Intracellular inhibition of carboxylesterases by benzil: modulation of CPT-11 cytotoxicity

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Abstract
Carboxylesterases are ubiquitous proteins responsible for the detoxification of xenobiotics. However, these enzymes also activate prodrugs, such as the anticancer agents capecitabine and CPT-11. As a consequence, overexpression of carboxylesterases within tumor cells sensitizes these cells to CPT-11. We have recently identified two classes of carboxylesterase inhibitors based on either a benzil (diphenylethane-1,2-dione) or a benzene sulfonamide scaffold and showed that these compounds inhibit carboxylesterases with $K_i$ in the low nanomolar range. Because both classes of inhibitors show reversible enzyme inhibition, conventional in vitro biochemical assays would not accurately reflect the in situ levels of carboxylesterase activity or inhibition. Therefore, we have developed a novel assay for the determination of intracellular carboxylesterase activity using 4-methylumbelliferyl-acetate as a substrate. These studies show that benzil and a dimethylbenzil analogue efficiently enter cells and inhibit human intestinal carboxylesterase and rabbit liver carboxylesterase intracellularly. This inhibition results in reduced cytotoxicity to CPT-11 due to the lack of carboxylesterase-mediated conversion of the prodrug to SN-38. These results suggest that intracellular modulation of carboxylesterase activity with benzil or its analogues may be applied to minimize the toxicity of normal cells to CPT-11.

Introduction
Carboxylesterases can be detected in virtually all organisms and are thought to act in a protective manner (1, 2). They cleave carboxylesters (RCOOR') to produce the corresponding alcohol (R'OH) and carboxylic acid (RCOOH). In agreement with their proposed function, carboxylesterases tend to be expressed in tissues that are exposed to xenobiotics (e.g., lung, small intestine, liver, and kidney). However, many therapeutically useful drugs are esters, and hence, their activity may depend on the expression and biodistribution of carboxylesterases that might activate or inactivate these agents. For example, the $\beta$-adrenergic blocking drug flesolol has a very short in vivo half-life due to its rapid cleavage by carboxylesterases (3). In contrast, the prodrug CPT-11 [irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin] is essentially inactive as an antitumor agent and requires activation to yield SN-38 (7-ethyl-10-hydroxyxycamptothecin) via carboxylesterase-mediated cleavage of a carbamate linkage (4–9).

CPT-11 has shown remarkable antitumor activity in animal models (10, 11) and has been approved for the treatment of colon cancer in adults (12–15). In addition, this drug is in phase I, II, and III trials for the treatment of a variety of other solid tumors (16–22). Because conversion of CPT-11 to SN-38 is required for antitumor activity, overexpression of carboxylesterases that mediate this hydrolysis increases sensitivity to the prodrug. This has been observed both in cells in culture (4, 7, 9) and in human tumor xenografts (4, 23). However, the dose-limiting toxicity for CPT-11 is diarrhea, and because high levels of carboxylesterase are expressed in the small intestine (5, 24), we have proposed that this toxicity may be due to direct activation of CPT-11 to SN-38 in gut epithelium. Based on this hypothesis, we have identified a series of specific carboxylesterase inhibitors (25) that may be used in conjunction with CPT-11 to ameliorate the diarrhea that occurs 48 to 96 hours following drug administration. We envisage that the inhibitor could be administered 8 to 96 hours after CPT-11, with the goal of inhibiting the intracellular conversion of CPT-11 to SN-38 in the intestinal cells, thereby reducing both the levels of active metabolite in the gut and this toxicity associated with CPT-11 therapy.

Recently, we identified benzil (diphenylethane-1,2-dione) as a potent general inhibitor of mammalian carboxylesterases, with $K_i$ in the low nanomolar range for a variety of esterified substrates (26). However, all of these above inhibitor studies were done using purified enzymes in vitro, and hence, the biological properties of the identified compounds were not assessed. In this study, we evaluated the ability of these inhibitors to accumulate within human tumor cells and directly inhibit carboxylesterases. Because these inhibitors act in a partially competitive and reversible fashion (25, 26), we have developed a novel assay that examines enzyme inhibition in situ.
Results from these studies indicate that benzil and a 3,4-dimethyl analogue inhibit mammalian carboxylesterases intracellularly and protect cells from CPT-11 toxicity.

