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Synthesis, β-glucuronidase inhibition and molecular docking studies of hybrid bisindole-thiosemicarbazides analogs

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ABSTRACT

Hybrid bisindole-thiosemicarbazides analogs (**1**–**18**) were synthesized and screened for β-glucuronidase activity. All compounds showed varied degree of β-glucuronidase inhibitory potential when compared with standard D-saccharic acid 1,4-lactone (IC_{50} = 48.4 ± 1.25 μM). Compounds **4**, **7**, **9**, **6**, **5**, **12**, **17** and **18** showed exceptional β-glucuronidase inhibition with IC_{50} values ranging from 0.1 to 5.7 μM. Compounds **1**, **3**, **8**, **16**, **13**, **2** and **14** also showed better activities than standard with IC_{50} values ranging from 7.12 to 15.0 μM. The remaining compounds **10**, **11**, and **15** showed good inhibitory potential with IC_{50} values 33.2 ± 0.75, 21.4 ± 0.30 and 28.12 ± 0.25 μM respectively. Molecular docking studies were carried out to confirm the binding interaction of the compounds.

1. Introduction

β-Glucuronidase (EC 3.2.1.31) is an inducible enzyme found in an aerobic *Escherichia*, *Bacteroides*, *Clostridia* and *Peptostreptococcus genera* which catalyzes the cleavage of β-glucuronosyl-*O*-bonds [1]. In human body, this enzyme is present in fluids and organs such as spleen, serum, bile, urine and kidney [2,3]. β-Glucuronidase improves activity in diverse pathological circumstances such as renal diseases [4], epilepsy [5], breast, larynx, testes [6] and in urinary tract infections [7–10]. Moreover, β-glucuronidase has been reported to be released in the synovial fluid in the inflammatory joint diseases, for instance, rheumatoid arthritis [11,12]. The involvement of β-glucuronidase in the colon cancer, and higher intestinal levels of the enzyme is correlated to the higher incidence of colon carcinoma [13]. Therefore, inhibition of β-glucuronidase enzyme is effective in preventing various diseases.

Bis(indolyl)methanes form an important class of heterocyclic compounds [14–18] that show a variety of pharmacological activities and play an important role in the treatment of chronic fatigue, fibromyalgia and irritable bowel syndrome [19,20]. They are also responsible for the promotion of beneficial estrogen metabolism and produce apoptosis in human cancer cells [21]. *Bis*(indolyl) methanes and its derivatives are also used as dietary supplements for humans [22–24]. It has inhibitory activity against bladder cancer growth [25], renal cell carcinoma growth [26], inhibit lung cancer [27] and colon cancer [28,29]. Some derivatives also show inhibitory activity against mammary tumor growth [30,31] and few analogs have potent antitumor activity [32]. They are applied as chemotherapeutic agents against tumors [33].

In this study, a novel series of hybrid analogs of *bis*(indolyl) methane and thiosemicarbazide had been synthesized and tested for their ability to inhibit β-glucuronidase activity. In our previous study, simple *bis*(indolyl)methane and thiourea derivatives had shown good inhibitory activity against β-glucuronidase [34,35]. The binding modes of active compounds were also identified to further understand how the molecules bind with β-glucuronidase protein.

2. Results and discussion

2.1. Chemistry

In the continuation working on active molecules based on indole [36–41]; we synthesized of bisindole-thiosemicarbazides

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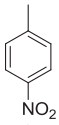
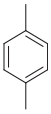
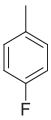
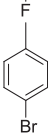
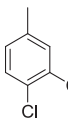
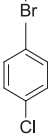
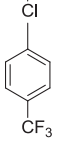
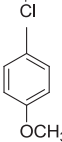
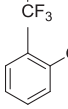
analogs **1**–**18** started with the synthesis of methyl 4-(di(1H-indol-3-yl)methyl)benzoate **I**. The indole (double molar equivalent) was treated to methyl 4-formylbenzoate in the presence of acetic acid to form methyl 4-(di(1H-indol-3-yl)methyl)benzoate **I**. The methyl 4-(di(1H-indol-3-yl)methyl)benzoate **I** was treated with methanolic hydrazine hydrate to form 4-(di(1H-indol-3-yl)methyl)benzohydrazide **II**. The 4-(di(1H-indol-3-yl)methyl)benzohydrazide **II** was treated with different isothiocyanates to form bisindole-thiosemicarbazides derivatives **1**–**18**.

2.2. In vitro β-glucuronidase inhibitory potential

Compounds **1**–**18** were screened for β-glucuronidase activity. All compounds showed varied degree of β-glucuronidase inhibitory potential when compared with standard D-saccharic acid 1,4-lactone (IC_{50} value 48.4 ± 1.25 μM). Compounds **2**, **3**, **4**, **6**, **12**, **14**, **16** and **17** showed exceptional β-glucuronidase inhibition with IC_{50} values 0.1 ± 0.001, 0.5 ± 0.001, 2.9 ± 0.01, 3.1 ± 0.04, 2.3 ± 0.01, 5.7 ± 0.01, 0.12 ± 0.001 and 0.20 ± 0.001 μM, respectively, which are many fold better than the standard inhibitor. Compound **1**, **5**, **8**, **9**, **10**, **11** and **18** showed potential 2–6 times better the standard with IC_{50} values 7.12 ± 0.04, 15.0 ± 0.38, 8.5 ± 0.06, 11.6 ± 0.15, 12.01 ± 0.11, 14.7 ± 0.16, and 9.40 ± 0.01 μM respectively. The remaining compounds **7**, **13**, and **15** also showed remarkable inhibitory potential with IC_{50} values 33.2 ± 0.75, 21.4 ± 0.30 and 28.12 ± 0.25 μM respectively.

