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Short communication

Synthesis and *in vitro* antimycobacterial activity of 2-methoxybenzanilides and their thioxo analogues

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ABSTRACT

A new series of *N*-(3/4-substituted phenyl) 4/5-chloro-2-methoxybenzamides and their thioxo analogues have been synthesised and evaluated for *in vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv, as well as the two atypical strains *Mycobacterium kansasii* and *Mycobacterium avium*. Five of the most active compounds were evaluated for cytotoxicity and their ability to inhibit mycobacterial isocitrate lyase, which is responsible for latent survival of *Mycobacterium*. The results showed that benzthioanilides were more active than the corresponding benzanilides. The most active compound, 4-chloro-2-methoxy-*N*-(3,4-dichlorophenyl)benzothioamide (**4e**), had a minimal inhibition concentration (MIC) against *M. tuberculosis* of 2 μmol L^{-1}, which was better than the activity of the previously published corresponding salicylanilide.

1. Introduction

In the last few years, tuberculosis (TB), caused by *Mycobacterium tuberculosis*, has again become one of the most dangerous and lethal infectious diseases. According to the new WHO report in 2010, there were an estimated 8.8 million new cases of TB, 1.45 million TB deaths and more than two billion people infected with *M. tuberculosis*, of which approximately 10% will most likely become ill during their life [1]. This situation is due to HIV infection (1.1 million of all TB causes and 0.35 million of TB deaths are in people who are HIV positive) [1], poor compliance of patients, population migration and increased drug resistance [2]. A particularly dangerous form of drug-resistant TB is multidrug-resistant tuberculosis (MDR–TB), caused by a strain of *M. tuberculosis* resistant to at least rifampicin and INH (isoniazid), the first line and most powerful antituberculotics, and extensively drug-resistant tuberculosis (XDR–TB), caused by the strains of *M. tuberculosis* also resistant to any fluoroquinolone and to at least one of the three injectable drugs kanamycin, capreomycin and amikacin [3]. For example, in 2010, there was an estimated 650,000 cases of MDR–TB, and 150,000 died from infection with this form in 2008 [1]. Although the absolute number of TB cases per year is slightly decreasing globally, the number of MDR–TB cases is increasing [1].

Recently, several pathways have been characterised as new possible drug targets, including cell wall metabolism, cellular respiration and protein processing enzymes [4]. One promising enzyme target in persistent and latent mycobacterial infection is isocitrate lyase (ICL), which is a key enzyme in the glyoxylate cycle essential for growth in macrophages. During steady state growth, ICL converts isocitrate to succinate and glyoxylate, followed by condensation of glyoxylate with acetyl-CoA to form malate by malate synthase [5]. The carbon conserving glyoxylate pathway has not been observed in the human body, therefore it has been selected as a potential drug target for new antituberculotic agents [6].

The need for new antituberculotics, especially ones with a new mode of action, demands intensive search for new compounds. Salicylanilides could fit this description, as they act on the bacterial two component system (TCS), which is not found in animal cells and is not a target for any current antituberculotic drugs [7–9]. They are currently well-known antibacterial [7] and antimycobacterial agents [10,11], but are too toxic for animal cells because they decouple oxidative phosphorylation in mitochondria. The phenolic OH group is responsible for this action, which seems to also be essential for antibacterial activity [7].

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On the other hand there is little information on the antimycobacterial properties of benzanilides, the possible metabolites of benzoxazoles [12]. According to Weidner-Wells et al. [13], who studied TCS inhibition by benzimidazoles and their isosteres benzoxazoles, neither the benzimidazole hydrogen bond donating ability nor the basicity of benzimidazole was necessary for their activity. In addition, they showed that benzoxazoles are also active even though the oxygen can only accept a hydrogen bond. Thus, it is possible that a hydrogen bond acceptor at the right position is sufficient for activity.

We prepared a series of 2-methoxybenzanilides and their thioxo analogues as the opened reverse form of 2-phenylbenzo[*d*]oxazoles, expecting that these compounds would show good activity even if they lacked a 2-OH group (Scheme 1).

2. Chemistry

Scheme 2 shows the preparation of substituted 2-methoxybenzanilides and their thioxo analogues. The substituents on both aromatic parts of the molecule were selected according to a previous study on salicylanilides [10]. For both synthetic steps, well known preparations were used: the reaction of substituted 2-methoxybenzoic acid with an appropriate aniline in the presence of PCl_3 and chlorobenzene as solvent [10], and thionation with P_4S_{10} in pyridine as solvent [14].

3. Pharmacology

3.1. In vitro antimycobacterial assay

The newly prepared compounds were tested in the Laboratory for Mycobacterial Diagnostics and TB at the Institute of Public Health in Ostrava for their *in vitro* antimycobacterial activity against *M. tuberculosis* 331/88, *Mycobacterium avium* 330/88, *Mycobacterium kansasii* 235/80 and clinically isolated *M. kansasii* 6509/96. The first line antituberculotic drug INH was used as a standard. The compound activity against *M. tuberculosis* and *M. avium* was evaluated after 14 and 21 days, and the compound activity against *M. kansasii* was evaluated after 7, 14 and 21 days. The minimal inhibition concentration (MIC) values represent the lowest concentration of the tested compounds at which the inhibition of mycobacterium growth was observed.

3.2. In vitro enzymatic assay of selected compounds

Five of the most active compounds (**4c**, **4d**, **4e**, **4g**, **4h**) against *M. tuberculosis* 331/88 after 14 days of incubation were tested for their ability to act as *in vitro* inhibitors of mycobacterial isocitrate lyase. This evaluation was performed at the Department of Biochemical Sciences, Faculty of Pharmacy in Hradec Králové, Charles University in Prague. The results are presented as % of inhibition and compared to the standard 3-nitropropionic acid.

3.3. In vitro cytotoxicity assay of selected compounds

Compounds **4c**, **4d**, **4e**, **4g** and **4h** were also tested for their *in vitro* cytotoxicity. This evaluation was performed at the Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University in Prague. For this purpose, the human liver cell line Hep G2 and a standard colorimetric test based on the reductive metabolic activity of the cells was used. The results are presented as the inhibitory concentration (IC_{50}), which is the concentration of the tested compound required for reduction of cell viability by 50% of the maximum (control) cell viability. To characterise the relationship between toxicity and antimycobacterial activity, the selectivity index (SI) was calculated. The SI is the ratio of IC_{50} to MIC after 14 days of incubation with *M. tuberculosis* 331/88.

4. Discussion and conclusion

Thirty-six novel compounds were synthesised and characterised by ^1H and ^{13}C NMR spectroscopy, IR spectroscopy, elemental analysis and melting point. The yields of thiobenzanilides were significantly lower (4.9–54.0%) than the yields of benzanilides (40.7–75.3%). This discrepancy was most likely due to the large excess of P_4S_{10} and long reaction time which were needed for complete conversion of amides to thioamides to simplify their isolation. It was possible that the P_4S_{10} formed some complex with the carbonyl/thiocarbonyl group of amides/thioamides in the similar way as Lewis acids with carbonyl groups of products of the Friedel–Crafts acylation.

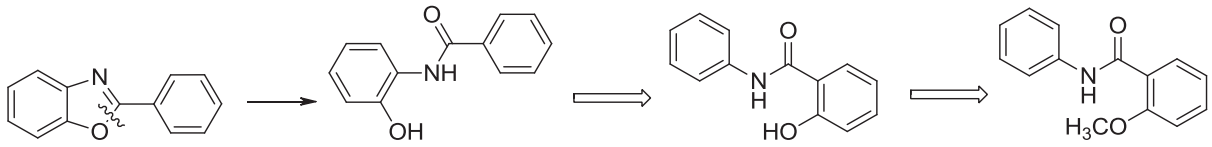
All compounds showed *in vitro* activity against all tested strains, with amides in the range of 4–500 μmol L^{-1} (Table 1) and thioamides in the range of 2–250 μmol L^{-1} (Table 2). In general, thioamides were more efficient than benzamides, but all prepared compounds were less active than their corresponding salicylanilides, except **3c**, **3g** and **4e**. For a comparison of the antimycobacterial activities of prepared compounds **3** and **4** and salicylanilides, see Tables 1–3 [10].

