

*Advances in Brief***Antibody-directed Enzyme Prodrug Therapy: Efficacy and Mechanism of Action in Colorectal Carcinoma¹**

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Abstract

In antibody-directed enzyme prodrug therapy, an enzyme conjugated to an antitumor antibody is given i.v. and localizes in the tumor. A prodrug is then given, which is converted to a cytotoxic drug selectively in the tumor. Ten patients with colorectal carcinoma expressing carcinoembryonic antigen received antibody-directed enzyme prodrug therapy with A5B7 F(ab')₂ antibody to carcinoembryonic antigen conjugated to carboxypeptidase G2 (CPG2). A galactosylated antibody directed against the active site of CPG2 (SB43-gal) was given to clear and inactivate circulating enzyme. A benzoic acid mustard-glutamate prodrug was given when plasma enzyme levels had fallen to a predetermined safe level, and this was converted by CPG2 in the tumor into a cytotoxic form. Enzyme levels derived from quantitative gamma camera imaging and from direct measurements in plasma and tumor biopsies showed that the median tumor:plasma ratio of enzyme exceeded 10000:1 at the time of prodrug administration. Enzyme concentrations in the tumor (median, 0.47 units g⁻¹) were sufficient to generate cytotoxic levels of active drug. The concentration of prodrug needed for optimal conversion (K_m) of 3 μM was achieved. Prodrug conversion to drug was shown by finding detectable levels of drug in plasma. There was evidence of tumor response; one patient had a partial response, and six

patients had stable disease for a median of 4 months after previous tumor progression (one of these six had a tumor marker response). Manageable neutropenia and thrombocytopenia occurred. Conditions for effective antitumor therapy were met, and there was evidence of tumor response in colorectal cancer.

Introduction

Systemic cancer therapy is limited by a lack of tumor selectivity and by drug resistance; ADEPT³ is designed to overcome both problems (1). An antibody directed against a tumor-associated antigen is linked to an enzyme and given i.v., resulting in selective accumulation of the enzyme in the tumor. When the discrimination between tumor and normal tissue enzyme levels is sufficient, a prodrug is given i.v., which is converted to an active cytotoxic drug by the enzyme within the tumor. Selectivity is achieved by the tumor specificity of the antibody and by delaying prodrug administration until there is a large differential between tumor and normal tissue enzyme levels. Drug resistance can be overcome by generating high levels of an alkylating agent in the tumor, and this is achieved through the capacity of each enzyme molecule to convert many molecules of prodrug into drug. ADEPT has shown antitumor activity in animal tumor models of human choriocarcinoma and colonic and breast carcinoma (2–4).

A convincing demonstration that such a complex system can be developed for clinical use requires evidence that each of the components of ADEPT functions by the mechanisms proposed. This can be provided by measuring antibody-enzyme conjugate concentration, enzyme activity, and prodrug and drug levels in tumor and normal tissues. We describe a clinical trial in which these parameters were measured along with conventional measurements of toxicity, efficacy, and immunogenicity.

Materials and Methods

Materials. Affinity-purified mouse monoclonal A5B7 IgG1 antibody (5) to CEA (Celltech PLC, Slough, Berks, United Kingdom) was digested to F(ab')₂ with cysteine-activated bromelain (6). Immunoreactivity was assessed by indirect ELISA against CEA. CPG2, a folate-depleting enzyme of bacterial origin, was produced by the Division of Biotechnology, Public Health Laboratory Service (Porton Down, United Kingdom; Ref. 7). CPG2 was covalently linked to the F(ab')₂ fragment of A5B7 by a stable thio-ether bond (8, 9) to make the antibody

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³ The abbreviations used are: ADEPT, antibody-directed enzyme prodrug therapy; CEA, carcinoembryonic antigen; CPG2, carboxypeptidase G2; A5CP, A5B7 F(ab')₂ antibody to CEA conjugated to CPG2; CMDA, benzoic acid mustard-glutamate prodrug; HAMA, human antimouse antibody; SPECT, single photon emission computerized tomographic; HPLC, high-pressure liquid chromatography; CTC, Common Toxicity Criteria; CT, computerized tomogram.

