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Synthesis, topoisomerase-targeting activity and growth inhibition of lycobetaine analogs

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ABSTRACT

The plant alkaloid lycobetaine has potent topoisomerase-targeting properties and shows anticancer activity. Based on these findings, several lycobetaine analogs were synthesized mainly differing in their substituents at 2, 8 and 9 position and their biological activities were evaluated. The topoisomerase-targeting properties and cytotoxicity of these structural analogs were assessed in the human gastric carcinoma cell line GXF251L. Performing a plasmid relaxation assay, an increased inhibition of topoisomerase I was found with *N*-methylphenanthridinium chlorides bearing a 8,9-methylenedioxy moiety or a methoxy group in 2-position. Furthermore, quaternized phenanthridinium derivatives bearing either a 2-methoxy or a 8,9-methylenedioxy moiety in conjunction with a 2-hydroxy or 2-methoxy group display potent topoisomerase II inhibition as shown by decatenation of kinetoplast DNA. In general, the *N*-methylphenanthridinium chlorides possess more potency in inhibiting topoisomerase I than topoisomerase II. All quaternized derivatives also exhibited potent inhibition of tumor cell growth in the low micromolar concentration range. Hence, *N*-methylphenanthridinium compounds were found to represent a promising class of compounds, potently inhibiting both, topoisomerases I and II, and may be further developed into clinically useful topoisomerase inhibitors.

1. Introduction

Many clinically used anticancer drugs are either naturally occurring molecules or their synthetic analogs. The phenanthridine-based alkaloid lycobetaine **1**(= ungeremine, Chart 1), a minor constituent from several species of the Amaryllidaceae family,[1–3] has attracted attention because of its growth inhibitory potential demonstrated in human tumor cell lines.[4–6] Furthermore, it exhibits cytotoxic activity against various carcinomas in mice or rats[7] and is able to reduce tumor development in mice with gastric cancer.[8] These anticancer activities might result from its DNA intercalative properties. Thereby, 1 shows preference for G/C-rich sequences but also exhibits binding affinity to the minor groove of DNA especially to A/T rich regions.[9] Intercalation into double-stranded DNA is known to interfere with catalytic activity of topoisomerase enzymes.[10,11]

DNA topoisomerases are crucial enzymes that regulate and adjust the topologic states of DNA and therefore are involved in all DNA processing steps, such as recombination, replication and transcription.[12,13] Two major classes of topoisomerases are known to be present in human cells, differing in their catalytic mechanisms. Type I topoisomerases introduce a transient single strand break in the phosphate backbone of the DNA and enable the 5′-hydroxy end of the cleaved strand to swivel around the intact strand.[14] Whereas topoisomerases of type II cleave both strands of DNA, creating a DNA-linked protein gate through which another intact double strand can be passed by using ATP.[15,16] Two isoforms of topoisomerase II have been identified in human cells, IIα and IIβ, differing in their cellular function and expression. Both, type I and type II enzymes are proficient in relaxing supercoiled DNA. However, only topoisomerase II is able to decatenate intertwined DNA molecules.[17]

Topoisomerases are essential for cell proliferation and their inhibition causes cell cycle arrest and might lead to cell death. Since many malignancies exhibit rapidly dividing cell populations, they are targets for cytotoxic and chemotherapeutic drugs expected to preferentially hit such rapidly dividing cells. The antitumor activity of topoisomerase-targeting agents is associated

Abbreviatons: A431, human epidermoid carcinoma cell line; kDNA, kinetoplast DNA; MCF-7, human breast adenocarcinoma cell line; ETO, etoposide; DMSO, dimethyl sulfoxide.

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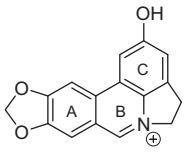


1

Chart 1. Structure of lycobetaine (**1**).

with their ability to inhibit the enzyme in the state when it is covalently linked to DNA.[18] Stabilization of the covalent topoisomerase-DNA intermediate entails the formation of double strand breaks when the replication or transcription machinery collides with the covalently bound enzyme–DNA complex. In consequence, an innocuous enzyme essential for all fundamental DNA processes is turned into a cellular poison by stabilizing the covalent complex of DNA and topoisomerase.[19,20]

Compound **1** has been shown to inhibit human topoisomerase II, a clinically relevant target for established antitumor drugs. We reported recently, that **1** acts as selective topoisomerase IIβ poison both, in cell-free test systems and in the human epidermoid carcinoma cell line A431 as detected by enzyme depletion in Western Blot analysis.[6] Here, we describe for the first time in vivo scavenging of the stabilized covalent topoisomerase-enzyme-intermediate by a technique called ICE bioassay which stands for in vivo complex of enzyme to DNA.

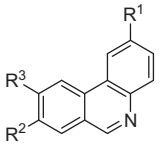
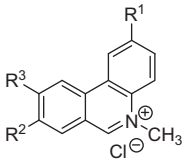
In the present study, we utilized the structural template of **1** to investigate the influence of substituent modification on its biological activity profile. We intended to simplify the synthetic approach to lycobetaine analogs while at the same time improving their biological activity. To keep the characteristic features of the parent molecule, representing a nearly planar 3-membered heteroaromatic ring system with a positively charged nitrogen atom, we replaced the ethylene bridge between phenanthridine atoms 4 and 5 by a methyl substituent attached to the N-atom at position 5. Our main interest focused on the molecular permutations of the 2-hydroxy- and the 8,9-methylenedioxy substituents. An efficient route for the preparation of lycobetaine analogs allowed the synthesis of a series of 2,8,9-substituted phenanthridines as well as *N*-methylphenanthridinium chlorides, providing several published (**6a/b**, **8a/b**, **10a**, **11a**, **18a**, **19a**, structures see Table 1)[21–24] as well as novel analogs (**7a/b**, **9a/b**, **10b**, **11b**, **16a/b**, **17a/b**, **18b**, **19b**). Structure–activity studies were performed with respect to topoisomerase targeting and growth inhibitory properties in human gastric carcinoma cells (GXF251L), since previous studies indicated enhanced sensitivity of gastric carcinomas against the parental compound **1** in vitro and in vivo.[6]

2. Material and methods

2.1. Chemicals and recombinant enzymes

Compound **1** was obtained as described before[2,25–27] and was characterized by HPLC and ^{1}H NMR spectroscopy. Compounds were dissolved in dimethyl sulfoxide just before the experiments to a final solvent concentration of 1% because of solubility problems. Etoposide (ETO) and the starting materials for the synthesis were purchased from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany) and Roth (Karlsruhe, Germany). Cell culture media and

Table 1
Chemical structure[a] of synthesized lycobetaine analogs. Inhibition of topoisomerases I/II[b] and human tumor cell growth[c]

Phenanthridines (a)				*N*-Methylphenanthridinium chlorides (b)			
							
