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Inhibition potential of phenyl linked benzimidazole-triazolothiadiazole modular hybrids against β-glucuronidase and their interactions thereof

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ABSTRACT

β-Glucuronidase is responsible for the catalytic deconjugation of β-D-glucuronides. β-Glucuronidase has evolved to be a viable molecular target for numerous therapeutic treatments. It plays a pivotal role in the metabolism of drugs and endogenous substances. Herein, we report the inhibitory potentials of newly developed and modular benzimidazole-triazolothiadiazole hybrids spaced through a phenyl linker (**1**–**26**) and their interactions with the β-glucuronidase. All analogues showed IC_{50} values in the range of 1.30 ± 0.10 to 44.10 ± 0.80 μM, and hence were found to have outstanding inhibitory potential as compare to the standard D-saccharic acid 1,4-lactone (IC_{50} = 48.4 ± 1.25 μM). These modular hybrids were successfully synthesized, rigorously characterized through various spectroscopic techniques. Molecular docking studies further revealed the potential interactions between the inhibitor and active amino acid site in β-glucuronidase. These findings helped in identifying the potential for new drug candidates. A Plausible structure activity relationship (SAR) were established which suggested that variation in the inhibitory potential was mainly based upon the substituents attached to the phenyl ring.

1. Introduction

Today's world is threatened with the danger of drug resistance and undesired consequences of existing drugs while this has earnestly endangered the human life in many facets [1]. At the same time, developing new and effective drugs became perplexing due to restricted monetary environment. Nonetheless, molecular target therapy is crafting its tactic for probing newer drugs with enriched potency. Among many targets explored, glycosyl hydrolases (GHs) are distinguished due to their role in many important biological processes [2]. Consequently, β-glucuronidase (EC 3.2.1.31) belongs to GH family 1, 2, 30, 79, 154. That is widely distributed in mammalian tissues, body fluids and microbiota; but predominantly retained in the endoplasmic reticulum [3]. Since the expression of β-glucuronidase is increased in necrotic areas and other body fluids of patients with various cancers such as breast [4], cervical [5], colon [6], lung [7], renal carcinoma and leukemia [8], when compared with healthy controls, this enzyme is a choice as a reliable biomarker for tumor diagnosis and clinical therapy assessment [9]. This over expression of the enzyme is also a worthy diagnostic tool for other diseases such as urinary tract infection [10], HIV [11], diabetes [12], neuropathy [13] and rheumatoid arthritis [14].

Adding to that, β-glucuronidase is substantially a potential molecular target for; 1) anticancer chemotherapy while considering its role in tumor growth and metastasis [15]. 2) Neonatal jaundice treatment due to its high expression in breast milk and role in enterohepatic bilirubin circulation (hyperbilirubinemia) [16]. 3) Diabetes mellitus management due to the positive correlations between the disease state and enzyme

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activity level and the associated periodontitis [17]. 4) Development of anti-inflammatory agents owing to their pro-inflammatory role following the significant release from degranulated mast cells and neutrophils [18]. Presumably, inhibition of β-glucuronidase could markedly alleviate these pathological complaints and their adverse effects hence forth taming the efficient regimens.

The hybridization of biologically active molecules is a commanding instrument for drug discovery employed to target a variety of diseases. It bids the viewpoint for better drugs to treat number of illnesses including cancer, malaria, tuberculosis and AIDS. Hybrid drugs can deliver combination therapies in a single multi-functional agent and, by doing so, be more specific and powerful than conventional classic treatments [19].

At the periphery of bio-inspired and logical drug design, this has become vital to synthesize hybrid molecules with two or more variable structural spheres with a different set of biological roles. The phrase of hybrid structures has earned much attention in our research group to mature the hybrid motifs for a biological target [20].

In the current manuscript we have successfully attempted to chemically hybridize two different pharmacophore units, i.e. benzimidazoles and the triazolothiadiazoles through a phenyl linker. Over the few decades of active research, benzimidazole has evolved to be an important heterocyclic moiety due to its wide range of pharmacological applications. It has evolved to be an important privileged heterocyclic scaffold in medicinal chemistry with wide range of biological potentials [21]. Similarly, triazolothiadiazole, another important class of fused heterocycles has been found to possess anticancer and antimicrobial profiles [22]. Based on the foregoing, we extrapolated the development of potent, specific and non- cytotoxic inhibitors of β-glucuronidase in order to improve the clinical efficacy of therapeutic agents and effective disease management while bearing in mind the physiological significance of both human and bacterial orthologs of the glycosyl hydrolase.

Our research group has been working extensively on heterocyclic molecular hybrids in search of potentials lead compounds [23–30]. We have reported benzimidazole analogues as effective α-glucosidase inhibitors [31,32] along with potent inhibitors for β-glucuronidase enzyme [33,34]. Herein, we decided to screen a library of benzimidazole based triazolo-thiadiazole analogues for β-Glucuronidase inhibitory potential (Fig. 1).

In search of potent enzyme inhibitors, we have recently reported different heterocyclic compounds along with other functionality in recent past [35–43] (Fig. 2).

2. Results and discussion

2.1. Chemistry

Twenty-six benzimidazole analogues (**1**–**26**) were synthesized by reacting 4-formylbenzoic acid with 4,5-dimethyl-*O*-phenylenediamine in DMF as solvent for 6 h to give 4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)benzoic acid which was reacted with thiocarbohydrazide to form 5-(4-(5,6-dimethyl-1*H*-benzo[*d*]imidazol-2-yl)phenyl)-4*H*-1,2,4-triazole-3-thiol [44] (**II**) which was treated with different substituted benzoic acid to afford (**1**–**26**) target compounds. The crude products were washed and recrystallized in ethanol to afford pure product in 75–85% yield (Scheme 1).

