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E-ring-modified 7-oxyiminomethyl camptothecins: Synthesis and preliminary in vitro and in vivo biological evaluation

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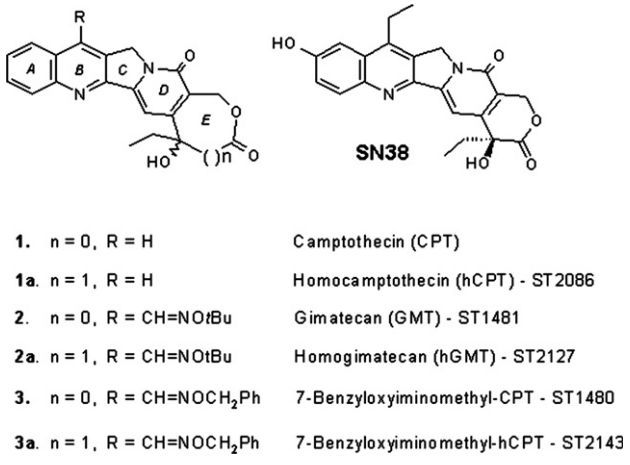
Abstract—In contrast to five-membered E-ring analogues, 7-oxyiminomethyl derivatives of homocamptothecins showed ability to form stable ternary complexes with DNA and topoisomerase I. The 7-oxyiminomethyl derivatives of homocamptothecins were evaluated as a racemic mixture. Following the isolation of the two enantiomers, the 20 (*R*)-hydroxy isomer confirms the best activity. By using a panel of human tumor cells, all tested homocamptothecins showed a potent antiproliferative activity, correlating to the persistence of the cleavable complex. No significant difference was observed between the natural scaffold and the corresponding homocamptothecin homologue. A selected compound of this series exhibited an excellent antitumor activity against human gastrointestinal tumor xenografts.

Camptothecin and its analogues exhibit a broad spectrum of antitumor activity, representing a very promising class of anticancer agents currently used in clinical practice and characterized by a selective activity as topoisomerase I inhibitor.[1]

Chemically, camptothecins are characterized by a pentacyclic moiety with an α-hydroxy lactone ring (E-ring, Fig. 1) which, under alkaline conditions, is present in its less potent open form inhibiting the topoisomerase I enzyme and in vivo potency. Modifications in the E-ring generally reduce or abolish this activity. In 1997, Lavergne et al., aiming to improve the drug's stability in human plasma, synthesized a novel seven-membered E-ring homologue, where the natural α-hydroxy-δ-lactone E-ring was replaced by a β-hydroxy-ε-lactone ring. These new derivatives, called *homocamptothecins* (hCPT),[2] showed an activity comparable to that of the natural scaffold (Fig. 1).



1.	n = 0, R = H	Camptothecin (CPT)
1a.	n = 1, R = H	Homocamptothecin (hCPT) - ST2086
2.	n = 0, R = CH=NO*t*Bu	Gimatecan (GMT) - ST1481
2a.	n = 1, R = CH=NOtBu	Homogimatecan (hGMT) - ST2127
3.	n = 0, R = CH=NOCH$_2$Ph	7-Benzyloxyiminomethyl-CPT - ST1480
3a.	n = 1, R = CH=NOCH$_2$Ph	7-Benzyloxyiminomethyl-hCPT - ST2143

Figure 1. Structures of corresponding CPT and hCPT homologues and Structure of SN38.

In this decade, a number of hCPT derivatives were synthesized not only by the total synthesis, but also by the following racemic and enantioselective synthetic strategies, starting from the natural scaffold. Some of these hCPTs, such as diflomotecan (currently in Phase II clinical trials),[3–5] were evaluated in many clinical trials. Since the introduction of a methylene group in the

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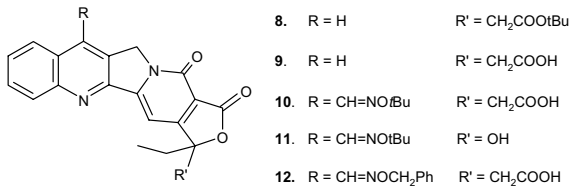


Figure 2. Structures of E-ring-modified CPTs [**8**: ST2084; **9**: ST2085; **10**: ST2196; **11**: ST2715; **12**: ST2285].