Structure-activity relationship suggested that the activity of a specific molecule is superficially directed by the substitution present at aromatic residues attach to the thiosemicarbazides. The *ortho*-fluoro analog **2** (IC_{50} = 0.1 ± 0.001 μM), *ortho*-chloro analog **3** (IC_{50} = 0.5 ± 0.001 μM), *ortho*-bromo analog **4** (IC_{50} = 2.9 ± 0.01 μM), *para*-fluoro analog **6** (IC_{50} = 3.1 ± 0.04 μM), *meta*-fluoro analog **12** (IC_{50} = 2.3 ± 0.01 μM), *meta*-nitro analog **14** (IC_{50} = 5.7 ± 0.01 μM), 3,4-dichloro analog **16** (IC_{50} = 0.12 ± 0.001 μM), and *para*-trifluoromethane analog **17** (IC_{50} = 0.20 ± 0.001 μM) respectively exhibited marvelous inhibitory potential among the series. Although the whole series of compounds is active, but it was observed that bringing about variation in substitution pattern of aromatic ring attach to thiosemicarbazides resulted in activities difference. Compound **2** (*ortho*-fluoro analog) is found to be the most active analog among the series with IC_{50} value 0.1 ± 0.001 μM. The most potent activity of this compound might be due to fluoro group an electronegative group which might be involved in hydrogen bonding. Compound **16** got the second position in series with IC_{50} value 0.12 ± 0.001 μM. The compound has 3,4-dichloro group substituent an electronegative group which

Table 1
The β-glucuronidase activity of bisindole analogs (**1**–**18**).

S. no.	R	IC_{50} (μM ± SEM[a])	S. no.	R	IC_{50} (μM ± SEM[a])
1		7.12 ± 0.04	**10**		**12.01 ± 0.11**
2		**0.1 ± 0.001**	**11**		14.7 ± 0.16
3		**0.5 ± 0.001**	**12**		2.3 ± 0.01
4		2.9 ± 0.01	**13**		21.4 ± 0.30
5		15.0 ± 0.38	**14**		5.7 ± 0.01
6		**3.1 ± 0.04**	**15**		28.12 ± 0.25
7		33.2 ± 0.75	**16**		**0.12 ± 0.001**
8		8.5 ± 0.06	**17**		**0.20 ± 0.001**
9		11.6 ± 0.15	**18**		9.40 ± 0.01
D-saccharic acid 1,4-lactone			**48.4 ± 1.25** μM		

[a] IC_{50} values are expressed as mean ± standard error of mean.

Table 2
Predicted docking scores of bisindole analogs (**1**–**18**).

Compound	Docking score	Compound	Docking score
1	−12.3022	**10**	−11.0304
2	−16.4534	**11**	−11.1638
3	−14.0556	**12**	−13.6479
4	−13.9201	**13**	−10.2442
5	−10.9523	**14**	−12.9388
6	−13.8253	**15**	−10.3289
7	−09.716	**16**	−15.0909
8	−11.9401	**17**	−15.0664
9	−10.2003	**18**	−12.6581

might be responsible for this potent inhibition. Compound **17** got the third position among the series with IC_{50} value 0.20 ± 0.001 μM. This compound has trifluoromethane group at *para* position of aromatic ring which might be helpful in this inhibition. The compounds **6** and **12** having *para*-fluoro and *meta*-fluoro group with IC_{50} values 3.1 ± 0.04 and 2.3 ± 0.01 μM, respectively also showed potent inhibition. The potential of these analogs are less than compound **2**. The slight activity difference among these analogs might be due to differences in position of fluoro group on the aromatic ring. Compound **3** (*ortho*-chloro analog) is also the most active analog among the series. The activity of compound might be due to chloro group an electronegative group. If we compared compound **3** with compound **8** a *para*-chloro analog with IC_{50} value 8.5 ± 0.06 μM. The changes in activity might be due to the difference in position of chloro group as well. Compound **1** *ortho*-methyl analog with IC_{50} value 8.5 ± 0.06 μM, **5** *para*-methyl analog with IC_{50} value 15.0 ± 0.38 μM and **11** a *meta*-methyl analog with IC_{50} value 14.7 ± 0.16 μM. The great activity difference is mainly because of the position difference of substituent. Similarly if we see to the compound **9** a *para*-methoxy analog with IC_{50} value 11.6 ± 0.15 μM, **15** a *meta*- methoxy analog with IC_{50} value 28.12 ± 0.25 μM and **18** a *ortho*-methoxy analog with IC_{50} value 9.40 ± 0.01 μM. We have found here that either electron withdrawing group or electron donating group at *ortho* position showed great inhibition, but the electron withdrawing group are superior to electron donating group overall. In case of bromo substituted analogs **4**, **7** and **13** and nitro-substituted analogs **10** and **14** we have observed similar results. The binding interactions of the most active compounds were confirmed through molecular docking studies.

2.3. Docking study

From the docking results a good correlation between the docking scores and biological activities of these newly synthesized compounds was observed (Tables 1 and 2). For example the docking scores of the most active compounds, compound **2**, **3**, **4**, **12**, **14**, **16** and **17** were found to be more negative as compare to the less active compounds, compound **5**, **7** and **15** (Tables 1 and 2). The docking conformations of all the compound showed that all the active derivatives showed significant binding interactions with the important catalytic site residues of the target protein. Compounds **2**, **3**, **4**, **12**, **14**, **16** and **17** give good interactions with the important active site residues of the enzyme. The docking conformation of the most active compound, compound **2** (*ortho*-fluoro analog) showed eight interactions with the important active site residues of *β*-glucuronidase. The *ortho*-fluoro analog makes arene-arene interaction with Tyr 508, the hydrogen atom of thiourea forms hydrogen bond with the Glu 451 while the indole and benzamide moiety of the compound forms week interactions with the His 509 residue of the protein (Fig. 1a). The second most active compound, compound **16** formed two arene-cation interactions. The *meta* and *para*-chloro analog forms arene-cation interaction with His 509 and the indole moiety of the compound forms arene-cation interaction with Arg 600 residues of the enzyme (Fig. 1b).

The third most active compound, compound **17** showed four interactions with the active site residues. Tyr 205 form hydrogen bond with the hydrogen of indole moiety and His 509 interact with the π electron system of trifluoromethyl benzene group of the compound (Fig. 2a). These three compounds have halogens atoms at the *ortho*, *meta*, and *para* positions of R-group and showed interactions with important residues of the binding pocket of *β*-glucuronidase. Fluorine which due to its smaller size and high electronegativity produces enough polarizability in the molecule and thus molecules having F at proper position showed good results.

