Synthesis of Novel Methyl 7-[(Hetero)arylamino]thieno[2,3-b]pyrazine-6-carboxylates and Antitumor Activity Evaluation: Effects in Human Tumor Cells Growth, Cell Cycle Analysis, Apoptosis and Toxicity in Non-Tumor Cells

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Abstract: Several novel methyl 7-[(hetero)arylamino]thieno[2,3-b]pyrazine-6-carboxylates were synthesized by Pd-catalyzed C–N Buchwald–Hartwig cross-coupling of either methyl 7-aminothieno[3,2-b]pyrazine-6-carboxylate with (hetero)arylhalides or 7-bromothieno[2,3-b]pyrazine-6-carboxylate with (hetero)arylamines in good-to-excellent yields (50% quantitative yield), using different reaction conditions, namely ligands and solvents, due to the different electronic character of the substrates. The antitumoral potential of these compounds was evaluated in four human tumor cell lines: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo-2), breast carcinoma (MCF7), and non-small-cell lung carcinoma (NCI-H460) using the SRB assay, and it was possible to establish some structure–activity relationships. Furthermore, they did not show relevant toxicity against a non-tumor cell line culture from the African green monkey kidney (Vero). The most promising compounds (Gl50 ≤ 11 µM), showed some selectivity either against AGS or CaCo-2 cell lines without toxicity at their Gl50 values. The effects of the methoxylated compounds 2b (2-OMeC6H4), 2f and 2g (3,4- or 3,5-diOMeC6H3, respectively) on the cell cycle profile and induction of apoptosis were further studied in the AGS cell line. Nevertheless, even for the most active (Gl50 = 7.8 µM) and selective compound (2g) against this cell line, it was observed that a huge number of dead cells gave rise to an atypical distribution on the cell cycle profile and that these cells were not apoptotic, which points to a different mechanism of action for the AGS cell growth inhibition.

Keywords: C–N Buchwald–Hartwig coupling; thieno[2,3-b]pyrazines; antitumor activity; gastric adenocarcinoma; cell cycle; apoptosis

1. Introduction

The Pd-catalyzed amination of aryl halides has become a fundamental tool in the synthesis of di(hetero)arylamines over the past two decades [1–10]. As this type of compounds plays important roles in the development of pharmaceuticals, agrochemicals, and organic compounds for materials science, the scope of application of the C–N Buchwald–Hartwig cross-coupling was developed and improved, using different ligands, bases, and catalysts, which promoted general methodologies that find applications either in academic research or industry. The choice of the catalyst system is largely dependent on the geometric and electronic character of the substrates. The use of different bases allowed the development of stronger or milder conditions depending on the functional moieties in the substrates. Over the years, some generations of ligands were developed: the monodentate phosphines PAr3-type or PR3 that have often been employed; the bidentate phosphines including...
the most commonly used rac-BINAP and Xantphos; and the dialkylbiarylphosphines that due to their structural variability can be tuned to promote the desired reactivity or selectivity [11–13].

The thieno[2,3-b]pyrazine skeleton has been found in natural products such as urothion and its derivatives [14] and in biologically active synthetic compounds. Some derivatives have been described as selective inhibitors of serine/threonine kinase 4 associated with interleukine-1 receptor (IRAK 4) [15], as inhibitors of ubiquitin-specific protease 28 (USP 28) and/or USP 25 [16], and as serine/threonine kinase B-Raf inhibitors [17], all useful in the prevention or treatment of inflammation, cell proliferation, and immune-related conditions and disease.

Our research group had already applied the C–N Buchwald–Hartwig cross-coupling to the synthesis of di(hetero)arylthieno[3,2-b]pyridines either functionalizing the pyridine [18,19] or the thiophene ring [20], with some of them showing to be promising as potential antitumor compounds.

In this work, due to the biological relevance of the thieno[2,3-b]pyrazine moiety and of di(hetero)arylamines in general, a series of novel methyl 7-(hetero)arylaminothieno[2,3-b]pyrazine-6-carboxylates were synthesized by Pd-catalyzed C–N Buchwald–Hartwig cross-coupling of either methyl 7-aminothieno[3,2-b]pyrazine-6-carboxylic acid with (hetero)arylhalides or 7-bromothieno[2,3-b]pyrazine-6-carboxylate with (hetero)arylamines in good-to-excellent yields, using different ligands and solvents, taking into account the electronic character of the coupling components. The antitumoral potential of the di(hetero)arylamines prepared against four human tumor cell lines and their toxicity using a non-tumor cell line were evaluated. The most promising compounds were submitted to cell cycle analysis and apoptosis induction studies in one of the cell lines studied.

**2. Results and Discussion**

**2.1. Synthesis of Methyl 7-((Hetero)arylamino)thieno[2,3-b]pyrazine-6-carboxylates 2a–2o**

The coupling component methyl 7-aminothieno[2,3-b]pyrazine-6-carboxylate 1a was prepared following the procedure previously described by Peinador et al. [21]. Compound 1a was treated with t-butylnitrite (t-BuONO) and CuBr2 in acetonitrile at room temperature, for 2h, to obtain the methyl 7-bromothieno[2,3-b]pyrazine-2-carboxylate 1b in 50% yield as another coupling component, following the reaction conditions earlier applied by us for the synthesis of a brominated thieno[3,2-b]pyridine [22] (Scheme 1).

![Scheme 1. Synthesis of methyl 7-bromothieno[2,3-b]pyrazine-2-carboxylate (1b) from amine 1a.](image)

The coupling components 1a and 1b were reacted with (hetero)arylhalides or (hetero)arylamines, respectively. Based on our research group experience [18] and that of others [23] in C–N Buchwald–Hartwig couplings, deactivated amine 1a (bearing an EWG in the adjacent position) as coupling component, Xantphos as ligand, Cs2CO3 as the base, and 1,4-dioxane as solvent were used (Table 1, reaction conditions A), while with the activated bromo compound 1b, for the cross-coupling reaction, rac-BINAP as the ligand, Cs2CO3 as the base, and toluene as solvent were used (Table 1, reaction conditions B). With these different conditions, taking into account the electronic character of the substrates, the corresponding di(hetero)arylamines 2a–2o were thus obtained in good-to-excellent yields.
Table 1. Synthesis of di(hetero)arylamines 2a–2o by C–N Buchwald–Hartwig cross-coupling.

