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-iodophenol 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 unresectable 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).
To address these requirements, an ADEPT system with the antibody-enzyme fusion protein MFECP1 and a bis-iodo phenol (BIP)\(^6\) mustard prodrug \([4-\text{bis}(2\text{-iodoethyl})\text{amino}]\text{phenyl} \text{oxycarbonyl-L-glutamic acid}\) was designed. MFECP1 is a recombinant fusion protein of carboxypeptidase G2 (CPG2), an enzyme derived from \textit{Pseudomonas aeruginosa}, directly linked to the NH\(_2\) terminus of the anti-CEA single-chain Fv antibody MFE-23 (9–11). Expression in \textit{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 \(\mu\)g/L. Patients with a serum CEA <10 \(\mu\)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 \(\mu\)mol/L), liver (bilirubin <30 \(\mu\)mol/L, alanine aminotransferase/aspartate aminotransferase ≤2× upper normal limit and ≤5× upper normal limit in case of liver metastases, international normalized ratio ≤1.5), and bone marrow function (hemoglobin ≥10 g/dL, platelet count ≥100 × 10\(^9\)/L, neutrophil count ≥2 × 10\(^9\)/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.

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\(^6\) 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 MFECP1 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 MFECP1 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 MFECP1 was measured by CEA binding (ELISA), enzyme activity, SDS-PAGE, and Western blot analysis. The dimeric nature of MFECP1 was confirmed by detection of a single MFECP1 peak on size exclusion chromatography in physiologic conditions. The mean specific activity was 128 ± 22.8 units/mg. For administration to patients, MFECP1 was diluted in 500 mL 0.9% sodium chloride and given over 90 to 120 minutes via a Hickman line.

Radiolabeling of MFECP1 with a tracer dose of 370 MBq 131I or 500 MBq 123I for patients undergoing single-photon emission computed tomography (SPECT) imaging to determine localization of MFECP1 was done using the chloramine-T method. TLC was used to assess iodine incorporation, and antigen binding was tested on a CEA column. 123I or 131I MFECP1 was given at the beginning of the infusion with MFECP1. Patients receiving radiolabeled MFECP1 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 MFECP1 fusion protein localizes in tumor and clears from normal tissue via liver, (b) to measure the dose-limiting toxicity (DLT) and maximum tolerated dose of one ADEPT cycle with MFECP1 and BIP prodrug, (c) to assess immunogenicity of a single administration of MFECP1, and (d) to obtain evidence of prodrug activation and preliminary evidence of efficacy.

**Study design**

Each patient was given a single dose of MFECP1 followed by three bolus doses of BIP prodrug 1 hour apart. The starting dose level of 5,000 units/m2 MFECP1 (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 × 3 on data from a previous clinical trial (8). In initial patients, a safe level of MFECP1 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 MFECP1 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 MFECP1 was based on mathematical prediction from blood elimination data after giving 5,000 units/m2 MFECP1.

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 MFECP1**

Serum samples were taken for estimation of their CPG2 enzyme activity by high-performance liquid chromatography 5 minutes after completion of infusion with MFECP1 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 MFECP1, samples were taken at the same time points for measurement of radioactivity in a gamma well counter.

**Tumor localization of MFECP1**

**Gamma camera imaging.** Patients who received a tracer dose of MFECP1 labeled with 125I or 131I 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 MFECP1 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 radioactive dose per kg of tissue was calculated, and the amount of enzyme in tumor was estimated from this.

**Assessment of MFECP1 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 MFECP1 and BIP prodrug. Fresh tissue was snap frozen and fixed in acetone. Tissues 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 MFECP1 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 MFECP1 were exposed to phosphorimage plates (18).

**Immune response**

Blood was taken before treatment and weekly for 8 weeks to assess the immune response to MFECP1. 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 × 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 (C0), area under the curve (AUC0→), clearance and volume of distribution in the body as steady state (Vss/Cl), was determined for each evaluable patients 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 MFEC1

MFEC1 was safe and well tolerated. Grade 1 rigor (one patient), fever (two patients), allergy (one patient), and hypertension (one patient) were seen.

Pharmacokinetics of MFEC1

Sixteen patients who received 5,000 units/m2 MFEC1 also received 123I-labeled MFEC1 and four patients received 131I-labeled MFEC1; administration of radiolabeled MFEC1 in the remaining patients was not possible for logistic reasons. MFEC1 was shown to clear rapidly through the liver with prominent hepatic tracer 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 MFEC1, respectively.

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 MFEC1, 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 MFEC1

Gamma camera imaging. MFEC1 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 MFEC1 and in five patients who received 131I-labeled MFEC1. 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 MFEC1. Immunohistochemistry with anti-CPG2 antibody showed MFEC1 localization in tumor in patients up to 19 hours after giving 5,000 units/m2 and 15 hours after 3,000 units/m2 MFEC1, respectively. Phosphorimaging confirmed uptake of radiolabeled MFEC1 in tumor in 15 hours after 3,000 units/m2 MFEC1. Figure 3 shows positive staining with anti-CEA antibody and anti-CPG2 antibody in a liver metastasis, confirming CEA expression and localization of MFEC1.

