Water Soluble 20(S)-Glycinate Esters of 10,11-Methylenedioxy camptothecins Are Highly Active against Human Breast Cancer Xenografts

Randy M. Wadkins, Philip M. Potter, Bogdan Vladu, Jennifer Marty, Gina Mangold, Steve Weitman, Govindarajan Manikumar, Mansukh C. Wani, Monroe E. Wall, and Daniel D. Von Hoff

Abstract

Water-soluble 20(S)-glycinate esters of two highly potent 10,11-methylenedioxy analogues of camptothecin (CPT) have been synthesized and evaluated for their ability to eradicate human breast cancer tumor xenografts. The glycinate ester moiety increases the water solubility of the 10,11-methylenedioxy analogues 4–16-fold. However, in contrast to CPT-11, a water-soluble CPT analogue that was recently approved for second line treatment of colorectal cancer, the 20(S)-glycinate esters do not require carboxylesterase for conversion to their active forms. The glycinate esters are hydrolyzed to their parent, free 20(S)-hydroxyl active analogues in phosphate buffer (pH 7.5) and in mouse and human plasma. The glycinate esters are also 20–40-fold less potent than CPT-11 in inhibiting human acetylcholinesterase. In vivo, we examined 20(S)-glycinate-10,11-methylenedioxy camptothecin, 20(S)-glycinate-7-chloromethyl-10,11-methylenedioxy camptothecin, and CPT-11. We found that the two 10,11-methylenedioxy analogues had antitumor activity against breast cancer xenografts that was comparable to that of CPT-11. Our results indicate that water-soluble 20(S)-glycinate esters of highly potent CPT analogues provide compounds that maintain biological activity, do not require interactions with carboxylesterases, and do not inhibit human acetylcholinesterase.

Introduction

In 1966, Wall et al. (1) discovered that CPT (2) was the component in the extract from the stem of the Chinese tree Camptotheca acuminata that was active against L1210 murine leukemia cells. Early clinical trials with the water-soluble, E-ring open lactone sodium salt of CPT afforded results that were inconclusive (2–4). It was soon discovered that this form of CPT was an inactive one, and clinical trials were discontinued.

Further development of topo I inhibitors for cancer therapy was stimulated by the characterization of CPT as a specific topo I inhibitor (5, 6). topo I relaxes DNA supercoiling by making transient single-strand breaks (7, 8). These breaks are coupled with the transient formation of a covalent DNA-enzyme intermediate termed the cleavable complex (5, 6). CPT and analogues specifically and reversibly stabilize cleavable complexes by inhibiting their religation (reviewed in Ref. 9). The mechanism of CPT cytotoxicity is thought to be the consequence of a collision between moving replication forks and CPT-stabilized cleavable DNA-topo I complexes (6, 10, 11).

CPT-11 (7-ethyl-10-[4-[1-piperidino]-1-piperidino]carbonyloxy camptothecin Irinotecan; Camptosar) was developed as a water-soluble CPT analogue (12). It has recently received regulatory approval in the United States and elsewhere for patients with metastatic colorectal carcinoma who have previously received 5-fluorouracil. This analogue of 10-hydroxycamptothecin possesses an ethyl group at the 7-position of CPT and a piperidino-1-piperidino carbamate ester at the 10-position. The latter moiety conveys water solubility and makes CPT-11 a prodrug that undergoes de-esterification by carboxylesterases in vivo to yield SN-38 (7-ethyl-10-hydroxycamptothecin).

In clinical use of CPT-11 in North America, neutropenia and diarrhea are the most common toxicities encountered, with diarrhea occurring in two forms. The most common form of diarrhea, a delayed-onset diarrhea, occurs usually after the second or third dose of CPT-11. The other form is a dose-related toxicity occurring during peritreatment period. This latter, acute form of diarrhea is usually associated with abdominal cramping, vomiting, flushing, visual (accommodation) disturbances, lacrimation, salivation, bradycardia, and diaphoresis (13, 14). These effects appear to be due to the cholineric actions of CPT-11, which is an inhibitor of AcChE in vitro. The inhibition of AcChE has recently been traced to the 1-(piperidino-1-piperidino) moiety of CPT-11 (15). The extreme effects of diarrhea can be mitigated both by the dosage control and regime and aggressive use of loperamide or atropine (16, 17).

