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FULL PAPER

Inhibition of human Topoisomerase II by new *N,N,N*-trimethylethanammonium iodide alkylcarbazole derivatives

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Abstract: Chemotherapy is used for treatment of all stages of breast cancer, including the metastatic disease and tends to be tailored for each person situation. However, chemotherapeutic agents are the leading cause of serious drug-related adverse effects and, often, resistance occurs. In this paper, we designed and synthesized a new series of *N*-alkylcarbazole derived by Ellipticine, an alcaloid with carbazole structure initially used in the treatment of metastatic breast cancer and then dismissed because of poor solubility in water and dramatic side effects. After the evaluation of the binding modes of our class of newly synthesized compounds with human Topoisomerase II (hTopo II) we performed hTopo II decatenation assay, obtaining that compound **4f** is a good inhibitor. Moreover, **4f** and **4g** showed a good anti-proliferative activity on breast cancer cells, causing apoptosis by activation of the caspases pathway. Interesting is the activity of these two compounds on triple negative MDA-MB-231 cells, which tend to be highly metastatic and aggressive, strictly connected with the observed inhibition of hTopo II.

Introduction

In the last few decades cancer, a multifactorial disease characterized by uncontrolled growth and invasion of abnormal cells, has emerged as one of the most alarming disease in the world. Particularly, breast cancer has become the second leading life-threatening disease for women accounting for high mortality rates after lung cancer [1]. It is a kind of malignant tumor that affects, with an overwhelming incidence, young women with a high mortality rate due, in several cases, to the development of resistance against chemotherapeutics [2]. In fact, breast cancer in younger women is often characterized by aggressive tumor subtypes that are less likely to be amenable to treatment at the time of diagnosis and have poorer survival outcomes than older women [3-4]. Therefore, the poor response to chemotherapy and the adverse effects of treatments currently used drives the search towards the development of new chemopreventive agents able to eradicate the cancer and with reduced undesirable side effects. Among candidates for treating breast cancer, heterocyclic compounds represent a remarkable type of anticancer drug candidates.

Carbazoles are an important class of indole-containing heterocycles with antitumor activities but, as well, antibacterial [5], anti-inflammatory, psychotropic, and anti-histamine properties [6]. Some derivatives showed interesting pharmaceutical properties interfering, as well, with DNA-dependent enzymes, as topoisomerases I/II and telomerase, or with other targets such as cyclin-dependent kinases and estrogen receptors and, for these reasons, they occupied an important place as antitumoral tools in preclinical and clinical trials [7-13].

Of particular interest the interaction with DNA or its metabolizing enzymes that induce the programmed cell death, namely apoptosis [14-18].

In particular, several analogues of carbazole derivatives structurally correlated to the Ellipticine, alcaloid from *Ochrosia elliptica* labil [19] introduced in the treatment of metastatic breast cancer [6, 20-23] and then dismissed because of poor solubility in water and dramatic side effects [24-25], were synthesized [26-30].

Trimethoxyphenylurea 1,4-dimethylcarbazoles [31], *N*-thioalkylcarbazoles [15], 1,4 dimethy-*N*-alkylcarbazoles [32], *N*-(1,4-dimethyl-9*H*-carbazol-3-yl)-*N'*-alkylguanidines [33], benzofuro [2, 3 -f]quinazolin-1 (2*H*)-ones [34] and hydrazine-carbazole derivatives [20] were also prepared and some of them showed an interesting anti-proliferative activity against breast cancer cells without affecting non-tumoral cell lines viability.

Several studies have reported that carbazole derivatives, as Ellipticine and Celiptium (Figure 1), are inhibitors of human Topoisomerases I and II [35-37].

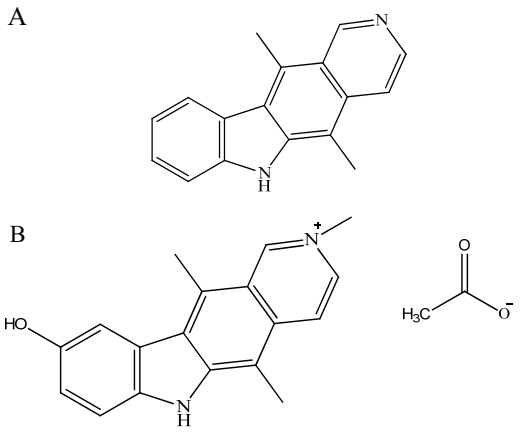
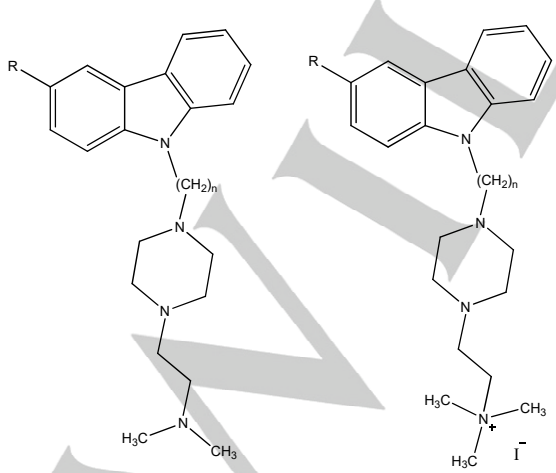


Figure 1. Structures of Ellipticine (A) and Celiptium (B).

hTopo I and II are essential enzymes, which play a crucial role during DNA transcription and replication and are over-expressed in tumor cells representing thus a valid target for the development of new molecules for the treatment of a wide spectrum of tumors [38-39]. In particular, the principal mechanism of Ellipticine's cytotoxicity is related to the inhibition of topoisomerase II, however it may bind to proteins [40], induce free radicals generation [41], inhibit cytochrome P4501A1 [42], uncouple oxidative phosphorylation [43], activate the transcription function of mutant p53 [44] and induce endoplasmic reticulum stress [45]. Recently some *N*-substituted carbazole derivatives, correlated to Ellipticine, have been prepared and investigated for their cytotoxic activity against different tumoral cell lines, revealing a remarkable induction of cell cycle arrest and apoptosis [46-48].
In this study, a new serie of *N*-alkylcarbazole derivatives and their quaternary ammonium iodide salts were designed, synthesized (Figure 2) and studied for their ability to inhibit human topoisomerase II by *in silico* and *in vitro* studies. Moreover, their antiproliferative activity was evaluated on two breast cancer cells lines (MCF-7 and MDA-MB-231).



R= H; n= 3,4,5,6,7
R= Cl; n= 5,6

Figure 2. General structure of *N*-alkylcarbazole derivatives and their quaternary ammonium iodide salts synthetized.

Solubility of synthesized compounds was improved through the formation of salts, as in the case of 9-hydroxyellipticine (Celiptium) which possesses a higher DNA affinity than Ellipticine and lacks of toxicity at therapeutic doses [49-51].
Some of these compounds have showed a noticeable cytotoxic activity against the breast tumoral cells, due to the inhibition of human Topoisomerase II and the activation of the apoptotic cell death. Overall, these compounds may be considered new and important tools for the exploration of the signaling pathways involved in breast cancer growth and progression.