2.2. In vitro β-glucuronidase inhibitory potential

Synthesized benzimidazole based triazolo-thiadiazole analogues (**1**–**26**) were screened against β-glucuronidase inhibitory potential. All analogues showed potent inhibitory potential with IC_{50} values ranging between 1.30 ± 0.10 to 44.10 ± 0.80 μM when compared with the standard drug D-saccharic acid 1,4-lactone (IC_{50} = 48.4 ± 1.25 μM). SAR studies were established by varied substituents on phenyl ring.

The utmost active analogue in series is analogue **2** having 2,3-dihydroxy substituents on phenyl part. The immense potential of this analogue is due to the intramolecular hydrogen bonding between adjacent hydroxyl groups. The comparison of analogue **2** (IC_{50} = 1.30 ± 0.10 μM) with analogue **3** (IC_{50} = 1.60 ± 0.10 μM), revealed almost similar activity due to intramolecular hydrogen bonding between adjacent hydroxyl groups.

Similarly, if we compare analogue **2** and **3** with analogue **11** (IC_{50} = 8.30 ± 0.20 μM) having 2,4-dihyroxy groups and analogue **12** (IC_{50} = 10.40 ± 0.30 μM) having 2,5-dihydroxy groups on phenyl ring four to five-fold less active due to the arrangement of dihydroxy group having no intramolecular hydrogen bonding (Fig. 3). The compound **5** (IC_{50} = 15.40 ± 0.10 μM), **6** (IC_{50} = 4.30 ± 0.10 μM) and **15** (IC_{50} = 19.50 ± 0.30 μM) having one hydroxy group and one methoxy group. The analogue **6** have higher activity as compare to analogues **5** and **15** due to the same reason intramolecular hydrogen binding play part in the interaction with enzyme (Fig. 3).

The analogue **18** is the second most active compound in the series with fluoro substituent on phenyl ring. If we compare analogue **18**, a 2-fluoro analogue with IC_{50} value 1.50 ± 0.10 μM with analogue **14**, a 4-fluoro analogue having IC_{50} value 5.50 ± 0.10 μM and **10**, a 3-fluoro analogue having IC_{50} value 7.30 ± 0.10. Analogue **18** is more potent which revealed that substituent position of fluorine at 2 position plays a vital role in this inhibitory potential compare to position 3 and 4 (Fig. 4).

By comparing analogue **1** (IC_{50} = 15.80 ± 0.50 μM) with analogue **4** (IC_{50} = 4.50 ± 0.10 μM) and analogue **19** with IC_{50} value (21.30 ± 0.50 μM), all these three compounds have nitro group, but at different position on phenyl ring which confirm that the position difference of substituents significantly affect their inhibitory potentials. The analogue **4** having highest activity among other analogues confirms that electron withdrawing substituent at position 2 play a key role in activity (Fig. 5).

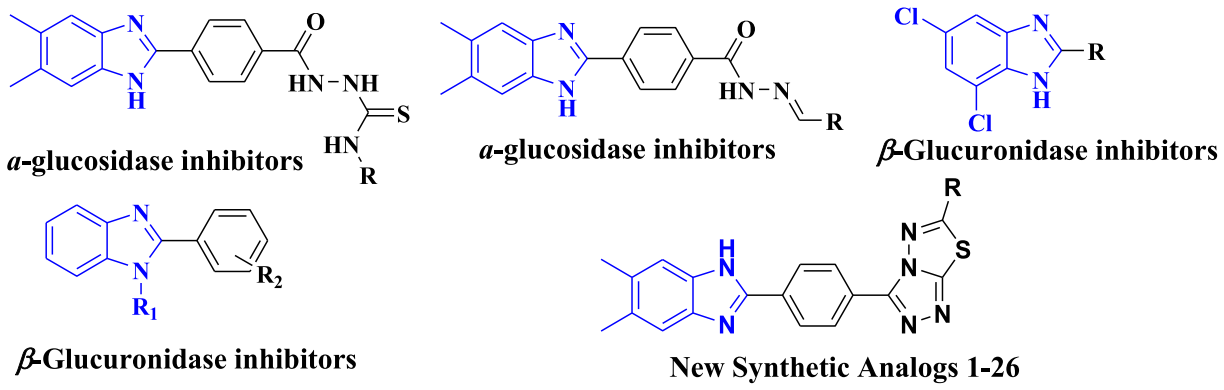


Fig. 1. Rationalization of the newly synthesized benzimidazole based triazolo-thiadiazole.

