Gut microbial \( \beta \)-glucuronidases reactivate estrogens as components of the estrobolome that reactivate estrogens

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Edited by Wolfgang Peti

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Gut microbial \( \beta \)-glucuronidase (GUS) enzymes have been suggested to be involved in the estrobolome, the collection of microbial reactions involving estrogens. Furthermore, bacterial GUS enzymes within the gastrointestinal tract have been postulated to be a contributing factor in hormone-driven cancers. However, to date, there has been no experimental evidence to support these hypotheses. Here we provide the first in vitro analysis of the ability of 35 human gut microbial GUS enzymes to reactivate two distinct estrogen glucuronides, estrone-3-glucuronide and estradiol-17-glucuronide, to estrone and estradiol, respectively. We show that certain members within the Loop 1, mini-Loop 1, and FMN-binding classes of gut microbial GUS enzymes can reactivate estrogens from their inactive glucuronides. We provide molecular details of key interactions that facilitate these catalytic processes and present the structures of two novel human gut microbial GUS enzymes related to the estrobolome. Further, we demonstrate that estrogen reactivation by Loop 1 bacterial GUS enzymes can be inhibited both in purified enzymes and in fecal preparations of mixed murine fecal microbiota. Finally, however, despite these in vitro and ex vivo data, we show that a Loop 1 GUS-specific inhibitor is not capable of reducing the development of tumors in the PyMT mouse model of breast cancer. These findings validate that gut microbial GUS enzymes participate in the estrobolome but also suggest that the estrobolome is a multidimensional set of processes on-going within the mammalian gastrointestinal tract that likely involves many enzymes, including several distinct types of GUS proteins.

The gastrointestinal (GI) \(^2\) microbiome harbors incredible metabolic potential and is intimately connected to human physiology. Possessing 150 times more genes than are found in the human genome, the gut microbiome encodes a vast number of enzymes that function in a variety of metabolic pathways, including the biosynthesis of essential vitamins and the breakdown of complex, nondigestible polysaccharides (1–4). The GI microbiota plays a potentially significant but enigmatic contribution to human health via the estrobolome, the aggregate of the enteric bacterial genes whose products are capable of metabolizing estrogens (5). It has been suggested that a woman’s estrobolome plays a key role in a number of hormonal disorders, including breast, endometrial, and ovarian cancers (5–8). Hypothesized to be especially important to metabolism within the estrobolome are bacterial species possessing \( \beta \)-glucuronidase (GUS) enzymes.

During phase II metabolism, UDP-glucuronosyltransferase enzymes (UGTs) append a glucuronic acid moiety to a variety of endo- and xenobiotics, typically inactivating them and marking them for excretion. GUS proteins within the GI tract can intercept this process, cleaving the glucuronic acid, allowing the reactivated compound to be recirculated throughout the body, thereby reversing phase II glucuronidation. In the case of SN-38, the active metabolite of the anticancer drug irinotecan, this process of glucuronidation and de-glucuronidation through the action of GUS has been shown to cause severe GI toxicity. By inhibiting the GUS enzymes responsible for this reactivation, the diarrhea associated with administration of SN-38 can be alleviated (9–12). The same has been established for lower GI damage caused by nonsteroidal anti-inflammatory compounds (NSAIDs) (13–17).

Similar to SN-38 and NSAIDs, estrogenic compounds are glucuronidated in the liver during phase II metabolism. Upon entry into the GI tract, they are exposed to GUS enzymes that could, in theory, cleave the sugar moiety, reactivating the parent compound and allowing the unconjugated estrogen to be reab-

\(^2\) The abbreviations used are: GI, gastrointestinal; GUS, \( \beta \)-glucuronidase; SSN, sequence similarity network; HMP, human microbiome project; UGT, UDP-glucuronosyltransferase; E1-3G, estrone-3-glucuronide; E2-17G, estradiol-17-glucuronide; pNPG, 4-nitrophenyl \( \beta \)-D-glucuronide; HR, +, hormone receptor–positive; LB, lysogeny broth; BisTris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; NSAID, nonsteroidal anti-inflammatory drug.
Gut microbial β-glucuronidases reactivate estrogens

![Diagram of estrogen glucuronidase enzymes](image)

**Figure 1. β-Glucuronidase enzymes reactivate estrogens.** Gut microbial β-glucuronidase enzymes within the GI deconjugate estrone-3- and estradiol-17-glucuronides to the aglycones estrone and estradiol, respectively. This reactivation allows unbound estrogens to be recirculated through the bloodstream, possibly contributing to a variety of hormonal disorders, including breast cancer and endometriosis.

sorbed in the bloodstream and undergo iterative rounds of enterohepatic recirculation (18, 19). This is potentially significant in oncology, as there is now general agreement that the concentrations of unbound estrogens are much higher in plasma and tissues of women with hormone-driven cancers (20–23). Therefore, an estrobolome enriched in β-glucuronidase enzymes that promote estrogen metabolite deconjugation reactions may result in greater reabsorption of free estrogens and a greater risk of hormone receptor–positive (HR +) cancers (Fig. 1).