![Chemical structure of di(hetero)arylamines](image)

| Entry | Precursor, Conditions | Compounds 2 | Entry | Precursor, Conditions | Compounds 2 |
|-------|-----------------------|-------------|-------|-----------------------|-------------|
| 1     | 1a, A                 | ![Chemical structure](image) | 9     | 1b, B                 | ![Chemical structure](image) |
| 2     | 1b, B                 | ![Chemical structure](image) | 10    | 1b, B                 | ![Chemical structure](image) |
| 3     | 1b, B                 | ![Chemical structure](image) | 11    | 1b, B                 | ![Chemical structure](image) |
| 4     | 1b, B                 | ![Chemical structure](image) | 12    | 1a, A                 | ![Chemical structure](image) |
| 5     | 1b, B                 | ![Chemical structure](image) | 13    | 1b, B                 | ![Chemical structure](image) |
| 6     | 1b, B                 | ![Chemical structure](image) | 14    | 1a, A                 | ![Chemical structure](image) |
Table 1. Cont.

![Chemical Structure](image)

| Entry | Precursor, Conditions | Compounds 2 | Entry | Precursor, Conditions | Compounds 2 |
|-------|-----------------------|-------------|-------|-----------------------|-------------|
| 7     | 1b, B                 | ![Chemical Structure](image) | 15    | 1a, A                 | ![Chemical Structure](image) |
| 8     | 1b, B                 | ![Chemical Structure](image) |       |                       | ![Chemical Structure](image) |

Reaction conditions A: Pd(OAc)$_2$ (10 mol.%), Xantphos (12 mol.%), Cs$_2$CO$_3$ (2.8 equiv.), 1,4-dioxane, 120 °C, 3–5 h. Reaction conditions B: Pd(OAc)$_2$ (6 mol.%), rac-BINAP (8 mol.%), Cs$_2$CO$_3$ (2 equiv.), toluene, 100 °C, 2–6 h.

From analysis of Table 1, it can be observed that the presence of either one methoxy group or a fluor atom in the anilines, in the coupling with 1b, gave the corresponding di(hetero)arylamines 2b–2d and 2i–2k, in good-to-high yields using reaction conditions B (entries 2–4 and 9–11). The di- and trimethoxylated anilines reacting with 1b, in the same conditions, gave the corresponding di(hetero)arylamines 2e–2h in high-to-excellent yields due to the high activation of both substrates for the C–N cross-coupling (entries 5–8). The reaction of amine 1a with the activated $p$-bromobenzonitrile gave compound 2n in quantitative yield using reaction conditions A (entry 14). Nevertheless, the couplings of the deactivated amine 1a using reaction conditions A, with bromobenzene, 3-bromopyridine, and 2-bromonitrobenzene gave the corresponding products 2a, 2l, and 2o only in good yields (entries 1, 12, and 15). The formation of diheteroarylamine 2m from 1b and pyrrole in 71% yield (entry 13) was also notable.

2.2. Cell Growth Inhibitory Effect of Compounds 2a–2o on AGS, CaCo-2, MCF7, NCI-H460 Cell Lines and on a Non-Tumor Cell Line (Vero)

The antitumor potential of the di(hetero)arylamines 2a–2o was evaluated using the sulfurhodamine B (SRB) assay [24,25] to establish some structure–activity relationships. Four human tumor cell lines (acquired from Leibniz-Institut DSMZ) were used: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo-2), breast adenocarcinoma (MCF7), and non-small-cell lung cancer (NCI-H460), as well as a non-tumor cell line from African green monkey kidney (Vero) to evaluate the toxicity of the compounds. Ellipticine was used as a positive control, and the results are presented in GI$_{50}$ values (µM) (Table 2).
Table 2. GI<sub>50</sub> concentrations of compounds 2a–2o and the positive control Ellipticine in four human tumor cell lines and in a non-tumor cell ¹.

| Compounds 2 | AGS      | CaCo-2   | MCF-7    | NCI-H460 | Vero     |
|-------------|----------|----------|----------|----------|----------|
| 2a          | 95 ± 9   | 38 ± 2   | 97 ± 2   | 122 ± 9  | 120 ± 6  |
| 2b          | 9.8 ± 0.2| 36 ± 2   | 127 ± 9  | 45 ± 1   | 21.29 ± 0.03 |
| 2c          | 98 ± 3   | 56 ± 1   | 160 ± 6  | 96 ± 1   | 94 ± 3   |
| 2d          | 59 ± 3   | 60 ± 2   | 136 ± 3  | 52.8 ± 0.3 | 127 ± 3 |
| 2e          | 34 ± 2   | 56 ± 2   | 140 ± 7  | 200 ± 3  | 138 ± 8  |
| 2f          | 9.2 ± 0.2| 8 ± 1    | 87 ± 1   | 41 ± 3   | 53 ± 3   |
| 2g          | 7.8 ± 0.2| 38 ± 4   | 182 ± 5  | 120 ± 10 | 144 ± 10 |
| 2h          | 33 ± 3   | 9.2 ± 0.3| 105.7 ± 0.5 | 77 ± 6   | 53 ± 5   |
| 2i          | 48 ± 4   | 88.4 ± 0.2 | 128 ± 5  | 76 ± 1   | 117 ± 10 |
| 2j          | 86 ± 6   | 142 ± 11 | 96 ± 6   | 69 ± 7   | 149 ± 4  |
| 2k          | 118 ± 2  | 62 ± 4   | 160 ± 7  | 84 ± 2   | 149 ± 7  |
| 2l          | 63.3 ± 0.1| 45 ± 3   | 116 ± 5  | 122 ± 11 | 110 ± 5  |
| 2m          | 88.6 ± 0.1| 43.3 ± 0.3 | 128 ± 2  | 49 ± 2   | 140 ± 12 |
| 2n          | 39 ± 3   | 10.9 ± 0.4| 103 ± 2  | 105 ± 8  | 48 ± 4   |
| 2o          | 55.0 ± 0.3| 31.2 ± 0.2 | 103 ± 3  | 43 ± 2   | 92 ± 8   |
| Ellipticine (positive control) | 0.9 ± 0.1 | 0.8 ± 0.1 | 1.020 ± 0.004 | 1.01 ± 0.01 | 0.6 ± 0.1 |

¹ GI<sub>50</sub> values correspond to the concentration that causes 50% of cell growth inhibition. Results are expressed as mean values ± standard deviation.

