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Multifaceted Properties of 1,4-Dimethylcarbazoles: Focus on Trimethoxybenzamide and Trimethoxyphenylurea Derivatives as Novel Human Topoisomerase II Inhibitors

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Abstract

Natural or synthetic carbazole derivatives have recently attracted the attention of the scientific world because of their multiple biological activity, leading to an increase of designed, synthesized and studied analogues. In this paper, four 1,4-dimethylcarbazole derivatives, analogues of Ellipticine, have been investigated for their ability to block cancer cells growth, with low effects on the proliferation of normal cells. DNA topoisomerases inhibition assays, docking simulations, stability studies and effects on a membrane model are reported. Particularly, compounds **2** and **3** have been found thermally stable and able to inhibit, strongly and selectively, the human DNA topoisomerase II. These properties confer a good and broad antitumoral activity *in vitro*, with very low cytotoxic effect on the proliferation of normal cell lines and without damaging, in contrast with Ellipticine, the cell membrane model. The presented outcomes set the most active compounds as good candidates for pre-clinical studies useful in cancer treatment.

Keywords

Human topoisomerases I/II; antitumor; docking simulation; thermal stability; large unilamellar vesicles.

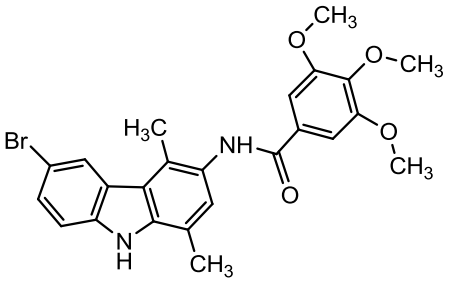
1. Introduction

In recent years, many research groups were interested in the multiple properties of different carbazole derivatives, whether isolated from plants such as those of genus *Ochrosia*, *Murraya*, *Glycosmis* and *Clausena*, or synthetically obtained (Knolker and Reddy, 2002b). The possibility to do some structural modifications, leading to new derivatives with peculiar biological properties or higher target selectivity, drew the interest toward this class of alkaloids. Therefore, an ever-increasing number of derivatives containing a structurally modified carbazole scaffold has been designed, synthesized and studied (Caruso et al., 2016; Saturnino et al., 2003; Tsutsumi et al., 2016). Amongst the plethora of biological activities exerted by carbazole derivatives, they should be enumerated at least the following: anti-cancer, antibacterial, antifungal, anti-inflammatory, anti-oxidative, hepatoprotective, neuroprotective, anti-HIV and sedative (Caruso et al., 2012; Du et al., 2015; Gu et al., 2014; Kong et al., 2016; Krahl et al., 2006; Saturnino et al., 2014; Zhu et al., 2013). According to the literature, these properties are often due to the inhibition of single or multiple key enzymes of cell metabolism, as for instance topoisomerases, telomerases, integrases, heparanase, protein-kinases. Amongst these biological targets, DNA and its metabolizing enzymes have been found to be the site of choice of many carbazole derivatives, through which they exert their effects (Bashir et al., 2015; Gluszynska, 2015).

Particularly, DNA topoisomerases are essential enzymes involved in the regulation of the topological state of DNA during its replication, transcription, recombination and chromatin remodeling (Brill et al., 1987). They are important for ensuing genomic integrity, however in pathological cases as, for exemple, tumor onset, these enzymes are over-expressed or their activity dramatically enhanced in order to ensure tumor cells surviving and sustain the uncontrolled proliferation (Larsen and Skladanowski, 1998). For these reasons, the possibility to interfere with these enzymes represents an effective strategy. Indeed, DNA topoisomerases I and II are good

targets of clinically significant classes of anticancer drugs able to interfere with the enzyme-DNA complexes producing permanent DNA damages and triggering cell death (Galsky et al., 2015; Yao et al., 2015).

In this paper, we report the ability of four carbazole derivatives **1**-**4**, structurally correlated to the alkaloid Ellipticine from *Ochrosia elliptica* Labill (Knolker and Reddy, 2002a), namely trimethoxybenzamide and N1-(trimethoxyphenyl)-N3-(dimethylcarbazole) urea derivatives (Panno et al., 2014) (Figure 1), to inhibit strongly and selectively the human topoisomerase II activity by direct enzyme assays and docking simulations. This inhibitory property is responsible to trigger cell death by apoptosis, as demonstrated by TUNEL assays. We have also evaluated their antitumor activity, compared with Ellipticine, using a panel of different cancer cells. All compounds under testing were very effective in diminishing tumor cells proliferation without affecting the viability of normal cells. Moreover, we have proved that these compounds are very stable, even at room temperature, and in contrast with their lead compound Ellipticine (Figure 1), they do not alter the stability of a model of artificial membranes (large unilamellar vesicules, LUVs). The versatility and the multifaceted features of these trimethoxybenzamide and trimethoxyphenylurea 1,4-dimethylcarbazole derivatives make them very interesting and promising anticancer candidates.



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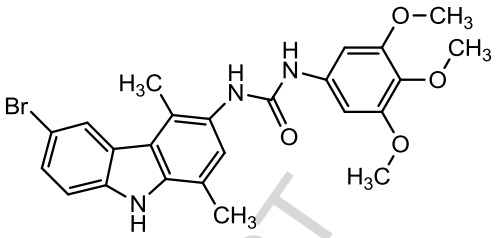

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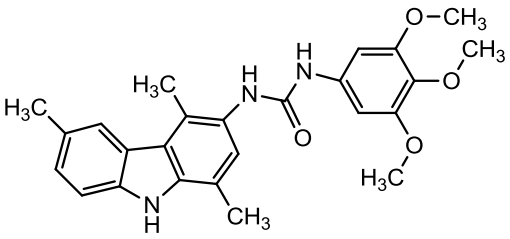

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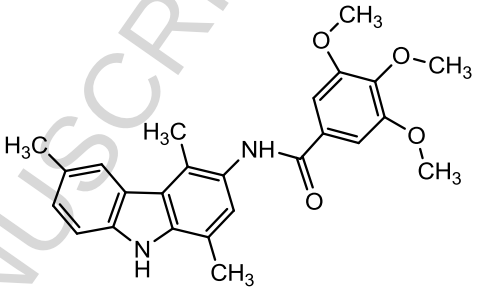

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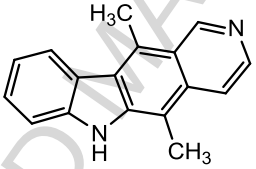


Ellipticine

Figure 1

2. Materials and Methods

2.1 Stability studies

Stability studies have been performed according to the procedures previously reported by Parisi et al. (Parisi et al., 2013)

2.2 Freeze–thaw stability

Reagent-grade DMSO has been purchased by Carlo Erba Reagenti (Milan, Italy). Absorption spectra have been recorded with a UV/Vis spectrophotometer V-530, Jasco - U.S. Aliquots of all the studied compounds (approximately 5 mg) have been put in vials and exposed at a temperature of 4°C for 2 days, and then heated at 45°C for 2 days per cycle; the freeze-thaw cycle has been repeated three times. At the end of each cycle, 4 mL of DMSO have been added to the samples and UV/Vis spectra have been recorded between 300 and 400 nm. The concentrations of the solutions after each cycle have been determined using calibration curves constructed as follows: 5 standard solutions of each carbazole dissolved in DMSO have been prepared and analyzed by UV/Vis spectroscopy. Correlation coefficient (R2) and slope of the regression equations have been calculated by the method of least square. The freeze-thaw stability has been evaluated by comparing the amount of compound recovered after each cycle to the starting amount of carbazole-derivative before freeze-thaw cycles. The procedure has been performed in triplicate and data were expressed as mean ± standard deviation.

