

A Phase I Study of Single Administration of Antibody-Directed Enzyme Prodrug Therapy with the Recombinant Anti – Carcinoembryonic Antigen Antibody-Enzyme Fusion Protein MFECP1 and a Bis-Iodo Phenol Mustard Prodrug

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Abstract Purpose: Antibody-directed enzyme prodrug therapy is a two-stage treatment whereby a tumor-targeted antibody-enzyme complex localizes in tumor for selective conversion of prodrug. The purpose of this study was to establish optimal variables for single administration of MFECP1, a recombinant antibody-enzyme fusion protein of an anti – carcinoembryonic antigen single-chain Fv antibody and the bacterial enzyme carboxypeptidase G2 followed by a bis-iodo phenol mustard prodrug. MFECP1 is manufactured in mannosylated form to facilitate normal tissue elimination.

Experimental Design: Pharmacokinetic, biodistribution, and tumor localization studies were used to test the hypothesis that MFECP1 localizes in tumor and clears from normal tissue via the liver. Firstly, safety of MFECP1 and a blood concentration of MFECP1 that would avoid systemic prodrug activation were tested. Secondly, dose escalation of prodrug was done. Thirdly, the dose of MFECP1 and timing of prodrug administration were optimized.

Results: MFECP1 was safe and well tolerated, cleared rapidly via the liver, and was less immunogenic than previously used products. Eighty-fold dose escalation from the starting dose of prodrug was carried out before dose-limiting toxicity occurred. Confirmation of the presence of enzyme in tumor and DNA interstrand cross-links indicating prodrug activation were obtained for the optimal dose and time point. A total of 28 of 31 patients was evaluable for response, the best response being a 10% reduction of tumor diameter, and 11 of 28 patients had stable disease.

Conclusions: Optimal conditions for effective therapy were established. A study testing repeat treatment is currently being undertaken.

Common epithelial tumors, such as colorectal and gastric cancer, are a major health burden. Curative options are currently not available if the disease is deemed nonresectable in spite of the recent introduction of new cytotoxic drugs (1, 2)

and monoclonal antibodies (3, 4). Response to treatment is limited due to drug resistance, lack of selectivity, and pathway redundancy. Antibody-directed enzyme prodrug therapy (ADEPT) aims to overcome these shortcomings by selective generation of a high concentration of drug in the tumor while sparing normal tissues. In this experimental two-stage system, a tumor-targeting antibody linked to an enzyme is given i.v. When cleared from normal tissue, prodrug is given and converted to active drug in tumor by the targeted enzyme (Fig. 1; ref. 5). ADEPT has potential to overcome drug resistance with minimal toxicity.

Carcinoembryonic antigen (CEA), an oncofetal antigen, provides an ideal target for ADEPT being abundantly expressed by a variety of adenocarcinomas, particularly colorectal cancers. Normal tissue expression in adults is limited to the luminal surface of the gut where it is inaccessible to systemically administered antibody-enzyme. The requirements for effective ADEPT have been established in previous trials and include rapid normal tissue elimination, sufficient tumor localization, effective prodrug conversion, and low immunogenicity of the antibody-enzyme (6–8).

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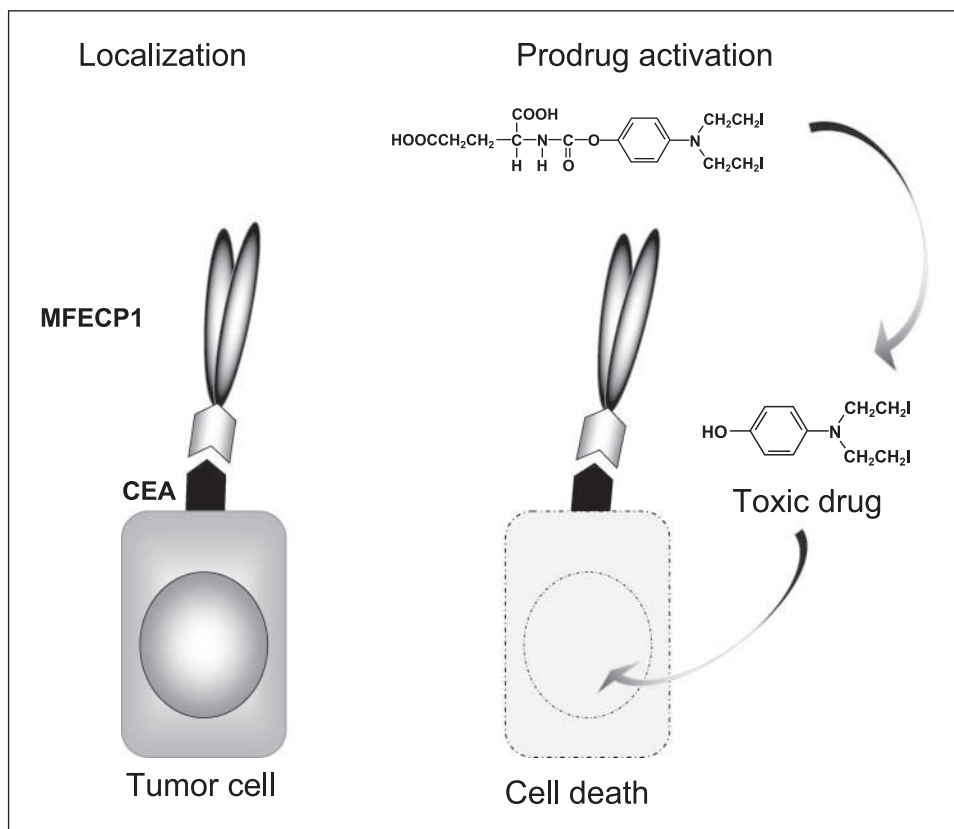


Fig. 1. An i.v. administered antibody-enzyme fusion protein MFECP1 localizes in tumor. This is followed by the BIP mustard prodrug after elimination of enzyme from circulation. CPG2 activates mustard prodrug by cleavage of the glutamate moiety.

