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New di(hetero)arylethers and di(hetero)arylamines in the thieno[3,2-*b*]pyridine series: Synthesis, growth inhibitory activity on human tumor cell lines and non-tumor cells, effects on cell cycle and on programmed cell death

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ABSTRACT

New fluorinated and methoxylated di(hetero)arylethers and di(hetero)arylamines were prepared functionalizing the 7-position of the thieno[3,2-*b*]pyridine, using copper (C–O) or palladium (C–N) catalyzed couplings, respectively, of the 7-bromothieno[3,2-*b*]pyridine, also prepared, with *ortho*, *meta* and *para* fluoro or methoxy phenols and anilines. The compounds obtained were evaluated for their growth inhibitory activity on the human tumor cell lines MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT15 (colon carcinoma), HepG2 (hepatocellular carcinoma) and HeLa (cervical carcinoma). The most active compounds, a di(hetero)arylether with a methoxy group in the *meta* position relative to the ether function and two di(hetero)arylamines with a methoxy group either in the *ortho* or in the *meta* position relative to the NH, were further tested at their GI_{50} concentrations on NCI-H460 cells causing pronounced alterations in the cell cycle profile and a strong and significant increase in the programmed death of these cells. The fluorinated and the other methoxylated compounds did not show important activity, presenting high GI_{50} values in all the cell lines tested. Furthermore, the hepatotoxicity of the compounds was assessed using porcine liver primary cells (PLP2), established by some of us. Results showed that one of the most active compounds was not toxic to the non-tumor cells at their GI_{50} concentrations showing to be the most promising as antitumoral.

1. Introduction

The thienopyridine skeleton has been reported as having interesting biological activities namely antitumoral [1–7] and antiangiogenic [8–12]. Sugano et al. reported that the (3-amino-6-thien-2-yl-thieno[2,3-*b*]pyridin-2-yl)arylmethanones **I** showed selectivity against a tumorigenic cell line, in very low EC_{50} values [1]. The methyl (3-amino-6-benzo[*d*]thiazol-2-ylamino)thieno[3,2-*b*]pyridine-2-carboxylate **II** was previously prepared by us and showed to be the most active compound in a series of methyl 3-amino-6-[(hetero)arylamino]thieno[3,2-*b*]thienopyridine-2-carboxylates [2]. In the 3-amino-6-[(hetero)aryl]thieno[3,2-*b*]pyridine-2-carboxylate series, compounds **III** and **IV** were shown to be the most promising compounds against MCF-7 (breast adenocarcinoma), A375-C5 (melanoma) and NCI-H460 (non-small cell lung cancer) cell lines. Indeed, compound **III** showed selectivity against MCF-7 and NCI-H460, presenting very low GI_{50} values (1 μM) and compound **IV** presented GI_{50} values in the 3–4 μM range for the three cell lines. The effects of compounds **III** and **IV** on the cell cycle profile of NCI-H460 cells pointed to different mechanisms of action for each compound [3]. Several methyl 3-amino-6-[(hetero)arylethynyl]thieno[3,2-*b*]pyridine-2-carboxylates have also been prepared by us and studied against the same human tumor cell lines, compounds **Va–c** being the most potent ones. Effects of these three

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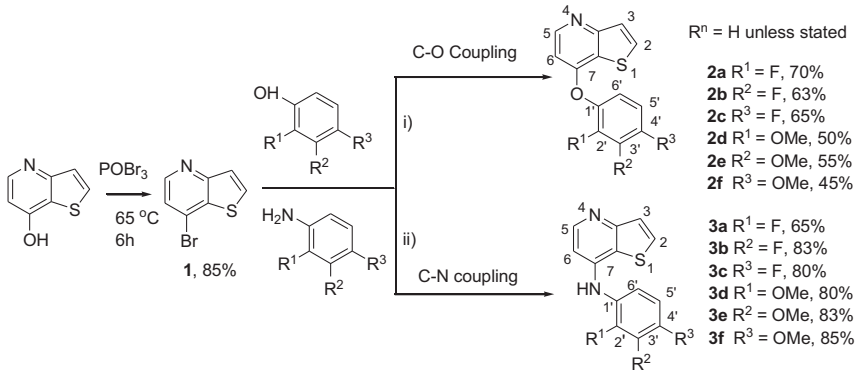
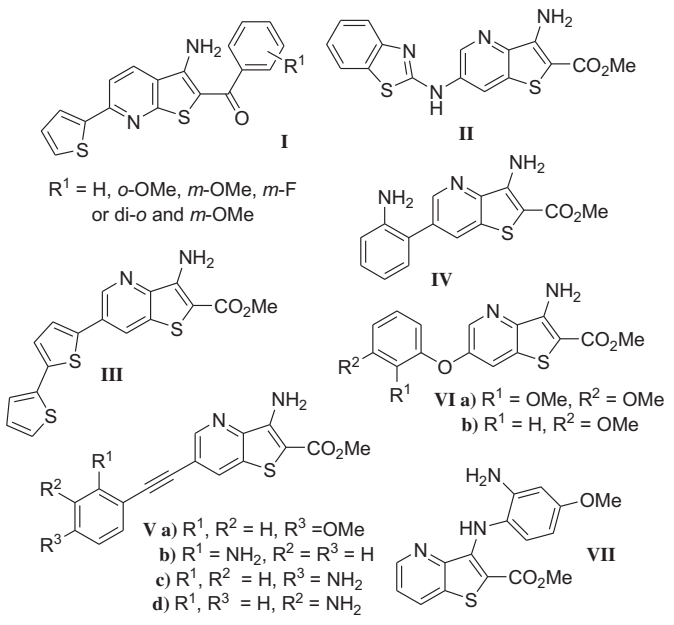


i) CuI (20 mol%), *N*, *N*-dimethylglycine (30mol%), Cs_2CO_3 (4 equiv.),dry dioxane, 110 ºC, 18h.
ii) $Pd(OAc)_2$ (6mol%), BINAP (8mol%), Cs_2CO_3 (2 equiv.), dry toluene, 100 ºC, 2h.