2.3 Thermal stability under stress conditions

The thermal stability has been evaluated by incubating in vials approximately 5 mg of **1**, **2**, **3** and **4** under stress conditions at different temperatures. The samples have been kept at 4°C for three days, then at 25°C for the successive three days and heated at 45°C for further three days. In order to determine the amounts of compounds preserved during the study, every three days the samples have been collected and dissolved by adding 4 mL of DMSO. Then, the solutions have been analyzed by UV/Vis spectroscopy and the concentrations were calculated using the calibration curves previously obtained. The experiment has been repeated 3 times for each compound and data were expressed as mean ± standard deviation.

2.4 Cell cultures

All the cell lines used in these studies have been purchased from American Type Culture Collection (ATCC, Manassas, VA, USA); media and additives have been purchased from Thermo Fisher Scientific (Milan, Italy), except when stated otherwise. MCF-7 cells have cultured in DMEM-F12 containing 10% fetal bovine serum (FBS), 1% L-Glutamine, 1 mg/ml penicillin–streptomycin. MDA-MB-231 cells have been cultured in DMEM containing 5% FBS, 1% L-Glutamine, 1 mg/mL penicillin–streptomycin. HeLa and Ishikawa cells were cultured in MEM containing 10% FBS, 1% L-Glutamine, 1% NEAA, and 1 mg/mL penicillin–streptomycin. R2C cells have been cultured in Ham's F-10 nutrient mix containing 15% horse serum, 2.5% FBS, 1% L-Glutamine and 1 mg/mL penicillin–streptomycin. HepG2 cells were cultured in DMEM containing 10% FBS, 1% L-Glutamine and 1 mg/mL penicillin–streptomycin. MCF-10A human mammary epithelial cells have been maintained in DMEM-F12 supplemented with 10% horse serum (HS), 1% L-Glutamine, 1% penicillin/streptomycin, 0.5 mg/ml hydrocortisone, 20 ng/ml hEGF (human epidermal growth factor) and 0.1 mg/ml cholera enterotoxin (Sigma–Aldrich, Milan, Italy) and 10 µg/ml insulin (complete medium). 3T3-L1 cells have been cultured in DMEM containing 4500 mg/L glucose,

supplemented with 1% penicillin/streptomycin and 10% newborn calf serum (NCS). Cells have been maintained at 37 °C in a humidified atmosphere with 5% CO_2 and have been screened periodically for Mycoplasma contamination.

2.5 Cell viability

Cell viability has been determined using the 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium Bromide (MTT, Sigma–Aldrich, Milan, Italy) assay (Carocci et al., 2013; Chimento et al., 2013; Sinicropi et al., 2009). Cells have been seeded on forty-eight well plates and grown in complete medium. Before being treated, cells have been starved in serum free medium for 24 h for allowing cell cycle synchronization. Seventy two hours after treatments, fresh MTT (Sigma), re-suspended in PBS, was added to each well (final concentration (0.5 mg/mL). After 2 hours incubation at 37°C, cells have been lysed with DMSO, and then optical density was measured at 570 nm using a microplate reader. At least six doses of the studied compounds, solubilized in DMSO (0.1% final concentration), have been evaluated and each experiment has been performed in triplicate. The absorbance values have been used to determine the IC_{50} for each cell line using GraphPad Prism 5 Software (GraphPad Inc., San Diego, CA). Data are representative of three independent experiments; standard deviations (SD) have been shown.

2.6 Human Topoisomerase I relaxation assay

hTopo I relaxation assays have been performed in a final volume of 20 μL as follows. 0.25 μg of supercoiled pHOT1 in TE buffer [TE: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA] (TopoGEN, Port Orange, FL, USA) has been added to a solution containing water (variable volume) and 1X assay buffer (10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.5 mM NaCl, 0.1% bovine serum albumin, 0.1 mM spermidine and 5% glycerol). Compounds have been added and the reaction initiated by

addition of recombinant hTopo I (2 U) (TopoGEN, Port Orange, FL, USA), incubated at 37 °C for 30 minutes and terminated by the addition of 5× stop buffer (5% sarkosyl, 25% glycerol, 0.125% bromophenol blue). After Proteinase K digestion (50 μg/mL) at 37 °C for 30 minutes, samples have been extracted with an equal volume of chloroform:isoamylic alcohol (24:1), vortexed and centrifuged for 30 seconds. The upper aqueous phase has been loaded onto a 1% agarose gel containing 1X TAE buffer (diluted from 50X buffer containing 242 g Tris base, 57.1ml glacial acetic acid and 100 ml of 0.5 M EDTA) without ethidium bromide (EB). At the end, 1X TAE buffer containing EB (0.5 μg/ml) has been used to stain agarose gel for 30 minutes and after wash with distilled water for 15 minutes, it has been visualized using a UV transilluminator.

2.7 Human Topoisomerase II decatenation assay

hTopo II decatenation assays have been performed in a final volume of 20 μL using 0.3 μg of kinetoplast DNA (kDNA) (topoGEN, Port Orange, FL, USA), 1X assay buffer [50 mM Tris-HCl, pH of 8, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM Dithiothreitol (DTT), 30 μg/mL bovine serum albumin (BSA)], and 1 mM ATP. The compounds have been added and the reaction started by adding 3 U of hTopo II (topoGEN, Port Orange, FL, USA) and incubating at 37 °C for 30 minutes. Then, 5X stop buffer has been added and the samples treated as described in the previous paragraph. The aqueous phase has been loaded on a 1% agarose gel containing 1X TAE buffer with EB (0.5 μg/ml) and visualized using an UV transilluminator.

2.8 Computer modelling and docking simulations

The crystal structure of *Saccharomyces cerevisiae* Topoisomerase II in complex with a short DNA fragment and the ATP-analogue AMPPNP (Schmidt et al., 2012) [PDB Code 4GFH] has been used

as a template to build a complete three dimensional model of human Topoisomerase II. Such model, hTopoII, was then used as a target for our molecular docking simulations. All the ligand three-dimensional structures have been built and energy minimized using the program MarvinSketch [ChemAxon ltd, Budapest, Hu]. In order to investigate the binding mode of our ligands we used the computer program GOLD v.5.2.2 [The Cambridge Crystallographic Data Center, CCDC, UK]. We have performed a "blind docking": the docking of the small molecule to the targets has been done without *a priori* knowledge of the location of the binding site by the system. We have chosen the ChemPLP docking scoring function; all program default parameters have been adopted in our case: for each molecule tested the number of islands has been set to 5, population size to 100, number of operations has been 100,000 with a selective pressure of 1.1. The results of the simulations allowed us to determine the binding modes of all the ligand tested. All the three-dimensional figures have been drawn using the program Chimera (Iacopetta et al., 2011; Pettersen et al., 2004).

2.9 Tunel assay

Cell death has been investigated by TUNEL assay, following the manufacturer instructions (CF™488A TUNEL Assay Apoptosis Detection Kit, Biotium, Hayward, CA, USA) with small modifications. Briefly, cells have been grown on glass coverslips and, after treatment, have been washed three times with PBS, then methanol-fixed at -20°C for 15 minutes. Fixed cells have been washed three times with 0.01% (V/V) Triton X-100 in PBS and incubated with 100 µL of TUNEL Equilibration Buffer for 5 minutes. After removal of Equilibration Buffer, 50 µL of TUNEL reaction mix containing 1 µL of terminal deoxynucleotidyl transferase (TdT) have been added to each sample and incubated for 2 hours in a humid chamber at 37ºC, protected from light. Samples have been washed 3 times, 5 minutes each, with 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 has been performed for 10 minutes in a humidified chamber at 37ºC,

protected from light. Cells have then washed three times with cold PBS and one drop of mounting solution has been added. Cells have been observed and imaged under an inverted fluorescence microscope (20X magnification) with excitation/emission wavelength maxima of 490 nm/515 nm (CF^{TM}488A) or 350 nm/460 nm (DAPI). Images are representative of three separate experiments.

