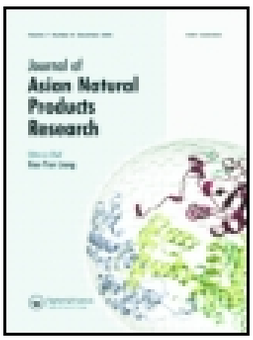

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Synthesis and antitumor activity of a series of lactone-opened camptothecin derivatives

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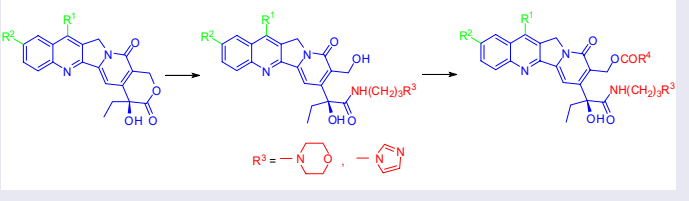
ABSTRACT

A series of E-ring lactone-opened camptothecin (CPT) derivatives bearing with terminal aza-heterocyclic groups were synthesized, and their antitumor activity was evaluated both *in vitro* and *in vivo*. Hydroxyl-amide analogues with morpholin-4-yl displayed excellent antitumor activity *in vitro* and efficient inhibition on tumor xenograph model in nude mice. Ester-amide compounds acted less active *in vitro* cytotoxicity and lower inhibition activity *in vivo*. Substitutions at 7- and 10- positions favored the antitumor activity.



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Camptothecin derivatives; E-ring lactone-opened; Synthesis; Antitumor activity

1. Introduction

Camptothecin (CPT, **1**, Figure 1), an natural antitumor alkaloid first isolated from Chinese tree *Camptotheca acuminate* by Wall and Wani in 1966 [1], has been drawing long-lasting interests. As potent inhibitors of DNA Topoisomerase I [2], CPT and some of its analogues form so-called "cleavable complex" with Topo I and DNA [3], which prevents DNA relegation and induces Topo I-mediated DNA breaks. Three CPT analogues, irinotecan (**2**) [4], topotecan (**3**) [5], and belotecan (**4**) [6], have been approved as chemotherapeutic drugs for treatment of human cancers in clinical and several other derivatives are in different phases of clinical developments [7–9].

The effect of phosphate groups on the hydrolysis of E-ring has been investigated by using UV-visible RP-HPLC (reverse-phase high-performance liquid chromatography) assay and kinetic solvent isotope effect (KSIE). It was concluded that P-CPT had a pH-dependent

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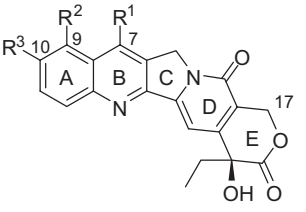
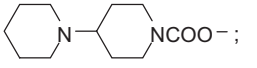


1 Camptothecin(CPT):R ^{1} =R ^{2} =R ^{3} =H;

2 Irinotecan:R ^{1} =Et,R ^{2} =H,R ^{3} =  ;

3 Topotecan:R ^{1} =H,R ^{2} =Me $_{2}$NCH$_{2}$-,R ^{3} =OH;

4 Belotecan:R ^{1} =Me $_{2}$CHNHCH$_{2}$CH$_{2}$-,R ^{2} =R ^{3} =H.

Figure 1. CPT and its famous derivatives.

equilibrium between lactone and carboxylate forms, similar to but not identical to CPT. But the early clinical trails with E-ring sodium salt of CPT (1a, Figure 2) were failed due to its low activity *in vivo* and unexpected side effects [10]. So intact lactone E-ring was once considered to be indispensable to maintain antitumor activity for CPT analogues [11,12]. However, further investigations on lactone-opened form of CPT derivatives demonstrated that E-ring-opened hydroxyl-amide compounds (**5**, Figure 2) or ester-amide compounds (**6**) were found to have comparable activity with CPT against human tumor cell lines P388, L1210, and B16 *in vivo* [13]. More recently, some CPT derivatives with lactone-opened ester-amide, conjugating with PEG (polyethylene glycol) through the C-21 acid functionality or through the 17-hydroxy group of CPT (**7**, **8**), were also exhibited high activity against a mammary carcinoma (MX-1) xenograph in nude mice [14]. Furthermore, the