Results and Discussion

Docking studies

First, in order to evaluate the binding modes and affinities between our class of newly synthesized compounds and hTopo II-Alpha, we performed molecular docking simulations using as ligands all the moieties synthesized. Their binding modes are reported in Figure 3. Using a "blind-docking approach" (no "a priori" information about the binding site was provided to the system) we discovered that all the compounds bind the ATPase domain, in proximity to the ATP binding site. Particularly, compounds bind to the same site of hTopo IIa positioning their carbazole moieties within a mainly hydrophobic pocket composed by residues Ile 125, Val 132, Leu 140, Ile 141, Thr 147, and forming a π-stacking with the aromatic ring of Tyr 151. Aminoacids involved in hydrogen bonds are Ser 149, Asp 152, Asp 154 and Glu 155. The compounds are posed in an almost identical orientation, with the exception of compound **4g**, which carbazole group is displaced of about 7Å towards the protein domain core occupying, therefore, the ATP catalytic site and interacting with residues Asn 91, Ala 92, Asp 94, Asn 95, Ile 141, Phe 142, Ser 148, Ala 167, Thr215 and Ile 217. The binding modes of all the other compounds tested *in silico*, however, do not seem to occupy directly the ATP binding cleft; the inhibitory activity of these molecules may be due to preventing the formation of the hTopo IIa homodimer by binding to an area that is usually occupied by the *N*-terminal helices of the ATPase domain (Figure 3, panels I and J) in the functional complex. Through our "in silico" screening, we identified compounds **3c**, **4c**, **3d**, **4d**, **3f**, **4f, 3g** and **4g** as good candidates to be tested *in vitro*, because they present good binding energies and a favorable clusterization of the simulation results. The lack of a significant *in vitro* inhibitory activity of **3c**, **4c**, **3d** and **4d**, however, may be due to the absence of the chlorine moiety, which is probably useful in stabilizing the interaction between the compounds and the protein. Interestingly, the carbazole moiety of **3f** and **4f** are positioned within the hydrophobic cleft described above, in different orientations being the two rings rotated of about 180 degrees. In this way, the chlorine atom in **4f** points towards the ATP binding cleft in hydrophobic contact with protein residues Ile125, His130, Val137, Leu140 and Ile141 and maintaining the polar bonds with Ser 149, Asp 152 and Glu 155. In the case of **3f,** the chlorine moiety is pointing outside the protein, towards the

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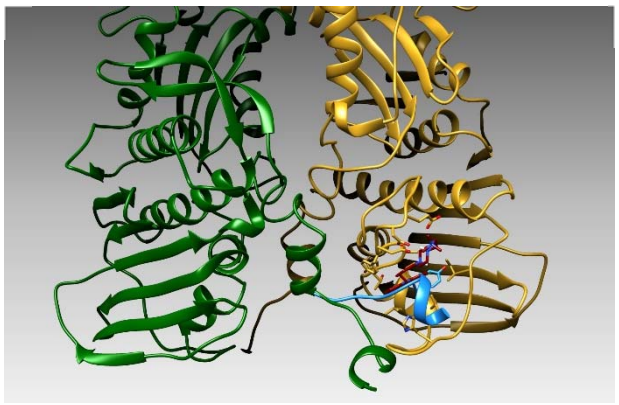

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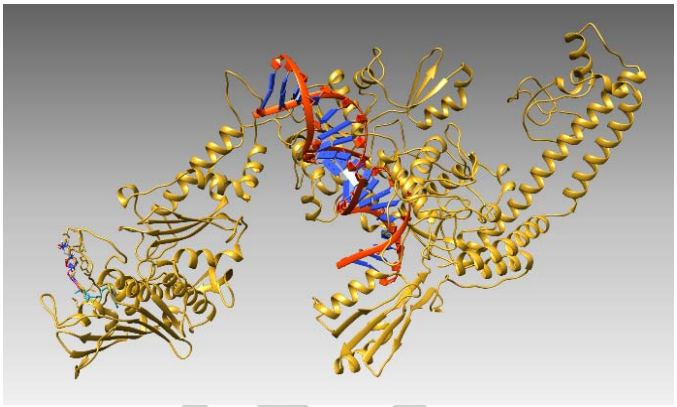

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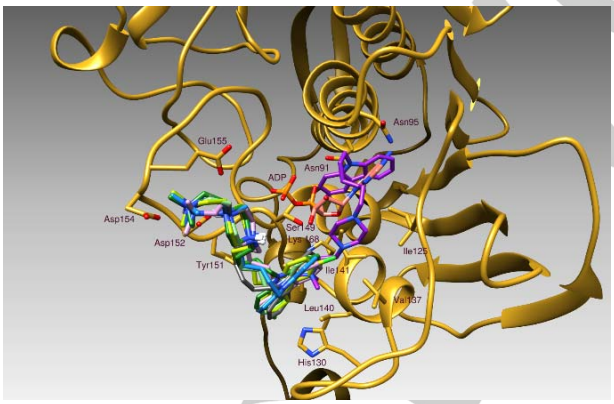

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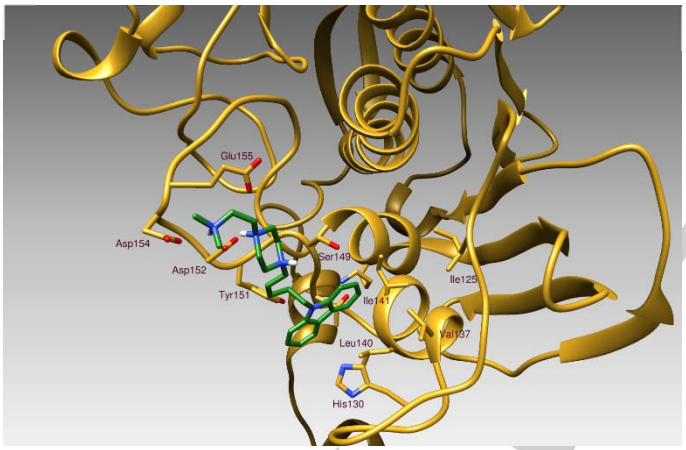

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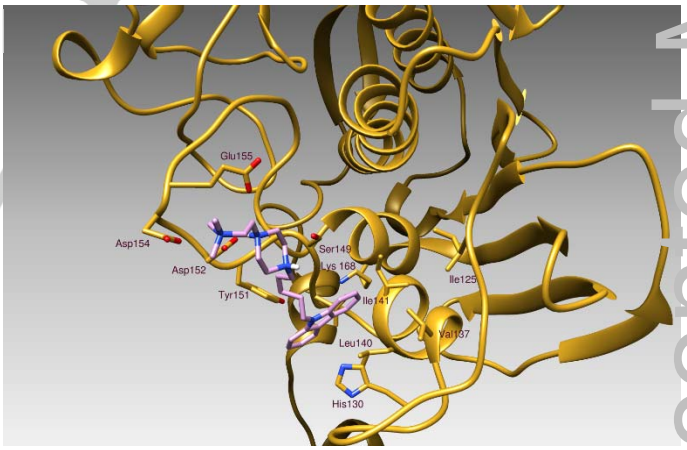

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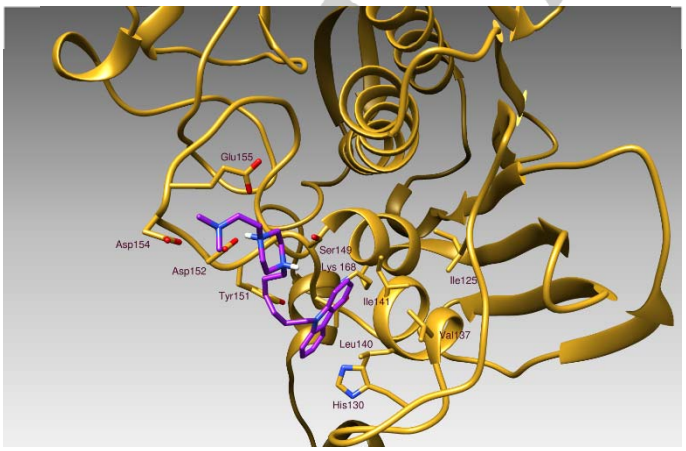

