Sustained Tumor Regression of Human Colorectal Cancer Xenografts Using a Multifunctional Mannosylated Fusion Protein in Antibody-Directed Enzyme Prodrug Therapy

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ABSTRACT

Purpose: Antibody-directed enzyme prodrug therapy (ADEPT) requires highly selective antibody-mediated delivery of enzyme to tumor. MFE-CP, a multifunctional genetic fusion protein of antibody and enzyme, was designed to achieve this by two mechanisms. First by using a high affinity and high specificity single chain Fv antibody directed to carcinoembryonic antigen. Second by rapid removal of antibody-enzyme from normal tissues by virtue of post-translational mannosylation. The purpose of this paper is to investigate these dual functions in an animal model of pharmacokinetics, pharmacodynamics, toxicity, and efficacy.

Experimental Design: MFE-CP was expressed in the yeast Pichia pastoris and purified via an engineered hexahistidine tag. Biodistribution and therapeutic effect of a single ADEPT cycle (1,000 units/kg MFE-CP followed by 70 mg/kg ZD2767P prodrug at 6, 7, and 8 hours) and multiple ADEPT cycles (9-10 cycles within 21-24 days) was studied in established human colon carcinoma xenografts, LS174T, and SW1222.

Results: Selective localization of functional enzyme in tumors and rapid clearance from plasma was observed within 6 hours, resulting in tumor to plasma ratios of 1,400:1 and 339:1, respectively for the LS174T and SW1222 models. A single ADEPT cycle produced reproducible tumor growth delay in both models. Multiple ADEPT cycles significantly enhanced the therapeutic effect of a single cycle in the LS174T xenografts (P = 0.001) and produced regressions in the SW1222 xenografts (P = 0.0001), with minimal toxicity.

Conclusions: MFE-CP fusion protein, in combination with ZD2767P, provides a new and successful ADEPT system, which offers the potential for multiple cycles and antitumor efficacy. These results provide a basis for the next stage in clinical development of ADEPT.

INTRODUCTION

Antibody-directed enzyme prodrug therapy (ADEPT) has shown feasibility as a treatment for cancer (1–3). ADEPT (Fig. 1) is designed to generate a potent cytotoxic drug selectively at tumor sites from its nontoxic precursor by a targeted enzyme not normally present in the body (4, 5). Higher concentration of drug at the tumor site may be obtained by this method than by direct administration of drug alone, as the enzyme provides an amplification effect. Furthermore, the drug is generated extracellularly and, being a small molecule, can diffuse throughout the tumor mass. Thus, antigen-negative tumor cells are killed alongside cells expressing the relevant antigen (6, 7).

Several antibody-enzyme prodrug systems have been developed since the original ADEPT approach was proposed (8). Both endogenous and nonendogenous enzymes have been used in this system (reviewed in ref. 9). In our studies, we have used the bacterial enzyme, carboxypeptidase G2 (CPG2), which has no known mammalian homologue, in combination with glutamated mustard prodrugs.

Our previous clinical studies with a chemical conjugate of anti–carcinoembryonic antigen (CEA) F(ab’)2 antibody with carboxypeptidase G2 enzyme have shown either an inadequate tumor to normal tissue ratios (10) or the need for a clearance system to accelerate removal of antibody enzyme from the circulation (3). The recombinant genetic fusion protein, composed of a single-chain Fv antibody and an enzyme expressed in Escherichia coli (11), gave improved tumor to normal tissue ratios in a colon carcinoma model (12) and the benefits of a uniform product. However, the tumor-to-normal tissue ratios were still only moderate, and it was proposed that a more rapid clearance of the fusion protein from the circulation would enhance the tumor selectivity of enzyme delivery.

Expression of the fusion protein in Pichia pastoris yields a protein glycosylated with branched mannose (13). We hypothesized that this would accelerate clearance of the fusion protein from the circulation, via hepatic mannose receptors, without inducing toxicity. It was also necessary to investigate whether a more rapid blood clearance would result in a proportional decrease in tumor concentrations of enzyme. Favorable results in these experiments made it feasible to investigate the efficacy of single and repeat doses of ADEPT, using a bis-iodo-phenol mustard prodrug (ZD2767P; ref. 14). This paper investigates these issues in two morphologically different human colon carcinoma xenografts (LS174T and SW1222) in
Materials and Methods

Construction of MFE-CP Gene

For details of MFE-23:CPG2 gene fusion, refer to Michael et al. (11). For construction of a recombinant transfer vector for P. pastoris expression, a six histidine tag (his tag) was engineered at the COOH terminus of the fusion protein to facilitate purification using immobilized metal affinity chromatography. This was achieved using PCR with the following primer sequence: CPG2-003631 5'-GAAGGCCGCAAGAAGCTGGTCA-GAAGGCCGG-3' and CPG2-4362 5'-ACCTGTAACTGCAGTTACTATTGCCAG-CAAGGCGG-3'. The resultant PCR product was cloned into pCR-Script SK (Stratagene, Amsterdam, NL). A SalI-EcoRI fragment, encoding the COOH-terminal region CPG2-His, was removed and inserted into pPM331 (11) in place of the authentic sequence. The MFE-23:CPG2-His6 gene fusion was inserted into pPICZaB (Invitrogen, Paisly, UK), as a SfiI-XbaI fragment. Following digestion with Pmel, the linearized vector construct was transformed into electrocompetent P. pastoris cells according to the conditions recommended by the supplier. Transformants were selected on zeocin and recombinant clones identified by PCR using the β factor 5'-TACTATTGCCAG-CATTGCTGC-3' and CPG2-005091 5'-CACCGCCTTGTC-GACCAGCTTGG-3' primers.

