

Synthesis, Antiproliferative Effect, and Topoisomerase II Inhibitory Activity of 3-Methyl-2-phenyl-1*H*-indoles

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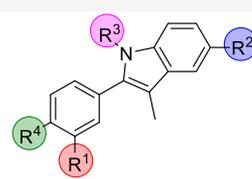
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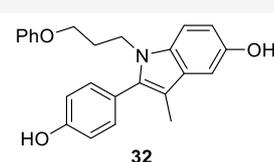
Supporting Information

ABSTRACT: A series of 3-methyl-2-phenyl-1*H*-indoles was prepared and investigated for antiproliferative activity on three human tumor cell lines, HeLa, A2780, and MSTO-211H, and some structure–activity relationships were drawn up. The GI_{50} values of the most potent compounds (**32** and **33**) were lower than 5 μM in all tested cell lines. For the most biologically relevant derivatives, the effect on human DNA topoisomerase II relaxation activity was investigated, which highlighted the good correlation between the antiproliferative effect and topoisomerase II inhibition. The most potent derivative, **32**, was shown to induce the apoptosis pathway. The obtained results highlight 3-methyl-2-phenyl-1*H*-indole as a promising scaffold for further optimization of compounds with potent antiproliferative and antitopoisomerase II activities.

KEYWORDS: 3-Methyl-2-phenyl-1*H*-indole, antiproliferative activity, topoisomerase II, apoptosis



R^1 and R^2 = H, OH or OCH_3
 R^3 = H, CH_3 , alkyl or phenoxyalkyl
 R^4 = OH, OCH_3 or arylcarboxyl



GI_{50} (HeLa) = 4.4 μM
 GI_{50} (A2780) = 2.2 μM
 GI_{50} (MSTO-211H) = 2.4 μM
 Inhibitory effect on topo II relaxation
 Induction of apoptosis

Cancer is one of the leading causes of mortality worldwide, and its incidence is rapidly growing.^{1,2} The use of conventional chemotherapeutic agents, such as alkylating cytostatics, nucleoside analogues, anthracyclines, and compounds that stabilize microtubules is often limited because of their severe side effects. Drugs such as tyrosine kinase inhibitors and monoclonal antibodies that target specific signaling oncoproteins have been developed to increase specificity and thus to reduce side effects, but these can also have limitations, such as development of resistance.³ Despite the considerable progress made with new targeted therapies, for many patients the therapeutic options remain limited. Thus, new chemotherapeutics with improved pharmacotoxicological profiles are urgently needed.⁴

One of the important targets for anticancer drug discovery is human DNA topoisomerase II (topo II).^{5–9} Topoisomerases are enzymes that catalyze changes in DNA topology, and they are crucial for processes such as DNA replication and transcription and chromosomal segregation. They introduce transient breaks in the DNA molecule by cleaving one (type I topoisomerases) or both (type II topoisomerases) strands.^{10,11} Topo II is a type II topoisomerase, and two isoforms, α and β , are expressed in mammalian cells. Topo II β is expressed constitutively throughout the cell cycle, whereas topo II α is predominantly in highly proliferating cells.

Based on their mechanism of action, compounds targeting topo II can be divided into two large groups: poisons¹² and catalytic inhibitors.^{6,13–16} Topo II poisons stabilize the covalent DNA–topo II complex, leading to DNA damage

and apoptotic cell death and are used in the clinic for the treatment of breast, lung, prostate, and hematological cancers and sarcomas.¹⁷ They included etoposide, doxorubicin, and mitoxantrone. A common side effect of DNA poisons is the risk of secondary malignancies and cardiotoxicity, which are mostly connected to the effects of these drugs on topo II β .¹⁸ On the other hand, catalytic inhibitors affect one of the steps in the catalytic cycle of the enzyme. In particular, they can block binding of the DNA molecule to the enzyme, compete for binding with ATP, inhibit cleavage of the DNA molecule, or prevent hydrolysis of ATP,¹² and interestingly, they do not increase DNA cleavage, which could indicate an improved safety profile.⁵