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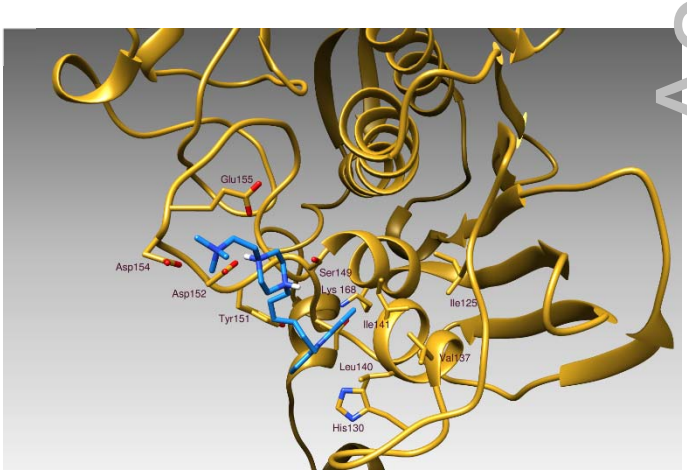


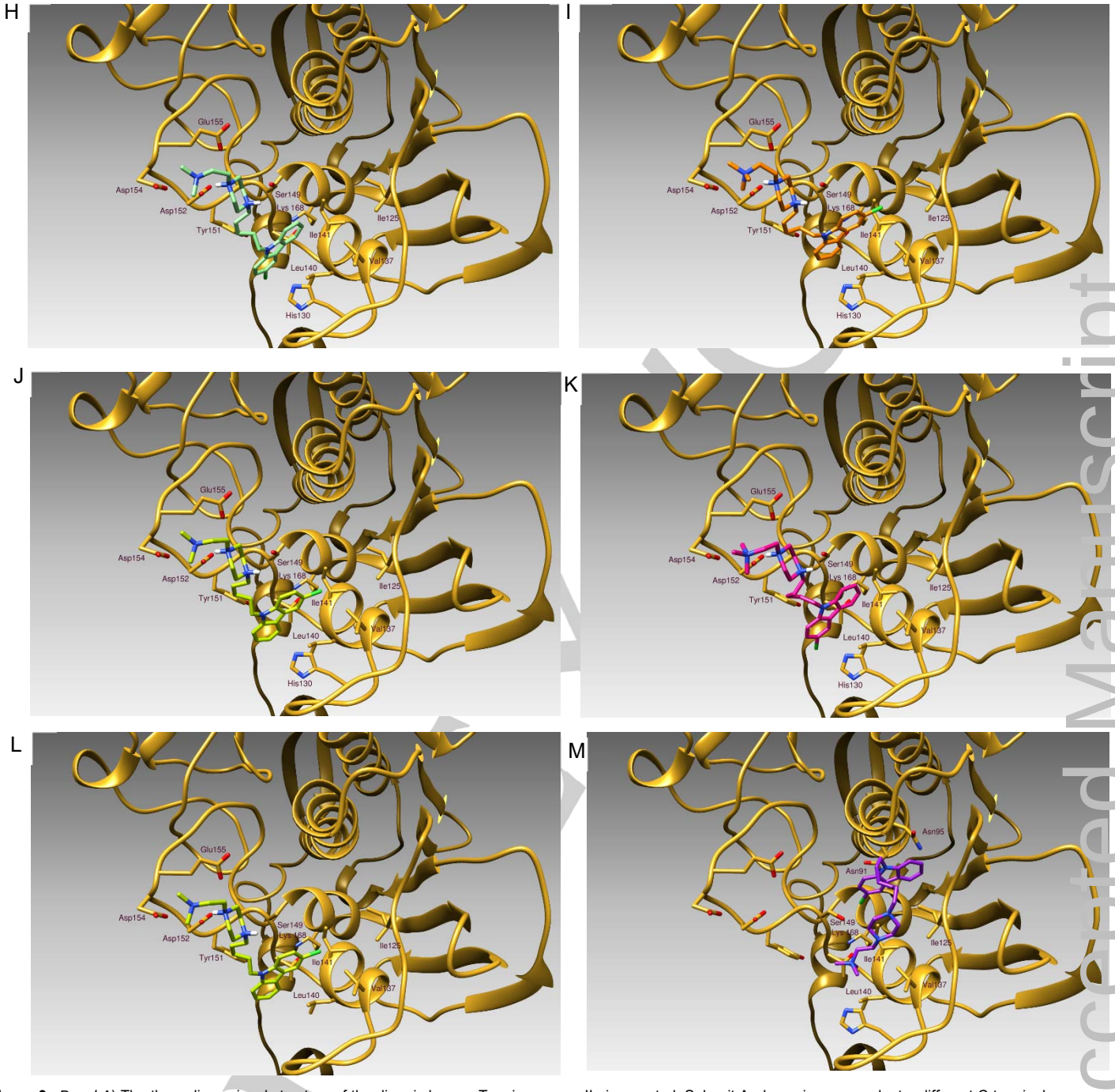
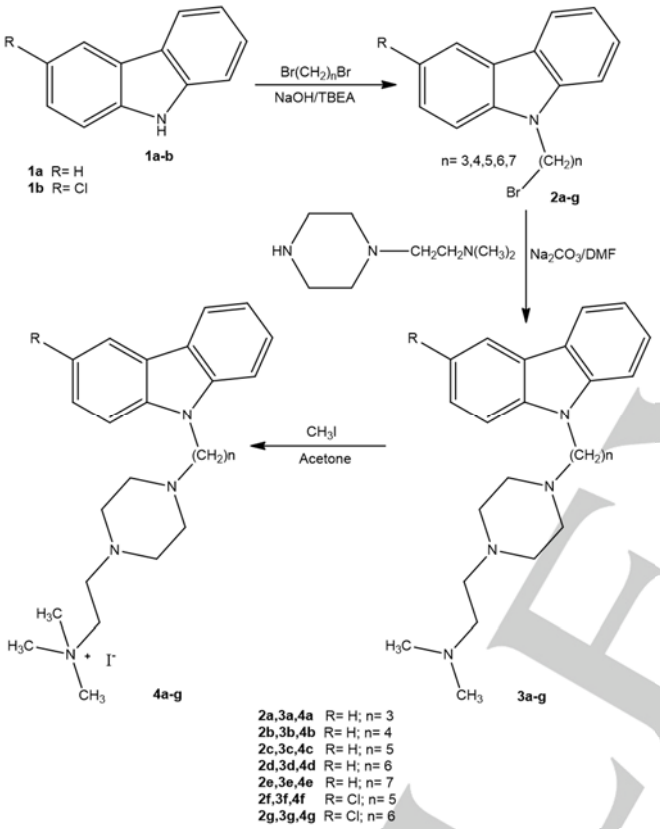


Figure 3. *Panel A*) The three dimensional structure of the dimeric human Topoisomerase IIa is reported. Subunit A, drawn in green, adopt a different C-terminal conformation according to the presence (or absence) of the ligand in its binding site. When in the dimeric conformation, the C-terminal alpha-helix (light blue) is positioned inside a cleft of the b subunit, close to the ATP binding site, interacting with different residues, thus conferring stability to the dimeric structure. When the ligand is bound, the C-term helix is pointing out (green helix) and the formation of the oligomer is prevented. *Panel B*) represents a model of the human Topoisomerase IIa, bound to a DNA fragment. On the left of the figure, the ATP binding domain. *Panel C*) different molecules of this series in their binding mode as forecasted by docking simulations. The crystallographic position of an ADP molecule is reported as reference. *Panel D-K* binding modes of the *N*-alkylcarbazole derivatives as determined by docking simulations. *Panel D*) molecule **3c**, *Panel E*) **4c**, *Panel F*) **3d**, *Panel G*) **4d**, *Panel H*) **3f**, *Panel I*) **4f**, *Panel J*) **3g**, *Panel K*) **4g**, *panel L*) **3g**, *Panel M*) **4g** (with the carbazole occupying the Adenine binding site).

solvent, where the carbazole is in stacking with Tyr 151 and forms hydrophobic contacts with Val132, Leu140 and Thr 147. Compound **4f**, therefore, is supposed to have a better inhibitory activity than **3f** on hTopo IIa *in vitro* due to its minor exposition to the solvent molecules.