The final construct (pPICZa01) and a cartoon of the MFE-CP fusion protein are shown in Fig. 2A.

Expression of MFE-CP in P. pastoris

Fermentation was done in a standard 10-L microbial stirred tank reactor (BioFlow 3000, New Brunswick Scientific, Hatfield, United Kingdom). Six liters of defined, glycerol-limited medium were inoculated with 300 mL of the same medium. Temperature was controlled at 30°C and pH at 5.0 (using ammonia solution). Dissolved oxygen was cascade controlled at 40% air saturation using agitation and oxygen supplementation. Growth proceeded batchwise until all the glycerol was depleted at which point a limited glycerol feed was started (108 mL/h of 50% v/v glycerol plus 12 mL/L PTM1 trace elements solution) and the pH raised to 6.5. After 9 hours, the glycerol feed was terminated and a methanol feed (100% methanol plus 12 mL/L PTM1 trace elements solution, Invitrogen) commenced at 22 mL/h to induce-product formation. Methanol feeds were increased to 35 mL/h after 2 hours and 53 mL/h after a further 2 hours. The fermentation ran for a further 36 hours before harvesting.

Purification of MFE-CP

The P. pastoris expressed fusion protein was purified by using immobilized metal affinity chromatography exploiting the his tag. This was achieved by capture using a chelating matrix preloaded with copper sulfate as described previously for bacterially expressed his-tagged MFE-23 (15, 16). Matrixes were Chelating Sepharose fast flow (Amersham Pharmacia, St Albans, United Kingdom) or Streamline 50 Chelating matrix in expanded bed (Amersham Pharmacia). Typically, the immobilized metal affinity chromatography chelating column was primed with 0.1 mol/L copper sulfate, washed with distilled water and equilibrated with 1 mol/L NaCl in PBS (NaCl/PBS). Fermentation media containing MFE-CP was added to the column in final concentration of 1 mol/L NaCl. Nonspecifically bound material was eluted by washing with NaCl/PBS followed by 40 mmol/L imidazole in NaCl/PBS. Specifically bound MFE-CP was subsequently eluted with 200 mmol/L imidazole in NaCl/PBS. The 200 mmol/L imidazole fraction was dialyzed overnight against PBS at 4°C and concentrated to ~2 mg/mL using an Amicon Stirred Cell Ultrafiltration System (Amicon, Stonehouse, Gloucestershire, United Kingdom) and a PM10 membrane (Millipore, Hertfordshire, United Kingdom) and aliquots were prepared in a sterile laminar flow cabinet (LaminAir HB 2448, Heraeus Instruments, Surrey, United Kingdom) and stored at −80°C.
Characterization of MFE-CP

MFE-CP was subjected to SDS-PAGE using 10% polyacrylamide gels; protein bands were visualized by staining with Coomassie blue and the remainder was used for protein electrophoretic transfer to polyvinylidene difluoride membrane (Trans-Blot Transfer Medium, Bio-Rad, United Kingdom). The polyvinylidene difluoride membranes were then used for Western blotting and lectin probing. For Western blotting, anti-hexahistidine antibody (Dianova, Hamburg, Germany) was used at a 1:500 dilution in PBS containing 1% milk powder to detect MFE-CP. The anti-hexahistidine antibody was incubated with the polyvinylidene difluoride membrane for 1 hour and detected using a 1:1,000 dilution of sheep anti-mouse IgG-alkaline phosphatase conjugate. For lectin blotting, polyvinylidene difluoride membranes were incubated with biotinylated concanavalin A (Vector Laboratories Ltd., Peterborough, United Kingdom) diluted 1:1,000 in Tween-Tris buffer saline [0.05% (v/v) Tween 20, 0.9% NaCl, and Tris-HCl (pH 7.4)] for 1 hour. The biotinylated concanavalin A was detected using avidin/alkaline phosphatase complex (Vector Laboratories). Alkaline phosphatase activity was visualized using 0.35 mmol/L of 5-bromo-4-chloro-3-indoyl phosphate.

