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Minireview

Exploring Drug Metabolism by the Gut Microbiota: Modes of Metabolism and Experimental Approaches

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ABSTRACT

Increasing evidence uncovers the involvement of gut microbiota in the metabolism of numerous pharmaceutical drugs. The human gut microbiome harbors 10–100 trillion symbiotic gut microbial bacteria that use drugs as substrates for enzymatic processes to alter host metabolism. Thus, microbiota-mediated drug metabolism can change the conventional drug action course and cause inter-individual differences in efficacy and toxicity, making it vital for drug discovery and development. This review focuses on drug biotransformation pathways and discusses different models for evaluating the role of gut microbiota in drug metabolism.

SIGNIFICANCE STATEMENT

This review emphasizes the importance of gut microbiota and different modes of drug metabolism mediated by them. It provides information on in vivo, in vitro, ex vivo, in silico and multi-omics approaches for identifying the role of gut microbiota in metabolism. Further, it highlights the significance of gut microbiota-mediated metabolism in the process of new drug discovery and development as a rationale for safe and efficacious drug therapy.

1. Introduction

Metabolism is the aggregate of all the chemical processes that occur in the body, and it comprises of anabolism and catabolism by enzymatic conversion of one chemical entity to another (Ritter et al., 2018). Drug metabolism involves an enzyme-catalyzed biochemical process that transforms lipophilic drugs into more polar and readily excretable metabolites, leading to the termination or alteration of the drugs biologic activity (Katzung, 2012). Drug metabolizing enzymes are present in abundance in the liver and play a crucial role in the metabolism of xenobiotics. They are also present in other sites, such as the kidneys, mucosa of the gastrointestinal tract, lungs, brain, and skin but contribute less to the drug metabolism (Krishna and Klotz, 1994). In addition to the metabolic enzymes present in host cells, similar enzymes produced by intestinal microorganisms are implicated in the metabolism of several drug compounds. Gut microbiota is the multitude of bacteria, archaea, eukarya, and viruses colonizing the adult human gastrointestinal tract with an approximate count of 100 trillion, that outnumbers the microbial count associated with body surfaces and is nearly 10 times greater than the total number of somatic and germ cells in the human body (Bäckhed et al., 2005). The collective genome of gut microbiota is termed microbiome and is approximately 100 times the number of genes compared with the human genome (Gill et al., 2006). Moreover, recent estimates show that the number of human cells ($3*10^{13}$) and bacterial cells ($3.8*10^{13}$) in the human body is of the same order, and the total mass occupied by the bacterial cells accounts for about 0.2 Kg (Sender et al., 2016).

Research in the field of metabolic capabilities of gut microbes has expanded concerning their effect on the efficacy and toxicity of drugs (Sousa et al., 2008). Co-evolving with the host, the microbiota is now viewed as a virtual organ with properties worthy of being combined with the host physiology (Evans et al., 2013). The United States National Insitutes of Health started an initiative, the Human Microbiome Project, to understand the gamut of human genetics and physiologic differentiation, the microbiome, and all aspects that affect the distribution transformation of the microorganisms in the body. It also involves the study of microflora related to human health and diseases. Another well-known European Union project on Metagenomics of the Human Intestinal Tract (MetaHIT) targets the gut microbiota in human health and associated metabolic activities of the microorganisms. Hence, the Human Microbiome Project and MetaHIT project are logical extensions of the Human Genome Project to characterize microbial populations colonizing healthy individuals (Turnbaugh et al., 2007; European Commission, n.d.). This review discusses gut microbes, their role in drug metabolism, and the experimental approaches used to elucidate them.

2. Gut Microbiota: Composition and Physiologic Functions

The fetal gut was previously considered sterile, but recent studies have indicated that early microbial exposure begins in utero as demonstrated by the distinct low diversity microbial compositions found in the placenta and meconium (Rautava et al., 2012; Nuriel-Ohayon et al., 2016). By the age of 2-5 years, gut microbiota in a child resembles that of an adult concerning diversity and composition. The gut microbial composition is shaped by the mode of delivery, type of infant feeding, gestational age,

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ABBREVIATIONS: Cgr, cardiac glycoside reductase; HFA, human flora-associated; H. pylori, Helicobacter pylori; MetaHIT, Metagenomics of the Human Intestinal Tract; PBPK, physiologically based pharmacokinetic modeling and simulation; p-cresol, para-cresol; SHIME, simulated human intestinal microbial ecosystem; SN-38G, SN-38 glucuronide.

antibiotic use, environment, lifestyle, and host genetics (Rodríguez et al., 2015). The upper gut has an antimicrobial climate due to gastric acid and bile salts and the slow rate of movement of intestinal contents, thus making the large intestine the prime site for microbial colonization (Macfarlane and Macfarlane, 2009). Gram-positive *Firmicutes* and gram-negative *Bacteroidetes* constitute the dominant bacterial phyla found in the gut, whereas methanogenic archaea, eukaryotes like yeasts and viruses, *Actinobacteria*, *Proteobacteria*, *Fusobacteria,* and *Verrucomicrobia* are the minor bacterial phyla that are identified in the gut (Eckburg et al., 2005; Lozupone et al., 2012). Disruption of healthy gut microbiota (dysbiosis) has been implicated in the pathogenesis of various inflammatory and metabolic disorders; nevertheless, healthy gut microbiota plays an essential role in human physiology by carrying out numerous metabolic functions, such as fermentation of indigestible carbohydrates, biosynthesis of vitamin K and vitamin B_{12}, production of hormonal mediators and neurotransmitters, metabolism of bile salts and xenobiotics. Microbes also perform protective functions like providing resistance to colonization by pathobionts, modulating intestinal barrier functions, and promoting immune homeostasis (LeBlanc et al., 2013; Natividad and Verdu, 2013; Bäumler and Sperandio, 2016; Pickard et al., 2017; Molinero et al., 2019).

3. Drug Metabolism by the Gut Microbiota

An emerging need to consider drug metabolism by gut microbiota as a crucial factor in drug discovery and development has been revealed. Drugs metabolized by gut microbiota may cause inter-individual differences in the drug response. Moreover, drug administration by diverse routes can encounter metabolism by gut microbes; for example, orally administered drugs remain unabsorbed in the upper gut. Instead, the intestinal microbes act upon a modified-release preparation that reaches the large intestine for their metabolism. Drugs that bypass the absorption process can still reach the gut microbiota through biliary excretion and undergo metabolism. In addition, rectally administered drugs are prone to microbial metabolism due to their proximity to the gut microbes (Sousa et al., 2008). The potpourri of microbial species metabolize many drugs leading to altered bioavailability, toxicity, and adverse drug reactions affecting therapeutic efficacy referred to as microbiome-derived metabolism.