	R^1	R^2	R^3	Effects on topoisomerases			Cell growth inhibition IC_{50} (μM)
				Topo I	Topo IIα	Topo IIβ	
6a	–H	–O–CH_2–O–		–			12.0 ± 1.6
6b	–H	–O–CH_2–O–		+++	–	–	1.9 ± 0.4
7a	–OCH_3	–OCH_3	–H	–	–	–	41.2 ± 4.5
7b	–OCH_3	–OCH_3	–H	+++	+	+	3.5 ± 0.7
8a	–OCH_3	–H	–OCH_3	–			23.7 ± 0.5
8b	–OCH_3	–H	–OCH_3	+++	++	++	2.5 ± 0.6
9a	–OCH_3	–OCH_3	–OCH_3	–			41.1 ± 8.9
9b	–OCH_3	–OCH_3	–OCH_3	+++	*	+	7.8 ± 0.8
10a	–OCH_3	–O–CH_2–O–		–			12.1 ± 1.3
10b	–OCH_3	–O–CH_2–O–		+++	+	++	0.9 ± 0.1
11b	–OCH_3	–H	–H	+++	*	*	4.0 ± 0.6
16a	–OH	–OCH_3	–H	–			40.6 ± 7.1
16b	–OH	–OCH_3	–H	–	–	–	8.4 ± 1.7
17a	–OH	–H	–OCH_3	–			> 50
17b	–OH	–H	–OCH_3	+++	–	–	5.4 ± 0.4
18a	–OH	–OCH_3	–OCH_3	–			26.0 ± 1.7
18b	–OH	–OCH_3	–OCH_3	+	–	–	18.8 ± 4.6
19a	–OH	–O–CH_2–O–		–			17.3 ± 6.4
19b	–OH	–O–CH_2–O–		+++	+	++	1.6 ± 0.2
1	Lycobetaine (chart 1)			+++	+	++	3.2 ± 0.7

[a] For better distinction, compounds are listed according to substituents in position 2, 8 and 9, respectively. *N*-Methylphenanthridinium chlorides are highlighted in gray.
[b] Effects of synthesized compounds on human recombinant topoisomerases (inhibition at: 10 μM (+++), 30 μM (++), 50 μM (+), 100 μM (*); no inhibition up to: 50 μM (–), 100 μM (–)).
[c] Growth inhibition of GXF251L cells after incubation for 72 h were determined by performing the sulforhodamine B assay. Quoted are the IC_{50} values of the respective compounds in respect to the solvent control DMSO.

supplements were received from GIBCO Invitrogen™ Life Technologies (Karlsruhe, Germany), Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany) and SARSTEDT AG & CO. (Nuembrecht, Germany). Recombinant topoisomerase I, IIα and IIβ were purified as described earlier[48] and were kindly provided by Professor Boege (Duesseldorf, Germany).

2.2. Cell culture

The human epidermoid carcinoma cell line A431 was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The human tumor xenograft cell line GXF251L (gastric carcinoma) was cultivated in RPMI-1640 medium and A431 cells in Minimum Essential Medium (MEM) supplemented with 1% L-glutamine, all containing 10% (v/v) heat inactivated fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin. Cell culture was performed in humidified incubators (37 °C, 5% CO_2) and cells were routinely tested for mycoplasm contamination.

2.3. In vivo complexes of enzyme to DNA (ICE)-bioassay

The ICE bioassay was performed with slight modifications as described previously.[28] Three million A431 cells were seeded into Petri dishes (diameter: 14 cm) and allowed to grow for 72 h. Cells were incubated with the solvent control (1%, v/v), **1** or ETO for 1 h under serum-free conditions. Afterwards, the medium was removed and cells were abraded at room temperature in 6 mL TE-buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 1% (w/v) *N*-laurylsarcosyl sodium salt). 4 mL cell lysate were layered onto a cesium chloride gradient in polyallomer tubes (14 mL—SW40, Beckman Coulter GmbH, Krefeld, Germany). One gradient is composed of four layers (2 mL/layer) cesium chloride with a decreasing density from the bottom to the top. The tubes were centrifuged at 100,000×g for 24 h at 20 °C. The gradients were fractionated (300 μL/fraction) from the bottom of the tubes. The DNA content in the single fractions were determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer, and all fractions were blotted onto a nitrocellulose membrane using a slot blot apparatus (Minifold II, Whatman®/Schleicher & Schuell, Dassel, Germany). Topoisomerase was detected using a rabbit polyclonal antibody against topoisomerase I (100 kDa) at a 1:300 dilution, topoisomerase IIα (170 kDa) and topoisomerase IIβ (180 kDa), respectively, at a 1:500 dilution. An anti-rabbit IgG peroxidase conjugate (1:2000) was used as secondary antibody. All antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). The respective chemoluminescent signals (LumiGLO, Cell Signaling Technology, USA) were analyzed using the LAS 3000 with the AIDA Image Analyzer 3.52 software for quantification (Raytest, Straubenhardt, Germany). Arbitrary Light Units were plotted as test over control [%].

2.4. Sulforhodamine B assay

To determine cell growth of GXF251L cells, the sulforhodamine B assay (SRB assay) was performed according to a modified method of Skehan et al.[29] Briefly, GXF251L cells were seeded in 24-well tissue culture plates at a density of 6000 cells per well and allowed to grow for 24 h. Subsequently, cells were incubated with the respective test compounds for 72 h in serum containing medium (10% (v/v) FCS). Incubation was stopped by addition of 100 μL trichloroacetic acid (50% (v/v) solution). After 1 h at 4 °C, plates were washed four times with water and dried over night. The dried plates were stained with a 0.4% solution of sulforhodamine B. Thereafter the dye was dissolved with Tris-buffer (10 mM, pH 10.5) and quantified photometrically at 570 nm. The growth inhibitory properties of compounds were determined as percent survival by the number of treated over control cells [%].

2.5. Relaxation assay

Nucleic extract was prepared from MCF-7 cells as described previously.[30]

Plasmid DNA (pUC18; 250 ng) was incubated at 37 °C for 30 min in a final volume of 30 μL containing 0.3 μL nucleic extract, 10 mM Tris/HCl, pH 7.9, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA and 0.03 mg/mL BSA. Incubation was terminated by adding 1/10 volume of 5% (w/v) SDS. Samples were digested with 1 mg/mL proteinase K at 37 °C for 30 min. Gel electrophoresis was performed at 4.5 V/cm in 1% (w/v) agarose gels with Tris/acetate/EDTA (TAE) buffer (40 mM Tris/HCL, 1 mM EDTA, pH 8.5 and 20 mM acetic acid). Following electrophoresis, the gel was stained with 10 μg/mL ethidium bromide for 15 min and the fluorescence of ethidium bromide was documented with the LAS-3000 system (Fujifilm raytest, Germany).

2.6. Decatenation assay

The catalytic activity of topoisomerase II was measured with Crithidia fasciculata kinetoplast DNA (kDNA) (obtained from TopoGen, Ohio, USA). The kDNA (200 ng) was incubated in a final volume of 30 μL containing 40 ng topoisomerase II, 50 mM Tris/HCl, pH 7.9, 120 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT, 0.5 mM EDTA and 0.03 mg/mL BSA at 37 °C for 60 min. The reaction was stopped by the addition of 1/10 volume of 1 mg/mL proteinase K in 10% (w/v) SDS and incubated for 30 min at 37 °C. Gel electrophoresis and detection were performed according to the relaxation assay.

