Discovering Specific Inhibitors for Intestinal E. coli β-Glucuronidase through In Silico Virtual Screening


1 Graduate Institute of Pharmacognosy, Taipei Medical University, 252 Wu Hsing Street, Taipei 11031, Taiwan
2 Institute of Biomedical Sciences, Academia Sinica, 128 Section 2, Academia Road, Nankang, Taipei 11529, Taiwan
3 Institute of Biomedical Science, National Sun Yat-Sen University, 70 Lienhai Road, Kaohsiung 80424, Taiwan
4 Department of Pharmacy, Chia Nan University of Pharmacy and Science, 60 Section 1, Erh-Ren Road, Tainan 71710, Taiwan
5 Department of Biomedical and Environmental Biology, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan
6 Graduate Institute of Medicine, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan
7 Center for Biomarkers and Biotech Drugs, Kaohsiung Medical University, 100 Shih-Chuan 1st Road, Kaohsiung 80708, Taiwan
8 School of Pharmacy, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan

Correspondence should be addressed to Tian-Lu Cheng; tlcheng@kmu.edu.tw and Chien-Shu Chen; cschen7@mail.cmu.edu.tw

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Glucuronidation is a major metabolism process of detoxification for carcinogens, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 1,2-dimethylhydrazine (DMH), of reactive oxygen species (ROS). However, intestinal E. coli β-glucuronidase (eβG) has been considered pivotal to colorectal carcinogenesis. Specific inhibition of eβG may prevent reactivating the glucuronide-carcinogen and protect the intestine from ROS-mediated carcinogenesis. In order to develop specific eβG inhibitors, we found that 59 candidate compounds obtained from the initial virtual screening had high inhibition specificity against eβGbunot human βG. In particular, we found that compounds 7145 and 4041 with naphthalenylidene-benzenesulfonamide (NYBS) are highly effective and selective to inhibit eβG activity. Compound 4041 (IC50 = 2.8 μM) shows a higher inhibiting ability than compound 7145 (IC50 = 31.6 μM) against eβG. Furthermore, the molecular docking analysis indicates that compound 4041 can bind to key residue (E413) at active site of eβG via hydrogen-bonding interactions. These novel NYBS-based eβG specific inhibitors may provide as novel candidate compounds, which specifically inhibit eβG to reduce eβG-based carcinogenesis and intestinal injury.

1. Introduction

Glucuronidation represents a major route of drug metabolism in the liver [1–3]. Many carcinogens and xenobiotics are detoxified by conjugation with a glucuronide acid to increase their water solubility, thus facilitating their excretion [4, 5]. For example, the 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 1,2-dimethylhydrazine (DMH), which lead to reactive oxygen species (ROS) mediated DNA damage, mutagenesis, and carcinogenesis, can be metabolized and detoxified to glucuronide form by UDP-glucuronosyltransferases (UGTs) [6–8]. However, when the glucuronide conjugates enter the intestine through enterohepatic circulation, they can be hydrolyzed and their toxicity can be reversed by E. coli β-glucuronidase (eβG) [9–11], which would induce intestinal injury [5, 12–14] and colon carcinogenesis. In addition, chemotherapy-induced diarrhea (CID) and intestinal damage have been clinically demonstrated in several chemotherapeutic drugs such as CPT-11 [15–17], 5-FU [18], and oxaliplatin [19]. There is a specific
βG inhibitor, glucaro-1,5-lactone, which has been shown to alleviate CPT-11-induced mucosal damage in the small intestine in vivo [5]. However, glucaro-1,5-lactone of the current treatments against CID is limited and not effective, since it preferentially inhibits human βG (hβG) activity [20], which may induce mucopolysaccharidoses (MPS) [21, 22]. The current treatments against CID are limited and not effective. Therefore, it is crucial to develop an eβG specific inhibitor, which cannot affect hβG activity, as an effective treatment against carcinogenesis and CID.

