Structure-activity relationship of alkyl 9-nitrocamptothecin esters

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ABSTRACT

AIM: To study the structure-activity relationship of alkyl 9-nitrocamptothecin esters. **METHODS**: Two alkyl 9-nitrocamptothecin (9NC) esters **5g** and **5h** were prepared by esterification reactions of 9NC with valeric anhydride and heptanoic anhydride, respectively. Eight 9NC esters **5a-5h** were tested for cytotoxicity against human leukemia cell lines HL-60 and U-937. Flow cytometry analysis was used to identify the cell cycle phase targeted by the esters and quantify the extent of ester-induced cell death (apoptosis). **RESULTS**: Esters **5b** and **5c** demonstrated great abilities to inhibit growth of the leukemia cells followed by induction of apoptosis; esters **5a**, **5e**, and **5g** induced slight perturbations in the cell cycle at high concentrations; and esters **5d**, **5f**, and **5h** were completely inactive against the cell lines tested. Thus these esters showed the cell anti-proliferative activity in an order of **5b≈5c>5a≈5e≈5g>5d≈5f≈5h**. Esters **5b**, **5c,** and **5e** were tested *in vivo* against various human carcinomas in nude mice grown as xenografts. Only **5b** and **5c** showed a significant antitumor activity. Particularly, ester **5b** demonstrated an antitumor activity against a broad spectrum of human carcinomas including breast, lung, colon, pancreas, stomach, ovarian, and melanoma, *etc*. **CONCLUSION**: These esters act like prodrugs of their parental 9-nitrocamptothecin. High drug doses need to be administered to animals in order to inhibit growth, and induce regression, of human tumor xenografts in nude mice. These compounds may be developed into potent anticancer drugs due to their low toxicity.

INTRODUCTION

Since the finding that the plant alkaloid camptothecin was very active against leukemia L1210 cells in 1966[1], the camptothecin chemistry has been a very interesting and an attractive field for chemical and medical researchers. Searching for high potency and low toxicity derivatives of camptothecin has been a continuous challenge in the research commol/Lunity. Over the years, many camptothecin derivatives have been prepared for biological evaluation. Of these compounds, 9-nitrocamptothecin (9NC) is more potent than its parent compound camptothecin and equally potent to another semisynthetic compound 9-aminocamptothecin in the ability to inhibit growth and subsequently induce regression of human tumors grown as xenografts in nude mice. Like all camptothecin derivatives, 9NC has a coplanar pentacyclic ring system that includes quinoline moiety, a lactam ring and a δ-lactone E-ring with an α-hydroxy group at the posi-

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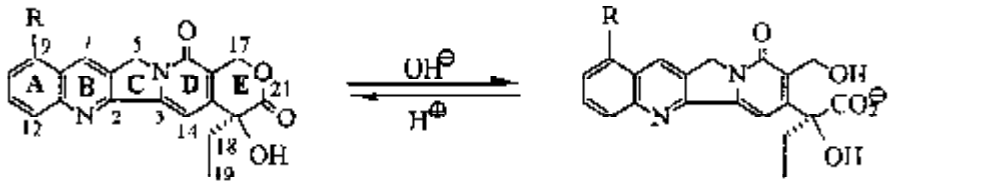
tion of carbon 20 with a *S*-configuration. Generally, the δ-lactone form is not stable and co-exists with its corresponding carboxylate form in equilibrium in aqueous solution, implying that the stability of the lactone form is pH-dependent. As shown in Chart 1, camptothecin lactone form **1** and 9NC lactone form **2** readily open in alkyaline environmol/Lent to yield the corresponding carboxylate forms **3** and **4**. Compounds **3** and **4** can be cyclized back to lactones **1** and **2** on acidification.

Compound **2** was first semi-synthesized by Wall *et al*[2] and was then prepared in our laboratory according to an improved procedure[3]. This compound demonstrated unprecedented antitumor activity against all human tumors grown as xenografts in nude mice, but the same compound tested in human did not exhibit the spectacular antitumor activities observed in the preclinical studies with animal models[4]. It has been established that the α-hydroxylactone moiety of the camptothecin molecule is a requirement for high topoisomerase I mediated cytotoxicity[5,6] due to a reversible covalent interaction between camptothecin and the DNA-enzyme complex[7]. Further, it has been reported that the open-ring form of camptothecin derivative has only one tenth of the potency of the lactone camptothecin[8]. High chemical reactivity of the α-hydroxycarbonyl group of the compound leads to a rapid equilibration *in vivo* between the lactone and the biologically inactive open-ring hydroxyacid formed. Physiological pH value of human plasma is 7.4. Unfortunately, under this slightly basic condition and the catalytic activity of human serum albumin, the majority of the compound in human plasma is the inactive carboxylate form. Thus the early human clinical trials showed no antitumor activity, but severe toxicities, because the compound was administered as a water-soluble sodium carboxylate form.

A pharmacokinetic study conducted in Burke's laboratory showed that the human serum albumin has a much higher affinity for the sodium carboxylate form than the lactone form[9]. Our studies have shown that about 50 % of 9NC is present in mouse plasma as active lactone form, while only about 3 % of the same compound is present in human plasma as the active form. Therefore, the results of the human clinical trials are much less spectacular than the results obtained from animal models. In order to overcome the instability of the drug in human plasma and to enhance the potency of the drug against human tumors in human clinical trials, we have modified the drug by replacement of the H-atom of the 20-hydroxy group with various acyl groups. Previously, we described the preparations, pharmacokinetics, structural characterizations, toxicity, and antitumor activity of six alkyl 9NC esters **5a-5f**[10,11]. From these studies, we have found that the antitumor activity of these 9NC alkyl esters is related to the length and the shape of the side alkyl ester chain. For the esters with the straight alkyl chains such as **5a**, **5b**, and **5c**, the activity increases as the lengths of the chains increase from C1 to C3. For the esters with the cyclic and the branched alkyl chains such as **5d**, **5e**, and **5f**, the activity decreases as the chains become bulkier. In order to further confirm these findings and to obtain the turning point of the chain length for the straight chain esters, we further prepared esters **5g** and **5h**, which have longer chains than the compounds reported previously. The biological studies we have conducted with all these esters (**5a-5h**) have indicated that, like their parental compound 9NC, some of these esters have substantial growth inhibitory effects on tumor cells; meanwhile, the toxicity of these esters in nude mice is remarkably decreased when compared with their parental 9NC. Thus, the purpose of this paper is to report the results of biological studies with esters **5a-5h** and to discuss the structure-activity relationship of these compounds.



