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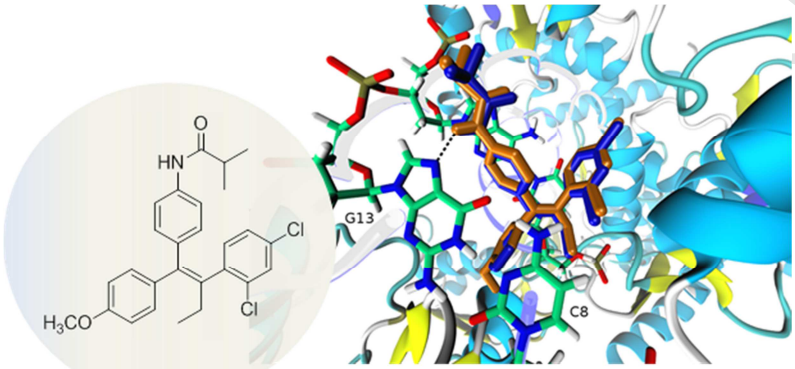
Graphical Abstract



4-(1,2-diarylbut-1-en-1-yl)isobutyranilide derivatives as inhibitors of topoisomerase II

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Abstract: The synthesis and biological evaluation of a new library of 4-(1,2-diarylbut-1-en-1-yl)isobutyranilides is described. The new compounds were found to be cytotoxic in the micromolar range in two human tumor cell lines, MCF-7 (mammary gland adenocarcinoma) and HeLa (cervix adenocarcinoma) and two human ovarian cancer cell lines (A2780 and OVCAR5). Detailed studies on the most active compound **6g** show that it was able to induce apoptosis and suggest topoisomerase II as a possible intracellular target. The relevance of the interaction of the most active compound with topoisomerase II is demonstrated and supported by docking studies.

Keywords: Tamoxifen derivatives, topoisomerase I and II, McMurry reaction

1. Introduction

Breast cancer is the second most common cancer in the world and ranks as the fifth cause of death from cancer overall. In less developed regions it is the most frequent cause of cancer death in women and the second cause in more developed regions after lung cancer.[1] Drugs that inhibit estrogen receptor (ER) or that block the production of estrogens still remain frontline interventions in the treatment and management of breast cancer.[2] Among them, tamoxifen (**1**, Figure 1), the lead compound of the selective estrogen receptor modulators (SERMs), is considered the gold-standard for

the targeted therapy of all stages of breast cancer, including male breast cancer,[3] and is used in more than 120 countries in the world.[4]

However, the adverse side effects associated to this drug[5-8] and the intrinsic or acquired resistance to the therapy represents serious obstacles in the use of tamoxifen. Indeed, a 30 % of ER-positive patients do not respond to tamoxifen therapy and the emergence of resistance to the treatment has been observed in 30-40% of patients taking the drug more than 5 years.[2,9]

The primary mechanism of action of tamoxifen and of its active metabolite 4-hydroxytamoxifen involves a competitive inhibition of estrogens to ER,[10] but high concentrations of tamoxifen are also effective in the absence of ER expression in breast cancer, melanoma, glioma, leukemia and pancreatic carcinoma.[11-13] Moreover, it has been observed that besides acting as SERM, tamoxifen can induce apoptosis in both ER-α-positive and ER-α-negative breast cancer cells through several distinct pathways including production of oxidative stress, induction of mitochondrial permeability transition, ceramide generation as well as changes in cell membrane fluidity.[14] Both ER-α-dependent and ER-α-independent pathways for tamoxifen-induced programmed cell death are critical for successful therapy and interestingly, it was also suggested that ER-α-independent induction of apoptosis could be a major mechanism of the observed antitumor effect of tamoxifen.[14,15] Tamoxifen also inhibits DNA topoisomerases and as a cationic drug is electrophoretically taken up by hepatic mitochondria to achieve high concentrations able to inhibit both β-oxidation and respiration.[16]

In this context, we have previously demonstrated for some tamoxifen analogs, an interesting cytotoxic activity on human breast cancer cell line MCF-7.[17] Most of these compounds exerted a significant antiproliferative effect also on two estrogen independent human tumour cell lines, suggesting a molecular target different from the estrogen receptor. In particular, the most interesting results were obtained for two derivatives carrying an isobutyramide moiety as side chain and notably, the isomer with the ethyl chain syn with respect to the anilide portion (Figure 1, Mod B) has shown a 4 fold more potent cytotoxic activity than the reference drug on both estrogen dependent (MCF-7) and estrogen independent (HeLa) human cell lines. Furthermore, the ability of these compounds to inhibit the relaxation activity of supercoiled

pBR322 DNA mediated by topoisomerase II was demonstrated, suggesting the nuclear enzyme as a potential intracellular target.

On the basis of these results, we report here the synthesis of a new series of tamoxifen analogs characterized by the triarylskeleton substituted with the isobutyramide moiety. We describe the synthesis of twenty-one novel compounds bearing different substituents in the rings A and B (Figure 1) and their antiproliferative activity on a panel of tumour cell lines. For the most cytotoxic derivatives, the effect on topoisomerases I and II relaxation is reported. Furthermore, cytofluorimetric analysis was performed to investigate the mechanism of cell death. Docking studies suggest a plausible explanation of the biological experimental evidences.

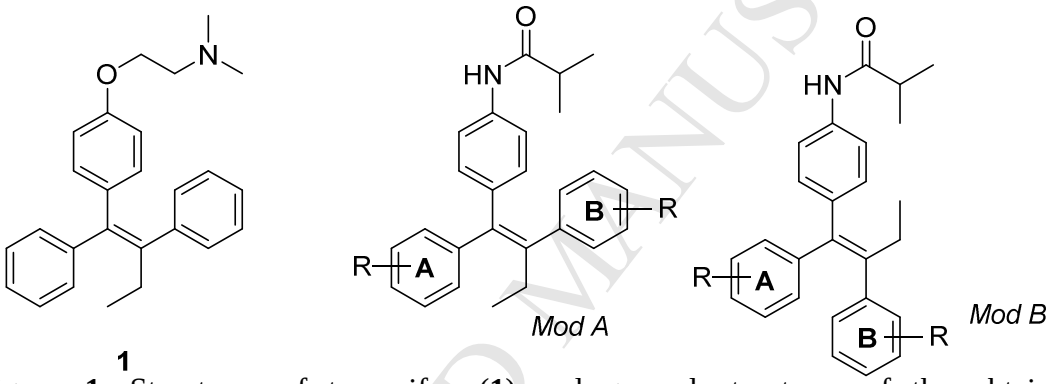


Figure 1. Structures of tamoxifen (**1**) and general structures of the obtained compounds.

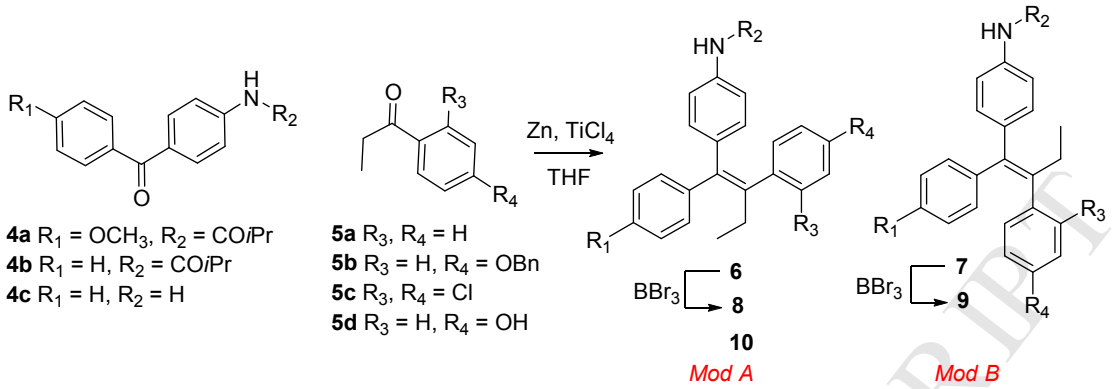
2. Results and discussion

2.1. Chemistry

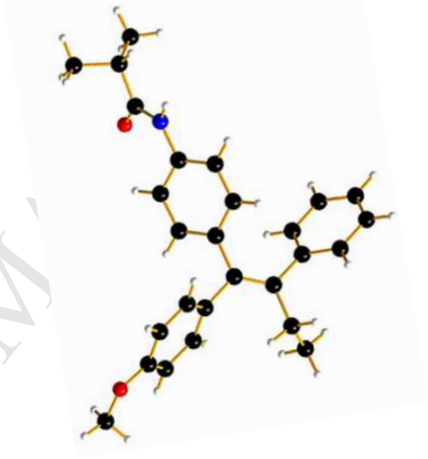
The preparation of the compound's library is based on the McMurry reaction (Scheme 1) in agreement with our previous results.[17]

Building blocks **4a** was easily prepared by reaction of *p*-anisaldehyde (**2**) with the lithium derivative of *N*-(4-bromophenyl)isobutyramide to provide the corresponding hydroxyl-amide (Scheme 2) in good yield (60%). The latter was then oxidized in the presence of Jones reagent to form the keto-amide **4a** in very good yield (85%). Keto-

amide **4b** was easily prepared by acylation of commercially available 4-aminobenzophenone (**4c**).



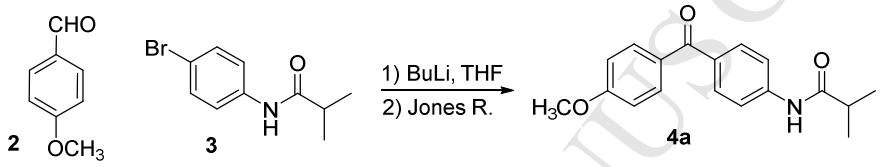
	R_1	R_2	R_3	R_4
6a/7a	H	CO*i*Pr	H	H
6b/7b	OCH_3	CO*i*Pr	H	H
6c/7c	H	CO*i*Pr	H	OBn
6d/7d	OCH_3	CO*i*Pr	H	OBn
6e/7e	OCH_3	CO*i*Pr	H	OH
6f/7f	H	CO*i*Pr	Cl	Cl
6g/7g	OCH_3	CO*i*Pr	Cl	Cl
8b/9b	OH	CO*i*Pr	H	H
8c/9c	H	CO*i*Pr	H	OH
8g/9g	OH	CO*i*Pr	Cl	Cl
10	H	H	H	H



Scheme 1. Synthetic scheme for the obtained compounds, X-ray structure for compound **6b**.