However, poor attention is given to understanding the pharmacokinetics of drugs affected by microbiome-derived metabolism (Javdan et al., 2020). The potential benefit of microbiome-derived metabolism is the activation of certain prodrugs, such as the conversion of azo drugs prontosil and neoprontosil to an active sulphanilamide moiety (Gingell et al., 1969). Oral co-administration of anti-viral drug sorivudine and anti-cancer drug 5-fluorouracil was the cause of acute deaths in eighteen patients. This lethality resulted from soaring levels of 5-fluorouracil due to the inhibition of hepatic enzyme-dihydropyrimidine dehydrogenase responsible for the metabolism of 5-fluorouracil by (E)-5-(2-bromovinyl) uracil, a metabolite of sorivudine produced by gut microbiota (Okuda et al., 1998).

4. Modes of Drug Metabolism by the Gut Microbiota

Gut microbiota can cause drug metabolism in several ways, including direct interaction with the drug or indirect action by intermeddling with the host metabolism. Fig. 1 depicts the different modes of drug metabolism by gut microbiota.

4.1 The Direct Effect of Gut Microbiota on Drug Metabolism

The gut microbiota expresses an extensive array of drug-metabolizing enzymes like oxidoreductases, hydrolases, and lyases (Koppel et al., 2017). The activity of these enzymes leads to alteration of bioactivity of the drug, such as activation, deactivation or reactivation of drug, or conversion of the drug to a toxic metabolite (Wilkinson et al., 2018; Hitchings and Kelly, 2019).

4.1.1 Activation of Drug. A prodrug is an inactive drug that converts into an active form after metabolism. Although the liver performs most of the metabolic processes, the enzymes produced by the gut microbiota also actuate the conversion of the prodrug to its active form.

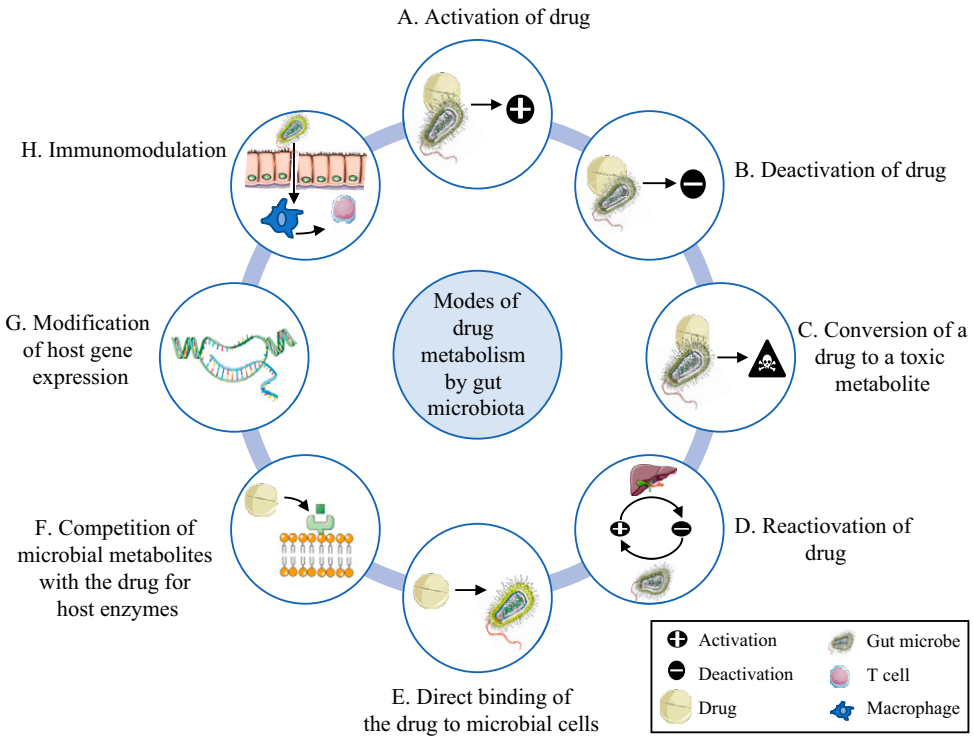

Fig. 1. The gut microbiota can perform metabolism of drugs by different modes of action: (A) activation of drug which includes conversion of prodrug to its active form; (B) deactivation of drug which results in loss in therapeutic efficacy of drug by inactivating it; (C) conversion of drug to a toxic metabolite leading to an adverse drug reaction; (D) reactivation of drug through enterohepatic recycling; (D) direct binding of drug to microbial cells due to adhesive proteins on the microbial cell surface; (F) competitive binding of microbial metabolite with the drug for host enzyme; (G) modification of the host gene expression and altered expression of crucial genes necessary for drug metabolism; (H) immunomodulation or translocation of microbes that stimulates differentiation of immune cells and develop autoimmunity.

For instance, prodrugs protonsil and neoprotonsil are transformed into the active sulfanilamide moiety by azoreductases, a gut microbial enzyme (Gingell et al., 1969). Substantial conversion of the neoprotonsil to sulfanilamide occurs in the gut, as evidenced by the excretion of a significant percentage of unchanged drug in bile after its intraperitoneal injection. Further, antibiotic-treated rats had less sulfanilamide excretion, implicating the role of gut microbial enzymes in azo-reduction of the drugs (Gingell et al., 1971). Azoreductases cleave azo bond in sulfasalazine, balsalazide, and olsalazine, releasing sulfapyridine and 5- amino salicylic acid. 5-Amino salicylic acid is an active moiety responsible for anti-inflammatory activity and used in the treatment of ulcerative colitis (Peppercorn and Goldman, 1972; Crouwel et al., 2021). Although probiotic treatment increased the azoreductase activity and plasma concentration, it failed to affect the pharmacokinetic parameters of sulfasalazine (Lee et al., 2012). Thioguanine and mercaptopurine are used in treatment of lymphoblastic leukemia as immunomodulating agents. E. *coli* strain DH5α in the gut microbiota metabolizes thioguanine and mercaptopurine to 6-thioguanine nucleotide by hypoxanthine phosphoribosyl transferase (Movva et al., 2016). The immunosuppressive action occurs due to interaction between DNA and phosphorylated 6-thioguanine nucleotide, an active metabolite of thiopurines, during replication (de Boer et al., 2018; Crouwel et al., 2021). In addition, colonic microbiota improved the chronic colitis by thioguanine even in the absence of host-mediated conversion of Thioguanine to 6-thioguanine nucleotide by hypoxanthine phosphoribosyl transferase, suggesting that the local gut microbiota mediated transformation can help in targeted therapy for Crohn's and ulcerative colitis (Oancea et al., 2017). Another interesting recent example is the Chinese herbal medicine, berberine, which undergoes drug activation upon conversion to oxyberberine by gut microbiota that helps treat gastric colitis (Li et al., 2020).

