table 2. Cycle 1 nonhematologic and hematologic toxicity

Cohort			Fatigue			Diarrhea			Nausea			Vomiting			Bilirubin			Anemia			Leukocytes			ANC			Fever/ Neutropenia			4 of 6		
(patients)	CPT	F	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4	2	3	4
Cohort 1 (3)	100	10	1																													
Cohort 2 (3)	100	30				2												1			1			1								
Cohort 3 (3)	100	50				1												1			1			1								
Cohort 4 (3)	100	60				1			2			1									1			1								
Cohort 5 (5)	100	70	1				**1**					1						1	1		4			4								**2**
Cohort 6 (6)	125	50	2	**1**		3	**1**		2			3									3			3								
Cohort 7a* (6)	125	60	1	**2**†		1	**1**		1	**1**		2				**1**					1	1		1	1							**1**
Cohort 7b (6)	125	60	1			1			2			1						1			2	2		1	3							**2**
Cohort 8																																
(9)	100	60	2			3			2			1						2			5	2	1	5	1	**2**			**1**			**1**
(1)	125	50				1																										

NOTE: DLTs are in boldface. Grades 2-4, possibly, probably, or definitely attributable to chemotherapy.
*Six patients were initially treated in this cohort, but then the cohort was expanded with six additional patients to better evaluate toxicity.
† One of these patients also had grade 3 bilirubinemia. Additional toxicity includes one patient in cohort 6 who developed grade 2 hiccups, one patient in cohort 7 who developed grade 2 alopecia, and one patient with grade 2 weight loss in the expanded cohort (irinotecan 100 mg/m^2, flavopiridol 60 mg/m^2).

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Table 3. Summary of DLTs and previous irinotecan-based therapy

Cohort (patients)	Irinotecan	Flavopiridol	Pts with DLT	Toxicity	Prior irinotecan therapy (irinotecan dose)	Toxicity with previous irinotecan schedule
Cohort 5 (5)	100	70	3	4 of 6	IFL* (125 mg/m^2 weekly)	None
				G 3 diarrhea	IFL (125 mg/m^2 weekly)	None
				4 of 6	IFL (125 mg/m^2 weekly)	Full records unavailable—tolerated by patient report
Cohort 6 (6)	125	50	1	G 3 fatigue and diarrhea	IFL (100 mg/m^2 weekly)	Grade 3 fatigue and diarrhea
Cohort 7a (6)	125	60	2	G 3 fatigue and G 3 hyperbilirubinemia	IFL (?dose)	Full records unavailable—tolerated by patient report
				G 3 fatigue, 4 of 6	IFL (125 mg/m^2 weekly)	None
Cohort 7b (6)	125	60	2	4 of 6	None	n/a
				4 of 6	IFL (100 mg/m^2 weekly)	None
Cohort 8 (9)	100	60	3	4 of 6	None	n/a
				G 4 neutropenia	None	n/a
				G 4 neutropenia + fever	Irinotecan 80 mg/m^2 weekly (3 on/1 off) + IHA FUDR†	None

*IFL: irinotecan 100-125 mg/m^2, fluorouracil 500 mg/m^2, leucovorin 20 mg/m^2 weekly for 4 weeks followed by a 2-week break.
† IHA FUDr: intrahepatic pump chemotherapy with floxuridine.

with therapy, requiring dose delays, which seemingly correlated with high bilirubin levels during the period of neutropenia. We note that SN-38, flavopiridol, and bilirubin are all glucoronidated by a common pathway involving the family of UGT1A glucuronidation enzymes (30, 31). Polymorphysms in the UGT1A1 promoter affect the efficiency of this enzyme, which can be reflected in a patient's baseline bilirubin such that an elevated serum bilirubin would suggest slow bilirubin glucuronidation, and therefore possibly reduced UGT1A enzymatic efficiency (32). To examine the hypothesis that slow glucuronidation may be associated with increased active drug levels, and consequently to increased toxicity of the combination regimen, we retrospectively explored the relationship between baseline serum bilirubin (as a marker of UGT1A1 activity) and toxicity. The median bilirubin across all dose levels on this study was 0.7 mg/dL. Using this level as a cutoff, the probability of experiencing DLT with a bilirubin of less than 0.7 mg/dL was significantly less than if the bilirubin was ≥0.7 mg/dL. Specifically, we observed 1 DLT of 18 patients (5.8%) with a bilirubin <0.7 mg/dL, whereas there were 10 DLTs of 27 patients (37%) with a baseline bilirubin of ≥0.7 mg/dL, $P = 0.03$. The baseline serum bilirubin also correlated with neutropenia in cycle 1 such that patients with a baseline serum bilirubin of <0.7 mg/dL had a significantly higher mean cycle 1 ANC as well as a significantly higher cycle 1 ANC nadir than if the serum bilirubin at baseline was ≥0.7 mg/dL (Wilcoxon $P = 0.01$ for both mean and nadir ANC). Note that of the 33 patients who had previously received irinotecan-based therapy, we were able to obtain the baseline bilirubin (before their previous irinotecan treatment) in 20 patients. We noted no significant differences between their previous bilirubin baseline and the baseline bilirubin before initiation of this study, suggesting that the association of bilirubin and toxicity is related to the combination therapy.

