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Research Article

Structural insights into β-glucuronidase inhibition to mitigate camptothecin chemoterapeutics side effects

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Abstract

Selective *Escherichia coli* β-glucuronidase (eGUS) inhibitors, which do not affect human β-glucuronidase (hGUS), greatly alleviate side effects (severe diarrhea) of worldwide used chemotherapeutics, camptothecin and its derivatives. We have studied the probable mechanism of the selectivity of eGUS inhibition. Structural superposition has shown high structural homology in tertiary structure of hGUS and eGUS. Active center models of the enzymes have been build, and the principal difference in quaternary structure of the two enzymes has been found. The four active centers of the human enzyme are positioned separately, each in the area of its own polypeptide chain; their amino acids are positioned distantly from those of the neighboring subunits. In contrast, each eGUS active center includes Phe365 amino acid residue derived from a neighboring polypeptide chain. The improved model of eGUS subunit substrate binding site suggests that future eGUS molecular docking and structure based *in silico* virtual screening studies should be performed with the dimeric structure rather than with a single subunit. All presently known natural eGUS inhibitors have been characterized, the possibility and expediency of obtaining of such inhibitors from herbal sources has been justified. The part of the research is aimed to advance towards "green" technologies in pharmaceutics.

Keywords: molecular docking, GUS, binding site, protein structure, selective inhibitors

1. Introduction

β-Glucuronidase, EC 3.2.1.31, (GUS) belongs to a family of enzymes that catalyze hydrolyses of glycosaminoglycans [1, 2]. Human and *E.coli* GUS have been studied extensively, their X-ray structures have been determined [3, 4], and the homology of the enzymes have been confirmed by cDNA sequence analysis [2]. According to the amino acid sequence similarity of mammalian and *E. coli* GUS to *E. coli* β -galactosidase, all three enzymes were included in glycosyl hydrolase family 2 [5]. In animals the glucuronidation plays important role in detoxification of endogenous metabolic garbage, steroid hormones, the remainders of medicines, xenobiotics etc. [6]. On the other hand, *E. coli* and other intestinal bacteria produce GUS activity that may cause hydrolyzes of excreted glucuronides and the liberation of xenobiotics. Elevated GUS activity is believed to be a primary factor in the etiology of colon cancer [7]. In fact, administration of a bacterial GUS inhibitor leads to a decrease in carcinogen induced colonic tumors [8]. It has been shown that use of most potent anticancer chemotherapeutics, such as camptothecin derivatives topotecan and irinotecan, promotes acute side effect, called CID (chemotherapy-induced diarrhea) [9]. The cause of the side effects was found to be bacterial (mainly *E.coli*) GUS, which converts the glucuronidated in the liver chemotherapeutics back into its toxic form in the intestine [10]. Therefore, discovering specific bacterial GUS inhibitors, which would not affect human GUS became challenging. Essential contribution for the solving the structure and eGUS inhibition has been made by R.M. Redinbo and colleagues [4, 11-16]. The authors identified 9 selective bacterial GUS inhibitors referred thereafter to as Redinbo inhibitors (RI). According to suggestion of the researchers selectivity of RI is associated with so-called bacterial loop, a stretch of amino acid residues 360-376, which present in eGUS and is absent in the mammalian enzyme [4]. They have shown that RI prevents also intestine injure caused by carboxylic acid-containing non-steroidal anti-inflammatory drugs diclofenac, indomethacin and ketoprofen [15, 16]. Recently, using *in silico* database virtual screening Cheng and coauthors [17] have identified another two selective inhibitors. In the present study we resume previous and own *in*

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silico investigations of human and *E.coli* GUS substrate binding site features that define the selectivity of some bacterial inhibitors. We also summarize all the known data regarding natural herbal eGUS inhibitors, validate the possibility of their use, and outline the ways to search for similar eGUS inhibitors. The study results provide new insights into GUS inhibition and aid identification of new selective *E.coli* GUS inhibitors.

2. Materials and methods

2.1. Molecular docking

GUS 3D structure was retrieved from Protein Data Bank at http://www.rcsb.org. For hGUS pdb entries 1BHG and 3HN3 were used. Pdb entries 3K4D and 3LPG represented eGUS. Ligands bound to the enzyme, as well as water molecules were removed from the original .pdb file. The obtained ligand free structures were checked for missing atoms, bonds and contacts. Molecular graphics and analyses were performed with the PyMol Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

Molecular structures of new inhibitors in .pdb format were generated using Avogadro v.1.1.0 for Windows. The geometry of a molecule was optimized using Ghemical force field. The optimization was achieved by conjugate gradient algorithm (number of steps 500). The minimization termination condition was set to the convergence criteria, RMS gradient of 0.0001 kcal/Å. mol in vacuo.