Table 1 Treatment schedule

Day 0	10,000 enzyme units m^{-2} (125 mg m^{-2}) of A5B7 F(ab') ₂ antibody-CPG2 conjugate in 500 ml of 0.9% saline over 2 h, 10 mg of conjugate labeled with 370 MBq of ¹³¹ I were given as an i.v. bolus at the end of conjugate infusion
Day +1	Infusion of SB43-gal antibody to CPG2, 7.5 mg m^{-2} in 500 ml of 0.9% saline over 6 h, 15 mg m^{-2} in 500 ml of 0.9% saline over 18 h, continued to day +4 at 8 mg m^{-2} in 1 liter of 0.9% saline over 24 h
Day +2	CMDA prodrug 200 mg m^{-2} infused i.v. over 2 min in fast running 1.26% NaHCO ₃ (given if CPG2 enzyme levels measured in blood were <0.2 enzyme units ml ⁻¹)
Day +3 and +4	Repeat day +2

Table 2 Patient demography and pretreatment status

Patient	Sex	Age (yr)	Performance status	Primary tumor	Prior surgery	Previous treatment	Assessable disease	Serum CEA
1	M	38	0	Colon	Hemicolectomy Ileostomy	Ctx: 5FU/fa ^a	Stoma site Liver Inguinal node	20
2	M	42	0	Colon	Hemicolectomy Hemi-hepatectomy	Ctx: 5FU/fa	Liver (CT portography)	10
3	F	63	0	Colon	Hemicolectomy	XRT: liver Ctx: 5FU/fa	Liver	581
4	M	57	0	Rectum	Anterior resection	Ctx: 5FU/fa, MMC	Lung	<3
5	M	31	0	Colon	Sigmoid colectomy	XRT: pelvis Ctx: 5FU/fa, 5FU/CDDP IFN- α	Omental mass Malignant ascites	<3
6	F	46	0	Appendix	Appendectomy Hemicolectomy	Ctx: 5FU/fa, 5FU/CDDP	Liver Ovary	<3
7	F	42	1	Sigmoid colon	Sigmoid colectomy Small bowel resection Hysterectomy Ureteric stent	Ctx: 5FU/fa	Para-aortic nodes	19
8	F	46	1	Colon	Hemicolectomy Oophorectomy	Ctx: 5FU/fa, 5FU/CDDP	Para-aortic nodes Lung Pelvic mass	<3
9	M	47	0	Colon	Low anterior resection	Ctx: 5FU/fa	Lung Liver	155
10	M	75	1	Rectum	Anterior resection	Ctx: 5FU/fa, oral 5FU/fa	Liver	419

^a Ctx, chemotherapy; XRT, radiotherapy; 5FU, 5fluorouracil; fa, folinic acid; MMC, mitomycin C; CDDP, cisplatin.

^b Reference ranges, CEA < 10 $\mu g l^{-1}$.

enzyme conjugate A5CP. Ten mg of the antibody-enzyme conjugate were radiolabeled with 10 mCi of ¹³¹I by the *N*-bromosuccinimide/L-tyrosine technique (10).

SB43 is an IgG1 mouse monoclonal antibody against CPG2 (11). Purified antibody samples were galactosylated (SB43-gal) according to a modification of the method described by Mattes (12, 13). CMDA, a synthetic benzoic acid mustard prodrug, undergoes cleavage of its terminal glutamic acid residue by CPG2 to generate the active alkylating agent 4-[(2-chloroethyl)[2(mesyloxy)ethyl]-amino] benzoic acid (14). Quality and safety of the product were determined using the Cancer Research Campaign Operation Manual (15).

Freeze-dried CMDA was reconstituted in 1.5 ml of DMSO, a solution that is stable in B-D syringes for >12 h. The CMDA/DMSO solution was injected in free running 1.26% sodium bicarbonate. The treatment schedule is given in Table 1.

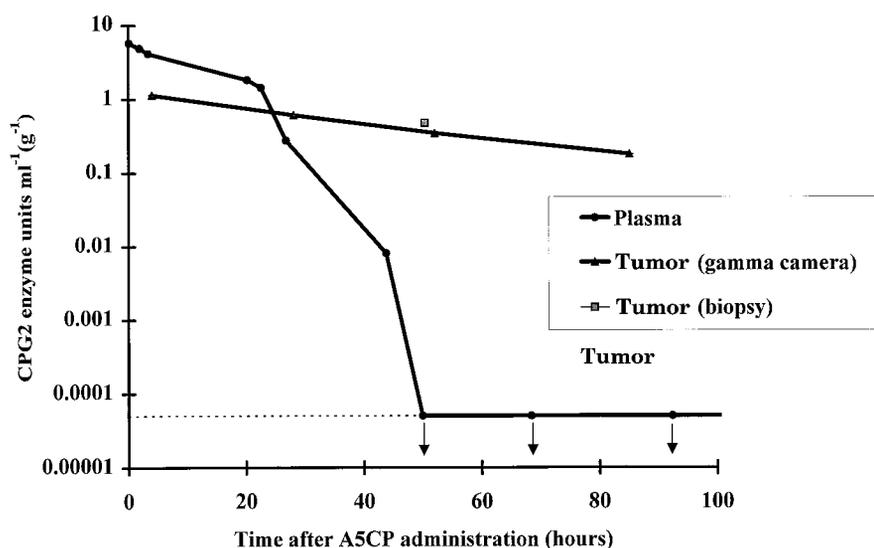
Methods. The catalytic activity of native and conjugated CPG2 was measured prospectively during the trial by a spectrophotometric methotrexate reduction assay (16, 17). Indirect assessment of A5CP concentrations were made by gamma counting of plasma samples (18).