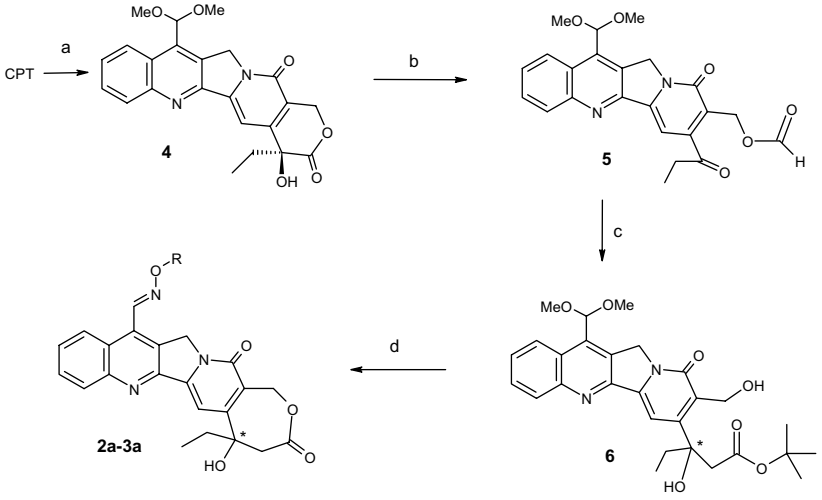
E-ring of CPT provides enhanced stability and decreased protein binding in human plasma without affecting its capability to poison topoisomerase I-DNA complex, the present study was undertaken to explore whether this modification improves the biological properties of the 7-oxyiminomethyl derivatives[6,7] (Fig. 1). The most advanced derivative of this series is gimatecan, currently in Phase II clinical trials.[8] This is the first study on 7-oxyiminomethyl-CPT modified in the E-ring.

The 7-oxyiminomethyl hCPT derivatives (Scheme 1) were prepared in enantiomeric mixture by the synthetic approach cited above (Lavergne).[2] CPT was treated with sulfuric acid, $FeSO_4 \cdot 7H_2O$ and 40% H_2O_2 to give the corresponding 7-hydroxymethyl derivative; this intermediate was converted in situ to 7-dimethylacetal derivative **4** by oxidation with MnO_2 in MeOH. $NaBH_4$ was added to a solution of **4** in MeOH. A subsequent reaction with $NaIO_4$ in CH_3COOH gave intermediate **5**. Under Reformatsky conditions **5**, with *tert*-butyl bromoacetate, gave β-hydroxyester **6** which, by treatment with *O*-*tert*-butyl hydroxylamine hydrochloride or *O*-benzyl hydroxylamine hydrochloride in CH_3COOH, at 80 °C, was directly converted into **2a** and **3a**, respectively.

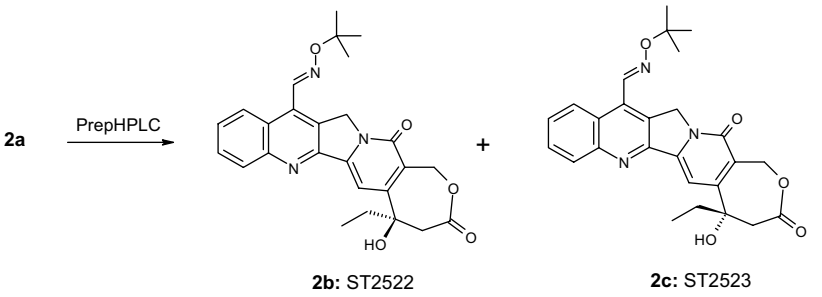
The racemic mixture of **2a** was separated by prep HPLC (Scheme 2) into the two single enantiomers (**2b** and **2c**), using a chiral stationary phase.

