Bioorganic & Medicinal Chemistry Letters 21 (2011) 2259–2263

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis, cytotoxicity and topoisomerase inhibition properties of multifarious aminoalkylated indeno[1,2-*c*]isoquinolin-5,11-diones

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ARTICLE INFO

Article history:
Received 17 January 2011
Revised 24 February 2011
Accepted 25 February 2011
Available online 1 March 2011

Keywords:
Cancer
DNA intercalators
Topoisomerases
Indenoisoquinolines

ABSTRACT

A number of mono- or diaminoalkylated indeno[1,2-*c*]isoquinolin-5,11-diones analogs of **1** were synthesized and evaluated for their DNA binding affinities, topoisomerase inhibition properties and antiproliferative activities against human cancer cell lines (HL60). Impact of the side chain connected to the aromatic D ring and to the N6 lactam position on the biological profile will be discussed.

Topoisomerase inhibitors occupy a place of choice in the arsenal of current chemotherapy strategies involved in the four-decade-long war against cancer,[1] a disease that is estimated to have affected 12.7 million people and to have taken away 7.6 million lives worldwide in 2008.[2] The identification of this ubiquitous enzyme in the early 1970s[3,4] led to the development of an array of DNA-intercalating and/or cytotoxic molecules capable of inhibiting the topoisomerases. FDA-approved topotecan[5] (HYCAMTIN®) falls into this class of compounds. It targets the single DNA strand regulating-topoisomerase type I (Topo I), whereas constitutionally diverse models such as daunorubicin/daunomycin[6] (CERUBEDINE®), mitoxantrone[7] (NOVANTRONE®) and etoposide[8] (VEPESID®) interfere with the duplex regulating-topoisomerase type II (Topo II) (Fig. 1).

Despite their efficiency, the therapeutic activity of these commercialized drugs is fraught with pharmacokinetic flaws, undesirable side effects and resistance from tumor cells, most widely by action of membrane ATP-dependent multidrug efflux pump mediated by P-glycoprotein (MDR1, ABCB1).[9] Besides, the potencies of Topo I inhibitors are seriously compromised by their chemical instabilities whilst, in case of Topo II inhibitors, the efficiencies of the drugs are lowered by the reduction of the expression of Topo II and by mutations of the targeted enzyme.[10]

Consequently a variety of new compounds overcoming such limitations and drawbacks were extensively developed during the late 1980s and 1990s, leading to the discovery by the National Cancer Institute of NSC 314622, a representative example of the class of indenoisoquinoline derivatives (Fig. 2).[11] Several structure–activity relationships and pharmacomodulations on the lead compound then led to the development of structurally diverse analogs with improved pharmacological activities on Topo I,[12] as exemplified by indimitecan or indotecan.[13–17] Recently an array of new indenoisoquinoline derivatives including **1**[18] and **2**[19] were shown to display good inhibition capacities on Topo II. Additionally, structurally related analogs such as indeno[1,2-*c*]quinolines **3** have recently proved to be potent dual inhibitors of Topo I and Topo II (Fig. 2).[20–22]

In a previous study from our group,[18] we reported that the most potent compound on Topo II in this series was a tetracyclic model equipped with two basic tertiary amino groups appended to the C8 and N6 lactam positions and linked to the tetracyclic intercalant moiety through ethoxy and ethyl group spacers respectively. We then surmised that a more thorough investigation of influence of multifarious aromatic D-ring substitution patterns coupled with varied lactam side chain at N6 could help to get better insight about Topo II inhibition activity. For this purpose, an array of models tailed with constitutionally diverse dimethylaminoalkyl chains at C7, C8 and C9 positions coupled with suitable spacing linkers at the N6 lactam position of the tetracyclic template were initially de-

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doi:10.1016/j.bmcl.2011.02.106