Protein structure files were energy minimized at Yasara Energy Minimization Server [18] and prepared for docking procedure by assigning Gastaiger charges, merging nonpolar hydrogens and saved as .pdbqt files using AutoDock Tools. The similar procedure was applied to create ligand .pdbqt files. Ligand preparation procedure included also defining the rotatable bonds of a ligand. Flexible ligand docks were performed by AutoDock Vina software [19]. All protein structures were structurally aligned to *E.coli* GUS dimer (pdb code 3LPG). Rectangular grid box for the ligand-binding site was of dimensions 30 x 30 x 30 Å with the center coordinates x = -31.8, y = 12, z = 0.6. Binding energy (affinity) of a ligand was calculated by the Vina module.

All calculations were performed on Intel Core2 Duo CPU 2.60 GHz running Microsoft Windows XP Professional, version 2002, service pack 3 OS and Linux Ubuntu 14.04.

2.2. Postdocking analysis

A number of approaches were applied for docking outputs post-processing in order to rank the inhibitory potential of compounds studied. High-ranking Vina outputs were analyzed by visual inspection of a ligand orientation and its interactions in the substrate cavity environment, compliance with the known ligand - enzyme biological assembly models. Crystal structure of eGUS complexed with the glucaro-D-lactam inhibitor (pdb code 3K4D) was used as a reference model.

3. Results and Discussion

3.1. The Structures

Both hGUS and eGUS are homotetramers composed of protomers of about 600 amino acid residues each [3, 4, 20]. Primary structure similarity of human and bacterial GUS is 46% [20].The essential difference in primary structure of the proteins is believed to be a short stretch of amino acid residues 360-376, so-called "bacterial loop", which was found in most bacterial and absent in mammalian GUS [4].

To better understand the molecular mechanisms of selectivity of bacterial inhibitors we performed systematic comparison of human and bacterial GUS at higher structural levels. The overall tertiary and domain structure of monomers as well as binding site are quite similar (Table 1). We have revealed that more significant difference between the two proteins is associated with their quaternary structure.

Structurally the tetramer may be considered as a complex of two dimers each composed of two monomers arranged in ‘head-to-head’ association. The substrate binding site is located in the 'head" part of a subunit (Fig.1).

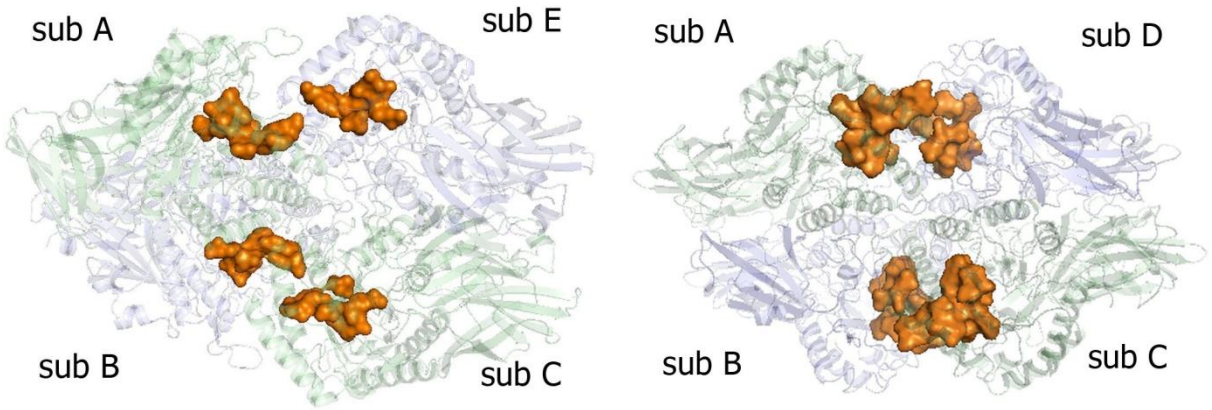

Fig. 1: Cartoon model of tetrameric structure of human GUS (hGUS) and reconstructed *E.coli* GUS (eGUS). Chain identifiers are as in original .pdb files. Substrate binding sites are shown as brown surfaces.

In contrast with relatively low sequence identity between human and *E.coli* GUS structural alignment reveals significant homology for Cα atoms of dimers, monomers, and 7 active site amino acids, whereas tetramers superposition show poor alignment (Table 1).

Molecular docking of various ligands to the dimeric forms of human and bacterial GUS revealed intimate details of the binding site structure. Fig.2 shows that 7 amino acid residues Asp163, His330, Asn412, Glu413, Tyr468, Tyr472, and Glu504 of bacterial GUS match their human enzyme analogs Asp207, His385, Asn450, Glu451, and Tyr504. On the other hand, the Phe365 residue in the binding site of a subunit belongs to polypeptide chain of the opposite subunit.

