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Short communication

Synthesis of novel spiropyrazoline oxindoles and evaluation of cytotoxicity in cancer cell lines

Ângelo Monteiro, Lídia M. Gonçalves, Maria M.M. Santos*

Instituto de Investigação do Medicamento (iMed.ULisboa), Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal

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ABSTRACT

A series of novel spiropyrazoline oxindole derivatives was synthesized by 1,3-dipolar cycloaddition reaction. The compounds were screened for their in vitro cytotoxic activity against MCF-7 breast cancer cell line (estrogen receptor positive (ER+) and human epidermal growth factor receptor 2 negative (HER2−)). Of the nineteen spiropyrazoline oxindoles tested, six compounds have a GI_{50} below 12 μM The most potent compounds in this series were also evaluated against MDA-MB-231 breast cancer cell line (ER− and HER2−). Two spiropyrazoline oxindoles were highly selective between MCF-7 tumor cells and MDA-MB-231 tumor cells. More importantly, they were noncytotoxic against HEK 293T non tumor derived cell lines.

1. Introduction

Cancer is one of the leading causes of mortality worldwide, causing 7.6 million deaths in 2008. Moreover, World Health Organization projects a rise in deaths from cancer to 13.1 million in 2030 [1]. Despite a more sophisticated understanding of the etiology of cancer and a greater emphasis placed on early detection of the disease, overall mortality rates from cancer have not diminished greatly and are not expected to decrease. As such, cancer continues to pose a major threat to human health and further research regarding new therapeutic strategies that more effectively combat cancer are needed. Furthermore, the increase cases of multidrug resistance (MDR) make a challenge for the development of new drugs a milestone on the treatment of various types of cancers (e.g. blood, breast, ovarian, lung, and lower gastrointestinal tract cancers). Various MDR mechanisms observed in cancer cells as well as various strategies developed to overcome these mechanisms have been extensively studied during the last few decades to enhance the efficacy of chemotherapy by suppressing or evading the MDR mechanisms [2].

We previously reported the potential use of spiroisoxazoline oxindoles as anticancer agents [3]. In fact, the spirooxindole system is the core structure of a variety of medicinal agents and natural products [4,5]. Spirooxindole derivatives were described with different biological activities, ranging from MDM2 antagonists, ion channel blockers and anti-inflammatory agents to antimalarials (Fig. 1) [6–9]. As a consequence, there is a huge interest in industry and academia to develop novel spirooxindoles with interesting biological activities. Pyrazoline is also a privileged unit in medicinal chemistry. We now report the study of spiropyrazoline oxindoles, containing a five membered ring (pyrazoline) with one more aromatic substituent (R^4) (oxygen atom in isoxazoline ring was replaced by a *N*–Ar group). To perform a structure-activity study, we synthesized and evaluated the antiproliferative activity of a small library of novel spiropyrazoline oxindoles containing different substituents at the pyrazoline ring and in the aromatic ring of the oxindole moiety (Fig. 2). Two breast cancer cell lines, one non-invasive estrogen receptor (ER) positive (MCF-7) and, one invasive ER-negative (MDA-MB-231), both derived from a metastatic adenocarcinoma on the mammary gland, were chosen as models for testing the new synthetized molecules. The ER-negative breast cancer [10] is specifically studied due to a lack of cellular targets compared with ER-positive breast cancer MCF-7 cell line, which can be effectively inhibited by targeting the estrogen receptor with anti-estrogen agents. The ER-positive breast cancer cells are described to often develop MDR [2,11]. This makes very important the search for potential new anticancer drugs independent from estrogens receptors to treat cancer.

* Corresponding author. Medicinal Chemistry Group, iMed.ULisboa, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal.
E-mail address: mariasantos@ff.ul.pt (M.M.M. Santos).

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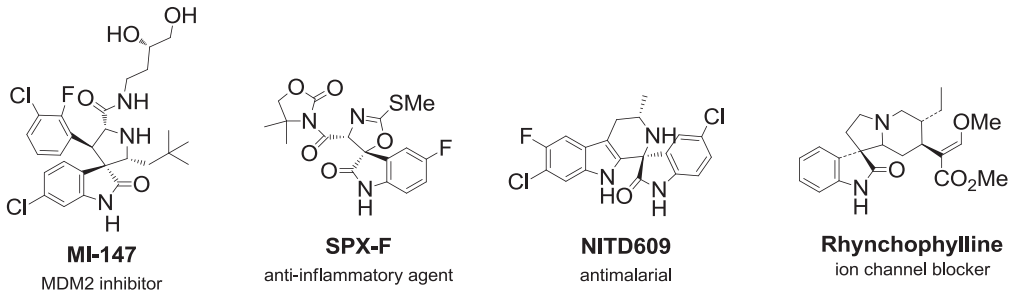


Fig. 1. Selected spirooxindoles with biological activity.

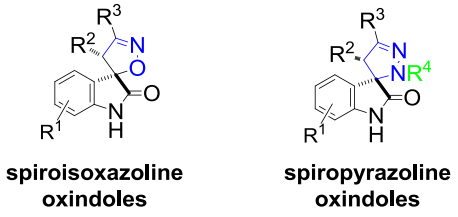


Fig. 2. Chemical structure of spiroisoxazoline and spiropyrazoline oxindoles.