In the thiobenzanilides, slightly higher activity against atypical strains of *Mycobacterium* was found for compounds having Cl, Br or CF_3 groups on the aniline and a 5-Cl substitution on the acyl aromate (Table 2). Conversely, the activity of the thioamides against *M. tuberculosis* was better if the Cl was in position 4 in the acyl part of the molecule (Table 2). Most of the thioamides exhibited activities against *M. avium* and *M. kansasii* 235/80 which are resistant to INH (Table 2).

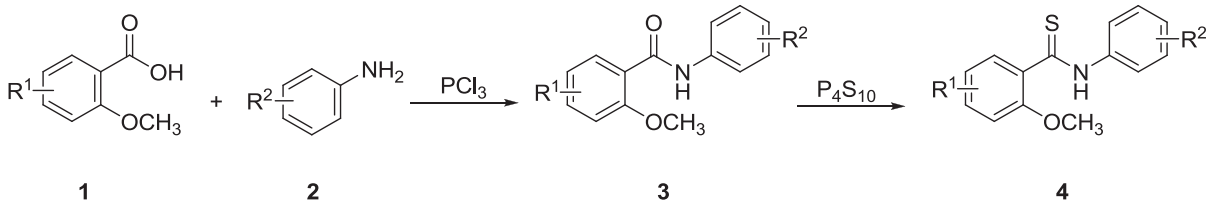
The results of *in vitro* enzymatic assays of the most active compounds showed that the tested derivatives were not effective isocitrate lyase inhibitors. The best inhibition activity was found for **4d** with 39 ± 5% inhibition. Inhibition by other compounds was within the range 17–27% (Table 4).

The best compounds in the cytotoxicity assay were **4c**, with an IC_{50} of 11.68 μmol L^{-1} and an SI for *M. tuberculosis* after 14 days of incubation of 2.92, and **4e**, with an IC_{50} of 6.50 μmol L^{-1} and an SI for My 331/88 after 14 days of 3.25 (Table 5).

Despite the prepared compounds showing antimycobacterial activity, use of the 2-methoxy group instead of the 2-hydroxy group decreased activity and *in vitro* cytotoxicity did not improve. The best compound in the cytotoxicity assay was **4e**, which was also more active against *M. tuberculosis* after 14 days than the corresponding salicylanilide. Compounds **3c** and **3g** were more active against *M. avium* than the corresponding salicylanilides.



Scheme 1. 2-Methoxybenzanilides as the opened reverse form of 2-phenylbenzo[*d*]oxazoles.



R^1= 4-Cl, 5-Cl; R^2= 3′-NO_2, 4′-NO_2, 3′-Cl, 4′-Cl, 3′,4′-di-Cl, 3′-Br, 4′-Br, 3′-CF_3, 4′-CF_3

Scheme 2. Synthesis of 2-methoxybenzanilides **3** and their thioxo analogues **4**.

5. Experimental protocols

All of the chemicals and solvents used in this study were purchased from Sigma–Aldrich, Prague, Czech Republic and Penta, Prague, Czech Republic, and were used without further purification.

5.1. Chemistry

5.1.1. General methods

The reactions were monitored and the purity of products was verified by thin layer chromatography using plates coated with 0.2 mm silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) and visualised using UV irradiation (254 and 366 nm). Column chromatography was performed using silica gel 60 with a particle size of 0.063–0.2 mm (Fluka, Prague, Czech Republic).

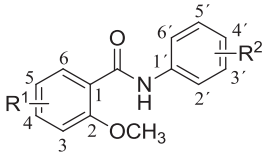
Melting points were determined on a Melting Point B-540 apparatus (Búchi Labortechnik AG, Flawil, Switzerland) in open capillaries and are uncorrected. IR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) over the range 400–4000 cm^{-1} using the ATR technique. The NMR spectra were measured in DMSO-d_6 or $CDCl_3$ solutions at ambient temperature on a Varian Mercury Vxbb 300 (300 MHz for ^{1}H and 75.5 MHz for ^{13}C, Varian Co., Palo Alto, CA, USA) and Varian Mercury (500 MHz for ^{1}H and 125 MHz for ^{13}C, Varian Co., Palo Alto, CA, USA) instruments. The chemical shifts (δ) are given in ppm and are indirectly related to tetramethylsilane as an internal standard. Elemental analysis (C, H, N, S) was performed on an automatic microanalyser CHNS–O CE instrument, FISONS EA 1110 (Thermo Fisher Scientific, Waltham, MA, USA). MS measurement was performed on an Agilent 500 Ion Trap LC/MS mass spectrometer using APCI ionization technique (Agilent Technologies, Santa Clara, CA, USA). For MS sample preparation was used methanol.

5.1.2. General procedure for the preparation of substituted 2-methoxybenzamides

Equimolar amounts of 4/5-chloro-2-methoxybenzoic acid (5.0 mmol), substituted aniline (5.0 mmol) and PCl_3 (5.0 mmol) were dissolved in 20.0 mL of chlorobenzene and stirred at 165 °C for 3 h. Then, the mixture was immediately filtered, evaporated and the residue was recrystallised from 96% EtOH/H_2O.

*5.1.2.1. 4-Chloro-2-methoxy-N-(3-nitrophenyl)benzamide (**3a**).* Yield: 64.5%, yellow solid; m. p. 185–186 °C; IR (ATR): 3331 (ν NH), 3071 (ν CH aromatic), 1670 (ν CO + δ NH, amide I), 1590 (ν CC aromatic),

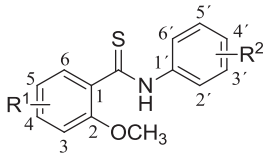
Table 1
Antimycobacterial activities of 2-methoxybenzanilide derivatives **3**.



	R^1	R^2	MIC [μmol L^{-1}]									
			M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80			*M. kansasii* My 6509/96		
			14d	21d	14d	21d	7d	14d	21d	7d	14d	21d
3a	4-Cl	3′-NO_2	125	125	125	125	125	250	250	125	125	125
3b	4-Cl	4′-NO_2	250	250	250	250	125	250	250	125	125	125
3c	4-Cl	3′-Cl	125	125	8	32	8	32	32	62.5	62.5	125
3d	4-Cl	4′-Cl	125	125	8	32	8	16	62.5	8	16	62.5
3e	4-Cl	3′,4′-diCl	125	125	125	125	125	125	125	125	125	125
3f	4-Cl	3′-Br	250	250	125	125	125	125	125	125	125	125
3g	4-Cl	4′-Br	62.5	125	8	32	4	8	32	4	8	32
3h	4-Cl	3′-CF_3	250	250	125	125	125	250	250	125	125	125
3i	4-Cl	4′-CF_3	125	125	62.5	125	125	125	125	32	125	125
3j	5-Cl	3′-NO_2	125	125	250	250	125	125	125	125	125	125
3k	5-Cl	4′-NO_2	125	125	125	125	125	125	125	125	125	125
3l	5-Cl	3′-Cl	250	250	250	250	62.5	250	250	125	250	250
3m	5-Cl	4′-Cl	62.5	62.5	62.5	62.5	62.5	250	250	62.5	125	125
3n	5-Cl	3′,4′-diCl	125	125	250	250	62.5	250	250	62.5	125	125
3o	5-Cl	3′-Br	125	125	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
3p	5-Cl	4′-Br	16	16	16	32	62.5	62.5	62.5	32	62.5	62.5
3q	5-Cl	3′-CF_3	4	16	125	125	4	4	16	4	4	16
3r	5-Cl	4′-CF_3	250	250	250	250	125	500	500	62.5	250	250
INH	–	–	0.5	0.5	>250	>250	>250	>250	>250	4	4	4

INH – isoniazid.

Table 2
Antimycobacterial activities of 2-methoxybenzthioanilide derivatives **4**.