Pharmacokinetics and pharmacodynamics. Plasma samples for pharmacokinetic analyses were obtained from all 45 patients, although cohort 1, cycle 1 pharmacology is not available. The one-compartment modeled flavopiridol plasma concentration-time profile (flavopiridol 60 mg/m^2) for cycle 1 (following irinotecan) and cycle 2 (preceding irinotecan) is presented in Fig. 1. Table 4 summarizes the flavopiridol pharmacokinetic variables for each dose level examined for cycles 1 and 2. Flavopiridol AUC increased significantly with dose ($P < 0.01$, using local regression), however, the rate of increase is dampened above flavopiridol 60 mg/m^2. There was no significant difference between flavopiridol AUC in cycles 1 and 2. Furthermore, at equivalent flavopiridol dosing, there was no significant difference in flavopiridol AUC when given with irinotecan 100 or 125 mg/m^2. At the flavopiridol MTD range (50-60 mg/m^2), the mean flavopiridol pharmacokinetic variables are as follows: AUC (7.36-14.04 μmol/L/h), clearance (12.8-26.1 L/h/m^2), and terminal half-life (5.1-7.7 hours). Flavopiridol C_{max} or AUC in cycle 1 did not correlate with toxicity of the combination regimen, including cycle 1 DLT, ANC nadir, and mean ANC in cycle 1.

We expanded the pharmacology blood draws in cohorts 7b and 8 to further characterize irinotecan pharmacokinetic. The cycles 1 and 2 irinotecan, SN-38, and SN-38G pharmacokinetic variables are presented in Table 5. The terminal half-lives for irinotecan, SN-38, and SN-38G in cycle 1 are 7.6, 16, and 18.1 hours, respectively. There was no significant difference between the pharmacokinetic variables for irinotecan and its metabolites between cycles 1 and 2.

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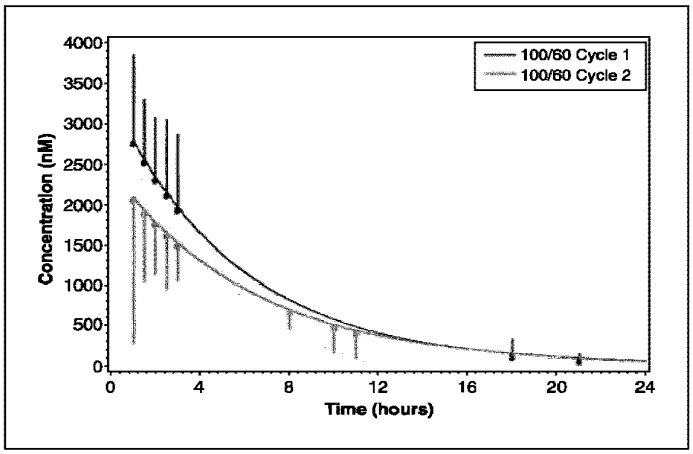


Fig. 1. Concentration-time profiles of flavopiridol at 60 mg/m^2 for cycle 1 and cycle 2 with CPT 100 mg/m^2. Data are fit to a one-compartment model (with SE bars) with the expanded pharmacology blood draws.

Antitumor activity. Forty-three patients were assessable for response assessment. We observed three patients with a partial response (two colorectal, one gastric), and 20 patients with stable disease, many of whom remained on study for prolonged periods of time, including 16 patients who remained on study for more than 6 months (36% of all enrolled patients) and 4 patients who remained on study for over 1 year. The median time on study for patients with partial responses or stable disease was 7.6 months with a range of 3.7 to 16.5 months. Notable durable stable responses included two patients with hepatocellular carcinoma who remained on study for 13.8 and 16.5 months, and two patients with adrenocortical cancer who remained on study with stable disease for 8.1 and 15.4 months. There was no association between the prior use of camptothecin and response to therapy.

Among the 27 evaluable patients with colorectal cancer, patients had a median of 2 prior treatment regimens with a range of 1 to 6 prior regimens. Twenty-three patients had received prior irinotecan, and nine patients had received both irinotecan and oxaliplatin before initiation of this clinical trial. We observed 14 patients (52%) with stable disease, one partial response, and 12 patients (44%) with disease progression as their best response. Patients with stable or responsive disease had durable periods of disease control with a median of 6.8 months (range 3.6-12.7 months).

Correlative studies. All 10 of the patients enrolled in the expanded cohort at the MTD (cohort 8) were eligible for and underwent computed tomography–guided biopsy of their tumor. These included six patients with colorectal cancer, and one each with gastric, pancreas, hepatocellular, and breast cancer. We planned pre- and posttreatment biopsies on each patient for a total of 20 biopsies. We were able to perform 19 biopsies (10 pretreatment and 9 posttreatment), with one patient not receiving a posttreatment biopsy because of a negative pretreatment biopsy. Seventeen biopsies (89%) showed tumor on H&E staining, and 15 biopsies (78%) were adequate for subsequent immunohistochemical analysis. Even following two treatments of irinotecan and flavopiridol, there was pathologic evidence of antitumor activity with tumor necrosis and apoptosis identified in H&E staining in one patient with radiographically stable disease (Fig. 2). The induction of apoptosis was confirmed by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (not shown). Two biopsies had no tumor on further sectioning. No biopsy-related complications were observed.

Tumors were evaluated for staining by immunohistochemistry for p53, p21, and Drg1, both at pretreatment and within 24 to 48 hours following the week 2 therapy (Table 6). As expected, p53 expression did not change when comparing the pre- and posttreatment biopsies. Therefore, a single result is reported. Matched samples for p21 staining are available for five patients. In the six patients who were p53 wild-type (negative p53 staining), p21 remained stable or was nondetectable on the posttreatment biopsy in three patients (patients 6, 8, and 9), all of whom had stable disease or a partial response. The change in p21 is uninterpretable in two patients (patients 2 and 7), both of whom also had stable disease. Matched samples for Drg1 staining are available for six patients. Figure 2 shows one patient with pre- and posttreatment biopsies stained for Drg1. We observed in five of six patients (84%) that the modulation of Drg1 correctly predicted outcome. Patients 1 and 3 (increase and no change) had disease progression, and patients 6, 7, and 8 (all with decreases in Drg1 following irinotecan and flavopiridol therapy) each had radiographic stable disease and remained on study for more than 7 months.