2.10 Large unilamellar vesicles (LUVs) preparation and characterization

Two formulations of LUVs, with or without cholesterol (Chol), have been prepared according to the thin film hydration method and resized by extrusion (Olson et al., 1979). Briefly, phosphatidylcholine (PC, from soybean, lipoid S 100, Lipoid GmbH, Ludwigshafen, Germany) and 1,2-Dioleoyl-sn-glycero-3[Phospho-rac-(1-glycerol)] (DOPG, DOPG-Na, lipoid PG 18:1/18/1, Lipoid GmbH, Ludwigshafen, Germany) have been mixed in a weight/weight (w/w) ratio of 4:1 (PC:DOPG) for the first formulation of LUVs, with the addition of Chol (Sigma-Aldrich, Saint-Quentin Fallavier, France), in a w/w ratio of 4:1:1 (PC:DOPG:Chol), for the second one. Then, lipids mix has been dissolved in an adequate amount of chloroform and after evaporation of organic solvent under a stream of nitrogen, the dried film was kept overnight in a vacuum desiccator to remove any residual trace of solvent. The resultant lipid film has been hydrated with a buffer A (50 mM HEPES, 10 mM NaCl, pH 7.4) containing carboxyfluorescein (CF) 50 mM and the mixture mixed by vortexing and then stirred for 1 hour at room temperature. This mixture has been extruded 13 times through polycarbonate filters with a pore diameter of 100 nm and the obtained LUVs have been separated from possible unincorporated material by passage through a Sepharose CL-4B loaded column, using buffer B (50 mM HEPES, 107 mM NaCl, ph 7.4) as eluent. The LUVs size and charge have been assessed by Dynamic Light Scattering (DLS, NanoZS, Malvern Instruments, Worcestershire, UK). CF-loaded LUVs formulated without cholesterol present an average diameter of 146.3 ± 1.5 nm associated with a polydispersity index (PDI) of 0.067 ± 0.005, and a ζ-potential value of -23.4 ± 2.5 mV. CF-loaded LUVs formulated with cholesterol present an average diameter

of 150.7 ± 1.6 nm with a PDI of 0.051 ± 0.027, and a ζ-potential value of -20.2 ± 1.5 mV. From these data, both LUVs formulations appear monodisperse in size (PDI<0.2) and present a negative surface charge related to the presence of DOPG moieties in the formulation. The entrapment of CF has been verified by dequenching of fluorescence after the addition of 10% w/w Triton X-100 measured with a microplate reader equipped with the appropriate filters (excitation 492 nm, emission 513nm). All the LUVs used in these experiments have been stored at 4°C and were stable for at least one month.

2.11 Liposomes CF Release Assay

CF release assay has been performed in a final volume of 200 µl, using 2 µl of the LUVs stock solution in buffer B and compounds to test, solubilized in DMSO, have been then added to the solution to reach a final concentration of 10 µM. The fluorescence has been recorded at different times, ranging from 0 to 4 hours, using a Synergy 2 microplate reader controlled by Gen 5 software (Biotek, Colmar, France) microplate reader at λex 485/20 nm and λem 528/20 nm. The fluorescence intensities at each time point have been normalized to the signal when LUVs have been disintegrated by the addition of 5 µl of a 20% (V/V) Triton X-100 solution. The percentage of CF leakage has been calculated using the following equation:

$$\text{CF release (\%)} = \frac{I_t - I_0}{I_T - I_0} \times 100$$

where I_0 and I_t correspond to the fluorescence intensity before and after the time (t) of compounds addition, respectively. I_T is the fluorescence intensity after LUVs disruption by the addition of 20% Triton X-100 solution. Each experiment has been performed in triplicate and the error bars correspond to the mean deviation of three independent experiments.

3. Results

3.1 Stability studies

Carbazole-derivatives are known to be morphologically and thermally stable compounds (Yu et al., 2014). In order to accomplish the requirements of drug regulatory agencies, the stability of carbazoles **1**-**4** has been investigated through accelerated thermal stability studies under constant temperature and humidity. Frozen states are often followed by thawing phases during drug storage that precedes the use. For this reason, stability after freeze-thaw cycles of compounds **1**-**4** series has been tested.

In addition, stability under accelerated thermal stress conditions has been investigated. This is a requirement of Food and Drug Administration (FDA) for the evaluation of pharmaceutical products stability stored under various conditions.

The data obtained from these studies have been calculated using equation (1) and expressed as recovery percentage ± deviation standard (see Table I and Table II).

$$Recovery\ (\%) = \frac{mol_n}{mol_0} \times 100 \qquad (1)$$

in which mol_n and mol_0 are the amount of carbazole compound recovered at t_n and the initial amount of carbazole-derivative sample used at the beginning of the experiment (t_0), respectively.

Table I. Freeze-thaw stability data.

Freeze-thaw Cycle (*t*)	**1** recovery (%)	**2** recovery (%)	**3** recovery (%)	**4** recovery (%)
0	100	100	100	100

1	98.36 ± 0.71	98.13 ± 0.65	98.58 ± 0.56	98.33 ± 0.53
2	97.78 ± 0.67	96.93 ± 0.73	97.58 ± 0.62	97.24 ± 0.48
3	96.15 ± 0.52	95.29 ± 0.48	96.62 ± 0.45	96.33 ± 0.57

Table II. Recovery results of compounds **1-4** under thermal stress conditions.

Step	Storage temperature (°C)			**1**	**2**	**3**	**4**
(*t*)	4	25	45	recovery (%)	recovery (%)	recovery (%)	recovery (%)
0	–	–	–	100	100	100	100
1	3 days	–	–	99.71 ± 0.49	98.64 ± 0.58	99.83 ± 0.72	99.41 ± 0.56
2	3 days	3 days	–	98.88 ± 0.63	96.34 ± 0.69	98.62 ± 0.47	97.24 ± 0.55
3	3 days	3 days	3 days	97.02 ± 0.58	92.18 ± 0.61	98.13 ± 0.68	94.74 ± 0.64

The wavelengths of maximum absorbance λ_{max} in DMSO obtained from UV spectra have been 336.5 nm, 348.5 nm, 347.0 nm and 334.0 nm for compounds **1**, **2**, **3** and **4**, respectively.

The same peaks have been detected for each compound before and after stability experiments, no other impurities were observed, even using derivative spectroscopy (data not shown). This evidence is, in fact, coherent with the recovery percentage showed in Table I and Table II.

