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Original article

Novel 6-[(hetero)arylamino]thieno[3,2-*b*]pyridines: Synthesis and antitumoral activities

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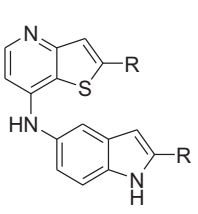
ABSTRACT

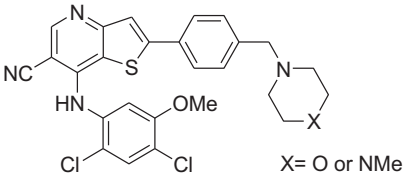
Several novel 6-[(hetero)arylamino]thieno[3,2-*b*]pyridines were prepared by palladium-catalyzed C–N Buchwald–Hartwig coupling of the methyl 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate with aryl and heteroarylamines, using different reaction conditions. The antitumoral activity of the di(hetero) arylamines obtained was evaluated against three representative human tumor cell lines, namely breast adenocarcinoma (MCF-7), melanoma (A375-C5), and non-small cell lung cancer (NCI-H460) and some structure–activity relationships were established within each series. The most promising compounds were shown to be a benzothiazole derivative with GI_{50} 3.5–6.9 μM followed by an indole derivative with GI_{50} 13–21 μM.

1. Introduction

Thienopyridine derivatives have attracted much attention because of their biological activities. 7-(Aryl)aminothieno[3,2-*b*] pyridines **I** were shown to be potent inhibitors of the VEGFR-2 (Vascular Endothelial Growth Factor Receptor-2) which has been identified as a key component of the signaling pathway responsible for the sprouting and maturation of new blood vessels from the tumor leading to tumor growth and metastasis [1]. Overexpression or overactivation of Src (the non-receptor tyrosine kinase) is implicated in various diseases such as cancer, osteoporosis and ischemic diseases. 7-[(2,4-Dichloro-5-methoxyphenyl)amino]thieno[3,2-*b*]pyridine-6-carbonitriles **II** with various groups at C-2 have shown to be inhibitors of Src kinase activity [2].



I



II

Here we describe the synthesis of novel methyl 6-(heteroaryl) aminothieno[3,2-*b*]pyridine-2-carboxylates by palladium-catalyzed C–N Buchwald–Hartwig coupling [3] of the methyl 6-bromothieno[3,2-*b*]pyridine-2-carboxylate (**1**), recently described by us [4], with arylamines and heteroarylamines using different conditions. All the methyl 6-[(hetero)arylamino]thieno[3,2-*b*] pyridine-2-carboxylates obtained were studied as potential antitumor compounds using three human tumor cell lines, representative of different types of tumors, and it was possible to establish some structure–activity relationships.

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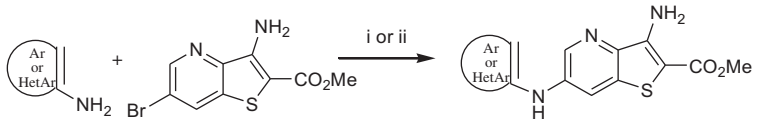
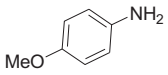
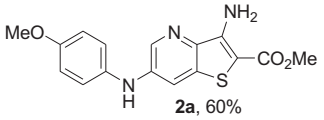
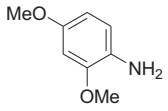
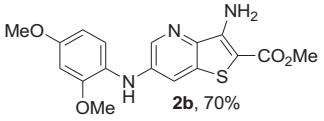
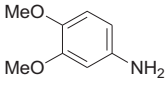
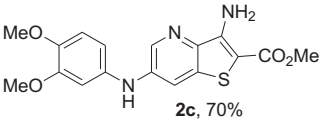
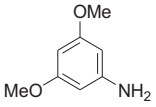
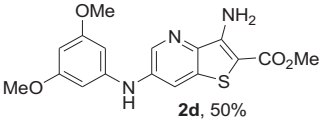
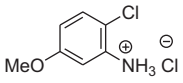
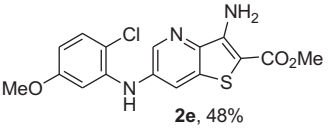
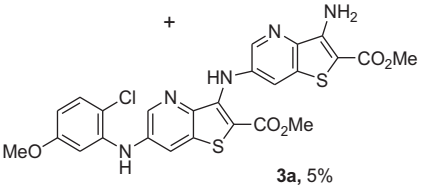
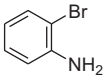
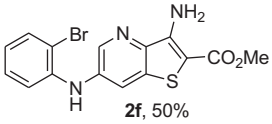
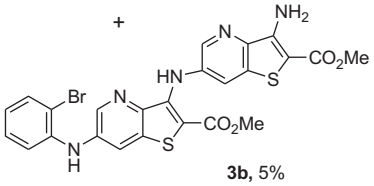
Table 1
Reaction scheme and conditions, aminated components and product structures and yields.



