ZD2767, an Improved System for Antibody-directed Enzyme Prodrug Therapy That Results in Tumor Regressions in Colorectal Tumor Xenografts


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ABSTRACT

ZD2767 represents an improved version of antibody-directed enzyme prodrug therapy. It consists of a conjugate of the F(ab')2, ASB7 antibody fragment and carboxypeptidase G2 (CPG2) and a prodrug, 4-[N,N-bis(2-iodoethyl)amino]phenoxycarbonyl L-glutamic acid. The IC50 of the prodrug against LoVo colorectal tumor cells was 47 µM, and cleavage by CPG2 released the potent bis-iodo phenol mustard drug (IC50 = 0.34 µM). The drug killed both proliferating and quiescent LoVo cells. Administration of the ZD2767 conjugate to nude mice bearing LoVo colorectal xenografts resulted in approximately 1% of injected ZD2767 conjugate localizing of tumor after 72 h, and blood and normal tissue levels of the conjugate were 1.5–10-fold lower. A single round of therapy involving the administration of the prodrug 72 h after the conjugate to athymic mice bearing established LoVo xenografts resulted in approximately 50% of the tumors undergoing complete regressions, tumor growth delays greater than 30 days, and little toxicity (as judged by body-weight loss). Similar studies using a control antibody-CPG2 conjugate that does not bind to LoVo tumor cells resulted in a growth delay of less than 5 days, confirming the tumor specificity of this approach. These studies demonstrate the potential of ZD2767 for the treatment of colorectal cancer.

INTRODUCTION

ADEPT3 is a two-step antibody-targeting strategy for the treatment of cancer (1–4). ADEPT results in the generation of a low-molecular-weight cytotoxic drug locally at the tumor site and outside the cell. The drug should be capable of diffusion to reach tumor cells not targeted directly by the conjugate due to either antigenic heterogeneity (5) or because the high-molecular-weight conjugate fails to diffuse away from the tumor vasculature and reach tumor cells distant from the blood supply (6). ADEPT can thus overcome problems of both antigenic heterogeneity and tumor access that have limited other forms of antibody-drug- and antibody-toxin-targeted therapy.

A number of bacterial and mammalian enzymes have been evaluated as potential candidates for ADEPT, and these include β-lactamases (7, 8), glucuronidase (9), and alkaline phosphatase (10). We have focused on developing ADEPT systems that incorporate the bacterial enzyme CPG2. This enzyme has no known mammalian equivalent and catalyzes the hydrolytic cleavage of reduced and nonreduced folates to pteroylglutamic acid (11). In a series of studies, we have shown that the combination of benzoic acid mustard-glutamate prodrugs and a conjugate of CPG2 linked to either the anti-β-hCG antibody, W14 (12, 13), or the anti-CEA antibody, ASB7 (14–16), results in significant antitumor activity in a number of tumor models. Clinical trials are ongoing with one of these benzoic acid mustard prodrugs in combination with ZD2767C in patients with advanced, drug-resistant, colorectal cancer, and encouraging responses have been reported (17). Use of ADEPT systems incorporating ZD2767C could be extended to a range of other major solid tumors because CEA is expressed in a large percentage of stomach, non-small cell lung, and pancreatic tumors and in a proportion of breast, ovarian, and small cell lung tumors (18).

These ADEPT systems that incorporate benzoic acid mustard prodrugs suffer from two potential drawbacks: (a) the drugs generated are not very potent (14, 19), and large doses of both conjugate and prodrug are required for antitumor activity; and (b) the drugs released have relatively long chemical (19) and biological (20) half-lives that potentially permit escape of the drug from the tumor site into the periphery, resulting in enhanced nonspecific toxicity. We have recently reported two prodrugs that are cleaved to release the 4-[N,N-bis(2-chloroethyl)amino]phenol drug (21). This drug is more potent and has a shorter chemical and biological half-life than the benzoic acid mustard drugs. These prodrugs, in combination with ZD2767C, produced improved antitumor activity compared with the benzoic acid mustard prodrugs (21).

Here we report the in vitro and in vivo antitumor activity of a new prodrug, ZD2767P, which is cleaved by CPG2 to release a very potent and highly reactive mustard drug (22). When compared to the original benzoic acid mustard (14) or the 4-[N,N-bis(2-chloroethyl)amino]-phenol prodrugs (21) described previously, ZD2767P, when used in combination with ZD2767C, results in improved antitumor activity in a colorectal tumor xenograft model.

