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Short communication

Thiosemicarbazide, a fragment with promising indolamine-2,3-dioxygenase (IDO) inhibition properties

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ABSTRACT

With the aim to explore the interest of the thiosemicarbazide scaffold for the inhibition of the indoleamine 2,3-dioxygenase (IDO), a promising therapeutic target for anticancer immunotherapy, a series of 32 phenylthiosemicarbazide derivatives was prepared and their IDO inhibition evaluated. Our study demonstrated that among these derivatives, compound **14** characterized with a 4-cyanophenyl group on the thiosemicarbazide was the more potent IDO inhibitor in this series being endowed with an IC_{50} of 1.2 μM. The SAR depicted showed that substitution in the 3- and 4-position relative to the phenylthiosemicarbazide are very promising whereas substitution in the 2-position always leads to less potent or inactive derivatives. In fact the study highlighted a novel interesting scaffold for IDO inhibition for further development.

1. Introduction

Immune dysregulation is one of the features of tumor growth and progression, a key event that allows for tumor evasion of the host immune system. Among the different mechanisms manipulated by tumors during their growth is the upregulation of the immunoregulatory enzyme indoleamine 2,3-dioxygenase [1]. Indeed, this enzyme plays a dual role in tumorigenesis by restricting the development of cancers that are driven by chronic inflammation, such as colon cancer [2], while promoting the development of tumors that are controlled by inflammation, such as certain types of skin tumors [3].

IDO is an extrahepatic cytosolic heme-containing dioxygenase that catalyses the initial and rate limiting step of L-tryptophan (L-Trp) along the kynurenine pathway. Through the local Trp depletion and the production of kynurenine and other downstream metabolites, IDO thus regulates immune response, suppressing effector T-cell functions and favoring the differentiation of regulatory T cells [4–5]. Moreover, it has been shown that an increased level of IDO expression in tumor cells is correlated with poor prognosis for survival in cancer. Indeed, it is now very clear that IDO is a very promising target for anticancer immunotherapy [6–8].

In the last years several small molecular-weight IDO inhibitors have been identified by natural products isolation [9,11], structure-based modifications [10–12], and in *silico* drug design [13–16]. However, to date, only two compounds have entered clinical trials: the 1-methyl-D-tryptophan (D-1MT) which was indeed shown not to be an IDO inhibitor in contrast to its L-isomer (L-1MT), and the hydroxylamidine INCB024360 (structure undisclosed) developed by Incyte corp [17,18]. Therefore, there exists a continuing need for the development of new IDO inhibitors.

In the present work, we investigated a series of thiosemicarbazide compounds that was designed by modification of the benzo[*d*]thiazol-2(3*H*)thione (**BTT**), a known IDO inhibitor (Fig. 1) [14]. Interestingly, the resulting phenylthiosemicarbazide series proved to be potent IDO inhibitors with inhibitory potency in the low micromolar range.

2. Results and discussion

As an initial step in this study, the **BTT** compound that was previously reported as an IDO inhibitor (IC_{50} = 50 μM) was docked inside the IDO binding cleft. It should be noted that the thiazole-

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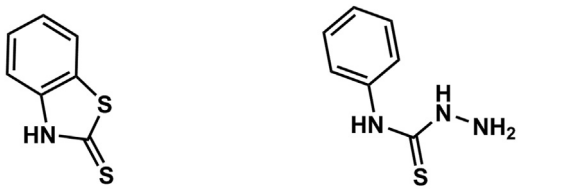
0223-5234/



benzo[*d*]thiazole-2(3*H*)-thione (BTT)
IC_{50} = 50 μM
LE = 0,60

***N*-phenylthiosemicarbazide (1)**
IC_{50} = 50 μM
LE = 0,55

Fig. 1. Structures of benzo[*d*]thiazole-2(3*H*)-thione (**BTT**), a known fragment endowed with IDO inhibition properties and the *N*-phenylthiosemicarbazide (**1**) investigated in this paper.

thione form was used for docking and not the thiazole-thiol tautomeric form, as in solution the thiazole-thione should predominate. Interestingly, in our docking experiments using the automated GOLD docking algorithm, the **BTT** compound was found deeply inserted inside the IDO binding cleft, interacting with the heme iron *via* its thione moiety, in contrast to a previously published docking of this compound that predicted a binding conformation stabilized through interaction of the thiazolic S-atom with the heme iron [14]. Moreover our study also showed that in this particular orientation, the substitution of the benzo[*d*]thiazole-2(3*H*)-thione in the 5-position with a chlorine atom is still possible and the compound very well stabilized inside the IDO binding cleft. This result is in agreement with previous data showing that the 5-chloro-benzo[*d*]thiazole-2(3*H*)-thione was also a good IDO inhibitor being characterized with an IC_{50} of 50 μM [14]. Indeed, in our modelling study, none of the conformations generated for the benzo[*d*]thiazole-2(3*H*)-thione substituted or not are found in an orientation stabilized through interaction between the thiazolic S-atom and the heme iron, as initially suggested. On the contrary these compounds are stabilized through an interaction between the thione moiety present in the inhibitor and the heme iron that is in agreement with the higher electron density around the thiole function as compared to the S atom of the thiazole ring.

Interestingly, during our analysis we observed that in such orientation, the thiazolic S-atom is just slightly too distant from the Ser167 residue to allow a strong H-bond interaction. We thus reasoned that extending that part of the molecule with a hydrogen bond donor group such as an amino could provide a better stabilization inside the IDO active site. This led us to design the thiosemicarbazide motif which presents some analogy to the thiazole-thione moiety. Moreover this fragment has known chelating properties and has not yet been investigated on IDO.

Docking of the phenyl-thiosemicarbazide (**1**) prototype provided the confirmation that a very similar binding is obtained when compared to **BTT**, with an additional stabilization between the terminal amino group of (**1**) and the hydroxyl group of Ser167. The overlay also demonstrates that the thione in **BTT** and in (**1**) are very well superimposed and shows that only small differences appear in the orientation of the aromatic ring (see Fig. 2).