Identity of MFE-CP and immunoreactivity of MFE-CP to CEA was assessed by ELISA using CEA-coated wells and probing with antibody to CPG2. Briefly, microtiter plates (Costar EIA/RIA 8 well strips, Costar Co., Cambridge, United Kingdom) were coated with 0.2 μg of CEA per well, blocked with 3% bovine serum albumin in PBS and washed with 0.05% (v/v) Tween 20 in PBS. Purified MFE-CP was diluted in PBS before duplicate aliquots (100 μL) of 10-fold serial dilutions were applied to CEA-coated wells and a control consisting of a mixture of MFE-23-His and CPG2 unfused (●, broken line). Results show that the ELISA profile was unchanged by storage at −80°C for 12 months (●). D, in vivo stability analysis of MFE-CP. Autoradiogram showing profile of radiolabeled MFE-CP resolved by SDS-PAGE before (strip 1) and after (strip 2) injection to mice bearing human tumors. The position of the 70-kDa major protein band in denatured MFE-CP is indicated with an arrow. Results confirm presence of MFE-CP in tumor and show stability of MFE-CP in vivo.
applied to the CEA-coated wells. MFE-CP that bound to the CEA was detected using rabbit anti-CPG2 serum (1:500), followed by goat anti-rabbit IgG-alkaline phosphatase (1:10,000) conjugate. Alkaline phosphatase labelling was detected using 100 μL of 1.4 mmol/L O-phenylenediamine dihydrochloride. The reaction was quenched with 50 μL 4 N HCl per well. Absorbance at 490 nm was read on a 96-well plate reader (Microplate Autoreader, Boots-Celltech Diagnostics Ltd., Slough, United Kingdom).

Enzyme activity of MFE-CP was measured as described previously (17) by incubating 10 μL of sample in 1 mL of assay buffer [100 mmol/L Tris-HCl (pH 7.3), 0.2 mmol/L ZnSO4, and 60 mmol/L methotrexate], at 37°C for 1 minute. CPG2 hydrolysis of methotrexate results in a change in absorbance at 320 nm which was measured by spectrophotometry (Beckman DU-64 spectrophotometer, Soft Pac Module KINETICS Software Package, Beckman Instruments Ltd., Bucks, United Kingdom). Enzyme activity was expressed in units, where 1 unit is the amount of enzyme required to hydrolyze 1 mmol of methotrexate per minute at 37°C.

MFE-CP protein was radiolabeled with 125I-iodine using the chloramine T method and separated from the free 125I using a PD10 column (Pharmacia Biotech, St Albans, United Kingdom). TLC of the radiolabeled fusion protein showed 99% incorporation of the radiolabel into the protein. Antigen binding of the radiolabeled fusion protein was measured by applying on to an affinity column containing CEA. Unbound material was washed through using PBS and bound material eluted with 3 mol/L ammonium thiocyanate, and the fractions were counted in a gamma counter (Wallac 1470 Wizard Automatic gamma counter).

In vivo stability was assessed by injecting tumor-bearing mice with radiolabeled MFE-CP. At 4 hours after injection, mice were sacrificed and tumor samples were homogenized on ice in PBS containing yeast protease inhibitors (Sigma, Gillingham, United Kingdom). A 20-μL fraction of the homogenized tumor material was transferred to 80 μL of SDS-sample buffer, mixed well, and boiled for 4 minutes. After boiling, particulate was removed by centrifugation. Finally, 10 μL of the boiled SDS-tumor protein sample was subjected to SDS-PAGE. Radiolabeled proteins within the polyacrylamide gel were visualized by autoradioluminography using a phosphor storage plate (Molecular Dynamics, Sunnyvale, CA), which was exposed to the polyacrylamide gel for 14 hours.

Prodrug

Bis-iodo-phenol prodrug (ZD2767P) was synthesized as described previously (14) and was a generous gift from AstraZeneca. ZD2767P has already been used in preclinical (18) and clinical (10) studies.

Xenografts

In vivo studies were done in nude mice bearing either the LS174T or SW1222 human colon adenocarcinoma xenograft. The human colonic adenocarcinoma cell lines LS174T and SW1222 were used to develop xenograft models in the flanks of female nude (nu/nu) mice (MF1; from our own breeding colony, 2- to 3-month-old, weighing 20-25 g). Subsequent passaging was by s.c. implantation of small tumor pieces (~ 1 mm³).

The LS174T xenograft is a moderately poorly differentiated adenocarcinoma, with small glandular acini. The SW1222 is organized into well-defined glandular structures around a central lumen. Both xenografts express CEA.

In vivo Studies

All experiments complied with the United Kingdom Coordinating Committee on Cancer Research Guidelines for the Welfare of Animals in Experimental Neoplasia.