We report here the design and synthesis of a series of 3-methyl-2-phenyl-1*H*-indole analogues with antiproliferative and topo II inhibitory effects (Figure 1A). The compounds were designed based on the 3-methyl-2-phenyl-1*H*-indole hit I (Figure 1A), which was identified through screening of an in-house library of compounds against three selected human tumor cell lines: cervix adenocarcinoma (HeLa), ovarian carcinoma (A2780), and biphasic mesothelioma (MSTO-211H). Compound I showed good antiproliferative activity,

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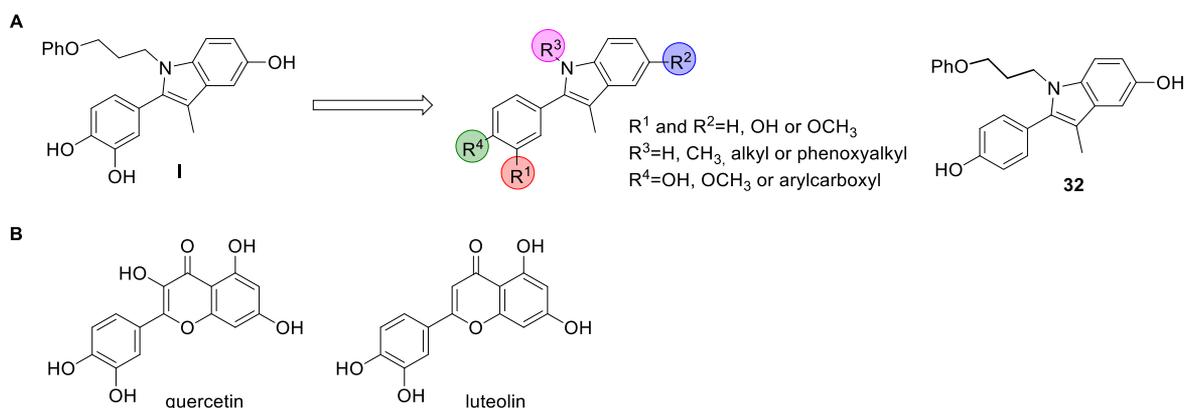
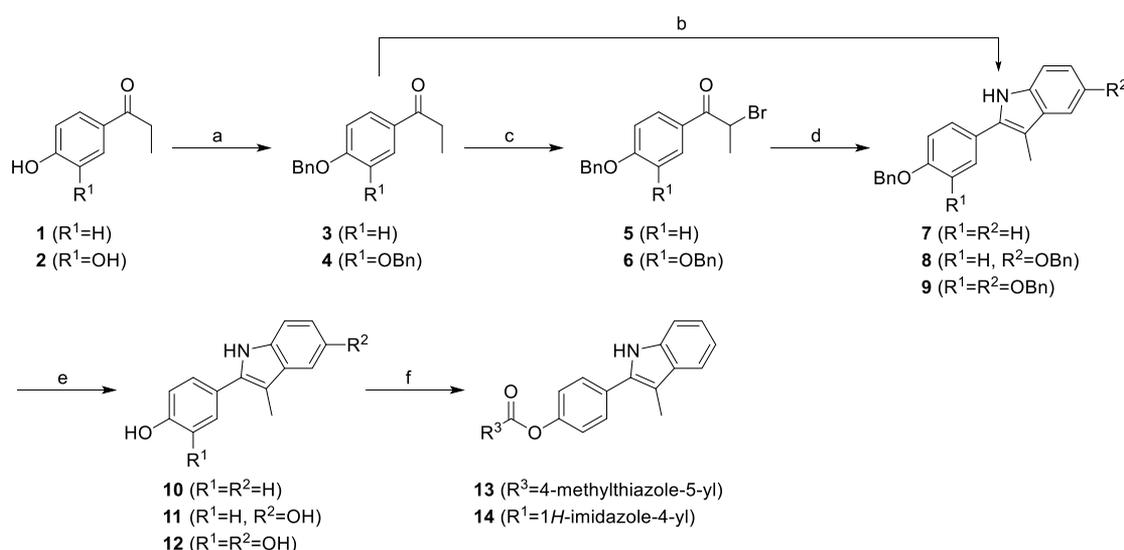


Figure 1. Design of the 3-methyl-2-phenyl-1*H*-indoles as antiproliferative compounds, based on screening hit I (A); structures of flavonoids quercetin and luteolin with topo II inhibitory activities (B).