According to what was expected with the hydroxyl group in specific C-20 configuration, 20(*R*)-hydroxyl-hGMT (**2b**) was more active than 20(*S*)-hydroxyl-hGMT (**2c**).

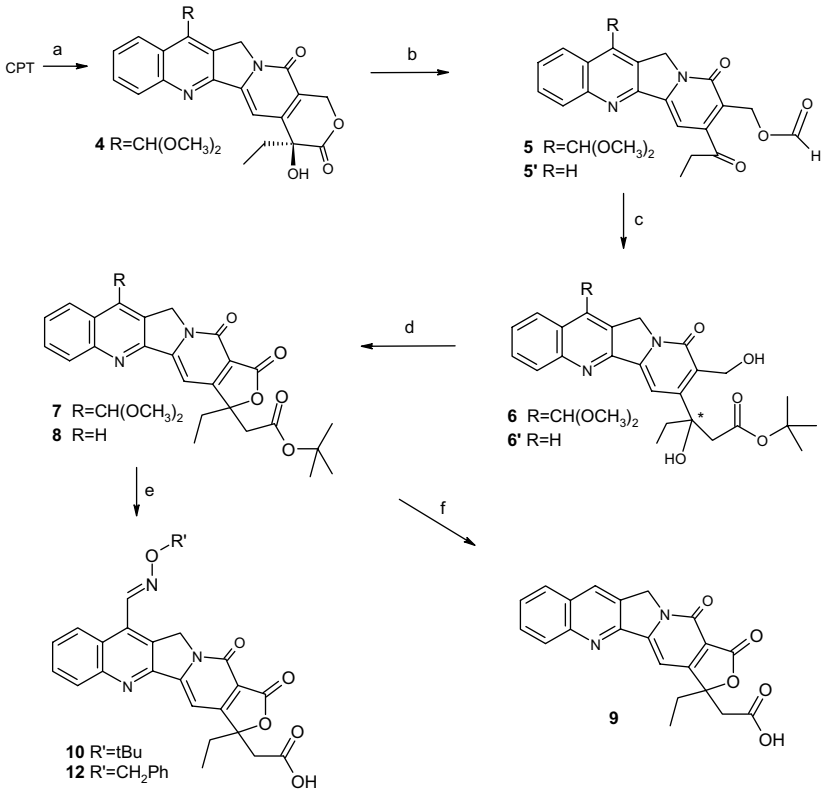
Another series with a modified E-ring was also prepared (**8**–**12**; Fig. 2). According to the previous observations,[9] the five-membered E-ring analogues exhibited a markedly reduced cytotoxic activity ($IC_{50} \gg 1$ μM, 2-h exposure against H460 cells).



Scheme 1. Synthesis of 7-oxyiminomethyl hCPT analogues **2a** and **3a**. Reagents and conditions: (a) 96% H_2SO_4, $FeSO_4 \cdot 7H_2O$, 40% H_2O_2, MnO_2, MeOH, 10–50 °C (83%). (b) i—$NaBH_4$, MeOH, rt (78%); ii—$NaIO_4$, CH_3COOH, rt (89%). (c) Zn, anhydrous Et_2O $(CH_3)_3SiCl$, $BrCH_2COO^tBu$, anhydrous THF, reflux (57%). (d) $RONH_2 \cdot HCl$, CH_3COOH, 80 °C (**2a** R = –*t*-Bu 56%, **3a** R = $-CH_2Ph$ 45%).



Scheme 2. Separation of the two enantiomers **2b** and **2c**. [Column (*S*,*S*)-DACH-DNB 5/100, eluent: CH_2Cl_2/*n*-hexane/MeOH (80:20:0.1); 1 mL/min, 22 °C, λ = 360 nm].