Chemistry

The *N*-alkylcarbazole derivatives (**3a-g** and **4a-g**) were achieved from the carbazole (**1a**) for **3a-e** and **4a-e** and from the 3-chloro-9*H*-carbazole (**1b**), commercially available, for **3f-g** and **4f-g**, in two steps of reaction for **3a-g** and in three steps for **4a-g** as shown in Scheme 1.



Scheme 1. Synthetic route for the preparation of **3a-g** and **4a-g** compounds.

The first step was the *N*-alkylation of the carbazole **1a** or **1b** with 1,3-dibromopropane or 1,4-dibromobutane or 1,5-dibromopentane or 1,6-dibromohexane or 1,7-dibromoheptane to obtain the intermediates **2a-g** respectively. These *N*-alkylated derivatives, in the second step were reacted to 1-[2-(dimethylamino) ethyl]piperazine to give the (9*H*-carbazol-9-yl)piperazin-1-yl)-*N,N*-dimethylethanamine derivatives (**3a-g**). Lastly, the compounds **3a-g,** in the third step were reacted with excess methyl iodide in acetone, the quaternary ammonium iodide derivatives **4a-g** were formed. All synthesized compounds were characterized by mass spectrometry, NMR analysis and the elemental analysis carried out on them, are in agreement with the proposed formulations.

Biology

Antitumor properties of *N*-alkylcarbazole derivatives

Once established, by *in silico* studies, that **4f** is the best hTopo II inhibitor out of our N-alkylcarbazole derivatives series (and with the knowledge that they could act not only as hTopo II inhibitors but, as well, they could interfere with other cellular metabolic pathways [6]), we screened the entire series to determine their antitumor properties in two models of breast cancer (namely the human ERα positive MCF-7 and the triple negative MDA-MB-231 cells). As evidenced in table 1, reporting the IC_{50} values calculated as described in the experimental section, six compounds (*i.e.* **3a**, **4a**, **3b, 4b, 3e** and **4e**) did not elicit inhibitory effects, whereas the four compounds **3c**, **4c**, **3d** and **4d** displayed a very poor antitumor activity on both the two breast cancer cell lines. A noticeable improvement has been recorded using compounds **3f**, **4f**, **3g** and **4g**. Particularly, compounds **4f** and **4g** exhibited the best antitumor properties, and between the two models of breast cancer we used in our experiments, the MDA-MB-231 cells resulted more sensitive to the treatment. We suppose that this peculiarity may be correlated with the higher duplication rate of MDA-MB-231 (a cell line that is aggressive and metastatic) with respect to MCF-7 cells. For this reason MDA-MB-231 cells need a faster DNA synthesis to proceed through mitosis, thus they must possess a very effective duplication machinery, including high levels of topoisomerases. Therefore, it is reasonable that, being the compound **4f** the best hTopo II inhibitor out of the series, it displays the antitumor activity mostly against MDA-MB-231 cells. Finally, it should be noted that the active compounds do not hamper the viability of the normal breast cells (MCF-10A), whereas Ellipticine, used as reference molecule, exerted dramatic effects.

Table 1. IC_{50} values of carbazole derivatives (**3a-g**, **4a-g**) and Ellipticine, expressed in micromolar (μM).

	MCF-7	MDA-MB-231	MCF-10A°
Ellipticine	1.35 ± 0.30	1.75 ± 0.15	1.50 ± 0.15
3a	>500	>500	>500
4a	>500	>500	>500
3b	>500	>500	>500
4b	>500	>500	>500
3c	357.0± 1.20	296.5± 1.10	> 500
4c	314.2± 0.90	270.3± 1.25	> 500
3d	381.0± 1.12	257.2± 1.35	> 500
4d	388.8± 1.27	221.9± 0.95	> 500
3e	>500	>500	>500
4e	>500	>500	>500
3f	35.9± 0.55	12.4± 0.75	> 500
4f	10.2± 0.45	4.4± 0.20	> 500
3g	77.4± 0.35	17.2± 0.25	>500
4g	20.4± 0.22	5.8± 0.28	> 500

Topoisomerase II inhibition assay and cells death by apoptosis induced by 4f.

Topoisomerase II (TopoII) is a nuclear enzyme that catalyzes DNA topological reactions via a DNA breakage/reunion mechanism and it essential to resolve topological problems that occur during DNA transcription, replication and chromosome segregation[52]. For these reasons, TopoII has become one of the most promising target in the anticancer drugs discovery field. Several studies demonstrated that carbazole derivatives show a good inhibitor TopoII activity[17, 53-54]. Given that the *in silico* studies results suggested interesting interactions with the hTopoII, we performed, in parallel, a direct enzymatic assay on all the synthetized series in order to verify the former hypotheses and establish whether our compounds are human topoisomerase II inhibitors or not. In particular, at a dose of 10μM, as indicated in figure 4 (panels A and B) the ten compounds **3a**, **4a**, **3b**, **4b**, **3c**, **4c**, **3d**, **4d, 3e** and **4e** were unable to block hTopo II enzymatic activity given that the enzyme binds its substrate (kDNA) and releases intact monomeric rings visible at the bottom of gel as two bands, representing the nicked open circular minicircles and fully closed circular rings (decatenation products). This happens, clearly, in the control experiment where the vehicle (DMSO) was added instead of the testing compounds (fig. 4, lane C) and hTopo II retains is full activity. Conversely, compound **3g** (10μM, fig.4, panel B) was able to partially inhibits hTopo II activity; indeed, two faint DNA bands are visible at the bottom of the gel but one band appears at the top of agarose gel, indicating that the enzyme is unable to fully cut the kDNA, which big size prevents the migration on agarose gel. hTopo II inhibition increases in the case of compounds **4g** and **3f**, (10μM, fig.4, panel B), which differs from compound **3g** for the presence of the quaternary ammonium iodide and the length of the alkyl chain (connecting carbazole and piperazine moieties) respectively. Finally, a total blockade of hTopo II activity was achieved exposing the enzyme to compound **4f** (10μM, fig. 4, panel B), which is the quaternary ammonium iodide derivative of compound **3f**; its inhibitory activity is evidenced by the disappearance of the bottom DNA bands and the presence of a clear kDNA band at the top of gel. We used, as control molecule, Ellipticine, which inhibitory activity on hTopoII is well established [15, 31, 34, 55-56] and, as expected, it is able to completely block hTopoII activity (panel C, lane E) but at a concentration five-fold higher with respect to the most active compound, **4f**. Overall, the obtained results well correlate with docking studies previously discussed, and indicate compound **4f** as the best candidate for poisoning hTopo II.

