Activity of a Novel Camptothecin Analogue, Homocamptothecin, in Camptothecin-resistant Cell Lines with Topoisomerase I Alterations

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Abstract

Homocamptothecin (hCPT), which differs from camptothecin (CPT) by the presence of an additional methylene group in the E-ring, was evaluated in CPT-resistant cell lines. Topoisomerase I (top1)-deficient leukemia P388/CPT45 cells were highly resistant to hCPT, which demonstrates that top1 is the primary target of hCPT. Three CPT-resistant cell lines with top1 point mutations (Chinese hamster lung fibroblast DC3F/C10, human prostate carcinoma DU-145/RC1, and human leukemia CEM/C2) and their top1 enzymes were cross-resistant to hCPT. The antiproliferative activity of hCPT was greater than that of CPT in both parental and CPT-resistant cell lines, particularly in the prostate cell lines. The top1 cleavage complexes formed in the presence of hCPT appear to be more stable than those induced by CPT. Together, these data indicate that hCPT is a specific top1 inhibitor, which shares a common binding site with CPT in the top1-DNA cleavage complexes. Because of its potency, hCPT might overcome resistance to CPT in some cancer cells.

Introduction

CPT derivatives are among the most promising anticancer drugs recently introduced in the clinic. Topotecan (Hycamtin; SmithKline Beecham) has been approved for the treatment of cisplatin-refractory ovarian carcinoma and for second-line therapy in small cell lung cancer. Irinotecan (CPT-11, Camptosar; Pharmacia) has been approved in the United States for the treatment of colorectal cancer. Current clinical trials indicate that CPT derivatives will be useful in a variety of other human malignancies. DNA top1 inhibitors such as CPT and its derivatives convert top1 into a cellular poison by inhibiting the religation step of the DNA nicking-closing reaction and thereby trapping top1 in a covalent complex with DNA. The cytotoxic lesions probably result from stable top1 covalent complexes associated with double-strand breaks that are generated after collision of DNA and RNA polymerases with the top1 cleavage complexes (for review, see Ref. 1). hCPT is a novel CPT analogue that differs from CPT by the presence of an additional methylene group in the E-ring. Thus, hCPT has a seven-member β-hydroxylactone E-ring (Fig. 1; Ref. 2). This modification enhances the stability of the lactone ring, which, in the case of CPT and its derivatives, opens to form an inactive carboxylate derivative at physiological pH (3, 4). Thus, hCPT has a slow and irreversible hydrolysis of the E-ring instead of the fast equilibrium observed in the case of CPT. This enhanced stability may account for the superior activity of hCPT in vivo and in the presence of purified top1 (2, 5). In addition, this E-ring modification decreases the drug binding to human serum albumin (3).

The different mechanisms of resistance to top1 inhibitors can be grouped into three categories (1): (a) the precleavage complex mechanisms related to drug metabolism and uptake; (b) top1 alterations that result in reduced levels of cleavage complexes; (c) the postcleavage complex mechanisms related to the multiple pathways leading to cell death, including apoptosis, cell cycle regulation and checkpoints, and DNA repair (for review, see Ref. 1). In this report, we examine the activity of hCPT in CPT-resistant cell lines with top1 alterations to evaluate the role of top1 in the cytotoxicity of the drug and the effect of known top1 point mutations on the activity of hCPT against top1.

Materials and Methods

Cell Culture, Chemicals, and Enzymes. Human prostate carcinoma DU-145, leukemia CEM cells and their CPT-resistant subclones, DU-145/RC1 (1) and CEM/C2 (6, 7), were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% FCS in a 5% CO2 incubator at 37°C. The CPT-resistant DU-145 subline DU-145/RC1 was established by Dr. Panayotis Pantazis and colleagues (Brown University, Providence, RI). Murine leukemia P388 and its CPT-resistant subclone, P388/CPT45, were provided by Michael R. Mattern and Randall K. Johnson (SmithKline Beecham) and cultured in RPMI 1640 containing 20% FCS and 10 μM β-mercaptoethanol (8, 9). The Chinese hamster lung fibroblast DC3F cell line and its CPT-resistant subline, DC3F/C10 (10), were grown in MEM with Earle’s salt, supplemented with 10% FCS, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (ABI, Columbia, MD). CPT was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Rockville, MD). hCPT was kindly provided by Drs. Dennis Bigg and Olivier Lavergne (Institut Henri Beaufour, Les Ulis, France). Other drugs were purchased from Sigma (St. Louis, MO). (α-32P)GdGTP was purchased from New England Nuclear (Boston, MA). Human top1 was purified from S99 cells by using a baculovirus construct (11).