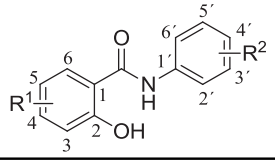
	R^1	R^2	MIC [μmol L^{-1}]									
			M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80			*M. kansasii* My 6509/96		
			14 d	21 d	14 d	21 d	7 d	14 d	21 d	7 d	14 d	21 d
4a	4-Cl	3′-NO_2	8	16	16	16	32	32	62.5	8	16	32
4b	4-Cl	4′-NO_2	8	32	16	16	16	32	32	16	16	32
4c	4-Cl	3′-Cl	4	16	16	16	16	32	32	16	32	32
4d	4-Cl	4′-Cl	4	16	32	32	32	62.5	62.5	16	32	62.5
4e	4-Cl	3′,4′-diCl	2	8	32	32	16	32	62.5	16	32	32
4f	4-Cl	3′-Br	32	32	125	125	125	125	125	125	125	125
4g	4-Cl	4′-Br	4	16	16	62.5	32	62.5	62.5	16	32	62.5
4h	4-Cl	3′-CF_3	4	8	32	32	16	32	32	16	32	32
4i	4-Cl	4′-CF_3	8	16	32	32	16	32	62.5	8	32	32
4j	5-Cl	3′-NO_2	250	250	250	250	62.5	250	250	62.5	250	250
4k	5-Cl	4′-NO_2	32	32	250	250	125	250	250	125	250	250
4l	5-Cl	3′-Cl	16	16	32	32	8	16	16	16	16	32
4m	5-Cl	4′-Cl	16	16	32	32	8	32	62.5	16	16	32
4n	5-Cl	3′,4′-diCl	8	8	32	62.5	32	62.5	62.5	62.5	125	125
4o	5-Cl	3′-Br	32	32	32	32	16	32	32	32	32	32
4p	5-Cl	4′-Br	16	16	16	16	62.5	250	250	16	32	32
4q	5-Cl	3′-CF_3	16	16	32	32	8	16	16	8	16	16
4r	5-Cl	4′-CF_3	16	16	16	16	8	16	32	8	16	16
INH	–	–	0.5	0.5	>250	>250	>250	>250	>250	4	4	4

INH – isoniazid.

1544 (δ NH + ν CN, amide II), 1520 (ν_{as} NO_2), 1477 (ν CC aromatic), 1354 (ν_s NO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.57 (1H, s, NH), 8.76 (1H, m, H2′), 8.04 (1H, m, H4′), 7.95 (1H, m, H6′), 7.64 (1H, t, J = 8.1 Hz, H5′), 7.63 (1H, d, J = 8.2 Hz, H6), 7.29 (1H, d, J = 1.8 Hz, H3), 7.14 (1H, dd, J = 8.2 Hz, J = 1.8 Hz, H5), 3.91 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.60 (1C, s, CONH), 157.55, 148.16, 140.18, 136.80, 131.19, 130.33, 125.96, 123.77, 120.68, 118.39, 113.99, 112.71, 56.68 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 307 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_4$ (306.70): C 54.83, H 3.62, N 9.13, Found: C 54.74, H 3.93, N 9.11.

*5.1.2.2. 4-Chloro-2-methoxy-N-(4-nitrophenyl)benzamide (**3b**).* Yield: 60.3%, yellow solid; m. p. 223–226 °C; IR (ATR): 3327 (ν NH), 3115 (ν CH aromatic), 1677 (ν CO + δ NH, amide I), 1593 (ν CC aromatic), 1551 (δ NH + ν CN, amide II), 1506 (ν_{as} NO_2), 1479 (ν CC aromatic), 1328 (ν_s NO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.70 (1H, s, NH),

Table 3
Antimycobacterial activities of corresponding salicylanilide derivatives [10] for comparison to prepared compounds **3** and **4**.



R^1	R^2	MIC[μmol L^{-1}]					
		M. tuberculosis My 331/88		*M. avium* My 330/88		*M. kansasii* My 235/80	
		14 d	21 d	14 d	21 d	14 d	21 d
4-Cl	3′-NO_2	8	8	16	16	16	16
4-Cl	3′-Cl	4	4	16	16	4	8
4-Cl	4′-Cl	4	4	8	8	4	8
4-Cl	3′,4′-diCl	4	4	16	16	8	8
4-Cl	4′-Br	4	4	16	16	4	4
4-Cl	4′-CF_3	4	4	8	8	4	4
5-Cl	3′-NO_2	8	8	16	16	8	8
5-Cl	4′-NO_2	4	8	8	8	4	4
5-Cl	3′-Cl	4	8	8	16	4	8
5-Cl	4′-Cl	4	4	8	8	8	8
5-Cl	3′,4′-diCl	4	8	16	16	4	4
5-Cl	4′-Br	8	16	8	8	4	4
5-Cl	4′-CF_3	2	2	8	8	1	1
INH		4	4	500	500	500	500

INH–isoniazid.

Table 4
Inhibition of isocitrate lyase by selected thioamides. Concentration of all of the tested compounds and 3-nitropropionic acid was 100 μmol L^{-1}.

	R^1	R^2	% Inhibition	± Standard deviation
4c	4-Cl	3′-Cl	20	±5
4d	4-Cl	4′-Cl	39	±5
4e	4-Cl	3′,4′-diCl	17	±1
4g	4-Cl	4′-Br	27	±5
4h	4-Cl	3′-CF_3	23	±6
3-Nitropropionic acid			67	±2.68

8.29–8.21 (2H, m, H3′, H5′), 8.00–7.91 (2H, m, H2′, H6′), 7.62 (1H, d, J = 8.2 Hz, H6), 7.29 (1H, d, J = 1.8 Hz, H3), 7.14 (1H, dd, J = 8.2 Hz, J = 1.8 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.82 (1C, s, CONH), 157.55, 145.21, 142.66, 136.88, 131.16, 125.14 (2C, s, C3′, C5′), 123.83, 120.69, 119.56 (2C, s, C2′, C6′), 112.73, 56.69 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 307 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_4$ (306.70): C 54.83, H 3.62, N 9.13, Found: C 54.90, H 3.81, N 9.21.

*5.1.2.3. 4-Chloro-2-methoxy-N-(3-chlorophenyl)benzamide (**3c**).* Yield: 71.3%, white solid; m. p. 116–118 °C; IR (ATR): 3355 (ν NH), 1668 (ν CO + δ NH, amide I), 1587 (ν CC aromatic), 1534 (δ NH + ν CN, amide II), 1482, 1460 (ν CC aromatic) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.28 (1H, s, NH), 7.91 (1H, t, J = 2.1 Hz, H2′), 7.65–7.57 (2H, m, H6, H4′), 7.36 (1H, t, J = 8.1 Hz, H5′), 7.27 (1H, d, J = 1.9 Hz, H3), 7.15 (1H, m, H6′), 7.13 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.22 (1C, s, CONH), 157.50, 140.52, 136.61, 133.27, 131.18, 130.63, 123.96, 123.55, 120.66, 119.31, 118.32, 112.68, 56.67 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 296 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2$ (296.15): C 56.78, H 3.74, N 4.73, Found: C 56.90, H 3.61, N 4.85.

*5.1.2.4. 4-Chloro-2-methoxy-N-(4-chlorophenyl)benzamide (**3d**).* Yield: 61.7%, white solid; m. p. 127–128 °C; IR (ATR): 3378 (ν NH), 3073 (ν CH aromatic), 1667 (ν CO + δ NH, amide I), 1596 (ν CC aromatic), 1540 (δ NH + ν CN, amide II), 1492, 1463 (ν CC aromatic) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.23 (1H, s, NH), 7.79–7.70 (2H, m, H3′, H5′), 7.61 (1H, d, J = 8.2 Hz, H6), 7.42–7.34 (2H, m, H2′, H6′), 7.27 (1H, d, J = 1.9 Hz, H3), 7.12 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.00 (1C, s, CONH), 157.48, 138.05, 136.49, 131.16, 128.82 (2C, s, C3′, C5′), 127.39, 124.10, 121.44 (2C, s, C2′, C6′), 120.64, 112.65, 56.64 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 296 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2$ (296.15): C 56.78, H 3.74, N 4.73, Found: C 56.98, H 4.01, N 4.76.

*5.1.2.5. 4-Chloro-2-methoxy-N-(3,4-dichlorophenyl)benzamide (**3e**).* Yield: 51.5%, white solid; m. p. 165–167 °C; IR (ATR): 3343 (ν NH), 3106 (ν CH aromatic), 1665 (ν CO + δ NH, amide I), 1584 (ν CC aromatic), 1525 (δ NH + ν CN, amide II), 1476, 1464, 1456 (ν CC aromatic) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.37 (1H, s, NH), 8.09 (1H, d, J = 2.4 Hz, H2′), 7.66 (1H dd, J = 8.8 Hz, J = 2.4 Hz, H6′), 7.64–7.56 (2H, m, H6, H5′), 7.28 (1H, d, J = 1.8 Hz, H3), 7.13 (1H, dd, J = 8.2 Hz, J = 1.8 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.30 (1C, s, CONH), 157.51, 139.15, 136.77, 131.21, 131.19, 130.86, 125.27, 123.71, 121.07, 120.69, 119.97, 112.70, 56.69 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 330 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{10}Cl_3NO_2$ (330.59): C 50.86, H 3.05, N 4.24, Found: C 50.95, H 3.37, N 4.23.