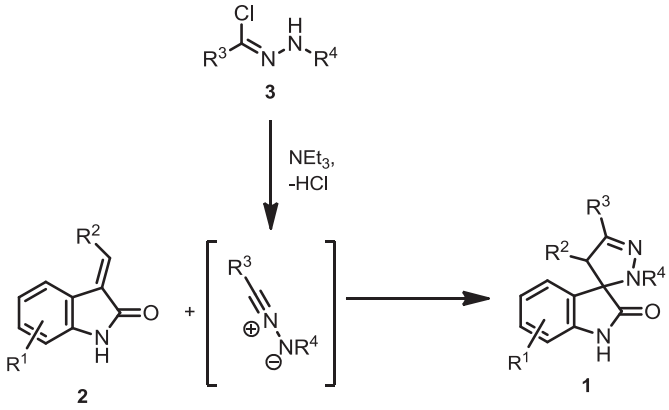
2. Results and discussion

2.1. Chemistry

Spiropyrazoline oxindoles **1** were synthesized by 1,3-dipolar cycloaddition reaction, one of the most versatile methods to obtain 5-membered heterocycles, between nitrile imines and 3-methylene indolinones **2** (Scheme 1). The nitrile amines were prepared in situ from hydrazonyl chlorides **3**.

The 3-methylene indolinones **2** containing aromatic groups at R^2 were synthesized by aldolic condensation of substituted indolin-2-ones with different aromatic aldehydes in the presence of piperidine in yields between 92 and 98% [12].

Specifically, the 3-methylene indolinone required for the synthesis of compounds **1a**–**c** and **1e** was obtained by aldol condensation between 5-chloro isatin with acetophenone in basic medium, followed by dehydration using dilute alcoholic hydrochloric acid as described in the literature [13] and, the 3-methylene indolinone required for the synthesis of compounds **1d** was easily prepared by Wittig reaction of 5-chloro isatin with (carbethoxymethylene)triphenylphosphorane in 92% yield [14].



Scheme 1. Strategy to obtain spiropyrazoline oxindoles **1**.

The hydrazonyl chlorides **3** required for the dipolar cycloaddition were obtained from reaction of *N*-chlorosuccinimide-dimethyl sulphide complex with the appropriate *N*-arylhydrazones at −78 °C [15].

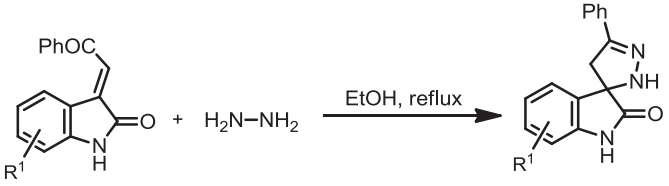
We started by synthesizing spiropyrazoline oxindoles **1a**–**c**, with the nitrogen of the pyrazoline ring unprotected, by nucleophilic addition of hydrazine to the appropriate 3-methylene indolinones **2** (Scheme 2) [13].

Then, spiropyrazoline oxindoles **1d**–**s** were synthesized from the appropriate 3-methylene indolinones **2** with hydrazonyl chlorides **3** in the presence of triethylamine (Scheme 3). The active nitrile amine required for the 1,3-dipolar cycloaddition was generated in situ by dehydrohalogenation of the corresponding hydrazonyl chloride in the presence of base. The reaction was regioselective, with the carbon end of the dipole adding to the β-position of 3-methylene indolinones. The relative configuration of the final products was established by comparison of their NMR spectra with NMR spectra of other spiropyrazoline oxindoles described in the literature, with published X-ray crystallography structure [16]. Using compound **1n** as an example, the proton chemical shift observed for the hydrogen of the pyrazoline ring was 5.14 ppm and the carbon chemical shift observed for the spiro carbon was 77.90 ppm.

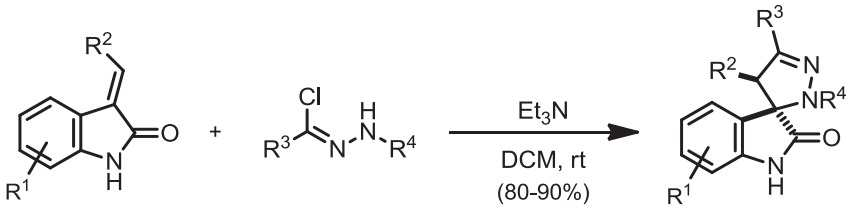
2.2. In vitro cytotoxicity

The in vitro cytotoxicity of compounds **1a**–**s** was evaluated by MTT assay in MCF-7 (human breast adenocarcinoma) tumor cell line. The GI_{50} values obtained (Table 1) allows the following observations:

1. Compounds **1a**–**c**, with an unsubstituted nitrogen and hydrogen at R^2, are inactive against MCF-7 cell line at tested concentrations ($GI_{50} > 100$ μM).
2. Of the nineteen compounds tested, six compounds (**1j**–**1o** and **1q**–**1r**) have a GI_{50} below 12 μM. These results show that the substitution of an isoxazoline ring [3] by a pyrazoline ring leads to an increase of activity as anticancer agents.
3. The order of inhibitory activity against MCF-7 cell line depends in significant extent on the nature of the substituent at R^2. Compounds **1d** and **1e** with a CO_2Et and a COPh substituent,



Scheme 2. Synthesis of spiropyrazoline oxindoles **1a**–**c**.



Scheme 3. Synthesis of spiropyrazoline oxindoles **1d**–**s**.

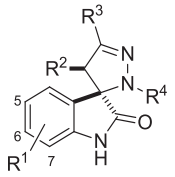
respectively, are inactive, while compound **1g** with a phenyl substituent has an GI_{50} of 37.7 μM. Also replacing the phenyl group by a 4-OMe-phenyl (PMP) group leads to loss of activity (**1h** versus PMP-substituted counterpart **1s**).

