Mechanistic insight into digoxin inactivation by Eggerthella lenta augments our understanding of its pharmacokinetics

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The human gut microbiota plays a key role in pharmacology, yet the mechanisms responsible remain unclear, impeding efforts toward personalized medicine. We recently identified a cytochrome-encoding operon in the common gut Actinobacterium *Eggerthella lenta* that is transcriptionally activated by the cardiac drug digoxin. These genes represent a predictive microbial biomarker for the inactivation of digoxin. Gnotobiotic mouse experiments revealed that increased protein intake can limit microbial drug inactivation. Here, we present a biochemical rationale for how the proteins encoded by this operon might inactivate digoxin through substrate promiscuity. We discuss digoxin signaling in eukaryotic systems, and consider the possibility that endogenous digoxin-like molecules may have selected for microbial digoxin inactivation. Finally, we highlight the diverse contributions of gut microbes to drug metabolism, present a generalized approach to studying microbe-drug interactions, and argue that mechanistic studies will pave the way for the clinical application of this work.

Introduction

Dating back to the 1960s, scientists and clinicians have been presenting sporadic reports that suggest the trillions of microbes that colonize the human gastrointestinal tract (the gut microbiota) can influence the fate of therapeutics.[1-3] The emerging field of metagenomics, which employs an extensive array of experimental and computational techniques to study the aggregate genomes (the human microbiome) and metabolic activities of these microbial communities has now set the stage to delve deeper into the mechanisms responsible for microbial drug metabolism. This "metagenomic" view of pharmacology promises to elucidate novel biology, while also contributing to efforts at personalized or precision medicine.[4-8]

A seminal example of the value of such studies comes from work by Redinbo and colleagues.[9-11] Their research has shown that inhibition of microbial β-glucuronidase activity ameliorates the enteric side effects of the chemotherapeutic drug, irinotecan, and multiple non-steroidal anti-inflammatory drugs. This work is the first to leverage mechanistic insight about microbial drug metabolism toward directly affecting a clinical outcome, as demonstrated by their in vivo mouse experiments, which show dramatic reductions in toxicity.

We sought to attain a similar degree of mechanistic insight into the bacterial inactivation of the cardiac drug, digoxin, with the long-term goal of discovering new ways of predicting or manipulating microbial drug metabolism. Digoxin is a natural cardiac glycoside that is used to treat atrial fibrillation and chronic heart failure. Positive inotropic effects of digoxin are realized by inhibition of the Na^+/K^+ ATPase in cardiac myocytes, causing an efflux of Na^+ and a net increase in Ca^{2+} (see ref. 12).

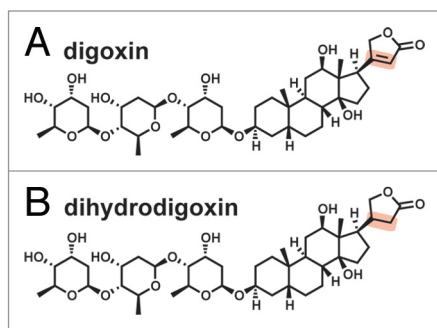Digoxin is particularly noteworthy to microbiologists, as it is well established that

Figure 1. Chemical structures of digoxin (**A**) and its cardioinactive metabolite, dihydrodigoxin (**B**). The double bond in the lactone ring (highlighted) becomes saturated, which reduces the affinity for its target, a Na^+/K^+ ATPase expressed in heart tissue.

Figure 2. Predicted structures or Cgr1 and Cgr2, and their proposed interaction. (**A**) Genome mining revealed structural and sequence homology between the Cgr1 protein and several structurally characterized members the NapC/NirT family of cytochrome c reductases such as the NrfH enzyme from the Gram-negative bacterium *Desulfovibrio vulgaris*. This protein anchors as a dimer in the cytoplasmic membrane and shuttles quinone (Q)-derived electrons to associated periplasmic nitrite reductases (purple and gray). (**B**) Cgr2 exhibits homology to FAD-binding fumarate reductases and may serve as the terminal electron reductase partner to Cgr1 by forming an complex with Cgr1 and receiving electrons from Cgr1 at the active site FAD redox cofactor. (**C**) The structural and electronic similarities between the unsaturated dicarboxylic acid of fumarate and the α,β-unsaturated lactone of digoxin suggests that digoxin is able to occupy the active site of Cgr2 and undergo reduction by the Cgr1/Cgr2 complex.

