J Comput Aided Mol Des (2014) 28:577–585
DOI 10.1007/s10822-014-9745-z

Structure-based design, synthesis and biological evaluation of β-glucuronidase inhibitors

Khalid M. Khan · Nida Ambreen · Muhammad Taha · Sobia A. Halim · Zaheer-ul-Haq · Shagufta Naureen · Saima Rasheed · Shahnaz Perveen · Sajjad Ali · Mohammad Iqbal Choudhary

Received: 21 November 2013 / Accepted: 15 April 2014 / Published online: 27 April 2014

Abstract Using structure-based virtual screening approach, a coumarin derivative (**1**) was identified as β-glucuronidase inhibitor. A focused library of coumarin derivatives was synthesized by eco-benign version of chemical reaction, and all synthetic compounds were characterized by using spectroscopy. These compounds were found to be inhibitor of β-glucuronidase with IC_{50} values in a micromolar range. All synthetic compounds exhibited interesting inhibitory activity against β-glucuronidase, however, their potency varied substantially from IC_{50} = 9.9–352.6 μM. Of twenty-one compounds, four exhibited a better inhibitory profile than the initial hit 1. Interestingly, compounds **1e**, **1k**, **1n** and **1p** exhibited more potency than the standard inhibitor with IC_{50} values 34.2, 21.4, 11.7, and 9.9 μM, respectively. We further studied their dose responses and also checked our results by using detergent Triton ×-100. We found that our results are true and not affected by detergent.

Electronic supplementary material The online version of this article (doi:10.1007/s10822-014-9745-z) contains supplementary material, which is available to authorized users.

K. M. Khan (✉) · N. Ambreen · S. Naureen · S. Rasheed · M. I. Choudhary
H. E. J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan
e-mail: khalid.khan@iccs.edu

M. Taha
Atta-ur-Rahman Institute for Natural Product Discovery, Universiti Teknologi MARA (UiTM), Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor D. E., Malaysia

M. Taha
Faculty of Applied Science, UiTM, 40450 Shah Alam, Selangor, Malaysia

Keywords β-Glucuronidase · Coumarin derivative · Docking study

Introduction

The lysosomal acid hydrolase, β-glucuronidase (E.C. 3.2.1.31) has been studied extensively both as model of enzyme sorting and transport and as an example of hormonally and developmentally regulated enzyme [1]. Human β-glucuronidase is reported to synthesize as an 80 kDa monomer (653 amino acids) before proteolysis removing 18 amino acids from the C-terminal end to form a 78 kDa monomer [2, 3]. β-glucuronidase exists as a 332 kDa homotetramer [4]. β-Glucuronidase contains several notable structural formations, including a type of β barrel known as a jelly roll barrel and a TIM barrel [5].

It catalyzes the stepwise degradation of β-D-glucuronic acid residues from the non-reducing termini of glycosaminoglycans (Hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin srulfate) to yield their respective

S. A. Halim · Zaheer-ul-Haq
Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

S. Perveen
PCSIR Laboratories Complex, Karachi, Shahrah-e-Dr. Salimuzzaman Siddiqui, Karachi 75280, Pakistan

S. Ali
Department of Chemistry, Karakoram International University, Gilgit-Baltistan, Gilgit 15100, Pakistan

glycosidase and hydrolysis results in release of alcohols and free glucuronic acid. Human β-glucuronidase is reported to be ubiquitously present in many organs and body fluids such as macrophages and other blood cells, liver, spleen, kidney, intestinal secretions, lung, muscle, bile, urine and serum [6]. To date, the only available three dimensional structures for β-glucuronidase is the X-ray crystal structure of human β-glucuronidase, determined by Jain et al. [5]. Structurally the enzyme is a tetramer of identical subunits from a single gene product. The native enzyme, human β-glucuronidase, has a molecular weight of 332,000 a.m.u., each monomer consisting of a single chain of 651 amino acid residues with four Asn-linked glycosylation sites. Each monomer is arranged into three structural domains, comprising residues 22–223, 224–342 and 343–632. The first domain is a highly distorted barrel-like structure with a jelly roll motif and two β-hairpin insertions. The second domain is similar in topology to the immunoglobulin constant domain, while the C-terminal domain forms an α/β or TIM-barrel motif. The last portion or the C-terminal domain is particularly important since it contains the active site of the enzyme. Amino acids Glu451, Glu540, and Tyr504 are proposed to be involved in catalytic process [5]. Enhanced activity of this enzyme have been reported in a variety of pathological conditions, including urinary tract infection [7–10], renal diseases [11], transplant rejection [12], epilepsy [13], and neoplasms of bladder [14], testes, larynx, and breast [15]. Furthermore, β-glucuronidase is reported to be released into the synovial fluid in inflammatory joint diseases such as rheumatoid arthritis, where these enzymes contribute to the symptoms of these diseases [16]. The over-expression of the enzyme also reported in some hepatic diseases and AIDS [17]. β-Glucuronidase is also found to be involved in the etiology of colon cancer and high intestinal level of the enzyme leads to higher incidence of colon carcinoma [18, 19]. This view has received support from the evidence that the administration of a bacterial β-glucuronidase inhibitor has resulted in a decrease in size of carcinogen-induced colonic tumors [20]. All these reports clearly suggest that the development of specific inhibitors of β-glucuronidase is of significant pharmacological importance. Stemming from our interest in the development of novel, potent β-glucuronidase inhibitors [21], we report herein the effective use of virtual screening protocol (using a combination of high-throughput docking with multiple scoring functions) for the identification of prospective ligands that bind to β-glucuronidase. In particular, recently we have reported an in-house molecular database, comprises of over 6,000 compounds of synthetic or natural origins [22]. These structurally characterized compounds fulfill the drug-like criteria according to Lipinski's rule of five [23], with the exceptions of a few naturally isolated compounds. This 6,000 compounds database was used for the search of potential candidates against β-glucuronidase. The libraries of 6,000 compounds were docked by GOLD docking program. Furthermore the top ranked docked poses of GOLD were re-scored by using four different scoring functions i.e. D-Score, G-Score, ChemScore and PMF score embedded in CScore module of Sybyl 7.3. Subsequently, consensus scoring was implemented and top 5 % (300 compounds) compounds (ranked by all the scoring functions) were selected and their binding modes were inspected visually. Analysis of docking results led us to choose four compounds (Fig. 2). These compounds were subjected to in vitro screening against β-glucuronidase inhibition.