Although it has been postulated that the GUS enzymes within the estrobolome play an integral role in estrogen reactivation and recirculation, this role has yet to be proven. Here we take a panel of 35 human gut microbial GUS enzymes and the glucuronides of two estrogens, estradiol (E2, dominant during reproductive years) and estrone (E1, dominant after menopause), and assess the ability of these enzymes to process these substrates. With this in vitro panel, we show that 17 of the 35 GUS enzymes tested are capable of reactivating estrogen conjugates. Guided by novel crystal structures of two human gut microbial GUS enzymes and 12 additional structures already in hand, we pinpoint structural features critical to estrogen glucuronide processing. Further, we examine the inhibition of key gut microbial GUS enzymes in vitro, ex vivo, and in vivo and test the estrobolome hypothesis with respect to breast tumor growth in an HR + mouse model. Taken together, our results validate gut microbial GUS enzymes as active members of the estrobolome but highlight the likely complex relationship between the estrobolome and tumorigenesis in vivo.

**Results**

**Identification of estrogen glucuronide–processing gut microbial GUS enzymes**

Gut microbial GUS enzymes have been shown to share a common fold but exhibit unique active-site architectures and differential activities with distinct substrates (24, 25). To gain greater insight into the specific sequence-structure-function relationships among GUS proteins, we generated a sequence similarity network (SSN) using sequences of β-glucuronidase enzymes found within the Human Microbiome Project (HMP). The resultant SSN clusters the 279 unique protein sequences based on sequence identity and homology (26).

Of the 279 unique GUS enzymes identified in the HMP, we have cloned, expressed, and purified 35 of these for in vitro study (Fig. 2A (triangles) and Table S1). Enzymes were chosen so that the prevalence of each loop category was comparable with what has been previously reported in the HMP (27). However, an exception to this is the Loop 1 enzymes, which are overrepresented in our panel of 35 proteins, as these have been previously shown to efficiently reactivate small-molecule drug substrates, a key focus of our work (27). Of the 35 enzymes examined, crystal structures have been reported for 18 (Table S1), and these structural data correlate with the family groupings present in the SSN.

To identify GUS enzymes capable of processing estrogen glucuronides, we employed an assay that couples the formation of glucuronic acid to its utilization by uronate dehydrogenase and subsequent reduction of NAD⁺ to NADH + H⁺, to define an end-point cleavage measurement (Fig. S1; see “Experimental procedures”). Of the 35 enzymes tested, 17 were capable of cleaving the glucuronide moiety of estrone (E1-3G), and 15 were capable of cleaving the glucuronide of estradiol (E2-17G).

We find that the GUS enzymes capable of cleaving estrogen cluster into three distinct categories: Loop 1 enzymes (red), mini-Loop 1 enzymes (green), and FMN-binding GUS enzymes (yellow). The GUS enzymes that processed glucuronides of estrogen are highlighted on the SSN (Fig. 2A, colored triangles).

These classes have been described in detail previously (27–30); briefly, the Loop 1 and mini-Loop 1 enzymes contain active site–proximal loops that provide favorable interactions for binding smaller substrate-glucuronides. The Loop 1 and mini-Loop 1 enzymes are most frequently associated with the cleavage of small-molecule glucuronides, including drug-glucuronide substrates. The FMN-binding GUS enzymes possess a flavin-mononucleotide cofactor at an allosteric site that aids in structural stability. To date, the exact role of FMN is unclear, but we have found that these enzymes are uniquely capable of small-molecule glucuronide cleavage.

We determined catalytic efficiency (M⁻¹ s⁻¹) values with E1-3G for the 17 enzymes identified. These rates range from 6.40 × 10⁶ to 1.08 × 10⁷ s⁻¹ M⁻¹ (Fig. 2B). The catalytic efficiencies were also determined for the 15 enzymes capable of processing estradiol-17-glucuronide. These rates range from 1.83 × 10⁴ to 1.26 × 10⁵ s⁻¹ M⁻¹ (Fig. 2C). Several of these enzymes have been examined previously with the standard GUS assay substrate 4-nitrophenyl β-D-glucuronide (pNPG), where the catalytic efficiencies range from 9.2 × 10³ to 3.6 × 10⁴, comparable with what we observe with the estrogen glucuronides (28). This result suggests that some gut microbial GUS enzymes may have evolved to be able to process estrogen glucuronide substrates efficiently to gain the six-carbon source of energy.

With the estrogen glucuronide substrates, we find that the Loop 1 enzymes are, in general, the fastest processors of both
estrone and estradiol glucuronide, with the FMN-binding GUSs the second fastest, and the mini-Loop 1 GUS enzymes as the slowest estrogen processors. In summary, we provide the first detailed in vitro data on the ability of human gut microbial GUS enzymes to process the glucuronides of estrone and estradiol, thus indicating that bacterial GUS proteins are active components of the estrobolome.