Scheme 1. Synthesis of di(hetero)arylethers **2a**–**f** and di(hetero)arylamines **3a**–**f** by copper (C–O) and palladium (C–N) catalyzed couplings, respectively.

compounds on the cell cycle profile of the NCI-H460 cells were observed and compound **Vb** was found to significantly induce apoptosis at its GI_{50} in this cell line [4]. Recently we have prepared methoxylated di(hetero)arylethers functionalizing the 6-position of the same thieno[3,2-*b*]pyridine moiety. The compounds with a methoxy group in *ortho* (**VIa**) or *meta* (**VIb**) relative to the ether function showed very low GI_{50} values (1–2.5 μM). These compounds are fluorescent and have been encapsulated into nanoliposomes for drug delivery purposes [5]. In another study, compound **Vd** showed a very low GI_{50} value (1.2 μM) in the HepG2 hepatocellular carcinoma cells with no hepatotoxicity to porcine liver primary cells. Effects of this compound on the cell cycle of HepG2 were verified [6]. By functionalizing the amino group of the 3-aminothieno[3,2-*b*]pyridine-2-carboxylate, several amino diarylamines were obtained. Among them, compound **VII** was the most active presenting very low GI_{50} values (1 μM) for all the cell lines referred above including HepG2, without hepatotoxicity to porcine liver primary cells. Effects on the cell cycle profile of the NCI-H460 cells treated with compound **VII** were observed [7].



Structures of compounds **I**-**VII**

Several *N*-(1*H*-indol-5-yl)-2-arylthieno[3,2-*b*]pyridin-7-amines [8], aminothieno[3,2-*c*]pyridine 1,3-diarylureas [9], thieno[3,2-*b*]pyridine arylethers phenylacetylthioureas [10], thieno[3,2-*b*]pyridine arylethers N^3-arylmalonamides [11], and *N*-(3-fluoro-4-(2-arylthieno[3,2-*b*]pyridin-7-yloxy)phenyl)-2-oxo-3-phenylimidazolidine-1-carboxamides [12] have been previously described as inhibitors of the vascular endothelial growth factor receptor (VEGFR-2), a mediator of the biological function of the vascular endothelial growth factor (VEGF), related to angiogenesis and metastasis.

In the present study, new fluorinated and methoxylated di(hetero)arylethers and di(hetero)arylamines were prepared by functionalizing the 7-position of the thieno[3,2-*b*]pyridine, using copper (C–O) or palladium (C–N) catalyzed couplings, respectively. The compounds were evaluated for their growth inhibitory activity towards human tumor cell lines, and the most active ones were further studied regarding their effects on NCI-H460 cell cycle profile and programmed cell death. The hepatotoxicity of the compounds was assessed using non-tumor porcine liver primary cells.

2. Results and discussion

2.1. Synthesis

The reaction of the 7-bromothieno[3,2-*b*]pyridine (**1**), also prepared by us from the commercial thieno[3,2-*b*]pyridin-7-ol and $POBr_3$, with methoxy or fluorophenols and anilines gave rise in moderate to good yields to the new di(hetero)arylethers **2a**–**f**, by a copper-catalyzed (C–O) Ullmann coupling with *N*,*N*-dimethyl glycine as the ligand [13], previously used by us in the synthesis of other di(hetero)arylethers [5], and in good to high yields to the new di(hetero)arylamines **3a**–**f** by a palladium-catalyzed (C–N) Buchwald–Hartwig coupling [14] with BINAP as the ligand, usually performed in our group [2] (Scheme 1).

2.2. Growth inhibitory activity on human tumor cell lines and on porcine liver primary cells

The tumor cell growth inhibitory activity of the di(hetero)arylethers **2a**–**f** and di(hetero)arylamines **3a**–**f** was evaluated in five human tumor cell lines: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung carcinoma), HCT15 (colon adenocarcinoma), HepG2 (hepatocellularcarcinoma) and HeLa (cervical carcinoma) using the sulforhodamine B assay as previously described [2–7]. This allowed the determination of the GI_{50} values (μM),

corresponding to the concentration of the compounds which inhibited 50% of cell growth (Table 1). Furthermore, the toxicity of the compounds towards non-tumor cells was studied using porcine liver primary cells (PLP2).

From the results obtained (Table 1) it is possible to establish some structure–activity relationships. Among the di(hetero)arylethers **2a**–**f**, compound **2e**, *m*-methoxylated, showed the lowest GI_{50} values in the different tumor cell lines (0.39–3.14 μM), in particular in the HCT15 cell line (0.39 μM). The other methoxylated compounds, **2d** and **2f**, were not very efficient inhibitors of cell growth. The fluorinated compounds **2a**–**c** presented no significant growth inhibitory activity against the five cell lines studied, presenting high GI_{50} values.

The di(hetero)arylethers **2a** and **2f** presented GI_{50} values in the PLP2 cells similar to the GI_{50} values obtained in most of the human tumor cell lines (except in the HepG2 and in the HeLa cells, in which the GI_{50} values were lower than in the primary cells). Nonetheless, the di(hetero)arylethers **2b**–**e** presented lower GI_{50} values in the tumor cell lines than in the PLP2.

