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N, N′ (4,5-Dihydro-1H-imidazol-2-yl)3-aza-1,10-decane-diamine and N, N′(4,5-dihydro-1H-imidazol-2-yl)3-aza-1, 10-dodecane-diamine antagonize cell proliferation as selective ligands towards topoisomerase II

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Abstract

New alkyl imidazoline derivatives have been synthesized as potential anticancer agents. The antiproliferative activity of these compounds, evaluated against representative human haematological and solid neoplastic cell lines, showed that N, N′-di (4,5-dihydro-1H-imidazol-2-yl)3-aza-1,10-decane-diamine (**8**) and N, N′-di (4,5-dihydro-1H-imidazol-2-yl)3-aza-1,10-dodecane-diamine (**9**) were the most active compounds; in fact, they inhibited the cell proliferation at submicromolar concentrations. In enzyme assays, compound **9** turned out to be an inhibitor of topoisomerase II at concentrations comparable with those of the reference topoisomerase II inhibitor, etoposide.

Introduction

Most of the antitumour agents currently used in the treatment of human malignancies have been targeted at topoisomerase II (topo II). DNA topoisomerases are a class of enzymes (Rangarajan et al 2000) involved in the regulation of DNA supercoiling. The function of this enzyme is that of relaxing a supercoiled DNA by allowing double-stranded DNA (dsDNA) chains to pass one through another following a cleavage, generating blunted DNA ends. The topo II inhibitors, referred to as 'poisons', etoposide, doxorubicin, mitoxantrone and amsacrine (Figure 1), convert this crucial enzyme into a potent cellular toxin that eventually drives cells to death (Froelich-Ammon & Oshero 1995; Wang et al 1997).

Several attempts have been made to exploit this enzyme towards subtype selective ligands. A large number of bisguanidine derivatives have been tested for their biological activity, such as antiparasitic, antiviral, and antineoplastic activity (Mueller et al 1992; Viossat et al 1996; Bailly et al 1997; Dardonville et al 2002, 2003).

In searching for agents with improved pharmacokinetic properties, potency or spectrum and lower side effects, structure–activity relationship studies have been very helpful in driving the synthesis of more active drugs that have a structure very close to that of the bisguanidines (Pauwels et al 1988; Perrault et al 1995; Oost et al 1997; Dardonville et al 2003). Prompted by the above findings, continuing in the research of guanidine derivatives endowed with anticancer activity, we became interested in novel compounds containing this function included into cyclic, rigid structures.

We report the synthesis of several 2-amino imidazolinium derivatives and their evaluation for in-vitro antitumoral efficacy against representative human cancer cell lines.

Materials and Methods

Alkyl imidazoline derivatives 1–10

Melting points were determined with a Gallenkam apparatus. ^{1}H NMR spectra were recorded on a Bruker Advance 300 and 600 MHz NMR spectrometer using TMS as internal standard and DMSO-d6. All chemical shifts were reported as δ (ppm) values. MS spectrometry

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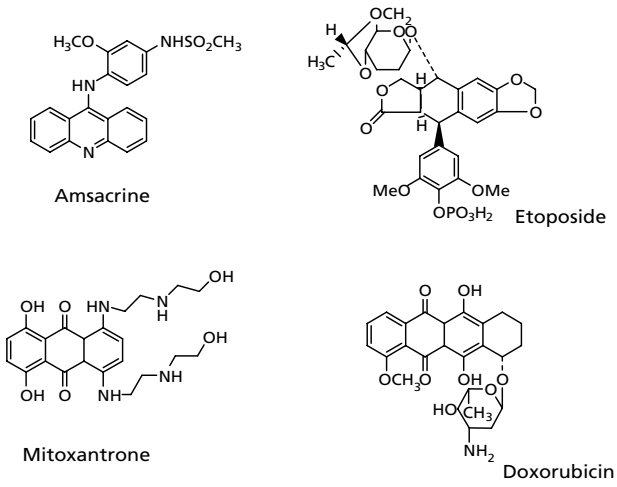


Figure 1 Structures of amsacrine, etoposide, mitoxantrone and doxorubicin.

analysis ESI-MS was carried out on a Finnigan LCQ Deca ion trap instrument.