Subsequently, keto-amides **4**, were treated under McMurry olefination conditions with various ketones (**7**), to provide a mixture of *E*/*Z* isomers (**6** and **7**) that were separated by flash column chromatography or by crystallization from average to very good yields (50 – 86%). Demethylation of compounds **6b**, **7b**, **6c**, **7c**, **6g** and **7g** with BBr_3 at -78 °C afforded their corresponding derivatives **8b**, **9b**, **8c**, **9c**, **8g** and **9g** in very good yields (75 – 80%) (Scheme 1). The stereochemical assignments of the *E*, *Z* isomers were determined on the basis of 2D-NOESY experiments as previously described.[17] For three of the aforementioned compounds (**6a**, **6b** and **6f**), the corresponding crystal structures were also obtained by single-crystal X-ray diffraction experiments down to T = 100(2) K and were in accordance with the 2D-NOESY

assignments (see Supporting Information). From the conformational viewpoint, in the solid state the three derivatives adopt a similar 'vane mill' motif, with the aromatic rings being significantly staggered with respect to the central double bond. Short intermolecular Cl···Cl contacts are also set up in **6f**, suggesting the possible presence of T-shaped halogen bonds[18] in the solid state for this structure. The interested reader can find full details of the crystallographic analysis within the Supporting Information (Section S2 SI). CCDC 1436893-1436895 contain the supplementary crystallographic data for this paper. The data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/getstructures.



Scheme 2. Preparation of **4a**.

A different approach to obtain the final products with a phenyl A ring was selected as presented in Scheme 1. Although, in this case, when 4-aminobenzophenone (**4c**) was reacted with propiophenone (**5a**) to furnish compound **10** only the *Z* stereoisomer was obtained. Acylation of **10** in the presence of isobutyroyl chloride provided the final compound **6a**. In our hands, the formation of the final product **8c** or **9c** directly from keto-amide **4b** and 4-hydroxy propiophenone (**5d**) resulted unsuccessful.

2.2. *In vitro* antiproliferative activity

The antiproliferative activity of the new tamoxifen derivatives was evaluated by an *in vitro* assay performed on two human tumor cell lines, MCF-7 (mammary gland adenocarcinoma) and HeLa (cervix adenocarcinoma) in comparison with the well-known drug tamoxifen (**1**), taken as reference. The obtained results, expressed as GI_{50} values, i.e. the concentration of compound able to inhibit 50% cell growth with respect to the control culture, are shown in Table 1.

Most of the synthesized tamoxifen analogues show an interesting antiproliferative activity, with GI_{50} values in the micromolar range with a higher cytotoxic ability on HeLa cells than on MCF-7 cells. An opposite behaviour, as expected, was found for

tamoxifen, which shows on breast cancer cells a GI_{50} value about half with respect to that obtained for HeLa.

Moreover, the comparison between the obtained results and the chemical structures of the new derivatives, allowed some interesting structure-activity relationships to be revealed. In detail, the analogues devoid of any aryl substituents (**6a** and **7a**) such as those characterized by a methoxy in R_1 (**6b** and **7b**) show a scarce antiproliferative effect. Interestingly, the insertion of a hydroxyl function in the same position (compounds **8b** and **9b**), renders the compounds more active, likely suggesting a role for the hydrophilicity and/or for a hydrogen bond donor in the cytotoxic effect.

To validate the role of hydrophilicity *c*logP[19] values for compounds **6-10** (Table 1) were calculated. The partition coefficient (*c*logP) is used to estimate the lipophilicity and solubility of chemical compounds, where a higher *c*logP value indicates greater lipophilicity and poor aqueous solubility. Actually, the above assumptions appear supported by the *c*logP values and by the presence of the hydroxyl in R_4. Indeed, as regard the *c*logP, it drops from 6.28 and 6.33 for **6a**, **7a** and **6b**, **7b**, respectively, to 5.80 for compounds **8b**, **9b**.

Moreover, a hydrogen bond donor in R_4 leads to a significant antiproliferative effect (**8c** and **9c**), considerably higher than the one obtained for the analogues **6c** and **7c** carrying a benzyloxy substituent in the same position, and even higher to those obtained for the analogues **8b** and **9b**. Indeed, the GI_{50} values of **8c** and **9c** are about half of those calculated for **8b** and **9b** highlighting for these latter isomers a significantly lower cytotoxicity. This latter result suggests that, in addition to the hydrogen bonding ability, also the aryl carrying the hydroxyl group could play an important role for the cell effect.

The addition to **8c** and **9c** of a methoxy group in R_1 position (**6e** and **7e**) does not appreciably affect the antiproliferative activity, thus indicating a more crucial role for the hydroxyl that the methoxy substituent underlining once again the importance of the occurrence of a hydrogen bond. Similarly, GI_{50} values of **6c** and **7c** appear comparable to those obtained for the corresponding methoxy analogues, **6d** and **7d**.

Derivatives bearing a chloride in both R_3 and R_4 (**6f, 7f**, **6g, 7g**, **8g** and **9g**), show an interesting antiproliferative effect; indeed, compound **6g** showed the lowest GI_{50} values in both cell lines. Among this latter series of analogues, neither the hydrophilicity nor the hydrogen bond donor group seem to be important for the cytotoxic effect, probably overhung by the prevailing effect of chloride.

Finally, the intermediate **10** appears unable to exert any effect on cells as **6a** and **7a**, thus substantiating a key role for linked functional groups to the triarylethylene skeleton.

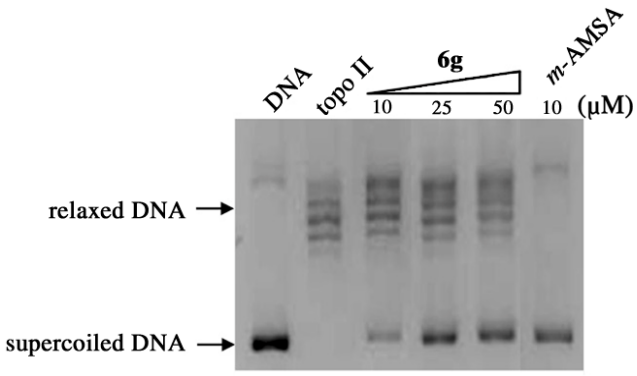
In addition, the antiproliferative activity of all the compounds was evaluated in A2780 (human ovarian carcinoma) and OVCAR5 (human ovarian epithelial carcinoma) cell lines (Table 1). Most of compounds were less active than in MFC7 and HeLa cells with IC_{50} values in the macromolar range.

Compound	GI_{50} (μM)[a]				*c*logP[b]
	MCF-7	HeLa	A2780	OVCAR5	
6a	>30	>30	>50	>50	6.28
7a	>30	28.1±1.0	31.6±3.4	>50	6.28
6b	>30	>30	>50	>50	6.33
7b	27.9±0.8	10.1±0.5	17.4±1.9	>50	6.33
6c	>30	10.2±2.3	8.8±0.5	>50	7.93
7c	>30	>30	>50	>50	7.93
6d	29.0±1.0	4.2±0.7	>50	>50	7.99
7d	>30	>30	>50	>50	7.99
6e	13.6±1.6	5.8±0.7	13.6±1.2	29.3±2.6	5.86
7e	9.0±0.7	3.7±0.1	5.7±0.5	23.6±2.5	5.86
6f	8.3±0.5	3.3±0.5	8.4±0.7	>50	7.56
7f	17.5±3.0	8.2±0.4	>50	>50	7.56
6g	6.6±0.8	2.2±0.7	3.8±0.5	>50	7.62
7g	13.2±1.3	5.5±2.0	8.2±0.6	>50	7.62
8b	14.7±1.7	9.9±0.5	34.0±4.0	42.0±4.6	5.80
9b	16.4±0.6	9.9±1.7	32.6±3.2	>50	5.80
8c	8.1±1.2	3.8±0.9	3.7±0.6	8.7±1.1	5.80
9c	8.5±0.8	5.1±0.8	4.6±0.3	12.1±0.9	5.80
8g	12.5±0.5	8.7±0.4	15.7±2.0	28.8±2.2	7.08
9g	26.6±1.0	20.3±0.2	28.1±2.7	32.3±7.6	7.08
10	>30	>30	>50	>50	5.05
1	6.5±0.8	12.0±0.9	30.3±5.0	32.0±2.8	6.1 (6.8)[20]

Table 1. Cell growth inhibition in the presence of examined compounds and tamoxifen as reference drug. [a] Values are the mean ± SD of at least three independent experiments. [b] *c*logP = calculated octanol–water partition coefficient.

2.3. Effect on topoisomerase relaxation activity

In a previous paper, we demonstrated for some cytotoxic tamoxifen derivatives, the ability to inhibit relaxation activity catalysed by topoisomerase II.[17] To verify if this enzyme could represent a possible cellular target also for the here reported compounds, we assayed the ability of compound **6g**, the most cytotoxic derivative, especially on the non estrogenic HeLa cells, and of two less active **6e** and **9c**, to affect the relaxation of pBR322 supercoiled DNA by topoisomerase II. Compound **1** was also tested in the same experimental conditions for comparison. Figure 2 shows the catalytic activity of the enzyme that converts plasmid DNA (DNA) into relaxed topoisomers (topo II). The well-known topoisomerase II inhibitor drug *m*-amsacrine (*m*-AMSA) was used as a reference at 10 μM concentration. In the presence of **6g**, the occurrence of a slight band corresponding to supercoiled DNA can be observed at 10 μM concentration, indicating a certain inhibition of the topoisomerase II activity. By increasing the concentration of **6g** up to 50 μM, a concurrent increase in the supercoiled DNA can be observed, thus confirming the inhibitory effect toward the enzyme. Compounds **6e** and **9c**, which show GI_{50} values appear approximately half with respect to **6g** (Table 1), demonstrate a similar effect also on the catalytic activity of the enzyme. Indeed, for both **6e** and **9c** an inhibitory effect comparable to that observed at 25 μM **6g** can be obtained only at 50 μM. Finally, compound **1**, which, as expected, induces only a weak antiproliferative effect on non-estrogenic HeLa cells causes a lower effect with respect to **6g**, confirming ER as its main biological target.



