Molecular Insights into Microbial β-Glucuronidase Inhibition to Abrogate CPT-11 Toxicity

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ABSTRACT

Bacterial β-glucuronidases expressed by the symbiotic intestinal microbiota appear to play important roles in drug-induced epithelial cell toxicity in the gastrointestinal (GI) tract. For the anticancer drug CPT-11 (irinotecan) and the nonsteroidal anti-inflammatory drug diclofenac, it has been shown that removal of the glucuronide moieties from drug metabolites by bacterial β-glucuronidases in the GI lumen can significantly damage the intestinal epithelium. Furthermore, selective disruption of bacterial β-glucuronidases by small molecule inhibitors alleviates these side effects, which, for CPT-11 (7-ethyl-10-[4-(1-piperidino)-1-piperidino]), can be dose limiting. Here we characterize novel microbial β-glucuronidase inhibitors that inhibit Escherichia coli β-glucuronidase in vitro with Kᵢ values between 180 nM and 2 μM, and disrupt the enzyme in E. coli cells, with EC₅₀ values as low as 300 nM. All compounds are selective for E. coli β-glucuronidase without inhibiting purified mammalian β-glucuronidase, and they do not impact the survival of either bacterial or mammalian cells. The 2.8 Å resolution crystal structure of one inhibitor bound to E. coli β-glucuronidase demonstrates that it contacts and orders only a portion of the “bacterial loop” present in microbial, but not mammalian, β-glucuronidases. The most potent compound examined in this group was found to protect mice against CPT-11-induced diarrhea. Taken together, these data advance our understanding of the chemical and structural basis of selective microbial β-glucuronidase inhibition, which may improve human drug efficacy and toxicity.

Introduction

CPT-11 [irinotecan; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]] is employed worldwide for the treatment of a variety of solid malignancies, but its efficacy is often limited by severe gastrointestinal (GI) toxicity (Rothenberg et al., 1996; Rougier et al., 1997, 1998; Cunningham et al., 1998; Saltz et al., 2000; Fuchs et al., 2003; Hu et al., 2006; Kurita et al., 2011). CPT-11 is most frequently used in first- and second-line treatment of metastatic colon cancers, typically in combination with other agents (Smith et al., 2006; Kambe et al., 2012). More recently, CPT-11 has been employed in preclinical and clinical trials against a range of other neoplasticities, including brain tumors, lung, breast, gastric, pancreatic, and gynecologic cancers (Matsumura et al., 2010; Han et al., 2012; Jo et al., 2012; Kim et al., 2012; Spigel et al., 2012; Zaniboni et al., 2012; Lee et al., 2013).

CPT-11 is a prodrug that is converted to SN-38 (7-ethyl-10-hydroxy-camptothecin) by carboxylesterase enzymes. SN-38, the active metabolite of CPT-11, induces antitumor effects by poisoning the human topoisomerase I catalytic cycle, leading to cell death (Kawato et al., 1991; Mathijssen et al., 2001; Ma and McLeod, 2003). The fate of SN-38 in vivo is further regulated by UDP-glucuronosyltransferase (UGT) enzymes in the liver and other tissues. UGT isoforms, in particular UGT1A1, catalyze the conjugation of a glucuronide group onto SN-38, producing the inactive compound SN-38 glucuronide (SN-38G), which is marked for elimination through the bile and into the GI tract (Nagar and Blanchard, 2006). However, as SN-38G passes through the GI tract, it acts as a substrate for bacterial β-glucuronidases present in intestinal symbiotic microbes (Tobin et al., 2003; Stein et al., 2010). These enzymes, in turn, reactivate SN-38 in situ, resulting in toxic injury to the intestinal epithelial cells (Araki et al., 1993). Indeed, the severity of CPT-11–induced diarrhea is correlated with levels of β-glucuronidase activity in the intestinal lumen, and the intestinal microbiota have been found to play an important role in the development of this toxicity (Takasuna et al., 1996, 1998; Mathijssen et al., 2001; Brandi et al., 2006). Although most early-onset diarrhea associated with CPT-11 treatment can be treated with antimotility agents, the delayed GI toxicity associated with SN-38 can be refractory to standard treatments (Saliba et al., 1998). It has now been convincingly demonstrated that the intestinal toxicity of CPT-11 limits dose intensification and optimized delivery of CPT-11 (Abigerges et al., 1994; Ducreux et al., 2003; Zhao et al., 2004).