The patients who progressed were either p53 mutant (patients 3, 4, and 5) or were p53 wild-type, but with a rising

Table 4. Flavopiridol pharmacokinetic variables for cycle 1 and cycle 2 for each dose level

			Cycle 1						Cycle 2					
Dose levels (patients)	Irinotecan	Flavopiridol	C_{max} (μmol/L)	SD	AUC (μmol/L/h)	SD	Clearance (L/h/m^2)	SD	C_{max} (μmol/L)	SD	AUC (μmol/L/h)	SD	Clearance (L/h/m^2)	SD
1 (3)	100	10							0.606	0.271	1.82	0.39	13.18	2.93
2 (3)	100	30	1.247	0.244	6.37	1.82	11.31	2.80	1.167	0.220	7.27	6.43	11.17	7.08
3 (3)	100	50	2.044	0.600	10.02	0.69	11.67	0.48	1.921	0.282	8.26	4.65	14.76	15.5
4 (12)	100	60	3.078	1.072	17.12	8.48	9.34	3.42	3.145	1.829	15.87	6.10	9.48	7.95
5 (5)	100	70	2.768	0.545	16.24	5.46	10.14	1.95	2.512	1.213	14.80	2.70	10.98	2.02
6 (7)	125	50	2.137	0.677	12.79	6.15	9.59	3.07	1.888	0.754	9.22	3.58	14.68	7.23
7 (12)	125	60	3.861	2.017	18.49	9.27	8.59	2.63	3.375	1.128	21.67	6.62	8.07	8.00

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Table 5. Pharmacokinetic variables for cycle 1 and cycle 2 for the expanded cohorts for which additional pharmacology blood draws were done for CPT and its metabolites

		Cycle 1						Cycle 2					
Cohort	CPT	C_{max} (mol/L)	SD	AUC (mol/L/h)	SD	*Clearance/F_M (L/h/m^2)	SD	C_{max} (mol/L)	SD	AUC (mol/L/h)	SD	Clearance/F_M (L/h/m^2)	SD
Irinotecan	100	1,928.39	412.80	7.02	3.75	28.47	17.28	1,821.38	687.13	13.03	7.59	28.45	36.04
	125	2,024.96	575.22	10.36	9.24	22.13	18.56	3,293.03	1,271.00	17.2	11.09	24.22	27.92
SN-38	100	74.52	58.71	0.40	0.25	856.15	449.8	52.66	31.09	0.60	0.41	1,146.22	1,772.3
	125	88.21	36.18	0.92	0.98	847.30	750.6	119.24	12.39	1.26	1.08	1,041.00	1,353.7
SN-38G	100	178.35	208.21	2.32	2.11	127.48	87.22	86.55	45.49	2.53	1.50	258.30	429.40
	125	195.86	173.30	5.01	5.63	91.79	72.52	195.86	21.75	4.05	3.97	234.04	302.84

*Clearance/F_M is the clearance divided by the formation fraction.

p21 and Drg1 following therapy (patient 1). Although the numbers are small, these data support the preclinical model that baseline wild-type p53 and pharmacodynamic changes in p21 and Drg1 may be predictive of response.

Discussion

Our preclinical research indicates that the effect of irinotecan can be greatly enhanced by flavopiridol in a sequential and schedule-dependent fashion (21). Based on these preclinical studies, we initiated this phase I clinical trial to establish the MTD of irinotecan followed 7 hours later by flavopiridol given weekly over 1 hour for 4 of 6 weeks. We observed that with irinotecan 100 mg/m^2, the recommended phase II dose of flavopiridol is 60 mg/m^2, and with irinotecan 125 mg/m^2, it is 50 mg/m^2.

This represents the first study in which flavopiridol was administered on a 1-hour weekly schedule, either as a single agent or in combination therapy. On this schedule, we achieved micromolar concentrations of flavopiridol, which is significantly higher than reported with continuous infusion schedules of 24 to 72 hours (26, 33). Assuming 10% free drug concentrations, the 2 to 3 μmol/L levels we achieved at the flavopiridol MTDs should result in free flavopiridol concentrations of 200 to 300 nmol/L, which have been shown *in vitro* to significantly potentiate the effects of chemotherapy (34).

The only other flavopiridol trial in which the 1-hour infusion of flavopiridol was tested clinically was reported by Tan and colleagues. This group reported on three different 1-hour infusion schedules of flavopiridol, including daily for 5 days, daily for 3 days, and over 1 hour for a single treatment but repeated every 3 weeks (35). The recommended phase II