2.7. Compounds and chemistry

Solvents and reagents obtained from commercial suppliers were at least of reagent grade and were distilled or dried according to prevailing methods prior to use, if necessary. The syntheses were done under argon atmosphere, when required. Argon 4.8 was purchased from Air Liquide (Duesseldorf, Germany) and was dried over phosphorus pentoxide. For monitoring the reactions, Alugram SIL G/UV254 sheets for TLC (Macherey & Nagel, Dueren, Germany) were used. Column chromatography was accomplished using Silica Gel 60 (Macherey & Nagel, 0.063–0.200 mm), for flash chromatography Silica Gel 60 (Macherey & Nagel, 0.040–0.063 mm) was used. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-400 (^1H NMR: 400 MHz, ^{13}C NMR: 100 MHz). Chemical shifts are reported in ppm from tetramethylsilane with solvent as the internal standard (^1H $CDCl_3$: δ 7.26; ^{13}C $CDCl_3$: δ 77.0; ^1H DMSO-d_6: δ 2.49; ^{13}C DMSO-d_6: δ 39.5). Purity of synthesized compounds was determined by elemental analyses and was found to be >95%. Elemental analyses were performed on an Element Analyzer Perkin–Elmer EA 240 or 2400 CHN at the University of Kaiserslautern, Department of Chemistry.

Synthetic procedures of *ortho*-lithiation, Suzuki cross coupling reaction, deprotection and quaternization are described representatively for compounds **5c**, **15**, **19a**, and **19b**. Detailed synthetic and analytical data of all compounds are provided in the Supplementary data.

2.7.1. 2-Pivaloylamino-5-triisopropylsilyloxyphenylboronic acid (5c)

Under argon atmosphere, *N*-pivaloyl-4-triisopropylsilyloxyaniline (11.89 g, 34 mmol) in dry diethyl ether (200 mL) was chilled

down to −15 °C. Within 15 min, a solution of *t*-butyllithium in n-pentane (50 mL, 1.7 M, 85 mmol) was added. After stirring the mixture for 3 h at −10 to −14 °C, trimethyl borate (11.6 mL, 102 mmol) was added within 10 min. The mixture solidified to become a glassy mass that on shaking by hand was resolubilized, warmed up to room temperature and stirred overnight. The mixture was quenched with saturated ammonium chloride (90 mL) and water (60 mL) and extracted with ethyl acetate (3 × 150 mL). The combined organic layers were dried over sodium sulfate and purified by flash chromatography on silica gel (EtOAc, MeOH) to afford pure **5c** (9.10 g, 23.1 mmol, 68%). ^{1}H NMR (400 MHz; $CDCl_3$): 1.07 (d, 18H, ^3J 7.2 Hz, CH(CH_3)$_2$), 1.20–1.25 (m, 3H, CH(CH$_3$)$_2$), 1.39 (s, 9H, C(CH$_3$)$_3$), 3.47 (s, 2H B(OH)$_2$), 6.75 (dd, 1H, ^3J 8.5 Hz, ^4J 2.7 Hz, C4-H), 7.00 (d, 1H, ^4J 2.6 Hz, C6-H), 7.13 (d, 1H, ^3J 8.4 Hz, C3-H), 9.90 (br, 1H, NH); ^{13}C {^{1}H} NMR (100 MHz; $CDCl_3$): 12.6, 17.9, 27.0, 38.8, 117.7, 119.4, 121.8, 122.4, 131.6, 154.6, 177.4. Anal. Calcd for $C_{20}H_{36}BNO_4Si$: C, 61.06; H, 9.22; N, 3.56. Found: C, 61.16; H, 9.38; N, 3.62.

2.7.2. 8,9-Methylenedioxy-2-triisopropylsilyloxy-phenanthridine (15)

To a degassed solution of 2-bromo-4,5-methylenedioxybenzaldehyde **3d** (2.06 g, 9 mmol) in dry 1,2-dimethoxyethane (25 mL) was added tetrakis(triphenylphosphine)palladium (0.468 g, 0.41 mmol, 4.5 mol%) and 3.89 g (9.9 mmol) of **5c**. The mixture was stirred at room temperature for 5 min, an aqueous solution of sodium carbonate decahydrate (7.73 g, 27 mmol, 25 mL) was added, and the resulting mixture was refluxed for 2 h. Then 4 N HCl (15 mL) was added slowly to the chilled solution, and the resulting mixture was refluxed for 30 min. An aqueous solution of 10 M NaOH was added to justify pH 10. Water (50 mL) and CH_2Cl_2 (250 mL) were added and the mixture was transferred to a separation funnel, shaken thoroughly and allowed to stand overnight. The solvent was removed in vacuo, the residue extracted with diethyl ether (2 × 150 mL), and filtered. The etheric solution was concentrated and subjected to column chromatography (silica gel). Eluting with EtOAc/*n*-hexane 1:1, followed by EtOAc, and EtOAc/MeOH (1:1) afforded protected phenanthridine **15** (3.00 g, 77%, R_f 0.7, EtOAc, whitish solid) and unprotected phenanthridine **19a** (0.29 g, 12%, R_f 0.4, EtOAc, slightly yellow solid, mp >250 °C decomposition). ^{1}H NMR data of **15** (400 MHz, $CDCl_3$); δ_H 1.16 (d, 18H, ^3J 7.3 Hz, CH(CH_3)$_2$), 1.30–1.40 (m, 3H, –CH(CH$_3$)$_2$), 6.14 (s, 2H, OCH$_2$O), 7.27 (dd, 1H, ^3J 8.9 Hz, ^4J 2.6 Hz, C3-H), 7.29 (s, 1H, C7-H), 7.74 (d, 1H, ^4J 2.1 Hz, C-1H), 7.75 (s, 1H, C10-H), 8.00 (d, 1H, ^3J 8.9 Hz, C4-H), 8.94 (s, 1H, C6-H), ^{13}C {^{1}H} NMR (100 MHz; $CDCl_3$): 12.8, 18.0, 99.9, 101.8, 105.4, 110.2, 122.5, 123.1, 125.4, 129.5, 131.3, 139.6, 148.2, 149.5, 151.0, 154.8. Anal. Calcd for $C_{23}H_{29}NO_3Si$: C, 69.84; H, 7.39; N, 3.54. Found: C, 69.53; H, 7.48; N, 3.52.

2.7.3. 2-Hydroxy-8,9-methylenedioxy-phenanthridine (19a)

To 936 mg (2.4 mmol) of **15** in THF (25 mL) a solution of Bu_4NF in THF (1 M, 3 mL) was added. The mixture was stirred for 2 h, poured into water (140 mL), extracted with EtOAc three times (200 mL each). The combined organic layers were dried over Na_2SO_4, the solvent was removed under reduced pressure to afford slightly yellow crystals of **19a** (483 mg, 84%). ^{1}H NMR (400 MHz, DMSO-d_6); δ_H 6.24 (s, 2H, OCH$_2$O), 7.22 (dd, 1H, ^3J 8.8 Hz, ^4J 2.1 Hz, C3-H), 7.57 (s, 1H, C7-H), 7.79 (d, 1H, ^4J 2.1 Hz, C-1H), 7.85 (d, 1H, ^3J 8.8 Hz, C-4H), 8.02 (s, 1H, C10-H), 8.92 (s, 1H, C6-H), 9.94 (s,1H, OH). ^{13}C {^{1}H} NMR (100 MHz; DMSO-d_6, 363 K): δ_c 99.7, 101.7, 104.8, 105.2, 118.5, 122.6, 125.0, 128.4, 130.6, 138.1, 147.7, 148.1, 150.6, 155.9. Anal. Calcd for $C_{14}H_9NO_3$: C, 70.29; H, 3.79; N, 5.85. Found: C, 70.04; H, 3.72; N, 5.85.