To address these requirements, an ADEPT system with the antibody-enzyme fusion protein MFECP1 and a bis-iodo phenol (BIP)⁶ mustard prodrug [4-[[bis(2-iodoethyl)amino]phenyl]oxycarbonyl-L-glutamic acid] was designed. MFECP1 is a recombinant fusion protein of carboxypeptidase G2 (CPG2), an enzyme derived from *Pseudomonas aeruginosa*, directly linked to the NH₂ terminus of the anti-CEA single-chain Fv antibody MFE-23 (9–11). Expression in *Pichia pastoris* added branched mannose at two sites on each enzyme monomer (12). The enzyme dimerizes to become functional, forming a stable glycosylated fusion protein. CPG2 converts BIP prodrug to active drug by cleavage of a glutamate moiety. The BIP prodrug was chosen due to the short half-life of its activated drug, which is expected to prevent leak back from tumor (13).

The design criteria of ADEPT with MFECP1 and BIP prodrug were met in studies in nude mice bearing human colon carcinoma xenografts. Tumor to normal tissue enzyme levels of 100:1 to 1,000:1 were observed, and sustained tumor regression without significant toxicity was obtained with repeated therapy given over 3 weeks (14).

This article describes a phase I clinical trial of single administration of MFECP1 and BIP prodrug. A three-phase trial design was used because ADEPT requires delivery of effective concentrations of enzyme to the tumor followed by administration of a potentially effective prodrug dose when enzyme levels in the blood are low enough to avoid systemic prodrug activation. Firstly, lack of significant toxicity of MFECP1 and a safe concentration of MFECP1 in blood for

prodrug administration was determined. Secondly, the prodrug dose was escalated, and finally, the effect of the dose of MFECP1 on the timing of prodrug administration was investigated. This design facilitated investigation of the toxicity of each component and of the range of combinations. The function of each component of the system was tested, and an optimal regimen was defined for investigation of efficacy in a subsequent trial of repeated therapy.

Materials and Methods

Eligibility criteria

Patients with nonresectable, locally recurrent, or metastatic histologically proven colorectal or other CEA-expressing cancer, who had been offered standard chemotherapy, were eligible for the study. Patients were required to have completed any anticancer therapy at least 4 weeks before study entry and toxicity of previous treatments had to have resolved; life expectancy had to be ≥ 4 months and WHO performance status 0 to 2. Patients were required to have measurable disease as defined by Response Evaluation Criteria in Solid Tumors criteria (15) and a serum CEA concentration of 10 to 1,000 $\mu\text{g/L}$. Patients with a serum CEA <10 $\mu\text{g/L}$ had their tumor sections checked for CEA expression by immunohistochemistry. Patients with $>50\%$ CEA-positive cells were considered eligible for treatment. Adequate renal (creatinine <120 $\mu\text{mol/L}$), liver (bilirubin <30 $\mu\text{mol/L}$, alanine aminotransferase/aspartate aminotransferase $\leq 2 \times$ upper normal limit and $\leq 5 \times$ upper normal limit in case of liver metastases, international normalized ratio ≤ 1.5), and bone marrow function (hemoglobin ≥ 10 g/dL, platelet count $\geq 100 \times 10^9/\text{L}$, neutrophil count $\geq 2 \times 10^9/\text{L}$) were required. Patients were required to have a negative test for human anti-mouse antibodies (HAMA) and human anti-CPG2 antibodies (HACA) before study entry. Patients with a history of allergy to iodine or mouse protein were excluded. Written informed consent was given by all participants.

⁶ BIP prodrug was formerly known as ZD2767P.

All patients were treated at the Department of Oncology at the Royal Free Hampstead NHS Trust (London, United Kingdom).

Study drugs

The genetic construct encoding MFECPI was cloned with COOH-terminal hexahistidine tag for purification by immobilized metal chromatography and expressed in *P. pastoris* as described previously (14). Further purification was achieved by size exclusion chromatography using Superdex 200 (GE Healthcare, Amersham, United Kingdom) and application to an endotoxin removal gel (Pierce, Chester, United Kingdom) at the Good Manufacturing Practice facility in the Department of Oncology, University College London (London, United Kingdom). Purified MFECPI was tested according to the European Union clinical trials directive for the manufacture of investigational medicinal products (16). This included testing for sterility, pyrogenicity, and likely contaminants from the process. Assessment of integrity and biological activity of MFECPI was measured by CEA binding (ELISA), enzyme activity, SDS-PAGE, and Western blot analysis. The dimeric nature of MFECPI was confirmed by detection of a single MFECPI peak on size exclusion chromatography in physiologic conditions. The mean specific activity was 128 (± 22.8) units/mg. For administration to patients, MFECPI was diluted in 500 mL 0.9% sodium chloride and given over 90 to 120 minutes via a Hickman line.

Radiolabeling of MFECPI with a tracer dose of 370 MBq ^{131}I or 500 MBq ^{123}I for patients undergoing single-photon emission computed tomography (SPECT) imaging to determine localization of MFECPI was done using the chloramine-T method. TLC was used to assess iodine incorporation, and antigen binding was tested on a CEA column. ^{123}I or ^{131}I MFECPI was given at the beginning of the infusion with MFECPI. Patients receiving radiolabeled MFECPI were administered 50 mg potassium iodide for 5 days starting on the day before treatment to prevent thyroid uptake of radioactive iodine.

BIP prodrug was manufactured and supplied by AstraZeneca (Macclesfield, United Kingdom) and prepared as described previously (8). The prodrug was administered as three bolus doses 1 hour apart into a fast running drip of 5% dextrose through a Hickman line. The split dose approach was adopted due to superior results *in vivo*.

