Chimmitecan, a Novel 9-Substituted Camptothecin, with Improved Anticancer Pharmacologic Profiles In vitro and In vivo

Min Huang,1 Heyong Gao,2 Yi Chen,1 Hong Zhu,1 Yujun Cai,1 Xiongwen Zhang,1 Zehong Miao,1 Huiliang Jiang,3 Jian Zhang,3 Hongwu Shen,4 Liping Lin,1 Wei Lu,2 and Jian Ding1

Abstract Purpose: This study aimed to evaluate antitumor activities and pharmacologic profiles of chimmitecan, a novel 9-small-alkyl – substituted lipophilic camptothecin, in comparison with irinotecan (CPT-11) and topotecan.

Experimental Design: The in vitro cytotoxicities of chimmitecan in human tumor cell lines and multidrug resistance (MDR) cells were evaluated by 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide and sulfhorhadamin B assays. DNA relaxation, cleavage, and cellular band depletion assay were combined to delineate its effects on topoisomerase I. DNA damage, cell cycle arrest, and apoptosis were assessed using comet assay, flow cytometry, and DNA ladder analysis, respectively. The in vivo antitumor activities were measured in nude mice bearing human tumor xenografts.

Results: Chimmitecan displayed more potent cytotoxicity than SN38 and topotecan. Neither a cross-resistance to chimmitecan in MDR cells nor an influence of human serum albumin in its cytotoxicity was observed. Chimmitecan exhibited comparable effects on topoisomerase I compared with the reference drugs, including inhibiting topoisomerase I catalytic activity and trapping and stabilizing covalent topoisomerase I-DNA complexes. Furthermore, nanomolar levels of chimmitecan caused impressive DNA damage, G2-M phase arrest, and apoptosis in human leukemia HL60 cells. I.v. administration of chimmitecan inhibited the growth of HCT-116, MDA-MB-435, BEL-7402, and A549 human carcinoma xenografts in nude mice, with greater potency than CPT-11 against the latter two tumors models. Chimmitecan presented potent efficacy in A549 tumor model when given orally.

Conclusions: Chimmitecan is a potent inhibitor of topoisomerase I and displays outstanding antitumor activity in vitro and in vivo. The substitution at the 9-position benefits chimmitecan a salient anti-MDR activity, stability in human serum albumin, improved solubility, and oral availability, which might favorably promise its therapeutic potential in clinical settings.

Camptothecin, a pentacyclic alkaloid originally isolated from Camptotheca acuminata by Wall and Wani in 1966 (1), is one of the most important lead compound in anticancer research. The antitumor activity of camptothecin is thought to be due to its ability to stabilize the reversible covalent DNA-topoisomerase I complex (2–4). Elucidation of the specific target and mechanisms of camptothecin has stimulated intensive efforts to identify novel analogues that overcome the drawbacks of the natural camptothecin molecule, which include low solubility in water; severe and unpredictable toxicity, including hemorrhagic cystitis; reversibility of the drug-target interaction; lactone instability; and drug resistance (5–7). One of the initial major strategies in this regard has been to improve the solubility of the natural camptothecin by chemical modification (5, 8). This approach has produced different series of water-soluble analogues or water-soluble produgs, among which topotecan and irinotecan (CPT-11) are the most successful.

Recent evidence indicates that the lipophilicity confers that camptothecins improved cytotoxicity and inhibition of topoisomerase I (7, 9, 10) over the water-soluble analogues. Therefore, considerable effort has been applied to the development of novel lipophilic derivatives in hopes of favoring for improved uptake, intracellular accumulation, lactone stability, drug-target interaction, and ternary complex stabilization (5). The 7-substituted lipophilic camptothecins are particularly potent and have improved antitumor profiles. Of these, gimatecan and BNP-1350 have been selected for...
clinical trials (5, 7, 10, 11). This has spurred an increase in the development of new camptothecin-based lipophilic analogues.

Except for the serial derivatives of topotecan, 9-nitrocamptothecin and 9-amino-camptothecin, the effects of modifications at C-9 have not been examined. In the present study, we synthesized a novel series of camptothecin analogues with small alkyl (allyl, propyl, ethyl, and methyl) groups at the 9-position and a hydroxy or a methyloxy group at the 10-position. Except 9-methyl-10-methoxy-camptothecin, all of the new analogues inhibited topoisomerase I activity and the growth of P388 mouse leukemia cells (IC50 = 0.04–0.5 μmol/L). In the further exploration, 9-allyl-10-hydroxy-camptothecin, designated as chimmitecan (Fig. 1), stood out for its especially favorable characteristics as a candidate antitumor compound.

This study compares antitumor efficiency of chimmitecan with that of topotecan and CPT-11 (or its active form SN38), and in particular, the contributive roles of C-7 and C-9 alkyl substitutions to the mother compounds. Our results indicated that a small alkyl 9-position substitution for camptothecin was critical for the equivalent or even superior antitumor efficacy compared with 7-substituted ones, promising chimmitecan a potential antitumor candidate for clinical development.