1: R = H, Camptothecin (Lactone)
2: R = NO_2, 9NC (Lactone)
3: R = H, Camptothecin (Carboxylate)
4: R = NO_2, 9NC (Carboxylate)

Chart 1. pH-Dependent equilibration between lactone form and carboxylate form of camptothecin and 9-nitrocamptothecin.

MATERIALS AND METHODS

General Dry nitrogen was routinely used as the reaction atmosphere in all reactions. All glassware was baked at (70±10) °C for a minimum of 2 h before being used. Melting points were obtained with an MEL-TEMP melting point apparatus and were uncorrected. The ^{1}H- and ^{13}C-NMOL/LR spectra of approximately 10 % (w/v) solution in $CDCl_3$ were obtained at 270.05 MHz with a JEOL GX-270 WB NMOL/LR spectrometer. Chemical shifts are reported in parts per million (δ scale), employing tetramethylsilane as an internal standard. In reporting the NMOL/LR data, we have used the following abbreviations: coupling constants in Hertz (J), singlet (s), doublet (d), triplet (t), broad singlet (bs), multiplet (m), and *etc*. Mass Spectra were recorded using a VG ZAB - SEQ mass spectrometer (VG Analytical Co, England) with a resolution of 10000. Routinely used solvents such as methylene chloride and THF were dried and freshly distilled. Silica gel (230-400 mesh, Aldrich) for column chromatography was used for all product separations. Eastman chromagram (Silica gel with fluorescent indicator on polyethylene) sheets were employed in thin-layer chromatography (TLC) operations. The numbering system used in reporting NMOL/LR data is shown in structure **5h** of Chart 2.

Drugs The starting camptothecin was purchased from The People's Republic of China and was subse-

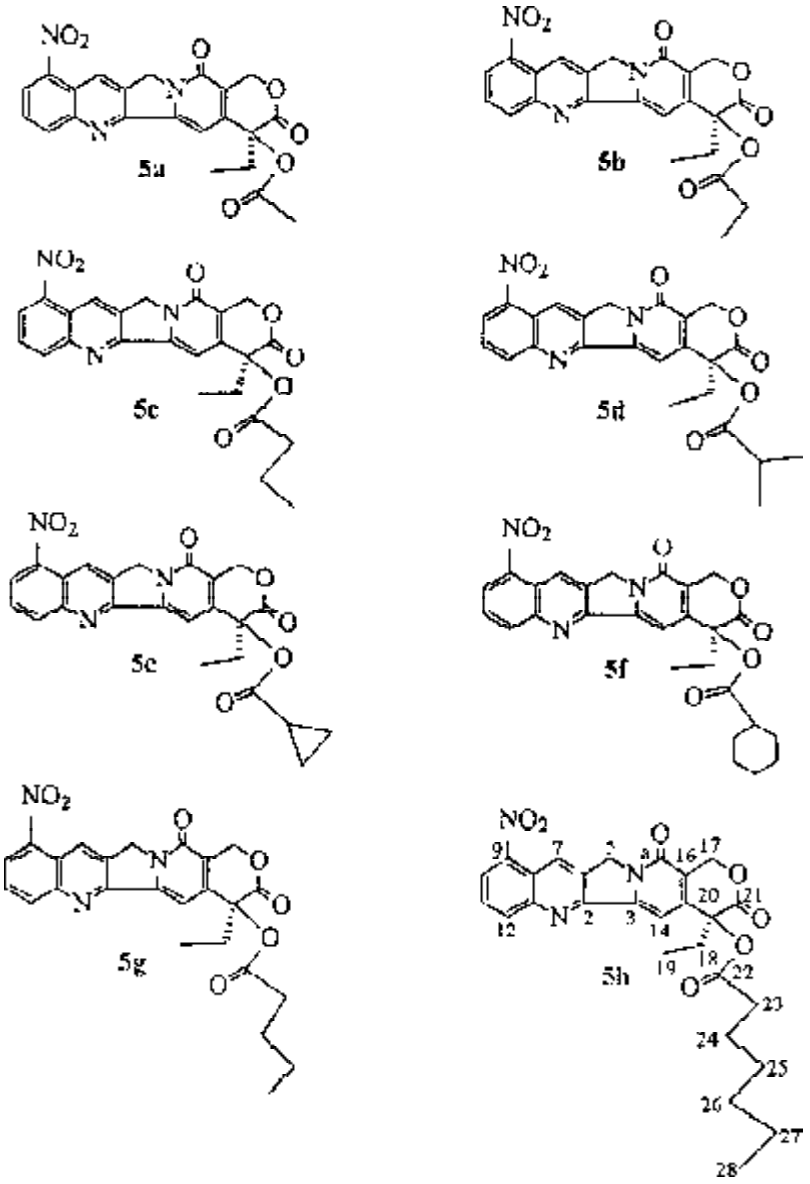
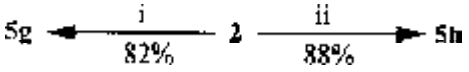


Chart 2. Structures of esters 5a-5h.

quently purified in our laboratory. Fresh 9NC prepared and purified according to our procedure[3] was used as starting material for the preparation reactions of **5g** and **5h** (Scheme 1). Esters **5a-5f** used in this study were freshly prepared according to our established procedure[11].



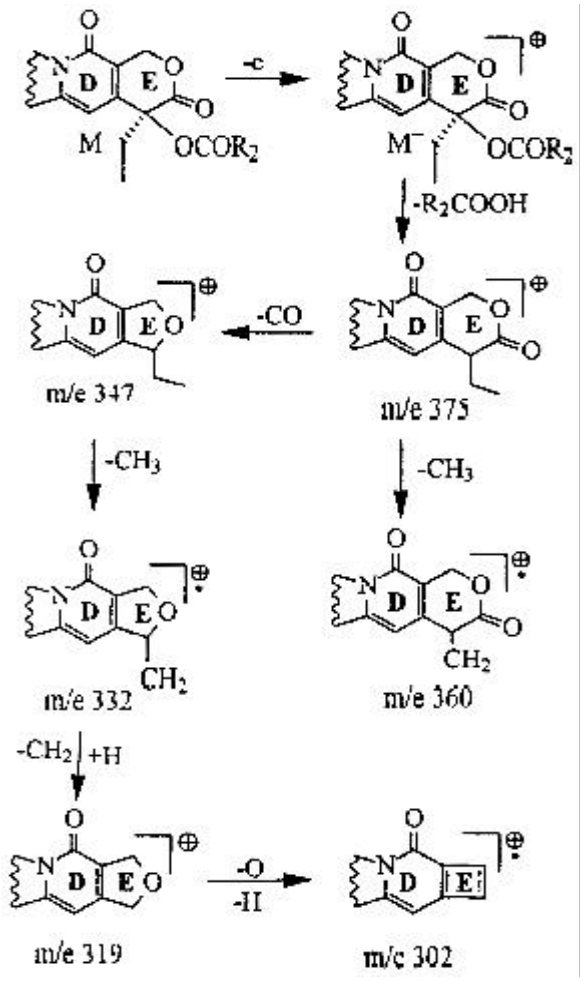
Scheme 1. Preparation of 5g and 5h.