Study objectives

The objectives of the trial were as follows: (a) to test the hypothesis that the MFECPI fusion protein localizes in tumor and clears from normal tissues via liver, (b) to measure the dose-limiting toxicity (DLT) and maximum tolerated dose of one ADEPT cycle with MFECPI and BIP prodrug, (c) to assess immunogenicity of a single administration of MFECPI, and (d) to obtain evidence of prodrug activation and preliminary evidence of efficacy.

Study design

Each patient was given a single dose of MFECPI followed by three bolus doses of BIP prodrug 1 hour apart. The starting dose level of 5,000 units/m² MFECPI (mean, 39 mg/m²; range, 30-72 mg/m²) was chosen based on *in vivo* data (14) and the starting dose of BIP prodrug of 12.42 mg/m² $\times 3$ on data from a previous clinical trial (8). In initial patients, a safe level of MFECPI in serum was determined followed by dose escalation of prodrug in subsequent patients. A modified Fibonacci dose escalation scheme was adopted; dose escalation of prodrug between patients was done in 2-fold increments until grade 3 toxicity was seen. Once the maximum tolerated dose of BIP prodrug was established, the hypothesis that a lower dose of MFECPI would clear more rapidly was tested due to favorable tumor to normal tissue ratios in animal models (14) at earlier time points. Reduction of the dose of MFECPI was based on mathematical prediction from blood elimination data after giving 5,000 units/m² MFECPI.

The trial was conducted according to the principles of Good Clinical Practice and managed and monitored by the Cancer Research UK Drug Development Office (London, United Kingdom). The trial was approved by the Local Ethics Committee and Medicines and Healthcare

Products and Regulatory Agency. A license for the administration of radioactive substances was obtained. Toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria.

Pharmacokinetics of MFECPI

Serum samples were taken for estimation of their CPG2 enzyme activity by high-performance liquid chromatography 5 minutes after completion of infusion with MFECPI and then up to hourly until CPG2 activity in serum had fallen below the threshold, initially <0.05 units/mL and then <0.005 units/mL. Additionally, in patients who received radiolabeled MFECPI, samples were taken at the same time points for measurement of radioactivity in a gamma well counter.

Tumor localization of MFECPI

Gamma camera imaging. Patients who received a tracer dose of MFECPI labeled with ^{123}I or ^{131}I were imaged on an ADAC Vertex Plus dual-headed gamma camera after 30 minutes, 4 to 6 hours, 24 hours, and 48 hours to determine localization of MFECPI in tumor. Planar and SPECT acquisitions were obtained. SPECT images were corrected for Compton scatter, reconstructed using standardized, attenuation-corrected OSEM software, and corrected for radioactive decay. Regions of interest were drawn on tumor and normal tissue (heart, lung, and liver) as described previously (8, 17). Percentage injected radioactivity dose per kg of tissue was calculated, and the amount of enzyme in tumor was estimated from this.

Assessment of MFECPI in tumor biopsies. Patients with a tumor lesion amenable to biopsy were asked to have a biopsy done. In consenting patients, biopsies were obtained after administration of MFECPI and BIP prodrug. Fresh tissue was snap frozen and fixed in acetone. Tissue sections were assessed on H&E-stained sections. CEA was shown in adjacent sections using a monoclonal anti-CEA antibody (A5B7, Centre for Applied Microbiology Research, Porton Down, United Kingdom) and binding of MFECPI with polyclonal anti-CPG2 antibody (University College London). Biotinylated secondary antibodies were used followed by avidin/biotin-peroxidase complex (Vector Laboratories, Peterborough, United Kingdom). Immunohistochemistry was scored semiquantitatively by counting of CEA- and CPG2-reactive cells. Photomicrographs were generated using a Zeiss Axioskop 2 microscope (Welwyn Garden City, United Kingdom). Sections of patients who had received radiolabeled MFECPI were exposed to phosphorimage plates (18).

Immune response

Blood was taken before treatment and weekly for 8 weeks to assess the immune response to MFECPI. HAMA and HACA were measured by ELISA as described previously (8). In brief, plates were coated with the monoclonal anti-CEA antibody A5B7 (Celltech Ltd., Slough, United Kingdom) for measuring HAMA and CPG2 (Centre for Applied Microbiology Research) for HACA. Peroxidase-labeled goat anti-human γ chain-specific IgG (Sigma, Poole, United Kingdom) was used as secondary antibody in both assays. Serum samples were diluted 1:100, and the cutoff for a positive result was set at an absorbance of 0.2 at 490 nm wavelength. This was obtained for the current clinical trial by assaying 50 pretreatment serum samples at 1:100 dilution, calculating the mean baseline absorbance, and adding $3 \times \text{SD}$. Pooled positive sera from a previous study were used as standard.

Pharmacokinetic studies of BIP prodrug

Plasma samples for analysis of BIP prodrug concentration were taken 2 minutes after each infusion and then 5, 10, 15, 30, and 60 minutes after the final infusion. A complete pharmacokinetic profile, including concentration of BIP prodrug, extrapolated to 0 minute (C_0), area under the curve (AUC_{INF}), clearance and volume of distribution in the body as steady state (V_{ss}), was determined for each evaluable patient using a noncompartmental model and WinNonlin software. The pharmacokinetic profile was obtained only for the prodrug because the estimated half-life of the active drug is in the order of seconds and therefore too short for pharmacokinetic studies to be done.

Prodrug activation

Single-cell comet assay was used to test for DNA interstrand cross-links (19). The assay acts as a surrogate for conversion of prodrug to active drug. A reduction in tail moment indicated the presence of interstrand cross-links. Comet assays were done on peripheral blood lymphocytes at ~60 minutes after the last prodrug administration and where applicable on tumor biopsies. Tumor samples were cut at 4°C and processed into single-cell suspension by gentle syringing using a 22-gauge needle (20). If a tumor biopsy was done, a blood sample was also taken within 10 minutes of the biopsy.

