

## Synthesis and Biological Evaluation of Novel Conjugates of Camptothecin and 5-Fluorouracil as Cytotoxic Agents

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Uma série de novos conjugados de camptotecina e 5-fluorouracil foi sintetizada pela primeira vez e suas atividades citotóxicas contra duas linhagens de células humanas tumorais (SGC-7901 e A-549) assim como a determinação farmacocinética *in vitro* da estabilidade de lactona foram investigadas. Dentre estes compostos, a maioria apresentou atividades citotóxicas comparáveis ou superiores a **2**, mas menos potentes quando comparados a **1**. Em particular, os conjugados **10b** e **10d** foram altamente ativos contra A-549 com valores de IC<sub>50</sub> de 0,45 e 0,38 μmol L<sup>-1</sup>, respectivamente. Além disto, a determinação farmacocinética *in vitro* dos níveis de lactona do composto representativo **10b** mostraram que o tempo de vida biológico de suas formas de lactona em plasma humano e de rato aumentaram significativamente quando comparados com o composto-mãe **1**. O método de relações quantitativas de estrutura-atividade (QSAR) foi então aplicado para o desenvolvimento de modelos lineares para prever as atividades citotóxicas destes derivados que ainda não foram sintetizados ou testados experimentalmente. Além disto, 'docking' molecular foi utilizado para esclarecer o modo de ligação destes derivados a DNA topoisomerase humana I. Ligações de hidrogênio importantes foram observadas entre estes derivados e seu receptor. Os resultados de modelagem molecular e estudos QSAR podem ser usados para guiar o design de novos conjugados com maior atividade antitumoral.

A series of novel conjugates of camptothecin and 5-fluorouracil were first synthesized and their cytotoxic activities against two human tumor cell lines (SGC-7901 and A-549) as well as *in vitro* pharmacokinetic determination of lactone stability were studied. Among these compounds, most tested conjugates showed comparable or superior cytotoxic activities to **2**, but less potent compared with **1**. Particularly, conjugates **10b** and **10d** were highly active against A-549 with IC<sub>50</sub> values of 0.45 and 0.38 μmol L<sup>-1</sup>, respectively. Also, the *in vitro* pharmacokinetic determination of lactone levels of representative compound **10b** showed that the biological life span of their lactone forms in human and mouse plasma significantly increased compared with their mother compound **1**. Quantitative structure-activity relationship (QSAR) method was then applied for developing linear models to predict the cytotoxic activities of these derivatives that have not yet been synthesized or experimentally tested. In addition, molecular docking was used to clarify the binding mode of these derivatives to human DNA topoisomerase I. The important hydrogen-bonding interactions were observed between these derivatives and their receptor. The results from molecular modeling and QSAR study can guide the design of novel conjugates with higher antitumor activity.

**Keywords:** camptothecin, 5-fluorouracil, cytotoxic agents, conjugates

### Introduction

A challenging research focus in current cancer therapy is the discovery of molecules that could be selective for tumor cells and that could be characterized by reduced undesirable effects. Among various strategies to improve

drug selectivity, conjugation of cytotoxic drug components has proven to be a promising approach to enhance the activity and selectivity of some monomeric leads by forming bivalent heterodimers.<sup>1,2</sup> This concept is now accepted as an effective strategy for designing ligands, inhibitors, and drugs that influence biological systems. On the basis of this theory, some interesting results have been reported by our group as well as others in recent years.<sup>3-7</sup> In our prior study,

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we explored the syntheses and evaluation of heterodimer conjugates by combination of an antimetabolite together with one or more different anticancer agents through various linkages, which has led to a large increase in potency over their monomeric counterparts.<sup>2,3,7</sup>

Camptothecin (CPT) (**1**) is a topoisomerase I targeting cytotoxic alkaloid with significant antineoplastic activity.<sup>8-10</sup> The therapeutic use of unmodified CPT was however suspended because of high toxicity stemming in part from instability of the active lactone form due to preferential binding of the carboxylate to serum albumin and problems with delivery due to poor water solubility. Included in approaches to improving the biological profile of CPTs are the development of prodrugs for preferential cellular uptake into tumor cells, enhanced solubility and lipophilicity, and stabilization of the camptothecin *E*-ring lactone. These efforts have led to the food and drug administration (FDA) approval of topotecan (**2**) and irinotecan (**3**) for colon and ovarian cancers treatment, respectively, and to the synthesis of several novel CPT derivatives that are currently in various stages of clinical trials.<sup>11-13</sup>

Antimetabolite 5-fluorouracil (5-FU, **4**) is one of the major anticancer agents used clinically for the treatment of stomach, colorectal, head, and neck cancers.<sup>14</sup> But 5-FU is poorly selective toward tumor, so its therapy causes high incidences of toxicity in the bone marrow, gastrointestinal tract, central nerve system and skin. To optimize the efficacy of 5-FU, it is often administrated by continuous infusion as well as in combination with other cytotoxic or with biochemical modulators.<sup>15,16</sup> Moreover, the considerable data from clinical studies showed that the combined use of CPT-related analogues and 5-FU resulted in higher response rates than for either agent alone and simultaneously circumvent some faults and decreased repair of camptothecin-induced DNA damage and increased induction of apoptosis, or increased DNA adduct formation in cancer cells.<sup>16,17</sup>

Herein, the aim of our work was to investigate the cytotoxicity, stability of the lactone ring and SAR studies of