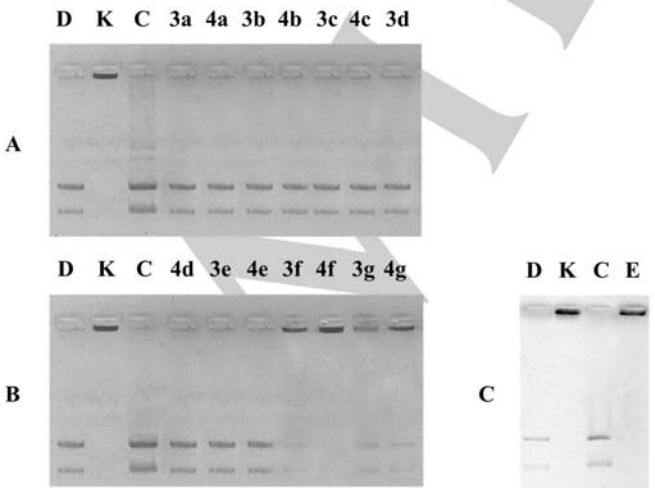


Figure 4. Human topoisomerase II decatenation assay**.** hTopo II has been incubated in absence (control) or presence of the compounds to test at 10μM for 1 h at 37 °C. The image is representative of three separated experiments. Panels A, B and C: D: decatenated DNA marker, K: Kinetoplast DNA; C: Control (DMSO); **3a-g** and **4a-g** assayed compounds, E, Ellipticine at 50μM.

It is well know that the biological functions of human topoisomerases are fundamental for insuring genomic integrity, thus a blockade of their activity results in DNA damage and, consequently, in cell death. This strategy has held the interest of researchers in cancer chemotherapy because many clinically active drugs that generate enzyme-mediated DNA damage as, for instance, topoisomerases targeting agents, were demonstrated to be effective, minimizing the risk of secondary malignancy and with low toxicity on the normal cells [57]. Moreover, DNA damage is one the stimuli that induce cell death by apoptosis, including nutrient depletion, heat shock and spindle disruption [11, 58], and is strictly related to the activation of one of the apoptosis pathways, *i.e.* the extrinsic and the intrinsic pathways [59].

Thus, we first performed a TUNEL assay with the aim to demonstrate that the observed hTopo II inhibition exerted by **4f** could induce MDA-MB-231 cells death by apoptosis. As evidenced in figure 5, the exposition of cancer cells for 24 h to a dose of 10 μM of **4f** is able to induce genomic DNA fragmentation, indicated with a green fluorescence (fig. 5, panel B, **4f**) that perfectly superposes with the blue fluorescence (fig. 5, panel C, **4f**) indicating cell nuclei. A control experiment, performed with the vehicle alone (DMSO) in the same conditions, did not produce the green fluorescence (panel B, CTRL), confirming that no DNA damage has occurred.

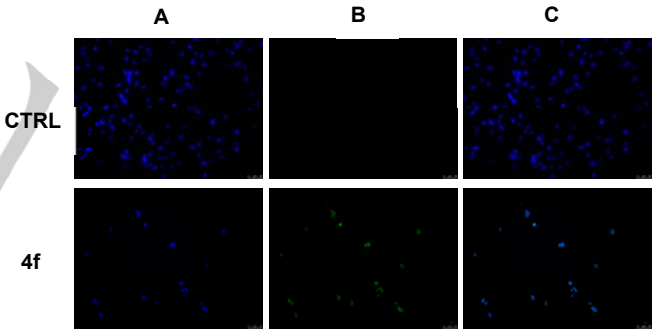


Figure 5. TUNEL assay. Apoptotic effect of **4f** used at 10 μM or vehicle (CTRL) on MDA-MB-231 cells determined after 24 hours of treatment. Panels A: DAPI (excitation/emission wavelength 350 nm/460nm), used to locate the nuclei of the cells; Panels B: CF^{TM}488 A (excitation/emission wavelength 490 nm/515nm), the green fluorescence indicates nuclei of cells undergoing apoptosis; Panels C: Shows the overlay channel.

Successively, we investigated which caspases pathways was involved using a cell-based luminescent assay. Particularly, we treated MDA-MB-231 cells for 24 hours with **4f** (or vehicle) at a concentration of 10 μM and then we evaluated the variation of caspases levels. Specifically, we measured caspase 8 and 9, which are involved respectively in the extrinsic and intrinsic pathway, and 3 and 7 (effectors or executioner caspases) that share the upstream activators and show some overlapping substrate specificity. Effectively, we noticed an increase of caspases 9 and 3/7 activity under **4f** treatment (fig. 6) with respect

to the control reaction, where cancer cells were added by the vehicle only (DMSO).

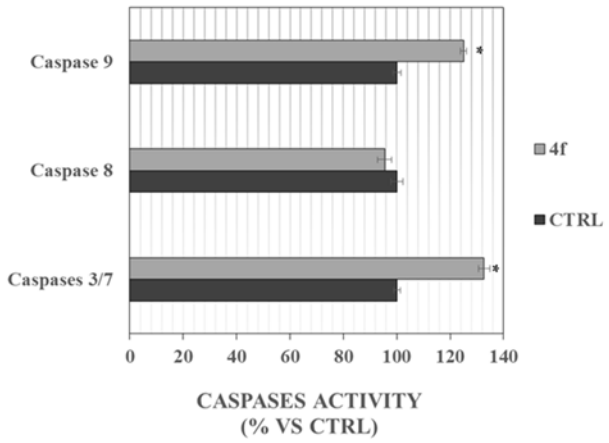


Figure 6. Caspases activity assay. The treatment of MDA-MB-231 cells with **4f** at the concentration of 10 μM for 24h caused the activation of caspases 3/7 and 9 (% over the CTRL, vehicle-treated cells). Columns mean, bars SD, *p<0.001.

Conclusions

A new series of *N,N,N*-trimethylethanammonium iodide alkylcarbazole derivatives has been designed and synthetized with the aim to test its hypothetical inhibitory effect on the human Topoisomerase II (hTopo II), first by *in silico* and then by *in vitro* studies. Amongst the whole series, four compound exhibited a noticeable inhibitory activity, being compound **4f** the most potent, even more that the reference molecule, Ellipticine. Moreover, the entire series has been screened against two models of breast cancer, finding out that the two compounds **3f** and **3g** possess a modest anticancer activity, whereas we noticed an improvement using **4g** and, mostly **4f**. These effects are more evident against MDA-MB-231 cells, which are more aggressive and metastatic and, they do not affect the proliferation of normal breast cells (MCF-10A). These observations strengthens our data regarding the inhibition of hTopo II, which blockade diminishes the uncontrolled duplication of these cells.

Experimental Section

Docking simulations

To preliminary screen the potential activity of these compounds against hTopo IIa "in silico", we performed docking simulations using the program Autodock (Goodsell et al., 1996) and its graphical interface ADT (Sanner et al, 1999). A single monomer of the dimeric crystallographic structure determined by X-ray crystallography of hTopo IIa in complex with AMP-PNP (Schmidt at al, 2012; PDB code 4GFH) was used as a target for the docking simulations. All the molecular structures of the ligands screened were built using the program Marvin (ChemAxon, www.chemaxon.com). The protein and the ligands were prepared using ADT. Polar hydrogens were added to the protein Kollman charged assigned and solvatation parameters added. The protein was considered as a rigid object while the ligands as fully flexible. A searching grid was extended all over the protein and affinity maps calculated. The search was carried out with a Lamarckian Genetic Algorithm: a population of 156 individuals with a mutation rate of 0.02 were evolved for 100 generations. Evaluation of the results was performed by listing the different ligand poses accordingly to the predicted binding energy. A cluster analysis based on root mean squares deviation values from the starting geometry was performed. The lowest energetic conformation of the most populated cluster was consider as the best candidate. When clusters are almost equipopulated and their energy distribution is spread, their corresponding molecules were considered as bad ligands [60-62].