Compound **1**, which was prepared according to the procedure described hereunder, was tested for IDO inhibition and displayed a promising IC_{50} of 50 μM and a very good ligand efficiency (LE) of 0.55 which prompted us to further investigate this series of compounds. The synthetic route for the thiosemicarbazide derivatives **1**–**30** is outlined in Scheme 1. The thiosemicarbazide moiety was linked with differently substituted aryls, varying the length and the nature of the substituents (Scheme 1). For the sake of an easily reproducible methodology, the compounds were prepared in good yields through the nucleophilic addition reaction of hydrazine hydrate with conveniently substituted corresponding isothiocyanates at room temperature under Argon [19]. For the synthesis of derivatives **31**–**32** we used the commercial 1-methyl-1-phenylhydrazine instead of hydrazine hydrate (Scheme 2). The structure and purity of all compounds were confirmed by means of ^{1}H and ^{13}C NMR, ^{19}F NMR (where applicable), LC–MS and elemental analysis (C, H, N, S).

All the synthesized thiosemicarbazide compounds **1**–**32** were evaluated for their capacity to inhibit IDO. To this end, a colorimetric in *vitro* IDO inhibition assay was used as previously described [20,21]. Briefly, the inhibitors were incubated 5 min at 37 °C with IDO and L-Trp, the natural substrate. Trichloroacetic acid (TCA) was then added to quench the reaction and the N-formylkynurenine generated was hydrolysed to kynurenine during the next 30 min. After that time, *p*-dimethylaminobenzaldehyde (*p*DMAB) was added to the reaction mixture to form a Schiff base with kynurenine. The IDO residual activity was measured at 490 nm which corresponds to absorption of the Schiff base formed. The IDO inhibitory potency of the compounds is indicated by IC_{50} values that were calculated by nonlinear regression analysis of the concentration–response curves obtained for each compound, in triplicate. The results are reported in Table 1. The compounds 1-methyl-tryptophan (1-MT) and **BTT** were used as reference IDO inhibitors.

A number of thiosemicarbazide compounds exhibit good inhibition of IDO activity. Among them, compound **14** displayed the most potent IDO inhibitory activity (IC_{50} = 1.2 μM) that is a substantial improvement compared to the positive control **1 MT** (IC_{50} = 100 μM) and a 41-fold improvement compared to **BTT** and compound **1**, our starting point in this study.

Structure–activity relationships in these thiosemicarbazide derivatives (**1**–**30**) demonstrated that the substitution of the aromatic ring in the 2-position (compounds **2**–**4**) always leads to non-potent IDO inhibitors (IC_{50} > 50 μM) whereas the substitution of the aromatic ring in the 3- (compounds **5**–**9**) or 4-positions (compounds **11**–**14** and **19**) with relatively small substituents are well tolerated and potent IDO inhibitors are obtained. Particularly, introducing a bromine, a fluorine or a cyano group in the 4-position of the aryl ring (compounds **11, 12** and **14**) leads to potent IDO inhibitors with IC_{50} of 1.8, 1.6 and 1.2 μM, respectively. On the contrary, introduction of larger groups is detrimental for IDO inhibition (**15**–**19**). Regarding the di-substitution, apart from compound **20** characterized by a 2,5-dichloro substitution which retains some IDO inhibition (IC_{50} = 18 μM), the presence of a substituent in the 2-position of the aromatic ring again leads to inactive compounds (**21**–**24**, **29**, **30**). Indeed only compounds possessing substituents in the 3,4- or 3,5-positions retain some IDO inhibition (**25**, **26**, **28**), although there are less potent than the monosubstituted derivatives.

The influence of the substitution of the terminal amino group of thiosemicarbazide was also investigated and showed that this substitution leads to inactive compounds (compare **31** and **25**, and **32** and **28**, respectively). This result is in agreement with the proposed binding orientation which involves the interaction of the terminal amino group of the thiosemicarbazide motif with the Ser167 residue thus preventing any substitution at this position.

3. Conclusions

In the present work, we aimed at exploring the interest of the thiosemicarbazide scaffold as an isostere of the thiazole-2(3*H*)-thione motif present in **BTT**, a known IDO inhibitor. Interestingly, our study confirms that potent IDO inhibitors can be obtained with appropriate substitution of the aromatic ring bearing the

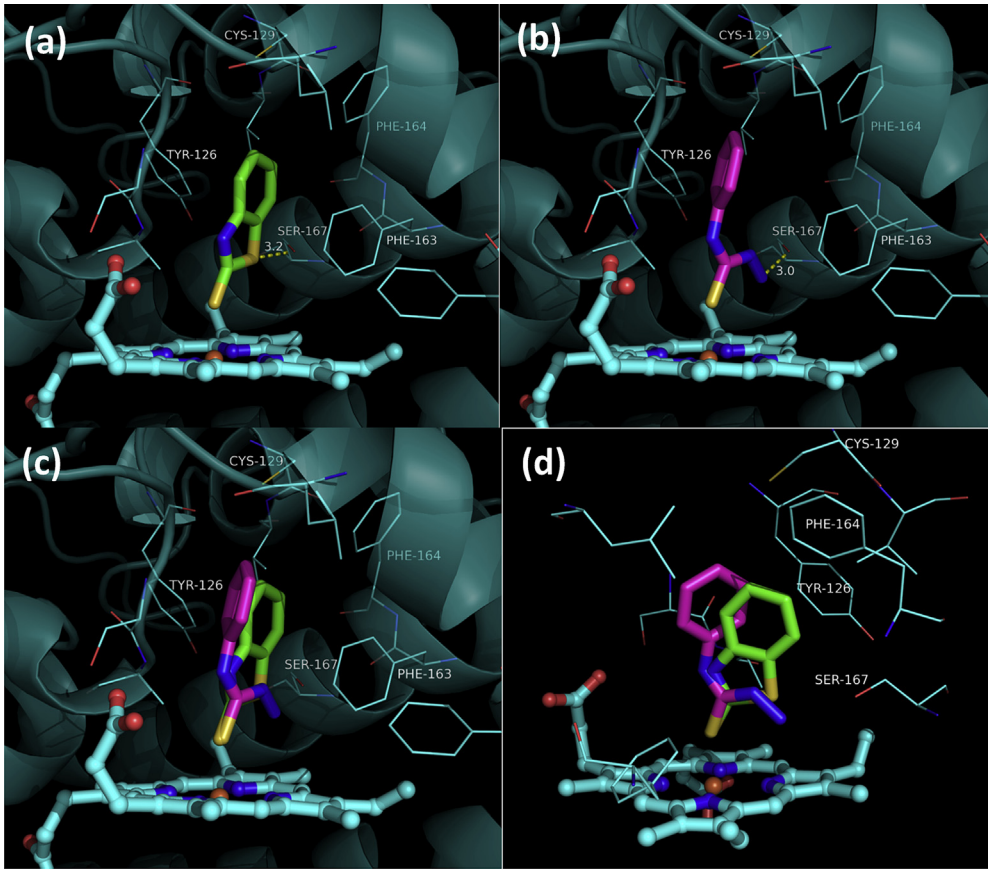
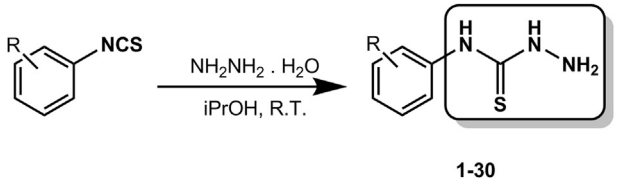
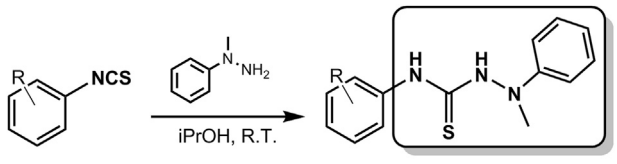