4. For compounds with a phenyl substituent at R^2 the order of inhibitory activity against MCF-7 cell line depends on the substituent on the aromatic ring of the oxindole and varies in the order Br > Cl > H (compare **1j**, **1g** and **1f**).
5. The presence of a Br on the aromatic ring of the oxindole seems to improve the activity compared with the Cl-substituted counterparts (e.g. **1j** and **1k** versus Cl-substituted counterparts **1g** and **1h**). The same trend was observed for compounds with a PMP or a *t*-Bu substituent at R^3 (spiropyrazoline oxindole **1o** and **1r** versus compounds **1m** and **1p**).
6. For 6-Br spiroxindoles containing phenyl groups at the nitrogen and the R^2, i.e. spiroxindoles **1k**, **1o** and **1r**, the activity is almost the same independently of the substituent at R^3 (Ph, PMP or *t*-Bu). However, for 7-Cl spiroxindoles containing phenyl groups at the nitrogen and the R^2, i.e. spiroxindoles **1i** and **1q**, the activity of the *t*-Bu derivative (**1q**) is ca 2.6-fold higher than that of its counterpart, with a phenyl group at R^3 (**1i**).

The most active compounds (GI_{50} lower than 30 μM) were also tested on human MDA-MB-231 tumor cell line (Table 2). Remarkably, spirooxindoles **1h**, **1j**, **1n**, **1o**, **1p** and **1r**, had higher potency against MCF-7 tumor cells than against breast MDA-MB-231 tumor cells. More remarkably, compounds **1j** and **1n**, exhibited more than 10-fold selectivity for MCF-7 cell line (Fig. 3).

Finally, in order to evaluate the cytotoxicity of these compounds on normal cells, we investigated the cytotoxicities of the most active compounds (GI_{50} lower than 30 μM) on normal human HEK 293T cell line (Table 2). To our delight, we found that six spiropyrazoline oxindoles (**1h**–**1j** and **1n**–**1p**) were noncytotoxic at concentrations up to 100 μM in human HEK 293T cell line. Only, spiropyrazoline oxindoles **1k**, **1q**, and **1r** led to cytotoxicity at concentrations <30 μM, but even in this case, the selectivity index was ~3, which indicates low toxicity.

Table 1
Cytotoxicity of compounds **1a**–**s** against cancer cell line MCF-7.



Compounds	R^1	R^2	R^3	R^4	MCF-7 GI_{50}, μM
1a	H	H	Ph	H	>100
1b	5-Cl	H	Ph	H	>100
1c	5-Br	H	Ph	H	>100
1d	5-Cl	CO_2Et	Ph	Ph	>100
1e	5-Cl	COPh	Ph	Ph	>100
1f	H	Ph	Ph	Ph	>100
1g	5-Cl	Ph	Ph	Ph	37.7 ± 14.1
1h	6-Cl	Ph	Ph	Ph	27.1 ± 8.2
1i	7-Cl	Ph	Ph	Ph	22.4 ± 3.5
1j	5-Br	Ph	Ph	Ph	7.3 ± 1.4
1k	6-Br	Ph	Ph	Ph	9.6 ± 1.0
1l	5-Cl	Ph	PMP	Ph	33.9 ± 15.4
1m	6-Cl	Ph	PMP	Ph	42.6 ± 18.8
1n	7-Cl	Ph	PMP	Ph	11.5 ± 1.6
1o	6-Br	Ph	PMP	Ph	7.0 ± 1.1
1p	6-Cl	Ph	t-Bu	Ph	21.7 ± 3.9
1q	7-Cl	Ph	t-Bu	Ph	8.6 ± 3.0
1r	6-Br	Ph	t-Bu	Ph	8.5 ± 1.8
1s	6-Cl	PMP	Ph	Ph	>100

3. Conclusion

Due to the lack of anticancer agents specific for tumours, the development of novel scaffolds of highly selective anticancer agents is still a very urgent topic of research. The studies herein presented led to the discovery of novel spiropyrazoline oxindoles active against tumor cells in vitro. Two compounds were highly selective between two breast cancer cell lines (MCF-7 and MDA-MB-231), and had minimal cytotoxicity in human cell line HEK 293T, thus representing useful lead compounds for the development of more potent and selective anticancer agents. Our finding thus adds, for the first time, the spiropyrazoline oxindole scaffold to the list of chemotypes of anticancer agents small-molecules. The mechanisms of cytotoxicity and lead optimization of the spirooxindole derivatives are currently under investigation.

4. Experimental section

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification.

Melting points were determined using a Kofler camera Bock monoscope M and are uncorrected.

The infrared spectra were collected on a Shimadzu FTIR Affinity-1 spectrophotometer.

Elemental analysis was performed in a Flash 2000 CHNS–O analyzer (ThermoScientific, UK), and results were within ±0.4% of the theoretical values.

Merck Silica Gel 60 F254 plates were used for analytical TLC; flash column chromatography was performed on Merck Silica Gel (200–400 mesh) and CombiFlash Rf from Teledyne ISCO (columns RediSep Rf, silica).

^1H and ^{13}C NMR spectra were recorded on a Bruker 400 Ultra-Shield instrument (400 MHz). ^1H and ^{13}C chemical shifts are expressed in δ (ppm) referenced to the solvent used and the proton coupling constants (J) are given in hertz.

4.1.1. General preparation of spiropyrazoline oxindoles *1a*–*c*

A mixture of the appropriate 3-methylene indolinone (0.35 mmol, 1 eq.) and hydrazine hydrate (0.42 mmol, 1.2 eq.) in

Table 2
Cytotoxicity of compounds **1h**–**k** and **1n**–**r** against cancer cell lines MCF-7 and MDA-MB-231 and non cancer cell line Hek 293T.