Chemistry

All reagents and solvents were purchased from Sigma Aldrich s.r.l. (Milan, Italy). The reactions were monitored by thin-layer chromatography (TLC), using silica gel aluminum plates Whatman K6F and Allumina (Merck), with fluorescence indicators and opportune solvents. A >95% purity could be inferred from the ^{1}H-NMR spectra. Melting points were taken on a Gallenkamp melting point apparatus and were uncorrected. The organic extracts were dried over sodium sulphate dry (Merck). The elemental analyses for C, H, N, were performed according to standard microanalytical procedures. Molecular weights were determined by a JEOL JMS GCMate spectrometer at ionising potential of 70 eV (EI) or by ESI mass spectrometry. ESI-MS analysis in positive and negative ion mode, were made using a Finnigan LCQ ion trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the Excalibur software for processing the data acquired. The sample was dissolved in a mixture of acetonitrile and methanol (50/50) and injected directly into the electrospray source, using a syringe pump, which maintains constant flow at 5 ml/min. The temperature of the capillary was set at 220 °C. ^{1}H NMR and ^{13}C NMR spectra were recorded at 298 K on a Bruker Avance 300 MHz spectrometer, using $CDCl_3$ and referred to internal tetramethylsilane [63-64].

Synthesis of *N*-alkylcarbazoles (2a-g)

TBEA (benzyltriethylamonium bromide) (2.73 mmol), was dissolved in an aqueous solution of NaOH 50% p/p (15 ml) and toluene (20 ml) and stirred for 15 min. After was added a solution of a suitable carbazole (**1a**) or 3-chloro-9*H*-carbazole (**1b**) (5.45 mmol) in toluene (20 ml). To this mixture was added 1,3-dibromopropane or 1,4-dibromobutane or 1,5-dibromopentane or 1,6-dibromohexane or 1,7-dibromoheptane (16.3 mmol) and was stirred at reflux for 48 h. After the reaction, the solvent was evaporated, poured into water and extracted with chloroform (2 x 50 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent removed by evaporation. The resulting residue was purified on a silica gel column chromatography in petroleum ether/ethyl acetate (1/9) to give as white/yellow solid (67% yield) (**2a-g**)[47, 51, 65].

General method for the synthesis of compounds 3a-g

1-[2-(Dimethylamino)ethyl]piperazine (l.35 mmol) and Na_2CO_3 (2.7 mmol) were dissolved in DMF (10 ml). A solution of *N*-bromoalkyl-substituted carbazole (**2a-g**) (1.35 mmol) in DMF (10 ml) was added at room temperature. The mixture was stirred for 20 h. After the reaction, the solvent was evaporated, poured into water and extracted with chloroform (2 x 50 ml). The organic layer was dried over anhydrous $MgSO_4$, filtered and the solvent removed by evaporation. Final product (**3a-g**) was a solid (Scheme 1).

2-(4-((9H-carbazol-9-yl)propyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3a)****:* White powder (68 % yield). Mp = 108 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.60 (d, 1H, *Ar*); 7.46-7.27 (m, 4H, *Ar*); 2.55-2.50 (m, 4H, 2 CH_2); 2.49-2.45 (m, 8H, CH_2-Pyperazine); 2.35 (t, 2H, CH_2N); 2.34-2.29 (m, 4H, 2 CH_2); 2.26 (s, 6H, 2 CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 139.48, 125.65, 123.32, 121.35, 119.32,

108.52, 53.54, 52.07, 51.66, 51.57, 50.04, 46.45, 43.34, 29.89. MS (ESI$^+$): 365 (M$^+$ +1). Anal. Calcd for $C_{23}H_{32}N_4$: C, 75.78; H, 8.85; N, 15.37. Found: C, 75.76; H, 8.88; N, 15.40.

2-(4-((9H-carbazol-9-yl)butyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3b)****:* White powder (20 % yield). Mp = 103 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.65 (d, 1H, *Ar*); 7.45-7.24 (m, 4H, *Ar*); 2.56-2.50 (m, 4H, 2 CH_2); 2.49-2.46 (m, 8H, CH_2-Pyperazine); 2.39 (t, 2H, CH_2N); 2.38-2.30 (m, 6H, 3 CH_2); 2.27 (s, 6H, 2 CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 132.45, 123.68, 121.43, 119.08, 118.98, 110.92, 61.50, 56.65, 53.90, 53.64, 52.09, 50.85, 45.34, 28.32, 27.10. MS (ESI$^+$): 379 (M$^+$ +1). Anal. Calcd for $C_{24}H_{34}N_4$: C, 76.15; H, 9.05; N, 14.80. Found: C, 76.18; H, 9.08; N, 14.76.

2-(4-((9H-carbazol-9-yl)pentyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3c)****:* White powder (33 % yield). Mp = 109 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 7.70 (d, 1H, *Ar*); 7,45 (d, 1H, *Ar*); 7.39 (d, 1H, *Ar*); 7.38-7.28 (m, 4H, *Ar*); 3.85-3.75 (m, 8H, 4 CH_2); 2.53-2.49 (m, 8H, CH_2-Pyperazine); 2.48-2.46 (m, 4H, 2 CH_2); 2.35 (t, 2H, CH_2N); 2.27 (s, 6H, 2 CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): 134.65, 123.78, 121.54, 120.18, 119.10, 111.93, 61.76, 56.69, 54.60, 53.80, 52.34, 50.70, 45.66, 30.45, 28.34, 25.09. MS (ESI$^+$): 393 (M$^+$ +1). Anal. Calcd for $C_{25}H_{36}N_4$: C, 76.49; H, 9.24; N, 14.27. Found: C, 76.51; H, 9.26; N, 14.24.

2-(4-((9H-carbazol-9-yl)hexyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3d)****:* White powder (60 % yield). Mp = 121 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.45-7.28 (m, 4H, *Ar*); 7.27 (d, 1H, *Ar*); 3.85 (t, 2H, CH_2N); 2.46-2.40 (m, 8H, CH_2-Pyperazine); 2.39-2.36 (m, 4H, 2 CH_2); 2.35 (t, 2H, CH_2N); 2.27 (s, 6H, 2 CH_3); 1.77-1.29 (m, 8H, 4 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): 133.67, 122.98, 121.79, 121.00, 119.19, 112.45, 61.89, 56.72, 54.65, 53.98, 52.36, 50.89, 45.98, 30.32, 28.45, 27.87, 25.65. MS (ESI$^+$): 407 (M$^+$ +1). Anal. Calcd for $C_{26}H_{38}N_4$: C, 76.80; H, 9.42; N, 13.78. Found: C, 76.82; H, 9.44; N, 13.75.

2-(4-((9H-carbazol-9-yl)heptyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3e)****:* White powder (24 % yield). Mp = 118 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.65 (d, 1H, *Ar*); 7.39-7.24 (m, 4H, *Ar*); 3.85 (t, 2H, CH_2N); 2.46-2.42 (m, 8H, CH_2-Pyperazine); 2.39-2.36 (m, 4H, 2 CH_2); 2.30 (s, 6H, 2 CH_3); 1.77-1.38 (m, 12H, 6 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): 139.53, 125.00, 122.83, 120.54, 118.19, 108.49, 59.89, 54.86, 52.65, 51.68, 50.65, 50.00, 46.87, 30.98, 27.99, 27.87, 27.65, 26.15, 25.95. MS (ESI$^+$): 421 (M$^+$ +1). Anal. Calcd for $C_{27}H_{40}N_4$: C, 77.10; H, 9.58; N, 13.32. Found: C, 77.13; H, 9.55; N, 13.29.