Pretreatment and follow-up studies

History, physical examination, performance status assessments, HAMA, and HACA measurements were done before treatment and once weekly for 8 weeks after treatment, and routine laboratory studies were done before treatment and then twice weekly for 4 weeks and once weekly for another 4 weeks. Routine laboratory studies included complete blood cell count, differential WBC count, electrolyte, and renal and liver function variables. Thyroid function was recorded before treatment and 4 weeks after treatment, and tumor markers were recorded before treatment and at 4 and 8 weeks after treatment. Cardiac enzymes were measured after prodrug administration. Tumor response was evaluated on CT at 4 and 8 weeks after treatment. Response was assessed according to the Response Evaluation Criteria in Solid Tumors (15). Patients were followed up for 8 weeks after treatment.

Results

Patient characteristics

Thirty-one patients (17 male and 14 female; median age, 64 years; range 35-78 years) with CEA-expressing tumors were treated in this study in the period from September 2001 until June 2004. Patients were suffering from colorectal (24), breast (2), peritoneal (1), or esophageal (1) cancer. In one patient, the primary tumor was unknown, and two patients had a gastric and a colonic cancer. Patients had widespread metastases, including liver (23), lung (22), peritoneum (2), lymph nodes (4), bone (1), and brain (1) metastases. Patients had received between one and five chemotherapy regimens before study entry. Performance status was 0, 1, and 2 in 15, 13, and 3 patients, respectively. The median CEA level was 131 µg/L (range, 1-598 µg/L).

Safety of MFECP1

MFECP1 was safe and well tolerated. Grade 1 rigor (one patient), fever (two patients), allergy (one patient), and hypertension (one patient) were seen.

Pharmacokinetics of MFECP1

Sixteen patients who received 5,000 units/m² MFECP1 also received ¹²³I-labeled MFECP1 and four patients received ¹³¹I-labeled MFECP1; administration of radiolabeled MFECP1 in the remaining patients was not possible for logistic reasons. MFECP1 was shown to clear rapidly through the liver with prominent hepatic tracer activity seen by 1 hour. There was subsequent reduction in hepatic radioiodine activity and excretion of tracer as expected through the urinary system and bowel. No unexpected areas of tracer uptake were identified (Fig. 2). Blood samples were analyzed in a laboratory gamma counter, the percentage injected activity was calculated, and a biexponential function was fitted to these values. The α half-life was 0.66 and 0.57 hours and the β half-life was 10.73 and 10.94 hours for ¹²³I- and ¹³¹I-labeled MFECP1, respectively.

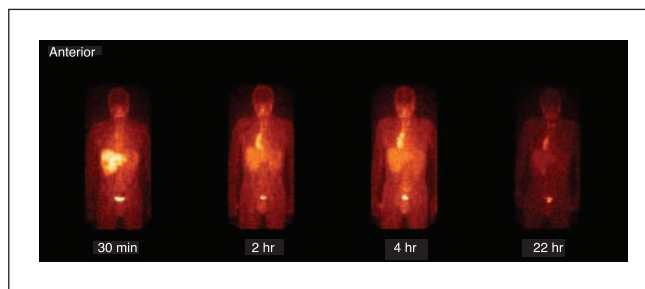


Fig. 2. The liver is the main organ for elimination of MFECP1. Radiolabeled MFECP1 is taken up by the liver 30 minutes after administration followed by rapid elimination. The uptake in the thorax in this patient is due to the gastric pull up following partial esophagectomy.

Measurements of CPG2 enzyme activity by high-performance liquid chromatography revealed an α half-life of 0.52 and 0.44 hours and a β half-life of 4.6 and 1.96 hours for 5,000 and 3,000 units/m² MFECP1, respectively.

The level for giving prodrug was initially set at <0.05 units/mL CPG2 activity in serum. This was lowered to <0.005 units/mL because DLT attributed to systemic prodrug activation was experienced at the starting dose of the prodrug (see under dose escalation and toxicity).

Tumor localization of MFECP1

Gamma camera imaging. MFECP1 localization was determined by gamma camera imaging. Quantitative assessment of CPG2 in tumor was possible in eight patients who received tracer doses of ¹²³I-labeled MFECP1 and in five patients who received ¹³¹I-labeled MFECP1. In the remaining patients, technical difficulties prevented quantitative assessment. Up to three tumor lesions were identified in individual patients. The median amount of CPG2 in tumor was calculated to be 0.18 units/g (range, 0.11-0.37 units/g), 0.07 units/g (range, 0.03-0.12 units/g), and 0.02 units/g (range, 0.01-0.04 units/g) after 4, 20, and 40 hours, respectively.

Biopsies. Seven patients consented to a tumor biopsy. Biopsies were done at 4.4 to 25.12 hours (median, 17.7 hours) after the end of the infusion of MFECP1. Immunohistochemistry with anti-CPG2 antibody showed MFECP1 localization in tumor in patients up to 19 hours after giving 5,000 units/m² and 15 hours after 3,000 units/m² MFECP1, respectively. Phosphorimaging confirmed uptake of radiolabeled MFECP1 in tumor 15 hours after 3,000 units/m² MFECP1. Figure 3 shows positive staining with anti-CEA antibody and anti-CPG2 antibody in a liver metastasis, confirming CEA expression and localization of MFECP1.

Immune response

HACA were detected in 36% (11 of 30) of patients. The median time to develop an immune response to CPG2 was 14 days (range, 7-42 days). None of the 30 patients developed HAMA.

Dose escalation and toxicity of BIP prodrug

The following dose levels of prodrug (×3) were explored: 12.4, 16.2, 21, 33.6, 67.2, 134.4, 268.8, 537.6, and 1,075 mg/m² in combination with 5,000 units/m² MFECP1; 268.8 and 537.6 mg/m² in combination with 3,000 units/m² MFECP1; and 200 mg/m² in combination with 1,500 units/m² MFECP1.

