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Original article

Synthesis and biological evaluation of *N*-substituted benzo[*c*]phenanthrolines and benzo[*c*]phenanthrolinones as antiproliferative agents

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ABSTRACT

Benzo[*c*]phenanthrolines and benzo[*c*]phenanthrolinones substituted by dialkylaminoalkyl side chains at position N5 and C6, respectively, were synthesised and their biological activity evaluated. They displayed interessant cytotoxicity associated with some DNA interactions. However, the low topoisomerase 1 affinity suggests that other cellular targets are responsible for the antiproliferative activity.

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1. Introduction

Topoisomerase 1 (Top1), a major enzyme involved in different cellular processes, is recognised as a clinically validated target for the research of new anticancer agents [1]. Among the different promising classes, non camptothecin tetracyclic aromatic structures derived from natural benzophenanthridine (BZP) alkaloids nitidine (**1**) and fagaronine (**2**) show promising activities, as illustrated by the currently developed drugs NSC 725776 **3** and Topovale (ARC 111) **4** (Scheme 1) [2].

Typically, it was shown in these series that the introduction of an aminoalkyl side chain onto the tetracyclic basic core improves DNA interactions and solubility in biocompatible solvents, inducing increased cytotoxicity and topoisomerase affinity [3]. Recently we focalised on the synthesis of aza-analogues of BZP with a pyridine cycle A, in the 1,8 and 1,7 benzo[*c*]phenanthrolinones series (Scheme 2) [4].

Unsubstituted compounds in both series were first shown to display cytotoxic activities within the same order of magnitude as the parent natural BZP alkaloids [4]. In a second step of this SAR program, the influence of the substitution of the basic cores by dialkylaminoalkyl side chains was envisioned. Compounds substituted on cycle A in position 1 or 2 exhibited potent cytotoxic activities, but a moderate Top1 affinity was observed despite good DNA interactions (Scheme 3) [5].

In the present study, new series of analogues were prepared in order to examine the influence of the introduction of basic side chains on cycle C on the cytotoxicity and Top1 inhibition (Scheme 4). Two series of compounds were envisaged for this purpose. The first one involves introduction of the dialkylaminoalkyl side chain at position N5 (A series). In the second series, the chain is located at position C6 (B series). We describe here the synthesis and the biological evaluation of these new substituted tetracyclic derivatives.

2. Chemistry

The different benzo[*c*]phenanthrolines and benzo[*c*]phenanthrolinones were synthetised following the route described in Schemes 5 and 6. The strategy is based on the construction of the four benzo[*c*]phenanthrolinone intermediates **5**–**8** through an intermolecular heteroaromatic Suzuki-Miyaura cross coupling, followed by a nitro reduction-*in situ* lactamisation sequence from readily available starting materials (Scheme 5) [6].

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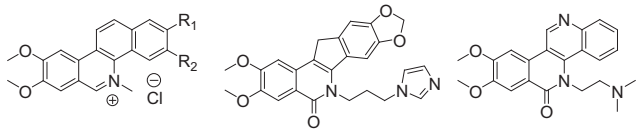


Scheme 1. Non-CPT Top1 inhibitors: examples.

The basic side chains were introduced either directly in position N5 (A series) or, after formation of the chloro derivatives at position C6 (B series) (Scheme 6). Following these two procedures, 23 new substituted benzo[*c*]phenanthrolinones and benzo[*c*]phenanthrolines were prepared (Tables 1 and 2).

3. Pharmacology

3.1. Cellular effects

3.1.1. Evaluation of the cytotoxicity

The N5-substituted benzo[*c*]phenanthrolinones and C6-substituted benzo[*c*]phenanthrolines were examined for their antiproliferative activity against human colon cancer cell line HT-29. The results (IC_{50}) are reported in Tables 1 and 2 for the N5-substituted and the C6-substituted series, respectively.

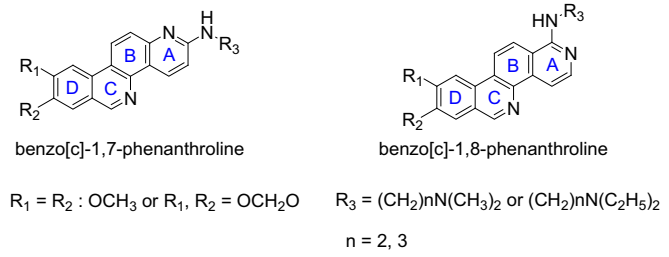
The cytotoxicity measurements indicate that methylenedioxy-substituted compounds in the N5-subtituted series are one order of magnitude more potent than the corresponding dimethoxy analogues (Table 1), compounds **23**–**28** compared with compounds (**17**–**22**). Similar results, but to a much lesser extend, are observed in the C6-substituted series (serie B, Table 2), unless for the methylenedioxy compound **36** being 2 fold less potent than its dimethoxy analogue **30**. In this series, the presence of a methylenedioxy or a dimethoxy substituent at position 8,9 on cycle D does not appear to have a dramatic influence on the biological activity.

The most potent compounds are the N5-substituted **23**, **28**, and the C6-substituted **35** compounds, followed by **29**, **32**, and **37** (one order of magnitude less than the first cited compounds), whereas compounds **17**, **19** and **20** (methoxy-substituted) display comparable cytotoxic activities comparable to that of the reference nitidine (**1**). Also, these results show that ethylamino side chains ($NCH_2CH_2N(R_2)$) are more appropriate than their propylamino counterparts ($NCH_2CH_2CH_2N(R_2)$) (up to 2 log difference) to observe potent cytotoxic activities.

3.1.2. Cell cycle blockade

The cell cycle effects were studied on the same HT-29 cell line. As shown in Fig. 1, all six methylenedioxy derivatives in the N5-substituted series (**23**–**28**) induced G2/M phases arrests in correlation with their sub-micromolar cytotoxicities. The two methylenedioxy derivatives in the C6 series **35** and **37** that were cytotoxic at sub-micromolar concentrations also displayed G2/M phases arrests at 48 h. By contrast, all other tested compounds that

Scheme 2. Benzo[c]phenanthroline skeletons.



Scheme 3. Introduction of alkylaminoalkyl side chains on cycle A.

presented cytotoxicities in the micromolar range, failed to modify the cell cycle at the tested concentrations, except compound **29** which induced strong G2/M phases cell cycle arrests. Such massive accumulation of G2/M phase arrests, in correlation with the cytotoxic effect, suggests that those compounds induce major perturbation of the integrity of the genomic DNA. It should be noted that similar observations were previously noted with fagaronine (**2**) [7].

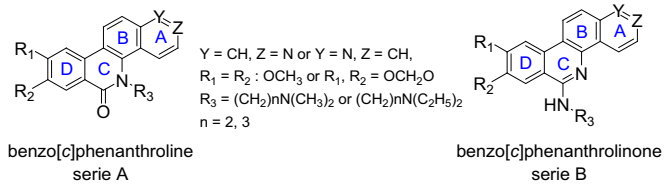
3.2. DNA binding properties and inhibition of topoisomerases 1 and 2

Several chemically related compounds exhibit a good cytotoxicity in correlation with a good Top1 inhibition and DNA binding potencies [8]. In order to address the pharmacological profile of the different substituted tetracycles **17**–**39**, modifications of Top1 and Top2 activities, and effects on DNA were studied.

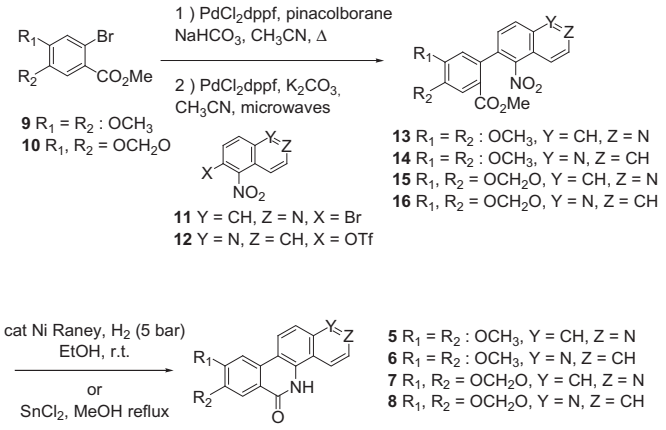
3.2.1. DNA binding studies

The ability of the two series of compounds to interact with DNA was assessed using UV/Vis spectrometry of the compound in absence or presence of CT-DNA (Fig. 2). The spectra of all tested compounds were modified in the presence of CT-DNA suggesting some drug/DNA interaction as evidenced by a strong hypochromic effect of the peak around 275–285 nm together with a bathochromic effect. In the dimethoxy-C6-substitued series, as well as with compounds **25** and **27**, an additionnal peak was observed around 320 nm that increased upon addition of CT-DNA (up arrows). The nature of this binding was then studied using Top1-induced DNA relaxation studies (Fig. 3) together with CD spectroscopy (Fig. 4). The dimethoxy-substituted compounds from the A or B series presented a strong decrease of the positive CD from the DNA (270–280 nm) associated with potent DNA intercalation profile from Top1-DNA relaxation studies. From these dimethoxy-substitued compounds, the B series (C6-substituted) were more efficient than the A series (N5-substituted) in relaxing the DNA upon Top1-DNA cleavage. In the methylenedioxy series, compounds from the B series were more potent to intercalate in the DNA helix than their respective counterparts in the A series for which binding seems to be more complex (Fig. 3).

The CD spectra also evidenced a weak positive peak generated at ~330 or 340 nm upon addition of all C6-substituted compounds, except for **36** and for the methylenedioxy-N5-substituted compounds. This positive band is associated with a negative peak at



Scheme 4. New series: side chain on cycle C in position N5 (serie A) or C6 (serie B).



Scheme 5. Preparation of the benzophenanthrolinone intermediates.

~300 nm (down arrow) in the dimethoxy-C6-substituted series (**29**–**34**), suggesting some differences in the DNA interaction between the dimethoxy- and methylenedioxy-subtituted series (Fig. 4).

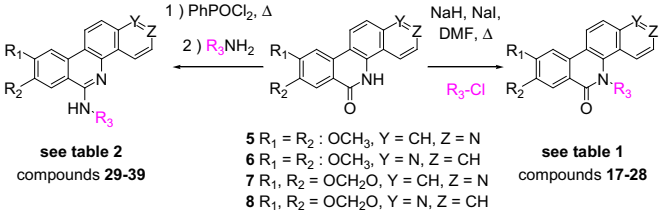
Neither the Top1-induced DNA relaxation nor the CD spectra measurements permit to conclude for a typical DNA intercalation or minor groove binding of the compounds. The DNA binding mode seems more complex, with some intercalation of part of the molecule between adjacent base pairs and other mode of binding.

3.2.2. Topoisomerases 1 and 2 inhibition assays

The potency of the two series of compounds to inhibit the activity of human Top1 and Top2 was next envisaged.

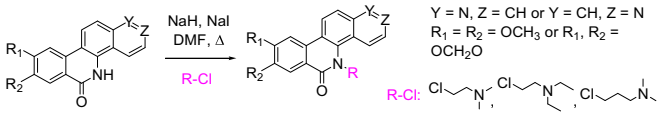
DNA cleavage assays were used to examine the effects of the different compounds on the catalytic activity of topoisomerases 1 and 2 (Figs. 5 and 6). For Top1, the assay was carried out with a 3′ end labelled DNA fragment and camptothecin (CPT) was used as the reference Top1 inhibitor (Fig. 5) [9]. After resolution on a denaturing polyacrylamide gel, the induction of Top1-DNA complexes was evidenced by the formation of specific DNA cleavage bands. In our case, no inhibition of the enzyme activity was observed for the different tetracycles when compared to camptothecin and the structure related compound nitidine (**1**). As evidenced by the increased intensity of the DNA cleavage bands, nitidine (**1**) presented some different base sequence preferences, which could ultimately lead to different pharmacological outcomes. Particularly, nitidine (**1**) generated cleavages at CG, CA and TA steps additionally to the typical CPT-induced cleavage at TG steps.

For Top2 experiments, a supercoiled plasmid DNA was used and the cleaved DNA products were resolved by gel electrophoresis on agarose (Fig. 6) [10]. In the presence of the reference Top2 inhibitor etoposide, supercoiled plasmid DNA (Sc) is cleaved to form linear (Lin) and nicked (Nck) DNAs after resolution. A weaker effect is observed with nitidine **1** [11]. For all the substituted tetracycles no significant Top2 poisoning effect was observed. As previously noted in these aza-series, topoisomerases inhibition does not constitute the



Scheme 6. Introduction of the aminoalkyl chains.

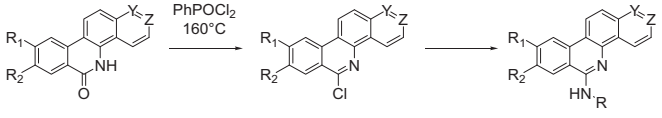
Table 1
Cytotoxicities of N5-substituted Analogues (Serie A).