Upon i.v. infusion, CPT-11 plasma concentrations are maximal immediately after the end of the infusion, whereas SN-38 plasma concentrations peak ~2 h later. Significant interpatient variability in plasma concentrations and area under the concentration-time curve are observed with CPT-11 (18–22). The variability in the kinetics of SN-38 formation, as well as in peak SN-38 concentrations, suggests that variations in carboxylesterase converting enzymes may, in part, account for these effects, and consequently, this variability may be a critical determinant of toxicity and response.

A number of other 7- and 10-substituted and 10,11-disubstituted CPTs have been developed (23–28). These include the highly potent MDCPT and CMMD (26, 28), the structures of which are given in Fig. 1. The latter compound is of particular interest because it is capable of forming a covalent complex with DNA through nucleophilic displacement of the chlorine moiety by DNA while it is in the cleavable complex. The cleavable complexes formed with MDCPT and CMMD show a longer lifetime than those formed with SN-38 (26), and longer lifetimes of cleavable complexes have been implicated as important for biological activity of CPT analogues (29). Thus, the development of water-soluble analogues of MDCPT was performed, with the specific intent of developing compounds that improve water solubility without sacrificing the stability of the E-ring ketone (critical for biological activity) and that do not require enzymatic conversion to the active compound.

Analogs of MDCPT and CMMD have been developed that would appear to satisfy this search. Substitution at the 20(S)-position
of CPT with a glycinate ester has recently been reported (24). The 20(S)-glycinate esters are more water-soluble than their parent compounds. The glycinites are converted by aqueous hydrolysis to their active 20(S)-hydroxy forms on a time scale comparable to in vivo conversion of CPT-11 to SN-38 by esterases. In this report, we describe the in vivo use of the glycinate esters of the highly potent MDCPT and CMMD analogues for treatment of human breast cancer xenografts and demonstrate that these analogues are as active as CPT-11 but may avoid some problems that are observed clinically with CPT-11.

**MATERIALS AND METHODS**

**CPT Analogues.** The synthesis of MDCPT, MDCPT-Gly, CMMD, and CMMD-Gly (Fig. 1) used in these studies has been described elsewhere (24, 27). CPT-11 and SN-38 for in vitro studies were synthesized as described previously (12). CPT-11 for the in vivo studies was obtained from the Cancer Therapy and Research Center pharmacy, as prepared by Pharmacia-Upjohn Pharmaceuticals (Kalamazoo, MI).

**Aqueous Solubility and Stability of CPT Analogues.** The enhanced solubility of the glycinate esters or CPT-11 analogues versus their unesterified parent compounds was determined using octanol-water partitioning. A ~10 µM solution of each analogue was prepared by diluting a DMSO stock solution of each into 2 ml of 1-octanol. The prepared solution was measured, and the solution subsequently vigorously extracted for 2 min with an equal volume of 0.1 M sodium acetate (pH 5.0). Following extraction, the fluorescence from the octanol solution was again measured, and the octanol-buffer partition coefficient p was determined from the ratio of the initial to final octanol solution fluorescence.

The kinetics of conversion of MDCPT-Gly and CMMD-Gly to their parent compounds was measured by HPLC. MDCPT-Gly or CMMD-Gly were dissolved in PBS (pH 7.5) at 1 mg/ml. The solutions were incubated at 37.5°C with stirring. Aliquots of the solution were removed at 0, 0.5, 1, 3, 6, and 24 h. Samples were extracted with CHCl3:methanol (4:1, v/v), and the organic layer was dried in vacuo. The resulting dry extract was redissolved in methanol and run on a C18 reverse-phase HPLC column, with methanol:water (3:2, v/v) as the mobile phase. Elution of MDCPT redissolved in methanol and run on a C18 reverse-phase HPLC column, with methanol:water (3:2, v/v) as the mobile phase. Detection of products was via fluorescence using an excitation wavelength of 365 nm and an emission wavelength of 445 nm.