At the starting dose for the BIP prodrug, $12.42 \text{ mg/m}^2 \times 3$ DLT with grade 3 thrombocytopenia, leukopenia, and neutropenia (Table 1) was seen in one patient. The enzyme level in serum was 0.017 units/mL, and DLT was attributed to systemic activation of prodrug. Following this, the MFECPI level in serum permitting prodrug administration was lowered to $<0.005 \text{ units/mL}$ from patient 4 onwards. Dose escalation of prodrug was carried out between patients, and DLT was again seen when the prodrug dose reached $1,075 \text{ mg/m}^2 \times 3$ with liver, renal, and myelotoxicity (Table 1). A biopsy of the kidney and liver from this patient confirmed acute tubular necrosis and hepatic necrosis, which is consistent with the toxicity profile seen in the preclinical model. All toxicities resolved but temporary hemodialysis was required.

In patients 4 to 23, who received $5,000 \text{ units/m}^2$ MFECPI and prodrug after MFECPI had cleared to $<0.005 \text{ units/mL}$, the median time interval between antibody-enzyme and prodrug was 19.1 hours (range, 5-37.2 hours). Because favorable tumor to normal tissue ratios had been seen in animal studies 6 hours after giving MFECPI, lowering the dose of MFECPI to shorten the time interval between MFECPI and prodrug was investigated. The dose reduction of MFECPI was calculated based on the patient elimination data available for $5,000 \text{ units/m}^2$. Patients 24 and 25 received $3,000 \text{ units/m}^2$ in combination with $537.6 \text{ mg/m}^2 \times 3$ prodrug. This dose of prodrug had been safe when given 16 to 18 hours after $5,000 \text{ units/m}^2$ MFECPI (patients 21 and 22) but caused dose-limiting liver and myelotoxicity (Table 1) given 9.7 and 13.8 hours after $3,000 \text{ units/m}^2$ MFECPI. When the combination of $1,500 \text{ units/m}^2$ MFECPI and $200 \text{ mg/m}^2 \times 3$ prodrug was given to patients 29 to 31, dose-limiting thrombocytopenia was seen in one patient receiving prodrug 7.9 hours after MFECPI (patient 30; Table 1). Toxicity at these time points may be due to enzyme retention in organs other than blood. In contrast, $268.8 \text{ mg/m}^2 \times 3$ prodrug given 13 hours (median; range, 11.6-15 hours) after $3,000 \text{ units/m}^2$ MFECPI (patients 26-28) was safe and was established as the maximum tolerated dose. Grade 1 and 2

toxicity for all doses included leukopenia (1), neutropenia (2), thrombocytopenia (8), anemia (1), raised bilirubin (2), raised alanine aminotransferase (4), raised aspartate aminotransferase (2), raised γ -glutamyl aminotransferase (1), coagulopathy (1), nausea (13), vomiting (7), diarrhea (1), fatigue (11), and anorexia (1). Toxicity of ADEPT with MFECPI and BIP prodrug was found to depend on the serum enzyme level, dose of prodrug, and time interval between fusion protein and prodrug.

Pharmacokinetics of BIP prodrug

Pharmacokinetic profiles were obtained in all patients. The mean half-life for the BIP prodrug in plasma was 10.3 minutes (± 3.45 minutes), the median half-life being 9.96 minutes. The rate of clearance was reduced as the prodrug dose increased, commensurate with the increased C_0 and AUC_{INF} . Table 2 summarizes the noncompartmental pharmacokinetic variables calculated for all patients, and Fig. 4 shows the plasma prodrug elimination profile following the third administration of prodrug.

Prodrug activation

Tumor was available for assessment of cross-link formation in a patient receiving $3,000 \text{ units/m}^2$ MFECPI and $268.8 \text{ mg/m}^2 \times 3$ prodrug 15 hours later. Reduction in tail moment by 58% was seen in this sample when compared with pretreatment peripheral blood lymphocytes, indicating a significant level of DNA interstrand cross-linking and prodrug activation in tumor. In this patient, no cross-linking was observed in lymphocytes taken at the same time as the biopsy. In the 31 patients studied, the reduction in tail moment of blood lymphocytes ranged from 0% to 27%, with only two patients giving $>20\%$ reduction.

Efficacy

Twenty-eight patients were evaluable for response at 8 weeks after treatment. The best response on CT was a 10% reduction of tumor diameter in a patient with peritoneal cancer. Eleven patients had stable disease after 8 weeks and 17 had progressive disease.

Discussion

Therapeutic selectivity of anticancer drugs is achieved due to a differential between tumor and normal tissue. ADEPT was designed to increase this differential by selectively achieving high concentrations of active drug in tumor due to activation of prodrug at the tumor site with a previously targeted enzyme. Alkylating agents have been chosen for ADEPT due to their dose-response relationship. Evidence of response has been seen in colorectal cancer, a tumor that is usually resistant to alkylating agents (7, 8). Alkylating agents induce DNA interstrand cross-links, which can be measured by comet assay and are formed as a result from ADEPT in a human colorectal carcinoma xenograft study (20).