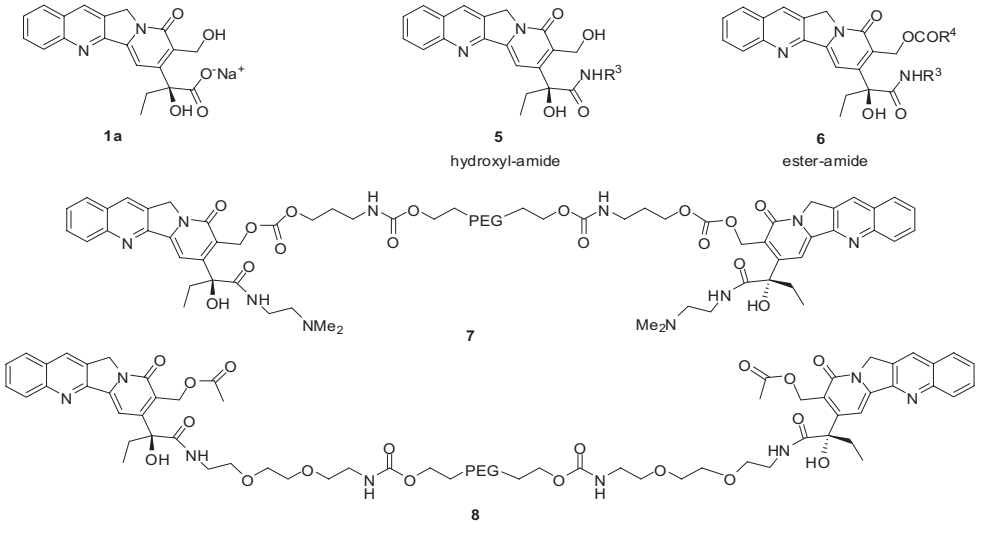


Figure 2. CPT derivatives with lactone-opened.

structure–activity relationship studies indicated that substitutions at 7- and 10-positions of CPT would enhance the antitumor activity [15]. All these survey implied the great potential in developing novel lactone-opened form of CPT derivatives.

In this paper, we firstly introduced aza-heterocyclic groups such as morpholin-4-yl or imidazol-1-yl to a series of CPT hydroxyl-amide and ester-amide analogues (**11a**, **11b**, and **12a–d**, Scheme 1). We hoped that these amide groups not only could be converted to amine salts and hence to enhance their aqueous solubility, but also could offer additional hydrogen-bonding sites for biological macromolecules, both of which may increase their biological activity.

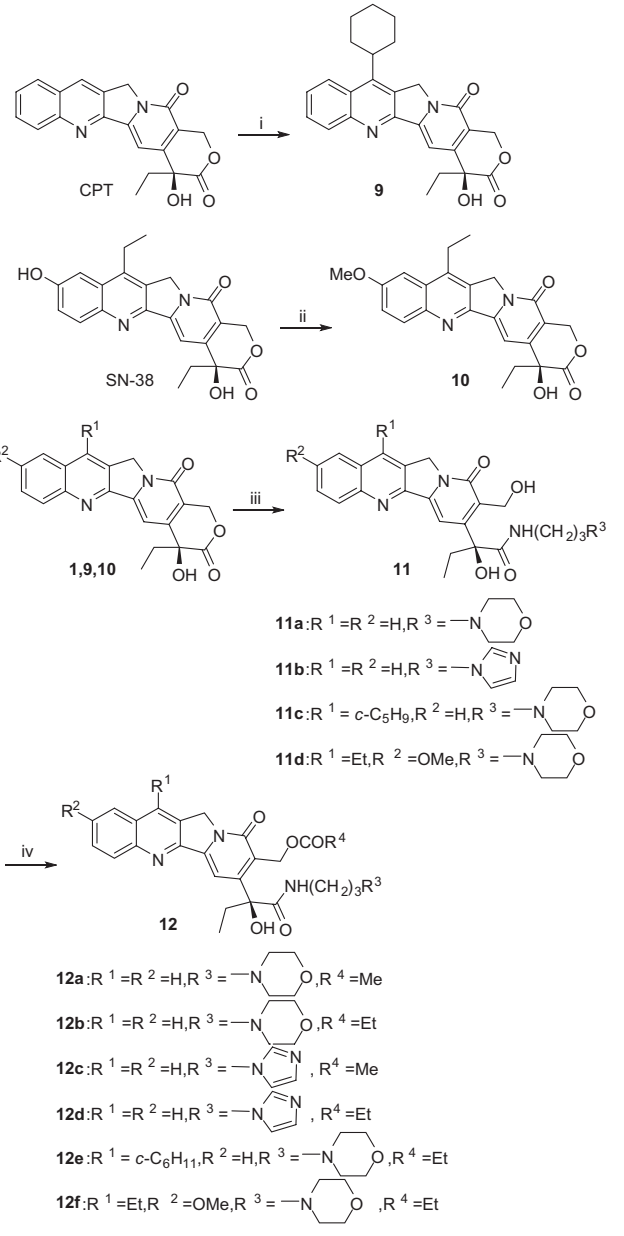
2. Chemistry

Synthesis of hydroxyl-amides (**11**) and ester-amides (**12**) derivatives is summarized in Scheme 1. Camptothecin was treated with excess of amines (3-morpholin-4-yl-propylamine or 3-imidazol-1-yl-propylamine) at 60 °C to give hydroxyl-amides **11**. Selective acylation of 17-hydroxy was processed with anhydride-pyridine condition and yielded the 17-esters (**12**). Preliminary in vitro antitumor activity evaluation towards compounds 11a-b and 12a-d provided instructive information for subsequent structure-activity relationships study, which secured access to the synthesis of compounds 11c-d and 12e-f based on the above mentioned procedure. The compound 7-cyclohexyl-CPT (**9**) was prepared with Sawada method [4], and 7-ethyl-10-methoxyl-CPT (**10**) was obtained from methylation of 7-ethyl-10-hydroxy-CPT with iodomethane and anhydrous potassium carbonate in refluxing acetone.