HAMA and anti-CPG2 antibody (HACPG2A) response were measured by ELISA (19).

Patients had torso SPECT gamma camera imaging performed at intervals of up to 72 h after radiolabeled conjugate injection on an IGE Gemini 700 camera. Enzyme in tumor and normal tissue was estimated by measuring radioactivity as a percentage of injected radioactivity. SPECT images were reconstructed using General Electric filtered back projection software and corrected for decay, attenuation, and Compton scatter. Estimates of radioactivity per unit mass were then made using region of interest analysis as described previously (18).

Prodrug and active drug concentrations in plasma were determined by HPLC, and their presence was confirmed by liquid chromatography mass spectroscopy (20, 21) and CPG2 in plasma and tumor biopsies by HPLC (21, 22). Fractional clearance estimates were made assuming an exponential clearance and a single-compartment model. An exponential curve fit using the least sum of squares method was used. Fractional half-life of clearance was calculated using the equation $t_{1/2} = \ln 2/K$ where K is slope of \ln plasma concentration versus time. These results are descriptive only as the concentration of the drug at the time

Fig. 1 Enzyme levels in plasma and tumor after the infusion of antibody-enzyme conjugate. Plasma levels were measured by methotrexate reduction spectrophotometric assay (●) and HPLC (↓). The arrow indicates that the level was below the limits of detection of this assay (---). Levels in tumor (▲) were estimated from SPECT gamma camera imaging. Each point is the median of 10 patients. The range includes 60% of data points. The enzyme level in tumor obtained from tumor biopsy and measured by HPLC (■) at 50.3 h is the median of five patients, and the range includes 60% of data points.



of administration (C_0) would theoretically be zero, and drug is being continually released from the prodrug by tumor localized antibody-enzyme conjugate. The pharmacokinetic details of the prodrug/drug system generated in this trial are reported in detail elsewhere (21). Area under the concentration *versus* time curve (AUC) to infinity was calculated by using the trapezoidal rule and by adding a tail by integrating the exponential curve-fit from the last measured time point to infinity.

Standard WHO criteria for response were used. Standard National Cancer Institute CTC (23) were used to evaluate toxicity. Survival times were calculated from the start of treatment. Duration of response was calculated from the onset of response to the date of disease progression.

Patients. The trial was performed with local ethical committee, Department of Health, United Kingdom, and Administration of Radioactive Substances Committee approval and according to Good Clinical Practice under the auspices of the Cancer Research Campaign Phase I/II Committee's Targeting Trials Group, by whose trials office the clinical data were monitored. All patients gave written informed consent. Eligibility criteria were unresectable or metastatic, histologically proven colorectal carcinoma; no antitumor treatment in the previous 4 weeks; measurable disease by plain X-ray, CT, or ultrasound scan; age >20 years; life expectancy >4 months; Eastern Cooperative Oncology Group performance status of 0–2; and normal hematological, renal, and cardiac indices unless abnormal due to tumor. Pretreatment serum CEA levels were required to be raised but <1000 $\mu\text{g l}^{-1}$; if they were not raised, then CEA had to be demonstrated immunohistochemically on tumor biopsy (24).

All patients had negative HAMA titers and negative reactions to intradermally administered A5CP and SB43-gal. All patients had previously been treated with 5-fluorouracil-based chemotherapy regimens and had either relapsed or shown no response. All patients had a triple lumen Hickman catheter inserted under direct radiographic screening into a subclavian vein. Cyclosporin A was given starting 2 days before A5CP

administration, initially as a continuous i.v. infusion at 5 mg kg^{-1} day for 7 days and then p.o. at 15 mg kg^{-1} /day in divided doses for 7 additional days. Dose adjustments were made to keep blood levels in the range 150–350 Ng ml^{-1} . Ondansetron and s.c. cyclizine were used as required. All patients were given a thyroid blockade against ^{131}I using potassium iodide.