The synthesis of target compounds was conveniently performed as outlined in Figure 2. The corresponding primary amines were reacted with (*S*)-methylisothiourea in dry CH_3CN to yield, after crystallization, the target compounds isolated as their iodide salts.

General procedure

A solution of 3 mmol of the corresponding diamine and 6 mmol of 2-methylthio-2-imidazoline hydriodide was heated under reflux in acetonitrile for 5–7 h. The reaction mixture was evaporated to dryness under vacuum. The residue was crystallized from a suitable solvent to give the imidazoline salts. All compounds gave correct analytical and spectral data. The characterization of compounds was as follows:

Crystallization from diethyl ether gave **1** as a white crystal, mp: 162°C, ^{1}H NMR (DMSO-d6): 1.53 (m, 2H, CH_2);1.60 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4NH); 2.66 (m, 4H, $2CH_2$); 2.70 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 446.1.

Crystallization from diethyl ether gave **2** as crystal, mp: 136°C, ^{1}H NMR (DMSO-d6): 1.55 (m, 4H, $2CH_2$); 1.60 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4NH); 2.65 (m, 4H, $2CH_2$); 2.69 (m, 4H, imidazoline) MS (ESI) m/z: 480.13.

Crystallization from diethyl ether gave **3**, mp: 156°C, ^{1}H NMR (DMSO-d6): 1.29 (m, 6H, $3CH_2$); 1.54 (m, 4H, $2CH_2$); 1.65 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4NH); 2.70 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 494.15.

Crystallization from diethyl ether gave **4**, mp: 196°C, ^{1}H NMR (DMSO-d6): 30 (m, 4H, $2CH_2$); 1.55 (m, 4H, $2CH_2$); 1.59 (m, 4H, $2CH_2$ imidazoline); 1.98 (s, 4H, 4NH); 2.66 (m, 4H, $2CH_2$); 2.69 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 508.18.

Crystallization from diethyl ether gave **5**, mp: 140°C, ^{1}H NMR (DMSO-d6): 1.28 (m, 6H, $3CH_2$); 1.55 (m, 4H, $2CH_2$); 1.63 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4 NH); 2.65 (m, 4H, $2CH_2$); 2.69 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 522.2.

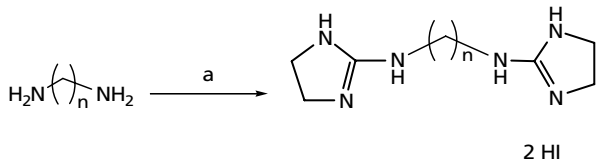
Crystallization from methanol gave **6**, mp: 60°C, ^{1}H NMR (DMSO-d6): 1.30 (m, 8H, $4CH_2$); 1.54 (m, 4H, $2CH_2$); 1.63 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4NH); 2.64 (m, 4H, $2CH_2$); 2.70 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 536.23.

Crystallization from methanol gave **7**, ^{1}H NMR (DMSO-d6): 1.30 (m, 10H, $5CH_2$); 1.53 (m, 4H, $2CH_2$); 1.58 (m, 4H, $2CH_2$ imidazoline); 1.98 (s, 4H, 4NH); 2.66 (m, 4H, $2CH_2$); 2.68 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 550.26.

Crystallization from methanol gave **8**, ^{1}H NMR (DMSO-d6): 1.32 (m, 12H, $6CH_2$); 1.54 (m, 4H, $2CH_2$); 1.60 (m, 4H, $2CH_2$ imidazoline); 1.99 (s, 4H, 4NH); 2.68 (m, 4H, $2CH_2$); 2,70 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 564.28.

Crystallization from diethyl ether gave **9** as a white crystal, mp: 70°C, ^{1}H NMR (DMSO-d6): 1.29 (m, 16H, $8CH_2$); 1.55 (m, 4H, $2CH_2$); 1.60 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4NH); 2.65 (m, 4H, $2CH_2$); 2.70 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 592.33.