9-Nitrocamptothecin-20-valerate (5g) To 10 mL valeric anhydride in a 50 mL round-bottomed flask, 9-nitrocamptothecin (300 mg, 0.8 mmol/L) and concentrate sulfuric acid (4-6 drops) were added. The mixture was stirred at (60±10) ºC for 24 h. After cooling down to room temperature, the mixture was poured onto 100 mL ice-water while stirring. The residue obtained by filtration was chromatographically separated with CH_2Cl_2-THF as eluent. The pure product (300 mg) was obtained by precipitation from petroleum ether as yellow powders, yield 82 %, mp 207 ºC, purity 99 % (HPLC). ^{1}H NMOL/LR: δ 0.92 (3H, t, J=7.32 Hz, C26-methyl protons), 0.98 (3H, t, J=7.50 Hz, C19-methyl protons), 1.35-2.05 (4H, m, C24- and C25-methylene protons), 2.10-2.30 (2H, m, C18-methylene protons), 2.45-2.52 (2H, m, C23-methylene protons), 5.35 (2H, s, C5-methylene protons), 5.37-5.71 (2H, dd, J=17.58, 17.95 Hz, C17-methylene protons), 7.22 (1H, s, C14-H), 7.91 (1H, t, J=7.91 Hz, C11-H), 8.50 (1H, d, J=10.81 Hz, C10-H), 8.53 (1H, d, J=9.53 Hz, C12-H), 9.28 (1H, s, C7-H). ^{13}C NMOL/LR: δ 4.50 (C26), 10.38 (C19), 20.20 (C24), 24.99 (C25), 30.16 (C18), 32.03 (C23), 50.21 (C5), 66.90 (C17), 75.50 (C20), 96.95, 121.02, 121.60, 125.90, 127.45, 128.49, 131.53, 136.56, 145.12, 145.99, 146.16, 148.92, 153.15, 157.56 (C2, C3, C6-C16, C16a), 169.10, 176.82 (C21, C22). MS m/e (relative intensity): 477 (M^+, 70), 375 (M - C_4H_9COOH, 100), 360 (M-C_4H_9COOH-CH_3, 100), 347 (M-C_4H_9COOH-CO, 100), 332 (M-C_4H_9COOH-CO-CH_3, 100), 319 (43), 302 (47), 286 (40), 274 (35), 258 (18), 245 (17), 216 (28). Precise mass ($C_{25}H_{23}N_3O_7$): Found, 477.154, required, 477.154.

9-Nitrocamptothecin-20-heptanoate (5h) Using 300 mg 9NC, 4-6 drop concentrate sulfuric acid, and 10 mL heptanoic anhydride, the reaction was carried out in the same manner as in preparing **5g**, yield 88 %, mp161 ºC, purity 99 % (HPLC). ^{1}H NMOL/LR: δ 0.81 (3H, t, J=7.50 Hz, C28-methyl protons), 0.98 (3H, t, J=7.01 Hz, C19-methyl protons), 1.20-1.80 (8H, m, C24-, C25-, C26-, and C27-methylene protons), 2.11-2.29 (2H, m, C18-methylene protons), 2.41-2.61 (2H, m, C23-methylene protons), 5.31 (2H, s, C5-methylene protons), 5.38-5.72 (2H, dd, J=17.70, 17.21 Hz, C17-methylene protons), 7.23 (1H, s, C14-H), 7.92 (1H, t, J=8.04 Hz, C11-H), 8.51 (1H, d, J=10.61, C10-H), 8.54 (1H, d, J=8.44 Hz, C12-H), 9.28 (1H, s, C7-H). ^{13}C NMOL/LR: δ 7.59 (C19), 14.10 (C28), 24.10 (C27), 27.16 (C26), 31.10 (C24), 32.69 (C25), 34.10 (C18), 36.50 (C23), 50.25 (C5), 67.00 (C17), 75.51 (C20), 96.82, 120.98, 121.56, 125.85, 127.43, 128.58, 131.55, 136.58, 145.10, 136.00, 146.18, 148.93, 153.10, 157.25 (C2, C3, C6-C16, C16a), 167.50, 176.10 (C21, C22). MS m/e (relative intensity): 505 (M^+, 60), 375 (M-$C_6H_{13}COOH$, 100), 360 (M-$C_6H_{13}COOH$-CH_3, 100), 347 (M-$C_6H_{13}COOH$-CO, 100), 332 (M-$C_6H_{13}COOH$-CO-CH_3, 100), 319 (38), 302 (47), 286 (42), 274 (32), 258 (18), 245 (15), and 216 (22). Precise mass ($C_{27}H_{27}N_3O_7$): Found, 505.185, required, 505.185.

The ^{1}H and ^{13}C NMOL/LR spectra of these two esters showed the corresponding characteristic absorptions for their side alkyl ester chains. The structures of eight 9NC esters **5a-5h** are shown in Chart 2. The detailed synthetic description, ^{1}H- and ^{13}C-NMOL/LR spectral data, and high-resolution mass data for esters **5a-5f** were previously reported[11]. The mass spectra of these eight 9NC esters showed a characteristic fragmentation. A proposed decomposition pattern for these esters is depicted in Scheme 2.

Topoisomerase I cleavage assay DNA topoisomerase I cleavage assays[12] were done as described by Hsiang *et al*. Briefly, YEpGDNA was linearized with BamH1 and then 3'-end-labeled with Klenow polymerase and [α-^{32}P]dCTP. Following phenol extraction and ethanol precipitation, the labeled DNA was resuspended in Tris 10 mmol/L-edetic acid 1 mmol/L, pH 8. The DNA cleavage assay was done in 20 μL reaction mixture containing Tris-Cl 40 mmol/L, pH 7.8, edetic acid 150 mmol/L, bovine serum albumin 30 mg/L, 20 mg of labeled YEpGDNA, and 5 ng of human topoisomerase I. Following incubation at 23 ºC for 15 min, the reactions were terminated by the addition of SDS and proteinase K to final concentrations of 1 % and 200 mg/L, respectively. The reaction mixtures were transferred to a 37 ºC water-bath for 1 h, then mixed with sucrose and bromophenol blue before loaded onto a 1 % agarose gel in Tris-phosphate 80 mmol/L-edetic



Scheme 2. Characteristic decomposition pathway of alkyl 9NC esters 5a-5h.

acid 8 mmol/L, pH 8. Gel drying and auto radiography were done as described[12].

