Development of an etoposide prodrug for dual prodrug-enzyme antitumor therapy

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Abstract
Enzyme-prodrug approaches to cancer therapy, theoretically, have the potential to mediate tumor-selective cytotoxicity. However, even if tumor-specific prodrug activation is achieved, enzyme-prodrug systems investigated thus far comprised a single enzyme and a specific prodrug. Although targeted, such systems constitute single-agent therapy, which may be ineffective and/or may promote development of drug resistance. Therefore, a goal of our laboratories was to design and characterize a novel dipiperidinylderivative of etoposide [1,4-dipiperidinyl-1-carboxylate-etoposide (dp-VP16)] that would act as a prodrug. We envisioned that dp-VP16 would be converted to the active chemotherapeutic agent VP-16 by the same rabbit carboxylesterase (rCE) that we have previously shown to efficiently activate the prodrug irinotecan (CPT-11). This dp-VP16 prodrug might then be used in combination with CPT-11, with both drugs activated by a single enzyme. We evaluated the ability of pure rCE and two human carboxylesterases, hCE1 and hiCE (hCE2), to activate dp-VP16 in vitro, and in neuroblastoma cell lines designed to express/overexpress each enzyme. In SK-N-AS neuroblastoma cell transfectants, expression of rCE or hiCE decreased the IC50 of dp-VP16 as a single agent by 8.3- and 3.4-fold, respectively, in growth inhibition assays. Purified hCE1 did not metabolize dp-VP16 in vitro and did not affect its IC50 in intact cells. The combination indices of sequential exposure to CPT-11 followed by dp-VP16 ranged from ~0.4 to 0.6, suggesting that this combination produced greater-than-additive cytotoxicity in neuroblastoma cells expressing rCE. These data provide proof-of-principle that enzyme-prodrug therapy approaches comprised of prodrugs with complementary mechanisms of cytotoxicity that are activated by a single enzyme can be developed. [Mol Cancer Ther 2006;5(6):1577–84]

Introduction
In principle, therapeutic approaches, such as antibody-, viral-, or neural progenitor cell–directed enzyme-prodrug therapy (antibody-directed enzyme-prodrug therapy, virus-directed enzyme-prodrug therapy, or neural progenitor cell–derived enzyme-prodrug therapy), have the potential to achieve tumor cell–selective activation of prodrugs and to produce tumor-specific cytotoxicity (1–6). Potentially, one of several “vectors” (antibodies, viruses, or neural progenitor cells, respectively) can be used to deliver a prodrug-activating enzyme selectively to tumor foci in vivo (antibodies or neural progenitor cells) or can be used to mediate tumor-selective expression of such an enzyme (viruses). In turn, the elevated level of enzyme at the tumor foci would be predicted to produce high local concentrations of active drug, thereby increasing the antitumor effect and decreasing the toxicity of the systemically given prodrug. The search for more efficient prodrug-activating enzymes/prodrugs for the treatment of cancer has led to investigations of a variety of combinations, including Herpes simplex virus-thymidine kinase/ganciclovir, Escherichia coli cytosine deaminase/5-fluorocytosine, E. coli purine nucleoside phosphorylase/2-fludarabine, cytochrome P450 reductase/cyclophosphamide, and carboxylesterase/irinotecan (CPT-11; reviewed in ref. 7).

We showed previously that rabbit liver carboxylesterase (rCE) efficiently converts the prodrug irinotecan (CPT-11) to the topoisomerase I inhibitor SN-38 (refs. 8, 9; Fig. 1A), and we are investigating the potential use of rCE/CPT-11 for enzyme-prodrug approaches to the treatment of metastatic neuroblastoma and other pediatric solid tumors (8, 10). However, antibody-directed enzyme prodrug therapy (1–3), virus-directed enzyme prodrug therapy (4–6), and neural progenitor cell–derived enzyme prodrug therapy3 approaches developed thus far comprised a single enzyme and a single prodrug; therefore, even if tumor cell–selective cytotoxicity is achieved, these approaches constitute single-agent therapy. One of the goals of our laboratories is to develop prodrugs of complementary mechanistic classes that are efficiently activated by a single

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3 Aboody et al., manuscript submitted for publication.
enzyme that might be combined to mediate tumor-selective combination therapy. Toward accomplishing this goal, a preliminary study showed that etoposide (VP-16) increased the cytotoxicity of CPT-11 in various solid tumor cell lines, including those derived from neuroblastoma. We, therefore, proposed to design and synthesize a prodrug of VP-16 that would be activated by rCE and to examine the cytotoxic potential of the combination of the two prodrugs and overexpression of carboxylesterase.