**Materials and Methods**

**Inhibitors and Drugs**

Benzil, 1-(3,4-dimethylphenyl)-2-phenylethane-1,2-dione (compound 1), and 4-chloro-N-(4-[[4-chlorophenyl]sulfonfonyl]amino)phenyl benzene sulphonamide (compound 2) were all obtained from Sigma Biochemical (St. Louis, MO). 1-(4-Chlorophenyl)-2-phenylethane-1,2-dione (compound 3) was purchased from Alfa Aesar (Ward Hill, MA), and 1,2-bis(4-chlorophenyl)ethane-1,2-dione (compound 4) was synthesized as described previously (26). The chemical structures of the compounds used in this article are indicated in Fig. 1.

G418 was obtained from Invitrogen (Carlsbad, CA), and CPT-11 was a kind gift from Dr. J.P. McGovern (Pfizer, New York, NY).

**In vitro Carboxylesterase Activity Assay**

Carboxylesterase activity in whole-cell sonicates was determined with a spectrophotometric assay using 3 mmol/L o-nitrophenyl acetate as a substrate (7, 27). Data were expressed as nmol of o-nitrophenol produced per minute per mg of protein.

**Cell Lines**

U373MG cells expressing human intestinal carboxylesterase (hiCE; refs. 5, 28) or rabbit liver carboxylesterase (rCE; ref. 7) were obtained by transfection of the parental cell line with the plasmid pIRESneo (BD Biosciences, Franklin Lakes, NJ) containing the appropriate cDNA. Pools of transfectedants were selected using 400 μg/ml G418, and carboxylesterase activity assays were done to confirm gene expression. Routinely, carboxylesterase activities of 400 and 1,000 nmol/min/mg were observed in cell extracts derived from U373hiCE and U373rCE cells, respectively. A control cell line U373IRES, which was obtained by transfection with the pIRESneo plasmid alone, was also constructed. These cells typically had a carboxylesterase activity of 6 to 10 nmol/min/mg.

**In situ CPT-11 Activation Assay**

CPT-11 activation in whole cells was assessed as described previously (4). Briefly, 10⁶ U373hiCE or U373rCE cells were resuspended in PBS and incubated with or without inhibitor (10 μmol/L) for 1 hour. CPT-11 was then added to a final concentration of 10 μmol/L, and cells were incubated at 37°C for 5 minutes. Cells were pelleted by centrifugation, the supernatant was aspirated, and, following two washes with cold PBS, methanolic extracts of the cells were prepared by adding 200 μL of cold acidified methanol (4, 29). SN-38 concentrations in these extracts were then determined using reverse-phase high-performance liquid chromatography. When U373IRES cells (cells lacking exogenous carboxylesterase expression) were used in these assays, the concentration of CPT-11 was increased to 100 μmol/L due to the very low conversion to SN-38 and the limits of detection of the high-performance liquid chromatography analyses.

**Quantitation of SN-38**

Concentrations of SN-38 in methanolic extracts were determined using reverse-phase high-performance liquid chromatography with fluorescent detection (4, 29). Data were expressed as pg SN-38 produced per 10⁶ cells.

**Growth Inhibition Assays**

Growth inhibition assays were done as described previously (7, 30). Briefly, cells were plated at a density of 2 × 10⁴ in individual wells of a six-well plate and allowed to attach overnight. After aspiration of the medium, inhibitor (10 μmol/L) was added for 1 hour before the addition of CPT-11. Two hours later, the drug and inhibitor were removed and fresh medium was applied to the cells. After 4 days (equivalent to three cell doublings), cells were harvested and counted using a Coulter Z2 counter (Beckman Coulter, Fullerton, CA). All data points were repeated in triplicate, and routinely, complete assays were done twice.

IC₅₀% (concentrations of CPT-11 that resulted in 50% growth inhibition) were calculated by determining the relative cell survival compared with control cells and plotting these data as a function of drug concentration. Numerical values were extrapolated from sigmoidal curve fits using Prism software (GraphPad, San Diego, CA).