MATERIALS AND METHODS

Materials

The prodrug ZD2767P was synthesized as described previously (22). The structures of ZD2767P and the corresponding 4-[N,N-bis(2-iodoethyl)amino]-phenol drug (ZD2767D) are shown in Fig. 1.

CPG2 from Pseudomonas sp. strain RS16 was cloned into Escherichia coli (23) and was produced as described previously (11). ASB7 antibody (IgG1), which reacts with CEA, was kindly supplied by the Department of Medical Oncology, CRC Laboratories, Chartering Cross Hospital (24). MOPC-21 (IgG1) control antibody, which has no known tissue reactivity, was generated from the hybridoma cell line P3X63Ag8 acquired from the European Collection of Animal Cell Cultures (ECACC No. 85011401). The F(ab')2 fragments of ASB7 and MOPC were prepared by papain digestion. For ASB7 and MOPC, an enzyme:antibody ratio of 1:70 for 24 h at 37°C and 1:20 for 4 h at 37°C was used, respectively. The F(ab')2 fragments were conjugated to CPG2 as described previously (25). Both conjugates had specific activities of 150–200 units CPG2/mg protein and consisted predominantly of one F(ab')2 fragment of ASB7 conjugated to one CPG2 molecule. The molecular weight of these conjugates is thus approximately 180,000.
DFMO INHIBITS NMBA-INDUCED ESOPHAGEAL CARCINOGENESIS

Fig. 1. Structure of ZD2767P and ZD2767D.

The LoVo colorectal tumor cell line was obtained from the European Collection of Animal Cell Cultures (ECACC No. 87060101).

Methods

Cytotoxicity Studies. The colorectal tumor cell line LoVo (CEA-positive and AS87-reactive) was incubated with prodrg, prodrg + CPG2 (1.0 unit/well) or drug, in 96-well microtiter plates (2,500 cells/well) for 1 min or 1 h. The cells were then washed and incubated for an additional 3 days at 37°C. Trichloroacetic acid was then added, and the amount of cellular protein adhering to the plates was assessed by the addition of SRB dye, according to Skehan et al. (26). Potency of the compounds is expressed as the concentration required to inhibit cell growth by 50% (IC50).

Quiescent Cell Studies. LoVo colorectal tumor cells were induced into quiescence by depletion of methionine from the medium [medium prepared using RPMI select amino kit (Life Technologies, Inc.) and leaving out the methionine component]. Cells were monitored for quiescence by a combination of FACScan analysis and measurement of the incorporation of [3H]thymidine, [3H]uridine, and [3H]leucine (Amersham) over a 4-h period. Six days after transferring LoVo cells to the methionine-depleted medium, quiescence was achieved, as judged by a shift in the FACScan cell-cycle profile from S phase to G phase and by the [3H]thymidine, [3H]uridine, and [3H]leucine levels having fallen to background levels (<5% of proliferating control cultures). After the induction of quiescence, LoVo cells remained viable in methionine-depleted medium for up to 7 days, as judged by <10% of cells staining with trypan blue, and cell levels remaining constant from SRB assay measurements (see above).

The cytotoxicity of the drug in quiescent and proliferating LoVo cells was measured as described above. Cells were maintained in quiescence throughout the assay by exposing drug and by washing drug away using the methionine-depleted medium. 6-Thioguanine (Sigma Chemical Co.) was used as a control in these studies and was exposed continuously (3 days) during the cytotoxicity assay with proliferating and quiescent cells.

Tumor Localization Studies. Athymic nude mice [nu/nu; Alpk (outbred)] were injected s.c. with 1 x 107 LoVo tumor cells. After 5–7 days, when the tumors were 8–10 mm in diameter and weighed 0.3–0.5 g, 5 mg/kg (1000 units/kg CPG2 activity) of ZD2767C or F(ab')2MOPC-CPG2 conjugate was injected i.v. Groups of 3 mice were killed 24, 48, and 72 h later, and the tumor and a sample of blood and other tissues (liver, lungs, and kidney) were removed, weighed, and frozen before determining the amount of CPG2 activity they contained, using a HPLC-based assay.