Regarding the di(hetero)arylamines **3a**–**f**, the most active compounds are **3d** and **3e**. These compounds, with *o*-methoxy or *m*-methoxy groups relative to the NH, presented the lowest GI_{50} values in the tumor cell lines studied but also presented low GI_{50} values in the non-tumor PLP2 cells. Nevertheless for compound **3d**, the GI_{50} for the HeLa cell line (1.40 μM) was very much lower than the GI_{50} (6.56 μM) for the PLP2 cells. Despite this, the GI_{50} values for this compound in the other cell lines, except for HepG2, were also lower (5.40–5.91 μM) than the GI_{50} value for PLP2, but they are very much closer. Also, for compound **3e**, which was the most active compound, the GI_{50} determined for most of the tumor cell lines (0.09–0.31 μM), with the exception of the HepG2 cells (5.02 μM), were slightly lower than the GI_{50} in PLP2 cells (1.94 μM). The fluorinated compounds **3a**–**c** presented much higher GI_{50} values than the methoxylated ones, for the inhibitory growth activity in the tumor cell lines studied and presented higher GI_{50} values in the PLP2 cells than in most of the tumor cell lines studied. On the other hand, the *p*-methoxylated compound **3f** presented similar GI_{50} values in the tumor cells and primary cells.

The most active compounds in the tumor cell lines, **2e**, **3d** and **3e**, were chosen to be further studied regarding their effect on cell cycle profile and programmed cell death on one of the most sensitive cell lines, NCI-H460. This cell line was also chosen since it represents non-small cell lung cancer, one of the cancers which currently cause most deaths worldwide.

2.3. Effects on cell cycle profile and programmed cell death of NCI-H460 cells

Treatment of NCI-H460 cells with the GI_{50} concentration of compounds **2e**, **3d** and **3e** showed that all the compounds caused pronounced alterations in the cell cycle profile of these cells (Fig. 1). In addition, a shoulder in the left of the G1 peak was evident following treatment with all the compounds, which was indicative of a sub-G1 peak and suggestive of cell death.

This was further confirmed with the TUNEL assay. Indeed, a strong and significant increase in programmed cell death was observed following 24 h and 48 h of treatment with the compounds (Fig. 2). When cells were treated with the compounds for 24 h, compounds **3d** and **3e** caused a stronger increase than compound **2e** in the levels of cell death. However, when cells were treated for 48 h all compounds caused similar effects in the levels of cell death, which was higher than 30%.