Structural rationale for estrogen glucuronide–processing gut microbial GUS enzymes

With concerted modeling and site-directed mutagenesis efforts, we have highlighted the residues involved in catalysis, providing a rationale for rate differences of the three categories. First, we modeled E1-3G and E2-17G into the active site of a tetrameric Loop 1 GUS from Clostridium perfringens (4JKM) (9). Because we know precisely where and how the glucuronide moiety binds (11, 12, 25, 27, 28), and these substrate molecules have a limited number of rotatable bonds, we know with reasonable certainty where these estrogens are within the active site. From these models, we have identified aromatic residues within the Loop 1 architecture that provide potential π-stacking interactions that would facilitate substrate binding to optimize cleavage (Fig. 3A).

One of these residues (Phe-363) comes from Loop 1 itself, providing an edge-to-face interaction. The second residue, Phe-368, is donated from the Loop 1 of an adjacent monomer, providing a face-to-face interaction. These residues, coupled with the completely conserved Tyr-472, create an aromatic cage, perhaps explaining why the Loop 1 enzymes exhibit the highest catalytic efficiencies that we measured in vitro (Fig. 3A). To test this hypothesis, mutants of F363A, F368A, and Y472A were created. The Y472A mutation is detrimental to structural stability and as a result is inactive. However, the mutations of F363A and F368A are both stable yet significantly reduce the catalytic efficiency of the molecule. Furthermore, the double mutation, F363A/F368A, eliminates activity of the enzyme entirely (Fig. 3B). These experiments help to confirm our modeling and establish the critical role the aromatic cage in a gut microbial GUS Loop 1 region to the turnover of estrogen glucuronides.

Second, we used the same model of estrogens overlaid with the structure from the tetrameric Bacteroides fragilis β-glucu-
Gut microbial β-glucuronidases reactivate estrogens

Figure 3. Structural rationale for estrogen glucuronide–processing gut microbial GUS enzymes. Shown in purple are E1-3G and E2-17G, respectively. Catalytic glutamates are highlighted in marine, whereas the N-K glucuronide recognition motif is highlighted in magenta. Statistical differences are annotated with *, **, and *** for p values of 0.001, 0.0001, and < 0.0001, respectively. A, active site of Loop 1 GUS from *C. perfringens*. Shown in cyan are the residues that contribute to the aromatic cage that allow the Loop 1 enzymes to be the fastest processors of estrogen glucuronides. B, activity differences between WT *C. perfringens* (Loop 1) and mutants. C, active site of mini-Loop 1 GUS from *B. fragilis*. Shown in cyan are the residues that contribute to an aromatic cage that allow the mini-Loop 1 enzymes to process estrogen glucuronides. D, activity differences between WT *B. fragilis* (mini-Loop 1) and mutants. E, monomer and active site of FMN-binding GUS from *R. gnavus*. The C-terminal domain (pink) is modeled from a previously resolved structure (SU69). F, activity differences between WT *R. gnavus* GUS (FMN) and mutants. As there are no obvious residues at the active site that contribute to binding, mutants were made to probe steric occlusion of Ser-344 and the role of the C-terminal domain. Data are presented as the average of 3 biological replicates ± SEM.

ronidase (3CMG) to identify structural features within the mini-Loop 1 architecture that aid in turnover. Like the Loop 1 GUS enzymes, the mini-Loop 1 possess the conserved tyrosine present in all GUS structures (e.g. Tyr-472 in *C. perfringens* Loop 1 GUS) and an aromatic residue in the mini-Loop 1, Tyr-389 (Fig. 3C). The tetramer of this GUS is different from the previously resolved Loop 1 GUS enzymes, however, in that the active-site interfaces do not overlap. Indeed, the loop of an adjacent monomer does not extend into the active site like it does in the Loop 1 architecture; thus, it only has the one aromatic residue rather than two, making the aromatic cage notably smaller, perhaps explaining the slower rates of the mini-Loop 1 enzymes compared with the Loop 1 enzymes (Fig. 3C). To test this, we created a Y389A mutation in *B. fragilis* GUS and found that it reduces the rate of estrone glucuronide cleavage by an order of magnitude, from a rate of $7.75 \times 10^3$ to $3.20 \times 10^2$ s\(^{-1}\) M\(^{-1}\) (Fig. 3D). Thus, structural modeling and mutagenesis provide a rationale for why the mini-Loop 1 enzymes are less efficient than the Loop 1 enzymes in processing estrone-3-glucuronide.

Third, we modeled the estrogens within the active site of a dimer from *Ruminococcus gnavus* 3 GUS (6MVG), an FMN-binding GUS (30). We have previously shown that although these enzymes have an open, planar active site with few aromatic residues, they contain roughly 150 residues that have yet to be resolved in any of the crystal structures we have determined to date (30). However, a model of this domain has been created based on a Loop 2 GUS from *Bacteroides uniformis* (SU61) (27), and this model places the C-terminal domain roughly 15 Å from the active sites of these enzymes. This potentially flexible C-terminal domain could influence catalytic activity of the FMN enzymes (Fig. 3E). Indeed, when this domain is eliminated (mutant: STP641), enzyme activity of *R. gnavus* 3 GUS is virtually eliminated (Fig. 3F). Thus, the C-terminal domains of the FMN-binding human gut microbial GUS enzymes play critical roles in substrate turnover that allow these FMN-binding GUS proteins to behave akin to Loop 1 GUS enzymes.