3.2 Effects on tumoral and non-tumoral cells viability

The inhibition of tumor cell growth of the four considered compounds (**1-4**) has been evaluated against a panel of cancer cells, namely: estrogen receptor positive (ER+) MCF-7 and triple negative MDA-MB-231 human breast cancer cell line (which do not express ER, PR, and do not have HER-2/Neu amplification), rat Leydig tumor cells R2C, human Ishikawa endometrial cancer cells, cervical cancer HeLa and human hepatoma HepG2 cells. The IC_{50} values were determined at 72

hours after treatment by the means of MTT assay and have been reported in table 3; Ellipticine has been used as reference molecule. All the studied compounds have been able to inhibit cancer cells growth, even though in a lesser extent with respect to Ellipticine, which have showed a better antitumor activity on all cell lines tested. However, Ellipticine was also found to affect dramatically the proliferation of non-malignant breast epithelial cells MCF-10A and mouse embryonic fibroblasts 3T3-L1, with IC_{50} values of 1.20±0.2 and 0.98±0.7 µM, respectively. On the contrary, the carbazoles **1-4** were found to be less cytotoxic than Ellipticine on the proliferation of normal cell lines (see table 1), maintaining a good inhibitory effect on all the different cancer cells used in these experiments. Amongst the studied compounds, the higher antitumor activity has been recorded for the compounds **3** and **2**, respectively, which also exhibited lesser cytotoxic effects on normal cells viability (IC_{50} values of 210.00±5.0 and 141.30±1.6 µM for compound **3** and 180.00±3.0 and 132.10±2.5 µM for compound **2,** calculated on MCF-10A and 3T3-L1 cells, respectively). However, compounds **1** and **4** were found to exert a minor inhibitory growth effect and to be a little more cytotoxic, particularly compound **1**, on MCF-10A cells (IC_{50} values of 72.00±1.0 and 150.00±1.2 µM for compounds **1** and **4**, respectively) and 3T3-L1 cells (IC_{50} values of 59.14±1.8 and 99.60±2.7 µM for compound **1** and **4,** respectively), compared with compounds **2** and **3**. Summing up, all the tested compounds have shown a good and broad antitumor activity, particularly compound **3**, on six different cancer cell lines without affecting the proliferation of two normal cell lines, if not at very high doses, differently from Ellipticine.

Table III: IC_{50} values expressed in µM

	MCF-7	MDA-MB-231	HeLa	ISK	R_2C	HepG2	MCF-10A	3T3-L1
1	8.89±1.5	5.45±0.2	7.48±1.2	11.82±1.3	15.87±1.0	22.00±1.3	72.00±1.0	59.14±1.8
2	3.79±0.5	2.50±0.8	5.41±0.5	6.77±0.5	11.50±1.4	13.00±2.5	180.00±3.0	132.10±2.5
3	2.78±0.9	1.93±0.7	4.08±0.7	5.66±0.4	6.90±1.5	8.00±1.7	210.00±5.0	141.30±1.6

4	7.31±1.2	6.35±1.0	6.20±1.7	8.89±1.2	8.50±1.3	12.00±2.0	150.00±1.2	99.60±2.7
Ellipticine	1.25±0.3	1.85±0.15	1.05±0.5	1.70±0.8	2.10±0.4	2.05±0.2	1.20±0.2	0.98±0.7

3.3 Inhibition assays on human Topoisomerase I and II

Compounds **1-4** have been evaluated for their ability to inhibit the important DNA-metabolizing enzymes topoisomerases, thus human Topoisomerase I (hTopo I) relaxation and Topoisomerase II (hTopo II) decatenation assays have been performed, as reported (Chimento et al., 2015; Rizza et al., 2016).

Firstly, the assay conducted on hTopo I has shown clearly a lack of any inhibitory activity of all tested compounds (figure 2, lanes 1-4), differently from Ellipticine (figure 2, lane E) that not only has been found able to inhibit the enzyme but, as well, to shift the super coiled plasmid DNA electrophoretic migration (see figure X, lanes SCD and E) because of its intercalating properties, as already reported elsewhere (Rizza et al., 2016; Stiborova and Frei, 2014). The hTopo I relaxation assay has been performed using compounds concentrations in the range 10 to 100 µM (figure 2 shows only the 10 µM concentration and same results have been obtained with increasing doses), but no inhibition has been detected.

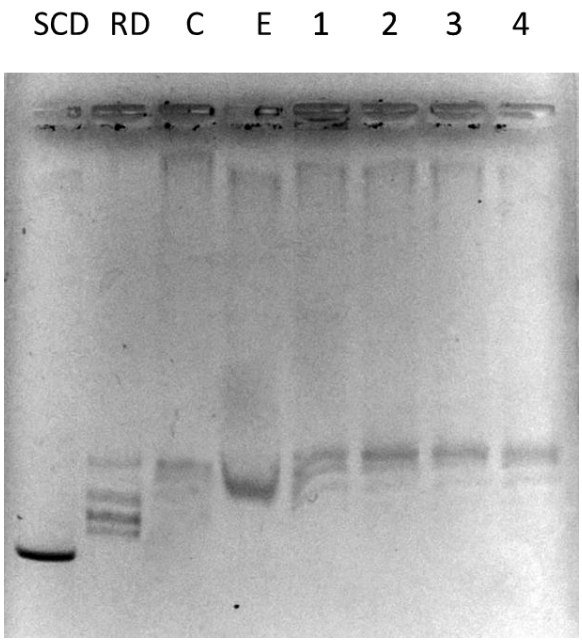


Figure 2

Conversely, in hTopo II decatenation assay, a different behavior has been noticed, because the compounds **2** and **3** have been able to completely inhibit the activity of hTopo II at concentration of 10 µM (Figure 3, lanes 2 and 3), whereas compound **1** was shown to exert a very slight inhibition (Figure 3, lane 1) and compound **4** was not able at all to inhibit the hTopo II. It should be noted that Ellipticine, used at the same concentration, could not been able to completely inhibit hTopo II activity (figure 3, lane E), given that small amounts of decatenation products (shifted, as well in this case, with respect to the control, lane C, figure 3) are visible at the bottom of the gel. Thus, compounds **2** and **3** have demonstrated a selective ability to inhibit completely the hTopo II activity in a better way than Ellipticine, used at the same concentration, whereas a very poor inhibition has been observed for the compound **1** and none for compound **4**.

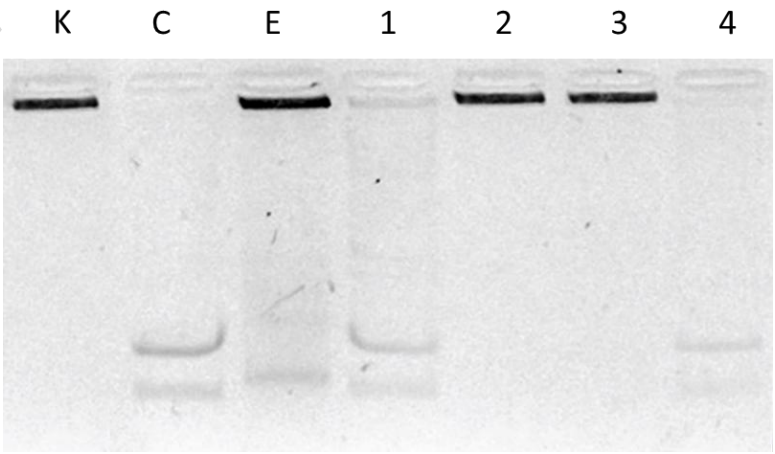


Figure 3

3.4 Docking studies

In order to investigate the binding modes between compounds **1-4** and hTopoII and to evaluate the possible binding energies, we have performed molecular docking simulations using as ligands the five molecules reported in Figure 1. Compounds **1-4** have been all scored with a higher fitness with respect to Ellipticine (Table IV).