Materials and Methods

**Materials.** Chimmitecan was produced by etherization of 10-hydroxy-camptothecin with allyl bromide in the presence of a base (usually K2CO3) followed by a Claisen rearrangement (12). The purity was 98.6% as determined by high-performance liquid chromatography. Camptothecin and 10-hydroxy-camptothecin were purchased from HSFY Pharmaceutical Co., Ltd. (Hubei, China). Topotecan, CPT-11, and SN38 were produced from 10-hydroxy-camptothecin, and 9-nitrocamptothecin was produced from camptothecin, and their purities were >98%. Doxorubicin and vincristine were purchased from Sigma (St. Louis, MO). All compounds were dissolved at 10 mmol/L in DMSO or normal saline as stock solutions for in vitro or in vivo studies, respectively, and stored at −20°C. Dilutions of the compounds were made up in normal saline before each experiment. The final DMSO concentration did not exceed 0.1% (v/v), and 0.1% DMSO was used as a vehicle control for in vitro experiments.

**Animals.** BALB/c nu/nu female mice ages 4 to 5 weeks were bred in the Shanghai Institute of Materia Medica (Shanghai, China). The animals were housed in sterile cages within laminar airflow hoods in a specific pathogen-free room with a 12-h light/12-h dark schedule and fed autoclaved chow and water ad libitum. All experiments were done according to institutional ethical guidelines on animal care.

**Cell lines.** The human gastric adenocarcinoma SGC-7901, hepatocellular carcinoma BEL-7402 and SMMC-7721, and the ovarian epithelial carcinoma HO-8910 cell lines were obtained from the cell bank of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Human promyelocytic leukemia HL-60, chronic myelogenous leukemia K562, lung adenocarcinoma A549, hepatocellular carcinoma HepG2, colorectal adenocarcinoma HT-29, oral epidermoid carcinoma KB, and cervical carcinoma HeLa cells were purchased from the American Type Culture Collection (Manassas, VA). Human gastric adenocarcinoma MKN-28 and MKN-45, colorectal carcinoma HCT-116 and HCT-15, breast carcinoma MCF-7, MDA-MB-435, and MDA-MB-468; and ovarian carcinoma OVCAR-5 and SK-OV-3 cell lines were from the Japanese Foundation of Cancer Research (Tokyo, Japan). The rhabdomyosarcoma cell line Rh30 was a gift from St. Jude Children’s Research Hospital (Memphis, TN). Doxorubicin-selected multidrug resistant (MDR) cell sublines K562/A02 (13) and MCF-7/ADR (14, 15) were purchased from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). The vincristine-selected MDR subline KB/vincristine (16) was obtained from Zhongshan University of Medical Sciences (Guangzhou, China). All cell lines except MCF-7 and MCF-7/ADR were maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 1-glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 μg/mL), and HEPES (10 mmol/L, pH 7.4) in a humidified atmosphere of 95% air/5% CO2 at 37°C. MCF-7 and the MCF-7/ADR cells were grown in the same medium supplemented with 1 mmol/L sodium pyruvate and 0.01 mg/mL bovine insulin.

**Cytotoxicity assays.** The cytotoxicity of chimmitecan was examined using a panel of human tumor cell lines, with SN38, topotecan, 9-nitrocamptothecin, and 10-hydroxy-camptothecin as reference compounds (see Table 1). Cells in 96-well plates were treated in triplicate with gradient concentrations of drugs 37°C for 72 h. For suspension cell lines, cytotoxicity was assessed by measuring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) to a colored product (17). Sulforhodamin B (Sigma) assay, as previously described (18), was applied to solid tumor cell lines. The cytotoxicity of the drugs was expressed as an IC50, defined as the concentration required for 50% inhibition of cell growth compared with control cells and determined by the Logit method from at least three independent experiments. In some experiments, 0.1% human serum albumin (HSA; Shanghai Institute of Biological Products, Shanghai, China) was added to the medium 15 min before the addition of drugs. The concentration of HSA was the highest possible not showing effects on cell proliferation.

**Cellular pharmacokinetic studies.** H160 cells (5 × 10⁷/mL) were pelleted and washed thrice with precooled PBS after treated with 10 μmol/L SN38, topotecan, or chimmitecan for indicated time. Washed cells were lysed as previously described (7). Protein concentrations were then determined using a bichinchoninic acid protein assay kit (Pierce, Rockford, IL). The lysates were mixed with an equal volume of methanol and then centrifuged for 12,000 × g for 15 min at 4°C. A 30-μL sample of the supernatant was then subjected to high-performance liquid chromatography using a Kromasil C18 column (4.6 × 150 mm; Waters, Milford, MA) with 0.02% ammonium formate in 63:37 (v/v) methanol/water delivered at 1 mL/min as the mobile phase. Elution of compounds was followed using a fluorescence detector with excitation and emission at 385 and 480 nm, respectively.

**Topoisomerase I–mediated supercoiled pBR322 relaxation.** DNA relaxation assay was done as previously described (19). The reaction buffer contained 35 mmol/L Tris-HCl (pH 8), 72 mmol/L KCl, 5 mmol/L MgCl2, 5 mmol/L DTT, 5 mmol/L spermidine, 0.01% bovine serum albumin, 0.25 μg supercoiled pBR322 DNA, and 2 units of topoisomerase I (GE Healthcare, Buckinghamshire, England) in a total volume of 20 μL. Reaction mixtures were incubated at 37°C for 30 min in the presence of drugs and terminated by adding 2 μL of...
10% SDS and proteinase K (50 μg/mL). Purified DNA samples were analyzed by electrophoresis on a 1% agarose gel. After staining with 0.5 μg/mL ethidium bromide, gel images were obtained using a ChemiGenius 2 Gel Documentation System (Syngene, Cambridge, United Kingdom).