Cytotoxicity Assays. The MIT assay was used to determine drug sensitivity. Cells (3,000–15,000) were seeded as a suspension (100 μlwell) in 96-well microtiter plates. The cells were incubated at 37°C in the continuous presence of drug for 3 or 5 days. Cell viability was assayed by adding of 50 μl of MTT dye (in PBS). After a 4-h incubation period, during which activated cells reduced the yellow MTT salt to purple formazan, the stain was eluted into the medium by the addition of 100 μl of 2-propanol (containing 0.04 N HCl) or DMSO. Optical densities were measured at 550 nm. Determinations for all experiments were made in triplicate, and the results were expressed as means and SDs.

Preparation of Nuclear Extracts. The method used is a modification of a method described previously (6). Briefly, log-phase cultures containing 107 cells were washed twice at 4°C using nucleus buffer (150 mM NaCl, 1 mM KH2PO4, 5 mM MgCl2, and 1 mM EGTA) and recovered by centrifugation at 200 × g for 10 min. Cell pellets were resuspended in nucleus buffer containing 0.03% Triton X-100. After incubation at 4°C for 10 min, nuclear pellets were recovered by centrifugation at 3,000 × g for 10 min. After washing with ice-cold nucleus buffer, pellets were recovered by centrifugation at 3,000 × g for 10 min. Salt extraction of the nuclear pellet was achieved by adjusting the final NaCl concentration to 0.35M and by gentle mixing at 4°C for 30 min. Supernatants containing salt-soluble material were collected as nuclear extract.

Sequencing of top1-mediated DNA Cleavage Sites. Top1-mediated cleavage sites were mapped in the 161-bp PfuI-HindIII fragment of pBlue-
denaturing polyacrylamide gels (7 M urea) in 1 and 0.1% bromphenol blue (pH 8.0). Reaction products were separated in 7% PAGE. 

**Fig. 1. Structure of 20-S-CPT and 20-R-hCPT.**

**Fig. 2. Schematic representation of human top1 with mutations leading to CPT resistance. The COOH-terminal domain, the linker region, the core domain, and the NH2 terminus domain are indicated from right to left as G, C, E, and L, respectively. Mutations (and the corresponding cell lines) used in the present study are shown: G364H (DU-145/RC1); G503S (DC3F/C10); and N722S (CEM/C2). Asterisks indicate the position of other CPT resistance mutations (for further details, see Ref. 1).**

**Table 1** Cross-resistance of CPT-resistant cell lines to hCPT

<table>
<thead>
<tr>
<th>CEM</th>
<th>CEM/C2</th>
<th>DC3F</th>
<th>DC3F/C10</th>
<th>DU-145</th>
<th>DU-145/RC1</th>
<th>P388</th>
<th>P388/CPT45</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 CPT (μM)a</td>
<td>0.009 ± 0.01</td>
<td>5.4 ± 2.9</td>
<td>0.07 ± 0.01</td>
<td>5.9 ± 1.8</td>
<td>0.06 ± 0.06</td>
<td>21.7 ± 7.6</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>RR</td>
<td>0.028 ± 0.0099</td>
<td>7.0 ± 3.5</td>
<td>0.02 ± 0.01</td>
<td>0.45 ± 0.32</td>
<td>0.004 ± 0.0003</td>
<td>0.67 ± 0.12</td>
<td>0.014 ± 0.005</td>
</tr>
<tr>
<td>IC50 hCPT (μM)b</td>
<td>0.06 ± 0.003</td>
<td>84</td>
<td>23</td>
<td>361</td>
<td>168</td>
<td>60 ± 26</td>
<td>4286</td>
</tr>
<tr>
<td>RRb</td>
<td>2500</td>
<td>23</td>
<td>23</td>
<td>168</td>
<td>168</td>
<td>4286</td>
<td></td>
</tr>
</tbody>
</table>

*a Drug concentrations producing 50% cell growth inhibition (IC50 ± SD) were calculated from at least three independent experiments such as those represented in Fig. 3.

*b Relative resistance (RR) was calculated as the IC50 ratio between CPT-resistant cells and the corresponding parental cells.

Because hCPT contains a seven-member α-hydroxylactone in place of the α-hydroxylactone found in CPT and its derivatives, this modification results in a more stable lactone and reduces the conversion of the drug to its carboxylate derivative (2), which, in the case of CPTs, is inactive against top1 (1) and is highly bound to human serum albumin (15). Decreased binding to human plasma protein has been observed consistently for hCPTs (3, 4). hCPTs are also more potent top1 inhibitors than the corresponding CPT derivatives both in the presence of purified top1 (2) and in cancer cells, including some

Discussion

hCPT contains a seven-member β-hydroxylactone in place of the six-member α-hydroxylactone found in CPT and its derivatives. This modification results in a more stable lactone and reduces the conversion of the drug to its carboxylate derivative (2), which, in the case of CPTs, is inactive against top1 (1) and is highly bound to human serum albumin (15). Decreased binding to human plasma protein has been observed consistently for hCPTs (3, 4). hCPTs are also more potent top1 inhibitors than the corresponding CPT derivatives both in the presence of purified top1 (2) and in cancer cells, including some
multidrug-resistant cells (2, 3). Until now, little was known about the activity of hCPT in CPT-resistant cells.