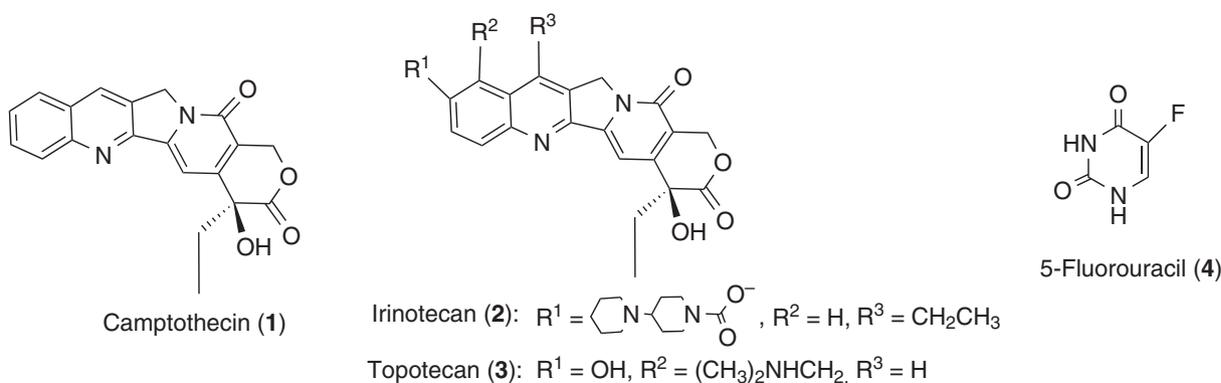
such novel conjugates and the effect of different dipeptide spacer linkers at the C-20-position.

## Experimental

Melting points were taken on a Kofler melting point apparatus and are uncorrected, infrared (IR) spectra were obtained on NIC-5DX spectrophotometer, mass spectral analysis was performed on a ZAB-HS and Bruker Daltonics APEXII49e instrument. Optical rotations were determined on PerkinElmer Model 341 spectropolarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-400 spectrometer at 400 MHz using tetramethylsilane (TMS) as reference (Bruker Company, USA). The starting camptothecin was isolated from a Chinese medicinal plant *C. acuminata* and was purified before being used. The [(5-fluorouracil-1-yl)acetyl]-L-amino acids **7a-7g** used for the experiments were prepared by following a modified previous procedure.<sup>18,19</sup>

### Synthesis of camptothecin-20-glycinate TFA salt (**9**)

*t*-Boc-glycine (377 mg, 2.1 mmol) was dissolved in 200 mL of anhydrous dichloromethane at room temperature, and to this solution were added CPT (250 mg, 0.72 mmol), dicyclohexylcarbodiimide (DCC) (444 mg, 2.1 mmol) and 4-dimethylaminopyridine (DMAP) (87 mg, 0.72 mmol) at 0 °C. The reaction mixture was stirred for 1 h and subsequently allowed to room temperature and left overnight. The solution was washed with 0.1 mol L<sup>-1</sup> hydrochloric acid (HCl), dried and evaporated under reduced pressure to yield a white solid, which was recrystallized from methanol to give camptothecin-20-ester of *t*-Boc-glycine **8**. The *t*-Boc protection group was removed by dissolving in a mixture of methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) (15 mL) and TFA (trifluoroacetic acid) (15 mL) and stirred at room temperature for 1 h. Solvent was removed and the solid was recrystallized from CH<sub>2</sub>Cl<sub>2</sub> and ether to give



**Figure 1.** Structures of camptothecin (**1**), irinotecan (**2**), topotecan (**3**) and 5-fluorouracil (**4**).

camptothecin-20-glycinate TFA salt **9**. Spectral data for **9** are identical to the reported by Greenwald.<sup>20</sup>

#### General procedure for synthesis of target compounds (10a-10g)

[(5-Fluorouracil-1-yl)acetyl]-L-amino acids **7a-7g** (0.047 mol) are dissolved in 50 mL of anhydrous dimethylformamide (DMF) and cooled to 0 °C. 1-Hydroxybenzotriazole (HOBt) (0.07 mol) and (0.056 mol) of *N*-ethyl-*N*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) are added, and the mixture is stirred for 30 min at 0 °C. Subsequently, (0.039 mol) of camptothecin-20-glycinate TFA salt **9** and finally 24.3 mL *N*-ethyl diisopropylamine are added. The mixture is stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the residue was separated by flash-column chromatography (gradient elution with mixtures of chloroform-methanol) on silica gel and monitored by TLC. Synthesized target compounds **10a-10g** were characterized by mp, IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR and HRMS analyses.

#### Spectral data of compound 10a

Yield: 73%; mp: 186-188 °C; [ $\alpha$ ]<sub>D</sub><sup>20</sup> -79° (c 0.5, DMF); IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$  463(NH), 3073, 1619, 1595, 1559 (ArH), 1762, 1702, 1664 (C=O), 1236 (C-F), 1184, 1155 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  0.89 (3H, t, H-18), 2.13 (2H, q, H-19), 4.04 and 4.23 (4H, 2d, 2×CH<sub>2</sub>-Gly-Gly), 4.29 (2H, s, 5-Fu-N-CH<sub>2</sub>), 5.47 (2H, s, H-17), 5.26 (2H, s, H-5), 7.14 (1H, s, H-14), 7.70 (1H, t, H-11), 7.84 (1H, s, 5-Fu-ring-H-6), 7.94 (1H, t, H-10), 8.12 (d, 1H, H-12), 8.46 (d, 1H, H-9), 8.66 (s, 1H, 1H, H-7), 11.80 (s, 1H, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  169.05, 168.81, 166.95, 157.58, 157.33, 156.45, 152.23, 149.66, 147.82, 145.92, 144.99, 138.07 and 140.36 (5-Fu-ring-5-C, *J*<sub>CF</sub> 229 Hz), 131.52, 131.02, 130.69, 130.04, 129.64, 128.85, 128.46, 127.90 and 127.64 (5-Fu-ring-C-6, *J*<sub>CF</sub> 26 Hz), 118.96, 95.11, 76.27, 66.31, 50.11, 49.45, 41.77, 30.25, 7.45. HRMS (*m/z*) calculated for C<sub>30</sub>H<sub>25</sub>N<sub>6</sub>O<sub>9</sub>F: 633.1740 [M+ H]<sup>+</sup>. Found: 633.1740.