Hydroxyl-amides (**11a–d)** were found to be difficult to purify [13] for being unstable in acidic or neutral medium. **11a** indeed was observed to turn back to CPT in $CHCl_3$ solution. However, after procedures optimization, it could be obtained as solid with up to 97.4% purity (2.1% of CPT as impurity, according to the high-performance liquid chromatography (HPLC) analysis) in 2 g scale. **11a** was stable enough in aqueous solution (5 mg **11a** in 1 ml water, the pH is 10.36), and no more CPT was observed after 40 h in this solution based on HPLC analysis. When the compound was dissolved in PBS (phosphate buffer solution, pH = 7.03), about a half of CPT generated after 40 h. Compound **12b,** which is more stable, can be purified on a silica gel column to get the product with up to 99.3% purity.

3. Results of biological assay and discussion

The primary test for inhibition rate of compounds **11a–b and 12a–d** was performed on four human cancer cell lines HL-60, A549, BGC-823, and SMMC-7721 by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay, using mitomycin and cisplatin as references. The results are summarized in Table 1. Cell lines HL-60 and A549 are more sensitive to these CPT analogues than cell lines BGC-823 and SMMC-7721. Hydroxyl-amides **11a** and **11b** generally showed much higher activity than their esterified counterparts. Derivatives substituted with morpholin-4-yl have higher inhibition rates than that with imidazol-1-yl on most of the cell lines. Among the ester-amides, propionate compounds displayed more active than acetate analogues (**12b** vs. **12a**, **12d** vs. **12c**) in inhibiting the tumor cell lines. Based on this preceding observation, modification of 7, 10-substituted CPTs (compounds **9** and **10**) was focused on morpholin-4-yl and 17-propionate derivatives.



Scheme 1. General chemical procedure. Reagents and conditions (i): cyclohexanecarbaldehyde, H_2O_2, H_2SO_4, HOAc-H_2O, 0 °C, 3 h; (ii): MeI, K_2CO_3, acetone, reflux over night; (iii) amine, 60 °C; iv: $(R^4CO)_2O$, Py, 40 °C.

The IC_{50} values of selected compounds against human cancer cell lines A549/ATCC and HT29 were listed in Table 2. On these two cell lines, activity of hydroxyl-amide compounds **11** displayed more active than references CPT, SN38, and topotecan, whereas only compound **12f** has comparable activity with the reference drugs for the ester-amide compounds. 7- and 10-substitutions were performed to enhance the activity, particularly on HT29 cell lines.

Table 1. Inhibition rate of lactone-opened CPT derivatives against four human tumor cell lines.

		Percentage of tumor cells inhibition[a] (%)			
compds	Conc. (M)	BGC-823	HL-60	A549	SMMC-7721
11a	1×10^{-7}	31.28	70.41	53.88	27.63
	1×10^{-6}	44.70	91.82	87.35	17.40
	1×10^{-5}	50.66	97.32	99.59	33.45
11b	1×10^{-7}	33.20	55.72	17.14	29.62
	1×10^{-6}	35.72	85.50	85.51	33.88
	1×10^{-5}	63.57	94.01	96.94	53.91
12a	1×10^{-7}	19.78	1.41	12.24	19.03
	1×10^{-6}	25.53	49.88	31.63	19.74
	1×10^{-5}	24.72	89.68	87.35	17.26
12b	1×10^{-7}	12.51	28.91	41.84	23.30
	1×10^{-6}	29.16	80.54	85.71	22.94
	1×10^{-5}	53.48	95.86	95.71	55.33
12c	1×10^{-7}	26.94	5.35	5.71	13.14
	1×10^{-6}	19.27	26.28	12.24	14.70
	1×10^{-5}	26.84	74.21	63.27	17.26
12d	1×10^{-7}	19.78	0.00	6.73	14.91
	1×10^{-6}	11.81	58.35	28.37	21.38
	1×10^{-5}	17.76	91.48	90.20	4.05
Mitomycin	1×10^{-7}			18.42	5.03
	1×10^{-6}	NT[b]	NT	41.93	49.14
	1×10^{-5}			89.46	61.68
Cisplatin	1×10^{-7}	11.00	24.82	NT	NT
	1×10^{-6}	40.90	46.37		
	1×10^{-5}	92.94	96.79		

[a]Evaluation standard of antitumor activity: high activity (inhibition rate % ≥ 50 in conc. 1×10^{-7} M, ≥ 50 in conc. 1×10^{-6} M, ≥ 50 in conc. 1×10^{-5} M); moderate (inhibition rate % < 50 in conc. 1×10^{-7} M, ≥ 50 in conc. 1×10^{-6} M, ≥ 50 in conc. 1×10^{-5} M); low activity (inhibition rate % < 50 in conc. 1×10^{-7} M, < 50 in conc. 1×10^{-6} M, ≥ 50 in conc. 1×10^{-5} M); poor activity (inhibition rate % < 50 in conc. 1×10^{-7} M, < 50 in conc. 1×10^{-6} M, < 50 in conc. 1×10^{-5} M).
[b]NT = No Tested.