**Results**

**Development of an In vivo Carboxylesterase Activity Assay**

Because all of the selective carboxylesterase inhibitors that have been recently identified act in a reversible fashion (25, 26, 31), a novel in situ assay was required to ensure that enzyme inhibition was occurring intracellularly. This was necessary because, during the preparation of cell extracts to measure carboxylesterase activity, the concentration of inhibitor would be vastly diluted, and hence, inhibition of carboxylesterase would not be apparent. Therefore, we developed an assay using 4-methylumbelliferone acetate (4-MUA) as a substrate that could detect carboxylesterase activity in situ. This ester was chosen as opposed to o-nitrophenyl acetate because the nonspecific hydrolysis of 4-MUA was considerably reduced (compared with o-nitrophenyl acetate) under the experimental conditions we used. 4-MUA is cleaved by carboxylesterase to yield the highly fluorescent product 4-methylumbelliferone, which facilitated the development of a fluorescence-based assay for this compound.

![Figure 1](https://example.com/figure1.png)  
Figure 1. Chemical structures of the compounds used in this study.
To assess intracellular carboxylesterase-mediated hydrolysis of 4-MUA, 10^7 cells were aliquoted into 1 mL PBS in a quartz cuvette, placed into a Hitachi (Schaumberg, IL) F-2000 fluorometer, and stirred gently throughout the assay with a micro-stir bar. 4-MUA was added to a final concentration of 0.75 mmol/L, and fluorescence was determined using an excitation wavelength of 365 nm and an emission wavelength of 460 nm. Data were recorded every 1 second for 2 minutes, and fluorescence intensity was plotted against time. Typical data plots are shown in Fig. 2.

For inhibitor studies, cells were preincubated with 10 μmol/L of the compound for 1 hour before assay. The inhibitor was dissolved in DMSO, and final concentrations of the solvent did not exceed 0.1%. As indicated in Fig. 2C, addition of benzil to the cells 1 hour before 4-MUA resulted in both a dramatically reduced rate of increase of fluorescence and in total fluorescence. This observation indicated that the inhibitor could enter cells and inhibit carboxylesterase activity intracellularly.

Data analysis of the graph plots (Fig. 2) was done in two different ways. Firstly, the fluorescence intensity at an arbitrary time point (20, 30, 40, or 115 seconds from the addition of 4-MUA) was determined and compared either with cells incubated with DMSO alone or with cells lacking exogenous carboxylesterase (U373IRES). The percentage inhibition of carboxylesterase activity was then calculated, and a typical data set for the 30-second time point is shown in Table 1. Secondly, the rate of change of fluorescence in the first 10 seconds was determined from the plots. These rates were then compared with values for cells lacking carboxylesterase or those treated with DMSO alone. In all cases, the value for U373IRES cells was subtracted from the test values, ensuring that the results reported in Table 1 were due to the exogenous carboxylesterase expressed within the cells rather than endogenous cellular esterases.

Intracellular Inhibition of hiCE

Using the in vivo 4-MUA assay described above, we monitored the ability of benzil and compounds 1 to 4 to inhibit hiCE expressed in U373MG cells. In all cases, cells were preincubated with 10 μmol/L inhibitor for 1 hour before the addition of 4-MUA. As indicated in the graphs depicted in Fig. 2, the rate in increase of fluorescence intensity was much greater in cells expressing hiCE compared with control cells (Fig. 2A and B). However, in the presence of benzil (Fig. 2C), this rate was considerably diminished as indicated by the almost linear rate of 4-methylumbelliferone generated. Similarly, intracellular inhibition of 4-MUA hydrolysis was also observed with compound 1 (Fig. 3C). Analysis of these data plots indicated that the levels of intracellular hiCE inhibition observed with benzil or compound 1 exceeded 88% (Table 1), suggesting that both these compounds are potent enzyme inhibitors that can act intracellularly. These properties would be essential for in vivo applications.

In contrast, the hiCE-specific inhibitor 2 (25) or the benzil analogues 3 or 4 (26) did not significantly reduce the levels of intracellular fluorescence (Fig. 3D–F). Because these compounds inhibit the activity of recombinant carboxylesterases in vitro (25, 26), the observed lack of enzyme inhibition in these assays was presumably due either to the inability of these compounds to enter cells or to the immediate intracellular inactivation of the inhibitor, leading to a failure to inhibit hiCE. As a consequence, the levels of enzyme inhibition produced by compounds 2, 3, or 4 (14–42%) were significantly lower that that seen for benzil (92%; Table 1).