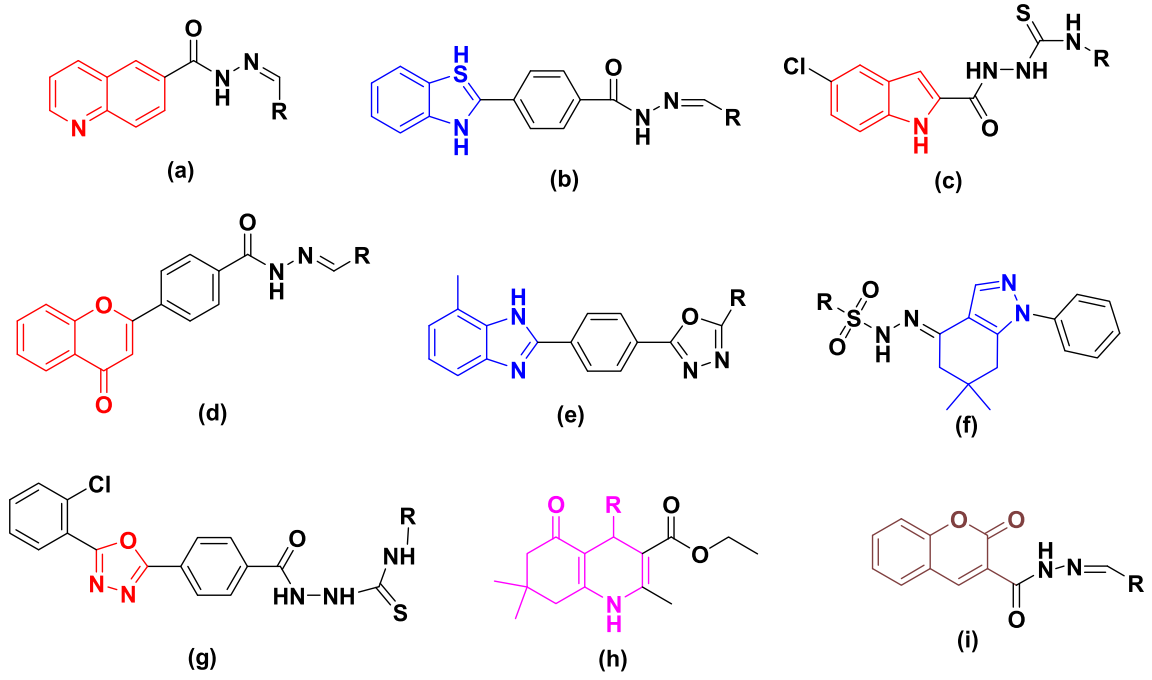


Fig. 2. The different classes of molecules (a–i) recently reported as enzyme inhibitors.

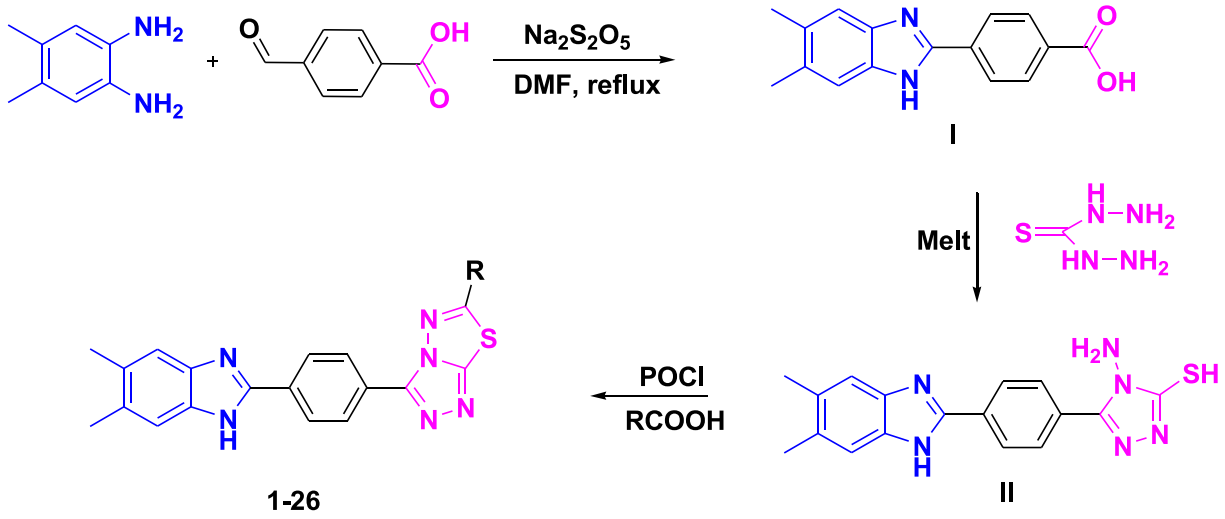
If we compare analogue **13** (IC_{50} = 16.40 ± 0.30 μM), **16** (IC_{50} = 28.20 ± 0.50 μM) and **23** (IC_{50} = 5.30 ± 0.20 μM). The analogue **23** is the most potent among these three which confirms that electron withdrawing substituent at position 2 play a key role in activity as compare to other position analogues **13** and **16** (Fig. 6).

The analogues **8** (IC_{50} = 34.20 ± 0.40 μM) and **9** (IC_{50} = 36.2 ± 0.40 μM) having methyl showed moderate activity compare with the dihydroxy or fluorine containing compounds which means electron donating substituents showed less activity. On the other hand, the analogues **7** (IC_{50} = 12.20 ± 0.40 μM) and **24** (IC_{50} = 16.5 ± 0.70 μM) having 2-methoxy and 4-methoxy respectively showed good activity (Fig. 7).

The analogues **17** having 2-4-dichloro (IC_{50} = 10.40 ± 0.40 μM), **20** having 2-chloro (IC_{50} = 6.20 ± 0.20 μM), **21** having 4-chloro (IC_{50} = 12.40 ± 0.40 μM) and **22** having 3-chloro (IC_{50} = 18.20 ± 0.20 μM) showed good activity. The analogue **20** showed highest activity when comparing with other analogues, which is due to the chloro substitution at position 2. Surprisingly analogue **17** having chloro at position 2 but at 4 as well which cause the decrease in its activity. The analogue **21** showed better activity compared to **22** this may be due to the 4-position of chloro in analogue **21** (Fig. 8).