Fig. 2. View of (a) benzo[*d*]thiazole-2(3*H*)-thione (BTT), (b) phenyl-thiosemicarbazide (**1**), (c and d) superimposition of both inside the IDO binding cleft. Pictures were made using Pymol from Delano Scientific.



1-30

Scheme 1. Synthesis of thiosemicarbazides **1**–**30.**



31-32

Scheme 2. Synthesis of thiosemicarbazides **31**–**32.**

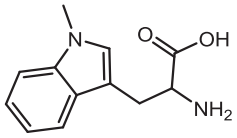
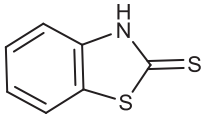
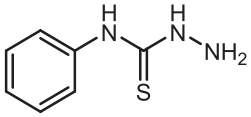
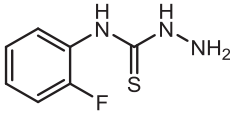
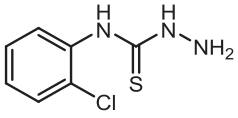
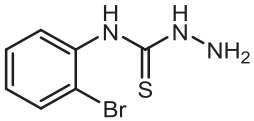
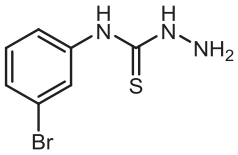
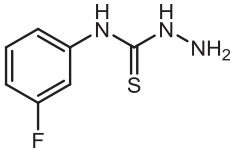
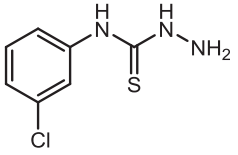
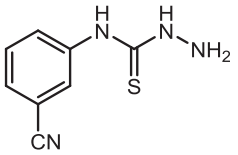
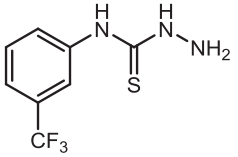
thiosemicarbazide motif. Substitution in the 4-position relative to the thiosemicarbazide, notably with groups such as the nitrile in compound **14**, the more potent compound in the present study, or halogens (**11**–**13**) seems very promising whereas substitution in the 2-position always leads to less potent derivatives. Interestingly, owing to their small size, these compounds are characterized with excellent ligand efficiencies (LE around 0.6 for the best derivatives) thus suggesting that these derivatives are very interesting starting points for further optimizations.

4. Experimental protocols

4.1. Materials and measurements

All chemical reagents and solvents were used as obtained from commercial sources (Sigma Aldrich, Acros, Maybridge, Apollo, Fisher Scientific). All reactions were performed under an inert argon (Alphagaz 2) atmosphere, unless stated otherwise. Melting points were determined with a Buchi B-540 capillary melting point apparatus in open capillaries and are uncorrected. Thin layer chromatography (TLC) was performed on silica gel plates (60F254, 0.2 mm thick, Merck) with visualization under ultraviolet light (254 and 365 nm). ^{1}H NMR spectra were recorded in DMSO-d_6 solution on a Jeol JNM EX 400 spectrometer at 400 MHz with tetramethylsilane (TMS) as internal standard. ^{13}C spectra were recorded on the same spectrometer in DMSO-d_6 solution at 100 MHz. Chemical shifts (δ) are expressed in ppm downfield from tetramethylsilane. Elemental analyses (C, H, N, S) were performed on a Thermo Finnigan- FlashEA 1112 apparatus. Analytical LC/MS analyses were performed on an Agilent 1100 series HPLC coupled with an MSD Trap SL system using UV detection at 254 and 361 nm. Mass spectra were recorded using electron spray ionization (ESI) operating in positive mode, unless stated otherwise. The following method was applied: injection of 10 μL of a 20 μg mL^{-1} acetonitrile solution onto a C18 3.5 μm Zorbax SB column (100 mm × 3 mm); separation using a gradient (flow rate of 0.5 mL min^{-1}) of acetonitrile in acetic acid (0.1% v/v in water) from 5% to 95% acetonitrile over 5 min, holding for 3 min, then reversing to 5% acetonitrile within 0.1 min and holding for an additional 5.4 min. Automated flash

Table 1
IDO inhibition by thiosemicarbazide derivatives (**1**–**32**).