Compound	R_1, R_2	Y, Z	Amino chain	IC_{50} μM (HT-29)
Nitidine 1				2.75 ± 0.15
17-k7755	$(OCH_3)_2$	Y = CH, Z = N	$NCH_2CH_2N(CH_3)_2$	1.42 ± 0.60
18-k7773	$(OCH_3)_2$	Y = CH, Z = N	$NCH_2CH_2CH_2N(CH_3)_2$	6.11 ± 0.50
19-k7758	$(OCH_3)_2$	Y = CH, Z = N	$NCH_2CH_2N(C_2H_5)_2$	1.82 ± 0.31
20-k7775	$(OCH_3)_2$	Y = N, Z = CH	$NCH_2CH_2N(CH_3)_2$	1.66 ± 0.29
21-k7792	$(OCH_3)_2$	Y = N, Z = CH	$NCH_2CH_2CH_2N(CH_3)_2$	4.98 ± 0.78
22-k7774	$(OCH_3)_2$	Y = N, Z = CH	$NCH_2CH_2N(C_2H_5)_2$	2.63 ± 0.38
23-k7896	OCH_2O	Y = CH, Z = N	$NCH_2CH_2N(CH_3)_2$	0.078 ± 0.016
24-k7953	OCH_2O	Y = CH, Z = N	$NCH_2CH_2CH_2N(CH_3)_2$	0.24 ± 0.125
25-k7952	OCH_2O	Y = CH, Z = N	$NCH_2CH_2N(C_2H_5)_2$	0.23 ± 0.11
26-k7893	OCH_2O	Y = N, Z = CH	$NCH_2CH_2N(CH_3)_2$	0.12 ± 0.03
27-k7969	OCH_2O	Y = N, Z = CH	$NCH_2CH_2CH_2N(CH_3)_2$	0.23 ± 0.08
28-k7895	OCH_2O	Y = N, Z = CH	$NCH_2CH_2N(C_2H_5)_2$	0.075 ± 0.023

essential target responsible for the cytotoxicity measured and introduction of an aminoalkyl side chain in position N5 or C6 does not influence the Top inhibition [4,5]. These results are interesting from a SAR point of view, when comparing these structures to ARC 111 (**4**), which exhibits a good Top1 inhibition correlated with a good cytotoxicity. This type of observation has been previously reported in analogous series and outlines the low level of tuning in these chemically related families [12]. Because of the DNA binding activity of these series of compounds, we evaluated the suppressor activity of the compounds on CPT-induced DNA cleavages (Fig. 7). Addition of increasing concentrations of the evaluated compounds to Top1 in the

Table 2
Cytotoxicities of C6-substituted Analogues (Serie B).



Compound	R_1, R_2	Y, Z	Amino chain	IC_{50} μM (HT-29)
Nitidine **1**				2.75 ± 0.15
29-k7121	$(OCH_3)_2$	Y = CH, Z = N	$NCH_2CH_2N(CH_3)_2$	1.02 ± 0.29
30-k7116	$(OCH_3)_2$	Y = CH, Z = N	$NCH_2CH_2CH_2N(CH_3)_2$	3.14 ± 0.99
31-k7119	$(OCH_3)_2$	Y = CH, Z = N	$NCH_2CH_2CH_2N(C_2H_5)$	5.47 ± 0.48
32-k7166	$(OCH_3)_2$	Y = N, Z = CH	$NCH_2CH_2N(CH_3)_2$	0.97 ± 0.20
33-k7164	$(OCH_3)_2$	Y = N, Z = CH	$NCH_2CH_2CH_2N(CH_3)_2$	3.33 ± 0.96
34-k7165	$(OCH_3)_2$	Y = N, Z = CH	$NCH_2CH_2CH_2N(C_2H_5)$	5.77 ± 0.51
35-k7887	OCH_2O	Y = CH, Z = N	$NCH_2CH_2N(CH_3)_2$	0.070 ± 0.021
36-k7968	OCH_2O	Y = CH, Z = N	$NCH_2CH_2CH_2N(CH_3)_2$	7.62 ± 1.03
37-k7894	OCH_2O	Y = N, Z = CH	$NCH_2CH_2N(CH_3)_2$	0.11 ± 0.01
38-k7772	OCH_2O	Y = N, Z = CH	$NCH_2CH_2CH_2N(CH_3)_2$	1.73 ± 0.09
39-k7970	OCH_2O	Y = N, Z = CH	$NCH_2CH_2CH_2N(C_2H_5)_2$	1.83 ± 0.42

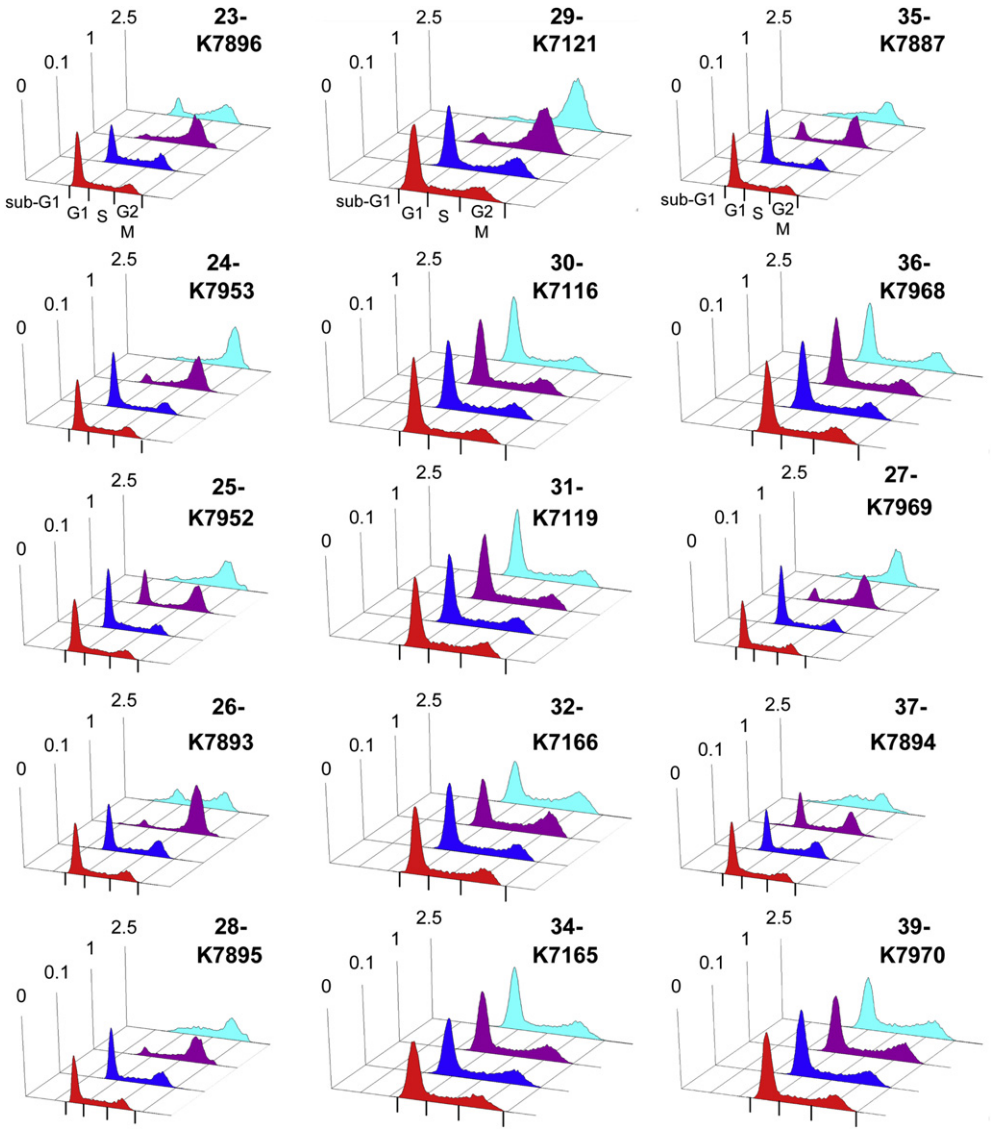


Fig. 1. Cell-cycle effect. Increasing concentrations (as indicated on the top of the plots, in μM) of the various drugs were added to HT-29 colon carcinoma cells for 48 h prior to IP staining and FACS analysis.

presence of CPT evidenced a concentration-dependant inhibition of CPT-induced poisoning effects. Particularly, the most cytotoxic compounds **23** and **35** are those presenting the highest inhibition of CPT-induced poisoning. A correlation between cytotoxic activities and the efficiency of the inhibition of CPT-induced poisoning was established for all tested compounds (Fig. 8). From this graph, it is clear that all strongly cytotoxic compounds (in bold, bellow the horizontal dotted lane fixed at 0.5 μM) are those presenting the highest inhibition of CPT-induced Top1-poisoning. Similarly, all dimethoxy-substituted derivatives in the A or B series (N5- or C6-substituted compounds) are cytotoxic at higher concentrations and are weaker inhibitors of CPT-induced poisoning. Such results suggest a correlation between the inhibition of CPT-induced poisoning and the cytotoxic effect of these series.

3.2.3. *Sequence-specific DNA binding*

The DNA relaxation profiles of the different tetracycles also suggested some intercalation. Consequently, a DNase I foot-printing analysis was carried out to explore the potency of compounds **17**–**39** to bind DNA in a sequence-specific manner (Figs. 9and10). A radio-labelled 265-bp DNA restriction fragment was incubated with increasing concentrations of the different substituted tetracycles. After digestion by a DNase I and resolution by gel electrophoresis, some sequence preferences at 5′-CTA sites were observed. It is noteworthy that benzo[*c*]phenanthrolines substituted by an aminoalkylamine chain on cycle A (position 1 or 2) present some preferential DNA intercalation sequences (5′-CT). Compounds presenting the highest abilities to interact with this sequence belong to the most cytotoxic series (methylenedioxy-substituted in the N5-series, compounds **23**–**38**) whereas the weakest (>3 μM) cytotoxic compounds **30**, **31** and **36** failed to bind this sequence.

4. Conclusion

Aza-analogues of the natural alkaloids nitidine (**1**) and fagaronine (**2**) were substituted on cycle C by different aminoalkylamino chain, in order to improve their biological activities. The anti-proliferative profile of the natural compounds has been amended, particularly in the case of methylenedioxy bearing benzo[*c*]phenanthrolinone derivatives. The pharmacological studies outline that these new analogues display a good DNA affinity, but appear to

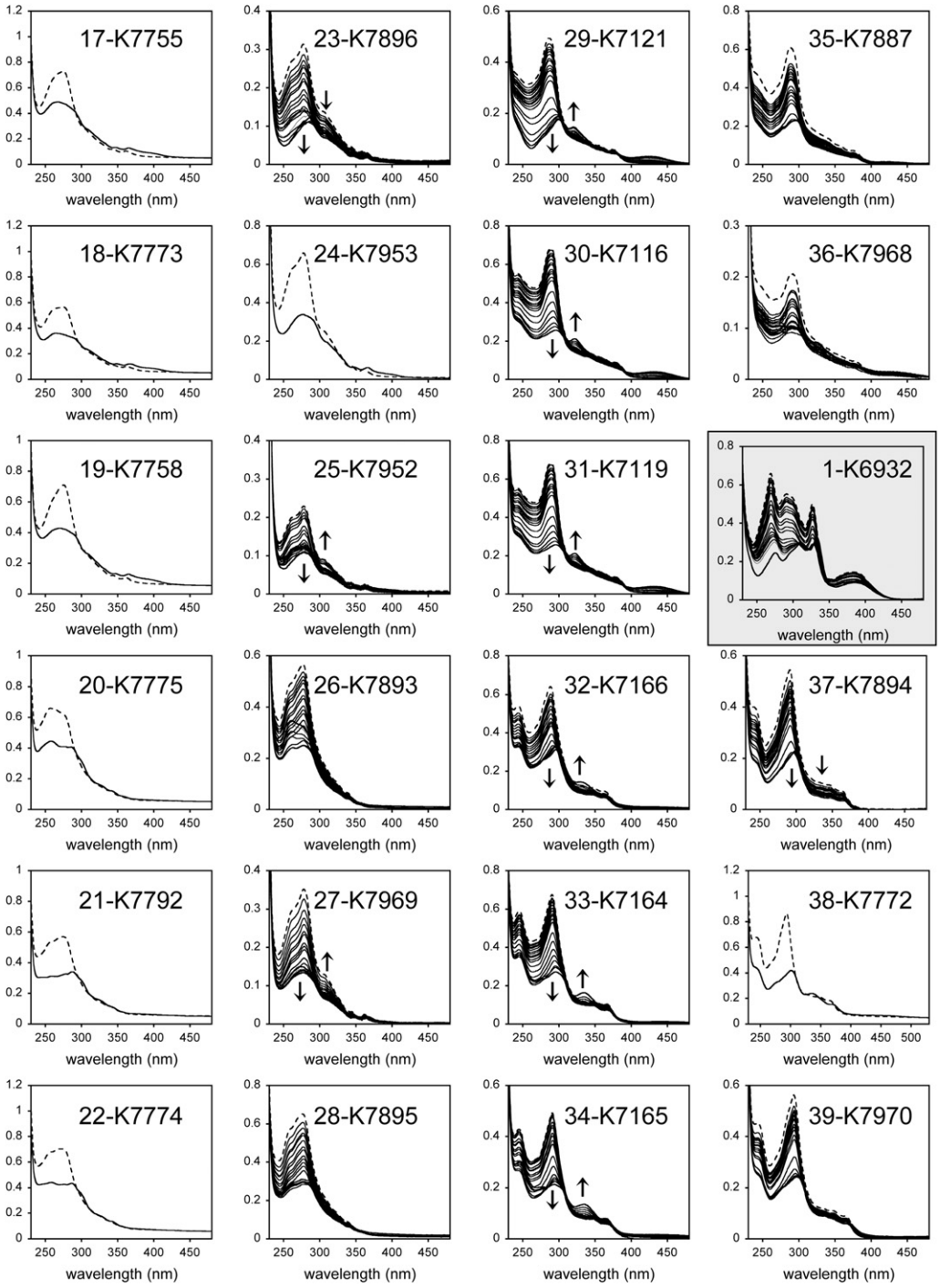


Fig. 2. UV/visible spectrometry. The various indicated compounds (20 μM) were incubated alone (top lanes), with a fixed concentration of CT-DNA (200 μM) or with increasing concentrations of CT-DNA (from 1 to 200 μM, top to bottom). Nitidine 1 (grey box) was used as a reference compound.