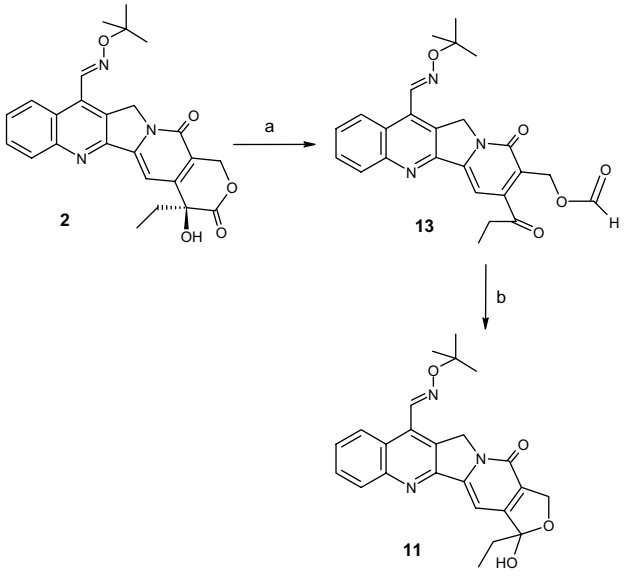


Scheme 3. Synthesis of five-membered E-ring CPT analogues **8**–**9**–**10**–**12**. Reagents and conditions: (a) 96% H_2SO_4, $FeSO_4{\cdot}7H_2O$, 40% H_2O_2, MnO_2, MeOH, 10 °C to 50 °C (**4**: 83%). (b) i—$NaBH_4$, MeOH, rt (R = H 75%, R = $CH(OCH_3)_2$ 78%); ii—$NaIO_4$, CH_3COOH, rt (R = H (**5′**) 80%, R = $CH(OCH_3)_2$ (**5**) 89%). (c) Zn, anhydrous Et_2O $(CH_3)_3SiCl$, $BrCH_2COOtBu$, anhydrous THF, reflux (**6**: 58%; **6′**: 60%). (d) PDC, CH_2Cl_2 rt (**7**: 74%; **8**: 73%). (e) $R'ONH_2{\cdot}HCl$, CH_3COOH, 80 °C (**10**: 68%, **12**: 61%). (f) TFA, CH_2Cl_2 rt (**9**: 100%).

An analogue approach was used to obtain intermediates **6**–**6′** in the synthesis of five-membered E-ring analogues **8**–**9**–**10**–**12** (Scheme 3). β-Hydroxyester **6**–**6′** (with R = $CH(OCH_3)_2$ or H), by treatment with PDC (Pyridinium DiChromate) in CH_2Cl_2 at rt, gave the five-membered E-ring intermediates **7** and **8**; this latter, under hydrolysis with TFA in CH_2Cl_2, gave **9** in quantitative yield. The subsequent reaction of **7** with *O-tert*-butyl hydroxylamine hydrochloride or *O*-benzyl hydroxylamine hydrochloride in CH_3COOH at 80 °C led to**10** and **12**, respectively.

Compound **2** was converted into β-hydroxyester (**13**) by a two-step reaction (Scheme 4), $NaBH_4$ in MeOH and subsequently $NaIO_4$ in CH_3COOH. A cyclization reaction with K_2CO_3 in MeOH/H_2O (10:1 v/v) gave **11**.

The antiproliferative effects of the hCPTs were determined after 72 h following a 1-h exposure to the drugs. Compared to topotecan [9-dimethylaminomethyl-10-hydroxy-camptothecin] and SN38 [7-ethyl-10-hydroxy-camptothecin], the hCPTs studied (Table 1) exhibited a higher potency in inhibiting cell growth.



Scheme 4. Synthesis of **11**. Reagents and conditions: (a) i—$NaBH_4$, MeOH, rt (78%); ii—$NaIO_4$, CH_3COOH, rt (89%). (b) K_2CO_3, MeOH/H_2O (10:1 v/v) (65%).