Crystallization from diethyl ether gave **10** as a white crystal, mp: 106°C, ^{1}H NMR (DMSO-d6): 1.32 (m, 2OH, $10CH_2$); 1.56 (m, 4H, $2CH_2$); 1.60 (m, 4H, $2CH_2$ imidazoline); 2.00 (s, 4H, 4NH); 2.70 (m, 4H, $2CH_2$); 2.75 (m, 4H, $2CH_2$ imidazoline) MS (ESI) m/z: 620.41.



1–10

Compound	n
1	3
2	4
3	5
4	6
5	7
6	8
7	9
8	10
9	12
10	14

Figure 2 Reagents and conditions: a, 2-methylmercapto-4,5-dihydroimidazole iodide, CH_3CN, reflux, 5 h.

Pharmacology

Cells

Cell lines were purchased from American Type Culture Collection (ATCC). Haematological tumour-derived cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 $U\,mL^{-1}$ benzylpenicillin and 100 $\mu g\,mL^{-1}$ streptomycin. Solid tumour-derived cells were grown in their specific media supplemented with 10% FCS and antibiotics. Cell cultures were incubated at 37°C in a humidified, 5% CO_2 atmosphere. The absence of mycoplasma contamination was checked periodically by the Hoechst staining method.

Antiproliferative assays

Exponentially growing cells were resuspended in growth medium containing eight serial dilutions of the drugs starting from 100 μM. Cell viability was determined after 96 h at 37°C by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method (Denizot & Lang 1986).

Antiviral assays

Activity of compounds against human immunodeficiency virus type-1 (HIV-1) was based on inhibition of virus-induced cytopathogenicity in MT-4 cells acutely infected with a multiplicity of infection (m.o.i.) of 0.01. Briefly, 50μL RPMI containing 1×10^4 MT-4 was added to each well of flat-bottom microtitre trays containing 50μL RPMI, without or with serial dilutions of test compounds. To this was added 20 μL HIV-1 suspension containing 100 CCID50 (the dose infecting 50% of the cell culture). After incubating for four days, cell viability was determined by the MTT method.

Antibacterial and antimycotic assays

Staphylococcus aureus, Salmonella spp and *Aspergillus fumigatus* were clinical isolates, *Candida albicans* 10231 was ATCC strain. Assays were carried out in Triptosio agar for *S. aureus* and *Salmonella* spp, and for *C. albicans* and *A. fumigatus* Sabouraud dextrose broth was used, with an inoculum of 10^3 bacteria mL^{-1} and 5×10^3 yeast mL^{-1}. *A. fumigatus* inocula were obtained from cultures grown at 37°C for one day and then diluting to 0.05 OD50 mL^{-1}. Minimum inhibitory concentrations (MIC) were determined after incubation at 37°C for 18 h in the presence of serial dilutions of the test compounds.

Linear regression analysis

Viral and cell growth at each drug concentration was expressed as percentage of untreated controls and the concentrations resulting in 50% (EC50, CC50) growth inhibition were determined by linear regression analysis.

Topo II catalytic assay

Inhibition of topo II catalytic activity was evaluated using a Topoisomerase-II kit (TopoGEN, INC, Columbus, OH). Purified human topoisomerase II (TopoGEN, Columbus, OH) was used as a source for topo II in the topo II assay kit. Assays were performed according to the manufacturer's instructions in the presence and absence of different concentrations of the compounds. Reaction products were analysed on a 1% agarose gel in the presence of 0.5 μg mL^{-1} ethidium bromide as required by the manufacturer's instructions.

Statistical analysis

Statistical analysis of the cytotoxicity effect of derivatives **1–10** are presented as mean ± s.d. of at least three different experiments. Multiple comparisons were performed using one-way analysis of variance, analysis of variance was performed using Tukey's multiple comparison test. The effect of compound **8** and **9** on the proliferation of wild-type and drug resistant cells was evaluated using Student's *t*-test. A significance level of $P < 0.05$ denoted significance in all cases.

Results and Discussion

The antiproliferative activity of test compounds was evaluated against a panel of cell lines derived from either haematological (CCRF-CEM, WIL-2NS and CCRF-SB) (Table 1) or solid (SKMEL28, MCF7, SKMES-1, HepG2, and DU145) human tumours (Table 2).