be weak substrates for Top1 and Top2 as previously observed for related benzophenanthroline derivatives. It is noteworthy that the more cytotoxic compounds (methylenedioxy-substituted in the A series) do not display efficient DNA intercalation potencies (Figs. 3 and 4) but inhibit CPT-induced Top1-poisoning effects and present common abilities to display specific DNA binding propensities.

In conclusion, from a structure-activity viewpoint, the replacement of the benzenic A ring in the natural BZP alkaloids by a pyridine in the benzophenanthroline and benzophenanthrolinone series resulted in a significant increase in the cytotoxicity associated with a loss of the topoisomerase affinity.

5. Experimental section

5.1. Chemistry

5.1.1. General

All reactions were carried out in a dried glassware under an argon atmosphere. All solvents were purchased with an analytical grade from SDS. CH_2Cl_2 was distilled from CaH_2. Methanol, acetonitrile and DMF were dried over molecular sieves. All other commercially available reagents were used as received. Flash chromatography was performed with silica gel CHROMATOGEL 60

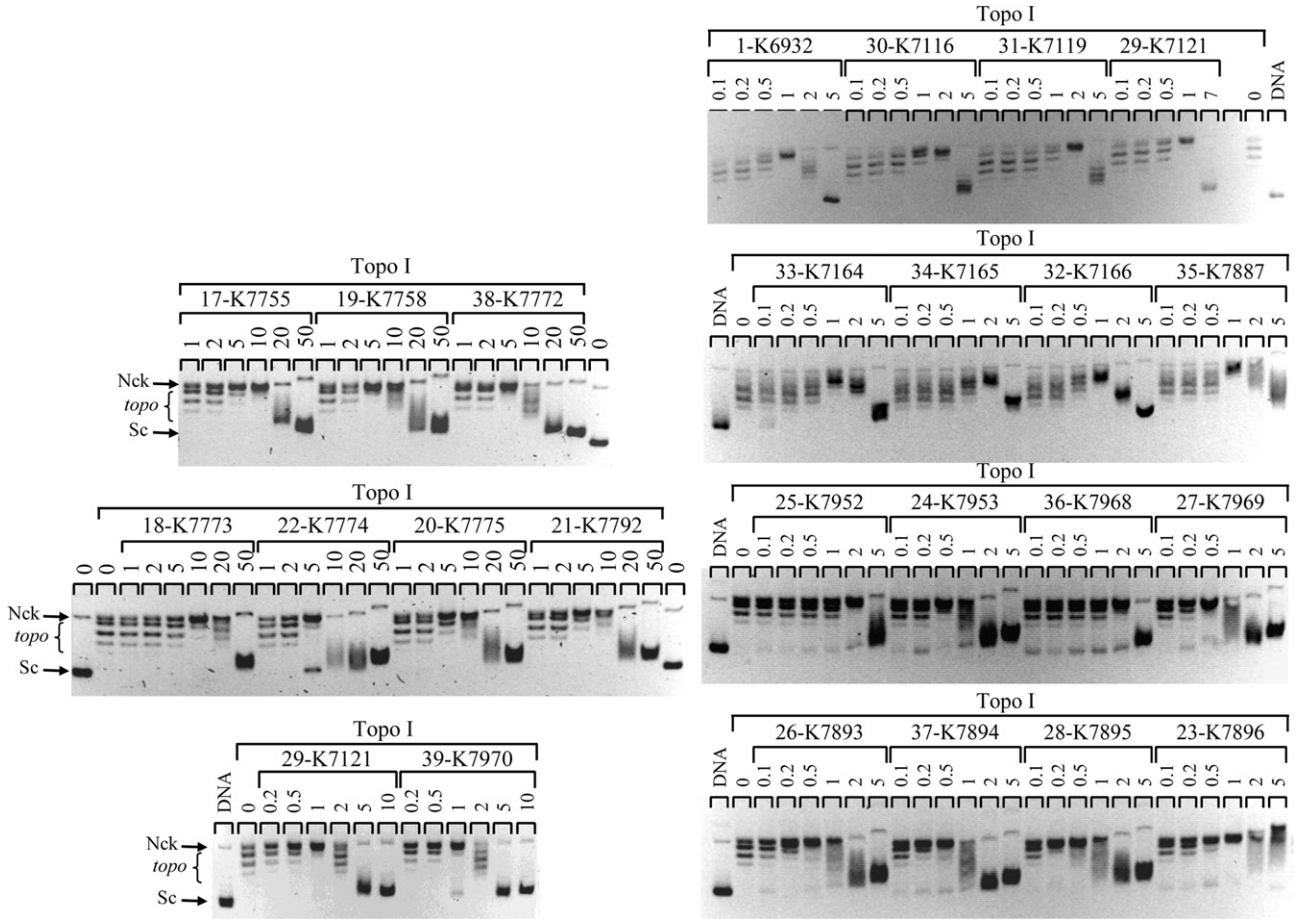


Fig. 3. Topoisomerase 1-induced DNA relaxation. Effect of compounds Y–Y on the relaxation of a supercoiled plasmid DNA by human Top1. Native supercoiled pUC19 vector (130 ng, lanes DNA) was incubated with 4 units of Top1 in the absence (lanes 0) or presence of tested compound at the indicated concentration (μM). Samples were separated by electrophoresis on a 1% agarose gel, which was stained post-electrophoresis with ethidium bromide after DNA migration. Gels were photographed under UV light. Nck, nicked; Sc, supercoiled; Rel, relaxed; Topo, topoisomer products.

(particle size 20–45 μ or 35–70 μ) supplied by SDS. Yield refers to chromatography and spectroscopically pure compounds, unless otherwise noted. ^{1}H NMR spectra were recorded on a BRUCKER AC 300 MHz spectrometer or a BRUCKER AVANCE 400 MHz spectrometer. Chemical shifts are reported in ppm and corrected to δ_H 7.26 for $CDCl_3$ as internal reference, coupling constants (J) in Hz. ^{13}C NMR spectra were recorded on a BRUCKER AC 300 spectrometer at 75 MHz. Chemical shifts are reported in ppm and corrected to δ_C 77.16 for $CDCl_3$ as internal reference. The assignments are based upon 1D and 2D spectra recorded using the following pulse sequences from the Bruker standard pulse program library: DEPT, COSY, HSQC, HMBC and NOESY. Mass spectra were measured with a ZQ 2000 Waters mass spectrometer (ESI). High resolution mass spectra were obtained on a Q-ToF1 ESI mass spectrometer (Waters). Infra-red spectra were recorded on a Nicolet FT-IR spectrometer and wavelengths (υ) are given in cm^{-1}. UV spectra were recorded on a Beckman DU 640 and wavelengths (λ) are given in nm.

5.1.2. General procedure for the preparation of the N5 dialkylaminoalkylbenzo[c]phenantholinone

At 0 °C, to a solution of a benzo[*c*]phenanthrolin-6-one (80 mg), NaI (1.5 equiv.) and the appropriate dialkylaminoalkylchloride hydrochloride (1.1 equiv.) in DMF (20 mL) was added NaH (60% mineral oil suspension, 3 equiv.) in small portions over 5 min. The mixture was allowed to warm to r.t. with stirring for 45 min. Then the flask was transferred into a preheated oil bath (65 °C), and heated at 110 °C for 3 h. After disappearance of the starting material (TLC monitoring), the mixture was cooled to r.t. and quenched by addition of a few drops of water. The solvent was removed under vacuum and the crude product was dissolved in 1 M aqueous HCl solution (50 mL). The aqueous layer was washed with CH_2Cl_2 (3 × 50 mL), basified with a 30% aqueous NaOH solution and extracted with CH_2Cl_2 (6 × 75 mL). The organic layers were combined, dried over $MgSO_4$ and concentrated under vacuum. The residue was purified by chromatography on silica gel (20/45 μ, eluent CH_2Cl_2/MeOH/NH_4OH 95/4.35/0.65 v/v/v).

*5.1.2.1. 5-[2′-(dimethylamino)ethyl]-8,9-dimethoxy benzo[c][1,8] phenanthrolin-6(5H)-one (**17**).* (49% yield) IR (CH_2Cl_2, cm^{-1}) 3403, 2934, 2854, 2778, 2356, 2343, 1731, 1645, 1615, 1591, 1523, 1486, 1455, 1417, 1329, 1274, 1209, 1035, 835, 789, 757, 723. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.29 (s, 1H), 8.84 (d, J = 5.6 Hz, 1H), 8.72 (d, J = 5.6 Hz, 1H), 8.32 (d, J = 8.9 Hz, 1H), 7.85 (d, J = 8.9 Hz, 1H), 7.77 (s, 1H), 7.72 (s, 1H), 4.91 (t, J = 5.9 Hz, 2H), 4.14 (s, 3H), 4.10 (s, 3H), 3.01 (t, J = 5.9 Hz, 2H), 2.50 (s, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.9, 153.0, 151.3, 150.3, 144.2, 137.7, 135.0, 130.4, 128.0, 122.9, 121.4, 120.9, 117.4, 115.1, 104.6, 102.4, 63.7, 57.8, 56.3, 56.2, 45.8 (2C). MS (ES$^+$) m/z 378 [M + H]$^+$. HRMS (ES$^+$) m/z calcd for $C_{22}H_{24}N_3O_3$ [M + H]$^+$ 378.1818, found 378.1807.