a subset of individuals receiving digoxin therapy excrete the inactive metabolite, dihydrodigoxin, in which the lactone ring is reduced[13,14] (**Fig. 1**). It has been over 40 years since Herrmann and Repke[15] first proposed that the saturation of the lactone ring of digoxin might be catalyzed by the gut microbiome, after demonstrating drug inactivation following ex vivo incubation with rat and human fecal samples. Lindenbaum and colleagues furthered the work by showing that there was an increase in the excretion of reduced metabolites following the administration of prolonged release digoxin formulations[16]; and that broad spectrum antimicrobial therapy blocked the formation of reduced digoxin metabolites, with a concomitant increase in the serum levels of the drug.[16] Both of these observations supported the hypothesis that the gut microbiota is responsible for digoxin inactivation. The final and key discovery of the Lindenbaum group was the isolation of *Eggerthella lenta* (originally classified as *Eubacterium lentum*), as the sole cultured gut bacterium capable of catalyzing the conversion to dihydrodigoxin in vitro.[17]

The isolation of *E. lenta* allowed us to frame our work around the following broad aims: (1) to identify the genes/gene products that encode digoxin inactivation capability; (2) to uncover the precise nature of the signals that control the expression of the digoxin inactivating genes; and (3) to determine if in vivo digoxin inactivation can be controlled by rational dietary interventions.
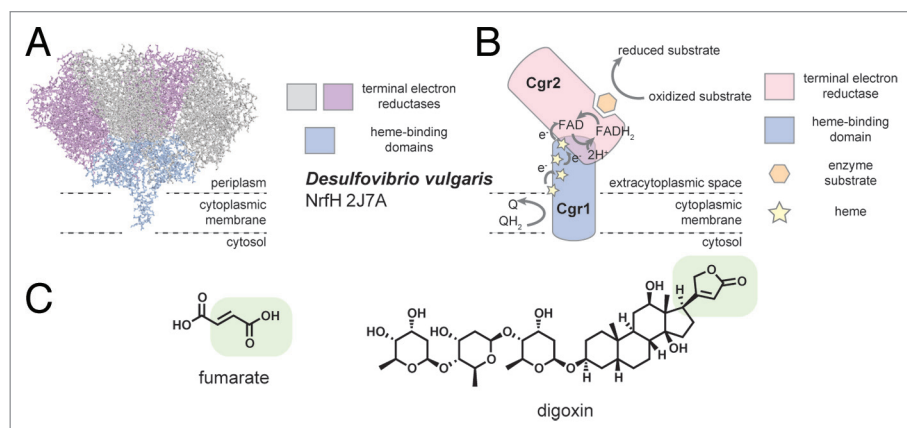
Mechanistic Insights into Bacterial Drug Inactivation

We began by using RNA-seq to identify *E. lenta* genes that are differentially expressed in the presence of digoxin. This resulted in the identification of a two-gene cytochrome-encoding operon that is significantly (>100 fold) upregulated in the presence of digoxin.[18] Comparative genomics supports the hypothesis that these genes encode the factors responsible for digoxin inactivation, as two *E. lenta* strains that lack the operon are unable to inactivate digoxin.[18] We now refer to these genes as the cardiac glycoside reductase (*cgr*) operon.

Based on sequence homology (PSI-BLAST)[19] and secondary structure predictions (HHPred),[20] the *cgr* operon is predicted to encode a protein homologous to the NapC/NirT family of cytochrome c reductases (Cgr1), as well as a protein related to fumarate reductase (Cgr2). Cytochromes from the NapC/NirT family, such as the Nrf enzyme of *Desulfovibrio vulgaris*[21] (**Fig. 2A**) are membrane-bound proteins that shuttle electrons from quinones to associated terminal electron reductase partner(s). As Cgr2 exhibits strong sequence and predicted structural