4.1.2 Deactivation of Drug. The active form of the drug can lose its therapeutic efficacy due to the deactivating action of gut microbial enzymes. Lindenbaum et al. performed an experiment in volunteers which showed that the formation of dihydrodigoxin, a reduced inactive metabolite of cardiac glycoside digoxin, was subject-dependent (Lindenbaum et al., 1981a). Further experimentation in volunteers displaying substantial levels of reduced metabolites showed that co-administration of erythromycin reduces the excretion of the dihydrodigoxin (Lindenbaum et al., 1981b). After examining hundreds of microbial isolates, *Eggerthelalenta*, a common anaerobic inhabitant of the gut, was the sole microbe responsible for this type of reduction. However, the mere presence of this microorganism in the gut cannot guarantee the inactivation of digoxin to dihydrodigoxin in in vivo (Dobkin et al., 1982; Saha et al., 1983). An investigation later showed that a two-gene cardiac glycoside reductase (*cgr*) *1* and *cgr2* operon was present in the strain of *E. lenta*, reducing digoxin, and their expression was necessary for the reduction reaction. Digoxin upregulates the *cgr* operon, whereas arginine inhibits it. Arginine repressed the expression of cgr operon, thus inhibiting digoxin reduction, which is explicated by higher digoxin levels in the serum and urine of gnotobiotic mice, and these mice are colonized with the digoxin-reducing *E. lenta* strain on a high-protein diet. The serum and urine levels of digoxin in mice colonized by non-reducing strains remain unaffected. This was a unique case in which dietary intervention reversed the metabolism by gut microbes (Haiser et al., 2013).

4.1.3 Conversion of a Drug to a Toxic Metabolite. Metabolizing enzymes produced by gut microbiota can form toxic intermediates, which, although rare, can lead to adverse drug reactions. For example, a study was performed on rats to investigate the involvement of gut microbiota in the metabolism of a hypnotic drug-nitrazepam and its teratogenic effect. Administration of large doses to pregnant rats displayed teratogenic effects. Even though nitrazepam was co-metabolized by liver and gut microbiota, antibiotic treatment sharply decreased the excretion of reduced metabolites and diminished the fetal aberrations (Takeno and Sakai, 1991). The investigation later concludes that nitrazepam-related teratogenicity depended on the reduction step, which converted nitrazepam to 7-aminonitrazepam. The reduction was catalyzed by an enzyme, nitroreductase, expressed by the gut bacterium *Clostridium leptum* (Rafii et al., 1997). There is unclear evidence about the role of gut microbiota in cisplatin-induced liver toxicity, but co-administration of antibiotics and cisplatin resulted in the reduction of hepatoxicity, thus confirming the role of the gut microbiota in cisplatin-induced liver toxicity (Gong et al., 2021).

4.1.4 Reactivation of Drug. Enterohepatic recycling includes biliary excretion followed by intestinal reabsorption of the drug or its metabolites. In biliary excretion, drugs and metabolites are secreted and concentrated in the bile. Later, they are released from the gall bladder and drained into the intestine, where reabsorption occurs. Fig. 2 represents the enterohepatic recycling of drugs, including those released by the de-conjugating action of bacterial enzymes. Reabsorption may convert the inactive form of the drug or its metabolite to an active form (Dobrinska, 1989). Enterohepatic cycling causes the plasma drug concentration-time profile to rise after specific time intervals, termed the "multiple peaking" phenomenon, and it significantly affects

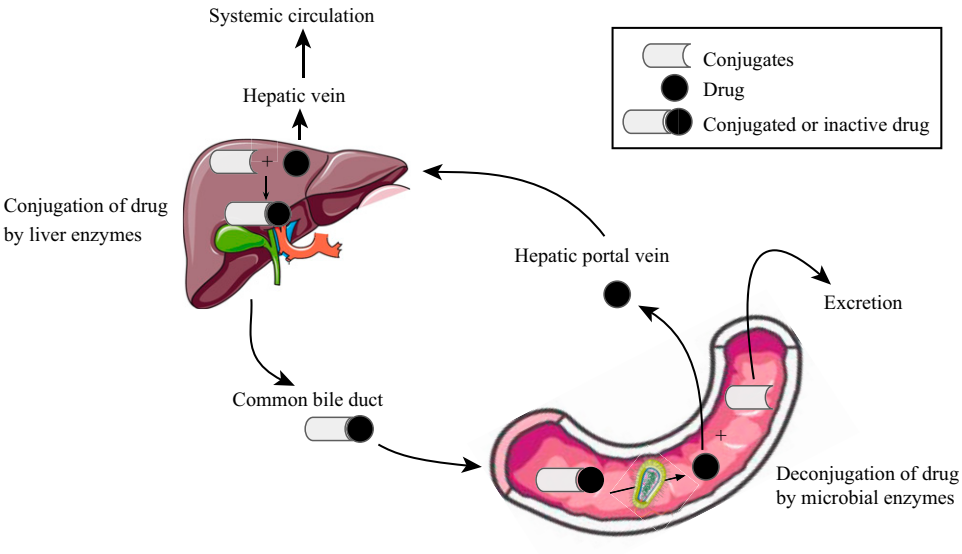

Fig. 2. Reactivation of drug by gut microbiota induced enterohepatic cycling. Enterohepatic recycling includes two processes: 1) biliary excretion followed by 2) intestinal absorption of drug or its metabolite. In the former part, drugs and metabolites which are formed due to action of liver enzymes are the inactivated or conjugated form of the drug and are secreted and concentrated in the bile; in the later part, the drug or the metabolites are released from the gall bladder and drained into the intestine where the action of microbial enzymes converts the inactivated or conjugated form of a drug to its active or deconjugated form. This activated or deconjugated form of a drug can be absorbed through the hepatic portal vein and get back into systemic circulation.

pharmacokinetic parameters (Malik et al., 2016). One of the plausible causes of intestinal injury is non-steroidal anti-inflammatory drugs, such as diclofenac, in which one of its metabolites, diclofenac-1-β-O-acyl glucuronide, cleaves to diclofenac by the action of bacterial β-glucuronidase in the gut. Recurrent exposure of enterocytes to the resulting product of glucuronide hydrolysis (aglycone) could cause local tissue injury and marks the beginning of enteropathy (Saitta et al., 2014). LoGuidice et al. undertook a study in mouse models of non-steroidal anti-inflammatory drug enteropathy and demonstrated that administration of selective bacterial β-glucuronidase inhibitors reduces the exposure of intestinal mucosa to the aglycone and provided protection against diclofenac-induced enteropathy (LoGuidice et al., 2012). Anti-cancer drug irinotecan (CPT-11) is an excellent example of the reactivation of drugs caused by gut microbial enzymes. SN-38 glucuronide (SN-38) is the prodrug irinotecan's active metabolite, glucuronidated in the liver to SN-38G and secreted in the bile. After reaching the intestine, β-glucuronidases of gut bacteria reconvert SN-38G to SN-38, responsible for the irinotecan-induced severe diarrhea (Takasuna et al., 1996).