*5.1.2.2. 5-[3′-(dimethylamino)propyl]-8,9-dimethoxy benzo[c][1,8] phenanthrolin-6(5H)-one (**18**).* (5% yield) IR (CH_2Cl_2, cm^{-1}) 3400,

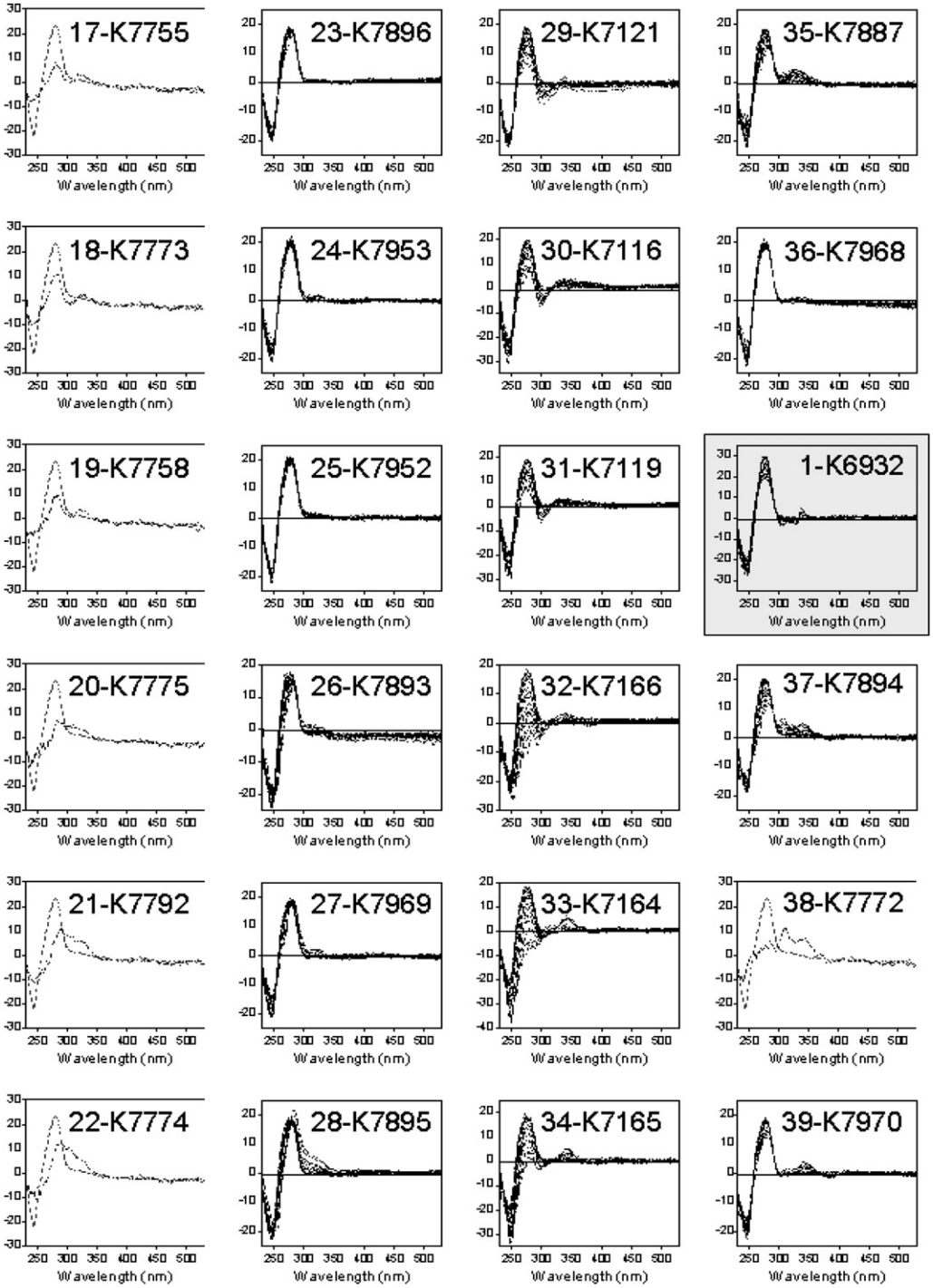


Fig. 4. Circular dichroism. A fixed concentration of CT-DNA vas incubated with he various indicated compounds at a fixed or at increasing concentrations. Nitidine 1 (grey box) was used as a reference compound.

2915, 1646, 1615, 1591, 1523, 1485, 1448, 1426, 1275, 1093, 1034; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.25 (s, 1H), 8.83 (d, J = 5.8 Hz, 1H), 8.66 (d, J = 5.8 Hz, 1H), 8.36 (d, J = 9.0 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.80 (s, 1H), 7.70 (s, 1H), 4.84 (t, J = 6.2 Hz, 2H), 4.14 (s, 3H), 4.11 (s, 3H), 3.02 (t, J = 7.5 Hz, 2H), 2.63 (s, 2 × 3H), 2.48–2.40 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.8, 153.1, 151.0, 150.4, 143.6, 137.4, 135.1, 130.3, 128.0, 122.8, 121.6, 121.1, 117.6, 115.0, 104.3, 102.4, 63.6, 56.6, 56.2 (2C), 44.2 (2C), 25.7; MS (ES^+) m/z 392 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{23}H_{26}N_3O_3$ $[M+H]^+$ 392.1974, found 392.1983; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 196 (3.7), 222 (4.1), 229 (4.1), 281 (4.4), 361 (3.4).

*5.1.2.3. 5-[2′-(diethylamino)ethyl]-8,9dimethoxy benzo[c][1,8]phenanthrolin-6(5H)-one (**19**).* (48% yield) IR (CH_2Cl_2, cm^{-1}) 3379, 2957, 2921, 2851, 1731, 1714, 1592, 1523, 1455, 1423, 1329, 1273, 1209, 1162, 1121, 1092, 1037, 789; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.24 (s, 1H), 8.83 (d, J = 5.8 Hz, 1H), 8.66 (d, J = 5.8 Hz, 1H), 8.33 (d, J = 9.1 Hz, 1H), 7.85 (d, J = 9.1 Hz, 1H), 7.77 (s, 1H), 7.71 (s, 1H), 4.93 (t, J = 6.0 Hz, 2H), 4.13 (s, 3H), 4.07 (s, 3H), 3.22 (t, J = 6.0 Hz, 2H), 2.86 (q, J = 7.2 Hz, 2x2H), 1.21 (t, J = 7.2 Hz, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.8, 152.9, 151.0, 150.3, 143.7, 137.5, 135.0, 130.3, 128.0, 122.7, 121.5, 121.0, 117.6, 115.0, 104.4, 102.4, 63.5, 56.2, 56.1,

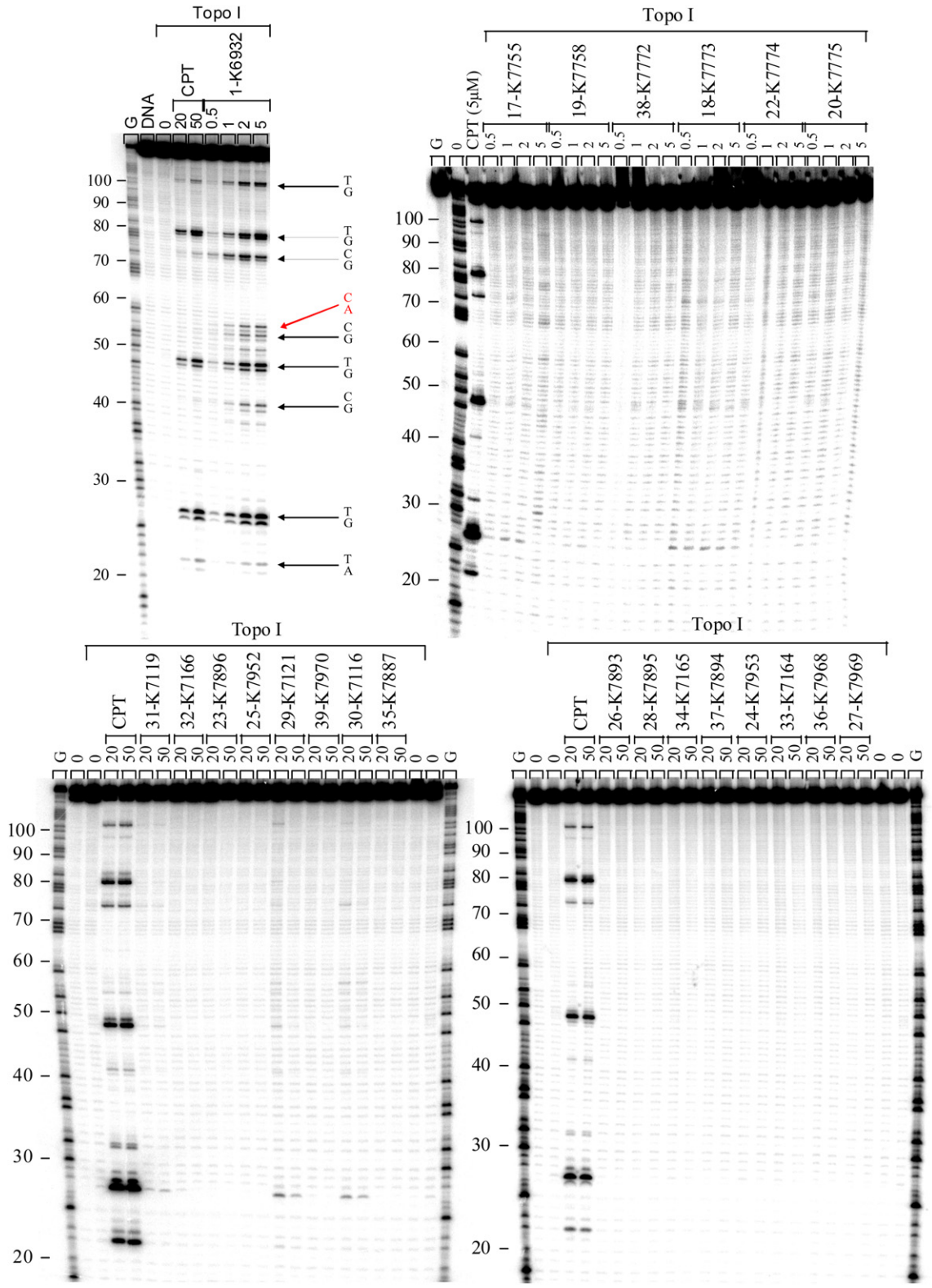


Fig. 5. Topoisomerase 1 poisoning assays. Potential cleavage of the 117-mer DNA fragment by Top1 was studied in the presence of the compounds Y–Y. The 3′-end labelled fragment (0) was incubated in the absence (lane Topol) or presence of the tetracycles at the indicated concentrations (μM). Camptothecin (CPT) was used as a positive control at concentrations of 20–50 μM Top1 cleavage reactions were analyzed on an 8% denaturing polyacrylamide gel, compared to the guanine track labelled (G).

50.9, 47.9 (2C), 11.2 (2C); MS (ES^+) *m/z* 405 $[M + H]^+$; HRMS (ES^+) *m/z* calcd for $C_{24}H_{28}N_3O_3$ $[M + H]^+$ 406.2131, found 406.2112; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 214 (3.8), 222 (4.3), 229 (4.3), 281 (4.6), 342 (3.6), 359 (3.5).

*5.1.2.4. 5-[2′-(dimethylamino)ethyl]-8,9-dimethoxy benzo[c][1,7] phenanthrolin-6(5H)-one (**20**).* (23% yield) IR (CH_2Cl_2, cm^{-1}) 3370, 2943, 1614, 1592, 1525, 1496, 1463, 1426, 1416, 1328, 1272, 1208, 1179, 1116, 1059, 1031, 1001, 820, 758; ^1H NMR (300 MHz, $CDCl_3$)

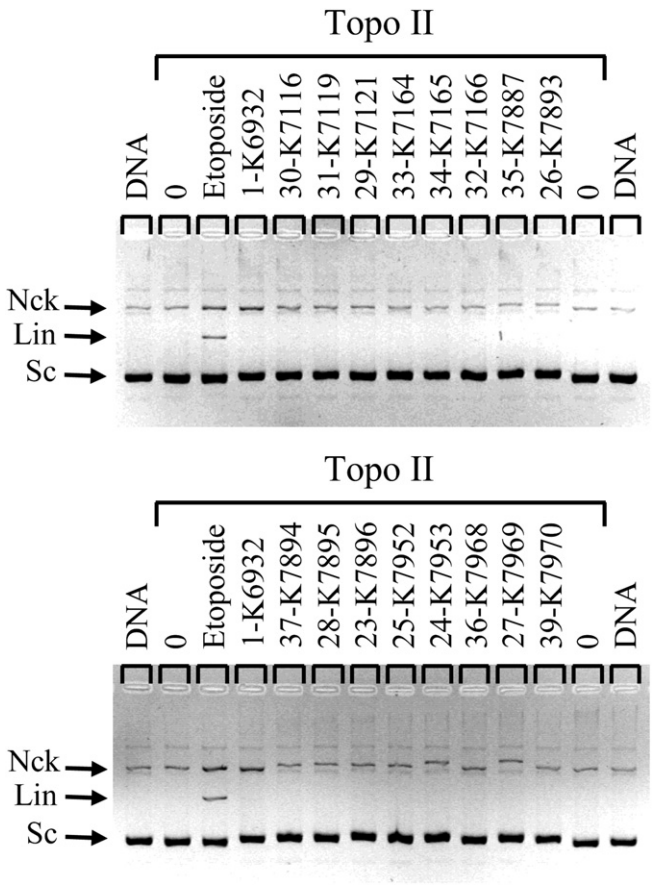


Fig. 6. Topoisomerase 2 poisoning assays. Gel shows the effect of tetracycles Y–Y on the cleavage of a supercoiled plasmid DNA by topoisomerase 2. Native supercoiled pUC19 plasmid DNA (350 ng) (lanes DNA) was incubated with 10 units topoisomerase 2 in the absence (lanes 0) or presence of the indicated drugs at 100 μM, and compared to the reference compound etoposide (50 μM). Reactions were stopped with sodium dodecylsulfate and treatment with proteinase K. DNA samples were separated by electrophoresis on 1% agarose gels containing ethidium bromide (1 μg/mL) and then photographed under UV light. Nck, nicked; Lin, linear; Sc, supercoiled DNA.