homology to FAD-binding fumarate reductase enzymes, we propose that it serves as the soluble reductase partner that interacts with the heme-binding domain of Cgr1, either in a transient or stable complex on the extracytoplasmic side of the membrane (**Fig. 2B**). Given the structural and electronic similarities between the α,β-unsaturated lactone of digoxin and the unsaturated carboxylic acid of fumarate (**Fig. 2C**), we hypothesize that digoxin and related cardiac glycosides can occupy the binding pocket of Cgr2 and undergo reduction by a similar mechanism. The use of digoxin as an alternative electron acceptor is also supported by the fact that reductases are generally induced by their substrate. Further biochemical characterization of the Cgr proteins is warranted. It will be particularly interesting to measure Cgr activity in the presence of related cardiac glycosides, as well as fumarate, to uncover the extent to which the enzyme processes these compounds and to confirm the natural and/or evolved role(s) for the proteins.

It may be useful to consider if the *cgr* operon has evolved to utilize cardiac glycosides, or if these compounds are cross-reacting with enzymes adapted to

a natural substrate of similar chemical structure, such as fumarate. Our initial structure-function analyses suggest the *cgr* operon is broadly responsive to compounds containing an α,β-unsaturated butyrolactone ring.[18] One possibility is that the prevalence of endogenous digitalis-like factors in mammals may have selected for the *cgr* operon.[22] Interestingly, these so-called "cardiotonic steroids" have also been shown to be present in the reduced state, and it has been hypothesized that these compounds may be attributable to the gut microbiota.[23] However, there is no obvious selective advantage at play, since we did not detect an increase in the in vitro growth rate or carrying capacity in the presence of digoxin, perhaps supporting the idea that this reduction may simply result from promiscuous enzyme activity, or only provide a fitness advantage under specific conditions. This potential in vivo selective pressure of digoxin on *E. lenta*, or lack thereof, might be better understood by studying the gut microbiota of patients or animal models receiving long-term digoxin therapy.

A Microbial Biomarker of Drug Pharmacokinetics

The Lindenbaum group attempted to correlate the presence and abundance of *E. lenta* in human fecal samples with digoxin inactivation but found that many individuals deemed "non-reducers" harbored strains of *E. lenta* in their feces.[17,24] We reasoned that the *cgr* operon might be a more suitable predictor of the drug inactivation phenotype, especially given the strain variation of *E. lenta* and the possibility that each individual might harbor multiple strains, as has been shown for other members of the gut microbiota.[25-27] Quantitative PCR of community DNA isolated from fecal samples from 20 healthy volunteers demonstrated a significant correlation between the "*cgr* ratio" (*cgr* abundance normalized by *E. lenta* 16S rDNA level) and ex vivo digoxin inactivation, discriminating low vs. high reducers with a sensitivity of 86%, specificity of 83%, and precision of 92%.[18] There was no

predictive value of the overall abundance of the *E. lenta* species.

This finding yields an intriguing scenario wherein clinical guidelines might be informed by the presence, abundance, and/or expression level of microbial genes known to play an important role in the metabolism of a given drug. In addition, clinicians might one day be able to rapidly stratify patient populations and identify individuals that are more likely to experience significant metabolism via their gut microbiota. If expanded to other drugs, this type of screening would almost certainly help manage clinical risk, and fill in some of the gaps that are seen with patient-to-patient variability with respect to drug responses in the clinic.

It is also noteworthy that digoxin, among other cardiac glycosides, has been implicated in several other signaling roles. Digoxin and ouabain were both recently shown to increase cholesterol synthesis by transcriptionally activating 3-hydroxy-3-methylglutaryl-coenzyme A reductase in human liver cells,[28] cardiac glycosides were shown to be inhibitors of HIF-1α—a hypoxia responsive transcription factor involved in tumor proliferation—resulting in decreased growth of tumor xenografts in mice,[29] and digoxin was identified as an inhibitor of RORγt transcription which blocks T_H17 differentiation, attenuating autoimmune disease.[30] Together, these findings point to a general role for this class of compounds in mediating a wide variety of signaling cascades and suggest that their metabolism by *E. lenta* may have much broader consequences than are currently appreciated.