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ABBREVIATIONS: CPT-11, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]; DMSO, dimethyl sulfoxide; GI, gastrointestinal; LB, lysogeny broth; PNPG, p-nitrophenyl β-D-glucuronide; SN-38, 7-ethyl-10-hydroxy-camptothecin; SN-38G, SN-38 glucuronide; UGT, UDP-glucuronosyltransferase.
The role microbial enzymes expressed by symbiotic bacteria plays in therapeutic metabolism has been appreciated since the early days of drug discovery (Sousa et al., 2008). We recently hypothesized that selective microbial β-glucuronidase inhibitors would alleviate CPT-11-induced toxicity. In vitro and cell-based assays demonstrated that a set of chemically similar compounds identified by high-throughput screening displayed potent inhibition of Escherichia coli β-glucuronidase and did not impact the viability of cultured bacterial or mammalian cells (Wallace et al., 2010). Crystal structures of inhibitors in complex with E. coli β-glucuronidase reveal that the small molecules bind to a loop present in bacterial β-glucuronidases but missing in the mammalian orthologs; accordingly, the inhibitors examined had no inhibitory effect in vitro on a mammalian β-glucuronidase (Wallace et al., 2010). Oral administration of one of the inhibitors to mice alleviated CPT-11-induced diarrhea and intestinal damage (Wallace et al., 2010). Furthermore, in a subsequent study, the administration of the same inhibitor protected mice from small intestinal ulceration caused by the nonsteroidal anti-inflammatory drug diclofenac, which is also subject to glucuronidation and reactivation in the intestinal lumen (LoGuidice et al., 2012).

Here we describe the inhibitory profile of four novel inhibitors that are chemically distinct from the originally reported set (Wallace et al., 2010; Ahmad et al., 2011). These compounds are shown to be in vitro inhibitors of E. coli β-glucuronidase but are nonlethal to cultured E. coli and mammalian cells. The X-ray crystal structure of E. coli β-glucuronidase in complex with one of the inhibitors reveals that it binds to the entrance of the enzyme’s active site and makes contact with, but does not completely order, the flexible bacterial loop unique to the microbial β-glucuronidases. We also show that the most potent of these new compounds protects mice from CPT-11-induced diarrhea. Taken together, these data enhance our understanding of the selective modulation of microbial β-glucuronidases, which may lead to the targeted alleviation of drug-induced toxicity in the human GI tract.

Materials and Methods

Expression and Purification of E. coli β-Glucuronidase. E. coli β-glucuronidase (EC 3.2.1.31) was expressed and purified as described previously (Wallace et al., 2010). Purified E. coli β-glucuronidase was stored in 20 mM HEPES, 50 mM NaCl, pH 7.4 at −10 mg/ml at −80°C. The Δ360–376 form of E. coli β-glucuronidase was created using polymerase chain reaction mutagenesis, confirmed by sequencing and purified as described previously (Wallace et al., 2010).

Inhibitor Compounds. Compounds (Fig. 1) identified from high-throughput screening (Wallace et al., 2010; Ahmad et al., 2011) were purchased from ASINEX, Inc. (Moscow, Russia). Each compound was provided as a solid powder and dissolved initially in 100% dimethyl sulfoxide (DMSO) at 25 mM.

Kinetic and Equilibrium Inhibition Assays. Inhibition assays were conducted by measuring the β-glucuronidase-catalyzed conversion of p-nitrophenyl β-D-glucuronide (PNPG) to p-nitrophenol. PNPG was acquired from Sigma-Aldrich (St. Louis, MO), and stored by dissolving in water at 250 mM. The conversion of increasing concentrations of PNPG to p-nitrophenol in the presence of 10 nM enzyme was measured in the presence of increasing concentrations of our putative inhibitors; zero-substrate and zero-inhibitor controls were carried out at the same time. Reactions were conducted in 96-well, clear-bottom assay plates (Costar, Tewksbury, MA) at 37°C in 50 μl of total volume. The reaction consisted of 10 μl of assay buffer (5% DMSO and 500 mM HEPES, pH 7.4), 5 μl of inhibitor solution (various concentrations), 5 μl of 100 nM enzyme, and 30 μl of substrate (various concentrations). Product formation was calculated by measuring the change in absorbance over time at 410 nm using a PHERAstar Plus microplate reader (BMG Labtech, Ortenberg, Germany). The acquired data were analyzed using Microsoft Excel (Microsoft, Redmond, WA) and SigmaPlot 11.0 (Systat Software, San Jose, CA). From these data, Ki values were calculated for each of the inhibitors.