We synthesized several derivatives of VP-16 and, based on preliminary data, selected one of the synthesized novel etoposide prodrug [1,4-dipiperidine-1'-carboxylate-VP-16 (dp-VP16); Fig. 1B] to be evaluated in combination with CPT-11, using neuroblastoma cell lines transfected to express rCE or human hCE1 or hiCE.

Materials and Methods

Chemistry: General

All anhydrous solvents and starting materials were purchased from Fisher Scientific, Inc. (Suwanee, GA) or Aldrich Chemical Co. (Milwaukee, WI). Flash column chromatography silica cartridges were obtained from Biotage, Inc. (Lake Forest, VA). Reactions were monitored by TLC on precoated Merck 60 F254 silica gel plates and visualized using UV light (254 nm), and preparative TLC was done using the same conditions. All 1H nuclear magnetic resonance spectra (NMR) were recorded on a Varian INOVA-500 (500 MHz) spectrometer (Palo Alto, CA). Chemical shifts were recorded in ppm (δ) relative to the residual solvent peak or internal standard (tetramethylsilane); coupling constants (J) are reported in Hertz (Hz). Mass spectra were recorded on a Bruker Esquire ESI-MS instrument (Bruker Daltonics, Billerica, MA). Purity of the target compound dp-VP16 (5) was confirmed by analytic high-performance liquid chromatography using an Alltech platinum C-18 reverse-phase column (4.5 mm × 150 mm; Columbia, MD) with UV detection at 215 nm using a gradient mixture of solvent A (0.1% trifluoroacetic acid/H2O) and solvent B (0.035% trifluoroacetic acid/acetonitrile) at a flow rate of 1.0 mL min−1. The mobile phase gradient elution profile followed are isocratic (0% B), 5 minutes; linear gradient (0-80% B), 30 minutes; and isocratic (80% B), 5 minutes. Using this profile, the retention times of the starting material (VP-16) and the product (dp-VP16) were 18.7 and 20.7 minutes, respectively.

**Electrospray Ionization-Mass Spectrometry Ion Trap System**

Mass spectrometric analyses of reaction products were done from RLP068 irradiation using a Bruker Esquire 4000 ion trap mass spectrometer (Bruker Daltonics) equipped with an electrospray source working in positive ion mode (11). The instrument was connected with the LC system outlet via Peek tubing. Mass spectrometry variables were the following: scan range m/z = 100 to 1,000, scan speed = 13,000 m/z s−1, nebulizer flow = 35 p.s.i., dry gas flow = 8.0 L/min, dry temperature = 300°C, capillary = −4 kV, skimmer = 40V, ion charge control target = 20,000, maximum accumulation time = 200 ms, spectra averages = 5, rolling averages = 2.

**Synthesis of 1,4’-Dipiperidine-1’-Carbonyl Chloride (3)**

To a solution of 1,4’-dipiperidine (800 mg, 4.76 mmol, 1) in dichloromethane (10 mL) and triethylamine (2 mL) at −10°C was added dropwise a solution of triphosgene (1.40 g, 4.78 mmol, 2) in dichloromethane (10 mL). The reaction mixture was allowed to gradually warm to room temperature over a 30-minute period and then concentrated in vacuo. The resulting residue was extracted with dichloromethane and washed with water (3×) and brine, dried over sodium sulfate/potassium carbonate, and concentrated in vacuo to give a crude product. Flash silica gel column purification using ethyl acetate as the eluent gave the pure compound 3 (313 mg, 29%). TLC (ethyl acetate/petroleum ether, 1:1): Rf = 0.1; 1H NMR (500 MHz, CDCl3): δ = 1.44 (m, 2H), 1.61 (m, 6H), 1.91 (d, 1H, J = 13 Hz), 2.53 (br, 5H), 2.89 (t, 1H, J = 12 Hz), 3.08 (t, 1H, J = 12.5 Hz), 4.34 (d, 1H, J = 14.5 Hz); electrospray ionization-mass spectrometry: m/z 231 (M’ + H).