Compounds	MCF-7	MDA-MB-231	Hek 293T	SI[a]
	GI_{50}, μM	GI_{50}, μM	GI_{50}, μM	
1h	27.1 ± 8.2	>50[b]	>50[b]	>2
1i	22.4 ± 3.5	16.9 ± 1.2	>100	>4
1j	7.3 ± 1.4	>100	>100	>13
1k	9.6 ± 1.0	8.3 ± 1.2	28.9 ± 1.2	3
1n	11.5 ± 1.6	>100	>100	>8
1o	7.0 ± 1.1	28.6 ± 1.1	>100	>14
1p	21.7 ± 3.9	>50[b]	>50[b]	>2
1q	8.6 ± 3.0	6.4 ± 1.2	17.8 ± 1.2	2
1r	8.5 ± 1.8	15.0 ± 1.2	20.9 ± 1.2	2.5

[a] Selectivity index toward MCF-7, which is expressed by the ratio GI_{50} Hek/GI_{50} MCF-7.
[b] Solubility issues at concentrations of 100 μM.

ethanol (2.5 ml) was refluxed for 1 h. The mixture was recrystallized from *i*-PrOH [13].

*4.1.1.1. Compound **1a**: obtained as a light yellow solid.* Yield 40% (42 mg). IR (KBr): 3296, 3186, 1705, 1618 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.78 (s, 1H), 7.69 (d, J = 7.3 Hz, 2H), 7.40 (m, 3H), 7.32 (d, J = 7.3 Hz, 1H), 7.25 (m, 2H), 7.02 (t, J = 7.5 Hz, 1H), 6.93 (d, J = 7.6 Hz, 1H), 3.72 (d, J = 16.7 Hz, 1H), 3.44 (d, J = 16.6 Hz, 1H). Anal. Calcd for $C_{16}H_{13}N_3O.0.2H_2O$: C 72.00, H 5.07, N 15.75, Found: C 71.74, H 4.75, N 15.38.

*4.1.1.2. Compound **1b**: obtained as a light orange solid.* Yield 35% (37 mg). IR (KBr): 3290, 3167, 1707, 1622 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.52 (s, 1H), 7.96 (s, 1H), 7.64 (d, J = 7.2 Hz, 2H), 7.45–7.32 (m, 3H), 7.29 (d, J = 6.1 Hz, 2H), 6.86 (d, J = 8.9 Hz, 1H), 3.43 (s, 2H). Anal. Calcd for $C_{16}H_{12}ClN_3O.0.4H_2O$: C 63.15, H 4.25, N 13.81, Found: C 62.77, H 4.00, N 13.39.

*4.1.1.3. Compound **1c**: obtained as a light orange solid.* Yield 37% (44 mg). IR (KBr): 3291, 3165, 1707, 1618 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.55 (s, 1H), 7.95 (m, 1H), 7.64 (d, J = 7.4 Hz, 2H), 7.49–7.31 (m, 5H), 6.81 (d, J = 8.1 Hz, 1H), 3.43 (s, 2H). Anal. Calcd for $C_{16}H_{12}BrN_3O$: C 56.16, H 3.53, N 12.28, Found: C 56.54, H 3.68, N 12.05.

*4.1.2. General preparation of spiropyrazoline oxindoles **1d**–**s***

The appropriate 3-methylene indolinone (0.3 mmol, 1 eq.) was dissolved in dry dichloromethane (2 ml) and the appropriate hydrazonyl chloride (0.6 mmol, 2 eq.) was added. Then, triethylamine (0.9 mmol, 3 eq.) was added and the reaction was stirred at room temperature for 3 h. The reaction was then portioned with 7.5 ml of water, the phases were separated and the aqueous phase was extracted twice with 10 ml of ethyl acetate. All organic extracts were combined, dried and concentrated to afford the crude product which was purified by flash column chromatography using ethyl acetate and *n*-hexane as eluent.

*4.1.2.1. Compound **1d**: purified by flash column chromatography using ethyl acetate/n-hexane 1:1 and then, recrystallized from dichloromethane. Obtained as a light yellow solid.* Yield 85% (0.11 g). IR (KBr): 3232, 1736, 1724, 1618, 1597, 1495 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 9.08 (s, 1H), 7.69 (d, J = 6.8 Hz, 2H), 7.46–7.31 (m, 3H), 7.21 (d, J = 7.1 Hz, 2H), 7.13 (t, J = 7.8 Hz, 2H), 6.92–6.75 (m, 4H), 4.93 (s, 1H), 3.90 (q, J = 7.1 Hz, 2H), 0.94 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.24 (C═O), 166.63 (C═O), 144.17 (Cq), 142.89 (Cq), 138.94 (Cq), 131.27 (Cq), 130.60 (CH), 129.20 (CH), 129.16 (CH), 128.76 (Cq), 128.67 (CH), 126.72 (Cq), 126.45 (CH), 125.98 (CH), 121.40 (CH), 114.78 (CH), 112.07 (CH), 74.03 (Cq), 62.90 (CH), 62.04 (CH_2), 13.65 (CH_3). Anal. Calcd for $C_{25}H_{20}ClN_3O_3$: C 67.34, H 4.52, N 9.42, Found: C 67.03, H 4.53, N 9.31.

*4.1.2.2. Compound **1e**: purified by flash column chromatography using ethyl acetate/n-hexane 1:2 and then, recrystallized from dichloromethane. Obtained as a light yellow solid.* Yield 76% (0.10 g). ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.36 (s, 1H), 7.69–7.57 (m, 4H), 7.48 (t, J = 7.4 Hz, 1H), 7.40–7.28 (m, 5H), 7.12 (t, J = 7.9 Hz, 2H), 7.03 (d, J = 1.6 Hz, 1H), 6.97 (dd, J = 8.3, 1.8 Hz, 1H), 6.93–6.79 (m, 3H), 6.51 (d, J = 8.3 Hz, 1H), 5.94 (s, 1H). Anal. Calcd for $C_{29}H_{20}ClN_3O_2$: C 72.88, H 4.22, N 8.79, Found: C 73.01, H 4.34, N 8.78.