**Topoisomerase I–mediated DNA cleavage assay.** The procedure of DNA cleavage assay was similar to topoisomerase I–mediated supercoiled pBR322, except that the amount of topoisomerase I was increased to 10 units, and electrophoresis was conducted on 1% agarose gels containing 0.5 μg/mL ethidium bromide (20).

**Reversal of cleavable complex formation.** Reversal of topoisomerase I–mediated cleavage of pBR322 was accomplished as described by Tanizawa et al. (21). Cleavable complexes were formed in the presence of sufficient compounds (10 μmol/L). The reaction mixture was the same as described for the assay of topoisomerase I–mediated DNA cleavage. After a 30-min incubation at 37°C, the reaction was adjusted to 0.5 mol/L NaCl. Aliquots were then removed after 5, 15, and 60 min, and the reaction was terminated and separated as described above. The intensity of bands was quantified using GeneTools Analysis Software, version 3.03.00 (Syngene). The intensity of nicked DNA (FII) at each time point was normalized to the total signal of all bands in each lane. The ratio of nicked DNA percentage at each time point to that at time 0 was plotted versus NaCl-treated time to present the reversal rate.

**Band depletion assay for topoisomerase I cleavable complexes.** Band depletion assay was done as previously described (22). Briefly, HL60 cells (5 × 10⁷/mL) were treated with drugs for 15 min at 37°C. The cells were pelleted and lysed with 0.2 mol/L NaOH containing 1 mM EDTA. Alkaline lysates were then neutralized with one-tenth volume of 2 mol/L HCl and one-tenth volume of a solution containing 10% NP40, 1 mol/L Tris (pH 7.4), 0.1 mol/L MgCl₂, 0.1 mol/L CaCl₂, 10 mmol/L DTT, 1 mmol/L EGTA, 100 μg/mL leupeptin, and 100 μg/mL aprotinin. The samples were analyzed by SDS-PAGE followed by immunoblotting with antibodies to topoisomerase I and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Molecular modeling.** The binding potential between drugs and topoisomerase I-DNA cleavable complexes was evaluated by molecular docking. In the present study, DOCK 4.0 (23, 24) was employed for conformational screening based on the X-ray crystal structure of the topoisomerase I-DNA complex in the Brookhaven Protein Database (25). Residues within 5 Å of the active center of the complex, a volume large enough to include the binding site, were extracted as the binding pocket for docking. During the docking simulation, Kollman-all-atom charges (26) were assigned to the protein, and Gasterger-Marsili charges (27) were assigned to the three compounds. After conformational screening, the energy scoring function was used to rank the molecules. The binding pocket for docking. During the docking simulation, Kollman-all-atom charges (26) were assigned to the protein, and Gasterger-Marsili charges (27) were assigned to the three compounds. After conformational screening, the energy scoring function was used to rank the molecules. The binding potential between drugs and topoisomerase I cleavable complexes was evaluated by molecular docking. In the present study, DOCK 4.0 (23, 24) was employed for conformational screening based on the X-ray crystal structure of the topoisomerase I-DNA complex in the Brookhaven Protein Database (25). For docking, the energy scoring function was used to rank the molecules. The binding potential between drugs and topoisomerase I cleavable complexes was evaluated by molecular docking. In the present study, DOCK 4.0 (23, 24) was employed for conformational screening based on the X-ray crystal structure of the topoisomerase I-DNA complex in the Brookhaven Protein Database (25).

**Cell cycle analysis.** HL60 cells (5 × 10⁷/mL) were exposed to drugs for 24 h, collected, washed twice with ice-cold PBS, and fixed overnight with 70% precooled (4°C) ethanol. Fixed cells were resuspended in PBS containing 0.02 mg/mL RNase A (Sino-American Biotechnology Co., Henan, China) and incubated at 37°C for 15 min. Next, the cells were stained with 0.01 mg/mL propidium iodide (Sigma) in the dark at 25°C for 30 min. The DNA content was analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Sunnyvale, CA) with CellQuest and ModFit LT3.0 software (Becton Dickinson).

**Cell apoptosis detection.** After treatment of cells with drugs for 12 h, DNA fragments were collected using miniprep spin columns as described by Steinfelder et al. (30). The samples were then separated by electrophoresis on 1.5% agarose gels.

**Antitumor activity against human tumor xenografts in nude mice.** Under sterile conditions, well-developed tumors were cut into 1-mm³ fragments and transplanted s.c. into the right flank of nude mice using a trocar. When the tumor reached a volume reached 100 to 200 mm³, the mice were randomly assigned into control and treatment groups. Control groups were given vehicle alone, and treatment groups received CPT-11 or chimmitecan (i.v. or p.o.). The sizes of the tumors were measured twice per week using microcalipers. The tumor volume (V) was calculated as follows: V = (length × width²) / 2. The individual relative tumor volume (RTV) was calculated as follows: RTV = V / V₀, where V₀ is the volume on each day, and V is the volume at the beginning of the treatment. The therapeutic effect of the compounds was expressed as the volume ratio of treatment to control (T/C, ref. 31): T/C (%) = 100% × (mean RTV of the treated group / mean RTV of the control group).