Scheme 1. Synthesis of Compounds 10–14^a

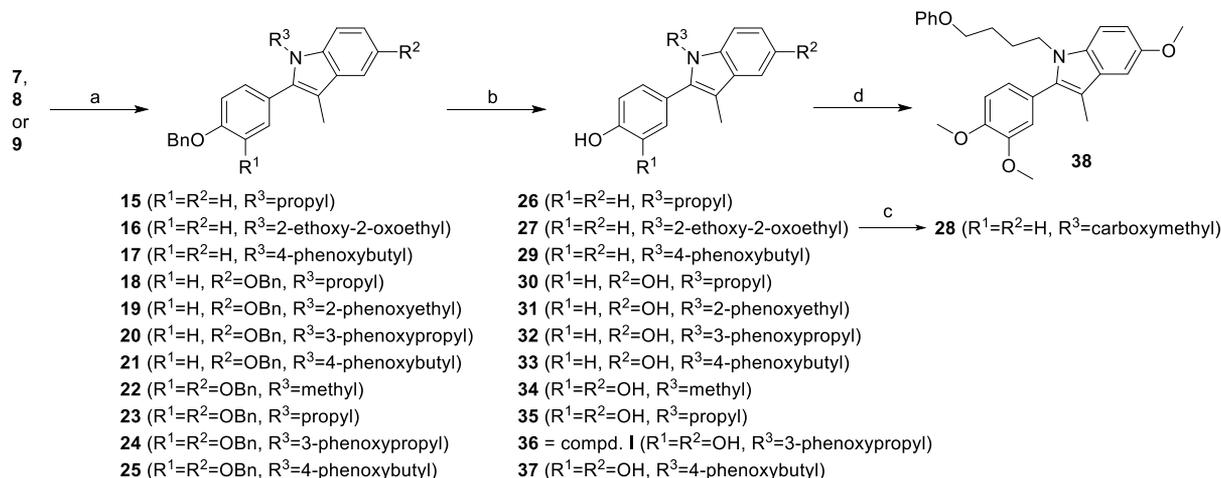


^aReagents and conditions: (a) benzyl bromide, K₂CO₃, acetone, reflux, 18 h; (b) for 7: phenylhydrazine, H₂SO₄, acetone, reflux, 24 h; (c) Br₂, glacial acetic acid, 0 °C, 1 h; (d) for 8, 9: 4-benzyloxyaniline-HCl, Et₃N, DMF, 120 °C, 2 h, then 150 °C, 3 h; (e) H₂, 10% Pd/C, EtOH, r.t., 18 h; (f) for 13: 4-methylthiazole-5-carboxylic acid, EDC, 4-dimethylaminopyridine, CH₂Cl₂, r.t., 15 h; for 14: 1*H*-imidazole-4-carboxylic acid, TBTU, NMM, DMF, r.t., 15 h.

with growth inhibition values (GI₅₀) in the micromolar range. Based on compound I, a series of analogues was designed and synthesized, through varying the substituents on R¹–R⁴ (Figure 1A). The antiproliferative activities of compounds were evaluated *in vitro* against HeLa, A2780, and MSTO-211H cells. Because of structural similarities of our compounds with some naturally occurring flavonoids that inhibit topo II, such as quercetin and luteolin (Figure 1B),^{19–21} the topo II inhibitory effects of the most potent analogues were investigated. For compound 32, cytofluorimetric analysis was performed to investigate the mechanism of cell death.

The designed compounds were prepared according to synthetic pathways presented in Schemes 1 and 2. Full synthetic procedures and analytical data are available in the Supporting Information. The synthesis of the central indole scaffold started with benzyl protection of hydroxyl group(s) of propiophenones 1 and 2, to obtain ethers 3 and 4 (Scheme 1). These ethers were α -brominated with bromine under acidic conditions, to yield 5 and 6, which were then reacted with 4-benzyloxyaniline in the Bischler–Möhlau indole synthesis, to