The crystal structures of hβG and eβG have been reported [16, 17]. In addition, eβG has a unique “bacteria loop” (LGIGFEAGNKPKELYSE) [16] which is absent in hβG. Some known drugs such as Amoxapine [23, 24] and Loxapine [23] and eβG inhibitors [16] have been also demonstrated to interact with the residues of bacterial loop and active sites of eβG [16, 24]. Those reports indicate that the area around the unique loop and the active site is an important target for eβG inhibitor selection.

For the development of specific eβG inhibitors, we demonstrated a crystal structure of recombinant eβG (provided by Steve R. Roffler, Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan) in complex with D-glucaro-1,5-lactone which revealed that the inhibitor was bound at the residues (E413, E504) of active site. And, we further compared the active center between eβG and hβG though overlap. We obtained candidate compounds that selectively inhibit eβG via computational screening by DOCK 4.0 program [25, 26] and X-ray crystal structure of eβG. A chemical database (SPECS) containing ∼300,000 commercially available compounds was computationally screened against a grid box enclosing the unique bacterial loop at eβG active site. To prove whether the candidate compounds can effectively inhibit eβG without affecting hβG activity, compounds were examined based on their specific inhibition for eβG versus hβG by in vitro βG activity-based assays. The binding motifs of eβG specific inhibitors were determined by molecular docking studies. The novel eβG specific inhibitor may provide a highly effective and selective agent to prevent eβG-based carcinogenesis and CID.

2. Materials and Methods

2.1. Expression and Purification of βG Protein. Plasmid pRESTB containing βG gene and a histidine tag at the N-terminus was constructed as described [27]. Recombinant βG (human and E. coli) was produced by isopropyl β-D-thiogalactopyranoside (IPTG) induction of BL21 (DE3) bacteria. βG was purified from bacterial supernatants by affinity chromatography on nickels Sepharose 6 Fast Flow (GE Healthcare). The column was washed by phosphate-buffered saline (PBS), with 50 mM imidazole, and βG was eluted by PBS with 250 mM imidazole. The purified βG was desalted on a Sephadex G-25 column equilibrated with PBS and stored at −80°C.

2.2. Virtual Screening of eβG Specific Inhibitors. The virtual screening was performed using the DOCK 4.0 program and the X-ray crystal structure of eβG (provide by Steve R. Roffler). The B-chain structure of protein, water molecules, and the co-crystallized inhibitor D-glucaro-1,5-lactone were removed. The remaining A-chain protein structure was used to prepare the target site for docking simulations. The active-site region of eβG was specified as the target site for ligand docking in virtual screening. Briefly, a molecular surface around the target site was generated with MS program using a 1.4 Å probe radius and this surface was used to generate with the SPHGEN program 43 overlapping spheres to fill the target site. A grid box enclosing the target site was created for grid calculations with dimensions 19.3 x 22.4 x 15.6 Å. The force filled scoring grids were calculated with the GRID program using a distance-dependent dielectric constant of 4r, an energy cutoff distance of 10 Å, and a grid spacing of 0.3 Å. The database for virtual screening was the SPECS compound collection, which included ∼300,000 commercially available compounds (downloaded from the ZINC database web site). The DOCK 4.0 program performs docking simulations using a distance-matching algorithm. The matching parameters used to run virtual screening were set as follows: distance tolerance = 0.5, distance minimum = 2.5, nodes maximum = 10, and nodes minimum = 4. The SPECS database was computationally screened against the active site of eβG using the force field scoring function based on interaction energy. Virtual screening was performed on a Silicon Graphics Octane workstation with dual 270 MHz MIPS R12000 processors.

For compound selection, the docking models of the 4724 top-ranked compounds (energy score values ≤ −42.00 kcal/mol) were visually inspected using the software PyMOL. Together with consideration of chemical diversity, the selection of compounds was assisted by analysis of the docking models with respect to shape fitting and hydrogen-bonding and hydrophobic interactions. Finally, we selected 59 compounds for enzyme inhibition assays against E. coli and human βGs. The compounds for testing were purchased from the SPECS Company. The SPECS ID number and docking energy score for compounds are listed in the supporting information.