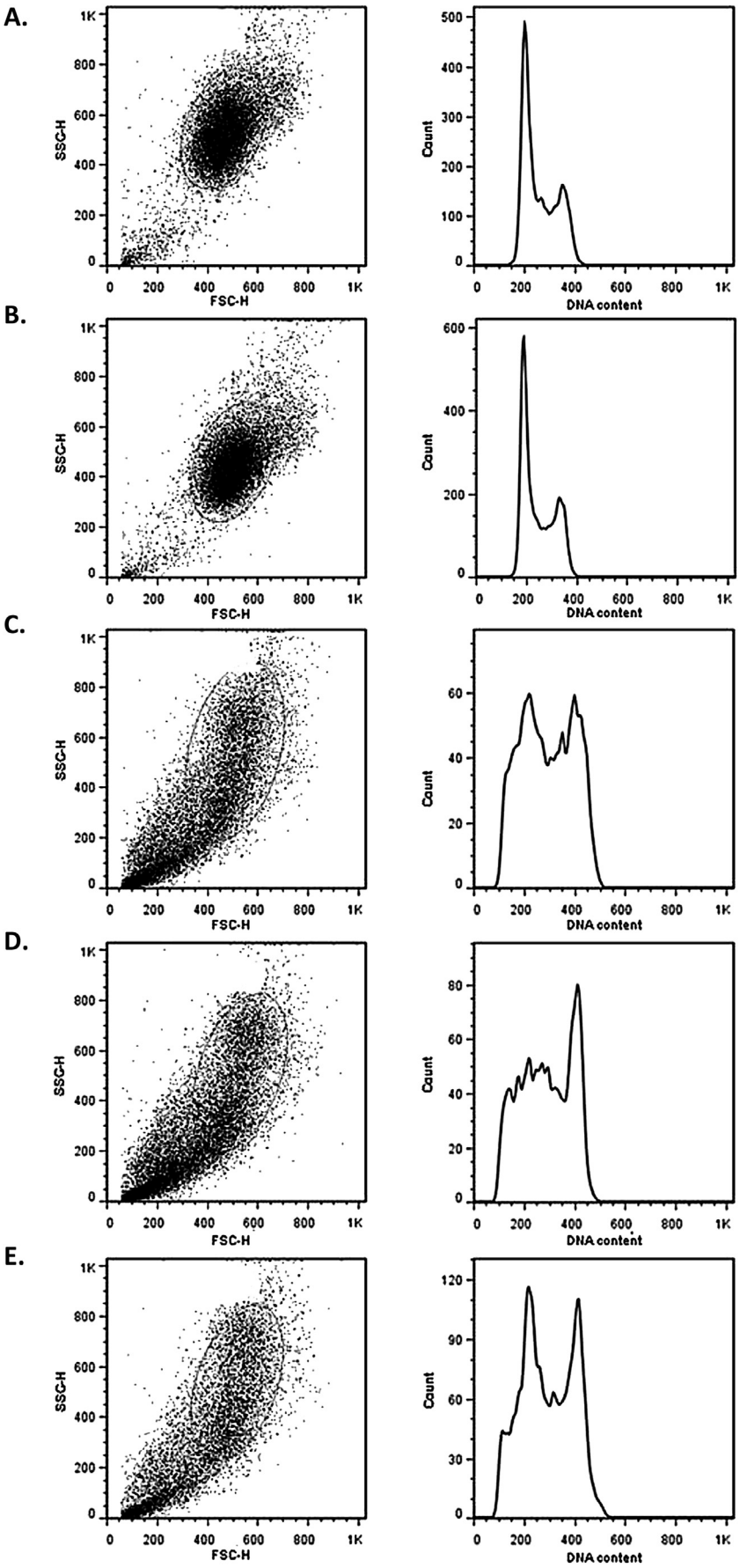
3. Conclusions

The fluorinated and methoxylated di(hetero)arylethers and di(hetero)arylamines were prepared by copper (C–O) or palladium (C–N) catalyzed couplings of 7-bromothieno[3,2-*b*]pyridine with *ortho*, *meta* and *para* fluoro or methoxy phenols or anilines in moderate to high yields, after chromatographic purification and were fully characterized. The compounds obtained were evaluated for their growth inhibitory activity on several human tumor cell lines. The most active compounds, were shown to be a di(hetero)arylether with a methoxy group in the *meta* position relative to the ether function and two di(hetero) arylamines with a methoxy group either in the *ortho* or in the *meta* position relative to the NH. These were further tested at their GI_{50} concentrations on NCI-H460 cells causing pronounced alterations in the cell cycle profile and a strong and significant increase in the programmed death of these cells. The fluorinated and the other methoxylated compounds presented high GI_{50} values in all the cell lines tested. Furthermore, the hepatotoxicity of the compounds was assessed using porcine liver primary cells (PLP2). The results showed that one of the most active compounds, the di(hetero)arylether **2e**, was nontoxic to non-tumor cells at their GI_{50} concentrations, compound **2e** being the most promising antitumoral. The hepatotoxicity of the two di(hetero)arylamines **3d** and **3e** needs to be further studied.

Table 1
Growth inhibitory activity of the synthesized di(hetero)arylethers **2a**–**f** and di(hetero)arylamines **3a**–**f** on various human tumor cell lines and in non-tumor porcine liver primary cells (PLP2).

GI_{50} (uM)[a]						
	HepG2	HeLa	MCF-7	NCI-H460	HCT15	PLP2
2a	16.43 ± 0.74	50.26 ± 2.47	74.29 ± 1.98	77.96 ± 3.98	97.22 ± 4.48	87.24 ± 4.29
2b	42.65 ± 3.12	34.51 ± 4.09	101.83 ± 8.40	102.65 ± 8.54	103.47 ± 1.61	>125
2c	45.10 ± 1.97	33.83 ± 2.30	98.48 ± 7.52	100.60 ± 5.90	88.08 ± 4.91	>125
2d	62.68 ± 4.22	28.94 ± 7.03	90.89 ± 5.01	94.17 ± 4.47	91.15 ± 5.21	>125
2e	2.70 ± 0.41	3.14 ± 0.12	1.56 ± 0.23	1.04 ± 0.04	0.39 ± 0.03	26.65 ± 1.98
2f	53.24 ± 4.65	49.4 ± 1.52	93.31 ± 4.86	86.77 ± 5.39	86.05 ± 4.24	94.45 ± 6.09
3a	25.8 ± 2.81	26.37 ± 2.93	94.37 ± 4.26	79.51 ± 1.94	86.65 ± 6.04	114.91 ± 6.92
3b	32.02 ± 2.33	69.24 ± 5.18	63.71 ± 3.78	77.47 ± 2.50	70.65 ± 5.67	119.46 ± 5.97
3c	26.26 ± 2.56	77.49 ± 3.83	80.74 ± 6.01	78.46 ± 6.56	91.16 ± 5.34	100.41 ± 4.41
3d	18.00 ± 1.29	1.40 ± 0.06	5.88 ± 0.86	5.40 ± 0.89	5.91 ± 0.21	6.56 ± 0.2
3e	5.02 ± 0.06	0.09 ± 0.00	0.25 ± 0.03	0.28 ± 0.03	0.31 ± 0.04	1.94 ± 0.16
3f	22.08 ± 2.11	20.42 ± 2.24	20.20 ± 1.31	19.44 ± 1.44	25.92 ± 1.83	22.73 ± 2.02
Ellipticine	5.38 ± 1.11	3.28 ± 0.67	4.32 ± 1.03	5.77 ± 0.95	1.91 ± 0.06	2.06 ± 0.03

[a] GI_{50} values correspond to the compound concentration which inhibited 50% of cell growth. Results are from three independent experiments (performed in triplicate), and are expressed as mean ± standard deviation (SD).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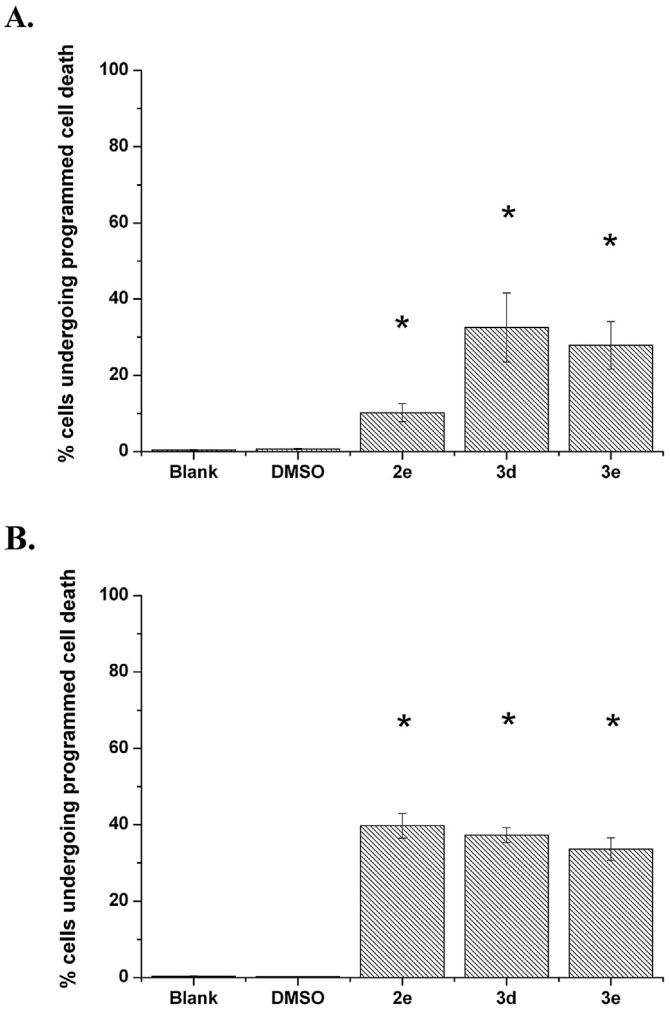

Fig. 2. Analysis of programmed cell death of NCI-H460 cells treated with the compounds. Cells were treated for 24 h (A) or 48 h (B) with medium, DMSO, or the GI_{50} concentrations of compounds **2e**, **3d** and **3e**. Levels of programmed cell death were determined with the TUNEL assay. Results are the mean ± SEM of three independent experiments. Statistical significance was tested by two tailed paired Student's *t*-Test using DMSO as a negative control. * Indicates $p < 0.05$.

