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Investigation of Host-Gut Microbiota Modulation of Therapeutic Outcome

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Nonstandard abbreviations

COMET – Consortium for Metabonomic Toxicology
CYP – Cytochrome P450
DGGE – Denaturing gradient gel electrophoresis
FISH – Fluorescence *in situ* hybridization
GC/TOFMS – Gas chromatography time-of-flight mass spectrometry
GMD – Golm Metabolome Database
HMDB – Human Metabolome Database
LC/MS – Liquid chromatography-mass spectrometry
MS – Mass spectrometry
NIST – National Institute of Standards and Technology
NMR – Nuclear magnetic resonance
qPCR – quantitative polymerase chain reaction
rRNA – ribosomal ribonucleic acid
SPF – Specific pathogen-free
TGGE – Temperature gradient gel electrophoresis
T-RFLP – Terminal restriction fragment length polymorphism

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Abstract

A broader understanding towards factors underlying inter-individual variation in pharmacotherapy is important for our pursuit of “personalized medicine”. Based on knowledge gleaned from the investigation of the human genetics, drug metabolizing enzymes and transporters, clinicians and pharmacists are able to tailor pharmacotherapies according to the genotype of patients. However, human host factors only form part of the equation that accounts for heterogeneity in therapeutic outcome. Notably, the gut microbiota possesses wide ranging metabolic activities that expands the metabolic functions of the human host beyond that encoded by the human genome. This review will first illustrate with examples the mechanisms in which gut microbes modulate pharmacokinetics and therapeutic outcome. Secondly, we discuss the application of metabonomics in deciphering the complex host-gut microbiota interaction in pharmacotherapy. Thirdly, we highlight an integrative approach with particular mention of the investigation of gut microbiota using culture-based and culture-independent techniques to complement the investigation of the host-gut microbiota axes in pharmaceutical research.

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Introduction

Our unique age, gender, genetic makeup, nutritional state, disease condition and environmental exposure influence therapeutic outcomes. The recognition of inter-individual differences in drug response has thus spawned the move towards "Personalized Medicine" (Mancinelli et al., 2000; Ginsburg and McCarthy, 2001; Woodcock, 2007; Hamburg and Collins, 2010). The ability to truly provide personalized medical care is dependent on mechanistic knowledge underlying heterogeneity in drug responses. With the emergence of 'omics' technologies, there has been a paradigm shift towards investigating diseases and drug therapies using the omics-based systems biology approach (Zhou et al., 2008; Lum et al., 2009; Bates, 2010; Chan and Ginsburg, 2011). The recommendation by the United States Food and Drug Administration (USFDA) to genotype patients prior to treatment with trastuzumab, clopidogrel, carbamazepine and irinotecan due to their differential efficacy and toxicity in stratified patient cohorts demonstrated the value of pharmacogenomics in predicting drug responses (Khoury et al., 2009; Leckband et al., 2013). Nonetheless, host genetics alone does not explain all variations in pharmacotherapy (Nebert et al., 2003).

In fact, human beings have been coined as "superorganisms" since our complex systems biology is dictated by two sets of genomes – the genetically inherited human genome and the environmentally acquired microbiome (Lederberg, 2000). It has been estimated that there are approximately 10^{12} parenchymal cells in human (excluding blood cells and neurons) and 10^{12} bacteria on the skin, 10^{10} in the mouth, and 10^{14} in the guts (Kumar et al., 2013). Considering the 10-fold higher proportion of microbial to human cells, the extragenomic influence by the microbiome on systems biology should not be underestimated (Savage, 1977; Holmes et al., 2012; Nicholson et al., 2012). Indeed, accumulating evidence revealed the diverse impacts of the microbiome on human health, including nutrition, physiology and host metabolism

(Nicholson et al., 2012; Guinane and Cotter, 2013). Dysbiosis of microbiome has also been linked to pathological conditions such as inflammatory bowel diseases (Morgan et al., 2012; Kostic et al., 2014), diabetes (Qin et al., 2012), obesity (Turnbaugh et al., 2006; Turnbaugh et al., 2009; Sweeney and Morton, 2013) and autism (Kang et al., 2013).

From an anatomical perspective, the gut microbiota forms an important external "organ" within the gastrointestinal ecosystem, comprising more than 400 different species of bacteria (Hao and Lee, 2004; Qin et al., 2010). Notably, these intestinal bacteria contributes about 1.5 kg of the human body weight (Toivanen et al., 2001), comparable to the weight of major human organs such as the liver (~1.5 kg) and brain (~1.4 kg) and exceeds that of the lung (~0.84 kg), kidney (~0.27 kg) and spleen (~0.14 kg) (Molina and DiMaio, 2012). Importantly, the gut microbiota performs functional activities not encoded by the host genome. For example, desert woodrats living in the Mojave desert of USA were found to harbor gut microbiota that facilitates their dietary consumption of highly toxic creosote bush that invaded their habitat 17,000 years ago (Karasov, 1989). The leaves of the creosote bush are covered with a phenolic-rich resin that is largely made up of nordihydroguaiaretic acid that is known to induce kidney cysts and liver damage in laboratory rodents (Lambert et al., 2002; Arteaga et al., 2005). Kohl *et al.* demonstrated that the distinct gut microbiota communities residing in Mojave woodrats confer tolerance to the creosote plant toxins by having higher abundance of microbes with genes that facilitate metabolism and detoxification of the aromatic toxic compounds compared to the naïve woodrats lacking similar ecological and evolutionary experience with creosote (Kohl et al., 2014). This example highlighted how microbial adaptation expands the host's enzymatic repertoire and confers the host with competitive advantage through access to nutrients that are otherwise toxic to other competitors incapable of performing microbial detoxification (Dearing et al., 2005).

Similarly, the metabolic crosstalk between the host and gut microbiota modulates the pharmacokinetics and pharmacodynamics of drugs (Sousa et al., 2008; Holmes et al., 2012; Haiser and Turnbaugh, 2013; Li and Jia, 2013; Carmody and Turnbaugh, 2014). In this review, we first offer an overview of how pharmacokinetics and therapeutic outcomes are affected by a number of key established host-gut microbiota interactions. Secondly, we discuss the application of metabonomics in deciphering the complex host-gut microbiota interaction underlying inter-individual variation in therapeutic outcome. Thirdly, we propose an integrative approach to investigate the gut microbiota with a special focus on culture-based and culture-independent techniques.