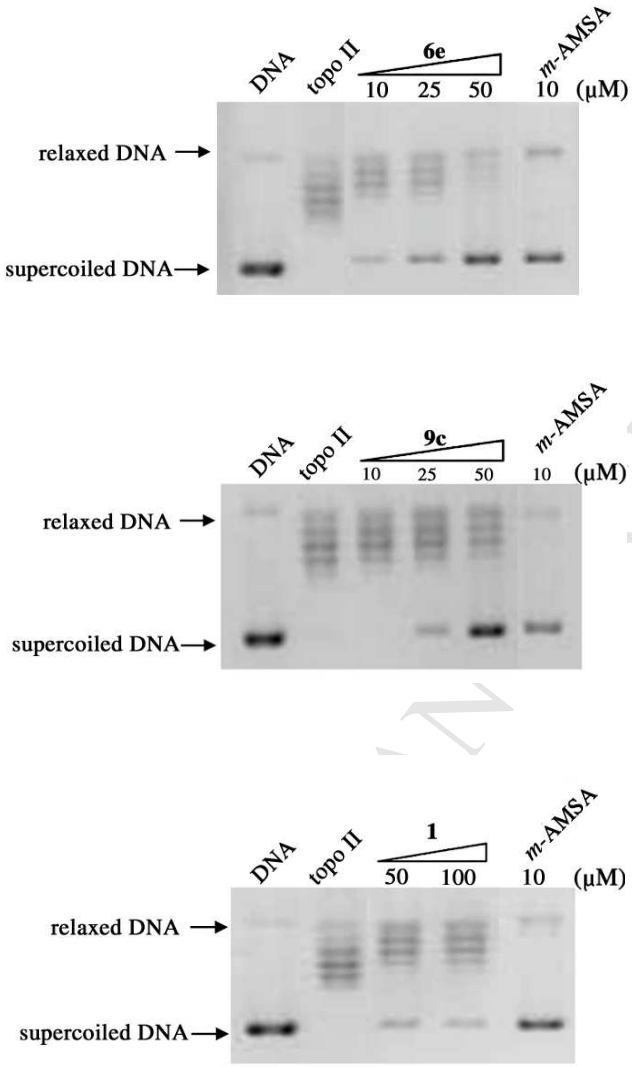


Figure 2. Effect of derivative **6g**, **6e**, **9c** and **1** on relaxation of supercoiled plasmid DNA by topoisomerase II. Supercoiled DNA (lane DNA) was incubated with topoisomerase II in the absence (lane topo II) and in the presence of test compound at indicated concentrations. *m*-AMSA was used as reference drug.

Based on the above results, it appeared of interest to establish if the effect on topoisomerase could be due to a poisoning effect rather than to an aspecific inhibition of the relaxation activity. In this connection, two of the most cytotoxic derivatives on HeLa cells, **6f** and **6g**, were tested for the ability to stabilize the cleavable complex and the obtained results are shown in Figure 3. Both compounds show the ability to act as topoisomerase II poisons, as demonstrated by the occurrence of the band corresponding to linear DNA.

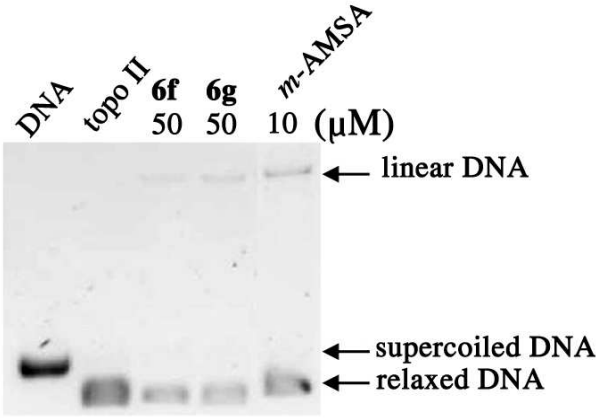


Figure 3. Effect of derivative **6f** and **6g** on the stabilization of covalent DNA-topoisomerase II complex. Supercoiled DNA (lane DNA) was incubated with topoisomerase II in the absence (lane topo II) and in the presence of test compounds at indicated concentrations. *m*-AMSA was used as reference drug.

Otherwise, **6g** appears unable to exert any effect on relaxation of supercoiled DNA catalyzed by topoisomerase I: indeed there are not differences in topoisomers pattern in the absence (topo I) or in the presence of 100 μM **6g** (Figure 4). Overall, these data point to the topoisomerase II as a possible intracellular target responsible for the cytotoxicity of **6g**, while exclude the involvement of topoisomerase I, in accordance with previous results obtained for analogues of 4-OH tamoxifen.[17]

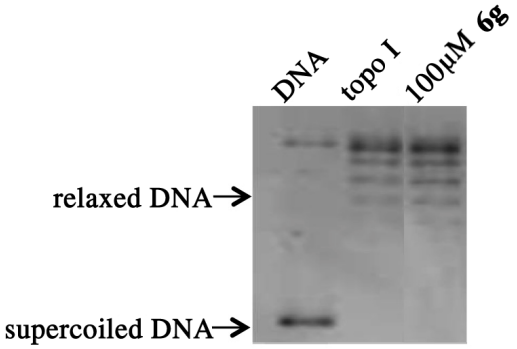


Figure 4. Effect of derivative **6g** on relaxation of supercoiled plasmid DNA by topoisomerase I. Supercoiled DNA (lane DNA) was incubated with topoisomerase I

in the absence (lane topo I) and in the presence of test compound at 100 μM concentration.

2.4. Determination of apoptosis

With the aim to investigate the mechanism of cell death induced by **6g**, a flow cytometric analysis was performed on HeLa cells labelled with Annexin V and propidium iodide (PI). Compound **1** was taken as reference. The results, shown as dot plots in Figure 5B, indicate that incubation of HeLa cells in the presence of 50 μM **6g** induces a significant decrease in viability, accomplished by a concurrent increase in apoptotic cells. In particular, upon treatment with **6g**, the percentage of viable cells diminishes from 95.3% in the control (Fig. 5A) to 31.7% while the percentage of apoptotic cells (both early and late fractions) increases from 3.8% to 66.2% (Figure 5A and 5B) indicating that tamoxifen analogue induces cell death through the activation of the apoptotic process. Also compound **1** causes apoptosis on cells (Figure 5C) and in these experimental conditions a slight difference in percentage of cell death (57.9% vs 66.2%) occurs.

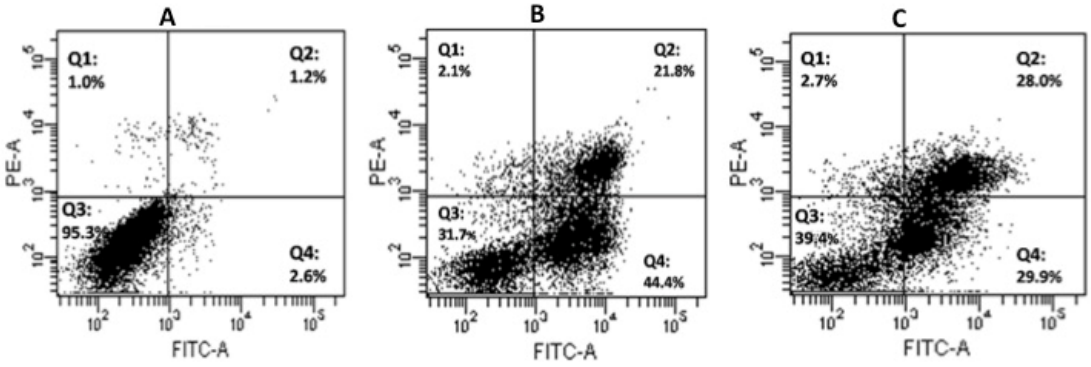


Figure 5. Dual parameter cytogram of FITC-labeled annexin V versus propidium iodide staining. HeLa cells were incubated in the absence (A) and in the presence of **6g** (B) or **1** (C) at 50 μM for 28 hours before staining.

As previously demonstrated for several cytotoxic compounds,[21-24] mitochondria can participate to intrinsic apoptosis process through the release of pro-apoptotic factors following mitochondrial membrane depolarization. In this connection, the effect of **6g** and for comparison of **1**, on mitochondria was investigated by staining whole HeLa

cells with the green fluorescent monomeric dye JC-1.[25] This lipophilic cationic fluorochrome selectively enters in mitochondria driven by the mitochondrial membrane potential, negative inside. The uptake in mitochondria, by increasing the concentration of JC-1, induces the formation of aggregates characterized by a high level of red fluorescence. A compound that induces a collapse in membrane potential provokes the leak of the dye from mitochondria to cytoplasm as monomers, resulting in a decrease of red fluorescence. The obtained results, shown in Figure 6 as dot plots, demonstrate that HeLa cells incubated with **6g** at 10, 15 and 25 μM undergoes a notable concentration-dependent decrease in red fluorescence with respect to the control. In particular, the percentage of cells with depolarized mitochondrial membrane (i.e. JC-1 monomers) increases from 4.0% in the untreated sample (control), to 80.3% following incubation for 28 hours in the presence of **6g** at 25 μM, the higher considered concentration. A dose-dependent mitochondrial depolarization is observed also for **1**, nevertheless for this latter compound a significant lower effect is observed in accordance with the cytotoxicity data shown in Table 1.