4. Materials and methods

4.1. Chemistry

Melting points (°C) were determined in a Stuart SMP3 and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Plus at 300 and 75.4 MHz, respectively or on a Bruker Avance III at 400 and 100.6 MHz, respectively. Two dimensional ^{1}H–^{13}C correlations were performed to attribute some signals. Mass spectra (MS) EI-TOF or ESI-TOF and HRMS on the M^+ or on the $[M + H]^+$ were performed by the mass spectrometry service of the University of Vigo, C.A.C.T.I., Spain. The reactions were monitored by thin layer chromatography (TLC) using Macherey–Nagel precoated aluminum silica gel 60 sheets (0.20 mm) with UV254 indicator. Column chromatography was performed on Panreac, Silica Gel 60, 230–400 mesh and the dry flash on silica was performed on Panreac, Silica Gel 60, 70–230 mesh. Ether refers to diethylether. Petroleum ether refers to the boiling range 40–60 °C.

4.1.1. 7-Bromothieno[3,2-b]pyridine (**1**)

From thieno[3,2-*b*]pyridin-7-ol (300 mg, 2.00 mmol) and $POBr_3$ (2.80 g, 10.0 mmol) and the mixture was heated at 65 °C for 6 h. After cooling, NaOH (aq) (5 mL), water (5 mL) and chloroform (5 mL) were added. The phases were separated and the aqueous phase was extracted with more chloroform (2 × 5 mL). The organic phase was dried ($MgSO_4$) and filtered. Removal of the solvent gave compound **1**as a yellow solid (363 mg, 85%), m.p. 67–68 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46 (1H, d, J = 5.2 Hz, 6-H), 7.67 (1H, d, J = 5.6 Hz, HetAr–H), 7.83 (1H, d, J = 5.6 Hz, HetAr–H), 8.51 (1H, d, J = 5.2 Hz, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 121.7 (6-CH), 125.9 (CH), 126.9 (C), 131.4 (CH), 135.7 (C), 147.5 (5-CH), 156.4 (C) ppm. HRMS (EI-TOF): calcd for $C_7H_4^{79}BrNS$ $[M]^+$ 212.9248. Found 212.9248. Calcd for $C_7H_4^{81}BrNS$ $[M]^+$ 214.9227. Found 214.9227.

4.1.2. General procedure for the synthesis of di(hetero)arylethers ***2a–f***

A dry Schlenk tube was charged under Ar with dry dioxane (3 mL), the fluoro or methoxyphenol (1.1 equiv.), *N*,*N*-dimethyl glycine (30 mol%), CuI (20 mol%), Cs_2CO_3 (2 equiv.) and compound **1**. The mixture was heated with stirring under Ar at 100 °C for 18 h. After cooling water (5 mL) and ethyl acetate (5 mL) were added. The phases were separated and the aqueous phase was extracted with more ethyl acetate (2 × 5 mL). The organic phase was dried ($MgSO_4$) and filtered. Removal of the solvent gave an oil which was submitted to column chromatography.

*4.1.2.1. 7-(2-Flurophenoxy)thieno[3,2-b]pyridine (**2a**).*
Compound **1** (100 mg, 0.470 mmol), 2-fluorophenol, and after purification by column chromatography using a solvent gradient from 50% to 65% ether/petroleum ether, compound **2a** was obtained as a white oil (81.0 mg, 70%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.54 (1H, d, J = 4.8 Hz, 6-H), 7.22–7.33 (4H, m, Ar–H), 7.64 (1H, d, J = 5.6 Hz, HetAr–H), 7.80 (1H, d, J = 5.6 Hz, HetAr–H), 8.54 (1H, br s, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 103.2 (6-CH), 117.5 (d, J = 18.1 Hz, 3′-CH), 122.4 (C), 123.6 (CH), 124.9 (CH), 125.2 (d, J = 4.0 Hz, 4′-CH), 127.4 (d, J = 7.0 Hz, 6′-CH), 131.3 (CH), 140.6 (d, J = 12.1 Hz, 1′-C), 148.6 (5-CH), 153.3 (C), 157.0 (d, J = 251.5 Hz, CF), 160.1 (C) ppm. HRMS (EI-TOF): calcd for $C_{13}H_8FNOS$ $[M^+]$ 245.0311. Found 245.0305.

*4.1.2.2. 7-(3-Flurophenoxy)thieno[3,2-b]pyridine (**2b**).* From compound **1** (100 mg, 0.470 mmol), 3-fluorophenol, and after purification by column chromatography using a solvent gradient from 30% to 50% ether/petroleum ether, compound **2b** was obtained as a white oil (72.0 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.64 (1H, d, J = 5.2 Hz, 6-H), 6.92–7.04 (3H, m, Ar–H), 7.39–7.44 (1H, m, Ar–H), 7.60 (1H, d, J = 5.6 Hz, HetAr–H), 7.77 (1H, d, J = 5.6 Hz, HetAr–H), 8.56 (1H, br s, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 104.7 (6-CH), 108.7 (d, J = 24.1 Hz, CH), 112.8 (d, J = 21.1 Hz, CH), 116.4 (d, J = 3.0 Hz, 6′-CH), 123.0 (C), 125.2 (CH), 130.9 (CH), 131.0 (d, J = 10.0 Hz, 5′-CH), 149.0 (5-CH), 154.9 (d, J = 11.1 Hz, 1′-C), 158.9 (C), 159.6 (C), 163.4 (d, J = 248.4 Hz, CF) ppm. HRMS (EI-TOF): calcd for $C_{13}H_8FNOS$ $[M^+]$ 245.0311. Found 245.0313.