2-(4-((3-chloro-9H-carbazol-9-yl)pentyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3f)****:* White powder (66 % yield). Mp = 112 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.17-8.10 (m, 4H, *Ar*); 7.46 (d, 1H, *Ar*); 7,44 (d, 1H, *Ar*); 7.33 (d, 1H, *Ar*); 4.20 (t, 2H, CH_2N) 2.48-2.30 (m, 8H, CH_2-Pyperazine); 2.28-2.25 (m, 4H, 2 CH_2); 2.24 (s, 6H, 2 CH_3); 1.94-1.92 (m, 8H, 4 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): 140.12, 128.38, 126.98, 125.18, 122.54, 121.93, 121.54, 120.12, 113.21, 111.10, 104.21, 61.32, 58.69, 58.20, 57.76, 56.34, 53.30, 47.10, 30.21, 28.21, 25.02. MS (ESI$^+$): 427 (M$^+$ +1). Anal. Calcd for $C_{25}H_{35}ClN_4$: C, 70.32; H, 8.26; N, 13.12. Found: C, 70.30; H, 8.24; N, 13.14.

2-(4-((3-chloro-9H-carbazol-9-yl)hexyl)piperazin-1-yl)-N,N-dimethylethanamine ***(3g)****:* White powder (33 % yield). Mp = 110 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34 (d, 1H, *Ar*); 7.70-7.68 (m, 2H, *Ar*); 7,46 (d, 1H, *Ar*); 7.41 (d, 1H, *Ar*); 7.18-7.14 (m, 2H, *Ar*); 4.873 (t, 2H, CH_2N); 2.70-2.66 (m, 8H, CH_2-Pyperazine); 2.38-2.35 (m, 4H, 2 CH_2); 2.21 (s, 6H, 2 CH_3); 1.98 (t, 2H, CH_2N); 1.28-1.12 (m, 8H, 4 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): 140.12, 129.32, 125.79, 125.18, 122.59, 121.15, 121.09, 119.54, 112.32, 110.21, 103.54, 61.37, 58.65, 57.98, 57.21, 53.10, 46.76, 29.54, 28.65, 27.96. MS (ESI$^+$): 441 (M$^+$ +1). Anal. Calcd for $C_{26}H_{37}ClN_4$: C, 70.80; H, 8.46; N, 12.70. Found: C, 70.82; H, 8.49; N, 12.68.

General method for the synthesis of compounds 4a-g

Excess methyl iodide (5 mL) was added to a mixture of **3** (1 mmol) in acetone (10 mL), the reaction mixture was heated at 30 °C for 1 h. After evaporation of all the solvent, the solid residue was recrystallized from methanol to give **4a-g**.

2-(4-((9H-carbazol-9-yl)propyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4a)****:* White solid (70% yield). Mp = 112 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7.70 (d, 1H, *Ar*); 7,60 (d, 1H, *Ar*); 7.46-7.27 (m, 4H, *Ar*); 2.55-2.50 (m, 4H, 2 CH_2); 2.49-2.45 (m, 8H, CH_2-Pyperazine); 2.35 (t, 2H, CH_2N); 2.34-2.29 (m, 4H, 2 CH_2); 2.28 (s, 9H, 3 CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 132.98, 121.98, 120.17, 119.76, 119.43, 111.21, 65.00, 60.32, 54.45, 53.89, 53.74, 52.12, 46.36, 28.10, 26.32. MS (EI) *m/z* (%): 379 (M$^+$ - I$^-$). Anal. Calcd for $C_{24}H_{35}IN_4$: C, 56.92; H, 6.97; N, 11.06. Found: C, 56.94; H, 6.99; N, 11.03.

2-(4-((9H-carbazol-9-yl)butyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4b)****:* White solid (75 % yield). Mp = 131 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.65 (d, 1H, *Ar*); 7.45-7.24 (m, 4H, *Ar*); 3.34 (t, 2H, CH_2N); 3.30 (s, 9H, 3 CH_3); 2.56-2.50 (m, 4H, 2 CH_2); 2.49-2.46 (m, 8H, CH_2-Pyperazine); 2.36-1.78 (m, 6H, 3 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 133.76, 122.28, 120.12, 119.32, 119.08, 111.12, 64.50, 60.65, 54.40, 53.74, 53.54, 52.75, 46.34, 28.12, 26.10. MS (EI) m/z (%): 393 (M$^+$ - I$^-$). Anal. Calcd for $C_{25}H_{37}IN_4$: C, 57.69; H, 7.17; N, 10.76. Found: C, 57.71; H, 7.15; N, 10.74.

2-(4-((9H-carbazol-9-yl)pentyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4c)****:* Yellow solid (55% yield). Mp = 145 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 7.70 (d, 1H, *Ar*); 7,45 (d, 1H, *Ar*); 7.39 (d, 1H, *Ar*); 7.38-7.28 (m, 4H, *Ar*); 3.85- 3.75 (m, 8H, 4 CH_2); 2.50-2.46 (m, 4H, 2 CH_2); 2.45-2.35 (m, 8H, CH_2-Pyperazine); 2.34 (t, 2H, CH_2N); 2.29 (s, 9H, 3 CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 133.86, 122.33, 120.14, 119.43, 119.21, 112.12, 64.76, 61.67, 54.50, 53.87, 53.23, 52.89, 46.31, 28.12, 26.15. MS (EI) *m/z* (%): 407 (M$^+$ - I$^-$). Anal. Calcd for $C_{26}H_{39}IN_4$: C, 58.42; H, 7.35; N, 10.48. Found: C, 58.43; H, 7.33; N, 10.51.

2-(4-((9H-carbazol-9-yl)hexyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4d)****:* White solid (78 % yield). Mp = 120 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.39 (d, 1H, *Ar*); 7.45-7.24 (m, 4H, *Ar*); 3.85 (t, 2H, CH_2N); 2.46-2.40 (m, 8H, CH_2-Pyperazine); 2.39-2.36 (m, 4H, 2 CH_2); 2.35 (t, 2H, CH_2N); 2.30 (s, 9H, 3 CH_3); 1.77-1.29 (m, 8H, 4 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.09, 126.76, 123.79, 120.97, 119.00, 109.67, 65.76, 54.65, 53.65, 51.78, 50.36, 50.21, 49.96, 30.87, 28.87, 28.09, 27.65, 27.00. MS (EI) *m/z* (%): 421 (M$^+$ - I$^-$). Anal. Calcd for $C_{27}H_{41}IN_4$: C, 59.12; H, 7.53; N, 10.21. Found: C, 59.15; H, 7.55; N, 10.23.

2-(4-((9H-carbazol-9-yl)heptyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4e)****:* Yellow solid (68 % yield). Mp = 151 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.36 (d, 1H, *Ar*); 8.00 (d, 1H, *Ar*); 7,70 (d, 1H, *Ar*); 7.65 (d, 1H, *Ar*); 7.39-7.24 (m, 4H, *Ar*); 3.85 (t, 2H, CH_2N); 3.29 (s, 9H, 3 CH_3); 2.46-2.42 (m, 8H, CH_2-Pyperazine); 2.40-2.36 (m, 4H, 2 CH_2); 1.77-1.38 (m, 12H, 6 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 139.87, 127.13, 124.00, 121.65, 120.12, 110.68, 66.46, 55.69, 54.25, 52.43, 50.56, 50.28, 50.12, 31.43, 29.86, 28.34, 27.87, 26.98. MS (EI) *m/z* (%): 435 (M$^+$ - I$^-$). Anal. Calcd for $C_{28}H_{43}IN_4$: C, 59.78; H, 7.70; N, 9.96. Found: C, 59.80; H, 7.68; N, 9.98.