Host-Gut Microbiota Modulation of Pharmacokinetics and Therapeutic Outcome

The Liver in Pharmacokinetic and Therapeutic Outcome

To understand pharmacokinetic and therapeutic outcome, pharmaceutical scientists have placed much attention in investigating the host (e.g. species, gene expression, genetic polymorphism, disease, gender and age), drug (e.g. chemical structure, dosage and frequency of administration) and other xeno-compounds (e.g. diet, supplements and other concomitant drugs) rather than the gut microbiota (Wilson and Nicholson, 2009). The liver being a major organ responsible for metabolizing xenobiotics has received special attention. The liver plays a central role in biotransformation of drugs and is equipped with a range of metabolizing enzymes and transporters necessary for its function. In Phase I functionalization reactions (e.g. oxidation, reduction and hydrolysis), polar functional groups are introduced to nonpolar molecules. Cytochrome P450 (CYP) is a major class of drug metabolizing enzymes in the liver responsible for Phase I metabolism (Wrighton and Stevens, 1992; Spatzenegger and Jaeger, 1995; Iyanagi, 2007). Phase II enzymes (e.g. UDP-glucuronosyltransferase, sulfotransferases or glutathione-S-transferases) catalyze conjugation reactions that add polar

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moieties such as glucuronic acid, sulfate or glutathione to the functional groups (Iyanagi, 2007). These processes facilitate the clearance of the xenobiotics via the urinary or biliary route with the aid of transporters or efflux pumps. Considering the key roles of the hepatic metabolizing enzymes and transporters in pharmacokinetics, factors such as induction, inhibition and genetic polymorphism that lead to alterations in the expression and functional activities of these enzymes and transporters, may contribute to inter-individual variation in pharmacokinetics and therapeutic outcome (Park and Breckenridge, 1981; Park et al., 1996; Ingelman-Sundberg et al., 1999; Ando et al., 2000; Shenfield, 2004; Bosch et al., 2006; Guengerich, 2006; Ieiri et al., 2006; Kerb, 2006; Tomalik-Scharte et al., 2007). However, these factors do not account for all forms of heterogeneity among individuals.

Gut Microbiota Modulates Pharmacokinetics and Therapeutic Outcome

In recent years, the interest to elucidate the roles of the gut microbiota on pharmacokinetics and therapeutic outcomes has rekindled. The gut microbiota is known to possess a diverse range of metabolic activities that are capable of modulating the fate of an administered drug and its therapeutic outcome (Scheline, 1968; Nicholson et al., 2005; Sousa et al., 2008; Clayton et al., 2009; Haiser and Turnbaugh, 2012; Haiser et al., 2013). Scheline has even proposed that the gut microbiota has the metabolic potential at least equivalent to the liver (Scheline, 1973).

Mechanistically, the gut microbiota is known to affect pharmacokinetics by partaking in the direct metabolism of xenobiotics or through its indirect interaction with the host enzymatic system (Scheline, 1968; Nicholson et al., 2005; Sousa et al., 2008; Clayton et al., 2009; Haiser and Turnbaugh, 2012; Haiser et al., 2013). The indirect interaction is facilitated through the production of microbial or mammalian-microbial co-metabolites that compete for metabolism of xenobiotics or act as signaling molecules that influence the host gene expression. Such interactions may complement or oppose the host's enzymatic activity,

culminating in wide-ranging therapeutic consequences. The following section illustrates the roles of the gut microbiota in modulating pharmacokinetic processes and the therapeutic implications. The list of affected drugs is presented in Table 1 (Sousa et al., 2008; Clayton et al., 2009; Kaddurah-Daouk et al., 2011; Haiser and Turnbaugh, 2013; Saitta et al., 2014).

Absorption

The efficacy of statins to control hypercholesterolemia and reduce the risk of cardiovascular disease exhibits great variation among individuals that can only be partly explained by genetic differences (Verschuren et al., 2011). Simvastatin is an inactive pro-drug widely prescribed for lowering cholesterol levels. Using metabonomics, Kaddurah-Daouk *et al.* performed a targeted analysis of a panel of metabolites in the cholesterol synthesis, dietary sterol absorption and bile acid formation pathways in the plasma of 100 individuals to determine metabolic phenotypes (metabotypes) predictive of variation in cholesterol lowering efficacy of statin (Kaddurah-Daouk et al., 2011). In their study, the pre-treatment concentrations of several primary and secondary bile acids were found to correlate with the on-treatment plasma simvastatin acid (active metabolite) levels. In addition, an association in the plasma levels of simvastatin acid and seven bile acids with a single nucleotide polymorphism, rs4149056, in the gene encoding SLCO1B1 was uncovered. Bile acids and statins share transporters such as organic anion transporter SLCO1B1 in the intestine and liver (Niemi et al., 2011). The authors postulated that genetic polymorphism might have limited the transport of these substrates, possibly through competition between simvastatin and bile acids for hepatic uptake by SLCO1B1 transporter. Such competition may hence influence the pharmacokinetics, efficacy and toxicity (e.g. myopathy) of simvastatin. Other drugs that are substrates to the same class of transporters may potentially be subjected to similar interaction with the bile acids. Considering the intricate role of bile acid in drug

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transport, the gut microbiota may potentially modulate systemic drug absorption through affecting bile acid metabolism (Macdonald et al., 1983; Dawson, 2011; Sayin et al., 2013).

Disposition

In the liver, the metabolism of xenobiotics comprises oxidative and conjugative reactions. On the other hand, it is notable that compared to the metabolic activities of the host, the reactions associated with the gut microbiota are primarily reductive reactions and hydrolytic cleavage of conjugates such as glucuronides and sulfate conjugates that have been secreted via the bile into gastrointestinal tract (Table 1) (Scheline, 1973; Goldman, 1978; Rowland, 1988). As such, the gut microbiota can generate metabolites that are otherwise not produced by the host. Nitrazepam, a benzodiazepine drug, has been reported to induce teratogenicity (Takeno and Sakai, 1991). Incubation of nitrazepam with bacterial suspensions prepared from rat cecal contents resulted in extensive reduction to 7-aminonitrazepam, that in turn yielded 7-acetylaminonitrazepam via hepatic acetylation. These metabolites were reported to cause fetal malformation. Suppression of the nitroreductase activity of the gut microbiota by antibiotics prior to administration of nitrazepam significantly decreased the urinary and fecal excretion of the two reduced metabolites from 30% to 2% and markedly reduced the incidence of teratogenicity. This underscores the roles of gut microbiota in nitrazepam-induced teratogenicity (Takeno and Sakai, 1991).

Lovastatin is a lactone pro-drug used in the treatment of hypercholesterolemia. In a recent study by Yoo *et al*, incubation of lovastatin with human and rat fecal preparations yielded the active β-hydroxy acid metabolite that is known to inhibit 3-hydroxy-3-methylglutaryl coenzyme-A reductase (Yoo et al., 2014). This suggested the role of gut microbes in activating lovastatin for eliciting its pharmacological effects. The administration of lovastatin to antibiotic-treated rats resulted in a reduced systemic exposure to and ~60% lower fecal

production of the active β-hydroxy acid metabolite (Yoo et al., 2014). The decreased bioavailability of the active metabolite due to antibiotic consumption diminishes the therapeutic efficacy of lovastatin for cholesterol control. In a wider context, the work illustrated that drug-drug interaction can occur between antibiotics and drugs that are subjected to direct microbial metabolism.