Preparation of liver homogenate A 6-month old super Swiss female mouse was sacrificed, and the liver was surgically removed and placed in a Dounce homogenizer standing on ice. Approximately 4 volumes of ice-cold KCl 0.15 mol/L were added, and the liver was homogenized with 20 strokes of a tight-fitting pestle. The resulting homogenate was centrifuged at 15 000×*g* for 15 min at 4 °C to pellet particulate material and the supernatant was transferred to a glass container standing on ice. The supernatant, hereafter called liver homogenate, was used for incubation of the 9NC esters as described below.

Incubation of 5b in liver homogenate For ester **5b**, 1 mL of homogenate was transferred from 4 °C to a micro centrifuge tube in a 37 °C water-bath and incubated for 10 min. Subsequently, 2 μL of ester stock (10 g ester per L of PEG) was added to the homogenate, mixed well, and incubated at 37 °C continued. The final concentrations of ester **5b** and PEG in the incubated mixture were 2.0 mg/L and 0.2 %, respectively. An aliquot was removed immediately after mixing the ester in the homogenate and before incubation at 37 °C (0-time aliquot). Other aliquots were subsequently removed at desired incubation times and were processed for HPLC determination of 9NC ester and 9NC and/or ability to induce apoptosis in cultured cells.

Processing of homogenate for HPLC and cell culture studies For HPLC determination, 0.2 mL of incubated homogenate was mixed well with 0.8 mL of ice-cold acetonitrile and stored at -20 °C until all samples were collected. 9NC and its esters were detected by fluorescence spectroscopy. Since 9NC and its esters do not fluoresce, they were converted to the corresponding 9AC congeners that highly fluoresce, prior to detection. 9AC and its esters have distinct retention times on the chromatography column. For detection of 9NC activity derived from the ester, 0.8 mL of the incubated homogenate was mixed with ice-cold acetone and stored at -20 °C until all samples were collected. Subsequently, the homogenate/acetone mixture was centrifuged at 15 000×*g* for 15 min to remove insoluble material. The clarified supernatant was freeze-dried, and the dry powder was dissolved in 0.1 mL Me_2SO prior to addition to the cell culture.

Growth inhibition assay and flow cytometry To assess the anti-proliferative activity of the various esters, identical cell cultures received equimolar concentrations of these esters and the cell number per mL was counted using the Trypan blue exclusion method. Stocks consisted of fine suspensions of esters in polyethylene glycol (PEG-400; Aldrich). Control cultures received only the carrier. The cell number was counted at 24 h, 72 h, and 120 h of treatment. The targeted cells included the HL-60 and U-937 cell lines, which have shown different levels of sensitivity to 9NC.

Flow cytometry analysis was used to identify the cell cycle phase targeted by the esters and quantify the extent of ester-induced cell death (apoptosis). Cell cycle perturbations and apoptotic fractions were determined with an Epics-Elite laser flow cytometer (Coulter Corp, Hialeah, FL) and analyzed with the Multicyle program (Phoenix Flow Systems, San Diego, CA). Flow cytometry methodology has been routinely used at our laboratory to study anticancer drugs including CPT and 9NC.

***In vivo* toxicity and antitumor activity** All the

animal experiments were performed on nude Swiss mice of the NIH, high-fertility strain. They were bred and raised in our laboratory under strict pathogen-free conditions. For the *in vivo* toxicity and antitumor activity determination, a tumor xenograft growing in a nude mouse, approximately 1 cm^3 in size, was surgically removed under sterile conditions, finely minced with iridectomy scissors, and suspended in cell culture medium at the ratio 1:10, v/v. One half of 1 mL of this suspension, containing about 50 mg of wet-weight tumor mince was subcutaneously inoculated on the upper half of the dorsal thorax of the mouse. Groups of four or five animals were used. The drug (9NC ester) was finely suspended in cottonseed oil and then injected into the stomach cavity of the mouse through the anterior abdominal wall using a 26 gauge needle. The weekly schedule previously established for intragastric injection of 9NC was once a day, 5 d on, and 2 d off. This schedule was employed throughout all the animal experiments. Treatment was initiated when the tumor had reached a volume of about 200 mm^3, ie, well-vascularized, measurable, and growing exponentially. Tumors growing in animals were checked and measured with a caliper once a week.

Statistical analysis Data were expressed as mean±SD and compared with *t*-test.

RESULTS AND DISCUSSION

The anti-proliferative activity was assayed in cultures of human HL-60 and U-937 leukemia cells. In these assays, untreated cells were used as negative control, ie, 100 % growth. Cells were treated with various concentrations of 9NC esters. For comparison, cells were also treated with two different concentrations of parental 9NC **2**. The results obtained from HL-60 and U-937 cells are summarized in Tab 1 and 2, respectively. When these tables are compared, it is obvious that U-937 cells are more resistant to the treatment with 9NC than HL-60 cells. For example, at 72 h, almost all HL-60 cells treated with 0.02 μmol/L 9NC were killed, but about 50 % of U-937 cells were still growing. This cell type-dependent effectiveness is also applied to 9NC esters **5a-5h**, ie, HL-60 cells are more sensitive to the treatment with these esters than U-937 cells. The data in Tab 1 and 2 also indicate that esters **5b** and **5c** are active, **5**a, **5e**, and **5g** are slightly active, while esters **5d**, **5f**, and **5h** are inactive. In order to achieve the anti-proliferative activity of 9NC, a higher concentration of **5b** or **5c** is required, implying that these esters are prodrugs of their parental 9NC.

Tab 1. Percent growth of HL-60 cells treated with esters 5a-5h. *n*=3. Mean±SD. [a]*P*>0.20, [b]*P*<0.02, [c]*P*<0.10 *vs* untreated group.