4.2 Effect on Drug Metabolism via Host Function Modulation

4.2.1 Binding of the Drug to Microbial Cells. Bacterial cells express adhesive proteins called adhesins and are responsible for binding to the host cells. Adhesins are also known to interact and directly bind to the drug molecules, decreasing microbial binding to host cells and altering drug pharmacokinetics. Parkinson's disease is characterized by the loss of dopaminergic neurons in the substantia nigra and the presence of intracellular aggregates of α-synuclein (Poewe et al., 2017). Levodopa, an immediate precursor of dopamine, is given to patients to relieve the symptoms. A small percentage of levodopa that escapes peripheral decarboxylation crosses the blood-brain barrier and is taken up by functioning dopaminergic neurons. Niehues et al. designed an in vitro study to test the hypothesis that the presence of Helicobacter (H.) pylori in patients with Parkinson's disease treated orally with levodopa can affect the plasma level of levodopa (Pierantozzi et al., 2001; Narożańska et al., 2014). The study revealed that adsorption of levodopa to bacteria surface is possible, and such interactions with levodopa result in the blocking of the proteins with adhesive properties, causing a lowered adhesion of H. pylori to gastric epithelial cells (Niehues and Hensel, 2009). The interaction between levodopa and H. pylori affects the pharmacokinetics of levodopa, i.e., it reduces the absorption and lowers plasma levels. Direct chemical transformation of levodopa by intestinal microbes has also been reported in patients with parkinsonism and rat models (Sandler et al., 1969; Goldin et al., 1973; Niehues and Hensel, 2009).

4.2.2 Alteration in the Absorption of Drugs Caused by Gut Microbiota. Gut microbiota and their metabolites can change the local microenvironment in the human gut, altering the absorption of drugs (Enright et al., 2016). Zou et al. screened 136 drug excipients for their inhibitory potential of the intestinal transporter, OATP2B1. Out of the 24 potent inhibitors of the OATP2B1 identified, 8 of them are azo dyes. Administration of FD&C Red No. 40 in mice, a red azo dye, decreased plasma levels of fexofenadine, a substrate for Oatp2B1, due to Oatp2B1 inhibition. However, the gut microbial isolates from diverse unrelated healthy humans metabolized azo dye to inactive metabolites that lack OATP2B1 inhibition. Thus, altering drug absorption by the gut microbiome proved beneficial in this case (Zou et al., 2020).

4.2.3 Competition of Microbial Metabolites with the Drug or Host Metabolites for Host Metabolic Enzymes. Competition for active sites of host metabolic enzymes among the drug or its metabolites and gut microbial metabolites varies the drug's therapeutic efficacy. Metabolism of acetaminophen occurs by three means: a) conjugation with glucuronide catalyzed by UDP-glucuronosyltransferases, b) sulfate conjugation catalyzed by sulfotransferases, and c) oxidative metabolism through the cytochrome P450 enzymes (McGill and Jaeschke, 2013). The oxidative metabolic product formed primarily by the CYP2E1, N-acetyl-p-benzoquinone imine, is highly reactive in nature as it covalently binds to the thiol groups on proteins causing cellular oxidative stress (Vermeulen et al., 1992). As a result, it rapidly conjugates with intracellular glutathione, producing a non-toxic glutathione conjugate and excreted as cysteine and mercapturic acid conjugates (Larson, 2007). Para-cresol (p-cresol) is a product of tyrosine fermentation produced by anaerobic gut microbes belonging to Coriobacteriaceae and Clostridium clusters XI and XIVa (Smith and Macfarlane, 1997; Saito et al., 2018). Clayton et al. used acetaminophen to test the applicability of pharmacometabonomic study in man. The research done on 99 healthy male volunteers inferred that the pre-dose urine level of p-cresol sulfate was inversely related to the post-dose ratio of acetaminophen sulfate to acetaminophen glucuronide. Thus, competition between p-cresol and acetaminophen for the active site of sulfotransferase reduces the effective systemic capacity of the individual to produce acetaminophen sulfate (Clayton et al., 2009). Moreover, computational chemistry has also established that cresols compete with acetaminophen for aryl sulfotransferase active site (DiGiovanni et al., 2013). This competition may lead to a toxic build-up of acetaminophen in case of acetaminophen overdose and result in hepatotoxicity. Another study deduced that the total excretion of acetaminophen conjugates remains constant to a great extent in germ-free and conventionally housed mice, i.e., lowered sulfonation was compensated by improved glucuronidation (Possamai et al., 2015). However, this subject matter requires further investigation.

4.2.4 Modification of Host Gene Expression. Despite the absence of direct contact between the liver and gut microbiota, they modulate hepatic gene expression of crucial genes playing a vital role in drug metabolism (Björkholm et al., 2009). Gene expression studies conducted on microarrays from germ-free (GF) and conventionally raised (CV) specific pathogen-free mice demonstrated differential expression of 112 genes among them, affecting the liver metabolic functions. In GF mice, genes regulated by constitutive androstane receptors showed higher expression. In contrast, CYP2B9 and CYP4A14 were expressed to a lower extent in CV mice. The enhanced metabolism with shorter-term pentobarbital-induced anesthesia further ascertained differential expression in GF compared with CV mice (Björkholm et al., 2009). Moreover, immunoblotting experiments in GF and CV rats revealed that gut microbiota moderately affects the levels of Phase II xenobiotic-metabolizing enzymes in the large intestine and liver but remain unaffected in the small intestine (Meinl et al., 2009). mRNA profiling in the four intestinal sections and liver for the expression of xenobiotic-processing genes (XPGs) in CV and GF mice elucidated that the GF mice expressed 116 XPGs in at least one intestinal section, but 133 XPGs were unaffected. Also, the liver and intestine of GF mice showed down-regulation of CYP3A that may result in altered metabolism of xenobiotics (Fu et al., 2016; Fu et al., 2017). Administration of a VSL3, a commercial probiotic mixture containing eight live strains of bacteria to CV and GF mice was investigated. In CV mice, VSL3 enhanced the mRNAs expression for CYP4V3, alcohol dehydrogenase 1, and carboxyesterase 2a, and declined for multiple phase II glutathione-S-transferases, whereas, in GF mice it reduced the mRNAs expression for UDP-glucuronosyltransferases 1a9 and 2a3 (Selwyn et al., 2016). In conclusion, gut microbiota modulates the expression of XPGs, leading to altered xenobiotic metabolism.