The results attained (Table 2) allow us to identify some promising antitumor compounds (GI<sub>50</sub> ≤ 11 µM), namely against AGS and CaCo-2 cell lines. Compounds 2f, 2h, and 2o showed lower GI<sub>50</sub> values against CaCo-2 (8, 9.2, and 10.9 µM, respectively), 2h and 2o being selective for this cell line among the cell lines used. The presence of a cyano group in the para-position of the phenyl ring (2n) led to a decrease in the GI<sub>50</sub> value (10.9 µM) comparing with compound 2a with a non-substituted phenyl ring (38 µM). It is noteworthy that for the CaCo-2 cell line, the lowest GI<sub>50</sub> values were obtained for di- and tri-methoxylated compounds 2f (8 µM) and 2h (9.2 µM), bearing simultaneous methoxy groups in the 3 and 4 positions on the phenyl ring relative to the amine, which seems to be an important feature for the inhibition of cell growth in this cell line.

Regarding the AGS cell line, the presence of methoxy groups is crucial for the cell growth inhibition, as observed for compounds 2b (2-OMeC<sub>6</sub>H<sub>4</sub>, 9.8 µM), 2f (3,4-diOMeC<sub>6</sub>H<sub>3</sub>, 9.2 µM), and 2g (3,5-diOMeC<sub>6</sub>H<sub>3</sub>, 7.8 µM). Compounds 2b and 2g were selective for this cell line, while compound 2f presented low GI<sub>50</sub> values (<10 µM) against both AGS and CaCo-2 cell lines (Table 2).

Notably, given the GI<sub>50</sub> values presented for compound 2 in the AGS and CaCo-2 cell lines, they did not show relevant toxicity in the Vero non-tumor cell line presenting higher GI<sub>50</sub> values. Despite the very low GI<sub>50</sub> values (≤1 µM) for the positive control Ellipticine in the human tumor cell lines tested, this is also toxic for the non-tumor line presenting a GI<sub>50</sub> = 0.6 µM (Table 2).

Compound 2g showed to be the most promising one due to its selectivity against the AGS cell line and the lowest GI<sub>50</sub> value presented (7.8 µM), together with the lower toxicity for the Vero cell line (GI<sub>50</sub> = 144 µM).

With these results in hand, AGS cell cycle profile effects and induction of apoptosis studies for compounds 2b, 2f, and 2g were performed.

2.3. Effects of Compounds 2b, 2f, and 2g on AGS Cell Cycle Profile

AGS cell cycle analysis was carried out using propidium iodide (PI) staining and flow cytometry [26] for compounds 2b, 2f, and 2g at their GI<sub>50</sub> concentrations (Table 2). This assay is based on the measurement of the DNA content in the PI-labeled nuclei. The results are presented in Figure 1.
Compounds 2b and 2f caused cell cycle arrest in G0/G1 phases, although this result was not statistically significant (Qi2 test). On the other hand, for the G2/M phase, these compounds did not present any difference in the percentage of cells compared to the blank.

In Figure 2, histograms of the AGS cell cycle profile for blank and compound 2f are presented. Compound 2b presented a similar histogram to the one obtained for the blank, and compound 2g caused a high percentage of cell death and an atypical distribution on cell cycle profile (results not shown).

**Figure 2.** Cell cycle of blank (a) and compound 2f (b). Note that 250,000; 500,000; 1,000,000; 1,757,072 and 1,777,215 correspond to 250,000; 500,000; 1,000,000; 1,757,072; 1,777,215.

### 2.4. Effect of Compounds 2b, 2f, and 2g on Induction of Apoptosis in AGS Cell Line

Apoptosis induction was performed using the Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Kit (BD Biosciences, San Jose, CA, USA), and measured by flow cytometry for compounds 2b, 2f, and 2g (Figure 3). FITC Annexin V staining was used to determine the percentage of cells within a population that are actively undergoing apoptosis [27].
Regarding the apoptotic process, compound 2g caused a large amount of cell death (Figures 3 and 4), which was in a similar range to what was observed in the cell cycle studies. In addition, compounds 2b and 2f also caused a moderated cell death compared to the blank (Figure 3).

For the tested compounds (2b, 2f, and 2g), a high number of cells in apoptosis was expected, due to the GI50 values obtained for the AGS cell line (Table 2). However, this was not verified, which led us to conclude that cytotoxicity against the AGS cell line involves mechanisms other than apoptosis.

3. Materials and Methods

3.1. Chemistry

Melting points (°C) were determined in a SMP3 Stuart apparatus. 1H, 13C, and 19F NMR spectra were recorded on a Bruker Advance III (Bruker, Bremen, Germany) at 400, 100.6, and 376.48 MHz, respectively (see Supplementary Materials), using the signals of the non-deuterated solvents of CHCl3 (7.27 ppm) of the CDCl3 or of DMSO (2.49 ppm) of the DMSO-d6, as internal reference relatively to TMS (0 ppm). DEPT (θ = 135°) and
bi-dimensional homo $^1$H–$^1$H (COSY) and heteronuclear correlations $^1$H–$^{13}$C (HMQC and HMBC) were used to attribute some signals. HRMS were obtained at the external service of mass spectrometry of the University of Vigo using EI M$^+$ or ESI [M + H]$^+$. Reactions were followed by thin-layer chromatography (TLC). Dry flash column chromatography on silica gel 0.035–0.070 mm, 60 A and Celite® 545 was used and this can be followed by column chromatography using solvent gradient to purify the compounds. Petroleum ether refers to the boiling range 40–60 °C. Ether refers to diethyl ether.