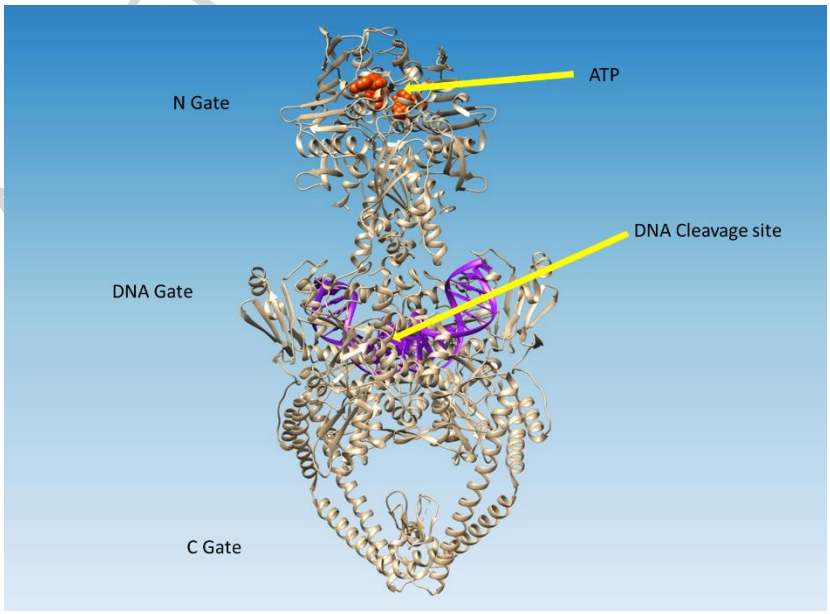
Table IV: Fitness score for the five molecules tested as valuated by GOLD

Compound	Gold Fitness
4	64.7390
1	63.9372
3	61.0058
2	58.9725
Ellipticine	53.4616

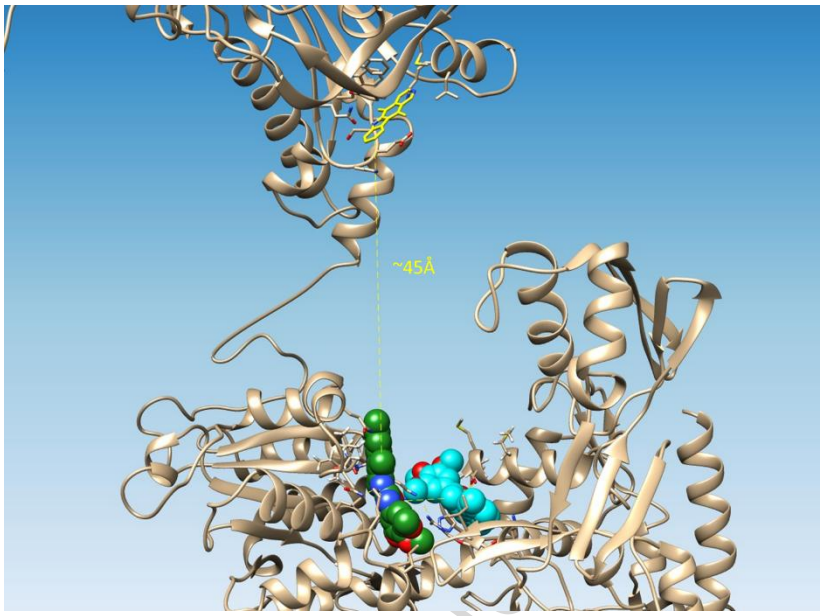
Moreover, a different binding pocket has been found for Ellipticine with respect to the four studied molecules; indeed, the latter bind to the Topoisomerase/Primase (TOPRIM) topological domain, in an area that is usually involved in DNA recognition and cleavage (Fig. 4 Panels A, B, C, D, E, F). Compounds **2** and **3** are accommodated in a position that is specific for DNA binding in hTopoII, therefore this binding modes may interfere with the regular activity of the protein. Particularly, compounds **2** and **3** bind hTopoII in an identical manner: their dimethylcarbazole moiety is involved with a hydrogen bond to the main chain oxygen atom of hTopoII residues Lys 448 and Ile 449 and through hydrophobic interactions with residue Lys 448, Leu 450 and Leu 511 side chains. Moreover, compounds **2** and **3** interact with hTopoII by hydrogen bonding formed by the ureido linker and the side chains of residues Asn 451 and Lys 614, while the trimethoxyphenyl groups are involved in hydrophobic interactions with residues Lys 614, Ile 617 and Thr 817. Additionally,

bromide group of compound **2** can form a halogen bond with Gln 459 side chain. Conversely, compound **1** binds in the same topological way but with a different "head to tail" position: its dimethylcarbazolic group occupies the region of the trimethoxyphenyl scaffold in the case of compounds **2** and **3,** giving a π-π stacking with Arg 620, and forming a hydrogen bond with Thr 817 and a halogen bond with Ser 820. This heterocycle portion also interacts with Ala 611, Phe 612 and Ile 617 by hydrophobic interactions. The amidic linker of compound **1** is involved in hydrogen bonds with Asn 451 and Lys 614 side chains while the trimethoxyphenyl group forms, in this case, hydrogen bonds with Gln 459 and interacts hydrophobically with Lys 448 and Leu 450 side chain atoms. Finally, compound **4** is positioned deeper in the protein core, closer to the winged helix domain (WHD), about 15Å far from the previously described binding site. This last compound has its dimethylcarbazolic group in stacking with Arg 672 and in hydrophobic contacts with Pro 683 and Ile 815. The amidic linker between the two aromatic groups is hydrogen bonded to Tyr 716 and Ser 722, while the trimethoxyphenyl group moiety makes a hydrogen bond with Asp 504 and a hydrophobic contact with Met 721.