2.7.4. 2-Hydroxy-5-methyl-8,9-methylenedioxy-phenanthridinium chloride (19b)

Dimethyl sulfate (4.0 mL, 5.33 g, 42.3 mmol) was added to a suspension of **15** (2.22 g, 5.6 mmol) in nitrobenzene (40 mL) and xylene (20 mL). Within 30 min, the mixture was heated to 175 °C and stirring was continued for another 30 min. The chilled mixture was poured into Et_2O (250 mL), the precipitate was allowed to settle down and filtered off, washed with Et_2O (2 × 50 mL) and dried in vacuo to afford 2-hydroxy-5-methyl-8,9-methylenedioxy-phenanthridinium methyl sulfate (1.87 g, 91%) as a whitish solid. It was dissolved in boiling water (300 mL), a solution of sodium chloride (8%, 100 mL) was added, and the mixture was chilled. The precipitate was filtered off, washed with ice-cold water, and dried in vacuo to yield pale yellow crystals of **19b** (948 mg, 64%, 3.3 mmol). ^{1}H NMR (400 MHz, DMSO-d_6); δ_H 4.51 (s, 3H, NCH_3), 6.45 (s, 2H, OCH_2O), 7.62 (dd, 1H, ^3J 9.5 Hz, ^4J 2.5 Hz, C3-H), 7.81 (s, 1H, C7-H), 8.16 (d, 1H, ^4J 2.5 Hz, C1-H), 8.28 (d, 1H, ^3J 9.5 Hz, C4-H), 8.37 (s, 1H, C10-H), 9.66 (s, 1H, C6-H), 11.03 (s 1H, OH). ^{13}C {^{1}H} NMR (100 MHz, DMSO-d_6/D_2O 1/1, 350 K) δ_c 46.0, 101.6, 105.2, 107.9, 108.0, 121.4, 121.9, 123.1, 127.9, 128.8, 134.3, 148.8, 151.2, 157.7, 158.5. Anal. Calcd for $C_{15}H_{12}ClNO_3$: C, 62.19; H, 4.17; N, 4.83. Found: C, 61.82; H, 4.17; N, 4.65.

3. Chemistry

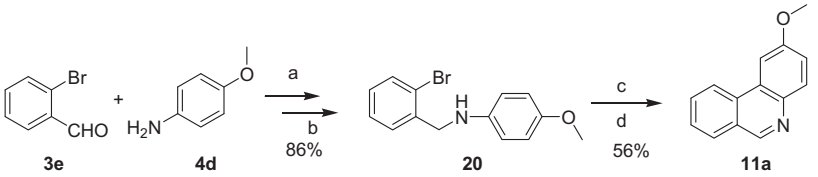
We started the phenanthridine syntheses applying a cyclization with potassium in liquid ammonia as crucial step, a method reported by Kessar et al.,[31,32] since intermediates for this cyclization could easily be prepared. For example, 2-methoxyphenanthridine **11a** was synthesized by cyclization of *N*-(4-methoxyphenyl)-2-bromobenzylamine (**20**) that was prepared via sodium borohydride reduction of the Schiff base formed by reaction of *p*-anisidine (**4d**) and 2-bromobenzaldehyde (**3e**) (Scheme 1).

However, yields of cyclization products decreased markedly when additional methoxy substituents were placed in 8- and/or 9-position. These phenanthridines (**6a–10a**) were found to be more efficiently synthesized via Suzuki cross-coupling reaction of appropriately substituted 2-bromobenzaldehydes and BOC protected *ortho*-aminophenylboronic acids[25] as depicted in Scheme 2.

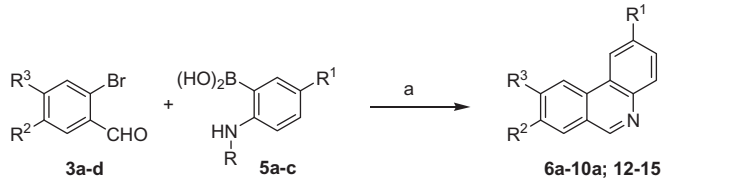
2-Hydroxyphenanthridines (**16a–19a**) were prepared analogously, the phenolic hydroxy group being TIPS protected during cyclization procedure and deprotected with tetrabutylammonium fluoride in THF (Scheme 2a). Furthermore, the more stable pivaloyl (PIV) protection of the aminogroup was found to be more effective for the syntheses of the 2-hydroxyphenanthridines.

Intermediates for the cross-coupling reactions were readily prepared referring to methods reported in the literature. The *ortho*-bromo benzaldehydes **3a,c,d** were prepared by direct bromination of the aldehydes **2a,c,d**[33–35] or were commercially available (**3e**). 2-Bromo-4-methoxybenzaldehyde **3b** was prepared via *ortho*-lithiation of anisaldehyde dimethylacetal and subsequent bromination with carbon tetrabromide as reported previously.[25] Phenyl carbamic acid *tert*-butylester and 4-methoxyphenyl carbamic acid *tert*-butylester **4a,b** were prepared by reacting aniline or *p*-anisidine and di-*tert*-butyl dicarbonate in THF.[36] *N*-(4-(triisopropylsilyloxy)phenyl)pivalamide **4c** was obtained by sequential reactions of p-aminophenol with triisopropylsilyl chloride and imidazole in dichloromethane followed by pivaloyl chloride and triethylamine in THF/Et_2O 1/1 with high yield. *ortho*-Lithiation of the protected aniline derivatives with *tert*-butyllithium,[37] reaction with trimethyl borate[38] and cautious hydrolysis afforded the TIPS/PIV- or BOC-protected boronic acids **5a–c**.

Suzuki cross coupling reactions of the bromobenzaldehydes **3a–c** with the boronic acids **5a–c** were performed in 1,2-dimethoxyethane in presence of tetrakis(triphenylphosphine)palladium



Scheme 1. Synthesis of 2-methoxyphenanthridine via KNH_2/NH_3 cyclization. Reagents and conditions: (a) EtOH, 30 min reflux; (b) $NaBH_4$, EtOH, 1 h reflux; (c) NH_3 (liquid), K, −78 °C, 30 min; (d) NH_4Cl (aq), air, 3 h.

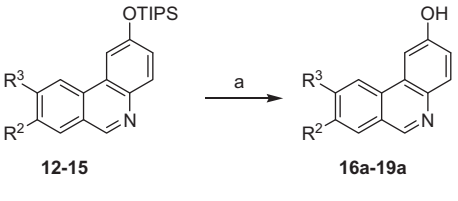


3	R^2	R^3
a	OMe	H
b	H	OMe
c	OMe	OMe
d	-OCH_2O-	

5	R^1	R
a	H	BOC
b	OMe	BOC
c	OTIPS	PIV

	R^1	R^2	R^3
6a	H	-OCH_2O-	
7a	OMe	OMe	H
8a	OMe	H	OMe
9a	OMe	OMe	OMe
10a	OMe	-OCH_2O-	
12	OTIPS	OMe	H
13	OTIPS	H	OMe
14	OTIPS	OMe	OMe
15	OTIPS	-OCH_2O-	

Scheme 2. Synthesis of phenanthridines via Suzuki coupling. Reagents and conditions: (a) Dimethoxyethane, $(Ph_3P)_4Pd^{(0)}$, Na_2CO_3 (aq), 3 h reflux, 4 N HCl, 30 min reflux.