We defined the substrate binding site as amino acid residues within 5 Å of the bound substrate, p-nitrophenyl-glucuronide (pNPG). The principal difference in structure of human and bacterial GUS tetramers was found to be partial overlapping subunits A/D and B/C of bacterial enzyme at the substrate binding site so that the Phe365 residues of each subunit protrudes up to the neighboring subunit active site. (Fig. 2). As a result each subunit substrate binding site includes seven GUS family conserved amino acid residues and Phe365 derived from the neighboring polypeptide chain. Four amino acid residues Ile363-Glu364 and Ile560-Leu561 derived from both neighboring subunits make the interface for the two binding sites (Fig.2).

Table 1: Root mean square deviation values for human and *E.coli* GUS superposition

Superimposed structures	rmsd, Å	number of Cα atoms aligned
tetramers	40.84	2278
dimers	1.4	1030
monomers	0.94	495
binding site AA*	0.76	7

*AA - amino acids; only 7 matched amino acids residues were considered

Such amino acid residue overlap, suggest that conformational changes of a subunit upon a ligand binding will most probably affect the conformation of the opposite polypeptide chain. Based on the sequence identity of eGUS subunits researchers consider usually only one (A) subunit for molecular dockings and /or virtual screenings. Our improved model of eGUS subunit substrate binding site, which indicates that each of four substrate binding site includes Phe365 of the neighboring subunit, suggests that eGUS molecular docking and structure based *in silico* virtual screening studies should be performed with the dimeric structure rather than with a single subunit as it was reported in paper [17].

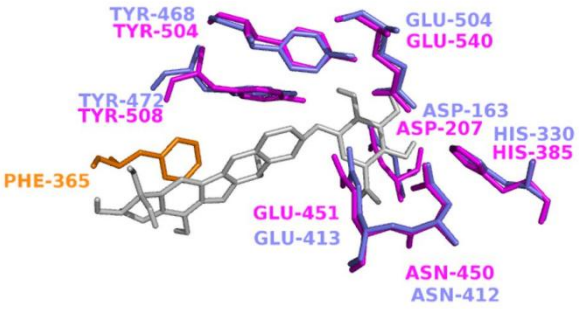

Fig. 2: Superposition of human (pink sticks) and bacterial (blue sticks) GUS substrate binding site. Unmatched Phe365 is shown in orange. Bound substrate, p-nitrophenyl glucuronide, pNPG [21] is presented as light grey stick model.

3.2. Molecular docking of natural GUS inhibitors.

All RIs, which are potent and selective eGUS inhibitors, are the compounds obtaining by chemical synthesis. It is obvious that availability of natural (herbal) analogous of such selective inhibitors would be a great advantage towards emerging the 'green technologies' in the pharmacology. We performed molecular dockings of all five known selective natural eGUS inhibitors to the dimeric form of hGUS and eGUS. These five natural inhibitors are *baicalin* and *wogonoside* obtained from *Scutellaria baicalensis* grown in South Siberia [23], *scoparic acid A* from *Scoparia Dulcis* [24], *mcusisoflavone A* from the figs of *Ficus mucuso* (Cameroon, Africa) [25] and *scopoletin* from *Fabiana imbricata* [26].

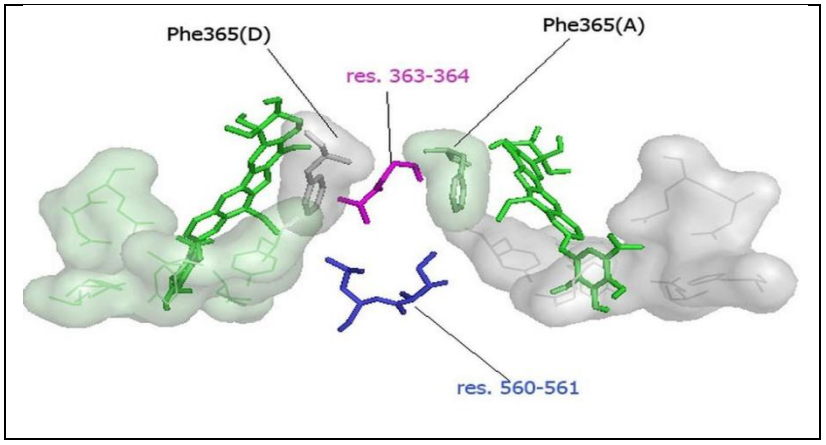

Fig. 3: The two adjacent substrate binding sites of eGUS subunits A/D. Substrate molecules of SN-38G [22] are shown as green sticks.