The analogue **25** having 4-pyridene (IC_{50} = 132.40 ± 0.80 μM), and **26** 3-pyridene (IC_{50} = 44.10 ± 0.80 μM) showed moderate activity. The analogue **25** showed higher activity than analogue **26** due to 4-pyridene ring (Fig. 9).

From the whole study it has been concluded that electron donating and electron withdrawing group on phenyl ring play dynamic role in the inhibition. However the number of substituents also affected the inhibitory potential. To understand the binding interaction of these compounds with enzyme, molecular docking study was executed.



Scheme 1: Synthesis of Phenyl Linked Benzimidazole-Triazolothiadiazole1-26..

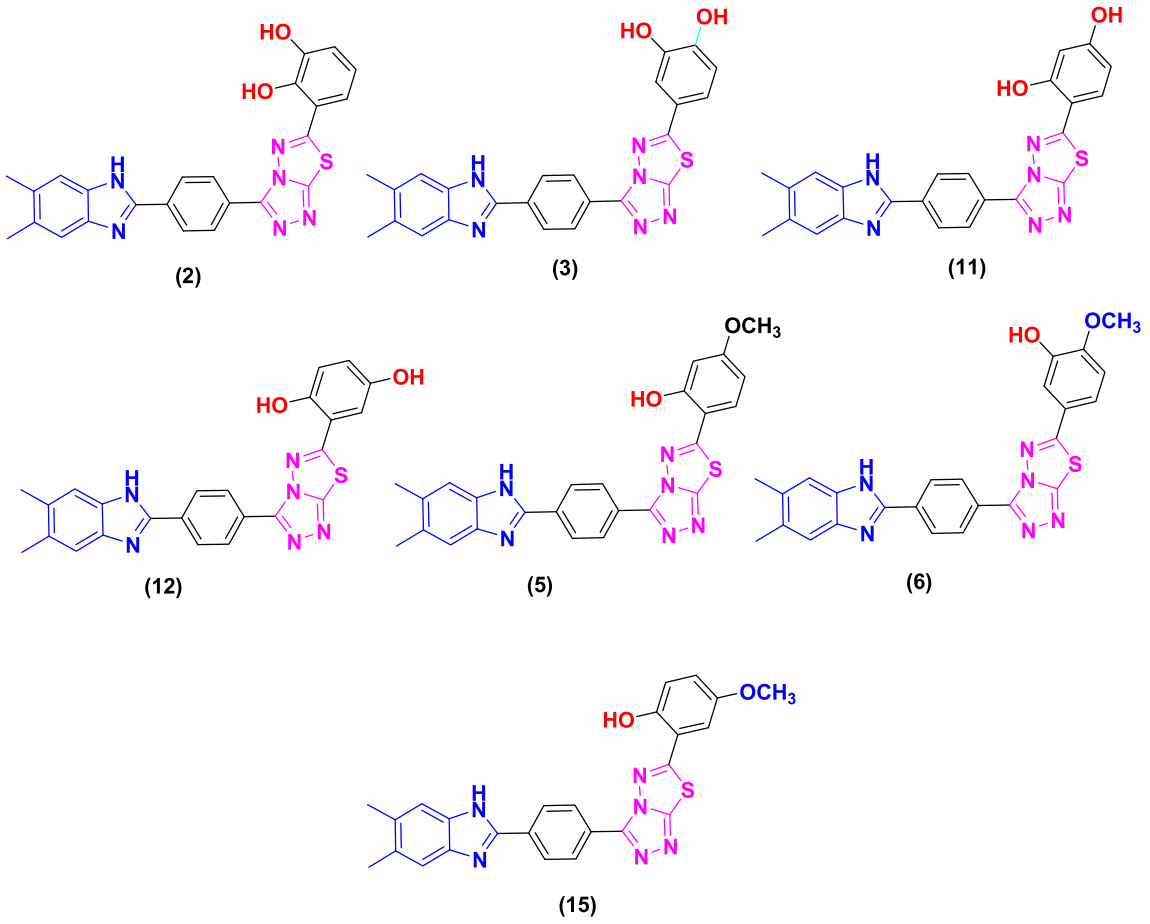


Fig. 3. The analogue having dihydroxy **2**, **3**, **11** and **12** and monohydroxy with methoxy substitution **5**, **6** and **15**.

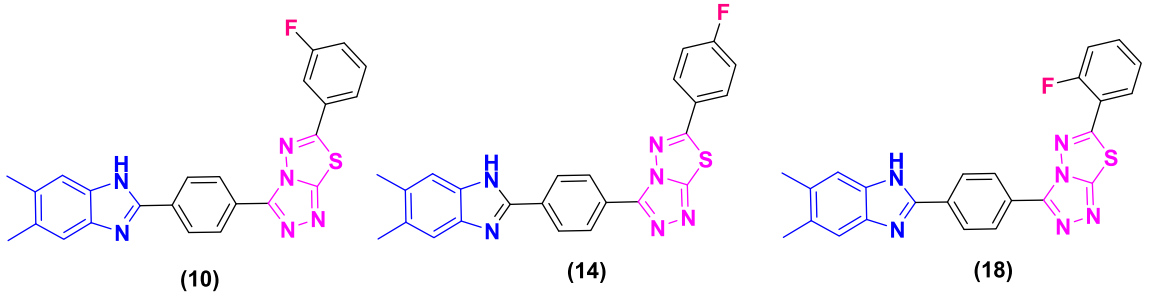


Fig. 4. The analogue **10**, **14** and **18** having fluorine at varied positions on phenyl ring.