Pilot clinical trials of ADEPT have been studied with A5CP, a chemical conjugate of CPG2 with the $F(ab')_2$ fragment of a monoclonal anti-CEA antibody (6-8). These trials used CMDA, a synthetic benzoic acid mustard prodrug (6, 7) or the BIP prodrug (8) used in our current trial. Taken together, these studies showed that, with A5CP, slow elimination of enzyme required an additional antibody clearance step to enable prodrug administration within 48 hours. Furthermore,

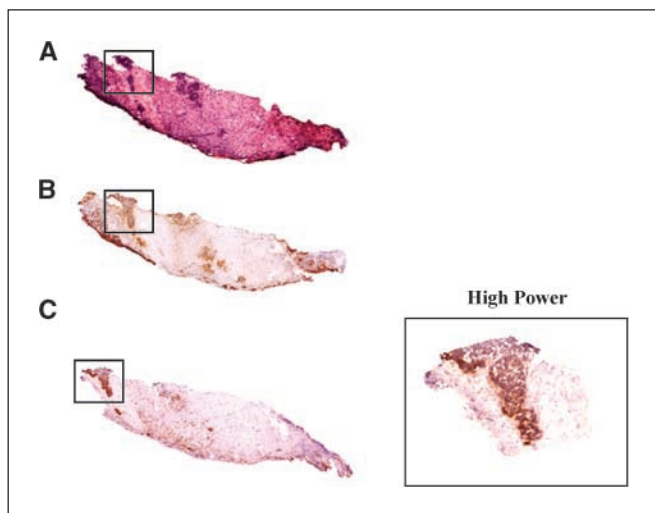


Fig. 3. Biopsy of a liver metastasis: H&E staining (A), staining with anti-CEA antibody (B), and staining with anti-CPG2 antibody (C). The H&E-stained section confirms presence of tumor in the biopsy, staining with anti-CEA antibody confirms CEA expression in tumor, and staining with anti-CPG2 antibody confirms localization of MFECPI in tumor. The biopsy was done 4.5 hours after the end of infusion with MFECPI.

Table 1. Summary of grade 3 and 4 toxicity

Treatment with MFECP1 (units/m ²) and BIP prodrug (mg/m ² ×3)	No. patients	Toxicity
5,000 MFECP1 + 12.42 BIP prodrug Plasma CPG2 0.0111 units/mL (median)	3	G3 thrombocytopenia (1), G3 neutropenia (1), G3 leukopenia (1)
5,000 MFECP1 + 1,075 BIP prodrug Plasma CPG2 <0.002 units/mL	1	G4 ALT/AST, G3 GGT, G4 Cr/urea, G3 anemia, G3 leukopenia, G3 thrombocytopenia
3,000 MFECP1 + 537.6 BIP prodrug Plasma CPG2 <0.002 units/mL	2	G4 ALT/AST (1), G3 anemia (1), G3 thrombocytopenia (1), G3 leukopenia (1), G3 fatigue (2)
1,500 MFECP1 + 200 BIP prodrug Plasma CPG2 <0.002 units/mL	1	G3 thrombocytopenia (1)

Abbreviations: G3, grade 3; G4, grade 4; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ -glutamyl aminotransferase.

the immune response to A5CP proved inhibitive to repeat administration without immunosuppression.

ADEPT with MFECP1 was designed to overcome the above limitations. Favorable results from tumor-bearing mice (14) led to the current clinical trial testing single administration of MFECP1 and the BIP prodrug. To achieve effective therapy with ADEPT, the trial was designed to determine initially a safe level of MFECP1 in serum followed by dose escalation of prodrug and optimization of dose and timing of both components. Pharmacokinetic data for the BIP prodrug, toxicity data for the combination therapy, biodistribution, tumor localization, and immunogenicity data for MFECP1, and evidence of prodrug activation were obtained.

Results of a pilot trial with A5CP and the BIP prodrug (8) provided guidance for the starting dose of prodrug and the threshold of CPG2 in serum. Our results showed dose-limiting myelotoxicity at the starting dose of 12.42 mg/m² ×3, and this was attributed to systemic prodrug conversion. The MFECP1 level was 0.017 units/mL in this patient. Subsequently, the threshold of MFECP1 in serum permitting prodrug administration was reduced to <0.005 units/mL. This allowed dose

escalation of prodrug >80-fold before DLT being reached. DLT consisted of liver, kidney, and myelotoxicity. All toxicity was reversible and the liver and kidney biopsies confirmed hepatic and acute tubular necrosis, which is consistent with the toxicity seen *in vivo*. The mean half-life of the prodrug was 10.3 (± 3.45) minutes and the median half-life was 9.96 minutes, showing consistency across the tested doses.

The optimal time point for giving prodrug is a balance defined by safe levels of MFECP1 in serum and sufficient amounts in tumor for effective prodrug conversion. This provides a potential challenge because molecules that clear rapidly tend to accumulate less in tumor. *In vivo* results from tumor-bearing mice showed rapid elimination of MFECP1 due to glycosylation of MFECP1, which leads to recognition and uptake of MFECP1 by the mannose receptors in liver sinusoidal cells. Results in our patients were consistent with these observations as MFECP1 cleared rapidly via liver as shown in Fig. 2. A short initial elimination phase was followed by a slower second phase. The relatively slow clearance of radioactivity from circulation compared with clearance of enzyme activity is most likely due to persistence of radioactive MFECP1

Table 2. BIP prodrug plasma pharmacokinetics

Dose (mg/m ² × 3) [no. patients]	Elimination half-life (min)	C ₀ (μ g/mL)	AUC _{INF} (μ g mL min)	Clearance (mL/min)	V _{ss} (mL)
12.42 [5]	8.5	4.2	34	824	7,568
16.15 [3]	7.0	5.8	55	671	5,889
21.0 [2]	11.5	8.9	97	424	6,398
33.6 [2]	8.2	14	97	663	6,128
67.2 [2]	9.0	20	177	609	6,800
134.4 [1]	11.0	61	708	380	5,522
200 [3]	10.6	66	741	527	6,904
268.8 [7]	9.9	90	1,160	490	5,847
537.6 [4]	14.4	235	3,408	302	5,448
1,075 [1]	16.3	*	8,228	254	6,065

*Plasma concentration increasing to 5 minutes maximum.