2.3. In Vitro βG-Activity Assay of eβG Specific Inhibitors. The candidate compounds were purchased from SPECS (The Netherlands). Each candidate was provided as a solid power and dissolved in 100% DMSO (Sigma-Aldrich) to 10 mM as stock. Candidates were screened for their inhibition specificity of eβG versus hβG, which were conducted at pH 7.3 or pH 5.4, in triplicate, respectively. 40 μL purified βG was treated with 10 μL compound solution at 37°C for 30 min and sequentially incubated with 50 μL of pNPG (Sigma-Aldrich) at 37°C for 30 min. Reactions were quenched with 5 μL of 2 N sodium hydroxide (Sigma-Aldrich). Each reaction consisted of 3.75 ng purified βG, 50 μM compound, and 5 mM pNPG in PBS containing 10% DMSO and 0.05% BSA (Sigma-Aldrich). βG-activities were measured by color development of pNP detected on a microplate reader at OD 405 nm. Results are displayed as percent of βG activity compared with
the untreated control. For IC50 determination, compounds at various concentrations (100 μM to 0.001 μM) were added.

2.4. Molecular Docking Studies of eβG Specific Inhibitors. The crystal structure of eβG for the virtual screening was also utilized in the docking studies of compounds 7145 and 4041. Hydrogen atoms were added to the A-chain protein structure, and the resulting structure was used in the docking simulations. The 3D structures of compounds were built and optimized by energy minimization using the MM2 force field and a minimum RMS gradient of 0.05 in the software Chem3D 6.0 (CambridgeSoft Corp., Cambridge, MA). Docking simulations were performed using the GOLD 5.0 program [28] on an HP xw6600 workstation with Intel Xeon E5450/3.0 GHz Quadcore as the processors. The GOLD program utilizes a genetic algorithm (GA) to perform flexible ligand docking simulations. In the present study, for each of the 30 independent GA runs, a maximum number of 100000 GA operations was performed on a single population of 100 individuals. Operator weights for crossover, mutation, and migration were set to 95, 95, and 10, respectively. The Gold-Score fitness function was applied for scoring the docking poses of compounds. The docking region was defined to encompass the active site of eβG. The best docking solution (with the highest GOLD fitness score) for a compound was chosen to represent the most favorable predicted binding mode to eβG.

3. Results

3.1. Comparison of the Active Site Structures of eβG and hβG. To identify the active site of eβG, recombinant full-length eβG was purified and shown to hydrolyze pNPG to PNP for detecting βG activity. The enzyme was crystallized in complex with an established inhibitor, D-glucaro-1, 5-lactone. Crystal structure of eβG (provided by Steve R. Roffler) in complex with D-glucaro-1, 5-lactone revealed that the inhibitor was bound at residues (E413 and E504) of the active site (Figure 1(a)). To compare the structures of the active centers between eβG and hβG (PDB ID 1BHG), βGs were analyzed by computer simulation technology. After superimposition, the crystallized structure of eβG is 45% similar to hβG. Moreover, there is a "bacterial loop" within eβG which is absent in hβG (Figure 1(b)). Similar results have been also shown in other reports [16]. This eβG unique loop of the active center is an ideal target site for screening compounds that can selectively inhibit eβG activity.

3.2. In Silico Virtual Screening of eβG Inhibitor Candidates. To identify potential eβG inhibitors that can selectively block eβG activity, but not hβG, the virtual screening proceeded based on the different structures of the active center between eβG and hβG. The SPECS database (~300,000 commercially available compounds) was computationally screened against the “grid box” which contains the bacterial loop of eβG and active site using the DOCK program (version 4.0). Fifty-nine candidate compounds were acquired from the initial virtual screening which was designed to target the bacterial loop of eβG and its active site. The docking energy scores of 59 candidate compounds measured by the DOCK program are −43 to −55 kcal/mol (Table S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2014/740815).