#### Spectral data of compound 10b

Yield: 69%; mp 189-191 °C; [ $\alpha$ ]<sub>D</sub><sup>20</sup> -80° (c 0.5, DMF); IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$  3359 (NH), 3067, 1600, 1558 (ArH), 1749, 1698, 1662 (C=O), 1237 (C-F), 1187, 1158 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  0.92 (3H, t, H-18), 1.24 (3H, d, *L*-Alanine-CH<sub>3</sub>), 2.15 (2H, q, H-19), 4.05 (2H, d, CH<sub>2</sub>-Gly), 4.25 (1H, m, *L*-alanine-CH),

4.35 (2H, s, 5-Fu-N-CH<sub>2</sub>), 5.31 (2H, s, H-5), 5.50 (2H, s, H-17), 7.17 (1H, s, H-14), 7.73 (1H, t, H-11), 7.85 (1H, s, 5-Fu-ring-H-6), 7.89 (1H, t, H-10), 8.15 (1H, d, H-12), 8.48 (1H, d, H-9), 8.71 (1H, s, H-7), 11.80 (1H, s, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  168.62, 167.17, 166.93, 157.56, 157.30, 156.42, 152.23, 149.54, 147.81, 145.95, 144.93, 138.15 and 140.42 (5-Fu-ring-5-C, *J*<sub>CF</sub> 228 Hz), 131.47, 130.84, 130.50, 130.32, 129.62, 128.83, 128.42, 127.88 and 127.60 (5-Fu-ring-C-6, *J*<sub>CF</sub> 28 Hz), 118.91, 95.08, 76.33, 66.28, 50.10, 49.37, 40.26, 30.37, 7.42. HRMS (*m/z*) calculated for C<sub>31</sub>H<sub>27</sub>N<sub>6</sub>O<sub>9</sub>F: 647.1896 [M+ H]<sup>+</sup>. Found: 647.1896.

#### Spectral data of compound 10c

Yield: 56%; mp 190-192 °C; [ $\alpha$ ]<sub>D</sub><sup>20</sup> -71° (c 0.5, DMF); IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$  3293 (NH), 3065, 1598, 1559 (ArH), 1750, 1699, 1662 (C=O), 1237 (C-F), 1188, 1161 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  0.92 (3H, t, H-18), 2.15 (2H, q, H-19), 3.03 (2H, m, *L*-phenylalanine-CH<sub>2</sub>), 4.08 (2H, d, CH<sub>2</sub>-Gly), 4.35 (2H, s, 5-Fu-N-CH<sub>2</sub>), 4.56 (1H, m, *L*-phenylalanine-CH), 5.30 (2H, s, H-5), 5.51 (2H, s, H-17), 7.19 (1H, s, H-14), 7.23 (m, 5H, *L*-phenylalanine-ArH), 7.73 (1H, t, H-11), 7.85 (1H, s, 5-Fu-ring-H-6), 7.88 (1H, t, H-10), 8.15 (1H, d, H-12), 8.54 (1H, d, H-9), 8.70 (1H, s, H-7), 11.77 (1H, s, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  171.25, 168.77, 166.91, 166.35, 157.49, 157.23, 156.45, 152.29, 149.50, 147.83, 145.95, 144.98, 137.92, 137.45, 131.50, 130.91, 130.58, 130.35, 129.69, 129.08, 128.87, 128.46, 127.98, 127.90, 127.64, 126.20, 118.94, 95.12, 76.27, 66.28, 53.85, 50.13, 49.13, 40.30, 37.65, 30.39, 7.43. HRMS (*m/z*) calculated for C<sub>37</sub>H<sub>31</sub>N<sub>6</sub>O<sub>9</sub>F: 745.209 [M+ Na]<sup>+</sup>. Found: 745.2014.

#### Spectral data of compound 10d

Yield: 52%; mp 186-188 °C; [ $\alpha$ ]<sub>D</sub><sup>20</sup> -90° (c 0.5, DMF); IR (KBr)  $\nu_{\max}/\text{cm}^{-1}$  3311(NH), 3067, 1599, 1557 (ArH), 1751, 1699, 1661 (C=O), 1236 (C-F), 1186, 1156 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)  $\delta$  0.83 (6H, dd, *L*-valine-CH(CH<sub>3</sub>)<sub>2</sub>), 0.92 (3H, t, H-18), 1.97 (1H, m, *L*-valine-CH(CH<sub>3</sub>)<sub>2</sub>), 2.15 (2H, q, H-19), 4.14 (2H, d, CH<sub>2</sub>-Gly), 4.36 (2H, s, 5-Fu-N-CH<sub>2</sub>), 4.42 (1H, m, *L*-valine-CH), 5.31 (2H, s, H-5), 5.50 (2H, s, H-17), 7.17 (1H, s, H-14), 7.73 (1H, t, H-11), 7.87 (1H, s, 5-Fu-ring-H-6), 7.89 (1H, t, H-10), 8.16 (1H, d, H-12), 8.60 (1H, d, H-9), 8.71 (1H, s, H-7), 11.78 (1H, s, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  171.89, 169.56, 167.25, 158.30, 158.05, 157.15, 152.87, 150.31, 148.49, 146.63, 145.78, 140.93 and 138.66 (5-Fu-ring-5-C, *J*<sub>CF</sub> 227 Hz), 132.20,

131.99, 131.66, 131.07, 130.26, 129.15, 129.11, 128.57 and 128.33 (5-Fu-ring-C-6,  $J_{CF}$  24 Hz), 119.61, 109.99, 95.58, 76.96, 66.99, 56.73, 50.80, 31.39, 31.20, 19.18, 18.62, 8.18. HRMS ( $m/z$ ) calculated for  $C_{33}H_{31}N_6O_9F$ : 675.2209 [M+H]<sup>+</sup>. Found: 675.2219.