3.1.1. Synthesis of Methyl 7-Bromothieno[2,3-b]pyrazine-6-carboxylate (1b)

$t$-Butyl nitrite (2.86 mmol, 341.3 µL) was added to a solution of CuBr$_2$ (2.15 mmol, 0.480 g) in acetonitrile (5 mL) at 0 °C, followed by addition of amine 1a (1.43 mmol, 0.300 g) in portions (three times, 5 min between each addition). The mixture was stirred for 2 h at room temperature. Then a saturated solution of NH$_4$Cl (20 mL) was added, and the aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 20 mL). The combined organic phases were washed with NH$_4$Cl$_{sat}$ (60 mL), water (60 mL), and brine (60 mL), dried (MgSO$_4$) and filtered, and the solvent was removed under reduced pressure. The resulting residue was purified by column chromatography (30% ether/70% petroleum ether) to give a solid that was crystallized from ether to give compound 1b as a white solid (0.196 g, 50%), m.p. 161–163 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 3.95 (s, 3H, OMe), 8.87 (d, $J$ = 2.0 Hz, 1H, HetArH), 8.97 (d, $J$ = 2.0 Hz, 1H, HetArH) ppm. $^{13}$C NMR (100.6 MHz, CDCl$_3$): $\delta$ = 53.3 (OMe), 115.0 (C), 131.0 (C), 145.0 (CH), 145.2 (CH), 146.0 (C), 153.5 (C), 160.5 (C=O) ppm. HRMS (EI): [M]$^+$ calculated for C$_8$H$_5$BrN$_2$O$_2$S: 271.9255, found: 271.9254; for C$_8$H$_5$BrN$_2$O$_2$S: 273.9235, found: 273.9243.

3.1.2. General Procedure for the Synthesis of Diarylamines Using Reaction Conditions A

To a dried Schlenk tube with dry 1,4-dioxane (2–3 mL), compound 1a (1 equiv.), (hetero)arylhalide (1.1 equiv.), Pd(OAc)$_2$ (10 mol.%), Xantphos (12 mol.%), and Cs$_2$CO$_3$ (2.8 equiv.) were added under argon. The reaction was stirred at 120 °C for 2–5 h. After cooling, the reaction mixture was passed through a pad of silica-gel covered with celite, using ether or AcOEt (50 mL). The removal of the solvent gave a solid that was washed with ether to isolate the product or the crude. The latter was submitted to column chromatography using a solvent gradient, increasing 10% each time, from 10/90 of ether/petroleum ether until the isolation of the product, unless stated.

3.1.3. General Procedure for the Synthesis of Diarylamines Using Reaction Conditions B

To a dried Schlenk tube with dry toluene (2–3 mL), compound 1b (1 equiv.), (hetero)arylamine (1.1 equiv.), Pd(OAc)$_2$ (6 mol.%), rac-BINAP (8 mol.%), and Cs$_2$CO$_3$ (2 equiv.) were added, under argon. The reaction was stirred at 100 °C for 2–6 h, and after cooling, the reaction mixture was passed through a pad of silica-gel covered with celite, using ether or AcOEt (50 mL). The removal of the solvent gave a solid that was washed with ether to isolate the product or the crude. The latter was submitted to column chromatography using a gradient of solvents, increasing 10% every time, from 10/90 of ether/petroleum ether until the isolation of the product, unless stated.

Methyl 7-(Phenylamino)thieno[2,3-b]pyrazine-6-carboxylate (2a)

From compound 1a (0.380 mmol, 80.0 mg), bromobenzene (0.420 mmol, 44.3 µL), following reaction conditions A and heating for 5 h, compound 2a was obtained as a yellow solid after dry flash column chromatography using 40% ether/petroleum ether (55.0 mg, 50%), m.p. 154–156 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 3.96 (s, 3H, OMe), 7.10–7.13 (m, 3H, ArH), 7.27–7.31 (m, 2H, ArH), 8.51 (d, $J$ = 2.0 Hz, 1H, HetArH), 8.58 (d, $J$ = 2.0 Hz, 1H, HetArH), 8.87 (broad s, 1H, NH) ppm. $^{13}$C NMR (100.6 MHz, CDCl$_3$): $\delta$ = 52.3 (OMe), 107.0 (C), 121.5 (2 × CH), 124.0 (CH), 129.0 (2 × CH), 140.6 (CH), 141.0 (C), 142.0 (C), 143.1 (C), 143.5 (CH), 155.5 (C), 165.2 (C=O). HRMS (ESI): [M + H]$^+$ calculated for C$_{14}$H$_{12}$N$_2$O$_2$S: 286.0645, found: 286.0649.
Methyl 7-[(2-Methoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2b)

From compound 1b (0.370 mmol, 100.0 mg), o-anisidine (0.400 mmol, 45.4 µL), following reaction conditions B and heating for 4 h, compound 2b was obtained as a pale yellow solid (75.0 mg, 65%), m.p. 186–188 °C. $^1$H NMR (400 MHz, DMSO- $d_6$): δ = 3.78 (s, 3H, 2′- OMe), 3.87 (s, 3H, OMe), 6.76–6.82 (m, 1H, ArH), 7.01–7.03 (m, 2H, ArH), 7.06 (broad d, $J = 7.6$ Hz, 1H, ArH), 8.64 (d, $J = 2.4$ Hz, 1H, HetArH), 8.75 (d, $J = 2.4$ Hz, 1H, HetArH), 8.77 (broad s, 1H, NH) ppm. $^{13}$C NMR (100.6 MHz, DMSO-$d_6$): δ = 52.4 (OMe), 56.0 (2′- OMe), 105.5 (C), 111.0 (CH), 120.0 (CH), 121.0 (CH), 123.5 (CH), 130.0 (C), 141.4 (CH), 142.0 (C), 142.2 (C), 144.5 (CH), 150.2 (2′-C), 154.0 (C), 164.2 (C=O) ppm. HRMS (ESI): [M + H]$^+$ calculated for C$_{13}$H$_{14}$N$_3$O$_3$: 316.0750, found: 316.0751.