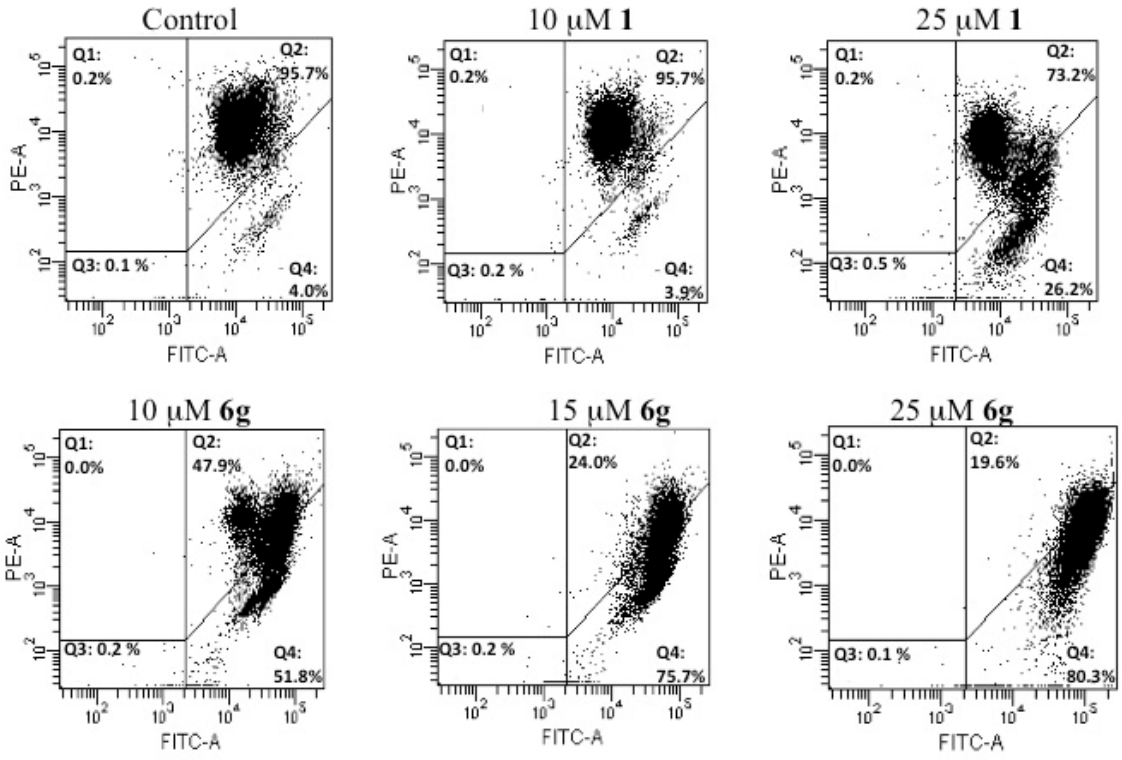


Figure 6. Mitochondrial membrane potential assessed by flow cytometry after JC-1 staining. HeLa cells were incubated in the absence (control) or in the presence of **1** or **6g** at indicated concentration for 28 hours before staining.

2.5. Molecular modeling

In order to better understand the intermolecular interactions between the Topoisomerase II/DNA complex and the ligands, molecular docking studies were performed. All the docked structures share the same binding site, located between four nucleobasis, namely G13, C8, A12 and T9 according to Wu *et al.*[26] Although the ligands exhibit many different binding modes, the main feature shared by all of them is the intercalation between the nucleobasis of at least one aromatic ring. This stacking interaction is likely to account for the inhibition of topoisomerase enzymatic activity caused by the analyzed compounds.

The binding energies calculated with the Autodock scoring function (Section S60 SI) are all largely negative, suggesting a strong binding affinity of tamoxifen derivatives for the proposed binding site, although it was not possible to effectively rank the compounds, due to the small binding energy differences observed among them, that are in general lower than the suggested significance threshold (2.5 kcal/mol). However, two of the most active compounds according to the biological studies, **6f** and **6g**, share the same orientation in the active site, stacking with the nucleobases and forming a H-bond with G13 (Figure 7). This orientation is also common to the compound **8g**.

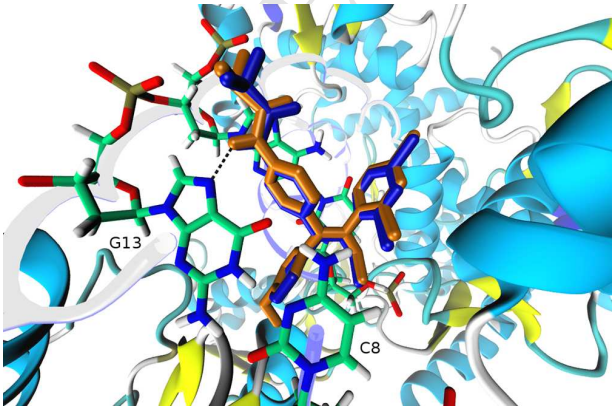


Figure 7. Docking poses of compounds **6f** (represented in blue) and **6g** (represented in orange). In both cases a H-bond between the amidic group of the molecules and Guanine 13 is observed.

3. Conclusions

A new library of 4-(1,2-diarylbut-1-en-1-yl)isobutyranilide derivatives has been described. The biological data on four different cancer cell lines showed interesting activity for most of the described compounds. The presence of the OH group or Cl moieties in the aromatic ring B seems to improve the cytotoxic activity while methoxy or hydroxyl substituents in the aromatic ring A of the tamoxifen skeleton don't affect positively the cytotoxicity of the compounds. Further biological experiments point out topoisomerase II as a possible intracellular target responsible for the cytotoxicity of these compounds and interestingly a correlation between antiproliferative effect and topoisomerase II inhibition is demonstrated for the most active derivatives. Moreover, the occurrence of cell death through the activation of the intrinsic apoptotic pathway is proved. Docking studies showed for the most active compounds the same orientation in the shared binding site by stacking and forming H-bond. In conclusion, interestingly, notwithstanding the common general triarylskeleton, the here reported tamoxifen analogs show a different biological profile with respect to the reference drug, being more cytotoxic on non estrogenic cell lines and affecting the catalytic activity of the topoisomerase II. Altogether, these results show 4-(1,2-diarylbut-1-en-1-yl) isobutyranilide as an interesting scaffold for the obtainment of new anticancer compounds.

4. Experimental section

4.1. General procedures

All reactions were carried out in oven-dried glassware and dry solvents under nitrogen atmosphere. Unless otherwise stated, all solvents were purchased from Sigma Aldrich and used without further purification. Substrates and reagents were purchased from Sigma Aldrich and used as received. Thin layer chromatography (TLC) was performed on Merck precoated $60F_{254}$ plates. Reactions were monitored by TLC on silica gel, with detection by UV light (254 nm) or by a solution of *p*-anisaldehyde with heating. Flash chromatography was performed using silica gel (240-400 mesh, Merck). All tested compounds possessed a purity of > 98% confirmed via elemental analyses (CHN) in Perkin Elmer 2400 instrument. ^{1}H-NMR spectra

were recorded on Bruker DRX-400 instruments and are reported relative to residual $CHCl_3$ and acetone-d_6. ^{13}C-NMR spectra were recorded on the same instruments (100 MHz) and are reported relative to $CDCl_3$ and acetone-d_6. Chemical shifts (δ) for proton and carbon resonances are quoted in parts per million (ppm) relative to tetramethylsilane (TMS), which was used as an internal standard. COSY, HSQC, HMBC and NOESY experiments were used in the structural assignment. MS spectra were recorded using electrospray ionization (ESI) technique on a Waters Micromass Q-Tof micro mass spectrometer.

4.2. Synthesis

4.2.1. Synthesis of *N*-(4-(4-methoxybenzoyl)phenyl)isobutyramide (4a). To a cold at -78 °C solution of *N*-(4-bromophenyl)isobutyramide (0.72 g, 3.0 mmol) in THF (17 mL), *n*-BuLi (5.6 mL, 1.6 M in Hexane, 9 mmol) was added dropwise and the new solution was stirred for 10 minutes at the same temperature. Then, *p*-anisaldehyde (**2**) (0.36 mL, 3.0 mmol) was added and the resulted solution was stirred for another 1 h at -78 °C and 1 h at r.t. After the completion of the reaction, the solvent was evaporated in vacuum, EtOAc was added and the organic layer was washed with sat. NH_4Cl, water and brine, dried with Na_2SO_4, filtered and evaporated. The residue was purified by flash column chromatography (Hex/EtOAc 6:4) to provide *N*-(4-(hydroxy(4-methoxyphenyl)methyl)phenyl)isobutyramide as oil (60% yield). R_f = 0.36 (Hex/EtOAc 6:4); ^{1}H NMR ($CDCl_3$) :δ = 7.51 – 7.48 (2H, m), 7.34 (1H, br. s), 7.29 (2H, d, *J* 6.8 Hz), 7.24 (2H, d, *J* 8.8 Hz), 6.86 (2H, d, *J* 8.8 Hz), 5.31 (1H, s), 3.80 (3H, s), 2.58 – 2.47 (1H, m), 1.26 (6H, d, *J* 6.8 Hz); ^{13}C NMR ($CDCl_3$): δ = 175.3, 158.9, 138.3, 137.2, 134.4, 128.5, 127.8, 119.8, 113.8, 79.06, 55.27, 36.66, 19.63; MS: 300.37 ($M+H^+$). To a cold at 0 °C solution of *N*-(4-(hydroxy(4-methoxyphenyl)methyl)phenyl)isobutyramide (0.35 g, 1.2 mmol) in acetone (49 mL), Jones reagent (9.1 mL) was added and the new solution was stirred for 30 minutes at the same temperature. After the completion of the reaction, 2-propanol was added and the solvent was evaporated in vacuum. Then, EtOAc was added and the organic layer was washed with water and brine, dried with Na_2SO_4, filtered and evaporated in vacuum till dryness to provide keto-amide **4a** as white amorphous solid (85% yield). R_f = 0.44 (Hex/EtOAc 1:1); ^{1}H NMR ($CDCl_3$): δ = 7.87 (1H, br. s), 7.80 (2H, d, *J* 8.8

Hz), 7.77 (2H, d, *J* 8.8 Hz), 7.69 (2H, d, *J* 8.8 Hz), 6.97 (2H, d, *J* 8.8 Hz), 3.90 (3H, s), 2.64 – 2.54 (1H, m), 1.27 (6H, d, *J* 6.8 Hz); ^{13}C NMR ($CDCl_3$): δ = 194.9, 176.0, 163.8, 142.4, 134.5, 132.8, 131.7, 131.3, 119.6, 114.3, 56.03, 37.35, 20.05; MS: 298.36 ($M+H^+$).