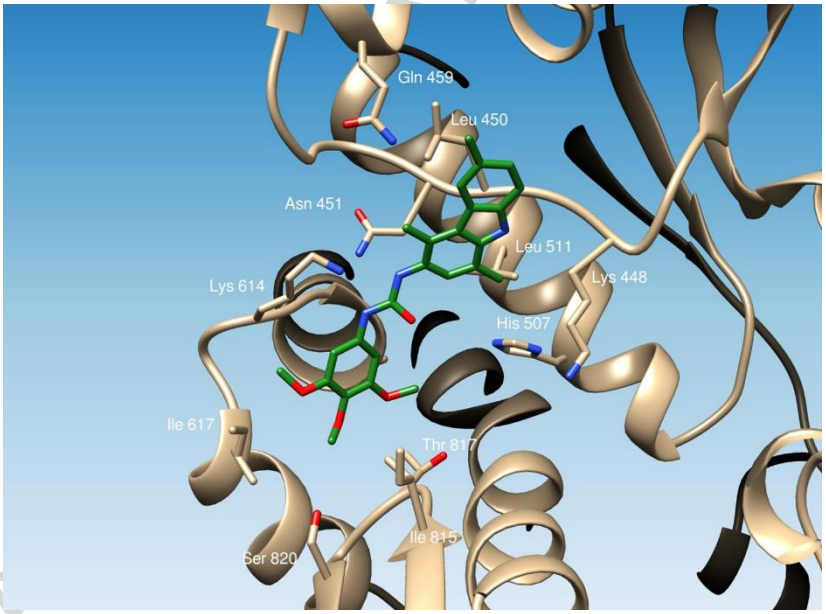
A



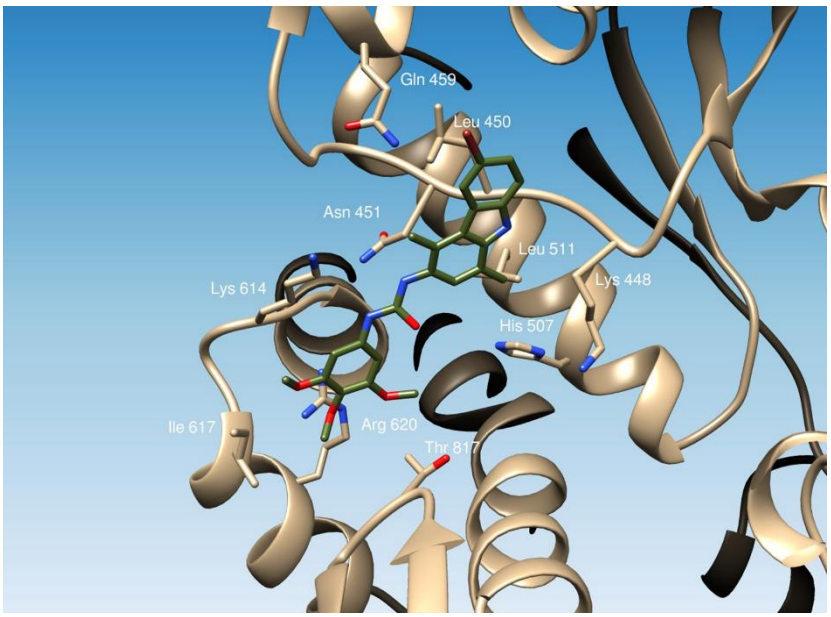
B



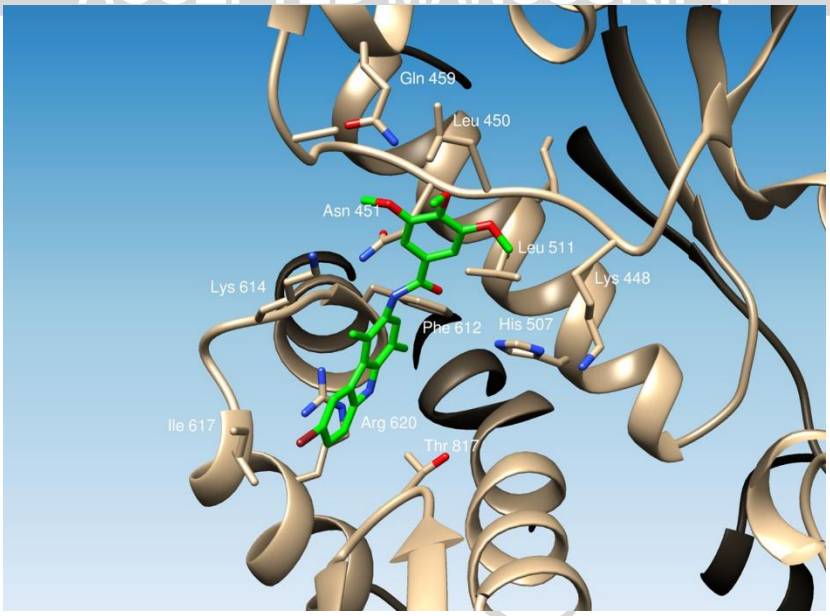
C



D



E



F

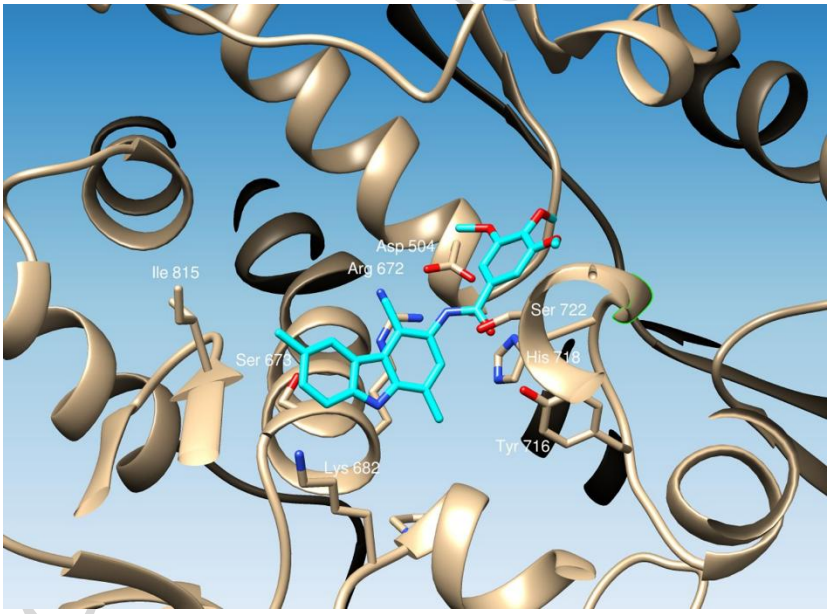


Figure 4

3.5 Compound 3 causes MCF-7 cell death by apoptosis

The most active compound (**3**) has been used in TUNEL assay conducted on MCF-7 cells, which have been plated in full media and then serum deprived for 24h, then treated at the dose of 10 µM or vehicle for 24h. At the end of the treatment time, cells have been washed with PBS, cold methanol fixed and subjected to TUNEL assay and after removal of enzyme and buffer, fixed cells have been DAPI stained (see Material and Methods). The obtained results shown in Figure 5, have evidenced a green nuclear fluorescence visible only in MCF-7 cells exposed to compound **3** (Figure 5, panel B, CF^{TM}488A) and not in the vehicle-treated cells (ctrl, Figure 5 panel B, CF^{TM}488A), indicating that this compound is able to kill MCF-7 cells by triggering apoptosis. Overlay channel (panel C) is also shown.

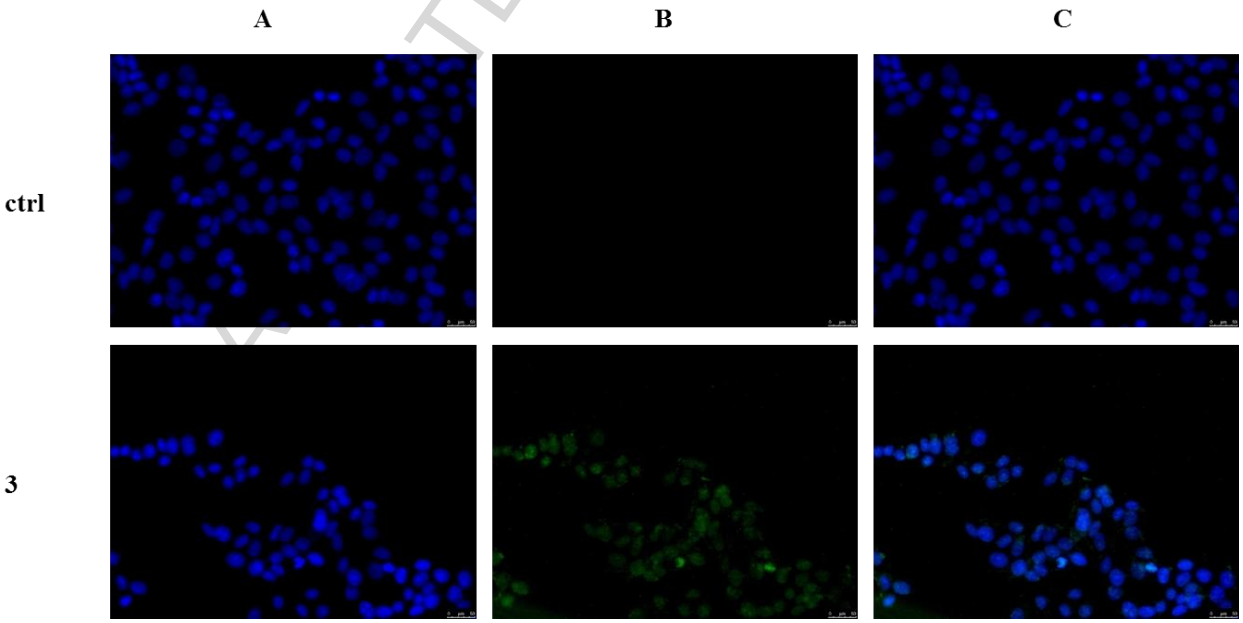
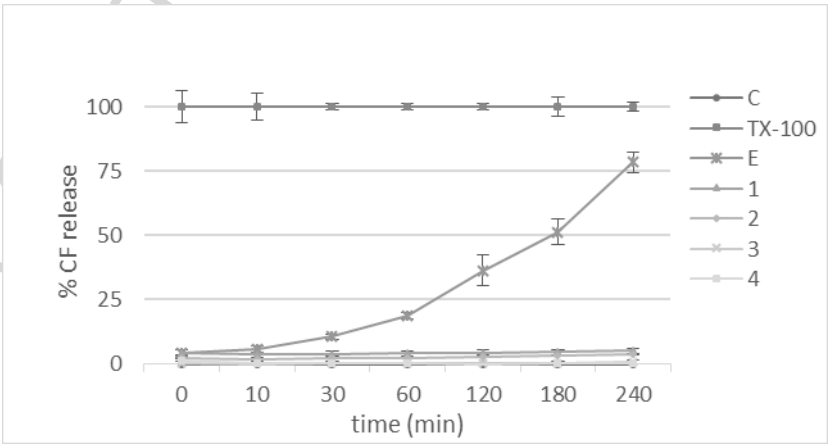