**In Vitro Growth-Inhibitory Activity.** MDCPT, CMMD, and SN-38 were evaluated in growth-inhibitory studies against human breast carcinoma lines MDA-231, ZR-75, and BT-20. These lines were maintained in Isovex’s MEM supplemented with 10% fetal bovine serum at 37°C in 5% CO2 incubators until they were plated for use in MTT assays. ZR-75 growth medium also contained 10 µg/ml bovine insulin.

Exponentially growing cells (1 × 10^3–2 × 10^5 cells, unless otherwise specified) in 0.1 ml of medium were seeded on day 0 in a 96-well microtiter plate. On day 1, 0.1-ml aliquots of medium containing graded concentrations of test analogues were added in triplicate to the cell plates. After incubation at 37°C in a humidified incubator with 5% CO2-95% air for 3 days, the plates were centrifuged briefly, and 100 µl of the growth medium were removed. Cell cultures were incubated with 50 µl of MTT (1 mg/ml in Dulbecco’s PBS) for 4 h at 37°C. The resulting purple formazan precipitate was solubilized with 200 µl of 0.04 N HCl in isopropyl alcohol. Absorbance was monitored in a Bio-Rad Model 3550 Microplate Reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

**Activity of MDCPT-Gly Against Breast Cancer.** CPT analogue and SN-38 were evaluated in growth-inhibitory studies against human breast carcinoma lines MDA-231, ZR-75, and BT-20. These lines were maintained in Isovex’s MEM supplemented with 10% fetal bovine serum at 37°C in 5% CO2 incubators until they were plated for use in MTT assays. ZR-75 growth medium also contained 10 µg/ml bovine insulin.

**AcChE Inhibition Assays.** Human AcChE was obtained from Sigma Chemical Co. (St. Louis, MO). Inhibition of the enzyme by the CPT analogues was determined using 3 mM o-nitrophenyl acetate, as described previously (30, 31). IC50s were determined using at least six concentrations of CPT analogue. Inhibition measurements were made within 10 min of addition of glycinate esters such that only trivial amounts of the nonglycinate parents were present in solution.

In vivo hydrolysis of MDCPT-Gly and CMMD-Gly glycinate esters was monitored by injecting B6D2F1 mice i.p. with the MTD of MDCPT-Gly or CMMD-Gly (10 or 15 mg/kg, respectively). At 0.25, 0.5, 1, 2, 4, and 24 h, blood samples were taken from three mice and combined, and the plasma was isolated. Plasma samples were snap-frozen with liquid nitrogen until they were processed.

A 50-µl aliquot of the plasma samples was mixed with 300 µl of 1 N HCl in methanol to precipitate plasma proteins. The samples were briefly centrifuged to remove precipitated protein. The clarified methanol solutions were analyzed by HPLC using a C8 column with 10 mM potassium phosphate buffer (pH 2.5): methanol (52:48) as the mobile phase. Detection of products was via fluorescence using an excitation wavelength of 365 nm and an emission wavelength of 445 nm. Under these conditions, the glycinate esters were present in four forms: protonated glycinate ester of the lactone, protonated glycinate of the hydroxy acid, and nonprotonated glycinate esters of the lactone and hydroxy acid. These four hydrophilic forms of MDCPT-Gly and CMMD-Gly eluted as broad peaks at 4–10 or 2–8 min, respectively, after injection. These broad peaks were integrated together and are considered total glycinate. The plasma total glycinate concentrations of both compounds peaked after 0.5 h and then decayed as a single exponential with time. The in vivo half-lives for the MDCPT-Gly and CMMD-Gly were 0.5 and 1.3 h, respectively. This conversion rate is only slightly faster than that described above for buffer.