As expected, the expansion of the lactone ring of CPT produced an increased antiproliferative activity (**1** vs **1a**). The cytotoxic potency of **2a** and **3a** was comparable to that of the six-membered CPT, **2** and **3**, respectively. The expansion of the lactone ring of gimatecan was slightly detrimental for cytotoxic potency (**2** vs **2a**) while an increased activity was observed for benzyl derivative (**3** vs **3a**). In addition, compared to **2a**, compound **3a** exhibited an increased cytotoxic potency. These features indicated the advantage to have a lipophilic substituent

Table 1. Antiproliferative activity of 7-modified homocamptothecins

Drug	IC_{50}[a] (μM)			
	H-460	HT29	HT29/mit	RI
Topotecan	1.18 ± 0.24	1.35 ± 0.66	46.29 ± 21	34
SN38	0.22 ± 0.013	0.67 ± 0.36	11.5 ± 2.7	17
1	0.18 ± 0.01	3.16 ± 1.2	8.81 ± 2.35	2.8
1a	0.0496 ± 0.047	0.44 ± 0.0002	8.55 ± 2.48	19
2	0.02 ± 0.005	0.056 ± 0.0223	0.070 ± 0.0022	1.2
2a	0.0368 ± 0.003	0.041 ± 0.0086	0.56 ± 0.0867	14
3	0.03 ± 0.002	0.056 ± 0.022	0.042 ± 0.014	0.75
3a	0.015 ± 0.0003	0.017 ± 0.0021	0.35 ± 0.022	20
2	0.015 ± 0.006[c]			
2b	0.0018 ± 0.0002[c]			
2c	>0.2[c]			

[a] IC_{50}, drug concentration required for 50% reduction of cell growth as compared to untreated controls after 1-h exposure to the drug. Means ± SD are reported from at least three experiments.
[b] RI, resistance index in HT29/mit.
[c] IC_{50}, determined after 2-h exposure.

in position 7 which could account for a more favorable intracellular accumulation. During two hours exposure, 20 (*R*) homogimatecan revealed a 10-fold increase in cytotoxic activity in comparison to gimatecan (**2** vs **2b**). Although effective in inhibiting the proliferation of HT29/mit, all compounds exhibited cross-resistance in this subline which overexpresses BCRP (Breast Cancer Resistance Protein), a transport system implicated in the resistance to conventional CPTs.[10] The results shown in Table 1 support that all tested homocamptothecins (**1a**, **2a**, and **3a**) are better substrates for BCRP than the corresponding CPT analogues with the natural E-ring.

Topoisomerase I-mediated DNA cleavage assays with purified human topoisomerase I were used to investigate the ability of hCPT to stimulate DNA damage. The hCPTs revealed an intensity of DNA damage comparable to that of SN38, used as reference compound (Fig. 3).

The cleavage pattern revealed additional sites not detected in the presence of SN38 (arrows in figure). Since the drug interaction with the DNA–enzyme complex is expected to be reversible, the persistence of the cleavable complex was evaluated after the addition of high salt concentrations (0.6 M NaCl), which favors the dissociation of the ternary drug–enzyme–DNA complex. As compared to SN38, **3a** and **2a** revealed a more stable ternary complex. Such stability was particularly evident for **2a**, which exhibited a DNA damage persistence of around 90% after 10 min (Fig. 4). From the inhibition data of topoisomerase I of the three compounds, **1a**, **2a**, and **3a**, it is evident that the presence of a bulky group in 7-position on B-ring of CPT scaffold, favors tertiary complex stability, as demonstrated by the introduction of a residual benzyloxyiminomethyl-group (**3a**) and *tert*-butyloxyiminomethyl (**2a**) in this position.

On the basis of their antiproliferative potency, **3a** and **2a** were selected for further preclinical development (Table 2). The antitumor activity of oral **2a** was studied in a