4.2.5 Immunomodulation. Gut microbiota can impact the efficacy of chemotherapeutic drugs by translocation and immunomodulation. For instance, the anti-cancer drug cyclophosphamide caused the shortening of small intestinal villi and disrupted the intestinal barrier function (Alexander et al., 2017). These disruptions led to the translocation of

several commensal gram-positive bacteria, such as Lactobacillus johnsonii, Lactobacillus murinus, and Enterococcus hirae, into the secondary lymphoid organs in the mice (Viaud et al., 2013), which resulted in stimulation of the differentiation of naive $CD4^+$ T cells to cause accumulation of type 17 T-helper and type 1 T-helper cell responses. GF mice and mice treated with the antibiotic vancomycin deplete gram-positive bacteria that lowers the 17 T-helper response and inhibit the antitumor effect of cyclophosphamide (Viaud et al., 2011, 2013). Effectiveness of gut microbiota in immune checkpoint-blocked cancer therapy is proven in periclinal models and cancer patients for *Akkermansia muciniphila*, *Bacteroides fragilis*, *Bifidobacterium spp*. and *Faecalibacterium spp* (Gopalakrishnan et al., 2018; Routy et al., 2018a; Routy et al., 2018b). The immune checkpoint-blocked therapy targets cytotoxic T lymphocyte protein-4, blockade of programmed death-1 (PD-1) protein, and programmed death-ligand 1 (PD-L1) (Sivan et al., 2015; Farrokhi et al., 2019). Table 1 summarizes the gut microbiota biotransformation reactions.

5. Experimental Approaches to Analyze the Metabolism of Drugs by Gut Microbiota

Different approaches are used to analyze gut-mediated metabolism. Table 2 gives examples of drug metabolism investigated using different approaches. Fig. 3 elucidates the experimental approaches to analyze the metabolism of drugs by gut microbiota.

5.1 In Vivo Approaches

5.1.1 Murine Models. Since the beginning of research in gut microbiota, mice models have been widely used due to their anatomic, physiologic, and genetic similarities to humans. Mice models allow approaches that require invasive sampling methods that would be considered unethical in human subjects. They have added advantages, such as small size, high reproductive rate, and low maintenance cost (Hugenholtz and de Vos, 2018). Similar to humans, gut microbiota in mice is also dominated by *Bacteroidetes* and *Firmicutes* phyla. Nevertheless, there lies a variation in the plethora of microorganisms at the genera level. *Prevotella*, *Faecalibacterium,* and *Ruminococcus* genera have a relatively higher abundance in the human gut, whereas *Lactobacillus*, *Alistipes*, and *Turicibacter* genera occupy a relatively higher proportion of mouse gut microbiota (Nguyen et al., 2015). Animal models cannot accurately recapitulate the human microbial repertoire, curbing direct extrapolating results from conventionally raised mice to humans.

Germ-free mice are reared in an isolator to ensure the complete absence of detectable microbes. Also, germ-free mice have enlarged cecum, reduced villous thickness and also show under-developed intestine-associated lymphoid tissue (Taguer and Maurice, 2016; Sun et al., 2019). Difficulty in generating and maintaining germ-free mice has led to an alternative antibiotic use to deplete mice gut microbiota. Some antibiotics can target a particular subset of microorganisms, such as metronidazole and clindamycin, which deplete anaerobes (Kennedy et al., 2018). Gnotobiotic mice, colonized by one or more defined microbial species, simplify the complex host-microbial interactions. Other animals, such as rats, pigs, dogs, and guinea pigs serve as an alternative to mice experiments. Thus, comparing metabolites in gnotobiotic mice and conventionally raised mice provides valuable insights into drug metabolism by gut microbes. Human flora-associated (HFA) animals were used to understand the role of human intestinal microbes in drug metabolism. They were created by inoculating germ-free animals with human fecal matter (Hirayama and Itoh, 2005). However, using the HFA animal models has several drawbacks; factors such as genetics and diet of the recipient animals have been found to affect colonization of the rodent gut by human bacterial communities. For instance, HFA mice display a lower *Firmicutes* to *Bacteriodetes* ratio than donor human or HFA rats (Wos-Oxley et al., 2012). However, despite its flaws, the establishment of human gut flora into the intestines of animals is known to yield a stable model with a better resemblance to microbial metabolism in the human gut.

5.1.2 Other Animal Models. Invertebrate *Caenorhabditis elegans*, a free-living bacterivorous nematode, is a simplified in vivo model to study drug metabolism by microbiota. This model was used to show that bacterial ribonucleotide metabolism can cause activation of fluoropyrimidine drugs like 5-fluorouracil (Scott et al., 2017). A vertebrate zebrafish also serves as a model of intermediate gut microbial diversity that is more complex than invertebrates and less complex than that of mammals. Some significant advantages of this alternative testing model include their small size, high fecundity, external fertilization, rapid development, transparency, and low cost. In addition, microbe-free embryos of zebrafish can be colonized with a required strain of microbial species by simple immersion in water (axenic zebrafish) and used as an in vivo model to study the effects of drug metabolism by microbiota (Catron et al., 2019).

5.2 In Vitro Approaches

5.2.1 Cell-Culture. In vivo studies utilizing human and animal models are inappropriate for routine and large-scale screening of xenobiotic metabolism by gut microbiota due to ethical, economic and time restrictions. In vitro models such as culturing representative strains of gut microbiota with the drug have been used to study microbial drug bio-transformation. Although both in vitro and ex vivo studies require laboratory experimentation setup, they differ in the source of the microbial sample used. In vitro studies use microbial samples that are isolated from an organism instead of laboratory microbial strains (National Academies Press, 2021). In vitro models allow differentiation of microbial xenobiotic metabolism from that of host metabolic activities. In vitro fermentation models can range from simple static batch cultures to multistage continuous culture systems that use chemostats to match the dynamic equilibrium of the gut. Culture models require suitable culture media and environmental conditions, such as the anaerobic chamber, temperature, and pH controls (Payne et al., 2012). Drugs are introduced into the fermentation systems, and aliquots removed at defined time points are quantitatively analyzed for the depletion of substrate and appearance of metabolites to determine the rate and extent of drug metabolism by gut microbiota. In such models, adaptation to specific culture conditions may cause deviation in the bacterial composition over time (Payne et al., 2011). These model designs prevent the extension of information regarding the drug's pharmacokinetics in the upper gastrointestinal tract; additionally, they neglect the interactions between the host tissue and microbiota, which are essential for the drugs co-metabolized by the host and microbes (Hu et al., 2019). An in vitro fermentation model for investigating the effect of exopolysaccharides on gut microbiota showed that exopolysaccharides are transformed into short-chain fatty acids. Furthermore, 16S rDNA sequencing elucidated that exopolysaccharides increased the abundance of *Ruminococcus*, *Dorea*, *Butyricicoccus*, and *Blautia* (Zhu et al., 2021).

