Three New Prodrugs for Suicide Gene Therapy Using Carboxypeptidase G2 Elicit Bystander Efficacy in Two Xenograft Models

Frank Friedlos, Lawrence Davies, Ian Scanlon, Lesley M. Ogilvie, Janet Martin, Stephen M. Stribbling, Robert A. Spooner, Ion Niculescu-Duvaz, Richard Marais, and Caroline J. Springer

ABSTRACT

Three new prodrugs, [prodrug 1: 4-[bis(2-iodoethyl)amino]-phenyloxy-carbonyl-l-glutamic acid; prodrug 2: 3-fluoro-4-[bis(2-chloroethyl)amino]-benzoyl-l-glutamic acid; and prodrug 3: 3,5-difluoro-4-[bis(2-iodoethyl)amino]benzoyl-l-glutamic acid] have been assessed for use with a mutant of carboxypeptidase G2 (CPG2, glutamate carboxypeptidase, EC 3.4.17.11) engineered to be tethered to the outer tumor cell surface (stCPG2(Q)3) as the activating enzyme in suicide gene therapy systems. All three of the prodrugs produce much greater cytotoxicity differentials between stCPG2(Q)3 and control β-galactosidase (β-gal) expressing breast carcinoma MDA MB 361 and colon carcinoma WiDr cells (70- to 450-fold) than was previously observed (19- to 27-fold) with 4-[2-chloroethyl]2-meslyoxyethyl)amino]benzoyl-l-glutamic acid (CMDA). Prodrug 1 is the most effective antimtumor agent in xenografts in mice inoculated with 100% stCPG2(Q)3-expressing MDA MB 361 cells, whereas prodrugs 2 and 3 are most effective when the percentage of stCPG2(Q)3-expressing cells is 50% or 10%. In nude mice bearing xenografts arising from inocula of 100% stCPG2(Q)3-expressing WiDr cells, prodrug 2 is the most effective antimtumor agent. All three of the prodrugs produced histological evidence of substantial bystander cell killing in WiDr xenografts in which only 10% or 50% of the cells inoculated were expressing stCPG2(Q)3. We conclude that all three of the prodrugs are more effective therapeutically with stCPG2(Q)3 than is the previously described prodrug CMDA and, also, that the optimal choice of prodrug varies among different tumor types and that prodrugs, optimized for their bystander effect, are effective when only low percentages of cells in a tumor express CPG2.

INTRODUCTION

Prodrugs have often been proposed as a solution to the major problem of nonspecific toxicity to normal tissues, such as the bone marrow and lining of the gut, often associated with conventional cytotoxic chemotherapy (1). Theoretically, prodrugs are relatively nontoxic molecules, capable of being converted to cytotoxic species only at the site of the tumor, affording enhanced antitumor selectivity. Several approaches to endogenous mechanisms of activation have been considered, including tumor-associated hypoxia (2), low pH (3), and enhanced enzyme levels (4). More recently, the use of exogenous activating enzymes by enzyme-antibody conjugates (5), fusion proteins (6), or catalytic antibodies (7), known as antibody-directed enzyme prodrug therapy (ADEPT) has been attempted and has proceeded to clinical testing (8–10). An alternative approach is the genetic manipulation of the tumor cells to express the activating enzyme. This has been variously called virally directed enzyme prodrug therapy (VDEPT), GDEPT (2), gene prodrug activation therapy (GPAT), or suicide gene therapy (11–13).

Several enzyme/prodrug systems have been designed for GDEPT but by far the most extensively studied are bacterial or yeast cytosine deaminase with 5-fluorocytosine, and herpes simplex virus thymidine kinase with ganciclovir, which have progressed to clinical trial (14, 15). Both the enzymes in these systems activate their respective prodrugs to intermediates that require subsequent metabolic processing to yield the drug. Also, the ultimate cytotoxins, being antimetabolites, are mainly cell phase specific, requiring active DNA synthesis before they can exert their cytotoxic effect. This is a disadvantage in tumors containing quiescent cells. We have used the Pseudomonas RS16 enzyme CPG2 (glutamate carboxypeptidase, EC 3.4.17.11), which hydrolyzes aromatic N-substituted glutamates to release benzoic acid, phenol, and aniline mustards (16, 17) or, in the case of self-immodate prodrugs, can release many conventional drugs, e.g., anthracycline antibiotics (18). The benefits of the CPG2 system with nitrogen mustard prodrugs are that the cytotoxin is not cell phase specific and that it is a one-step activation, thus avoiding the potential problem of rate limitation in the subsequent metabolism of the product.