Materials and methods

The 3D coordinates of the compounds were generated by Molecular Operating Environment [24] (MOE). The "Washing" module of MOE was applied to generate the physiologically relevant protonation state of compounds. The structures were minimized by MMFF94 force field and charges were added. These compounds were only considered in one tautomeric or protonation state during docking. Our in-house database [25, 26] comprises over 6,000 3D structures of small molecules stored in mol format [27].

Docking protocol

The 2.6 Å resolution X-ray crystal structure of human β-D-glucuronidase (PDB code 1BHG, the Protein Data Bank: http://www.rcsb.org/pdb) was retrieved for docking studies. The protein structure was checked for missing atoms, bonds and contacts. Hydrogen was added to enzyme structure. The B-chain of protein and hetero-atoms including cofactors were removed from the crystal structure. All the docking calculations were performed on Xeon 3.0 GHz Linux work station running under SUSE9.2, using GOLD [28] as the computational software.

GOLD4.0

The configuration file was defined by the following process. The docking site was defined as all atoms within 10 Å of a specified central point (x, y, z coordinates: 80.43, 84.41, 90.48). For each independent GA run, a maximum number of 1,000 GA operations was performed on a single population of 50 individuals. Operator weights for cross over, mutation and migration were set to 100, 100 and 0 respectively. To allow poor non bonded contacts at the start of each GA run, the maximum distance between hydrogen

donors and fitting points were set to 5.0 Å and non-bonded VdW energies were cut-off at 10 Å.

To build a protein model whose active site exists in the probable substrate-bound conformation, the known substrate *p*-nitrophenyl β-glucuronide was first docked into the active site of β-glucuronidase using the GOLD. From the docking results, a high-scoring docked pose of *p*-nitrophenyl β-glucuronide having the glucuronyl moiety oriented properly toward the key residues Glu451 and Glu540 involved in the enzyme catalysis reaction was chosen to represent its most probable binding mode. The obtained docking model was further subjected to energy minimization for refinement. Prior to energy minimization, the Glu451 residue in the active site was assigned a protonated form that was consistent with its role in acting as a proton donor during catalysis. In addition, a structural subset was specified as the ligand and the amino acid residues within a 10 Å radius of the ligand. The complex structure was then refined through a two-step minimization, allowing (1) only the ligand and the side chains of the subset to relax and then (ii) only all the atoms of the subset to relax. The energy minimization calculations were performed in SYBYL 7.1 using the Tripos force field, the Powell method, a distance-dependent dielectric constant of 1*r*, and a non-bonded cutoff of 8 Å. The minimization was terminated when the energy gradient convergence criterion of 0.01 kcal/(mol.Å) was reached.

The modeled complex structure of β-glucuronidase with the substrate *p*-nitrophenyl β-glucuronide was used for the prediction of a favorable binding mode of database compounds to β-glucuronidase by docking. The top ranked GOLD docked poses of all the compounds were further re-scored using the CScore module implemented in SYBYL7.2 to estimate the binding affinity of the compounds. To support the virtual screening results dose dependent graphs are also given here. The O.D of compounds and their respective % inhibition at different concentration are given in dose–response curves.

2D similarity searching

MOE [24] was employed for similarity searching. We selected Molecular ACCess System (MACCS) structural keys [24] (166 bit positions) with the general form of Tanimoto coefficient [24]. MACCS consists of 166 structural substructures/patterns with 1–10 non-hydrogen atoms. As a default parameter, 85 % of overlap was selected.