5.2.2 Simulators. Complex simulators of the intestine, such as the simulated human intestinal microbial ecosystem (SHIME) reactor have been developed to mimic the gut microbial environment. SHIME is a validated five-stage model in which a two-step fill-and-draw system simulates the small intestine while the latter three reactors mimic the large intestine. When inoculated with microorganisms, each reactors hosts intestinal region-specific distinctive microbial communities. All the reactors are connected in series employing pumping systems and placed under well-defined and controlled conditions (Molly et al., 1993). One validation test for the SHIME model involves converting prodrug sulfasalazine to its active moiety 5-aminosalicylic acid. Results

TABLE 1

Biotransformation reactions performed by gut microbiota

Type of Reaction	Drug Exemplar	Class of Drug	Produced Drug Metabolites	Outcome	Microorganism	Reference
Azo-bond reduction	Sulfasalazine	Anti-inflammatory	5-Aminosalicylic acid and sulphapyridine	Activation of prodrug	Unknown	(Peppercorn and Goldman, 1972)
Alkene double bond reduction	Digoxin	Cardiac glycoside	Dihydrodigoxin	Reduction of therapeutic efficacy	*Eggerthelalenta*	(Lindenbaum et al., 1981b; Dobkin et al., 1982; Saha et al., 1983)
Carbonyl double bond reduction	Tacrolimus	Immuno-suppressant	9-Hydroxytacrolimus	Low and variable exposure to the immunosuppressant	*Faecalibacteriumprausnitzii*	(Guo et al., 2019)
Nitro reduction	Nitrazepam	Hypnotic	7-Aminonitrazepam	Produces 7-acetylaminonitrazepam having teratogenic potential	*Clostridium leptum*	(Rafii et al., 1997)
N-oxide reduction	Loperamide oxide	Anti-diarrheal	Loperamide	Activation of prodrug	Unknown	(Lavrijsen et al., 1995)
Sulfoxide reduction	Sulindac	Non-steroidal anti-inflammatory drug	Sulindac sulfide	Activation of prodrug	Aerobes *Escherichia coli,Enterobacter* and anaerobes *Clostridia*	(Strong et al., 1987)
Hydrolysis	Sorivudine	Anti-viral	(E)-5-(2-bromovinyl) uracil	Lethal effects due to combination of Sorivudine and 5-fluorouracil	*Bacteroides* species *B. Vulgatus, B.Thetaiotaomicron, B. fragilis, B. uniformis and B. eggerthii*	(Nakayama et al., 1997; Okuda et al., 1998)
Proteolysis	Calcitonin	Synthetic hormone	Unknown	Degradation of peptide drug causing loss of therapeutic efficacy	Unknown	(Sousa et al., 2008)
De-conjugation	Irinotecan metabolite	Anti-cancer	SN-38	Intestinal toxicity	Unknown	(Takasuna et al., 1996)
De-hydroxylation	Levodopa	Anti-parkinsonian	m-Tyramine and m-hydroxyphenyl acetic acid	Reduced therapeutic activity	Unknown	(Sandler et al., 1969; Goldin et al., 1973)
De-methylation	Imipramine	Tricyclic anti-depressant	Desipramine	Conversion to active metabolite causing variation in response to imipramine therapy	Aerobes *Escherichia coli Klebsiella pneumoniae* and anaerobes *Fusobacterium fusiforme*	(Clark et al., 1983)
Acetylation	5-Aminosalicylic acid	Metabolite of sulfasalazine	N-Acetyl-5-aminosalicylic acid	Therapeutic inactivation of the drug	Unknown	(Allgayer et al., 1989)
De-acetylation	Aspirin	Non-steroidal anti-inflammatory drugs and anti-thrombotic	Salicylic acid and hydroxylated salicylic acid	Decrease in anti-thrombotic effect of the drug	Unknown	(Kim IS et al., 2016)
De-amination	5-Fluorocytosine	Anti-fungal	5-Fluorouracil	Possible cause of toxicity of the drug	Unknown	(Harris et al., 1986)
Oxidation	Lovastatin	HMG-CoA reductase inhibitor	Hydroxylated and hydroxy acid metabolites	Inter-individual variability in pharmacokinetics	Unknown	(Yoo et al., 2014)
De-nitration	Glyceryl trinitrate	Anti-anginal	Glyceryl-1,3-dinitrate, glyceryl-1,2-dinitrate, glyceryl-1-mononitrate,glyceryl-2-mononitrate	Decrease in the therapeutic activity of the drug	Unknown	(Sousa et al., 2008)
Thiazole ring opening	Levamisole	Anthelmintic	Levametabol I, II and III	The activity of metabolites not reported	*Bacteroidetes* and *Clostridium spp*. under anaerobic conditions	(Shu et al., 1991)

TABLE 2

Examples of investigation of drug metabolism by gut microbiota using different approaches

Sr. No.	Type of Study	Drug	Class of Drug	Result	Reference
1	In vivo murine model	Deleobuvir	Hepatitis C	Plasma exposure of reduced metabolite of deleobuvir (CD 6168) was ninefold lower in pseudo-germ free rats than control rats	(McCabe et al., 2015)
2	In vivo nematode model C. elegans	Doxorubicin	Anti-cancer	Reduced toxicity of doxorubicin was attributable to its deglycosylation by capsulated bacilli *Raoultellaplanticola* under anaerobic conditions	(Yan et al., 2018)
3	In vitro	Flucytosine	Anti-fungal	Extensive conversion of flucytosine to fluorouracil can occur in the human intestinal microflora by *E. coli.* Hence, fluorouracil exposure and fluorouracil-related toxicity may occur in the flucytosine-treated patient	(Vermes et al., 2003)
4	Ex vivo	Epacadostat(EPA)	Anti-cancer	Amidine metabolite of EPA (M11) and N-dealkylated form of M11 (M12) were formed in negligible quantities when EPA was incubated with human microsomes from multiple tissues, hepatocytes, recombinant human cytochrome P450s. Whereas, M11 was formed when EPA was incubated with human fecal homogenates, and the amount of M11 depleted upon addition of antibiotic ciprofloxacin to the fecal homogenates which confirmed the role of gut microbiota in its metabolism	(Boer et al., 2016)
5	In silico	Brivudine	Anti-viral	PBPK model accurately predicts and separates the host and microbiome mediated metabolism of brivudine to its hepatotoxic metabolite bromovinyluracil	(Zimmermann et al., 2019b)
6	Multi-omic	Simvastatin	HMG-CoA reductase inhibitor	Metabolomic study revealed that the levels of bacterially derived bile acids assist in predicting the efficacy of simvastatin in lowering low-density lipoprotein cholesterol and raise the possibility of competitionbetween simvastatin and bile acids for SLCO1B1 transporter may influence both the pharmacokinetics and pharmacodynamics ofsimvastatin, and possibly the risk of muscle toxicity	(Kaddurah-Daouk et al., 2011)

stated that only a small percentage of 5-aminosalicylic acid was released up to the end of vessel 2 (small intestine). At the same time, a complete transformation happened at the beginning of vessel 3 (large intestine) (Molly et al., 2009). The disadvantage of this model is that the bacterial miscellany in the colon mucosa is under-represented in the fecal inoculum (Durbán et al., 2011). Therefore, an up-gradation, mucosal-SHIME, was introduced in the SHIME model, which had an advantage over the older SHIME model. This optimization of SHIME considers the luminal microbiota and the colon mucosa-associated microbial communities by incorporating mucin-covered microcosms (Van den Abbeele et al., 2012).