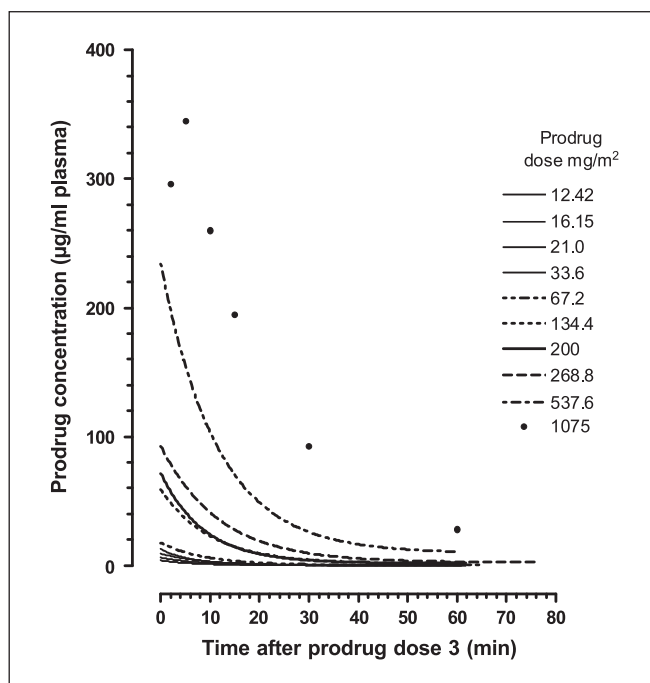


Fig. 4. Mean plasma elimination curves for mustard prodrug showing consistency across tested doses.

breakdown products, which are enzymatically inactive. Prodrug administration was possible in all patients except one within 24 hours despite some variation in elimination. Immunohistochemical staining of biopsies and SPECT imaging revealed presence of enzyme in tumor up to 19 and 24 hours, respectively. The estimated amount of CPG2 levels in tumor by SPECT compares favorably with a previous study (8) using a slowly clearing chemical conjugate of antibody-enzyme, whereas higher levels were seen in a study with a chemical conjugate and a clearing antibody (7). Selective localization of MFECPI seems to be related to presence of antigen, although confirmation of this is needed using coregistration of images. Reduction of the dose of MFECPI was explored with the aim of reducing the interval between MFECPI and prodrug because favorable tumor to normal tissue ratios of MFECPI were seen 6 hours after administration with MFECPI in mice (14). This resulted in faster elimination from circulation but also in unforeseen DLT in patients receiving prodrug doses, which had previously been found safe and well tolerated when given at a later time point. Toxicity may potentially be due to enzyme retention in other organs at these time points, which is not obvious by measuring CPG2 in serum. Hence, the toxicity of ADEPT with MFECPI and BIP prodrug is dependent on the serum enzyme level, dose of prodrug, and time interval between fusion protein and prodrug. In patients, 14 to 18 hours proved to be optimal.

DNA interstrand cross-links in tumor measured by comet assay were chosen as a surrogate to provide evidence of activation of prodrug. A tumor biopsy of a liver metastasis was obtained in one patient with metastatic colon cancer, who received the maximum tolerated dose of 3,000 units/m² MFECPI followed by 268.8 mg/m² × 3 prodrug at the optimal time interval. Immunohistochemical staining and phosphor-imaging confirmed retention of MFECPI in tumor, and comet

assay results provided evidence of prodrug activation for this dose and time interval. Further evidence of activity was seen in one patient suffering from widespread peritoneal cancer with a 10% reduction of tumor diameter on CT imaging.

ADEPT is designed to overcome drug resistance in solid tumors. Drug resistance is thought to depend on several factors, including genetic abnormalities of cancer cells and alterations in the critical pathways of cell cycle checkpoint controls, DNA repair, and apoptosis. Initially, sensitive cancer cells can be destroyed by effective chemotherapy but, if they survive, develop resistance to further treatment due in part to drug-induced mutations in their DNA. High doses of chemotherapy are able to overwhelm these protective mechanisms in some cases. In ADEPT, repair of DNA interstrand cross-links at 24 hours has been reported (20). Repeat administration with up to three treatments weekly was required for efficacy with MFECPI and BIP prodrug in tumor-bearing mice (14) and therefore validated that clinical responses are more likely to be observed on repeat treatment in man.

Thus far, the development of HAMA and HACA has been an obstacle to repeated treatment with ADEPT without immunosuppression. After giving the chemical conjugate A5CP, 100% of patients developed HAMA to A5B7 monoclonal antibody and 97% developed HACA (7, 8). In the current study, the same assay was used and none of the patients treated with MFECPI developed HAMA to A5B7 and only 36% of patients developed HACA after MFECPI. An anti-MFE-23 ELISA is currently being developed to measure the immune response specifically to the single-chain Fv moiety of MFECPI. However, an immune response to the single-chain Fv part of the fusion protein is likely to be a more tractable problem than an immune response to the CPG2 moiety. Single-chain Fv antibodies have been reported to be less immunogenic than larger antibody fragments, and particularly, no antibody formation to MFE-23 had been detected after single administration (10). Furthermore, antibody fragments are readily mutated by a variety of established methods to obtain humanized forms and this has already been achieved for MFE-23 (21). Foreign enzymes, such as CPG2, are more problematic (22). Potentially, they can be made less immunogenic by understanding the immune response and by modifying the enzyme accordingly. A particularly challenging aspect is that the modifications introduced do not interfere with structure, function, or stability of the enzyme.

Encouragingly, when given in form of MFECPI, CPG2 proved also to be significantly less immunogenic than in previous trials. Further investigations into this have been reported (23). In brief, a clinically relevant immunogenic epitope at the COOH terminus of CPG2, the CM79-identified epitope, has been identified using phage display technology, surface enhanced laser desorption/ionization affinity mass spectrometry, and bioinformatics algorithms (24). Because MFECPI carries a hexahistidine tag at the COOH terminus of MFECPI, which can potentially adopt any conformational state, it has been hypothesized that this potentially masks the CM79-identified epitope. Comparison of patient sera after giving MFECPI and A5CP, in which the enzyme lacks the COOH-terminal hexahistidine tag, confirmed a significantly reduced immune response to the CM79-identified epitope after MFECPI. Masking of the clinically relevant epitope may thereby contribute to the reduction of the immune response to MFECPI.