Fig. 1. Analysis of cell cycle profile by flow cytometry of NCI-H460 cells treated with the compounds. Cells were treated for 24 h with medium (A), DMSO (B), or the GI_{50} concentrations of compounds **2e** (C), **3d** (D) or **3e** (E). The left panel corresponds to dot plots of forward *versus* side scatter (FSC *versus* SSC) and shows the gated population. In the right panel, the histograms indicate the cell cycle profile of the gated population following the exclusion of cellular aggregates and debris. All images are representative of three independent experiments.

*4.1.2.3. 7-(4-Flurophenoxy)thieno[3,2-b]pyridine (**2c**).* From compound **1** (100 mg, 0.470 mmol) and 4-fluorophenol, and after purification by column chromatography using a solvent gradient from 40% to 60% ether/petroleum ether, compound **2c** was obtained as a white oil (75.0 mg, 65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.54 (1H, d, J = 5.2 Hz, 6-H), 7.13–7.20 (4H, m, Ar–H), 7.60 (1H, d, J = 5.6 Hz, HetAr–H), 7.76 (1H, d, J = 5.6 Hz, HetAr–H), 8.52 (1H, br s, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 103.9 (6-CH), 116.9 (d, J = 23.1 Hz, 3′ and 5′-CH), 122.6 (d, J = 9.0 Hz, 2′ and 6′-CH), 124.9 (C), 125.2 (CH), 130.9 (CH), 148.9 (5-CH), 149.5 (d, J = 3.0 Hz, 1′-C), 158.8 (C), 160.2 (d, J = 244.5 Hz, CF), 160.5 (C) ppm. HRMS (EI-TOF): calcd for $C_{13}H_8FNOS$ [M^+] 245.0311. Found 245.0314.

*4.1.2.4. 7-(2-Methoxyphenoxy)thieno[3,2-b]pyridine (**2d**).* From compound **1** (140 mg, 0.488 mmol), 2-methoxyphenol, and after purification by column chromatography using a solvent gradient from 25% ether/petroleum ether to 30% ether/petroleum ether, compound **2d** was obtained as a yellow oil (55.0 mg, 50%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.76 (3H, s, OMe), 6.43 (1H, d, J = 5.7 Hz, 6-H), 7.02–7.07 (2H, m, Ar–H), 7.19–7.22 (1H, m, Ar–H), 7.27–7.29 (1H, m, Ar–H), 7.57 (1H, d, J = 5.4 Hz, HetAr–H), 7.73 (1H, d, J = 5.4 Hz, HetAr–H), 8.46 (1H, d, J = 5.7 Hz, 5-H) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 55.8 (OMe), 103.3 (6-CH), 113.0 (CH), 121.2 (CH), 121.9 (C), 123.00 (CH), 125.1 (CH), 127.1 (CH), 130.5 (CH), 141.9 (C), 148.9 (5-CH), 151.8 (C), 158.6 (C), 160.4 (C) ppm. HRMS (EI-TOF): calcd for $C_{14}H_{11}NO_2S$: [M^+] 257.0511. Found 257.0513.

*4.1.2.5. 7-(3-Methoxyphenoxy)thieno[3,2-b]pyridine (**2e**).* From compound **1** (140 mg, 0.488 mmol) and 3-methoxyphenol, and after purification by column chromatography using a solvent gradient from 30% ether/petroleum ether to 70% ether/petroleum ether, compound **2e**was obtained as a yellow oil (61.0 mg, 55%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, s, OMe), 6.62 (1H, d, J = 5.6 Hz, 6-H), 6.75 (1H, apparent t, J = 2.4 Hz, 2′-H), 6.77–6.79 (1H, m, Ar–H), 6.83–6.86 (1H, m, Ar–H), 7.34 (1H, apparent t, J = 8.4 Hz, 5′-H), 7.58 (1H, d, J = 5.2 Hz, HetAr–H), 7.74 (1H, d, J = 5.2 Hz, HetAr–H), 8.51 (1H, d, J = 5.6 Hz, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 55.5 (OMe), 104.4 (6-CH), 106.8 (2′-CH), 111.6 (CH), 112.9 (CH), 122.8 (C), 125.2 (CH), 130.5 (5′-CH), 130.6 (CH), 149.1 (5-CH), 154.8 (C), 158.9 (C), 160.1 (C), 161.1 (C) ppm. HRMS (EI-TOF): calcd for $C_{14}H_{11}NO_2S$: [M^+] 257.0511. Found 257.0509.

*4.1.2.6. 7-(4-Methoxyphenoxy)thieno[3,2-b]pyridine (**2f**).* From compound **1** (140 mg, 0.488 mmol) and 4-methoxyphenol, and after purification by column chromatography using a solvent gradient from 30% ether/petroleum ether to 70% ether/petroleum ether, compound **2f** was obtained as a yellow solid (50.0 mg, 45%), m.p. 70.5–72.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.86 (3H, s, OMe), 6.53 (1H, d, J = 5.2 Hz, 6-H), 6.98 (2H, d, J = 9.2 Hz, 3′ and 5′-H), 7.13 (2H, d, J = 9.2 Hz, 2′ and 6′-H), 7.59 (1H, d, J = 5.6 Hz, HetAr–H), 7.75 (1H, d, J = 5.6 Hz, HetAr–H), 8.49 (1H, d, J = 5.2 Hz, 5-H) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 55.6 (OMe), 103.6 (6-CH), 115.1 (3′ and 5′-CH), 122.2 (2′ and 6′-CH), 122.5 (C), 125.1 (CH),130.7 (CH), 147.0 (C), 148.9 (5-CH), 157.4 (C), 158.5 (C), 161.1 (C) ppm. HRMS (EI-TOF): calcd for $C_{14}H_{11}NO_2S$: [M^+] 257.0511. Found 257.0512.