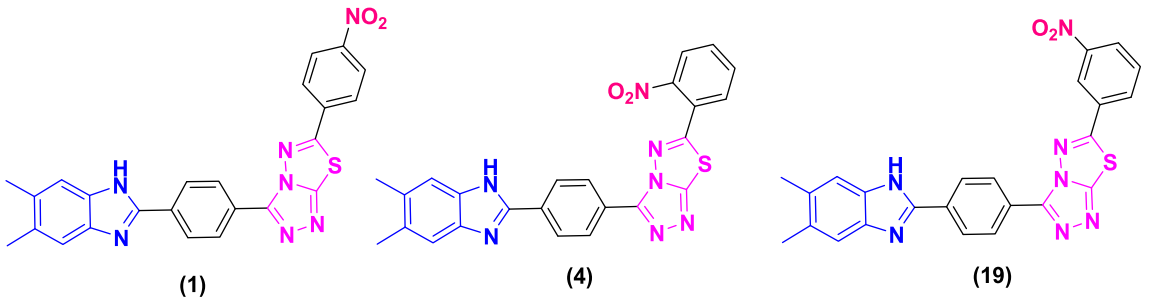


Fig. 5. The analogue **1**, **4** and **19** having nitro group at varied position on phenyl ring.

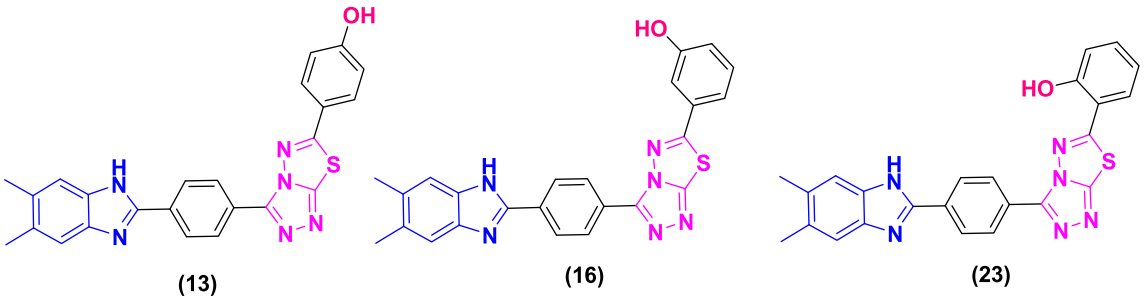


Fig. 6. The analogue **13**, **16** and **23** having hydroxyl group at varied position on phenyl ring.

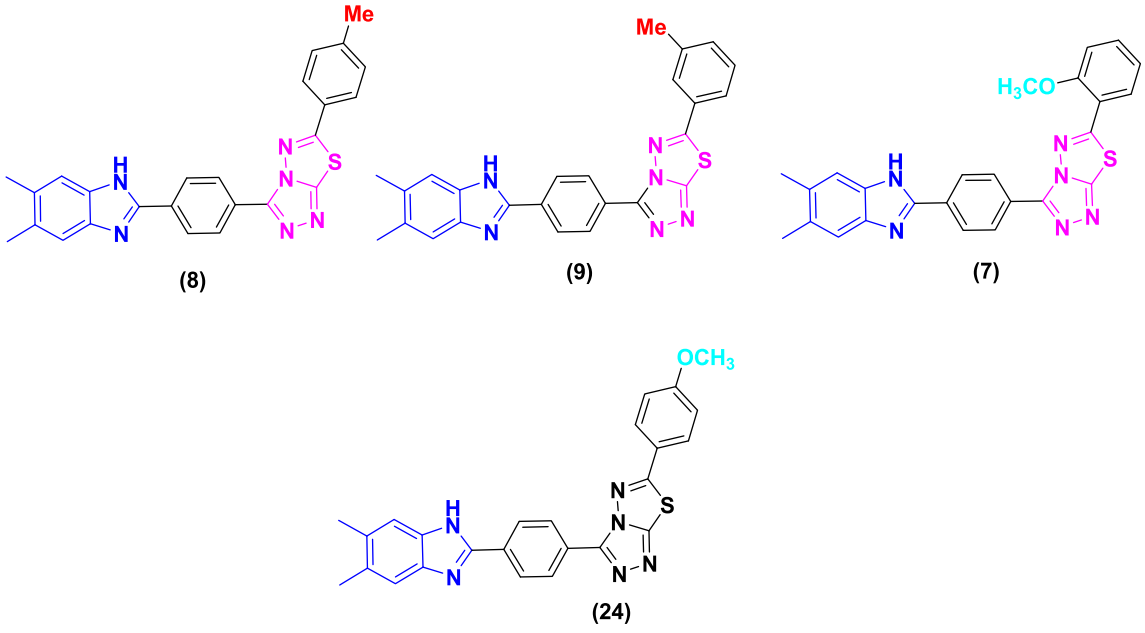


Fig. 7. The analogue having methyl **8** and **9** and methoxy **7** and **24**.