Table 1 Cytotoxicity against leukaemia/lymphoma

Compound	[a]IC50 (μM)			
	[b]MT-4	[c]CCRF-CEM	[d]WIL-2NS	[e]CCRF-SB
1	> 100	> 100	> 100	> 100
2	> 100	> 100	> 100	> 100
3	> 100	> 100	> 100	> 100
4	≥ 100	> 100	> 100	> 100
5	23 ± 4	23 ± 3	60 ± 3	36 ± 0.1
6	6 ± 0.1	8 ± 0.1	32 ± 3	18 ± 2
7	2.9 ± 0.1	2 ± 0.1	7 ± 0.5	4 ± 0.5
8	0.7 ± 0.1*§	0.5 ± 0.1*§	2 ± 0.1*§	1.1 ± 0.4*§
9	0.2 ± 0.05*§	0.3 ± 0.05*§	0.6 ± 0.05*§	0.5 ± 0.02*§
10	3 ± 0.1	4.5 ± 0.6	7 ± 0.5	6 ± 0.4
6-Mercaptopurine	0.1 ± 0.02	1 ± 0.05	3 ± 0.05	1 ± 0.1
Camptothecin	0.004 ± 0.0004	0.003 ± 0.0005	0.005 ± 0.0005	0.003 ± 0.0005

[a]Inhibitory concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± s.d.) for three independent determinations. Data was performed using one-way analysis of variance and Tukey's multiple comparison test. [b]$CD4^+$ human T-cells containing an integrated HTLV-1 genome. [c]$CD4^+$ human acute T-lymphoblastic leukaemia. [d]Human splenic B-lymphoblastoid cells. [e]Human acute B-lymphoblastic leukaemia. *$P < 0.001$ vs **5, 6, 7, 10**. §$P > 0.05$ vs 6-mercaptopurine, camptothecin.

Table 2 Cytotoxicity against solid tumour-derived cell lines

Compound	[a]IC50 (μM)				
	[b]SK-MEL-28	[c]MCF7	[d]SKMES-1	[e]HepG2	[f]DU145
1	> 100	> 100	> 100	> 100	> 100
2	> 100	> 100	> 100	> 100	> 100
3	> 100	> 100	> 100	> 100	> 100
4	88 ± 5	> 100	90 ± 2	70 ± 4	≥ 100
5	33 ± 1	≥ 100	27 ± 1	22 ± 2	21 ± 0.5
6	8.9 ± 0.1	28 ± 1	5.4 ± 0.5	3.3 ± 0.1	3.8 ± 0.5
7	4 ± 0.1	8.6 ± 0.5	2.9 ± 0.2	1.7 ± 0.1	2.4 ± 0.1
8	6 ± 0.2*§#	2.5 ± 0.2*§#	0.8 ± 0.08*§#	0.4 ± 0.05*§#	0.5 ± 0.05*§#
9	0.4 ± 0.03*°¡	0.4 ± 0.01*°¡	0.2 ± 0.02*°¡	0.2 ± 0.04*°¡	0.1 ± 0.001*°¡
10	6 ± 0.3	10 ± 1	6 ± 0.2	4 ± 0.7	3 ± 0.1
6-Mercaptopurine	15 ± 1	3 ± 0.5	58 ± 2	8 ± 0.5	2 ± 0.05
Camptothecin	0.04 ± 0.005	0.04 ± 0.01	0.01 ± 0.004	0.03 ± 0.005	0.01 ± 0.005

[a]Inhibitory concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± s.d.) for three independent determinations. Data was performed using one-way analysis of variance and Tukey's multiple comparison test. [b]Human skin melanoma. [c]Human breast adenocarcinoma. [d]Human lung squamous carcinoma. [e]Human hepatocellular carcinoma. [f]Human prostate carcinoma. *$P < 0.001$ vs **5, 6, 7, 10**. §$P < 0.001$ vs 6-mercaptopurine, #$P < 0.05$ vs camptothecin, °$P < 0.001$ vs 6-mercaptopurine, ¡$P > 0.05$ vs camptothecin.