4.2.2. Synthesis of *N*-(4-benzoylphenyl)isobutyramide (4b). To a cooled at 0 °C solution of 4-aminobenzophenone (**4c**, 5.0 g, 25.0 mmol) in *t*-butyl methyl ether (123 mL) and 40% NaOH (3 mL), isobutyryl chloride was added (3.2 mL, 30 mmol) and the new solution was stirred for 30 minutes at r.t. After the completion of the reaction, the solvent was evaporated in vacuum, EtOAc was added and the organic layer was washed with water and brine, dried with Na_2SO_4, filtered and evaporated in vacuum till dryness to provide compound **4b** as white amorphous solid (85% yield). R_f = 0.25 (Hex/EtOAc 7:3); ^{1}H NMR ($CDCl_3$): δ = 7.82 (2H, d, *J* 8.8 Hz), 7.81 (1H, br. s), 7.78 (2H, d, *J* 7.6 Hz), 7.71 (2H, d, *J* 8.8 Hz), 7.60 (1H, d, *J* 7.6 Hz), 7.49 (2H, d, *J* 7.6 Hz), 2.64 – 2.54 (1H, m), 1.28 (6H, d, *J* 7.2 Hz); ^{13}C NMR ($CDCl_3$): δ = 195.8, 175.8, 142.3, 137.9, 132.8, 132.2, 131.6, 129.8, 128.3, 118.8, 36.78, 19.55; MS: 268.34 ($M+H^+$).

4.2.3. General procedure for the synthesis of analogues 6 and 7.

4.2.3.1. Synthesis of (*Z*)-*N*-(4-(1,2-diphenylbut-1-en-1-yl)phenyl)isobutyramide (6a) and (*E*)-*N*-(4-(1,2-diphenylbut-1-en-1-yl)phenyl)isobutyramide (7a). To a cold at -10 °C suspension of Zn (1.2 g, 18 mmol) in THF (18 mL), $TiCl_4$ (0.82 mL, 7.5 mmol) was added dropwise and the new mixture was stirred for another 10 minutes in the same temperature and after was refluxed for 2 h. Then, a solution of keto-amide **4b** (0.50 g, 1.9 mmol) and propiophenone (**5a**, 0.28 g, 2.1 mmol) in THF (37 mL), was added to the cooled suspension of the titanium reagent at 0 °C, and the reaction mixture was refluxed for 2.5 h. After the completion of the reaction, the reaction mixture was cooled to r.t. and poured into a 10% aq K_2CO_3 solution (15 mL). Vigorous stirring was maintained for 5 min, and the dispersed insoluble material was removed by vacuum filtration using celite. The organic layer was separated and the aqueous layer was extracted 3 times with EtOAc. The combined EtOAc extracts were washed with water and brine, dried with Na_2SO_4, filtered and evaporated. The residue was purified by flash column chromatography (Hex/EtOAc 8:2) to provide a mixture of the 2 diastereomers **6a** and **7a** (86% total yield). The 2 diastereomers were

separated by crystallization from MeOH to provide compound **6a** as crystals and compound **9a** as white amorphous solid in a ratio **6a**/**7a** = 3. **6a:** R_f = 0.30 (Hex/EtOAc 8:2); ^{1}H NMR ($CDCl_3$): δ = 7.38 – 7.35 (2H, m), 7.31 – 7.25 (3H, m), 7.20 (2H, d, *J* 8.4 Hz), 7.19 (2H, d, *J* 7.2 Hz), 7.15 – 7.14 (3H, m), 7.05 (1H, br. s), 6.84 (2H, d, *J* 8.4 Hz), 2.49 (2H, q, *J* 7.6 Hz), 2.47 – 2.38 (1H, m), 1.21 (6H, d, *J* 7.2 Hz), 0.956 (3H, t, *J* 7.6 Hz); ^{13}C NMR ($CDCl_3$): δ = 175.3, 144.1, 143.0, 142.8, 139.7, 139.0, 136.4, 131.9, 130.3, 130.1, 128.7, 128.5, 127.2, 126.8, 119.4, 37.20, 29.61, 20.07, 13.94; MS: 370.52 ($M+H^+$); Anal. Calcd for $C_{26}H_{27}NO$: C, 84.51; H, 7.37; N, 3.79. Found: C, 83.65; H, 7.16; N, 3.65. . **7a:** R_f = 0.30 (Hex/EtOAc 8:2); ^{1}H NMR (acetone-d_6): δ = 9.11 (1H, br. s), 7.72 (2H, d, *J* 8.8 Hz), 7.38 (2H, t, *J* 8.8 Hz), 7.30 (1H, t, *J* 8.8 Hz), 7.20 (2H, d, *J* 8.8 Hz), 7.03 – 6.99 (3H, m), 6.93 (2H, d, *J* 8.4 Hz), 6.83 (2H, d, *J* 8.8 Hz), 2.68 – 2.61 (1H, m), 2.53 (2H, q, *J* 7.6 Hz), 1.19 (6H, d, *J* 7.2 Hz), 0.948 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.0, 143.3, 142.3, 138.7, 138.4, 138.3, 137.8, 130.8, 130.6, 129.5, 128.2, 127.8, 126.6, 125.7, 118.8, 35.72, 28.82, 19.02, 12.89; MS: 370.51 ($M+H^+$); Anal. Calcd for $C_{26}H_{27}NO$: C, 84.51; H, 7.37; N, 3.79. Found: C, 83.64; H, 7.14; N, 3.66.

4.2.3.2. Synthesis of (*E*)-*N*-(4-(1-(4-methoxyphenyl)-2-phenylbut-1-en-1-yl)phenyl)isobutyramide (6b) and (*Z*)-*N*-(4-(1-(4-methoxyphenyl)-2-phenylbut-1-en-1-yl)phenyl)isobutyramide (7b). According to the general procedure, compounds **6b** and **7b** were obtained from keto-amide **4a** and propiophenone (**5a**) after purification by flash column chromatography (Hex/EtOAc 7:3) and evaporation of the solvent as a mixture of diastereomers (74% yield). The 2 diastereomers were separated by crystallization from MeOH to provide compound **6b** as crystals and compound **7b** as white amorphous solid in a ratio **6b**/**7b** = 0.15. **6b:** R_f = 0.42 (Hex/EtOAc 7:3); ^{1}H NMR ($CDCl_3$): δ = 7.28 – 7.21 (9H, m), 7.04 (1H, br. s), 6.90 (2H, d, *J* 8.4 Hz), 6.83 (2H, d, *J* 8.0 Hz), 3.85 (3H, s), 2.52 (2H, q, *J* 7.2 Hz), 2.47 – 2.40 (1H, m), 1.21 (6H, d, *J* 6.8 Hz), 0.960 (3H, t, *J* 7.2 Hz); ^{13}C NMR ($CDCl_3$): δ = 175.4, 159.2, 143.2, 142.6, 140.1, 138.7, 136.7, 136.4, 131.9, 131.2, 130.3, 128.4, 126.6, 119.5, 114.4, 55.84, 37.21, 29.61, 20.05, 13.94; MS: 400.54 ($M+H^+$); Anal. Calcd for $C_{27}H_{29}NO_2$: C, 81.17; H, 7.32; N, 3.51. Found: C, 80.35; H, 7.11; N, 3.37. **7b:** R_f = 0.42 (Hex/EtOAc 7:3); ^{1}H NMR ($CDCl_3$): δ = 7.55 (2H, d, *J* 8.0 Hz), 7.28 (1H, br. s), 7.23 – 7.13 (7H, m), 6.79 (2H, d, *J* 8.4 Hz), 6.56 (2H, d, *J* 8.4 Hz), 3.70 (3H, s), 2.58 – 2.52 (1H, m), 2.50 (2H, q, *J* 7.8 Hz), 1.29 (6H, d, *J* 6.8 Hz), 0.947 (3H,

t, *J* 6.8 Hz); ^{13}C NMR ($CDCl_3$): δ = 175.7, 158.4, 143.3, 142.3, 140.6, 138.6, 137.3, 136.2, 132.4, 130.7, 130.3, 128.4, 126.6, 120.3, 113.6, 55.63, 37.26, 29.59, 20.12, 13.97; MS: 400.53 ($M+H^+$); Anal. Calcd for $C_{27}H_{29}NO_2$: C, 81.17; H, 7.32; N, 3.51. Found: C, 80.34; H, 7.12; N, 3.38.