The result of molecular docking of the natural inhibitors and some other ligands is presented in Fig. 4. The figure shows the poses of docked ligands inside the substrate pocket of the enzyme. The substrate pocket of eGUS has dumbbell shape and almost all of its amino acid residues are situated under the surface of the protein globule. The pocket has openings on the both its sides. Therefore, it could be called substrate "channel" rather than "pocket". In the Fig. 4 the substrate binding cavity is shown transparent that allows observing orientation and pose of each docked ligand inside the cavity. Dumbbell shaped "channel" has average size 8 x15 Å.

The Fig.4 shows that relatively small ligand molecules such as GDL [27], scopoletin, the standard inhibitor, D-glucaro-1, 4-lactone [28] as well as the glycon part of pNPG are positioned deep into the bottom part of the substrate cave. The wall of this part is formed by amino acid residues most important for the catalysis. These are the two catalytic residues Glu413, Glu540, as well as important Tyr468, Tyr472. The most effective inhibitors baicalin, wogonoside, and Redinbo inhibitors are positioned vertically along almost the whole substrate cave. Generally this effective inhibitors make hydrogen bonds with such amino acid residues as Glu413 (most often), Asp163 (often), Arg562 (less often), and with Ile363, Ser557, Trp549. Principally RIs, baicalin and wogonoside bind to the enzyme substrate cave by nonpolar hydrophobic interactions. Inhibitors RI-5 and RI-6 do not form polar bonds inside the substrate cave. The mechanism of action of such inhibitors consists most probably in spatial blocking of substrate 'entrance opening' or in blocking the reaction products exit through the 'exit opening'. Such a molecular mechanism can be easily suggested by the analysis of Fig.4 data. Less effective inhibitor, scoparic acid A poses is in the upper

part of the substrate cave, where it forms less important polar bonds with Ser360 and nonpolar bonds with distant residues Arg417, Met447, Phe448 and Val473.

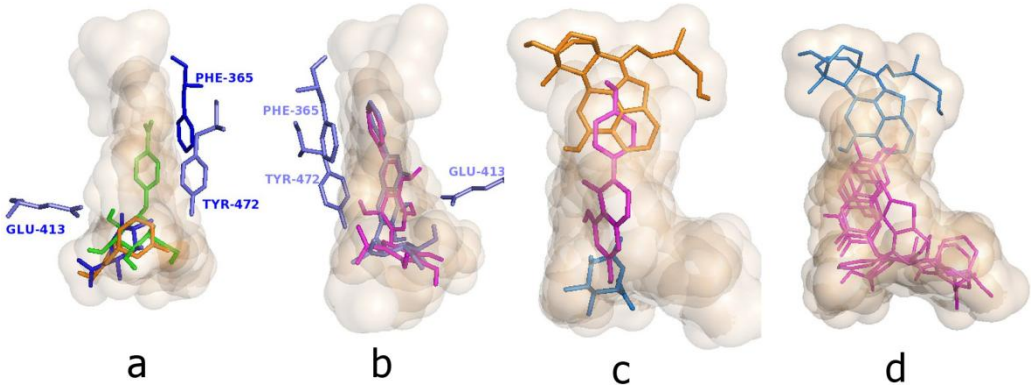

Fig. 4: Poses of different ligands inside the substrate cave of eGUS.

Color scheme: a: pNPG, green, scopoletin, orange and GDL, blue; b: baicalin, blue, wogonoside, pink; c: scoparic acid A, orange, mucusisoflavone, pink and GDL, blue; d: scoparic acid A, blue, RIs, pink. Positions of amino acid residues Phe365, Glu413, and Tyr472 in a and b (all in blue color) serve as the binding cave markers.

4. Conclusions

hGUS and eGUS show similarity in primary, secondary and tertiary structure, however there are some pharmacologically important compounds that selectively inhibit bacterial enzyme. We have found the important difference in quaternary structure of the enzymes that might be the clue for the molecular mechanism of selectivity of the inhibitors. Each of four substrate binding site of hGUS is composed of amino acid residues of a single polypeptide chain. In contrast, a substrate binding site of eGUS is build of seven GUS family conserved amino acids plus Phe365 of the symmetry mate polypeptide chain. Our improved model of eGUS subunit substrate binding site suggests that eGUS molecular docking and structure based *in silico* virtual screening studies should be performed with the dimeric structure rather than with a single subunit We have also characterized all five documented natural eGUS inhibitors, which may have an important application as auxiliary remedy in cancer chemotherapy, justified the possibility and expediency of obtaining such inhibitors from plant sources. Research and development of methods for obtaining such inhibitors might be an important step towards "green" technologies in pharmaceutics.

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