Blocking Microbial Drug Metabolism via Dietary Interventions

While attempting to improve the laboratory growth of *E. lenta*, Sperry and Wilkins[31] discovered that growth of *E. lenta* requires the amino acid arginine, and it is likely that arginine serves as the main source of nitrogen and carbon for *E. lenta*. Dobkin et al.[17] reported that while arginine enhances growth it simultaneously inhibits digoxin inactivation. Thus, elevated levels of

arginine from dietary, host, or microbial sources might be exploited to prevent this undesirable microbial activity. To test this hypothesis, we colonized germ-free mice with *E. lenta* prior to digoxin administration. The animals were split between two otherwise identical diets: one completely lacking a protein source, and the other providing 20% kcal from protein (i.e., casein). Remarkably, we found that increasing dietary protein significantly elevates both serum and urinary digoxin levels, and that this only occurs in mice colonized with the type strain, which is capable of reducing digoxin.[18]

These results suggest that host diet might provide one avenue with which to tune the rate of microbial drug metabolism, and provide further evidence for the intimate links between nutritional status and our associated microbial communities.[32-34] Of course, the two diets tested in this study reflect dramatic changes to protein consumption, which are unlikely to occur in human patients. Additional work is necessary to determine the relative impacts of diets designed with protein sources containing high or low arginine concentrations (e.g., soy vs. animal protein, respectively), supplemented with pure arginine, or subjected to various methods of food processing.

These studies also prompt some additional questions—why and how does arginine block digoxin metabolism? RNA-seq and qRT-PCR revealed that *cgr* expression is significantly elevated in low arginine conditions relative to high arginine; however, it remains unclear how arginine represses the *cgr* operon and the degree to which this is sufficient to explain the observed decrease in digoxin inactivation. These questions might be elucidated through the computational analysis of transcription factor binding sites, a more in-depth analysis of the transcriptional responses to arginine, screening *E. lenta* genomic libraries for transcription factors, the use of tagged arginine (and digoxin) to isolate interacting proteins from cell lysates, or even mutagenesis of *E. lenta*. Successful heterologous expression of the *cgr* operon, and subsequent purification of the
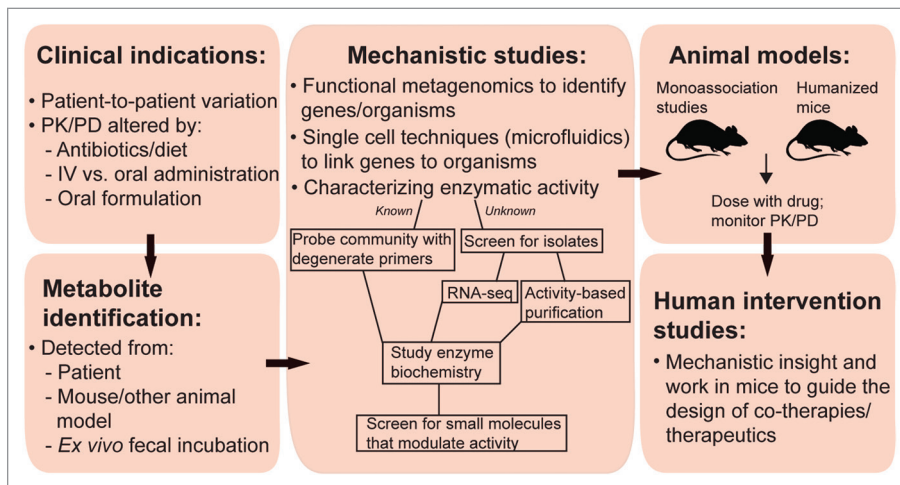
Figure 3. Approaches for studying the role of the microbiome in therapeutic drug metabolism. Initial evidence often comes from clinical data such as unexplained patient-to-patient variation in the response to therapeutics and/or altered pharmacokinetics (PK) and pharmacodynamics (PD) in response to antibiotic treatment, dietary intake, IV vs. oral routes of drug administration, or varying oral formulations to delay absorption. Drug metabolites may then be identified directly from patient samples, from mouse or other animal models, or after ex vivo incubation with fecal samples. Mechanistic insight can be garnered by combining a number of complementary methods: functional metagenomics, microfluidics, and screening gut microbial communities for relevant enzymatic activities. Determining the signals that activate genes, and biochemical characterization of the relevant gene products, will enrich findings from these studies. From there, animal models will determine the translational potential, while human intervention trials could be utilized to work out co-therapy strategies.
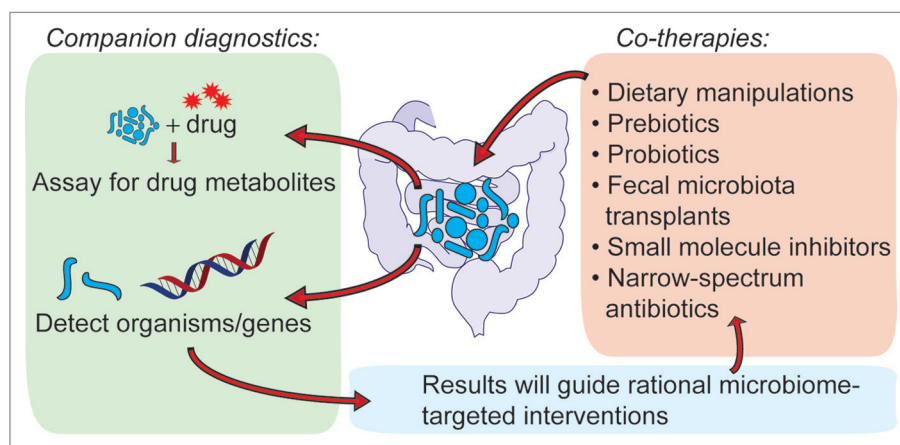