δ 9.45 (dd, J = 8.1, 1.8 Hz, 1H), 8.99 (dd, J = 4.1, 1.8 Hz, 1H), 8.53 (dd, J = 9.2, 1.8 Hz, 1H), 8.08 (d, J = 9.2 Hz, 1H), 7.85 (s, 1H), 7.74 (s, 1H), 7.56 (dd, J = 8.1, 4.1 Hz, 1H), 4.91 (t, J = 6.0 Hz, 2H), 4.14 (s, 3H), 4.10 (s, 3H), 2.99 (t, J = 6.0 Hz, 2H), 2.46 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.1, 152.9, 150.3, 149.7, 148.6, 138.8, 133.0, 130.6, 126.6, 125.4, 123.5, 121.2, 118.2, 114.4, 104.5, 102.2, 64.4, 58.2, 56.1 (2C), 46.2 (2C); MS (ES^+) m/z 378 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{22}H_{24}N_3O_3$ $[M + H]^+$ 378.1818, found 378.1818; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 222 (4.68), 282 (4.98), 338 (3.98), 353 (3.65).

*5.1.2.5. 5-[3′-(dimethylamino)propyl]-8,9-dimethoxy benzo[c][1,7] phenanthrolin-6(5H)-one (**21**).* (13% yield) IR (CH_2Cl_2, cm^{-1}) 3369, 2944, 1660, 1614, 1592, 1525, 1496, 1428, 1415, 1332, 1272, 1208, 1178, 1059, 1037, 820, 788; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.47 (ddd, J = 8.3, 1.8, 0.7 Hz, 1H), 8.99 (d, J = 4.3, 1.8 Hz, 1H), 8.53 (d, J = 9.2 Hz, 1H), 8.08 (dd, J = 9.2, 0.7 Hz, 1H), 7.84 (s, 1H), 7.72 (s, 1H), 7.58 (d, J = 8.3, 4.3 Hz, 1H), 4.84 (t, J = 6.5 Hz, 2H), 4.16 (s, 3H), 4.10 (s, 3H), 2.63 (t, J = 7.4 Hz, 2H), 2.35 (s, 2 × 3H), 2.27–2.20 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3, 152.9, 150.3, 149.8, 148.6, 138.9, 133.1, 130.6, 126.6, 125.3, 123.6, 121.1, 118.1, 114.5, 104.3, 102.2, 64.6, 56.9, 56.1 (2C), 45.6 (2C), 27.4; MS (ES^+) m/z 392 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{23}H_{26}N_3O_3$ $[M + H]^+$ 392.1974, found 392.1978; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 222 (4.5), 282 (4.7).

*5.1.2.6. 5-[2′-(diethylamino)ethyl]-8,9-dimethoxy benzo[c][1,7]phenanthrolin-6(5H)-one (**22**).* (35% yield) IR (CH_2Cl_2, cm^{-1}) 3381, 2966, 2925, 1659, 1592, 1524, 1428, 1272, 1116, 1087, 1060, 1037, 786; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.47 (dd, J = 8.4, 1.5 Hz, 1H), 9.00 (dd, J = 4.3, 1.5 Hz, 1H), 8.55 (d, J = 9.3 Hz, 1H), 8.09 (d, J = 9.3 Hz, 1H), 7.86 (s, 1H), 7.74 (s, 1H), 7.59 (dd, J = 8.4, 4.3 Hz, 1H), 4.95 (t, J = 6.1 Hz, 2H), 4.17 (s, 3H), 4.10 (s, 3H), 3.21 (t, J = 6.1 Hz, 2H), 2.86 (q, J = 7.2 Hz, 2 × 2H), 1.23 (t, J = 7.2 Hz, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.1, 153.0, 150.3, 149.8, 148.6, 138.8, 133.0, 130.6, 126.6, 125.5, 123.6, 121.2, 118.2, 114.4, 104.4, 102.2, 64.4, 56.1 (2C), 51.2, 48.1 (2C), 11.9 (2C); MS (ES^+) m/z 405 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{24}H_{28}N_3O_3$ $[M + H]^+$ 406.2131, found 406.2126; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 222 (4.4), 282 (4.6).

*5.1.2.7. 5-[2′-(dimethylamino)ethyl]-8,9-methylene dioxybenzo[c] [1,8]phenanthrolin-6(5H)-one (**23**).* (14% yield) IR (CH_2Cl_2, cm^{-1}) 3363, 2911, 2845, 2763, 1620, 1576, 1489, 1467, 1260, 1195, 1032, 939, 836, 759; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.30 (s, 1H), 8.87 (d, J = 5.7 Hz, 1H), 8.74 (d, J = 5.7 Hz, 1H), 8.31 (d, J = 9.0 Hz, 1H), 7.87 (d, J = 9.0 Hz, 1H), 7.86 (s, 1H), 7.76 (s, 1H), 6.18 (s, 2H), 4.91 (t, J = 5.8 Hz, 2H), 2.99 (t, J = 5.8 Hz, 2H), 2.48 (s, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3, 151.6, 151.3, 148.6, 144.2, 137.9, 135.0, 132.2, 128.1, 122.8, 121.5, 121.2, 117.5, 116.5, 102.7, 102.0, 100.4, 64.5, 58.2, 46.1 (2C); MS (ES^+) m/z 362 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{21}H_{20}N_3O_3$ $[M + H]^+$ 362.1505, found 362.1487; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 281 (4.6), 342 (3.4), 360 (3.4).

*5.1.2.8. 5-[3′-(dimethylamino)propyl]-8,9-methylene dioxybenzo[c] [1,8]phenanthrolin-6(5H)-one (**24**).* (13% yield) IR (CH_2Cl_2, cm^{-1}) 3386, 2946, 2911, 2816, 2764, 1625, 1585, 1493, 1470, 1455, 1332, 1261, 1233, 1197, 1036, 838, 763; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.30 (s, 1H), 8.88 (d, J = 5.7 Hz, 1H), 8.74 (d, J = 5.7 Hz, 1H), 8.31 (d, J = 9.0 Hz, 1H), 7.88 (d, J = 9.0 Hz, 1H), 7.86 (s, 1H), 7.74 (s, 1H), 6.19 (s, 2H), 4.82 (t, J = 6.4 Hz, 2H), 2.62 (t, J = 7.1 Hz, 2H), 2.35 (s, 6H), 2.19 (td, J = 7.1, 6.4 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.4, 151.5, 151.3, 148.6, 144.2, 138.0, 135.0, 132.2, 128.1, 122.7, 121.5, 121.1, 117.5, 116.6, 102.5, 102.0, 100.5, 64.8, 56.8, 45.6 (2C), 27.4; MS (ES^+) m/z 376 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{22}H_{22}N_3O_3$ $[M + H]^+$ 376.1661, found 376.1659; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 215 (4.0), 222 (4.6), 230 (4.3), 281 (4.7), 343 (3.6), 362 (3.5).

*5.1.2.9. 5-[2′-(diethylamino)ethyl]-8,9-methylene dioxybenzo[c][1,8] phenanthrolin-6(5H)-one (**25**).* (22% yield) IR (CH_2Cl_2, cm^{-1}) 3385, 2917, 2840, 1620, 1587, 1470, 1328, 1263, 1195, 1129, 1035; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.31 (s, 1H), 8.89 (d, J = 5.7 Hz, 1H), 8.74 (d, J = 5.7 Hz, 1H), 8.32 (d, J = 9.0 Hz, 1H), 7.90 (d, J = 9.0 Hz, 1H), 7.89 (s, 1H), 7.75 (s, 1H), 6.20 (s, 2H), 4.89 (t, J = 6.3 Hz, 2H), 3.13 (t, J = 6.3 Hz, 2H), 2.79 (q, J = 7.1 Hz, 2x2H), 1.18 (t, J = 7.1 Hz, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.2, 151.7, 151.3, 148.7, 144.3, 138.0, 135.0, 132.4, 128.1, 123.0, 121.6, 121.2, 117.5, 116.6, 102.6, 102.1, 100.6, 64.7, 51.2, 48.0 (2C), 11.8 (2C); MS (ES^+) m/z 390 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{23}H_{24}N_3O_3$ $[M + H]^+$ 390.1818, found 390.1815; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 219 (4.3), 222 (4.3), 228 (4.0), 281 (4.3).

*5.1.2.10. 5-[2′-(dimethylamino)ethyl]-8,9-methylene dioxybenzo[c] [1,7]phenanthrolin-6(5H)-one (**26**).* (17% yield) IR (CH_2Cl_2, cm^{-1}) 3371, 2915, 2854, 2357, 2342, 1658, 1478, 1457, 1257, 1031; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.46 (dd, J = 8.4, 1.4 Hz, 1H), 9.00 (dd, J = 4.3, 1.4 Hz, 1H), 8.48 (d, J = 9.3 Hz, 1H), 8.07 (d, J = 9.3 Hz, 1H), 7.90 (s, 1H), 7.77 (s, 1H), 7.57 (dd, J = 8.4, 4.3 Hz, 1H), 6.19 (s, 2H), 4.92 (t, J = 5.9 Hz, 2H), 3.00 (t, J = 5.9 Hz, 2H), 2.48 (s, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3, 151.6, 150.5, 148.7, 148.2, 139.0, 133.0, 132.6, 126.5, 125.6, 123.7, 121.2, 118.6, 115.8, 102.6, 102.0, 100.3, 64.3, 58.1, 46.0 (2C); MS (ES^+) m/z 362 $[M + H]^+$; HRMS (ES^+) m/z calcd for $C_{21}H_{20}N_3O_3$ $[M + H]^+$ 362.1505, found 362.1492; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 222 (4.2), 239 (4.2), 281 (4.6).

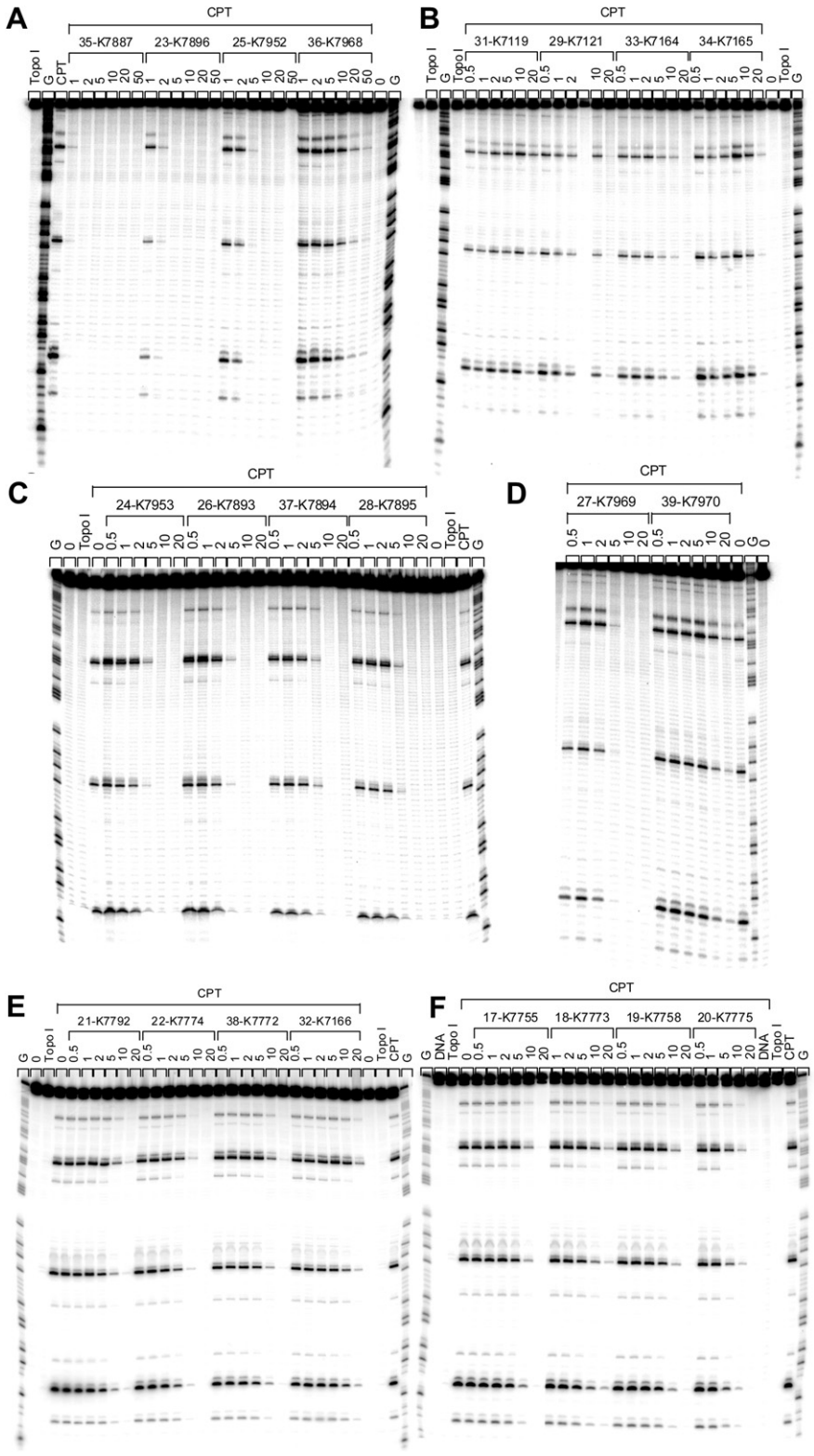


Fig. 7. Inhibition of CPT-induced poisoning of Top1. The 3′-end-labelled 117 bp DNA fragment was incubated alone (0) or with Top1 (4 units), in the presence of Top1-poisoning compound campthotecin (CPT, 20 μM) together with increasing concentrations (μM) of the indicated compounds. Top1 cleavage reactions were analyzed on a 8% denaturing polyacrylamide gel, compared to the guanine track labelled (G).