As shown in Tables 1 and 2, all derivatives specifically inhibited the proliferation of cells derived from haematological tumours at concentrations significantly lower than those inhibitory for solid tumours. Statistical analysis demonstrated that compounds **8** and **9** were statistically different ($P<0.001$) compared with other compounds, but they were not statistically different ($P>0.05$) compared with the reference drugs. Compounds **8** and **9** were the most potent against haematological and solid tumour-derived cell lines, being active in the range 0.1–1 μM.

Compounds **8** and **9** were evaluated also against cell lines derived from normal human tissues (CCL-75, MRC-5 and CRL7065) (Table 3).

Drug resistance is a relevant therapeutic problem caused by the emergence of tumour cells that confer resistance to a variety of anticancer drugs. The most common mechanisms of drug resistance are related to the over-expression of glycoproteins, capable of mediating the efflux of different drugs, such as doxorubicin, vincristine, and etoposide or to the altered contents of target enzymes, such as topoisomerases I and II. Compounds **8** and **9** were evaluated in-vitro against the wild type nasopharyngeal carcinoma cell line (KB^{WT}) and it was deemed interesting to investigate the susceptibility of drug-resistant KB subclones. Therefore, **8** and **9**, representative compounds of the series, were tested against cell lines (KB^{MDR} and KB^{V20C}) over-expressing the drug efflux pump MDR1/P-glycoprotein responsible for the MDR phenotype (Endicott & Ling 1989; Dardonville et al 2002; Dardonville & Brun 2004), and against an etoposide-resistant KB cell line (KB^{7D}) (Twentyman 1993), that besides a twofold decrease in topo II levels, over-expresses a protein referred to as multidrug resistance associated protein (MRP), which is known to reduce the uptake of etoposide and other antineoplastic agents (MDR phenotype). Compounds **8** and **9** proved to be fully inhibitory to all these resistant cell lines, thus suggesting that they were not subjected to the pump mediating the efflux of many antitumour drugs, and that they could interfere with the DNA synthesis.

Table 3 Cytotoxicity against "normal" cell lines

Compound	[a]IC50 (μM)		
	[b]CCL-75	[c]MRC-5	[d]CRL7065
1	> 100	> 100	> 100
2	> 100	> 100	> 100
3	> 100	> 100	> 100
4	> 100	> 100	> 100
5	> 100	> 100	> 100
6	> 100	> 100	> 100
7	> 100	> 100	> 100
8	60 ± 1*§	70 ± 3*§	43 ± 2*§
9	30 ± 1*§	20 ± 0.5*§	19±1*§
10	15 ± 0.5	18 ± 0.2	9 ± 0.5
6-Mercaptopurine	> 100	> 100	> 100
Camptothecin	2 ± 0.05	0.3 ± 0.005	0.5 ± 0.005

[a]Inhibitory concentration required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± s.d.) for three independent determinations. Data was analysed using one-way analysis of variance and Tukey's multiple comparison tests. [b]Human lung fibroblasts. [c]Human lung fibroblasts. [d]Human foreskin fibroblasts. *$P < 0.001$ vs **5, 6, 7, 10**. §$P > 0.05$ vs 6-mercaptopurine, camptothecin.

To assess whether the antiproliferative activity of **8** and **9** correlated with inhibition of the topoisomerase II catalytic activity, a cell-free system (see biological assays) was used (Minderman et al 2000). This inhibition effect was compared with that of the topo II inhibitor etoposide (VP16) and topo-I inhibitor camptothecin, used as reference compounds. Like VP16, only compound **9** was able to

Table 4 Effect of compounds **8** and **9** on the proliferation of wild-type and drug-resistant KB cells