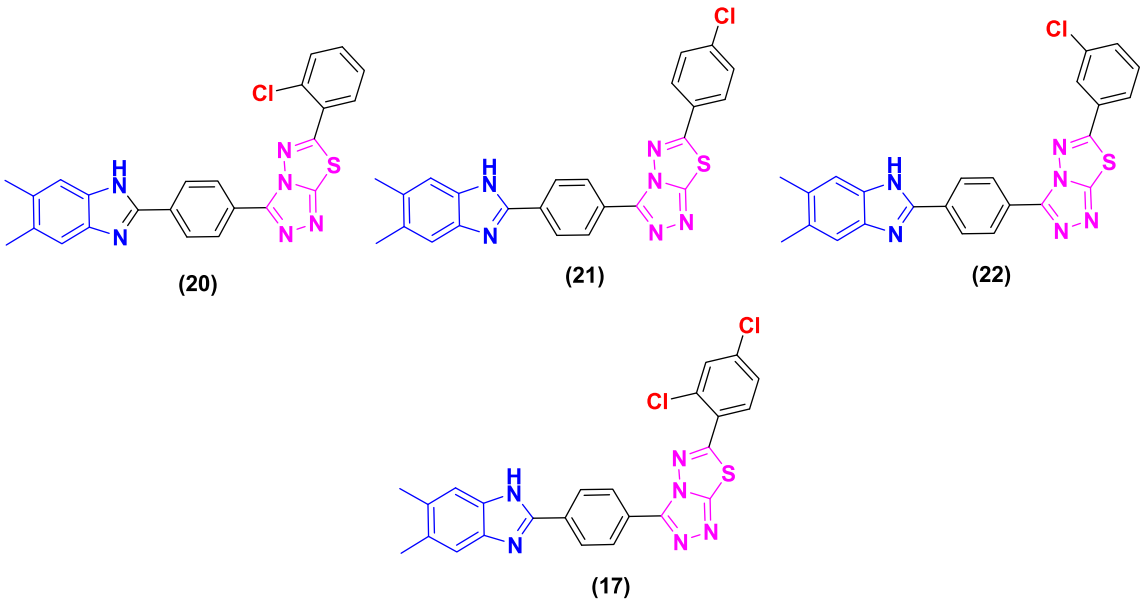


Fig. 8. The analogue **17** and **20**–**22** having chlorine at varied position on phenyl ring.

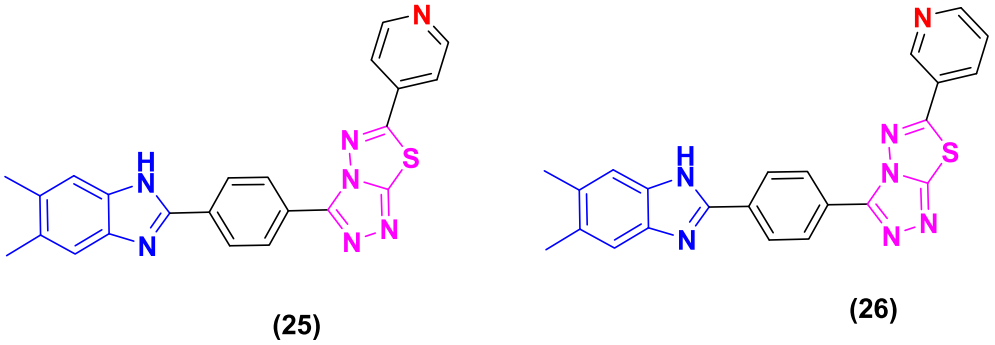
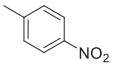
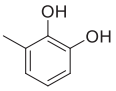
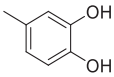
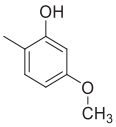
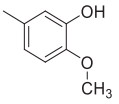
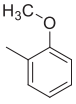
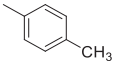
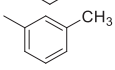
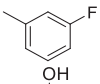
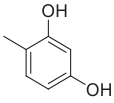
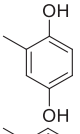
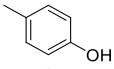
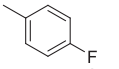
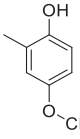
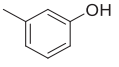
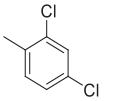
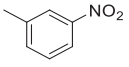
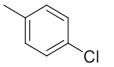
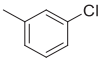
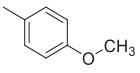


Fig. 9. The analogue having pyridine ring **25** and **26**.

Table 1
Different substituents and β-Glucuronidase inhibitory potential of benzimidazole based triazolo-thiadiazole analogues.

No.	R	IC_{50} values	S. No	R	IC_{50} values
1.		15.80 ± 0.50	**14**		**5.50 ± 0.10**
2.		1.30 ± 0.10	**15**		**19.50 ± 0.30**
3.		1.60 ± 0.10	**16**		**28.20 ± 0.50**
4.		4.50 ± 0.10	**17**		**10.40 ± 0.40**
5.		15.40 ± 0.10	**18**		**1.50 ± 0.10**
6.		4.30 ± 0.10	**19**		**21.30 ± 0.50**
7.		12.20 ± 0.40	**20**		**6.20 ± 0.20**
8.		34.20 ± 0.40	**21**		**12.40 ± 0.40**
9.		36.2 ± 0.40	**22**		**18.20 ± 0.20**
10.		7.30 ± 0.10	**23**		**5.30 ± 0.20**
11.		8.30 ± 0.20	**24**		**16.5 ± 0.70**
12.		10.40 ± 0.30	**25**		**32.40 ± 0.80**
13.		16.40 ± 0.30	**26**		**44.10 ± 0.80**
Standard Drug		**D-saccharic acid 1,4-lactone**			48.4 ± 1.25