*4.1.3. General procedure for the synthesis of di(hetero)arylamines **3a–f***

A dry Schlenk tube was charged under Ar with dry toluene (3 mL), the fluoro or methoxyanilines (1.1 equiv.), $Pd(OAc)_2$ (6 mol %), BINAP (8 mol%), Cs_2CO_3 (2 equiv.) and compound **1**. The mixture was heated with stirring under Ar at 100 °C for 2 h. After cooling water (5 mL) and ethyl acetate (5 mL) were added. The phases were separated and the aqueous phase was extracted with more ethyl acetate (2 × 5 mL). The organic phase was dried ($MgSO_4$) and filtered. Removal of the solvent gave a solid which was submitted to a *dry flash* in silica using ether or AcOEt and a solid was obtained.

*4.1.3.1. N-(2-Fluorophenyl)thieno[3,2-b]pyridin-7-amine (**3a**).* From compound **1** (200 mg, 0.940 mmol) and 2-fluoroaniline, compound **3a** was obtained as a yellow solid (148 mg, 65%), m.p. 134–135 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.43 (1H, d, J = 5.2 Hz, 6-H), 7.24–7.37 (4H, m, Ar–H), 7.42 (1H, d, J = 5.6 Hz, HetAr–H), 7.95 (1H, d, J = 5.6 Hz, HetAr–H), 8.26 (1H, d, J = 5.2 Hz, 5-H), 8.76 (1H, br s, NH) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 102.4 (6-CH), 116.4 (d, J = 20.0 Hz, 3′-CH), 119.1 (C), 124.9 (d, J = 6.0 Hz, CH), 125.0 (CH), 126.9 (d, J = 8 Hz, CH), 127.2 (d, J = 12.0 Hz, 1′-C), 127.50 (CH), 130.1 (CH), 146.8 (C), 148.01 (5-CH), 157.4 (C), 156.7 (d, J = 246.5 Hz, CF) ppm. HRMS (EI-TOF): calcd for $C_{13}H_9FN_2S$ [M^+] 244.0470. Found 244.0473.

*4.1.3.2. N-(3-Fluorophenyl)thieno[3,2-b]pyridin-7-amine (**3b**).* From compound **1** (200 mg, 0.940 mmol) and 3-fluoroaniline, compound **3b** was obtained as a yellow solid (180 mg, 83%), m.p. 186–187 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.86–6.89 (1H, m, Ar–H), 7.00 (1H, d, J = 5.2 Hz, 6-H), 7.06–7.13 (2H, m, Ar–H), 7.37–7.39 (1H, m, Ar–H), 7.46 (1H, d, J = 5.6 Hz, HetAr–H), 8.00 (1H, d, J = 5.6 Hz, HetAr–H), 8.36 (1H, d, J = 5.2 Hz, 5-H), 9.00 (1H, br s, NH) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 103.2 (6-CH), 107.6 (d, J = 24.0 Hz, CH), 109.4 (d, J = 22.0 Hz, CH), 116.7 (d, J = 2.0 Hz, 6′-CH), 120.8 (C), 125.1 (CH), 130.3 (CH), 130.7 (d, J = 10.0 Hz, 5′-CH), 142.4 (d, J = 10 Hz, 1′-C), 145.2 (C), 148.3 (5-CH), 157.7 (C), 162.6 (d, J = 244.0 Hz, CF) ppm. HRMS (EI-TOF): calcd for $C_{13}H_9FN_2S$ [M^+] 244.0470. Found 244.0470.

*4.1.3.3. N-(4-Fluorophenyl)thieno[3,2-b]pyridin-7-amine (**3c**).* From compound **1** (200 mg, 0.940 mmol) and 4-fluoroaniline, compound **3c** was obtained a yellow solid (180 mg, 80%), m.p. 184–185 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 6.75 (1H, d, J = 5.2 Hz, 6-H), 7.20–7.24 (2H, m, 3′ and 5′-H), 7.29–7.32 (2H, m, 2′ and 6′-H), 7.42 (1H, d, J = 5.6 Hz, HetAr–H), 7.96 (1H, d, J = 5.6 Hz, HetAr–H), 8.27 (1H, d, J = 5.2 Hz, 5-H), 8.78 (1H, br s, NH) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 101.6 (6-CH), 115.9 (d, J = 22.0 Hz, 3′ and 5′-CH), 119.5 (C), 124.7 (d, J = 8.0 Hz, 2′ and 6′-CH), 125.1 (CH), 130.0 (CH), 136.2 (d, J = 3 Hz, 1′-C), 146.5 (C), 148.1 (5-CH), 157.5 (C), 158.6 (d, J = 240.4 Hz, CF) ppm. HRMS (EI-TOF): calcd for $C_{13}H_9FN_2S$ [M^+] 244.0470. Found 244.0474.

*4.1.3.4. N-(2-Methoxyphenyl)thieno[3,2-b]pyridin-7-amine (**3d**).* From compound **1** (100 mg, 0.470 mmol) and 2-methoxyaniline, compound **3d** was obtained a yellow solid (95.0 mg, 80%), m.p. 130–131 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.73 (s, 3H, OMe), 6.37 (1H, d, J = 5.2 Hz, 6-H), 6.97–7.01 (1H, m, Ar–H), 7.12–7.14 (1H, m, Ar–H), 7.22–7.29 (2H, m, 2× Ar–H), 7.38 (1H, d, J = 5.6 Hz, HetAr–H), 7.91 (1H, d, J = 5.6 Hz, HetAr–H), 8.20 (1H, br s, 5-H), 8.43 (1H, br s, NH) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 55.4 (OMe), 102.2 (6-CH), 112.2 (CH), 118.6 (C), 120.6 (CH), 124.6 (CH), 126.9 (CH), 127.0 (CH), 127.53 (C), 130.17 (CH), 147.29 (5-CH), 147.85 (C), 154.27 (C), 156.69 (C) ppm. HRMS (EI-TOF): calcd for $C_{14}H_{12}N_2OS$ [M^+] 256.0670. Found 256.0675.