A related assay was employed to calculate the IC50 values in the following manner. An analogous 50 μl reaction, consisting of enzyme (1 nM final), buffer, PNPG (1 nM final), and variable concentrations of the inhibitors, was incubated at 37°C for 6 hours to allow the reaction to reach equilibrium and then quenched with 100 μl 0.2 M sodium carbonate. The percent inhibition for each concentration of inhibitor was calculated based on changes in absorbance and used to plot a dose-response curve. The IC50 value was calculated as the concentration of inhibitor that produced 50% in vitro inhibition. The
same assay was performed with purified bovine liver β-glucuronidase with PNPG as the substrate.

We also tested the ability of these compounds to inhibit β-glucuronidase in E. coli cells. We grew chemically competent E. coli cells, transformed with the pET-28a vector containing the β-glucuronidase gene, to an optical density (OD)₆₀₀ of 0.6 in lysogeny broth (LB) medium and used a small aliquot in an assay similar to the in vitro IC₅₀ assay described above. The cells (39 μl) were incubated with 1 μl of variable concentrations of inhibitor and 10 μl of 1 mM PNPG at 37°C for 6 hours. The reaction was quenched with 100 μl 0.2 M sodium carbonate. The amount of substrate turnover and therefore the amount of inhibition is calculated from the change in absorbance compared with zero-inhibitor controls. EC₅₀ values were calculated as the amount of inhibitor necessary to produce 50% inhibition. The in vitro and cell-based assays employed here were similar to those reported previously (Wallace et al., 2010).

Additional Glycosidase Enzymes. Mammalian (bovine liver) β-glucuronidase (lyophilized powder, EC 3.2.1.31), Prunis dulcis β-glucosidase (lyophilized powder, EC 3.2.1.21), Helix pomatia β-mannosidase (ammonium sulfate suspension, EC 3.2.1.25), E. coli β-galactosidase (lyophilized powder, EC 3.2.1.23), and bovine liver β-galactosidase (lyophilized powder, EC 3.2.1.23) were purchased from Sigma-Aldrich. The assays were conducted as previously published, using the appropriate p-nitrophenyl-glycosidase compound as the primary substrate for enzyme activity detection (Graef et al., 1977). Each of the four inhibitors was tested for an effect on each glycosidase enzyme activity by adding a concentration range of 0 to 100 μM to the reaction mixture. The reaction was allowed to proceed for 6 hours at 37°C and then quenched with 100 μl 0.2 M sodium carbonate. Absorbance was measured at the appropriate wavelength, and the data were analyzed using Microsoft Excel and SigmaPlot 11.0.

TABLE 1
In vitro and cell-based assays for β-glucuronidase activity and inhibitor efficacy

<table>
<thead>
<tr>
<th>E. coli β-Glucuronidase In Vitro</th>
<th>E. coli Cell Based</th>
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<tbody>
<tr>
<td>Kᵢ</td>
<td>IC₅₀</td>
</tr>
<tr>
<td>nM</td>
<td>nM</td>
</tr>
<tr>
<td>Inhibitor 5</td>
<td>217 ± 42.6</td>
</tr>
<tr>
<td>Inhibitor 6</td>
<td>668 ± 28.8</td>
</tr>
<tr>
<td>Inhibitor 7</td>
<td>1920 ± 21.0</td>
</tr>
<tr>
<td>Inhibitor 8</td>
<td>957 ± 22.8</td>
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</tbody>
</table>

NI, no inhibition.