Finally, the ratio of the cleavage rate of estrone-3-glucuronide over estradiol-17-glucuronide was calculated for each of the enzymes tested (Table 1). Interestingly, the Loop 1 GUS and mini-Loop 1 GUS orthologs show preference for E1-3G over E2-17G. Furthermore, whereas two of the three mini-Loop 1 GUS proteins tested show some activity with E2-17G, the *B. fragilis* enzyme does not process E2-17G at all. Two distinctions between these substrates may be involved in these differences. First, E1-3G contains an aromatic ring immediately adjacent to the ether-linked glucuronic acid moiety. The planar feature of this aromatic ring may enhance activity because it can form favorable interactions with the GUS active sites in Loop 1 and some mini-Loop 1 enzymes. In contrast, the more flexible cyclopentane ring in E2-17G cannot form potential π-π stacking interactions at the enzyme active site and thus serves as a less optimal substrate.

Second, the methyl group on E2-17G appears to interfere with optimal positioning of the catalytic glutamates, which would certainly reduce catalytic activity with GUS enzymes (Fig. S2). In contrast, this methyl group in E1-3G is directed away from the active-site glucuronic acid, singularly allowing these catalytic residues more ready access to the ether link-
age to be cleaved in E1-3G compared with E2-17G. Thus, differences in aromatic ring and methyl group positioning between these two estrogen glucuronides likely explain why the 3-glucuronide of E1 is a more efficient substrate for the Loop 1 and mini-Loop 1 GUS enzymes compared with E2-17G.

In contrast, the FMN-binding GUS enzymes that exhibit no preference for the 3- versus 17-positions have more open active sites and may not encounter the same steric hindrance as the Loop 1 and mini-Loop 1 GUS enzymes. Taken together, we provide structure-guided insights into the observed differences in estrogen glucuronide substrate turnover between the three categories of GUS enzymes found to process these compounds.

**Novel GUS structures reveal unique active-site architectural motifs**

We were particularly intrigued with the observation that although the mini-Loop 1 enzyme from Faecalibacterium prausnitzii and the Loop 1 enzyme from uncultured Clostridium sp. process these estrogenic compounds, they were hundreds of times slower than every other mini-Loop 1 and Loop 1 enzyme examined (Fig. 1, A (squares), B, and C). We reasoned that there may be unique features to the *F. prausnitzii* and uncultured *Clostridium* sp. GUS proteins that make them slow with these estrogen glucuronide substrates.

Using a multiple-sequence alignment of all 279 GUS enzymes identified from the HMP, we found that 14 contained a loop insert of roughly 25 residues, like in *Eubacterium eligens* GUS (6BJW) (29), that were near the active site, adjacent to the Loop 1 and mini-Loop 1 GUS enzymes compared with E2-17G.

In contrast, the FMN-binding GUS enzymes that exhibit no preference for the 3- versus 17-positions have more open active sites and may not encounter the same steric hindrance as the Loop 1 and mini-Loop 1 GUS enzymes. Taken together, we provide structure-guided insights into the observed differences in estrogen glucuronide substrate turnover between the three categories of GUS enzymes found to process these compounds.

**Table 1**

<table>
<thead>
<tr>
<th>Gut microbial β-glucuronidases reactivate estrogens</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ratio of catalytic efficiencies of E1-3G to E2-17G</strong></td>
</tr>
<tr>
<td>Red, green, and yellow labels represent Loop 1, mini-Loop 1, and FMN-binding GUS enzymes, respectively.</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>E. eligens (L1)</strong></td>
</tr>
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<td><strong>R. hominis (mL1)</strong></td>
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<td><strong>B. fragilis (mL1)</strong></td>
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<td><strong>L. rhamnosus (L1)</strong></td>
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</table>

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**Table 1**

**Ratio of catalytic efficiencies of E1-3G to E2-17G**

Red, green, and yellow labels represent Loop 1, mini-Loop 1, and FMN-binding GUS enzymes, respectively.
steric occlusion by a loop not present in other mini-Loop 1 enzymes likely explains its poor functioning with the two estrogen glucuronides of interest in this report.