Evaluation of the Validity of the Different Data Analyses

Because we could analyze the data obtained from the fluorescent 4-MUA assay using two different approaches, we sought to determine which method would be the most appropriate for subsequent inhibitor studies. Therefore, we analyzed the results obtained from these assays using benzil and a series of analogues to inhibit hiCE in U373hiCE cells. As indicated in Table 1, the percentage of intracellular carboxylesterase inhibition calculated either from the single time point data or from the rate analyses was in very close agreement for all of the inhibitors. For
Inhibiting CPT-11 Metabolism

In situ

To confirm that the inhibitors that reduced 4-MUA metabolism in situ would also prevent conversion of CPT-11 to SN-38, we determined the intracellular levels of SN-38 produced following incubation of cells expressing carboxylesterases with CPT-11 in the presence of selected inhibitors. In these studies, we used U373 cells expressing either hiCE (U373hiCE) or rCE (U373rCE). We included cells expressing rCE because this enzyme has been shown to be the most efficient at activating CPT-11 (4, 7). As a control for these studies, cells lacking exogenous carboxylesterase expression (U373RES) were used. As indicated in Table 2, preincubation of cells expressing hiCE or rCE with either benzil or compound 1 before incubation with 10 μmol/L CPT-11 significantly reduced the intracellular conversion of the drug to SN-38. For example, the amounts of SN-38 produced in U373hiCE following incubation with compound 1 were reduced by as much as ~97% or >30-fold. Hence, the in situ metabolism data obtained using CPT-11 as a substrate are comparable with fluorescence assays using 4-MUA (Fig. 2).

In cells lacking exogenous carboxylesterase expression (U373RES), very low levels of SN-38 were produced even following incubation with 100 μmol/L CPT-11. This concentration of drug was necessary to visualize the very small amounts of SN-38 produced. Under these conditions, addition of benzil or compound 1 resulted in essentially no change in the yield of the active metabolite (Table 2). This suggests that the enzyme responsible for CPT-11 activation in these cells is not sensitive to inhibition by these inhibitors. Overall, these studies indicate that intracellular inhibition of the conversion of CPT-11 to SN-38 can be achieved using benzil or compound 1.

### Table 1. A representative analysis of intracellular carboxylesterase inhibition using the fluorescent 4-MUA assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Carboxylesterase activity (nmol/min/mg ± SD)</th>
<th>Inhibitor</th>
<th>In vitro $K_i$ for hiCE (nmol/L ± SE)</th>
<th>Fluorescence intensity at 30 s (arbitrary unit)</th>
<th>Inhibition based on value of 30 s (%)</th>
<th>Rate of change of fluorescence (arbitrary unit/s)</th>
<th>Inhibition based on rate analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U373RES</td>
<td>8.4 ± 0.5</td>
<td>None (DMSO)</td>
<td>—</td>
<td>202</td>
<td>—</td>
<td>5.2</td>
<td>—</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>149.9 ± 21.1</td>
<td>None (DMSO)</td>
<td>—</td>
<td>1,470</td>
<td>88.1</td>
<td>91.8</td>
<td>—</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>149.9 ± 21.1</td>
<td>Benzil</td>
<td>14.7 ± 1.9</td>
<td>353</td>
<td>88.1</td>
<td>91.8</td>
<td>—</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>149.9 ± 21.1</td>
<td>Compound 1</td>
<td>4.1 ± 0.4</td>
<td>317</td>
<td>90.9</td>
<td>93.4</td>
<td>—</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>149.9 ± 21.1</td>
<td>Compound 2</td>
<td>53.3 ± 5.5</td>
<td>1,115</td>
<td>28.0</td>
<td>40.3</td>
<td>25.5</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>149.9 ± 21.1</td>
<td>Compound 3</td>
<td>18.2 ± 3.6</td>
<td>1,014</td>
<td>36.0</td>
<td>32.3</td>
<td>42.4</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>149.9 ± 21.1</td>
<td>Compound 4</td>
<td>106 ± 22</td>
<td>1,241</td>
<td>18.1</td>
<td>45.5</td>
<td>14.4</td>
</tr>
</tbody>
</table>

*R* results were determined using o-nitrophenyl acetate as a substrate and are taken from refs. 25, 26.