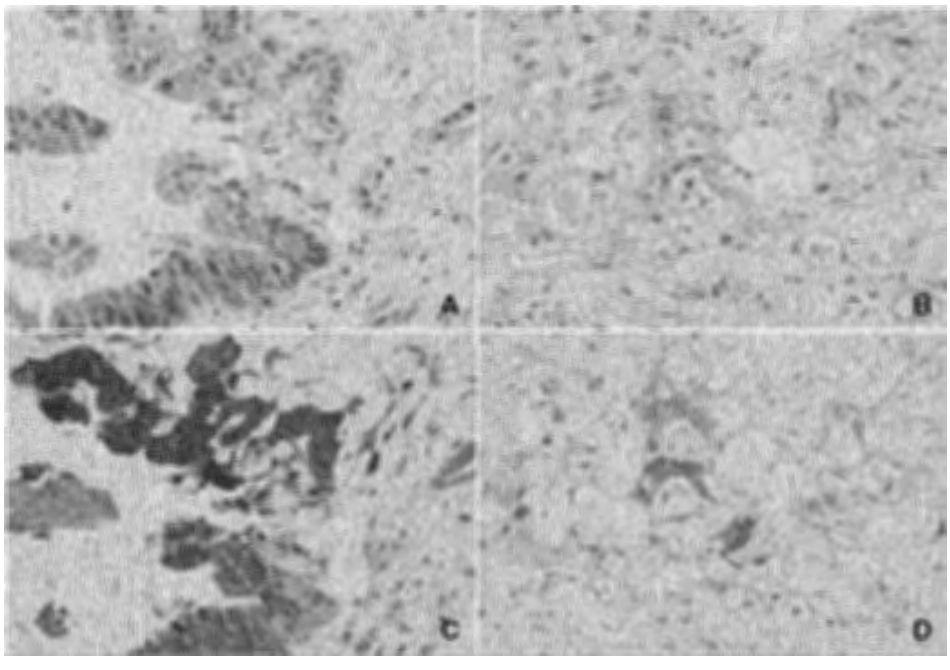


Fig. 2. Pre- and posttreatment biopsy H&E and Drg1 stains at 400× magnification. *A* and *C*, H&E and Drg1 stains, respectively, of the pretreatment baseline biopsy obtained within 2 weeks of study entry. *B* and *D*, H&E and Drg1 stains, respectively, of the posttreatment biopsy that was obtained within 48 hours of the week 2 treatment of CPT and flavopiridol. *B* and *D*, significant treatment effects including vacuolization, chromatin clumping, and apoptotic bodies.

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Table 6. Results of Biopsies at the expanded cohort at the MTD

Patient	p53	p21		p21 Interpretation	Drg1		Drg1 interpretation	Prior irinotecan	Best response*	Time on study (mo)
		Pre	Post		Pre	Post				
1	Neg†	20	60	Increase	5	30	Increase	No	POD	2.3
2	Neg	NT‡	70	?	NT	75	?	Yes	SD	16.5
3	Pos	30	25	No change	20	10	No change	Yes	POD	2.7
4	Pos	NT	10	?	NT	30	?	No	POD	2.7
5	Pos	5	10	No change	40	0	Decrease	Yes	POD	2.7
6	Neg	20	25	No change	80	30	Decrease	Yes	SD	8.3
7	Neg	0	NT	?	70	50	Decrease	Yes	SD	9.3
8	Neg	40	30	No change	100	10	Decrease	Yes	SD	7.1
9	Neg	NT	0	No change or Decrease	NT	0	No change or decrease	No	PR	7.6

NOTE: One patient had no tumor on the pretreatment biopsy and a second biopsy was not attempted.
*POD, progression of disease; SD, stable disease; PR, partial response.
† Neg, negative p53 staining.
‡ NT, no tumor.

dose for flavopiridol administered 1 hour on an every-3-week schedule was 62.5 mg/m^2. We observed similar DLTs, including neutropenia, diarrhea, and fatigue, with the more frequent weekly dosing. However, we did not observe several of the nonhematologic toxicities previously reported, such as anorexia, hypotension, hypophosphatemia, and hypoalbuminemia (35). In both Tan's study and our study, flavopiridol doses above 70 mg/m^2 were associated with significant and unacceptable toxicity. At the MTD in our study, the pharmacokinetic variables of flavopiridol, including C_{max}, AUC, clearance, and terminal half-life, are consistent with previous reports of similar short infusion flavopiridol schedules (35, 36). Thus, our study suggests that patients are able to receive another flavopiridol dose 1 week later, presumably because of having recovered from the previous dose 1 week earlier. Furthermore, the rate of increase in AUC with flavopiridol doses above 60 mg/m^2 was less pronounced, suggesting an apparent threshold for flavopiridol dosing above which clearance of the drug is possibly increased. This will need further confirmatory evaluation in larger pharmacology studies with this drug in this schedule. Interestingly, thrombotic events, which were a cause for concern with flavopiridol when administered as a 3-day continuous infusion, were rarely observed with flavopiridol administered over 1 hour in both studies.

It remains to be determined what is the best schedule to administer irinotecan with flavopiridol. Even though we could not administer the combination for 4 consecutive weeks in all patients, we still believe the weekly schedule of irinotecan with flavopiridol is superior to an every-3-week irinotecan schedule as it provides more frequent opportunity to potentiate chemotherapy with this CDK inhibitor. Notably, we did not observe a significant pharmacokinetic interaction between irinotecan and flavopiridol.

We found a significant correlation between baseline serum bilirubin levels and the probability of toxicity of the combination therapy, in particular myelosuppression. Transient hyperbilirubinemia was previously observed with 1-hour flavopiridol administration as well, suggesting a shared pathway with bilirubin and flavopiridol metabolism (35, 37, 31). Similarly, bilirubin and irinotecan share this metabolic pathway as well (30, 38, 39). In one recent study, there was a significant association between an elevated baseline serum bilirubin and the occurrence of grades 3 or 4 neutropenia in patients receiving a weekly schedule of irinotecan (40). In our study, patients with a baseline serum bilirubin below the median were significantly less likely to have DLT or neutropenia. Although a significant portion of the toxicity predicted by the baseline serum bilirubin may have been attributable to irinotecan, virtually all of the patients enrolled on this study previously received irinotecan-based therapy and tolerated it, and their previous baseline bilirubin value was not significantly different than when they initiated this study. This suggests that the serum bilirubin may be a sensitive indicator of toxicity of the combination of irinotecan and flavopiridol because both active moieties are metabolized and inactivated by the same pathway, the activity of which can be assayed by the serum bilirubin level. Such data place into question the current definitions of DLT with the combination regimen, and indicate that, perhaps, the optimal dose of the combination for individual patients may be defined by their baseline serum bilirubin. This hypothesis would require prospective confirmatory evaluation.

Notably, the UGT1A1 polymorphisms were examined with regard to flavopiridol toxicity previously (36). In that study, UGT1A1 polymorphisms were not associated with flavopiridol pharmacokinetics or flavopiridol-induced diarrhea. It is possible that UGT1A9 is the primary enzyme responsible for flavopiridol glucuronidation (41). Examination of polymorphisms in the promoter region of UGT1A1 and UGT1A9 is planned in future studies examining irinotecan and flavopiridol combinations.