For example, compound **12** having a *meta*-fluoro group, also showed good interactions with the active pocket of protein. In compound **12** the aromatic ring of *meta*-fluoro group forms π-interaction with the Arg 600 residue and the indole group made two arene-arene interactions with the His 509 (Fig. 2b). Bromo and

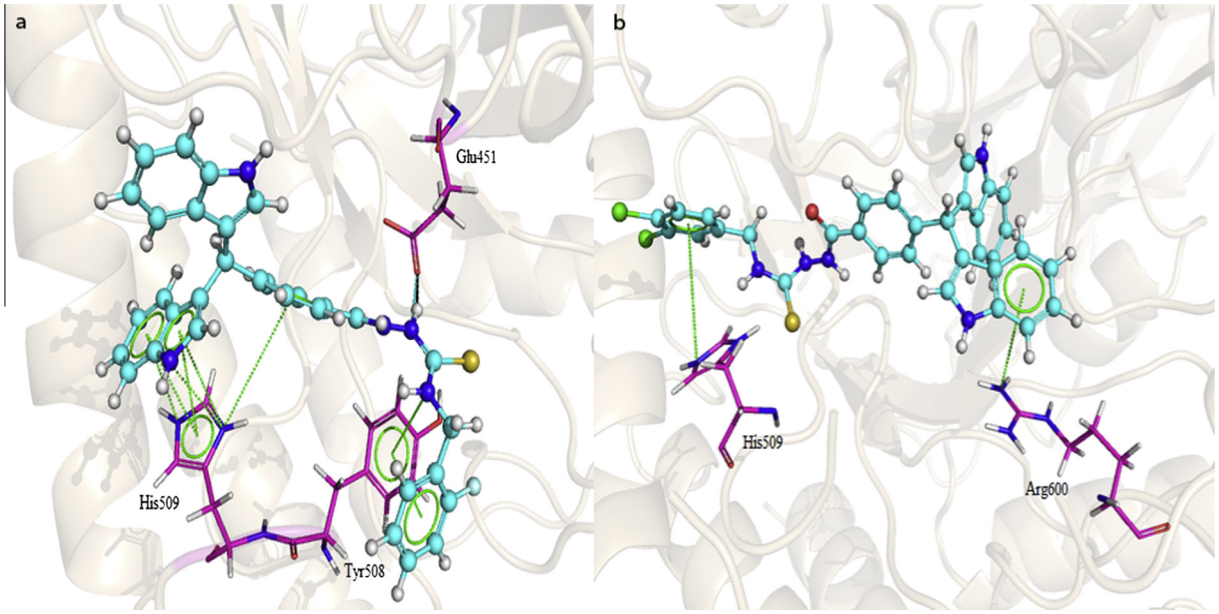


Fig. 1. Docking conformation of compound **2** (a) and compound **16** (b) in the active site of *β*-D-glucuronidase.

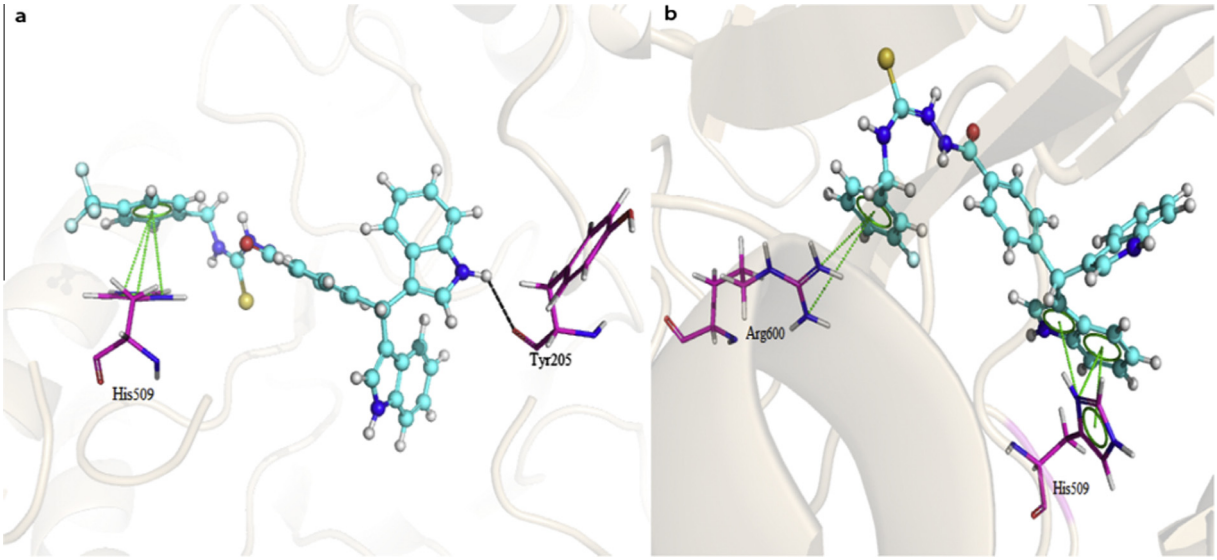


Fig. 2. Docking conformation of compound **17** (a) and compound **12** (b) in the active site of β-D-glucuronidase.

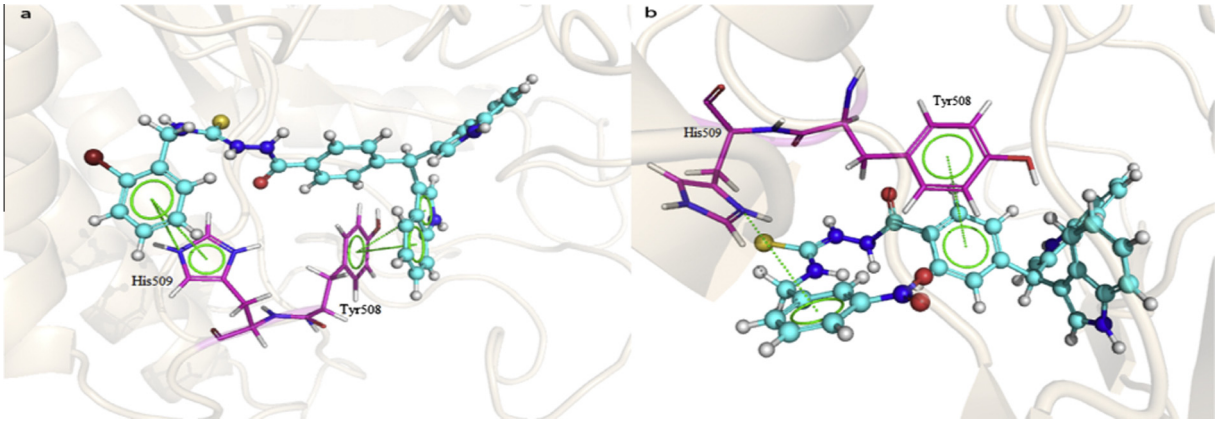


Fig. 3. Docking conformation of compound **4** (a) and compound **14** (b) in the active site of *β*-D-glucuronidase.