Figure 5

3.6 Ellipticine damages artificial membranes differently from trimethoxybenzamide and trimethoxyphenylurea 1,4-dimethylcarbazoles

With the aim to evaluate whether trimethoxybenzamide and trimethoxyphenylurea 1,4-dimethylcarbazole derivatives could interact with biologic membrane components, a simple artificial model of membranes has been used (*i.e.* liposomes). In these assays, large unilamellar vesicles (LUVs) (see Material and Methods), with or without cholesterol, containing the fluorescent dye carboxyfluorescein (CF), have been formulated. The exposure of LUVs to all the four compounds, at the dose of 10µM, did not produce CF leakage (see figure 6, panels A and B), indicating that these compounds do not interact with any component of LUVs, formulated with or without cholesterol. On the contrary, Ellipticine (same concentration of compounds **1**-**4**) has been shown to be able to produce CF leakage in LUVs, as already reported, mostly in LUVs without cholesterol. Membrane destabilizing effects of Ellipticine are already observed by time 0 and increase quite exponentially in the time (Figure 6, panels A and B), reaching the maximum of leakage at the end of experiment (4 hours).

A



B

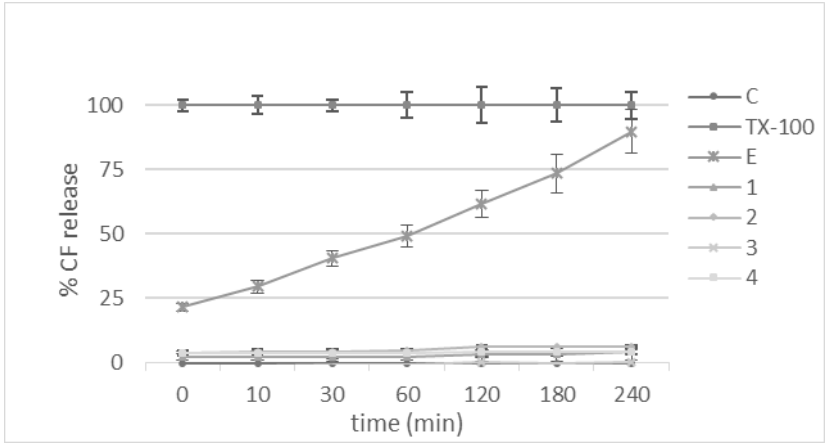


Figure 6

4. Discussion

During the development process of a new drug and its evaluation as candidate for commercial diffusion, stability is an important parameter that needs to be assessed. Environmental conditions, handling and storage can severely affect the stability and quality of a drug, which must accomplish the requirements of drug regulatory agencies. In this point of view, the stability under accelerated thermal stress conditions, namely under constant temperature and humidity and after freeze-thaw cycles, of carbazoles **1**-**4** has been evaluated. All of the four compounds have been found to be very stable. Indeed, they have been completely preserved during the whole time. In particular, compound **3** has shown the best performance, especially under thermal stress. These results suggest a possible storage of these derivatives for long time, also at room temperature. Next, considered that Ellipticine and its derivatives may play pleiotropic biological effects (Caruso et al., 2009; Gluszynska, 2015; Saturnino et al., 2013), amongst them antiproliferative effects, we evaluated the ability of compounds **1**-**4** to affect the viability of different cancer cells. In these assays we have used two human breast cancer cell line (MCF-7 and the most aggressive and metastatic MDA-MB-231), human endometrial (Ishikawa) and cervical (HeLa) cancer cells, human hepatoma cells (HepG2) and rat Leydig tumor cells (R2C), representative of common and frequent tumoral types. We have found that all the considered compounds possess a good inhibitory effect on the proliferation of all cancer cells used. In particular, for the compound **1** IC_{50} values had a range from 5.5 to 22 μM; for compound **2** from 2.5 to 13 μM; for compound **3** from 1.93 to 8 μM; and for compound **4** from 6.20 to 12 μM. As shown in table III, compound **3** was the most active and proven to be, as well, the most thermally stable compound in this study. Ellipticine, the lead compound of these molecules, was used as a reference drug in viability studies. It possesses very good IC_{50} values, ranging from 1.05 to 2.1 μM on all the tested cancer cells. Although Ellipticine

antitumor activity is higher than those of compounds **1-4**, yet a higher cytotoxicity was noticed in normal cells, namely breast epithelial cells MCF-10A and mouse embryonic fibroblasts 3T3-L1, differently from compounds **1-4** that have exerted a very lesser cytotoxicity. However, compounds **3** and **2** were found to be the most effective on the entire cancer cells panel and less toxic on non-tumoral cells, respectively, followed by compound **4**, whereas compound **1** showed the lowest activity on cancer cells proliferation and the higher inhibitory effects on normal cells (table III).

It is worthy to note that Ellipticine and many other its derivatives, used in cancer therapy, bind and regulate different cellular targets, and in most cases they possess multiple activity on the same target. Indeed, the studied compounds **1-4** have been previously reported, by some of us, as inhibitors of tubulin polymerization (Panno et al., 2014). With the aim to evaluate whether compounds **1-4** could share with Ellipticine the ability to inhibit human DNA topoisomerases, we have performed inhibition assay on hTopoI and hTopoII. DNA topoisomerases are fundamental for the DNA metabolism, maintain genomic integrity and represent the main target of several anticancer drugs that, interfering with their activity, produce irreversible DNA damages and cell death (McClendon and Osheroff, 2007). Using direct enzymatic assays on purified hTopoI and hTopoII, we have observed that compounds **2** and **3** exert a complete inhibition of hTopoII activity in our experimental conditions, even better than Ellipticine and without sharing its intercalating properties. Conversely, compound **1** seems to have minor effects, retaining a very poor ability to inhibit hTopoII in cutting catenated DNA. Indeed, even though the decatenation products have been released (two DNA bands visible at the bottom of gel, representing the nicked open circular minicircles and fully closed circular rings), a small amount of kDNA is still visible at the top of the gel (figure 3). Finally, compound **4** has not been able to inhibit hTopoII activity, given that no noticeable kDNA band is present at the top of gel (figure 3). The inhibition assays conducted on hTopo I have demonstrated that all the compounds have no effects, indicating a selectivity toward hTopo II. As well in this case, Ellipticine differs from our compounds, being able to inhibit hTopoI and maintaining its intercalative properties as demonstrated by the electrophoretic shift of the super

coiled plasmid DNA used as hTopoI substrate (figure 4). Thus, the antiproliferative activity observed in viability assays can be ascribed not only to the tubulin polymerization inhibition ability (Panno et al., 2014) but synergistically and most importantly to the high and selective inhibition property exerted on hTopoII, which has been demonstrated by these studies for the first time. In addition to these assays, docking simulations have been conducted on the complete three-dimensional model of hTopoII using all the studied compounds. Our compounds may bind in two different sites that are both located in the DNA gate but are positioned about 15Å away each other. The first site, occupied by the three compounds, **1**, **2** and **3**, is located in the TOPRIM topological domain while the second, occupied by compound **4**, is close to the WHD domain. The compounds **2** and **3** adopt an identical binding mode, whereas compound **1** is positioned in a "head to tail" conformation. This site is adopted by the protein to covalently bind and cleave DNA and therefore the presence of our molecules may interfere with the correct hTopoII functions. However, binding mode of compound **1** doesn't seem to interfere dramatically with the DNA recognition and cleavage mechanism. This may be due to an unspecific binding of compound **1,** demonstrating the importance of the length of the fragment connecting the 1,4-dimethylcarbazole nucleus with the trimethoxyphenyl group. On the other hand, compound **4** is located relatively far away (about 15Å) from the previously descripted DNA binding site, thus does not interfere with the correct functions of hTopoII.