Toxicity of Inhibitors toward Cultured Cells. The toxicity of inhibitors to bacterial cells was examined by incubating chemically competent E. coli cells, transformed with the pET-28a vector containing the β-glucuronidase gene, and grown to an OD₆₀₀ of 0.6 in LB medium with each compound, as well as DMSO and ampicillin as controls, for 6 hours. Cell survivability was measured by plating a 10⁻² dilution of the cells on LB media enriched with kanamycin. After overnight incubation, colonies were counted to quantify cell viability. We used a similar assay to assess each inhibitor’s toxicity toward cultured HCT116 human colon cancer cells, which were grown and cultured in Dulbecco’s modified Eagle’s medium. Aliquots of HCT116 cells were incubated with 100 μM of each lead for 24 hours. The resulting viability of the cells was quantified by using the CellQuanti-Blue Cell Viability Assay Kit (BioAssay Systems, Hayward, CA). Cells were incubated with CellQuanti-Blue Reagent, and fluorescence was measured.

Fig. 2. Impact of increasing concentrations of Inhibitors 5–8 on the activity of bovine β-glucuronidase.
Crystal Structure Determination. Crystals of *E. coli* β-glucuronidase were obtained at 2 mg/ml protein with 30-fold molar excess Inhibitor 8 (Fig. 1) in 17% polyethylene glycol 3350 (w/v), 250 mM magnesium acetate, and 0.02% sodium azide (w/v) at 16°C. Crystals first appeared after 5 days and were immediately cryoprotected with perfluoropolyether vacuum pump oil (Sigma-Aldrich) and flash-cooled in liquid nitrogen. Diffraction data were collected on the 22-BM beam line at SER-CAT (Advanced Photon Source, Argonne National Laboratory, Lemont, IL). Data in space group C2 were indexed and scaled using HKL2000 (Otwinski and Minor, 1997) to 2.83 Å resolution. The asymmetric unit contained two monomers. The structure was determined with Phaser as part of the software suite PHENIX (PHENIX Project, Berkeley, CA) (McCoy et al., 2007) using molecular replacement with the recent apo *E. coli* β-glucuronidase structure (PDB ID 3K46) as a search model. The structure was refined using simulated annealing and torsion angle refinement with the maximum likelihood function target in the program central nervous system (crystallography and NMR system) and monitored using both the crystallographic R and cross-validating R-free statistics (Brünger, 1997). The software suite PHENIX (PHENIX Project) (Adams et al., 2002) was also employed for grouped B factor (with two groups: main chain and side chain) and translation, libration, screw refinement. The model was manually adjusted using Coot (Emsley and Cowtan, 2004) and 2Fo–Fc and Fo–Fc electron density maps. The ligand model and definition files were generated using PRODRG (GlycoBioChem, Dundee, Scotland, UK) (Schüttelkopf and van Aalten, 2004), and were placed into electron density in the active site of both monomers in the asymmetric unit.

Animal Studies. Animal experiments were performed according to the Institutional Animal Care and Use guidelines approved by the Institutional Animal Care and Use Committee (IACUC # 20070715 and 20100711) of the Albert Einstein College of Medicine, Bronx, NY. CPT-11 was purchased from LC Laboratories (catalog number: I-4122; Woburn, MA), as a hydrochloride salt (>99% high-performance liquid chromatography purified grade). CPT-11 (20 mg/ml) and Inhibitor 5 (100 μg/ml) were dissolved in 0.25% (w/v) carboxymethylcellulose sodium salt (Sigma-Aldrich; C5013) to make stock solutions. As a vehicle control, all animals received an equivalent volume (compared with experimental groups) of 0.25% (w/v) carboxymethylcellulose sodium salt solution. BALB/cJ mice female