Under our HPLC conditions, the released active form of the drugs eluted as a single peak at 12–14 and 10–11 min for MDCPT and CMMD, respectively. In vivo, only a small amount of free CMMD could be detected relative to the CMMD-Gly. We interpret this to mean that, because CMMD is a reactive compound, it covalently binds with serum proteins or other macromolecules and cannot be efficiently extracted from plasma. MDCPT, however, could be easily detected. Plasma levels of MDCPT peaked at 2 h following i.p. injection. Hence, disappearance of the glycinites corresponded with appearance of the active form of the drug.

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Table 2. Inhibition of human acetylcholinesterase by CPT analogues

<table>
<thead>
<tr>
<th>CPT analogues</th>
<th>IC₅₀ (µM)</th>
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<tbody>
<tr>
<td>MDCPT-Gly</td>
<td>19.5 ± 6.5</td>
</tr>
<tr>
<td>CMMD-Gly</td>
<td>10.3 ± 2.5</td>
</tr>
<tr>
<td>SN-38</td>
<td>19.4 ± 10.3</td>
</tr>
<tr>
<td>CPT-11</td>
<td>0.52 ± 0.07</td>
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</tbody>
</table>

*Concentration of analogue needed to inhibit human AcChE activity by 50%, at 3 mM o-nitrophenyl acetate.

Table 3. Growth-inhibitory activity of CPT analogues against selected breast cancer cell lines

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ vs. cell line (µM)</th>
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</thead>
<tbody>
<tr>
<td>CPT</td>
<td>216.8 ± 22.1</td>
</tr>
<tr>
<td>MDCPT</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>CMMD</td>
<td>10.1 ± 0.8</td>
</tr>
<tr>
<td>SN-38</td>
<td>9.7 ± 5.6</td>
</tr>
<tr>
<td>ZR-75</td>
<td>85.1 ± 16.8</td>
</tr>
<tr>
<td>MDA-231</td>
<td>5.1 ± 2.1</td>
</tr>
<tr>
<td>BT-20</td>
<td>12.8 ± 6.7</td>
</tr>
<tr>
<td></td>
<td>330.4 ± 20.4</td>
</tr>
<tr>
<td></td>
<td>6.8 ± 0.8</td>
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<tr>
<td></td>
<td>1.4 ± 1.8</td>
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<td>177.8 ± 25.4</td>
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</table>

In Vivo Activity of CMMD-Gly and MDCPT-Gly. Female nude mice weighing ~20 g were implanted s.c. by trocar with fragments of either MDA-231 or MX-1 human breast carcinomas harvested from s.c. growing tumors in nude mice hosts. When tumors were ~5 mm × 5 mm in size (10 days after inoculation for MDA-231 and 12 days after inoculation for MX-1), the animals were pair-matched into treatment and control groups. Each group contained eight mice with tumors, each of which was ear-tagged and followed individually throughout the experiment. The administration of drugs or vehicle began the day the animals were pair-matched (day 1), and all injections were performed i.p. The glycinate esters were formulated for injection in 0.25% methylcellulose-2% Tween 80. For MDA-231, MDCPT-Gly was administered at 1.0 and 0.5 mg/kg on a qd×5 schedule and at 10.0 and 5.0 mg/kg on a qd×1 schedule. CMMD-Gly was administered at 7.5 and 3.75 mg/kg on a qd×5 schedule and at 15.0 and 7.5 mg/kg on a qd×1 schedule. These concentrations were the MTD and half-MTD for each compound. CPT-11 was used as a positive control and was given at 0.1 mg/kg on a once-a-week for 3 weeks schedule. For MX-1, MDCPT-Gly was administered at 0.5 and 0.25 mg/kg on a qd×1 schedule and at 5.0 and 2.5 mg/kg on a qd×1 schedule. CMMD-Gly was administered at 7.5 and 3.75 mg/kg on a qd×5 schedule and at 15.0 and 7.5 mg/kg on a qd×1 schedule. CPT-11 was used as a positive control and was given at 100 mg/kg on a once-a-week for 3 weeks schedule.