Localization of Fusion Protein by CPG2 Activity. CPG2 activity in tumor and normal tissues was measured by an indirect high-performance liquid chromatography assay (12) by using methotrexate as a substrate. CPG2 cleaves methotrexate to form 2,4-diamino-N-10-methylpteroic acid, a metabolite of methotrexate. At selected time points, tissues were collected from mice injected with fusion protein and blotted, weighed and homogenized in PBS containing 0.2 mmol/L ZnCl2 to obtain 10% or 20% (w/v) homogenate. The homogenate was further diluted as appropriate. A 600-μL aliquot of each homogenate was incubated in a shaking water bath set at 30°C for 20 minutes. The homogenate was incubated with methotrexate (6 μL of 10 mmol/L methotrexate in DMSO) for 30 minutes at 30°C. The reaction was terminated by adding 500 μL of ice-cold stop solution (90% methanol + 10% ammonium formate) to 500 μL of homogenate. The mixture was centrifuged (Heraeus Biofuge – 14,000 rpm for 10 minutes). The solution (700 μL) was removed and placed into high-performance liquid chromatography autosampler vials. The samples (60 μL) were analyzed on the high-performance liquid chromatography using a P4000 pump, an AS 3000 autosampler and a SpectroFocus scanning detector set at 320 nm wavelength with a flow rate of 0.1 mL/min (ThermoQuest Limited, Manchester, United Kingdom). The mobile phase, consisting of 55% ammonium formate 1 mL/min (ThermoQuest Limited, Manchester, United Kingdom). The mobile phase, consisting of 55% ammonium formate + 45% methanol with a flow rate of 1 mL/min, was used to elute 2,4-diamino-N-10-methylpteroic acid. Quantitative analysis of CPG2 activity was done by comparing 2,4-diamino-N-10-methylpteroic acid peak areas with the calibration curves, which were prepared using tissues from control untreated mice, added to known enzyme activity, processed, and analyzed in the same way as experimental samples.

Plasma Clearance. Nude mice bearing LS174T xenografts were injected (1,000 units/kg i.v.) with either MFE-CP (P. pastoris), MFE-23:CPG2 (E. coli) or ASB7-F(ab')2-CPG2 (chemical conjugate). Blood samples were taken at 1, 2, 3, 4, 6, and 24 hours after protein injection and plasma assayed for CPG2 activity.

Plasma clearance was assumed to follow a single exponential model. The variables of the model were determined for each preparation by best fit to the experimental data using Newton’s method in the Microsoft Excel “solver” optimization tool.

Tissue Distribution. Groups of four mice bearing either LS174T or SW1222 xenografts were injected with fusion protein (1,000 units/kg i.v.). Plasma, tumor, liver, kidney, lung, and spleen for CPG2 activity were collected at 2, 4, and 6 hours after fusion protein administration in the LS174T xenografts and at 6 hours only for the SW1222 xenografts. In addition, effect of dose escalation of MFE-CP on biodistribution was studied in LS174T xenografted nude mice within the dose range of 400 to 2400 units/kg.
Microscopic Localization of Fusion Protein

Radioluminography (Phosphor Image Analysis). To investigate the distribution of MFE-CP within the tumor mass, four nude mice bearing LS174T xenografts were injected with 125I-labeled MFE-CP (0.3 mL, 68.5 MBq/mg, i.v.). Tumor, liver, and kidney were collected at 4 hours after injection, fixed in 10% neutral formalin for 24 hours, and paraffin processed for routine histology. Sections (5 μm) were cut at 100-μm intervals through the tumor tissue in order to examine the extent of penetration. After dewaxing, sections were exposed to a phosphor storage plate for 21 days. The plate was scanned using a Storm 860 phosphorimaging reader (Molecular Dynamics, Kent, United Kingdom), and the digitized images of radio-labeled fusion protein distribution were analyzed using Image-Quant for Windows NT software. Sections exposed to the phosphor plates were subsequently stained by H&E and scanned using a desktop scanner, in order to relate morphology to fusion protein distribution.

Immunohistochemistry. We subsequently used immunohistochemistry to investigate whether the CPG2 enzyme was retained within viable areas of the tumor at the time of prodrug administration. Four nude mice bearing LS174T xenografts were injected with MFE-CP (1,000 units/kg i.v.). At 6 hours after injection the mice were sacrificed and tumor, kidney, and liver samples were snap frozen in isopentane (cooled in liquid N2). Cryostat sections (5 μm) were fixed in acetone (10 minutes) and washed in PBS. Normal goat serum (3% in PBS) was then applied for 20 minutes before incubating sections for 40 minutes with rabbit polyclonal antibody directed against CPG2 (diluted 1/160 in PBS). After washing, biotinylated goat anti-rabbit antibody and an avidin-biotin complex were applied sequentially (Vectastain Kit, Vector Laboratories). Visualization of fusion protein retention was achieved using diaminobenidine tetrahydrochloride at 1 mg/mL. After counterstaining in haematoxylin, dehydrating and mounting, sections were examined using light microscopy (Axioskop 2, Zeiss Ltd., Hertfordshire, United Kingdom) and images were taken using an Axiocam digital camera (Zeiss). Primary antibody omission control (tumor section) and normal tissues (liver and kidney) removed from the same animals were done to confirm the specificity of the reaction for CPG2.

Immunofluorescence Staining. Mice bearing LS174T tumors were injected with MFE-CP fusion protein (1,000 units/kg, i.v.). At 1 hour after injection, mice were sacrificed and tumors removed, snap frozen in isopentane (cooled over liquid nitrogen), sectioned at 10 μm and stored at −80°C. Frozen sections were fixed in acetone for 10 minutes and incubated at room temperature for 1 hour with a biotinylated anti-CEA mouse monoclonal antibody (20 μg/mL). These were then incubated with 20 μg/mL of a goat anti-biotin FITC-conjugated antibody (Vector Laboratories) and with 50 μg/mL of an anti-CPG2 antibody labeled with Alexa Fluor 546 (Invitrogen), according to the manufacturer's instructions. Sections were mounted in a mountant medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories) and viewed under a fluorescence microscope using UV, FITC, and rhodamine filters.