*5.1.2.11. 5-[3′-(dimethylamino)propyl]-8,9-methylene dioxybenzo[c][1,7]phenanthrolin-6(5H)-one (**27**).* (6% yield) IR (CH_2Cl_2, cm^{-1}) 3374, 2916, 2845, 1606, 1470, 1447, 1250, 1135, 1035; ^1H NMR (300 MHz, $CDCl_3$) δ 9.48 (dd, $J = 8.3$, 1.4 Hz, 1H), 9.01 (dd, $J = 4.3$, 1.4 Hz, 1H), 8.48 (d, $J = 9.2$ Hz, 1H), 8.08 (d, $J = 9.2$ Hz, 1H), 7.91 (s, 1H), 7.75 (s, 1H), 7.59 (dd, $J = 8.3$, 4.3 Hz, 1H), 6.20 (s, 2H), 4.84 (t, $J = 6.3$ Hz, 2H), 2.74 (t, $J = 7.6$ Hz, 2H), 2.43 (s, 2 × 3H), 2.28 (qu, $J = 2$H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.3, 151.6, 150.5, 148.7, 148.2,

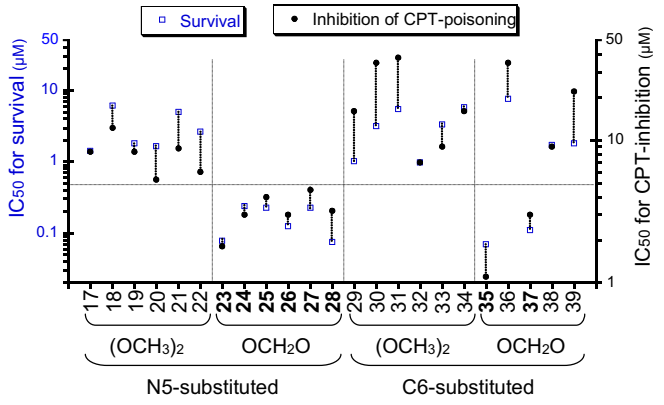


Fig. 8. Correlation analysis between the cytotoxic activity (IC50 for survival on HT-29 cells as determined in Tables 1 and 2) and the inhibition for CPT-induced Top1-poisoning (IC50 calculated from quantification from Fig. 7). Compounds in bold correspond to the most cytotoxic compounds (bellow 0.5 μM as delimited by the horizontal dashed lane).

139.0, 133.1, 132.6, 126.5, 125.6, 123.7, 121.2, 118.6, 115.7, 102.4, 102.0, 100.3, 64.3, 56.7, 45.1, 26.8 (2C); MS (ES^+) *m/z* 376 $[M + H]^+$; $[M + H]^+$; HRMS (ES^+) *m/z* calcd for $C_{22}H_{22}N_3O_3$ $[M + H]^+$ 376.1661, found 376.1657; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 214 (3.9), 222 (4.3), 232 (4.2), 282 (4.5).

*5.1.2.12. 5-[2′-(diethylamino)ethyl]-8,9-methylene dioxybenzo[c][1,7]phenanthrolin-6(5H)-one (**28**).* (17% yield) IR (CH_2Cl_2, cm^{-1}) 3379, 2959, 2910, 2845, 2355, 2333, 1603, 1527, 1467, 1440, 1325, 1293, 1255, 1184, 1124, 1032, 934, 825; ^1H NMR (300 MHz, $CDCl_3$) δ 9.44 (dd, J = 8.3, 1.5 Hz, 1H), 9.00 (dd, J = 4.3, 1.5 Hz, 1H), 8.43 (d, J = 9.2 Hz, 1H), 8.05 (d, J = 9.2 Hz, 1H), 7.85 (s, 1H), 7.70 (s, 1H), 7.57 (dd, J = 8.3, 4.3 Hz, 1H), 6.18 (s, 2H), 4.88 (t, J = 6.2 Hz, 2H), 3.14 (t, J = 6.2 Hz 2H), 2.80 (q, J = 7.1 Hz, 2x2H), 1.18 (t, J = 7.1 Hz, 2 × 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.2, 151.5, 150.4, 148.7, 148.1, 138.9, 133.0, 132.5, 126.5, 125.6, 123.6, 121.1, 118.5, 115.7, 102.4, 101.9, 100.2, 64.4, 51.3, 48.0 (2C), 11.9 (2C); MS (ES^+) *m/z* 390 $[M + H]^+$; HRMS (ES^+) *m/z* calcd for $C_{23}H_{23}N_3O_3Na$ $[M + Na]^+$ 412.1637, found 412.1656; U.V. λ_{max} (nm) (logϵ) (CH_2Cl_2) 239 (4.5), 282 (4.7).

5.1.3. General procedure for the C6 chlorination of benzo[c]phenanthroline core

Benzo[*c*]phenanthroline dissolved in $PhPOCl_3$ was heated at 160 °C for 3 h. Then the reaction mixture was cooled to rt and poured into ice. pH was adjusted to 8–9 with an aqueous ammonia solution and the solution was left for 2 h. The precipitate formed was then filtered, washed with water and dried. Purification by flash chromatography on silica gel (20/45 μ, eluent CH_2Cl_2/MeOH 95/5 v/v) afforded the corresponding chloro compound as a white-grey solid.

5.1.3.1. 6-Chloro-8,9-dimethoxybenzo[c][1,7] phenanthroline. (52 % yield). IR 1647, 1615, 1493 (υ CN), 1258, 1150, 732 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.42 (dd, 1H, J = 8.0 Hz, 1 Hz), 9.01 (dd, 1H, J = 4.0 Hz, 1 Hz), 8.45 (d, 1H, J = 9.0 Hz), 8.15 (d, 1H, J = 9.0 Hz), 7.74 (s, 1H), 7.64 (s, 1H), 7.60 (dd, 1H, J = 8.0 Hz, 4.0 Hz), 4.13 (s, OCH_3), 4.09 (s, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.8, 150.8, 149.7, 148.3, 139.4, 133.1, 130.5, 128.7, 126.7, 123.2, 122.1, 120.9, 120.7, 106.5, 102.2, 56.4, 56.3. MS (IC, NH_3) *m/z* 325, 327 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{18}H_{13}ClN_2O_2$ $[M + H]^+$ 324.0666, found 324.0670.

5.1.3.2. 6-Chloro-8,9-dimethoxybenzo[c][1,8] phenanthroline. (54% yield). IR 2927, 2844 (υ CN), 1655, 1620, 1521, 1487, 1165, 1016 (υ CCl); 973 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.38 (s, 1H), 9.01 (d, 1H, J = 6.0 Hz), 8.84 (d, 1H, J = 6.0 Hz), 8.50 (d, 1H, J = 9.0 Hz), 8.09 (d, 1H, J = 9.0 Hz), 7.97 (s, 1H), 7.86 (s, 1H), 4.23 (s, OCH_3), 4.19 (s, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.7, 151.1, 149.7, 145.0, 138.4, 135.2, 132.4, 130.8, 125.8, 123.3, 120.7, 106.5, 102.3, 56.4, 56.3. MS (CI, NH_3) *m/z* 325, 327 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{18}H_{13}ClN_2O_2$ $[M + H]^+$ 324.0666, found 324.0668.

5.1.3.3. 6-Chloro-8,9-methylenedioxybenzo[c][1,7] phenanthroline. (74% yield). IR 2250 (ν CN), 1477, 1736, 1470, 1296, 1036 (ν C–Cl) 956, 826 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.60 (dd, 1H, J = 8.0 Hz, 1 Hz), 9.05 (d, 1H, J = 4.0 Hz, 1 Hz), 8.60 (d, 1H, J = 9.0 Hz), 8.22 (d, 1H, J = 9.0 Hz), 7.99 (s, 1H); 7.82 (s, 1H), 7.68 (dd, 1H, J = 8.0 Hz, 4.0 Hz), 6,24 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.1, 149.9, 147.5 (2C), 142.3, 135.7, 133.7, 128.2, 126.5, 127.2, 123.8, 121.8, 121.1, 121.0, 104.0, 102.4, 99.9. MS (CI, NH_3) *m/z* 309, 311 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{17}H_9ClN_2O_2$ $[M + H]^+$ 308.0353, found 308.0351.

5.1.3.4. 6-Chloro-8,9-methylenedioxybenzo[c][1,8] phenanthroline. (57% yield). IR 2912, 2334 (ν CN), 1477, 1263, 1042 (ν C–Cl), 957 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.35 (s, 1H), 8.74 (d, 1H, J = 6.0 Hz), 8.32 (d, 1H, J = 9.0 Hz), 8.11 (d, 1H, J = 6.0 Hz), 8.01 (s, 1H), 7.87 (d, 1H, J = 9.0 Hz), 7.74 (s, 1H), 4.17 (s, OCH_3), 4,10 (s, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.7, 152.5 (2C), 150.7, 144.7, 131.9, 131.3, 129.2, 128.2, 125.6, 125.6, 120.8, 118.9, 117.1, 104.3, 102.2, 100.0. MS (IC, NH_3) *m/z* 309, 311 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{17}H_9ClN_2O_2$ $[M + H]^+$ 308.0353, found 308.0357.

5.1.4. General procedure for the preparation of the C6 dialkylaminoalkylbenzo[c]phenanthroline

The chlorinated benzo[*c*]phenanthroline was refluxed in an excess of the appropriate dialkylaminoalkylamine. After the reaction was completed (TLC monitoring) the excess of dialkylaminoalkylamine was removed under vacuum. The residue was dissolved in CH_2Cl_2 and washed successively with an aqueous NaOH solution (10% m/v) and water. The organic layer was dried over MgSO4, filtered and concentrated in vacuo. The residue was purified by chromatography on silica gel (20/45 μ, CH_2Cl_2/MeOH/NH_4OH 90/8.75/1.25 v/v/v) to afford the corresponding compound.

*5.1.4.1. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-dimethoxybenzo[c][1,8]phenanthroline (**29**).* Yield 56%. IR 3354 (υ NH), 2923 (υ CH), 2361, 1588, 1543, 1482, 1272 (υ CN), 1209, 1035 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.26 (s, 1H), 8.92 (d, 1H, J = 6.0 Hz), 8.68 (d, 1H, J = 6.0 Hz), 8.32 (d, 1H, J = 9.0 Hz), 7.84 (s, 1H), 7.74 (d, 1H, J = 9.0 Hz), 7.40 (s, 1H), 6.32 (bs, NH), 4.14 (s, OCH_3), 4.10 (s, OCH_3), 3.98 (q, 2H, J = 6.0 Hz), 2.83 (t, 2H, J = 6.0 Hz), 2.43 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.0, 152.1, 151.2, 149.9, 143.6, 139.5, 134.9, 129.1, 128.4, 121.5, 120.4, 118.5, 117.6, 114.2, 103.1, 58.3, 56.3, 56.0, 45.3, 45.1, 39.0. MS (ES^+) *m/z* : 377 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{23}H_{24}N_4O_2$ $[M + H]^+$ 377.1899, found 377.1903.

*5.1.4.2. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-dimethoxybenzo[c][1,8]phenanthroline (**30**).* Yield 87%. IR 3307 (υ NH), 2927 (υ CH), 2356, 1619 (δ NH), 1588, 1545, 1482, 1272 (υ CN), 1209, 1028 cm^{-1} ^1H NMR (400 Hz, $CDCl_3$) δ 9.21 (s, 1H), 8.90 (d, 1H, J = 6.0 Hz), 8.65 (d, 1H, J = 6.0 Hz), 8.20 (d, 1H, J = 9.0 Hz), 7.75 (bs, NH), 7.70 (s, 1H), 7.63 (d, 1H, J = 9.0 Hz), 7.12 (s, 1H), 4.07 (s, 3H), 4.00 (s, 3H), 3.90 (td, 2H, J = 6.0 Hz, 1.0 Hz), 2.59 (t, 2H, J = 6.0 Hz), 2.35 (s, 6H), 2.01–1.93 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.4, 151.8, 151.1, 149.7, 143.3, 139.8, 134.8, 129.0, 128.4, 121.5, 119.8, 118.1, 117.8, 114.3, 103.1, 102.8, 59.9, 56.0, 55.8, 45.7 (2C) 42.6, 25.1. MS (ES^+) *m/z* 391 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{23}H_{26}N_4O_2$ $[M + H]^+$ 391.2056, found 391.2064.