Results

Study Population. Ten patients (six male and four female) with a median age of 46 years were enrolled into the study. All received one course of ADEPT, with two patients who had a negative HAMA and HACPG2A after cycle 1 receiving a second treatment. The pretreatment status of the patients is summarized in Table 2. Concentrations of the components of ADEPT were measured in tumor and normal tissues to determine whether conditions for effective therapy were met. The results were related to toxicity and of the components of ADEPT were measured in tumor and normal tissues to determine efficacy assessments.

Antibody and Enzyme in Plasma. A5CP cleared from the plasma as shown in Fig. 1. Indirect measurements of enzyme activity in plasma by HPLC and spectrophotometric assay correlated with the estimates of enzyme made from measurements of radiolabeled A5CP until clearing antibody was given. Thereafter, active enzyme measurements gave lower values, consistent with enzyme inactivation by SB43-gal in addition to clearance of complexed A5CP. The concentration of enzyme was less than the limits of detection (5×10^{-5} units of enzyme ml^{-1} of plasma) 48 h after A5CP administration.

Enzyme Levels in Tumor and Normal Tissues. Quantitative SPECT gamma camera imaging showed ^{131}I -labeled A5CP localization in the tumor (Fig. 2, a and b), reaching median peak levels of 6.8% of the injected radioactivity kg^{-1} 4 h after administration. Initially, blood and other normal tissue levels of radioactivity exceeded those in the tumor, but this trend was reversed after SB43-gal administration (Fig. 3).

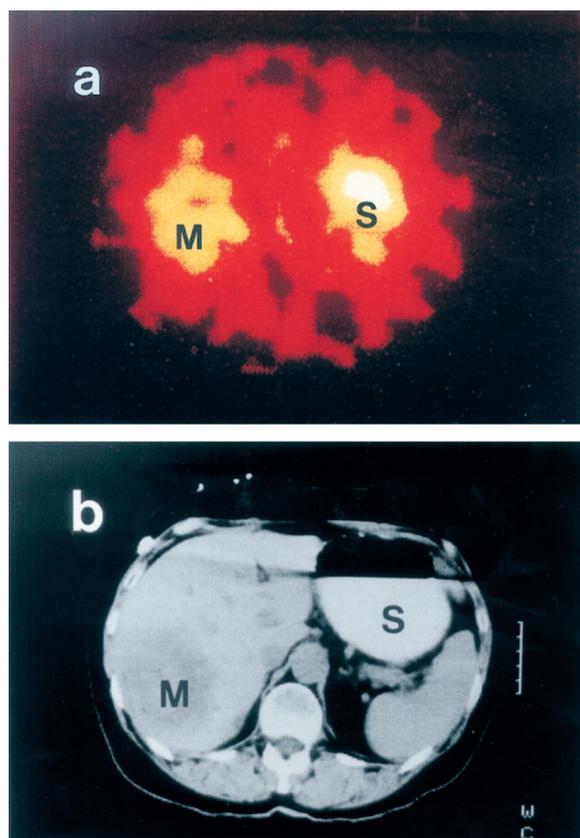


Fig. 2 A5CP localization in liver metastasis. *a*, SPECT image in patient three 52 h after A5CP administration, showing the localization of the conjugate within a liver metastasis (*M*). Free ^{131}I is also seen in the stomach (*S*). *b*, corresponding CT scan through the liver showing the large posterolateral metastasis (*M*). *S*, stomach.

Enzyme activity was measured indirectly in tumor biopsies taken immediately before the start of prodrug administration from five patients (two liver metastases, one peritoneal metastasis, and one ovarian metastasis biopsied under ultrasound control and one metastasis of a stomal recurrence). Median enzyme concentration was 0.47 (range, 0.32–0.62) enzyme units g^{-1} , which correlated well with the concentration of 0.34 (range, 0.19–0.63) calculated from the amount of radioactivity measured by gamma camera imaging (Fig. 1). This showed that the enzyme in the tumor was inaccessible to inactivation by SB43-gal and that gamma camera estimates of enzyme concentration in tumor were valid.

Normal liver was obtained in biopsies from two patients, and no enzyme activity was detectable in them by HPLC. However, there was retention of some radioactivity, suggesting that enzyme activity was lost in the residual radiolabeled material.