Therapy Studies. Mice bearing either the LS174T or SW1222 colorectal tumor were given ADEPT when the tumors reached 0.1 to 0.2 cm³ and were in exponential growth.

Single-Cycle ADEPT. MFE-CP fusion protein was given i.v. (1,000 units/kg) followed by the prodrug (70 mg/kg, i.p.) at 6, 7, and 8 hours. Control groups included fusion protein alone, prodrug alone, and no treatment.

Multicycle ADEPT. The ADEPT cycle was given five times in the first week and then twice per week for 2 weeks in the LS174T xenografts. For the SW1222, the ADEPT cycle was given four times in the first week followed by twice per week for 3 weeks. Control groups included prodrug alone, fusion protein alone, one-cycle ADEPT, and no treatment.

Tumors were measured on the day of fusion protein injection and subsequently on 3rd or 4th day until tumor volume reached 1.5 cm³ and the mice were culled. The measurements were carried out in three dimensions (length, width, and height) and the tumor volume estimated as length × width × height/2.

Toxicity Studies. Mice were weighed before fusion protein injection and then twice weekly for signs of toxicity.

Statistical Analysis. The natural logarithm of tumor size at each time point was used to calculate the slope for each mouse based on a least-square fit on Microsoft Excel 5.0 (19). This allowed comparison of tumor growth for groups of mice given different treatments. Comparison between groups was carried out using the unpaired t test assuming equal variances.

RESULTS

Characterization of Purified MFE-CP

The yield of MFE-CP after immobilized metal affinity chromatography purification was in the range of 65 to 110 mg/L of cell-free fermentation broth. Catalytic activity of the purified material was estimated as 42 to 66 units/mg as measured by MFE-CP hydrolysis of methotrexate. SDS-PAGE analysis and Coomassie stain revealed a major protein band in the purified MFE-CP at ~70 kDa. This is illustrated in Fig. 2B, strip 1, and is indicated with an arrow. This band corresponds to the expected molecular weight of MFE-CP monomer (69.6 kDa) in denaturing conditions, where the naturally occurring dimeric form of CPG2 becomes monomeric. A diffuse band above the main band was also stained with Coomassie blue. This is consistent with the higher molecular weight heterogeneously glycosylated species obtained with P. pastoris expression as we have previously described with our studies using tandem mass spectrometry (13). A similar staining profile was also seen when the bands were probed with the mannose-binding lectin concanavalin A (Fig. 2B, strip 3), giving further indication of glycosylation. The protein bands stained with Coomassie were identified as His-tagged MFE-CP by specific probing with anti-his antibody (Fig. 2B, strip 4). Lower molecular weight species were also observed with Coomassie stain (Fig. 2B, strip 1), these may have been breakdown products as they were also reactive with the anti-his antibody used for Western blotting (Fig. 2B, strip 4) and seemed to be glycosylated when probed with lectin (Fig. 2B, strip 3). However, the lower molecular weight species did not increase with storage because a similar Coomassie staining profile was seen after 12 months at −80°C (Fig. 2B,
In vivo units/g (±SD, 0.2), whereas the corresponding plasma value from liver and other tissues was rapid. clearance for this fusion protein. However, subsequent clearance CP2 activity at this time, indicating that this was the route of after administration (Table 1). Liver showed highest level of mice showed high levels of CP2 activity retention at 2 hours (Fig. 3). MFE-CP cleared from plasma with a 
conjugate were determined in LS174T xenografted nude mice shows that 125I-MFE-CP in the viable tumor remained intact 
autoradioluminography. Results are illustrated in Fig. 2 
tumor at 4 hours was separated by SDS-PAGE and visualized by 
125I-MFE-CP prior to injection (Fig. 2 
NOTE. Results are expressed as mean ± SD of four mice per time point (cut-off for plasma, 0.0002 units/mL; tissues, 0.002 units/g).

Table 1 Biodistribution of CP2 activity in LS174T xenografted female nude mice given MFE-CP (1,000 units/kg, i.v.) at 2, 4, and 6 hours after injection

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Plasma</th>
<th>Liver</th>
<th>Kidney</th>
<th>Lung</th>
<th>Spleen</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.15 ± 0.26</td>
<td>3.15 ± 0.23</td>
<td>0.75 ± 0.13</td>
<td>0.97 ± 0.21</td>
<td>0.97 ± 0.1</td>
<td>1.52 ± 0.76</td>
</tr>
<tr>
<td>4</td>
<td>0.0022 ± 0.0002</td>
<td>0.006 ± 0.003</td>
<td>0.007 ± 0.002</td>
<td>0.013 ± 0.0004</td>
<td>0.017 ± 0.004</td>
<td>1.34 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>0.0011 ± 0.0004</td>
<td>&lt;0.002</td>
<td>0.004 ± 0.002</td>
<td>0.004 ± 0.0025</td>
<td>0.005 ± 0.0001</td>
<td>1.55 ± 0.2</td>
</tr>
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</table>

Based on these data, an interval time of 6 hours was used between the fusion protein and prodrug injections in the therapy studies.