nitro-substituted analogs (compound **4** and **14**) also showed interactions with the binding site residues of the enzyme. In case of compound **4**, the bromine substituted moiety showed interactions with His 509 whereas the indole moiety makes interactions with the Tyr 508 residues of the enzyme (Fig. 3a). In case of compound **14**, the docking conformation showed that the nitro-substituted moiety interacts with His 509 and the aromatic ring of benzamide moiety makes arene-arene interaction with Tyr 508 residues of the enzyme (Fig. 3b).

3. Conclusion

The use of *β*-glucuronidase for glucuronide hydrolysis is a common practice in bio-analytical techniques. The present study reports the synthesis and biological evaluation of a series of new hybrids bisindole-thiosemicarbazide derivatives (**1**–**18**). All synthesized derivatives showed excellent *β*-glucuronidase inhibitory potential if compared with the standard D-saccharic acid 1, 4-lactone. The results of our synthesized derivatives encouraged us to further develop potential *β*-glucuronidase inhibitors based on bisindole in future.

4. Experimental

4.1. Chemistry

NMR experiments were performed on Ultra Shield Bruker FT NMR 500 MHz; CHN analysis was performed on a Carlo Erba Strumentazion-Mod-1106, Italy. Electron impact mass spectra (EI-MS) were recorded on a Finnegan MAT-311A, Germany. Thin layer chromatography (TLC) was performed on pre-coated silica gel aluminum plates (Kieselgel 60, 254, E. Merck, Germany). Chromatograms were visualized by UV at 254 and 365 nm.

4.2. Synthesis of methyl 4-(di(1H-indol-3-yl)methyl)benzoate (*I*)

Methyl 2-hydroxybenzoate (4.03 g, 27 mmol) and indole (6.32 g, 53 mmol) were mixed in glacial acetic acid (100 mL). The mixture was refluxed for 8 h. Upon completion, reaction mixture was treated with sodium carbonate and extracted using ethyl acetate (50 mL × 4). The solvent was evaporated under reduced pressure and the product was crystallized with methanol. Yield is 9.51 g (92.7%). White solid, m.p. 219–221 °C; ^{1}H NMR (500 MHz,

DMSO-d_6): δ δ 7.95 (d, J = 7.4 Hz, 2H), 7.72 (dd, J = 7.5, 1.6 Hz, 2H), 7.54 (dd, J = 7.5, 1.5 Hz, 2H), 7.41 (d, J = 8.0 Hz, 2H), 7.39 (td, J = 8.0, 1.5 Hz, 2H), 7.27 (td, J = 7.4, 1.5 Hz, 2H), 6.96 (s, 2H), 5.72 (s, 1H), 3.95 (s, 3H); ^{13}C NMR (DMSO-d_6, 125 MHz): δ 167.38, 1402, 138.42, 138.42, 130.81, 128.61, 128.61, 128.19, 128.19, 126.97, 126.97, 123.48, 123.48, 121.67, 121.67, 121.42, 121.42, 118.42, 118.42, 113.39, 113.39, 110.18, 110.18, 52.13, 37.27; HREI-MS: m/z calcd for C25H20N2O2 [M]+ 380.1525; Found 380.1532; Anal. Calcd for $C_{25}H_{20}N_2O_2$, C, 78.93; H, 5.30; N, 7.36 found C, 78.94; H, 5.28; N, 7.35.

4.3. Synthesis of 4-(di(1H-indol-3-yl)methyl)benzohydrazide (**II**)

A mixture of compound **I** (7.00 g, 18.4 mmol) and hydrazine hydrate (20 mL) were refluxed for 6 h in 100 mL methanol. When reaction has completed, methanol was evaporated and the residue was washed with diethyl ether, filtered, dried, and crystallized from ethanol and to give a brownish solid, (5.85 g, 83.7%). m.p. 253–255 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.45 (s, 2H), 7.89 (d, J = 7.5 Hz, 2H), 7.72 (dd, J = 7.3, 1.6 Hz, 2H), 7.54 (dd, J = 7.4, 1.5 Hz, 2H), 7.41 (d, J = 7.5 Hz, 2H), 7.39 (dd, J = 7.3, 1.6 Hz, 2H), 7.28 (dd, J = 7.3, 1.6 Hz, 2H), 7.08 (s, 2H), 5.66 (s, 1H), 3.82 (s, 2H); ^{13}C NMR (150 MHz, DMSO-d_6): δ 167.54, 142.19, 138.56, 138.56, 134.94, 129.49, 129.49, 126.93, 126.93, 126.87, 126.87, 123.43, 121.49, 121.49, 121.35, 121.35, 118.12, 118.12, 113.39, 113.39, 110.09, 110.09, 37.36; HREI-MS: m/z calcd for $C_{24}H_{20}N_4O$ [M]+ 380.1637; Found 380.1645; Anal. Calcd for $C_{24}H_{20}N_4O$, C, 75.77; H, 5.30; N, 14.73; found C, 75.77; H, 5.30; N, 14.73;

4.4. Synthesis of 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-phenylhydrazinecarbothioamides (**1**–**18**)

Equal amount of bisindole hydrazide **II** (1 mmole) and isothiocyanates (1 mmole) were mixed in the presence of ethanol (20 mL) and catalytic amount of triethylamine and refluxed for 6 h. The completion of reaction was confirmed by TLC. The crude products were further recrystallized from methanol to afford pure compounds with the yield between 72% and 92%.