Figure 4. Integrating the gut microbiome into personalized or precision medicine. Rapid personalized clinical diagnostics may one day include ex vivo incubation of a patients' microbiota with potential drug therapy cocktails, or "microbiota typing" using culture-dependent or -independent methods (e.g., sequencing, quantitative PCR). Patient populations might be stratified based on the results from these tests, and appropriate co-therapies may be administered. Potential therapeutic strategies include dietary supplements, prebiotics, probiotics, fecal microbiota transplantation, small molecule modulators of microbial gene expression/enzyme activity, or antibiotics.

encoded proteins, would enable testing of any direct interactions with arginine. It will also be important to characterize the degree to which other members of the gut microbiota can promote or inhibit this activity through competition for arginine or other metabolic interactions. As has been demonstrated for the β-glucuronidase inhibitors, this type of mechanistic information might be used

to design more sophisticated methods of targeting *E. lenta* in vivo.

A Framework for Studying Microbial Drug Metabolism

The high degree of inter-individual variation in the abundance of the *cgr* operon provides a contrast to other well-studied microbial drug metabolism enzymes (e.g., β-glucuronidases and azoreductases), which are considered to be more widely distributed across multiple bacterial taxa and consistently found in the human gut microbiome.[8] Follow-up studies of healthy controls and cardiac patients will be necessary to determine the extent to which *cgr* abundance is predictive of in vivo digoxin pharmacokinetics and the degree to which this association is stable during the course of therapy. Human intervention studies might be designed to test the ability to limit microbial digoxin reduction by modifying dietary intake.

Our results provide the first example of a host-associated microbial operon that predicts drug inactivation, although Westman et al.[35] recently used an activity-based purification scheme to identify the enzyme complex responsible for the inactivation of the antineoplastic compound doxorubicin. An experimental and computational platform for the mechanistic dissection of microbial drug metabolism is now emerging (**Fig. 3**). This framework could be more broadly applied to drugs that impact the active members of the human gut microbiome,[36] in addition to the >40 known drugs subject to microbial modification.[37] A critical component to furthering this work will be to elucidate the molecular mechanisms responsible via functional metagenomics, single cell methods (e.g., flow cytometry and microfluidics), in-depth enzymatic characterization, and numerous other complementary approaches (**Fig. 3**). The insights gained from this work promise to aid in the rational design of companion diagnostics such as metabolite, gene, and organism screening, which will ultimately inform co-therapies aimed at modulating the microbiota in a clinically meaningful way (**Fig. 4**). These targeted therapies could provide an attractive alternative

to broad-spectrum antibiotics, which, although they have been shown to prevent microbial drug inactivation in patients[16] can have rapid[36] and long-lasting[38] impacts on the gut microbiome. Considered in light of recent links between anticancer treatment and the gut microbiome,[39,40] these studies emphasize that a comprehensive view of pharmacology must encompass the dynamic metabolic activities and structure of our associated microbial communities.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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