Selectivity. Tumor:plasma ratios of enzyme were $>10,000:1$ (based on undetectable levels in the blood) at the time when prodrug administration was started. The validation of gamma camera estimates of enzyme concentration given by the biopsy studies supports the view that effective enzyme levels persist in the tumor for at least up to 85 h. Tumor:liver ratios in

the two patients biopsied also exceeded 10,000:1 (based on concentrations below the limits of detection in normal liver).

Prodrug and Drug Levels in Plasma. Prodrug cleared from plasma with a biological half-life of 16 min (range, 5–27 min; Fig. 4*a*). Drug was detected in all cases within 3 min of the end of prodrug administration; the half-life recorded for drug was longer than for the prodrug at 46 min (range, 7–85 min; Fig. 4*b*). There was variation in the concentrations of prodrug and drug between patients and between days in the same patient. Calculated area under the curve to infinity correlated linearly for prodrug and drug for a given administration and patient. This showed that the prodrug was activated by the enzyme, and the available data on enzyme distribution suggest that this was principally occurring in the tumor with diffusion of drug into the circulation.

Toxicity. Neutropenia and thrombocytopenia occurred with median times to nadir of 35 and 25 days, respectively. Four patients reached CTC grade 3 or 4 toxicity for both parameters and required platelet transfusion, whereas five patients had grade 1 or 2 toxicity. One patient had a febrile neutropenic episode requiring hospitalization and antibiotic therapy. Nine patients had nausea or vomiting (CTC grade 3 or 4 in severity in four of these patients). This was attributed to the combination of DMSO and cyclosporin administered. Transient rises in urea and creatinine were seen in four patients (all grade 1 or 2 toxicity); again, these were probably attributable to cyclosporin A.

Immune Response. HAMA and human anti-CPG2 antibody were found in all patients after 2 weeks, preventing further therapy.

Responses. There was evidence of antitumor activity. One patient had a partial response lasting 4 months, six patients had stable disease after previous tumor progression with a median progression-free survival of 4 months (range, 2–16 months), and one of these patients had a decline in serum CA19/9 levels sustained for 4 months. Three patients had progressive disease. The tumor response in the patient with a partial response is shown in Fig. 5, *a–d*.

Discussion

The conditions needed for effective ADEPT were achieved in patients, and tumor response was achieved after only one treatment. Tumor localization of the enzyme at a median of 0.5 units g^{-1} (Fig. 1) was in the range that produced an antitumor effect in animal models (3). The very favorable tumor:plasma and tumor:liver ratios of enzyme recorded ($>10,000:1$) make it unlikely that there is a significant amount of enzyme remaining in normal tissues at the time of prodrug administration.

The levels of radioactivity in the tumor gave a reasonably accurate indication of the enzyme level in the tumor at the time of prodrug administration, as judged by comparison of enzyme measurements in biopsies and SPECT gamma camera data. Although there was only approximately 1.5% and 1% of injected radioactivity kg^{-1} in the tumor after 52 and 85 h, respectively, this represented a satisfactory level of active enzyme for prodrug activation, as indicated above. Blood and other normal tissue levels of radioactivity remained close to those in the tumor until 85 h after administration. However, enzyme activity

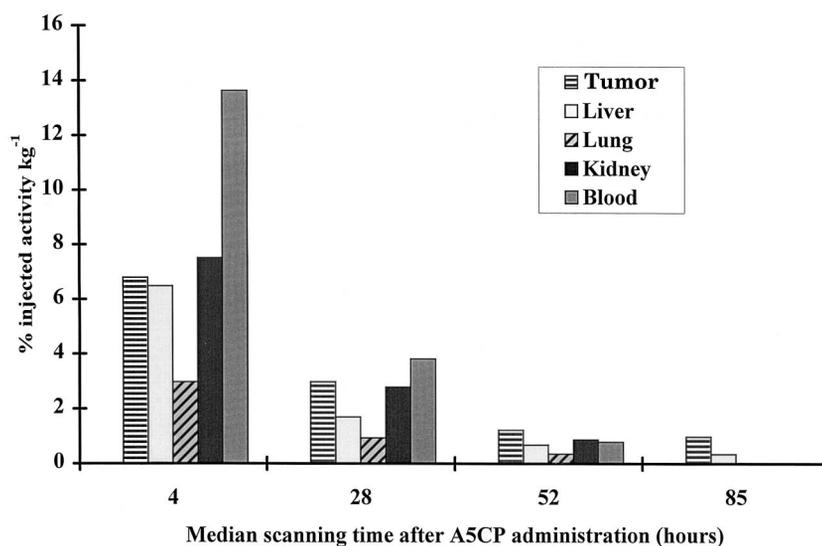


Fig. 3 Distribution of radiolabeled A5CP in tumor and normal tissue over time as judged by SPECT imaging. Each point is the median of 10 patients. The range includes 60% of data points.