Bioassay techniques with determination of IC_{50} values

The concentration of test compounds that inhibited the enzyme activity by 50 % (IC_{50} values) were determined by monitoring the effect of increasing concentrations of compounds in the assay described below. The IC_{50} values were calculated from inhibition data by using EZ-Fit Enzyme Kinetic Program (Perrella Scientific Inc., Amherst, USA).

β-Glucuronidase assay protocol

β-Glucuronidase activity was determined by measuring the absorbance at 405 nm of *p*-nitrophenol formed from the substrate by the spectrophotometeric 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-β-D-glucuronide [29]. All assays were run in triplicate. 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.

Assay for antiglycation

Bovine serum albumin (BSA) was purchased from Research Organics (Cleveland, USA), while others chemicals {glucose anhydrous, trichloroacetic acid (TCA) sodium azide (NaN_3), dimethyl sulfoxide (DMSO), sodium dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydroxide (NaOH) were purchased from the Sigma-Aldrich. Sodium phosphate buffer (pH 7.4), was prepared by mixing Na_2HPO_4 and NaH_2PO_4 (67 mM) containing sodium azide (3 mM). Phosphate buffer saline (PBS) was prepared by mixing NaCl (137 mM), Na_2HPO_4 (8.1 mM), KCl (2.68 mM), and KH_2PO_4 (1.47 mM) and pH 10 was adjusted with NaOH (0.25 mM). BSA (10 mg/mL) and glucoses anhydrous (50 mg/mL) solutions were prepared in sodium phosphate buffer. Test samples were prepared in DMSO (1 mM/mL).

In 96-well plate assays, each well contained 60 µL reaction mixtures (20 µL BSA (10 mg/mL), 20 µL of glucose anhydrous (50 mg/ml) and 20 µL test sample). Glycated control contain 20 µL BSA, 20 µL glucose and 20 µL sodium phosphate buffer, while blank control contains 20 µL BSA and 40 µL sodium phosphate buffer [30]. Reaction mixture was incubated at 37 °C for 7 days. After incubation, 6 µL TCA 100 % was added in each well and centrifuged (15,000 rpm) for 4 min at 4 °C. After centrifugation, the pellets were rewashed with 60 µL of TCA (5 %). The supernatant containing glucose, standard inhibitor and interfering substance, was removed and pellet

containing, AGE-BSA were dissolved in 60 μL of PBS. Fluorescence spectrum (ex. 370 nm), and change in fluorescence intensity (370–440 nm), based on AGEs formation, were monitored by using Spectrofluorimeter RF-1500 (Shimadzu, Japan).

% inhibition was calculated through the following formula:

$$\% \text{ Inhibition } = 1 - [(\text{Fluorescence of test sample})/(\text{Fluorescence of glycated})] \times 100$$

Phosphodiesterase I inhibition assay

Activity against snake venom was assayed by taking 33 mM *Tris*-HC1 buffer pH 8.8, 30 mM Mgacetate with 0.000742 U/well final concentration of enzyme using a microtiter plate assay and 0.33 mM *bis*-(*p*-nitropheny1) phosphate (Sigma N-3002) as substrate. From Merck Cystein and EDTA were used as positive controls ($1C_{50} = 748 \pm 0.015$, 274 ± 0.007 μM, respectively). After 30 min pre-incubation of the enzyme with the test samples, enzyme activity was monitored spectrophotometrically at 37 °C on a microtitre plate reader (SpectraMax, Molecular Devices) by following the rate (change in OD/min) of release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 410 nm. All assays were conducted in triplicate [31–34].

Results and discussion

Structure based virtual screening experiment was performed targeting the active site amino acid residues (Glu451, Glu540, and Tyr504) of β-glucuronidase. The crystal structure of human β-glucuronidase was retrieved from the Protein Data Bank (PDB code: 1BHG). To scrutinize the performance of our molecular modeling process, the chromogenic derivative of known physiological substrate *p*-nitro phenyl β-glucuronide [35] was first docked into the active site of β-glucuronidase using GOLD [28]. The purpose of such initial modeling was to build a protein model whose active site exists in the probable substrate-bound conformation. The modeled substrate-bound structure of human β-glucuronidase showed that the glycosidic bond of *p*-nitrophenyl D-glucuronide was properly oriented towards the catalytic residues Glu451 and Glu540 (Figure S1 in supporting information). It has been proposed for human β-glucuronidase that during the catalysis, Glu451 acts as an acid/base catalyst while Glu540 serve as a nucleophilic residue [36]. This modeled protein structure was then used in the prediction of a favorable binding mode. Ranking of database compounds was achieved via GOLD docking program. GOLD was used with two scoring functions GOLD-Score [21] and ASP. [22]. Additionally, the CScore module of Sybyl7.3 [25] were utilized to re-score the top scored docked poses obtained by GOLD, by using four scoring functions (D-score, G-score, ChemScore, and PMF-score).