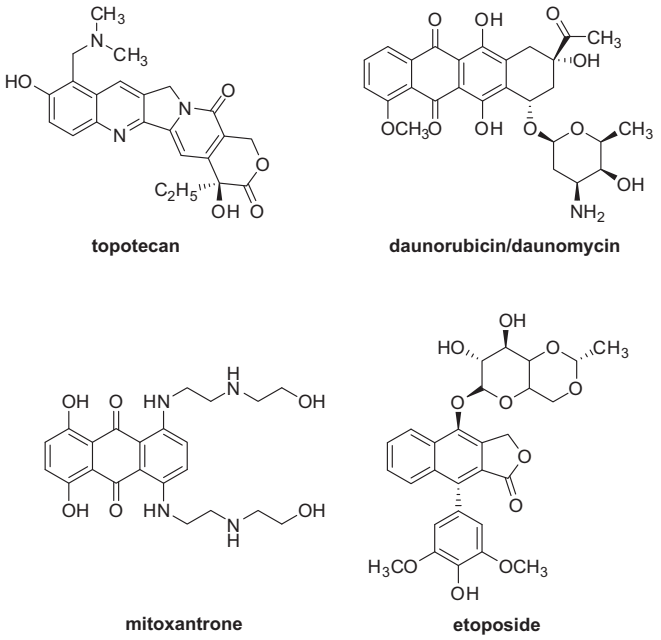


Figure 1. Structures of FDA-approved Topo I and Topo II inhibitors.

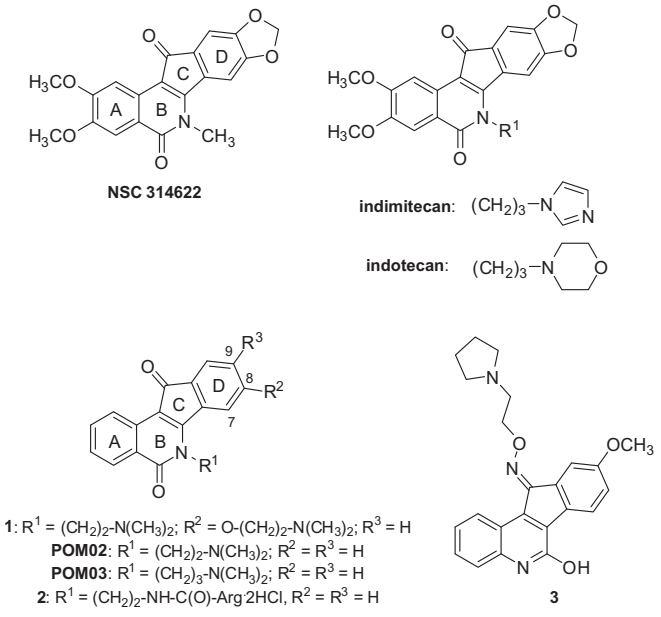
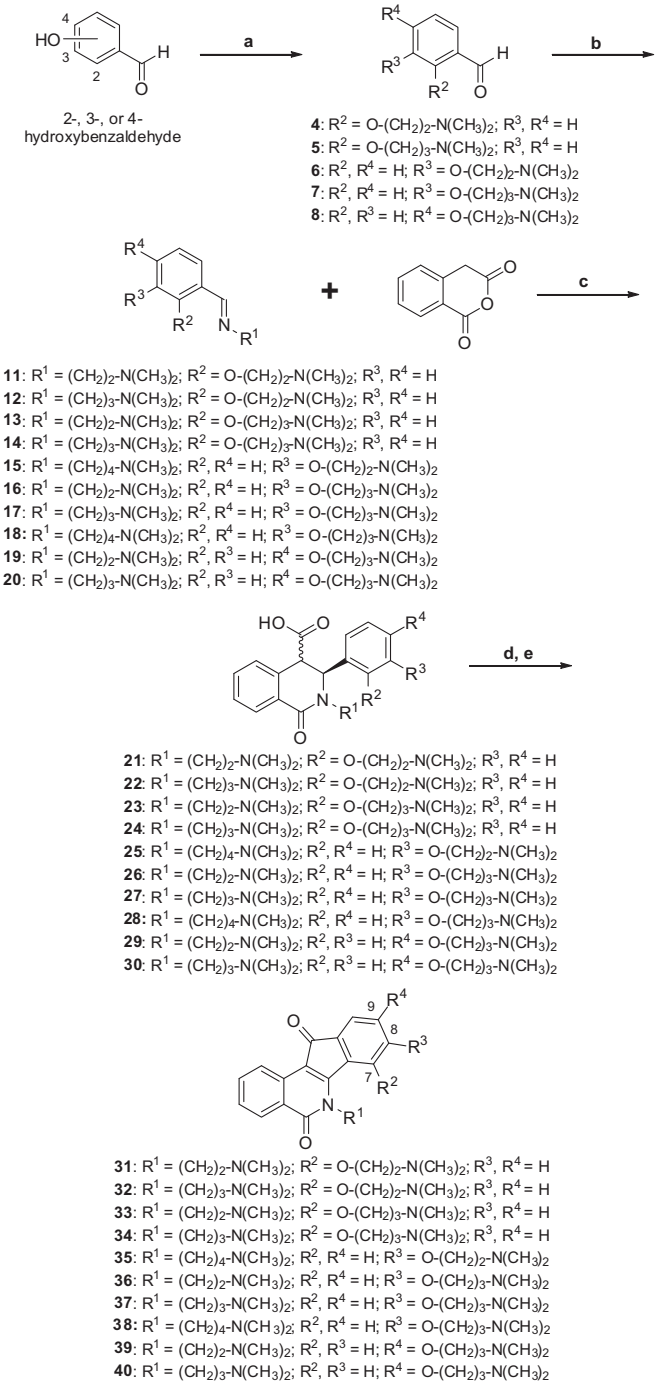