Methyl 7-[(3-Methoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2c)

From compound 1b (0.370 mmol, 100 mg), m-anisidine (0.400 mmol, 45.4 µL), following reaction conditions B and heating for 2 h, compound 2c was obtained as a pale yellow solid (92.0 mg, 80%), m.p. 171–173 °C. $^1$H NMR (400 MHz, DMSO- $d_6$): δ = 3.65 (s, 3H, 3′- OMe), 3.77 (s, 3H, OMe), 6.48 (apparent dd, $J = 8.0$ and 2.0 Hz, 1H, ArH), 6.56 (broad d, $J = 8.0$ Hz, 1H, ArH), 6.60 (apparent t, $J = 2.0$ Hz, 1H, 2′-H), 7.05 (apparent t, $J = 8.0$ Hz, 1H, 5′-H), 8.66 (d, $J = 2.4$ Hz, 1H, HetArH), 8.72 (d, $J = 2.4$ Hz, 1H, HetArH), 8.93 (broad s, 1H, NH) ppm. $^{13}$C NMR (100.6 MHz, DMSO-$d_6$): δ = 52.4 (OMe), 55.0 (3′- OMe), 105.4 (2′-CH), 108.0 (CH), 109.1 (C), 112.0 (CH), 129.0 (5′-CH), 141.0 (C), 142.0 (CH), 142.3 (C), 143.2 (C), 144.5 (CH), 154.0 (C), 159.5 (3′-C), 163.4 (C=O) ppm. HRMS (ESI): [M + H]$^+$ calculated for C$_{15}$H$_{14}$N$_3$O$_3$: 316.0750, found: 316.0754.

Methyl 7-[(4-Methoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2d)

From compound 1b (0.370 mmol, 100.0 mg), p-anisidine (0.400 mmol, 49.6 mg), following reaction conditions B and heating for 4 h, compound 2d was obtained as a pale yellow solid (87.0 mg, 75%), m.p. 147–149 °C. $^1$H NMR (400 MHz, DMSO- $d_6$): δ = 3.72 (s, 3H, 4′- OMe), 3.83 (s, 3H, OMe), 6.81 (d, $J = 8.8$ Hz, 2H, 3′ and 5′-H), 7.05 (d, $J = 8.8$ Hz, 2H, 2′ and 6′-H), 8.60 (d, $J = 2.4$ Hz, 1H, HetArH), 8.71 (d, $J = 2.4$ Hz, 1H, HetArH), 8.88 (broad s, 1H, NH) ppm. $^{13}$C NMR (100.6 MHz, DMSO-$d_6$): δ = 52.2 (OMe), 55.2 (4′- OMe), 105.0 (C), 114.0 (3′ and 5′-CH), 123.0 (2′ and 6′-CH), 134.4 (C), 141.3 (CH), 142.0 (C), 143.0 (C), 144.3 (CH), 154.3 (C), 155.5 (4′-C), 164.0 (C=O) ppm. HRMS (ESI): [M + H]$^+$ calculated for C$_{15}$H$_{14}$N$_3$O$_3$: 316.0750, found: 316.0753.

Methyl 7-[(2,4-Dimethoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2e)

From compound 1b (0.220 mmol, 60.0 mg), 2,4-dimethoxyaniline (0.240 mmol, 34.4 µL), following reaction conditions B and heating for 2 h, compound 2e was obtained as a red solid (76.0 mg, quantitative yield), m.p. 159–161 °C. $^1$H NMR (400 MHz, CDCl$_3$): δ = 3.78 (s, 3H, 2′- OMe), 3.83 (s, 3H, 4′- OMe), 3.95 (s, 3H, OMe), 6.41 (dd, $J = 8.4$ and 2.4 Hz, 1H, 5′-H), 6.52 (d, $J = 2.4$ Hz, 1H, 3′-H), 7.05 (d, $J = 8.4$ Hz, 1H, 6′-H), 8.46 (d, $J = 2.4$ Hz, 1H, HetArH), 8.53 (d, $J = 2.4$ Hz, 1H, HetArH), 8.79 (broad s, 1H, NH) ppm. $^{13}$C NMR (100.6 MHz, CDCl$_3$): δ = 52.1 (OMe), 55.5 (4′- OMe), 55.6 (2′- OMe), 99.0 (3′-CH), 103.3 (5′-CH), 103.5 (C), 123.0 (C), 124.0 (6′-CH), 140.4 (CH), 142.2 (C), 143.3 (CH), 144.2 (C), 153.0 (2′-C), 155.5 (C), 157.4 (4′-C), 165.4 (C=O) ppm. HRMS (ESI): [M + H]$^+$ calculated for C$_{16}$H$_{16}$N$_3$O$_5$: 346.0856, found: 346.0856.