4.2.3.3. Synthesis of (*Z*)-*N*-(4-(2-(4-(benzyloxy)phenyl)-1-phenylbut-1-en-1-yl)phenyl) isobutyramide (6c) and (*E*)-*N*-(4-(2-(4-(benzyloxy)phenyl)-1-phenylbut-1-en-1-yl)phenyl) isobutyramide (7c). According to the general procedure, compounds **6c** and **7c** were obtained from keto-amide **4b** and 1-(4-(benzyloxy)phenyl)propan-1-one (**5b**) after purification by flash column chromatography (Hex/EtOAc 7:3) and evaporation of the solvent as white amorphous solids in a ratio **6c**/**7c** = 0.33 (62% yield). **6c:** R_f = 0.41 (Hex/EtOAc 7:3); ^{1}H NMR (acetone-d_6): δ = 9.08 (1H, br. s), 7.71 (2H, d, *J* 8.8 Hz), 7.47 (2H, br. d, *J* 6.8 Hz), 7.39 (2H, t, *J* 6.4 Hz), 7.28 (1H, t, *J* 6.8 Hz), 7.18 (2H, d, *J* 8.8 Hz), 7.09 (2H, d, *J* 8.8 Hz), 7.05 – 6.98 (3H, m), 6.94 (2H, dd, *J* 8.4 Hz *J* 1.6 Hz), 6.84 (2H, d, *J* 8.8 Hz), 5.05 (2H, s), 2.69 – 2.59 (1H, m), 2.50 (2H, q, *J* 7.6 Hz), 1.19 (6H, d, *J* 6.8 Hz), 0.950 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.7, 158.0, 144.2, 142.1, 139.2, 139.0, 138.8, 138.2, 135.2, 131.4, 131.3, 130.2, 130.0, 129.0, 128.4, 128.0, 126.3, 120.0, 114.8, 70.15, 36.44, 29.24, 19.72, 13.71; MS: 476.64 ($M+H^+$); Anal. Calcd for $C_{33}H_{33}NO_2$: C, 83.33; H, 6.99; N, 2.94. Found: C, 82.49; H, 6.79; N, 2.81. **7c:** R_f = 0.45 (Hex/EtOAc 7:3); ^{1}H NMR (acetone-d_6): δ = 8.89 (1H, br. s), 7.48 (2H, br. d, *J* 6.8 Hz), 7.43 – 7.30 (8H, m), 7.27 (2H, d, *J* 8.0 Hz), 7.13 (2H, d, *J* 8.8 Hz), 6.88 (2H, d, *J* 8.8 Hz), 6.84 (2H, d, *J* 8.8 Hz), 5.07 (2H, s), 2.60 – 2.51 (1H, m), 2.46 (2H, q, *J* 7.2 Hz), 1.13 (6H, d, *J* 6.8 Hz), 0.933 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 174.8, 157.4, 143.9, 141.1, 138.2, 137.5, 134.6, 130.9, 130.8, 129.3, 128.4, 128.1, 127.7, 127.6, 126.5, 118.2, 114.2, 69.52, 35.70, 28.44, 18.98, 12.98; MS: 476.63 ($M+H^+$); Anal. Calcd for $C_{33}H_{33}NO_2$: C, 83.33; H, 6.99; N, 2.94. Found: C, 82.48; H, 6.80; N, 2.82.

4.2.3.4. Synthesis of (*E*)-*N*-(4-(2-(4-(benzyloxy)phenyl)-1-(4-methoxyphenyl)but-1-en-1-yl)phenyl) isobutyramide (6d) and (*Z*)-*N*-(4-(2-(4-(benzyloxy)phenyl)-1-(4-methoxyphenyl)but-1-en-1-yl)phenyl) isobutyramide (7d). According to the general procedure, compounds **6d** and **7d** were obtained from keto-amide **4a** and 1-(4-(benzyloxy)phenyl)propan-1-one (**5b**) after purification by flash column

chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as white amorphous solids in a ratio **6d**/**7d** = 1 (69% yield). **6d:** R_f = 0.50 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 8.87 (1H, br. s), 7.48 (2H, br. d, *J* 6.8 Hz), 7.48 – 7.38 (4H, m), 7.34 (1H, t, *J* 7.2 Hz), 7.17 (2H, d, *J* 8.8 Hz), 7.11 (2H, d, *J* 8.8 Hz), 6.95 (2H, d, *J* 8.8 Hz), 6.86 (2H, d, *J* 8.8 Hz), 6.83 (2H, d, *J* 8.8 Hz), 5.06 (2H, s), 3.83 (3H, s), 2.60 – 2.53 (1H, m), 2.49 (2H, q, *J* 7.6 Hz), 1.13 (6H, d, *J* 7.2 Hz), 0.940 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 174.8, 158.6, 157.3, 140.8, 138.5, 137.8, 137.6, 137.4, 136.1, 134.8, 130.9, 130.8, 130.4, 128.4, 127.7, 127.6, 118.1, 114.2, 113.5, 69.51, 54.60, 35.71, 28.82, 18.99, 13.03; MS: 506.67 ($M+H^+$); Anal. Calcd for $C_{34}H_{35}NO_3$: C, 80.76; H, 6.98; N, 2.77. Found: C, 79.93; H, 6.78; N, 2.65. **7d:** R_f = 0.46 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 9.08 (1H, br. s), 7.70 (2H, d, *J* 8.4 Hz), 7.48 (2H, d, *J* 7.2 Hz), 7.40 (2H, t, *J* 7.2 Hz), 7.34 (1H, t, *J* 7.2 Hz), 7.16 (2H, d, *J* 8.4 Hz), 7.10 (2H, d, *J* 8.8 Hz), 6.86 (2H, d, *J* 8.8 Hz), 6.83 (2H, d, *J* 8.8 Hz), 6.61 (2H, d, *J* 8.8 Hz), 5.07 (2H, s), 3.69 (3H, s), 2.68 – 2.61 (1H, m), 2.48 (2H, q, *J* 7.2 Hz), 1.19 (6H, d, *J* 6.8 Hz), 0.935 (3H, t, *J* 7.2 Hz); ^{13}C NMR (acetone-d_6): δ = 175.6, 158.5, 158.0, 141.3, 139.6, 138.9, 138.6, 138.3, 136.5, 135.7, 132.3, 131.4, 130.1, 128.9, 128.2, 128.1, 119.7, 115.0, 113.5, 70.37, 55.08, 36.42, 29.42, 19.57, 13.53; MS: 506.66 ($M+H^+$); Anal. Calcd for $C_{34}H_{35}NO_3$: C, 80.76; H, 6.98; N, 2.77. Found: C, 79.94; H, 6.77; N, 2.67.

4.2.3.5. Synthesis of (*E*)-*N*-(4-(2-(4-hydroxyphenyl)-1-(4-methoxyphenyl)but-1-en-1-yl)phenyl) isobutyramide (6e) and (*Z*)-*N*-(4-(2-(4-hydroxyphenyl)-1-(4-methoxyphenyl)but-1-en-1-yl)phenyl) isobutyramide (7e). According to the general procedure, compounds **6e** and **7e** were obtained from keto-amide **4a** and 1-(4-hydroxyphenyl)propan-1-one (**5d**) after purification by flash column chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as oils in a ratio **6e**/**7e** = 1 (56% yield). **6e:** R_f = 0.26 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 8.86 (1H, br. s), 8.16 (1H, br. s), 7.37 (2H, d, *J* 8.4 Hz), 7.16 (2H, d, *J* 8.4 Hz), 7.00 (2H, d, *J* 8.4 Hz), 6.94 (2H, d, *J* 8.8 Hz), 6.82 (2H, d, *J* 8.8 Hz), 6.68 (2H, d, *J* 8.8 Hz), 3.83 (3H, s), 2.59 – 2.52 (1H, m), 2.47 (2H, q, *J* 7.2 Hz), 1.12 (6H, d, *J* 6.8 Hz), 0.940 (3H, t, *J* 7.2 Hz); ^{13}C NMR (acetone-d_6): δ = 174.8, 158.5, 155.7, 141.1, 138.7, 137.3, 136.2, 133.3, 130.9, 130.7, 130.4, 118.1, 114.8, 113.4, 54.59, 35.70, 28.83, 18.99, 13.06; MS: 416.55 ($M+H^+$); Anal. Calcd for $C_{27}H_{29}NO_3$: C, 78.04; H, 7.03; N, 3.37. Found: C, 77.28; H, 6.84; N, 3.23. **7e:** R_f = 0.28 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ =

9.13 (1H, br. s), 8.25 (1H, br. s), 7.70 (2H, d, *J* 8.4 Hz), 7.15 (2H, d, *J* 8.4 Hz), 6.99 (2H, d, *J* 8.8 Hz), 6.82 (2H, d, *J* 8.8 Hz), 6.68 (2H, d, *J* 8.8 Hz), 6.61 (2H, d, *J* 8.8 Hz), 3.68 (3H, s), 2.68 – 2.61 (1H, m), 2.46 (2H, q, *J* 7.6 Hz), 1.19 (6H, d, *J* 6.8 Hz), 0.932 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.6, 158.5, 156.4, 141.7, 139.8, 138.8, 137.8, 136.7, 134.2, 132.2, 131.3, 130.1, 119.9, 115.5, 113.5, 55.09, 36.42, 29.08, 19.54, 13.47; MS: 416.56 ($M+H^+$); Anal. Calcd for $C_{27}H_{29}NO_3$: C, 78.04; H, 7.03; N, 3.37. Found: C, 77.28; H, 6.83; N, 3.24.

4.2.3.6. Synthesis of (*Z*)-*N*-(4-(2-(2,4-dichlorophenyl)-1-phenylbut-1-enyl)phenyl)isobutyramide (6f) and (*E*)-*N*-(4-(2-(2,4-dichlorophenyl)-1-phenylbut-1-enyl)phenyl)isobutyramide (7f). According to the general procedure, compounds **6f** and **7f** were obtained from keto-amide **4b** and 1-(2,4-dichlorophenyl)propan-1-one (**5c**) after purification by flash column chromatography (Hex/EtOAc 8:2) and evaporation of the solvent as white amorphous solids in a ratio **6f**/**7f** = 2.2 (50% yield). **6f:** R_f = 0.30 (Hex/EtOAc 8:2); ^{1}H NMR (acetone-d_6): δ = 8.89 (1H, br. s), 7.42 – 7.39 (5H, m), 7.34 – 7.25 (5H, m), 6.92 (2H, d, *J* 8.8 Hz), 2.57 – 2.51 (1H, m), 2.45 (2H, q, *J* 7.6 Hz), 1.11 (6H, d, *J* 6.8 Hz), 0.949 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.5, 143.1, 142.0, 140.4, 138.8, 138.3, 137.7, 134.7, 133.1, 134.1, 130.3, 129.7, 129.5, 128.9, 127.6, 127.4, 118.8, 36.35, 28.40, 19.59, 12.91; MS: 439.41 ($M+H^+$); Anal. Calcd for $C_{26}H_{25}Cl_2NO$: C, 71.23; H, 5.75; N, 3.20. Found: C, 70.50; H, 5.59; N, 3.06. **7f:** R_f = 0.24 (Hex/EtOAc 8:2); ^{1}H NMR (acetone-d_6): δ = 9.15 (1H, br. s), 7.74 (2H, d, *J* 8.8 Hz), 7.39 (1H, d, *J* 1.6 Hz), 7.29 – 7.24 (4H, m), 7.06 – 7.00 (4H, m), 6.92 (1H, t, *J* 8.4 Hz), 2.68 – 2.61 (1H, m), 2.49 (2H, q, *J* 7.6 Hz), 1.18 (6H, d, *J* 6.8 Hz), 0.968 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.6, 143.2, 142.1, 140.5, 139.4, 138.7, 137.7, 134.7, 133.1, 131.1, 129.9, 129.4, 128.0, 127.2, 126.9, 119.8, 36.43, 28.24, 19.58, 12.82; MS: 439.41 ($M+H^+$); Anal. Calcd for $C_{26}H_{25}Cl_2NO$: C, 71.23; H, 5.75; N, 3.20. Found: C, 70.48; H, 5.60; N, 3.05.