Compound	Structure	IDO IC_{50} (μM)	Ligand efficiency[a] (LE) = 1.4 × pIC_{50}/HAC
L-1MT		100	0.35
BTT		50	0.60
1		~50	0.55
2		>50	–
3		>50	–
4		>50	–
5		2.7	0.65
6		3.7	0.63
7		15	0.56
8		3.8	0.58
9		41	0.41

(*continued on next page*)

Table 1 (*continued*)

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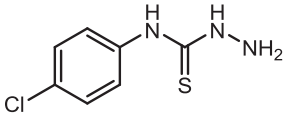
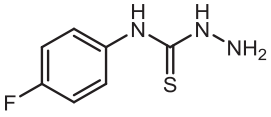
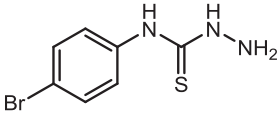
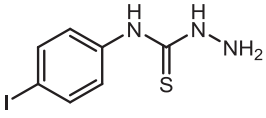
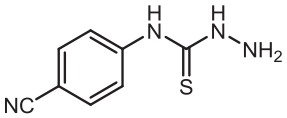
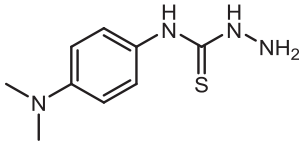
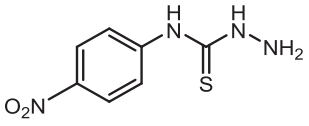
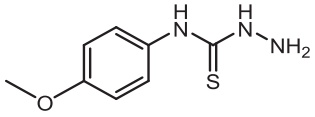
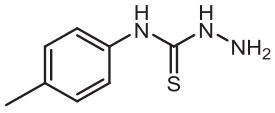
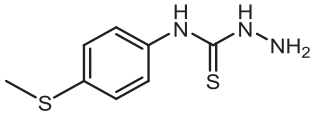
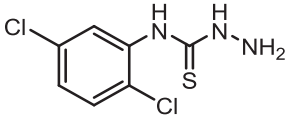
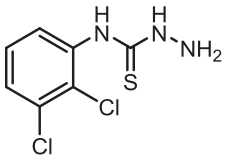
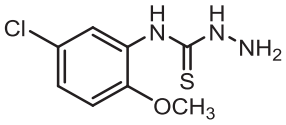
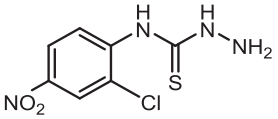
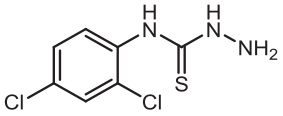
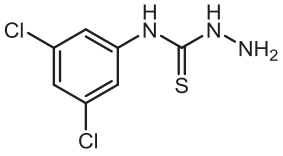
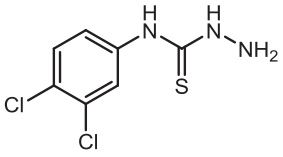
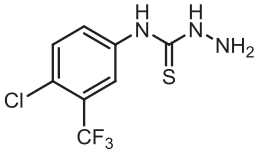
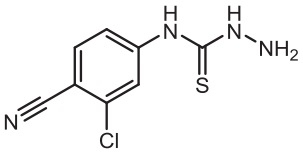
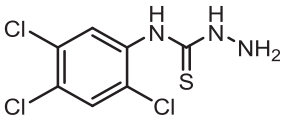
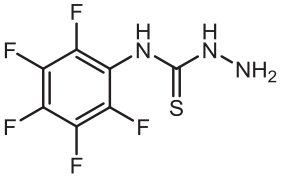
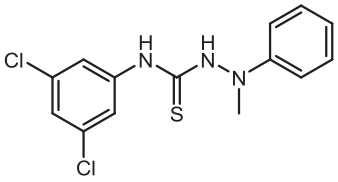
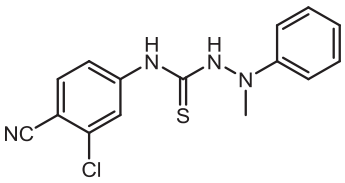
10		>50	–
11		1.8	0.67
12		1.6	0.68
13		3.5	0.64
14		1.2	0.64
15		>50	–
16		>50	–
17		>50	–
18		>50	–
19		34	0.48
20		18	0.51
21		>50	–

Table 1 (*continued*)

Compound	Structure	IDO IC_{50} (μM)	Ligand efficiency[a] (LE) = 1.4 × pIC_{50}/HAC
22		>50	–
23		>50	–
24		>50	–
25		13	0.53
26		17	0.51
27		>50	–
28		34	0.45
29		>50	–
30		>50	–
31		>50	–

(*continued on next page*)

Table 1 (*continued*)

Compound	Structure	IDO IC_{50} (μM)	Ligand efficiency[a] (LE) = 1.4 × pIC_{50}/HAC
32		>50	–

[a] HAC = heavy atom count (number of non-hydrogen atoms).

chromatography was performed on a Biotage AB SP1 system equipped with prepacked flash KP-Sil silica cartridges. The following gradient of ethyl acetate in cyclohexane (unless stated otherwise) was used for elution: the elution started with an ethyl acetate/cyclohexane ratio of 12/88 for one column volume (CV); the ratio increased to 62/38 over 5 CV, kept for 1 CV, then increased to 100/0 over 7 CV, and finally kept at this value over 5 CV. The product detection was by UV absorption at 254 and 320 nm. The products were precipitated by concentration of the pooled column fractions combined with the addition of cyclohexane. The precipitate formed was filtered off, washed twice with cyclohexane, and dried at 40 °C in vacuum to yield an analytically pure sample. All new compounds were determined to be >95% pure by LC/MS.

4.2. General procedure for the synthesis of thiosemicarbazide derivatives (**1**–**30**)

To a stirred solution of hydrazine hydrate (85%, 2.4 mmol) in 20 mL of isopropanol, appropriately substituted isothiocyanate (2.0 mmol) was added at room temperature. Precipitate was formed immediately. Stirring was continued for 3 h. Then the mixture was filtered and the precipitate was washed with isopropanol three times to give the desired product.

4.2.1. 4-Phenylthiosemicarbazide (**1**)

Yield 91%. Mp. 138–141 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.80 (s, 1H, NH), 9.24 (s, 1H, NH), 7.57–7.54 (m, 2H, H3′, H5′), 7.26 (t, J = 7.7 Hz, 2H, H2′, H6′), 7.05 (t, J = 7.2 Hz, 1H, H4′), 5.10 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 181.7, 139.2, 128.9, 128.8, 124.5, 121.6, 121.3. LC–MS R_t 4.7 min; m/z [MH^+] 168. Anal. Calcd. for $C_7H_9N_3S$: C, 50.28; H, 5.42; N, 25.13; S, 19.17. Found: C, 49.83; H, 5.31; N, 25.34; S, 19.19.4.2.