Methyl 7-[(3,4-Dimethoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2f)

From compound 1b (0.220 mmol, 60.0 mg), 3,4-dimethoxyaniline (0.240 mmol, 37.0 mg) following the reaction conditions B and heating for 2h, compound 2f was obtained as a red solid (65.0 mg, 86%), m.p. 167–169 °C. $^1$H NMR (400 MHz, CDCl$_3$): δ = 3.79 (s, 3H, 3′- OMe), 3.89 (s, 3H, 4′- OMe), 3.96 (s, 3H, OMe), 6.68 (dd, $J = 8.8$ and 2.4 Hz, 1H, 6′-H), 6.74 (d, $J = 2.4$ Hz, 1H, 2′-H), 6.79 (d, $J = 8.8$ Hz, 1H, 5′-H), 8.49 (d, $J = 2.0$ Hz, 1H, HetArH), 8.56 (d, $J = 2.0$ Hz, 1H, HetArH), 8.92 (broad s, 1H, NH) ppm. $^{13}$C NMR (100.6 MHz, CDCl$_3$): δ = 52.2 (OMe), 55.9 (3′- OMe), 56.1 (4′- OMe), 105.0 (C), 107.4 (2′-CH), 111.1 (5′-CH),
Methyl 7-[(3,5-Dimethoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2g)

From compound 1b (0.200 mmol, 55.0 mg), 3,5-dimethoxyaniline (0.220 mmol, 33.9 mg), following reaction conditions B and heating for 2 h, compound 2g was obtained as a yellow solid (56.0 mg, 80%), m.p. 196–198 °C. 1H NMR (400 MHz, CDCl3): δ = 3.74 (s, 6H, 3' and 5'-OMe). 3.96 (s, 3H, OMe), 6.22-6.26 (m, 3H, 4', 2' and 6'-H), 8.56-8.60 (m, 2H, HetArH), 8.78 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 52.4 (OMe), 55.3 (3' and 5'-OMe), 96.0 (4'-CH), 100.0 (2' and 6'-CH), 108.4 (C), 141.0 (CH), 142.1 (C), 142.7 (C), 144.0 (CH), 155.4 (C), 161.0 (3' and 5'-C), 165.1 (C=O). HRMS (ESI): [M + H]+ calculated for C16H16N2O3S: 346.0856, found: 346.0856.

Methyl 7-[(3,4,5-Trimethoxyphenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2h)

From compound 1b (0.220 mmol, 60.0 mg), 3,4,5-trimethoxyaniline (0.240 mmol, 44.3 mg), following reaction conditions B and heating for 2 h, compound 2h was obtained as a dark orange solid (85.0 mg, quantitative yield), m.p. 194–196 °C. 1H NMR (400 MHz, DMSO-d6): δ = 3.62 (s, 3H, 4'-OMe), 3.63 (s, 6H, 3' and 5'-OMe), 3.85 (s, 3H, OMe), 6.43 (s, 2H, 2' and 6'-H), 8.69 (d, J = 2.0 Hz, 1H, HetArH), 8.75 (d, J = 2.0 Hz, 1H, HetArH), 8.93 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 52.4 (OMe), 55.7 (3' and 5'-OMe), 60.1 (4'-OMe), 98.4 (2' and 6'-CH), 107.0 (C), 133.2 (4'-C), 137.4 (C), 141.4 (CH), 141.5 (C), 142.0 (C), 144.4 (CH), 153.0 (3' and 5'-C), 154.2 (C), 164.0 (C=O) ppm. HRMS (ESI): [M + H]+ calculated for C16H16N2O3S: 376.0962, found: 376.0964.

Methyl 7-[(2-Fluoroanilino]thieno[2,3-b]pyrazine-6-carboxylate (2i)

From compound 1b (0.240 mmol, 65.0 mg), 2-fluoroaniline (0.260 mmol, 25.3 µL), following reaction conditions B and heating for 2 h, compound 2i was obtained as a pale yellow solid after dry flash column chromatography till 10% ether/petroleum ether (59.0 mg, 82%), m.p. 137–139 °C. 1H NMR (400 MHz, CDCl3): δ = 3.97 (s, 3H, OMe), 7.03–7.13 (m, 3H), 7.19 (broad apparent t, J = 8.0 Hz, 1H, 5'-H), 8.49 (d, J = 2.0 Hz, 1H, HetArH), 8.58 (d, J = 2.0 Hz, 1H, HetArH), 8.81 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 52.3 (OMe), 106.5 (C), 115.5 (d, J = 20.0 Hz, 3'-CH), 123.6 (d, J = 4.0 Hz, CH), 123.8 (d, J = 1.0 Hz, 5'-CH), 124.7 (d, J = 7.0 Hz, CH), 128.9 (d, J = 13.0 Hz, 1'-C), 141.0 (CH), 142.0 (C), 143.0 (C), 144.0 (CH), 155.3 (C), 155.5 (d, J = 245.0 Hz, C-F), 165.2 (C=O) ppm. 19F NMR (376.48 MHz, CDCl3): δ = −125.5 (s). HRMS (ESI): [M + H]+ calculated for C18H18F3N3O2S: 304.0551, found: 304.0555.

Methyl 7-[(3-Fluoroanilino]thieno[2,3-b]pyrazine-6-carboxylate (2j)

From compound 1b (0.220 mmol, 60.0 mg), 3-fluoroaniline (0.240 mmol, 23.2 µL), following reaction conditions B and heating for 2 h, compound 2j was obtained as a yellow solid (59.0 mg, 89%), m.p. 170–172 °C. 1H NMR (400 MHz, CDCl3): δ = 3.97 (s, 3H, OMe), 6.76–6.82 (m, 2H, ArH), 6.85–6.89 (m, 1H, 6'-H), 7.20–7.25 (m, 1H, 5'-H), 8.56 (d, J = 2.0 Hz, 1H, HetArH), 8.61 (d, J = 2.0 Hz, 1H, HetArH), 8.84 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 52.4 (OMe), 108.2 (d, J = 25.0 Hz, CH), 109.0 (C), 110.1 (d, J = 22.0 Hz, CH), 116.5 (d, J = 2.0 Hz, 6'-CH), 129.7 (d, J = 9.0 Hz, 5'-CH), 141.0 (CH), 142.0 (C), 142.2 (C), 142.8 (d, J = 10.0 Hz, 1'-C), 144.0 (CH), 155.4 (C), 163.0 (d, J = 245.0 Hz, C-F), 165.1 (C=O) ppm. 19F NMR (376.48 MHz, CDCl3): δ = −112.7 (s). HRMS (ESI): [M + H]+ calculated for C18H18F3N3O2S: 304.0551, found: 304.0555.