#### Spectral data of compound **10e**

Yield: 45%; mp 176-178 °C;  $[\alpha]_D^{20}$  -77° (*c* 0.5, DMF); IR (KBr)  $\nu_{max}/cm^{-1}$  3291(NH), 3069, 1598, 1557 (ArH), 1751, 1700, 1661 (C=O), 1237 (C-F), 1188, 1160 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub> 400 MHz)  $\delta$  0.81 (6H, m, 2×L-leucine-CH<sub>3</sub>), 0.91 (3H, t, H-18), 1.48 (2H, m, L-leucine-CH<sub>2</sub>), 1.59 (1H, m, L-leucine-CH(CH<sub>3</sub>)<sub>2</sub>), 2.13 (2H, q, H-19), 4.06 (2H, d, CH<sub>2</sub>-Gly), 4.19 (1H, m, L-leucine- $\alpha$ -CH), 4.37 (2H, s, 5-Fu-N-CH<sub>2</sub>), 5.31 (2H, s, H-17), 5.50 (2H, s, H-5), 7.17 (1H, s, H-14), 7.73 (1H, t, H-11), 7.97 (1H, s, 5-Fu-ring-H-6), 8.17 (1H, t, H-10), 8.40 (1H, d, H-9), 8.59 (1H, d, H-12), 8.71 (1H, s, H-7), 11.79 (1H, s, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  169.53, 167.67, 167.04, 158.29, 158.04, 157.17, 152.96, 150.32, 148.55, 146.68, 145.80, 140.98 and 138.71 (5-Fu-ring-5-C,  $J_{CF}$  227 Hz), 132.23, 131.92, 131.59, 131.07, 130.36, 129.61, 129.17, 128.62 and 128.36 (5-Fu-ring-C-6,  $J_{CF}$  26 Hz), 119.62, 95.79, 76.98, 66.99, 56.72, 51.58, 50.16, 41.91, 31.16, 24.72, 23.58, 22.22, 19.20, 8.20. HRMS ( $m/z$ ) calculated for  $C_{34}H_{33}N_6O_9F$ : 689.2366 [M+H]<sup>+</sup>. Found: 689.2366.

#### Spectral data of compound **10f**

Yield: 68%; mp 192-194 °C;  $[\alpha]_D^{20}$  -67° (*c* 0.5, DMF); IR (KBr)  $\nu_{max}/cm^{-1}$  3307 (NH), 3066, 1599, 1541 (ArH), 1752, 1702, 1660 (C=O), 1236 (C-F), 1180, 1154 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub> 400 MHz)  $\delta$  0.91 (3H, t, H-18), 1.92 (3H, s, L-methionine-CH<sub>3</sub>), 2.13 (3H, m, L-methionine-CH<sub>2</sub>SCH<sub>3</sub> and H-19), 2.41 (2H, m, L-methionine-CH<sub>2</sub>CH<sub>2</sub>SCH<sub>3</sub>), 4.09 (2H, d, CH<sub>2</sub>-Gly), 4.30 (1H, m, L-methionine- $\alpha$ -CH), 4.38 (2H, s, 5-Fu-N-CH<sub>2</sub>), 5.29 (2H, s, H-17), 5.48 (2H, s, H-5), 7.15 (1H, s, H-14), 7.72 (1H, t, H-11), 7.94 (1H, s, 5-Fu-ring-H-6), 8.15 (1H, t, H-10), 8.44 (1H, d, H-9), 8.54 (1H, d, H-12), 8.68 (1H, s, H-7), 11.78 (1H, s, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  171.32, 168.80, 166.91, 166.57, 157.56, 157.30, 156.44, 152.23, 149.63, 147.82, 145.94, 144.99, 140.28 and 138.01 ((5-Fu-ring-5-C,  $J_{CF}$  217 Hz), 131.49, 131.15, 130.81, 130.35, 129.64, 128.873 and 128.61 (5-Fu-ring-C-6,  $J_{CF}$  26 Hz), 127.89, 127.63, 118.92, 95.07, 76.28, 66.27, 51.73, 50.111, 49.52, 40.34, 32.09, 30.43, 29.35, 14.47, 7.46. HRMS ( $m/z$ ) calculated for  $C_{33}H_{31}N_6O_9SF$ : 729.1749 [M+Na]<sup>+</sup>. Found: 729.1738.

#### Spectral data of compound **10g**

Yield: 62%; mp 192-194 °C;  $[\alpha]_D^{20}$  -75° (*c* 0.5, DMF); IR (KBr)  $\nu_{max}/cm^{-1}$  3421, 3308 (NH,OH), 3072, 1589, 1562 (ArH), 1755, 1699, 1659 (C=O), 1238 (C-F), 1182, 1162 (ester linkage-O-C); <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub> 400 MHz)  $\delta$  0.91 (3H, t, H-18), 1.05 (3H, m, L-threonine-CH(OH)CH<sub>3</sub>), 2.17 (2H, q, H-19), 4.07 (2H, d, CH<sub>2</sub>-Gly), 4.12 (1H, m, L-threonine-CH(OH)CH<sub>3</sub>), 4.19 (m, 1H, L-threonine- $\alpha$ -CH), 4.28 (2H, s, 5-Fu-N-CH<sub>2</sub>), 5.50 (2H, s, H-17), 5.31 (2H, s, H-5), 7.19 (1H, s, H-14), 7.75 (1H, t, H-11), 7.87 (1H, s, 5-Fu-ring-H-6), 7.95 (1H, t, H-10), 8.18 (1H, d, H-12), 8.38 (1H, d, H-9), 8.71 (1H, s, H-7), 11.79 (1H, s, 5-Fu-ring-NH); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>)  $\delta$  170.30, 168.81, 166.96, 166.79, 157.56, 157.30, 156.46, 152.28, 149.61, 147.82, 145.93, 144.99, 137.95 and 140.22 (5-Fu-ring-5-C,  $J_{CF}$  227 Hz), 131.54, 131.22, 130.88, 130.38, 129.68, 128.87, 128.47, 127.91 and 127.66 (5-Fu-ring-C-6,  $J_{CF}$  25 Hz), 118.96, 95.20, 76.23, 66.52, 58.24, 50.14, 49.47, 40.43, 30.50, 19.81, 7.45. HRMS ( $m/z$ ) calculated for  $C_{32}H_{29}N_6O_{10}F$ : 699.1821 [M+Na]<sup>+</sup>. Found: 699.1825.