The high levels of resistance (>4000-fold; Table 1) of the top1-deficient p388/CPT45 cells (8, 9, 13) to hCPT and the cross-resistance of the three cell lines with top1 point mutations demonstrate that top1 is the cellular target of hCPT. Although all of the CPT-resistant cells were cross-resistant to hCPT, hCPT was more efficient than CPT in both parental and CPT-resistant cell lines. hCPT appears to be relatively more potent than CPT in the diverse mammalian cell lines examined: human prostate carcinoma; human leukemia; murine leukemia; and Chinese hamster lung fibroblasts (see Fig. 3 and Table 1). It is noticeable that, in the parental human prostate DU-145 cells, hCPT was 15-fold more effective than CPT. Recently Philippart et al. (5) compared the antiproliferative activity of CPT, topotecan, and SN-38 against that of two new hCPTs, BN80245 and BN80915, in 48 colon cancer cell types. The two hCPT derivatives exerted greater antiproliferative activity than the three anti-top1 reference compounds, with the highest activity observed for BN80915. We find that the IC_{50} values for hCPT in two of the CPT-resistant cell lines, DC3F/C10 and DU-145/RC1, are 10-fold lower than that in the CEM/C2 cell line (0.45, 0.67, and 7 μM, respectively). Thus, hCPT can be effective at submicromolar concentrations in two of the CPT-resistant cell lines (Table 1), which suggests that because of its enhanced potency, hCPT could potentially overcome resistance to CPT in some cancer cells.

As shown in Fig. 2, the top1 point mutations leading to CPT resistance cluster in different regions that are highly conserved among eukaryotic top1s. According to crystal structure data, the top1 residues that are mutated in CPT-resistant cells and belong to the core domain are in close proximity to the catalytic tyrosine (Y723), the other residues involved in CPT resistance in the COOH-terminal domain and the bound DNA (1, 16). It is interesting to note that, in the present study, the three mutations led to resistance to both CPT and hCPT in the top1-DNA cleavage Fig. 3. Cross-resistance of CPT-resistant cell lines to hCPT. Parental cells [CEM, DC3F, DU-145, and P388 (•)] and their top1-altered derivative cell lines [CEM/C2, DC3F/C10, DU-145/RC1, and P388/CPT45 (○)] were treated with the indicated concentrations of CPT (top panels) or hCPT (bottom panels). Cell proliferation was measured by MTT assay after 72 h of continuous drug exposure. SDs were calculated from at least three independent experiments.

Fig. 4. Reduced DNA cleavage induced by hCPT in nuclear extracts from CPT-resistant cell lines. Nuclear extracts from wild-type (WT; parental) and CPT-resistant cells with top1 point mutations were used for cleavage assay. A 32P-end-labeled PvuII-HindIII 161-bp fragment from pBluescript was incubated with purified top1 or nuclear extracts (A, CEM and CEM/C2; B, DC3F and DC3F/C10; C, DU-145 and DU-145/RC1) in the presence or absence of CPT or hCPT at room temperature for 30 min. The mutations are indicated in parentheses at the top of the figure (see Fig. 1). CPT was used at 1 μM, and 0.1, 1, and 10 represent the concentrations of hCPT (μM). Lane C, control DNA without top1 or drug. Reactions were stopped with SDS and resolved by 7% sequencing gels. Imaging and quantification were performed with a PhosphorImager.
assays. This cross-resistance suggests that the two drugs share a common binding site in the top1-DNA complex. In the case of CPTs, this binding site has been proposed to be at the enzyme-DNA interface, with the drug stacked along the base pairs flanking the DNA cleavage site (16–18). We also found that, in the DNA segment examined, there was no difference in DNA cleavage patterns between CPT and hCPT. It should be noted, however, that some differences have been reported in the sequence specificity of drug-induced DNA cleavages in other DNA fragments (14). It is also noticeable that the top1 cleavage complexes formed in the presence of hCPT are less reversible in high salt than those induced by CPT (see Fig. 5; Ref. 14). Greater stability of top1 cleavage complexes can contribute to the greater cytotoxicity of top inhibitors (1). Together, these observations indicate that hCPT and CPT probably bind to a common binding site in the top1-DNA complexes and exert their antiproliferative activity in a top1-dependent manner. Stability of the lactone ring and enhanced molecular interactions with the top1-DNA complexes probably account for the greater antiproliferative activity of hCPT in all cell lines examined, including the CPT-resistant cell lines.

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References
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