1 **2a-g** and **4a-f**

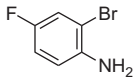
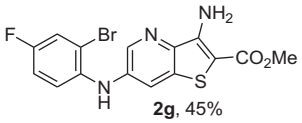
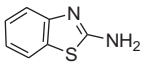
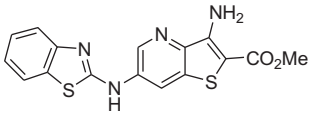
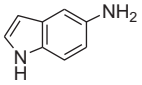
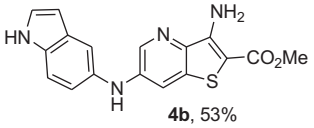
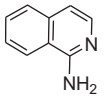
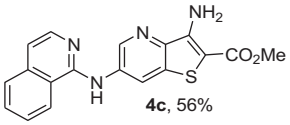
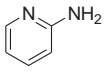
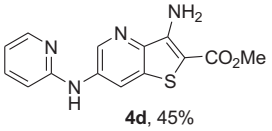
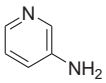
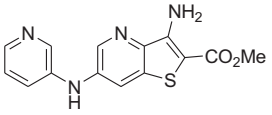
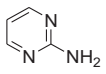
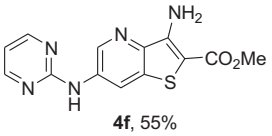
i) $Pd(OAc)_2$ (16 mol%), *rac*-BINAP (18 mol %), Cs_2CO_3 (1.8 equiv.), dry toluene, 110 ºC, under Ar.
ii) $Pd(OAc)_2$ (16 mol%), xantphos (18 mol%), Cs_2CO_3 (1.8 equiv.), dry dioxane, 120 ºC, under Ar.

.

Aminated Component	Reaction Conditions	Di(hetero)arylamine
	i, 21 h	 **2a**, 60%
	i, 4 h	 **2b**, 70%
	i, 6 h	 **2c**, 70%
	i, 21 h	 **2d**, 50%
	i, 22 h	 **2e**, 48% +  **3a,** 5%
	i, 25 h	 **2f**, 50% +  **3b,** 5%

(*continued on next page*)

Table 1 (*continued*)

Aminated Component	Reaction Conditions	Di(hetero)arylamine
	i, 22 h	 **2g**, 45%
	ii, 4 h	 **4a**, 54%
	ii, 5 h	 **4b**, 53%
	ii, 3 h	 **4c**, 56%
	ii, 4 h	 **4d**, 45%
	ii, 4 h	 **4e**, 45%
	ii, 2 h	 **4f**, 55%

2. Results and discussion

2.1. Chemistry

Several methyl 6-(arylamino)thieno[3,2-*b*]pyridine-2-carboxylates **2a**–**g** were prepared by C–N Buchwald–Hartwig coupling of compound **1** with different methoxylated and/or halogenated anilines, in good yields, using the most general conditions, already used by us in this type of coupling [5] for halogenated electron-deficient rings and anilines (Table 1, i). When 2-chloro-5-methoxyaniline hydrochloride and 2-bromoaniline were used as coupling components the minor products **3a** and **3b** (Table 1), were also formed resulting from the reaction of compound **1** with the 3-amino group of the di(hetero)arylamines **2e** and **2f** previously formed, respectively.

Based in earlier experiences on C–N Buchwald–Hartwig couplings using deactivated amines [5,6] as coupling components we have used Xantphos as the ligand, Cs_2CO_3 as the base and dioxane as solvent, on the coupling of compound **1** with different heteroarylamines. The corresponding 6-(heteroarylamino)thieno[3,2-*b*]pyridines **4a**–**f** were obtained in good yields (Table 1, ii), thus extended the scope of the reactivity of compound **1** in this type of coupling. The use of BINAP as the ligand did not afford the diheteroarylamines **4**, so the use of Xantphos is crucial in the coupling with the heteroarylamines used.

2.2. Biological activity

The *in vitro* growth inhibitory activity of all the di(hetero)arylamines **2** and **4** prepared, was evaluated using three human tumor cell lines, representing different tumor types, namely breast adenocarcinoma (MCF-7), melanoma (A375-C5), and non-small cell lung cancer (NCI-H460), after a continuous exposure of 48 h. The results are summarized in Table 2. Doxorubicin was used as a positive control.

Table 2
Growth inhibitory activity of compounds **2** and **4** on the three human tumor cell lines in study.

Compound	GI_{50} (μM)[a]		
	MCF-7	A375-C5	NCI-H460
2a	123.6 ± 6.1	>150.0	95.0 ± 7.8
2b	26.3 ± 0.8	27.3 ± 0.1	26.3 ± 1.8
2c	30.0 ± 3.7	26.3 ± 0.7	26.2 ± 2.1
2d	>150.0	>150.0	>150.0
2e	22.1 ± 0.8	25.8 ± 2.2	21.7 ± 1.6
2f	28.9 ± 1.0	34.1 ± 2.4	30.9 ± 1.5
2g	28.9 ± 1.2	34.0 ± 3.3	31.3 ± 1.8
4a	6.0 ± 0.1	3.5 ± 0.0	6.4 ± 0.5
4b	18.1 ± 3.4	17.3 ± 2.8	15.8 ± 2.5
4c	>150.0	>150.0	>150.0
4d	>150.0	>150.0	>150.0
4e	82.4 ± 13.2	>150.0	49.4 ± 6.7
4f	>150.0	>150.0	>150.0

Doxorubicin was used as positive control (GI_{50}: MCF-7 = 43.3 ± 2.6 nM; A375-C5 = 130.2 ± 10.1 nM; NCI-H460 = 35.6 ± 1.6 nM).