#### Cytotoxicity assays

Cytotoxicity assays are performed on human gastric tumor SGC-7901 and human lung carcinoma A-549. Cells (6000-10.000) in 100  $\mu$ L culture medium *per* well were seeded into 96 well microtest plates (Falcon, CA). Cells were treated in triplicate with gradient concentration of test compounds and incubated at 37 °C for 48 h (or 72 h). The microculture [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT (Sigma, St. Louis, MO) assay was performed to measure the cytotoxic effects. The drug concentration required for 50% growth inhibition (IC<sub>50</sub>) of tumor cells was determined from the dose-response curve.

#### *In vitro* determination of lactone levels in human and mouse plasma for **1** and **10b**

To 0.8 mL pre-incubated human and mouse plasma, test compounds (0.2 mL, 100  $\mu$ g mL<sup>-1</sup>) in acetonitrile were added. The mixture was incubated at 37 °C, and 100  $\mu$ L aliquots were taken at 2, 4, 6, 8 and 24 h. To precipitate plasma protein, 400  $\mu$ L acetonitrile (-20 °C) were added, vortexed for 20 s, and centrifuged at 10.000 rpm for 5 min. Supernatant was transferred to a glass vial and stored at -20 °C immediately until high performance liquid chromatography (HPLC) analysis. HPLC (HP1100) analysis: 20  $\mu$ L of solution obtained above were injected onto a C-18 column (Zobax SB, 4.6×150 mm) and chromatographed with methanol:water 0.1% acetic acid as mobile phase.

Camptothecin and **10b** were detected (detector: DAB) at 254 nm. The percent of lactone was determined by the ratio of lactone levels measured at different time points to the lactone levels measured at starting point ( $t = 0$  h).

#### Computational details

##### General

The crystal structure for complex CPT-DNA-Topo I (3.0 Å resolution,  $R_{\text{cryst}} = 0.244$ ) was obtained from the Brookhaven protein databank with PDB code 1T8I. All docking calculations were performed with discovery studio 2.1 software package and QSAR models were performed with Codessa software. All performances were completed on Pentium IV computer.

##### Docking analysis

The protein atoms were then typed using the CharMm force field. Ligand conformations were randomly generated and energy minimized using the CharMm force field. We set the maximum number of conformations generated to 300 (MaxConfs 300) with a 20 kcal mol<sup>-1</sup> energy window to generate lowest energy conformers *per* ligand. The maximum number of conformations was chosen to ensure adequate coverage of conformational space. For protein, the binding site can be found using as volume of selected ligand from protein complex with a value of 9 Å distance for the site opening based on binding site module. Then, LibDock procedure was applied to position conformation of these compounds correctly in the active site. The procedure was performed using LibDock module. The binding results could be displayed by scoring ligand poses and several scoring functions can be used for measuring the goodness of a docking study to find a top ranked pose for ligands. In this study, relatively energy, absolute energy and Libdock score can be obtained and the latter was used as the final criteria.

##### QSAR study

The structures of the compounds were drawn with the ISIS DRAW 2.3 program. The final geometries were obtained with the semi-empirical PM3 method in the HYPERCHEM program. All calculations were carried out at restricted Hartree-Fock level with no configuration interaction. The molecular structures were optimized using the Polak-Ribiere algorithm until the root-mean-square gradient was 0.001, the resulted geometry was transferred into software Codessa, which can calculate 470 descriptors for each compound. Then, heuristic method

was employed to select the optimal subset from original calculated descriptors. The step involves correlation of the given property with (i) the top descriptor in the above list with each of the remaining descriptors and (ii) the next one with each of the remaining descriptors, etc. The best pairs, as evidenced by the highest F-values in the two-parameter correlations, are chosen. Then, as shown above, three descriptors were finally obtained. Thus, the linear functions can be developed based on the three descriptors using multiple linear regression module of Codessa software.

## Results and Discussion

Reaction of 5-fluorouracil **4** with chloroacetic acid in the presence of potassium hydroxide gave the corresponding 5-fluorouracil-1-acetic acid **5** in almost excellent yields,<sup>18</sup> which upon treatment with *p*-nitrophenol using *N,N*-dicyclohexylcarbodiimide (DCC) afforded activated ester **6**. Sequential treatment of **6** with a series of L-amino acids in alkaline DMF-H<sub>2</sub>O solvent produced the corresponding [(5-fluorouracil-1-yl)acetyl]-L-amino acids **7a-7g** (Scheme 1).<sup>19</sup> Camptothecin **1** was isolated from a Chinese medicinal plant *Camptotheca acuminata* and served as the starting material for the preparations of all the derivatives. Conversion of the isolated available camptothecin to camptothecin-20-ester of *N*-Boc-glycine derivative **8** in the presence of *N,N*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as catalyst was accomplished by a modified version of Greenwald's method,<sup>20</sup> followed by removal of the *N*-Boc group of **8** with TFA in CH<sub>2</sub>Cl<sub>2</sub> (1:1), forming the TFA salt **9**. The desired compounds **10a-10g** were obtained by treating the TFA salt **9** with the corresponding [(5-fluorouracil-1-yl)acetyl]-L-amino acids **7a-7g** using EDCI as the coupling agent (Scheme 2). The desired compounds **10a-10g** were purified by standard flash chromatography on silica gel and characterized by mp, IR, <sup>1</sup>H NMR, <sup>13</sup>C NMR and HRMS analyses.