*C* results are taken from a representative experiment.
Inhibition of Intracellular Carboxylesterase Results in Resistance to CPT-11

Having determined that benzil and compound 1 could inhibit intracellular carboxylesterase, resulting in reduction of both 4-MUA and CPT-11 hydrolysis, we evaluated whether this inhibition would result in decreased sensitivity to CPT-11. Therefore, growth inhibition assays using combinations of benzil or compound 1 and CPT-11 were done. As indicated in Fig. 4, U373IRES, U373hiCE, or U373rCE cells treated with benzil or compound 1 alone did not show any cytotoxicity toward the inhibitor at concentrations up to 100 μmol/L. This is consistent with the hypothesis that these ethane-1,2-diones are essentially nontoxic. However, pretreatment of cells expressing carboxylesterase with either compound significantly increased the IC50s for CPT-11 (Table 3; Fig. 4C–E). Indeed, these IC50s were similar to those obtained in growth inhibition assays using cells lacking exogenous carboxylesterase expression (U373IRES).

For example, with cells expressing hiCE, the IC50 for CPT-11 increased 17-fold following treatment with compound 1 (Table 3; Fig. 4D). Similarly, with U373rCE, incubation of cells with benzil resulted in a 113-fold increase in this variable (Fig. 4F). More importantly, however, the IC50s for both carboxylesterase-expressing cell lines pretreated with either of the inhibitors before incubation with CPT-11 were similar to cells that lacked carboxylesterase expression (U373IRES; Table 3; Fig. 4A and B). These data suggest that almost complete carboxylesterase inhibition had occurred intracellularly, resulting in little CPT-11 activation following drug exposure. These data, therefore, are consistent with the hypothesis that inactivation of the intracellular carboxylesterase prevents the formation of SN-38 and reduces the toxicity of the prodrug CPT-11.

Discussion

The dose-limiting toxicity of CPT-11 is delayed diarrhea that usually occurs 48 to 96 hours following drug administration. Although this diarrhea can usually be managed by supportive care, hospitalization and electrolyte replacement is sometimes required. Several mechanisms for this diarrhea have been proposed. Firstly, patients that show polymorphisms in the UGT1A1 gene, which encodes a glucuronidase responsible for detoxifying SN-38, are at an increased risk for CPT-11-induced toxicity (32). Secondly, it is thought that bacteria in the gut that have glucuronidase activity can hydrolyze the SN-38 glucuronide to produce SN-38 (33, 34). Because these metabolites are cleared from the circulation via the bile, it is likely that high concentrations of SN-38 would be present within the small intestine that would result in direct injury to the epithelial and crypt cells, resulting in diarrhea.

Thirdly, it is known that the small intestine has high levels of carboxylesterase that can activate CPT-11 (5). We have shown that, in a mouse model, very high levels of CPT-11 are present in the bile for up to 24 hours after drug administration (24). In addition, studies by Slatter et al. (35) indicated that high concentrations of CPT-11 could be detected in the bile of cancer patients treated with this drug. Therefore, it is highly likely that CPT-11 would be deposited into the lumen of the small intestine via the bile following i.v. infusion. This would then be a substrate for the carboxylesterase present within the gut epithelia (hiCE), leading to the production of SN-38 and hence cytotoxicity, resulting in diarrhea.

In an attempt to ameliorate the toxicity that may occur via the latter route, we screened a series of compounds for their ability to selectively inhibit hiCE. We identified a series of benzene sulfonamides, of which compound 2 showed the lowest Ki for the inhibition hiCE (25). In addition, we determined that benzil is a potent general inhibitor of mammalian carboxylesterases (26). However, all of the biochemical studies were done in vitro and no assessment of the biological properties of these compounds has been done. In this article, we determined the ability of these compounds to enter mammalian cells and inhibit carboxylesterases intracellularly and evaluated whether they altered cellular sensitivity to CPT-11.

Because both the benzene sulfonamides and the benzil analogues are competitive reversible inhibitors of