4.4.1. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(o-tolyl) hydrazinecarbothioamide (**1**)

Yield: 0.43 g (81%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.30 (s, 2H), 9.85 (s, 2H), 9.70 (s, 1H), 7.80 (t, J = 7.8 Hz, 2H), 7.50 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.30 (d, J = 7.9 Hz, 2H), 7.30–7.25 (m, 4H), 7.11 (t, J = 7.8 Hz, 2H), 6.92 (t, J = 7.4 Hz, 2H), 6.85 (s, 2H), 5.93 (s, 1H), 2.45 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6):δ 180.87, 166.91, 142.37, 138.72, 138.72, 137.96, 134.51, 132.74, 129.92, 129.56, 129.56, 127.76, 127.50, 127.50, 126.98, 126.98, 124.63, 123.51, 123.51, 123.08, 121.45, 121.45, 121.41, 121.41, 118.28, 118.28, 113.39, 113.39, 110.05, 110.05, 37.35, 17.31; Anal. Calcd for $C_{32}H_{27}N_5OS$, C = 72.56, H = 5.14, N = 13.22; Found C = 72.58, H = 5.13, N = 13.24; HR MS Calcd for $C_{32}H_{27}N_5OS$: 529.1936 found 529.1941.

4.4.2. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(2-fluorophenyl) hydrazinecarbothioamide (**2**)

Yield: 0.46 g (86%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.91 (s, 2H), 10.85 (s, 2H), 8.70 (s, 1H), 7.81 (d, J = 8.0 Hz, 2H), 7.50 (t, J = 8.5 Hz, 4H), 7.41 (d, J = 8.2 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 7.30–7.25 (m, 2H), 7.11 (t, J = 7.5 Hz, 2H), 6.96 (t, J = 7.5 Hz, 2H), 6.91 (s, 2H), 5.97 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6):δ 180.87, 166.90, 158.99 (d, J = 262.3 Hz), 142.38, 138.62, 134.52, 129.46, 127.82, 127.61, 127.53, 126.94, 125.70, 124.43, 123.96, 123.52, 121.47, 118.22, 117.25, 117.04, 113.37, 110.07, 37.30; Anal. Calcd for $C_{31}H_{24}FN_5OS$, C = 69.77, H = 4.53, N = 13.12; Found C = 69.79, H = 4.51, N = 13.14; HR MS Calcd for $C_{31}H_{24}FN_5OS$: 533.1686 found 533.1675.

4.4.3. N-(2-Chlorophenyl)-2-(4-(di(1H-indol-3-yl)methyl)benzoyl) hydrazinecarbothioamide (**3**)

Yield: 0.40 g (73%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.02 (s, 2H), 10.48 (s, 2H), 8.82 (s, 1H), 8.02 (s, 1H), 7.82 (d, J = 7.9 Hz, 2H), 7.50 (t, J = 7.0 Hz, 2H), 7.43 (d, J = 7.0 Hz, 2H), 7.38 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 7.8 Hz, 2H), 7.12 (t, J = 7.5 Hz, 2H), 7.04–6.96 (m, 3H), 6.89 (s, 2H), 5.95 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6):δ 180.87, 166.97, 142.39, 138.52, 138.52, 134.64, 134.42, 129.85, 129.36, 129.36, 129.28, 127.73, 127.73, 127.23, 126.84, 126.84, 125.34, 125.08, 123.53, 123.53, 121.48, 121.48, 121.35, 121.35, 118.12, 118.12, 113.47, 113.47, 110.19, 110.19, 37.40; Anal. Calcd for $C_{31}H_{24}ClN_5OS$, C = 67.69, H = 4.40, N = 12.73; Found C = 67.67, H = 4.42, N = 12.75; HR MS Calcd for $C_{31}H_{24}ClN_5OS$: 549.1390 found 549.1398.

4.4.4. N-(2-Bromophenyl)-2-(4-(di(1H-indol-3-yl)methyl)benzoyl) hydrazinecarbothioamide (**4**)

Yield: 0.42 g (71%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.05 (s, 2H), 11.20 (s, 2H), 8.70 (s, 1H), 7.83 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 8.0 Hz, 2H), 7.50 (t, J = 7.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.15 (t, J = 7.5 Hz, 2H), 6.93–6.88 (m, 3H), 6.89 (s, 2H), 5.96 (s, 1H);^{13}C NMR (125 MHz, DMSO-d_6):δ 181.86, 167.92, 142.39, 138.63, 138.63, 136.56, 134.523, 132.69, 129.41, 129.41, 128.72, 127.73, 127.73, 126.84, 126.84, 125.07, 124.09, 123.42, 123.42, 121.67, 121.67, 121.35, 121.35, 118.28, 118.28, 117.44, 113.30, 113.30, 110.17, 110.17, 37.40; Anal. Calcd for $C_{31}H_{24}BrN_5OS$, C = 62.63, H = 4.07, N = 11.78; Found C = 62.61, H = 4.09, N = 11.76; HR MS Calcd for $C_{31}H_{24}BrN_5OS$: 593.0885 found 593.0879.

4.4.5. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(p-tolyl) hydrazinecarbothioamide (**5**)

Yield: 0.47 g (89%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.70 (s, 2H), 10.07 (s, 2H), 8.46 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 7.63 (d, J = 7.5 Hz, 2H), 7.52 (d, J = 7.9 Hz, 2H), 7.43 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 7.5 Hz, 2H), 7.26 (d, J = 7.5 Hz, 2H), 7.12 (t, J = 7.6 Hz, 2H), 6.95 (t, J = 7.5 Hz, 2H), 6.87 (s, 2H), 5.94 (s, 1H), 2.35 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.39, 166.80, 142.39, 138.67, 138.67, 137.62, 134.50, 132.72, 130.36, 130.36, 129.48, 129.48, 127.54, 127.54, 126.97, 126.97, 123.62, 123.62, 121.37, 121.37, 121.25, 121.25, 120.97, 120.97, 118.12, 118.12, 113.39, 113.39, 110.18, 110.18, 37.27, 21.02; Anal. Calcd for $C_{32}H_{27}N_5OS$, C = 72.56, H = 5.14, N = 13.22; Found C = 72.57, H = 5.11, N = 13.26; HR MS Calcd for $C_{32}H_{27}N_5OS$: 529.1936 found 529.1945.