	R^2	R^3
12	OMe	H
13	H	OMe
14	OMe	OMe
15	-OCH_2O-	

	R^2	R^3
16a	OMe	H
17a	H	OMe
18a	OMe	OMe
19a	-OCH_2O-	

Scheme 2a. Removing of the TIPS protecting group. Reagents and conditions: (a) THF, 1 M Bu_4NF/THF, 1 h, rt.

and sodium carbonate. Acid hydrolysis of the reaction mixtures yielded 2-unsubstituted (**6a**), 2-methoxy- (**7a–10a**), or a mixture of TIPS-protected (**12–15**) and unprotected (**16a–19a**) 2-hydroxyphenanthridines (Scheme 2), which could easily be separated by column chromatography. Removing of unnecessary TIPS protecting groups was readily achieved with tetrabutylammonium fluoride in THF (Scheme 2a).

5-Methylphenanthridinium chlorides **6b–11b, 16b–19b** were synthesized in analogy to the published procedure by quaternization of 2-methoxy- or TIPS-protected 2-hydroxyphenanthridines with dimethyl sulfate in a mixture of nitrobenzene/toluene and subsequent aqueous transformation of the methyl sulfate into the chloride[39] (Scheme 3).

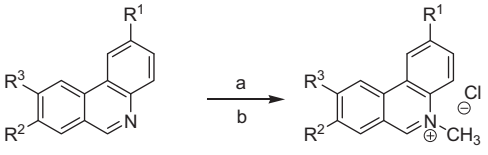
4. Biological evaluation

4.1. Lycobetaine stabilizes the covalent topoisomerase II/DNA-intermediate

We reported previously that **1** acts as topoisomerase II poison with preference to the IIβ isoform.[6] As these investigations were mainly taken using cell free test systems, we focused in the present study on the topoisomerase-targeting properties of this alkaloid in carcinoma cells. The ICE-bioassay was performed using the human epidermoid carcinoma cell line A431. Therefore, lysed cells were centrifuged in a cesium chloride density gradient following drug treatment to separate free and DNA-bound enzyme. The DNA content of the gradient fractions was determined photometrically, obtaining a maximum (60–80 ng/μL) at fraction 5–7 (Fig. 1A). After immunoblotting the amount of topoisomerase was determined in the DNA-peak.

Treatment of A431 cells for 1 h with **1** leads to enhanced levels of topoisomerase II in the DNA peak fractions in a concentration dependent manner (Fig. 1B). Clearly recognizable effects of **1** are observed at a concentration of 30 μM for topoisomerase IIβ and 50 μM for topoisomerase IIα, respectively. In all tested concentrations the amount of topoisomerase IIβ covalently linked to DNA was slightly, but not significantly in excess of topoisomerase IIα.

However, **1** did not stabilize topoisomerase I-DNA complexes in A431 cells (data not shown), indicating that this alkaloid acts as topoisomerase II, but not topoisomerase I poison in vitro. Therefore, **1** analogs were synthesized having various substituents on the phenanthridine core to identify potential structural features which may lead to an enhanced topoisomerase-targeting activity and moreover, an increased growth inhibition of cancer cells.



	R^1	R^2	R^3
6a	H	-OCH_2O-	
7a	OMe	OMe	H
8a	OMe	H	OMe
9a	OMe	OMe	OMe
10a	OMe	-OCH_2O-	
11a	OMe	H	H
12	OTIPS	OMe	H
13	OTIPS	H	OMe
14	OTIPS	OMe	OMe
15	OTIPS	-OCH_2O-	

	R^1	R^2	R^3
6b	H	-OCH_2O-	
7b	OMe	OMe	H
8b	OMe	H	OMe
9b	OMe	OMe	OMe
10b	OMe	-OCH_2O-	
11b	OMe	H	H
16b	OH	OMe	H
17b	OH	H	OMe
18b	OH	OMe	OMe
19b	OH	-OCH_2O-	

Scheme 3. Synthesis of phenanthridinium chlorides. Reagents and conditions: (a) $(CH_3)_2SO_4$, Xylene/$PhNO_2$, 1 h, reflux; (b) NaCl (aq), 1 h, 0 °C.

4.2. Compound screening for growth inhibition in vitro

All synthesized analogs (**6–19**) were tested for growth inhibition of the human gastric tumor cell line GXF251L by performing the sulforhodamine B assay. An overview of the results of selected compounds (**1**, **10b**, **18**–**19b**) is given in Figure 2A, while Figure 2B illustrates the obtained IC_{50}-values of all tested compounds. The calculated IC_{50}-values are summarized in Table 1, the lead compound **1** is included for comparison purposes.

Eighteen of the nineteen synthesized compounds exhibited strong to moderate growth inhibition of the gastric cell line GXF251L, only **17a**, carrying a hydroxy group at position 2 and a methoxy substituent at position 8 showed no effect on tumor cell growth up to the maximum tested concentration of 50 μM. The lead structure **1** inhibits growth of GXF251L cells with a mean IC_{50}-value of 3.2 ± 0.7 μM.[3,6] The inhibitory potential of the tested compounds having a 8,9-methylenedioxy substituent at the phenanthridine core (**6b**, **10b** and 1**9b**) and of the derivative with methoxy substituents in position 2 and 9 (**8b**) exceed that of **1**, while **10b** shows the strongest growth inhibitory potency of all tested derivatives with an IC_{50} <1 μM.

Quaternized derivatives generally show enhanced growth inhibitory potential (IC_{50}-values 0.9 to 18 μM) compared to the respective phenanthridine analogs (IC_{50}-values 12–41 μM). Among the phenanthridines, the compounds with an 8,9-methylenedioxy-bridge (**6a**, **10a** and **19a**) possess the strongest inhibitory potential, albeit not as potent as the quaternized derivatives. Overall, quaternization of the phenanthridines results in a 5- to 10-fold increase of tumor cell growth inhibition.

4.3. Impact of lycobetaine derivatives on the catalytic activity of human topoisomerase I

To investigate the impact of the synthesized compounds on the catalytic activity of human topoisomerase I, a cell-free relaxation assay was performed. Topoisomerase I activity was determined as relaxation of supercoiled pUC18 plasmid DNA by nucleic extract from MCF-7 cells. The assay was performed by exclusion of ATP to rule out topoisomerase II activity.

Compound **1** effectively inhibits the catalytic activity of topoisomerase I at a concentration of 10 μM (Fig. 3, lane 5). All *N*-methylphenanthridinium derivatives are found to inhibit topoisomerase I activity, whereas no inhibition could be observed for the non quaternized phenanthridines (Table 1). Almost all