4.2.2. 4-(2-Fluorophenyl)-3-thiosemicarbazide (**2**)

Yield 77%. Mp. 164–166 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.33 (s, 1H, NH), 8.02 (s, 1H, NH), 7.43–7.34 (m, 1H, H6′), 7.23–7.10 (m, 3H, H3′, H4′, H5′), 4.89 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.7, 155.8, 127.8, 127.3, 126.5, 124.2, 115.7. LC–MS R_t 4.7 min; m/z [MH^+] 186. Anal. Calcd. for $C_7H_8FN_3S$: C, 45.39; H, 4.35; N, 22.69; S, 17.31. Found: C, 45.45; H, 4.52; N, 22.57; S, 17.88.

4.2.3. 4-(2-Chlorophenyl)-3-thiosemicarbazide (**3**)

Yield 89%. Mp. 131–133 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.43 (s, 1H, NH), 9.15 (s, 1H, NH), 8.36 (d, J = 8.2 Hz, 1H, H3′), 7.45 (d, J = 8.0 Hz, 1H, H6′), 7.28 (t, J = 7.8 Hz, 1H, H5′), 7.14 (t, J = 7.7 Hz, 1H, H4′), 4.98 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 179.6, 136.5, 129.8, 129.6, 127.3, 126.6, 126.1. LC–MS R_t 5.2 min; m/z [MH^+] 202. Anal. Calcd. for $C_7H_8BrN_3S$: 41.69; H, 4.00; N, 20.84; S, 15.90. Found: C, 41.53; H, 3.38; N, 20.65; S, 16.08.

4.2.4. 4-(2-Bromophenyl)-3-thiosemicarbazide (**4**)

Yield 87%. Mp. 103–105 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.82 (s, 1H, NH), 7.51 (s, 1H, NH), 7.35–7.31 (m, 2H, H3′, H5′), 7.20–7.17 (m, 2H, H2′, H6′), 5.00 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 181.8, 139.3, 129.6, 125.7, 122.8, 121.6, 121.2. LC–MS R_t 5.4 min; m/z [MH^+] 246; 248. Anal. Calcd. for $C_7H_8BrN_3S$: 34.16; H, 3.28; N, 17.07; S, 13.03. Found: C, 35.03; H, 3.38; N, 17.34; S, 13.08.

4.2.5. 4-(3-Bromophenyl)-3-thiosemicarbazide (**5**)

Yield 87%. Mp. 103–105 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.82 (s, 1H, NH), 7.51 (s, 1H, NH), 7.35–7.31 (m, 2H, H3′, H5′), 7.20–7.17 (m, 2H, H2′, H6′), 5.00 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 181.8, 139.3, 129.6, 125.7, 122.8, 121.6, 121.2. LC–MS R_t 5.5 min; m/z [MH^+] 246; 248. Anal. Calcd. for $C_7H_8BrN_3S$: 34.16; H, 3.28; N, 17.07; S, 13.03. Found: C, 35.03; H, 3.38; N, 17.34; S, 13.08.

4.2.6. 4-(3-Fluorophenyl)-3-thiosemicarbazide (**6**)

Yield 86%. Mp. 135–138 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.27 (s, 1H, NH), 7.82 (s, 1H, NH), 7.51–7.40 (m, 1H, H5′), 7.24–7.30 (m, 1H, H2′), 7.20–7.10 (m, 1H, H6′), 6.98–6.87 (m, 1H, H4′), 4.87 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 179.5, 161.3, 140.2, 130.1, 119.4, 110.7, 110.1. LC–MS R_t 5.0 min; m/z [MH^+] 186. Anal. Calcd. for $C_7H_8FN_3S$: C, 45.39; H, 4.35; N, 22.69; S, 17.31. Found: C, 45.45; H, 4.54; N, 22.97; S, 17.41.

4.2.7. 4-(3-Chlorophenyl)-3-thiosemicarbazide (**7**)

Yield 66%. Mp. 170–173 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.27 (s, 1H, NH), 7.96 (s, 1H, NH), 7.53 (s, 1H, H6′), 7.29 (t, J = 15.3 Hz, 1H, H3′), 7.24–7.20 (m, 1H, H2′), 7.11–7.09 (d, J = 6.4 Hz, 1H, H4′), 4.99 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 179.7, 141.1, 132.6, 130.1, 124.2, 123.3, 122.4. LC–MS R_t 6.5 min; m/z [MH^+] 202. Anal. Calcd. for $C_7H_8ClN_3S$: C, 41.69; H, 4.00; N, 20.84; S, 15.90. Found: C, 41.45; H, 3.63; N, 20.57; S, 15.28.

4.2.8. 4-(3-Cyanophenyl)-3-thiosemicarbazide (**8**)

Yield 76%. Mp. 152–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.38 (s, 1H, NH), 8.21 (s, 1H, NH), 7.97 (s, 1H, H2′), 7.91–7.88 (m, 1H, H4′), 7.60–7.64 (m, 2H, H5′, H6′), 4.98 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 179.8, 140.8, 130.0, 129.0, 128.0, 127.1, 119.3, 111.1. LC–MS R_t 4.7 min; m/z [MH^+] 193. Anal. Calcd. for $C_8H_8N_4S$: C, 49.98; H, 4.19; N, 29.14; S, 16.68. Found: C, 49.61; H, 4.14; N, 28.89; S, 16.69.

4.2.9. 4-[3-(Trifluoromethyl)phenyl]-3-thiosemicarbazide (**9**)

Yield 65%. Mp. 117–120 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.33 (s, 1H, NH), 8.22 (s, 1H, NH), 7.97 (s, 1H, H2′), 7.98–7.86 (m, 1H, H4′), 7.50 (t, J = 15.57 Hz, 1H, H5′), 7.38 (d, J = 6.64 Hz, 1H, H6′), 4.98 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.8, 140.8, 129.8, 129.0, 128.0, 127.1, 123.3, 121.1. LC–MS

R_t 5.7 min; *m/z* [MH^+] 236. Anal. Calcd. for $C_8H_8F_3N_3S$: C, 40.85; H, 3.43; N, 17.86; S, 13.63. Found: C, 40.43; H, 3.36; N, 17.84; S, 13.16.