Table 1 Inhibition of β-glucuronidase by compounds **1**–**5**, calculated as ($IC_{50} \pm$ SEM)

Compound ID	($IC_{50} \pm$ SEM)
1	113.6 ± 4.00
2	172 ± 00
3	194 ± 1.88
4	266 ± 12.8
d-Saccharic acid[a]	48.4 ± 1.25

SEM standard error of the mean

[a] standard drug

The activity of target protein was determined as described in supporting information. [a]D-Saccharic acid (standard inhibitor for β-glucuronidase inhibitory activity). The ranks and scores of compounds **1**–**4**, obtained by all the above mentioned scoring functions are reported in supporting information (Table 1).

In order to evaluate the efficacy of scoring functions to identify the known inhibitors from the random database, five known inhibitors (Fig. 2) [37–41] were included in 6,000 compounds. All the above mentioned scoring functions were able to identify the known inhibitors in the top 5 % of the screened database. After the docking/scoring protocol, top 5 % of the ranked database (predicted by all the above mentioned scoring functions) was selected and the docked poses were inspected visually. Analysis of docking results led us to choose four compounds (Fig. 2).

These compounds were subjected to in vitro screening against β-glucuronidase inhibition. All the selected compounds showed biological activities in micro-molar range. The IC_{50} value of compounds **1**–**4** ranged between 113.6 ± 4.00 to 266 ± 12.8 μM (Table 1).

In addition 2D similarity searches were conducted by using the Tanimoto coefficient with MACCS keys (166 bits) as implemented in MOE [24]. The chemical structures of 5,7,8,3′,4′,5′-hexahydroxyflavone-*O*-β-D-glucuronide and Scopoletin were used as queries (Figure S2). Compounds **1**–**4** were returned with the best Tanimoto values ranging from 0.66 to 0.88. 2D Similarity profile is summarized in Table-1 in supplementary information. Detailed information about virtual screening strategy and the flow chart of high throughput docking (Figure S3) is described in the Supporting Information.

The binding mode of compound **1**, obtained by GOLD, is presented in Fig. 1a, while the binding mode of compounds **2**–**4** is shown in supporting information (Figure S4). Molecular docking investigations have clearly demonstrated that all the compounds displayed a good steric and chemical complementarities with the ligand binding site. Compound **1** was found to be completely surrounded

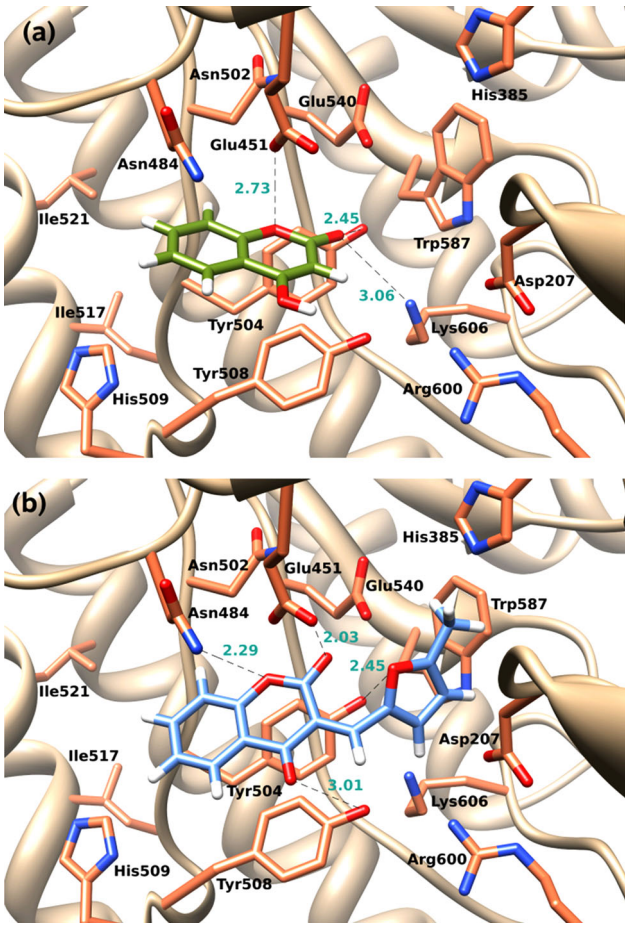


Fig. 1 Binding mode analysis of compound **1** (**a**) and compound **1p** (**b**) in the catalytic binding site of β-glucuronidase. Hydrogen bonds are indicated as *dashed lines*

by the catalytic amino acid residues in the active site of the enzyme and mediating strong hydrogen bond with OE2 of Glu451 (2.73 Å). The carbonyl oxygen of the compound **1** created a hydrogen bond with hydroxyl group of Tyr504 (2.45 Å) and the side chain amino nitrogen of Lys606 (3.06 Å). Additionally, the benzopyrone moiety of compound **1** depicted π–π interaction with the side chain phenol ring of Tyr504. In addition, several hydrophobic interactions with Tyr205, Asp207, Asn484, Asn450, Asn502, Tyr504, Tyr508, Glu540, Ag600 and Lys606 which contribute to stabilization of compound **1** in complex with β-glucuronidase.