Many data have evidenced the over-expression of one of both types of topoisomerases in cancer cells, so that the blockade of these enzymes prevents DNA lesion repair, induces DNA damage and triggers cell death, for instance by apoptosis (Ferlin et al., 2009; Hosoya and Miyagawa, 2014). Thus, in order to establish if the most active compound could induce cell death by apoptosis, we performed a TUNEL assay on MCF-7 cells treated with compound **3** or vehicle alone. Effectively, the obtained data have evidenced nuclear morphological changes, with shrunken, irregularly shaped or degraded nuclei and, most importantly, a green fluorescence is visible in the compound **3**-treated cells for 24 hours. The green fluorescence overlaps perfectly nuclear DAPI staining, indicating that

nuclear DNA has been fragmented and cells are undergoing apoptosis (figure 5, ctrl and **3**, panels A, B, C). On the contrary, the vehicle-treated cells have shown larger and round nuclei with regular contours and no evident fluorescence has been observed. Finally, it is noteworthy that Ellipticine and some its derivatives possess an amphipathic character, which enable them to interact with membrane components, *e.g.* phospholipids, and to have a strong affinity for the negatively charged phosphatidylglycerol (Terce et al., 1982, 1983). Interaction with these components may lead to membrane destabilization that could produce many effects and, amongst them, the loss of the important properties of cytoplasmic or organelles membranes in a living system (eukaryotic or prokaryotic cells), inducing cell death (Serrano-Puebla and Boya, 2015; Sun et al., 2016). For this purpose, we have used liposomes as simple biomimetic membranes to study the possible membrane destabilizing behavior of compounds **1**-**4**. We first obtained stable large unilamellar vesicles (LUVs) using a mixture of two common phospholipids (see Materials and Methods) constituting the biological cytoplasmic membrane. As cholesterol may influence membrane rigidity and fluidity, LUVs were enriched or not with cholesterol, and were filled with a fluorescent probe (CF).

This model allows to evaluate the ability of the examined compounds to permeabilize negative charged membranes, since in case of bilayer disruption, entrapped CF is released in the experimental time. In our experiments, Ellipticine showed a high ability to produce CF leakage immediately after its addition to the LUVs suspension and all along the experiment (figure 6, panels A and B). This effect is more evident on LUVs without cholesterol (figure 6, panel B), as it has been reported (Terce et al., 1983). Indeed, the presence of cholesterol in LUVs membrane partially hinders the permeabilization, probably because the higher rigidity of the membrane. Additionally, the further characterization of LUVs by light scattering after exposition to Ellipticine, has not evidenced changes in LUVs architecture (size and charge, data not shown), suggesting that, differently from TX-100 able to solubilize LUVs, Ellipticine alters the permeability, probably creating pores, which allows the exit of the fluorescent dye. Conversely, none of our compounds has produced the same effects on LUVs, indicating that they do not alter the model membrane

permeability. These outcomes suggest that Ellipticine, may damage also cell membranes without distinguishing tumor and normal cells (non-selective toxic effect) whereas compounds **1-4** did not show a similar behavior.

5. Conclusion

Up to date many carbazole derivatives have been designed and tested, with the aim to explore their multiple biological activities for increasing the selectivity and diminishing the toxic side effects. In the present study, we have investigated some biological properties of four carbazole derivatives **1-4.** The results indicated that compounds **2** and **3** possess strong hTopo II inhibitory effect and that the most active compound **3** causes cell death by apoptosis. It was also shown that compounds under testing are thermally stable and do not confer permeability to LUVs membranes, and are different from Ellipticine, which interacts with phospholipids and allows CF leakage. Thus, in conclusion, under the adopted experimental conditions, these compounds showed multiple properties, a wide antitumor activity and low cytotoxic effects on the normal cells proliferation, the matter that opens new perspectives for the N1-(trimethoxyphenyl)-N3-(1,4-dimethylcarbazole)urea derivatives in oncology.

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Figure captions

Figure 1 *N*-(6-Bromo-1,4-dimethyl-9*H*-carbazol-3-yl)-3,4,5-trimethoxybenzamide (**1**), *N*-(6-bromo-1,4-dimethyl-9*H*-carbazol-3-yl)-*N'*-(3,4,5-trimethoxyphenyl) urea (**2**), *N*-(1,4,6-trimethyl-9*H*-carbazol-3-yl)-*N'*-(3,4,5-trimethoxyphenyl) urea (**3**), 3,4,5-trimethoxy-*N*-(1,4,6-trimethyl-9*H*-carbazol-3-yl) benzamide (**4**) and Ellipticine.

Figure 2 hTopo I relaxation assay. hTopoI has been incubated in the absence (control) or presence of the compounds to test at the concentration of 10 μM, then the activity of hTopoI has been estimated and visualized on agarose gel (see material and methods). SCD, Supercoiled DNA; RD, relaxed DNA marker; C, control (DMSO); E, Ellipticine; 1-4, tested compounds.

Figure 3 hTopo II decatenation assay. hTopoII has been incubated in absence or presence of the compounds to test at the concentration 10 μM. The activity of hTopoII has been evaluated by loading the reaction products, after purification, on agarose gel (see material and methods). K, kinetoplast DNA; C, control (DMSO); E, Ellipticine; 1-4, assayed compounds.

Figure 4 hTopoII docking simulations. Panel A) Ribbon representation of a dimeric hTopoII (coloured in tan) in complex with DNA (purple) end two ATP molecules (orange). Panel B) A close up showing the different binding sites as suggested by docking simulations. In yellow Ellipticine, in dark green compound **3** binding site (shared with **1** and **2**), in cyan compound **4** binding site. The distance from Ellipticine and compound **3** binding site is approximatively 45Å, while this last is about 14Å far from compound **4** binding site. Panels C), D), E) and F) show the interaction of hTopo II with compounds **3**, **2**, **1** and **4**, respectively. The amino acids involved in ligand binding are drawn as sticks and properly labeled.

Figure 5 TUNEL assay. MCF-7 cells have been treated with compound **3** (at 10µM concentration, panels A, B, C) for 24h or veichle (ctrl, panels A, B, C). After treatment, cells have been cold methanol fixed and subjected to TdT reaction. Then, cells have been washed, dyed with DAPI and observed and imaged under an inverted fluorescence microscope (20X magnification, excitation/emission wavelength 490 nm/515 nm for CF^{TM}488A, ctrl and **3**, panels A, and 350 nm/460 nm for DAPI, ctrl and **3**, panels B). ctrl and **3**, panels C, overlay. Representative fields have been shown.

Figure 6 time course of CF leakage. Large unilamellar vesicles (LUVs) CF loaded, without (panel A) or with (panel B) cholesterol, have been exposed to Ellipticine (E) or to the four compounds (1-4), at the dose of 10µM. TX-100 has been used to disrupt LUVs. Error bars correspond to the mean deviation of three independent experiments.

Graphical abstract