*4.1.2.3. Compound **1f**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a light yellow solid.* Yield 78% (0.11 g). IR (KBr): 3198, 1719, 1618, 1597, 1495 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.97 (s, 1H), 7.75–7.62 (m, 2H), 7.37–7.21 (m, 3H), 7.21–

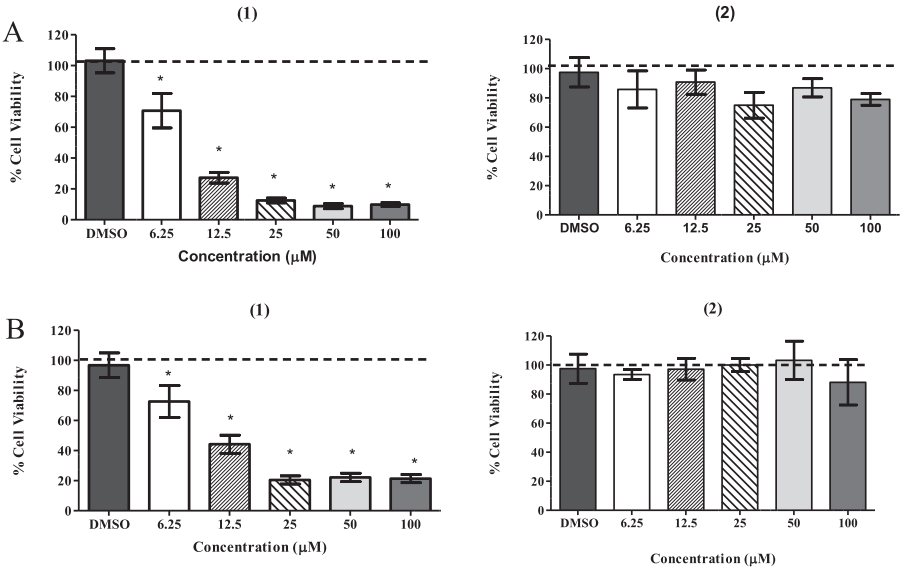


Fig. 3. Effects of compounds **1j** (A), and **1n** (B) on MCF-7 (1) and MDA-MB-231 (2) cell viability using the MTT assay for 48 h of exposition. Results are given in % relative to control and represent means ± SD of three independent experiments performed in duplicate and carried out independently (* indicates $P < 0.05$ antiproliferative effect significantly higher in MCF-7 than in MDA-MB-231).

6.88 (m, 10H), 6.87–6.69 (m, 2H), 6.55 (t, J = 7.6 Hz, 1H), 6.35 (d, J = 7.5 Hz, 1H), 5.16 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.39 (C=O), 148.93 (Cq), 144.33 (Cq), 140.24 (Cq), 134.78 (Cq), 131.81 (Cq), 129.48 (CH), 129.29 (CH), 129.03 (CH), 128.76 (CH), 128.66 (CH), 128.47 (CH), 128.02 (CH), 126.95 (CH), 126.54 (CH), 125.65 (Cq), 122.40 (CH), 121.08 (CH), 115.43 (CH), 110.62 (CH), 77.47 (Cq), 62.67 (CH). Anal. Calcd for $C_{28}H_{21}N_3O$: C 80.94, H 5.09, N 10.11, Found: C 80,88, H 5.34, N 9.95.

*4.1.2.4. Compound **1g**: purified by flash column chromatography using ethyl acetate/n-hexane 1:4 and then, recrystallized from dichloromethane. Obtained as a light yellow solid.* Yield 82% (0.11 g). IR (KBr): 3171, 1730, 1595, 1493 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.68 (d, J = 6.7 Hz, 2H), 7.42–7.27 (m, 3H), 7.28–7.08 (m, 6H), 7.02 (d, J = 6.5 Hz, 2H), 6.89 (d, J = 8.3 Hz, 1H), 6.83–6.79 (m, 3H), 6.14 (d, J = 1.8 Hz, 1H), 5.45 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.32 (C=O), 149.54 (Cq), 143.94 (Cq), 140.42 (Cq), 134.62 (Cq), 131.15 (Cq), 129.46 (CH), 129.09 (CH), 129.05 (CH), 128.65 (CH), 128.03 (CH), 127.10 (Cq), 126.61 (CH), 125.60 (CH), 125.55 (Cq), 120.70 (CH), 114.39 (CH), 111.74 (CH), 75.84 (Cq), 60.81 (CH). Anal. Calcd for $C_{28}H_{20}ClN_3O.0.5CH_2Cl_2$: C 69.63, H 4.31, N 8.55, Found: C 69.19, H 4.30, N 8.55.

*4.1.2.5. Compound **1h**: purified by flash column chromatography using ethyl acetate/n-hexane 1:4 and then, recrystallized from dichloromethane. Obtained as a light yellow solid.* Yield 83% (0.11 g). IR (KBr): 3171, 1722, 1611, 1597, 1495 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.95 (s, 1H), 7.68 (d, J = 5.4 Hz, 2H), 7.28 (m, 3H), 7.13 (m, 5H), 6.94 (m, 4H), 6.85 (t, J = 7.0 Hz, 1H), 6.78 (s, 1H), 6.55 (d, J = 8.1 Hz, 1H), 6.23 (d, J = 8.1 Hz, 1H), 5.11 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.62 (C=O), 149.19 (Cq), 144.22 (Cq), 141.12 (Cq), 135.39 (Cq), 134.44 (Cq), 131.56 (Cq), 129.18 (CH), 128.99 (CH), 128.93 (CH), 128.57 (CH), 128.35 (CH), 127.39 (CH), 126.98 (CH), 124.09 (Cq), 122.65 (CH), 121.48 (CH), 115.52 (CH), 111.48 (CH), 76.84 (Cq), 62.60 (CH). Anal. Calcd for $C_{28}H_{20}ClN_3O.0.15H_2O$: C 74.29, H 4.53, N 9.29, Found: C 73.96, H 4.33, N 9.08.