The binding mode analysis of compound **2** (Figure S4a in the Supporting Information) revealed that the compound is stabilized by hydrogen bonds with important active site amino acid residues. The hydrogen bonding was observed between the carbonyl oxygen of the compound **2** and the binding site residues Arg600 and Lys606. The carbonyl oxygen of **2** accepts hydrogen bonds with the side chain side chain guanidinium moiety of Arg600 and the side chain amino group of Lys606 with the distance of 2.29 and 1.79 Å, respectively. The phenyl ring of the compound **2** is stabilized by the π–π interactions provided by the side chain phenol ring of Tyr 504 and Tyr508 (Fig. 2).

Similar to the binding mode of compound **2**, the carbonyl moiety of compound **3** also mediates hydrogen bonding interactions with the side chains of Arg600 and Lys606. The observed hydrogen bond distance between the side chain guanidinium (Nε2) of Arg600 was 2.84 Å, while the hydrogen bond distance between the carbonyl moiety and the side chain amino nitrogen (Nζ) was 1.41 Å. In addition, compound **3** also mediates hydrogen bonding with side chain of Asp207 (1.85 Å).

The predicted docked pose of compound **4** revealed that it is hydrogen bonded to the imidazole ring of His385. The Nε2 of imidazole ring of His385 served as hydrogen bond acceptor and accepted the phenolic hydroxyl hydrogen of compound **4** with the distance of 1.94 Å. Furthermore the phenol group of Tyr508 provides π-π interaction to the benzene of chromen moiety of compound **4**. The predicted binding mode of compound **4** is presented in Figure S4b in the Supporting Information.

Subsequently, we decided to scrutinize the inhibitory potential of these compounds against other available enzymes. Compound **1** was randomly screened against phosphodiesterase inhibition and for anti-glycation potential. Compounds **2** and **4** were tested for urease inhibitory activity (screening protocols are described in the Supporting Information). Compound **1** found to be inactive against urease and phosphodiesterase. Similarly, no anti-glycation activity was observed. However, interestingly compounds **2** and **4** showed anti-urease activities in micromolar range.

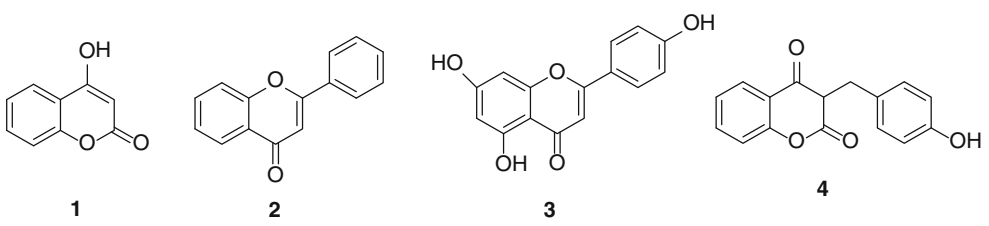
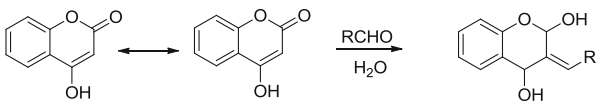
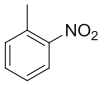
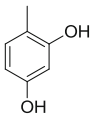
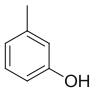
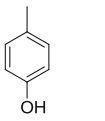
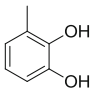
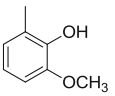
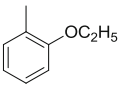
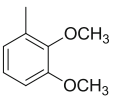
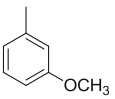
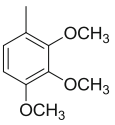
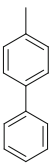


Fig. 2 The structures of compounds **1**-**4**, identified by virtual screening

Scheme 1 β-Glucuronidase inhibitory activities of coumarin diones (**1a–1u**). *SEM* standard error of the mean, *N/D* not determined



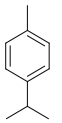
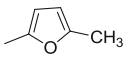
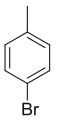
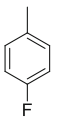
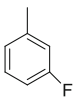
1 **1a-1u**

Compounds	R	IC_{50} ± SEM (μM)
1a		282.3 ± 2.32
1b		56.2 ± 0.60
1c		174.3 ± 1.63
1d		266.1 ± 2.8
1e		34.2 ± 0.49
1f		61.6 ± 1.52
1g		151.6 ± 4.36
1h		87.7 ± 2.6
1i		85.7 ± 2.13
1j		226 ± 5.37
1k		21.4 ± 0.16

According to this preliminary study, compound **1** was identified as a specific inhibitor of β-glucuronidase; Results are summarized in scheme.