*4.2.10. 4-(4-Chlorophenyl)-3-thiosemicarbazide (**10**)*

Yield 81%. Mp. 178–181 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.77 (s, 1H, NH), 7.56 (s, 1H, NH), 7.51 (s, 1H, H2′), 7.30–7.27 (m, 1H, H4′), 7.23–7.21 (m, 1H, H6′), 7.12–7.09 (m, 1H, H5′), 4.79 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 181.7, 146.9, 133.5, 133.2, 130.5, 126.6, 126.2. LC–MS R_t 5.4 min; *m/z* [MH^+] 202. Anal. Calcd. for $C_7H_8ClN_3S$: 41.69; H, 4.00; N, 20.84; S, 15.90. Found: C, 41.83; H, 4.38; N, 20.91; S, 15.78.

*4.2.11. 4-(4-Fluorophenyl)-3-thiosemicarbazide (**11**)*

Yield 96%. Mp. 175–178 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.67 (s, 1H, NH), 9.11 (s, 1H, NH), 7.78–7.38 (m, 2H, H2′, H6′), 7.09 (t, J = 8.8 Hz, 2H, H3′, H5′), 4.73 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 180.2, 159.5, 135.7, 126.5, 126.3, 115.4, 114.9. LC–MS R_t 4.8 min; *m/z* [MH^+] 186. Anal. Calcd. for $C_7H_8FN_3S$: C, 45.39; H, 4.35; N, 22.69; S, 17.31. Found: C, 45.45; H, 4.47; N, 22.70; S, 17.86.

*4.2.12. 4-(4-Bromophenyl)-3-thiosemicarbazide (**12**)*

Yield 90%. Mp. 166–169 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 10.27–9.37 (m, 1H, NH), 9.21 (s, 1H, NH), 7.62 (d, J = 7.2 Hz, 2H, H2′, H6′), 7.42 (d, J = 8.7 Hz, 2H, H3′, H5′), 4.80 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 176.3, 138.0, 131.3, 131.2, 125.3, 125.2, 115.0. LC–MS R_t 5.5 min; *m/z* [MH^+] 246; 248. Anal. Calcd. for $C_7H_8BrN_3S$: C, 34.16; H, 3.28; N, 17.07; S, 13.03. Found: C, 34.46; H, 3.38; N, 17.17; S, 13.33.

*4.2.13. 4-(4-Iodophenyl)-3-thiosemicarbazide (**13**)*

Yield 89%. Mp. 182–184 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.11 (s, 1H, NH), 7.98 (s, 1H, NH), 7.34–7.29 (m, 2H, H3′, H5′), 7.24–7.20 (m, 2H, H2′, H6′), 4.92 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 176.7, 139.7, 137.2, 137.1, 122.5, 121.3, 96.4. LC–MS R_t 5.7; *m/z* [MH+] 295. Anal. Calcd. for $C_7H_8IN_3S$: C, 28.68; H, 2.75; N, 14.34; S, 10.94. Found: C, 28.82; H, 2.86; N, 14.11; S, 10.73.

*4.2.14. 4-(4-Cyanophenyl)-3-thiosemicarbazide (**14**)*

Yield 80%. Mp. 175–177 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.47 (s, 1H, NH), 9.23 (s, 1H, NH), 8.02 (d, J = 8.1 Hz, 2H, H3′, H5′), 7.70 (d, J = 8.4 Hz, 2H, H2′, H6′), 5.00 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.3, 142.4, 131.5, 131.3, 120.8, 120.4, 119.3, 102.2. LC–MS R_t 6.0 min; *m/z* [MH^+] 193. Anal. Calcd. for $C_8H_8N_4S$: C, 49.98; H, 4.19; N, 29.14; S, 16.68. Found: C, 49.53; H, 4.36; N, 28.84; S, 16.19.

*4.2.15. 4-[(4-Dimethylamino)phenyl]-3-thiosemicarbazide (**15**)*

Yield 71%. Mp. 190–192 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.33 (s, 1H, NH), 8.83 (s, 1H, NH), 7.27 (d, J = 8.5, 2H, H2′, H6′), 6.69–6.55 (m, 2H, H3′, H5′), 4.56 (s, 2H, NH_2), 2.83 (s, 6H, 2 X CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.3, 148.1, 132.2, 127.2, 126.9, 113.2, 113.2, 41.9, 41.8. LC–MS R_t 2.7 min; *m/z* [MH^+] 211. Anal. Calcd. for $C_9H_{14}N_4S$: 51.40; H, 6.71; N, 26.64; S, 15.24. Found: C, 51.83; H, 6.58; N, 26.54; S, 15.58.

*4.2.16. 4-(4-Nitrophenyl)-3-thiosemicarbazide (**16**)*

Yield 76%. Mp. 188–190 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.52 (s, 1H, NH), 8.95 (s, 1H, NH), 7.50–7.44 (m, 2H, H2′, H6′), 4.75 (s, 2H, NH_2), 3.70 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 183.0, 143.7, 143.2, 125.1, 125.0, 121.0, 120.9. LC–MS R_t 4.3 min; *m/z* [MH^+] 198. Anal. Calcd. for $C_8H_{11}N_3OS$: C, 48.71; H, 5.62; N, 21.30; S, 16.25. Found: C, 48.76; H, 5.57; N, 21.50; S, 16.26.

*4.2.17. 4-(4-Methoxyphenyl)-3-thiosemicarbazide (**17**)*

Yield 86%. Mp. 154–156 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.61 (s, 1H, NH), 9.33 (s, 1H, NH), 8.20–8.09 (m, 4H, H2′, H3′, H5′, H6′), 4.73 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.7, 154.8, 133.2, 122.4, 122.3, 114.2, 114.1, 56.2. LC–MS R_t 4.3 min; *m/z* [MH^+] 198. Anal. Calcd. for $C_7H_8N_4O_2S$: C, 39.62; H, 3.80; N, 26.40; S, 15.11. Found: C, 39.45; H, 3.57; N, 26.50; S, 15.26.

*4.2.18. 4-(4-Methylphenyl)-3-thiosemicarbazide (**18**)*

Yield 90%. Mp. 142–144 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.70 (s, 1H, NH), 9.02 (s, 1H, NH), 7.45 (d, J = 7.1, 2H, H2′, H6′), 7.06 (d, J = 8.1 Hz, 2H, H3′, H5′), 4.67 (s, 2H, NH_2), 2.23 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.8, 137.7, 132.7, 130.6, 130.3, 121.0, 120.9, 21.1. LC–MS R_t 4.5 min; *m/z* [MH^+] 182. Anal. Calcd. for $C_8H_{11}N_3S$: C, 53.01; H, 6.12; N, 23.18; S, 17.69. Found: C, 53.45; H, 6.27; N, 23.30; S, 17.86.