Mice were weighed twice weekly, and tumor measurements were taken by calipers twice weekly, starting on day 1. These tumor measurements were converted to tumor weight (in mg) by the formula L² × W/2 (where L is length and W is width), and from these calculated tumor weights, the termination date was determined. The experiment was terminated when control tumors reached a size of 2000 mg. Upon termination, all mice were weighed and sacrificed, and their tumors were excised. Tumors were weighed, and the mean tumor weight per group was calculated. In this model, the value [mean treated tumor weight (T)]/ [mean control tumor weight (C)] × 100% was subtracted from 100% to give the TGI for each group.

With these agents, the final weight of a given tumor was subtracted from its own weight at the start of treatment on day 1. This difference divided by the initial tumor weight is the percentage shrinkage. A mean percentage tumor shrinkage was calculated from data from the mice in a group that experienced regressions.

RESULTS

Aqueous Solubility of CPT Analogues. The octanol:buffer partition coefficient p is given for the six CPT analogues in Table 1. The piperidino-1-piperidino carbamate ester of CPT-11 doubled the aqueous solubility of this compound relative to SN-38. Both MDCPT and CMMD were substantially less soluble than SN-38 in an aqueous solution. However, addition of the 20(S)-glycinate in MDCPT-Gly and CMMD-Gly increased the solubility of these compounds 16- and 4-fold, respectively. The glycinate showed similar solubility to SN-38 but were slightly less soluble than CPT-11.

Inhibition of AcChE. As shown in Table 2, CPT-11 was a potent inhibitor of human AcChE. This is presumably due to the piperidino-1-piperidino carbamate group of CPT-11 because SN-38 was 37-fold less effective as an inhibitor of AcChE. In contrast to CPT-11, both MDCPT-Gly and CMMD-Gly were much less potent (20–38-fold) as inhibitors of AcChE.

Cellular Growth Inhibition by CPT Analogues. The three parent CPT analogues MDCPT, CMMD, and SN-38 were examined for their ability to inhibit growth of three breast carcinoma cell lines in vitro. The IC₅₀ values for growth inhibition are given in Table 3. MDCPT and CMMD were substantially more active against BT-20 cells than SN-38. MDCPT and CMMD were also significantly more potent against MDA-231 cells than was SN-38. However, all three compounds were of similar toxicity to ZR-75 cells.

In Vivo Studies of the Antitumor Activity of Water-soluble Forms of MDCPT and CMMD. The results of the glycinate compounds against MX-1 human breast tumor and MDA-231 human breast tumor are given in Figs. 2 and 3, respectively, and are compared...
to those of CPT-11. MDCPT-Gly and CMMD-Gly both showed considerable antitumor activity against the MX-1 human breast tumor xenograft model (Fig. 2). MDCPT-Gly at 0.25 and 0.5 mg/kg on a qd×5 schedule resulted in three of eight and eight of eight complete responses, respectively. MDCPT-Gly at 5 mg/kg on a qd×1 schedule produced seven complete responses, whereas one complete response was noted at 2.5 mg/kg using the same dosing schema. MDCPT-Gly was, in general, well tolerated, with one toxic death at 5 mg/kg on a qd×1 schedule.

CMMD-Gly at 3.75 and 7.5 mg/kg on a qd×5 schedule resulted in complete responses in all animals bearing MX-1 tumors. When this agent was administered on the qd×1 schedule, three and one complete responses were seen at 7.5 and 15 mg/kg, respectively. The mean final tumor weight was 868.4 mg at 7.5 mg/kg and 177.5 mg at 15 mg/kg (mean weight of controls, 1227.4 mg) using the qd×1 schedule. CMMD-Gly was well tolerated with no toxic deaths or substantial change in body weight. CPT-11 (100 mg/kg) was used as a positive control and produced seven of eight complete responses using a once-a-week for 3 weeks schedule.

