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TITLE
Capturing the complex interplay between drugs and the intestinal microbiome

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INTRODUCTION

Predicting drug interactions, disposition, and side effects is central to the practice of clinical pharmacology. Until recently, the human microbiome has been an underappreciated player in the dynamics of drug metabolism. It is now clear that humans are 'superorganisms' with about tenfold more microbial cells than human cells and harboring an immense diversity of microbial enzymes. Owing to the advent of new technologies, we are beginning to understand the human microbiome's impact on clinical pharmacology.

MAIN TEXT

The majority of the human microbiome, approximately 1 trillion cells, resides in the intestines. The intestinal microbiome is a complex community of microorganisms, typically containing hundreds of different species, many of which can be cultured in the laboratory (1). The composition of individuals' microbiomes are remarkably resilient given that they reside in a transient environment with a continual flux of variable food sources. Microbiome composition varies considerably from person-to-person, and inter-individual variation is often greater than day-to-day variation within the same individual. Moreover, an individual's microbiome composition can return to its original state after extreme perturbations such as oral antibiotics. This suggests that there are external forces selecting for a specific microbiome composition, such an individual's immune system.

The human microbiome is a complex system of interacting microorganisms coupled to another complex system, the human. For this reason, it is perhaps unsurprising that microbiome composition has been associated with a wide array of ailments, including arthritis, asthma, autism, behavior, obesity, and differing treatment outcomes. Disentangling correlation from causation is a common challenge in human microbiome

studies, but new technologies are helping to elucidate the mechanistic basis underlying the microbiome's effects. Recent work is moving toward building a framework of microbiome function within the host, with the goals of controlling and manipulating the microbiome for the benefit of human health.

The intestinal microbiome partakes in a multi-way interaction with its host and their food or drug intake. There are three main ways in which drugs interplay with the intestinal microbiome (Fig. 1a):

1. Drugs may stimulate or inhibit the growth of a subset of microorganisms.

2. Drugs can be transformed or catabolized by members of the intestinal microbiome.

3. Drugs, or their byproducts, can elicit a response from the microbiome, such as production of new compounds.

In this article we describe modern methods for studying these three processes with microbiome samples. While these methods are broadly applicable, we focus on green tea (GT) because it is a mixture of natural products with hallmarks of complex interactions with the intestinal microbiome (2). Similar methods could be applied to study other natural products, drugs, or drug-drug interactions.

Microbiome surveys to screen for drug effects

Many drugs have effects on microbiome composition even though they are not widely assumed to have antimicrobial properties. The activity of non-antibiotic drugs is often species specific (3), and may be difficult to determine given the large number of microorganisms that inhabit human intestine. Culture-based assays serve as models for confirming a drug's ability to modulate membership in the intestinal microbiome. For example, green tea is well known for its antimicrobial properties and health benefits, which

are attributed to the catabolization of green tea polyphenols by the gut microbiome (2).

Different concentrations of green tea can inhibit the growth of microorganisms that are

well-known for their prevalence in the gut. Using *in vitro* techniques, we have found that

Escherichia coli is inhibited on media containing a concentration equivalent to tea brewed

with 1 tea bag (~0.5 g/L catechins), while 1.5 tea bags are required to inhibit the growth of

Enterococcus faecium. Nevertheless, culture-based assays are laborious and limited by the

fact that we do not yet know how to culture many microorganisms from the human

intestine (1).

The most common approach for studying the intestinal microbiome is to perform

sequencing of the 16S ribosomal RNA (rRNA) gene of fecal samples before and after

treatment with a drug (Fig. 1b). Sequencing the 16S rRNA has become popular due to the

gene's ubiquity across all prokaryotes, ease of selective amplification with PCR primers

targeting conserved regions, and variable regions that may provide genus-level resolution.

Many companies now offer services to amplify and sequence the 16S rRNA gene from

thousands of samples in microbiome studies. This allows comparison of microbiome

samples originating from large cohorts across many timepoints. The resulting sequences can

be used to identify many of the organisms that are present, compare diversity across

samples, or construct a phylogenetic tree depicting the evolutionary relationships among

microorganisms. For example, 16S rRNA sequencing has been applied to study i) the shift in

intestinal microbiomes before and after drinking green tea (4) and ii) the impact of

polybrominated diphenyl ethers, persistent environmental contaminants, on the regulation

of primary bile acids metabolized by the mouse gut microbiome (5).

While 16S rRNA sequencing has a relatively low cost per sample, there are many challenges associated with processing and interpreting the immense number of resulting DNA sequences. As is common for observational studies, it is often impossible to distinguish correlation from causation in microbiome surveys. Furthermore, experimental issues (e.g., PCR artifacts and sequencing errors) can confound the analysis of 16S rRNA amplicon sequences. The 16S rRNA gene is also an imperfect marker of microbiome composition, as it does not offer species-level resolution and ignores many microorganisms (e.g., eukaryotes and viruses). For this reason, metagenome sequencing has become increasingly popular (Fig. 1b). Although it is more costly per sample, metagenomics provides the ability to investigate microbial functions and even assemble some genomes without culturing.