**Statistical analysis.** Data were presented as X ± SD, and differences were considered significant when P < 0.05 as determined by Student’s t test.

**Results**

**Chimmitecan exhibits potent antitumor activity in vitro**

Chimmitecan is highly cytotoxic to human tumor cell lines. Chimmitecan was highly cytotoxic (IC₅₀s in the nanomolar range) to a wide variety of tumor cells, including leukemia, lung cancer, gastric cancer, hepatocellular cancer, colon cancer, breast cancer, ovarian cancer, and cervical cancer, and rhabdomyosarcoma cells (Table 1). Meanwhile, chimmitecan showed distinct selectivity against cells derived from different tissues. Cell lines derived from lung, colon, and breast cancers were more sensitive to chimmitecan than those from hepatic and gastric cancers. For example, the average IC₅₀ against liver cancer was 261.5 nmol/L, which is >30-fold higher than that against lung cancer (7.6 nmol/L). In general, chimmitecan exhibited a lower average IC₅₀ but similar selectivity against cells derived from different tissues compared with the other four reference analogues (SN38, topotecan, 9-nitro-camptothecin, and 10-hydroxy-camptothecin).

**Chimmitecan retains its potency in MDR cell lines.** We next examined the cytotoxicity of chimmitecan and reference compounds (camptothecin, SN38, topotecan, 10-hydroxy-camptothecin, doxorubicin, and vincristine) in three MDR-expressing sublines: K562/A02, MCF-7/ADR, and KB/VCR (Table 2). For each of these cell lines, we calculated the resistance factor as the ratio of the IC₅₀ of resistant cells to that of the parental cells (K562, MCF-7, and KB). The resistance factor values for doxorubicin in K562/A02 and MCF-7/ADR sublines and for vincristine in the KB/VCR subline were 140.24, 143.11, and 93.54, respectively, which are much higher than the resistance factor values for the four
The presence of 0.1% HSA reduced the cytotoxicity of camptothecin towards the inactive carboxylate (32, 33). We therefore conclude that camptothecin (Fig. 2A). Our data suggested that chimmitecan displays similar cellular pharmacokinetics but distinct cellular accumulation compared with topotecan and SN38.

Cytotoxicity of chimmitecan is unaffected by HSA. HSA has been reported to preferentially bind to the carboxylate form of camptothecin, thus shifting the lactone/carboxylate equilibrium towards the inactive carboxylate (32, 33). We therefore examined the influence of HSA on cytotoxicity of camptothecin. The presence of 0.1% HSA reduced the cytotoxicity of camptothecin by ~3-fold (IC_{50} increased from 14 to 40 nmol/L) in HL60 cells (Fig. 2B). In contrast, HSA had little effect on the cytotoxicity of chimmitecan, topotecan, or SN38.