[a] The lowest concentrations causing 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 48 h, expressed as means ± SEM of three independent experiments performed in duplicate.

From the analysis of Table 2 it is possible to infer that for the di(hetero)arylamines **2** the presence of two methoxy groups in the *ortho* and *para* positions (**2b**) and in the *meta* and *para* positions (**2c**) decreases significantly the GI_{50} values (25–34 μM) relatively to the monomethoxylated **2a** and to the dimethoxylated with two methoxy groups in the *meta* positions (**2d**). For the *ortho*-halogenated compounds (**2e**–**g**) the GI_{50} values are comparable to those obtained for compounds **2b** and **2c**, but are slightly lower for the chlorinated compound bearing also a methoxy group (**2e**) (21–28 μM). The presence of a fluorine atom in compound **2g** gave identical GI_{50} values to the corresponding unsubstituted *ortho*-brominated compound **2f** (28–37 μM).

The most active compounds are two of the diheteroarylamines **4**. The benzothiazole derivative **4a** presents the lowest GI_{50} values (3.5–6.9 μM) and the indole derivative **4b** is the second most active with GI_{50} values 13–21 μM.

3. Conclusions

Several new 6-[(hetero)aryl]aminothieno[3,2-*b*]pyridines were prepared from the methyl 3-amino-6-bromothieno[3,2-*b*]pyridine-2-carboxylate by C–N Buchwald–Hartwig using different ligands for the coupling of arylamines and heteroarylamines. The di(hetero) arylamines prepared were evaluated as antitumorals in three representative human tumor cell lines and it was possible to establish some structure–activity relationships. For the aniline derivatives the most active compounds are a methoxylated *ortho*-chloro derivative (GI_{50} 21–28 μM) and the dimethoxylated derivatives with methoxy groups in the *ortho* and *para* positions and in the *para* and *meta* positions (GI_{50} 25–34 μM) relative to the N–H bond. For the heterocyclic amine derivatives the most active is the benzothiazole derivative, presenting very low GI_{50} values, followed by the indole derivative. These compounds are also the most promising within all the compounds studied. Further studies with the latter compounds will be envisaged in order to determine their mechanism(s) of action.

4. Experimental

4.1. Synthesis

Melting points were determined on a Stuart SMP3 apparatus and are uncorrected. The ^{1}H NMR spectra were measured on a Varian Unity Plus at 300 MHz on a Bruker Avance III at 400 MHz. The ^{13}C NMR spectra were measured in the same instruments at 75.4 and 100.6 MHz respectively. Mass spectra (EI) and HRMS were performed by the mass spectrometry service of University of Vigo-Spain.

Column chromatography was performed on Macherey–Nagel silica gel 230–400 mesh. Petroleum ether refers to the boiling range 40–60 °C. Ether refers to diethyl ether.

The ligand *rac*-BINAP [2,2′-bis(diphenylphosphane)-1,1′-binaphtyl, 97%], Xantphos [9,9-dimethyl-4,5-bis(diphenylphosphane)xanthene] and the base Cs_2CO_3 were purchased from Sigma Aldrich.

4.1.1. General procedure for the synthesis of the di(hetero) arylamines ***2a–g***

A dry Schlenk tube was charged under Argon with dry toluene (3–5 mL), the thienopyridine **1**, $Pd(OAc)_2$ (16 mol%), *rac*-BINAP (18 mol%), Cs_2CO_3 (1.8 equiv.), the aniline (1.2 equiv) and the mixture was heated at 110 °C for several hours (Table 1). The reactions were followed by TLC. After cooling, water (5 mL) and ethyl acetate (5 mL) were added. The phases were separated, the aqueous phase was extracted with more ethyl acetate (3 × 5 mL), the organic phases were collected, dried ($MgSO_4$) and filtered. Removal of the solvent gave an oil which was submitted to column chromatography using solvent gradient to isolate the products as yellow solids after some washes with petroleum ether.

*4.1.1.1. Methyl 3-amino-6-(4-methoxyphenylamino)thieno[3,2-b]pyridine-2-carboxylate (**2a**).* From compound **1** (150 mg, 0.540 mmol) and 4-methoxyaniline (74.0 mg, 0.648 mmol), using solvent gradient in the column chromatography from 50% ether/petroleum ether till neat ether, compound **2a** was isolated (100 mg, 60%), m.p.199–201 °C ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.84 (3H, s, OMe), 3.88 (3H, s, OMe), 5.82 (1H, br s, NH), 6.13 (2H, br s, NH_2), 6.94 (2H, d, J = 8.8 Hz, 3′ and 5′-H), 7.15 (2H, d, J = 8.8 Hz, 2′ and 6′-H), 7.39 (1H, d, J = 2.8 Hz, HetAr-H), 8.23 (1H, br s, HetAr-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 50.93 (OMe), 55.11 (OMe), 111.51 (CH), 114.57 (3′and 5′-CH), 123.51 (2′ and 6′-CH), 132.64 (C), 135.65 (C), 137.21 (CH), 138.59 (C), 141.40 (C), 147.51 (C), 156.27 (C), 165.05 (C=O), ppm. MS (EI): *m*/*z* (%) 329 (M^+, 36), 257 (100). HRMS M^+ calct. for $C_{16}H_{15}N_3O_3S$ 329.0834; found 329.0835.