In enterohepatic recirculation, a solute entering the gastrointestinal tract is absorbed intestinally by the enterocytes; it is then taken up into the hepatocytes via the portal vein and is biliary secreted into the intestines, where it can undergo further intestinal reabsorption to be channeled back to the systemic circulation (Roberts et al., 2002). Drugs subjected to enterohepatic recirculation are often characterized by multiple C_{max} peaks, longer apparent half-life in plasma concentration-time profile and larger apparent volume of distribution (Roberts et al., 2002). Examples of drugs subjected to enterohepatic recirculation include irinotecan (Mathijssen et al., 2001), morphine (Walsh and Levine, 1975) and indomethacin (Duggan et al., 1975). Enterohepatic recirculation is often associated with hepatic conjugation and intestinal deconjugation of parent drug. Irinotecan is a pro-drug that is administered intravenously as a first line therapy for colorectal cancers (Pommier, 2006). However, the utilization of irinotecan in chemotherapy is affected by its dose-limiting gastrointestinal toxicity characterized by severe diarrhea in patients (Rothenberg et al., 1996). Being a pro-drug, irinotecan is known to require hydrolysis by carboxylesterase in tissue and serum to generate the active metabolite, SN-38, for its pharmacologic effect (Mathijssen et al., 2001). The active SN-38 is subjected to Phase II glucuronidation by hepatic UDP-glucuronosyltransferase 1A1 to form the inactive SN-38G which is biliary secreted into the intestines (Mathijssen et al., 2001). Bacteria are reported to be the main contributor of intestinal source of β-glucuronidase enzyme for deglucuronidation (Rod and Midtvedt, 1977; Oleson and Court, 2008). Indeed, it has been discovered that the hydrolysis of SN-38G to

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SN-38 by microbial β-glucuronidase is responsible for the undesirable side effect of severe diarrhea (Takasuna et al., 1996; Mathijssen et al., 2001). The manipulation of the microbial β-glucuronidase activity with β-glucuronidase inhibitors and antibiotics has been shown to prevent the production of toxic SN-38 metabolites and protect the intestines of mice against injury (Takasuna et al., 1996; Wallace et al., 2010; Roberts et al., 2013). This underscores the therapeutic potential of targeted manipulation of gut microbes in minimizing the toxicity associated with irinotecan.

Phase II conjugation is a means of drug clearance, hence, the hydrolytic deconjugating activity of the gut microbiota may potentiate the pharmacological or toxicological effects of parent drugs by augmenting their systemic exposure if the aglycones are subjected to enterohepatic recirculation (Scheline, 1973; Goldman, 1978; Rowland, 1988). In a study by Clayton *et al*. (Clayton et al., 2009), the team identified that in patients administered with 1 g of acetaminophen, individuals that have high pre-dose urinary levels of p-cresol sulfate had low post-dose urinary ratio of acetaminophen sulfate to acetaminophen glucuronide. The authors attributed the reduced metabolic clearance of acetaminophen sulfate in implicated individuals to the generation of higher levels of microbially derived p-cresol that competes with acetaminophen for Phase II sulfonation in the liver (Clayton et al., 2009). Extrapolating this finding, one may hypothesize that the gut microbiota potentially affects the disposition of other drugs whereby sulfonation is an essential metabolic pathway. Additionally, the study by Clayton *et al.* illuminated an alternative mechanism of host-gut microbiota interaction mediated via microbial metabolites or co-metabolites (Clayton et al., 2009). This mode of interaction completely bypasses the need for direct contact between the drug and gut microbiota, yet enabling the gut microbiota to exert a remote influence on the fate of drug disposition by effecting host metabotypic changes that in turn modulate the host's enzymatic activities. In light of the extensive influence of the gut microbiota on the host metabotype (Li

et al., 2008; Zheng et al., 2011), it is believed that the work by Clayton *et al*. sets a new direction to investigate the pervasive influence of gut microbiota on the pharmacokinetics of drugs.

From the above examples, it is clear that the gut microbiota possesses diverse metabolic capabilities that augment or reduce the metabolic status of the host and produce active, inactive or toxic metabolites, some of which are not synthesized endogenously. Collectively, the current evidences emphasize the importance of the gut microbiota in pharmaceutical research and personalized medicine.

Elucidating Host-Gut Microbiota Interactions: A Need (Not Want)

It has been reported that ~3936 molecular entities have been approved for human use by major markets worldwide including US (Huang et al., 2011). Despite the vast metabolic potential of the gut microbiota, it has been estimated that only about 40 marketed drugs have been identified as substrates to date (Sousa et al., 2008; Haiser and Turnbaugh, 2013). The relatively few examples related to microbial xenobiotic metabolism is a reflection of our limited knowledge in this field.

Many recent metagenomics studies observed large variation in the gut microbiota composition amongst individuals across different age groups and populations (Group et al., 2009; Turnbaugh et al., 2009; Yatsunenko et al., 2012). Considering the profound metabolic roles exerted by the microbes as described earlier, such variations in gut microbial composition may hold important clinical implications. Digoxin used in treating heart failure is known to possess narrow therapeutic index and requires therapeutic drug monitoring. It has been long known that digoxin can be reduced and inactivated by gut *Eggerthella lenta* into dihydrodigoxin and dihydrodigoxigenin (Saha et al., 1983; Robertson et al., 1986). 10% of the population is known to harbor these enteric bacteria that metabolize more than 40% of the

orally ingested dose to inactive metabolites prior to its absorption (Li and Jia, 2013). Co-administration of antibiotics that eradicate the gut bacteria (e.g. erythromycin and tetracycline) has been reported to decrease microbial metabolism of digoxin (Lindenbaum et al., 1981). However, not all patients harboring high abundance of *Eggerthella lenta* will reduce digoxin (Saha et al., 1983). In a recent discovery by Haiser *et al.*, the authors found that the concerted influence of colonization by distinct digoxin-reducing strains of *Eggerthella lenta*, microbial interaction and host diet (arginine) determine the pharmacokinetics of digoxin (Haiser et al., 2013). Accordingly, the variation in the abundance of these specific strains of bacteria leads to inter-individual differences in the metabolism of digoxin and influence its efficacy and toxicity outcomes. This case study further emphasized the need to scrutinize the genetic and metabolic functions represented by the microbial communities to derive a more refined understanding of the role of gut microbiota in pharmacotherapy.

Definitely, more of such studies are needed to unveil the underlying mechanisms of host-gut microbiota interaction in modulating pharmacokinetics and therapeutic outcome. Such mechanistic understanding will aid the systematic characterization of drugs that are susceptible to the metabolic influence of gut microbiota and provide insights for their therapeutic management (Jia et al., 2008; Wallace et al., 2010; Haiser and Turnbaugh, 2012; Holmes et al., 2012; LoGuidice et al., 2012; Nicholson et al., 2012; Haiser and Turnbaugh, 2013; Maurice et al., 2013; Roberts et al., 2013).

Metabonomics: Mining the Metabolome to Study Host-Microbiota Interaction

Most of the established microbial influence on pharmacokinetics and therapeutic outcomes is attributed to its direct enzymatic actions on drug. Beyond this direct action, recent studies illuminated the insidious modulatory roles of the microbial or co-metabolites produced by the

microbes on the host enzymatic activities. There is now an increasing awareness of the symbiotic relationship between the host and gut microbiota and their combinatorial metabolic capacities. The host metabotype is a culmination of both host systems biology and gut microbiota biology (Martin et al., 2007; Claus et al., 2008; Martin et al., 2009; Wikoff et al., 2009; Claus et al., 2011; Zheng et al., 2011; Nicholson et al., 2012; Zhao et al., 2013). Even though the gut microbiota resides in the intestines, the vena portae and lymphatic system facilitate its interaction with the host through the transportation of metabolites via the enterohepatic circulation system. As the metabotypes are reflected within the host metabolome in biological matrices (e.g. plasma, urine and feces), it renders the metabolome an important source for elucidating the interaction between the host and gut microbiota.