Compound	Concentration/ μmol·L^{-1}	24 h[a]	72 h[b]	120 h[c]
Untreated		100±0	100±0	100±0
9NC **2**	0.02	39.6±0.2	0.18±0.02	0±0
	0.20	0.76±0.13	0±0	0±0
Ester **5a**	0.02	72.3±0.5	85.8±0.4	99.3±0.7
	0.05	86.5±0.4	82.9±0.6	96.8±0.6
	0.10	84.6±0.3	74.4±0.3	55.5±0.4
	0.20	91.2±0.8	28.6±0.6	11.4±0.5
5b	0.02	100.8±1.2	84.7±0.5	94.0±0.4
	0.05	77.3±0.5	41.9±0.7	19.6±0.3
	0.10	68.5±0.6	13.4±0.4	1.28±0.23
	0.20	48.8±0.5	0.87±0.12	0±0
5c	0.02	60.4±0.6	70.2±0.7	44.4±0.4
	0.05	65.4±0.4	26.3±0.6	2.14±0.05
	0.10	51.9±0.8	1.79±0.08	0±0
	0.20	40.4±0.6	0±0	0±0
5d	0.02	78.2±0.5	88.1±0.6	94.6±0.4
	0.20	60.3±0.7	76.3±0.3	93.0±0.5
5e	0.025	79.3±0.3	104.9±0.9	98.7±0.7
	0.20	97.1±0.6	91.1±0.5	88.6±0.5
	0.40	85.6±0.5	97.0±0.6	44.3±0.8
	0.80	96.2±0.4	67.1±0.5	8.5±0.3
5f	0.80	97.0±0.5	92.7±0.6	112.3±0.8
5g	0.20	77.0±0.8	48.4±0.6	6.8±0.3
5h	0.20	95.0±0.7	80.8±0.8	93.6±0.5

The apoptosis-inducing activity of esters **5a**, **5b** and **5c** was detected and compared in cultures of the human leukemia HL-60 cells. Unmodified 9NC served as the positive control agent. Drug-induced cell cycle perturbations and apoptosis were monitored by fluorescence cytometry at various periods of treatment in order to compare the time of treatment required for the drugs, at equal concentrations, to exhibit activity. The results are shown in Fig 1. No apoptotic (AP) cells were detected in the untreated culture at 24 h (A) and 72 h (A1), but at 120 h (A2) the presence of a "shoulder" on the left side of the G1-peak indicated that a fraction of cells had entered apoptosis. In contrast to the untreated culture, there was a rapid accumulation of cells at late S-phase, and a distinct apoptotic fraction of 16 % at 24 h (B) in the culture treated with 20 nmol/L 9NC. In the same culture and at 72 h (B1) there was

Tab 2. Percent growth of U-937 cells treated with esters 5a-5h. *n*=3. Mean±SD. [a]*P*>0.10, [b]*P*<0.01, [c]*P*<0.05 *vs* untreated group.

Compound	Concentration/ μmol·L^{-1}	24 h[a]	72 h[b]	120 h[c]
Untreated		100±0	100±0	100±0
9NC **2**	0.02	80.7±0.5	50.1±0.6	29.1±0.4
	0.20	35.1±0.3	0.20±0.08	0±0
Ester **5a**	0.02	115.3±0.9	123.4±0.8	105.3±0.8
	0.05	124.6±0.7	97.6±0.6	102.3±0.9
	0.10	111.5±0.8	93.3±0.7	77.3±0.6
	0.20	117.6±0.6	79.9±0.7	44.2±0.5
5b	0.02	109.2±0.5	95.4±0.8	98.8±0.8
	0.05	140.0±0.9	89.7±0.7	63.7±0.4
	0.10	105.3±0.8	54.1±0.5	19.6±0.4
	0.20	103.8±0.6	19.4±0.3	3.5±0.3
5c	0.02	109.2±0.5	94.6±0.6	78.0±0.4
	0.05	138.5±0.7	63.6±0.6	22.9±0.6
	0.10	111.5±0.8	26.4±0.4	5.22±0.3
	0.20	76.9±0.7	12.3±0.5	1.93±0.22
5d	0.02	79.7±0.6	99.4±0.7	92.8±0.4
	0.20	77.4±0.5	65.3±0.4	71.9±0.5
5e	0.025	76.0±0.7	108.1±0.8	118.7±0.6
	0.20	80.2±0.5	65.9±0.6	63.4±0.4
	0.40	79.3±0.5	65.9±0.4	33.5±0.7
	0.80	81.0±0.7	35.2±0.6	14.6±0.3
5f	0.80	71.7±0.4	90.0±0.6	113.0±0.6
5g	0.20	82.8±0.5	57.8±0.4	33.7±0.4
5h	0.20	108.8±0.8	96.5±0.6	66.1±0.5

a dramatically decreased G1-fraction, and practically no G2-fraction as the majority of the cells was included in the AP-fraction. Only dead cells and cell fragments were present in the 9NC-treated culture at 120 h as confirmed by microscopy observations.

Unlike the 9NC-treated cells, cells treated with the same concentration of **5a** (C,C1,C2) and **5b** (D,D1,D2) yielded histograms identical to those of the untreated cells (A,A1,A2) indicating the complete ineffectiveness of these esters. However, ester **5c** was ineffective at 24 h (E), but induced a small accumulation of cells at late-S/G2 at 72 h (E1), and a larger accumulation of cells at late-S/G2 at 120 h (E2). A small AP fraction (18 %) of cells was also observed at this time. Cells exposed to an increased 9NC concentration of 100 nmol/L for 24 h (F) appeared to be blocked from entering the S-phase, whereas the cells in the S-phase at the time of treatment did not progress to G2 but rather died (accumulation of cells with DNA content less than the DNA content in G1). At this concentration, no live 9NC-treated cells were detected at 72 h. In contrast, 100 nmol/L **5a** had no apparent effect at 24 h and 72 h, but induced cell accumulation in the S-phase at 120 h (G, G1,G2). The effectiveness of 100 nmol/L **5b** was apparent at 24 h, and increased as the treatment went longer. At 120 h, a large portion (30 %) of cells entered AP-fraction (H, H1, H2). Of all these three esters tested with a concentration of 100 nmol/L in this section, **5c** was the most effective one, since this concentration induced generation of a large late-S/G2-fraction at 24 h (I), and its subsequent decrease or elimination at 72 h as the cells progressed to the AP-fraction (I1). At this **5c** concentration, no live cells were detected in the culture at 120 h. Cells treated with 200 nmol/L 9NC generated a histogram (J) similar to that of cells treated with 100 nmol/L 9NC (F) indicating that drug concentrations of 100 nmol/L or above induced cell death. Further, at this concentration of 200 nmol/L, **5a** induced time-dependent changes in the cell cycle fractions (K, K1, K2) that are typically induced by low 9NC concentrations. However, even at 120 h, there was only a small AP-fraction present (K2) indicating the low potency of this ester. As shown with the lower concentrations, both **5b** and **5c** at 200 nmol/L were less potent than 9NC but more potent than **5a** at the same period of treatment.