Table 2. Cytotoxicity of 7- and 10- modified CPT analogues on human cell lines A549/ATCC and HT29.

	IC_{50}[a] (μM)			IC_{50}[a] (μM)	
Compounds	A549/ATCC	HT29	Compds	A549/ATCC	HT29
11a	0.03	0.05	**12a**	2.88	2.93
11c	0.01	0.01	**12e**	1.06	1.09
11d	0.02	0.02	**12f**	0.2	0.27
SN38	0.06	0.08	Topotecan	0.5	0.23
CPT	0.05	0.12			

[a]Measured with sulphorhodamine (SRB) method.

Tables 3 and 4 depict the *in vivo* antitumor efficacy of hydroxyl-amide **11a** and ester-amide **12a,** respectively. Compound **11a** was tested in a human tumor xenograft model (A549 cell line, Table 3) in mice using HCPT (10-Hydroxylcamptothecin) as reference drug. The result indicated that compound **11a** had better ability in inhibiting the growth of the transplanted tumor than HCPT under the same conditions. No mice died in this series of treatments. Compound **12a** was treated on transplanted HT-29 tumor cell line with irinotecan as reference. It could markedly inhibit the growth of the tumor, but displayed higher toxicity (several mice died in the treatment) and lower efficacy in reducing tumor growth compared with irinotecan.

The activity of lactone-opened hydroxyl-amides was once ascribed to the reform of CPT. However, according to our investigation, only less than 50% of compound **11a** turned back

Table 3. Antitumor efficacy of compound **11a** against mouse tumor A549 using intravenous injection (iv).

Tumor	Compounds	Dose(mg/kg)	Lethala[a] toxicity	TV[b](X±SD, mm^3)		RTV[c] X ± SD	T/C[d] (%)
				D_0	D_n		
A549	NS	–	0/10	128 ± 71	390 ± 342	2.8 ± 1.0	
	HCPT	8	0/5	131 ± 54	258 ± 137	2.0 ± 0.5	71.4
	11a	10	0/5	132 ± 134	211 ± 237	1.5 ± 0.4	53.6*
	11a	5	0/5	126 ± 72	407 ± 365	2.9 ± 1.3	103.6
	11a	2.5	0/5	120 ± 23	200 ± 73	1.7 ± 0.5	60.7*

[a]Number of dead mice/total number of mice in this series of treatment for 17 days.
[b]Tumor volume (TV) of D_0 (the day when the chemicals were given) and D_n (the day of the optimal treatment, which was the 17th day in the experiments), $TV = 1/2 \times a \times b^2$ (in which a is the length of tumors, b is the width of tumors).
[c]RTV is the relative tumor volume, $RTV = V_n/V_0$ (in which V_0 is the TV at the day when the chemicals were given and V_n is the TV of final measurement after 17 days).
[d]The evaluation index of antitumor activity was relative tumor growth ratio T/C (%). $T/C = T_{RTV}/C_{RTV} \times 100\%$. ($T_{RTV}$: tested group's and positive control group's RTV; C_{RTV}: common negative control group's RTV).
*$p < 0.01$ vs. HCPT.

Table 4. Antitumor efficacy of compound **12a** against mouse tumor HT29 using intravenous injection (iv).

Tumor	Compounds	Dose(mg/kg)	Lethala[a] toxicity	TV[b] (X ± SD, mm^3)		RTV[c] X ± SD	T/C[d] (%)
				D_0	D_{21}		
HT-29	NS	–	0/10	230 ± 43	1101 ± 287	4.85 ± 1.21	
	12a	10	0/5	218 ± 16	638 ± 129	2.98 ± 0.78	61.4*
	12a	20	3/5	204 ± 27	486 ± 377	2.12 ± 1.70	43.7*
	12a	40	1/5	209 ± 27	373 ± 153	1.85 ± 0.69	38.1*
	Irinotecan	5	0/5	219 ± 23	685 ± 111	3.19 ± 0.79	65.8*
	Irinotecan	10	0/5	218 ± 22	541 ± 164	2.48 ± 0.69	51.1*
	Irinotecan	20	0/5	210 ± 23	587 ± 222	2.74 ± 0.87	56.5*

[a]Number of dead mice/total number of mice in this series of treatment for 21 days.
[b]Tumor volume (TV) of D_0 (the day when the chemicals were given) and D_n (the day of the optimal treatment, which was the 21st day in the experiments), $TV = 1/2 \times a \times b^2$ (in which a is the length of tumors, b is the width of tumors).
[c]RTV is the relative tumor volume, $RTV = V_n/V_0$ (in which V_0 is the TV at the day when the chemicals were given and V_n is the TV of final measurement after 21 days).
[d]The evaluation index of antitumor activity was relative tumor growth ratio T/C (%).$T/C = T_{RTV}/C_{RTV} \times 100\%$. ($T_{RTV}$: tested group's and positive control group's RTV; C_{RTV}: common negative control group's RTV).
*$p < 0.01$ vs. NS.

to CPT within 40 h in PBS, while the activity of **11a** both *in vitro* and *in vivo* presented to be superior to CPT itself (even superior to reference drug HCPT which is considered to be more efficient than CPT). So, we have reason to believe that hydroxyl-amide **11a** does not only play as a pre-drug of CPT, but also exhibit its own intrinsical antitumor property. It implies that compound **11a** is worthy of further study.