Against MDA-231 (Fig. 3), MDCPT-Gly at 1 mg/kg on a qd×5 schedule was too toxic (eight of eight toxic deaths) to evaluate the efficacy of this agent. Using this schedule at 0.5 mg/kg, we observed no antitumor activity. A mean final tumor weight of 2414.5 mg compared to 1959.5 mg in vehicle controls and 507.3 mg in animals treated with CPT-11 resulted. MDCPT-Gly at 10 mg/kg on a qd×1 schedule was, again, too toxic to evaluate antitumor activity of this agent. When this agent was administered at 5 mg/kg on the qd×1 schedule, the final tumor weight (673.0 mg; TGI = 68.0) was significantly (P < 0.05) decreased compared to controls and was comparable to animals treated with CPT-11 (507.3 mg; TGI = 73.0). There were no partial or complete responses observed with administration of MDCPT-Gly using this tumor model.

CMMD-Gly at 3.75 mg/kg on a qd×5 schedule was inactive against the MDA-231 tumor model. When this agent was administered at 7.5 mg/kg on the same schedule, modest antitumor activity was observed with a final tumor weight of 1080.5 and TGI of 46.5. CMMD-Gly at 7.5 and 15 mg/kg on a qd×1 schedule was inactive against this tumor model. No partial or complete responses were noted with CMMD-Gly and the MDA-231 tumor model. This agent was well tolerated, with no toxic deaths or substantial change in body weight.

Administration of CPT-11 resulted in a TGI of 73.3 with one partial response of 53%. The mean final tumor weight following CPT-11 (507.3, CPT-11 versus 1959.5, controls) administration was significantly less than that of vehicle-treated controls.

DISCUSSION

In this report, we examined the use of 20(S)-glycinate esters of highly potent 10,11-methylenedioxy analogues of CPT, MDCPT, and CMMD. The parent compounds MDCPT and CMMD were as active or were significantly more active than SN-38 against three breast cancer cell lines. However, MDCPT and CMMD were substantially less water soluble than SN-38 or CPT-11, making them difficult to use in animal studies. In contrast, the glycinate esters of these compounds are significantly more water soluble, and have been used for in vivo studies against MX-1 and MDA-231 breast cancer models. Importantly, the glycinate esters are not inhibitors of human AcChE, nor do they require enzymatic conversion to the parent, active analogues.

The glycinate esters of MDCPT and CMMD were examined in vivo by comparing MDCPT-Gly, CMMD-Gly, and CPT-11 against MX-1 and MDA-231 human tumor xenografts (Figs. 2 and 3). In these studies, MDCPT-Gly and CMMD-Gly showed similar activity against both tumor models and were either equivalent to CPT-11 (in the MX-1 model; Fig. 2) or only slightly less active than CPT-11 (in the MDA-231 model; Fig. 3). Although CMMD is capable of topo I-mediated alkylation of DNA (26, 28), this did not result in enhanced activity of CMMD against tumor xenografts in the animal models using our dosing schedule.

A number of attempts have been made to modify the 20(S)-OH group of CPT, either for improved water-solubility or to prevent opening of the E-ring lactone. These included replacing the 20(S)-OH group with hydrogen and fluorine and derivatizing the 20(S)-OH group with acetylation (24, 25, 32, 33). None of these modifications were successful because all of these changes have resulted in inactive, less cytotoxic compounds. Our results suggest that 20(S)-glycinate esters of CPT and its more potent analogues can be synthesized and used clinically when water solubility is needed in an analogue. The 20(S)-glycinate analogues enhance water solubility without sacrificing the activity of the compounds. Furthermore, the poor inhibition of AcChE by the glycinate esters as compared to CPT-11 suggests that...
the cholinergic phenomena associated with CPT-11 may be avoided by using glycinate esters as solubilizing moieties.

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