Table 1. Cytotoxicity of chimmitecan against a panel of human tumor cell lines

<table>
<thead>
<tr>
<th>Origins</th>
<th>Cell lines</th>
<th>Topotecan</th>
<th>SN38</th>
<th>9-Nitro-camptothecin</th>
<th>10-Hydroxy-camptothecin</th>
<th>Chimmitecan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>HL-60</td>
<td>23 ± 1</td>
<td>7 ± 1</td>
<td>21 ± 3</td>
<td>34 ± 14</td>
<td>9.2 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>A549</td>
<td>28 ± 7</td>
<td>6 ± 2</td>
<td>32 ± 4</td>
<td>16 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td></td>
<td>NCI-H23</td>
<td>122 ± 36</td>
<td>234 ± 116</td>
<td>70 ± 15</td>
<td>156 ± 31</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Stomach</td>
<td>SGC-7901</td>
<td>489 ± 97</td>
<td>465 ± 75</td>
<td>98 ± 7</td>
<td>536 ± 61</td>
<td>318 ± 51</td>
</tr>
<tr>
<td></td>
<td>MKN-28</td>
<td>412 ± 188</td>
<td>303 ± 56</td>
<td>116 ± 6</td>
<td>302 ± 188</td>
<td>57 ± 46</td>
</tr>
<tr>
<td></td>
<td>MKN-45</td>
<td>239 ± 38</td>
<td>601 ± 258</td>
<td>160 ± 27</td>
<td>268 ± 157</td>
<td>78 ± 13</td>
</tr>
<tr>
<td>Colon</td>
<td>HCT-15</td>
<td>14 ± 4</td>
<td>6 ± 4</td>
<td>9.6 ± 0.5</td>
<td>15 ± 4</td>
<td>10 ± 2</td>
</tr>
<tr>
<td></td>
<td>HCT-116</td>
<td>38 ± 2</td>
<td>13 ± 5</td>
<td>14 ± 1</td>
<td>6.5 ± 0.2</td>
<td>7 ± 2</td>
</tr>
<tr>
<td></td>
<td>HT-29</td>
<td>203 ± 73</td>
<td>115 ± 58</td>
<td>95 ± 36</td>
<td>69 ± 23</td>
<td>49 ± 16</td>
</tr>
<tr>
<td>Liver</td>
<td>BEL-7402</td>
<td>420 ± 80</td>
<td>136 ± 35</td>
<td>59 ± 11</td>
<td>141 ± 11</td>
<td>313 ± 24</td>
</tr>
<tr>
<td></td>
<td>SMMC-7721</td>
<td>1,383 ± 172</td>
<td>379 ± 66</td>
<td>1,380 ± 25</td>
<td>1,401 ± 142</td>
<td>466 ± 108</td>
</tr>
<tr>
<td>Breast</td>
<td>MDA-MB-435</td>
<td>50 ± 6</td>
<td>3 ± 2</td>
<td>19 ± 6</td>
<td>11 ± 1</td>
<td>9.9 ± 0.3</td>
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<tr>
<td></td>
<td>MDA-MB-468</td>
<td>176 ± 97</td>
<td>96 ± 37</td>
<td>65 ± 30</td>
<td>88 ± 19</td>
<td>17 ± 9</td>
</tr>
<tr>
<td></td>
<td>MCF-7</td>
<td>387 ± 46</td>
<td>73 ± 16</td>
<td>63 ± 20</td>
<td>52 ± 10</td>
<td>35 ± 25</td>
</tr>
<tr>
<td>Ovary</td>
<td>SKOV3</td>
<td>58 ± 8</td>
<td>45 ± 9</td>
<td>47 ± 6</td>
<td>43 ± 9</td>
<td>15 ± 5</td>
</tr>
<tr>
<td></td>
<td>OVCAR5</td>
<td>34 ± 10</td>
<td>23 ± 2</td>
<td>28 ± 11</td>
<td>41 ± 10</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Others</td>
<td>Hela</td>
<td>141 ± 70</td>
<td>176 ± 11</td>
<td>24 ± 10</td>
<td>176 ± 71</td>
<td>12 ± 5</td>
</tr>
<tr>
<td></td>
<td>Rh30</td>
<td>8 ± 2</td>
<td>9 ± 3</td>
<td>6 ± 2</td>
<td>5.5 ± 0.5</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>Average</td>
<td>IC_{50}, nmol/L</td>
<td>282</td>
<td>191</td>
<td>127</td>
<td>283</td>
<td>83</td>
</tr>
</tbody>
</table>

NOTE: Cells seeded in 96-well plates were treated with various concentrations of drugs for 72 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or sulforhodamin B assay (see Materials and Methods). IC_{50} values were shown as mean ± SD of three independent experiments. Topotecan, SN38, 9-nitro-camptothecin, and 10-hydroxy-camptothecin were used as reference drugs.

Table 2. Cytotoxicity of chimmitecan and reference drugs against pairs of resistant and sensitive cell lines

<table>
<thead>
<tr>
<th>Compounds</th>
<th>K562 (μmol/L)</th>
<th>K562/A02 (μmol/L)</th>
<th>MCF-7 (μmol/L)</th>
<th>MCF-7/ADR (μmol/L)</th>
<th>KB (μmol/L)</th>
<th>KB/VCR (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td>0.49 ± 0.08</td>
<td>69 ± 9</td>
<td>0.97 ± 0.02</td>
<td>138 ± 7</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Vincristine</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>0.007 ± 0.002</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Camptothecin</td>
<td>0.16 ± 0.03</td>
<td>0.15 ± 0.05</td>
<td>0.04 ± 0.03</td>
<td>0.05 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.22 ± 0.04</td>
<td>1.5 ± 0.2</td>
<td>0.33 ± 0.05</td>
<td>2 ± 1</td>
<td>0.034 ± 0.002</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>SN38</td>
<td>0.02 ± 0.01</td>
<td>0.25 ± 0.02</td>
<td>0.069 ± 0.007</td>
<td>0.64 ± 0.06</td>
<td>0.07 ± 0.01</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>10-Hydroxy-camptothecin</td>
<td>0.15 ± 0.07</td>
<td>0.17 ± 0.07</td>
<td>0.039 ± 0.007</td>
<td>0.086 ± 0.006</td>
<td>0.026 ± 0.002</td>
<td>0.27 ± 0.05</td>
</tr>
<tr>
<td>Chimmitecan</td>
<td>0.03 ± 0.02</td>
<td>0.046 ± 0.007</td>
<td>0.042 ± 0.003</td>
<td>0.092 ± 0.006</td>
<td>0.015 ± 0.001</td>
<td>0.22 ± 0.06</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with indicated compounds for 72 h, and IC_{50} were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or sulforhodamin B assays. Abbreviation: NT, not tested.
SN38, and topotecan reached a maximum following only a 15-min exposure. During 15 to 120 min, the concentration of chimmitecan within the cells decreased gradually from 0.22 to 0.14 nmol/mg protein. Although similar trends were observed for SN38 (from 9.8 to 7.8 nmol/mg protein) and topotecan (from 0.05 to 0 nmol/mg protein), the intracellular concentration of chimmitecan was approximately an order of magnitude lower than that for SN38 but an order of magnitude higher than that for topotecan.