Metabolomics to pinpoint drug-microbiome interactions

While microbiome surveys have implicated the microbiome in human health, metabolomics holds the potential to shed further light on its role in the host. Metabolomics involves the use of a mass spectrometer to identify compounds according to their mass-to-charge ratio (m/z) in cell-free supernatant. Untargeted metabolomics can detect compounds at very small abundances, making it possible to screen for transformations that are mediated by members of the intestinal microbiome (Fig. 1c). These transformations may cause off-target effects or alter the potency of drugs in individuals with specific microbiomes. For example, the bioactive compounds in green tea are known to undergo a variety of transformations into metabolic derivatives with largely unknown effects (2). In this manner, the intestinal microbiome may play a role in promoting or disrupting the beneficial effects of drugs and natural products.

Untargeted metabolomics studies generally collect mass spectra on a variety of samples from different conditions. For example, we have applied metabolomics to study the exometabolome of common intestinal microorganisms in monoculture and co-culture during growth in the presence or absence of green tea (Fig. 2a). Using this approach, we have observed the secretion of a number of compounds by *E. coli* that are induced only in the presence of green tea (Fig. 2b). Tandem mass spectrometry can be used to fragment specific compounds of interest within the sample. In conjunction with the initial molecular weight (MS1), the secondary fragmentation spectrum (MS2) can be compared to extensive databases of known MS2 spectra to identify molecules of interest, such as caffeine in green tea (Fig. 2c). This data can be used in conjunction with molecular networking to identify microbially-mediated transformations to drugs or natural products.

However, untargeted metabolomics of the bacterial exometabolome is not without drawbacks. In addition to the inability to quantify many compounds, mass spectra are rife with low-intensity peaks from unknown compounds (i.e., noise), and may be missing peaks from many present compounds for a wide variety of reasons. Notwithstanding these limitations, it is possible to use the entire MS1 spectrum to compare samples with ordination techniques such as principle coordinates analysis (PCoA). Many replicates are typically used to verify that inter-sample differences are greater than variability from experimental sources. This approach allows the reduction of complex mass spectra with many unknowns into a manageable framework for comparing samples across treatment conditions (Fig. 2d), and it may reveal the underlying compounds that are important for further investigation. Ordination techniques can be used in conjunction with dose gradients to uncover changes in the exometabolome that are elicited in response to increasing levels of drugs or natural products.

Conclusions

The impact of the human microbiome on clinical pharmacology has only recently been widely recognized. It is now commonplace for researchers to investigate the microbiome's influence on drug interactions, disposition, and side effects. Yet, it remains challenging to determine the functional basis behind the microbiome's role due to the abundance of interactions among members of the microbiome, their human host, and a person's food or drug intake. Although the microbiome is indirectly considered during clinical trials, they are unlikely to capture the full variability among human microbiomes or the breadth of potential drug interactions. Experiments in laboratory (e.g., *in vitro*) systems offer a means of studying these interactions in a controlled setting, albeit one removed from the human host. New technologies continue to emerge that enable clinical pharmacologists to probe these interactions in high-throughput. Although few well-characterized examples currently exist of the complex interplay between drugs and the intestinal microbiome, we are optimistic that many will emerge in the future as the importance of the microbiome becomes increasingly clear.

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FIGURE LEGENDS

Figure 1. Schematic of experimental designs for capturing the interplay between drugs and the intestinal microbiome. (**a**) Drugs can change the microbiome's composition, and the microbiome can transform or respond to drugs. (**b**) Microbiomics experimental pipelines typically involve extracting DNA from samples and amplifying marker gene sequences before performing reference-free (e.g., clustering) or referenced-based (e.g., classification) analyses. (**c**) Metabolomics studies of the exometabolome generally involve processing cell-free extracts before performing tandem mass spectrometry with or without prior separation by chromatography. The resulting MS1/MS2 data can be used to identify compounds and generate molecular networks that inform drug transformations mediated by the microbiome. For example, the red MS1 peak may result in an MS2 spectra that allows it to be related to other peaks through a molecular network.

Figure 2. Studying the exometabolome of gut bacterial strains and how their interactions change in response to green tea (GT). (**a**) Monocultures and co-cultures of *Escherichia coli* and *Enterococcus faecium* grown in the presence and absence of green tea. A negative control is used for background subtraction in subsequent steps. (**b**) Mass spectra of the exometabolome of *E. coli* grown in the presence of green tea with removal of m/z features found in the medium. Peaks are colored based on whether they were found in the green tea extract alone (black), *E.coli* grown on the medium without green tea (orange), or found only when grown in the presence of green tea (green). (**c**) Molecular structure of caffeine and MS2 spectrum of the precursor ion (top) compared to a caffeine standard (bottom). (**d**) Principle coordinates analysis (PCoA) of captured exometabolomes of two gut microbiota strains (*E. coli* and *E. faecium*) in replicate monocultures (circles and squares) and co-cultures (triangles) grown on the medium alone (orange) or medium supplemented with green tea extract (green). All samples have peaks in the medium and green tea background subtracted before analysis. Samples cluster according to the growth medium and strains, revealing differences in the exometabolome elicited by growth in the presence of green tea.