4.4.6. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(4-fluorophenyl) hydrazinecarbothioamide (**6**)

Yield: 0.51 g (96%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.90 (s, 2H), 10.20(s, 2H), 8.44 (s, 1H), 7.84–7.76 (m, 4H), 7.52 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.32–7.26 (m, 2H), 7.11 (t, J = 7.6 Hz, 2H), 6.92 (t, J = 7.5 Hz, 2H), 6.86 (s, 2H), 5.93 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6):δ 180.38, 165.90, 161.53 (d, J = 262.3 Hz), 159.43, 141.38, 137.62, 137.62, 134.51, 129.36, 129.36, 127.51, 127.51, 126.97, 126.97, 123.42, 123.42, 122.49, 122.49, 121.87, 121.87, 121.35, 121.35, 118.28, 118.28, 115.35, 115.35, 113.38, 113.38, 110.09, 110.09, 36.30; Anal. Calcd for $C_{31}H_{24}FN_5OS$, C = 69.77, H = 4.53, N = 13.12; Found C = 69.77, H = 4.52, N = 13.14; HR MS Calcd for $C_{31}H_{24}FN_5OS$: 533.1686 found 533.1679.

*4.4.7. N-(4-Bromophenyl)-2-(4-(di(1H-indol-3-yl)methyl)benzoyl) hydrazinecarbothioamide (**7**)*

Yield: 0.48 g (81%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.90 (s, 2H), 9.88 (s, 2H), 8.33 (s, 1H), 7.81 (d, J = 8.2 Hz, 2H), 7.56 (d, J = 8.3 Hz, 2H), 7.46 (d, J = 8.1 Hz, 2H), 7.39 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.12 (t, J = 7.6 Hz, 2H), 6.92 (t, J = 7.4 Hz, 2H), 6.87 (s, 2H), 6.83 (d, J = 8.4 Hz, 2H), 5.96 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.47, 166.80, 142.28, 138.32, 138.32, 137.92, 134.58, 131.26, 129.36, 129.36, 127.51, 127.51, 126.97, 126.97, 123.42, 123.42, 123.18, 123.18, 121.46, 121.46, 121.25, 121.25, 118.27, 118.27, 114.51, 114.51, 113.38, 113.38, 110.09, 110.09, 35.32; Anal. Calcd for $C_{31}H_{24}BrN_5OS$, C = 62.63, H = 4.07, N = 11.78; Found C = 62.63, H = 4.10, N = 11.75; HR MS Calcd for $C_{31}H_{24}BrN_5OS$: 593.0885 found 593.0871.

*4.4.8. N-(4-Chlorophenyl)-2-(4-(di(1H-indol-3-yl)methyl)benzoyl) hydrazinecarbothioamide (**8**)*

Yield: 0.43 g (78%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.92 (s, 2H), 10.03 (s, 2H), 8.41 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 7.76 (d, J = 7.0 Hz, 2H), 7.50 (t, J = 8.0 Hz, 4H), 7.42 (d, J = 8.0 Hz, 2H), 7.31 (d, J = 8.0 Hz, 2H), 7.12 (t, J = 7.5 Hz, 2H), 6.95 (t, J = 7.5 Hz, 2H), 6.89 (s, 2H), 5.92 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 182.38, 166.91, 142.39, 139.82, 138.65, 138.65, 134.49, 129.56, 129.56, 129.17, 129.17, 128.17, 127.56, 127.56, 126.98, 126.98, 123.42, 123.42, 121.85, 121.85, 121.42, 121.42, 121.17, 121.17, 118.12, 118.12, 113.38, 113.38, 110.17, 110.17, 37.36; Anal. Calcd for $C_{31}H_{24}ClN_5OS$, C = 67.69, H = 4.40, N = 12.73; Found C = 67.68, H = 4.41, N = 12.77; HR MS Calcd for $C_{31}H_{24}ClN_5OS$: 549.1390 found 549.1384.

*4.4.9. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(4-methoxyphenyl) hydrazinecarbothioamide (**9**)*

Yield: 0.46 g (84%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.60 (s, 2H), 9.90 (s, 2H), 8.36 (s, 1H), 7.80 (d, J = 8.2 Hz, 2H), 7.68(d, J = 8.0 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.12 (t, J = 7.6 Hz, 2H), 7.04 (d, J = 8.0 Hz, 2H), 6.91 (t, J = 7.5 Hz, 2H), 6.87 (s, 2H), 5.94 (s, 1H), 3.81 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.12, 165.87, 154.87, 142.28, 138.67, 138.67, 134.42, 133.06, 129.36, 129.36, 127.57, 127.57, 126.95, 126.95, 123.53, 123.53, 122.12, 122.12, 121.48, 121.48, 121.15, 121.15, 118.24, 118.24, 114.23, 114.23, 113.38, 113.38, 110.17, 110.17, 56.02, 37.27; Anal. Calcd for $C_{32}H_{27}N_5O_2S$, C = 70.44, H = 4.99, N = 12.83; Found C = 70.46, H = 5.01, N = 12.85; HR MS Calcd for $C_{32}H_{27}N_5O_2S$: 545.1885 found 545.1874.

*4.4.10. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(4-nitrophenyl) hydrazinecarbothioamide (**10**)*

Yield: 0.51 g (91%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.08 (s, 2H), 10.70 (s, 2H), 8.52 (s, 1H), 8.32 (d, J = 8.0 Hz, 2H), 8.01 (d, J = 7.0 Hz, 2H), 7.82 (d, J = 8.0 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 8.0 Hz, 2H), 7.12 (t, J = 7.5 Hz, 2H), 6.93 (t, J = 7.5 Hz, 2H), 6.89 (s, 2H), 5.96 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.32, 166.91, 143.57, 143.34, 142.48, 138.65, 138.65, 134.51, 129.56, 129.56, 127.52, 127.52, 126.91, 126.91, 125.07, 125.07, 123.53, 123.53, 121.68, 121.68, 121.35, 121.35, 120.95, 120.95, 118.12, 118.12, 113.38, 113.38, 110.17, 110.17, 37.35; Anal. Calcd for $C_{31}H_{24}N_6O_3S$, C = 66.41, H = 4.31, N = 14.99; Found C = 66.43, H = 4.34, N = 15.01; HR MS Calcd for $C_{31}H_{24}N_6O_3S$: 560.1631 found 560.1638.