Our preclinical model suggested that the potentiation of irinotecan by flavopiridol is related to suppression of p21 and Drg1 following irinotecan therapy (21, 25). p21 is both an inhibitor of CDKs, causing both G_1 and G_2 cell cycle arrests, as well as an inhibitor of apoptosis by binding to and inhibiting caspase 3. It has recently been reported that a decrease in p21

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expression following preoperative chemoradiotherapy for rectal cancer was associated with improved disease-free survival (42). Thus, induction of p21 seems to be a novel mechanism for chemotherapy resistance. Interestingly, irinotecan induces p21, especially in tumors with an intact (or wild-type) p53 pathway (21). Drg1 is a recently identified gene product that is up-regulated in differentiation and may be a metastatic suppressor in colon cancer (43) and in breast and prostate cancers (44, 45). With regard to resistance to irinotecan, similarly, Drg1 is transcriptionally induced by irinotecan treatment and is associated with resistance to irinotecan-based therapy. Subsequent administration of flavopiridol is associated with a decrease in Drg1 and subsequent sensitivity to irinotecan (25). We elected to explore this hypothesis in this phase I study by conducting pre- and posttreatment biopsies at the expanded MTD. We observed that patients who showed disease control indeed were p53 wild-type and either had a low p21 level that did not increase or a decrease in Drg1 expression. Patients with disease progression had an increase in p21 and Drg1 or were p53 mutants. Although preliminary, these data support the preclinical model that sensitivity to the combination is related to the p53-p21 DNA damage/repair axis. These preliminary studies have led to ongoing translational laboratory research aimed at better defining the role of these proteins in increasing irinotecan sensitivity, and suggest that flavopiridol achieves this by suppressing discrete molecular events that prevent apoptosis in irinotecan-treated tumor cells. Confirmatory prospective evaluation is again necessary for definitive conclusions.

This combination has proven to be safe and tolerable, and is associated with interesting clinical activity with patients having stable or responsive disease and remaining on study for prolonged periods of time. There are several patients who have remained on study for over 12 months without cumulative toxicity. Overlapping glucuronidation pathways may be important in drug metabolism and consequent toxicity of the combination therapy. Finally, our correlative studies support the biological basis for the combination. This combination is being evaluated in a phase II clinical trial in hepatocellular carcinoma, and in combination with cisplatin and with 5-fluorouracil/leucovorin in separate phase I studies. Laboratory efforts to better understand resistance and sensitivity pathways are ongoing.

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British Journal of Cancer (2009) 100, 1021 – 1025
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Minireview

Childhood exposure to external ionising radiation and solid cancer risk

S Sadetzki*[,1,2] and L Mandelzweig[1]

[1]Cancer and Radiation Epidemiology Unit, Sheba Medical Center, Gertner Institute for Epidemiology and Health Policy Research, Tel Hashomer 52621, Israel; [2]Department of Epidemiology and Preventive Medicine, Sackler Faculty of Medicine, Tel Aviv University, Ramat Aviv, Israel

The increasing use of ionising radiation for diagnostic purposes has raised concern about potential iatrogenic damage, especially in children. In this review, we discuss some aspects of radiation-induced cancer in relation to age at exposure and measures that should be taken for limiting exposure in this sensitive population.
British Journal of Cancer (2009) **100,** 1021 – 1025. doi:10.1038/sj.bjc.6604994 www.bjcancer.com
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Keywords: ionising radiation; cancer risk; childhood exposure

Wilhelm Roentgen's discovery of X-rays in 1895, followed by Henri Becquerel's discovery of radioactivity in 1896, led to the introduction of man-made radiation. Among the important applications of these new sources of ionising radiation (IR) was their use in medical diagnosis and treatment, which spread quickly throughout the twentieth century.

Within a short time after these discoveries, reports of acute adverse effects of radiation, such as skin damage among radiation workers, began to appear. The first case of death associated with radiation-induced skin cancer was reported in 1904, while in 1911 the first case of radiation-induced leukaemia was described in a physician (Finch, 2007). Later, in the 1940s, reports of studies on mortality among physicians showed an excess of leukaemia and other cancer (attributed to exposure to IR) among radiologists and dermatologists in comparison with other physicians (Dublin and Spiegelman, 1948).

In 1913, the German Roentgen Society provided professional guidelines aimed at reducing the dangers of radiation exposure to medical workers. Among the recommendations of the British X-ray and Radium Protection Committee published several years later were a limitation of maximum work schedules, required amounts of leisure time and special accommodations for the workers (Committee for Review and Evaluation of the Medical Use Program of the Nuclear Registry Commission, 1996). The International Commission on Radiological Protection (ICRP), established in 1928, introduced important concepts such as 'tolerance dose', which served as an upper limit for the exposure of workers, and 'effective dose', which considers the overall effect of irradiation at different areas of the body (Committee for Review and Evaluation of the Medical Use Program of the Nuclear Registry Commission, 1996).

*Correspondence: Dr S Sadetzki, Cancer and Radiation Epidemiology Unit, Sheba Medical Center, Gertner Institute for Epidemiology and Health Policy Research, Tel Hashomer 52621, Israel;
E-mail: siegals@gertner.health.gov.il
Revised 10 February 2009; accepted 27 February 2009

During the second half of the twentieth century, epidemiological data on radiation-induced cancer began to accumulate. Among the important sources of such data were a series of papers from the Life Span Study (LSS) cohort of atomic bomb (A-bomb) survivors. In addition, follow-up studies of cohorts treated with radiotherapy for benign conditions or cancer, as well as children exposed to lower doses because of repeated diagnostic procedures, contributed to our understanding of the dose–response relationship between exposure and risk.