Figure 2. Structures of potent indenoisoquinolines.

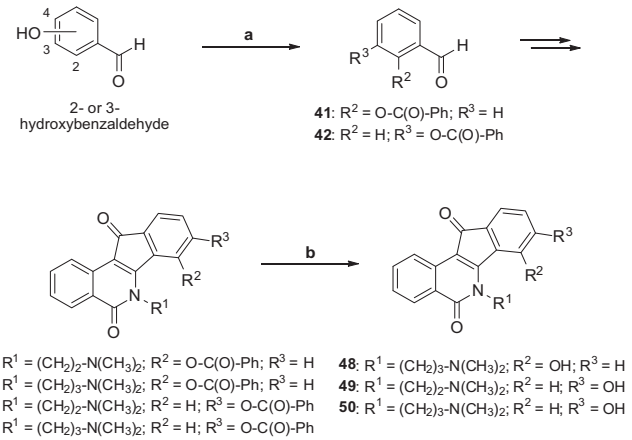


Scheme 1. Reagents and conditions: (a) appropriate dimethylamino alkyl chloride Cl-$(CH_2)n$-$N(CH_3)_2$ (n = 2 or 3), K_2CO_3,DMF, reflux, 6 h; (b) R^1-NH_2, $MgSO_4$, $CHCl_3$, rt, 18 h. For the synthesis of compounds **15** and **18**, TEA was also added; (c) THF (MeOH for the synthesis of compounds **25**, **26** and **28**), 0 °C, 1 h; (d) $SOCl_2$, reflux, 18 h; (e)$AlCl_3$, CH_2Cl_2, 0 °C, 3 h.

signed. These newly synthetized compounds were subsequently evaluated for DNA interaction measurements, Topo I and Topo II inhibition activities and for cytotoxicity against HL60 sensitive and resistant leukemic cell lines.

The targeted indenoisoquinoline-dione derivatives **31**–**40** were readily assembled as depicted in Scheme 1.[18] The first fact of the synthesis was the preliminary O-alkylation of 2-, 3- or 4-hydroxybenzaldehyde with 2-dimethylaminoethyl or 3-dimethylaminopropyl chlorides afforded the corresponding hydroxybenzaldehyde derivatives **4**–**8**. Subsequent condensation of benzaldehyde analogs **4**–**8** with dimethylaminoethyl-, propyl- and butylamine provided the imines **11–20**, which were then allowed to react with homophthalic anhydride to afford a diastereomeric mixture of 3-aryl-4-carboxyisoquinolones **21**–**30**. The *cis*/*trans* ratio of these isoquinolones ranged from 1:2 to 1:6.6 in favor of the *trans*-diastereoisomer. It is likely that the formation of these lactamic compounds proceeds via the mechanism proposed by Cushman and co-workers[23] and Gonzalez-Lopez et al.[24] but it is worth mentioning that this mechanism could not account for the favored formation of the *trans*-diastereoisomer when increasing the side chain length.