**Synthesis of dp-VP16 (5)**

To a solution of VP-16 (4; 60 mg, 0.102 mmol; Sigma, St. Louis, MO) in dichloromethane (30 mL) was added 4-dimethylaminopyridine (25 mg, 0.205 mmol) and triethylamine (0.5 mL), and the mixture was stirred at room temperature for 30 minutes. To this reaction mixture was added dropwise a solution of 1,4-dipiperidinyl carbonyl chloride (23 mg, 0.100 mmol, 3) in dichloromethane (1 mL) and the solution was left to stir overnight at room temperature. To this reaction mixture, 5 mL of methanol was added to quench the reaction. The mixture was stirred...

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*K.J. Yoon and M.K. Danks, unpublished data.*

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Figure 1. Metabolism of CPT-11 (A) and proposed metabolism of dp-VP16 (B) by carboxylesterases.
for 5 minutes at room temperature, and then the mixture was concentrated in vacuo. The resulting residue was triturated with water (10 mL) and then filtered to give the crude product as pale white powder-like crystals. This product was purified by preparative TLC developed with methanol to give pure product 5 (31 mg, 38%). TLC (methanol): $R_f = 0.2$; $^1$H NMR (500 MHz, CDCl$_3$): $\delta = 1.40$ (d, 3H, $J = 4.9$ Hz), 1.46 (br, 2H), 1.60 (br, 6H), 1.84 (d, 2H, $J = 12.5$ Hz), 2.31 (d, 1H, $J = 2.2$ Hz), 2.53 (br, 4H), 2.63 (d, 1H, $J = 2.0$ Hz), 2.64 (br, 1H), 2.80 (br, 1H), 2.89 (m, 1H), 2.96 (br, 1H), 3.27 (dd, 1H, $J = 14.2$ and 5.1 Hz), 3.35 (m, 1H), 3.36 (m, 1H), 3.45 (m, 1H), 3.58 (m, 1H), 3.69 (s, 6H), 3.76 (m, 1H), 4.17 (tt, 1H, $J = 10.5$ and 2.2 Hz), 4.23 (t, 1H, $J = 8.3$ Hz), 4.25 (br, 1H), 4.36 (br, 1H), 4.43 (dd, 1H, $J = 10.7$ and $J = 9.0$ Hz), 4.65 (d, 1H, $J = 5.1$ Hz), 4.68 (d, 1H, $J = 7.6$ Hz), 4.76 (q, 1H, $J = 5.1$ Hz), 4.90 (d, 1H, $J = 3.4$ Hz), 6.00 (dd, 2H, $J = 9.8$ and 1.2Hz), 6.28 (br, 1H), 6.57 (s, 1H), 6.83 (s, 1H), 6.83 (s, 1H); electrospray ionization-mass spectrometry: $m/z = 783$ (M$^+$ + H); tandem mass spectrometry (783): 595 and 577.

### Enzymatic Activation of Novel Prodrug dp-VP16 Analyzed by Liquid Chromatography-Mass Spectrometry

Recombinant rCE, hCE1, and hiCE were produced in baculovirus-infected cell culture media and isolated to >95% purity, as described previously (12). For in vitro metabolism assays, the enzymatic activity of recombinant carboxylesterases was first determined using the general carboxylester substrate ortho-nitrophenyl acetate, as reported previously (10). Briefly, carboxylesterase activity was determined spectrophotometrically by incubating purified carboxylesterases with 3 mmol/L substrate in 50 mmol/L HEPES (pH 7.4) and monitoring generation of ortho-nitrophenol at 420 nm. Using the extinction coefficient of ortho-nitrophenol, the enzymatic activity was calculated, and the concentration of CPT-11 required to inhibit cell growth by 50% (IC$_{50}$) was determined using the calibration curve and the concentration of CPT-11 for 4 hours or to dp-VP16 (10$^{-4}$ to 10$^{-6}$ mol/L) for 5 days, to determine their potency as single agents. Subsequently, cells were exposed to CPT-11 for 4 hours followed by dp-VP16 for 5 days, combining equimolar concentrations of the two prodrugs over the concentration range indicated above. Following the 5-day exposure to dp-VP16, the number of remaining viable cells was quantitated with a Coulter counter. The percentage of surviving cells compared with untreated controls was calculated, and the concentration of CPT-11 required to inhibit cell growth by 50% (IC$_{50}$) was determined using GraphPad Prism software as published previously (10). Growth inhibition assays were done in duplicate for each drug as a single agent and for both drugs in combination, with two samples for each condition in replicate assays.