*4.1.3.5. N-(3-Methoxyphenyl)thieno[3,2-b]pyridin-7-amine (**3e**).* From compound **1** (100 mg, 0.470 mmol) and 3-methoxyaniline, compound **3e** was obtained a yellow solid (99.0 mg, 83%), m.p. 146–147 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, s, OMe), 6.67 (1H, br s, NH), 6.74–6.77 (1H, m, 4′-H), 6.84–6.85 (1H, m, 2′-H), 6.87–6.89 (1H, m, 6′-H), 6.98 (1H, d, J = 5.2 Hz, 6-H), 7.31 (1H, apparent, J = 8.4 Hz, 5′-H), 7.55 (1H, d, J = 5.6 Hz, HetAr–H), 7.65 (1H, d, J = 5.6 Hz, HetAr–H), 8.39 (1H, d, J = 5.2 Hz, 5-H), ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 55.4 (OMe), 102.4 (6-CH), 108.5 (2′-CH),

110.3 (4′-CH), 114.9 (6′-CH), 120.7 (C), 125.5 (CH), 128.8 (CH), 130.3 (5′-CH), 140.20 (C), 146.7 (C), 147.52 (5-CH), 156.5 (C), 160.63 (C) ppm. HRMS (EI-TOF): calcd for $C_{14}H_{12}N_2OS$ [M^+] 256.0670. Found 256.0678.

*4.1.3.6. N-(4-Methoxyphenyl)thieno[3,2-b]pyridin-7-amine(**3f**).* From compound **1** (100 mg, 0.470 mmol) and 4-methoxyaniline, compound **3f** was obtained a yellow solid (102 mg, 85%). m.p. 184–185 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.76 (3H, s, OMe), 6.61 (1H, d, J = 5.2 Hz, 6-H), 6.97 (2H, d, J = 8.8 Hz, 3′ and 5′-H), 7.21 (2H, d, J = 8.8 Hz, 2′ and 6′-H), 7.39 (1H, d, J = 5.6 Hz, HetAr–H), 8.21 (1H, br s, 5-H), 8.60 (1H, br s, NH), ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 55.3 (OMe), 101.1 (6-CH), 114.4 (3′ and 5′-CH), 118.9 (C), 125.1 (CH), 125.6 (2′ and 6′-CH), 129.8 (CH), 132.4 (C), 147.5 (C), 148.1 (5-CH), 156.4 (C), 157.4 (C) ppm. HRMS (EI-TOF): calcd for $C_{14}H_{12}N_2OS$ [M^+] 256.0670. Found 256.0677.

4.2. Growth inhibitory activity on human tumor cell lines and on porcine liver primary cells

Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma) from DSMZ (Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), NCI-H460 (non-small cell lung carcinoma), HCT15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma) from ECACC (European Collection of Cell Cultures). Cells were routinely maintained as adherent cell cultures at 37 °C, in a humidified air incubator containing 5% CO_2. Cells were cultured in: i) RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine, for the MCF-7, NCI-H460 and HCT15 cell lines and ii)DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, for HeLa and HepG2 cells). To assess the growth inhibitory activity of the compounds, the Sulforhodamine B assay was performed according to a procedure previously described by the authors [2–7]. For this, each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT15 cells or 1.0×10^4 cells/well for HeLa and HepG2 cells) in 96-well plates.

For the evaluation of toxicity towards non-tumor cells, a primary cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure previously established by some of us [6,7]; these cells were designated as PLP2 cells. Cell culture was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultured in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were treated for 48 h with the different compounds solutions and the procedure for SRB assay was followed, as described above. The results were expressed in GI_{50} values (concentrations that inhibited 50% of the net cell growth). Ellipticine was used as positive control.

4.3. Analysis of cell cycle profile and programmed cell death

NCI-H460 cells (1×10^5 cells/well) were plated in 6-well plates and incubated at 37 °C for 24 h. Cells were then treated with complete medium (blank), with the compounds (**2e**, **3d** and **3e**) at their previously determined GI_{50} concentrations or with their respective vehicle (DMSO, control). Cells were harvested following a 24 h and/or 48 h and further processed according to the following protocols. For cell cycle profile analysis, following 24 h treatment cells were fixed in 70% ice-cold ethanol and kept at 4 °C for at least 12 h until analysis. Prior to analysis, cells were incubated with Propidium Iodide (5 μg/mL) and RNase A in PBS (100 μg/mL) for 30 min on ice [15]. Cellular DNA content was analyzed using a FACS Calibur (BD) flow cytometer. Cell cycle profile was subsequently analyzed using the Flow Jo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion [4,7].

For programmed cell death analysis, following 24 h and 48 h treatment cells were fixed in 4% paraformaldehyde for 30 min and further kept in PBS at 4 °C until analysis. Cytospins were then prepared and cells permeabilized in ice cold 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Programmed cell death was assessed using the "in situ cell death detection kit-fluorescein" (Roche, Basel Switzerland). Briefly, cells were incubated with the TUNEL reaction mix (enzyme diluted 1:20) for 1 h, as previously described [16]. Slides were mounted with Vectashield Mounting Media with DAPI (Vector Laboratories). Cells were observed in a DM2000 microscope (LEICA) and a semi-quantitative evaluation of the levels of programmed cell death was performed by counting a minimum of 500 cells per slide, except in the cases of **2e** and **3d** treatment at 48 h, in which only ~300 cells were counted.

4.4. Statistical analysis

Results were expressed as mean ± standard deviation (SD) or standard error (SEM) of at least three independent experiments, performed in duplicate. Statistical significance was tested with a two tailed paired Student's t-Test in relation to control (DMSO treatment). $^*p < 0.05$.

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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.2013.09.023.

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