*5.1.2.6. 4-Chloro-2-methoxy-N-(3-bromophenyl)benzamide (**3f**).* Yield: 73.9%, white solid; m. p. 136–137 °C; IR (ATR): 3355 (ν NH), 3081 (ν CH aromatic), 1670 (ν CO + δ NH, amide I), 1588 (ν CC aromatic), 1536 (δ NH + ν CN, amide II), 1484, 1461 (ν CC aromatic) cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz): δ 10.25 (1H, s, NH), 8.05 (1H, m, H2′), 7.65 (1H, m, H4′), 7.61 (1H, d, J = 8.2 Hz, H6), 7.32–7.25 (3H, m, H3, H5′, H6′), 7.12 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 164.16 (1C, s, CONH), 157.50, 140.64, 136.61, 131.18, 130.90, 126.44, 123.92, 122.17, 121.73, 120.65, 118.70, 112.67, 56.66 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 340 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNO_2$ (340.60): C 49.37, H 3.26, N 4.11, Found: C 49.33, H 3.60, N 4.17.

*5.1.2.7. 4-Chloro-2-methoxy-N-(4-bromophenyl)benzamide (**3g**).* Yield: 66.8%, white solid; m. p. 124–126 °C; IR (ATR): 3350 (ν NH), 3104 (ν CH aromatic), 1668 (ν CO + δ NH, amide I), 1589 (ν CC aromatic), 1539 (δ NH + ν CN, amide II), 1488, 1460 (ν CC aromatic) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.23 (1H, s, NH), 7.74–7.65 (2H, m, H3′, H5′), 7.60 (1H, d, J = 8.2 Hz, H6), 7.56–7.48 (2H, m, H2′, H6′), 7.27 (1H, d, J = 1.9 Hz, H3), 7.12 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.02 (1C, s, CONH), 157.48, 138.47, 136.50, 131.74 (2C, s, C3′, C5′), 131.16, 124.11, 121.81 (2C, s, C2′, C6′), 120.64, 115.44, 112.66, 56.65 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 340 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNO_2$ (340.60): C 49.37, H 3.26, N 4.11, Found: C 49.61, H 3.58, N 4.15.

*5.1.2.8. 4-Chloro-2-methoxy-N-(3-trifluoromethylphenyl)benzamide (**3h**).* Yield: 73.2%, white solid; m. p. 117–119 °C; IR (ATR): 3355 (ν NH), 1670 (ν CO + δ NH, amide I), 1592 (ν CC aromatic), 1558 (δ NH + ν CN, amide II), 1493 (ν CC aromatic) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.43 (1H, s, NH), 8.22 (1H, m, H2′), 7.91 (1H, m, H4′), 7.63 (1H, d, J = 8.1 Hz, H6), 7.56 (1H, t, J = 8.1 Hz, H5′), 7.44 (1H, m, H6′), 7.28 (1H, d, J = 1.8 Hz, H3), 7.13 (1H, dd, J = 8.1 Hz, J = 1.8 Hz, H5), 3.91 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.44 (1C, s, CONH), 157.53, 139.84, 136.67, 131.17, 130.16, 129.87 (1C, q, J = 31.2 Hz, C3′), 124.51 (1C, q, J = 270.6 Hz, CF_3), 123.91, 123.51, 120.65, 120.19 (1C, q, J = 4.0 Hz, C2′), 115.91 (1C, q, J = 4.1 Hz, C4′), 112.69, 56.67 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 330 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2$ (329.70): C 54.64, H 3.36, N 4.25, Found: C 54.30, H 3.48, N 4.47.

Table 5
Cytotoxicity and selectivity index for selected compounds.

	R^1	R^2	IC_{50} [μmol L^{-1}]	SI for My 331/88, 14 d
4c	4-Cl	3′-Cl	11.68	2.92
4d	4-Cl	4′-Cl	2.93	0.73
4e	4-Cl	3′,4′-diCl	6.50	3.25
4g	4-Cl	4′-Br	3.55	0.89
4h	4-Cl	3′-CF_3	1.81	0.45

SI (selectivity index) = IC_{50}/MIC.

*5.1.2.9. 4-Chloro-2-methoxy-N-(4-trifluoromethylphenyl)benzamide (**3i**).* Yield: 67.8%, white solid; m. p. 141–142 °C; IR (ATR): 3368 (ν NH), 1673 (ν CO + δ NH, amide I), 1594 (ν CC aromatic), 1541 (δ NH + ν CN, amide II), 1483, 1464 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.46 (1H, s, NH), 7.97–7.88 (2H, m, H3′, H5′), 7.75–7.66 (2H, m, H2′, H6′), 7.62 (1H, d, J = 8.2 Hz, H6), 7.28 (1H, d, J = 1.9 Hz, H3), 7.13 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.90 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.51 (1C, s, CONH), 157.53, 142.66, 136.67, 131.16, 126.26 (2C, q, J = 3.9 Hz, C3′, C5′), 125.25 (1C, q, J = 269.5 Hz, CF_3), 124.02, 123.80 (1C, q, J = 31.8 Hz, C4′), 120.66, 119.77 (2C, s, C2′, C6′), 112.70, 56.67 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 330 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2$ (329.70): C 54.64, H 3.36, N 4.25, Found: C 54.43, H 3.25, N 4.44.

*5.1.2.10. 5-Chloro-2-methoxy-N-(3-nitrophenyl)benzamide (**3j**).* Yield: 47.5%, white solid; m. p. 190–192 °C; IR (ATR): 3318 (ν NH), 1670 (ν CO + δ NH, amide I), 1596 (ν CC aromatic), 1552 (δ NH + ν CN, amide II), 1530 (ν_{as} NO_2), 1474, 1458, 1433 (ν CC aromatic), 1338 (ν_s NO_2) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.66 (1H, s, NH), 8.76 (1H, t, J = 2.2 Hz, H2′), 8.03 (1H, m, H4′), 7.96 (1H, m, H6′), 7.63 (1H, t, J = 8.2 Hz, H5′), 7.62 (1H, d, J = 2.8 Hz, H6), 7.56 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 7.22 (1H, d, J = 8.8 Hz, H3), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.17 (1C, s, CONH), 155.51, 148.15, 140.14, 131.81, 130.38, 128.97, 126.65, 125.94, 124.42, 118.48, 114.24, 113.99, 56.53 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 307 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_4$ (306.70): C 54.83, H 3.62, N 9.13, Found: C 54.56, H 3.91, N 8.88.

*5.1.2.11. 5-Chloro-2-methoxy-N-(4-nitrophenyl)benzamide (**3k**).* Yield: 49.3%, yellow solid; m. p. 209–211 °C; IR (ATR): 3318 (ν NH), 1677 (ν CO + δ NH, amide I), 1596 (ν CC aromatic), 1553 (δ NH + ν CN, amide II), 1513 (ν_{as} NO_2), 1478, 1454 (ν CC aromatic), 1330 (ν_s NO_2) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.79 (1H, s, NH), 8.29–8.21 (2H, m, H3′, H5′), 8.00–7.92 (2H, m, H2′, H6′), 7.61 (1H, d, J = 2.8 Hz, H6), 7.57 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 7.22 (1H, d, J = 8.8 Hz, H3), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.38 (1C, s, CONH), 155.50, 145.16, 142.72, 131.89, 128.94, 126.69, 125.17 (2C, s, C3′, C5′), 124.43, 119.59 (2C, s, C2′, C6′), 114.25, 56.54 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 307 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_4$ (306.70): C 54.83, H 3.62, N 9.13, Found: C 54.38, H 3.21, N 8.95.