A number of improvements are required before GDEPT can become a successful therapy. Many gene-delivery systems are currently in development, including conditionally replicating (19) and nonreplicating viruses (20), nonviral delivery, (21) and bacterial vectors (22). However, these systems are unlikely to be able to transduce more than a portion of the cells of the tumor, variously reported to be between 15–50% (23–25), requiring the action of a bystander effect. This is the ability of those cells expressing the therapeutic gene to convert an excess of prodrug to active drug that can migrate to, and kill, neighboring non-enzyme-expressing cells, thereby mitigating the limitations of the gene delivery system. Another area of improvement of GDEPT is focused on the optimization of the bystander effect. This can be addressed by engineering the enzymes or by designing better prodrugs. We have shown that engineering CPG2 to be displayed at the outer cell surface conferred on the cells an improved sensitivity to the prodrug CMDA compared with cytoplasmic expression of CPG2 and resulted in a greater bystander effect (26). Accordingly, in the present study, we have chosen to use only stCPG2(Q)3. CPG2 is particularly amenable to prodrug optimization, having a broad substrate specificity that can be further expanded by the self-immolative strategy, and we have previously exemplified a number of prodrug alternatives (13, 17, 18, 27). Here, we describe the cytotoxicity properties of three prodrugs 1, 2, and 3 (Fig. 1) for CPG2. One novel prodrug (prodrug 3) is reported here for the first time, and the other two have not been previously assessed in GDEPT. We report on their in vivo antitumor efficacy in the bystander xenograft model with stCPG2(Q)3-transfected MDA MB 361 breast carcinoma cells, de...
scribed previously in (26), and one new stCPG2(Q)3-engineered WiDr xenograft of colorectal carcinoma.

MATERIALS AND METHODS

Prodrugs

The synthesis of 4-[bis(2-iodoethyl)amino]-phenyl氧carbonyl-L-glutamic acid (prodrug 1, also called ZD2767P), and 3-fluoro-4-[bis(2-chloroethyl)aminobenzoyl-L-glutamic acid (prodrug 2) have been described previously (17, 27). Prodrug 3 was synthesized from commercially available compounds according to the following procedure. (The structures of all three prodrugs and the corresponding drugs that are released by the enzymatic action of CPG2 are shown in Fig. 1.)

3.5-Difluoro-4-[bis(2-hydroxyethyl)amino]benzonic Acid. A solution of 3,4,5-trifluorobenzonitrile (5 g, 32 mmol) and diethanolamine (8 ml, 85 mmol) in N,N-dimethylethlamide was stirred for 10 days. The solvent was then evaporated, the residue partitioned between CHCl3 (200 ml) and H2O (200 ml), and the organic layer dried (MgSO4) and evaporated to dryness. The residue was chromatographed using CHCl3-CH3OH as eluant to give, after recrystallisation from toluene, 2.7 g (35%) of pure white crystals: mp 146.5°C; 1H NMR δ 3.33 (t, 4H, NCH2), 3.48 (t, 4H, OCH2, J = 6 Hz), 4.43 (bs, 2H, OH), 7.57 (dd, 2H, H2,F3, and H6,F5, J = 6, 10 Hz); MS m/z 150 [M]+. Anal. (C11H13NO4F2) C,H,N.

In Vivo Cytotoxicity Assays

MDA MB 361 (5 × 106) or WiDr (2 × 106) cells were seeded into 6-well tissue culture dishes, yielding similarly confluent monolayers at 48 h. Prodrugs were dissolved in DMSO at 100 times the highest dose immediately before treatment and were administered in a two-stage protocol, as described previously (29). After exposure to prodrug, the cells were harvested, diluted, reseeded, and grown until the control wells reached confluence and the extent of cell growth was assayed by sulforhodamine-B dye (30). The results are expressed as the percentage of control growth against log dose, and the IC50 determined by nonlinear regression to a log dose-effect sigmoid, constraining the minimum to be positive (GraphPad Prism, GraphPad Software Inc., San Diego, CA).