give indoles 8–9. Indole 7 was obtained by Fischer indole synthesis from ketone 3 and phenylhydrazine under acidic conditions. Removal of the benzyl protecting groups of 7–9 to obtain phenols 10–12 was achieved by hydrogenation, using palladium on charcoal as catalyst. In addition, esters 13 and 14 were prepared from phenol 10: for 13, with 4-methylthiazole-5-carboxylic acid using 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) as coupling reagent, and for 14, from 1*H*-imidazole-4-carboxylic acid using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium tetrafluoroborate (TBTU). Moreover, the benzyl protected indoles 7–9 were reacted with different haloalkanes using NaH as the base in *N,N*-dimethylformamide (DMF), to give *N*-alkylated indoles 15–25, which were deprotected by catalytic hydrogenation to obtain the final compounds 26, 27, and 29–37 (Scheme 2). Carboxylic acid derivative 28 was obtained by alkaline hydrolysis of ester 27. In addition, methylation of 37 with methyl iodide in the presence of cesium carbonate gave ether 38.

Scheme 2. Synthesis of Compounds 26–38^a

^aReagents and conditions: (a) for **15**, **18**, **23**: 1-bromopropane; for **16**: ethyl bromoacetate; for **17**, **21**, **25**: (4-bromobutoxy)benzene; for **20**, **24**: (3-bromopropoxy)benzene; for **19**: (2-bromoethoxy)benzene; for **22**: methyl iodide; NaH, DMF, r.t., 3 h; (b) H_2 , 10% Pd/C, THF/EtOH, r.t., 18 h; (c) 1 M NaOH; (d) methyl iodide, Cs_2CO_3 , acetone, 60 °C, 48 h.

The antiproliferative effect exerted by the new compounds **10–14** and **26–38** and by the well-known inhibitors of topo II *m*-amsacrine (*m*-AMSA) and quercetin was assayed on three human tumor cell lines: HeLa, A2780, and MSTO-211H. The results are expressed as GI_{50} values, i.e. the concentration (μM) of compound able to inhibit the growth in 50% of cells with respect to a control culture. The obtained data are shown in Table 1.

Most of the tested compounds had significant antiproliferative effect on the selected cell lines, showing GI_{50} values ranging from 2.0 to 18 μM . In general, the analogues **10–14**, with the unsubstituted R^3 ($R^3 = H$), showed a rather low antiproliferative effect. Nevertheless, their GI_{50} values allowed us to draw some preliminary structure–activity relationships. In particular, it can be seen that **10** (unsubstituted at R^1 and R^2 ; hydroxyl group at R^4) did not have any effect at the tested concentrations. The introduction of a hydroxyl group at R^2 (**11**) induced a small increase in antiproliferative activity against HeLa and A2780 cells, while the insertion of a hydroxyl group at both R^1 and R^2 (**12**) resulted in a GI_{50} value of 2.2 μM on A2780 cells. Finally, the data obtained for compounds **13** and **14** suggest that large arylcarboxy substituents at R^4 do not improve the cell effect.

As regards compounds **26–38**, characterized by alkyl substituents at R^3 , they appeared generally more potent than **10–14**. This can be seen by comparing the cell growth inhibition data of **10** ($GI_{50} > 20 \mu M$, on all tested cell lines) with those of **26**, **27**, and **29**. Interestingly, **28** with a carboxymethylene substituent at R^3 was not active ($GI_{50} > 20 \mu M$), which indicated that introduction of groups with low pK_a is detrimental for cellular activity. Similarly as for **10**, **26**, **27**, and **29**, also the activity of **12** (R^1 , R^2 , and $R^4 = OH$) on HeLa and MSTO-211H cells increased when alkyl substituents were introduced at R^3 (**12**, $GI_{50} > 20 \mu M$; **34–37**, GI_{50} from 3.4 to 18 μM). On the other hand, the activity of **12** on A2780 cells ($GI_{50} = 2.2 \mu M$) was higher compared to that of **34** (methyl group at R^3 ; $GI_{50} = 14 \mu M$), **35** (propyl group at R^3 ; $GI_{50} = 8.4 \mu M$), **36** (phenoxypropyl group at R^3 ; $GI_{50} = 7.4 \mu M$), and **37** (phenoxybutyl group at R^3 ; $GI_{50} = 4.3 \mu M$).