*4.2.19. [4-(4-Methylthio)phenyl]-3-thiosemicarbazide (**19**)*

Yield 96%. Mp. 166–167 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.42 (s, 1H, NH), 9.10 (s, 1H, NH), 7.55 (d, J = 7.5 Hz, 2H, H3′, H5′), 7.25–7.10 (m, 2H, H2′, H6′), 4.91 (s, 2H, NH_2), 2.43 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.7, 136.9, 133.4, 124.3, 124.2, 123.8, 123.7, 16.5. LC–MS R_t 4.7 min; *m/z* [MH^+] 214. Anal. Calcd. for $C_8H_{11}N_3S_2$: C, 45.04; H, 5.20; N, 19.70; S, 30.06. Found: C, 45.45; H, 5.47; N, 19.70; S, 30.36.

*4.2.20. 4-(2,5-Dichlorophenyl)-3-thiosemicarbazide (**20**)*

Yield 79%. Mp. 156–158 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 9.64 (s, 1H, NH), 8.70 (s, 1H, NH), 7.50 (d, J = 8.6 Hz, 1H, H4′), 7.25–7.11 (m, 2H, H3′, H6′), 4.97 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 184.6, 138.2, 132.8, 130.2, 128.9, 124.8, 123.5. LC–MS R_t 5.7 min; *m/z* [MH^+] 236. Anal. Calcd. for $C_7H_7Cl_2N_3S$: C, 35.61; H, 2.99; N, 17.80; S, 13.58. Found: C, 35.94; H, 3.00; N, 17.96; S, 12.98.

*4.2.21. 4-(2,3-Dichlorophenyl)-3-thiosemicarbazide (**21**)*

Yield 96%. Mp. 123–126 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 9.60–9.47 (m, 1H, NH), 8.22 (s, 1H, NH), 7.48–7.20 (m, 3H, H4′, H5′, H6′), 4.42 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 184.1, 136.4, 132.9, 127.8, 127.4, 126.5, 122.4. LC–MS R_t 5.8 min; *m/z* [MH^+] 236. Anal. Calcd. for $C_7H_7Cl_2N_3S$: C, 35.61; H, 2.99; N, 17.80; S, 13.58. Found: C, 35.89; H, 3.07; N, 17.61; S, 13.04.

*4.2.22. 4-(5-Chloro-2-methoxyphenyl)-3-thiosemicarbazide (**22**)*

Yield 97%. Mp. 167–170 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 10.05 (s, 1H, NH), 9.41 (s, 1H, NH), 9.02 (s, 1H, H4′), 7.12–6.98 (m, 2H, H3′, H6′), 4.88 (s, 2H, NH_2), 3.82 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 178.4, 148.3, 129.8, 123.8, 123.2, 119.8, 118.4, 112.8. LC–MS R_t 5.9 min; *m/z* [MH^+] 232. Anal. Calcd. for $C_8H_{10}ClN_3OS$: C, 41.97; H, 4.35; N, 18.14; S, 13.84. Found: C, 40.92; H, 4.28; N, 17.84; S, 13.76.

*4.2.23. 4-(2-Chloro-4-nitrophenyl)-3-thiosemicarbazide (**23**)*

Yield 84%. Mp. 191–194 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.95 (s, 1H, NH), 9.16 (d, J = 9.2 Hz, 1H, H3′), 8.36 (d, J = 2.6 Hz, 1H, H5′), 8.19 (dd, J = 9.2, 2.7 Hz, 2H, NH, H6′), 5.00 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 184.8, 142.5, 138.8, 126.0, 124.4, 124.3, 119.7. LC–MS R_t 5.8 min; *m/z* [MH^+] 247. Anal. Calcd. for $C_7H_7ClN_4O_2S$: C, 34.08; H, 2.86; N, 22.71; S, 13.00. Found: C, 34.98; H, 2.99; N, 22.91; S, 12.78.

*4.2.24. 4-(2,4-Dichlorophenyl)-3-thiosemicarbazide (**24**)*

Yield 64%. Mp. 169–171 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.50 (s, 1H, NH), 8.37 (s, 1H, NH), 7.63 (s, 1H, H3′), 7.38–7.36 (m, 2H, H5′, H6′), 4.99 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-

d_6): δ (ppm) = 179.7, 135.9, 134.1, 129.9, 127.9, 127.5, 126.3. LC–MS R_t 5.9 min; *m/z* [MH^+] 236. Anal. Calcd. for $C_7H_7Cl_2N_3S$: C, 35.61; H, 2.99; N, 17.80; S, 13.58. Found: C, 35.49; H, 2.99; N, 17.84; S, 13.33.

*4.2.25. 4-(3,5-Dichlorophenyl)-3-thiosemicarbazide (**25**)*

Yield 77%. Mp. 157–159 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.43 (s, 1H, NH), 7.93 (s, 1H, NH), 7.24 (s, 1H, H4′), 7.00–6.97 (m, 2H, H2′, H6′), 4.88 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 179.5, 142.4, 133.5, 133.3, 123.5, 121.8, 119.5. LC–MS R_t 6.1 min; *m/z* [MH^+] 236. Anal. Calcd. for $C_7H_7Cl_2N_3S$: C, 35.61; H, 2.99; N, 17.80; S, 13.58. Found: C, 35.69; H, 3.03; N, 17.85; S, 13.96.

*4.2.26. 4-(3,4-Dichlorophenyl)-3-thiosemicarbazide (**26**)*

Yield 79%. Mp. 169–172 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 8.82 (s, 1H, NH), 7.52 (s, 1H, NH), 7.36 (s, 1H, H2′), 7.30–7.27 (m, 1H, H5′), 7.16–7.12 (m, 1H, H6′) 4.79 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.3, 141.6, 132.7, 131.9, 126.2, 123.6, 120.1. LC–MS R_t 5.9 min; *m/z* [MH^+] 236. Anal. Calcd. for $C_7H_7Cl_2N_3S$: C, 35.61; H, 2.99; N, 17.80; S, 13.58. Found: C, 35.63; H, 3.01; N, 17.75; S, 13.66.