*4.1.1.2. Methyl 3-amino-6-(2,4-dimethoxyphenylamino)thieno[3,2-b] pyridine-2-carboxylate (**2b**).* From compound **1** (150 mg, 0.540 mmol) and 2,4-dimethoxyaniline (100 mg, 0.648 mmol), using solvent gradient in the column chromatography from 50% ether/petroleum ether till neat ether, compound **2b** was isolated (135 mg, 70%), m.p. 156–158 °C ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.84 (3H, s, OMe), 3.85 (3H, s, OMe), 3.88 (3H, s, OMe), 5.95 (1H, br s, NH), 6.13 (2H, br s, NH_2), 6.52 (1H, dd, J = 8.9 and 2.8 Hz, 5′-H), 6.58 (1H, d, J = 2.8 Hz, 3′-H), 7.26 (1H, d, J = 8.9 Hz, 6′-H), 7.47 (1H, d, J = 2.8 Hz, HetAr-H), 8.29 (1H, d, J = 2.8 Hz, HetAr-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.38 (OMe), 55.65 (OMe), 55.68 (OMe), 96.19 (C), 99.69 (3′-CH), 104.02 (5′-CH), 112.23 (CH), 121.49 (6′-CH), 122.87 (C), 136.04 (C), 138.38 (CH), 139.14 (C), 141.42 (C), 148.01 (C), 152.34 (C), 156.87 (C), 165.53 (C=O) ppm. MS (EI): *m*/*z* (%) 359 (M^+, 100), 312 (48). HRMS M^+ calct. for $C_{17}H_{17}N_3O_4S$ 359.0940, found 359.0940.

*4.1.1.3. Methyl 3-amino-6-(3,4-dimethoxyphenylamino)thieno[3,2-b] pyridine-2-carboxylate (**2c**).* From compound **1** (150 mg, 0.540 mmol) and 3,4-dimethoxyaniline (100 mg, 0.648 mmol), and using solvent gradient in the column chromatography from 50% ether/petroleum ether till neat ether, compound **2c** was isolated (125 mg, 70%), m.p. 187–189 °C ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.87 (3H, s, OMe), 3.88 (3H, s, OMe), 3.91 (3H, s, OMe), 5.85 (1H, br s, NH), 6.13 (2H, br s, NH_2), 6.73–6.80 (2H, m, Ar-H), 6.89 (1H, d, J = 8.7 Hz, 5′-H), 7.43 (1H, d,

$J = 2.4$ Hz, HetAr-H), 8.25 (1H, d, $J = 2.4$ Hz, HetAr-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 51.41 (OMe), 56.00 (OMe), 56.20 (OMe), 96.23 (C), 106.80 (CH), 112.06 (5′-CH), 112.51 (CH), 114.24 (CH), 133.59 (C), 136.05 (C), 137.78 (CH), 139.22 (C), 141.63 (C), 146.24 (C), 147.91 (C), 149.89 (C), 165.49 (C=O), ppm. MS (EI): *m/z* (%) 359 (M^+, 53), 257 (53), 236 (69), 155 (100). HRMS M^+ calct. for $C_{17}H_{17}N_3O_4S$ 359.0940, found 359.0939.

4.1.1.4. Methyl 3-amino-6-(3,5-dimethoxyphenylamino)thieno[3,2-b] pyridine-2-carboxylate ***(2d)****.* From compound **1** (150 mg, 0.540 mmol) and 3,5-dimethoxyaniline (100 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% ether/petroleum ether till neat ether compound **2d** was isolated (97 mg, 50%), m.p. 218–220 °C ^{1}H NMR ($CDCl_3$ + DMSO-d_6, 400 MHz): δ 3.56 (6H, s, 2 × OMe), 3.64 (3H, s, OMe), 5.91 (1H, t, $J = 2.4$ Hz, 4′-H), 6.03 (2H, br s, NH_2), 6.13 (2H, d, $J = 2.0$ Hz, 2′ and 6′-H), 7.50 (1H, d, $J = 2.4$ Hz, HetAr-H), 7.71 (1H, br s, NH), 8.19 (1H, d, $J = 2.4$ Hz, HetAr-H) ppm. ^{13}C NMR ($CDCl_3$ + DMSO-d_6, 100.6 MHz): δ 50.76 (OMe), 54.73 (2 × OMe), 93.47 (4′-CH), 95.53 (C), 96.75 (2′and 6′-CH), 113.74 (CH), 135.18 (C), 138.43 (C), 138.68 (CH), 139.66 (C), 142.89 (C), 147.34 (C), 161.03 (2 × C), 164.75 (C=O) ppm. MS (EI): *m/z* (%) 359 (M^+, 100), 327 (40), 255 (41), 236 (60). HRMS M^+ calct. for $C_{17}H_{17}N_3O_4S$ 359.0940; found 359.0947.