In Vivo Analyses

All experiments were conducted in accordance with University Home Office regulations and United Kingdom Co-ordinating Committee on Cancer Research guidelines (31). Xenografts were established in nude (nu/nu) female BALB/C mice (20–22 g) by s.c. inoculation (0.2 ml) in the right flank of a suspension of MDA MB 361 or WiDr cells (105 and 8 × 106 cells, respectively) in PBS. The inocula, (5–10 animals/group), consisted of mixtures of β-gal- and stCPG2(Q)3-expressing cells, comprising 0, 10, 50, or 100% stCPG2(Q)3-expressing cells. After 4 days, mice were divided randomly into control and treated groups, and those in the treated groups received prodrugs (day 0). Prodrugs were dissolved in DMSO and diluted 20-fold in 1.26% (w/v) sodium bicarbonate just before injection. Each course of prodrg treatment consisted of three i.p. injections over a 2-h (1) or 24-h (2, 3) period, to a total preestablished maximum tolerated dose of 300, 1200, and 600 mg/kg for...
prodrugs 1, 2, and 3 respectively. Additional courses of prodrug were administered on days 7, 14, 28, and 36 (± 1 day) and, when palpable tumor remained, on days 42, 56, and 63. When eventual tumor regrowth followed a prolonged tumor-free period, an additional course was administered. Animals were culled if the tumor exceeded 1.5 cm in any dimension. At the time of cull, the tumors were excised and analyzed for specific CPG2 activity by enzyme assay as described previously (32). In a separate experiment, WiDr xenografts consisting of 0, 10%, and 50% stCPG2(Q)3-expressing cells were established as above and were treated with similar courses of the prodrugs. Courses were repeated on days 7 and 14. Twenty-four h after the end of the last course, the tumors were excised and fixed in formalin-saline. After paraffin mounting, sections (5 μm) were cut, stained with H&E, and examined microscopically (×40). Representative fields of view were photographed.

RESULTS

In Vitro Cytotoxicity of Prodrugs in MDA MB 361 and WiDr Cells Expressing β-gal or stCPG2(Q)3. We compare the cytotoxicity (Table 1) of the prodrugs 1, 2, and 3 (Fig. 1) for efficacy in GDEPT toward MDA MB 361 breast carcinoma and WiDr colon carcinoma cell lines engineered for stable expression of stCPG2(Q)3, with parental cells expressing β-gal as controls. Relative dye binding in control wells is similar for β-gal- and stCPG2(Q)3-expressing cells, implying equal growth rates. Cell monolayers are exposed to a range of concentrations of the prodrugs, and the resultant IC50 values are shown in Table 1. When the survival of the prodrug-treated cells did not fall below 50% of the control untreated cells, a value of greater than the highest dose used is quoted. In both the MDA MB 361 and WiDr cell lines, prodrug 1 is the most potent, toward both the β-gal- and stCPG2(Q)3-expressing variants (Table 1). Prodrug 3 is more potent than 2 in β-gal-expressing MDA MB 361 and WiDr cells, but very similar in both MDA MB 361 and WiDr stCPG2(Q)3-expressing cells. In MDA MB 361 and WiDr, the magnitude of the dose modification factor ranks prodrug 1 > prodrug 2 > prodrug 3, and is as high as 450-fold in the case of WiDr stCPG2(Q)3 treated with prodrug 1 (Table 1).