It is also noteworthy that these diastereoisomers could not be separated despite many attempts with multiple purification conditions, as already previously mentioned.[14,18] Subsequent treatment of the mixture with thionyl chloride followed by intramolecular



Scheme 2. Reagents and conditions: (a) benzoyl chloride, TEA, CH_2Cl_2; (b) R^1-NH_2, $MgSO_4$, $CHCl_3$, rt, 18 h; (c)homophthalic anhydride, THF or MeOH, 0 °C, 1 h; (d) $SOCl_2$, reflux, 18 h; (e) $AlCl_3$, CH_2Cl_2, 0 °C, 3 h; (f) LiOH, CH_2Cl_2/MeOH (1:1), rt, 1 h.

Table 1
Variations in ΔT_m and in apparent DNA affinity constants

Compound	ΔT_m poly(dAT)$_2$[a]	K_{app} 10^6 M^{-1} [b]
Daunorubicin	21.9[c]	–
31	30.5	55.79
32	42.7	52.67
33	44.7	130.28
34	41.3	107.67
35	33.9	25.69
36	31.4	28.71
37	40.6	33.38
38	37.1	41.63
39	36	71.99
40	46.9	89.31
48	17.1	–
49	7.7	–
50	16.6	–

[a] Variation in melting temperature $\Delta T_m = T_m^{\text{drug-DNA complex}} - T_m\text{DNA}^{\text{alone}}$. Drug/DNA ratio = 0.25.

[b] Apparent binding constant measured by fluorescence using [BET]/[DNA] = 1.26, K_{app} in 10^6 M^{-1}.

[c] ΔT_m of daunorubicin measured at ratio = 1. K_{app} of this compound could not be measured due to the interferences of fluorescence spectra of DNA and BET.

Friedel–Crafts acylation delivered the targeted N6- and D ring-substituted indenoisoquinolines **31–40**, albeit in moderate yield.[25] The low overall yield may be attributable to the deleterious formation of diastereoisomeric isoquinolones not prone to cyclize, namely those lacking electron-donating groups on the A ring, associated with the generation of the regioisomers as byproducts upon the ultimate intramolecular acylation reaction. The phenolic derivatives **48–50** were readily constructed following a similar strategy combined with an alkaline hydrolysis reaction at the final stage to remove the benzoxylcarbonyl protecting group (Scheme 2).

The ability of the drugs to protect poly(dAT)$_2$ DNA against thermal denaturation was used as an indicator of the relative capacity of the new molecules to bind and to stabilize the DNA double helix. These experiments were perfomed in poly(dAT)$_2$ especially rich in AT. Variations of $\Delta T_m = T_m^{\text{drug-DNA complex}} - T_m^{\text{DNA alone}}$ ranged from 7.7 °C to 46.9 °C (Table 1). As expected, indenoisoquinolines **48–50**, bearing phenolic functions and tested as control molecules, displayed lower ΔT_m values (7.7–17.7 °C) than the analogs **31–40** bearing two cationic side chains (29–46.9 °C). C8-aminoalkoxy-substituted products were found to less stabilize DNA from heat denaturation compared to their C7-aminoalkoxy- and C9-aminoalkoxy counterparts (**36** vs **33** vs **39** and **37** vs **34** vs **40**). Insertion of an additional methylene unit either on the aminoalkoxy side chain on the aromatic D ring or on the N6 lactam side chain resulted in an upward shift in ΔT_m values. This trend was also observed for the N-substituted indenoisoquinoline derivatives (**49** vs **50**). However, further increase in the N6 lactam side chain length did not end up with the highest ΔT_m values (**36**, **37** vs **38**), suggesting that the presence of three methylene units may be the optimum for the duplex stabilization.