##### Cytotoxic activities

The cytotoxicities of compounds **10a-10g** were measured on two different human cancer cell lines (human gastric tumor SGC-7901 and human lung carcinoma A-549) using MTT assay *in vitro*. Irinotecan (**2**) and CPT (**1**) were used as reference compounds. The IC<sub>50</sub> values are shown in Table 1.

As it can be seen in Table 1, against the two cell lines, all of the conjugates were less potent than **1** in cytotoxic assay, which was almost the common feature of prodrugs, and whereas most of compounds exhibited comparable

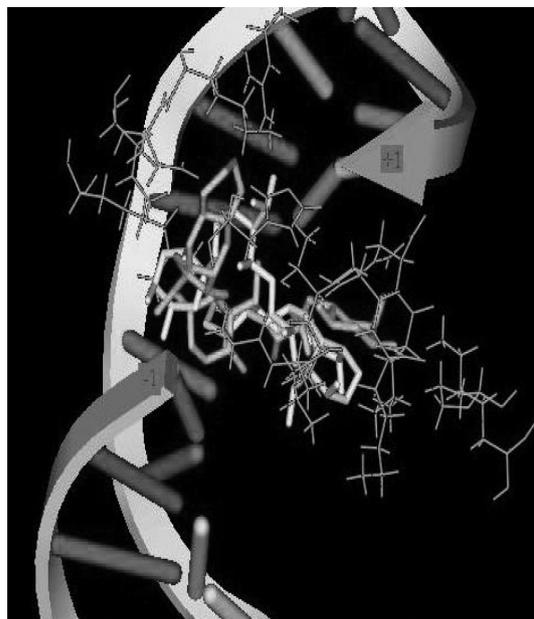


containing hydroxyl and methylthio groups in the dipeptide side chains decreased the activity significantly, this decrease may be caused by hydrogen bonding between the nitrogen or oxygen atoms and the enzyme/DNA might cause the loss of activity, or the relatively polar moieties might impede an important hydrophobic interaction between the molecules and topoisomerase I/DNA. This investigation also further highlighted the fact that the constitution of the dipeptide spacers has a major impact on cytotoxic activity of such analogues. Hence, a systemic, predictable correlation could be made between the nature of dipeptides and anticancer activities. Further pharmacological and toxicological evaluation of these promising compounds is in progress. The detailed explanation of SAR would be discussed in the following QSAR studies.

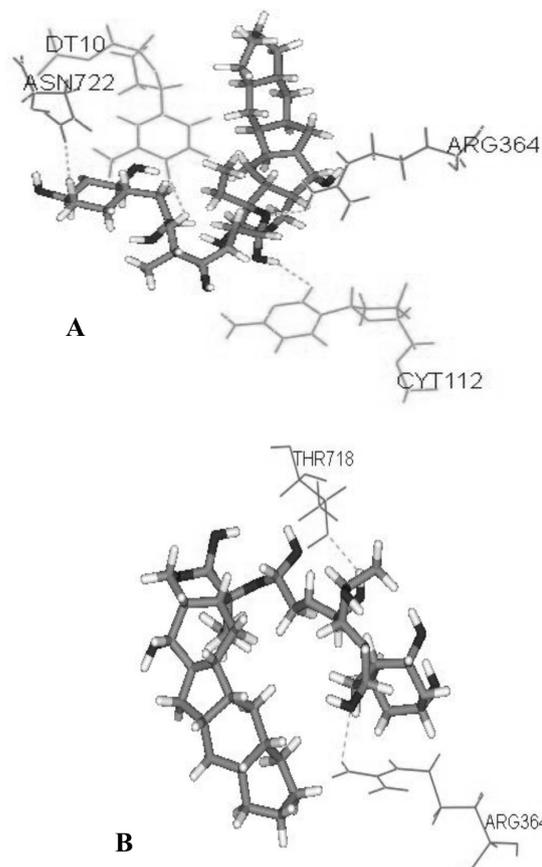
### Molecular docking

Human DNA topoisomerase I (Topo I) is molecular target of CPT derivatives. These compounds can bind to a transient Topo I-DNA covalent complex, leading to an accumulation of DNA strand breaks upon replication, ultimately causing cell death.<sup>21</sup> Thus, we were interested in understanding the interactions of camptothecin derivatives with topoisomerase I by investigating the binding behavior of the two compounds **10b** and **10g**, which can be treated as the representation for these derivatives based on their cytotoxicities on two different human cancer cell lines listed in Table 1. In the present paper, a ligand-receptor docking studies were carried out to explore the probable binding modes within the active site of protein topoisomerase I.

The crystal structure of topoisomerase I (code: 1T8I) was extracted from Protein Data Bank. The hydrogen atoms, missing atoms and residues were added to complete the protein chain. Compounds **10b** and **10g** were minimized and the lowest energy conformation of each ligand was then located. The whole docking studies were performed using Libdock method based on discovery studio 2.1 software in our molecular modeling process. As shown in Figure 2, it can be seen that the two compounds can intercalate at the site of DNA cleavage with base-stacking interactions with both the -1 (upstream) and +1 (downstream) base pair. The orientations of **10b** and **10g** in the active site cavity are perpendicular to the main axis of the DNA while the direction of camptothecin moiety is reverse. Previous studies indicated that hydrogen-bonding interaction between ligand and receptor always played key roles during the formation of drug-DNA-enzyme ternary complex.<sup>22,23</sup> As shown in Figure 3, ternary complex of compound **10b**-DNA-enzyme formed four hydrogen bonds, while the **10g**-DNA-enzyme



**Figure 2.** Base pairs forming stack interaction between Topoisomerase I and compounds **10b** and **10g**. Amino acid residues are colored purple. Compounds **10b** and **10g** are represented by green and yellow color respectively. The backbone of DNA is expressed as white arrows and the base pairs as ladders.