Dose escalation of fusion protein (400-2,400 mg/kg) in the LS174T xenograft model showed increased levels of CP2 activity in tumors without retention in normal tissues (Table 2). In addition, enzyme levels of approximately 1.2 units/g were maintained in the tumor for up to 8 hours after MFE-CP injection (Table 3), and adequate levels of enzyme for a therapeutic level of prodrug conversion remained for at least 24 hours.

Microscopic Localization of Fusion Protein 
Radioluminography. Prior to injection, 125I-MFE-CP showed 78% binding to an immobilized CEA column (Activated CH Sepharose 4B affinity column, Pharmacia Biotech).

Figure 4 shows typical fusion protein distribution in tumor and normal tissues, for one of the four mice examined. The fusion protein (seen as black dots) had penetrated throughout the tumor by 4 hours, but there was selective retention within the viable regions, as indicated by haematoxylin and eosin staining. Eosin is a general cytoplasmic stain, whereas haematoxylin stains nuclei. Viable regions of tumor stain purple and necrosis stains pink, allowing the discrimination of these two regions.

The radiolabeled fusion protein had, however, cleared from liver and kidney by 4 hours after injection (Fig. 4).
The effect of dose escalation of i.v. given MFE-CP fusion protein on the biodistribution of CPG2 activity in LS174T xenografted female nude mice at 6 hours after injection

<table>
<thead>
<tr>
<th>Dose (units/kg)</th>
<th>Plasma</th>
<th>Tumor</th>
<th>Liver</th>
<th>Kidney</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
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<tr>
<td>400</td>
<td>nd</td>
<td>0.28 ± 0.14</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>600</td>
<td>0.00087 ± 0.0003</td>
<td>0.31 ± 0.038</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>800</td>
<td>0.00073 ± 0.0001</td>
<td>0.95 ± 0.28</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>1,200</td>
<td>0.0017 ± 0.0005</td>
<td>1.6 ± 0.88</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>0.0075 ± 0.006</td>
</tr>
<tr>
<td>2,400</td>
<td>0.032 ± 0.04</td>
<td>2.54 ± 0.66</td>
<td>0.009 ± 0.006</td>
<td>&lt;0.002</td>
<td>0.173 ± 0.13</td>
<td>0.0046 ± 0.00001</td>
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NOTE. Results are expressed as mean ± SD of four mice per group.

Table 2

Retention of CPG2 activity over time in plasma and tumors of LS174T xenografted nude mice given MFE-CP injection (1,000 units/kg i.v.)

<table>
<thead>
<tr>
<th>Time after MFE-CP administration (h)</th>
<th>Tumor</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Tumor</td>
<td>1.24 ± 0.009</td>
<td>1.2 ± 0.0007</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.0011 ± 0.00005</td>
<td>0.00082 ± 0.00009</td>
</tr>
</tbody>
</table>

NOTE. Results are expressed as mean ± SD for four mice per time point for tumors.

Table 3

Abbreviation: nd, not detected or that the observed value was below the limit of quantification of the analytical method (plasma, 0.0002 units/mL; tissue, 0.002 units/g).

**Immunochemistry**. The selective localization of CPG2 to tumors was also confirmed by immunohistochemistry at 6 hours after injection (Fig. 4B). In agreement with the radioluminographs the enzyme had been targeted to, and was retained in, viable regions of the tumor and around blood vessels as the antibody binds to the nearest antigen following extravasation. Negative control for tumor with primary antibody omission showed no reaction. No retention of enzyme was observed in kidney or liver tissue (Fig. 4B).

**Immunofluorescence Staining**. The fluorescence images (Fig. 4C) show colocalization of CEA and the i.v. injected fusion protein MFE-CP (1,000 units/kg) in the LS174T xenografted nude mice. Tumor cells surrounding the lumen of a glandular acini show expression of CEA (Fig. 4C, I), localization of MFE-CP (Fig. 4C, II) and registered image showing colocalization of CEA and the MFE-CP fusion protein (Fig. 4C, III).

**Therapy Studies**. The effect of various treatment regimes on mean tumor growth over time, for mice bearing either the LS174T or the SW1222 xenograft, is shown in Fig. 5.

**Single-Cycle ADEPT**. Administration of a single ADEPT cycle to mice bearing the LS174T xenograft resulted in ~9 days growth delay (P ≤ 0.00004) compared with untreated controls (Fig. 5A). In the case of the SW1222 xenograft, the growth delay was extended (P = 0.0004) to ~14 days (Fig. 5B). In both cases, there was no statistical difference between the untreated mice and any of the other control groups.

**Multicycle ADEPT**. Giving multiple cycles of ADEPT significantly enhanced the therapeutic effect of single-cycle ADEPT in both xenograft models (P = 0.001 in LS174T and P = 0.0001 in SW1222). A tumor growth delay of ~21 days was observed in the LS174T model (Fig. 5A). However, in the SW1222 xenografts, significant tumor regressions were achieved (Fig. 5B). In both cases, there was no statistical difference between the untreated mice and any of the other control groups.