3.3. Screening of eβG Inhibitor Candidates by In Vitro βG Activity Assay. To prove whether these 59 candidate compounds can effectively inhibit eβG without affecting hβG activity, 50 μM compounds were examined for their specific inhibition for eβG versus hβG by in vitro βG-based activity assays, in which the conversion of pNPG to PNP was detected by measuring the increases in PNP absorbance at OD 405 nm. The result showed that all the 59 candidate compounds displayed selective inhibition against eβG activity. The inhibiting ability against eβG activity, especially, was >95% in 7 candidates of eβG specific inhibitors (Table S1). Based on these results, we concluded that the pocket site in the unique loop and active site of eβG are an ideal site to screen eβG specific inhibitors through virtual screening. We found that compound 7145 (4-tert-butyl-N-(4-oxo-1(4H)−naphthalenylidene-benzensulfonylamide) can inhibit >95% eβG activity and does not hamper hβG activity at 50 μM condition (Table S1). The result indicated that the derivatives of naphthalenylidene-benzensulfonylamide (NYBS) might effectively and specifically inhibit the eβG activity. Based on the NYBS structure, we performed the substructure search and then found compound 4041 (4-methyl-N-(4-oxo-3-(1H-1,2,4-triazol-5-ylsulfonyl)-1(4H)-naphthalenylidene)/benzensulfonylamide). In particular, compound 4041 has been shown to be a more potent eβG antagonist than compound 7145. Figure 2 and Table 1 show that while compound 4041 (IC50 = 2.8 μM) can selectively inhibit >80% eβG activity, at 10 μM, the inhibition of compound 7145 (IC50 = 31.6 μM) is 55% inhibition. Compared to D-saccharic acid 1,4-lactone (saccharolactone), which showed higher inhibition on hβG, our candidate compounds displayed specificity against eβG activity (Figure 2). Based on these results, we concluded that the derivatives of NYBS may provide a novel specific inhibitor to reduce eβG-based intestinal injury and CID.

3.4. Molecular Docking Studies of eβG Specific Inhibitors. To predict the binding modes of compounds 7145 and 4041 in the active site of eβG, we performed molecular docking studies using the GOLD 5.0 program. As depicted in Figure 3, the docking model revealed that compound 7145 forms four hydrogen bonds to eβG. Three of the hydrogen bonds, to residues Y468, Y472, and R562, arise from the SO2 group of compound 7145. The carbonyl group on the bicyclic 4-oxo-1(4H)−naphthalenylidene ring can form one hydrogen bond with H296. Compound 7145 makes hydrophobic interactions with the surrounding residues, including W549, F554, F164, V355, V446, M447, F448, Y468, and Y472. The residue L361 in the bacterial loop makes hydrophobic contact with compound 7145. Compound 7145 showed a GOLD fitness score of 62.09.

The docked orientation of compound 4041 is considerably different from that of compound 7145.
Table 1: The structure, IC\textsubscript{50}, and GOLD fitness scores of compound 7145 and compound 4041 docked into the active site of eβG.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>GOLD fitness score\textsuperscript{a}</th>
<th>IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7145</td>
<td><img src="image" alt="Structure of 7145" /></td>
<td>62.09</td>
<td>31.6</td>
</tr>
<tr>
<td>4041</td>
<td><img src="image" alt="Structure of 4041" /></td>
<td>64.91</td>
<td>2.8</td>
</tr>
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\textsuperscript{a}Docking simulations were performed using the GOLD 5.0 program.

Figure 1: The crystal structure of eβG and hβG. (a) The crystal structure of eβG bound with the inhibitor D-glucaro-1,5-lactone in the active site was used in the virtual screening. A docking box (red line) was defined to enclose the active site for virtual compound screening. (b) The eβG (green) and hβG (purple) were modeled by superimposing. The eβG contains a "bacterial loop" (yellow) not found in the hβG. The E413 and E504 are two catalytic residues in the active site of eβG.