2.3. Molecular docking study

IC_{50} values of benzimidazole based triazolo-thiadiazole analogues as β-glucuronidase inhibitors are shown in Table 1. It is obvious form Table 1 that the inhibitory efficiency of the benzimidazole based triazolo-thiadiazole analogues depends on the number, type and positions of the substituents in group R of the basic skeleton. To shed light on the binding interaction between the synthesized compound and β-glucuronidase enzyme active sites of lysosomal β-glucuronidase (pdb 1BHG) and a bacterial β-glucuronidase (pdb 5G05), molecular docking study has been performed on three selected compounds (**2**, **13** and **23**). Table 2 summarized the calculated binding energies of the stable complexes ligand-β-glucuronidase, number of established intermolecular hydrogen bonding between the synthesized compounds (**2**, **13** and **23**) and active site residues of lysosomal β-glucuronidase (pdb 1BHG) and a bacterial β-glucuronidase (pdb 5G05).

All the complexes formed between the docked compounds (**2**, **13** and **23**) and the amino acids into the pocket binding sites of β-glucuronidase show negative bending energies, which is an indication that the inhibition of lysosomal and bacterial β-glucuronidases by the selected compounds is thermodynamically favorable (Table 2). The stability of the complexes formed between the docked selected compounds and the amino acids into the active sites of lysosomal and bacterial β-glucuronidases is relatively in well accordance with the observed results (Table 2). All the selected compounds form hydrogen bonding with the active amino acids of β-glucuronidase. It is obvious that the higher inhibition efficiency of **2** compared with **13** and **23** is refer to the existence of hydroxyl groups in *ortho* and *meta* position. In case of lysosomal β-glucuronidase, the two hydroxyl groups of 2 form three conventional hydrogen bonding with ASN A174 and LEU A176 of distances 2.02, 2.26 and 2.92 Å (Fig. 10). However, for bacterial β-glucuronidase, the two hydroxyl groups form two hydrogen bonding with Glu A 245 of distance 1.85 and 2.29 Å (Fig. 10).

3. Conclusion

Current study culminated a highly modular benzimidazole-triazolothiadiazole hybrids spaced through a phenyl linker (**1**–**26**) as an effective class of β-glucuronidase inhibitors. Further, the virtual interactions of this enzyme with various hybrid substrates were also investigated using molecular docking as a principal tool. Newly synthesized analogues displayed various degree of potential as β-glucuronidase inhibitors while minimum inhibitory concentrations (IC_{50}) in the range of 1.30 ± 0.10 to 44.10 ± 0.80 μM. These results were some of the most astounding of our research work since we found that our newly developed hybrid structure was many a times more potent than the standard inhibitor (D-saccharic acid 1,4-lactone; IC_{50} = 48.4 ± 1.25 μM). In silico investigations further disclosed the potential interactions among the inhibitor and active amino acid site in β-glucuronidase. These conclusions helped us in detecting and identifying the likelihood for newer drug candidates. A Plausible SAR suggested that substituent/s residing on phenyl ring played a pivotal role in the inhibitory potentials of these hybrid structures.

4. Material and methods

4.1. Synthetic chemistry procedures and characterization

All procedures and characterization data of synthetic chemistry are provided in the Supporting information.

4.2. Molecular docking details

Autodock package was carried for binding mode of synthesized benzimidazoletriazole-thiadiazole analogues; and human lysosomal β-glucuronidase and bacterial β-glucuronidase from acidobacteriumcapsulatum [44]. Coordinates of human lysosomal β-glucuronidase and bacterial β-glucuronidase and their corresponding originated docked ligands were downloaded from the RCSB data bank web site (PDB codes 1BHG and 5G0Q) [45,46].

4.3. β-Glucuronidase bioassay

Previously known method was used for determining β-Glucuronidase activity [46] using spectroscopic analysis by measuring the absorbance of p-nitro-phenol formed substrate. Total volume of the complete reaction was 250 μL. The reaction mixture was including 10 μL β-Glucuronidase (E.C. 3.2.1.31, from bovine liver, G-0251) enzyme solutions (2.5 unit/mL), 185 μL acetate buffers (pH = 7.0) and incubated for 30 min at 37 °C. The plates were recorded on a multiplate reader (SpectaMax plus 384) after the addition of 50 μL of 0.4 mM *p*-nitrophenyl-β-D-glucuronide. Experiments were performed for triplicate [47]. To avoid molecules precipitation, compound concentrations were decreased, and the volume of the reaction was increased (200 μL). Precipitation probability was less; thus, the addition of detergents was not needed.

4.4. Statistics analysis IC_{50}

The IC_{50} values, concentration required to inhibit the enzyme activity by 50% were calculated by a non-linear regression graph plotted between percentage inhibition (x axis) versus concentrations (y axis), using a Graph Pad Prism Software (Version 5).

Declaration of competing interest

The authors declare no competing financial interests.

Table 2
IC_{50}, free binding energies, number of conventional hydrogen bonding, and number of closest residues to the docked synthesized derivatives (**2**, **13** and **23**) within the binding sites of lysosomal β-glucuronidase (pdb 1BHG) and bacterial β-glucuronidase (pdb 5G05).