*4.2.27. 4-[4-Chloro-3-(trifluoromethyl)phenyl]-3-thiosemicarbazide (**27**)*

Yield 89%. Mp. 151–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 9.42 (s, 1H, NH), 8.36 (s, 1H, NH), 7.98 (d, *J* = 7.5 Hz, 1H, H5′), 7.61 (s, 1H, H2′), 7.27–7.25 (m, 1H, H6′), 4.89 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 179.6, 137.7, 130.7, 127.5, 125.1, 123.8, 120.4. LC–MS R_t 6.1 min; *m/z* [MH^+] 272. Anal. Calcd. for $C_8H_7ClF_3N_3S$: C, 35.63; H, 2.62; N, 15.58; S, 11.89. Found: C, 35.91; H, 3.00; N, 15.77; S, 11.76.

*4.2.28. 4-[(3-Chloro-4-cyano)phenyl]-3-thiosemicarbazide (**28**)*

Yield 88%. Mp. 194–197 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 9.66 (s, 1H, NH), 8.46 (s, 1H, NH), 8.15 (s, 1H, H2′), 7.99 (d, *J* = 8.9 Hz, 1H, H5′), 7.82 (d, *J* = 8.7 Hz, 1H, H6′), 6.46 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.3, 142.7, 139.3, 132.6, 121.4, 118.5, 114.7, 99.9, 13.6. LC–MS R_t 5.6 min; *m/z* [MH+] 227. Anal. Calcd. for $C_8H_7ClN_4S$: C, 42.39; H, 3.11; N, 24.72; S, 14.14. Found: C, 42.66; H, 3.38; N, 24.54; S, 14.51.

*4.2.29. 4-(2,3,4-Trichlorophenyl)-3-thiosemicarbazide (**29**)*

Yield 74%. Mp. 170–172 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 9.69 (s, 1H, NH), 9.12 (s, 1H, NH), 8.84 (s, 1H, H3′), 7.88 (s, 1H, H6′), 4.80 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 176.9, 137.1, 130.1, 129.7, 129.1, 128.8. LC–MS R_t 6.5 min; *m/z* [MH^+] 272. Anal. Calcd. for $C_7H_6Cl_3N_3S$: C, 31.07; H, 2.24; N, 15.53; S, 11.85. Found: C, 31.74; H, 2.21; N, 15.51; S, 12.07.

*4.2.30. 4-(2,3,4,5,6-Pentafluorophenyl)-3-thiosemicarbazide (**30**)*

Yield 63%. Mp. 184–186 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 9.62 (s, 1H, NH), 8.98 (s, 1H, NH), 6.40 (s, 2H, NH_2). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 182.1, 150.0, 149.3, 139.2, 138.5, 138.3, 119.3. ^{19}F NMR (376 MHz, DMSO-d_6): δ (ppm) = 164.7 (t, *J* = 22.0, 2F), 157.2 (t, *J* = 22.0, 1F), 144.5 (d, *J* = 22.0, 2F). LC–MS R_t 5.5 min; *m/z* [MH^+] 258. Anal. Calcd. for $C_7H_4F_5N_3S$: C, 32.69; H, 1.57; N, 16.34; S, 12.47. Found: C, 33.05; H, 1.46; N, 16.40; S, 12.67.

*4.3. General procedure for the synthesis of thiosemicarbazide derivatives (**31**–**32**)*

To a stirred solution of 1-methyl-1-phenylhydrazine (2.4 mmol) in 20 mL of isopropanol, appropriately substituted isothiocyanate (2.0 mmol) was added at room temperature. Precipitate was formed immediately. Stirring was continued for 3 h. Then the mixture was filtered and the precipitate was washed with isopropanol three times to give the desired product.

*4.3.1. 4-(3,5-dichlorophenyl)-1-(methylphenyl)-3-thiosemicarbazide (**31**)*

Yield 89%. Mp. 177–179 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 10.08 (s, 2H, 2NH), 7.80 (s, 2H, H2′, H6′), 7.32 (s, 1H, H4′), 7.27 (t, *J* = 7.7 Hz, 2H, H3″, H5″), 6.87 (dd, *J* = 15.7 Hz, 8.0 Hz, 3H, H2″, H4″, H6″), 3.03 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 181.7, 149.5, 140.4, 133.3, 133.2, 129.6, 129.5, 123.5, 121.1, 119.2, 119.1, 114.3, 114.1, 44.8. LC–MS R_t 7.4 min; *m/z* [MH^+] 326. Anal. Calcd. for $C_{14}H_{13}Cl_2N_3S$: C, 51.54; H, 4.02; N, 12.88; S, 9.83. Found: C, 52.05; H, 4.05; N, 12.70; S, 9.87.

*4.3.2. 4-(3,5-dichlorophenyl)-1-(methylphenyl)-3-thiosemicarbazide (**32**)*

Yield 89%. Mp. 177–179 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) = 10.08 (s, 2H, 2NH), 7.80 (s, 2H, H2′, H6′), 7.32 (s, 1H, H4′), 7.27 (t, *J* = 7.7 Hz, 2H, H3″, H5″), 6.87 (dd, *J* = 15.7 Hz, 8.0 Hz, 3H, H2″, H4″, H6″), 3.03 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) = 181.7, 149.5, 140.4, 133.3, 133.2, 129.6, 129.5, 123.5, 121.1, 119.2, 119.1, 114.3, 114.1, 44.8. LC–MS R_t 7.4 min; *m/z* [MH^+] 326. Anal. Calcd. for $C_{14}H_{13}Cl_2N_3S$: C, 51.54; H, 4.02; N, 12.88; S, 9.83. Found: C, 52.05; H, 4.05; N, 12.70; S, 9.87.

4.4. Enzymatic assay

The IDO inhibition assay was performed using a reported procedure [12,15].

4.5. Molecular modelling

Molecular modelling experiments were performed on a windows workstation [22]. The compounds were built using the Chembiodraw 3D module and minimized using the MMFF94 forcefield. The GOLD docking software was used using the IDO X-ray structure (pdb code 2D0T) with an active site definition of 10Å around PIM chosen as center of the active site. For each compounds 30 conformations were generated and ranked according to the GOLDSCORE.

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