These preliminary studies suggested that compound **1** possess a significant inhibitory activity and can serve as useful starting point for further studies. We therefore

Scheme 1 continued

Compounds	R	IC_{50} ± SEM (μM)
1l		48.1 ± 3.20
1m	—$CH_2(CH_2)_7CH_3$	352.6 ± 6.43
1n		11.7 ± 0.48
1o		75.9 ± 3.6
1p		9.9 ± 0.34
1q		108 ± 0.54
1r		176 ± 0.56
1s		96.2 ± 1.65
1t		87.7 ± 0.96
1u	—$CH_2(CH_2)_5CH_3$	N/D

decided to make a small library of derivatives of compound **1**. We assume that different derivatives of compound **1** (4-hydroxy coumarin) can further enhance/improve the potency and selectivity of the ligand against β-glucuronidase. In particular, coumarins are a large class of compounds, which are reported to have a large number of biological and therapeutically important properties, including antiviral, antifungal, anticoagulant, antithrombotic, tuberculostatic, antioxidant and antitumor activities [29]. Due to the physiological importance of coumarins, we synthesized a small focused library of compound **1** derivative, with the aim of increasing the potency and selectivity against the β-glucuronidase enzyme.

Several methods are reported for the synthesis of coumarin analogues [42–45]. In these reactions, different types of chemicals are used that can be hazardous for environment. To reduce the use and generation of hazardous chemicals, we have utilized environmental friendly version of organic reaction. Eco-benign versions of organic reactions are potential candidates for the synthesis of biologically active compounds because of low cost, non-inflammability and non toxicity [22]. However, so far as we know, there are no reports concerning the use of eco-benign synthesis of coumarin analogues. Scheme 1

The synthesis was achieved by a single step chemical reaction. A mixture of aryl aldehyde (1.0 g, 10 mmol) and 4-hydroxy coumarin (1.6 g, 10 mmol), in water (10 mL) was stirred at 100 °C. Heating and stirring was continued for 1–2 h at 100 °C. The reaction was monitored by TLC. After the completion of reaction, the solid was filtered and washed subsequently with boiling water and hexane. After drying in vacuum, the coumarin diones were obtained as solid products.

The inhibitory potential of the coumarin derivatives were assessed against β-glucuronidase activity by measuring the absorbance at 405 nm of *p*-nitrophenol released

from the substrate by the spectrophotometeric 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 and 10 μL of enzyme solution which 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-β-D-glucuronide (26). All assays were run in triplicate.

All synthetic compounds exhibited interesting inhibitory activity against β-glucuronidase, however, their potency varied substantially from $IC_{50} = 9.9–352.6$ μM. Of the twenty-one compounds, four exhibited a better inhibitory profile than the initial hit **1**. Interestingly, compounds **1e, 1 k, 1n** and **1p** exhibited more potency than the standard inhibitor with IC_{50} values 34.2, 21.4, 11.7, and 9.9 μM, respectively. The results are summarized in Scheme 1. We repeat our experiment with Triton ×-100 as detergent as procedure described in [46] and we got same results.

The binding mode analysis revealed that the coumarin moiety of all these derivatives oriented similar to compound **1**, in the binding site of β-glucuronidase. Compounds **1p**, the most potent analogue of the series with an IC_{50} of 9.9 μM, showed that electron rich five-membered heterocyclic ring substitutions is the primary determinant of the inhibitory activity. The docking analysis revealed the electron rich heterocyclic ring interact with the electropositive side chain of His385. Furthermore, methyl substituent of **1p** exhibited additional Van der Waals interactions with His385, and thus contribute in the stabilization of the ligand. Additionally, the side chain indole ring of Trp587 is involved in π–π stacking with the furan moiety of compound **1p**. Moreover, the first carbonyl oxygen of compound **1p** form a hydrogen bond with the side chain hydroxyl hydrogen of Tyr504 (3.01 Å), while another carbonyl oxygen of compound **1p** is involved in hydrogen bonding with the side chain hydrogen of Glu451 (2.03 Å). The hydrogen bond distance between the ring oxygen of the coumarin moiety and the side chain amidic nitrogen of Asn484 was found to be 2.29 Å. While the hydrogen bond distance between the side chain hydroxyl of Tyr405 and the furan oxygen was about 2.45 Å. As shown in Fig. 1b, compound **1p** is also stabilized by several hydrophobic interactions with Asp207, Asn502, Tyr508, His509, Ile517, Ile521, Trp587, Arg600, and Lys606 in the active site of β-glucuronidase. Therefore, it is observed in a detailed binding mode analysis that the binding of the inhibitors in the active site of β-glucuronidase can be facilitated by the introduction of multiple hydrogen bonds with the side chain and backbone groups. Furthermore, the hydrophobic interactions with the residues near the active site can play a significant role in stabilizing the inhibitors in the active site of β-glucuronidase. This may serve as key features for future designing of the selective β-glucuronidase inhibitors.

In order to elucidate the β-glucuronidase binding mechanism and orientation of coumarin derivatives in more detail, molecular dynamic simulation study is in process and the results will be published later.

Conclusion

In summary, we have employed a computer-aided drug design strategy, involving structure based virtual screening and in vitro enzyme assay in a consecutive manner to identify potent β-glucuronidase inhibitors. Compound **1** was identified as a starting point for the development of more potent and selective inhibitors. Subsequently, we synthesized and tested a focused library of coumarin derivatives based on the structure of **1** which exhibited a significant potency with IC_{50} values ranging between 9.9 and 352.6 μM. The proposed scaffold of β-glucuronidase inhibitors offers the possibility of convenient further modifications that could give rise to structures with improved inhibitory activity and selectivity toward the enzyme.