In Vivo CPG2 Levels in Tumors during GDEPT Therapy of Established stCPG2(Q)3 MDA MB 361 and WiDr Xenografts. The concentrations of CPG2 in the non-prodrug-treated tumors remain proportional to the percentage of stCPG2(Q)3-expressing cells in the original inoculum (Fig. 2). The specific activity of the 100% stCPG2(Q)3-expressing tumors is 0.5 units/g in the MDA MB 361 (Fig. 2A), which is consistent with previous observations (33), and four times greater, 2 units/g, in the WiDr (Fig. 2B). Treatment with the prodrugs variously alters this relationship. Treatment with prodrug 3 does not change the activity, the slopes of the lines (expressed as units of CPG2 activity per gram of tumor wet-weight per percentage of stCPG2(Q)3-expressing cells in the original inoculum) for both the MDA MB 361 (0.005) and WiDr (0.02) tumors being the same as for their respective controls. After treatment with prodrug 2, the slopes are reduced to 0.003 (MDA MB 361) and 0.01 (WiDr). Prodrug 1 has the greatest effect. No tumors were available for assay in the group of MDA MB 361 inoculated with 100% stCPG2(Q)3 because all of these had been completely cured. In the WiDr tumors, the slope of the line after treatment with prodrug 1 was reduced to 0.002, one-tenth of that in the control.

In Vivo Effect of Prodrugs on Tumor Histology during GDEPT Therapy of Established MDA MB 361 and WiDr Xenografts. Mice bearing 4-day-old WiDr xenografts were treated with the three prodrugs according to the schedule as detailed. Twenty-four h after the last dose, tumors were excised, fixed, sectioned, mounted, stained with H&E, and examined microscopically. Representative views of each of the sections are photographed (Fig. 3). The appearance of control tumors from mice that had received no prodrug (not illustrated) are indistinguishable from those 0% stCPG2(Q)3-expressing tumors that had received prodrug 3, being comprised of a dense, tightly packed cell mass with few interstitial spaces. Treatment with the other prodrugs produced a number of “holes” in the section, indicating a slight degree of antitumor effect in the absence of CPG2, prodrug 1 being more effective in this respect than prodrug 2. When the tumor arises from an inoculum of 10% stCPG2(Q)3-expressing cells, the sections of tumors from mice treated with prodrugs 2 and 3 are visibly more “ragged” than those arising from an inoculum of 0% stCPG2(Q)3-expressing cells, prodrug 2 being more effective than prodrug 3. Treatment with prodrug 1 produces little additional effect in the tumors arising from an inoculum of 10% compared with 0% stCPG2(Q)3-expressing cells. When the tumors arise from an inoculum of 50% stCPG2(Q)3-expressing cells, a marked change in the appearance of the sections is seen. After treatment with all three of the prodrugs, little of the original structure remains, the sections consist-

<table>
<thead>
<tr>
<th>Table 1 The cytotoxicity of prodrugs 1, 2, and 3 against MDA MB 361 or WiDr cells engineered to express β-gal or stCPG2(Q)3</th>
<th>IC50 (μM)</th>
<th>IC50 ratio β-gal:stCPG2(Q)3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type</td>
<td>Prodrug</td>
<td>β-gal</td>
</tr>
<tr>
<td>MDA MB 361</td>
<td>1</td>
<td>45.15 (27.0–75.5)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&gt;4000</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>WiDr</td>
<td>1</td>
<td>171.3 (104.3–281.3)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&gt;5000</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1663 (358.8–7713)</td>
</tr>
</tbody>
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*Values in parentheses are 95% confidence intervals.
ing mostly of an open lattice network, with a few embedded foci that resemble the original dense appearance. These sections probably consist mostly of a pad of fat and connective tissue remaining at the site of inoculation, still constituting a palpable lump but with few of the actual xenograft cells remaining. Such an extensive effect in these tumors indicates a substantial bystander effect.

**In Vivo Therapeutic Effect of Prodrugs during GDEPT Therapy of Established MDA MB 361 and WiDr Xenografts.** In MDA MB 361 xenografts arising from an inoculum of 100% stCPG2(Q)3-expressing cells, prodrug 1 produces the greatest increase (424 days) in median survival, with prodrugs 2 and 3 also producing significant increases in median survival of 360 and 146 days, respectively (Fig. 4A). The median survival of all of the non-prodrug-treated controls for all of the inocula was 33.2 days. In MDA MB 361 xenografts arising from an inoculum of 50 and 10% stCPG2(Q)3-expressing cells, prodrug 1 becomes progressively less effective, whereas prodrugs 2 and 3 remain effective when only 10% of the cells in the inoculum express stCPG2(Q)3. In WiDr xenografts arising from inocula of 10% stCPG2(Q)3-expressing cells, none of the prodrugs had any therapeutic effect. In WiDr xenografts arising from inocula of 50% stCPG2(Q)3-expressing cells, prodrug 1 was somewhat more effective, whereas prodrug 2 produced the largest extension of life span (372.5 days compared with 30.1 days in the non-prodrug-treated controls) in tumors arising from 100% stCPG2(Q)3-expressing cells (Fig. 4B).