**Chimmitecan exerts comparable effects on topoisomerase I compared with topotecan and SN38**

**Chimmitecan inhibits the catalytic activity of topoisomerase I.** We examined the effect of chimmitecan on the catalytic activity of topoisomerase I by measuring the topoisomerase I–mediated relaxation of supercoiled pBR322. Chimmitecan reduced the amount of relaxed DNA, while simultaneously increasing the amount of supercoiled DNA, and it caused a significant and dose-dependent inhibition of topoisomerase I catalytic activity (Fig 3A). The intensity of the supercoiled DNA band at 12.5 μmol/L chimmitecan was comparable with that observed in the presence of 100 μmol/L topotecan, SN38, or camptothecin, suggesting that chimmitecan is a more potent inhibitor of topoisomerase I–mediated relaxation than topotecan, SN38, and camptothecin.

**Chimmitecan traps the topoisomerase I-DNA cleavable complex.** A cell-free DNA cleavage assay was next applied to detect drug-trapped topoisomerase I-DNA cleavable complex. The band of nicked DNA (FII) represents topoisomerase I–mediated single-strand breakage of plasmid DNA due to drug trapping of the cleavage complex. It was found that chimmitecan efficiently trapped the topoisomerase I-DNA complex at concentrations as low as 0.1 μmol/L (Fig. 3B).

To extend the findings in a cell-free system, band depletion assay (22) was applied to further confirm the effect of chimmitecan on topoisomerase I-DNA complex in HL60 cells. In this assay, free topoisomerase I is detected as a 100-kDa band. The cleavable complexes, which are trapped by alkaline lysis as covalent topoisomerase I-DNA complexes, are measured from the reduction of the topoisomerase I band. As shown in Fig. 3C, SN38, topotecan, and chimmitecan (5 μmol/L) were all able to reduce the intensity of the topoisomerase I band, and chimmitecan was slightly more effective than topotecan and comparable with SN38.

**Chimmitecan stabilizes the topoisomerase I-DNA cleavable complexes.** The topoisomerase I–mediated primary DNA lesions are potentially reversible before cellular processing of the cleavable complex. Therefore, stabilization of the ternary complex is a critical determinant of drug activity (34, 35). Because of salt-induced dissociation of the ternary complex and religation the nicked DNA strand (21), we next adjusted the reaction to 0.5 mol/L NaCl to induce the reversal of drug-induced cleavable complexes to evaluate the stability of the complex. It was found that chimmitecan induced more cleavable complex than equimolar SN38 and topotecan (Fig. 3D) at time 0. The time course curve shown in Fig. 3E indicates that chimmitecan and SN38 dissociated at a comparable rate from cleavable complexes, which was slower than that of topotecan.

**Chimmitecan shows high affinity for topoisomerase I-DNA complex.** To help clarify the reason for the increased stability of chimmitecan-trapped complex, we used automated molecular docking to predict the conformation of each analogue when bound to the topoisomerase I-DNA complex. The most probable binding conformations of the three drugs were obtained, and the ranking of the three drugs as determined using the DOCK program was listed in Figure 3F. We noticed that chimmitecan and SN38 shared a great part of similar interacting mode with topoisomerase I-DNA complex, whereas topotecan adopts a distinct conformation (Fig. 4). In addition, although chimmitecan and SN38 form the same intermolecular

**Table 3. Intracellular content of SN38, topotecan, and chimmitecan in HL60 cells**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>SN38</th>
<th>Topotecan</th>
<th>Chimmitecan</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6.6 ± 1.9</td>
<td>0.04 ± 0.01</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>15</td>
<td>9.8 ± 0.9</td>
<td>0.053 ± 0.003</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>30</td>
<td>8.5 ± 3.0</td>
<td>0.02 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>60</td>
<td>8.5 ± 2.2</td>
<td>0.00 ± 0.00</td>
<td>0.145 ± 0.004</td>
</tr>
<tr>
<td>120</td>
<td>7.8 ± 1.2</td>
<td>0.00 ± 0.00</td>
<td>0.14 ± 0.01</td>
</tr>
</tbody>
</table>

NOTE: Intracellular drug contents were determined after exposure to 10 μmol/L drugs for indicated time using high-performance liquid chromatography assay with a fluorescence detector. The values are expressed as mean ± SD of three independent experiments.
hydrogen bond with the topoisomerase I-DNA complex, chimmmitecan provides more hydrophobic bonding with the binding site. The best conformation of topotecan, in contrast, makes few hydrophobic contacts with the side chains of the binding motif. Ranking according to Dock energy and conformational analyses together indicate that advanced stability of chimmmitecan or SN38 trapped topoisomerase I-DNA complex is due to their higher affinity for the complex than topotecan.

Chimmitecan induces DNA damage, cell cycle arrest, and apoptosis in HL60 cells

Camptothecins are supposed to stabilize otherwise transient topoisomerase I-DNA intermediates, inducing DNA strand breaks and, thus, cell cycle arrest and apoptosis (36, 37). After confirmation of its impressive effects on topoisomerase I, we accordingly investigated the ability of chimmmitecan to cause these downstream events.

Chimmitecan induces DNA damage. The comet assay is a sensitive method for evaluating DNA damage. The alkaline one is applied to detect both single-strand and double-strand breaks (38, 39). Following exposure to chimmmitecan for 1 h, obvious “comet tails” were detected in HL60 cells. The untreated control group showed only obscure “halos” without clear directions (Fig. 5A). Semiquantitative analysis with Komet 5.5 software indicated that 200 nmol/L chimmmitecan caused severe DNA damage, similar to that caused by 200 nmol/L SN38 but stronger than that caused by 200 nmol/L topotecan (Fig. 5B).