The compounds with a hydroxyl group at R^2 (**11**, **12**, **30–37**) were generally more potent than those with unsubstituted

R^2 (**10**, **13**, **26–29**), as demonstrated by the comparison between **26** (unsubstituted R^2) and **30** (hydroxyl group at R^2). A similar behavior can also be observed for **29** (unsubstituted R^2) with respect to **33**, even if the differences in GI_{50} values appeared notably reduced.

The presence of an OH group at R^1 had unfavorable effects on the antiproliferative activity as demonstrated by the higher potencies of **30** ($R^1 = H$) versus **35** ($R^1 = OH$), **32** ($R^1 = H$) versus **36** ($R^1 = OH$), and **33** ($R^1 = H$) versus **37** ($R^1 = OH$). The good antiproliferative profile of **30** ($GI_{50} = 7.9 \mu M$, 2.3 μM and 2.9 μM for HeLa, A2780 and MSTO-211H, respectively) was further improved by the addition of a phenoxy group to the propyl side chain of R^3 , to obtain the 3-phenoxypropyl derivative **32**. Promising activity was maintained even when the length of the alkyl linker was increased from propylene to butylene, as for **33**. The GI_{50} values of **32** and **33** were in the low micromolar range for all cell lines tested, and these values were the lowest among all of the studied analogues, thus highlighting **32** and **33** as the most promising compounds of the series. The comparison between the antiproliferative activities of **32** (3-phenoxypropyl substituent at R^3) and **31** (3-phenoxyethyl substituent at R^3) showed a significant decrease in activity for the latter in all tested cell lines. This result indicates that the appropriate length of the side chain at R^3 is crucial for the biological effects.

Finally, alkylation of the hydroxyl groups of **37** to obtain the trimethoxy derivative **38** resulted in a significant decrease in the antiproliferative effect, which indicates the possibility of hydrogen bonding between the OH groups and the biological target.

Based on the structural similarities of the prepared compounds with some flavonoid inhibitors of topo II (Figure 1B) and with the aim to investigate the molecular mechanism responsible for the antiproliferative activity, we evaluated the effect of the most interesting analogues on the relaxation of supercoiled plasmid DNA catalyzed by topo II.^{22,23}

In the series of compounds with unsubstituted R^3 (**10–14**), the activities of the most potent derivatives, **11** and **12**, were analyzed and *m*-AMSA (Figure 2) and quercetin (Figure 1 SI) were used as references.

Table 1. Chemical Structures and Cell Growth Inhibition in the Presence of Compounds 10–14 and 26–38 and of *m*-AMSA and Quercetin as Reference

Compd.	R ¹	R ²	R ³	R ⁴	HeLa	A2780	MSTO-211H
10	H	H	H	OH	>20	>20	>20
11	H	OH	H	OH	16±2	11±2	>20
12	OH	OH	H	OH	>20	2.2±0.5	>20
13	H	H	H		>20	13±1	>20
14	H	H	H		>20	>20	>20
26	H	H	CH ₂ CH ₂ CH ₃	OH	>20	9.0±2.2	7.1±1.4
27	H	H	CH ₂ COOCH ₂ CH ₃	OH	>20	13±1	>20
28	H	H	CH ₂ COOH	OH	>20	>20	>20
29	H	H	CH ₂ CH ₂ CH ₂ CH ₂ OPh	OH	11±2	3.1±0.6	3.3±0.5
30	H	OH	CH ₂ CH ₂ CH ₃	OH	7.9±1.9	2.3±0.6	2.9±0.8
31	H	OH	CH ₂ CH ₂ OPh	OH	15±0.3	4.7±0.9	7.6±1.2
32	H	OH	CH ₂ CH ₂ CH ₂ OPh	OH	4.4±1.0	2.2±0.4	2.4±0.4
33	H	OH	CH ₂ CH ₂ CH ₂ CH ₂ OPh	OH	4.0±0.6	2.0±0.6	2.9±0.2
34	OH	OH	CH ₃	OH	>20	14±3	>20
35	OH	OH	CH ₂ CH ₂ CH ₃	OH	>20	8.4±1.3	>20
36 ^b	OH	OH	CH ₂ CH ₂ CH ₂ OPh	OH	18±1	7.4±0.7	7.1±1.2
37	OH	OH	CH ₂ CH ₂ CH ₂ CH ₂ OPh	OH	10±1	4.3±1.3	3.4±0.9
38	OCH ₃	OCH ₃	CH ₂ CH ₂ CH ₂ CH ₂ OPh	OCH ₃	>20	17±4	13±2
<i>m</i> -AMSA ^c					0.031±0.016	0.027±0.005	0.019±0.001
Quercetin ^c					17.7±0.9	19.7±2.0	>20

^aValues are the mean ± SD of at least three independent experiments. ^bCompound I. ^cReference compounds.