**DISCUSSION**

In this study, we examine three new prodrugs, prodrugs 1, 2, and 3, for use in GDEPT with CPG2 using the cell surface tethered variant enzyme stCPG2(Q)3. When assessed for potency in an in vitro cytotoxicity assay, prodrugs 2 and 3 are of similar potency toward both MDA MB 361 and WiDr β-gal-expressing control cells, having IC$_{50}$ values in the 1–10 mM range. We have previously assessed only one prodrug, CMDA, for efficacy with CPG2 in GDEPT systems, and found IC$_{50}$ values of 3.1 and 3.2 mM for CMDA in MDA MB 361 and WiDr β-gal-expressing control cells respectively (26, 28). When taken together, these data indicate that MDA MB 361 and WiDr cells have a similar intrinsic sensitivity to bifunctional alkylating agents. The
phenol mustard prodrug 1 is more potent than the benzoic acid mustard prodrugs 2 and 3, having an IC₅₀ in the β-gal-expressing control cells in the <200 μM range. On activation by CPG2, all three of the new prodrugs produce substantial cytotoxicity differentials between stCPG2(Q)₃- and control β-gal-expressing MDA MB 361 and WiDr cells (70- to 450-fold), with prodrug 1 producing the greatest cytotoxic differential in both cell lines. These cytotoxicity differentials between stCPG2(Q)₃- and control β-gal-expressing cells are, for all three prodrugs, substantially greater than we previously showed with the prodrug CMDA (19-fold for MDA MB 361 (26), 27-fold for WiDr (28)).

We test the three new prodrugs for therapeutic efficacy in the previously described stCPG2(Q)₃- and control β-gal-expressing MDA MB 361 xenograft models and in the newly described stCPG2(Q)₃- and control β-gal-expressing WiDr xenograft model. To assess the ability of the prodrugs to mount a bystander effect when activated by CPG2, we assess their therapeutic effect in xenografts arising from inocula composed of mixtures of stCPG2(Q)₃- and control β-gal-expressing cells. At the termination of each experiment, the tumors were excised, and the specific activities of CPG2 assayed and expressed per gram of wet weight of tumor. The concentrations of CPG2 observed in the tumors at the time of cull suggest that the prodrugs can act as selective agents against stCPG2(Q)₃-expressing cells in a mixed population with differing efficiency. Prodrug 1 greatly reduces the specific activity of CPG2 in tumors that arose from inocula in which only 10 or 50% of the cells expressed CPG2. This suggests that prodrug 1, although the most potent and generating the greatest cytotoxic differential between stCPG2(Q)₃- and control β-gal-expressing cells, is mounting only a limited bystander effect. This is because a drug with a poorer bystander effect will kill only the stCPG2(Q)₃-expressing cells in which it is generated, leaving the non-CPG2-expressing bystanders to regrow, giving rise to a tumor with little remaining CPG2. In this situation, it would be expected that tumors that regrew from inocula of 10 and 50% mixtures of stCPG2(Q)₃-expressing cells display reduced CPG2-specific activity, because the drug derived from prodrug 1 has acted as a selective agent. That the same reduced concentration of CPG2 is seen in tumors that regrew from an inoculum of 100% stCPG2(Q)₃-expressing WiDr cells suggests that, at the time of prodrug treatment, the actual percentage of stCPG2(Q)₃-expressing cells was less than the inoculated 100%. This may be because, although the cell lines were cloned in and periodically reselected in G418, they are selection-free once they are in vivo, and the expression of CPG2 may become extinguished in a proportion of the cells. Tumors that regrew after treatment with prodrugs 2 and 3 possess concentrations of CPG2 that were essentially unchanged, which suggests that these prodrugs mount a considerable bystander effect, to the extent that only 10% of the cells of the tumor express CPG2, all of the cells experience similar cytotoxicity. Indeed, the results with prodrug 1 suggest that the actual percentage may be less than 10%, indicating an even more powerful bystander effect.