Taken together, a series of E-ring lactone-opened CPT analogues were prepared, and their biological activity was evaluated. Hydroxyl-amide analogues with terminal morpholin-1-yl group and ester-amide compounds with propionate presented higher inhibition rate than their congeners. Substitutions at 7- and 10- positions usually enhance the activity. The superiority of biological evaluation of compound **11a** indicated that it had distinct antitumor property known from CPT itself.

4. Experimental

IR spectra were obtained on Bruker Equinox 55 spectrophotometer (Thermo Scientific, Massachusett America); NMR spectra were recorded on Bruker 300-MHz or 400-MHz spectrometer (Bruker Biospin, Billerica America); Chemical shifts (δ values) and coupling constants are given in ppm and Hz, respectively. MS (ESI) were recorded on a Thermo Finnigan TSQ Quantum Ultra AM mass spectrometer (Thermo Finnigan, State of California America). All reagents and solvents were reagent grade or were purified by standard methods before use.

4.1. Synthesis of compounds

4.1.1. Synthesis of 7-cyclohexylcamptothecin (9) with Sawada method

To a stirred solution of CPT (35 mg, 0.1 mmol) cooled with ice bath in acetic acid (AcOH) (1.5 ml), deionized water (1.5 ml) and conc. H_2SO_4 (0.4 ml) was added cyclohexanecarbaldehyde (0.3 mmol) and 30% H_2O_2 (0.3 mmol). The stirring was continued for additional 3 h. The reaction mixture was then diluted with ice water (6 ml) and extracted with CH_2Cl_2 (10 ml × 3). Combined organic layers were dried on Na_2SO_4, evaporated under reduced pressure, and purified on silica gel column chromatography (Eluent: $CHCl_3$~$CHCl_3$/MeOH = 100/1) to give a yellow powder in 49% yield; ^{1}H NMR (300 MHz, $CDCl_3$, TMS) δ: 1.20 (t, J = 6.9 Hz, H_3-18), 1.43-2.03 (m, H_{10}-cyclohexyl and H_2-19), 3.65 (br, H_1-OH), 3.73–3.75 (m, H_1-cyclohexyl), 5.31 (d, J = 16.5 Hz, H_1-17), 5.43 (s, H_2-5), 5.76 (d, J = 16.5 Hz, H_1-17), 7.68 (t, J = 7.7 Hz, H_1-10), 7.78 (s, H_1-14), 7.81 (t, J = 8.1 Hz, H_1-11), 8.28–8.30 (m, H_1-9 and H_1-12); MS (ESI): m/z 431.2[M+H]$^+$.

4.1.2. Synthesis of 7-ethyl-10-methoxylcamptothecin

To a suspension of 7-ethyl-10-hydroxyl-camptothecin (SN38, 100 mg, 0.28 mmol) in 10 ml acetone, MeI (0.1 ml, 6 equiv.) and K_2CO_3 (190 mg, 5equiv.) were added. The reaction mixture was stirred and heated to reflux over night. After cooled to room temperature, the reaction mixture was then diluted with ice water (15 ml) and extracted with CH_2Cl_2 (10 ml × 3). Combined organic layers were dried on Na_2SO_4, evaporated and purified on silica gel column chromatography (Eluent: $CHCl_3$~$CHCl_3$/MeOH = 100/1) to give a yellow powder in 75% yield; ^{1}H NMR (300 MHz, DMSO-d_6 : $CDCl_3$=1:10, TMS) δ: 0.96 (t, J = 7.3 Hz, H_3-18), 1.39 (t, J = 7. 5 Hz, H_3-7-Et), 1.88-1.90 (m, H_2-19), 3.21 (q, J = 7.5 Hz, H_2-7-Et), 4.00 (s, H_3-10-OMe), 5.26 (s, H_2-5), 5.29 (d, J = 15.6 Hz, H_1-17), 5.54 (d, J = 15.6 Hz, H_1-17), 7.39-7.44 (m, H_1-14, H_1-11), 7.95 (s, H_1-9), 8.06 (d, J = 9.0 Hz, H_1-12). MS (ESI): m/z 407.3 [M+H]$^+$.

4.1.3. General procedure for synthesis of hydroxyl-amides (11a-d)

Suspension of CPTs (**1**, **9,** or **10**) in amine (3-morpholin-4-yl-propylamine or 3-imidazol-1-yl-propylamine, 5 ml amine per 1 g CPTs) was stirred at 60 °C for 1.5 h (or until total conversion of CPTs according to TLC analysis) under N_2 atmosphere, the clear mixture was then cooled to room temperature, saturated with diethyl ether (Et_2O), and stored in refrigerator at −4 °C over night then filtrated, and the solid was dried to give hydroxyl-amide.