*5.1.2.12. 5-Chloro-2-methoxy-N-(3-chlorophenyl)benzamide (**3l**).* Yield: 71.7%, white solid; m. p. 133–135 °C; IR (ATR): 3326 (ν NH), 3080 (ν CH aromatic), 1667 (ν CO + δ NH, amide I), 1588 (ν CC aromatic), 1525 (δ NH + ν CN, amide II), 1483, 1463 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.35 (1H, s, NH), 7.91 (1H, t, J = 2.1 Hz, H2′), 7.65–7.58 (2H, m, H6, H4′), 7.54 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 7.36 (1H, t, J = 8.1 Hz, H5′), 7.20 (1H, d, J = 8.8 Hz, H3), 7.15 (1H, m, H6′), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.71 (1C, s, CONH), 155.47, 140.44, 133.26, 131.66, 130.61, 128.97, 126.76, 124.42, 123.62, 119.33, 118.32, 114.21, 56.50 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 296 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2$ (296.15): C 56.78, H 3.74, N 4.73, Found: C 56.41, H 3.64, N 4.66.

*5.1.2.13. 5-Chloro-2-methoxy-N-(4-chlorophenyl)benzamide (**3m**).* Yield: 70.0%, white solid; m. p. 135–137 °C; IR (ATR): 3335 (ν NH), 3085 (ν CH aromatic), 1665 (ν CO + δ NH, amide I), 1591 (ν CC aromatic), 1535 (δ NH + ν CN, amide II), 1491, 1477 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.32 (1H, s, NH), 7.79–7.70 (2H, m, H3′, H5′), 7.59 (1H, d, J = 2.8 Hz, H6), 7.54 (1H, dd, J = 8.9 Hz, J = 2.8 Hz, H4), 7.43–7.35 (2H, m, H2′, H6′), 7.19 (1H, d, J = 8.9 Hz, H3), 3.86 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.55 (1C, s, CONH), 155.45, 138.02, 131.57, 128.97, 128.85 (2C, s, C3′, C5′), 127.48, 126.95, 124.42, 121.45 (2C, s, C2′, C6′), 114.19, 56.50 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 296 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NO_2$ (296.15): C 56.78, H 3.74, N 4.73, Found: C 56.40, H 4.16, N 4.66.

*5.1.2.14. 5-Chloro-2-methoxy-N-(3,4-dichlorophenyl)benzamide (**3n**).* Yield: 50.2%, white solid; m. p. 175–177 °C; IR (ATR): 3343 (ν NH), 3099 (ν CH aromatic), 1674 (ν CO + δ NH, amide I), 1587 (ν CC aromatic), 1529 (δ NH + ν CN, amide II), 1476, 1469 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.45 (1H, s, NH), 8.09 (1H, d, J = 2.3 Hz, H2′), 7.65 (1H, dd, J = 9.0 Hz, J = 2.3 Hz, H6′), 7.62–7.57 (2H, m, H6, H5′), 7.54 (1H, dd, J = 8.9 Hz, J = 2.7 Hz, H4), 7.20 (1H, d, J = 8.9 Hz, H3), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.83 (1C, s, CONH), 155.48, 139.09, 131.80, 131.19, 130.87, 128.99, 126.55, 125.36, 124.43, 121.07, 119.96, 114.23, 56.52 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 330 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{10}Cl_3NO_2$ (330.59): C 50.86, H 3.05, N 4.24, Found: C 50.38, H 3.26, N 4.17.

*5.1.2.15. 5-Chloro-2-methoxy-N-(3-bromophenyl)benzamide (**3o**).* Yield: 68.3%, white solid; m. p. 133–134 °C; IR (ATR): 3350 (ν NH), 1661 (ν CO + δ NH, amide I), 1592 (ν CC aromatic), 1534 (δ NH + ν CN, amide II), 1479, 1459 (ν CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.72 (1H, s, NH), 8.20 (1H, d, J = 2.8 Hz, H6), 7.88 (1H, t, J = 1.9 Hz, H2′), 7.55 (1H, m, H4′), 7.42 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 7.25 (1H, m, H6′), 7.21 (1H, t, J = 7.9 Hz, H5′), 6.96 (1H, d, J = 8.8 Hz, H3), 4.05 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 161.86 (1C, s, CONH), 155.63, 139.29, 133.03, 132.13, 130.25, 127.28, 127.15, 123.19, 122.67, 122.57, 118.85, 113.05, 56.68 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 340 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNO_2$ (340.60): C 49.37, H 3.26, N 4.11, Found: C 49.04, H 3.56, N 4.04.

*5.1.2.16. 5-Chloro-2-methoxy-N-(4-bromophenyl)benzamide (**3p**).* Yield: 40.7%, white solid; m. p. 139–141 °C; IR (ATR): 3337 (ν NH), 3084 (ν CH aromatic), 1666 (ν CO + δ NH, amide I), 1594, 1586 (CC aromatic), 1535 (δ NH + ν CN, amide II), 1488, 1477 (CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.31 (1H, s, NH), 7.74–7.66 (2H, m, H3′, H5′), 7.59 (1H, d, J = 2.8 Hz, H6), 7.57–7.48 (3H, m, H4, H2′, H6′), 7.19 (1H, d, J = 8.9 Hz, H3), 3.86 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 163.54 (1C, s, CONH), 155.44, 138.42, 131.74 (2C, s, C3′, C5′), 131.56, 128.95, 126.94, 124.41, 121.81 (2C, s, C2′, C6′), 115.53, 114.18, 56.49 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 340 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNO_2$ (340.60): C 49.37, H 3.26, N 4.11, Found: C 49.12, H 3.59, N 4.00.

*5.1.2.17. 5-Chloro-2-methoxy-N-(3-trifluoromethylphenyl)benzamide (**3q**).* Yield: 53.2%, white solid; m. p. 107–108 °C; IR (ATR): 3342 (ν NH), 1665 (ν CO + δ NH, amide I), 1597 (ν CC aromatic), 1559 (δ NH + ν CN, amide II), 1479 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.51 (1H, s, NH), 8.21 (1H, m, H2′), 7.90 (1H, m, H4′), 7.68–7.50 (3H, m, H4, H6, H5′), 7.44 (1H, m, H6′), 7.21 (1H, d, J = 8.9 Hz, H3), 3.87 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 164.01 (1C, s, CONH), 155.50, 139.80, 131.71, 130.19, 129.83 (1C, q, J = 31.4 Hz, C3′), 128.96, 126.78, 124.80 (1C, q, J = 270.6 Hz, CF_3), 124.41, 123.50, 120.27 (1C, q, J = 4.0 Hz, C2′), 115.95 (1C, q, J = 4.3 Hz, C4′), 114.23, 56.52 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 330 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2$ (329.70): C 54.64, H 3.36, N 4.25, Found: C 54.81, H 2.98, N 4.16.

*5.1.2.18. 5-Chloro-2-methoxy-N-(4-trifluoromethylphenyl)benzamide (**3r**).* Yield: 75.3%, white solid; m. p. 131–133 °C; IR (ATR): 3336 (ν NH), 1675 (ν CO + δ NH, amide I), 1605 (ν CC aromatic), 1545 (δ NH + ν CN, amide II), 1481 (ν CC aromatic) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 10.54 (1H, s, NH), 7.97–7.89 (2H, m, H3′, H5′), 7.75–7.66 (2H, m, H2′, H6′), 7.60 (1H, d, J = 2.7 Hz, H6), 7.55 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H4), 7.21 (1H, d, J = 8.8 Hz, H3), 3.87 (3H, s, OCH_3); ^{13}C

NMR (DMSO-d_6, 75 MHz): δ 164.05 (1C, s, CONH), 155.48, 142.59, 131.71, 128.94, 126.86, 126.25 (2C, q, J = 3.9 Hz, C3′, C5′), 125.17 (1C, q, J = 268.9 Hz, CF_3), 124.42, 124.06 (1C, q, J = 31.8 Hz, C4′), 119.77 (2C, s, C2′, C6′), 114.22, 56.51 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 330 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NO_2$ (329.70): C 54.64, H 3.36, N 4.25, Found: C 54.26, H 3.11, N 4.21.

5.1.3. General procedure for the preparation of substituted 2-methoxybenzthioamides

A suspension of 2.0 mmol of the starting benzamide (**3a**–**3r**) and an equimolar amount of P_4S_{10} (2.0 mmol) in 25.0 mL of pyridine was stirred and heated at reflux temperature (155 °C) until the starting benzamide disappeared by TLC (3–8 h). More P_4S_{10} was added if necessary. The unreacted P_4S_{10} was removed by the addition of ice water. The mixture was then extracted three times with 50 mL of chloroform and the organic phase was collected, dried with Na_2SO_4 and evaporated. The oil residue was purified by column chromatography on silica gel with toluene, toluene/hexane (9/1) for **4h** or toluene/hexane (4/1) for **4o** as a mobile phase.