Previously, there is a lack of systematic scientific techniques for elucidating the complex host-gut microbiota interaction. Fortunately, this situation has greatly improved with scientific advancement in analytical sciences, molecular biology and bioinformatics that when used in combination, greatly support the endeavor of systems biology-based investigation of host-gut microbiota interaction in pharmaceutical research. Metabonomics is defined as ‘the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification’ (Nicholson et al., 1999). Metabonomics is an attractive platform which provides access to this wealth of information embedded within the metabolome and valuable insights into the shared responsibility between the host and gut microbiota in modulating therapeutic outcome (Nicholson et al., 1999; Lindon et al., 2003; Nicholson and Wilson, 2003; Clayton et al., 2006; Lindon et al., 2007; Han et al., 2010; Nicholson et al., 2012). The birth of metabonomics dates back to the mid-1980s (Nicholson et al., 1999). Through the use of powerful analytical instruments such as nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), scientists are able to identify and quantify the small metabolites

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(<1500 Da) present within the metabolome (Li et al., 2008; Pasikanti et al., 2008; Wikoff et al., 2009; Yip and Chan, 2013).

The Metabonomics Workflow and its Considerations

The typical metabonomics workflow is illustrated in Fig. 1A. Fig. 1B further illustrates the various processes involved in a metabonomics study using gas chromatography time-of-flight mass spectrometry (GC/TOFMS) as an example. In brief, the workflow starts with the research question that defines the experimental design and the choice of biological matrix. As many investigations are clinically translational in nature, urine and blood samples are analyzed most frequently due to the ease of obtaining these samples (Dunn et al., 2011). The urinary and blood metabolic profiles are known to reflect variation owing to the host, drug, environment (e.g. diet, stress and lifestyle) and gut microbiota. The samples are prepared according to the type of biological matrix being analyzed and the instrument used for performing data acquisition (Beckonert et al., 2007; Dunn et al., 2011). For example, urine is known to contain high concentration of urea which will impose gas chromatographic interference. Hence, incubation with urease is performed to remove this interference during sample preparation (Pasikanti et al., 2008; Chan et al., 2011). For serum and plasma which are rich in proteins, the proteins are first precipitated by organic solvents (Bruce et al., 2009). As for tissues, they are usually homogenized before sample extraction (Want et al., 2013). For samples that will be subjected to GC/TOFMS analysis, they are typically subjected to chemical derivatization such that the derivatized analytes are sufficiently volatile and thermally stable for analysis (Kaal and Janssen, 2008).

Metabolites are downstream end products of transcription and translation and are known to regulate gene expression and function as building blocks of more complex biological molecules. Such metabolites include amino acids, organic acids, amines, sugars, nucleotides, fatty acids and steroids which span huge polarity and molecular weight ranges. Unlike DNA,

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RNA and proteins that are made up of chemically well-defined building blocks, the metabolites are chemically diverse and no single analytical technique is able to measure the full complement of these metabolites. In addition, the large number of metabolites within the metabolome and the wide dynamic range of their abundances further contribute to the analytical challenges. As such, various analytical techniques such as NMR spectroscopy, GC/MS and liquid chromatography-mass spectrometry (LC/MS) are often used complementarily to broaden the metabolic coverage. This complementary approach has been adopted to profile the human metabolomes in urine, blood and cerebrospinal fluid and the findings are made available in the Human Metabolome Database (HMDB) (Wishart et al., 2008; Psychogios et al., 2011; Mandal et al., 2012; Bouatra et al., 2013; Wishart et al., 2013). Metabonomics typically generates rich data in which meaningful interpretation is made possible by systematic preprocessing of the data to workable formats for further downstream analysis using statistical tools (Wishart, 2009; Chan et al., 2011; Dunn et al., 2011; Enot et al., 2011). In applying metabonomics in pharmaceutical research, one has to be mindful of the presence of interfering chromatographic peaks derived from drugs or their metabolites which can confound the statistical differentiation of the metabolic profiles of the drug treatment group from the control during chemometric multivariate data analysis. Manual exclusion of these peaks during the data preprocessing step is often tedious and erroneous. In such cases, a useful strategy that our group has adopted is to pool quality control samples solely from the drug-naïve controls prior to sample preparation and data acquisition (Yip and Chan, 2013). These pooled quality control samples can function as references whereby their metabolic features are used for the alignment of the metabolic features derived from other samples during data preprocessing (Chan et al., 2011). While this method ensures complete exclusion of all drug-related metabolic features, one needs to be mindful that selected

metabolites that are highly elevated in the drug treatment group but fall below the detection limit within the control may be accidentally excluded at times.

The identities of the marker metabolites are confirmed using authentic standards and/or relevant database libraries like HMDB, National Institute of Standards and Technology (NIST) and Golm Metabolome Database (GMD). Metabonomics has been applied to identify marker metabolites implicated in gastroenterological diseases (Yoshida et al., 2012), central nervous disorders (Quinones and Kaddurah-Daouk, 2009), cancers (Chan et al., 2009; Mal et al., 2009; Pasikanti et al., 2010a; Pasikanti et al., 2010b; Mal et al., 2012) and kidney diseases (Weiss and Kim, 2012). Metabonomics has also been employed in preclinical toxicological screening and its usefulness has been comprehensively evaluated by the Consortium for Metabonomic Toxicology (COMET) which comprises scientists from the Imperial College London and several pharmaceutical companies (Lindon et al., 2003; Lindon et al., 2005; Ohta et al., 2009; Aa et al., 2011; Zgoda-Pols et al., 2011).

Metabonomics Revealed Host-Gut Microbiota Modulation of Drug Disposition

More recently, metabonomics has been used to understand the interaction between the host and gut microbiota. Through the use of different experimental designs such as antibiotic-perturbed rodents (Zheng et al., 2011; Zhao et al., 2013), germfree versus conventional rodents (Claus et al., 2008; Wikoff et al., 2009) or through gut microbiota colonization of axenic mice (Martin et al., 2007; Claus et al., 2011), metabonomic analysis of the biological matrices (e.g. urine, plasma, fecal extracts, intestines, liver and kidney) revealed extensive gut microbiota modulation of the host systemic metabolism. Table 2 illustrates a list of metabolites with differential abundance among hosts having different gut microbial community composition. It has been found that the gut microbial communities exert profound influence on the host's metabolism and partake in the metabolism of short chain fatty acids, amino acids, primary and secondary bile acids, tryptophan and carbohydrate.

The alterations of such metabolites (e.g. bile acids) as a result of combinatorial activities of the host and gut microbiota suggested broad metabolic implications on drug metabolism. In a study by Toda *et al.*, the authors determined the effects of the intestinal microbiota on CYP expression by comparing the specific pathogen-free (SPF) and germfree mice (Toda et al., 2009). SPF mice are free of specific infectious microorganisms and parasites but are otherwise colonized with an undefined microbiota. They observed a higher mRNA expression of a majority of CYPs in the livers of SPF mice. Using a mouse CYP3A substrate, triazolam, the authors demonstrated a higher metabolic activity of liver microsomes extracted from the SPF mice compared with germfree mice, correlating with the higher CYP3A expression in the SPF mice. Nuclear receptors like aryl hydrocarbon receptor, constitutive androstane receptor, farnesoid X receptor and pregnane X receptor which modulate the expressions of the CYPs were also highly expressed in the livers of these SPF mice as compared to germfree mice. Interestingly, cholesterol-derived metabolites such as bile acids (e.g. lithocholic acid), steroid hormones and bilirubin have been proposed to function as activators of liver nuclear receptors and the levels of these metabolites were known to be mediated by microbial metabolism (Hylemon, 1985; Li and Chiang, 2013). As such, these studies demonstrated that the gut microbiota can modulate Phase I metabolism through influencing nuclear receptor and CYP expressions (Bjorkholm et al., 2009; Toda et al., 2009; Claus et al., 2011).