*4.1.1.5. Methyl 3-amino-6-(2-chloro-5-methoxyphenylamino)thieno [3,2-b]pyridine-2-carboxylate (****2e****) and methyl 3-amino-6-[6-(2-chloro-5-methoxyphenylamino)-2-(methoxycarbonyl)thieno[3,2-b]pyridin-3-ylamino]thieno[3,2-b]pyridine-2-carboxylate (****3a****).* From compound **1** (150 mg, 0.540 mmol) and 2-cloro-5-metoxianilina (126 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 10% AcOEt/petroleum ether till 30% AcOEt/petroleum ether, compound **2e** was isolated (94.0 mg, 48%), m. p. 138–140 °C ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.77 (3H, s, OMe), 3.90 (3H, s, OMe), 6.18 (2H, broad s, NH_2), 6.25 (1H, broad s, NH), 6.52 (1H, dd, $J = 8.8$ and 2.8 Hz, 4′-H), 6.86 (1H, d, $J = 2.8$ Hz, 6′-H), 7.32 (1H, d, $J = 8.8$ Hz, 3′-H), 7.78 (1H, d, $J = 2.4$ Hz, Ar-H), 8.45 (1H, d, $J = 2.4$ Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.54 (OMe), 55.59 (OMe), 97.88 (C), 103.23 (6′-CH), 107.69 (4′-CH), 114.82 (C), 118.04 (CH), 130.45 (3′-CH), 135.46 (C), 137.81 (C), 139.12 (C), 140.41 (CH), 141.34 (C), 147.59 (C), 159.22 (C), 165.37 (C=O) ppm. MS (EI): *m/z* (%)365 (M^+ ^{37}Cl, 35), 363 (M^+ ^{35}Cl, 100). HRMS M^+ calct. for $C_{16}H_{14}ClN_3O_3S$: M^+ ^{37}Cl 365.0415; found 365.0412. M^+ ^{35}Cl calct. 363.0444; found 363.0436.

Compound **3a** was also isolated as a minor product using 60% AcOEt/petroleum ether in the column chromatography as a yellow solid (10 mg, 5%), m.p. 205–207 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.78 (3H, s, OMe), 3.90 (3H, s, OMe), 3.94 (3H, s, OMe), 6.18 (2H, broad s, NH_2), 6.27 (1H, broad s, NH), 6.55 (1H, dd, $J = 8.8$ and 2.8 Hz, 4′-H), 6.91 (1H, d, $J = 2.8$ Hz, 6′-H), 7.33 (1H, d, $J = 8.8$ Hz, 3′-H), 7.71 (1H, d, $J = 2.4$ Hz, HetAr-H), 7.81 (1H, d, $J = 2.4$ Hz, HetAr-H), 8.35 (1H, d, $J = 2.4$ Hz, HetAr-H), 8.48 (1H, d, $J = 2.4$ Hz, HetAr-H), 8.94 (1H, broad s, N–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.50 (OMe), 52.17 (OMe), 55.65 (OMe), 97.95 (C), 104.32 (6′-CH), 107.18 (C), 108.28 (4′-CH), 115.56 (C), 116.10 (CH), 120.61 (CH), 130.57 (3′-CH), 134.62 (C), 135.86 (C), 137.46 (C), 137.58 (C), 138.35 (C), 140.22 (CH), 141.13 (C), 141.43 (CH), 141.60 (C), 143.30 (C), 147.78 (C), 159.19 (C), 165.20 (C=O), 165.45 (C=O) ppm. MS (EI): *m/z* (%) 571 (M^+ ^{37}Cl, 42), 569 (M^+ ^{35}Cl, 100) HRMS M^+ calct. for $C_{25}H_{20}ClN_5O_5S_2$: M^+ ^{37}Cl 571.0565; found 571.0573. M^+ ^{35}Cl calct. 569.0594; found 569.0574.

*4.1.1.6. Methyl 6-(2-bromophenylamino)-3-aminothieno[3,2-b]pyridine-2-carboxylate (****2f****) and methyl 3-amino-6-[6-(2-bromophenylamino)-2-(methoxycarbonyl)thieno[3,2-b]pyridin-3-ylamino]thieno [3,2-b]pyridine-2-carboxylate (****3b****).* From compound **1** (150 mg, 0.540 mmol) and 2-bromoaniline (120 mg, 0.648 mmol) and using neat ether in the column chromatography, compound **2f** was isolated (102 mg, 50%), m.p.163–165 °C ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.90 (3H, s, OMe), 6.17 (2H, broad s, NH_2), 6.24 (1H, broad s, N–H), 6.89–6.93 (1H, m, Ar-H), 7.25–7.34 (2H, m, Ar-H), 7.60 (1H, dd, $J = 8$ and 1.6 Hz, Ar-H), 7.73 (1H, d, $J = 2.4$ Hz, Ar-H), 8.43 (1H, broad s, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.52 (OMe), 97.71 (C), 114.11 (C), 117.32 (CH), 117.70 (CH), 123.24 (CH), 128.44 (CH), 133.42 (CH), 135.48 (C), 138.23 (C), 139.48 (C), 140.14 (CH), 141.20 (C), 147.64 (C), 165.37 (C=O) ppm. MS (EI): *m/z* (%) 379 (M^+ ^{81}Br, 100), 377 (M^+ ^{79}Br, 95), 347 (51), 345 (45). HRMS M^+ calct. for $C_{15}H_{12}BrN_3O_2S$: M^+ ^{81}Br 378.9813; found 378.9805. M^+ ^{79}Br calct.: 376.9834; found 376.9839.