### Calculation of Combination Index

Cells were treated with 10-fold serial dilutions of each drug as a single agent or with a fixed ratio (1:1) of the two drugs sequentially. The concentrations of prodrugs that produced a specific level (fraction) of growth inhibition were calculated using CalcuSyn software (Biosoft, Ferguson, MO), according to the Chou and Talalay method, and the following equation (18–21).

\[ \text{Combination index (CI)} = \frac{(D_1)_{12}}{(D_1)_{2} + (D_2)_{1} + (D_1)_{2} + (D_2)_{1}} \]

where, $(D_1)_{12}$ and $(D_2)_{1}$ in the numerators are the concentrations of prodrugs 1 (CPT-11) and 2 (dp-VP16) that produced x% growth inhibition as single agents. $(D_1)_{2}$ and $(D_2)_{2}$ in the denominators are the concentrations of $D_1$ and $D_2$ in combination that decreased survival by the same x%. Using the above equation, a CI < 1 indicates that the two agents exert a greater-than-additive (synergistic) cytotoxicity; a CI = 1 indicates an additive effect; and a CI > 1 is interpreted as less than additive (antagonistic; ref. 21). CalcuSyn software (Biosoft) also facilitates determination of
the median effect dose ($D_{m}$), an alternate nomenclature for the concentration of compound needed to inhibit cell growth by 50%. We used the $D_{m}$ values generated by CalcuSyn to corroborate IC$_{50}$ values determined using GraphPad Prism.

**Results**

**Design of VP-16 Prodrug**

One of the long-range goals of our laboratories is to identify prodrugs that will produce additive or synergistic cytotoxicity when combined with CPT-11, components of carboxylesterase-mediated enzyme-prodrug activation as a novel approach for the treatment of solid tumors. The specific goal of this study was to design a VP-16 prodrug that would be activated by rCE. Having shown previously (14, 15, 17) that a dipiperidinyl side chain attached by a carboxamid linkage confers preferential metabolism of CPT-11 to its active form SN-38 (Fig. 1A) by rCE > hiCE > hCE1, we attached this side chain via carbamate linkage to the 4" position on the E-ring of the anticancer agent VP-16 (Fig. 1B). We predicted that attachment of a side chain in this position would maximize steric hindrance that might prevent the dipiperidinyl side chain entering into the active catalytic gorge of rCE. Consequently, addition of a side chain at this position would be unlikely to limit access of the active amino acids of the enzyme to the carbamate linkage that must be cleaved to convert dp-VP16 to VP-16.

**Synthesis of dp-VP16 (5)**

The synthesis of dp-VP16 is outlined in (Fig. 2). This synthesis first required the preparation of 3 (1,4'-dipiperidinyl-1'-carbonyl chloride). 1,4'-Dipiperidinyl was reacted with excess triphosphate in dichloromethane using triethylamine as a base. Under these reaction conditions, the major byproduct diethylamide carbonyl chloride was also detected. As this byproduct could potentially react with VP-16 in next reaction step, it was necessary to purify compound 3 to purity by silica gel column chromatography before the next step. The preparation of compound dp-VP16 was then completed using a modification of the method of Henegar et al. (22) by coupling 3 with VP-16 in dichloromethane. VP-16 contains three hydroxyls at G2, G3, and E4" positions that may react with compound 3. It was anticipated that the E4"-OH is most reactive and could be selectively attacked. This assumption proved correct. The generation of additional byproducts was further reduced by using VP-16 in excess to compound 3. The optimum method of purification of the desired product from starting materials and regioisomers was found to be by preparative TLC. Using this method, it was possible to obtain dp-VP16 in an acceptable yield. The structure of compound dp-VP16 was confirmed by $^1$H 500-MHz NMR and tandem mass spectrometry analysis. Diagnostic tandem mass spectrometry fragmentation of the 783 molecular ion of dp-VP16 gave ions at 577 and 595 corresponding to the loss of an unreacted glycone portion and confirming the desired structure.