*4.4.11. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(m-tolyl) hydrazinecarbothioamide (**11**)*

Yield: 0.38 g (72%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.46 (s, 2H), 10.20 (s, 2H), 8.43 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 7.53 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 7.5 Hz, 2H), 7.29 (s, 2H), 7.11 (t, J = 7.6 Hz, 2H), 6.97 (t, J = 7.5 Hz, 2H), 6.88 (s, 2H), 5.94 (s, 1H), 3.40 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 183.38, 171.90, 152.38, 140.26, 138.52, 138.52, 138.37, 134.32, 129.47, 129.47, 128.50, 127.52, 127.52, 126.97, 126.97, 126.59, 123.59, 123.59, 121.73, 121.57, 121.57, 121.35, 121.35, 120.17, 118.28, 118.28, 113.27, 113.27, 110.08, 110.08, 37.31, 21.23; Anal. Calcd for $C_{32}H_{27}N_5OS$, C = 72.56, H = 5.14, N = 13.22; Found C = 72.58, H = 5.10, N = 13.24; HR MS Calcd for $C_{32}H_{27}N_5OS$: 529.1936 found 529.1929.

*4.4.12. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(3-fluorophenyl) hydrazinecarbothioamide (**12**)*

Yield: 0.44 g (83%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.88 (s, 2H), 10.23 (s, 2H), 8.45 (s, 1H), 7.80 (d, J = 7.5 Hz, 2H), 7.50 (t, J = 8.5 Hz, 3H), 7.42 (d, J = 8.0 Hz, 2H), 7.31(d, J = 7.5 Hz, 2H), 7.12 (t, J = 7.5 Hz, 2H), 7.04–6.96 (m, 5H), 6.86 (s, 2H), 5.93 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.38, 166.90, 160.02 (d, J = 262.3 Hz), 162.38, 150.11, 137.62, 137.62, 134.51, 129.81, 129.81, 129.36, 127.52, 127.52, 126.91, 126.91, 123.82, 123.82, 121.67, 121.67, 121.25, 121.25, 118.45, 118.28, 118.28, 113.47, 113.47, 112.29, 111.21, 110.09, 110.09, 37.20; Anal. Calcd for $C_{31}H_{24}FN_5OS$, C = 69.77, H = 4.53, N = 13.12; Found C = 69.77, H = 4.51, N = 13.13; HR MS Calcd for $C_{31}H_{24}FN_5OS$: 533.1686 found 533.1690.

*4.4.13. N-(3-Bromophenyl)-2-(4-(di(1H-indol-3-yl)methyl)benzoyl) hydrazinecarbothioamide (**13**)*

Yield: 0.47 g (79%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.82 (s, 2H), 10.20 (s, 2H), 8.33 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.1 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.26 (t, J = 7.5 Hz, 2H), 7.20 (s, 1H), 7.15–7.10 (m, 3H), 6.92 (t, J = 7.5 Hz, 2H), 6.88 (s, 2H), 5.94 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 176.38, 169.90, 142.28, 140.92, 138.52, 138.52, 134.59, 129.76, 129.76, 129.49, 127.43, 127.43, 126.34, 126.34, 125.71, 123.62, 123.62, 122.50, 122.02, 121.46, 121.46, 121.35, 121.35, 120.77, 118.32, 118.32, 113.38, 113.38, 110.17, 110.17, 37.35; Anal. Calcd for $C_{31}H_{24}BrN_5OS$, C = 62.63, H = 4.07, N = 11.78; Found C = 62.61, H = 4.08, N = 11.76; HR MS Calcd for $C_{31}H_{24}BrN_5OS$: 593.0885 found 593.0877.

*4.4.14. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(3-nitrophenyl) hydrazinecarbothioamide (**14**)*

Yield: 0.54 g (96%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.05 (s, 2H), 10.65 (s, 2H), 8.55 (s, 1H), 8.24 (d, J = 7.5 Hz, 1H), 8.13 (d, J = 7.0 Hz, 1H), 7.82 (d, J = 8.0 Hz, 2H), 7.74 (t, J = 8.0 Hz, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.2 Hz, 2H), 7.34 (d, J = 8.0 Hz, 2H), 7.13 (t, J = 7.5 Hz, 2H), 7.02–6.96 (m, 3H), 6.89 (s, 2H), 5.94 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 178.38, 166.91, 150.52, 142.39, 141.37, 138.42, 138.42, 134.57, 129.56, 129.33, 129.33, 127.58, 127.58, 126.97, 126.97, 126.64, 123.58, 123.58, 121.87, 121.87, 121.55, 121.55, 118.28, 118.28, 118.12, 115.95, 113.38, 113.38, 110.19, 110.19, 32.35; Anal. Calcd for $C_{31}H_{24}N_6O_3S$, C = 66.41, H = 4.31, N = 14.99; Found C = 66.42, H = 4.32, N = 15.00; HR MS Calcd for $C_{31}H_{24}N_6O_3S$: 560.1631 found 560.1624.

*4.4.15. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(3-methoxyphenyl) hydrazinecarbothioamide (**15**)*

Yield: 0.43 g (79%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.70 (s, 2H), 9.91(s, 1H), 9.62 (s, 1H), 8.35 (s, 1H), 7.80 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 80 Hz, 2H), 7.41(d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.25 (t, J = 8.0 Hz, 2H), 7.18 (s, 1H), 7.14–7.08 (m, 3H), 6.92 (t, J = 7.5 Hz, 2H), 6.89 (s, 2H), 5.94 (s, 1H) 3.89 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.49, 166.80, 158.49, 142.48, 140.44, 138.72, 138.72, 134.51, 129.45, 129.45, 128.97, 127.52, 127.52, 126.92, 126.92, 123.50, 123.50, 121.67, 121.67, 121.35, 121.35,

118.42, 118.42, 113.57, 113.57, 112.96, 110.47, 110.47, 110.47, 109.16, 56.13, 32.57; Anal. Calcd for $C_{32}H_{27}N_5O_2S$, C = 70.44, H = 4.99, N = 12.83; Found C = 70.45, H = 4.98, N = 12.81; HR MS Calcd for $C_{32}H_{27}N_5O_2S$: 545.1885 found 545.1878.