The histological appearance of sections cut from WiDr xenografts arising from inocula in which only 10% of the cells expressed CPG2 is supportive of the proposal that, although prodrug 1 is more potent than prodrugs 2 and 3, it exerts a poorer bystander effect. We see that although prodrug 1 produces some visible damage in tumors composed solely of control β-gal-expressing cells, it produces no additional effect in tumors arising from inocula of 10% CPG2-expressing cells. By contrast, prodrugs 2 and 3 produce a visible effect in tumors arising from inocula of 10% CPG2-expressing cells, which indicates the presence of a bystander effect when compared with no effect produced in tumors composed entirely of control β-gal-expressing cells. When 50% of the cells of the inoculum expressed CPG2, a marked bystander effect is seen with all three prodrugs.

When the therapeutic effect is examined, in MDA MB 361 xenografts arising from 100% stCPG2(Q)₃-expressing cells, the most therapeutically effective prodrug was 1, consistent with its being the most potent prodrug, and having the greatest cytotoxic differential. However, in tumors arising from inocula of 50% and 10% stCPG2(Q)₃-expressing cells, prodrug 1 becomes progressively less effective. This is consistent with the histological and CPG2 activity findings that suggest that prodrug 1 exerts a relatively small bystander effect. Prodrugs 2 and 3, although not as effective as prodrug 1 in tumors arising from inocula of 100% stCPG2(Q)₃-expressing cells, retain their efficacy in tumors arising from inocula of 50% and 10% stCPG2(Q)₃-expressing cells, consistent with their correspondingly larger bystander effect, as evidenced in the histological and CPG2 activity sections of the study. In WiDr xenografts, the pattern is less clear, with a much smaller therapeutic effect being seen in tumors arising from inocula of 50% stCPG2(Q)₃-expressing cells, and none at all in tumors arising from inocula of 10% stCPG2(Q)₃-expressing cells.

The extent of the bystander efficacy as compared with the cytotoxic potency may reflect the stability of the active drugs. The phenol mustard drug deriving from prodrug 1 will be the most reactive of the drugs, and will, thus, tend to react primarily at its site of production, becoming inactivated before it can diffuse to bystander cells. The less reactive, and less potent, drugs will be able to diffuse to a greater extent before inactivation. Clearly, there is a balance between a drug being too reactive with a poor bystander effect, or being insufficiently reactive and leaking from the tumor to cause systemic toxicity. The high cytotoxic differential of prodrug 1 may reflect a larger difference in lipophilicity between prodrug and drug than for prodrugs 2 and 3, which could also influence bystander efficacy. The optimum lipophilicity will enable a drug to permeate nearby cells efficiently, but not to be so completely sequestered in their lipid membranes that it cannot reach bystander cells. Future experiments will endeavor to establish the quantitative relationship between the half-life, lipophilicity, cytotoxic potency, cytotoxic differential, and bystander efficacy of these and other prodrugs and drugs. This will enable the design of prodrugs with the optimum combination of characteristics for use in GDEPT.

In summary, we have synthesized and studied three new prodrugs for use with CPG2 in GDEPT. In one tumor model, the prodrugs with the poorer cytotoxic differential but greater bystander effect produce a relatively larger extension of life span in tumors composed of a low percentage of stCPG2(Q)₃-expressing cells. This model is, thus, more relevant to GDEPT, in which expression of the transgene is more likely in only a small proportion of the tumor cells, than in the commonly used systems, in which 100% of the cells of the tumor express the transgene. These results show the need to optimize several properties of prodrugs for GDEPT for performance in different tumor types, and for tumors composed of different percentages of cells expressing the therapeutic enzyme. These results also highlight the versatility of prodrug design available in CPG2-based GDEPT that is precluded in many other commonly used GDEPT systems.

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