4.2.3.7. Synthesis of (*E*)-*N*-(4-(2-(2,4-dichlorophenyl)-1-(4-methoxyphenyl)but-1-enyl)phenyl) isobutyramide (6g) and (*Z*)-*N*-(4-(2-(2,4-dichlorophenyl)-1-(4-methoxyphenyl)but-1-enyl)phenyl) isobutyramide (7g). According to the general procedure, compounds **6g** and **7g** were obtained from keto-amide **4a** and 1-(2,4-dichlorophenyl)propan-1-one (**5c**) after purification by flash column chromatography

(Hex/EtOAc 6:4) and evaporation of the solvent as white amorphous solids in a ratio **6g**/**7g** = 1 (58% yield). **6g:** R_f = 0.54 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 8.90 (1H, br. s), 7.41 – 7.38 (3H, m), 7.29 (1H, d, *J* 8.4 Hz), 7.26 (1H, d, *J* 2.0 Hz), 7.23 (2H, d, *J* 8.8 Hz), 6.97 (2H, d, *J* 8.8 Hz), 6.89 (2H, d, *J* 8.4 Hz), 3.83 (3H, s), 2.58 – 2.52 (1H, m), 2.48 (2H, q, *J* 7.6 Hz), 1.11 (6H, d, *J* 6.8 Hz), 0.956 (3H, t, *J* 7.2 Hz); ^{13}C NMR (acetone-d_6): δ = 175.5, 159.5, 141.6, 140.7, 138.7, 138.1, 137.9, 135.2, 134.7, 134.7, 132.9, 131.0, 130.4, 129.5, 127.4, 118.8, 114.2, 55.30, 36.37, 28.46, 19.63, 13.01; MS: 469.42 ($M+H^+$); Anal. Calcd for $C_{27}H_{27}Cl_2NO_2$: C, 69.23; H, 5.81; N, 2.99. Found: C, 68.52; H, 5.65; N, 2.86. **7g:** R_f = 0.51 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 9.10 (1H, br. s), 7.73 (2H, d, *J* 8.8 Hz), 7.40 – 7.39 (1H, m), 7.29 (1H, d, *J* 8.4 Hz), 7.26 (1H, d, *J* 2.0 Hz), 7.23 (2H, d, *J* 8.4 Hz), 6.90 (2H, d, *J* 8.8 Hz), 6.63 (2H, d, *J* 8.8 Hz), 3.68 (3H, s), 2.68 – 2.61 (1H, m), 2.48 (2H, q, *J* 7.6 Hz), 1.19 (6H, d, *J* 6.8 Hz), 0.954 (3H, t, *J* 7.2 Hz); ^{13}C NMR (acetone-d_6): δ = 175.1, 158.2, 141.0, 140.1, 138.7, 137.3, 137.1, 134.8, 134.1, 133.5, 132.3, 130.6, 129.4, 128.8, 126.7, 118.9, 112.8, 54.41, 35.78, 27.82, 19.04, 12.36; MS: 469.43 ($M+H^+$); Anal. Calcd for $C_{27}H_{27}Cl_2NO_2$: C, 69.23; H, 5.81; N, 2.99. Found: C, 68.51; H, 5.64; N, 2.85.

4.2.3.8. Synthesis of (*Z*)-4-(1,2-diphenylbut-1-en-1-yl)aniline (10). According to the general procedure, compound **10** was obtained from 4-aminobenzophenone (**4c**) and propiophenone (5**a**) after purification by flash column chromatography (Hex/EtOAc 8:2) and evaporation of the solvent as white amorphous solid (40% yield). R_f = 0.24 (Hex/EtOAc 8:2); ^{1}H NMR ($CDCl_3$): δ = 7.37 (2H, t, *J* 7.2 Hz), 7.31 – 7.27 (3H, m), 7.23 – 7.13 (5H, m), 6.69 (2H, d, *J* 8.8 Hz), 6.40 (2H, d, *J* 8.8 Hz), 2.48 (2H, q, *J* 7.6 Hz), 0.950 (3H, t, *J* 7.6 Hz); ^{13}C NMR ($CDCl_3$): δ = 144.7, 144.0, 143.5, 141.4, 139.4, 134.7, 132.3, 130.4, 130.1, 128.6, 128.4, 127.0, 126.5, 115.2, 29.55, 13.99; MS: 300.42 ($M+H^+$); Anal. Calcd for $C_{22}H_{21}N$: C, 88.25; H, 7.07; N, 4.68. Found: C, 87.35; H, 6.87; N, 4.50.

4.2.4. General procedure for the demethylation of compounds 6 and 7.

4.2.4.1. Synthesis of (*E*)-N-(4-(1-(4-hydroxyphenyl)-2-phenylbut-1-enyl)phenyl)isobutyramide (8b). To a cold at -78 °C solution of compound **6b** (0.050 g, 0.125 mmol) in CH_2Cl_2 (8.9 mL), BBr_3 (0.625 mmol, 1.0 M in CH_2Cl_2, 5 equiv per bond, 0.625 mL) was added dropwise and the reaction was kept for another

1 h at the same temperature. Then, the solution was cooled at 0 °C, neutralized with MeOH and HCl 1 N was added. The organic phase was extracted twice with EtOAc. The combined organic extracts were washed with water and brine, dried with Na_2SO_4, filtered and evaporated. The residue was purified by flash column chromatography (Hex/EtOAc 6:4) to provide compound **8b** as oil (75% yield). R_f = 0.34 (Hex/EtOAc 6:4); ^{1}H NMR ($CDCl_3$): δ = 7.28 (1H, br. s), 7.20 – 7.11 (9H, m), 6.83 (4H, br. d, *J* 7.6 Hz), 2.53 – 2.45 (3H, m), 1.21 (6H, d, *J* 6.8 Hz), 0.951 (3H, t, *J* 7.6 Hz); ^{13}C NMR ($CDCl_3$): δ = 175.4, 155.2, 143.2, 142.3, 140.6, 138.5, 136.7, 136.3, 131.9, 131.4, 130.3, 128.4, 126.6, 119.6, 115.8, 37.26, 29.62, 20.06, 13.95; MS: 386.50 ($M+H^+$); Anal. Calcd for $C_{26}H_{27}NO_2$: C, 81.01; H, 7.06; N, 3.63. Found: C, 80.17; H, 6.83; N, 3.47.

4.2.4.2. Synthesis of (*Z*)-N-(4-(1-(4-hydroxyphenyl)-2-phenylbut-1-enyl)phenyl)isobutyramide (9b). According to the general procedure, compound **9b** was obtained from compound **7b** after purification by flash column chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as oil (75% yield). R_f = 0.34 (Hex/EtOAc 6:4); ^{1}H NMR ($CDCl_3$): δ = 7.53 (2H, d, *J* 8.0 Hz), 7.29 (1H, br. s), 7.21 (2H, d, *J* 8.4 Hz), 7.18 (2H, d, *J* 8.0 Hz), 7.14 – 7.11 (3H, m), 6.72 (2H, d, *J* 8.4 Hz), 6.50 (2H, d, *J* 8.4 Hz), 2.59 – 2.53 (1H, m), 2.49 (2H, q, *J* 7.2 Hz), 1.29 (6H, d, *J* 6.4 Hz), 0.942 (3H, t, *J* 6.8 Hz); ^{13}C NMR ($CDCl_3$): δ = 175.5, 153.8, 142.4, 141.5, 140.0, 137.6, 136.4, 135.4, 132.1, 130.2, 129.7, 127.9, 126.0, 119.6, 114.4, 36.79, 29.04, 19.67, 13.62; MS: 386.53 ($M+H^+$); Anal. Calcd for $C_{26}H_{27}NO_2$: C, 81.01; H, 7.06; N, 3.63. Found: C, 80.16; H, 6.84; N, 3.51.

4.2.4.3. Synthesis of (*Z*)-*N*-(4-(2-(4-hydroxyphenyl)-1-phenylbut-1-en-1-yl)phenyl)isobutyramide (8c). According to the general procedure, compound **8c** was obtained from compound **6c** after purification by flash column chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as oil (80% yield). R_f = 0.28 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 8.90 (1H, br. s), 8.23 (1H, s), 7.40 – 7.37 (4H, m), 7.29 (1H, t, *J* 7.2 Hz), 7.25 (2H, d, *J* 8.4 Hz), 7.01 (2H, d, *J* 8.8 Hz), 6.82 (2H, d, *J* 8.8 Hz), 6.69 (2H, d, *J* 8.4 Hz), 2.58 – 2.52 (1H, m), 2.43 (2H, q, *J* 7.6 Hz), 1.12 (6H, d, *J* 6.8 Hz), 0.930 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.0, 156.4, 144.7, 142.2, 138.9, 138.0, 134.0, 131.3, 129.9, 128.6, 127.0, 119.0, 115.4,

36.36, 29.24, 19.48, 13.41; MS: 386.53 ($M+H^+$); Anal. Calcd for $C_{26}H_{27}NO_2$: C, 81.01; H, 7.06; N, 3.63. Found: C, 80.19; H, 6.85; N, 3.48.