Methyl 7-[(4-Fluoroanilino]thieno[2,3-b]pyrazine-6-carboxylate (2k)

From compound 1b (0.400 mmol, 110.0 mg), 4-fluoroaniline (0.440 mmol, 42.0 µL), following reaction conditions B and heating for 2 h, compound 2k was obtained as an orange solid (86.0 mg, 70%), m.p. 171–173 °C. 1H NMR (400 MHz, DMSO-d6): δ = 3.83 (s, 3H,
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OMe), 7.02–7.07 (m, 2H, 3' and 5'-H), 7.08–7.12 (m, 2H, 2' and 6'-H), 8.64 (d, J = 2.4 Hz, 1H, HetArH), 8.74 (d, J = 2.4 Hz, 1H, HetArH), 8.97 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, DMSO-d6): δ = 52.4 (OMe), 107.3 (C), 114.8 (d, J = 220.0 Hz, 3' and 5'-CH), 122.2 (d, J = 9.0 Hz, 2' and 6'-CH), 138.1 (C), 138.12 (C), 141.5 (CH), 142.0 (C), 144.4 (CH), 154.2 (C), 158.1 (d, J = 238.4 Hz, C-F), 163.5 (C=O) ppm. HRMS (ESI): [M + H]+ calculated for C14H11FN3O2S: 304.0551, found: 304.0556.

Methyl 7-[(Pyridin-3-yl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2I)

From compound 1a (0.380 mmol, 80.0 mg), 3-bromopyridine (0.420 mmol, 40.5 µL), following reaction condition A and heating for 2 h, compound 2I was obtained as an orange solid after dry flash column chromatography till 80% ethyl acetate/petroleum ether (60.0 mg, 55%), m.p. 150–152 °C. 1H NMR (400 MHz, CDCl3): δ = 3.98 (s, 3H, OMe), 7.23 (dd, J = 8.4 and 4.8 Hz, 1H, 5'-H), 7.42–7.45 (m, 1H, 4'-H), 8.35 (dd, J = 4.8 and 1.2 Hz, 1H, 6'-H), 8.46–8.47 (m, 1H, 2'-H), 8.50 (d, J = 2.0 Hz, 1H, HetArH), 8.60 (d, J = 2.0 Hz, 1H, HetArH), 8.92 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 52.4 (OMe), 108.0 (C), 123.0 (5'-CH), 128.4 4'-CH), 137.3 (C), 141.0 (CH), 141.5 (C), 142.2 (C), 143.6 (2'-CH), 144.0 (CH), 145.0 (6'-CH), 155.5 (C), 165.3 (C=O) ppm. HRMS (ESI): [M + H]+ calculated for C13H11N4O2S: 287.0597, found: 287.0592.

Methyl 7-[(1H-pyrrol-1-yl)thieno[2,3-b]pyrazine-6-carboxylate (2m)

From compound 1b (0.380 mmol, 80.0 mg), 1H-pyrrol (0.420 mmol, 40.5 µL), following reaction conditions B and heating for 6 h, compound 2m was obtained as an pale yellow solid after dry flash column chromatography till 30% ether/petroleum ether (54.0 mg, 71%), m.p. 166–168 °C. 1H NMR (400 MHz, CDCl3): δ = 3.93 (s, 3H, OMe), 6.45 (apparent t, J = 2.0 Hz, 2H, 2 × HetArH), 7.13 (apparent t, J = 2.0 Hz, 2H, 2 × HetArH), 8.79 (d, J = 2.0 Hz, 1H, HetArH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 53.0 (OMe), 110.0 (2 × CH), 123.0 (2 × CH), 126.0 (C), 136.0 (C), 143.3 (CH), 144.0 (CH), 144.3 (C), 154.3 (C), 161.1 (C=O) ppm. HRMS (ESI): [M + H]+ calculated for C12H10N3O2S: 260.0488, found: 260.0486.

Methyl 7-[(4-Cyanophenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2n)

From compound 1a (0.380 mmol, 80.0 mg), 4-bromobenzonitrile (0.400 mmol, 76.5 mg), following reaction conditions A and heating 3.5 h, compound 2n was obtained as a yellow solid (119 mg, quantitative yield), m.p. 214–216 °C. 1H NMR (400 MHz, CDCl3): δ = 3.99 (s, 3H, OMe), 7.11 (d, J = 8.8 Hz, 2H, 2' and 6'-H), 7.54 (d, J = 8.8 Hz, 2H, 3' and 5'-H), 8.60 (d, J = 2.0 Hz, 1H, HetArH), 8.66 (d, J = 2.0 Hz, 1H, HetArH), 8.90 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 53.0 (OMe), 105.3 (C), 112.0 (C), 119.3 (C), 120.0 (2' and 6'-CH), 133.0 (3' and 5'-CH), 140.5 (C), 141.1 (CH), 142.0 (C), 144.0 (CH), 145.2 (C), 155.3 (C), 165.0 (C=O) ppm. HRMS (ESI): [M + H]+ calculated for C15H11N4O2S: 311.0597, found: 311.0598.

Methyl 7-[(2-Nitrophenyl)amino]thieno[2,3-b]pyrazine-6-carboxylate (2o)

From compound 1a (0.450 mmol, 95.0 mg), 2-nitrobenzene (0.500 mmol, 101.0 mg), following reaction conditions A and heating 3 h, compound 2o was obtained as a yellow solid after dry flash column chromatography till 100% ethyl acetate (90.0 mg, 60%), m.p. 225–227 °C. 1H NMR (400 MHz, CDCl3): δ = 4.01 (s, 3H, OMe), 6.96–7.04 (m, 2H, ArH), 7.35–7.40 (m, 1H, ArH), 8.20 (dd, J = 8.0 and 1.6 Hz, 1H, 3'-H), 8.61 (d, J = 2.0 Hz, 1H, HetArH), 8.67 (d, J = 2.0 Hz, 1H, HetArH), 10.65 (broad s, 1H, NH) ppm. 13C NMR (100.6 MHz, CDCl3): δ = 53.0 (OMe), 117.5 (C), 120.4 (CH), 121.0 (CH), 126.0 (3'-CH), 134.0 (CH), 136.4 (C), 137.5 (C), 138.4 (CH), 142.5 (C), 144.0 (CH), 155.0 (C), 163.4 (C=O). HRMS (ESI): [M + H]+ calculated for C14H11N4O2S: 331.0496, found: 331.0496.
3.2. In Vitro Antitumor Evaluation