**Fig. 3.** Impact of Inhibitors 5–8 at 100 μM on the survival of *E. coli* (A) and HCT116 cells (B). Ampicillin and 10% Triton-X100 are used as positive controls for microbial and human cell lethality, respectively. Additionally, 2% DMSO, in which the inhibitors are solubilized, is also shown. DMEM, Dulbecco’s modified Eagle’s medium.
(8–10 weeks old) were obtained from the Jackson Laboratories (Bar Harbor, ME). The mice were housed in conventional metabolic cages (N = 1/cage) and kept in a room under controlled temperature (20–22°C) and 12-hour day-night cycle. Animals had free access to water and conventional food without fortification. Mice were divided into four groups of 9 animals each: group 1, vehicle controls received equivalent volume of 0.25% (w/v) carboxymethylcellulose sodium salt solution intraperitoneally and by oral gavage (∼100 μl twice per day); group 2, Inhibitor 5 gavaged (10 μg/d) twice per day (every 10 hours) starting on day 1 with oral gavage of 0.25% (w/v) carboxymethylcellulose sodium salt solution and intraperitoneally once per day; group 3, CPT-11 injected (50 mg/kg) intraperitoneally once daily in the morning with oral gavage of 0.25% (w/v) carboxymethylcellulose sodium salt solution; group 4, CPT-11 injected (50 mg/kg) intraperitoneally once daily in the morning and Inhibitor 5 gavaged (10 μg/d) twice per day (every 10 hours). Total injected volume was identical for each animal. Mice were weighed and examined daily for signs of diarrhea (fecal staining of skin, lose watery stool) and bloody diarrhea (black sticky stool). Body weight, stool consistency, and blood in stool were monitored daily using methods previously published (Cooper et al., 1993; Wallace et al., 2010). Previous studies indicate that dosing at ∼60–80 mg/kg/day of CPT-11 for 4 days allows for observation of delayed diarrhea around 15 days (Reagan-Shaw et al., 2008). A dosing scheme of 50 mg/kg/day, once daily for 9 days, was chosen with the intention of accelerating the onset of diarrhea while preventing death. As outlined previously, 50 mg/kg CPT-11 in mice is roughly equivalent to the 5 mg/kg typical human CPT-11 dose based on differences in body surface area (Brandi et al., 2006; Reagan-Shaw et al., 2008). GI symptoms in group 3 started as early as day 2 and up to day 10 and included decreased appetite, bowel movements, mobility, and body weight. All animals were euthanized on day 11.

**Results**

**In Vitro and Cell-Based E. coli β-Glucuronidase Inhibition.** Four compounds were chosen from high-throughput screening results (Wallace et al., 2010; Ahmad et al., 2011) for further functional and structural characterization (Fig. 1). These compounds (Inhibitors 5–8) are relatively distinct in structure from one another and are also distinct from the four chemically similar compounds (Inhibitors 1–4) reported previously (Wallace et al., 2010). In in vitro assays with purified E. coli β-glucuronidase, we found that all four compounds functioned as inhibitors, with IC50 and K<sub>i</sub> values ranging from 180 nM (Inhibitor 5) to 4 μM (Inhibitor 7) (Table 1), similar to those observed previously with Inhibitors 1–4 (Wallace et al., 2010). Only Inhibitors 5, 6, and 8, however, were effective against the β-glucuronidase target in living E. coli cells. Inhibitor 7, the weakest in vitro compound, showed no impact in cells, whereas Inhibitors 5, 8, and 6 displayed 300 nM, 1.2 μM, and 7.3 μM EC50 values, respectively (Table 1). The relatively potent in-cell EC50 values compared with the in vitro IC50 values are likely attributable to differences in compound entry, metabolism, export, or partitioning within living cells; such features will be examined in future studies. In summary, we have identified novel compounds that exhibit in vitro and in-cell inhibition of E. coli β-glucuronidase; this information expands our understanding of chemical moieties capable of disrupting bacterial β-glucuronidase activity.

**Selectivity for Bacterial β-Glucuronidase.** We next tested the ability of Inhibitors 5–8 to disrupt the activity of β-glucuronidase from bovine liver, a mammalian enzyme ortholog that processes larger glucosaminoglycan substrates relative to the bacterial β-glucuronidases (Ray et al., 1999).

We found that Inhibitors 5–8 failed to impact the activity of the bovine liver enzyme at concentrations up to 100 μM (Fig. 2), demonstrating that Inhibitors 5–8 are selective for the E. coli β-glucuronidase. We also examined the in vitro effects of these inhibitors on other glycosidase enzymes. We tested each inhibitor against four commercially available enzymes: a plant β-glucosidase (from the almond tree P. dulcis), a mollusk β-mannosidase (from H. pomatia), and the β-galactosidases from both E. coli and bovine sources. In all cases, Inhibitors 5–8 failed to exhibit inhibition at compound concentrations up to 100 μM (unpublished data). Similarly, the previously reported Inhibitors 1–4 also failed to exert an effect on these glycosidases (unpublished data). Thus, we conclude that the β-glucuronidases Inhibitors 1–8 are selective for the bacterial β-glucuronidase and do not inhibit either mammalian β-glucuronidase or members of the related family of sugar-cleaving enzymes from a range of sources.