Tissues were homogenized in PBS [170 mm NaCl, 3.4 mm KCl, 12 mm Na2HPO4, and 1.8 mm KH2PO4 (pH 7.2)], using a motor-driven homogenizer and 5-mm probe (Kinematica AG). Samples of tissue homogenate or plasma (0.6-ml reaction volume) were incubated with 0.1 mm methotrexate for 5 min at 30°C. The reaction was stopped by diluting the sample 2.5-fold in ice-cold methanol + 0.1% trifluoroacetic acid. Samples were centrifuged (8,800 g) for 4 min, and 60-μl aliquots of the supernatants were analyzed by HPLC to detect production of methotrexate metabolite, 4-[(2,4-diamino-6-pteridinyl methyl) amino] benzoic acid (Aldrich Chemical Co.). Standard curves were produced by incubating known concentrations of CPG2 (0.002–0.01 unit/ml) in control tissue homogenates or plasma for 5 min with 0.1 mm methotrexate. An ISS200 automated HPLC system (Perkin Elmer Cetus) was used, and separation of methotrexate and its metabolite was achieved with a Spherisorb S5 SCX cation exchange column (5 μm particle size, 4.6 mm x 10 cm; Hitrom), using a mobile phase of 70% methanol, 30% ammonium formate, and 0.1% trifluoroacetic acid; a flow rate of 1 ml/min; and a detection wavelength of 300 nm.

The amount of CPG2 activity in the tumor, blood, and normal tissues was calculated from the amount of metabolite produced and the standard curves. Results were expressed as the percentage of injected enzyme activity present/g of tissue at the various time points.

Antitumor Studies. Groups of 7–10 female athymic nude mice were injected s.c. with 1 x 107 LoVo tumor cells. When the tumors were 4–5 mm in diameter, ZD2767C (500–2000 units CPG2 enzyme activity/kg) or PBS was injected i.v. Seventy-two h later, prodrg was injected i.p. (three doses at 1-h intervals). The length of the tumors in two directions was then measured three times a week, and the tumor volume was calculated using the formula:

\[ \text{Volume} = \frac{\pi D^3}{6} \]

in which \( D \) = larger diameter and \( d \) = smaller diameter of the tumor.

Tumor volume was expressed relative to the tumor volume at the time of initiation of the prodrg arm of the therapy. At this stage, tumors measured 7–8 mm in diameter and had a calculated weight (assuming a density of 1.0) of approximately 0.2–0.3 g. The antitumor activity was compared to control groups given PBS instead of either ZD2767C or ZD2767P. Other groups of tumor-bearing mice received F(ab')2MOPC-CPG2 conjugate followed by ZD2767P, or they were given ZD2767P or ZD2767D alone at the same time as ZD2767P was administered in the combination arm of the study. ZD2767P and ZD2767D were both prepared in ice-cold PBS to minimize breakdown of the compounds before administration to the mice. As a positive control, some mice received 30 mg/kg of 5-fluorouracil (Sigma Chemical Co.) daily for 15 days, starting at the same time as prodrg was administered in the ZD2767 studies. Tumor growth delays were calculated by determining the time it took treated tumors to increase their volume by 4-fold and subtracting from this time it took control (PBS)-treated tumors to increase their tumor volume 4-fold.

Toxicity was monitored throughout the studies by measuring body weight and by monitoring the condition of the animals. Statistical significance of the antitumor effects was judged using the ANOVA (one way) test (27).

RESULTS

In Vitro Cytotoxicity of ZD2767 Prodrug and Drug. The cytotoxic potency of ZD2767P and ZD2767D released by CPG2 was investigated on human LoVo colorectal tumor cells. The drug (ZD2767D) had an IC50 versus LoVo cells of 0.34 ± 0.11 μM

Fig. 2. In vitro cytotoxicity of ZD2767P and ZD2767D in LoVo colorectal tumor cells. LoVo cells were incubated for 1 h with ZD2767D (A) or ZD2767P either alone (B) or with 1 unit CPG2 (C). Cytotoxicity was assessed using the SRB assay after an additional 3 days. Points, means of triplicate determinations.
crease in A! in Zn—IDFMO+ rats compared with Zn—IDFMO animals cannot be accounted for by food restriction in the former group, bearing in mind that both DFMO-untreated zinc-sufficient and -deficient animals had similar Al.

Zinc, an essential trace element, is known to play an important role in programmed cell death that occurs in embryos and in vivo in normal tissues including small intestine, thymus, prostate, and testis (revised in Refs. 44 and 45). Yet, the role of dietary zinc deficiency and the molecular mechanism(s) whereby it influences apoptosis, especially under conditions leading to tumor formation, are still unknown.