Cytotoxicity of esters **5a-5c** was also tested against leukemia U-937 cells by using the flow cytometry method. The corresponding histograms are shown in Fig 2. The mother compound **2** was also used as a positive control. At a concentration of 50 nmol/L, 9NC induced significant cell cycle perturbations at 24 h, appearance of a large AP fraction (45 %) at 72 h, and almost no live cells were detected at 120 h. Ester **5a** did not show any activity at this concentration level, whereas both **5b** and **5c** showed a delayed activity. At a higher concentration of 200 nmol/L, ester **5a** induced a slight cell cycle perturbation, but there was no obvious cell death by apoptosis. Esters **5b** and **5c** behaved similarly at this concentration. No live cells were detected at 120 h of treatment with these two esters.

The cycloalkyl esters **5e** and **5f** were also tested using the flow cytometry method. Fig 3 shows the results of treatment of HL-60 cells with these two esters. In order to show the effectiveness of these two esters, the cells had to be treated with higher concentrations and for longer periods. For example, 400 nmol/L and

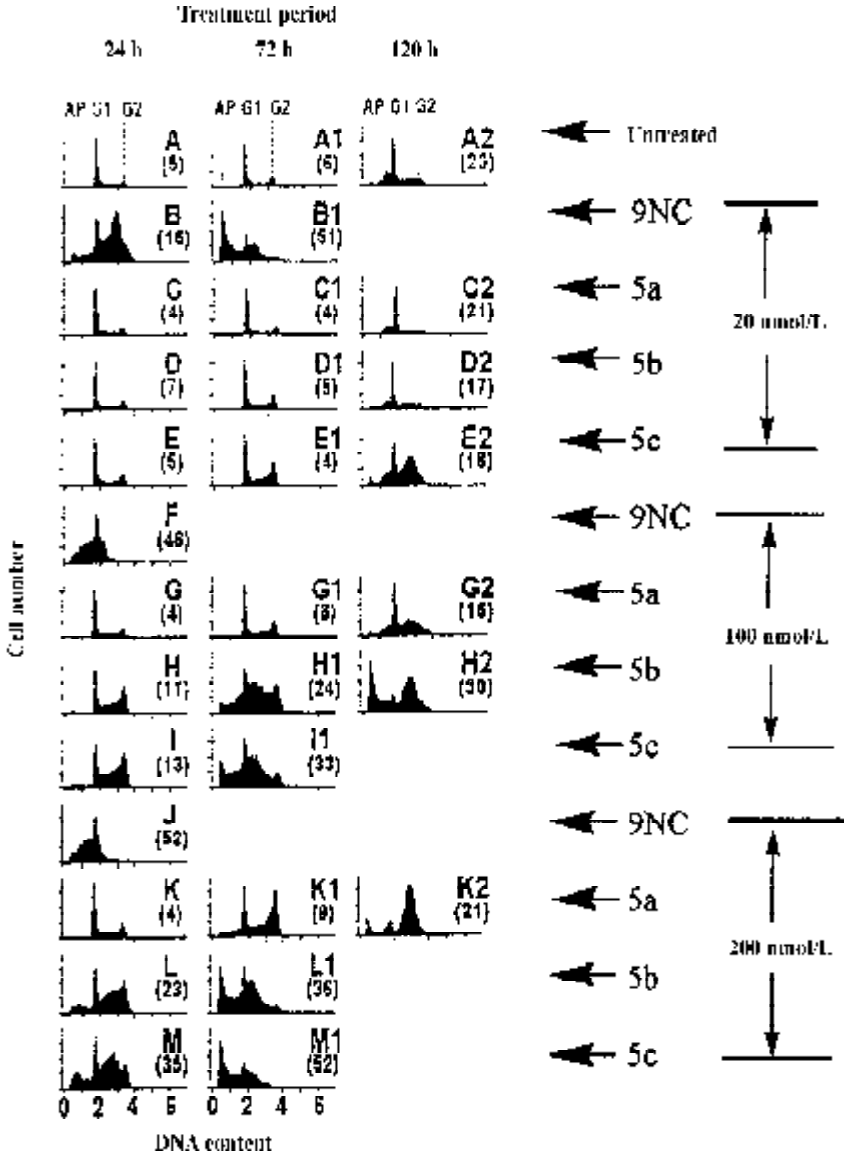


Fig 1. Flow cytometry histograms of HL-60 cells treated with esters 5a- 5c. Identical cultures of HL-60 cells were treated with 20, 100, and 200 nmol/L of 9NC and esters. Cells were removed at 24 h, 72 h, and 120 h time points and analyzed by flow cytometry. G1=G0+G1; G2=G2+M; AP=apoptotic fraction. The numbers in parenthesis indicate the percent of apoptotic cells in the culture.

800 nmol/L of ester **5e** induced small AP fractions during a 120 h treatment period. Further, ester **5f** was completely ineffective even at 800 nmol/L and 120 h of treatment period. Ester **5d** was also tested against this cell line and was ineffective (histogram not shown).

It is apparent from these experimental results that the lengths of the side alkyl ester chains of these 9NC esters affect the cell anti-proliferative activity. Of five esters with straight alkyl chains tested, ester **5b** with a C2 chain and **5c** with a C3 chain are active. Ester **5g** with C4 side chain is slightly active. When the length of the side chain of the ester increases further, the activity vanishes. It is also clear from these experimental results that the shapes of the side alkyl chains of these 9NC esters affect the activity. For example, esters **5c**, **5d**, and **5e** contain a straight, a branched, and a cyclic C3 chain, respectively. Their activities are significantly different. **5c** is active, **5e** is slightly active, and **5d** is inactive. The activity of these three esters decreases when the size of the side chain increases, suggesting that the compound carrying a less steric side chain is more active. Thus, the *in vitro* inhibitory studies showed that the anti-proliferative activity decreased in the order of **5b≈5c>5a≈5e≈5g>5f≈5d≈5h**.

As concluded above, of eight alkyl 9NC esters, **5b** and **5c** are potent. However, the tests of *in vivo*