**Lethality to Bacterial or Mammalian Cells.** Eliminating toxic drug metabolites in the GI tract by disrupting microbial β-glucuronidases requires that inhibitors are not harmful to human or bacterial cells. Indeed, it is increasingly well established that symbiotic microbiota are essential for GI health (Human Microbiome Project Consortium, 2012). We tested whether Inhibitors 5–8 were lethal to cultured E. coli cells or HCT116 human colon cancer cells at compound concentrations up to 100 μM. We found that all four inhibitors did not affect the survival of either E. coli or HCT116 cells (Fig. 3). Thus, they appear to satisfy the criteria that successful compounds are relatively nontoxic to bacterial cells or human epithelial cells.

**Structure of E. coli β-Glucuronidase Complexed with Inhibitor 8.** To further our understanding of the molecular basis of E. coli β-glucuronidase inhibition, we determined the 2.83 Å resolution crystal structure of the enzyme in complex with Inhibitor 8 (PDB ID 4JHZ) (Table 2). The asymmetric unit contained two β-glucuronidase monomers (Fig. 4A), whereas C2 crystallographic symmetry produced the physiologically relevant β-glucuronidase tetramer (Wallace et al., 2010). The apo (unliganded) structure of E. coli β-glucuronidase

**TABLE 2**

<table>
<thead>
<tr>
<th>Structure of E. coli β-glucuronidase complexed with Inhibitor 8</th>
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<tbody>
<tr>
<td>X-ray source</td>
</tr>
<tr>
<td>Space group</td>
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<tr>
<td>Unitcell: a, b, c (Å); α, β, γ(°)</td>
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<td>Resolution range (Å)</td>
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<td>Wilson B factor</td>
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<tr>
<td>Molecules per AU</td>
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<tr>
<td>No. of waters per AU</td>
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<tr>
<td>No. of protein residues per AU</td>
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<tr>
<td>Average B-factor</td>
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<tr>
<td>RMS (bond lengths)</td>
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<tr>
<td>RMS (bond angles)</td>
</tr>
<tr>
<td>Ramachandran favored (%)</td>
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<tr>
<td>Ramachandran outliers (%)</td>
</tr>
</tbody>
</table>

APS ANL, Advanced Photon Source, Argonne National Laboratory; AU, asymmetric unit; RMS, root mean square.
was used as a molecular replacement search model to eliminate any previous ligand model bias. After careful refinement of the protein model structure, electron density at 1.0 σ level in the composite omit map was present for Inhibitor 8 in both monomers of the asymmetric unit (Fig. 4B). After refinement of the atomic positions as well as their thermal displacement parameters (B factors), the ligands had an occupancy of 0.93 and 0.91, with an average B-factor of 66.4 and 76.5 Å² for chain A and B, respectively.

Inhibitor 8 was placed in an orientation that both satisfies the electron density and makes chemically reasonable interactions with the neighboring amino acid side chains. The compound binds to the entrance to the active site cleft of E. coli β-glucuronidase (Fig. 4A), 3.5 Å from the catalytic residue Glu413 and contacts Asp163, Val446, Phe448, Tyr472, and Arg562 within the active site region of the enzyme (Fig. 5). Notably, in chain A it directly contacts Leu361 of the bacterial loop, a region that is unique to the microbial forms of the β-glucuronidase relative to the mammalian β-glucuronidases (Jain et al., 1996; Wallace et al., 2010). Inhibitor 8 binds in a manner similar to Inhibitors 2 and 3 elucidated previously in complexes with E. coli β-glucuronidase (Wallace et al., 2010), occupying a similar location in the enzyme and contacting five of the same residues (Fig. 5). Indeed, the root mean square deviation between the Ca positions of the Inhibitor 2- and Inhibitor 8-bound structures of E. coli β-glucuronidase is 0.54 Å. However, although most residues contacted by Inhibitor 8 are in the same position in the Inhibitor 2 complex structure, the side chain of Leu361 shifts by 3.6 Å. This observation suggests that the bacterial loop is capable of conforming to the presence of different bound inhibitors. Electron density in the simulated annealing omit and 2F₀–Fᵣ maps is less complete for Leu361 in chain B compared with chain A.