Table 2. Intracellular levels of SN-38 following incubation of cells with CPT-11

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Carboxylesterase activity (nmol/min/mg SD)</th>
<th>Inhibitor</th>
<th>SN-38 produced (pg 10^6 cells ± SD)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U373IRES</td>
<td>11.5 ± 0.3</td>
<td>None</td>
<td>4.5 ± 0.3</td>
<td>—</td>
</tr>
<tr>
<td>U373IRES</td>
<td>11.5 ± 0.3</td>
<td>Benzil</td>
<td>4.0 ± 0.1</td>
<td>11.1</td>
</tr>
<tr>
<td>U373IRES</td>
<td>11.5 ± 0.3</td>
<td>Compound 1</td>
<td>4.1 ± 0.4</td>
<td>8.9</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>335.4 ± 19.2</td>
<td>None</td>
<td>37.1 ± 5.5</td>
<td>—</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>335.4 ± 19.2</td>
<td>Benzil</td>
<td>12.4 ± 0.4</td>
<td>66.6</td>
</tr>
<tr>
<td>U373hiCE</td>
<td>335.4 ± 19.2</td>
<td>Compound 1</td>
<td>1.2 ± 0.2</td>
<td>96.8</td>
</tr>
<tr>
<td>U373rCE</td>
<td>662.7 ± 87.0</td>
<td>None</td>
<td>22.5 ± 0.5</td>
<td>—</td>
</tr>
<tr>
<td>U373rCE</td>
<td>662.7 ± 87.0</td>
<td>Benzil</td>
<td>7.0 ± 0.2</td>
<td>68.9</td>
</tr>
<tr>
<td>U373rCE</td>
<td>662.7 ± 87.0</td>
<td>Compound 1</td>
<td>1.9 ± 0.1</td>
<td>92.6</td>
</tr>
</tbody>
</table>

*U373hiCE and U373rCE cells were incubated with 10 μmol/L CPT-11. However, due to the very low levels of carboxylesterase activity in U373IRES cells, assays with this line were done using 100 μmol/L CPT-11.
carboxylesterases (25, 26), measurements of inhibition of carboxylesterase activity using the in vitro o-nitrophenyl acetate assay would be inappropriate. This is due to the fact that, in the preparation of the sample to measure the carboxylesterase activity, the inhibitor would be greatly diluted and hence the enzyme would seem to be active. Therefore, we developed an in situ cell-based assay using 4-MUA as a substrate. By direct examination of the production of the fluorescent product 4-methylumbelliferone within cells, we could evaluate carboxylesterase activity directly and therefore determine the effectiveness of different carboxylesterase inhibitors (Fig. 2). Using this assay, it was apparent that both benzil and the 3,4-dimethylphenyl derivative (compound 1) could readily accumulate within cells and prevent carboxylesterase-mediated hydrolysis of 4-MUA and CPT-11 (Figs. 3 and 4).

Because the graphs obtained from the 4-MUA assay were hyperbolic in nature, we examined two different analytic methods to assess intracellular inhibition. These consisted of the initial rate of product formation similar to that for in vitro enzyme analysis and a calculation of the amount of product formed at discrete time points during the assay. The percentage of enzyme inhibition calculated by these two approaches, as indicated in Table 1, showed significant agreement between these results ($r^2 = 0.99$). We did not determine a traditional $K_m$ or $K_d$ (the point on the curve at half the maximal value) because this would result in a value in time, and it would be unclear how this variable could be compared between cell lines expressing different levels of carboxylesterases. However, to confirm that these percentage inhibition data that we obtained from these assays were valid for the metabolism of biologically relevant substrates, growth inhibition studies were done using CPT-11 in the presence of selected inhibitors.

The data presented in Fig. 4 and Table 3 confirm that benzil and compound 1 enter cells and inhibit either hiCE or rCE intracellularly, preventing the conversion of CPT-11 to SN-38 in situ. This is exemplified by the increase in the $IC_{50}$ for CPT-11 following exposure of the cells to the inhibitor. Importantly, the $IC_{50}$ for inhibitor-treated cells as indicated in Table 1, showed significant agreement between these results ($r^2 = 0.99$). We did not determine a traditional $K_m$ or $K_d$ (the point on the curve at half the maximal value) because this would result in a value in time, and it would be unclear how this variable could be compared between cell lines expressing different levels of carboxylesterases. However, to confirm that these percentage inhibition data that we obtained from these assays were valid for the metabolism of biologically relevant substrates, growth inhibition studies were done using CPT-11 in the presence of selected inhibitors.