The competitive ethidium displacement method was applied to our newly designed compounds.[26–28] Apparent DNA binding affinities K_{app} using CT DNA were quantified by means of fluorescence. The values of compounds **48–50** could not be obtained due to the high insolubility of the molecules. The K_{app} values ranged from 25.69×10^6 M^{-1} to 130.28×10^6 M^{-1} (Table 1). C7-aminoalkoxy-substituted indenoisoquinolines **31–34** displayed the highest K_{app} values, followed by compounds bearing the aromatic D ring side chain at C9 position (**33** vs **39** and **34** vs **40**). And similar to what was observed for ΔT_m values, C8-aminoalkoxy-substituted counterparts exhibited the lowest affinities, with K_{app} values 2.5- to 2.7-fold less than those of C9-aminoalkoxy counterparts (**39** vs **36** and **40** vs **37**). Incrementing one methylene unit either on D ring side chain or on the N6-lactam side chain resulted, once again, in the increase in K_{app} values. But the amplitude of the variation depended on the position of the side chain on which the addition of a CH_2 group was performed. In fact, increasing the length of the N6-lactam side chain did not bring significant variations in K_{app} values (**31** vs **32**, **33** vs **34**, **36** vs **37** and **38**). The previous study showed also this trend.[18] On the other hand, increase in the D ring side chain length brought about significant raise in the DNA affinity constants (**31** vs **33**, **32** vs **34**). These results show that, unlike ΔT_m values, lengthening the spacer up to four methylene units did produce highest K_{app} values (**36** and **37** vs **38**).

A conventional DNA relaxation assay was used to assess the effects of the compounds in the three series on the catalytic activity of recombinant Topo I (Fig. 3A).[29] Negatively supercoiled plasmid DNA was first treated with the enzyme in the presence of the tested drug at different concentrations and the DNA relaxation products were then resolved by electrophoresis. All tested compounds modified the relaxation of the DNA at 1 μM or higher concentrations of the INDO. But the compounds were not able to stabilize the cleavage complex, as opposed to CPT. Nevertheless, these results were consistent with the binding (unwinding of closed circular duplex DNA) of our compounds through intercalation mode on DNA.

The same tests, with Topo I being replaced with Topo II, were performed to evaluate effects on the catalytic activity of Topo II. In these experiments supercoiled plasmid DNA was treated with Topo II in the presence of graded concentrations of the tested drug and the DNA relaxation products were then resolved by electrophoresis. The reference drug, etoposide, produced a marked level of DNA double stranded breaks, corresponding to linear DNA (Fig. 3B and C). The activity of the compounds was expressed semi-quantitatively as follows on Table 2: 0: no specific inhibition activity, + and ++: weak activity, +++: good activity, ++++ as active as etoposide. Inhibition of Topo II was clearly detected with our new derivatives. Etoposide produced a marked level of DNA double stranded breaks (linear DNA).

The agarose gel resulting from the assay revealed that some of our C8-substituted analogs **36** and **37** showed the ability to stabilize the cleavage complex DNA-Topo II (Fig. 3B). None of C7 and C9-aminoalkoxy-substituted INDOs was able to specifically inhibit Topo II. As illustrated in the Figure 3C, the previously reported indenoisoquinoline **1** displayed the same potency as the etoposide (++++), whereas compounds **36** and **37** exhibited good potencies (+++) in Topo II inhibition among newly synthesized derivatives. The length of the N6 lactam side chain had an impact on the Topo II inhibition abilities, as compounds **35** (+) and **38** (+) were shown