Chimmitecan causes G2-M arrest. Perturbation of the cell cycle by a 24-h drug treatment was assessed by flow cytometry. As the chimmmitecan concentration was increased from 0 to 10 nmol/L, the proportion of G2 phase cells increased from 10.6% to 39.2% (Fig. 5C). An equal concentration (10 nmol/L) of topotecan or SN38 was less effective in this regard than chimmmitecan (Fig. 5E).

Chimmitecan induces apoptosis. DNA fragmentation of apoptotic cells (40) was detected in HL60 cells following a 12-h drug treatment. Obvious DNA fragments were detected
Fig. 4. Bindings of three drugs to topoisomerase I-DNA complex. A, chimmitecan; B, SN38; C, topotecan. Topoisomerase I (blue) and DNA helices (orange). Residues in purple represent hydrophobic interaction with drugs. Dash line, hydrogen bond between drugs and topoisomerase I.

Chimmitecan exhibits potent anticancer activity

Chimmitecan displays comparable anticancer activity compared with CPT-11. Given its encouraging in vitro activity, we examined the antitumor efficiency of chimmitecan in vivo. Specifically, we measured the antitumor activity of chimmitecan in four human tumor xenograft nude mice models: A549 lung cancer, MDA-MB-435 breast cancer, BEL-7402 hepatocellular cancer, and HCT-116 colon cancer (Fig. 6A). The i.v. administration of chimmitecan (twice for HCT-116 and thrice for the other tumors per week) caused a dramatic and dose-dependent suppression of tumor growth in all four tumor models. At a dosage of 15 mg/kg, the T/C (%) values were 17.6% (HCT-116), 24.2% (MDA-MB-435), 28.2% (BEL-7402), and 23.0% (A549). CPT-11 also showed evident antitumor efficiency in these four models, although, when given at the same dosage and with the same schedule, chimmitecan was more potent than CPT-11 in the BEL-7402 and A549 models. In the MDA-MB-435 and HCT-116 models, however, CPT-11 achieved better responses.

Chimmitecan exhibits antitumor activity when given orally. Finally, we examined the antitumor effect of chimmitecan with oral administration in the A549 nude mice model. As indicated in Fig. 6B, when given thrice per week for 2 weeks, chimmitecan inhibited the growth of the A549 xenograft at dosages as low as 4.5 mg/kg, and significant antitumor activity was observed at a dosage of 9 mg/kg (T/C = 22.2%), which was similar to results obtained by i.v. administration.

Discussion

Despite the crucial role in clinical cancer therapy, camptothecins are challenged with many problems and thirsty for further optimization. Our efforts focus on the design and screening of novel camptothecin analogues based on the previous studies in this field. Structure-activity studies of camptothecins have revealed that positions 7, 9, and 10 in the A and B rings of camptothecin can be modified without a loss of activity (41, 42). X-ray crystallography of the topoisomerase I-DNA complex further shows that these positions face the major groove of the DNA. Modification of the substituents at these positions is not expected to sterically interfere with drug binding by the complex (25), which provides us a wide space for chemical manipulations. Unlike the initial modifications aiming at improving water solubility, recent efforts have focused on the development of lipophilic derivatives (43). Although poorly soluble in water, they were designed to overcome the major limitations of the first-generation camptothecins. Of these, 9-substituted analogues have shown potent antitumor activity and favorable pharmacologic profiles, and some are now in the early stages of clinical development (7, 9, 44). Herein, we attempt to investigate 9-substituted ones, in hopes of probing some other favorable direction in chemical modulation of camptothecin, which may lead to identification of more promising anticancer drug candidates.

Because insertion of a hydroxyl group at the 10-position seems to enhance the stability of cleavable complexes and the antitumor activities (41, 45), we thus challenge the 9-substituted lipophilic manipulation to the structure of mother compound 10-hydroxy-camptothecin. Interestingly, an allyl substitution at the 9-position enables chimmitecan a special affinity for many conventional solvents, including chloroform, glacial acetic acid, dimethylformamide, 1,4-dioxane, and methanol, permitting the formation of similar stable colloids with these solvents. The peculiar physiochemical features endow chimmitecan better solubility in aqueous solutions than other lipophilic camptothecins, including SN38 and 10-hydroxy-camptothecin. In addition, chimmitecan is also easier to produce than the other camptothecin derivatives we synthesized, which assures its supply for further development.

Lipophilic derivatives of camptothecin, such as SN38 and 9-nitro-camptothecin, are known to be much more cytotoxic than water-soluble derivatives (8, 46). In the present study, we found that, in addition to being much more cytotoxic in all of the tested cell lines than the water-soluble analogue topotecan, chimmitecan exhibited more potent cytotoxicity than SN38, 9-nitro-camptothecin, and 10-hydroxy-camptothecin in over half of the cell lines. Moreover, the resistance factor values revealed that chimmitecan did better in the three MDR sublines.
than SN38 and topotecan. These results suggest that the structural modification of camptothecin at the 9-position provides not only improved cytotoxicity but also favorable anti-MDR properties.