4.1.3.1. Compound 11a. Yellow powder, yield in 80%; IR (KBr, cm^{-1}) *v*: 1651 (*CO*NH); ^1H NMR (300 MHz, $CDCl_3$, TMS) *δ*: 1.07 (t, *J* = 7.2 Hz, H_3-18), 1.75–1.77 (m, H_2-$CH_2CH_2CH_2$), 2.24–2.26 (m, H_1-19), 2.42–2.44 (m, H_1-19), 2.43–2.45 (m, H_6-NCH_2), 3.40–3.42 (m, H_2-$CONHCH_2$), 3.74–3.76 (m, H_4-OCH_2), 4.88 (d, *J* = 12.7 Hz, H_2-17), 5.04 (s, H_2-5), 7.47 (t, *J* = 8.4 Hz, H_1-10), 7.48 (s, H_1-14), 7.56 (d, *J* = 7.2 Hz, H_1-9), 7.67 (t, *J* = 5.2 Hz, H_1-11), 7.87 (s, H_1-7), 7.97 (d, *J* = 8.5 Hz, 1H), 8.20 (br, H_1-CONH). MS (ESI): *m/z* 493.4 $[M+H]^+$.

4.1.3.2. Compound 11b. Yellow powder, yield in 93%; IR (KBr, cm^{-1}) *v*: 1651 (*CO*NH); ^1H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.06 (t, *J* = 6.4 Hz, H_3-18), 2.04–2.06 (m, H_2-19), 2.30–2.39 (m, H_2-$CH_2CH_2CH_2$), 3.32–3.34 (m, H_2-$CONHCH_2$), 4.01 (t, *J* = 7.0 Hz, H_2-CH_2-imidazol-1-yl), 4.92 (d, *J* = 12.6 Hz, H_2-17), 5.04 (s, H_2-5), 6.94 (s, H_1-Imidazo-1-yl), 7.01 (s, H_1-Imidazo-1-yl), 7.45 (t, *J* = 7.2 Hz, H_1-10), 7.51 (d, *J* = 7.1 Hz, H_1-9), 7.56 (s, H_1-14), 7.65 (d, *J* = 9.1 Hz, H_1-12), 7.70 (t, *J* = 7.4 Hz, H_2-11), 8.03 (br, H_1-CONH), 8.05 (s, H_1-7). MS (ESI): *m/z* 474.2$[M+H]^+$.

4.1.3.3. Compound 11c. Yellow powder, yield in 99%, IR(KBr, cm^{-1}): *v* 1655 (*CO*NH); ^1H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.08 (t, *J* = 6.6 Hz, H_3-18), 1.74–1.76 (m, H_2-19), 1.44–2.13 (m, H_{10}-cyclohexyl), 2.29–2.31 (m, H_2-$CH_2CH_2CH_2$), 2.47 (s, H_6-NCH_2), 3.39-3.43 (m, H_1 of cyclohexyl and H_2 of $CONHCH_2$), 3.74–3.76 (m, H_4-OCH_2), 4.80 (d, *J* = 12.6 Hz, H_1-17), 5.04 (d, *J* = 12.6 Hz, H_1-17), 5.11 (s, H_2-5), 7.45 (t, *J* = 7.5 Hz, H_1-10), 7.55 (s, H_1-14), 7.60 (t, *J* = 8.1 Hz, H_1-11), 7.92 (d, *J* = 8.1 Hz, H_1-12 and H_1-9), 8.21 (br, H_1-CONH). MS (ESI): *m/z* 575.5$[M+H]^+$.

4.1.3.4. Compound 11d. Yellow powder, yield in 80%, IR (KBr, cm^{-1}): *v* 1651 (*CO*NH); ^1H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.08 (t, *J* = 7.2 Hz, H_3-18), 1.25 (3H, t, *J* = 7.5 Hz, H_3-7-Et), 1.76–1.78 (m, H_2-19), 2.27–2.29 (m, H_2-$CH_2CH_2CH_2$), 2.42–2.44 (m, H_2-NCH_2), 2.49–2.51 (m, H_4-NCH_2), 2.82 (q, *J* = 7.5 Hz, H_2-7-Et), 3.41-3.43 (m, H_2-$CONHCH_2$), 3.76 (t, *J* = 4.2 Hz, H_4-OCH_2), 3.93 (s, H_3-10-OMe), 4.85 (d, *J* = 12.9 Hz, H_1-17), 4.91 (s, H_2-5), 4.94 (d, *J* = 12.9 Hz, H_1-17), 6.89 (d, *J* = 2.7 Hz, H_1-9), 7.34 (d, *J* = 9.3 Hz, 2.4 Hz, H_1-11), 7.46 (s, H_1-14), 7.93 (d, *J* = 9.3 Hz, H_1-12), 8.07 (br, H_1-CONH). MS (ESI): *m/z* 551.3 $[M+H]^+$.

4.1.4. General procedure for synthesis of ester-amides (12a-f)

To a stirred solution of hydroxyl-amide (0.30 mmol) in pyridine (3 ml) was added anhydride (0.2 ml). The mixture was warmed to 40 °C in water bath and stirred over night. The solvents were removed under reduced pressure, and the residue was purified on silica gel column chromatography (eluent: ethyl acetate/petroleum ether = 1/1 ~ 3/1) to give ester-amide.