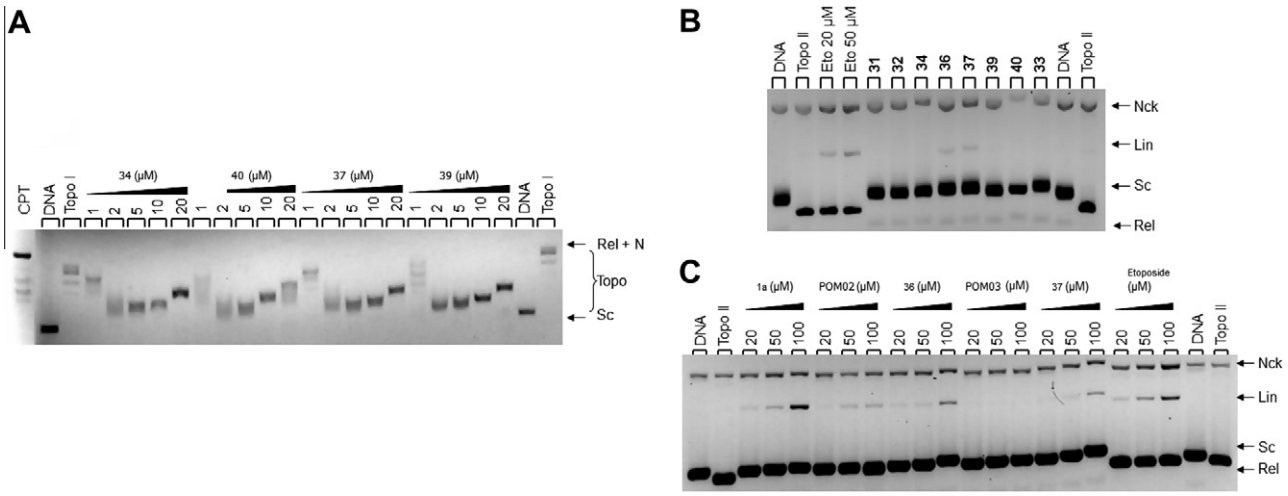


Figure 3. (A) Effects of the compounds **34**, **37**, **39** and **40** on the relaxation of plasmid DNA by Topo I. Native supercoiled pUC19 (130 ng, lane DNA) was incubated with 8 units of Topo I in the absence (lane Topo I) or presence of tested compounds at the indicated concentration (1–20 μM). Camptothecin (CPT) was used at 20 μM. DNA samples were separated by electrophoresis on a 1% agarose gel which was stained with ethidium bromide after DNA migration. Gels were photographed under UV light. Nck: nicked; Sc: supercoiled; Rel: relaxed; Topo, topoisomer products. B and C. Effects of novel indenoisoquinoline derivativeson the relaxation of plasmid DNA by Topo II. Native supercoiled pUC19 (350 ng, lane DNA) was incubated with 8 units of Topo II in the absence (lane Topo II) or presence of tested compounds at the indicated concentration (B: 50 μM and C: 20–100 μM). Etoposide was used at indicated concentrations. DNA samples were separated by electrophoresis on a 1% agarose gel containing 1 μg/mL ethidium bromide. Gels were photographed under UV light. Nck: nicked; Sc: supercoiled; Lin: linear; Rel: relaxed.

Table 2
Cytotoxicities and Topo II inhibitory activity of the novel INDOs

Composé	IC_{50} (in μM)		RRI[b]	Topo II inhibition
	HL60[a]	HL60/MX2		
MTX	0.063 ± 0.020	1.51 ± 0.08	24	–
Eto	1.4 ± 0.0	15.75 ± 1.85	11.25	++++
1	0.66 ± 0.02	1.5 ± 0.3	2.3	++++
31	1.10 ± 0.10	1.3 ± 0.10	1.2	0
32	1.4 ± 0.0	2.4 ± 0.0	1.4	0
33	2.6 ± 0.0	2.3 ± 0.2	0.9	0
34	5.60 ± 0.25	4.90 ± 0.25	0.9	0
35	3.7 ± 0.2	6.85 ± 0.25	1.85	+
36	1.1 ± 0.0	1.4 ± 0.0	1.3	+++
37	1.00 ± 0.14	3.6 ± 1.0	3.6	+++
38	2.2 ± 0.3	2.65 ± 0.05	1.2	+
39	2.8 ± 0.5	3.2 ± 0.0	1.1	0
40	5.05 ± 0.05	3.5 ± 0.2	0.6	0
48	–	–	–	–
49	1.4 ± 0.0	4.15 ± 0.35	2.9	++
50	0.625 ± 0.050	1.55 ± 0.35	2.4	++