*5.1.3.1. 4-Chloro-2-methoxy-N-(3-nitrophenyl)benzothioamide (**4a**).* Yield: 23.0%, yellow solid; m. p. 160–162 °C; IR (ATR): 3304 (ν NH),1589, 1561 (CC aromatic), 1524 (ν_{as} NO_2), 1477, 1462 (CC aromatic), 1345 (ν_s NO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.16 (1H, s, NH), 9.13 (1H, t, J = 2.2 Hz, H2′), 8.30 (1H, m, H4′), 8.11 (1H, m, H6′), 7.72 (1H, t, J = 8.2 Hz, H5′), 7.51 (1H, d, J = 8.2 Hz, H6), 7.23 (1H, d, J = 1.9 Hz, H3), 7.09 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.85 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 195.25 (1C, s, CSNH), 154.98, 147.70, 140.69, 135.34, 132.30, 131.26, 130.33, 128.94, 120.83, 120.34, 116.84, 112.38, 56.58 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 323 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_3S$ (322.77): C 52.10, H 3.44, N 8.68, S 9.93, Found: C 52.31, H 3.53, N 8.94, S 10.07.

*5.1.3.2. 4-Chloro-2-methoxy-N-(4-nitrophenyl)benzothioamide (**4b**).* Yield: 20.0%, yellow solid; m. p. 153–154 °C; IR (ATR): 3364 (ν NH), 1589, 1561 (CC aromatic), 1510 (ν_{as} NO_2), 1491, 1473 (CC aromatic), 1318 (ν_s NO_2) cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.20 (1H, s, NH), 8.39–8.26 (4H, m. H2′, H3′, H5′, H6′), 7.49 (1H, d, J = 8.2 Hz, H6), 7.23 (1H, d, J = 1.9 Hz, H3), 7.09 (1H, dd, J = 8.2 Hz, J = 1.9 Hz, H5), 3.84 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 195.83 (1C, s, CSNH), 154.96, 145.41, 144.17, 135.43, 132.58, 131.31, 124.71 (2C, s, C3′, C5′), 122.47 (2C, s, C2′, C6′), 120.36, 112.38, 56.57 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 323 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_3S$ (322.77): C 52.10, H 3.44, N 8.68, S 9.93, Found: C 52.38, H 3.62, N 8.51, S 9.79.

*5.1.3.3. 4-Chloro-2-methoxy-N-(3-chlorophenyl)benzothioamide (**4c**).* Yield: 50.2%, yellow solid; m. p. 124–125 °C; IR (ATR): 3290 (ν NH), 1588, 1565, 1479, 1460 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.53 (1H, s, NH), 8.41 (1H, d, J = 8.6 Hz, H6), 7.85 (1H, t, J = 2.0 Hz, H2′), 7.62 (1H, m, H4′), 7.35 (1H, t, J = 8.0 Hz, H5′), 7.26 (1H, m, H6′), 7.06 (1H, dd, J = 8.6 Hz, J = 1.9 Hz, H5), 6.98 (1H, d, J = 1.9 Hz, H3), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.78 (1C, s, CSNH), 154.99, 140.16, 138.57, 136.34, 134.36, 129.81, 126.88, 126.84, 124.19, 122.38, 121.77, 112.09, 56.70 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 312 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NOS$ (312.21): C 53.86, H 3.55, N 4.49, S 10.27, Found: C 54.11, H 3.71, N 4.72, S 10.02.

*5.1.3.4. 4-Chloro-2-methoxy-N-(4-chlorophenyl)benzothioamide (**4d**).* Yield: 54.0%, yellow solid; m. p. 128–129 °C; IR (ATR): 1590, 1565, 1527, 1488, 1460 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.52 (1H, s, NH), 8.42 (1H, d, J = 8.6 Hz, H6), 7.75–7.66 (2H, m, H3′, H5′), 7.43–7.34 (2H, m, H2′, H6′), 7.07 (1H, dd, J = 8.6 Hz, J = 1.9 Hz, H5), 6.98 (1H, d, J = 1.9 Hz, H3), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.64 (1C, s, CSNH), 155.02, 138.53, 137.61, 136.35, 132.01, 128.96 (2C, s, C3′, C5′), 126.79, 125.52 (2C, s, C2′, C6′), 121.76, 112.07, 56.68 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 312 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NOS$ (312.21): C 53.86, H 3.55, N 4.49, S 10.27, Found: C 53.65, H 3.45, N 4.56, S 10.15.

*5.1.3.5. 4-Chloro-2-methoxy-N-(3,4-chlorophenyl)benzothioamide (**4e**).* Yield: 39.0%, yellow solid; m. p. 122–123 °C; IR (ATR): 3300 (ν NH), 1583, 1542, 1477, 1459 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.56 (1H, s, NH), 8.40 (1H, d, J = 8.6 Hz, H6), 7.97 (1H, d, J = 2.5 Hz, H2′), 7.59 (1H, dd, J = 8.7 Hz, J = 2.5 Hz, H6′), 7.46 (1H, d, J = 8.7 Hz, H5′), 7.06 (1H, dd, J = 8.6 Hz, J = 1.9 Hz, H5), 6.98 (1H, d, J = 1.9 Hz, H3), 4.01 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.87 (1C, s, CSNH), 155.00, 138.80, 138.36, 136.42, 132.58, 130.33, 130.15, 126.52, 125.77, 123.56, 121.84, 112.09, 56.75 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 346 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{10}Cl_3NOS$ (346.66): C 48.51, H 2.91, N 4.04, S 9.25, Found: C 48.27, H 3.05, N 4.15, S 9.02.

*5.1.3.6. 4-Chloro-2-methoxy-N-(3-bromophenyl)benzothioamide (**4f**).* Yield: 53.4%, yellow solid; m. p. 129–130 °C; IR (ATR): 3303 (ν NH), 1588, 1560,1547, 1477 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.52 (1H, s, NH), 8.41 (1H, d, J = 8.6 Hz, H6), 7.97 (1H, m, H2′), 7.69 (1H, m, H4′), 7.41 (1H, m, H6′), 7.28 (1H, t, J = 8.1 Hz, H5′), 7.06 (1H, m, H5), 6,98 (1H, m, H3), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): 193.81 (1C, s, CSNH), 155.00, 140.29, 138.59, 136.35, 130.07, 129.79, 127.04, 126.82, 122.91, 122.21, 121.78, 112.09, 56.72 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 356 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNOS$ (356.67): C 47.14, H 3.11, N 3.93, S 8.99, Found: C 46.96, H 3.34, N 3.79, S 8.94.

*5.1.3.7. 4-Chloro-2-methoxy-N-(4-bromophenyl)benzothioamide (**4g**).* Yield: 29.0%, yellow solid; m. p. 123–124 °C; IR (ATR): 1592, 1566, 1486, 1462 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.52 (1H, s, NH), 8.42 (1H, d, J = 8.6 Hz, H6), 7.69–7.62 (2H, m, H3′, H5′), 7.58–7.54 (2H, m, H2′, H6′), 7.07 (1H, dd, J = 8.6 Hz, J = 2.0 Hz, H5), 6.98 (1H, d, J = 2.0 Hz, H3), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.57 (1C, s, CSNH), 155.00, 138.55, 138.10, 136.36, 131.93 (2C, s, C3′, C5′), 126.81, 125.77 (2C, s, C2′, C6′), 121.78, 119.88, 112.08, 56.70 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 356 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNOS$ (356.67): C 47.14, H 3.11, N 3.93, S 8.99, Found: C 47.20, H 3.31, N 3.96, S 9.37.

*5.1.3.8. 4-Chloro-2-methoxy-N-(3-trifluoromethylphenyl)benzothioamide (**4h**).* Yield: 37.0%, yellow solid; m. p. 109–110 °C; IR (ATR): 3289 (ν NH), 1591, 1566, 1483, 1450 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.64 (1H, s, NH), 8.44 (1H, d, J = 8.6 Hz, H6), 8.05–7.95 (2H, m, H2′, H4′), 7.60–7.50 (2H, m, H5′, H6′), 7.08 (1H, dd, J = 8.6 Hz, J = 1.9 Hz, H5), 7.00 (1H, d, J = 1.9 Hz, H3), 4.03 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 194.17 (1C, s, CSNH), 155.07, 139.56, 138.75, 136.46, 131.28 (1C, q, J = 32.5 Hz, C3′), 129.38, 127.67, 126.67, 123.66 (1C, q, J = 270.8 Hz, CF_3), 123.41 (1C, q, J = 3.8 Hz, C2′), 121.86, 121.09 (1C, q, J = 3.8 Hz, C4′), 112.12, 56.75 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 346 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NOS$ (345.77): C 52.10, H 3.21, N 4.05, S 9.27, Found: C 52.31, H 3.02, N 4.37, S 9.16.