In addition, the metabolic products of the enteric bacteria are known to impose a huge burden on the Phase II metabolic processes of the host. Using metabonomics, Wikoff *et al*. revealed large effects of gut microbiota on the metabolic profiles of the mouse serum (Wikoff et al., 2009). Compared to the germfree mice, conventional mice were observed to have an exclusive presence of numerous sulfated, glycine-conjugated and glucuronide adducts. For example, indoxyl sulfate was identified exclusively in the serum of conventional mice. This

metabolite is formed as a result of the Phase II sulfonation of the bacterial metabolite indole, which is derived from the enzymatic conversion of tryptophan by bacterial tryptophanase (Wikoff et al., 2009). Apart from the indole derivatives, numerous metabolites apparently resulting from Phase II processing of microbial metabolites (e.g. hippuric acid, p-cresol sulfate, phenylacetylglycine) suggested a broad, drug-like Phase II metabolic response of the host to the microbial metabolites (Wikoff et al., 2009). This metabolic burden posed by microbial metabolites is not trivial as many drugs are rendered more polar for renal and biliary clearance by hepatic Phase II metabolism. It is clear that the complex interaction between the host and gut microbiota plays a pertinent role in influencing the metabotype, which in turn underpins the variable Phase II metabolic capacities among individuals with different gut microbiota composition.

Integrative Approach to Investigate Host-Gut Microbiota Interaction

As described earlier, metabonomics enables us to capture global perturbation of microbial metabolites and co-metabolites within the host system. From the metabonomics findings, scientists can glean complementary insights on the host-gut microbiota interaction that accounts for variation in pharmacokinetics and therapeutic outcome. To elucidate the complex nature of interaction between the host and gut microbiota more completely, it is necessary to adopt an integrative approach which encompasses the study of the host, microbiota and the drug (Fig. 2). This can include pharmacogenomics studies to evaluate for genetic polymorphism and *in vitro* assays for functional characterization of drug target receptor, metabolic enzyme and transporter of the host. The ability to characterize the microbial composition and to identify the implicated gut microbes and their metabolic activities plays a major part towards expanding our understanding of their involvement in pharmacology. The integration of the host and gut microbiota biology using

pharmacokinetics, pharmacology, toxicology, metabonomics, host genomics and gut microbiota metagenomics data forms a holistic strategy to elucidate the complex host-gut microbiota interaction in pharmaceutical research. The following section summarizes the techniques that support the investigation of the gut microbiota.

Culture-based and Culture-independent Techniques

Both the culture-based technique which has traditionally been used to study microbial metabolism in pharmaceutical research and the more modern culture-independent approach to study the gut microbiota are valuable techniques. The initial work on the gut microbiota since the 1970s relies on culture-based techniques (Tuohy and McCartney, 2006). Through the use of selective growth media and conditions, more than 400 to 500 bacterial species have been identified within the human gut. The majority of gut bacteria resides in the colon and it is a major site for microbial metabolism of many endogenous and exogenous compounds (Tuohy and McCartney, 2006; Sousa et al., 2008). However, the anatomical location of the human colon prevents the direct examination of the functional activities of the microbiota. As such, *in vitro* determination of drug metabolism using culture-based technique has a particular place in pharmaceutical research as it circumvents the challenge of invasive procedures, ethical drawbacks, cost and laborious nature associated with *in vivo* investigation (Sousa et al., 2008). In this regard, static batch culture is an example of an *in vitro* method that has been frequently employed for elucidating microbial metabolism of drugs (Coates et al., 1988; Sousa et al., 2008). Such cultures attempt to closely simulate the colonic environment by placing specific strains of bacteria, cecal or intestinal contents (of animals) or feces (of animal or human) into a suitable medium under careful control of factors such as anaerobic condition and pH. Drug is added to such cultures and samples are taken intermittently to quantify the amount of drug and its metabolites. It is notable that for the majority of the 40 drugs where their pharmacokinetics is associated with microbial activity, the culture-based

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technique has been applied to establish the role of gut microbiota in their metabolism (Sousa et al., 2008). However, some key limitations exist with the use of culture-based techniques. Inherent to the nature of bacteria cultivation technique, this method provides an incomplete view of the phylogenetic diversity of gut microbial community as some bacteria remain uncultivable (Tuohy and McCartney, 2006). To date, less than 30% of gut bacteria species have been cultured (Fraher et al., 2012). The symbiotic relationship among gut microbiota species adds to the difficulties in characterizing and reproducing appropriate growth environment for these bacteria (Miura et al., 1980; Stams, 1994; Doern, 2000). Such constraints impede culture efforts and pose challenges in creating an environment that supports the growth of all intestinal or fecal bacteria to mimic the actual colonic environment. Furthermore, culture-based techniques might be over simplistic as they do not consider other dynamic processes that occur in intact physiological conditions such as metabolic exchange and interaction between the host and gut microbial community and absorption of fermented products (Sousa et al., 2008). Hence, *in vivo* studies are conducted complementarily with *in vitro* studies to obtain deeper insights that are otherwise not obtainable from *in vitro* culture-based investigations alone. These may involve investigating microbial metabolism by comparing bile metabolites with fecal metabolites or lower gut metabolites with upper gut metabolites or comparison of drug metabolism between germfree or antibiotic-treated animals with conventional animals (Meuldermans et al., 1994; Sousa et al., 2008; Yoo et al., 2014).

Since the 1990s, the development of molecular biology gave rise to culture-independent techniques which revolutionized our knowledge of the gut microbiota (Fraher et al., 2012). The application of molecular tools has greatly improved our understanding of the microbial community by analyzing the structural diversity and functional activities of the microbes, even for those that cannot be cultured. The two key culture-independent approaches are based

on the analysis of the 16S ribosomal ribonucleic acid (rRNA) genes and the whole genome of the microbes respectively (Blaut et al., 2002; Tuohy and McCartney, 2006; Brugere et al., 2009; Dave et al., 2012; Fraher et al., 2012; Kuczynski et al., 2012; Weinstock, 2012).

Present throughout the cytoplasm of a bacterial cell are 70S ribosomes that are made up of two subunits, the 30S and 50S subunits. The 30S subunit is known to contain the 16S rRNA. The 16S rRNA gene is universally present in bacteria and has highly conserved domains flanking nine hypervariable regions which possess considerable sequence diversity to be used for distinguishing bacteria (Blaut et al., 2002; Chakravorty et al., 2007). As such, the 16S rRNA gene has been considered as a phylogenetic and evolutionary marker for bacteria identification (Blaut et al., 2002). Majority of the culture-independent techniques for the analysis of the gut microbiota are based on the analysis of the 16S rRNA genes. Such techniques include quantitative polymerase chain reaction (qPCR), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence *in situ* hybridization (FISH), DNA microarrays and sequencing of 16S rRNA amplicons (Blaut et al., 2002; Tuohy and McCartney, 2006; Fraher et al., 2012). Amongst these techniques, qPCR has been widely employed in gut microbiota investigation as the method is fast, sensitive, quantitative, enables phylogenetic discrimination and targeted analysis of specific bacteria of interest through primer design (Matsuki et al., 2002; Rinttilä et al., 2004; Tuohy and McCartney, 2006; Fraher et al., 2012). However, it is subjected to inherent bias of PCR-based techniques and does not allow identification of unknown bacteria (Polz and Cavanaugh, 1998; Acinas et al., 2005; Tuohy and McCartney, 2006). On the other hand, 16S rRNA gene sequencing facilitates detection of unknown bacteria and yields information on the proportion of various microbes that in turn allows inference on the composition of microbial communities (Fraher et al., 2012). The development of next-generation sequencing techniques (e.g. 454