Both **11** and **12** are able to inhibit topo II relaxation activity at 100 μM concentration (Figure 2A). In detail, compound **12** showed an electrophoretic pattern with almost solely supercoiled DNA, which suggests that it completely inhibited topo II activity. For compound **11**, as well as the presence of supercoiled DNA, some topoisomers were formed, thus suggesting a slightly lower inhibitory activity. To further confirm these effects, topo II was incubated in the presence of **11** and **12** at 25 μM and 50 μM (Figure 2B). At the lower concentration, only **12** inhibited the activity of topo II, while **11** had no measurable effect. Interestingly, these results correlate with the antiproliferative effects on A2780 cells,

where **11** showed a GI₅₀ value (GI₅₀ = 11 μM) about 5 times higher with respect to **12** (GI₅₀ = 2.2 μM) (Table 1).

As regards the compounds having alkyl substituents at the R³ position (**26–38**), the effects on topo II activity of 100 μM **26–29** (unsubstituted at R¹ and R²) are shown in Figure 3. For **26–28**, the electrophoretic patterns appeared comparable to that of topo II alone, with a series of topoisomers as a result of the enzyme relaxation activity. On the other hand, as well as a weak formation of topoisomers, in the presence of **29**, a strong band corresponding to supercoiled DNA also appears, indicative of an inhibitory effect, and in agreement with the antiproliferative data shown in Table 1.

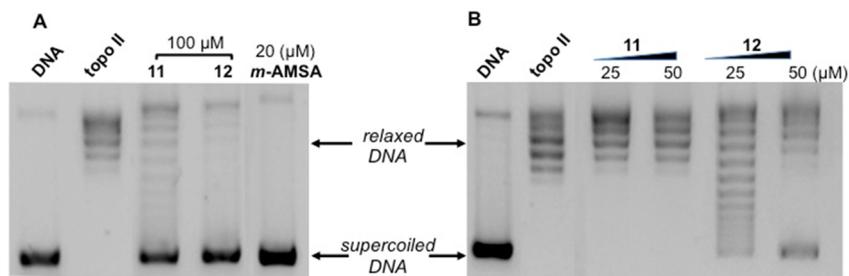


Figure 2. Effect of compounds 11 and 12 on relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II. Supercoiled DNA (DNA) was incubated with the enzyme in the absence (topo II) and in the presence of 11 and 12 at 100 μM (A) or 25 μM and 50 μM (B). Twenty μM *m*-AMSA was used as reference.

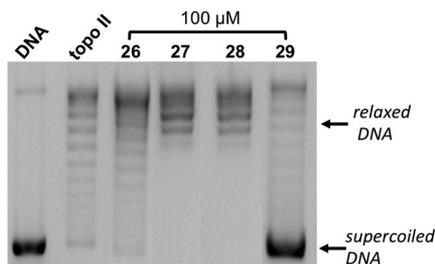


Figure 3. Effect of compounds 26–29 on relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II. Supercoiled DNA (DNA) was incubated with topo II in the absence (topo II) and in the presence of 26–29 at 100 μM .

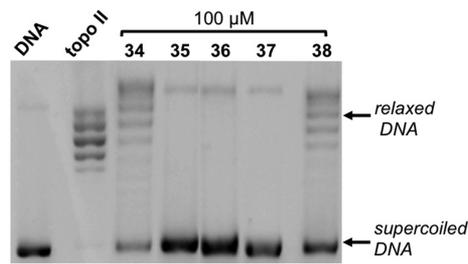


Figure 5. Effect of compounds 34–38 on the relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II. Supercoiled DNA (DNA) was incubated with topo II in the absence (topo II) and in the presence of 34–38 at 100 μM .