In 1977, the ICRP introduced a system of dose limitations based on the principle of keeping exposures to radiation 'As Low As is Reasonably Achievable' (ALARA). This system included (1) justification – no practice (causing exposures of people to radiation) shall be adopted unless its introduction produces a positive net benefit (should not cause more harm than good), (2) optimisation – all exposures should be kept as low as reasonably achievable, economic and social factors being taken into account, and (3) dose limits – the dose equivalent to individuals shall not exceed the limits recommended for the appropriate circumstances (US Department of Energy, 1997).

CHILDHOOD EXPOSURE TO IR

Specific concern over the potentially harmful effects of exposure to radiation in children began to develop in the 1940s when it was suggested that fluoroscopy use in infants should be restricted according to clinical indications, and that attention should be given to calibrating the machines to limit the exposure (Buschke and Parker, 1942). In the 1950s, Alice Stewart proposed that exposure to weak irradiation could initiate malignant changes in a foetus or very young child. This hypothesis was based on the findings of a case–control study which showed that almost twice as many mothers of children who died from leukaemia or malignant disease before the age of 10 had undergone X-ray examinations of the abdomen during pregnancy (pelvimetry) in comparison with mothers of controls (Stewart *et al*, 1956). Later analyses showed that this excess risk was inversely related to foetal

age and increased with the number of films taken (Bithell and Stewart, 1975).

An increased susceptibility of children to radiation-induced cancer is biologically plausible because of the fact that their tissues are still growing and therefore the dividing cells are more prone to somatic genetic damage. In addition, children have a longer life expectancy during which oncogenic effects may develop (Brody *et al*, 2007; Shah and Platt, 2008).

The purpose of this review is to summarise data on the relationship between childhood exposures to external IR and cancer risk, with specific attention to the need for adequate measures for limiting the risk of exposure in this sensitive population.

ASSOCIATION BETWEEN AGE AT EXPOSURE AND RISK

Estimates of excess risk associated with exposure to IR are generally presented in terms of excess relative risk per gray (ERR per Gy) and excess absolute risk (EAR per Gy). The ERR associated with an exposure represents the ratio of the difference between the rate of the disease in the exposed and unexposed groups to the rate in the unexposed group. The ERR per dose is defined on the basis of the assumption that the ERR is proportional to the dose and represents the ERR for a unit dose of radiation. Excess absolute risk (EAR) per dose refers to the additional risk that a radiation dose contributes beyond the background risk.

A recent publication from the LSS on risk of solid cancer (malignancies that form tumours in solid organs, as opposed to leukaemia, which affects the blood) among atomic bomb survivors exposed to radiation doses ranging from <0.005 to 4 Gy included data showing the effect of age at exposure on excess risk for all solid cancer and by specific sites (Preston *et al*, 2007). For all solid cancer, the ERR per Gy decreased with increasing age at exposure, with estimates at attained age 70 of 0.72 (90% CI: 0.52–0.98), 0.64 (90% CI: 0.51–0.79), 0.41 (90% CI: 0.33–0.50), and 0.41 (90% CI: 0.29–0.53) for survivors aged 0–9, 10–19, 20–39, and 40+, respectively, at the time of the bombings. The EARs per 10^4 PY of solid cancer at attained age 70 in the LSS were also higher for those who were younger at the time of exposure, with rates of 90 (90% CI: 68–113), 52 (90% CI: 43–60), and 30 (90% CI: 22–39) for ages 10, 30, and 50, respectively.

The pattern of decreasing risk with increasing age at exposure was particularly striking for thyroid cancer (Preston *et al*, 2007). This inverse association is supported by data from cohorts exposed to radiotherapy for cancer during childhood (Sigurdson *et al*, 2005), tinea capitis (Sadetzki *et al*, 2006), and other benign conditions, as shown in Figure 1A. The results of a pooled analysis, including individuals from six studies who were under the age of 15 at the time of radiation exposure, yielded an ERR per Gy of 7.7 (95% CI: 2.1–28.7) and an EAR per 10^4 PY Gy of 4.4 (95% CI: 1.9–10.1) for risk of thyroid cancer. A trend for decreasing risk with increasing age at irradiation was observed (Ron *et al*, 1995). The investigators suggested that these findings might indicate a greater radiation effect during periods of rapid cell proliferation, which occur when the thyroid gland is developing.

For many years, brain tissue was considered to be resistant to radiation damage. Four studies that have quantified the risk between IR and brain cancer have reported estimations that vary in terms of the magnitude of the risk per Gy. The Israeli tinea capitis study found an ERR per Gy of 1.98 (95% CI: 0.73–4.69) for glioma after a mean dose to the brain of 1.5 Gy (range: 1.0–6.0 Gy) (Sadetzki *et al*, 2005); a pooled analysis of two Swedish cohorts of infants who received radium treatment for haemangiomas (mean absorbed intracranial dose: 7 cGy; range: 0–11.5 Gy) estimated the ERR per Gy for all brain tumours as 2.7 (95% CI: 1.0–5.6) (Karlsson *et al*, 1998); a childhood cancer survivor study reported an ERR per Gy of 0.33 (95% CI: 0.07–1.71) for glioma (Neglia *et al*, 2006) after radiation doses ranging from <1 Gy to >45 Gy; and the LSS found an ERR per Gy for glioma of 0.6 (95% CI: -0.2 to 2.0) (Preston *et al*, 2002b). Evidence of increasing risk of brain cancer with younger age at exposure has been provided by the first three aforementioned studies (Figure 1B), whereas for the LSS, the risk was non-significantly higher for those exposed before age 20. It is interesting to note that even among infants, the risk of those treated for haemangioma when they were <5 months old was much greater than that of those treated over the age of 5 months (Figure 1B).

Radiation effects on female breast cancer rates have been studied extensively. The results of pooling eight cohorts showed that age at exposure is an important modifier of the breast cancer ERRs with higher risks for younger ages, so that exposure after age 50 carries a lower risk than exposure earlier in life. No simple unified summary model adequately described the excess risks in all populations, suggesting that host factors (e.g. earlier benign breast disease or reproductive factors) are determinants of the variations in the ERR with age at exposure (Preston *et al*, 2002a).