*5.1.3.9. 4-Chloro-2-methoxy-N-(4-trifluoromethylphenyl)benzothioamide (**4i**).* Yield: 22.0%, yellow solid; m. p. 95–96 °C; IR (ATR): 1589, 1565, 1482, 1464 (CC aromatic) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 10.66 (1H, s, NH), 8.43 (1H, d, J = 8.6 Hz, H6), 8.00–7.89 (2H, m, H3′, H5′), 7.73–7.64 (2H, m, H2′, H6′), 7.08 (1H, dd, J = 8.6 Hz, J = 1.9 Hz, H5), 7.00 (1H, d, J = 1.9 Hz, H3), 4.02 (3H, s, OCH3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.95 (1C, s, CSNH), 154.97, 142.03, 138.76, 136.40, 126.91, 126.06 (2C, q, J = 3.8 Hz, C3′, C5′),

123.93 (2C, s, C2′, C6′), 121.89, 112.15, 56.75 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 346 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NOS$ (345.77): C 52.10, H 3.21, N 4.05, S 9.27, Found: C 52.33, H 3.39, N 4.26, S 9.39.

*5.1.3.10. 5-Chloro-2-methoxy-N-(3-nitrophenyl)benzothioamide (**4j**).* Yield: 5.3%, yellow solid; m. p. 158–159 °C; IR (ATR): 3291 (ν NH), 1560 (ν CC aromatic), 1531 (ν_{as} NO_2), 1479, 1459 (ν CC aromatic), 1344 (ν_s NO_2) cm^{-1}; ^1H NMR (DMSO-d_6, 500 MHz): δ 12.23 (1H, s, NH), 9.13 (1H, t, J = 2.2 Hz, H2′), 8.29 (1H, m, H4′), 8.12 (1H, m, H6′), 7.73 (1H, t, J = 8.2 Hz, H5′), 7.51–7.45 (2H, m, H4, H6), 7.17 (1H, d, J = 8.5 Hz, H3), 3.82 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 194.55 (1C, s, CSNH), 153.04, 147.70, 140.56, 134.78, 130.36, 128.98, 128.94, 127.85, 124.10, 120.93, 116.90, 113.98, 56.47 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 323 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_3S$ (322.77): C 52.10, H 3.44, N 8.68, S 9.93, Found: C 51.92, H 3.28, N 8.37, S 9.78.

*5.1.3.11. 5-Chloro-2-methoxy-N-(4-nitrophenyl)benzothioamide (**4k**).* Yield: 17.0%, yellow solid; m. p. 171–172 °C; IR (ATR): 3276 (ν NH), 1594 (CC aromatic), 1565 (ν_{as} NO_2), 1493, 1477 (CC aromatic), 1322 (ν_s NO_2) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 12.28 (1H, s, NH), 8.38–8.27 (4H, m, H2′, H3′, H5′, H6′), 7.52–7.44 (2H, m, H4, H6), 7.16 (1H, d, J = 8.8 Hz, H3), 3.81 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 195.09 (1C, s, CSNH), 153.02, 145.32, 144.25, 135.06, 130.42, 128.98, 124.72 (2C, s, C3′, C5′), 124.12, 122.53 (2C, s, C2′, C6′), 113.98, 56.46 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 323 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}ClN_2O_3S$ (322.77): C 52.10, H 3.44, N 8.68, S 9.93, Found: C 52.37, H 3.35, N 8.85, S 10.05.

*5.1.3.12. 5-Chloro-2-methoxy-N-(3-chlorophenyl)benzothioamide (**4l**).* Yield: 11.2%, yellow solid; m. p. 103–104 °C; IR (ATR): 3243 (ν NH), 1592, 1480, 1463 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.55 (1H, s, NH), 8.42 (1H, d, J = 2.7 Hz, H6), 7.86 (1H, t, J = 2.0 Hz, H2′), 7.64 (1H, m, H4′), 7.38 (1H, dd, J = 8.9 Hz, J = 2.7 Hz, H4), 7.36 (1H, t, J = 8.0 Hz, H5′), 7.27 (1H, m, H6′), 6.93 (1H, d, J = 8.9 Hz, H3), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.31 (1C, s, CSNH), 153.19, 140.10, 134.51, 134.41, 132.13, 129.86, 129.60, 126.95, 126.84, 124.06, 122.27, 113.08, 56.77 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 312 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NOS$ (312.21): C 53.86, H 3.55, N 4.49, S 10.27, Found: C 53.54, H 3.27, N 4.65, S 10.35.

*5.1.3.13. 5-Chloro-2-methoxy-N-(4-chlorophenyl)benzothioamide (**4m**).* Yield: 44.0%, yellow solid; m. p. 106–107 °C; IR (ATR): 3292 (ν NH), 1592, 1552, 1477 and 1460 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.54 (1H, s, NH), 8.43 (1H, d, J = 2.5 Hz, H6), 7.77–7.66 (2H, m, H3′, H5′), 7.44–7.33 (3H, m, H4, H2′, H6′), 6.93 (1H, d, J = 8.8 Hz, H3), 3.99 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.17 (1C, s, CSNH), 153.22, 137.56, 134.51, 132.08, 129.62, 129.01 (2C, s, C3′, C5′), 126.84, 125.41 (2C, s, C2′, C6′), 113.08, 56.76 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 312 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}Cl_2NOS$ (312.21): C 53.86, H 3.55, N 4.49, S 10.27, Found: C 53.74, H 3.65, N 4.52, S 10.39.

*5.1.3.14. 5-Chloro-2-methoxy-N-(3,4-chlorophenyl)benzothioamide (**4n**).* Yield: 4.9%, yellow solid; m. p. 122–123 °C; IR (ATR): 3301 (ν NH), 1584, 1536, 1475, 1463 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.57 (1H, s, NH), 8.44 (1H, d, J = 2.7 Hz, H6), 7.98 (1H, d, J = 2.5 Hz, H2′), 7.63 (1H, dd, J = 8.7 Hz, J = 2.5 Hz, H6′), 7.49 (1H, d, J = 8.7 Hz, H5′), 7.40 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H4), 6.94 (1H, d, J = 8.8 Hz, H3), 4.00 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.47 (1C, s, CSNH), 153.23, 138.35, 134.66, 132.70, 132.33, 130.43, 130.28, 129.35, 127.00, 125.71, 123.50, 113.11, 56.84 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 346 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{10}Cl_3NOS$ (346.66): C 48.51, H 2.91, N 4.04, S 9.25, Found: C 48.41, H 2.75, N 4.25, S 9.02.

*5.1.3.15. 5-Chloro-2-methoxy-N-(3-bromophenyl)benzothioamide (**4o**).* Yield: 50.2%, yellow solid; m. p. 103–105 °C; IR (ATR): 3238 (ν NH), 1590, 1532 (CC aromatic), 1478, 1463 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.54 (1H, s, NH), 8.41 (1H, d, J = 2.7 Hz, H6), 7.98 (1H, t, J = 1.9 Hz, H2′), 7.69 (1H, m, H4′), 7.45–7.35 (2H, m, H4, H6′), 7.29 (1H, t, J = 8.1 Hz, H5′), 6.93 (1H, d, J = 8.8 Hz, H3), 3.96 (3H, s, OCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 193.33 (1C, s, CSNH), 153.20, 140.23, 134.48, 132.12, 130.11, 129.85, 129.61, 126.88, 126.83, 122.77, 122.24, 113.09, 56.78 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 356 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNOS$ (356.67): C 47.14, H 3.11, N 3.93, S 8.99, Found: C 47.38, H 3.34, N 3.96, S 9.36.