Second, the 2.2 Å resolution structure of the GUS from the uncultured _Clostridium_ sp. (PDB code 6U7J, Fig. 4B) initially revealed no obvious differences in quaternary or tertiary structure from the other Loop 1 enzymes, as the predicted novel loop contains ordered β-sheets, similar to _Escherichia coli_ GUS (PDB code 3LPF) (11). From _E. coli_ GUS, we know that deletion of this region does not impact substrate turnover with pNPG or diclofenac-glucuronide (28). However, inspection of the active site reveals a unique positioning of a critical aromatic residue situated on the Loop 1 region expected to coordinate estrogen binding in other Loop 1 and mini-Loop 1 GUS enzymes (e.g. for Loop 1 enzymes _EeGUS_: Phe-374 and _CpGUS_: Phe-363; for the mini-Loop 1 enzymes _BfGUS_: Tyr-389). As expected, UNC10201652 potently inhibits E1-3G and E2-17G processing by several “standard” Loop 1 gut microbial GUS enzymes, particularly those that showed efficient activities with these substrates _in vitro_, such as _E. eligens_ and _C. perfringens_ (Fig. 5A). UNC10201652 does not inhibit the activities of the Loop 1 GUS enzymes from _F. prausnitzii_ or _Lactobacillus rhamnosus_, however, which corroborates previous observations regarding these enzymes (28). Furthermore, it does not affect the activities of the unique gut microbial GUS enzymes from _F. prausnitzii_ or the uncultured _Clostridium_ sp. examined above. Variability in UNC10201652 potency may be a result of differences in the amino acid sequences at the Loop 1 region, as previously hypothesized (28). In addition, UNC10201652 may be ineffective against _F. prausnitzii_ (mini-Loop 1) because of the novel loop identified here.

**In vitro inhibition of gut microbial GUS enzymes that process estrogen glucuronides**

We have developed a series of chemotypes that are selective for and potently and nonlethally inhibit human gut microbial GUS enzymes (9–12, 29). To date, these compounds are selective for the Loop 1 group of microbial GUS enzymes (28, 29). Of the compounds examined, one inhibitor, UNC10201652, is particularly potent, as it contains a piperazine ring that interrupts the catalytic cycle of Loop 1 microbial GUS enzymes and is capable of forming a long-lived inhibitor-glucuronide moiety that remains bound at the enzyme’s active site (30). Thus, we chose to examine UNC10201652 (Fig. S4) for its ability to inhibit estrogen glucuronide processing by a range of Loop 1 and non-Loop 1 gut microbial GUS enzymes.

**Table 2**

Crystallography data collection and refinement statistics

Data were refined using Phenix version 16.1. Values in parentheses are for the highest-resolution shell.

<table>
<thead>
<tr>
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<th><em>F. prausnitzii</em> (PDB code 6U7J)</th>
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</table>
Finally, we tested the ability of UNC10201652 to inhibit estrogen glucuronide conversion by living *E. coli* cells. We have previously constructed a variant of *E. coli* K-12 MG1655 cells in which the *gus* gene was truncated to remove the amino acids between the two conserved catalytic glutamates (GUSΔ413–504). As expected, in this knockout strain, we see no GUS activity in cultured cells (Fig. 5B). We find with WT *E. coli* K-12 MG1655 cells that UNC10201652 shows EC_{50} values of 155 ± 7 and 148 ± 8 nM toward E1-3G and E2-17G processing, respectively (Fig. 5B), similar to what has been observed previously with this inhibitor and other substrates (29). Thus, taken together, we have demonstrated that our most potent gut microbial GUS inhibitor to date is effective in vitro and in cells against several key estrogen glucuronide–processing Loop 1 GUS enzymes, but does not inhibit all of the β-glucuronidases that would appear to participate in the complete estrobolome.

**In fimo evaluation of estrobolome modulation**

Next we sought to examine the reactivation of estrogen glucuronides by gut microbial GUS enzymes present in mammalian intestinal contents. We use the Latin term *in fimo* to describe studies on fecal samples obtained *ex vivo*. In this *in fimo* study, we obtained fecal sample preparations from 11 BALB/c mice (6 female, 5 male) and tested their ability to deconjugate E1-3G and E2-17G *in fimo*. Fecal samples were homogenized, sonicated, and then subjected to centrifugation, after which the resulting supernatant was used to quantify GUS activity.

After incubation of the estrogen conjugate and the *in fimo* specimen for 1 h, we find that every fecal sample is capable of processing these estrogen glucuronides to varying degrees to produce the parent compounds, estrone and estradiol. This indicates that GUS enzymes are indeed active members of the estrobolome (Fig. 6, A and B). Further, we tested the ability of UNC10201652 to inhibit estrogen glucuronide conversion by...
these in fimo preparations. We find that upon the addition of 10 μM UNC10201652, we can inhibit in fimo the formation of estrone and estradiol from E1-3G and E2-17G, respectively. All samples tested showed a trend toward reduction in these reaction rates. The variability in response to UNC10201652 likely arises from the differential levels of Loop 1 GUS enzymes present in each sample. Taken together, these data support the conclusion that gut microbial GUS enzymes are active components of the estrobolome and that such enzymes may be amenable to control using targeted small-molecule inhibitors.

In vivo model of the estrobolome hypothesis

We have previously shown for both irinotecan and NSAIDs that targeted Loop 1 GUS inhibitors effectively alleviate GI toxicity associated with these drugs (11, 13, 14). This is despite the fact that we have also shown that other GUS orthologs, including the mini-Loop 1 and FMN GUS enzymes, also reactivate these glucuronides (28). As such, we hypothesized that our inhibitor could similarly be used to prevent tumor growth in an HR+ breast cancer model.