The role of polyamines in apoptosis is complex. Several studies have shown that high levels of polyamines or polyamine analogues can induce apoptosis and that this phenomenon may be related to the generation of reactive oxygen species (46—51). Similarly, increased polyamine synthesis may mediate myc-induced apoptosis in murine myeloid cells deprived of interleukin 3 (52). In contrast, spermine protects against apoptosis, and a reduction in cellular polyamines is associated with glucocorticoid-induced apoptosis in thymocytes (53, 54). Polyamines also prevent apoptosis in cultured neurons (55) and in mouse T cells exposed to herbimycin A (56). Apoptosis of tumor cells has been observed in response to inhibitors of polyamine synthesis other than DFMO (57, 58). Thus, it appears that the effect of polyamines on apoptosis may be biphasic, with an increased cell death rate occurring both at high and at low polyamine levels. Our results provide strong evidence that apoptosis in zinc-deficient esophageal cells is enhanced by low levels of polyamines brought about by daily food intake, resulting in a marked decrease in body weight, greater in zinc-sufficient (27—32%) than in -deficient animals (9—18%). In this regard, calorie restriction is known to lead to a decline in cell proliferation or LI in mouse esophagus, among other tissues (41, 42). Our previous results (20) also showed that zinc-sufficient rats, pair-fed to deficient animals (and therefore —40% calorie restricted relative to ad libitum zinc-sufficient rats) exhibited reduced LI in the esophagus (20). On the contrary, rats fed a zinc-deficient diet ad libitum displayed sustained increased cell proliferation in the esophagus (Refs. 20, 25, and 31 and the present experiment). On the basis of these findings and the fact that the weight loss caused by DFMO in deficient rats was not statistically significant, we conclude that the antiproliferation effect of DFMO in zinc-deficient esophagus is specific. However, it cannot be ruled out that calorie restriction, brought about by a reduced food intake in Zn+IDFMO+ rats, does not contribute in part to the decreased LI observed in the esophagi of these animals. Follow-up studies with lower DFMO doses will be in order.

The effect of food restriction per se on the rate of apoptosis in the rat esophagus has not been reported. Other investigators (43) have demonstrated that a 40% dietary restriction in mice induced a relative increase in the rate of apoptosis in hepatocytes compared to ad libitum fed mice. In the present study, animals in all four experimental groups were fed ad libitum. Relative to their respective DFMO-untreated counterparts, Zn—/DFMO+ animals, with a 9% decrease in body weight, had a higher rate of apoptosis in the esophagus than Zn+/DFMO+ rats, with a 27% decrease. Therefore, the substantial in Fig. 2. In contrast, the prodrug (ZD2767P) had an IC50 of 47 ± 31 μM (mean ± SD for 14 determinations). Thus, attachment of the glutamate residue to ZD2767D via a carbamate linkage decreased its cytotoxic potency by over 100-fold. If CPG2 enzyme was added to ZD2767D during the 1-h incubation period, its cytotoxicity increased to match that of ZD2767D (IC50 = 0.32 ± 0.28 μM, mean ± SD for three determinations; Fig. 2), thus confirming that CPG2 can catalyze the release of active drug from the prodrug in vitro and can result in enhanced cell-killing.

ZD2767D can kill LoVo tumor cells after a very short exposure. When ZD2767D was incubated with LoVo tumor cells for 1 min, and then residual drug was washed away, an IC50 value of 1.57 ± 0.41 μM (mean ± SD for four determinations) was obtained.

To investigate whether ZD2767D could kill both proliferating and quiescent LoVo tumor cells, quiescent cultures of LoVo tumor cells were prepared. This was achieved by depleting the cell culture medium of methionine. After 6 days in methionine-depleted medium, the LoVo tumor cells were judged quiescent based on FACS analysis showing a shift of the cells from S phase into G phase and because incorporation of radioactive precursors of DNA (³H]thymidine), RNA (³H]uridine), and protein (³H]leucine) had all fallen to < 5% of equivalent proliferating LoVo tumor cells grown in complete medium. ZD2767D was then exposed for 1 h to either the proliferating or quiescent tumor cells. After the 1-h exposure, the proliferating cells were cultured for an additional 3 days in complete medium, and the quiescent cells were cultured for 3 days in methionine-depleted medium to maintain quiescence (confirmed in control cultures). The cytotoxic effects of the drug in the quiescent cells could be determined because the SRB assay is a dye-binding assay measuring cell protein and does not rely on the incorporation of radioactive precursors of DNA, RNA, or protein. ZD2767D had an IC50 against the quiescent LoVo tumor cells of 1.9 ± 1.2 μM (mean ± SD for four determinations), and thus the quiescent cells were only 5—6-fold less sensitive to ZD2767D compared to the proliferating LoVo tumor cells (IC50 = 0.34 μM). As a control in these studies, the S-phase-specific cytotoxic drug 6-thioguanine was used. The quiescent cells were not killed with this drug at concentrations up to 100 μM, whereas the proliferating cells had an IC50 of 0.8 μM.