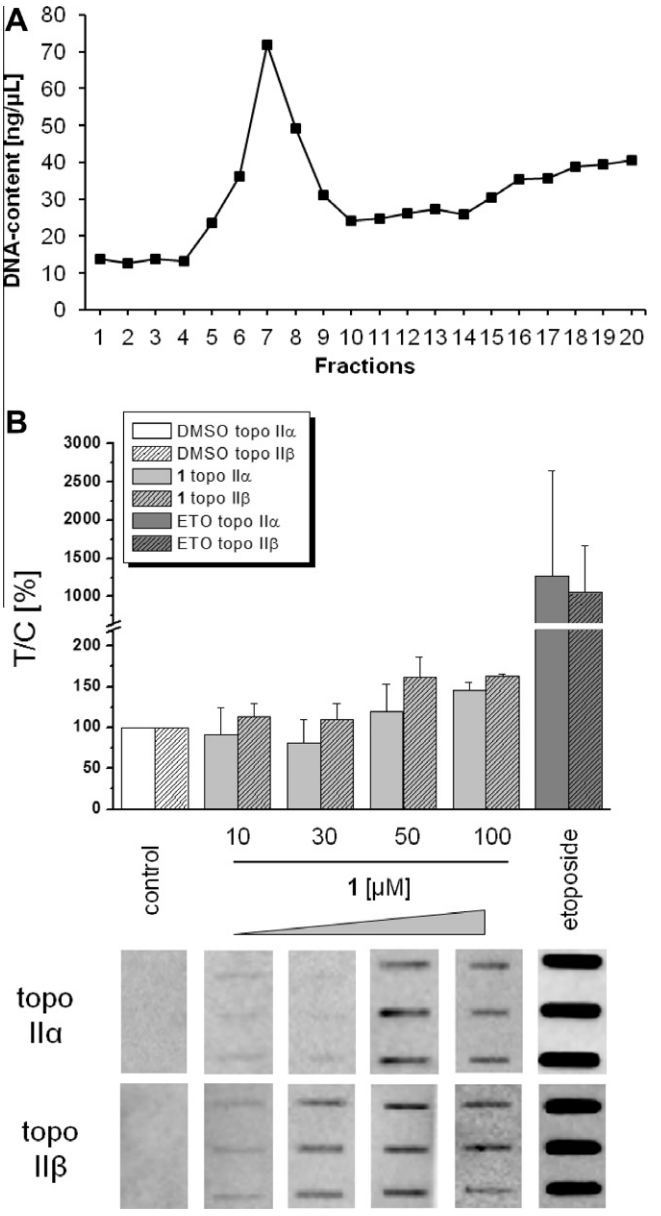


Figure 1. Detection of covalent topoisomerase II/DNA intermediates in the ICE assay. A431 cells were treated with **1** (increasing concentrations from 10 to 100 μM) and the topoisomerase II-poison etoposide (50 μM), respectively, in serum-free medium for 1 h. (A) DNA-content measured as absorbance at 260 nm. (B) The level of topoisomerase II/DNA intermediates was calculated as test over control cells (treated with 1% DMSO) with respect to DNA content × 100 (T/C, %). The data presented are the mean ± SD of three independent experiments. Representative, DNA-containing sections (Fractions 6–8) of the according immunoblots are shown.

synthesized *N*-methylphenanthridinium chlorides (**6b–11b**, **17b** and **19b**, Table 1) affect topoisomerase I activity at a concentration of 10 μM and completely block the enzyme at a concentration of 30 μM (Fig. 3). These derivatives exhibit a 8,9-methylenedioxy bridge or a methoxy-substituent in position 2. The synthesized compounds with a hydroxy group in position 2 (**18b** (Fig. 3, lane 13–15) and **16b** (Table 1)) however, show no interaction with the enzyme up to a concentration of 50 μM. An exception is derivative **17b** bearing a hydroxy group in position 2 in addition to a methoxy substituent on position 9: this compound inhibits topoisomerase I at a concentration of 10 μM (Table 1).

In summary, phenanthridines do not affect topoisomerase I unless they are quaternized as *N*-methylphenanthridinium chlorides

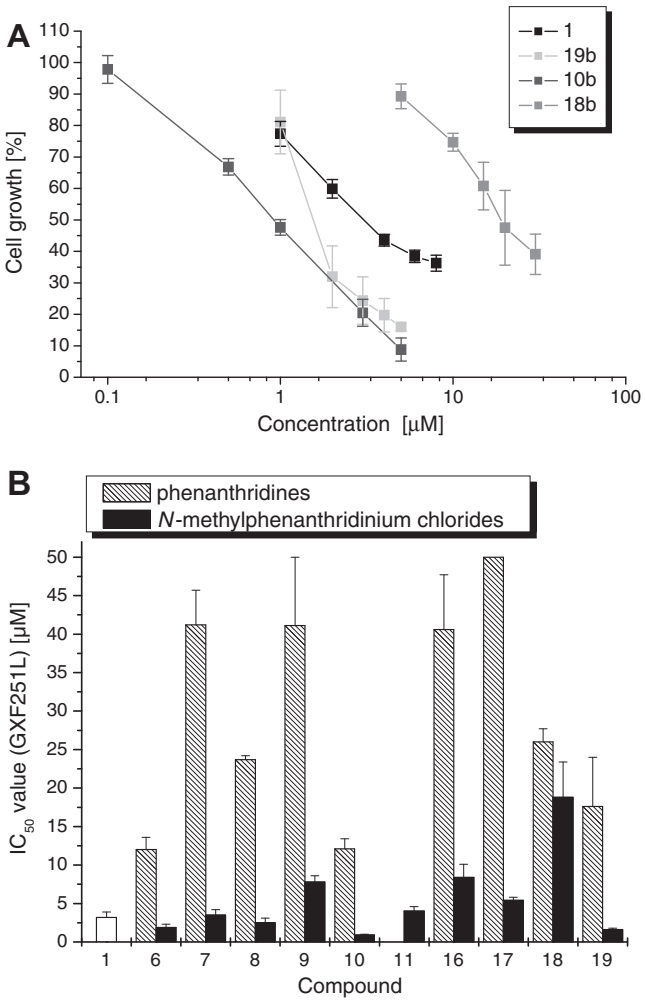


Figure 2. Effects of **1** and its analogs on cell growth of GXF251L cells after 72 h of exposure in the sulforhodamine B (SRB) assay. (A) Growth inhibitory potential of certain lycobetaine analogs in comparison to **1** itself, calculated as survival of treated cells over control cells × 100 [T/C, %]. The concentrations are plotted on the abscissa using a log scale. The data presented are the mean ± SD of three independent experiments. (B) Acquired IC_{50}-values of the tested compounds are represented.

and bear a 8,9-methylenedioxy-group or a methoxy substituent in position 2 of the phenanthridine core.

4.4. Inhibition of topoisomerase II

Decatenation assays were performed with the synthesized compounds to identify pharmacophores responsible for the inhibition of the catalytic activity of recombinant topoisomerase IIα and IIβ. Catenated DNA is not able to migrate into an agarose gel, in contrast to DNA-minicircles, which are released from the catenated DNA network by catalytically active topoisomerase II.

Compound **1** inhibits the catalytic activity of topoisomerase IIβ at a concentration of 30 μM, weak interference with the IIα isoform could also be observed at 50 μM (Fig. 4, lane 3–5). Substitution of the 4,5-ethylene bridge of **1** with a methyl group (**19b**) and introduction of a methoxy-group at position 2 (**10b**) do not result in significant activity change with respect to the tested topoisomerase isoforms (Fig. 4). Replacement of the hydroxy or methoxy group in position 2 with a hydrogen (**6b**) leads to a considerable loss of inhibitory activity in the tested concentration range (Table 1).

Replacing the 8,9-methylenedioxybridge with two methoxy groups as well as introducing an additional methoxy group in position 2 (**9b**) results in diminished activity (topoisomerase IIβ 50 μM, IIα 100 μM). Derivatives bearing a 2-methoxy group exhibit similar topoisomerase inhibitory properties (**7b**, **8b** and **11b**; Table 1). Compound **8b** with two methoxy residues at position 2 and 9 affects the activity of topoisomerase II isoforms already at 30 μM, whereas methoxy groups in position 2 and 8 (**7b**) show lower inhibitory properties (50 μM). In addition, removal of the functional groups in position 8 and 9 (**11b**) weaken the efficacy towards topoisomerase II even more (100 μM). Replacement of the methoxy group in favor of a hydroxy group in position 2 (**16b–18b**) results in the loss of topoisomerase II inhibition.