5.2.3 Microfluidic Devices (Organ-on-Chip Systems). Organ-on-a-chip models consist of continuous perfusion of microchambers that are amenable to the inhabitation of cultured living cells to simulate tissue- and organ-level physiology (Bhatia and Ingber, 2014). Gut-on-a-chip models comprised two hollow chambers separated by an extracellular matrix coated by a porous membrane of polyester or polycarbonate and lined with human intestinal epithelial cells. In some studies, the microenvironment of the cells is recreated by control of culture medium flow rate through the microchannels and, in other studies, by applying cyclic strain to cause peristalsis like deformations (Kim et al., 2012). A handful of microbiota-related studies have been done using microfluidic devices that demonstrate the ability of differentiated epithelial cells to sustain the growth of intestinal inhabitants. *Lactobacillus rhamnosus* GG host-pathogen interactions and interaction among components of the intestine, the immune system and bacteria (Kim et al., 2010, 2012; HJ Kim et al., 2016). These models with fluidic control can permit controlled delivery of desired concentration of drugs dissolved in medium to the intestinal epithelium co-cultured with microbes to investigate microbiota-based drug metabolism in a biomimetic system (Tanaka et al., 2006; Vickerman et al., 2008). However, replicating all the intestinal wall layers and co-culturing total microbiota rather than single microbial species or microbial consortia are some of the challenges yet to be explored (Lee et al., 2019). Shah et al. presented a modular, microfluidics-based model HuMix (human–microbial crosstalk) that allows co-culturing of human and microbial cells under representative gastrointestinal human-microbe interface conditions. The individual transcriptional responses from human epithelial cells co-cultured with *Lactobacillus rhamnosus* GG under anaerobic conditions inside HuMix are in accordance with in vivo data.

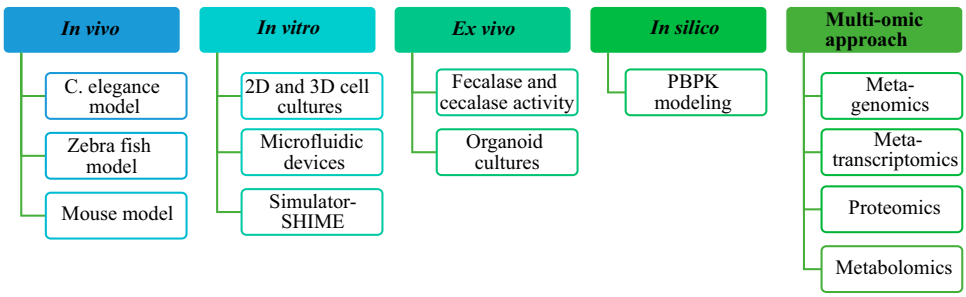

Fig. 3. Different experimental approaches to analyze the metabolism of drugs by gut microbiota.

In addition, human epithelial cells co-cultured with *Bacteroides caccae* and LGG elicit a transcriptional response differing from a co-culture containing only LGG, suggesting the use of HuMix in molecular interactions between host and host microbiome (Shah et al., 2016).

5.3 Ex Vivo Approaches

In contrast to in vitro studies, ex vivo studies use biologic material comprising microbes in an artificial setting. For example, multiple studies use ex vivo fermentation systems inoculated with human or animal fecal homogenates or animal caecal contents to screen drug metabolism by microbes (van de Seeg et al., 2018). This allows drug screening against a greater diversity of microorganisms present in the fecal/caecal contents.

5.3.1 Fecalase or Caecalase Assay. This research tool makes use of a cell-free extract of fecal or caecal contents, fecalase or caecalase assays. Lysis of bacterial cells in fecal suspension by passing through a homogenizer or sonication followed by removing cell debris by centrifugation yields a stable extract that retains the enzyme fraction of bacteria (Tamura et al., 1980). The fecalase assay assesses the conversion of amlodipine to pyridine metabolite. The pyridine metabolite concentration increased with incubation time, suggesting the role of gut microbiota in amlodipine metabolism and further confirmed by a pharmacokinetic study demonstrating an increase in bioavailability of amlodipine in antibiotic-treated mice compared with control mice (Yoo et al., 2016). However, diet and physiologic factors have affected fecalase and caecalase activity, leading to intra- and inter-individual differences (Yeo et al., 2012).

5.3.2 Organoids. Scientific advancement has led to the development of three-dimensional ex vivo multicellular tissue constructs derived from human stem cells containing organ-specific cell types termed organoids (de Souza, 2017; Min et al., 2020). Intestinal and gastric organoids have been used to examine the crosstalk between infectious pathogens such as *Salmonella typhimurium, Helicobacter pylori*, rotavirus, and the luminal epithelium (Finkbeiner et al., 2012; Bartfeld et al., 2015; Forbester et al., 2015). Engineered gastrointestinal organoids microinjected with microbiota can decipher the mechanism of drugs action and microbiota-based metabolism. Cell culture and simulator models are insufficient to represent host cell impact on microbiota physiology that can be overcome using the organoid model (Hill et al., 2017). The intestinal epithelial organoid is a novel model to study host-microbiota interactions. Investigation on the effect of short-chain fatty acids produced by commensal gut bacteria with the metabolites of *Akkermansia muciniphila and Faecalibacterium prausnitzii* revealed that *Akkermansia muciniphila* metabolites affect transcription factors and genes (Fiaf, Gpr43, histone deacetylases, and peroxisome proliferator-activated receptor-gamma) associated with cellular lipid metabolism and growth. In contrast, *Faecalibacterium prausnitzii* has a weak effect on host transcription (Lukovac et al., 2014).