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Pyrosequencing®, Illumina® and SOLiD™) has led to significant reduction in sequencing cost and time (Kuczynski et al., 2012), rendering these techniques to be widely adopted in research. In a study to examine the long term effects of exogenous microbiota transplantation combined with and without an antibiotic pretreatment using rat model, Maichanh *et al.* combined the application of qPCR and 16S rRNA gene sequencing to determine the degree to which the gut microbiota can be experimentally manipulated (Manichanh et al., 2010). qPCR was employed to elucidate the bacterial load present in the rat fecal samples and a decrease in bacterial load was confirmed following the administration of antibiotics. Using 16S rRNA gene sequencing, Maichanh *et al.* discovered that the exogenous transplant of cecal materials from different strains of donor rats led to a change in the fecal bacterial diversity of the recipients (without antibiotic pretreatment) such that it highly resembles the donor samples and the effect persist even up to 3 months following a single inoculation. Interestingly, pretreatment with antibiotics did not facilitate the establishment of the exogenous microbiota in the recipient rats. Instead, it culminated into a greater reshaping effect, leading to a gut microbiome composition that is distant from both the donor and long-term antibiotic treatment animal. Although this result is highly counterintuitive to the authors' original hypothesis of antibiotic helping to enhance the reshaping effect of microbiota transplantation, the authors suggested that their findings should be taken into consideration during the design of future fecal microbiota transplantation studies (Manichanh et al., 2010). Integrated approach that combines 16S rRNA gene sequencing with metabonomics has also been employed in system biology-based investigation. Using the integrated approach, Lu *et al.* revealed that arsenic significantly perturbed the gut microbiome composition of C57BL/6 mice. Correlation analysis further demonstrated that the abundance of selective perturbed bacteria was highly correlated with the altered gut microbiota-related metabolites (Lu et al., 2014).

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The other culture-independent technique known as whole-genome shotgun sequencing involves sequencing the entire genomes of the microbes (Dave et al., 2012; Fraher et al., 2012). This method involves random fragmentation of microbial DNA, sequencing of DNA fragments and reconstruction of overlapping sequence to form a continuous sequence (Fraher et al., 2012). Through whole-genome shotgun sequencing of the microbiome, microbial genes that encode for metabolic functions can be identified. This in turn provides insights into the potential functional activities of the microbes. In other words, whole-genome shotgun sequencing provides information on both genetic diversity and functions of the gut microbiota while 16S rRNA gene sequencing provides only information on genetic diversity. However, whole-genome shotgun sequencing comes at a higher cost and is computationally more intensive. Other drawbacks include the requirement for large amount of DNA for analysis unless genome amplification is performed and many genes identified may not have known function currently.

Conclusion

Currently, there remains limited knowledge pertaining to the roles of gut microbiota in modulating therapeutic outcome. The diverse metabolic capabilities of these gut microbes and their variable composition in human gut provide the impetus to scrutinize the intricate involvement of the gut microbes in accounting for inter-individual variability. With the advancement in omics technologies (e.g. metabonomics and metagenomics), we envisage that the deliberate and systematic integration of these technologies will empower scientists to better investigate the roles of gut microbiota in pharmacokinetics and pharmacotherapy. Novel drug candidates targeting the bacteria or their enzymes may be designed to modulate efficacy or toxicity (Jia et al., 2008; Holmes et al., 2012). The mitigation of irinotecan-induced gastrointestinal toxicity using β-glucuronidase inhibitors is one example (Wallace et

al., 2010). Manipulation of gut microbiota may also be effected via the administration of probiotics, prebiotics, antibiotics and fecal transplant (Jia et al., 2008; Holmes et al., 2012; Rohlke and Stollman, 2012). Considering the pervasive influence of the gut microbes on pharmacokinetics and therapeutic outcome, careful manipulation and engineering of the gut microbes may present exciting opportunities in personalized medicine.

Authorship contributions

Wrote or contributed to the writing of the manuscript: Yip and Chan

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Footnote

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Figure Legends

Fig. 1 (A) A typical metabonomics workflow and (B) flow diagram of various processes typically involved in GC/TOFMS-based metabonomics (Chan et al., 2011).

Fig. 2 Integrative approach to study host-gut microbiota interaction in pharmaceutical research.

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Tables

TABLE 1 Drugs whose pharmacokinetics or therapeutic outcomes are mediated by gut microbiota.

Reaction	Drug	Bacteria or its enzymes	Consequences of gut microbiota metabolism on drug pharmacokinetics and therapeutic outcomes
Reduction	Prontosil (Gingell et al., 1971)	Azoreductase enzymes	Activation of azo-bond containing pro-drug to sulfanilamide
	Neoprontosil (Gingell et al., 1971)		
	Sulfasalazine (Peppercorn and Goldman, 1972)		Activation of azo-bond containing pro-drug to 5-aminosalicylic acid
	Balsalazide (Chan et al., 1983)		
	Olsalazine (Wadworth and Fitton, 1991)		
	Nitrazepam (Rafii et al., 1997)	Nitroreductase	Co-metabolism of nitrazepam produces 7-acetylaminonitrazepam responsible for teratogenic activity: Step 1: Nitroreduction of nitrazepam to 7-aminonitrazepam by gut microbiota Step 2: 7-aminonitrazepam is converted to 7-acetylaminonitrazepam in the liver
	Clonazepam (Elmer and Remmel, 1984)	Not reported	Nearly complete reduction to 7-aminoclonazepam
	Misonidazole	Not reported	Reduction to 1-(2-aminoimidazol-1-yl)-3-

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Reaction	Drug	Bacteria or its enzymes	Consequences of gut microbiota metabolism on drug pharmacokinetics and therapeutic outcomes
	(Koch et al., 1980)		methoxypropan-2-ol
	Omeprazole (Watanabe et al., 1995)	Not reported	*In vitro* reduction by gut microbiota to omeprazole sulfide metabolite. However, there was no alteration of oral *in vivo* pharmacokinetics as omeprazole is fully absorbed before reaching hindgut.
	Sulfinpyrazone (Strong et al., 1987)	Not reported	Reduction to sulfinpyrazone sulfide metabolite (solely by gut microbiota)
	Sulindac (Strong et al., 1987)	Not reported	Reduction to sulindac sulfide metabolite
	Digoxin (Lindenbaum et al., 1981; Haiser et al., 2013)	*Eggerthella lenta*	Reduction to inactive metabolites (e.g. dihydrodigoxin or dihydrodigoxigenin) by gut microbiota reduce therapeutic efficacy
	Zonisamide (Kitamura et al., 1997)	*Clostridium sporogenes*	Reduction to 2-sulphamoylacetylphenol
	Metronidazole (Koch et al., 1979)	*Clostridium perfringens*	Reduction to N-(2-hydroxyethyl)-oxamic acid and acetamide
Hydrolysis	Lactulose (Sahota et al., 1982)	*Lactobacillus*, *Bacteroides* and *Clostridium*	Therapeutic activity depends on its metabolism by intestinal bacteria to form lactic and acetic acids
	Sorivudine (Okuda et al., 1998)	*Bacteroides* species (e.g. *Bacteroides eggerthii* and *Bacteroides*	A major metabolite of sorivudine, (E)-5-(2-bromovinyl)uracil, generated from microbial metabolism of the drug was found to inactivate a key hepatic enzyme