measurements in plasma and liver biopsies after the second antibody administration showed no measurable enzyme activity. This was consistent with inactivation of enzyme activity in normal tissues by the anti-enzyme antibody SB43-gal, whereas inactivation did not occur in the tumor. Thus, the levels of radioactivity in blood and normal tissues overestimated enzyme activity after the administration of SB43-gal.

The drug present in plasma was probably generated in the tumor, and it is interesting that the two patients with the highest plasma drug levels had large burdens of tumor. Treating smaller tumors may be advantageous because the potential for release of the drug into the circulation would be reduced in proportion to the targeted tumor volume, whereas the drug concentration in the tumor would be similar.

The characteristics of the CPG2 enzyme in prodrug activation are known (25) and indicate a K_m for prodrug conversion of 3 μM . This optimal level of prodrug concentration was maintained for approximately 2 h in the plasma (Fig. 4a). Also the IC_{50} of the drug is 200 μM (25), and this value was never exceeded in the blood in any of the study patients (Fig. 4b). Despite this, drug in the circulation appeared to cause myelosuppression. Given that no active enzyme was found in plasma by HPLC assay at any of the time points when prodrug was given, it is likely that the presence of drug in the circulation was the result of “leakback” from tumor. It is also probable that higher concentrations of drug were present in the tumor than were measured in the blood, and it is possible that a change in prodrug regimen (for instance, to an infusion over 3 or more days) would prevent plasma drug concentration from rising to a toxic level. The possibility that the prodrug itself caused myelosuppression was excluded in a previous study in which the prodrug was given alone (26).

The prodrug CMDA is converted to the cytotoxic parent drug 4-[(2-chloroethyl)[2-(mesyloxy)ethyl]amino]benzoic acid (14), which was found to be most effective *in vivo* in ADEPT experimental models, leading to complete regressions in transplanted human tumor xenografts that were resistant to all con-

ventional cytotoxic agents (2). Relevant antibody-enzyme conjugates (2000 units/kg, i.v. injection) were administered to animals with transplanted choriocarcinoma xenografts, followed 72, 94, and 99 h later by CMDA (400 mg/kg, i.v. injection). Control groups of animals received saline alone, CMDA alone, or irrelevant antibody-enzyme conjugate followed by CMDA at the same doses and time points as the test groups. All control animals were dead by day 110, whereas 9 of 12 of the ADEPT test animals were still tumor free at day 300 ($P < 0.01$).

It is not known how the relatively small amount of drug leads to bone marrow suppression 25–35 days after injection. However, we have noticed that after CMDA incubation with cells in culture, the cells do not die immediately but appear to be primed for death and die later.⁴

We appreciated that the CMDA-derived drug might have a long half-life from the outset, but CMDA was a practical prodrug for synthesis and testing of the principles of ADEPT. The relatively long half-life of the drug found in plasma is consistent with it causing the dose-limiting myelosuppression. ADEPT with the same antibody and enzyme but with a drug with a shorter half-life has been shown to be an effective therapy for colorectal cancer in an animal model and is being developed for clinical use (27, 28).

This study shows how measurement of the parameters required for the function of ADEPT gives insight into its substantial ability for selective delivery of cancer therapy. The tumor responses demonstrate that generation of an alkylating agent at the tumor site can overcome the drug resistance usually seen with this class of drug in colorectal cancer (29). Although it was not possible to measure drug directly in the tumor because of the small size of the biopsies, the plasma drug levels and the absence of enzyme in normal tissues suggest that this was the result of generation of high concentrations of drug in the tumor.

⁴ Unpublished observations.