Localization of ZD2767C to LoVo Tumor Xenografts. Before initiating antitumor studies with ZD2767, the ability of ZD2767C to localize to LoVo tumor xenografts was investigated. LoVo tumor xenografts were established by implantation of 10⁶ tumor cells s.c into athymic nude mice. After 5—7 days, the tumors weighed approximately 0.3—0.5 g. Immunohistology with A587 on frozen cryostat sections of the xenografts showed that approximately 60% of the tumor cells were expressing CEA, as judged by reactivity with A587 (results not shown). The amount of ZD2767C that localized to these tumor xenografts and remained in blood and normal tissues at various time intervals after conjugate administration was determined by measuring active enzyme levels. This was achieved by monitoring the conversion of methotrexate, a known substrate for CPG2 (11), in tissue homogenates and blood, using a HPLC-based assay (see "Materials and Methods"). The results are shown in Fig. 3.

Approximately 4% of the injected dose of ZD2767C was present/g of tumor 24 h after injection of the conjugate. However, at this time point, a similar level of enzyme activity was present in the bloodstream. By 48 h, the level in the tumor had fallen by approximately 50% but exceeded the level in the blood by 3-fold. After 72 h, the level in the tumor had fallen only slightly from the amount present at 48 h and exceeded the amount/g of blood by 10-fold and the amount/g of normal tissues by 20—50-fold. Based on this data, a time interval of 72 h between ZD2767C and ZD2767P administration was used in the antitumor studies.

Similar localization studies with a control F(ab')₂ MOPC-CPG2 conjugate resulted in less than 0.2% of the injected dose of conjugate localizing/g of tumor at 24, 48, and 72 h. At the 72-h time point, only 0.13% injected dose of the F(ab')₂ MOPC-CPG2 conjugate was present/g of tumor (11-fold lower than the ZD2767C conjugate) and the tumor: blood ratio at 72 h was 0.4. Thus, the control F(ab')₂ MOPC-CPG2 conjugate did not localize specifically to the LoVo tumors.

Antitumor Activity of ZD2767. Administration of ZD2767P (3 x 70 mg/kg given as three i.p. injections hourly over a 2-h period) 72 h after administration of ZD2767C (2.5 mg/kg, 500 units CPG2/kg) resulted in tumor regressions and a prolonged significant (P < 0.05) growth delay (Fig. 4). The mean growth delay from five separate studies using this dosing regime was 35 days, and approximately 50% of the tumors (22 of 39) in these five studies regressed completely, as judged by the lack of macroscopic tumor remaining at the site of implantation. With time, the majority of these tumors reappeared. This dose of ZD2767C in combination with ZD2767P only resulted in a transient body-weight loss of 6—7% (maximal loss, day 2—3). By day 7, the mice had generally regained this body-weight loss.

Fig. 3. Localization of ZD2767C to LoVo tumor xenografts. Athymic nude mice bearing LoVo tumors (0.3—0.5 g) were injected with ZD2767C. At 24, 48, and 72 h, blood (B), tumor (C), liver (D), kidney (E), and lungs (F) were removed, and the enzyme activity present in them was determined using a HPLC assay as described in "Materials and Methods." Results were expressed as percentage of the injected dose of enzyme activity/g of tissue and are the values obtained from five tissues pooled at each time point before enzyme measurement.
In four separate antitumor studies, ZD2767P alone or ZD2767C alone has no antitumor activity in this xenograft model (Fig. 4). Similarly, ZD2767D administered at a dose (3 × 2 mg/kg) that caused 10% body-weight loss also gave no significant antitumor activity (four separate studies). Administration of a control F(ab')2 MOPC-CPG2 conjugate (which does not localize specifically to the LoVo tumor xenografts) along with ZD2767P only resulted in a mean growth delay of 4 days (five separate studies; Fig. 4) and caused similar toxicity (7% body-weight loss). The residual enzyme level in the blood (0.05 unit CPG2/ml plasma) at 72 h was the same in both the ZD2767C and MOPC conjugate arms of the study.