**Conversion of Prodrug by Carboxylesterases**

To evaluate metabolism of this novel VP-16 prodrug (dp-VP16) by carboxylesterases, reaction mixtures containing dp-VP16 and one of three recombinant carboxylesterases (rCE, hiCE, or hCE1) or no enzyme were incubated at 37°C overnight in vitro. Liquid chromatography-mass spectrophotometry analysis was used to calculate the percent conversion of dp-VP16 to VP-16 by each enzyme (23). The amount of VP-16 produced by rCE > hiCE > hCE1, with percentages of conversion of 19.3 ± 5.4%, 10.5 ± 0.2%, and 0% (undetectable), respectively. The VP-16 produced in control reactions containing no enzyme and in reactions containing hCE1 was below the level of detection, suggesting that dp-VP16 was stable for at least 18 hours and was not a substrate for this hCE1. Furthermore, there was no evidence, by liquid chromatography-mass spectrophotometry, of nonenzymatic degradation of dp-VP16 in HEPES for 24 hours at 37°C. We next evaluated enzymatic activation of dp-VP16 in intact cells, using cell lines transfected to express rCE, hiCE, or hCE1 using growth inhibition assays.

**Determination of IC$_{50}$ Doses in Neuroblastoma Cell Lines Transfected to Express rCE, hiCE, or hCE1**

We first determined the level of expression of rCE, hiCE, and hCE1 in transfected SK-N-AS and SK-N-SH neuroblastoma cells. The levels of enzyme activity expressed by SK-N-AS cells transfected to express rCE or hCE1 were 581 ± 82 and 394 ± 103 units, respectively; and SK-N-SH transfectants expressed 473 ± 88 and 447 ± 141 units of carboxylesterase activity, respectively. In contrast to the comparatively similar levels of rCE and hCE1 expressed by these neuroblastoma cell transfectants (400–600 units, quantitated using ortho-nitrophenyl acetate as a substrate), for unknown reasons, neuroblastoma cell lines and multiple other types of cell lines transfected to express...
hiCE express lower levels (SK-N-AS IRES hiCE, 200 ± 11 units and SK-N-SH IRES hiCE, 146 ± 31 units) of carboxylesterase activity. Therefore, it is difficult to compare directly the efficiency of prodrug activation in situ by the three enzymes, but the data support several conclusions. For CPT-11, data in Fig. 3A verify the expected increased sensitivity to CPT-11 mediated by the transfected cDNAs: rCE > hiCE > hCE1 > control transfectants (Fig. 3A). Based on data presented in Fig. 3A and levels of carboxylesterase expression, we predicted that neuroblastoma cells transfected to express rCE would have a lower IC50 for dp-VP16 than cells transfected to express hCE1, and this is in fact the case (Fig. 3B). IC50 values obtained from these growth inhibition data show that the concentrations of dp-VP16 required to decrease cell survival by 50% were 3.5, 11.7, 14.8, and 17.3 μmol/L for SK-N-AS rCE, SK-N-AS IRES hiCE, SK-N-AS IRES hCE1, and vector control SK-N-AS IRES neo cells, respectively (Table 1). The IC50 for VP-16 itself in untransfected SK-N-AS cells is 1.1 μmol/L. These results indicate that dp-VP16 is ~15-fold less potent than VP-16 in SK-N-AS cells expressing only endogenous carboxylesterase (compare IC50s of 1.1-17.3 μmol/L), but that dp-VP16 is only ~3-fold less potent than the parent VP-16 in cells transfected to overexpress rCE. These results indicate that rCE activates dp-VP16 in intact cells. The IC50 for cells expressing rCE was significantly different (P < 0.05) than the IC50 for the control vector transfectants.

![Figure 3](image-url)  
**Figure 3.** Dose-response curves for SK-N-AS cells transfected with control plasmid (neo) or with the plasmid containing the cDNA encoding rCE, hiCE, or hCE1 following exposure to CPT-11 for 4 h (A) or to dp-VP16 for 5 d (B) as single agents.

Table 1. **IC50 values**

<table>
<thead>
<tr>
<th>Drug</th>
<th>neo</th>
<th>rCE</th>
<th>hiCE</th>
<th>hCE1</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPT-11</td>
<td>21.4</td>
<td>1.3</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>dp-VP16</td>
<td>17.3</td>
<td>3.5</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>CPT-11 → dp-VP16</td>
<td>12.3</td>
<td>0.1</td>
<td>1.1</td>
<td>NT</td>
</tr>
</tbody>
</table>

NOTE: Each value was calculated using GraphPad Prism software. Values represent mean ± SD of replicate experiments.
Abbreviation: NT; not tested.