5.4 In Silico Approaches

Technological advances have resulted in efficacious computational tools to predict drug pharmacokinetics in the human body. Despite the difficulty in modeling complex biochemical reactions, multiple computational tools have been developed to predict microbial xenobiotic metabolism. The complexity arises from the non-specificity of many microbial enzymes for substrates and vast microbiota diversity at the species and community level (Klünemann et al., 2014). Physiologically based pharmacokinetic modeling and simulation (PBPK) models describe the whole-body drug kinetics using compartments corresponding to the body's organs connected by a circulating blood system (Zhuang and Lu, 2016). However, PBPK models were unable to account for microbial metabolism until the recent inclusion of the microbiome component in the intestinal compartment. Therefore, a PBPK model was devised that accurately predicts and separates brivudine's host and microbiome mediated metabolism to its hepatotoxic metabolite bromovinyluracil. For constructing the model, the kinetic parameters of the drug and its metabolite were obtained in various compartments over time in the presence and absence of microbial drug metabolism (Zimmermann et al., 2019b). Additionally, a generalized approach was developed by building models parametrized with drug and metabolite kinetics in germ-free and conventionally raised mice for two additional drugs, sorivudine and clonazepam (Zimmermann et al., 2019b). An example of a searchable resource is MicrobeFDT, in which networks of food compounds and drugs with similar structures are created and linked to microbial enzymes with known toxicities. Based on the postulation, if a microbial enzyme acts on one compound from a group, it may act on related compounds found in the group (Guthrie et al., 2019). A PBPK model-based study indicated that hydrolysis of intestinal glucuronide impacts the pharmacokinetics of aglycone. A PBPK model was developed to investigate the effect of intestinal glucuronide on the pharmacokinetics of an active compound, SN-38 glucuronide, considering the liver and gut as the major eliminating organs. This two-compartment model suggested that hydrolysis of glucuronide in the gut increased the local intestinal exposure to the SN-38, but the systemic exposure was insignificant (Wu, 2012; Guthrie and Kelly, 2019). Thus, results from *in silico* prediction tools can complement and improvise the experimental setup of in vitro studies.

5.5 Multi-omics Approach

An ever-increasing knowledge of the microbiome impact on drug efficacy, disposition, and toxicity, pharmacomicrobiomics (microbiome-drug interactions) were pioneered as an extension of pharmacogenetics (Mariam et al., 2010; Aziz et al., 2018; Sharma et al., 2019). Genetic diversity of the gut microbial communities has come to light due to the advent of next-generation sequencing techniques, such as 16S rRNA gene amplicon sequencing and shotgun metagenomics (Sanschagrin and Yergeau, 2014). Metagenomics, in addition, gives a peek at the potential functional capabilities of the microorganisms (Haiser and Turnbaugh, 2013). Nevertheless, the mere abundance of the gene cannot guarantee its expression. Meta-transcriptomics, the sequencing of gene transcripts and meta-proteomics, the measurement of expressed proteins also form part of pharmacomicrobioics. Apart from solely detecting the microbial species present in large numbers, these techniques also distinguish the metabolically active gut microbes (Ursell and Knight, 2013; Xiong et al., 2015). Targeted or untargeted metabolomics and metabonomic analysis coupled with in vivo and in vitro experimental designs have proven valuable to determine the gut microbial contribution to drug metabolism (Aura et al., 2011; Yip and Chan, 2015). Metabolomic analysis has been used for targeted analysis of drug metabolites and to discover unique biomarkers that have helped predict the host response or the host metabolism of a drug. For instance, the pre-dose level of bacterial metabolite, *p*-cresol, influences the hepatic sulfate conjugation of acetaminophen (Clayton et al., 2009). A high-throughput screening led to the identification of bacterial gene products that metabolize drugs. Integrated data obtained from relevant omic approaches, such as untargeted metabolomics and metagenomics, supports identifying the gene products. Moreover, the study demonstrated the ability of 76 gut bacterial species to metabolize 271 drugs belonging to a wide range of chemical classes (Zimmermann et al., 2019a). Even though the omic methods provide valuable information, using these techniques in elucidating drug metabolism by gut microbiota still has a long way to go.

6. Toxicological Assessment of Drug Metabolites

As discussed earlier, microbial action may result in drug toxicity, and a meticulous study has been done on the intestinal toxicity caused by the activity of gut microbes on the anti-cancer drug irinotecan. SN-38, the active metabolite of the prodrug irinotecan, is glucuronidated in the liver to SN-38G and secreted in the bile. In the intestine, bacterially-derived β-glucuronidases converts SN-38G to SN-38, responsible for the irinotecan-induced severe diarrhea (Takasuna et al., 1996). The toxicity of drug metabolites produced by gut microbes is mainly evaluated by comparative clinical observations and histologic examination of tissues of germ-free/antibiotic-treated and conventionally raised/HFA animals. Other tools, such as toxicity predicting software and omics technology for studying toxico-microbiomics, are also used (Abdelsalam et al., 2020). The toxicity of short-lived reactive metabolites is analyzed by trapping the electrophilic metabolites with nucleophiles like glutathione and cyanide ions followed by the mass spectrometric analysis adducts formed (Tang and Lu, 2010). Manipulation in the gut microbial composition or metabolic activity can lower the production of toxic metabolites or improve the therapeutic outcomes. Wallace and co-author demonstrated that the administration of selective bacterial β-glucuronidase inhibitor protected mice from irinotecan-induced toxicity without killing the bacteria. This inhibitor would benefit cancer patients as it will selectively inhibit the bacterial β-glucuronidase and prevent the formation of SN-38 that kills the microbiota essential for human health; hence, it will alleviate irinotecan-induced toxicity (Wallace et al., 2010).

7. Impact of Drugs on the Gut Microbiome

Several drugs, such as proton pump inhibitors, statins, and angiotensin-converting enzyme inhibitors, have demonstrated the ability to change the gut environment, thus affecting the composition, growth, and functions of gut microbial communities (Weersma et al., 2020). For instance, metformin affects the composition and function of gut microbiota. Germ-free mice treated with gut microbiota from metformin-treated individuals showed lower blood glucose levels than those treated with gut microbiota from placebo-treated donors. This showed that metformin-altered gut microbiota improves glucose metabolism (Wu et al., 2017). High-throughput screening of over 1000 drugs covering a wide range of therapeutic classes was performed against 40 gut microbial strain isolates. Around 835 drugs acted upon molecular targets in human cells, and the rest were anti-infectives. Using drug concentrations similar to that estimated to be found in the gut for many drugs, the study showed that 27% of non-antibiotics suppressed the growth of at least one of the tested microbial strains (Maier et al., 2018). Thus, the interplay between gut microbiota and drugs is complex and bidirectional.

8. Conclusion

Although gut microbiota-mediated drug metabolism was first discovered in the mid-20th century, research in this field has been challenging due to the extensive and variable repertoire of gut microbes in individuals and the complicated mechanisms by which the microbes carry out drug metabolism. Furthermore, Microbially-derived drug metabolites can have a different potency than parent drugs or, in some cases, even a toxic potential, thus causing deviations from the expected therapeutic outcomes of the drug. Integrating experimental, computational, and multi-omic approaches will deepen our understanding of gut microbial composition and recognize gut microbiota-mediated drug metabolism. Evaluation of drug metabolism by gut microbiota in new chemical entity drug discovery and development is rational for safe and efficacious drug therapy. Once the role of gut microbes in drug metabolism is established, the host-microbiota symbiotic relation exploration will help develop refined and personalized drug therapies with maximum therapeutic benefits and minimal toxicological effects.

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