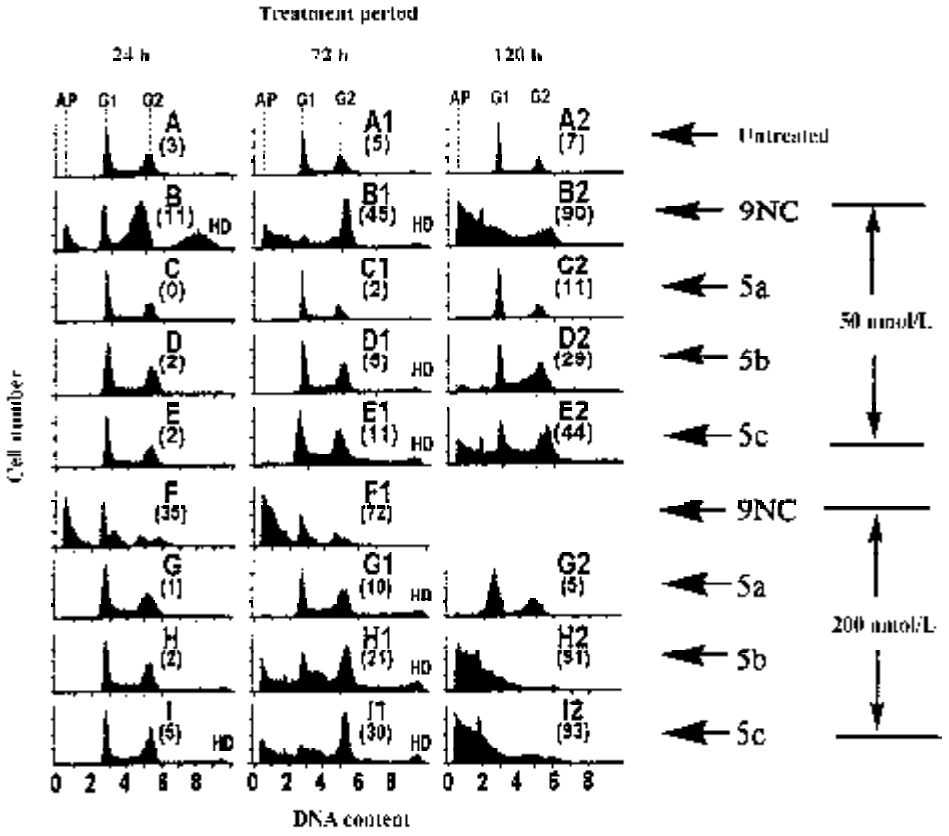


Fig 2. Flow cytometry histograms of U-937 cells treated with esters 5a-5c. Identical cultures of U-937 cells were treated with 50 nmol/L and 200 nmol/L of 9NC and esters as described in Fig 1.

antitumor activity show that **5b** is more active than **5c**. Fig 4 shows that ester **5b** inhibits tumor growth more effectively than **5c** when human DOY lung carcinomas grown in nude mice as xenografts are treated with **5b** (CZ112) and **5c** (CZ116), respectively, at a dose of 8 mg/kg. A higher dose is required to achieve greater inhibition. As shown in Fig 5, a greater tumor growth inhibition is observed when human SPA lung carcinomas in nude mice are treated with a higher dose of **5b** (20 mg/kg). What is more impressive is that no toxicity is observed at this effective dose level. Fig 6 shows no loss of body weights during treatment. Generally, 10 %-15 % loss of body weight can be considered as a sign of toxicity. Ester **5b** seems to have a broad spectrum of antitumor activity. This compound has been tested against various human carcinomas in nude mice such as colon, lung, breast, ovarian, stomach, pancreas, and melanoma. Fig 7 shows the activity of **5b** against human BRO-melanoma in nude mice, and Fig 8 shows the activity of the same compound against human BRE-stomach carcinoma in nude mice. Again, no toxicity is observed in these cases. Fig 9 shows the changes of body weights of mice during the treatment period. Losses of body weights are not observed.

It is established that camptothecins are all the "topoisomerase I-poisoning" agents. Topoisomerase I cleavage assays with these 9NC-esters fail to exhibit such kind of activity. These esters seem acting as prodrugs of their parental compound. In order for the esters to exhibit antitumor activity, topoisomerase I is required, but not sufficient. Another metabolic enzyme, presumably an esterase, has to play a role. The side ester chain cleavage by enzymatic digestion gives rise to parental compound, which then interacts with topoisomerase I to exhibit the biological activity. Since an enzyme-catalyzed cleavage is involved, the fact that these 9NC-esters show the delayed activity is expected because the metabolic conversion of 9NC-esters to 9NC needs a certain amount of time. Fig 10 shows that 9NC is not detected for several hours when ester **5b** is mixed with mouse liver homogenate.

The interaction of an ester with the enzyme requires a compatible steric condition. Only those esters with a suitable side alkyl chain meet the requirement

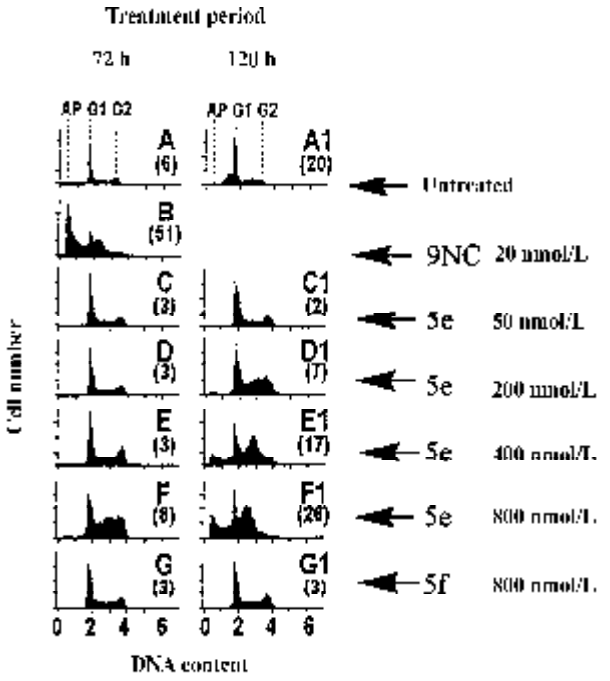


Fig 3. Flow cytometric determination of potency of esters 5e-5f. Identical cultures of HL-60 cells were treated with 20 nmol/L of 9NC, various concentrations of 5e as indicted, and 800 nmol/L 5f. Cell samples were removed for analysis at 72 h and 120 h time points.