The activity of ZD2767 was compared with the activity of 5-fluorouracil given as 15 daily doses of 30 mg/kg i.p. (maximum tolerated dose for 5-fluorouracil using this dosing schedule) in the LoVo tumor xenograft model. ZD2767 gave major tumor regressions and a prolonged growth delay. In contrast, 5-fluorouracil did not give any tumor regressions and only gave a small, but significant (P < 0.05), growth delay of 6–7 days (Fig. 5).

The effect of dose of ZD2767C on the antitumor activity in the LoVo tumor xenograft model was explored. The ZD2767C dose administered to athymic mice bearing LoVo tumors was varied over a 20-fold dose range (0.5–10 mg/kg = 100–2000 units CPG2/kg), and ZD2767P was administered 72 h later. The dose of prodrug administered was varied so that similar levels of toxicity, as judged by body-weight loss, were seen at each conjugate dose. The mean results of three separate experiments are shown in Table 1. Significant (P < 0.05) tumor growth delays were seen at all conjugate doses.

**DISCUSSION**

The major finding to emerge from these studies is that we have identified an improved ADEPT system consisting of a prodrug, ZD2767P, and a conjugate, ZD2767C, that results in complete tumor regressions and prolonged growth delays in a colorectal tumor xenograft model.

The drug (ZD2767D) released from ZD2767P by CPG2 is at least 300-fold more potent than the original benzoic acid mustard drugs used in the initial CPG2-based ADEPT systems (12, 14, 19). Consequently, less drug needs to be converted at the tumor site by CPG2 to achieve cell kill. The k_{p} /k_{a} turnover number of ZD2767P for CPG2 is approximately 10-fold lower than that of the benzoic acid mustard prodrugs (22), but this is easily countered by the increased drug potency; consequently, significantly less conjugate is required at the tumor site to achieve antitumor activity. The lower enzyme-turnover of ZD2767P compared to the original benzoic acid mustard prodrugs may also lead to improved tumor selectivity, based on predictions from a computer model (28) and recent experimental results (21). The rationale for this is that a prodrug with a low turnover number optimizes use of the higher concentration of conjugate at the tumor site.

ZD2767D has a short chemical half-life of approximately 2 min in plasma. This half-life should be sufficient for diffusion locally within the tumor (29) but should minimize peripheral toxicity if the drug escapes from the tumor into the circulation. In contrast, the drug generated from the benzoic acid mustard prodrug currently in clinical trials has a chemical half-life of 58 min (19) and a biological half-life of approximately 20 min in mouse (20) and man (17), and thus, in this case, drug escaping from the tumor is likely to contribute to toxicity. ZD2767D kills the LoVo cells even with very short 1 min exposure, suggesting the lipophilic drug (clogP = 3.6) is rapidly taken up by the tumor cells. We have also demonstrated that ZD2767D can kill both proliferating and quiescent LoVo tumor cells *in vitro*, in accord with literature data indicating mustard alkylation agents are non-cell-cycle specific (30). We believe that this is an important property of the drug because in major solid tumors in man, a large proportion of the tumor cells are not actively dividing (31), and the number of rounds of therapy that can be used with ZD2767 will be limited by the immune response to the conjugate (17). In addition to their ability to kill quiescent cells, another important property of alkylation agents is that it is reported to be difficult to achieve high levels of drug resistance because they are not subject to multi-drug resistance (30).

The ability of ZD2767C to localize to LoVo tumor xenografts has been assessed in these studies by measuring the enzyme activity in tumor, blood, and normal tissues. Measurement of enzyme activity provides a functional measure of ability to turn over prodrug at the different tissue sites. The levels of enzyme/g of tumor and /g of blood increased linearly with ZD2767C dose over the dose ranges (100–2000 units CPG2 conjugate activity/kg) used in these studies (results not shown), indicating we had not reached antigen saturation even at the highest ZD2767C dose. The number of units of CPG2 conjugate enzymic activity localized/g of LoVo tumor is similar to that achieved in colorectal tumors in patients (32) given scaled doses (300–600 units CPG2 conjugate activity/kg) of the conjugate in initial clinical trials of ADEPT using ZD2767C. This indicates that the LoVo tumor xenograft mouse model is a realistic model of the clinical situation in terms of conjugate enzyme activity available for prodrug activation.