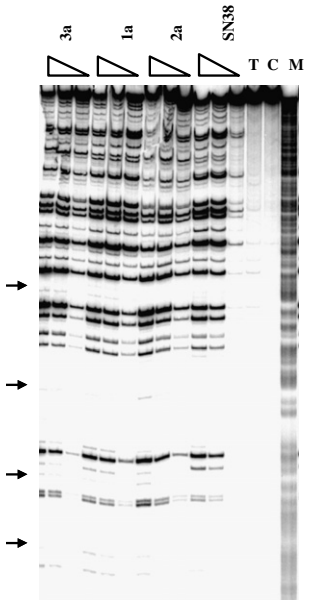


Figure 3. Topoisomerase I-mediated DNA cleavage assays. The samples were reacted with 1, 10, and 50 μM drug at 37 °C for 30 min. Reaction was then stopped by adding 1% SDS, 0.3 mg/mL of proteinase K, and incubating for 45 min at 42 °C before loading onto a denaturing 8% polyacrylamide gel. C, control DNA; T, reaction without drug; M, purine markers. Arrows indicate the additional sites observed for hCPTs. The experiment was repeated three times, reporting the results of a representative value.

panel of human tumor xenografts. The maximum tolerated dose (MTD) was determined with an intermittent q4dx4 schedule. In animals bearing MKN-28 gastric carcinoma, **2a** was well tolerated up to 12 mg/kg (Table 2). Derivative **3a** was substantially less effective under a comparable range of doses. Therefore, **2a** was also tested against CoBA colon carcinoma and pancreatic carcinoma, Panc-1. Against CoBa, a dose of 15 mg/kg was effective without lethal toxicity, but with substantial body weight loss (Table 2 and Fig. 5). A 20 mg/kg dose was highly toxic in most treated mice (3/4). With a dose of 14 mg/kg against the pancreatic carcinoma model

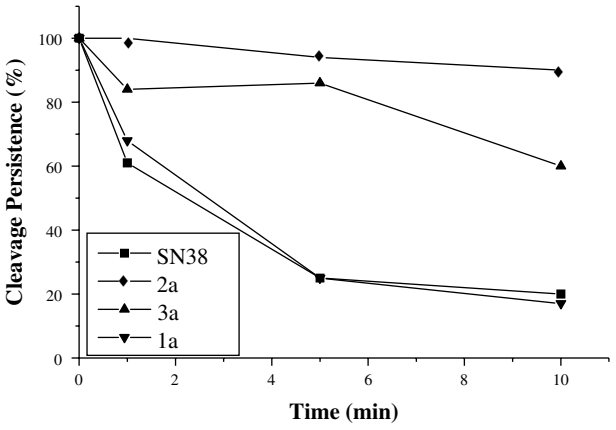


Figure 4. Cleavage persistence assay. The samples were reacted for 30 min with 10 μM drug. DNA cleavage was then reversed by adding 0.6 M NaCl. The 100% value refers to the extent of DNA cleavage after 30 min of incubation. Each value was obtained by densitometric analysis. The experiment was repeated three times, reporting the results of a representative value.

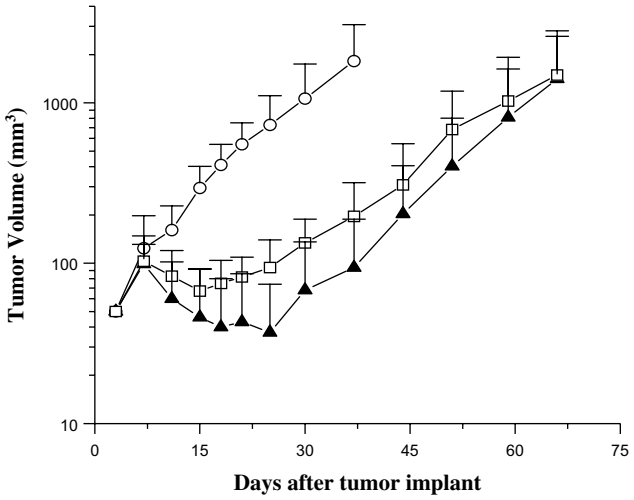


Figure 5. In vivo antitumor activity of **2a**. (ST2127) on sc-growing CoBa colon carcinoma. (○) Untreated control tumors; (▲) oral **2a**, 15 mg/kg, q4dx4. (□) CPT11, 50 mg/kg, q4dx4. Bars, standard deviation.

Panc-1, there was excellent antitumor activity with no evidence of lethal toxicity. Indeed, 9/10 treated tumors exhibited complete tumor regression (Table 2). The activity of **2a** was superior to that of irinotecan at maximum tolerated doses under similar treatment conditions.