HSA can modulate the anticancer potencies of camptothecin by binding to the carboxylate, driving the equilibrium of lactone hydrolysis towards the carboxylate, thus reducing the effective serum concentration of active camptothecin (9, 32). In the current studies, we found that the cytotoxicity of camptothecin in HL60 cells was dramatically reduced even with addition of a very diluted concentration of HSA. In contrast, the cytotoxicity of chimmitecan, like SN38 and topotecan, is hardly affected. This agrees with other reports that specific modifications at the 7- and 9-positions, such as in CPT-11, topotecan, and SN38, enhance drug stability in the presence of HSA (47). However, 9-amino-camptothecin is highly unstable upon binding to HSA (48). Overall, it seems that substitutions at the 9-position have variable effects on the stability of camptothecins in the presence of HSA. In particular, the 9-allyl substitution permits its stability in the presence of HSA.

Increased cellular accumulation and rapid uptake are two of the goals for the rational development of camptothecin derivatives. Our cellular pharmacokinetics analysis showed that the intracellular content of chimmitecan, SN38, and topotecan reached a maximum after only 15-min exposure, indicative of a rapid uptake of the three compounds. The decline thereafter may have been due to conversion of the compounds from the lactone to the carboxylate form (49). The distinct difference in intracellular content among these three drugs reflected their respective lipophilic properties. Topotecan, which is a water-soluble

![Figure 5](image_url)
analogue, was detected at <0.1 nmol/mg protein in cells. On the other hand, SN38, with high lipophilicity, was accumulated at >100-fold higher levels than topotecan. The cellular concentration of chimmitcane was between those of SN38 and topotecan because it is more soluble than SN38 and other lipophilic analogs. These findings support the hypothesis that lipophilicity promotes the cellular accumulation of camptothecins (7).

Stabilization of the cleavable complex is another challenge for the design of new lipophilic camptothecin derivatives, aiming at overcoming the relevant limitations of known molecules of this class. In our study, the addition of a small alkyl group at the 9-position provides chimmitcane with the ability to potently inhibit topoisomerase I catalytic activity and to trap and stabilize the topoisomerase I-DNA cleavable complex. Using molecular modeling, we found that chimmitcane exhibited a higher affinity for the binding pocket on the topoisomerase I-DNA complex than topotecan and SN38. Although chimmitcane and SN38 form similar intermolecular hydrogen bonds with the complex, chimmitcane can form more hydrophobic interactions with the docking sites, which may help to stabilize the ternary complex. These binding simulations should help clarify why chimmitcane is more cytotoxic than topotecan and SN38, and, thus, how it potently triggers events leading to the inhibition of tumor cell growth, including DNA damage, G2-M arrest, and apoptosis.

The excellent properties of chimmitcane combine to help achieve its impressive antitumor activities in nude mice models and distinguish it from CPT-11. These differences are undoubtedly due to the inclusion of a small lipophilic group at the C-9 position. Thus, lipophilic C-9 substitutions are expected to be

**Fig. 6.** Antitumor activity of chimmitcane in vivo. Activity of chimmitcane was determined in four human tumor xenografts including non–small cell lung carcinoma A549, hepatic carcinoma BEL-7402, breast carcinoma MDA-MB-435, and colon carcinoma HCT-116 models in nude mice. Tumor fragments were implanted s.c. into right flank on nude mice. The animals were randomly divided to groups when tumor volume reached 100 to 200 mm³ and received i.v. (A) or p.o. (B) injection of normal saline (■), CPT-11 (●), or chimmitcane at low (△) or high (◇) dose, respectively. A. CPT-11 groups in all the tumor models were treated at dose of 15 mg/kg, except at 10 mg/kg for HCT-116 group. Chimmitcane at 10 mg/kg (low) or 15 mg/kg (high) doses was given to BEL-7402 group, or at 7.5 mg/kg (low) or 15 mg/kg (high) doses for the other three models. The two compounds were evaluated with same schedules in A549 (q3w × 5), BEL-7402 (q3w × 3), MDA-MB-435 (q3w × 4), and HCT-116 (q2w × 3) models. B. mice bearing A549 carcinoma were treated with chimmitcane at 4.5 or 9 mg/kg thrice a week for 2 weeks. *, P < 0.05; **, P < 0.01, CPT-11 or chimmitcane treatments versus normal saline treatment.
important for the development of camptothecin analogues with improved antitumor activities.

Optimal effects of drugs in this class can be achieved by administering them in multiple daily doses, which ensures prolonged exposure to the drug and intracellular drug levels. Thus, oral administration is highly appreciated due to its alleviation in pains and the convenience it conveys. The oral availability is consequently another advantage of camptithecin.

In summary, we have shown for the first time that chimmitecan, a novel 9-substituted lipophilic camptothecin derivative, is a potent inhibitor of topoisomerase I and therefore displays outstanding antitumor activity in vitro and in vivo. In particular, that the substitution at the 9-position benefits chimmitecan a salient anti-MDR activity, stability in HSA, improved solubility, easy preparation, and oral availability, encouragingly promise its extraordinary effects on the treatment of cancer than the analogues clinically available. More importantly, our results in turn probably offer a favorable approach in the exploration of lipophilic camptothecin optimization.

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