Compounds 30–33 (hydroxyl substituent at R²) were characterized by low micromolar GI₅₀ values (Table 1), and actually they showed significant topo II inhibitory activity at 100 μM concentration (Figure 4A). In particular, in these experimental conditions, for 30, 32, and 33 a marked band of supercoiled DNA was observed. For 31, a slightly lower inhibitory activity was detected, in agreement with its lower antiproliferative effect with respect to 30, 32, and 33 (Table 1). In addition, incubation of the enzyme with 25 μM and 50 μM concentrations (Figure 4B) highlighted 32 as the most effective and 31 as the least effective of this group of compounds. Indeed, in the presence of 32 a complete block of DNA relaxation can be observed at 50 μM , while no effect was obtained in the presence of 31 at the same concentration.

Finally, topo II inhibition by 34–38, which showed intermediate antiproliferative profiles, was also investigated (Figure 5). Incubation of topo II in the presence of 100 μM 34–38 indicates compounds 34 and 38 as the less active derivatives of this series, in agreement with their relatively low antiproliferative effect, while a more pronounced inhibitory

activity is exerted by 35–37, according with their more significant inhibition of cell proliferation (Figure 5; Table 1).

The interesting biological profile of 32 prompted us to investigate more in depth its effects on cells, and in particular the mechanism involved in cell death. For this purpose, the most sensitive A2780 cells were treated with increasing concentrations of 32, incubated with the fluorescent probe JC-1, and then analyzed by flow cytometry, as previously described.²⁴ The green monomeric fluorescent dye JC-1 accumulates in the mitochondrial matrix driven by the membrane potential, negative inside. This uptake leads to an increase in JC-1 concentration in mitochondria that promotes the formation of aggregates, which are characterized by high red fluorescence. The activation of the apoptosis pathway can result in collapse of mitochondrial transmembrane potential and release of mitochondrial proapoptotic factors in the cytoplasm. This collapse allows the leakage of JC-1, with a consequent decrease in red fluorescence. Figure 6 shows that A2780 cells incubated in the presence of 32 underwent a concentration-dependent decrease of mitochondrial trans-

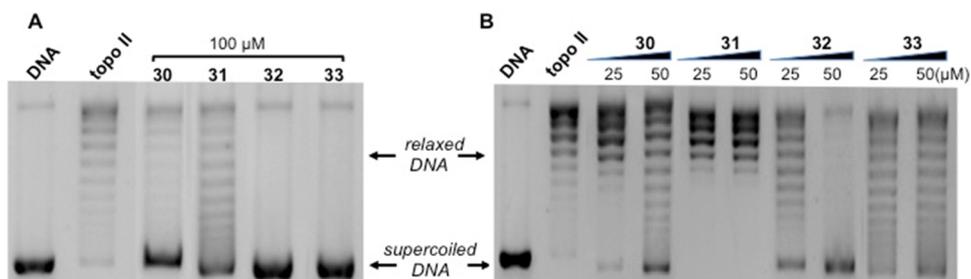


Figure 4. Effect of compounds 30–33 on the relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II. Supercoiled DNA (DNA) was incubated with topo II in the absence (topo II) and in the presence of 30–33 at 100 μM (A), or at 25 μM and 50 μM (B).

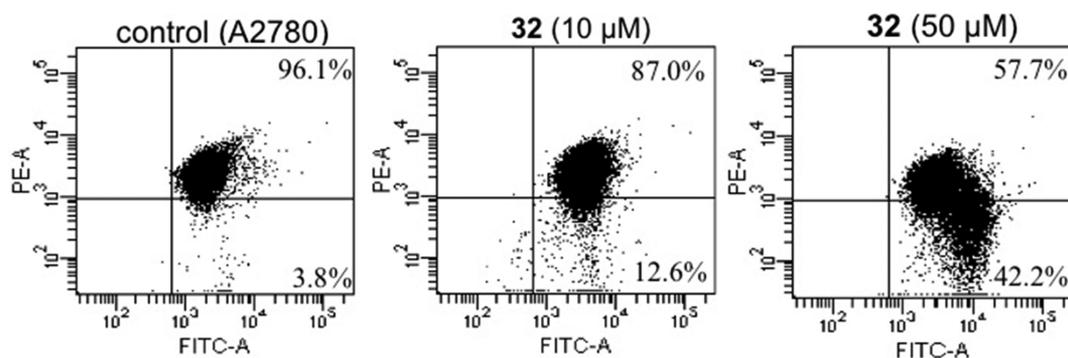


Figure 6. Mitochondrial membrane potential assessed by flow cytometry in A2780 cells after JC-1 staining. Cells were incubated for 28 h in the absence (control) and in the presence of compound 32 at the indicated concentrations. The percentages of cells with polarized (above) and depolarized (below) mitochondrial membranes are shown.

membrane potential, with about 40% of cells showing depolarized mitochondria at 50 μM concentration.