Among the many strategies adopted in the last years aimed at improving lactone stability of CPT, the synthesis of five- and seven-membered E-ring CPT appears to be the most promising.[12,3–5] The results reported in the present manuscript indicated that the elimination of a methylene group of the lactone ring is detrimental for the activity of the studied CPTs. In contrast to five-membered E-ring analogues, 7-oxyiminomethyl derivatives of hCPT exhibit a cytotoxic activity comparable to that of the corresponding CPT with the natural lactone ring. The racemic hGMT (**2a**) separates into the two single enantiomers (**2b** and **2c**) and the 20(*R*) isomer shows more in vitro activity than the corresponding 20(*S*). In addition, compared to GMT (**2**), 20(*R*) hGMT (**2b**), reveals about a 10-fold increase in cytotoxic potency. However, in contrast to GMT, hCPTs appear to be the substrates of BCRP, which is expected, since it was already reported that the expansion of the lactone ring increase the recognition of the drug by BCRP.[11] As already reported for other compounds of this series (e.g., GMT), the tested hCPTs exhibit stable ternary complexes with DNA and topoisomerase I. On the basis of the antiproliferative data reported in Table 1, and of stabilization of the cleavable complex (Fig. 4), it is evident that the inhibition of the intracellular target is the crucial event responsible for cytotoxic potency. Indeed, the two potent hCPTs (**2a** and **3a**) exhibited an increased capability to stabilize the cleavable complex. Although the stabilization of the cleavable complex is recognized

Table 2. Effects of **3a** (ST2143) and **2a** (ST2127) given orally, q4dx4, against human gastrointestinal tumors xenografted sc in athymic nude mice

Tumor model	Drug	Dose (mg/kg)	TVI%[a]	CR[b]	LCK[c]	BWL%[d]	Tox[e]
MKN28	3a	4	17	0/8	0	0	0/4
Gastric carcinoma	3a	8	60	0/8	0.4	6	0/4
	3a	16	47	0/8	0.2	0	0/4
	2a	3	71	0/8	0.6	0	0/4
	2a	6	47	0/8	0.2	0	0/4
	2a	12	81	0/8	0.6	2	0/4
Panc-1	2a	14	99	9/10	1.4	5	0/5
Pancreatic carcinoma	2a	15	99	7/8	1.6	16	1/5
	CPT11	400	99	5/10	1.6	7	0/5
CoBa	2a	15	95[f]	0/8	1.3	21	0/4
Colon carcinoma	2a	20	—	—	—	18	3/4
	Gimatecan	2	90	0/8	1.1	18	0/4
	CPT11 (iv)	50	86	0/8	1.1	2	0/4

[a] Tumor volume inhibition % in treated control mice.
[b] Complete responses: that is, disappearance of tumor lasting at least ten days.
[c] Gross log10 cell kill to reach an established tumor volume; the highest change is reported.
[d] Body weight loss % induced by drug treatment.
[e] Dead/treated mice.
[f] $P < 0.05$ vs CPT11, by Student's t test.

to be one of the most important feature for CPT activity, differences in cellular uptake and subcellular distribution may contribute to determine antitumor activity. Such observations suggest that optimal drug efficacy can be achieved from a balance between cellular pharmacokinetics and intrinsic ability to induce DNA cleavage.

A selected compound of this series (**2a**) produces remarkable antitumor activity against human gastrointestinal tumors. The antitumor potency and efficacy of the novel hGMT were apparently superior to that of CPT11. The advantages of **2a** over GMT remain to be documented.

Acknowledgments

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Supplementary data

Materials and methods: Growth Inhibition Assay; Topoisomerase I–Dependent DNA Cleavage Assay; Antitumor activity studies. References for material and methods. NMR spectra: ST2084; ST2085; ST2086; ST2127; ST2143; ST2196; ST2285; ST2715. Polarimetric Analysis of the two single enantiomers ST2522; ST2523. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.03.074.

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