**Toxicity**. The toxicity produced by the various therapeutic regimens for LS174T and SW1222-bearing mice, as reflected in changes in weight over time, is shown in Fig. 5C and D.

The mean values from six mice per group is expressed as a percentage of their pretreatment value and shown until their original value was reached or the group had been culled. In the control no treatment group, the weights continued to rise throughout the experiment. In the control groups receiving either fusion protein or the prodrg alone, the weights remained constant. In the groups receiving a single ADEPT cycle the weight loss was <2% for either xenograft model, or this was quickly regained with no other signs of significant toxicity. For groups receiving multiple ADEPT cycles, there was again no increase in toxicity for either xenograft model. Less than 2% weight loss was observed, indicating that there was potential for giving increased numbers or higher doses of the prodrg in the clinic.

Although there was no weight cell count done in the current study, we have previously shown good correlation between the effect of antibody-targeted therapy on systemic toxicity (by blood cell counts) and on mouse weight (20).

**DISCUSSION**

ADEPT has the potential to enhance the efficacy of chemotherapeutic drugs without dose-limiting normal tissue toxicity. This potential can only be realized by selective delivery of enzyme to tumors.

This has been achieved by an engineered recombinant MFE-CP fusion protein with desired characteristics for stability, purification, and clearance. This fusion protein has been expressed in the yeast expression system, P. pastoris, which has several advantages in that the large yields of correctly folded and stable proteins can be adapted to scaled-up production in...
large biomass fermentors. In addition, proteins expressed in this system tend to be glycosylated (21), which may contribute to protein stability (22) and rapid clearance.

Expressed in the yeast, *P. pastoris*, the fusion protein (MFE-CP) is glycosylated at 2 of 3 potential N-glycosylation sites with 5 to 13 mannose residues (13). We predicted that this fusion protein would have good tumor localization due to the high affinity of MFE for CEA and the rapid clearance characteristics of the three phase (2, 3) system due to clearance of MFE-CP through mannose receptors in the liver. This was
confirmed with *in vivo* studies where it was shown that the fusion protein is relatively stable in the tumor but is rapidly eliminated from plasma and other normal tissues, probably via the mannose receptors on endothelial and Kupffer cells in the liver (23). However, we currently have no experimental evidence to show fusion protein internalization after binding to either CEA or the mannose receptor, but *in vitro* models to test this are being developed.

Although several antibody-enzyme/prodrug systems have been developed by other groups (reviewed in ref 9), to our knowledge, MFE-CP is the first mannosylated fusion protein to be used for ADEPT.

Biodistribution studies of radiolabeled MFE-CP using autoradiography and phosphor image analysis as well as the immunofluorescence techniques have shown that, despite the high molecular weight of the fusion protein and the diffusion barriers observed for solid tumors (24, 25), MFE-CP is able to penetrate through tumor tissue and show preferential localization in the CEA positive viable regions, where it can be most beneficial. However, whereas our microscopic studies of MFE-CP distributions show that the fusion protein is in viable tissue, they cannot indicate whether the localized CPG2 is active. The catalytic activity of tissue-localized MFE-CP has therefore, been studied by *in vitro* turnover of methotrexate to its metabolite (2,4-diamino-N-10-methylpteroyl acid). This method measures the ability of enzyme to convert prodrug to drug in different tissues and gives indications of potential toxicity.

The human colon carcinoma xenograft models LS174T and SW1222 showed high levels of CPG2 activity in tumors with rapid clearance from blood, giving tumor to plasma ratios of 1,400:1 and 339:1, respectively, at 6 hours after MFE-CP administration. This difference in tumor to plasma ratio is caused by slightly lower plasma levels in LS174T (0.0011 versus 0.0038 units/mL for SW1222). In contrast, tumor to plasma ratios of 1.6:1 and 0.77:1, respectively, were observed at 6 hours for the *E. coli* fusion protein and the chemical conjugate, respectively. Higher tumor to plasma ratios with the chemical conjugate in our studies (26, 27) and by others (28–30) have been achieved by employing a clearance step in a three-phase system. Although effective, this was thought to be too complicated for further...
development. Also, chemical conjugation (31) resulted in heterogeneity of the final product, a problem which had not been eliminated despite application of methods such as reverse proteolysis (32) and site-specific conjugation (33). These studies and our clinical experience of ADEPT identified a need to develop an enzyme delivery molecule that would encompass the clearance characteristics of the three-phase system, but would be less complex and consist of a uniform product with predictable properties. This has been addressed by applying molecular and structural biology to inform design of the recombinant antibody–enzyme fusion protein, MFE-CP.

An advantage of the glycosylated fusion protein (MFE-CP) for ADEPT is that in spite of the rapid clearance of MFE-CP, high levels of CPG2 activity were retained in the tumor, resulting in high (up to 1,400:1) tumor to plasma ratios within 6 hours. Although high tumor-to-normal tissue ratios have been reported for other fusion proteins, ratios of 100:1 have only been achieved either after 24 hours (34) or after 7 days (35).