Compound **3b** was also isolated as a minor polar product using neat ether in the column chromatography as a yellow solid (10 mg, 5%), m.p. 201–203 °C, after some washes with petroleum ether. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.90 (3H, s, OMe), 3.94 (3H, s, OMe), 6.17 (2H, broad s, NH_2), 6.28 (1H, broad s, N–H), 6.92–6.97 (1H, m, Ar-H), 7.27–7.32 (2H, m, Ar-H), 7.38 (1H, dd, $J = 8.0$ and 1.6 Hz, Ar-H), 7.62 (1H, dd, $J = 8$ and 1.2 Hz, Ar-H), 7.71 (1H, d, $J = 2.4$ Hz, Ar-H), 7.74 (1H, d, $J = 2.4$ Hz, Ar-H), 8.33 (1H, d, $J = 2.4$ Hz, Ar-H), 8.47 (1H, d, $J = 2.4$ Hz, Ar-H), 8.94 (1H, broad s, N–H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.49 (OMe), 52.15 (OMe), 97.94 (C), 106.93 (C), 114.91 (C), 115.45 (CH), 118.74 (CH), 120.61 (CH), 123.95 (CH), 128.47 (CH), 133.54 (CH), 134.61 (C), 135.92 (C), 137.45 (C), 138.00 (C), 138.76 (C), 139.89 (CH), 140.94 (C), 141.47 (CH), 141.60 (C), 143.34 (C), 147.77 (C), 165.21 (C=O), 165.44 (C=O) ppm. MS (EI): *m/z* (%) 585 (M^+ ^{81}Br, 100), 583 (M^+ ^{79}Br 95), 521 (61), 519 (54). HRMS M^+ calct. for $C_{24}H_{18}BrN_5O_4S_2$: M^+ ^{81}Br 584.9963; found 584.9947. M^+ ^{79}Br 582.9984; found 582.9969.

*4.1.1.7. Methyl 6-(2-bromo-4-fluorophenylamino)-3-aminothieno[3,2-b]pyridine-2-carboxylate (****2g****).* From compound **1** (150 mg, 0.540 mmol) and 2-bromo-4-fluoroaniline (0.07 mL, 0.648 mmol) and using solvent gradient in the column chromatography from 10% AcOEt/petroleum ether till 30% AcOEt/petroleum ether, compound **2g** was isolated (97.0 mg, 45%), m.p. 175–177 °C ^{1}H NMR ($CDCl_3$, 300 MHz): δ 3.90 (3H, s, OMe), 6.03 (1H, broad s, N–H), 6.17 (2H, broad s, NH_2), 7.02–7.09 (1H, m, Ar-H), 7.29–7.34 (1H, m, Ar-H), 7.37–7.41 (1H, m, Ar-H), 7.58 (1H, d, $J = 2.4$ Hz, Ar-H), 8.36 (1H, d, $J = 2.4$ Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 75.4 MHz): δ 51.53 (OMe), 96.46 (C), 115.50 (C), 115.53 (d, $J = 22$ Hz, CH), 115.91 (CH), 120.31 (d, $J = 5$ Hz, CH), 120.50 (d, $J = 22$ Hz, CH), 135.58 (C), 135.72 (d, $J = 3$ Hz, C), 138.97 (C), 139.29 (CH), 140.87 (C), 147.65 (C), 157.88 (d, $J = 247$ Hz, CF), 165.37 (C=O) ppm. MS (EI): *m/z* (%) 397 (M^+ ^{81}Br, 99), 395 (M^+ ^{79}Br, 98), 365 (51) 363 (49). HRMS M^+ calct. for $C_{15}H_{11}BrFN_3O_2S$: M^+ ^{81}Br 396.9719; found 396.9709. M^+ ^{79}Br 394.9728; found 394.9739.

4.1.2. General procedure for the synthesis of the diheteroarylamines ***4a–f***

A dry Schlenk tube was charged, under Argon, with dry dioxane (3–5 mL), the thienopyridine **1**, $Pd(OAc)_2$ (16 mol%), Xantphos (18 mol%), Cs_2CO_3 (1.8 equiv), the heteroarylamine (1.1 equiv) and the mixture was heated at 110 °C for several hours (Table 1). The reaction work-up was done following the general procedure for the synthesis of compounds **2**.