Among Canadian women who were examined by fluoroscopy during treatment for tuberculosis (range of radiation dose: $<0.01-\geqslant 10$ Sv), the ERR per Sv for breast cancer mortality was shown to decrease with increasing age at exposure ($P=0.0003$) (Howe and McLaughlin, 1996). In the latest results of the LSS, a statistically significant age-at-exposure effect of -19% per decade increase in ERR (90% CI: -33 to -4%) was noted for breast cancer. However, the addition of attained age to the model improved the fit significantly and reduced the age-at-exposure effect estimate to 0, whereas both attained age and age at exposure had a significant joint effect on the EAR (Preston *et al*, 2007). Differences between studies still remain regarding the influence of age at exposure around menarche and menopause on the risk.

A strong inverse association between age at exposure and risk has also been noted for non-melanoma skin cancer ($P<0.001$) in the LSS (Preston *et al*, 2007). This finding is supported by data from the tinea capitis study, in which the fitted relative risk for basal cell carcinoma decreased from 19.0 (95% CI: 14.4–28.4) for irradiation at age $\leqslant 4$, to 5.9 (95% CI: 3.3–11.0) for exposure at age 5–9, and 2.0 (95% CI: 1.3–4.0) among those exposed at ages 10–15 (Ron *et al*, 1991).

It is interesting to note that for lung cancer a different picture was observed, in which the ERR increased with increasing age at exposure. This finding may be attributed to the additive joint effects of radiation and smoking (Pierce *et al*, 2003; Preston *et al*, 2007).

The evaluation of childhood cancer risk in the LSS study was limited by the fact that the tumour registries were only established in 1958, and therefore, data on cancer incidence during the first 13 years after the bombings were unavailable. An assessment of risk among survivors diagnosed during adolescence identified eight cases of solid cancers between the ages of 13 and 19, yielding an ERR per Gy of 19.8 (90% CI: 6–77) for age <20 years. It has been suggested that if follow-up of the cohort had been initiated at an earlier stage, the risks associated with childhood cancer would have been extremely high (Preston *et al*, 2007).

AGE AT EXPOSURE AND TEMPORAL PATTERNS OF RISK (ATTAINED AGE AND LATENCY PERIOD)

Age at exposure, time since exposure, and attained age are collinear variables, and their combined effect differs from the individual effects of each factor. Thus, the association between these factors and radiation-induced cancer risk can only be estimated for two of the three at any one time, and the true causal effect of age at exposure on radiation-induced cancer cannot be assessed (Lagarde, 2006).

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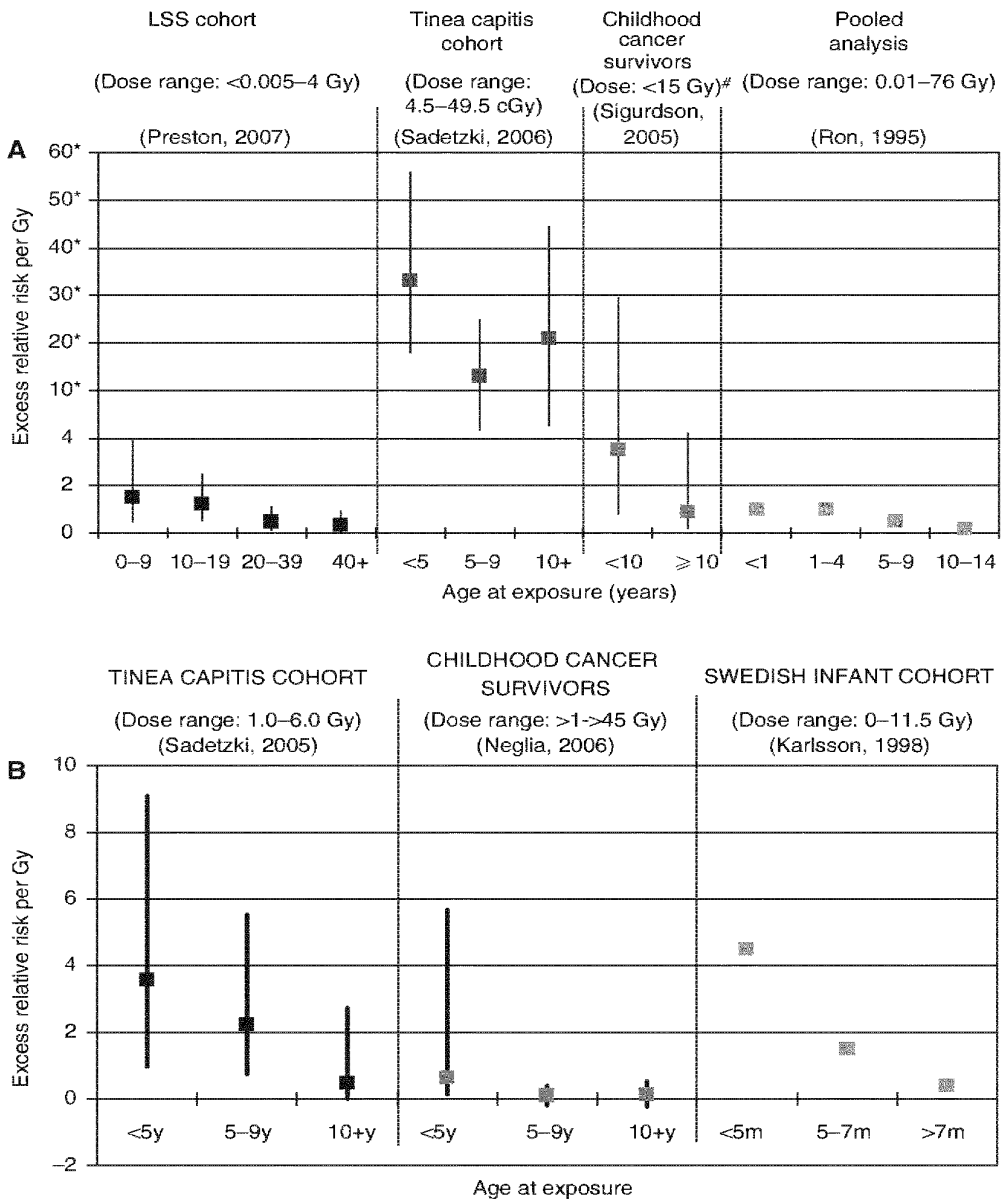