*5.1.4.3. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-dimethoxybenzo[c][1,8]phenanthroline (**31**).* Yield 68%. IR 3346 (υ NH), 2966 (υ CH),

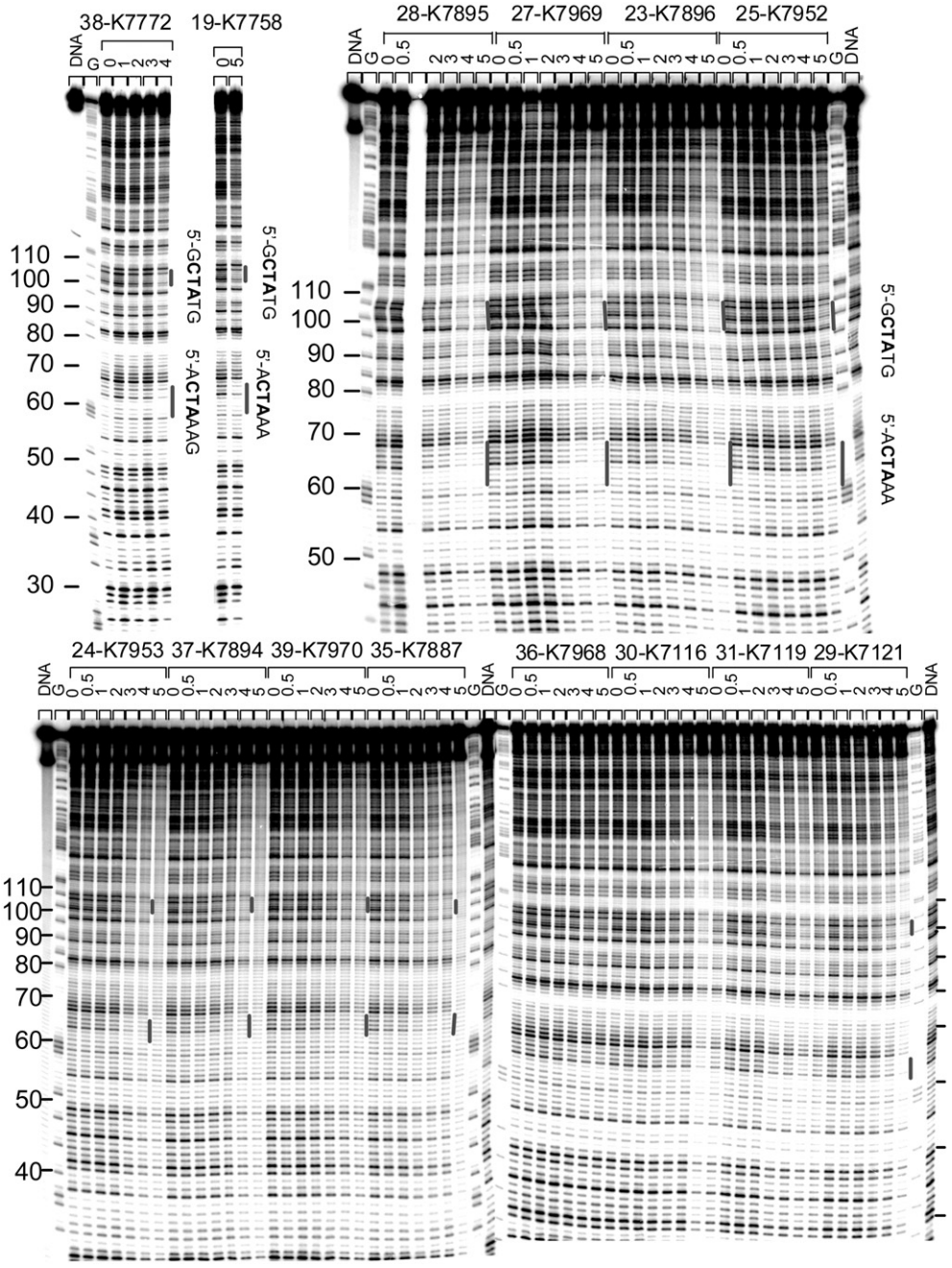


Fig. 9. Sequence-specific DNA binding. Appropriate concentrations of the various benzophenanthrolines were incubated with the 265 bp 3′-end-radio-labelled DNA fragment prior to DNA digestion using DNase I (0.01 unit/ml). The digestion reaction was stopped by freeze drying and lyophilisation of the samples. DNA samples were separated by electrophoresis on a 8% denaturing polyacrylamide gel. Each resolved band on the autoradiograph was assigned to a particular bond within the DNA fragment from comparison with its position relative to the guanines sequencing standard (G-track).

1617 (δ NH), 1588, 1544, 1482, 1422, 1385, 1273 (υ CN), 1209, 1164, 1033, 838, 792 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.23 (s, 1H), 8.91 (d, 1H, $J = 6.0$ Hz), 8.67 (d, 1H, $J = 6.0$ Hz), 8.25 (d, 1H, $J = 9.0$ Hz), 7.76 (bs, 2H), 7.68 (d, 1H, $J = 9.0$ Hz), 7.21 (s, 1H), 4.10 (s, 3H), 4.03 (s, 3H), 3.95–3.94 (m, 2H), 2.72 (t, 2H, $J = 6.0$ Hz), 2.66 (q, 4H, $J = 6.0$ Hz), 2.00–1.98 (m, 2H), 1.09 (t, 6H, $J = 6.0$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.3, 151.9, 151.1, 149.7, 143.4, 139.8, 134.8, 129.1, 128.4, 121.5, 119.9, 118.1, 117.8, 114.3, 103.1 (2C) 56.1, 56.0, 52.8, 47.4 (2C), 43.0, 24.9, 11.6 (2C). MS (ES^+) m/z 419 $[M + H]^+$. HRMS (ES^+) m/z calcd for $C_{25}H_{30}N_4O_2$ $[M + H]^+$ 419.2369, found 419.2401.

*5.1.4.4. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-dimethoxybenzo [c][1,7]phenanthroline (**32**).* Yield 48%. IR 3345 (υ NH), 2926 (υ CH), 2854, 1614 (δ NH), 1587, 1525, 1494, 1463, 1429, 1380, 1270 (υ CN), 1208, 1059, 819 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.50 (d, 1H, $J = 8.0$ Hz, 1 Hz), 8.96 (dd, 1H, $J = 6.0$ Hz, 1.0 Hz), 8.48 (d, 1H, $J = 9.0$ Hz), 7.94 (d, 1H, $J = 9.0$ Hz), 7.84 (s, 1H), 7.52 (dd, 1H, $J = 8.0$ Hz, 6.0 Hz), 7.32 (s, 1H), 6.21 (bs, NH), 4.14 (s, OCH_3), 4.09 (s, OCH_3), 3.97 (q, 2H, $J = 6.0$ Hz), 2.83 (t, 2H, $J = 6.0$ Hz), 2.41 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.0, 152.2, 150.0, 149.5, 148.9, 140.4, 133.1, 129.4, 126.3, 123.7, 123.2, 120.6, 116.0, 113.4, 103.0, 102.9, 58.3,

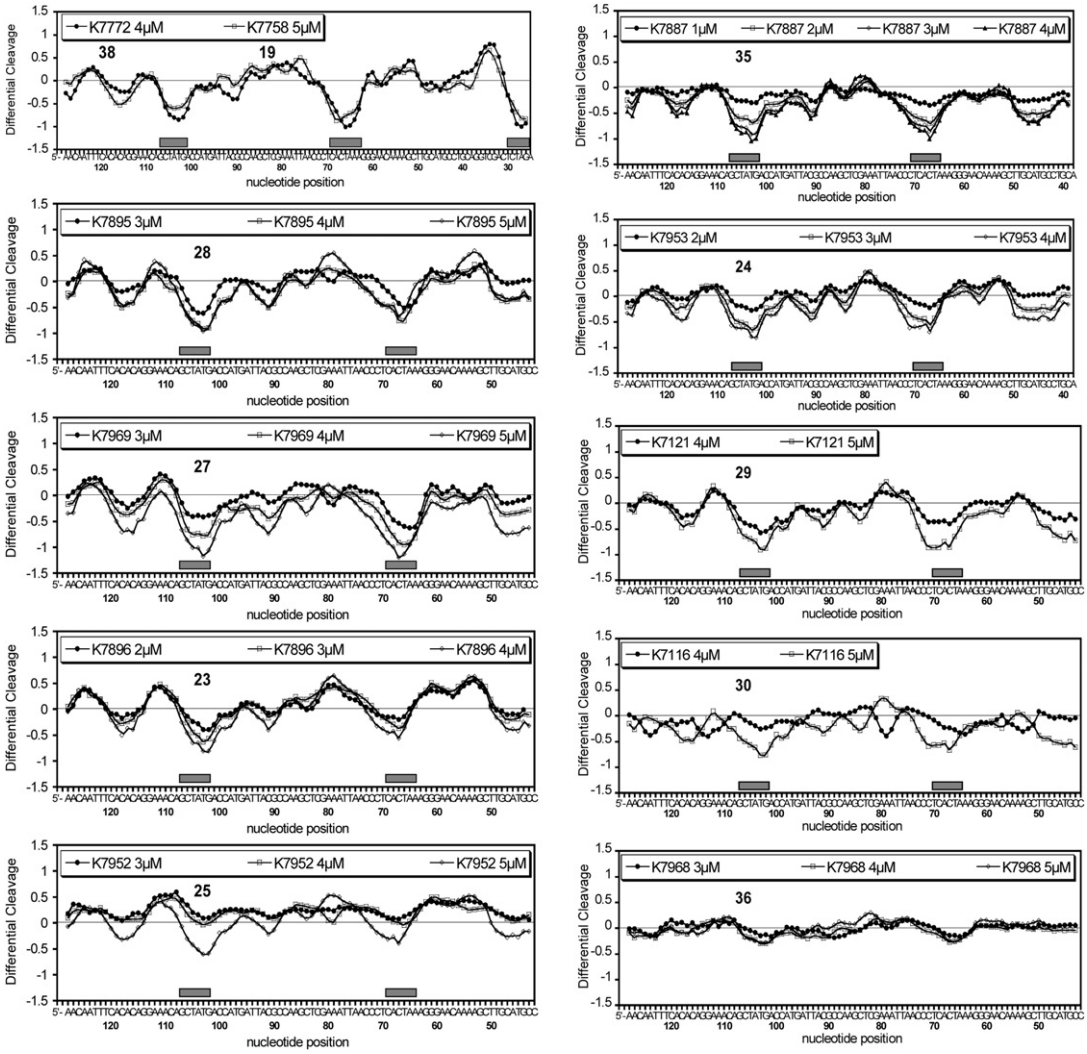


Fig. 10. Sequence-specific DNA binding. Densitometric analysis for each compounds were obtained from quantification of the band inhibition from Fig. 9. Grey boxes correspond to the portions of DNA that are protected by the evaluated compounds from DNase I cleavage.

56.3, 56.0, 45.3 (2C), 39.0. MS (ES^+) *m/z* 377 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{23}H_{24}N_4O_2$ $[M + H]^+$ 377.1899, found 377.1906.

*5.1.4.5. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-dimethoxybenzo [c][1,7]phenanthroline (**33**).* Yield 54%. IR 3304 (υ NH), 2932 (υ CH), 2849, 2765, 2357, 1611 (δ NH), 1587, 1548, 1494, 1428, 1382, 1271 (υ CN), 1208, 1179, 1058, 818, 790 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.51 (dd, 1H, $J = 9.0$ Hz, 1 Hz), 8.94 (dd, 1H, $J = 6.0$ Hz, 1 Hz), 8.43 (d, 1H, $J = 10.0$ Hz), 7.91 (d, 1H, $J = 10.0$ Hz), 7.86 (bs, NH), 7.81 (s, 1H), 7.52 (dd, 1H, $J = 9.0$ Hz, 6.0 Hz), 7.16 (s, 1H), 4.12 (s, OCH_3), 4.03 (s, OCH_3), 3.95 (q, 2H, $J = 6.0$ Hz), 2.61 (t, 2H, $J = 6.0$ Hz), 2.39 (s, 6H), 2.02–1.96 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.4, 151.9, 149.9, 149.3, 148.9, 140.9, 133.3, 129.3, 126.4, 123.8, 122.6, 120.5, 115.5, 113.5, 102.9, 102.7, 59.9, 56.0, 55.8, 45.7 (2C), 42.7, 25.1. MS (ES^+) *m/z* 391 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{24}H_{26}N_4O_2$ $[M + H]^+$ 391.2056, found 391.2072.