Compound no.	Free binding energy (kcal/mol)	Conventional H-Bonds (HBs)	Number of closest residues to the docked ligand in the active site	IC_{50} ± SEM
1BHG				
2	−8.33	3	ASN A: 174; LEU A: 176; THR A: 177; TRP A: 98; ARG A: 55; ASN A: 54; TYR A: 188; PHE A:200; PHE A:51; ASP A: 53	1.30 ± 0.10
13	−7.92	2	SER A:128; GLU A:143;TYR A:129; TYR A:98; TRP A: 98; ARG A: 55; ASN A: 54; TYR A: 188; PHE A:200; PHE A:51	16.4 ± 0.30
23	−8.01	2	SER A:128; GLU A:143; TRP A: 98; ARG A: 55; TYR A: 188	5.30 ± 0.20
5G0Q				
2	−9.87	4	GLU A:173; PRO A:248; PRO A:247; GLY A:246; GLU A:245; TYR A: 292; LEU A: 176; GLN A: 293; ASN A: 80; GLU A:287; PRO A:104; HIS A:327	1.30 ± 0.10
13	−9.79	2	GLU A:173; ASP A:175; LEU A: 176; TYR A: 292; TYR A: 243; GLU A:287; ASN A: 80; PRO A:104; HIS A:327	16.4 ± 0.30
23	−9.84	2	GLU A:173; ASP A:175; LEU A: 176; TYR A: 292; TYR A: 243; GLU A:287; PRO A:104; HIS A:327	5.30 ± 0.20

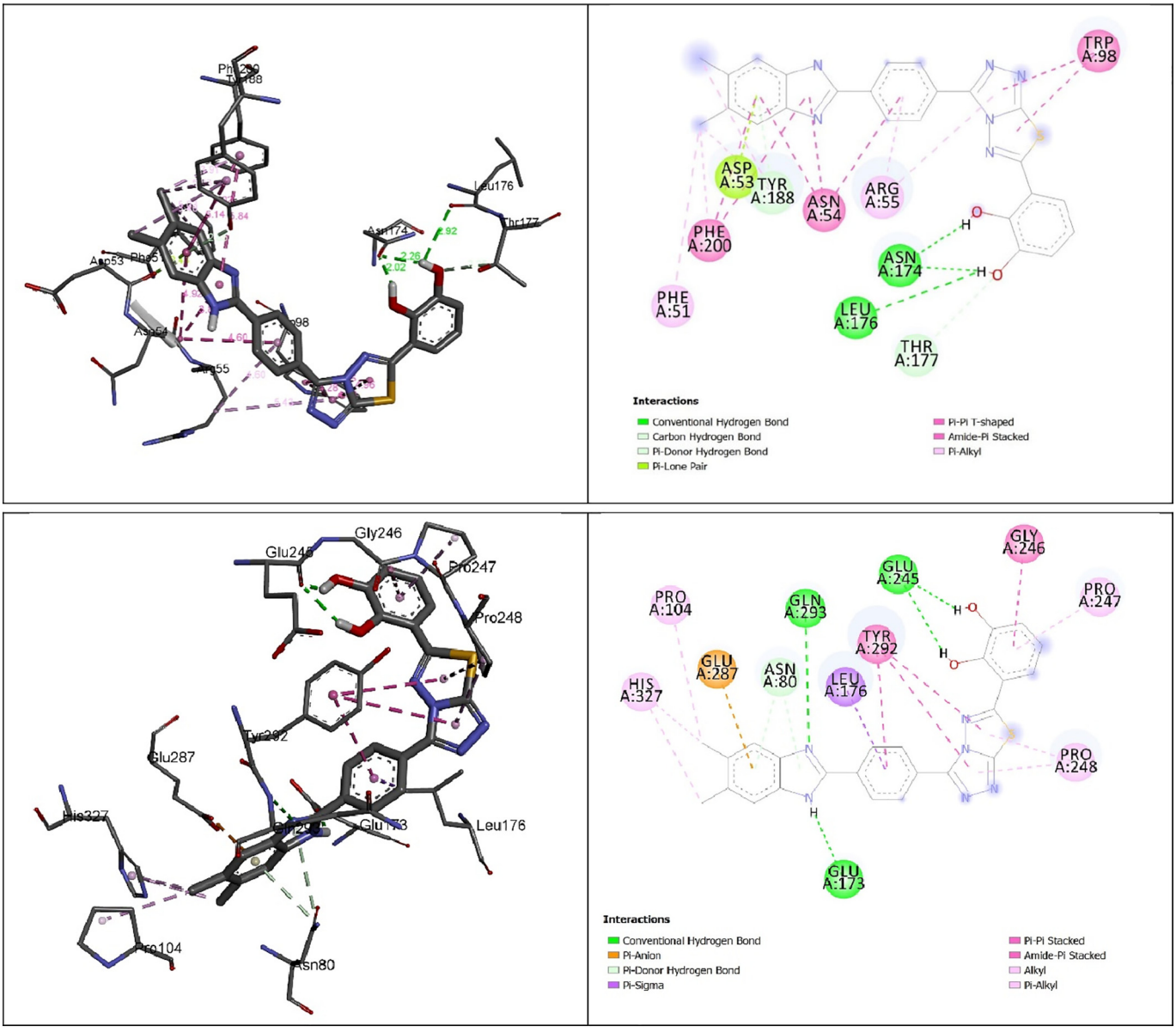


Fig. 10. 3D (right) and 2D (left) closest interactions between active site residues of β-glucuronidase and synthesized compounds **2** (up) and **13 (bottom)**.

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Appendix A. Supplementary data

Supplementary data consist of synthesized compounds characterization and synthetic procedure and spectroscopy spectra. Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2020.06.006.

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