Figure 1 (**A**) Excess relative risk of thyroid cancer by age at exposure among selected cohorts exposed to ionising radiation. Dose ranges refer to doses to the thyroid gland. (**B**) Excess relative risk of brain cancer by age at exposure among selected cohorts treated with radiotherapy. Dose ranges refer to doses to the brain. *Please note the change in the scale of the *y*-axis. #ERR estimates presented here are for survivors aged < 10 and ⩾ 10 years who were exposed to low doses.

Analyses of the LSS provided evidence of a decrease in ERRs with increasing attained age, for any age at exposure. The age-at-exposure effect is shown by a 20% decrease in attained-age-specific ERRs per decade increase in age at exposure. In contrast, EARs were shown to increase throughout life regardless of the age at exposure, and there have been indications that for solid cancers, attained-age-specific EARs are higher for those exposed at younger ages (Preston *et al*, 2003).

REDUCING EXPOSURE TO IR IN CHILDREN

A profound discussion on the dose–response association is beyond the scope of this review. Scientific consensus groups as well as regulatory agencies have endorsed the use of the linear, no-threshold model in determining standards of protection from the risk of solid cancer associated with exposure to IR at low dose levels (Committee for Review and Evaluation of the Medical Use Program of the Nuclear Registry Commission, 1996). The data described above highlight the need for applying the ALARA principle for reducing exposure to IR, with specific attention to children, who represent a subgroup with increased radiosensitivity. The importance of this approach has increased as a result of the growing use of diagnostic procedures that usually involve low-dose radiation, and specifically for computed tomography (CT) scanning, which commonly involves 30–90 mSv per 2–3 scans. This dose is comparable to the low doses of radiation (5–150 mSv), to which a subgroup of 25 000 A-bomb survivors was

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exposed. The statistically significant increase in risk of solid cancer associated with the latter doses suggests that the organ doses corresponding to a CT study may also increase the risk of cancer (Brenner and Hall, 2007).

It should also be noted that a dramatic increase in the use of CT over time has been observed, as indicated by estimates of 2 million per year in the United States in 1980 rising to 69 million in 2007 (Hall and Brenner, 2008). Data from the United States and Europe have shown that although CT constitutes only 5–10% of all imaging procedures, 40–67% of all exposure to medical diagnostic radiation can be attributed to this procedure (Sadetzki, 2007).

An important development in the attempt to reduce unnecessary exposure of children to IR was the recent establishment of the Alliance for Radiation Safety in Pediatric Imaging. This coalition, which comprises members of leading medical societies, agencies and regulatory groups, aims to influence patient care and change practice through an educational and awareness campaign entitled 'Image Gently'. The campaign includes a four-point strategy regarding performing pediatric CT scans, including the need to reduce or 'child-size' the amount of radiation used, scan only when necessary, scan only the indicated region, and refrain from multiphase scanning unless it is necessary (Goske *et al*, 2008).

Other strategies aimed at protecting pediatric patients include consideration of alternatives to CT, such as ultrasound and magnetic resonance imaging, and shielding of the breast areas (including the nipples and breast buds in young girls), thyroid, and gonads when possible during CT scans (Shah and Platt, 2008).

The need to increase the awareness of professionals regarding the late health effects of exposure to IR has been shown in a study of radiography conducted in five Israeli neonatal care units, which identified unnecessary overexposure to IR in a substantial proportion of body regions beyond those that were ordered. This problem was particularly prevalent in radiographs of the abdomen, in which the neck and upper chest were exposed in 45 and 64%, respectively, which did not comply with international recommendations. Other problems encountered included the need to repeat up to 20% of the radiographs, thereby increasing the overall exposure of the infant to radiation, and the lack of shielding of the gonads in most of the X-rays taken. Correction of this situation does not require investment of resources. By simply raising awareness among the staff with better guidelines regarding exposure to IR, these issues can be resolved (Bader *et al*, 2007).

Another measure that has been proposed for limiting exposure to diagnostic radiation in children is the incorporation of personal information on lifetime IR exposure in computerised medical systems. Such information could include the numbers and types of procedures carried out, the ages at which they were carried out, and calculations of cumulative doses absorbed by different organs. Consideration of these factors could be important in determining to which diagnostic examinations the patient should be referred (Sadetzki, 2007).

Thus, there are also legal and ethical implications of exposure to diagnostic IR that need to be addressed, such as the right of a parent to be informed of the risks involved in the procedures to which his or her child has been referred.

CONCLUSION

The use of IR in medicine has provided a wide range of possibilities for diagnosis and treatment. Constant refinement of the techniques used in radiological examinations has produced greater resolution in imaging, facilitating greater accuracy of assessments. However, along with the important benefits of medical applications of IR, there are risks that need to be considered as well. Children constitute a subgroup at greater risk from exposure to radiation because of their greater degree of radiosensitivity and because of the many years of life ahead of them during which they could develop radiation-induced cancer. In order to minimise the risks, it is essential that procedures involving low doses of IR be used with discretion, in a responsible way. Although the risks involved may be relatively small, the ancient concept expressed by Hippocrates of '*Primum non nocere*' (to help, or at least to do no harm) should serve as a guide when assessing the appropriateness of radiation-based imaging procedures in pediatric patients.

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