In Fig. 6B, Fig. S5 and Tables S2 and S3. This result may be because only one inhibitor chemotype was examined, at a single dose, with one dosing regimen; at this point, however, we only have potent gut microbial GUS inhibitors that target Loop 1 GUS enzymes. Therefore, such a compound may be insufficient to block the reactivation of estrogens in the gut that potentially participate in tumor growth in this model.

Gut microbial β-glucuronidases reactivate estrogens

Figure 5. Inhibition by UNC10201652. A, in vitro inhibition of all 17 estrogen-reactivating enzymes identified in this study. UNC10201652 is only effective toward the Loop 1 GUS architecture. B, EC50 plot of WT and Δgus E. coli K-12 MG1655 cells incubated with UNC10201652. As expected, the knockout strain shows no activity with estrogen glucuronide substrates. WT cells exhibit sub-micromolar EC50 values. L1, Loop 1; mL1, mini-Loop 1. Data are presented as the average of 3 biological replicates ± SEM.
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**Discussion**

The estrobolome, first defined in 2011 as the aggregate of all enteric bacteria capable of metabolizing estrogen, is predicted to impact endogenous estrogen metabolism by modulating the enterohepatic circulation of estrogens, thus affecting plasma estrogen levels (5). This initial review and subsequent contributions to the literature suggest that gut microbial β-glucuronidase enzymes are especially important in total estrogen circulation (5–8). Further, it has been hypothesized that an estrobolome rich in deconjugating GUS enzymes would be a contributing factor in breast cancer.

Here, we probe the potential roles of gut microbial GUS proteins in the estrobolome by testing a panel of 35 human gut microbial GUS enzymes with two endogenous small-molecule estrogens: estradiol-17-glucuronide and estrone-3-glucuronide. Guided by the structures of 14 GUS enzymes, including novel structures reported here, we have identified characteristics of GUS enzymes that contribute to deconjugation. In general, specific members of three subtypes of GUS enzymes are able to process these glucuronides: Loop 1 GUS, mini-Loop 1 GUS, and FMN-binding GUS. In addition to defining key residues involved in estrogen glucuronide processing, we demonstrated that we can inhibit estrogen reactivation by Loop 1 GUS enzymes using a targeted microbial GUS inhibitor, UNC10201652. Despite these promising preliminary data, UNC10201652 alone was not able to impact tumor development in the PyMT mouse model of breast cancer.

We have previously demonstrated that GUS orthologs other than the Loop 1 enzymes are capable of processing irinotecan and NSAIDs, yet our targeted Loop 1 inhibitors effectively alleviate gut toxicity associated with these drugs (11, 13, 14, 28). As such, we hypothesized that the same paradigm could apply to inhibiting hormone reactivation in the GI and possibly impact tumor growth in the PyMT mouse model of HR+ breast cancer. However, we see no difference in mammary lesion size between mice treated with vehicle or UNC10201652. Lack of impact on breast tumor lesions may be because only a single inhibitor chemotype, at a single dose, with one dosing regimen was examined. This inhibition may not be sufficient to disrupt estrogen regeneration in the GI tract that has been hypothesized to be reabsorbed and trafficked to the developing tumors in breast tissues. Future studies could focus on variabilities in dosing and regimen to answer this question more completely. However, ultimately, the inability to combat breast tumor formation in the PyMT model is most likely the consequence of the complexity of breast tumor formation.

There is increasing evidence from epidemiological, animal, and in vitro studies that endogenous estrogens are involved in breast carcinogenesis (34, 35). As such, steroid hormone biosynthetic pathways have been under investigation for decades, and there is abundant information on their metabolism in humans. Cytochromes P450, UGTs, sulfotransferases, and catechol-O-methyltransferases are just a few of the major enzymatic families that metabolize estrone, for example (37–41).
Some of the major metabolites of estrone are highlighted in Fig. 7. It is possible that estrone and estradiol are first glucuronidated in the liver and, after being transported to the gut, are immediately acted on by GUS enzymes. These GUS enzymes then reactivate the parent estrogens and allow them to be further metabolized into methoxy-estrogens, semiquinones, quinones, etc. Such considerations might explain why targeting GUS has no impact on tumorigenesis in the PyMT model. However, it must be noted that sulfonation and hydroxylation play much larger roles in human metabolism than glucuronidation does. The action of sulfatase enzymes is perhaps more likely the preferred deconjugation step of estrogenic metabolites in the gut. Future studies will be required to evaluate the role of sulfatases in gut estrogen metabolism.