**Figure 3.** Stereoview of hydrogen bonds (marked in green dotted lines) formed between topoisomerase I and the compounds **10b** (A) and **10g** (B). The nucleotides forming hydrogen-bonds are colored blue and the residues are colored carmine.

ternary complex formed two H-bonds, respectively. For the complex with compound **10b** (Figure 3A), camptothecin and 5-fluorouracil moieties formed H-bonds with amino ARG364 and ASN722, in addition, dipeptides linkage moiety produced other two H-bonds with nucleotides. For the complex with compound **10g** (Figure 3B), the two H-bonds formed between the dipeptides linkage moiety with amino acid residues, THR718 and ARG364. As we know, the more hydrogen bond interactions always indicated stronger binding affinity. Thus, compound **10b** has higher potency to form the hydrogen bonding with protein compared with compound **10g**.

From above it can be seen that amino acid residue ARG364 is the necessary amino acid when hydrogen bond formed for both compounds **10b** and **10g**. Furthermore, it can be deduced that amino acid residue ARG364 is likely to play a key role for the process of compounds binding to topoisomerase I.

#### QSAR study

To gain insight into the structural and biological activities of these derivatives, QSAR (quantitative structure-activity relationships) analysis has been widely accepted to account for the various types of activities of the molecule and they are found useful in predicting the activities of other derivatives hitherto unknown. In the present paper, heuristic method (HM)<sup>24</sup> available in the framework of the Codessa program was undertaken to perform a complete search for the best multilinear correlations with a multitude of descriptors.

A large pool of 470 descriptors, including constitutional, topological and quantum chemistry properties for compounds **10a-10g** was calculated using Codessa software. After correlation analysis of the descriptors, some of them were removed to eliminate redundant information. Three descriptors were then obtained to develop the predictive model and their physical-chemical meanings were listed in Table 2. Thus, in the present paper, the cytotoxic activities against human gastric tumor cell SGC-7901 and human lung carcinoma A-549 were regarded as the dependent variable and the selected descriptors were treated as independent variable for constructing the linear regression function 1 and 2 with statistical results squared correlation coefficient ( $R^2$ ), and squared correlation coefficient for leave-one-out cross-validation  $R^2_{cv}$ . In addition, the predicted cytotoxicity activities based on QSAR models were shown in Table 3 and Figure 4.

For cytotoxic activity against human gastric tumor cell SGC-7901 (equation 1):

$$IC_{50}(SGC-7901) = 2.77 \times 10^4 + 3.86 \times 10^5 \text{MaxRE}_{HN} - 2.01 \times 10^1 \text{FNSA}_2 + 5.37 \times 10^{-2} \text{HASA}_2$$

$$n = 7, R^2 = 0.9989, R^2_{cv} = 0.9893, F = 598.82 \quad (1)$$

For cytotoxic activity against human gastric tumor cell A-549 (equation 2):

$$IC_{50}(A-549) = -7.03 \times 10^3 + 1.69 \times 10^{-1} \text{HASA}_1 + 1.42 \times 10^3 \text{MinEE}_{C-N} - 2.05 \times 10^1 \text{ABIC}_2$$

$$n = 7, R^2 = 0.9975, R^2_{cv} = 0.9903, F = 403.54 \quad (2)$$

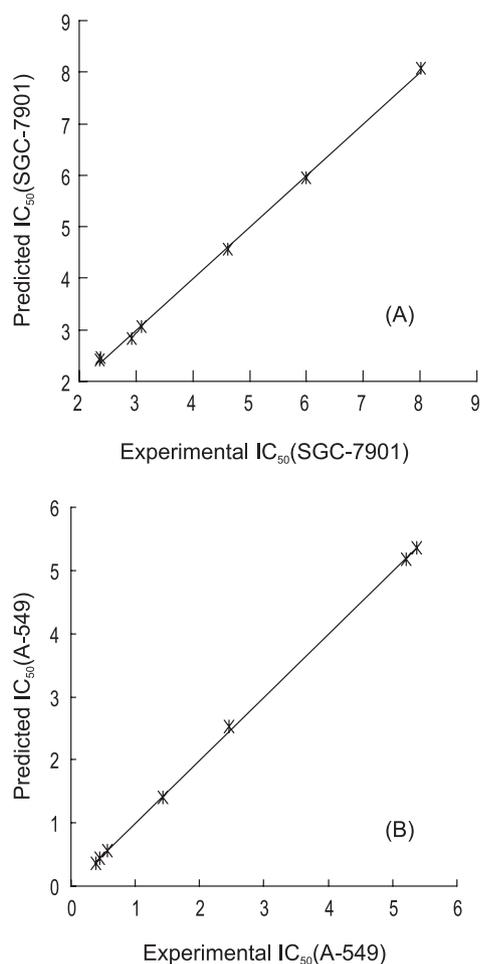
**Table 2.** Physical-chemical meanings for selected descriptors

Dependent variable	Independent variable	Chemical-physical meaning
$IC_{50}(SGC-7901)$	$\text{MaxPC}_N$	Max partial charge for a N atom [Zefirov's PC]
	$\text{FNSA}_2$	FNSA-2 Fractional PNSA (PNSA-2/TMSA) [Zefirov's PC]
	$\text{HASA}_2$	HASA-2 [Quantum-Chemical PC]
$IC_{50}(A-549)$	$\text{HASA}_1$	HASA-1 [Zefirov's PC]
	$\text{MinEE}_{C-N}$	Min exchange energy for a C-N bond
	$\text{ABIC}_2$	Average Bonding Information content (order 2)