Acknowledgments The authors are thankful to Higher Education Commission (HEC) Pakistan for providing financial support under "National Research Program for Universities" to Project No. 20-1394.

References

1. Paigen K (1989) Mammalian beta-glucuronidase: genetics, molecular biology, and cell biology. Prog Nucl Acid Res Mol Biol 37:155–205
2. Islam MR, Grubb JH, Sly WS (1993) C-terminal processing of human beta glucuronidase. The propeptide is required for full expression of catalytic activity, intracellular retention, and proper phosphorylation. J Biol Chem 268:22627–22633
3. Shipley JM, Grubb JH, Sly WS (1993) The role of glycosylation and phosphorylation in the expression of active human β-glucuronidase. J Biol Chem 268:12193–12198
4. Kim HW, Mino K, Ishikawa K (2008) Crystallization and preliminary X-ray analysis of endoglucanase from *Pyrococcus horikoshii*". Acta Crystallogr Sect F Struct Biol Cryst Commun 64:1169–1171
5. Jain S, Drendel WB, Chen ZW, Mathews FS, Sly WS, Grubb JH (1996) Structure of human beta-glucuronidase reveals candidate lysosomal targeting and active-site motif. Nat Struct Biol 3:375–381
6. Sperker B, Mürdter TE, Schick M, Eckhardt K, Bosslet K, Kroemer HK (1997) Interindividual variability in expression and activity of human beta-glucuronidase in liver and kidney: consequences for drug metabolism. J Pharmacol Exp Ther 281:914–920
7. Bank N, Bailine SH (1965) Urinary beta-glucuronidase activity in patients with urinary-tract infection. N Engl J Med 272:70–75