*Significantly different from value for SK-N-AS IRES neo cells (P < 0.05).

**Sequential Treatment of CPT-11 Followed by dp-VP16 of Neuroblastoma Cell Lines**

The cytotoxic effect of exposure to the two prodrugs in combination was then investigated using SK-N-AS and SK-N-SH IRES transfectants, as the rCE enzyme seemed to activate dp-VP16 most efficiently of the three enzymes, and was expressed at relatively high levels in both neuroblastoma cell lines. To circumvent potential enzyme binding site competition, we exposed cells to the two prodrugs sequentially. Data in Table 1 report the IC50 of the drugs as single agents or in combination. Data in Fig. 4 compare the cell survival dose-response curves of transfected SK-N-AS (Fig. 4A) or SK-N-SH (Fig. 4B) neuroblastoma cells following exposure to CPT-11 or dp-VP16 as single agents, or to a constant 1:1 ratio of CPT-11 followed by dp-VP16. Because we used an equimolar ratio of CPT-11 and dp-VP16, the data on the graph showing the effect of the combination indicate the concentration of both dp-VP16 and CPT-11. For example, the IC50 for the combination of CPT-11 and of dp-VP16 for SK-N-AS IRES rCE cells was 0.1 μmol/L for each drug. Calculations with GraphPad Prism and CalcuSyn software both indicated that at the ratio of 1:1, dp-VP16 decreased the IC50 of CPT-11 by 4.7- and 3.5- fold in SK-N-AS IRES rCE and SK-N-SH IRES rCE cells, respectively. In contrast, no potentiation was seen in SK-N-AS control transfectants. In addition, notably, the IC50 of dp-VP16 decreased by ~35-fold (compare, e.g., an IC50 of 3.5 μmol/L for dp-VP16 as a single agent with SK-N-AS IRES rCE cells with an IC50 of 0.1 μmol/L when combined CPT-11; Fig. 4A, right). These results indicate that the two prodrugs were not antagonistic and were potentially additive or greater than additive, under the conditions of the assay. To evaluate the interactive effects of the two drugs, we calculated CIs by the Chou-Talalay method using CalcuSyn software (24).

**Determination of Additive or Greater-than-Additive Effect of the Combination of CPT-11 and dp-VP16, as Reflected by Calculation of the CI**

The combination index (CI) equation of Chou and Talalay is based on a multiple drug effect equation derived from enzyme kinetic models (18–21). Using this approach, a CI = 1 indicates an additive effect (18). A CI < 1 denotes greater-than-additive toxicity (synergism), and a CI > 1...
describes less than additive toxicity (antagonism). Each CI value derives from two individual experiments with duplicate determinations at each concentration, following sequential exposure to the constant ratio of 1:1 of the two prodrugs. CI values for the IC50s of the combination of the two drugs for SK-N-AS and SK-N-SH rCE-transfected cell lines were 0.40 ± 0.01 and 0.61 ± 0.06, respectively, and for the IC50s were 0.51 ± 0.02 and 0.57 ± 0.01, respectively. These results suggest that the combination exerted a greater-than-additive cytotoxic effect. Together, the data indicate that dp-VP16 is stable and less potent (by ~15-fold) than VP-16 itself; dp-VP16 is activated to a very limited extent in neuroblastoma cells that express endogenous levels of carboxylesterase; dp-VP16 is converted to VP-16 by rCE in intact cells; and exposure of neuroblastoma cells to CPT-11 followed by dp-VP16 produces greater-than-additive cytotoxicity. The study described here comprises proof-of-principle that dual prodrug-enzyme approaches to therapy can be developed.

Discussion
Enzyme-prodrug approaches to cancer therapy have the potential to achieve tumor-selective cell death (7, 25). The current study provides proof-of-principle that combinations of prodrugs, activated by a single enzyme, might comprise a novel, effective therapeutic approach. Theoretically, the advantage of such an approach would be increased tumor specificity and antitumor efficacy as well as, perhaps, circumvention or delay of the development of drug resistance.