[a] The cytotoxicity IC_{50} values are the concentrations corresponding to 50% growth inhibition.
[b] Relative resistance index: $IC_{50}^{(MX\text{-}resistant)}/IC_{50}^{(MX\text{-}sensitive)}$.

to be poor Topo II inhibitors (data not shown). As for the 8-hydroxylated indenoisoquinolines **49** and **50**, they also displayed weak Topo II inhibition (++), emphasizing that the aromatic D ring side chain seems to play a major role in Topo II inhibition (data not shown). These results undoubtedly illustrate the prominence of the C8 position on the D ring in Topo II inhibition. C7 and C9 substituted derivatives did not show any inhibition activities. The side chains lengths were proved to be key-points for the inhibition activity. The optimal side chain length at both aromatic D ring and N6-lactam positions seems to be two methylene units. Three methylene units were well tolerated, except in the case of models with an INDO bearing three methylene units at the N6 lactam position and two CH_2 groups in the C8 position of the aromatic D ring, as previously observed.[18] On the other hand, spacers longer than four CH_2 groups were proved to be deleterious for the inhibition activity, as evidenced by indenoisoquinolines **35** and **38**. The tests also revealed no connections between DNA affinities and Topo II inhibition abilities.

The antiproliferative activities of our compounds were tested using two human leukemia[30] cell lines, HL60 and HL60/MX2, respectively sensitive and resistant to the antitumor drug mitoxantrone. The assays on HL60/MX2 cell line, displaying altered catalytic activity and reduced levels of Topo II, were performed to gain an insight into the involvement of Topo II inhibition in the cytotoxicity of our derivatives. Table 2 shows that most of our newly synthesized compounds **31**–**40**, **48**–**50** displayed micromolar IC_{50} values, ranging from 0.625 μM to 5.6 μM. The most cytotoxic compound **50** presented submicromolar IC_{50} value, close to that of **1**, which remained our most potent compound in HL60 cell line (0.66 μM, ++++). No obvious relationship could be observed between the structures of the indenoisoquinoline derivatives and cytotoxicities. The inhibition of cleavage complex is unlikely the main contributor to the high cytotoxicity of compound **50** (0.625 μM), thus suggesting the possible involvement of additional targets other than Topo II or differences in cellular uptake. The weak resistance index RRI obtained for our derivatives may be explained by their binding to the enzyme at a site different from that of mitoxantrone or by specific kinetics. As for cell cycle effects and apoptosis, in light of the work on structurally related analogs from other groups, one possibility could be the cell cycle arrest, by our products, either in the S or the G2-M phase, depending on the concentration of the drug, whilst the cytotoxicity is p53-dependant. Cell cycle arrest in sub-G1 phase, as shown by Tseng et al. with the indeno[1,2-*c*]quinolines could be another possibility, subsequently followed by caspase-3 activation and PARP cleavage.[22] Phosphorylation of the histone protein H2AX at Ser^{139} residue following DNA double-strand break event can also be expected from our compounds.[22,30]

In conclusion, 13 structurally and constitutionally new analogs of the indeno[1,2-*c*]isoquinoline **1** have been designed and evaluated against DNA, Topo II and HL60 cell line. Despite the fact that no lead compound could emerge from this study, the C8 position has been confirmed as the sole position on the D-ring capable of inhibiting the topoisomerase by stabilizing the ternary cleavage complex. C7 and C9 positions, more favorable to the binding to the DNA, are not active against Topo II. The most potent compound remains the lead **1** whereas analogs **36** and **37** equipped with an aminopropoxy side chain on the D ring displayed also good poten-

cies against Topo II and HL60 cell line. These results suggested that Topo II inhibition may be favored with shorter N6 lactam side chain. Lengthening the N6 side chain beyond three methylene units proved to be deleterious to the Topo II inhibition activity. No correlation has been observed between DNA binding ability, Topo II inhibition and cytotoxicity, suggesting Topo II is not the only mechanism involved in the antiproliferative activity.

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