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Reaction	Drug	Bacteria or its enzymes	Consequences of gut microbiota metabolism on drug pharmacokinetics and therapeutic outcomes
		vulgatus)	involved in the metabolism of 5-fluorouracil. Co-administration of sorivudine and 5-fluorouracil resulted in drug interactions that led to death.
Deconjugation of drugs excreted in bile as inactive conjugates	Digitoxin (Volp and Lage, 1978)	β-glucuronidase	Hydrolysis of glucuronide
	Indomethacin (Saitta et al., 2014)		Hydrolysis of glucuronide of indomethacin release the aglycone which leads to gastrointestinal toxicity
	Morphine (Walsh and Levine, 1975)		Hydrolysis of glucuronide
	Irinotecan (Roberts et al., 2013)		Hydrolysis of SN-38 glucuronide of irinotecan (pro-drug) release SN-38 in the intestines which leads to gastrointestinal toxicity
Removal of succinate group	Succinylsulfathiazole (Sousa et al., 2008)	Not reported	Activation of pro-drug to sulfathiazole
Dehydroxylation	L-Dopa (Goldin et al., 1973)	Not reported	Alteration of L-dopa pharmacokinetics by gut microbiota metabolism to form m-tyramine and m-hydroxyphenylacetic acid
Acetylation	5-Aminosalicylic acid (Dull et al., 1987; Delomenie et al.,	N-acetyltransferase	Acetylation to form acetylated 5-aminosalicylic acid

Reaction	Drug	Bacteria or its enzymes	Consequences of gut microbiota metabolism on drug pharmacokinetics and therapeutic outcomes
	2001)		
Deacetylation	Phenacetin (Smith and Griffiths, 1974)	Not reported	Formation of p-phenetidin from deacetylation reaction is correlated with toxicities such as methemoglobinemia and nephritis
Cleavage of N-oxide bond	Ranitidine (Basit and Lacey, 2001)	Not reported	Susceptible to N-oxide bond cleavage by gut bacteria
	Nizatidine (Basit et al., 2002)	Not reported	Susceptible to N-oxide bond cleavage by gut bacteria
Proteolysis	Insulin (Tozaki et al., 1997)	Not reported	Susceptible to proteolysis
	Calcitonin (Tozaki et al., 1997)	Not reported	Susceptible to proteolysis
Denitration	Glyceryl trinitrate (Abu Shamat and Beckett, 1983; Sousa et al., 2008)	Not reported	Generate glyceryl-1,3-dinitrate, glyceryl-1,2-dinitrate, glyceryl-1-mononitrate and glyceryl-2-mononitrate
	Isosorbide dinitrate (Sousa et al., 2008)	Not reported	Generate isomeric mononitrates and isosorbide
Amine formation and hydrolysis of amide linkage	Chloramphenicol (Holt, 1967)	Not reported	Metabolized to metabolites such as p-aminophenyl-2-amino-1,3-propanediol. Aplasia of the marrow, the most serious complication of chloramphenicol, has been proposed to be due to the activity of the intestinal microbiota.

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Reaction	Drug	Bacteria or its enzymes	Consequences of gut microbiota metabolism on drug pharmacokinetics and therapeutic outcomes
Thiazole ring-opening	Levamisole (Shu et al., 1991)	*Bacteroides* and *Clostridium* species	Generate levametabol-I, II and III metabolites
Isoxazole scission	Risperidone (Meuldermans et al., 1994)	Not reported	Scission of the isoxazole in the benzisoxazole ring system of risperidone is a major metabolic pathway contributed by the gut microbiota
N-demethylation	Methamphetamine (Caldwell and Hawksworth, 1973)	Not reported	Converted to amphetamine by gut microbiota. May be inconsequential to man since the parent drug is quite efficiently absorbed in the upper gastrointestinal tract.
Competition of microbial metabolite for Phase II drug clearance	Acetaminophen (Clayton et al., 2009)	Bacteria like *Clostridium difficile* is a p-cresol producer	High pre-dose levels of microbial metabolite p-cresol compete for clearance by hepatic sulfotransferase and diminish the host's metabolic capacity for Phase II sulfonation of acetaminophen.
Competition of microbial metabolite for hepatic uptake of drug	Simvastatin (Kaddurah-Daouk et al., 2011)	Bacteria like *Lactobacillus* is involved in production of coprostanol	Microbially derived secondary bile acids may compete with simvastatin for hepatic uptake by SLCO1B1 transporters, thereby affecting the pharmacokinetics and pharmacodynamics of simvastatin, and increasing the risk of myopathy.

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TABLE 2 List of metabolites modulated in host harboring different gut microbial community. The biological matrices in which differential abundance of these microbial-related metabolites are reported and the respective analytical platforms (NMR, LC or GC/MS) used for detection of these metabolites are provided to facilitate design and interpretation of metabonomic studies on host-gut microbiota interaction. Readers are encouraged to refer to literature such as (Nicholson et al., 2012) for the related bacteria and biological functions or consequences of the metabolites.

Metabolites	Biological matrix#	Analytical platform	Host	References
Bile acids				
Chenodeoxycholic acid	P, K, H, F (#refer to abbreviations at table footnote)	LC	Rat	(Swann et al., 2011; Zheng et al., 2011)
Cholic acid	P, L, K, H, F	NMR, LC	Mice, Rat	(Swann et al., 2011; Zheng et al., 2011; Zhao et al., 2013)
Deoxycholic acid	P, L, K, F	NMR, LC	Mice, Rat	(Swann et al., 2011; Zhao et al., 2013)
Glycochenodeoxycholate	P, K, H, L	LC	Rat	(Swann et al., 2011)
Glycocholic acid	P, K, H, L	LC	Rat	(Swann et al., 2011)
Glycodeoxycholic acid	P, K, H, L	LC	Rat	(Swann et al., 2011)
Hyocholic acid	K, H	LC	Rat	(Swann et al., 2011)

Metabolites	Biological matrix#	Analytical platform	Host	References
Hyodeoxycholic acid	K, H, L, F	LC	Rat	(Swann et al., 2011; Zheng et al., 2011)
Taurochenoxycholic acid	P, L, K, H	LC	Rat	(Swann et al., 2011)
Taurocholic acid	P, L, K, H, F	NMR, LC	Mice, Rat	(Swann et al., 2011; Zhao et al., 2013)
Taurodeoxycholic acid	P, K, H	LC	Rat	(Swann et al., 2011)
Tauro-α-muricholic acid	K	LC	Rat	(Swann et al., 2011)
Tauro-β-muricholic acid	P, L, K, H, F	NMR, LC	Mice, Rat	(Swann et al., 2011; Zhao et al., 2013)
Tauro-ω-muricholic acid	K	LC	Rat	(Swann et al., 2011)
Ursodeoxycholic acid	P, K, H, L	LC	Rat	(Swann et al., 2011)
α-Muricholic acid	L, K, H	LC	Rat	(Swann et al., 2011)
β-Muricholic acid	P, L, K	LC	Rat	(Swann et al., 2011)
ω-Muricholic acid	K, H	LC	Rat	(Swann et al., 2011)
Choline metabolites				
Betaine	K	NMR	Mice	(Claus et al., 2008)
Dimethylamine	U	NMR	Mice	(Claus et al., 2008)
Trimethylamine	U	NMR	Mice	(Claus et al., 2008)
Trimethylamine N-oxide	U, L	NMR	Mice	(Claus et al., 2008)
Phenolic, benzoyl and phenyl derivatives				
2-(4-hydroxyphenyl)propionic	F	NMR	Mice	(Zhao et al., 2013)