Further, the metabolites outlined in Fig. 7 do not include the incredible, diverse, and surprising amount of novel chemistry that occurs in the gastrointestinal tract. For example, the sterol scaffold–related bile acids are a good example of our growing understanding of the biotransformations that occur during mammalian-microbial symbiosis. Bile acids can be conjugated, deconjugated, hydrolyzed, epimerized, oxidized, methylated, etc., to play roles in absorption and digestion (42–45). Therefore, like bile acids, it may be that endogenous estrogens are transformed and repurposed for use in other areas of the body, like distal mucosal or receptor sites. We therefore propose that the estrobolome acts as an estrogen reservoir in the gut and is capable of creating estrogenic metabolites for local and nonlocal functions, rather than only simple reactivation. Future studies with other model systems and deeper bioanalytical investigations will be required to unravel how gut estrogen metabolism may affect hormone-dependent tumor growth in vivo. Taken together, these observations support the conclusion that the estrobolome’s effects on breast cancer development are likely complex and multivariant.

Conclusion

Guided by initial data with 35 gut microbial GUS enzymes and then by the subsequent detailed analysis of 14 GUS proteins, including the resolution of relevant novel crystal structures, we have pinpointed molecular characteristics that contribute to estrogen deconjugation. These are key initial data to validate the fact that gut microbial GUS enzymes are functional members of the estrobolome. Furthermore, we postulate that the gut may serve as a reservoir for estrogenic metabolites capable of acting locally and perhaps distantly in systemic homeostasis and the development of disease.

Experimental procedures

Gene synthesis, expression, and purification of GUSs

All genes were codon-optimized for heterologous expression in E. coli, synthesized by BioBasic, and incorporated into a pLIC-His vector via ligation-independent cloning, and resultant plasmids were transformed into BL21-G E. coli cells. Glycerol stocks were made from overnight cultures and snap-frozen and stored at −80 °C. Verification of successful transformation and sample integrity were determined by DNA sequencing.

Cultures of 100 ml of LB with ampicillin were inoculated with glycerol stock and incubated overnight at 37 °C with shaking at 225 rpm. For protein expression, 50 ml of the overnight culture, ~40 µl of Antifoam 204, and 750 µl of 2000× ampicillin were added to 1.5 liter of LB in a 2.5-liter Erlenmeyer flask and incubated at 37 °C at 225 rpm. At an optical density of ~0.6, the temperature was reduced to 18 °C, and the culture was induced with isopropyl β-D-galactopyranoside (100 µM) and incubated overnight with shaking at 225 rpm. Cultures were spun down in a Sorvall Instruments RC-3B centrifuge at 4500 × g for 25 min in 1-liter round, flat-bottom plastic bottles. Cultures were resuspended in LB and transferred to a 50-ml Falcon tube and spun down in a Thermo Scientific Sorvall ST 40R centrifuge.
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Centrifuge for 15 min at 5000 × g. Supernatant was discarded, and cell pellets were stored at −80 °C until purification.

Cell pellets were lysed in 30 ml of Nickel A buffer (20 mM KH₂PO₄, 500 mM NaCl, 50 mM imidazole (pH 7.4)) with DNase, lysozyme, and a Roche EDTA-free protease inhibitor tablet. The resultant cell slurry was sonicated on a Fisher sonic dismembrator model 500 twice with 1-s pulses for 1.5 min. The resultant lysate was subsequently spun down on a Beckman Coulter J2-HC centrifuge for 1 h at 17,000 rpm. The supernatant was subject to filtration with a 0.22-μm filter prior to purification.

Protein was first purified with an Aktaexpress FPLC (Amer- sham Bioscience) via a nickel-nitrilotriacetic acid column. Protein was eluted in one step using Nickel B buffer (20 mM Hepes, 50 mM NaCl, 50 mM imidazole (pH 7.4)). The eluent was then subject to size-exclusion chromatography on a HiLoad™ 16/60 Superdex 200 gel filtration column. Size exclusion buffer was utilized for elution (20 mM Hepes, 50 mM NaCl, pH 7.4). Fractions were collected, and an SDS-polyacrylamide gel was performed to assess purity and stability of the enzyme. Protein concentration was determined on an ND-1000 spectrophotometer, and then cultures were snap-frozen in liquid nitrogen and stored at −80 °C.

Site-directed mutagenesis of GUSs

All mutants were created via site-directed mutagenesis. Mutagenesis primers were synthesized by Integrated DNA Technologies. Mutant plasmids were sequenced by Eton Bioscience to confirm successful mutagenesis (Table S4).

SSN construction

The sequence similarity network diagram of GUS enzyme sequences was generated using the Enzyme Function Initiative-Enzyme Similarity Tool (EFI-EST) online tool (26). The sequences obtained from the GUS rubric were used in combination with the EFI-EST “FASTA” tool to create a sequence with 279 nodes. Each node represents sequences bearing ≥90% sequence identity to each other. A BLAST E-value of 1 × 10⁻²²⁰ was employed.