(Figure 3). For compound 4041, the bicyclic 4-oxo-1(4H)-naphthalenylidene ring points towards M447 and makes a π-π stacking interaction with Y472. In contrast, the bicyclic ring of compound 7145 is oriented in the opposite direction to M447 and located very close to the experimental binding position of the inhibitor D-glucaro-1,5-lactone. Compound 4041 is hydrogen bonded to residues Y472 and R562 through the SO\textsubscript{2} group and to E413 through the 1,2,4-triazole moiety. Compound 4041 makes hydrophobic interactions with the surrounding residues, including V446, M447, Y472, and L561. The residues I361 and I363 in the bacterial loop make hydrophobic contact with compound 4041 (Figure 4). Compound 4041 has a GOLD fitness score of 64.91 higher than that of compound 7145. Figure 5 shows...
Figure 2: Specific inhibition of compounds 7145 and 4041 against εβG. Compound 7145 and compound 4041 acquired from ligand docking in virtual screening were evaluated based on their selective inhibition for recombinant εβG versus hβG. 10 μM of compound 7145, compound 4041, saccharolactone, and 10% DMSO (control) was incubated with purified εβG (○) and hβG (■), respectively. βG activity was determined by hydrolysis of the pNPG substrate. Error bars represent SD; N = 3.

Figure 3: Binding model of compound 7145. Predicted binding mode of compound 7145 in the active site of εβG from the docking study. Compound 7145 (yellow) and some amino acid residues (cyan) interacting with the inhibitor are shown as stick structures. The red dashed lines indicate hydrogen-bonding interactions. The residue L361 (purple) in the bacterial loop makes hydrophobic contact with compound 7145.

Figure 4: Binding model of compound 4041. Predicted binding mode of compound 4041 in the active site of εβG from the docking study. Compound 4041 (yellow) and some amino acid residues (cyan) interacting with the inhibitor are shown as stick structures. The red dashed lines indicate hydrogen-bonding interactions. The residues L361 and I363 (purple) in the bacterial loop make hydrophobic contact with compound 4041.

Figure 5: Binding model of compound 4041 in the crystal structure. Overlay of the docking pose of compound 4041 (yellow) with the bound orientation of an εβG-specific inhibitor (gray) observed in the cocrystal structure of εβG.

4. Discussion

In this study, we have obtained potent and selective εβG inhibitors from in silico virtually screening and further confirmed their inhibition specificity by in vitro βG activity-based assay. All the 59 candidate compounds from the initial screening showed high effective and selective inhibition against εβG. We identified the two most promising compounds, compound 7145 and its derivate compound 4041, showing IC₅₀ values of 31.6 μM and 2.8 μM, respectively. Importantly, compound 4041 with naphthalenylidenbenzenesulfonyamide displayed inhibition selectivity against εβG by binding to the active site at E413 and the unique loop of εβG at L361 and I363.

an overlay of the docking pose of compound 4041 with the bound orientation of an εβG-specific inhibitor [16] observed in the cocrystal structure of εβG. In comparison to the cocrystallized inhibitor, compound 4041 has similar binding interactions with εβG, including hydrogen bonding to the catalytic residue E413 and close contact with the bacterial loop. Based on these results, we suggest that binding to the active site and the bacterial loop of εβG may provide selective abilities of inhibitors against εβG.
High-throughput screening (HTS) allows researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite costly, and the screening process is time-consuming; thus, the limitations have become more apparent. Hence, virtual screening has become an important tool to access novel drugs. Costly, and the screening process is time-consuming; thus, a compound library is quite small. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite costly, and the screening process is time-consuming; thus, the limitations have become more apparent. Hence, virtual screening has become an important tool to access novel drugs. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery. However, this method is limited by the size of compound library. Generally, a compound library is quite small. However, this method is limited by the size of screening million compounds for lead identification in drug discovery. HTS, allowing researchers to screen millions of compounds for lead identification in drug discovery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Ta-Chun Cheng and Kuo-Hsiang Chuang contributed equally.

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