2-(4-((3-Chloro-9H-carbazol-9-yl)pentyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4f)****:* White solid (56% yield). Mp = 115 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.13-8.10 (m, 4H, *Ar*); 7.46 (d, 1H, *Ar*); 7,44 (d, 1H, *Ar*); 7.33 (d, 1H, *Ar*); 4.20 (t, 2H, CH_2*N*); 2.48-2.30 (m, 8H, CH_2-Pyperazine); 2.29 (s, 9H, 3 CH_3); 2.28-2.25 (m, 4H, 2 CH_2); 1.94-1.92 (m, 8H, 4 CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 139.67, 138.01, 135.65, 135.2, 131.23, 128.34, 125.89, 123.11, 121.87, 117.21, 112.67, 112.45, 64.32, 60.65, 57.32, 56.87, 55.87, 54.60, 52.76, 46.87, 29.65, 27.87, 25.11. MS (EI) *m/z* (%): 441 (M$^+$ - I$^-$). Anal. Calcd for $C_{26}H_{38}ClIN_4$: C, 54.89; H, 6.73; N, 9.85. Found: C, 54.91; H, 6.70; N, 9.87.

2-(4-((3-Chloro-9H-carbazol-9-yl)hexyl)piperazin-1-yl)-N,N,N-trimethylethanammonium iodide ***(4g)****:* White solid (78 % yield). Mp = 113 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.34 (d, 1H, *Ar*); 7.70 (d, 1H, *Ar*); 7,69 (d, 1H, *Ar*); 7.46 (d, 1H, *Ar*); 7.41 (d, 1H, *Ar*); 7.18 (d, 1H, *Ar*); 7.14 (d, 1H, *Ar*); 4.73 (t, 2H, CH_2N); 2.71 (s, 8H, CH_2-Pyperazine); 2.37 (s, 4H, 2 CH_2); 2.27 (s, 9H, 3 CH_3); 1.60-1.57 (m, 2H, CH_2); 1.28-1.25 (m, 6H, 3 CH_2); 1.18-1.08 (m, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ 140.01, 128.71, 125.34, 125.12, 121.23, 121.11, 119.87, 112.65, 109.65, 103.38, 63.76, 58.21, 57.91, 56.87, 54.37, 48.09, 29.43, 28.30, 27.32. MS (EI) m/z (%): 455 (M+ - I^-). Anal. Calcd for $C_{27}H_{40}ClIN_4$: C, 55.63; H, 6.92; N, 9.61. Found: C, 55.65; H, 6.89; N, 9.63.

Biology

Cell culture and cell proliferation assay

Human estrogen receptor (ER)-positive MCF-7 and triple negative MDA-MB-231 breast cancer cells were cultured in DMEM-F12 medium containing 2 mmol/L L-glutamine, 1mg/ml penicillin-streptomycin and 5% Newborn Calf Serum (NCS) or 5% Fetal Bovine Serum (FBS), respectively. MCF-10A human mammary epithelial cells, were cultured in DMEM/F12 medium, supplemented with 5% horse serum (HS, Thermo Fisher Scientific, Milan, Italy), 100 U mL-1 penicillin/streptomycin, 0.5 mg mL-1 hydrocortisone, 20 ng mL-1 human epidermal growth factor (hEGF), 10 mg mL-1 insulin, and 0.1 mg mL-1 cholera enterotoxin (Sigma–Aldrich, Milan, Italy). The cell lines used in these experiments were obtained from American Type Culture Collection (ATCC, Manassas, VA), were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and periodically screened for contamination. Cells were seeded in 48-well and grown in complete medium, then starved in serum free medium for 24h for allowing cell cycle synchronization, before being treated. After 72h exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich, Milan, Italy) was added and incubated for 2h at 37 °C. The cells were lysed with DMSO, and then optical density was measured at 570 nm using a microplate reader. IC_{50} values were calculated for each cell line using GraphPad Prism 5 Software (GraphPad Inc., San Diego, CA). Data are representative of three independent experiments and standard deviations (SD) were shown [66].

Human topoisomerase II decatenation assay

hTopo II decatenation assays were performed in a final volume of 20 µL as reported by Iacopetta et al, using 0.3 µg of kinetoplast DNA (kDNA) (topoGEN, Port Orange, FL, USA) compounds to test and 3 Units of hTopo II (topoGEN, Port Orange, FL, USA). Products reactions were analyzed by agarose gel electrophoresis, without ethidium bromide (EB). At the end, EB (0.5 µg/mL) has been used to stain agarose gel and after wash with distilled it was observed using a UV transilluminator. The experiment was repeated thrice.

Tunel assay

Apoptosis was detected by the TUNEL assay, according to the guidelines of the manufacturer (CF™488A TUNEL Assay Apoptosis Detection Kit, Biotium, Hayward, CA, USA).(8) Briefly, cells were grown on glass coverslips and, after treatment, they were washed trice with PBS, then methanol-fixed at -20 °C for 15 min. Fixed cells were washed trice with 0.01% (V/V) Triton X-100 in PBS and incubated with 100 µL of TUNEL equilibration buffer for 5 min. After its removal, 50 µL of TUNEL reaction mixture containing 1 µL of terminal deoxynucleotidyl transferase (TdT) were added to each sample and incubated in a dark and humidified chamber for 2 h at 37 °C. Samples were washed trice with ice-cold phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 5 mg/mL bovine serum albumin (BSA). 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (0.2 µg/mL) counterstain was performed (10 min, 37 °C, dark and humidified conditions). After three additional washes with cold PBS, one drop of mounting solution was added, then they were observed and imaged under a fluorescence microscope (Leica DM 6000) (20X magnification) with excitation/emission wavelength maxima of 490 nm/515 nm (CF^{TM}488A) or 350 nm/460 nm (DAPI). Representative fields were shown. The experiments were repeated three times.

Caspase Assay

Caspases -3/7, -8 and -9 activities were measured with the Caspase-Glo Assay, according to the guidelines of manufacturer (Caspase-GloR 3/7, 8 and 9 Assay Systems, Promega Corporation, Madison, WI, USA). Cells were grown in white-walled 96-well plates and, after treatment, 100µL of different caspases reagents were added to each well, as previously described in Iacopetta *et al* [67]. Results were plotted as percentage (%) over the vehicle-treated cells (CTRL), after background readings subtraction.

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Keywords: topoisomerase II; caspases; *N*-alkylcarbazoles; apoptosis; docking simulation.

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FULL PAPER

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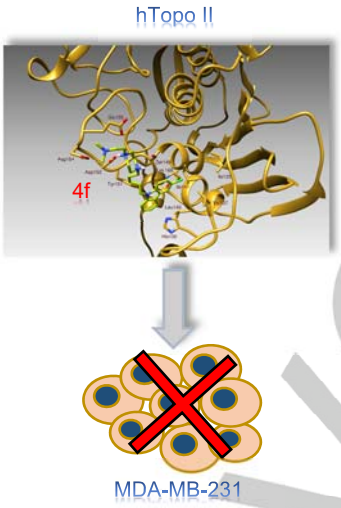
Inhibition of human Topoisomerase II by new *N,N,N*-trimethylethanammonium iodide alkylcarbazole derivatives



New N,N,N-trimethylethanammonium iodide alkylcarbazole derivatives. The synthesized compounds have been tested as hTopo II inhibitors by *in silico* and *in vitro* studies. Compound **4f** is the most potent inhibitor and is able to trigger the intrinsic apoptotic pathway in MDA-MB-231 breast cancer cells