Compound	[a]IC50 (μM)			
	[b]KB^{WT}	[c]KB^{MDR}	[d]KB^{7D}	[e]KB^{V20C}
8	1.1 ± 0.1	2.1 ± 0.05*	0.8 ± 0.5*	1.3 ± 0.1*
9	0.3 ± 0.05	0.4 ± 0.01*	0.2 ± 0.02*	0.3 ± 0.05*
Doxorubicin	0.06 ± 0.01#	1.8 ± 0.05#	3 ± 0.5#	0.4 ± 0.02#
VP16	0.8 ± 0.1#	> 100	> 100	2.4 ± 0.2#
VCR	0.004 ± 0.0005#	1 ± 0.1#	0.05 ± 0.002#	0.2 ± 0.005#

[a]Inhibitory concentration required to reduce cell proliferation by 50%, as determined with the MTT method, under condition allowing untreated controls to undergo at least three consecutive rounds of multiplication. Data represent mean values (± s.d.) for three independent determinations. Statistical analysis was performed using Student's *t*-test. [b]KB human nasopharyngeal carcinoma. [c]KB subclones passaged in the presence of doxorubicin 0.09 μM. [d]KB subclones passaged in the presence of etoposide 7 μM. [e]KB subclones passaged in the presence of vincristine 0.02 μM. *$P > 0.05$ vs KB- wild-type, #$P < 0.0001$ vs KB-wild-type.

inhibit the formation of supercoiled DNA from relaxed DNA (Figure 3).

Title compounds were also assayed for other biological activity. However, none of them were able to prevent the HIV-induced cytopathogenicity in MT-4 cells at non-cytotoxic concentrations (data not shown). Furthermore, they were evaluated for their capability to inhibit the multiplication of various human pathogenic fungi (*C. albicans, C. parapsilosis, C. paratropicalis, A. fumigatus* and *Criptococcus neoformans*) and bacteria (*S. aureus,* group D *Streptococcus, Salmonella* spp and *Shigella* spp), but none of the compounds were active (data not shown).

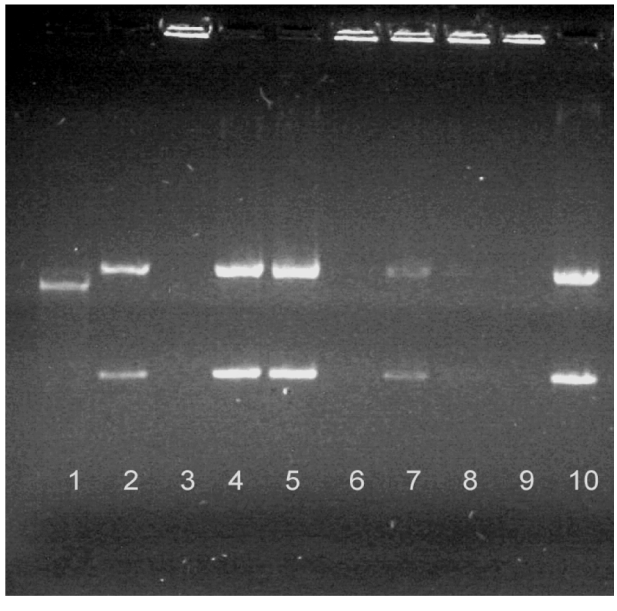


Figure 3 Inhibitory effects of etopside (VP16), camptothecin, and compounds **8** and **9** on the activity of topoisomerase II. The first five lanes represent controls; lane 1: marker linear KDNA; lane 2: marker decatenated KDNA; lane 3: kinetoplast DNA (KDNA); lane 4: topo II activity; lane 5: topo II activity in the presence of DMSO (0.1%); lane 6: topo II activity in the presence of VP16 100 μM; lane 7: topo II activity in the presence of VP16 10 μM; lane 8: topo II activity in the presence of camptothecin 100 μM; lane 9: topo II activity in the presence of **8** 100 μM; lane 10: topo II activity in the presence of **9** 100 μM).

Conclusion

New data has been described about the antiproliferative activity and cytotoxicity of a new imidazoline class; biological data suggested that, in general, change of the physicochemical properties of the title compounds, due to an increase of lipophilicity, in an exact range, were beneficial to activity. In experiments with human cancer cell lines, derivatives **8** and **9** significantly reduced cell proliferation. Further investigation in animal models is necessary to establish whether the pharmacodynamic and pharmacokinetic properties of the title compounds improved.

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