The optimal time for prodrug administration is a balance between retaining sufficient conjugate at the tumor site to activate sufficient prodrug for tumor-cell killing versus ensuring that the level of enzyme
in the blood and normal tissues does not cause excessive toxicity. From the antitumor results, we have been able to define a time interval (72 h) when this criterion is met. ZD2767 consistently produced tumor regressions and prolonged growth delays in this LoVo tumor xenograft model at dose levels of conjugate and prodruk that only resulted in modest toxicity, as judged by body-weight loss. In other instances, including the combination of the benzoic acid mustard prodruks and ZD2767C, the use of a clearing system has been used to improve antitumor activity (15, 16, 33). The clearing systems accelerate the blood clearance of the conjugate, enabling prodruk to be administered when tumor levels are higher. The major disadvantage of introducing a clearing system is that it adds an additional level of complexity to exploiting the ADEPT approach in the clinic, particularly from a commercial standpoint.

We have used only a single course of ADEPT therapy to achieve significant antitumor activity. In the majority of previous ADEPT studies involving nonalkylating agent drugs, multiple courses of ADEPT therapy have been used to achieve major antitumor effects. The ability of a single course of therapy to result in complete tumor regressions may, in part, be related to the ability of the drug component to kill both the proliferating and quiescent cell populations. Because only approximately 60% of the tumor cells expressed target antigen, the fact that half the tumors treated macroscopically disappeared indicates that the ADEPT approach killed both antigen-positive and antigen-negative tumor cells. Tumors that regrew had a similar level of staining with ASB7, as judged by immunohistochemistry (results not shown), indicating that there had not been any selective elimination of antigen-positive cells.

The results with the MOPC-CPG2 conjugate support the fact that the antitumor effects achieved by ZD2767 are due to activation of prodruk at the tumor site. Similar levels of enzyme activity were present in the blood stream at the time of prodruk administration with both the MOPC and ZD2767 conjugate, and thus the minimal antitumor effect seen with the MOPC conjugate demonstrates that little of the ZD2767 activity was due to prodruk activation in the blood stream. The lack of activity with the active drug given systemically confirms this view.

ZD2767 proved to be significantly better than 5-fluorouracil given as 15 daily doses at its maximum tolerated dose in this tumor model. 5-Fluorouracil and 5-fluorouracil/leucovorin combinations are the most widely used chemotherapy regimens in colorectal cancer and achieve a 15–30% response rate (34). The antitumor effects with ZD2767 are much larger than can be achieved with the original CMDA benzoic acid mustard prodruk in combination with the ZD2767 conjugate in either LoVo or LS174T colorectal tumor xenografts (14). In both these tumor models, using the same dosing schedule used for ZD2767, only 8–10-day growth delays were seen with CMDA prodruk doses, which caused similar toxicity to that seen in the therapy studies reported here. This is very encouraging because the CMDA prodruk, in combination with ZD2767C, has shown promising antitumor activity in patients with advanced, drug-resistant, colorectal cancer in a pilot clinical trial (17).

Antitumor activity with ZD2767 could be achieved with a 20-fold dose range of conjugate. The amount of conjugate localized to the LoVo xenografts was linear over this dose range (results not shown), which demonstrates that the absolute conjugate dose delivered to the tumor is not critical to achieve antitumor effects with ZD2767P. This is important because it is likely that the conjugate dose delivered to tumors in patients will vary due to a number of factors (such as antigen-expression levels and the extent of tumor vascularity).

Indeed, radiiodinated ASB7 (antibody component of ZD2767 conjugate) localization to primary colorectal tumors in patients has been found to vary over a 20-fold dose range (35), supporting the need for antibody-based therapeutics to work over a large range of localization levels.

In conclusion, these studies demonstrate that ZD2767 represents a potent ADEPT system for the treatment of CEA-positive tumors and is now in preclinical development for the treatment of colorectal cancer.

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ZD2767, an Improved System for Antibody-directed Enzyme Prodrug Therapy That Results in Tumor Regressions in Colorectal Tumor Xenografts

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