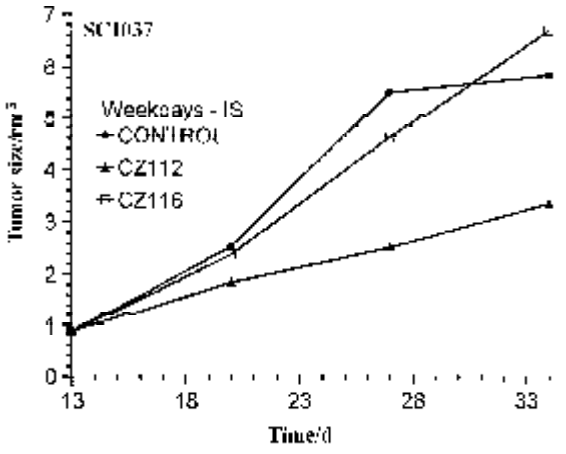


Fig 4. Antitumor activity of ester 5b (CZ112) and 5c (CZ116) against human lung (DOY) xenografts in nude mice at a dose of 4 mg/kg.

and show the biological activity. Ester **5d** with a branched side alkyl chain, ester **5f** with a cyclohexyl chain, and ester **5h** with C6 chain completely block this kind of interaction due to the steric effects of their bulky side chains. In conclusion, the lengths and shapes of the side alkyl chains of these 9NC esters have effects on the antitumor activity. For drug development with these esters, attention should be paid to selecting the

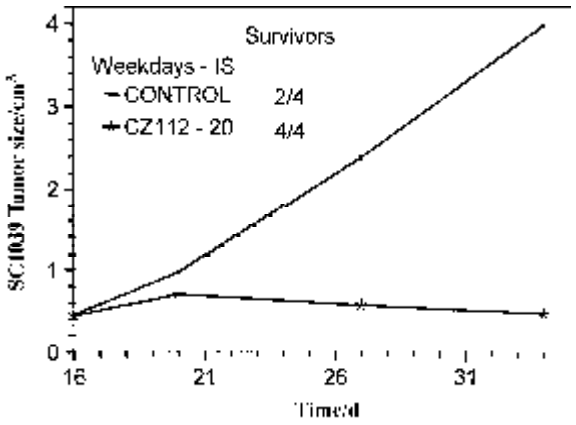


Fig 5. Antitumor activity of 5b (CZ112) against human lung (SPA) xenografts in nude mice at a dose of 20 mg/kg.

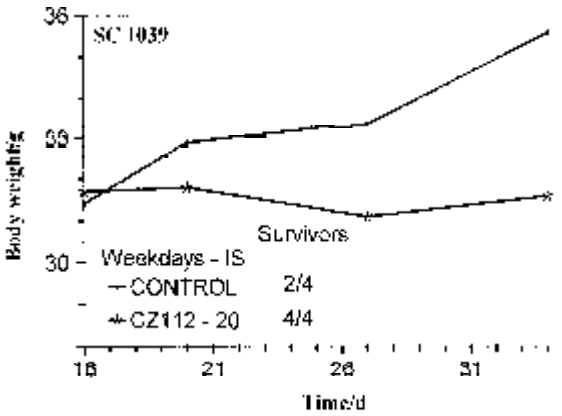


Fig 6. Toxicity of 5b (CZ112) in nude mice at a dose of 20 mg/kg.

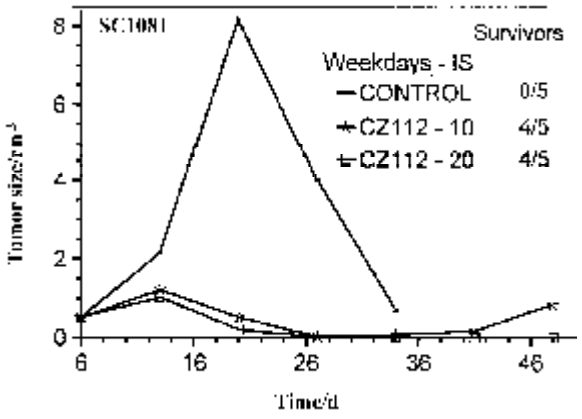


Fig 7. Antitumor activity of 5b (CZ112) against human melanoma (BRO) xenografts in nude mice at a dose of 10 mg/kg and 20 mg/kg.

chains. The 9NC esters act like prodrugs. A higher dose of the compound is needed in order to obtain the same antitumor activity as its parental compound. The toxicity of these esters shown in nude mice is much

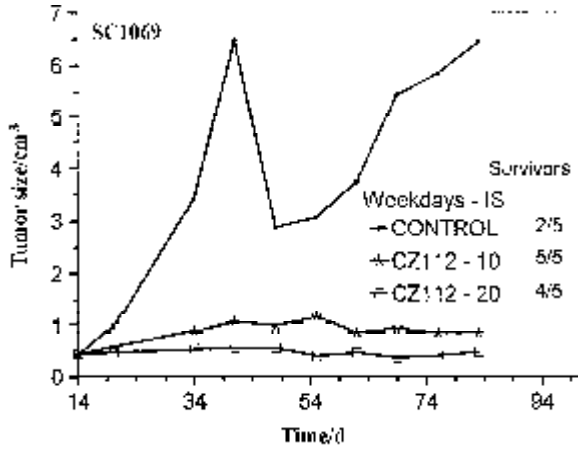


Fig 8. Antitumor activity of 5b (CZ112) against human stomach (BRE) xenografts in nude mice at a dose of 10 mg/kg and 20 mg/kg.

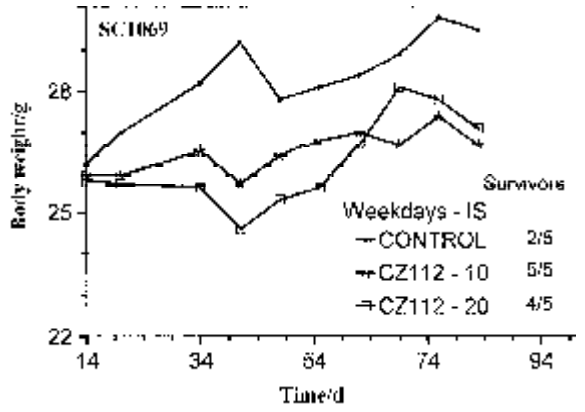


Fig 9. Toxicity of 5b (CZ112) in nude mice at a dose of 10 mg/kg and 20 mg/kg.

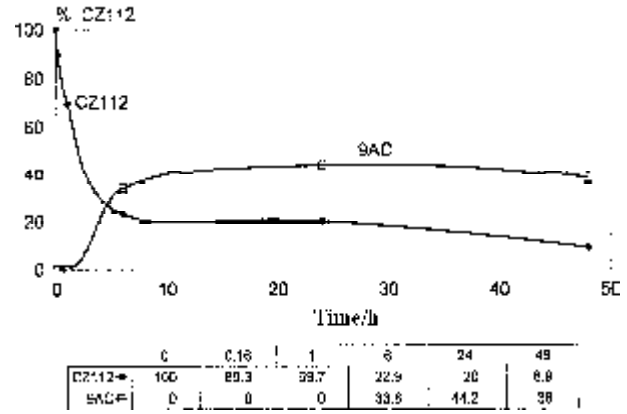


Fig 10. % Conversion of ester 5b to parental 9NC in mouse liver homogenate (1 μg of CZ112 per mL liver homogenate).

lower than their parent compound, meaning these esters can be developed to highly effective and lowly toxic agents for cancer treatment. The results reported in this paper can be used as a basis for the further development of this type of camptothecin derivatives.

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