In vitro estrogen-processing coupled assay

E1-3G and E2-17G were purchased as solids (Toronto Research Chemicals) and resuspended in DMSO to a concentration of 10 mM. In vitro assays were conducted at 37 °C in a 50-μl total volume. Reactions consisted of 10 μl of assay buffer (50 mM HEPES, 50 mM NaCl, various pH), 10 μl of enzyme (various concentrations), and 30 μl of E1-3G or E2-17G (various concentrations) diluted in assay buffer. The pH of each reaction was chosen based on the optimal pH determined for each GUS with pNPG (28). Reactions were quenched at six time intervals with 50 μl of 25% trichloroacetic acid. After centrifugation at 13,000 × g for 10 min, the resultant supernatant was subjected to HPLC analysis. The concentration of E1-3G or E2-17G remaining at each time point was quantified on an Agilent 1260 Infinity II liquid chromatograph system. Samples were separated on an Agilent InfinityLab Poroshell 120 C18 column (4.6 × 100 mm, 2.7-μm particle size) at 38 °C. The flow rate was 0.9 ml/min, and the injection volume was 10 μl. LC conditions were set at 98% water with 0.1% formic acid (A) for 2 min and then ramped linearly over 10 min to 98% acetonitrile with 0.1% formic acid (B) and held until 14 min. At 15 min, the gradient was switched back to 100% A and allowed to re-equilibrate until 17 min. E1 and E2 were monitored at 280 nm. The concentration of E1 and E2 were determined from a standard curve (0–500 μM E1-3G/E2-17G in assay buffer). Resultant progress curves were fit by a custom linear regression analysis program in MATLAB. Initial velocities were then plotted against substrate concentration and fit with linear regression in Microsoft Excel to determine catalytic efficiency (kcat/Km). Control reactions were performed in which enzyme was substituted with buffer. Background hydrolysis was not observed at each pH tested. Reactions were performed in triplicate for each enzyme. Data were calculated from the average absorbance of three biological replicates ± S.E.

Crystallization and structure determination

Both structures were derived from crystals grown at 20 °C via the sitting-drop method in Hampton Research 3-well Midi Crystallization Plates (Swissci) by an Art Robbins Instruments Crystal Phoenix robot with the following drop conditions. For F. prausnitzii GUS, 100 nl of 10 mg/ml protein were added to 100 nl of 0.1 M BisTris-HCl, pH 8.5, and 1.8 M magnesium sulfate. For uncultured Clostridium sp. GUS, 100 nl of 12 mg/ml protein were added to 100 nl of 0.2 M calcium chloride, 0.1 M Tris-HCl, pH 8.5, and 20% (w/v) PEG 4000.

Crystal specimens were cryo-protected in the crystallization conditions as described above with the addition of 20% glycerol, and diffraction data were collected at 100 K on APS Beamline I19.
In vitro inhibition assay

Reactions consisted of 10 μl of GUS (15 nm final), 5 μl of inhibitor (1 μM final for inhibitor UNC10201652 and 10 μM final for all other inhibitors), 30 μl of estrone-3-glucuronide or estradiol-17-glucuronide (900 μM final), and 10 μl of assay buffer (25 mM NaCl, 25 mM HEPES, pH 7.4 final). Reactions were initiated by the addition of nPGP and then incubated for 1 h, after which the end-point absorbance was determined. Final percentage inhibition was determined via HPLC as described above.

In-cell inhibition assay

WT E. coli K-12 MG1655 was grown overnight in 10 ml of LB, and a 100-μl portion was subcultured the following morning in 5 ml of fresh LB. Cells were grown to an optical density of ~0.6 and used for the cell-based assay. Reactions were carried out in Costar 96-well black clear-bottom plates. Reaction volumes consisted of 90 μl of cells premixed with 700 μM estrone-3-glucuronide or estradiol-17-glucuronide and various concentrations of 10 μl of inhibitor. This reaction was incubated for 24 h at 37 °C with a low evaporation lid. Incubations were quenched by the addition of 50 μl of 0.2 M sodium carbonate. Absorbance values were measured at 410 nm in a BMG Labtech PHERAstar plate reader. Percentage inhibition and EC_{50} values were determined as described previously (29).

In fimo estrogen deconjugation and inhibition

All animal studies were approved by the University of North Carolina Institutional Animal Care and Use Committee, in accordance with the Care and Use of Laboratory Animals guidelines set by the National Institutes of Health. BALB/c mice were individually housed in specific pathogen-free conditions according to the guidelines set by the National Institutes of Health. PyMT males, a gift from the Jeffery Pollard laboratory, and FVB females were put in one cage for genotyping pups. Males are kept for continuous breeding with FVB females. PyMT females were used for UNC10201652 assays. Two groups of 11 mice each were individually housed in a specific pathogen-free vivarium maintained with a 12-h/12-h light/dark cycle, in specific pathogen-free conditions with sterile ventilator cages containing corn bedding, with ad libitum access to chow and water. Beginning at 4 weeks old, female PyMT mice were gavaged with UNC10201652 for a final concentration of 100 μl/20 μg/mouse (final DMSO 0.67%) every day except weekends for 9 weeks. On the last day, animals were deeply anesthetized with CO2 followed by manual cervical dislocation to collect samples for histology analysis and evaluation of hyperplasia and adenoma in breast, liver, lung, and kidney tissue.

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References


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