The ability of 32 to induce apoptosis was further confirmed by the analysis of hypodiploid cells stained with propidium iodide. Indeed, an important hallmark of apoptosis is the activation of endonucleases that cleave DNA at internucleosomal sites, thus creating a large number of small fragments of nucleic acid. Then, on DNA content histograms, apoptotic cells show DNA content as sub- G_0 or hypodiploid peak.^{25,26}

Figure 7 shows the results obtained in A2780 cells incubated with 10 μM and 50 μM 32, stained with propidium iodide and

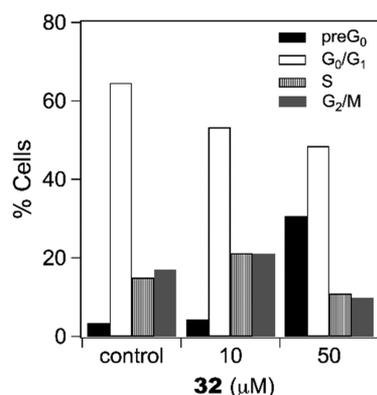


Figure 7. Flow cytometry analysis of A2780 cells treated for 28 h with compound 32 at the indicated concentrations and stained with propidium iodide.

analyzed for DNA/cell content. Compound 32 induced a significant increase in the sub- G_0 phase, thus indicating apoptotic events in about 30% of cells treated with the higher concentration (Figure 7).

A series of 3-methyl-2-phenyl-1H-indoles (10–14 and 26–38) was prepared and evaluated for the antiproliferative effect on three human tumor cell lines, HeLa, A2780, and MSTO-211H. The role of different substituents on the two aromatic moieties of these compounds was discussed. In particular, compounds with an unsubstituted R^1 were generally more potent than those with a hydroxyl or a methoxy group at R^1 . On the other hand, at R^2 and R^4 , hydroxyl groups were generally preferred. The most active compounds contained alkyl or phenoxyalkyl substituents at R^3 , with the size of these substituents important for the activity. The most interesting compound was 32, showing GI_{50} values of 4.4 μM , 2.2 μM ,

and 2.4 μM on HeLa, A2780, and MSTO-211H cell lines, respectively. Notably, for six compounds (12, 29–31, 33, 37), GI_{50} values lower than 5 μM against at least one cell line were obtained.

For selected compounds (11, 12, 26–38), the effect on the relaxation of supercoiled plasmid DNA catalyzed by topo II was also measured. The obtained results highlighted a good correlation between the inhibition of enzyme and the antiproliferative effect. For the most promising compound 32, cytofluorimetric analysis showed the ability to induce a concentration-dependent collapse of mitochondrial transmembrane potential. Moreover, a significant increase in the sub- G_0 phase was measured in cells incubated with 32, confirming the occurrence of apoptosis. Overall, these data highlight 3-methyl-2-phenyl-1H-indole as a promising scaffold for the design of compounds with significant antiproliferative and antitopo II activities and provide new ideas for further optimization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.9b00557>.

General information—chemistry; synthetic procedures and analytical data; ^1H and ^{13}C spectra of representative compounds; materials and methods—biological studies; Figure 1 SI: Effect of quercetin on the relaxation of supercoiled pBR322 DNA by human recombinant topoisomerase II (topo II); references (PDF)

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Author Contributions

The manuscript was written using contributions from all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DMF, *N,N*-dimethylformamide; DIAD, diisopropylazodicarboxylate; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; EDTA, ethylenediaminetetraacetic acid; *m*-AMSA, *meta*-amsacrine; NMM, *N*-methylmorpholine; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; THF, tetrahydrofuran; topo II, human DNA topoisomerase II

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