3.2.1. Cell Growth Inhibition Assay (SRB Assay)

The cell growth inhibition of compounds 2a–2o DMSO/water solutions (3.9–250 µM) was evaluated against four human tumor cell lines (acquired from Leibniz-Institut DSMZ), namely: gastric adenocarcinoma (AGS), colorectal adenocarcinoma (CaCo-2), breast carcinoma (MCF7), and non-small-cell lung carcinoma (NCI-H460), as well as a non-tumor culture from African green monkey kidney (Vero). Each cell line was prepared in 96-well plates, at the required density (1.0 × 10^4 cells/well) and incubated for 24 h to achieve cell attachment. The solutions of the compounds were applied and incubated for another 48 h. GI_{50} values (µM) corresponding to the compound concentration that inhibited 50% of cell growth were determined using the sulforhodamine B assay [24,25]. Two separate tests were carried out for each compound, in duplicate, with the effects expressed as mean values and standard deviation (SD). Ellipticine was used as a positive control.

3.2.2. Flow Cytometric: Cell Cycle Analysis

AGS cells were seeded in six-well plates (4 × 10^5 cells/well) and incubated with the compounds 2b, 2f, and 2g, at their GI_{50} concentration for each sample, for 72 h. Cell cycle analysis was performed using propidium iodide (PI) staining and flow cytometry. This assay is established on the measurement of the DNA content of nuclei labeled with PI. Following this, the cells were staining according to the protocol PI/RNASE Solution (Immunostep, Spain, Salamanca). The harvested cells corresponding to 2 × 10^5 to 1 × 10^6 cells, were centrifuged for 5 min at 300g, and the supernatant was removed. The cells were fixed and added to 200 µL of 70% ethanol and left in the ethanol at 4 °C for 30 min. Following this, the cells were washed once in 2 mL phosphate-buffered saline (PBS) and centrifuged for 5 min at 300g, and resuspended in 500 µL of PI solution (PI/RNASE) and incubated in the dark, at room temperature, for 15 min. Cell cycle phase distribution was evaluated using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA). The DNA content of at least 20,000 cells was counted per sample, and the percentage of cells in different phases (G0/G1, S, and G2/M phases) of the cell cycle was evaluated using BD Accuri C6 software (BD Biosciences, San Jose, CA, USA) [26]. Ellipticine was used as a positive control at its GI_{50} concentration for AGS cell line.

3.2.3. Flow Cytometric: Apoptosis Detection

AGS cells were seeded in six-well plates (4 × 10^5 cells/well) and incubated with compounds 2b, 2f, and 2g at their GI_{50} concentration for each sample, for 72 h. Apoptosis detection was performed using the Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Kit (BD Biosciences, San Jose, CA, USA) and flow cytometry. FITC Annexin V staining is used to determine the percentage of cells within a population that are actively undergoing apoptosis. The cells were washed twice with PBS and resuspended cells in 1 × Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2) at a concentration of 1 × 10^6 cells/mL. Following this, 100 µL of the solution (1 × 10^6 cells) was transferred to a 5 mL culture tube and 5 µL of FITC Annexin V (BD Biosciences, San Jose, CA, USA) and 5 µL of PI (BD Biosciences, San Jose, CA, USA) were added to each tube, and they were incubated in the dark, at room temperature, for 15 min. Finally, 400 µL of 1 × Binding Buffer was added to each tube. Next, 30,000 cells were acquired using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA), and the percentage of cell distribution was evaluated with Accuri C6 software (BD Biosciences, San Jose, CA, USA) [27]. Ellipticine was used as a positive control at its GI_{50} concentration for AGS cell line.

3.2.4. Statistical Analysis

Statistical analysis of data was performed using SPSS Statistics (version 23 for Windows; IBM Corp., Armonk, NY, USA). Statistical differences between groups were assessed by the chi-square test and differences with a p < 0.05 were considered significant.
4. Conclusions

A series of novel di(hetero)arylamines were synthesized by C–N Buchwald–Hartwig cross-coupling of either aminated or brominated thieno[2,3-b]pyrazines with (hetero)arylhalides or (hetero)arylamines, respectively, in good-to-excellent yields, using different reaction conditions, taking into account the electronic character of the substrates. The antitumor potential of the compounds obtained was evaluated against four human tumor cell lines (AGS, CaCo-2, MCF7, and NCI-H460). Despite the variety of the compounds, the results showed that the most promising ones were the mono- or dimethoxylated, 2b (2-OMeC₆H₄), 2f (3,5-diOMeC₆H₃), and 2g (3,4-diOMeC₆H₃) which presented the lowest GI₅₀ values and selectively inhibited the cell growth of the AGS and/or CaCo-2 cell lines among the human tumor cell lines tested. Moreover, compound 2h (3,4,5-triOMeC₆H₂) and 2n (4-CNC₆H₄) showed to be selective against CaCo-2 cell line presenting GI₅₀ = 9.2 and 10.9 µM, respectively. The toxicity of the compounds was evaluated in a non-tumor cell line (Vero), and they did not show relevant toxicity at their GI₅₀ concentrations (presenting high GI₅₀ values). Effects on the cell cycle profile and induction of apoptosis were evaluated for compounds 2b, 2f, and 2g in the AGS cell line. Nevertheless, even for the most active compound against this cell line (GI₅₀ = 7.8 µM), it was observed that a huge number of dead cells gave rise to an atypical distribution on the cell cycle profile and that these cells were not apoptotic, which indicates that a different mechanism of action for the AGS cell growth inhibition is involved.

Supplementary Materials: The following are available online, ¹H, ¹³C, and ¹⁹F NMR spectra of the novel compounds 1b and 2a–2o are presented.

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