4.1.4.1. Compound 12a. Yellow powder, 82% yield; IR (KBr, cm^{-1}) *v* 1734 (Me*CO*O), 1621 (CONH). ^1H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.10 (t, *J* = 7.2 Hz, H_3-18), 1.76-1.78 (m, H_1-19), 2.06 (s, H_3-COMe), 2.29–2.31 (m, H_2-$CH_2CH_2CH_2$), 2.29–2.31 (m, H_1-19), 2.52–2.54 (m, H_1-19, H_6-NCH_2), 3.31–3.42 (m, H_2-$CONHCH_2$), 3.77–3.79 (m, H_4-OCH_2), 5.15 (d, *J* = 19.4 Hz, H_2-5), 5.47 (d, *J* = 11.6 Hz, H_2-17), 7.43 (t, *J* = 7.4 Hz, H_1-10), 7.58 (s, H_1-14), 7.63 (d, *J* = 8.1 Hz, H_1-9), 7.69 (t, *J* = 7.2 Hz, H_1-11), 8.04 (d, *J* = 8.3 Hz, H_1-12), 8.14 (s, H_1-7), 8.17 (br, H_1-CON*H*). MS (ESI): *m/z* 535.4 $[M+H]^+$.

4.1.4.2. Compound 12b. Yellow powder, 82% yield; IR (KBr, cm^{-1}) *v* 1730 (Et*CO*), 1655 (*CO*NH); ^{1}H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.11 (t, *J* = 7.2 Hz, H_3-18), 1.21 (t, *J* = 7.1 Hz, H_3-COEt), 1.76-1.78 (m, H_2-19), 2.30–2.32 (m, H_1-$CH_2CH_2CH_2$), 2.40–2.15 (m, H_1-$CH_2CH_2CH_2$), 2.55–2.56 (m, H_6-NCH_2), 3.31-3.33 (m, H_1-$CONHCH_2$), 3.43–3.45 (m, H_1-$CONHCH_2$), 3.48 (q, *J* = 7.0 Hz, H_2-COEt), 3.79 (t, *J* = 4.5 Hz, H_4-OCH_2), 5.13 (d, *J* = 19.2 Hz, H_2-5), 5.47 (d, *J* = 11.6 Hz, H_2-17), 7.45 (t, *J* = 7.4 Hz, H_1-10), 7.57 (s, H_1-14), 7.60 (d, *J* = 8.1 Hz, H_1-9), 7.68 (t, *J* = 7.5 Hz, H_1-11), 8.03 (d, *J* = 8.5 Hz, H_1-12), 8.12 (s, H_1-7), 8.21 (s, H_1-CON*H*). MS (ESI): *m/z* 549.3[M+H]$^+$.

4.1.4.3. Compound 12c. Yellow powder, 88% yield. IR (KBr, cm^{-1}) *v* 1722 (Me*CO*), 1651 (*CO*NH); ^{1}H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.07 (t, *J* = 7.2 Hz, H_3-18), 2.06 (s, H_3-COMe), 2.09–2.11 (m, H_2-19), 2.32–2.46 (m, H_2-$CH_2CH_2CH_2$), 3.32–3.34 (m, H_2-$CONHCH_2$), 4.07 (t, *J* = 7.0 Hz, H_2-CH_2-imidazol-1-yl), 5.19–5.21 (m, H_2-5), 5.52 (d, *J* = 11.5 Hz, H_2-17), 7.01 (s, H_1-imidazol-1-yl), 7.10 (s, H_2- imidazol-1-yl), 7.54 (t, *J* = 7.2 Hz, H_1-10), 7.61 (s, H_1-14), 7.76 (d, *J* = 7.1 Hz, H_2-9), 7.79 (t, *J* = 7.4 Hz, H_1-11), 7.82 (br, H_1-CON*H*), 8.12 (d, *J* = 9.1 Hz, H_1-12), 8.23 (s, H_1-7). MS (ESI): *m/z* 516.2 [M+H]$^+$.

4.1.4.4. Compound 12d. Yellow powder, 74% yield; IR (KBr, cm^{-1}) *v* 1726 (Et*CO*), 1652 (*CO*NH); ^{1}H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.01 (t, *J* = 7.1 Hz, H_3-18), 1.23 (t, *J* = 7.1 Hz, H_3-COEt), 2.03–2.05 (m, H_2-19), 2.30–2.47 (m, H_2-COEt and H_2-$CH_2CH_2CH_2$), 3.30–3.32 (m, H_2-$CONHCH_2$), 4.04 (t, *J* = 7.0 Hz, H_2-CH_2-imidazol-1-yl), 5.15–5.17 (m, H_2-5) 5.52 (d, *J* = 11.6 Hz, H_2-17), 6.98 (s, H_1-imidazol-1-yl), 7.06 (s, H_1-imidazol-1-yl), 7.51 (t, *J* = 7.2 Hz, H_1-10), 7.59 (s, H_1-14), 7.60 (br, H_1-CON*H*), 7.67 (d, *J* = 7.1 Hz, H_1-9), 7.73 (t, *J* = 7.4 Hz, H_1-11), 8.01 (d, *J* = 9.0 Hz, H_1-12), 8.18 (s, H_1-7). MS (ESI): *m/z* 530.3 [M+H$^+$].