*4.1.2.6. Compound **1i**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 87% (0.12 g). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.31 (s, 1H), 7.67 (d, J = 6.6 Hz, 2H), 7.36–7.30 (m, 3H), 7.21–7.12 (m, 6H), 7.02 (d, J = 7.5 Hz, 2H), 6.83–6.78 (m, 3H), 6.56 (t, J = 7.9 Hz, 1H), 6.21 (d, J = 7.5 Hz, 1H), 5.47 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.70 (C=O), 149.63 (Cq), 143.96 (Cq), 139.20 (Cq), 134.58 (Cq), 131.17 (Cq), 129.61 (CH), 129.08 (CH), 128.65 (CH), 128.60 (CH), 127.99 (CH), 127.01 (Cq), 126.61 (CH), 124.35 (CH), 122.63 (CH), 120.77 (CH), 114.54 (CH), 114.38 (Cq), 76.48 (Cq), 61.04 (CH). Anal. Calcd for $C_{28}H_{20}ClN_3O \cdot 0.15H_2O$: C 74.29, H 4.53, N 9.29, Found: C 74.07, H 4.69, N 8.89.

*4.1.2.7. Compound **1j**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 82% (0.10 g). IR (KBr): 3180, 1718, 1618, 1593, 1493 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.01 (s, 1H), 7.69 (d, J = 7.6 Hz, 2H), 7.37–7.30 (m, 4H), 7.22 (m, 3H), 7.14 (t, J = 7.9 Hz, 2H), 7.01 (d, J = 7.4 Hz, 2H), 6.85–6.79 (m, 4H), 6.24 (s, 1H), 5.45 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.15 (C=O), 149.57 (Cq), 143.95 (Cq), 140.76 (Cq), 134.62 (Cq), 132.27 (CH), 131.14 (Cq), 129.10 (CH), 129.06 (CH), 128.66 (CH), 128.61 (CH), 128.36 (CH), 128.04 (CH), 127.38 (Cq), 126.61 (CH), 120.71 (CH), 114.38 (CH), 113.25 (CH), 112.20 (Cq), 75.84 (Cq), 60.78 (CH). Anal. Calcd for $C_{28}H_{20}BrN_3O_2.0.55H_2O$: C 66.68, H 4.23, N 8.33, Found: C 66.29, H 4.09, N 8.18.

*4.1.2.8. Compound **1k**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from dichloromethane. Obtained as a white solid.* Yield 85% (0.11 g). IR (KBr): 3172, 1720, 1609, 1597, 1493 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 11.01 (s, 1H), 7.66 (d, J = 7.5 Hz, 2H), 7.36–7.31 (m, 3H), 7.20 (m, 3H), 7.14 (t, J = 7.9 Hz, 2H), 7.01–7.02 (m, 3H), 6.82–6.78 (m, 3H), 6.73 (d, J = 8.1 Hz, 1H), 6.16 (d, J = 8.1 Hz, 1H), 5.43 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.49 (C=O), 149.62 (Cq), 143.99 (Cq), 143.17 (Cq), 134.68 (Cq), 131.17 (Cq), 129.07 (CH), 129.02 (CH), 128.65 (CH), 128.02 (CH), 127.39 (CH), 126.59 (CH), 124.45 (Cq), 124.08 (CH), 122.37 (Cq), 120.72 (CH), 114.51 (CH), 113.24 (CH), 75.65 (Cq), 60.73 (CH). Anal. Calcd for $C_{28}H_{20}BrN_3O.0.05H_2O$: C 67.90, H 4.10, N 8.49, Found: C 67.61, H 4.13, N 8.25.

*4.1.2.9. Compound **1l**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 33% (0.05 g). IR (KBr): 3117, 1740, 1711, 1599, 1499 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.97 (s, 1H), 7.61 (d, J = 8.5 Hz, 2H), 7.27–7.08 (m, 6H), 7.00 (d, J = 6.7 Hz, 2H), 6.89 (dd, J = 18.2, 8.4 Hz, 3H), 6.78 (dd, J = 12.6, 7.8 Hz, 3H), 6.12 (s, 1H), 5.41 (s, 1H), 3.74 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.36 (C=O), 159.90 (Cq), 149.44 (Cq), 144.20 (Cq), 140.31 (Cq), 134.71 (Cq), 129.30 (Cq), 128.95 (CH), 128.50 (CH), 128.14 (CH), 127.88 (CH), 127.26 (CH), 125.52 (Cq), 125.46 (CH), 123.65 (Cq), 120.31 (CH), 114.23 (CH), 114.08 (CH), 111.59 (CH), 75.66 (Cq), 61.02 (CH), 55.20 (OCH_3). Anal. Calcd for $C_{29}H_{22}ClN_3O_2.1.1H_2O$: C 69.80, H 4.90, N 8.42, Found: C 69.42, H 4.91, N 8.55.