This study focused on the combination of the prodrug CPT-11, currently in clinical use, and a novel VP-16 prodrug for several reasons. First, CPT-11 and VP-16 are each relatively effective in treating neuroblastoma (26, 27), but neither drug is curative. Second, in vitro, sequential exposure of several neuroblastoma cell lines to CPT-11 and VP-16 produced additive or greater-than-additive cytotoxicity. Third, we have shown previously that CPT-11 is preferentially activated by an rCE compared with the human carboxylesterases hiCE and hCE1, and our goal was to develop a second prodrug with a mechanism of cytotoxicity complementary to that of CPT-11, which would also be selectively activated by the rabbit enzyme.

Other types of VP-16 analogues have been synthesized and characterized as potential prodrugs, but none of the compounds previously reported have the desired characteristics of stability in the absence of enzyme and selective conversion to the active form by a specific enzyme. Intriguingly, one of these prodrugs (Pro-VP-16 IV), 2-(((3-methoxybenzyloxy) carbonyl) methyl) amino) ethyl (methyl), was a carbamate derivative of VP-16 but was uniquely designed to be a substrate for tyrosine hydroxylase, an enzyme frequently expressed by neuroblastoma (28). Other previously reported VP-16 prodrugs, similar to those reported here, were designed to be activated by carboxylesterases and contained 4-propylcarbonoxy moieties (29, 30) attached via an ester linkage. These prodrugs (Pro-VP-16 I and Pro-VP-16 II) differ from dp-VP16 in this study in that Pro-VP-16 I and Pro-VP-16 II had a relatively short aliphatic chain with or without a single

Figure 4. Dose-response curves for exposure of neuroblastoma cells to CPT-11 alone, dp-VP16 alone, or to CPT-11 followed by dp-VP16. SK-N-AS (A) and SK-N-SH (B) cells transfected with control plasmid (pIREmneo, left) or with this vector encoding rCE (right).
five-membered ring (propylcarbonoxy moieties) attached by a carbamate linkage. Important for their potential development as prodrugs, these compounds were acid labile, and active VP-16 was generated even in the absence of enzyme, an undesirable characteristic for the prodrug in an enzyme-prodrug approach. Perhaps having greater potential was a VP-16 glucuronide-prodrug having a glucuronic acid (the substrate of the activating enzyme) and spacer (carbamate linkage) attached to the 4′ position (31). This glucuronic conjugate seemed to be stable in the absence of enzyme and efficiently cleaved by E. coli β-glucuronidase. However, the efficiency of prodrug activation by human compared with E. coli enzymes, an important factor in achieving tumor-selective prodrug activation, has not yet been reported.

Our laboratories have shown that larger side chains with up to two six-membered rings have the flexibility to enter into the active site gorge of carboxylesterases. Such side chains also permit access of the active amino acids at distal end of the catalytic gorge (32). These two properties contribute to selective metabolism by specific carboxylesterases. As it was our goal to design a VP-16 prodrug that would be activated by rCE, we chose to attach a dipiperidine side chain to the E4′ position of VP-16. The hypothesis to be tested was that the same enzymes that activate CPT-11 would activate dp-VP16, and those enzymes that activate CPT-11 less efficiently would also activate dp-VP16 relatively poorly. The unknown factor influencing whether dp-VP16 would be a substrate for rCE was whether the two -OMe (methoxy) substituents at the 3′ and 5′ positions on the VP-16 E ring to which the dipiperidine side chain was attached would prevent entrance of the side chain into the catalytic cleft of rCE. Such steric hindrance would decrease the ability of rCE to hydrolyze the COON linker moiety to produce active VP-16 and preclude further development of similar compounds.

However, the data support the hypothesis proposed that dp-VP16 is a substrate for the two carboxylesterases that have been shown to most efficiently activate CPT-11 (rCE and hiCE) and is a poor substrate for the enzyme for which CPT-11 is not a substrate (hCE1). This study provides proof-of-principle that combination enzyme-prodrug approaches to therapy represent viable approaches toward the development of tumor-selective therapy. While providing solid proof-of-principle, however, the combination of CPT-11 and VP-16 has been reported to exert synergistic, additive, or antagonistic cytotoxic effects in cell lines derived from different types of tumors (33, 34).

Therefore, this specific combination may be useful only for certain tumor types. Ongoing studies focus on the design and characterization of a prodrug which, when activated, would alkylate DNA, a mechanistic combination that has been reported to be synergistic in cell lines derived from several types of solid tumors (35–37). The data presented here strongly support the conclusion that such combination enzyme-prodrug approaches to therapy are feasible.

References

Development of an etoposide prodrug for dual prodrug-enzyme antitumor therapy


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