**Table 3.** Experimental and predicted activities

Compounds	Experimental activity		Predicted activity	
	$IC_{50}(SGC-7901)$	$IC_{50}(A-549)$	$IC_{50}(SGC-7901)$	$IC_{50}(A-549)$
<b>10a</b>	3.10	2.45	3.07	2.53
<b>10b</b>	2.35	0.45	2.43	0.45
<b>10c</b>	4.62	0.57	4.56	0.57
<b>10d</b>	2.92	0.38	2.84	0.36
<b>10e</b>	2.38	1.43	2.47	1.40
<b>10f</b>	5.99	5.21	5.96	5.18
<b>10g</b>	8.01	5.37	8.06	5.36

Among the selected descriptors which were used to build QSAR model,  $FNSA_2$ ,  $HASA_2$  and  $HASA_1$  were all related with charged partial surface area (CPSA) descriptors, which were originally designed to capture information about the features of molecules responsible for polar intermolecular interactions in terms of functional group portions. By combining the atomic surface areas and charges for specific atom types, CPSA descriptors



**Figure 4.** Plot of calculated versus experimental cytotoxicity activity against human gastric tumor SGC-7901(A) and human lung carcinoma A-549 (B).

can convey information about the specific atom types (such as N, O) or encode the hydrogen-bonding ability of a molecule. In addition, as we know, hydrogen bonds are formed when a hydrogen atom is shared between two molecules. A hydrogen atom covalently attached to a very electronegative atom (N, O, or P) shares its partial positive charge with a second electronegative atom (N, O, or P). Thus, for the parameter “Max partial charge for N atom”, it is also related with the ability of hydrogen-bonding.

Then, from inspection of predictive model, it indicated that the hydrogen bonding interactions may be the most relevant factor controlling the binding behavior and the cytotoxicity activity. Considering the good statistical results, the development QSAR model is suitable to do a good prediction for cytotoxicity activity and it can give us some insight into the factors that govern the cytotoxicity activity and binding behavior with topoisomerase I.

#### *In vitro* determination of lactone levels in human and mouse plasma for **1** and **10b**

The results of *in vitro* determination, based on literature method,<sup>25</sup> of lactone levels in human and mouse plasma buffer for **10b** and CPT (**1**) are shown in Table 4.

As shown in Table 4, the percent lactone of CPT (**1**) in human blood is 28.4% after 4 h, 10.9% after 8 h, and 3.8% after 24 h. In other words, the active form of **1** is significantly decreased in a relative short time period after oral administration. This is the opposite to what is observed in mice, in which the active drug form (*i.e.*, the closed lactone form) lasts for a relative long time period. For example, the lactone form of **1** in mouse plasma is still 40.5% even after 4 h. The lactone form of **10b** in human plasma is much more stable than its mother compound **1**. For example, 70.3% of **10b** is still detected as the lactone form even after 4 h. CPT **1**, in terms of lactone level, shows a difference between mouse and human. Similarly, this kind of difference is also observed for prodrug **10b**. These results further show that the biological life span of lactone

**Table 4.** Comparison of percent lactone of the representative prodrug **10b** and **1** in human and mouse plasmas

	time (h)					
	0	2	4	6	8	24
Human plasma						
lactone for <b>10b</b> (%)	100.0	81.9	70.3	54.3	42.5	10.5
lactone for CPT (%)	100.0	61.4	28.4	19.4	10.9	3.8
Mouse plasma						
lactone for <b>10b</b> (%)	100.0	83.8	80.7	73.1	52.6	17.9
lactone for CPT (%)	100.0	55.1	40.5	24.5	14.6	5.3

forms of these compounds in human and mouse plasma significantly increased when compared with their mother compound CPT **1**. Meanwhile, the cleavage of **10b** in both mouse and human plasmas was monitored by HPLC that clearly showed that compound **10b** was cleaved to afford mainly its parental compound of CPT in both mouse and human plasmas. The cleavage of **10b** is the result of the combination of chemical and enzymatic effects in both mouse and human plasmas. It was also the case in pH 7.4 phosphate buffer.

Another important aspect, which has to be addressed, is that these compounds would be cleaved to release active camptothecin and 5-fluorouracil in both mouse and human plasma and such an assumption was supported by employing HPLC chromatography experimental method, which simultaneously implied that these conjugates might act as mutual prodrugs and exert their cytotoxicities by hydrolyzing to the parent compounds camptothecin and 5-fluorouracil, and they could have synergetic effects in biological systems.

## Conclusions

In summary, we firstly synthesized a series of novel conjugates of camptothecin and 5-fluorouracil joined by dipeptide linkages based on the effective combination principle. Most of compounds exhibited potent cytotoxic activity against tumor cell replication. Particularly, conjugates **10b** and **10d** were highly active against A-549 with  $IC_{50}$  values of 0.45 and 0.38  $\mu\text{mol L}^{-1}$  respectively. Compound **10b** selected for the *in vitro* determination of lactone levels showed that their biological life span in human and mouse plasma was much longer than that of their parent compounds. Moreover, some interesting QSAR equations have been developed for cytotoxic activity and the correlation between the actual *versus* predicted activities are in good agreement particularly by taking into account the molecular graphic models. Molecular docking study was further used to clarify the binding mode of these derivatives. Three hydrogen bonds are formed between the dipeptide linkages of conjugates and the active site of Topo I. The results obtained from QSAR studies indicated that the hydrogen bonding interactions may be the most relevant factor controlling the binding behavior and the cytotoxic activity. Considering the good statistical results, the development of QSAR model is suitable to do a good prediction for cytotoxicity activity and it allows for the rational design of more potent conjugates.

These results are encouraging and suggested that the design and syntheses of these compounds should be beneficial for therapeutic values of camptothecin analogues and the approach should be applicable for other antitumor

agents, and it is worthwhile to explore the antitumor potential of these and similar types of compounds. In-depth mechanistic studies and the development of new camptothecin/5-fluorouracil conjugates are actively underway in our laboratory.

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