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**Figure 4.** Growth inhibition assays using U373MG cells with CPT-11. A and B, data from U373iRES treated with benzil and compound 1, respectively. C and D, data from U373hiCE treated with benzil and compound 1, respectively. E and F, data from U373rCE treated with benzil and compound 1, respectively. •, cells treated with CPT-11 alone; ▲, cells treated with CPT-11 + inhibitor; ▼, cells treated with inhibitor alone.
was similar to cells that had not been transfected with a carboxylesterase known to activate CPT-11. Hence, benzil or compound 1 can completely reverse the cytotoxicity that is conferred by carboxylesterase expression within these cells. Disappointingly, compound 2, which we identified as a selective hiCE inhibitor (25), did not inhibit hiCE intracellularly. Because we know that this benzene sulfonamide inhibits the enzyme in vitro (25), we concluded that this compound cannot enter cells. We have hypothesized that this class of inhibitors may be useful in ameliorating the delayed diarrhea that occurs in humans following CPT-11 administration by preventing the conversion of CPT-11 to SN-38 in the gut. However, the studies presented here would indicate that the compound is poorly bioavailable, and hence, in contrast to benzil and compound 1, compound 2 must be chemically modified before it has suitable properties for in vivo application. Such studies are currently under way.

Ideally, the development of carboxylesterase inhibitors that would be selectively taken up by normal cells, but not by tumor cells, would be extremely beneficial. These compounds would then accumulate in normal cells and organs, resulting in minimal hydrolysis of CPT-11 and therefore reduced toxicity. In contrast, tumor carboxylesterases would still convert the drug to SN-38, resulting in tumor-specific cytotoxicity and presumably enhanced antitumor activity. Although this hypothesis is attractive, it is currently unclear what the chemical properties of a tumor-specific carboxylesterase inhibitor would be. In addition, the level of accumulation in normal (and tumor) cells would likely be modulated by a variety of transport processes. Hence, future studies with these compounds will evaluate their ability to selectively accumulate in normal versus tumor cells.

In the described study, we have developed a novel rapid assay for determining intracellular carboxylesterase inhibition and evaluated the efficacy of five benzil-based and benzene sulfonamide–based inhibitors. Results indicate that benzil and a 3,4-dimethyl analogue (1) enter cells and inhibit hiCE and rCE intracellularly. This prevents the cytotoxicity of CPT-11. Therefore, these inhibitors may have use in ameliorating the gut toxicity of CPT-11, as the intestinal epithelia express relatively high levels of endogenous carboxylesterases. We are currently developing appropriate animal models to evaluate the applicability of such approaches.

Acknowledgments

We thank Dr. J.P. McGovren for the gift of CPT-11.

References


Table 3. IC50 for cells treated with CPT-11 in combination with carboxylesterase inhibitors

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Carboxylesterase activity (nmol/min/mg ± SD)</th>
<th>Inhibitor</th>
<th>CPT-11 IC50 (μmol/L)</th>
<th>r2 for curve fit</th>
<th>Fold change in IC50 compared with cells not treated with inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRES 8.6 ± 1.1</td>
<td>None</td>
<td>25.1</td>
<td>1.0</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>IRES 8.6 ± 1.1</td>
<td>Benzil</td>
<td>37.2</td>
<td>0.99</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>IRES 12.4 ± 0.4</td>
<td>Compound 1</td>
<td>22.9</td>
<td>1.0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IRES 408 ± 6</td>
<td>None</td>
<td>0.84</td>
<td>0.96</td>
<td>—</td>
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</tr>
<tr>
<td>hiCE 408 ± 6</td>
<td>Benzil</td>
<td>10.6</td>
<td>0.99</td>
<td>12.6</td>
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</tr>
<tr>
<td>hiCE 204 ± 12</td>
<td>None</td>
<td>1.4</td>
<td>0.99</td>
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<tr>
<td>hiCE 204 ± 12</td>
<td>Compound 1</td>
<td>24.3</td>
<td>1.0</td>
<td>17.4</td>
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<tr>
<td>rCE 1,124 ± 121</td>
<td>None</td>
<td>0.3</td>
<td>0.97</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>rCE 1,124 ± 121</td>
<td>Benzil</td>
<td>33.9</td>
<td>0.98</td>
<td>113</td>
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<td>rCE 1,124 ± 121</td>
<td>Compound 1</td>
<td>27.9</td>
<td>1.0</td>
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Intracellular Inhibition of Carboxylesterases
Molecular Cancer Therapeutics

Intracellular inhibition of carboxylesterases by benzil: modulation of CPT-11 cytotoxicity

Janice L. Hyatt, Lyudmila Tsurkan, Monika Wierdl, et al.

*Mol Cancer Ther* 2006;5:2281-2288.

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