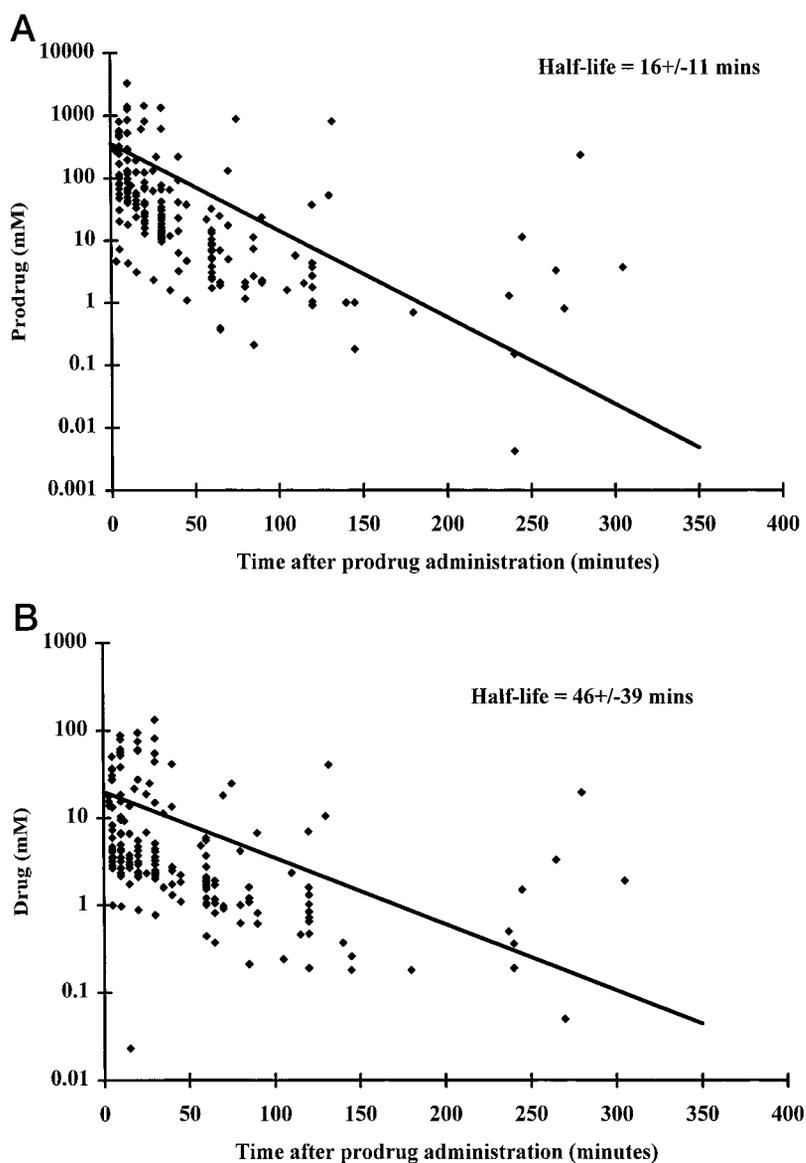


Fig. 4 Prodrug and drug pharmacokinetics. Plasma concentration of the prodrug (CMDA; *a*) and drug (*b*) after bolus administration of CMDA. Each point indicates a separate determination. The *thick line* reflects a modeled "best-fit" exponential clearance, and the half-life was determined from this.

Conventional Phase I clinical trials in which tumor response and toxicity are studied in dose escalation studies are unlikely to be adequate for investigating such a complex system. For instance, a poor antitumor effect at the maximum tolerated dose of the prodrug could be caused by inadequate levels of enzyme in the tumor, excess enzyme in normal tissues, failure to continue administering prodrug while favorable tumor and normal tissue enzyme levels persist, an inappropriate prodrug regimen, or primary drug resistance. Little could be done about the latter, but knowledge of the first four components could lead to redesign of a clinical protocol to overcome the problems.

The immunogenicity of CPG2 is not surprising, considering its bacterial origin. It has the important advantage over most mammalian enzymes that it has no human equivalent that could activate the prodrug endogenously. Administration of cyclosporin A delays the production of a human antibody response to

A5B7 antibody (30). With ADEPT, production of human antibodies directed against CPG2 and A5B7 antibody limits therapy to two or three doses, but this does not appear to prevent useful antitumor activity, as shown here and in a previous study (26). The proteins used in ADEPT studies were expected to be immunogenic in patients. In a previous clinical trial of ADEPT (26) with SB43gal and CMDA, 11 patients received antibody-enzyme conjugate without any immunosuppressive agent. All patients had detectable HAMA and anti-CPG2 antibodies in serum within 10 days after a single treatment with antibody-enzyme conjugate (19). Six patients received cyclosporin A 48 h before the ADEPT regimen (31). Two patients, who had very large hepatic metastases, received cyclosporin A p.o. and developed fatal hepato-renal failure after the first cycle of therapy. Subsequent patients receiving cyclosporin A by continuous i.v. infusion showed temporary increases in creatinine and urea