*4.1.2.1. Methyl 3-amino-6-(benzo[d]thiazol-2-ylamino)thieno[3,2-b] pyridine-2-carboxylate (****4a****).* From compound **1** (150 mg, 0.540 mmol) and 2-aminobenzo[*d*]thiazole (80.0 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% AcOEt/petroleum ether till AcOEt, compound **4a** was isolated (103 mg, 54%), m.p. 167–169 °C ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.91 (3H, s, OMe), 6.14 (2H, broad s, NH_2), 7.01 (1H, broad s, N–H), 7.19 (1H, app dt, $J = 8.0$

and 1.6 Hz, Ar-H), 7.42 (1H, dd, J = 8.0 and 1.2 Hz, Ar-H), 7.55–7.63 (3H, m, Ar-H), 8.35 (1H, d, J = 2.0 Hz, Ar-H) ppm. ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 51.76 (OMe), 99.80 (C), 109.56 (C), 115.48 (C), 115.75 (CH), 124.68 (CH), 128.50 (CH), 131.80 (C), 132.64 (CH), 134.94 (C), 137.66 (CH), 139.86 (C), 144.80 (CH), 144.94 (C), 147.10 (C), 165.19 (C=O) ppm. MS (EI): m/z (%) 356 (M^+, 30), 273 (100), 230 (44). HRMS M^+ calct. for $C_{16}H_{12}N_4O_2S_2$: 356.0402; found 356.0414.

*4.1.2.2. Methyl 3-amino-6-(1H-indol-5-ylamino)thieno[3,2-b]pyridine-2-carboxylate (**4b**).* From compound **1** (150 mg, 0.540 mmol) and 5-aminoindole (80.0 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% AcOEt/petroleum ether till AcOEt, compound **4b** was isolated (96.0 mg, 53%), m.p. 227–229 °C ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.74 (3H, s, OMe), 6.38 (1H, m, Ar-H), 6.72 (2H, broad s, NH_2), 6.98 (1H, dd, J = 8.8 and 2.0 Hz, 6′-H), 7.32–7.34 (1H, m, Ar-H), 7.38–7.41 (2H, m, Ar-H), 7.50 (1H, d, J = 2.4 Hz, Ar-H), 8.31 (1H, d, J = 2.4 Hz, Ar-H), 8.55 (1H, broad s, N–H), 11.06 (1H, broad s, N–H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.03 (OMe), 92.45 (C), 100.98 (CH), 109.26 (CH), 112.21 (CH), 112.60 (CH), 116.98 (CH), 126.09 (CH), 128.25 (C), 132.23 (C), 133.03 (C), 135.69 (C), 136.95 (C), 137.58 (CH), 143.29 (C), 148.66 (C), 164.64 (C=O) ppm. MS (EI): m/z (%) 338 (M^+, 100). HRMS M^+ calct. for $C_{17}H_{14}N_4O_2S$: 338.0837; found 338.0840.

*4.1.2.3. Methyl 3-amino-6-(isoquinolin-1-ylamino)thieno[3,2-b]pyridine-2-carboxylate (**4c**).* From compound **1** (150 mg, 0.540 mmol) and 1-aminoisoquinoline (87.0 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% AcOEt/petroleum ether till AcOEt, compound **4c** was isolated (106 mg, 56%), m.p. 222–224 °C ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.80 (3H, s, OMe), 6.85 (2H, broad s, NH_2), 7.31 (1H, d, J = 5.6 Hz, Ar-H), 7.68 (1H, broad t, Ar-H), 7.74 (1H, broad t, Ar-H), 7.88 (1H, broad d, J = 8.0 Hz, Ar-H), 8.10 (1H, d, J = 5.6 Hz, Ar-H), 8.58 (1H, broad d, J = 8.0 Hz, Ar-H), 9.02 (1H, d, J = 2.0 Hz, Ar-H), 9.09 (1H, d, J = 2.0 Hz, Ar-H), 9.67 (1H, broad s, N–H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.27 (OMe), 94.70 (C), 113.93 (CH), 118.70 (C), 118.79 (CH), 123.17 (CH), 126.65 (CH), 126.99 (CH), 130.40 (CH), 134.15 (C), 136.95 (C), 137.64 (C), 139.89 (C), 140.24 (CH), 141.00 (CH), 148.31 (C), 151.95 (C), 164.60 (C=O) ppm. MS (EI): m/z (%) 350 (M^+, 93), 349 ($M^+ - 1$, 100), 317 (62). HRMS M^+ calct. for $C_{18}H_{14}N_4O_2S$: 350.0837; found 350.0838.

*4.1.2.4. Methyl 3-amino-6-(pyridin-2-ylamino)thieno[3,2-b]pyridine-2-carboxylate (**4d**).* From compound **1** (150 mg, 0.540 mmol) and 2-aminopyridine (57.0 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% AcOEt/petroleum ether till AcOEt, compound **4d** was isolated (75.0 mg, 45%), m.p. 198–200 °C, after some washes with petroleum ether. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.78 (3H, s, OMe), 6.80 (2H, broad s, NH_2), 6.85–6.89 (1H, m, Ar-H), 6.92–6.96 (1H, m, Ar-H), 7.63–7.68 (1H, m, Ar-H), 8.25–8.27 (1H, m, Ar-H), 8.65 (1H, d, J = 2.4 Hz, Ar-H), 9.01 (1H, d, J = 2.4 Hz, Ar-H), 9.70 (1H, broad s, NH) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.21 (OMe), 94.02 (C), 111.74 (CH), 115.63 (CH), 115.65 (CH), 134.69 (C), 137.71 (C), 138.09 (CH), 138.85 (C), 139.17 (CH), 147.24 (CH), 148.39 (C), 155.02 (C), 164.59 (C=O) ppm. MS (EI): m/z (%) 300 (M^+, 100) 299 ($M^+ - 1$, 51) 267(54). HRMS M^+ calct. for $C_{14}H_{12}N_4O_2S$: 300.0681; found 300.0681.