Optimal tumor localization with MFE-CP was observed within 6 hours, but measurable enzyme activity in the tumors was retained for >24 hours. An increase in dose of MFE-CP over a 6-fold dose range resulted in an increase in enzyme activity in tumors, without retention of activity in normal tissues. In preliminary experiments, both the LS174T and the SW1222 xenografts showed similar optimal levels of enzyme activity in tumors at the time of prodrug administration (i.e., at 6-8 hours after fusion protein injection). This was favorable, because not only was the CPG2 activity optimal at this time, but it was also localized within CEA positive and viable regions of the tumor (Fig. 4). This is advantageous in our system because the drug would be generated within these viable regions for maximum therapeutic effect.

In the subsequent therapy experiments, therefore, the prodrug was injected at 6, 7, and 8 hours after fusion protein administration, using mice bearing either LS174T or SW1222 xenografts. A single cycle of ADEPT resulted in a reproducible tumor growth delay for both models.

In vitro studies (36) have shown that the mechanism of cell death induced by the current ADEPT system seems to be due to the generated drug inducing DNA lesions resulting in apoptosis. Damage to the tumor from ADEPT in vivo, as assessed by chromosomal aberration and single cell gel electrophoresis assay, in the present study (preliminary data not shown) as well as our previous studies (37), produced cross-links in DNA, but significant repair occurred within 24 hours. We therefore investigated the effect of multiple ADEPT cycles and found a significant enhancement of the growth delay produced by a single cycle in the LS174T tumors. Complete regression of tumors was obtained in the SW1222 model, without toxicity. It is possible that the multicycle therapy attacked previously untreated tumor cells and also increased the DNA damage of cells already damaged by earlier cycles. Although the mice bearing SW1222 xenografts received an extra ADEPT cycle compared with LS174T, it is unlikely that this completely explains the increased response to treatment. This model has been shown to be more responsive to radiotherapy in vivo (38) than LS174T. It also showed greater growth delay following a single-cycle ADEPT (present study). The reasons for this intrinsically greater sensitivity, however, are not clear.

Because we have used a bacterial enzyme to avoid endogenous activation of prodrug, this system poses a limitation by virtue of being immunogenic in man. With the chemical conjugate, immune responses to both antibody and enzyme developed after only one treatment (39), although use of the immunosuppressive agent cyclosporine A (40) in patients allowed three cycles to be given, at weekly intervals, before development of an immune response. However, recent work suggests that immune suppression may not be an advantage in this system (41), and that ADEPT may synergize with the immune system to give an enhanced therapeutic effect. One of the advantages of our current system would be that due to rapid clearance of MFE-CP from plasma, repeat cycles of ADEPT are feasible in patients, and three or four cycles may be given within 10 days before an immune response develops. Although our earlier clinical studies with radiolabeled MFE-23 alone showed no evidence of HAMA in patients receiving a single dose of this antibody (42), it is possible that repeat administrations of MFE-CP may result in an immune response to the fusion protein. However, the immunogenicity of the antibody component of the fusion protein poses less of a problem as it may be overcome by humanization. The immunogenicity of CPG2 may also be modified. By applying molecular modelling and phage technology, we have identified a major B-cell immunodominant epitope on the COOH terminus of CPG2 (43). However, this epitope seems to be masked by the His-tag on the MFE-CP fusion protein and this effect on immunogenicity of CPG2 is currently being investigated.

The ADEPT approach can be applied to any tumor type using an appropriate targeting antibody and the same matching pair of enzyme and prodrug (44–46). Alternatively, new enzyme/prodrug molecules may also be applied (reviewed in ref 9).

Our studies show that with one course of multicycle ADEPT, sustained regressions can be achieved in the xenograft systems. Complete eradication of tumors may be possible with repeated courses of treatment if an alternative administration route to the limitations of mouse tail vein injection is employed. However, repeated courses of ADEPT cycles may be given in clinical studies as the fusion protein can be infused.

The therapeutic effect of ADEPT may be enhanced by using prodrug/drug molecules which cause DNA lesions that are not repaired, or by combination therapy. In our previous studies, the combination of ADEPT with 5,6-dimethylxanthenone-4-acetic acid, an antivascular agent which targets established tumor blood vessels, significantly increased the period of tumor growth delay (47). However, the importance of dose, timing, and sequence of agents being used in combinations, as well as potential toxicity, need further study.

In summary, we have produced a glycosylated fusion protein which seems to have the favorable pharmacokinetics required for successful ADEPT. The primary aim of an ADEPT approach is to generate cytotoxic drug selectively within tumors and minimize damage to normal tissues. We have shown that, with MFE-CP fusion protein, enzyme activity rapidly clears from blood and other normal tissues but is selectively retained within tumors. MFE-CP in combination with an alkylating agent (ZD2767P prodrug) produced antitumor effects in two colorectal
carcinoma xenografts without apparent toxicity. These studies have informed the design for a proposed clinical trial with repeated ADEPT cycles in patients with CEA-positive tumors.

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Sustained Tumor Regression of Human Colorectal Cancer Xenografts Using a Multifunctional Mannosylated Fusion Protein in Antibody-Directed Enzyme Prodrug Therapy

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