The full bacterial loop of E. coli β-glucuronidase, residues 360–376, was visualized in the Inhibitor 2 and 3 cocrystal structures (Wallace et al., 2010); in complex with Inhibitor 8, although only residues 372–376 are ordered with average B factors less than 80 Å² (Fig. 6A). In the absence of a bound ligand, the bacterial loop is not observed, as it gave no interpretable electron density in the structure of apo E. coli β-glucuronidase reported previously (Wallace et al., 2010). Thus, we conclude that E. coli β-glucuronidase can be inhibited effectively by compounds, like Inhibitor 8, capable of contacting only a portion of the loop unique to the microbial β-glucuronidases. In support of this conclusion, we found that a form of E. coli β-glucuronidase in which the bacterial loop was deleted, Δ360–376, was not inhibited by up to 100 μM of Inhibitors 5–8 (Fig. 6B). The Δ360–376 E. coli β-glucuronidase exhibited comparable Kᵦᵦ (260 μM for Δ360–376, 360 μM for wild-type) values, but the Vₘₐₓ (2.44 nmol/s for Δ360–376, 398 nmol/s for wild-type) and kᵢₜᵢₜ (0.244 second⁻¹ for Δ360–376, 39.8 second⁻¹ for wild-type) values were reduced by approximately two orders of magnitude. Thus, the bacterial loop is essential for full activity of E. coli β-glucuronidase, as well as its selective inhibition.
The body weight of each mouse was also recorded each day during the course of the experiment (Fig. 7B). Although Inhibitor 5 alleviated most of the toxicity associated with the CPT-11 treatment, it had no effect on the average body weight of the mice. Moreover, group 2, which only received treatment with Inhibitor 5, showed comparable body weights to the vehicle-only group, but had significantly higher body weights, starting at day 6, than group 4, which received treatment with Inhibitor 5 in addition to CPT-11. These data indicate that the presence of Inhibitor 5 significantly reduces the incidence of acute GI toxicity caused by CPT-11, and they suggest that Inhibitor 5 does not impact the systemic effects of this anticancer drug, as measured by weight loss.

**Discussion**

Research endeavors such as the Human Microbiome Project continue to expand our appreciation of the roles the symbiotic microbiota play in mammalian physiology (Human Microbiome Project Consortium, 2012). It also appears that widespread use of antibiotics may have subtle but serious side effects on human health (Maurice et al., 2013). Additionally, for patients with colorectal cancer, the use of antibiotics to treat CPT-11-induced toxicity may lead to the increased
prevalence of subdominant bacterial species with higher overall β-glucuronidase activity in the GI tract and thus, increased toxicity (Brandi et al., 2006). Studies have also shown that the long-term use of neomycin in mice leads to increased serum bilirubin that may interfere with proper hepatic CPT-11 metabolism (Vítek et al., 2005). These observations lead to the conclusion that the selective, non-lethal inhibition of components of the GI microbiome will be an important method of enhancing drug efficacy and tolerance. In this report, we interrogate the roles bacterial β-glucuronidases play in mammalian GI drug toxicity by pharmacologically targeting this enzyme with potent and selective inhibitors.

Each of the compounds described, Inhibitors 5–8, exhibited strong in vitro inhibition of E. coli β-glucuronidase (Table 1) but did not affect the viability of either cultured bacterial or mammalian cells (Fig. 3). Only Inhibitor 7 failed to disrupt β-glucuronidase activity in living E. coli cells. Furthermore, because compounds were ineffective against mammalian β-glucuronidase (Fig. 2) and a range of enzymes from the glycosidase family, each inhibitor displayed selectivity toward E. coli β-glucuronidase. The selectivity of our inhibitors is particularly critical for their use in conjunction with CPT-11 because human β-glucuronidase expressed by tumor cells appears to play an important role in the antitumor efficacy of CPT-11 through the reactivation of SN-38G to SN-38 in the tumor microenvironment (Tobin et al., 2006; Huang et al., 2011). Of the four compounds described here, only Inhibitor 5 (Kᵢ and IC₅₀ values of 180 and 540 nM, respectively) displayed potency comparable with our previously-characterized inhibitors, which have a scaffold not shared by the four inhibitors outlined in this study (Wallace et al., 2010). As such, these data advance our understanding of the chemical moieties capable of selective bacterial β-glucuronidase inhibition.