*4.1.2.10. Compound **1m**: purified by flash column chromatography using ethyl acetate/n-hexane 1:4 and then, recrystallized from dichloromethane. Obtained as a light yellow solid.* Yield 32% (0.04 g). IR (KBr): 3210, 1714, 1701, 1597, 1497 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 10.99 (s, 1H), 7.59 (d, J = 8.6 Hz, 2H), 7.22–7.17 (m, 3H), 7.12 (t, J = 7.9 Hz, 2H), 7.00 (d, J = 6.8 Hz, 2H), 6.94–6.86 (m, 3H), 6.82–6.73 (m, 3H), 6.58 (d, J = 8.1 Hz, 1H), 6.20 (d, J = 8.1 Hz, 1H), 5.38 (s, 1H), 3.73 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.75 (C=O), 159.94 (Cq), 149.58 (Cq), 144.31 (Cq), 143.00 (Cq), 134.87 (Cq), 133.82 (Cq), 129.03 (CH), 128.63 (CH), 128.21 (CH), 127.96 (CH), 127.05 (CH), 124.24 (Cq), 123.73 (Cq), 121.17 (CH), 120.39 (CH), 114.37 (CH), 114.15 (CH), 110.45 (CH), 75.44 (Cq), 61.01 (CH), 55.27 (OCH_3). Anal. Calcd for $C_{29}H_{22}ClN_3O_2.1.1H_2O$: C 69.80, H 4.90, N 8.42, Found: C 69.42, H 4.91, N 8.55.

*4.1.2.11. Compound **1n**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 75% (0.10 g). IR (KBr): 3204, 1732, 1608, 1595, 1497 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.62 (s, 1H), 7.59 (d, J = 8.8 Hz, 2H), 7.21–7.05 (m, 6H), 6.98 (s, 2H), 6.91 (d, J = 7.9 Hz, 2H), 6.83 (m, 3H), 6.56 (t, J = 7.9 Hz, 1H), 6.30 (d, J = 7.5 Hz, 1H), 5.14 (s, 1H), 3.78 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.78 (C=O), 160.30 (Cq), 149.06 (Cq), 144.49 (Cq), 137.57 (Cq), 134.51 (Cq), 129.35 (CH), 129.29 (CH), 129.13 (CH), 128.80 (CH), 128.51 (CH), 128.24 (CH), 127.51 (Cq), 124.99 (CH), 124.23 (Cq), 123.30 (CH), 121.39 (CH), 115.70 (CH), 115.22 (Cq), 114.00 (CH), 77.90 (Cq), 63.22 (CH), 55.39 (CH_3). Anal. Calcd for $C_{29}H_{22}ClN_3O_2$: C 72.57, H 4.63, N 8.76, Found: C 72.34, H 4.56, N 8.66.

*4.1.2.12. Compound **1o**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 91% (0.12 g). IR (KBr): 3220, 1747, 1711, 1608, 1597, 1496 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.76 (s, 1H), 7.61 (d, J = 8.5 Hz, 2H), 7.22–7.06 (m, 5H), 6.92 (m, 5H), 6.83 (m, 8.2 Hz, 3H), 6.71 (d, J = 8.1 Hz, 1H), 6.17 (d, J = 8.1 Hz, 1H), 5.07 (s, 1H), 3.78 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.46 (C=O), 160.33 (Cq), 149.20 (Cq), 144.54 (Cq), 141.24 (Cq),

134.54 (Cq), 129.23 (CH), 129.15 (CH), 128.92 (CH), 128.52 (CH), 128.32 (CH), 127.74 (CH), 125.54 (CH), 124.86 (Cq), 124.25 (Cq), 123.28 (Cq), 121.31 (CH), 115.51 (CH), 114.12 (CH), 114.04 (CH), 77.37 (Cq), 62.75 (CH), 55.41 (CH_3). Anal. Calcd for $C_{29}H_{22}BrN_3O_2.0.2H_2O$: C 65.96, H 4.28, N 7.96, Found: C 65.60, H 4.39, N 7.70.

*4.1.2.13. Compound **1p**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from dichloromethane. Obtained as a white solid.* Yield 80% (0.10 g). IR (KBr): 3213, 1724, 1608, 1597, 1491 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.37 (s, 1H), 7.50–7.02 (m, 6H), 6.81 (m, 3H), 6.74 (m, 1H), 6.66 (s, 1H), 6.55 (dd, $J = 8.2$, 1.7 Hz, 1H), 6.12 (d, $J = 8.2$ Hz, 1H), 4.43 (s, 1H), 1.19 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.91 (C═O), 161.88 (Cq), 145.72 (Cq), 141.38 (Cq), 135.18 (Cq), 134.99 (Cq), 128.99 (CH), 128.37 (CH), 127.39 (CH), 124.32 (Cq), 122.43 (CH), 121.41 (CH), 116.29 (CH), 111.05 (CH), 77.05 (Cq), 62.36 (CH), 34.91 ($\underline{C}(CH_3)_3$), 29.49 ($C(\underline{C}H_3)_3$). Anal. Calcd for $C_{26}H_{24}ClN_3O.0.5CH_2Cl_2$: C 67.37, H 5.34, N 8.90, Found: C 67.14, H 5.39, N 8.84.

*4.1.2.14. Compound **1q**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 87% (0.11 g). IR (KBr): 3177, 1752, 1712, 1618, 1598, 1498 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 87.89 (s, 1H), 7.36 (s, 2H), 7.25 (m, 1H), 7.09 (m, 4H), 6.88–6.78 (m, 3H), 6.68 (s, 1H), 6.53 (t, $J = 7.9$ Hz, 1H), 6.15 (d, $J = 7.6$ Hz, 1H), 4.49 (s, 1H), 1.19 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.50 (C═O), 161.89 (Cq), 145.63 (Cq), 137.96 (Cq), 134.94 (Cq), 129.24 (CH), 129.01 (CH), 128.34 (CH), 127.46 (Cq), 124.85 (CH), 123.06 (CH), 121.55 (CH), 116.54 (CH), 115.12 (Cq), 78.17 (Cq), 62.59 (CH), 34.90 ($\underline{C}(CH_3)_3$), 29.48 ($C(\underline{C}H_3)_3$). Anal. Calcd for $C_{26}H_{24}ClN_3O.0.4H_2O$: C 71.43, H 5.73, N 9.61, Found: C 71.11, H 5.70, N 9.30.