This article reports the first clinical use of the recombinant antibody-enzyme fusion protein MFECP1 for ADEPT. MFECP1 is safe, well tolerated, clears rapidly, localizes in tumor, and is less immunogenic compared with a previously used chemical antibody-enzyme conjugate. The *P. pastoris* expression system is capable of producing large quantities of functional protein and is becoming an accepted industry standard for recombinant protein manufacture. Several *P. pastoris*-generated protein-based biopharmaceutical agents have been tested in clinical trials (25, 26). To our knowledge, the MFECP1 antibody-enzyme fusion protein we describe here is first of its kind in terms of multifunctional complexity in that the molecular design criteria of CEA binding *in vivo* and rapid elimination from normal tissues are successfully shown in patients.

The trial has established safe doses of MFECP1 and BIP prodrug for single administration by addressing the complexity of ADEPT in a systematic manner. Evidence of prodrug

activation and activity of the combination has been obtained. This study is the first clinical report of ADEPT with a recombinant targeting agent and forms an important platform for testing the exciting clinical potential of repeat therapy with ADEPT.

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References

- Douillard JY, Cunningham D, Roth AD, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. *Lancet* 2000;355:1041–7.
- De Gramont A, Figer A, Seymour M, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 2000;18:2938–47.
- Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335–42.
- Cunningham D, Humblet Y, Siena S, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med* 2004;351:337–45.
- Bagshawe KD, Springer CJ, Searle F, et al. A cytotoxic agent can be generated selectively at cancer sites. *Br J Cancer* 1988;58:700–3.
- Bagshaw KD, Sharma SK, Springer CJ, et al. Antibody-directed enzyme prodrug therapy: a pilot scale clinical trial. *Tumor Targeting* 1995;1:17–29.
- Napier MP, Sharma SK, Springer CJ, et al. Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma. *Clin Cancer Res* 2000;6:765–72.
- Francis RJ, Sharma SK, Springer C, et al. A phase I trial of antibody directed enzyme prodrug therapy (ADEPT) in patients with advanced colorectal carcinoma or other CEA producing tumours. *Br J Cancer* 2002;87:600–7.
- Chester KA, Begent RH, Robson L, et al. Phage libraries for generation of clinically useful antibodies. *Lancet* 1994;343:455–6.
- Begent RH, Verhaar MJ, Chester KA, et al. Clinical evidence of efficient tumor targeting based on single-chain Fv antibody selected from a combinatorial library. *Nat Med* 1996;2:979–84.
- Mayer A, Tsiompanou E, O'Malley D, et al. Radioimmunoguided surgery in colorectal cancer using a genetically engineered anti-CEA single-chain Fv antibody. *Clin Cancer Res* 2000;6:1711–9.
- Medzihradsky KF, Spencer DI, Sharma SK, et al. Glycoforms obtained by expression in *Pichia pastoris* improve cancer targeting potential of a recombinant antibody-enzyme fusion protein. *Glycobiology* 2004;14:27–37.
- Springer CJ, Dowell R, Burke PJ, et al. Optimisation of alkylating agent prodrugs derived from phenol and aniline mustards: a new clinical candidate prodrug (ZD2767P) for antibody-directed enzyme prodrug therapy (ADEPT). *J Med Chem* 1995;38:5051–65.
- Sharma SK, Pedley RB, Bhatia J, et al. Sustained tumor regression of human colorectal cancer xenografts using a multifunctional mannosylated fusion protein in antibody-directed enzyme prodrug therapy. *Clin Cancer Res* 2005;11:814–25.
- Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–16.
- Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations, and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. *Official Journal L* 2001;121:34–44.
- Green AJ, Dewhurst SH, Begent RH, et al. Accurate quantification of 131-I distribution by gamma camera imaging. *Eur J Nucl Med* 1990;16:361–5.
- Flynn AA, Green AJ, Boxer GM, et al. A novel technique using radioluminography, for the measurement of uniformity of radiolabelled antibody distribution in a colorectal cancer xenograft model. *Int J Radiat Oncol Biol Phys* 1999;43:183–9.
- Spanswick VJ, Hartley JM, Ward TH. Measurement of drug-induced DNA interstrand crosslinking using the single-cell gel electrophoresis (comet) assay. In: Brown R and Boeger-Brown U, editors. *Methods in molecular medicine: cytotoxic drug resistance mechanisms*. Totowa, NJ: Humana Press; 1998. p.143–154.
- Webley SD, Francis RJ, Pedley RB, et al. Measurement of the critical DNA lesions produced by antibody-directed enzyme prodrug therapy (ADEPT) *in vitro*, *in vivo*, and in clinical material. *Br J Cancer* 2001;84:1671–6.
- Graff CP, Chester K, Begent R, Wittrup KD. Directed evolution of an anti-carcinoembryonic antigen scFv with a 4-day monovalent dissociation half-time at 37 degrees C. *Protein Eng Des Sel* 2004;17:293–304.
- Chester KA, Baker M, Mayer A. Overcoming the immunologic response to foreign enzymes in cancer therapy. *Expert Rev Clin Immunol* 2005;1:549–59.
- Mayer A, Sharma SK, Tolner B, et al. Modifying an immunogenic epitope on a therapeutic protein: a step towards an improved system for antibody-directed enzyme prodrug therapy (ADEPT). *Br J Cancer* 2004;90:2402–10.
- Spencer DI, Robson L, Purdy D, et al. A strategy for mapping and neutralizing conformational immunogenic sites on protein therapeutics. *Proteomics* 2002;2:271–9.
- Herbst RS, Hess KR, Tran HT, et al. Phase I study of recombinant human endostatin in patients with advanced solid tumors. *J Clin Oncol* 2002;20:3792–803.
- Malkin EM, Diemert DJ, McArthur JH, et al. Phase I clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *Infect Immun* 2005;73:3677–85.