In general, derivatives with a 8,9-methylenedioxy-group and 2-methoxy-residue, respectively, exhibit the strongest inhibitory properties on topoisomerase II.

5. Discussion

Topoisomerases are ubiquitous enzymes regulating the topological state of DNA and play essential roles in a number of fundamental DNA processes. As they generate DNA strand breaks during their catalytic cycle, they also possess the potential to fragment the genome.[20,40,41] Due to the mode of interaction with topoisomerases, two groups of inhibiting drugs can be defined, topoisomerase poisons and pure catalytic inhibitors. By definition, topoisomerase poisons stabilize the covalent DNA-topoisomerase intermediate, preventing the release and resealing of the DNA strand and consequently resulting in DNA damaging properties.[42–44] In contrast, pure catalytic inhibitors abrogate catalytic activity of topoisomerases without stimulating DNA cleavage.[45,46]

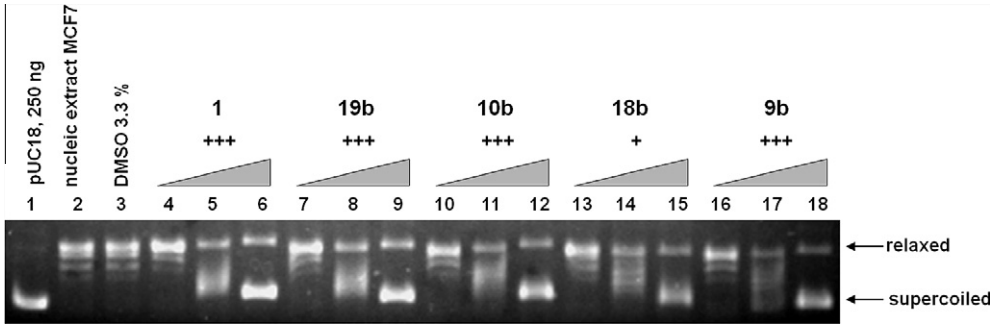


Figure 3. Impact of **1** and its analogs on the catalytic activity of topoisomerase I (relaxation assay). Active topoisomerase I converts the supercoiled pUC18 plasmid DNA into the relaxed form. Lane 1: supercoiled pUC18 plasmid DNA (250 ng). Lane 2: pUC18 + nucleic extract from MCF-7-cells. Lane 3: pUC18, nucleic extract + solvent control DMSO. Lanes 4–6: pUC18, nucleic extract + **1** at 1, 10, 30 μM. Lanes 7–9: pUC18, nucleic extract + **19b** at 1, 10, 30 μM. Lanes 10–12: pUC18, nucleic extract + **10b** at 1, 10, 30 μM. Lanes 13–15: pUC18, nucleic extract + **18b** at 30, 50, 100 μM. Lanes 16–18: pUC18, nucleic extract + **9b** at 1, 10, 30 μM. Shown is a representative gel out of three independent experiments with similar results.

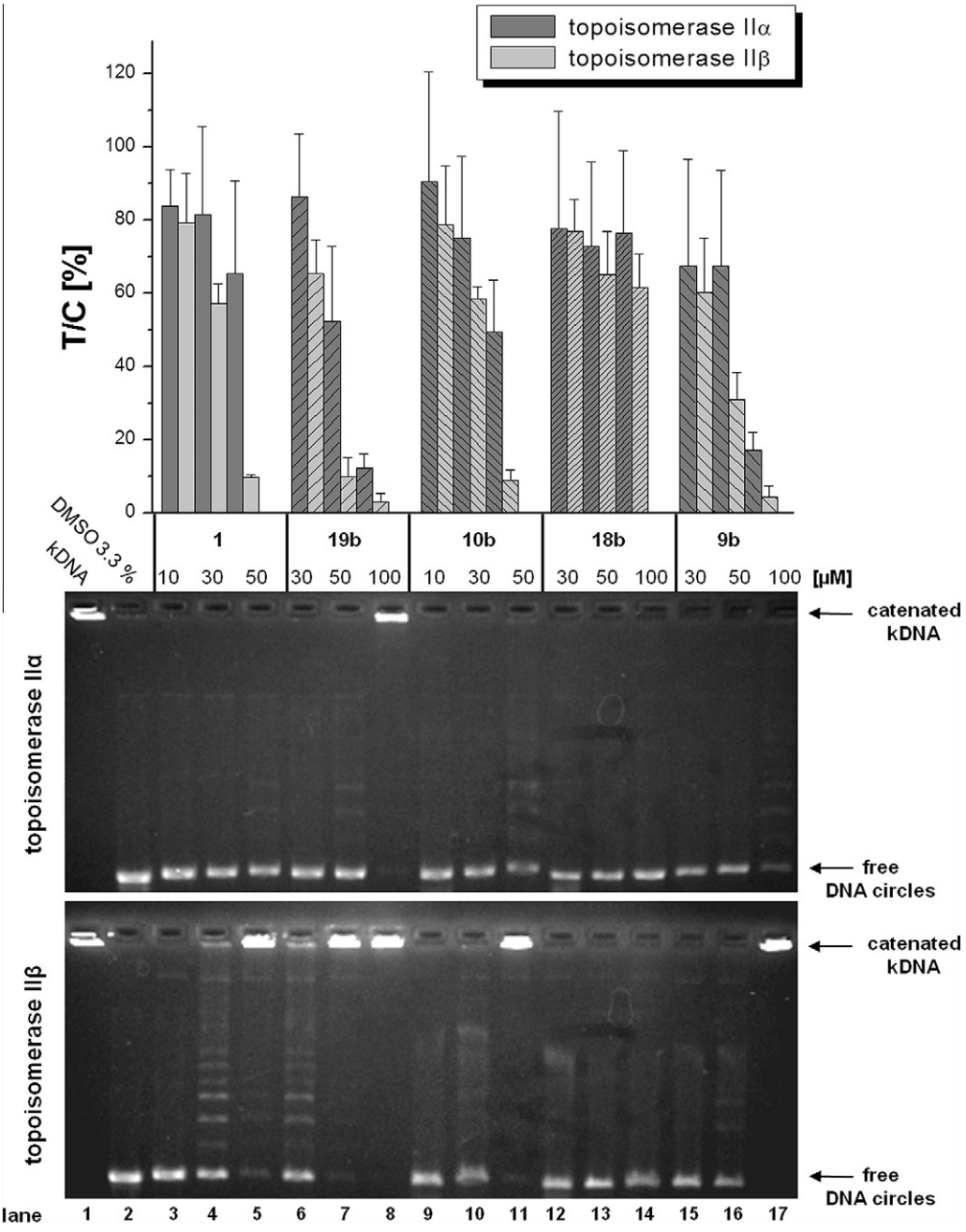


Figure 4. Catalytic activity of recombinant human topoisomerase II determined by decatenation of kDNA. Topoisomerase II (40 ng) was incubated for 60 min at 37 °C in the absence (3.3% DMSO, lane 2) or presence of **1** (lane 3–5) and the corresponding 5-methylphenanthridinium chlorides (lane 6–17), respectively. The reaction was stopped with 10% (w/v) SDS, and after digestion with proteinase K, samples were separated in 1% agarose gels in the absence of ethidium bromide. The ethidium bromide stained gel was documented under UV-light by digital photography. Fluorescence signals of decatenated kDNA treated with topoisomerase II and the respective compounds were calculated as test over control (T/C, %) in respect to the solvent control DMSO. The data plotted show the means ± SD of three independent experiments. A representative gel out of the three identical experiments with similar outcome is represented. Lane 1 shows catenated kDNA not exposed to the enzyme. Active topoisomerase II releases single free DNA circles from the catenated DNA network when it is treated with the solvent control DMSO (lane 2). Lane 3–17 show incubation of human recombinant topoisomerase II with synthesized lycobetaine analogs.