*4.1.2.15. Compound **1r**: purified by flash column chromatography using ethyl acetate/n-hexane 1:4 and then, recrystallized from diethyl ether. Obtained as a white solid.* Yield 92% (0.11 g). IR (KBr): 3218, 1723, 1610, 1598, 1490 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.48 (s, 1H), 7.34 (s, 2H), 7.26 (m, 1H), 7.15 (s, 1H), 7.07 (t, $J = 7.9$ Hz, 2H), 6.90 (s, 1H), 6.81 (m, 3H), 6.73–6.58 (m, 2H), 6.05 (d, $J = 8.1$ Hz, 1H), 4.43 (s, 1H), 1.18 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.88 (C═O), 161.89 (Cq), 145.70 (Cq), 141.50 (Cq), 134.93 (Cq), 129.01 (CH), 128.38 (CH), 127.65 (CH), 125.35 (CH), 124.86 (Cq), 123.15 (Cq), 121.39 (CH), 116.21 (CH), 113.87 (CH), 77.09 (Cq), 62.29 (CH), 34.90 ($\underline{C}(CH_3)_3$), 29.49 ($C(\underline{C}H_3)_3$). Anal. Calcd for $C_{26}H_{24}BrN_3O.0.05H_2O$: C 65.70, H 5.12, N 8.84, Found: C 65.32, H 5.02, N 8.64.

*4.1.2.16. Compound **1s**: purified by flash column chromatography using ethyl acetate/n-hexane 1:3 and then, recrystallized from dichloromethane. Obtained as a light yellow solid.* Yield 78% (0.10 g). IR (KBr): 3171, 1720, 1611, 1597, 1491 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.90 (s, 1H), 7.68 (m, $J = 7.0$, 1.9 Hz, 2H), 7.34–7.26 (m, 3H), 7.11 (t, $J = 7.9$ Hz, 2H), 6.97–6.80 (m, 5H), 6.78 (d, $J = 1.3$ Hz, 1H), 6.69 (d, $J = 8.6$ Hz, 2H), 6.60 (dd, $J = 8.1$, 1.5 Hz, 1H), 6.29 (d, $J = 8.1$ Hz, 1H), 5.06 (s, 1H), 3.73 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.59 (C═O), 159.42 (Cq), 149.49 (Cq), 144.32 (Cq), 141.15 (Cq), 135.34 (Cq), 131.63 (Cq), 130.37 (CH), 129.16 (CH), 128.95 (Cq), 128.54 (CH), 127.52 (CH), 126.99 (CH), 126.41 (Cq), 124.24 (Cq), 122.72 (CH), 121.43 (CH), 115.53 (CH), 114.26 (CH), 111.44 (CH), 76.90 (Cq), 62.00 (CH), 55.33 (OCH_3). Anal. Calcd for $C_{29}H_{22}ClN_3O_2$: C 72.57, H 4.62, N 8.75, Found: C 72.28, H 4.94, N 8.36.

4.2. In vitro cytotoxicity

Cytotoxicity was assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), a yellow, water-soluble tetrazolium dye that is converted by mitochondrial dehydrogenases in viable cells to a water-insoluble, purple formazan [17]. The day before experiments cells obtained from the American Type Culture Collection HEK 293T – human embryonic kidney epithelial cell line (ATCC CRL-11268), two breast cancer cell lines MCF-7 (ATCC HTB-22™) (estrogen receptor positive (ER+) and human epidermal growth factor receptor 2 negative (HER2−)) and MDA-MB-231 (ATCC HTB-26™) (ER−, and HER2−) were seeded at 2×10^4 cells per well in 96 well tissue culture plates, in 100 μl of RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units of penicillin G (sodium salt), 100 μg of streptomycin sulfate and 2 mM L-glutamine, at a concentration that allows cells to grow exponentially during the assay. Moreover, the cell lines have the doubling time of 24–30 h for Hek 293T, 29 h for MCF-7, and 38 h for MDA-MB-231, during the 48 h of the assay all cell lines duplicate.

Tested compounds were dissolved in dimethyl sulfoxide (DMSO) serially diluted in the culture medium and added to the cells. The final concentration of DMSO in culture medium during treatment did not exceed 0.5% (v/v), and the same concentration of DMSO was added to the control. Each compound concentration was tested in triplicate in a single experiment which was repeated at least 3 times, controls contained equivalent concentrations of DMSO. Cells were incubated at 37 °C in humidified 5% CO_2 atmosphere. After 48 h, cell media was removed and replaced with fresh medium, the MTT dye solution was added to each well (5 mg/mL in 10 mM phosphate buffer solution at pH 7.4), and after 3 h of incubations the media was removed and intracellular formazan crystals were solubilized and extracted with DMSO. After 15 min at room temperature absorbance was measured at 570 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Germany), and the percentage of viable cells was determined for each compound concentration as described previously [18].

GI_{50}s were determined by non-linear regression using GraphPad PRISM software as previously published.

Statistical analysis of the experimental data was performed by applying one-way ANOVA tests using the software package GraphPad PRISM. Differences were considered to be significant when $P < 0.05$.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.04.023.

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