*4.4.16. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(3,4-dichlorophenyl)-hydrazinecarbothioamide (**16**)*

Yield: 0.50 g (85%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.52 (s, 2H), 9.37 (s, 1H), 9.24 (s, 1H), 8.25 (s, 1H), 7.82 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 8.0 Hz, 2H), 7.37 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 7.5 Hz, 2H), 7.23 (s, 1H), 7.13 (t, J = 7.5 Hz, 2H), 6.93–6.98 (m, 2H), 6.88 (s, 2H), 6.76 (d, J = 8.0 Hz, 2H), 5.94 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 180.76, 166.80, 142.39, 138.52, 138.52, 136.47, 134.51, 132.94, 129.45, 129.45, 127.72, 127.55, 127.38, 127.38, 126.97, 126.97, 126.39, 123.62, 123.62, 122.38, 121.67, 121.67, 121.41, 121.41, 118.12, 118.12, 113.33, 113.33, 110.13, 110.13, 37.15; Anal. Calcd for $C_{31}H_{23}C_{l2}N_5OS$, C = 63.70, H = 3.97, N = 11.98; Found C = 63.71, H = 3.99, N = 11.99; HR MS Calcd for $C_{31}H_{23}C_{l2}N_5OS$: 583.1000 found 583.1010.

*4.4.17. 2-(4-(di(1H-indol-3-yl)methyl)benzoyl)-N-(4(trifluoromethyl) phenyl)hydrazinecarbothio amide (**17**)*

Yield: 0.45 g (78%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.04 (s, 2H), 11.90 (s, 2H), 8.63 (d, J = 4.2 Hz, 2H), 8.42 (s, 1H), 7.81 (d, J = 8.0 Hz, 2H), 7.68 (s, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 7.12 (t, J = 7.5 Hz, 2H), 6.96 (t, J = 7.5 Hz, 2H), 6.89 (s, 2H), 5.96 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 179.48, 167.50, 143.53, 142.28, 138.72, 138.72, 134.42, 129.45, 129.45, 127.52, 127.52, 126.84, 126.84, 126.33, 126.33, 126.02, 124.53 (d, J = 248.9 Hz), 123.40, 123.40, 121.48, 121.48, 121.15, 121.15, 120.62, 120.62, 118.12, 118.12, 113.38, 113.38, 110.17, 110.17, 37.35; Anal. Calcd for $C_{32}H_{24}F_3N_5OS$, C = 65.85, H = 4.14, N = 12.00; Found C = 65.81, H = 4.15, N = 12.01; HR MS Calcd for $C_{32}H_{24}F_3N_5OS$: 583.1654 found 583.1662.

*4.4.18. 2-(4-(Di(1H-indol-3-yl)methyl)benzoyl)-N-(2-methoxyphenyl) hydrazinecarbothioamide (**18**)*

Yield: 0.44 g (81%); ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.80 (s, 2H), 9.95 (s, 2H), 8.85 (s, 1H), 8.16 (d, J = 2.0 Hz, 1H), 8.06 (d, J = 8.0 Hz, 1H), 7.82 (d, J = 7.5 Hz, 2H), 7.70–7.66 (m, 1H), 7.50 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.0 Hz, 2H), 7.32 (d, J = 8.0 Hz, 2H), 7.11 (t, J = 7.5 Hz, 2H), 6.94 (t, J = 7.5 Hz, 2H), 6.89 (s, 2H), 5.96 (s, 1H), 3.90 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 181.85, 167.96, 151.17, 140.39, 136.68, 136.68, 135.52, 132.16, 129.48, 129.48, 127.43, 127.43, 126.96, 126.96, 124.92, 123.47, 123.47, 122.52, 122.29, 121.48, 121.48, 121.35, 121.35, 118.21, 118.21, 113.38, 113.38, 111.21, 110.09, 110.09, 56.58, 37.25; Anal. Calcd for $C_{32}H_{27}N_5O_2S$, C = 70.44, H = 4.99, N = 12.83; Found C = 70.43, H = 4.97, N = 12.84; HR MS Calcd for $C_{32}H_{27}N_5O_2S$: 545.1885 found 545.1874.

4.5. Bioassay

β-Glucuronidase activity was determined by measuring the absorbance at 405 nm of p-nitrophenol formed from the substrate by the spectrophotometric method. The total reaction volume was 250 μL. The reaction mixture contained 185 μL of 0.1 M acetate buffer, 5 μL of test compound solution, 10 μL of enzyme solution was incubated at 37 °C for 30 min. The plates were read on a multiplate reader (SpectraMax plus 384) at 405 nm after the addition of 50 μL of 0.4 mM p-nitrophenyl-*b*-D-glucuronide. All assays were run in triplicate [42–45]. Furthermore to avoid precipitation, the concentration of the compounds was decreased and the reaction volume was high (200 μL) so the chance of precipitation was less hence the addition of detergents was not needed.

4.6. Molecular docking

Molecular docking studies were performed in order to get more insight into the binding mode of *Bis*(indolyl)methanes derivatives within the active site of β-D-glucuronidase and to obtain further validations for our experimental results. All the docking calculations were performed on Intel (R) xenon (R) CPU E5620@2.40 GHz system having 3.8 GB RAM with the open 11.4 (X 86_64) operating platform. The software package MOE (Molecular Operating Environment) (www.Chemcomp.comwas) used for docking. LigPlot implemented in MOE was used to examine the interactions between the enzyme and Ligands.

The crystal structure of β-D-glucuronidase (PDB code 1BHG) was retrieved from protein data bank for docking studies. The structure was checked for missing atoms, bonds and contacts. From the original protein data bank file the B-chain of protein and hetero-atoms including cofactors were removed. After 3D protonation of the enzyme the energy of the retrieved protein molecule was minimized using the default parameters of MOE energy minimization algorithm (gradient: 0.05, Force Field: MMFF94X). Energy minimization was terminated when the root mean square gradient falls below the 0.05.

The 3D structures of these newly synthesized *Bis*(indolyl) methanes derivatives were constructed using MOE-Builder tool and hydrogens were added. Then, these molecules were energy minimized using the default parameters of MOE energy minimization algorithm (gradient: 0.05, Force Field: MMFF94X). All the minimized molecules were saved in the mdb file format. The prepared ligands were used as input file for MOE-Dock in the next step.

MOE docking program was used to analyze the binding modes of the ligands with the protein molecule. To find the correct conformations of the ligands and to obtain minimum energy structures, ligands were allowed to be flexible. The default parameters of MOE-Dock program were used for the molecular docking of the ligands. The top ranked pose of each compound was selected on the basis of docking score (S) for further analysis. At the end of docking, the best conformations on the basis of score were analyzed for hydrogen bonding/π-π interactions.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2016.07.008.

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