4.1.4.5. Compound 12e. Yellow powder, 38% yield; IR (KBr, cm^{-1}): *v* 3423, 1728 (EtCO), 1650 (CONH), 1591; ^{1}H NMR ($CDCl_3$, 400 MHz, TMS) *δ*: 1.07–1.01 (m, H_3-18 and H_3-COEt), 1.56–1.94 (m, H_{10}-cyclohexyl), 2.08–2.51 (m, H_2-19, H_2-COEt and H_2-$CH_2CH_2CH_2$), 2.75 (s, H_6-NCH_2), 3.31–3.33 (m, H_1-CH of cyclohexyl), 3.43–3.45 (m, H_2-$CONHCH_2$), 3.89 (s, H_4-OCH_2), 5.17 (d, *J* = 18.6 Hz, H_1-17), 5.29 (d, *J* = 18.6 Hz, H_1-17), 5.39 (d, *J* = 11.6 Hz, H_1-5), 5.56 (d, *J* = 11.6 Hz, H_1-5), 7.42 (t, *J* = 7.3 Hz, H_1-10), 7.53 (s, H_1-14), 7.63 (t, *J* = 7.4 Hz, H_1-11), 7.90 (d, *J* = 9.9 Hz, H_1-9), 7.96 (d, *J* = 8.0 Hz, H_1-12), 8.09 (s, H_1-CONH). MS (ESI): *m/z* 631.4 [M+H]$^+$.

4.1.4.6. Compound 12f. Yellow powder, 78% yield; IR (KBr, cm^{-1}) *v*: 1726 (Et*CO*), 1652 (*CO*NH); ^{1}H NMR ($CDCl_3$, 300 MHz, TMS) *δ*: 1.08–1.10 (m, H_3-18 and H_3-COEt), 1.27 (t, *J* = 6.9 Hz, H_3-7-Et), 1.74–1.76 (m, H_2-$CH_2CH_2CH_2$), 2.28–2.30 (m, H_2-COEt and H_1-19), 2.51–2.53 (m, H_1-19 and H_6-NCH_2), 2.90–2.92 (m, H_2-7-Et), 3.19–3.43 (m, H_2-$CONHCH_2$), 3.78–3.80 (m, H_4-OCH_2), 3.89 (s, H_3-10-OMe), 4.90–5.03 (d, *J* = 18.9 Hz, H_2-5), 5.23 (d, *J* = 11.4 Hz, H_1-17), 5.45 (br, H_1-OH), 5.61 (d, *J* = 11.4 Hz, H_1-17), 6.73 (s, H_1-14), 7.27 (d, *J* = 9.0 Hz, H_1-11), 7.44 (s, H_1-9), 7.86 (d, *J* = 9.0 Hz, H_1-12), 8.33 (br, H_1-CONH). MS (ESI): *m/z* 607.3[M+H]$^+$.

4.2. Assessment of antitumor activity

4.2.1. Cytotoxicity assays

Inhibition rate assays were performed on human lung cancer cell line A549, human gastric cancer line BGC-823, human liver cancer cell line SMMC-7721, and human leukemia cell line HL-60. 180 μl (20~40 thousand cell per ml) per well was plated in 96-well plates. After culturing for 24 h, compounds **11a–b** and **12a–d** were added with different concentrations, and 0.1% DMSO (dimethyl sulfoxide) was used for control. After 48 h of incubation, 20 μl MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) solution (5 mg/ml) was added to each well, and after shaking for 1 min, the plate was incubated for further 4 h. Formazan crystals were dissolved with DMSO (150 μl per well). The absorbance (OD) was quantitated with microplate spectrophotometer at 570 nm. Wells containing no drugs were used as blanks for the spectrophotometer. The survival of cells was expressed as percentage of untreated control wells. The prototypical inhibitors mitomycin and cisplatin were included as reference standards; the results of these assays were used to obtain the corresponding inhibition rates.

Inhibition of compounds **11c–d**, **12 e–f** and reference drugs SN38, CPT and topotecan (different concentrations were: 100, 33, 10, 3.3, 1, 0.33, 0.1, 0.033, 0.01, 0.0033, 0.001, 0.00033 μM) on A549/ATCC and HT29 cell lines were incubated for 72 h with the same method. IC_{50} is defined as the inhibitory drug concentration causing a 50% decrease of cell growth over that of untreated control.

4.2.2. Experimental transplantation tumor in mice

BALA/cA-nude mice, ♀, 6–7 weeks, were purchased from Shanghai SLAC Laboratory Animal CO. LTD. According to the protocol of transplantation tumor research [16], A549 or HT29 tumor tissues were chopped into 1.5 mm^3, which was subcutaneously transplanted into right axillary fossa of nude mice. The diameter of transplantation tumor was measured with vernier caliper, and the tumor-transplanted mice were randomly divided into groups when tumor size grew to 100–300 mm^3, i.e. positive control group and treated groups (iv, 3 times per week). Mice in the negative control (normal subject, NS) group were treated with normal saline.

Disclosure statement

No potential conflict of interest was reported by the authors.

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