*5.1.4.6. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-dimethoxybenzo [c][1,7]phenanthroline (**34**).* Yield 55%. IR 3345 (υ NH), 2965 (υ CH), 2928, 2845, 1587, 1548, 1494, 1429, 1381, 1272 (υ CN), 1209, 1058, 814, 787 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.53 (dd, 1H, $J = 8.0$ Hz, 1.0 Hz), 8.95 (dd, 1H, $J = 6.0$ Hz, 1.0 Hz), 8.44 (d, 1H, $J = 9.0$ Hz), 7.91 (d, 1H, $J = 9.0$ Hz), 7.81 (bs, 2H), 7.51 (dd, 1H, $J = 8.0$ Hz, 6.0 Hz), 7.18 (s, 1H), 4.11 (s, 3H), 4.01 (s, 3H), 3.94 (td, 2H, $J = 6.0$ Hz, 1.0 Hz), 2.71 (t, 2H, $J = 6.0$ Hz), 2.65 (q, 4H, $J = 8.0$ Hz), 1.99–1.95 (m, 2H), 1.16 (t, 6H, $J = 8.0$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.4, 152.0, 149.9, 149.2, 148.9, 140.9, 133.3, 129.5, 126.4, 123.8, 122.6, 120.5, 115.5, 113.6, 103.0, 102.9, 56.1, 56.0, 53.1, 47.5 (2C), 43.3, 25.0, 11.8 (2C). MS (ES^+) *m/z* 419 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{25}H_{30}N_4O_2$ $[M + H]^+$ 419.2369, found 419.2401.

*5.1.4.7. 6-[(2′-(Dimethylamino)ethyl)amino]-8,9-methylene dioxybenzo[c][1,8]phenanthroline (**35**).* Yield 14%. IR 3358 (υ NH), 2917, 2779, 1626 (υ CO), 1580 (δ NH), 1538, 1504, 1470, 1378 (υ CN), 1305, 1263, 1230, 1040 (υ OCO), 840, 758 cm^{-1} ^1H NMR (400 MHz, $CDCl_3$) δ 9.25 (s, 1H), 8.91 (d, 1H, $J = 5.7$ Hz), 8.69 (d, 1H, $J = 5.7$ Hz), 8.24 (d, 1H, $J = 9.0$ Hz), 7.85 (s, 1H), 7.74 (d, 1H, $J = 9.0$ Hz), 7.37 (s, 1H), 6.18 (bs, NH), 6.13 (s, 2H), 3.93 (q, 2H, $J = 5.5$ Hz), 2.79 (t, 2H, $J = 5.5$ Hz), 2.40 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.1, 151.2, 150.7, 148.4, 143.7, 139.6, 134.8, 131.0, 128.4, 121.6, 120.5, 118.8, 117.6, 115.3, 102.0, 101.0, 100.6, 58.0, 45.2 (2C), 38.9. MS (ES^+) *m/z* 361 $[M + H]^+$. HRMS (ES^+) *m/z* calcd for $C_{21}H_{20}N_4O_2$ $[M + H]^+$ 361.1586, found 361.1602.

*5.1.4.8. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-methylenedioxybenzo[c][1,8]phenanthroline (**36**).* Yield 5%. ^1H NMR (400 MHz, $CDCl_3$) δ 9.26 (s, 1H), 8.92 (d, 1H, $J = 5.6$ Hz), 8.69 (d, 1H, $J = 5.6$ Hz),

8.29 (d, 1H, J = 9.0 Hz), 7.91 (s, 1H), 7.87 (bs, NH), 7.73 (d, 1H, J = 9.0 Hz), 7.35 (s, 1H), 6.18 (s, 2H), 4.05–3.96 (m, 2H), 2.74 (t, 2H, J = 6.0 Hz), 2.49 (s, 6H), 2.11–2.09 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.6, 151.2, 150.7, 148.5, 143.6, 139.5, 134.7, 131.0, 128.6, 121.7, 120.2, 118.6, 117.6, 115.6, 101.9, 101.0 (2C), 57.6, 44.6 (2C), 41, 24.5. MS (ES^+) m/z 375 $[M + H]^+$. HRMS (ES^+) m/z calcd for $C_{22}H_{22}N_4O_2$ $[M + H]^+$ 375.1743, found 375.1789.

*5.1.4.9. 6-[(2′-(Dimethylamino)ethyl)amino]-8,9-methylene dioxybenzo[c][1,7]phenanthroline (**37**).* Yield 17%. IR 3347 (υ NH), 2917, 2840, 2356, 2337, 1625 (υ CO), 1581 (δ NH), 1547, 1498, 1474, 1450, 1430, 1369 (υ CN), 1120, 1044 (υ OCO) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.51 (dd, 1H, J = 8.0 Hz, 1.5 Hz), 8.97 (dd, 1H, J = 4.3 Hz, 1.5 Hz), 8.42 (d, 1H, J = 9.0 Hz), 7.93 (d, 1H, J = 9.0 Hz), 7.90 (s, 1H), 7.52 (dd, 1H, J = 8.0 Hz, 4.3 Hz), 7.40 (s, 1H), 6.19 (bs, NH), 6.16 (s, 2H), 3.94 (q, 2H, J = 5.7 Hz), 2.81 (t, 2H, J = 5.7 Hz), 2.41 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.1150.8, 150.2, 149.0, 148.0, 140.6, 133.1, 131.3, 126.2, 123.9, 123.4, 120.7, 116.4, 114.7, 101.9, 100.9, 100.7, 58.1, 45.1 (2C), 38.6. MS (ES^+) m/z 361 $[M + H]^+$. HRMS (ES^+) m/z calcd for $C_{21}H_{20}N_4O_2$ $[M + H]^+$ 361.1586, found 361.1590.

*5.1.4.10. 6-[(3′-(Dimethylamino)propyl)amino]-8,9-methylenedioxybenzo[c][1,7]phenanthroline (**38**).* Yield 17%. IR 3286 (υ NH), 2911, 2846, 2347, 1622 (υ CO), 1601, 1582 (δ NH), 1544, 1539, 1473, 1449, 1429, 1366 (υ CN), 1258, 1238, 1045 (υ OCO) cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.51 (dd, 1H, J = 8.3 Hz, 1.5 Hz), 8.95 (dd, 1H, J = 4.3 Hz, 1.5 Hz), 8.37 (d, 1H, J = 9.1 Hz), 7.89 (d, 1H, J = 9.1 Hz), 7.84 (s, 1H),, 7.80 (bs, NH), 7.52 (dd, 1H, J = 8.3 Hz, 4.3 Hz), 7.16 (s, 1H), 6.14 (s, 2H), 3.92 (q, 2H, J = 5.7 Hz), 2.63 (t, 2H, J = 5.7 Hz), 2.41 (s, 6H), 2.08–2.06 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.7, 150.5, 150.1, 149.0, 147.9, 141.1, 133.3, 131.2, 126.3, 123.9, 122.8, 120.5, 115.9, 114.9, 101.7, 100.9, 100.3, 59.9, 45.6 (2C) 42.8, 25.2. MS (ES^+) m/z 375 $[M + H]^+$. HRMS (ES^+) m/z calcd for $C_{22}H_{22}N_4O_2$ $[M + H]^+$ 375.1743, found 375.1795.

*5.1.4.11. 6-[(3′-(Diethylamino)propyl)amino]-8,9-methylene dioxybenzo[c][1,7]phenanthroline (**39**).* Yield 5%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.52 (dd, 1H, J = 8.3 Hz, 1.7 Hz), 8.95 (dd, 1H, J = 4.3 Hz, 1.7 Hz), 8.40 (d, 1H, J = 9.1 Hz), 8.09 (bs, NH), 7.90 (s, 1H), 7.89 (d, 1H, J = 9.1 Hz), 7.52 (dd, 1H, J = 8.3 Hz, 4.3 Hz), 7.38 (s, 1H), 6.16 (s, 2H), 4.02–3.93 (m, 2H), 2.83–2.73 (m, 6H), 2.07–2.05 (m, 2H), 1.18 (t, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.4, 150.6, 150.1, 149.0, 148.3, 141.0, 133.3, 131.2, 126.4, 124.0, 122.8, 120.5, 116.0, 114.8, 101.8, 100.9, 100.8, 49.7, 46.8 (2C) 46.0, 24.5, 11.2 (2C). MS (ES^+) m/z 403 $[M + H]^+$. HRMS (ES^+) m/z calcd for $C_{24}H_{26}N_4O_2$ $[M + H]^+$ 403.2056, found 403.2092.

5.2. Biology

5.2.1. Cytotoxicity (Tables 1 and 2)

HT-29 colon carcinoma cells were grown as specified and the cytotoxicity was assessed as previously described [13,14].

5.2.2. Cell cycle analysis

For flow cytometric analyses of DNA content were obtained as described in Lemster et al. [13], with the following modifications. Cells in exponential growth phase (300,000 per well) were plated for 24 h before being treated with graded concentrations (0.1, 1 and 2.5 μM) of the various compounds for 48 h. Cells were then collected and fixed as described and analyzed on a Becton Dickinson FACScan flow cytometer using the LYSYS II software. Excitation and emission wavelengths for PI were 488 nm and 620 nm (channel Fl-3), respectively. Each experiment was performed in duplicate.

5.2.3. Topoisomerase 1-induced DNA relaxation assay (Fig. 1)

The experiments were conducted as presented elsewhere [15]. Supercoiled pUC19 plasmid DNA (130 ng) was incubated with 4 units of human recombinant topoisomerase 1 (TopoGen) at 37 °C for 45 min in 20 mL of relaxation buffer (50 mM Tris pH 7.8, 50 mM KCl, 10 mM $MgCl_2$, 1 mMDTT,1 mM EDTA, 1 mM ATP) in the presence of graded concentrations (from 0.1 to 5 μM) of the tested compounds. The reactions were stopped by the addition of SDS (0.25%) and proteinase K (250 mg/mL) followed by an incubation of 30 min at 50 °C. Then 3 mL of the electrophoresis dye mixture was added to each DNA samples and loaded on a 1% agarose gel. After resolution at room temperature for 2 h at 120 V gels run were stained using an ethidium bromide containing bath, washed and photographed under UV light.

5.2.4. Topoisomerase 1 inhibition assays

Agarose gel experiments were performed as specified in 5.2.3., unless the DNA samples were run on an ethidium bromide containing agarose gel (1%) in the same condition as above, before they were washed and photographed under UV light. In order to identify the sequence selectivity of the cleavage sites induced by topoisomerase I poisoning effect, the localisation of DNA cleavage sites that could be generated with the various tested compounds was addressed using a 3′-end-labelled 117 base pairs DNA fragment as already published with the following modifications [16]. The cleavage reactions to identify DNA poisoning effect were conducted in the presence of CPT (as a positive reference) or increasing concentrations of the tested drugs; whereas gels performed to compete for CPT poisoning inhibition were conducted in the presence of a fixed concentration of CPT (20 μM) together with increasing concentration of the tested drugs at the concentrations specified in the figures, then stopped and treated as published elsewhere [16].

5.2.5. Topoisomerase 2 inhibition assays (Fig. 2B)

Supercoiled pUC19 plasmid DNA (350 ng) was incubated with 50 μM of the various tetracyclic derivatives or etoposide, as control, prior to the addition of 10 units of human topoisomerase 2 (TopoGen) at 37 °C for 45 min in the appropriate cleavage buffer. SDS (0.25%) and proteinase K (250 mg/mL) were then added to stop the reaction during 30 min at 50 °C. DNA samples were loaded on 1% agarose gels containing ethidium bromide for 2 h at 120 V in TBE buffer. After migration, gels were washed and photographed under UV light.

5.2.6. DNase I footprinting (Fig. 3)

Experiments were performed as previously described and using a 265 bp 30-end-labelled DNA fragment obtained from double digestion of the pBS plasmid (Stratagene, La Jolla, CA) at *EcoRI* and *PvuII* restriction sites followed by radiolabelling of one strand by incorporation of α-[^{32}P]-dATP (3000 Ci/mmol, GE Healthcare, Buckinghamshire, England) at the *EcoRI* restriction site using AMV reverse transcriptase (Ozyme, France). Appropriate concentrations of the various substituted tetracycles were incubated with the 265-bp radio-labelled DNA fragment for 15 min at 37 °C to ensure equilibrium prior to DNA digestion using DNase I (0.01 unit/ml) in 20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$ (pH 7.3), for 3 min. The digestion reaction was stopped by freeze drying and lyophilisation of the samples. The DNA pellets were dissolved in 5 mL of denaturing loading buffer (80% formamide solution containing tracking dyes), heated at 90 °C for 4 min and chilled in ice for another 4 min prior to be loaded on a 8% denaturing polyacrylamide gel for 90 min at 65W in TBE buffer. The data were collected using a Molecular Dynamics STORM 860 PhosphorImager and analyzed using the ImageQuant version 4.1 software. Each resolved band on the

autoradiograph was assigned to a particular bond within the DNA fragment from comparison with its position relative to the guanines sequencing standard (G-track) classically obtained using DMS and piperidine treatment of the same DNA fragment.

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