*5.1.3.16. 5-Chloro-2-methoxy-N-(4-bromophenyl)benzothioamide (**4p**).* Yield: 50.3%, yellow solid; m. p. 116–117 °C; IR (ATR): 3299 (ν NH), 1594, 1551, 1479, 1458 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.53 (1H, s, NH), 8.43 (1H, d, J = 2.8 Hz, H6), 7.72–7.62 (2H, m, H3′, H5′), 7.58–7.50 (2H, m, H2′, H6′), 7.38 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 6.93 (1H, d, J = 8.8 Hz, H3), 3.99 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.09 (1C, s, CSNH), 153.20, 138.06, 134.50, 132.09, 131.96 (2C, s, C3′, C5′), 129.63, 129.00, 126.84, 125.63 (2C, s, C2′, C6′), 113.08, 56.77 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 356 $[M + H]^+$; Anal. Calcd. for $C_{14}H_{11}BrClNOS$ (356.67): C 47.14, H 3.11, N 3.93, S 8.99, Found: C 47.19, H 3.38, N 4.15, S 9.21.

*5.1.3.17. 5-Chloro-2-methoxy-N-(3-trifluoromethylphenyl)benzothioamide (**4q**).* Yield: 31.3%, yellow solid; m. p. 93–94 °C; IR (ATR): 3303 (ν NH), 1573, 1540, 1484, 1453 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.66 (1H, s, NH), 8.43 (1H, d, J = 2.8 Hz, H6), 8.01, 7.55 (4H, m, H2′, H4′, H5′, H6′), 7.39 (1H, dd, J = 8.8 Hz, J = 2.8 Hz, H4), 6.94 (1H, d, J = 8.8 Hz, H3), 4.01 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.66 (1C, s, CSNH), 153.25, 139.49, 134.52, 132.24, 131.28 (1C, q, J = 32.5 Hz, C3′), 129.41, 128.18, 127.48, 126.85, 124.79 (1C, q, J = 270.9 Hz, CF_3), 123.43 (1C, q, J = 3.7 Hz, C2′), 120.90 (1C, q, J = 3.9 Hz, C4′), 113.10, 56.78 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 346 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NOS$ (345.77): C 52.10, H 3.21, N 4.05, S 9.27, Found: C 52.02, H 3.37, N 3.89, S 8.98.

*5.1.3.18. 5-Chloro-2-methoxy-N-(4-trifluoromethylphenyl)benzothioamide (**4r**).* Yield: 8.1%, yellow solid; m. p. 102–103 °C; IR (ATR): 3260 (ν NH), 1593, 1550, 1476, 1463 (CC aromatic) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 10.68 (1H, s, NH), 8.42 (1H, d, J = 2.7 Hz, H6), 8.00–7.90 (2H, m, H3′, H5′), 7.74–7.64 (2H, m, H2′, H6′), 7.40 (1H, dd, J = 8.8 Hz, J = 2.7 Hz, H4), 6.94 (1H, d, J = 8.8 Hz, H3), 4.01 (3H, s, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 193.50 (1C, s, CSNH), 153.18, 141.97, 134.54, 132.27, 129.69, 128.43 (1C, q, J = 32.9 Hz, C4′), 126.94, 126.08 (2C, q, J = 3.7 Hz, C3′, C5′), 123.80 (2C, s, C2′, C6′), 123.78 (1C, q, J = 270.2 Hz, CF_3), 113.13, 56.81 (1C, s, OCH_3); LRMS-APCI$^+$: m/z 346 $[M + H]^+$; Anal. Calcd. for $C_{15}H_{11}ClF_3NOS$ (345.77): C 52.10, H 3.21, N 4.05, S 9.27, Found: C 51.85, H 2.98, N 3.79, S 9.17.

5.2. In vitro antimycobacterial assay

The *in vitro* antimycobacterial activity of the prepared compounds was determined against *M. tuberculosis* My 331/88 (10^{-3} dilution of strain), *M. avium* My 330/88 (10^{-5} dilution of strain), *M. kansasii* My 235/80 (10^{-4} dilution of strain) and *M. kansasii* 6509/96 (10^{-4} dilution of strain). All of the strains were obtained from the Czech National Collection of Type Cultures (CNCTC) with the exception of *M. kansasii* 6509/96, which was clinically isolated. The antimycobacterial activity of the compounds was determined in a Šula's semisynthetic medium (SEVAC, Prague,

Czech Republic) via the micromethod for the determination of the minimum inhibitory concentration (MIC) at 37 °C after 14 and 21 days, and after 7, 14 and 21 days for *M. kansasii*. The tested compounds were added to the medium as DMSO solutions and INH was used as a standard in sterile water solution. The following concentrations of the tested compounds were used: 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1 and 0.5 μmol L^{-1}. INH was used in the concentration range 0.5–250 μmol L^{-1}.

5.3. In vitro enzymatic assay

The gene encoding isocitrate lyase was amplified by PCR from mycobacterium H37Rv genomic DNA. The amplified DNA was digested using *Nde*I and *Hind*III, cloned into the pET-28b(+) plasmid vector Novagen (Merck KGaA, Darmstadt, Germany) and the recombinant plasmid was transformed into *E. coli* HB101. DNA sequencing was used to confirm that the inserted coding sequence had no mutations. For bacterial expression, 25 mL culture volumes were inoculated with BL21(DE3) cells containing the recombinant plasmid and allowed to grow until $OD_{595} = 0.6$ was achieved. The culture was then induced with 1 mmol L^{-1} isopropyl-β-D-thio-galactopyranoside solution and incubated at 30 °C for an additional 4 h. The cells were harvested by centrifugation at 4 °C and resuspended in BugBuster Protein Extraction Reagent Novagen (Merck KGaA, Darmstadt, Germany). Histidine-tagged protein was purified using an Äkta purifier (Amersham Biosciences, Valley Stream, NY, USA) and confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie staining.

Isocitrate lyase activity was assayed according to the protocol reported by Dixon and Kornberg [15]. The enzyme assay was optimised in the final volume of 1 mL. The reaction buffer contained 50 mmol L^{-1} KH_2PO_4, 4 mmol L^{-1} $MgCl_2 \cdot 6H_2O$, 4 mmol L^{-1} phenylhydrazine hydrochloride, 12 mmol L^{-1} cysteine, H_2O and KOH to pH 7. The isocitrate cleavage was measured by the change in absorbance at 324 nm associated with the formation of glyoxylate phenyl hydrazone. Each tested compound was dissolved in DMSO to prepare a 10 mmol L^{-1} solution, and 10 μL of this solution was added to 939 μL reaction buffer and 1 μL of recombinant isocitrate lyase. ICL was used as phosphate buffer and glycerol solution with concentration 0.58 mg mL^{-1}. Finally the reaction was started by the addition of 0.2 μmol of (+)-potassium Ds-threo-isocitrate in solution. 3-Nitropropionic acid served as the positive control. The inhibitory activity of DMSO alone (10 μL) was subtracted from the activities of the evaluated compounds.

5.4. In vitro cytotoxicity assay

Measurement of the *in vitro* cytotoxicity of the most active compounds was carried out in the human hepatic cell line Hep G2 (passage 12) (Health Protection Agency Culture Collections - ECACC, Salisbury, UK) using the CellTiter 96 AQueous One Solution Assay method (Promega G3580, East Port, Prague, Czech Republic). To start, 10, 000 cells were placed in each well of a 96-well plate (NUNC, Schoeller, Prague, Czech Republic). The incubation medium was composed of Minimum Essentials Eagle Medium (Sigma–Aldrich, Prague, Czech Republic), 1% glutamine, 10% foetal bovine serum (PAA, Biotech, Prague, Czech Republic) and 1% nonessential amino acids. The incubation period was 24 h and carried out in an incubation device (Shel Lab, Cornelius, OR, USA) at 37 °C with a 5% atmosphere of CO_2. The cells were then inspected microscopically. Each of the tested compounds was dissolved in DMSO and evaluated at eight concentrations in quadruplicate. A control for determination of 100% viability, a control for action of 1% DMSO, a control for determination of 0% viability (incubation with 10% DMSO), a control for determination of possible interaction of the tested compound with the reagent, and a control for the background of the incubation medium were also prepared. The tested compounds and control samples were incubated with the cells for 24 h at 37 °C. After the incubation period, the reagent from the kit was added to the wells and the mixture was maintained at 37 °C for 1.5 h. Then, the absorbance at 490 nm was measured with a plate analyser Infinite M200 (Tecan Group, Männedorf, Switzerland). The results were statistically evaluated in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and the IC_{50} value for each tested compound was determined using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA, USA).

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