*4.1.2.5. Methyl 3-amino-6-(pyridin-3-ylamino)thieno[3,2-b]pyridine-2-carboxylate (**4e**).* From compound **1** (150 mg, 0.540 mmol) and 3-aminopyridine (57.0 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% AcOEt/petroleum ether till AcOEt, compound **4e** was isolated (75.0 mg, 45%), m.p. 210–212 °C ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.77 (3H, s, OMe), 6.78 (2H, broad s, NH_2), 7.30–7.34 (1H, m, Ar-H), 7.63–7.67 (1H, m, Ar-H), 7.96 (1H, d, J = 2.4 Hz, Ar-H), 8.16 (1H, m, Ar-H), 8.41 (1H, d, J = 2.4 Hz, Ar-H), 8.47 (1H, d, J = 2.8 Hz, Ar-H), 8.99 (1H, broad s, N–H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.20 (OMe), 94.10 (C), 113.43 (CH), 124.02 (CH), 124.27 (CH), 135.23 (C), 138.23 (C), 138.89 (CH), 139.01 (C), 139.42 (C), 140.45 (CH), 142.33 (CH), 148.27 (C), 164.60 (C=O) ppm. MS (EI): m/z (%) 300 (M^+, 100), 268 (60). HRMS M^+ calct. for $C_{14}H_{12}N_4O_2S$: 300.0681; found 300.0682.

*4.1.2.6. Methyl 3-amino-6-(pyrimidin-2-ylamino)thieno[3,2-b]pyridine-2-carboxylate (**4f**).* From compound **1** (150 mg, 0.540 mmol) and 2-aminopyrimidine (57.0 mg, 0.648 mmol) and using solvent gradient in the column chromatography from 50% AcOEt/petroleum ether till AcOEt, compound **4f** was isolated (90.0 mg, 55%), m.p. 271–273 °C ^{1}H NMR (DMSO-d_6, 400 MHz): δ 3.79 (3H, s, OMe), 6.82 (2H, broad s, NH_2), 6.97 (1H, t, J = 4.8 Hz, 4′-H), 8.58 (2H, d, J = 4.8 Hz, 3′ and 5′-H), 7.82 (1H, d, J = 2.4 Hz, Ar-H), 8.91 (1H, d, J = 2.4 Hz, Ar-H), 10.24 (1H, broad s, N–H) ppm. ^{13}C NMR (DMSO-d_6, 100.6 MHz): δ 51.27 (OMe), 94.62 (C), 113.60 (4′-CH), 117.33 (CH), 134.29 (C), 136.88 (C), 139.80 (CH), 148.25 (C), 158.23 (3′ and 5′-CH), 159.60 (C), 164.55 (C=O) ppm. MS (EI): m/z (%) 301 (M^+, 100), 269 (42). HRMS M^+ calct. for $C_{13}H_{11}N_5O_2S$: 301.0633; found 301.0633.

4.2. In vitro antitumor evaluation

4.2.1. Material and methods

4.2.1.1. Reagents. Fetal bovine serum (FBS), L-glutamine, phosphate buffered saline (PBS) and trypsin were from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was from Cambrex (New Jersey, USA). Acetic acid, dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin, ethylenediaminetetraacetic acid (EDTA), sulforhodamine B (SRB) and trypan blue were from SigmaChemical Co. (Saint Louis, USA). Trichloroacetic acid (TCA) and Tris were sourced from Merck (Darmstadt, Germany).

4.2.1.2. Solutions of the compounds. Stocks solutions of the tested compounds were prepared in DMSO and kept at −70 °C. Appropriate dilutions were freshly prepared in the test medium just prior to the assays. The effect of the vehicle solvent (DMSO) on the growth of the cell lines was evaluated by exposing untreated control cells to the maximum concentration of DMSO used in the assays (0.25%). No influence was found (data not shown).

4.2.1.3. Cell cultures. Three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) were used. MCF-7 and A375-C5 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK), and NCI-H460 was kindly provided by the National Cancer Institute (NCI, Bethesda, USA). They were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 5% heat-inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL), at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were obtained by plating 1.5×10^5 cells/mL for MCF-7 and 0.75×10^5 cells/mL for A375-C5 and NCI-H460, followed by a 24 h incubation.

4.2.1.4. Growth inhibition assay. The effects on the *in vitro* growth of human tumor cell lines were evaluated according to the procedure adopted by the NCI (USA) in their "*In vitro* Anticancer Drug Discovery Screen", using the protein-binding dye sulforhodamine B to assess cell growth [7,8]. Briefly, exponentially growing cells were exposed for 48 h, in 96-well microtiter plates, to five serial dilutions of each test compound, starting from a maximum concentration of 150 μM (if possible). Following the exposure period adherent cells were fixed *in situ*, washed and stained with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (Bio-tek Instruments Inc., Powerwave XS, Wincoski, USA). Dose-response curves were obtained for each test compound

and cell line, and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of the compounds that inhibited 50% of the net cell growth was calculated as described elsewhere [7]. Doxorubicin was tested in the same manner to be used as a positive control.

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