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 MFEC1; 268.8 and 537.6 mg/m2 in combination with 3,000 units/m2 MFEC1; and 200 mg/m2 in combination with 1,500 units/m2 MFEC1.
At the starting dose for the BIP prodrug, 12.42 mg/m² × 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 MFECP1 level in serum permitting prodrug administration was lowered to <0.005 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 mg/m² 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 units/m² MFECP1 and prodrug after MFECP1 had cleared to <0.005 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 MFECP1, lowering the dose of MFECP1 to shorten the time interval between MFECP1 and prodrug was investigated. The dose reduction of MFECP1 was calculated based on the patient elimination data available for 5,000 units/m². Patients 24 and 25 received 3,000 units/m² in combination with 537.6 mg/m² × 3 prodrug. This dose of prodrug had been safe when given 16 to 18 hours after 5,000 units/m² MFECP1 (patients 21 and 22) but caused dose-limiting liver and myelotoxicity (Table 1) given 9.7 and 13.8 hours after 3,000 units/m² MFECP1. When the combination of 1,500 units/m² MFECP1 and 200 mg/m² × 3 prodrug was given to patients 29 to 31, dose-limiting thrombocytopenia was seen in one patient receiving prodrug 7.9 hours after MFECP1 (patient 30; Table 1). Toxicity at these time points may be due to enzyme retention in organs other than blood. In contrast, 268.8 mg/m² × 3 prodrug given 13 hours (median; range, 11.6-15 hours) after 3,000 units/m² MFECP1 (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 MFECP1 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 C₀ and AUCINF. 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 units/m² MFECP1 and 268.8 mg/m² × 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 31 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 monoclonal anti-CEA antibody (6–8). These trials used CMDA, a synthetic benzoic acid mustard prodrug (6, 7) or monoclonal anti-CEA antibody (6–8).
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 1. Summary of grade 3 and 4 toxicity**

<table>
<thead>
<tr>
<th>Treatment with MFECP1 (units/m²) and BIP prodrug (mg/m² × 3)</th>
<th>No. patients</th>
<th>Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,000 MFECP1 + 12.42 BIP prodrug</td>
<td>3</td>
<td>G3 thrombocytopenia (1), G3 neutropenia (1), G3 leukopenia (1)</td>
</tr>
<tr>
<td>Plasma CPG2 0.0111 units/mL (median)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5,000 MFECP1 + 1,075 BIP prodrug</td>
<td>1</td>
<td>G4 ALT/AST, G3 GGT, G4 Cr/urea, G3 anemia, G3 leukopenia, G3 thrombocytopenia</td>
</tr>
<tr>
<td>Plasma CPG2 &lt;0.002 units/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,000 MFECP1 + 537.6 BIP prodrug</td>
<td>2</td>
<td>G4 ALT/AST (1), G3 anemia (1), G3 thrombocytopenia (1), G3 leukopenia (1), G3 fatigue (2)</td>
</tr>
<tr>
<td>Plasma CPG2 &lt;0.002 units/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,500 MFECP1 + 200 BIP prodrug</td>
<td>1</td>
<td>G3 thrombocytopenia (1)</td>
</tr>
<tr>
<td>Plasma CPG2 &lt;0.002 units/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Plasma concentration increasing to 5 minutes maximum.

**Table 2. BIP prodrug plasma pharmacokinetics**

<table>
<thead>
<tr>
<th>Dose (mg/m² × 3)</th>
<th>[no. patients]</th>
<th>Elimination half-life (min)</th>
<th>C₀ (µg/mL)</th>
<th>AUC₀_INF (µg mL min)</th>
<th>Clearance (mL/min)</th>
<th>Vₜ (mL)</th>
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<td>12.42 [5]</td>
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<td>4.2</td>
<td>34</td>
<td>824</td>
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<td>16.15 [3]</td>
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<td>5.8</td>
<td>55</td>
<td>671</td>
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<td>21.0 [2]</td>
<td>11.5</td>
<td>8.9</td>
<td>97</td>
<td>424</td>
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<td>33.6 [2]</td>
<td>8.2</td>
<td>14</td>
<td>97</td>
<td>663</td>
<td>6,128</td>
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</tr>
<tr>
<td>67.2 [2]</td>
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<td>20</td>
<td>177</td>
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<td>134.4 [1]</td>
<td>11.0</td>
<td>61</td>
<td>708</td>
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<tr>
<td>200 [3]</td>
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<td>268.8 [7]</td>
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</tbody>
</table>

*Abbreviations: G3, grade 3; G4, grade 4; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl aminotransferase."
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 MFECP1 seems to be related to presence of antigen, although confirmation of this is needed using coregistration of images. Reduction of the dose of MFECP1 was explored with the aim of reducing the interval between MFECP1 and prodrug because favorable tumor to normal tissue ratios of MFECP1 were seen 6 hours after administration with MFECP1 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 MFECP1 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 MFECP followed by 268.8 mg/m² × 3 prodrug at the optimal time interval. Immunohistochemical staining and phosphor-imaging confirmed retention of MFECP1 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 MFECP1 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 MFECP1 developed HAMA to A5B7 and only 36% of patients developed HACA after MFECP1. An anti-MFE-23 ELISA is currently being developed to measure the immune response specifically to the single-chain Fv moiety of MFECP1. 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 MFECP1, 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 MFECP1 carries a hexahistidine tag at the COOH terminus of MFECP1, 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 MFECP1 and A5CP, in which the enzyme lacks the COOH-terminal hexahistidine tag, confirmed a significantly reduced immune response to the CM79-identified epitope after MFECP1. Masking of the clinically relevant epitope may thereby contribute to the reduction of the immune response to MFECP1.
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 vitro and rapid elimination from normal tissues are successfully shown in patients.

The trial has established safe doses of MFECP1 and BIP produg 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

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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