We successfully determined the X-ray crystal structure of one of our inhibitors (Inhibitor 8) in complex with E. coli β-glucuronidase (Fig. 4). This adds to our knowledge of E. coli β-glucuronidase inhibition that started with the structures of the complexes of Inhibitors 2 and 3 reported previously (Wallace et al., 2010). Similar to those structures, Inhibitor 8 binds at the entrance to the active site cleft and forms related contacts, including one with the catalytic glutamic acid residue, Glu413. Importantly, Inhibitor 8 also makes a hydrophobic contact with Leu361, which is part of the bacterial loop

![Graph A](image1.png)  
**Fig. 7.** (A) Groups of nine BALB/cJ mice were dose with either vehicle, Inhibitor 5, CPT-11, or pretreated with Inhibitor 5 followed by CPT-11 and then examined for incidences of CPT-11-induced toxicity (i.e., bloody diarrhea). (B) The mice were weighed daily. Administration of CPT-11 incited the loss of body weight during treatment. Oral administration of Inhibitor 5 had no protective effects on body weight during CPT-11 treatment. Inhibitor 5 only and CPT-11 + Inhibitor 5 groups were analyzed for statistical significance. **P < 0.01; ***P < 0.001.
unique to the microbial enzymes. Overlapping the structures of the complexes with Inhibitor 2 and Inhibitor 8, it is apparent that Leu361 has shifted to facilitate binding, suggesting that bacterial loop flexibility is involved in inhibition (Fig. 6). The observation that the bacterial loop in the Inhibitor 8 complex structure is disordered for the eight residues C-terminal to Leu361 supports that conclusion. Mutagenesis studies in which the bacterial loop has been deleted confirm its importance in microbial β-glucuronidase inhibition (Fig. 6).

Lastly, only the previously reported Inhibitor 1 had been examined to date in drug-induced GI toxicity studies in mice (Wallace et al., 2010; LoGuidice et al., 2012). In this report, we tested the in vivo efficacy of our most effective compound outlined here, Inhibitor 5, in a mouse model of CPT-11 toxicity (Fig. 7). Groups of BALB/cJ mice were dosed with Inhibitor 5, CPT-11, or a combination of both. The group that received CPT-11 alone developed bloody diarrhea by day 8, and 100% of the mice showed signs of this toxicity by day 10. The group that was treated with Inhibitor 5 orally, along with CPT-11 intraperitoneally, had significantly fewer incidents of bloody diarrhea compared to the CPT-11 group, highlighting the protective effects of Inhibitor 5. It is also interesting to note that the mice that received CPT-11 lost body weight at the same rate regardless of treatment with Inhibitor 5. This suggests that Inhibitor 5 protected against CPT-11–induced GI toxicity but that the systemic pharmacodynamics of CPT-11 in the mouse are not impacted. A more comprehensive examination of tumor xenographs and pharmacokinetics will be required to substantiate this conclusion.

We have described the inhibitory profile of a range of chemically distinct compounds and assessed their potential as oraly delivered pharmacological agents. They are in vitro inhibitors to E. coli β-glucuronidase and, with the exception of Inhibitor 7, are also effective in E. coli cells. Moreover, our inhibitors appear to be selective for bacterial β-glucuronidase and nonlethal to bacterial and mammalian cells. The structural data presented here, along with the data from our previously characterized inhibitors, will allow us to better understand their mechanism of selective microbial β-glucuronidase inhibition. As such, they may facilitate the development of therapeutics capable of alleviating drug-induced GI toxicity generated by symbiotic microbial β-glucuronidases.

Authorship Contributions

Participated in research design: Roberts, Wallace, Kumar, Mani, Redinbo.

Conducted experiments: Roberts, Wallace, Kumar.

Performed data analysis: Roberts, Wallace, Kumar, Mani, Redinbo.

Wrote or contributed to the writing of the manuscript: Roberts, Kumar, Mani, Redinbo.

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Graef V, Furuya E, and Nishikaze O (1977) Hydrolysis of steroid glucuronides with human beta-glucuronidase and, with the use of beta-glucuronidase inhibitors, will allow us to better understand their mechanism of selective microbial β-glucuronidase inhibition. As such, they may facilitate the development of therapeutics capable of alleviating drug-induced GI toxicity generated by symbiotic microbial β-glucuronidases.

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