We have demonstrated previously that **1** acts as a topoisomerase II poison.[6] It has later been reported that **1** also acts similarly on type IV topoisomerase from *Escherichia coli*.[47] Furthermore, we found that the primary target of **1** is the IIβ isoform. The selective inhibition of topoisomerase IIβ by **1** was shown in cell-free test systems as well as in the human epidermoid carcinoma cell line A431 using the immunoband depletion assay.[6]

In the ICE assay, we could confirm that **1** acts as topoisomerase II poison (Fig. 1). In contrast to the results of the immunoband depletion assay,[6] the present data shows only a slight preference of **1** to topoisomerase IIβ. This may be due to the fact that in the previous study different concentrations and incubation periods were chosen. Concentrations used in the present study were based on those used in the cleavage assay. The ICE system used in this study, excludes potential confounding by further mechanisms like enzyme degradation that may have influenced the immunoband depletion results. In addition we showed that **1** does not act as topoisomerase I poison in A431 cells, while it inhibits the catalytic activity of topoisomerase I in the cell-free relaxation assay.

Our present results allow to conclude that **1** acts as topoisomerase II poison with a slight preference to the IIβ isoform, additionally affecting topoisomerase I as a pure catalytic inhibitor.

Based on these findings, we performed studies to identify potential structural features of **1** which are influencing biological activity.

The phenanthridine core was used as a basis for the modifications in 2, 8 and 9 position to investigate the influence of different functional groups in those positions with respect to catalytic activity of human topoisomerases and growth inhibition of human gastric carcinoma cells. Results are summarized in Table 1. The data implicate that the quaternized compounds are more potent compared to the non quaternized respective phenanthridines. A similar effect was shown in previous studies where the reactivity of the charged iminium moiety has been associated with enhanced cytotoxicity of certain structure-related benzo[*c*]phenanthridines.[48,49] Yet, the iminium charge seems not to be crucial for the biological activity of these compounds, as it could be demonstrated that uncharged analogs of the benzo[*c*]phenanthridinium nitidine[50] and certain benzo[*i*]phenanthridines[51] also act as topoisomerase inhibitors and exhibit cytotoxic activity. Furthermore, it has been reported that the position of the nitrogen heteroatom is decisive for the biological activity of benzo[*i*]phenanthridine derivatives.[52] Thus, the iminium moiety of the *N*-methylphenanthridinium chlorides investigated in this study may play an important role in the interaction with cellular targets resulting in cytotoxicity and topoisomerase inhibition. Similar conclusions have been drawn from a structure–activity relationship analysis of isolated natural compounds with a lycorine (tetrahydrolycobetaine) structure.[53] In addition to the importance of the quarternized nitrogen, the substituents in positions 2, 8 and 9 also contribute to the observed biological activity.

Moreover, the data listed in Table 1 clearly indicate that appropriately substituted *N*-methylphenanthridinium chlorides are able to inhibit both, topoisomerase I and II. Efficient topoisomerase I inhibition (at a concentration of 10 μM) is achieved by derivatives bearing either a 8,9-methylenedioxy moiety or a methoxy residue at position 2. The more active analogs targeting topoisomerase II were those possessing a methylenedioxy substituent at the 8,9-position and concomitantly a hydroxy or methoxy group at position 2.

Molecular inspection of a related group of compounds, the benzo[*i*]phenanthridines reveals the 2,3-methylenedioxy substituent on the A-ring of benzo[*i*]phenanthridines to be similarly positioned as the 8,9-methylenedioxy moiety at the A-ring of the **1** analogs (Chart 1), although the nitrogen is located at a neighboring position.[51,52] Comparing the structure activity studies of the benzo[*i*]phenanthridines and our data suggests that the methylenedioxy moiety is a favorable substituent for enhancement of topoisomerase inhibitory activity.

Of note, some *N*-methylphenanthridinium chlorides (**9b**, **10b** and **19b**) show a preference to the IIβ isoform. Furthermore, the 4,5-ethylene bridge within the lycobetaine molecule is found to play an important role for the inhibition of topoisomerase II activity, whereas no effect on topoisomerase I inhibition is observed. Altogether, the synthesized lycobetaine analogs possess more potency in inhibiting topoisomerase I than in topoisomerase II. This is in line with findings reported for **1**, showing stronger inhibition of plasmid (pBR322) relaxation by topoisomerase I compared to topoisomerase IIα.[47]

These results are in accordance with several studies on the structurally related group of benzophenanthridines concerning topoisomerase-targeting properties. Benzo[*c*]phenanthridines, like fagaronine and nitidine, have been shown to possess cytotoxic properties and moreover, to inhibit topoisomerase I at low concentrations (enzyme inhibition starting at concentrations of 6–18 μM) and topoisomerase II at higher concentrations (approximately 80% inhibition of DNA unknotting in the presence of 40 μM nitidine or fagaronine).[54,55] Substituted benzo[*i*]phenanthridines have been described to exert similar biological activity as benzo[*c*]phenanthridines except for inhibition of topoisomerase II.[12,51,52,56,57] Previously work has also shown even slight modifications in either substituents or substituent pattern to significantly influence specificity for either topoisomerase I or II.[58,59]

The poor cytotoxic activities of the phenanthridines **6a**–**19a** in comparison to the quaternized compounds may be explained in part by the lack of topoisomerase inhibition. Since phenanthridine derivatives were also able to inhibit growth in GXF251L cells with IC_{50}-values in the low micromolar range, their cytotoxic effects appear not solely to depend on topoisomerase inhibition.

We identified three lycobetaine analogs (**8b**, **10b**, **19b**) possessing stronger biological activity compared to the parent compound **1**. All of these three compounds show enhanced inhibition of cell growth while exhibiting the same topoisomerase-targeting properties as **1** (**10b**, **19b**) or even exceeding the inhibitory properties of **1** (**8b**), however not showing a preference to a type II isoenzyme.

In summary, we identified potential pharmacophores of phenanthridinium alkaloids derived from the topoisomerase II poison **1** which play an important role for the interaction with topoisomerases. Preferred inhibition of topoisomerase I appears to require a 8,9-methylenedioxy-moiety or a methoxy group in position 2. In contrast, improved topoisomerase II inhibition requires a methoxy or 8,9-methylenedioxy or hydroxy group in position 2. A hydroxy moiety instead of a methoxy residue at position 2 results in a general decrease of biological activity, particularly regarding topoisomerase II, while compounds bearing a 8,9-methylenedioxy-bridge still exhibit topoisomerase-targeting properties independently of hydroxylation at position 2. The data present a promising platform for further development of novel phenanthridine analogs with preference to topoisomerase isoenzymes.

Acknowledgment

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Supplementary data

Supplementary data (synthesis details, NMR data of intermediates and further products, as well as elemental analysis data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.011.

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