8. Roberts AP, Frampton J, Karim SM, Beard RW (1967) Estimation of beta-glucuronidase activity in urinary-tract infection. N Engl J Med 276:1468–1470
9. Ronald AR, Silverblatt F, Clark H, Cutler RE, Turck M (1971) Failure of urinary beta-glucuronidase activity to localize the site of urinary tract infection. Appl Microbiol 21:990–992
10. Kallet HA, Lapco L (1967) Urine beta glucuronidase activity in urinary tract disease. J Urol 97:352–356
11. Gonick HC, Kramer HJ, Schapiro AE (1973) Urinary β-glucuronidase activity in renal disease. Arch Intern Med 132:63–69
12. Schapiro A, Paul W, Gonick H (1968) Urinary beta-glucuronidase in urologic diseases of the kidneys. J Urol 100:146–157
13. Plum CM (1967) Beta-glucuronidase activity in serum, cerebrospinal fluid and urine in normal subjects and in neurological and mental patients. Enzymol Biol Clin 8:97–112
14. Hradec E, Petrík R, Pezlarová J (1965) The activity of beta-glucuronidase in cases of bladder neoplasms. J Urol 94:430–435
15. Boyland E, Gasson JE, Williams DC (1957) Enzyme activity in relation to cancer; the urinary beta-glucuronidase activity of patients suffering from malignant disease. Br J Cancer 11:120–129
16. Caygill JC, Pitkeathly DA (1966) BTS/BLF/BALR early career investigator of the year: a study of β-acetylglucosaminase and acid phosphatase in pathological joint fluids. Ann Rheum Dis 25:137–144
17. Weissmann G, Zurier RB, Spieler PJ, Goldstein IM (1971) Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. J Exp Med 134:149S–165S
18. Reddy BS (1976) Dietary factors and cancer of the large bowel. Semin Oncol 3:351–359
19. Goldin BR, Gorbach SL (1976) The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. J Nat Cancer Inst 57:371–375
20. Takada H, Hirooka T, Hiramatsu Y, Yamamoto M (1982) Effect of β-glucuronidase inhibitor on azoxymethane-induced colonic carcinogenesis in rats[1]. Cancer Res 42:331–334
21. Khan KM, Shujaat S, Rahat S, Hayat S, Choudhary MI (2002) Beta-N-cyanoethyl acyl hydrazide derivatives: a new class of beta-glucuronidase inhibitors. Chem Pharm Bull 50:1443–1446
22. Khan KM, Rahim F, Wadood A, Taha M, Khan M, Naureen S, Ambreen N, Hussain S, Perveen S, Choudhary MI (2014) Evaluation of bisindole as potent β-glucuronidase inhibitors: synthesis and in silico based studies. Bioorg Med Chem Lett 24:1825–1829
23. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (1997) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Del Rev 23:3–25
24. MOE (The Molecular Operating Environment), Version 2006.08 (2007) software, Chemical Computing Group Inc., Montreal, Canada.http://www.chemcomp.com
25. SYBYL Molecular Modeling Software Packages, Version 6.9 (2004) Tripos Associates, St Louis, MO
26. http://www.iccs.edu/pcmd/compound_bank.php
27. Abid O, Babar TM, Ali FI, Ahmed S, Wadood A, Rama NH, Uddin R, Ul-Haq Zaheer, Khan A, Choudhary MI (2010) Identification of novel urease inhibitors by high-throughput virtual and in vitro screening. Med Chem Lett 1:145–149
28. GOLD Version 4.0. (2001) Astex Technology, Cambridge
29. Collins RA, Ng TB, Fong WP, Wan CC, Yeung HW (1997) Inhibition of glycohydrolase enzymes by aqueous extracts of Chinese medicinal herbs in a microplate format. Biochem Mol Biol Int 42:1163–1169
30. Yamaguchi F, Ariga T, Yoshimura Y, Nakazawa H (2000) Antioxidative and anti-glycation activity of garcinol from *Garcinia indica* fruit rind. J Agric Food Chem 48:180–185
31. Mamillapalli R, Haimovitz R, Ohad M, Shinitzky M (1998) Enhancement and inhibition of snake venom phosphodiesterase activity by lysophospholipids. FEBS Lett 436:256–258
32. López-Gómez J, Costas MJ, Meireles RJ, Fernández A, Romero A, Ávalos M, Cameselle JC (1998) Glycine-enhanced inhibition of rat liver nucleotide pyrophosphatase/phosphodiesterase-I by EDTA: a full account of the reported inhibition by commercial preparations of acidic fibroblast growth factor (FGF-1). FEBS Lett 421:77–79
33. Razzell WE, Khorana HG (1959) Studies on polynucleotides III. Enzymic degradation. Substrate specificity and properties of snake venom phosphodiesterase. J Biol Chem 234:2105–2113
34. Nakabayashi T, Matsuoka Y, Ikezawa H, Kimura Y (1994) Alkaline phosphodiesterase I release from eucaryotic plasma membranes by phosphatidylinositol-specific phospholipase C–IV. The release from *Cavia porcellus* organs. Int J Biochem 26:171–179
35. Kato K, Yoshida K, Tsukamoto H, Nobunaga M, Masuya T, Sawada T (1960) Synthesis of p-nitro phenyl beta-D-glucopyranosiduronic acid and its utilisation as substrate for assay of beta-glucuronidase activity. Chem Pharm Bull 8:239–242
36. Islam MR, Tomatsu S, Shah GN, Grubb JH, Jain S, Sly WS (1999) Active site residues of human beta-glucuronidase. Evidence for Glu(540) as the nucleophile and Glu(451) as the acid-base residue. J Biol Chem 274:23451–23455
37. Kawasaki M, Hayashi T, Arisawa M, Morita N, Berganza L (1988) H.8-hydroxytricetin 7-glucuronide. A β-glucuronidase inhibitor from *Scoparia dulcis*. Photochemistry 27:3709–3711
38. Schmeda-Hirschmann G, Loyola JI, Reyes S, Hubert E, Rodriguez M, Rodriguez J, Dutra-Behrens M (1994) β-Glucuronidase inhibition and diuretic activity of *Fabiana imbricata* R. & P. (Solanaceae). Phytother Res 8:485–487
39. Hayashi T, Kawasaki M, Okamura K, Tamada Y, Morita N, Tezuka Y, Kikuchi T, Miwa Y, Taga T (1992) Scoparic acid A, A β-glucuronidase inhibitor from *Scoparia dulcis*. J Nat Prod 55:1748–1755
40. Cenci di Bello I, Dorling P, Fellows L, Winchester B (1984) Specific inhibition of human β-D-glucuronidase and α-L-iduronidase by a trihydroxy pipecolic acid of plant origin. FEBS Lett 176:61–64
41. Levy G, Conchi J (1966) β-Glucuronidase and the hydrolysis of glucuronides. In: Dutton GJ (ed) Glucuronic Acid. Academic Press, Free and Combined New York, pp 301–364
42. Borges F, Roleira F, Milhazes N, Santana L, Uriarte E (2005) Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis and biological activity. Curr Med Chem 12:887–916
43. Nicolaides DN, Fylaktakidou KC, Litinas KE, Hadjipavlou-Litina D (1996) Synthesis and biological evaluation of some 4-(isoxazolinyl or 1,2,4-oxadiazolyl) coumarins. J Heterocycl Chem 33:967–971
44. Burgart YV, Shcherbakov KV, Saloutin VI, Chupakhin ON (2004) 5,6,7,8-Tetrafluoro-4-hydroxycoumarin derivatives in reactions with o-phenylenediamin. Russ Chem Bul 53:1237–1239
45. Lu J, Li Y, Bai Y, Tian M (2004) Green chemistry approaches to the synthesis of 5-arylidenethiobarbituric acids by a condensation reactions between aromatic aldehydes and thiobarbituric acid: comparison of water, microwave irradiation, and grinding. Heterocycles 63:583–589
46. Ferreira RS, Bryant C, Ang KKH, McKerrow JH, Shoichet BK, Renslo AR (2009) Divergent modes of enzyme inhibition in a homologous structure–activity series. J Med Chem lett 52:5005–5008