4.2.4.4. Synthesis of (*E*)-*N*-(4-(2-(4-hydroxyphenyl)-1-phenylbut-1-en-1-yl)phenyl)isobutyramide (9c). According to the general procedure, compound **9c** was obtained from compound **7c** after purification by flash column chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as oil (80% yield). R_f = 0.36 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 9.08 (1H, br. s), 8.18 (1H, s), 7.70 (2H, d, *J* 8.8 Hz), 7.17 (2H, d, *J* 8.8 Hz), 7.06 – 7.00 (3H, m), 6.99 (2H, d, *J* 8.8 Hz), 6.93 (2H, d, *J* 8.0 Hz), 6.66 (2H, d, *J* 8.8 Hz), 2.70 – 2.60 (1H, m), 2.48 (2H, q, *J* 7.2 Hz), 1.19 (6H, d, *J* 6.8 Hz), 0.951 (3H, t, *J* 7.2 Hz); ^{13}C NMR (acetone-d_6): δ = 174.8, 155.8, 143.7, 141.8, 138.7, 138.3, 137.8, 133.1, 130.7, 130.6, 129.6, 127.3, 125.5, 118.9, 114.8, 35.75, 28.62, 19.02, 13.01; MS: 386.52 ($M+H^+$); Anal. Calcd for $C_{26}H_{27}NO_2$: C, 81.01; H, 7.06; N, 3.63. Found: C, 80.18; H, 6.86; N, 3.49.

4.2.4.5. Synthesis of (*E*)-*N*-(4-(2-(2,4-dichlorophenyl)-1-(4-hydroxyphenyl)but-1-en-1-yl)phenyl) isobutyramide (8g). According to the general procedure, compound **8g** was obtained from compound **6g** after purification by flash column chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as oil (80% yield). R_f = 0.32 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 8.90 (1H, br. s), 8.43 (1H, br. s), 7.40 – 7.38 (3H, m), 7.28 (1H, d, *J* 8.0 Hz), 7.25 (1H, d, *J* 2.0 Hz), 7.14 (2H, d, *J* 8.4 Hz), 6.89 (2H, d, *J* 8.8 Hz), 6.88 (2H, d, *J* 8.4 Hz), 2.58 – 2.52 (1H, m), 2.49 (2H, q, *J* 7.6 Hz), 1.11 (6H, d, *J* 6.8 Hz), 0.953 (3H, t, *J* 7.6 Hz); ^{13}C NMR (acetone-d_6): δ = 175.6, 157.2, 141.9, 140.8, 138.6, 138.3, 137.6, 134.7, 134.2, 132.9, 131.0, 130.4, 129.4, 127.4, 118.7, 115.6, 36.37, 28.45, 19.63, 13.04; MS: 455.41 ($M+H^+$); Anal. Calcd for $C_{26}H_{25}Cl_2NO_2$: C, 68.73; H, 5.55; N, 3.08. Found: C, 68.03; H, 5.40; N, 2.95.

4.2.4.6. Synthesis of (*Z*)-*N*-(4-(2-(2,4-dichlorophenyl)-1-(4-hydroxyphenyl)but-1-en-1-yl)phenyl) isobutyramide (9g). According to the general procedure, compound **9g** was obtained from compound **7g** after purification by flash column chromatography (Hex/EtOAc 6:4) and evaporation of the solvent as oil (80% yield). R_f = 0.32 (Hex/EtOAc 6:4); ^{1}H NMR (acetone-d_6): δ = 9.13 (1H, br. s), 8.18 (1H, br. s), 7.72 (2H, d, *J* 8.4 Hz), 7.40 – 7.38 (1H, m), 7.27 (1H, d, *J* 8.0 Hz), 7.26 (1H, d, *J*

2.0 Hz), 7.22 (2H, d, *J* 8.8 Hz), 6.80 (2H, d, *J* 8.8 Hz), 6.54 (2H, d, *J* 8.8 Hz), 2.70 – 2.60 (1H, m), 2.47 (2H, q, *J* 7.6 Hz), 1.18 (6H, d, *J* 6.8 Hz), 0.947 (3H, t, *J* 7.2 Hz); ^{13}C NMR (acetone-d_6): δ = 175.7, 156.6, 141.9, 140.9, 139.3, 138.1, 137.4, 134.7, 134.4, 134.2, 132.8, 131.3, 130.1, 129.4, 127.4, 119.5, 114.9, 36.40, 28.40, 19.67, 13.03; MS: 455.40 ($M+H^+$); Anal. Calcd for $C_{26}H_{25}Cl_2NO_2$: C, 68.73; H, 5.55; N, 3.08. Found: C, 68.05; H, 5.41; N, 2.94.

4.3. Biological evaluation

4.3.1. Cell cultures

HeLa (human cervix adenocarcinoma cells) and MCF-7 (human mammary gland adenocarcinoma cells) were grown in Nutrient Mixture F-12 [HAM] (Sigma Chemical Co.) and Dulbecco's Modified Eagle's Medium (Sigma Chemical Co.), respectively. 10% Heat-inactivated fetal calf serum (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B (Sigma Chemical Co.) were added to both media. Ovarian cancer cell lines A2780 and OVCAR5 were cultured in RPMI1640 (Lonza), with the addition of 10% Fetal Bovine Serum (FBS, Lonza) and 2mM Glutamine (Lonza). The cells were cultured at 37°C in a moist atmosphere of 5% carbon dioxide in air.

4.3.2. Inhibition growth assay

HeLa and MCF-7 ($4\text{-}5\times10^4$) cells were seeded into each well of a 24-well cell culture plate. After incubation for 24 h various concentrations of the test agents and tamoxifen (**1**, T-5648 Sigma-Aldrich) were added and the cells were then incubated in standard conditions for a further 72 h. A trypan blue assay was performed to determine cell viability. Cytotoxicity data were expressed as GI_{50} values, i.e. the concentration of the test agent inducing 50% reduction in cell number compared with control cultures. A2780 and OVCAR5 were plated at a concentration of 15 000 cell/mL. 72 h later cells were treated with the different compounds, after 72 h cell survival was assessed by MTS assay and dose-response curves were plotted.

4.3.3. Topoisomerase-Mediated DNA Relaxation

Supercoiled pBR322 plasmid DNA (0.25 µg, Fermentas Life Sciences) was incubated with 1U topoisomerase II (human recombinant topoisomerase II α, USB Corporation) or 2U topoisomerase I (human recombinant topoisomerase I, TopoGen) and the test compounds as indicated, for 60 min at 37 °C in 20 µL reaction buffer.

Reactions were stopped by adding 4 µL stop buffer (5% sodium dodecyl sulfate (SDS), 0.125% bromophenol blue, and 25% glycerol), 50 µg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel at room temperature. The gels were stained with ethidium bromide 1 µg/mL in TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.3.4. Topoisomerase II-mediated DNA cleavage

Supercoiled pBR322 plasmid DNA (0.25 µg) was incubated with 10 U topoisomerase II (human recombinant topoisomerase II α, USB) and the test compounds, as indicated, for 60 min at 37 °C in 20 µL reaction buffer.

Reactions were stopped by adding 4 µL stop buffer (5% SDS, 0.125% bromophenol blue and 25% glycerol), 50 µg/mL proteinase K (Sigma) and incubating for a further 30 min at 37 °C. The samples were separated by electrophoresis on a 1% agarose gel containing ethidium bromide 0.5 µg/mL in TBE buffer (0.09 M Tris-borate and 0.002 M EDTA), transilluminated by UV light, and fluorescence emission was visualized by a CCD camera coupled to a Bio-Rad Gel Doc XR apparatus.

4.3.5. Evaluation of apoptotic cell death by Annexin V-FITC and propidium iodide staining

To detect phosphatidylserine translocation from the inner face to the outer surface of plasma membrane a FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen) was used.

HeLa cells (2.0×10^5) were seeded into each cell culture plate. After incubation for 24 h the test agents were added to the complete medium at the indicated concentrations and cells were incubated for a further 28 h. After treatment, cells were centrifuged and resuspended at 10^6 cells/mL in binding buffer. Cell suspensions (100 µL) were added with Annexin V-FITC and propidium iodide (PI) as indicated by the supplier's

instructions, and incubated in the dark for 15 min at room temperature. The populations of Annexin V-positive/PI-negative cells (early apoptosis) and Annexin V-positive/PI-positive cells (late apoptosis) were evaluated by FACSCanto II flow cytometer (Becton-Dickinson, Mountain View, CA).

4.3.6. Determination of mitochondrial membrane potential on whole cells

The mitochondrial membrane potential was evaluated by using the BD™ MitoScreen Kit (BD Pharmigen) containing the membrane-permeable lipophilic cationic fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1), according to Cossarizza et al.[25] HeLa cells (2.0×10^5) were seeded into each cell culture plate. After incubation for 24 h the test agents were added to the complete medium at the indicated concentrations and cells were incubated for a further 28 h. After treatment, cells were centrifuged, resuspended in JC-1 Working Solution and incubated for 30 min at 37°C in CO_2 incubator. Following incubation, cells are washed twice, resuspended in Assay Buffer and analyzed by a FACSCanto II flow cytometer (Becton-Dickinson, Mountain View, CA).

4.4. Molecular modeling

All the synthesized compounds were docked in the etoposide binding site in the crystal structure of the topoisomerase II beta/DNA complex (3QX3.pdb)[26] using AutoDock 4.2 software.[27] A Lamarkian genetic algorithm[28] was employed for the docking simulation, performing 100 independent runs per molecule. In each run, a population of 50 individuals evolved along 27000 generations and a maximum number of 25 million energy evaluations was performed. The best fit (lowest docked energy) solutions of the 100 independent runs were stored for subsequent analysis. The visual inspection of docked structures was carried out using VMD.[29]

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Highlights

- We describe the synthesis of a new library of 4-(1,2-diarylbut-1-en-1-yl)isobutyranilide derivatives
- Anti-proliferative activity in 4 cell lines indicates activity at low micromolar range
- The most active compound was able to induce apoptosis
- The most active compound seems to target topoisomerase II