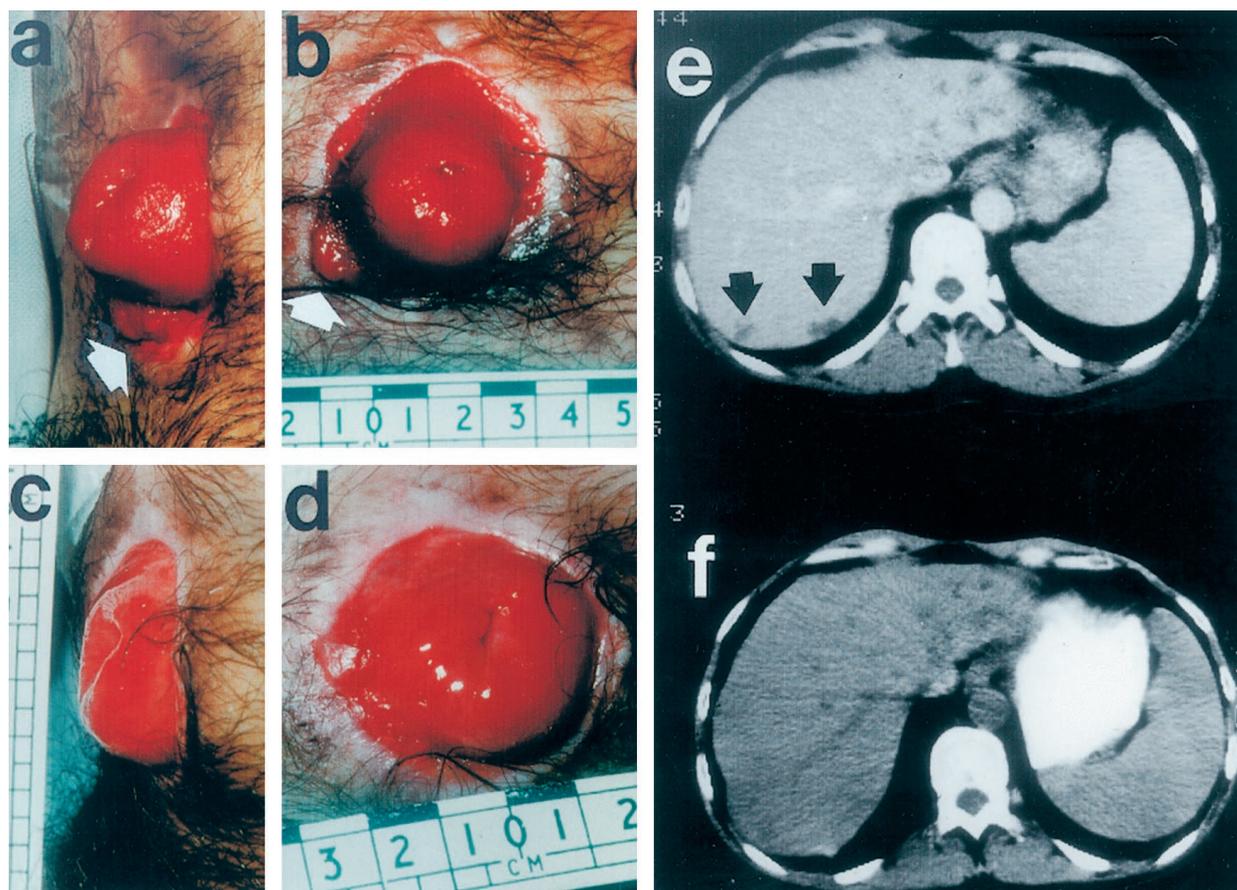


Fig. 5 Response to treatment in patient 1. Stomal metastasis of rectal cancer (*a* and *b*) before and (*c* and *d*) 56 days after ADEPT therapy; a substantial reduction in the volume of tumor is seen. CT (*e*) showing hepatic metastases of rectal cancer (*arrows*) and resolution (*f*) 56 days after ADEPT.

levels. Two patients had antibody responses after a second cycle of therapy. Two patients had no detectable antibody responses until cyclosporin A was discontinued and were able to receive three cycles of ADEPT during a 21-day period.

Other immunosuppressive or tolerizing agents may also be considered for their potential to delay the antibody response (32, 33). Humanization of the antibody may reduce immunogenicity, and it is possible that less immunogenic enzymes can be identified.

The data presented here support the proposed mechanism of action of ADEPT and justify additional studies to develop the system for treatment for colorectal and other cancers.

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