Metabolites	Biological matrix#	Analytical platform	Host	References
acid				
3-(3,4-Dihydroxyphenyl)lactic acid	U	LC	Rat	(Zheng et al., 2011)
3-(3-Hydroxyphenyl)propanoic acid	F, U	GC	Rat	(Zheng et al., 2011)
3-Hydroxycinnamic acid	U	NMR	Mice	(Claus et al., 2008)
4-Hydroxyphenylpropionic acid	U	NMR, LC	Mice, Rat	(Claus et al., 2008; Zheng et al., 2011)
4-Hydroxyphenylpyruvic acid	U	LC	Rat	(Zheng et al., 2011)
5-Phenylvaleric acid	F	LC	Rat	(Zheng et al., 2011)
Aminophenol	F	LC	Rat	(Zheng et al., 2011)
Benzoyl glucuronide	U	LC	Rat	(Zheng et al., 2011)
Cinnamoylglycine	P	LC	Mice	(Wikoff et al., 2009)
Hippuric acid	U, P	NMR, LC, GC	Mice, Rat	(Claus et al., 2008; Yap et al., 2008; Wikoff et al., 2009; Zheng et al., 2011)
Hydroxyphenylacetylglycine	U	LC	Rat	(Zheng et al., 2011)
Hydroxyphenyllactic acid	F	LC	Rat	(Zheng et al., 2011)
m-Hydroxyphenylacetic acid	F, U	GC	Rat	(Zheng et al., 2011)

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Metabolites	Biological matrix#	Analytical platform	Host	References
N-Acetyl-L-phenylalanine	U	LC	Rat	(Zheng et al., 2011)
p-Aminobenzoic acid	F	LC	Rat	(Zheng et al., 2011)
p-Cresol	U	GC	Rat	(Zheng et al., 2011)
p-Cresol sulfate	P	LC	Mice	(Wikoff et al., 2009)
Phenol	U	GC	Rat	(Zheng et al., 2011)
Phenyl sulfate	P	LC	Mice	(Wikoff et al., 2009)
Phenylacetic acid	F, U	LC	Rat	(Zheng et al., 2011)
Phenylacetylglycine	P, U	NMR, LC	Mice	(Claus et al., 2008; Wikoff et al., 2009)
Phenylalanine	F, P, L, K, D, J, I, C	NMR, LC, GC	Mice, Rat	(Claus et al., 2008; Yap et al., 2008; Wikoff et al., 2009; Zheng et al., 2011; Zhao et al., 2013)
Phenylalanyl-hydroxyproline	F, U	LC	Rat	(Zheng et al., 2011)
Phenylethanolamine	F, U	LC	Rat	(Zheng et al., 2011)
Phenylglycine	F	LC	Rat	(Zheng et al., 2011)
Phenyllactic acid	F, U	LC, GC	Rat	(Zheng et al., 2011)
Phenylpropionylglycine	P	LC	Mice	(Wikoff et al., 2009)
p-Hydrocinnamic acid	F, U	GC	Rat	(Zheng et al., 2011)
p-Hydroxybenzaldehyde	F	GC	Rat	(Zheng et al., 2011)
p-Hydroxybenzoic acid	U	GC	Rat	(Zheng et al., 2011)

Metabolites	Biological matrix#	Analytical platform	Host	References
p-Hydroxyphenylacetic acid	F, U	NMR, GC	Mice, Rat	(Zheng et al., 2011; Zhao et al., 2013)
Polyamines				
Cadaverine	F, U	GC	Rat	(Zheng et al., 2011)
Putrescine	F, U	NMR, GC	Mice, Rat	(Zheng et al., 2011) (Claus et al., 2008)
Spermidine	F	LC	Human	(Matsumoto and Benno, 2007)
Spermine	F	LC	Human	(Matsumoto and Benno, 2007)
Short chain fatty acids				
Acetic acid	F, U, L, K, D, J, I, C	NMR	Mice	(Claus et al., 2008; Yap et al., 2008)
Butyric acid	F, U	LC, GC	Rat	(Zheng et al., 2011)
Hexanoic acid	F	GC	Rat	(Zheng et al., 2011)
Isobutyric acid	F, U	GC, LC	Rat	(Zheng et al., 2011)
Isovaleric acid	F, U	NMR, LC	Mice, Rat	(Claus et al., 2008; Zheng et al., 2011)
Propionic acid	F, U	NMR, GC	Mice, Rat	(Yap et al., 2008; Zheng et al., 2011)
Tryptophan, indole derivatives				
2-Indoleacetaldehyde	F, U	LC	Rat	(Zheng et al., 2011)

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Metabolites	Biological matrix#	Analytical platform	Host	References
2-Indolecarboxylic acid	F, U	LC	Rat	(Zheng et al., 2011)
3-Indolepropionic acid	F	LC	Rat	(Zheng et al., 2011)
3-Methyldioxyindole	F	LC	Rat	(Zheng et al., 2011)
5-Hydroxyindoleacetic acid	U	LC	Rat	(Zheng et al., 2011)
5-Hydroxyindoleacetylglycine	U	LC	Rat	(Zheng et al., 2011)
5-Hydroxytryptophan	U	NMR	Mice	(Claus et al., 2008)
5-Hydroxytryptophol	F, U	LC	Rat	(Zheng et al., 2011)
6-Hydroxymelatonin sulfate	F, U	LC	Rat	(Zheng et al., 2011)
Hydroxykynurenine	U	LC	Rat	(Zheng et al., 2011)
Indole	U	LC	Rat	(Zheng et al., 2011)
Indole-3-propionate	P	LC	Mice	(Wikoff et al., 2009)
Indoleacetic acid	U	LC	Rat	(Zheng et al., 2011)
Indolelactic acid	U	LC	Rat	(Zheng et al., 2011)
Indoxyl	F	LC	Rat	(Zheng et al., 2011)
Indoxyl sulfate	P, U	LC, GC	Mice, Rat	(Wikoff et al., 2009; Zheng et al., 2011)
Kynurenic acid	U	GC	Rat	(Zheng et al., 2011)
N-Acetyltryptophan	P	LC	Mice	(Wikoff et al., 2009)
N-Methyltryptamine	U	LC	Rat	(Zheng et al., 2011)
Serotonin	P	LC	Mice	(Wikoff et al., 2009)
Tryptamine	F, U	LC	Rat	(Zheng et al., 2011)

Metabolites	Biological matrix#	Analytical platform	Host	References
Tryptophanol	U	LC	Rat	(Zheng et al., 2011)
Vitamins				
Biotin	U	LC	Rat	(Zheng et al., 2011)
Pantothenic acid	U	LC	Rat	(Zheng et al., 2011)
Pyridoxal	F	LC	Rat	(Zheng et al., 2011)
Pyridoxine	F	LC	Rat	(Zheng et al., 2011)
Riboflavin	F	LC	Rat	(Zheng et al., 2011)
Thiamine	F	LC	Rat	(Zheng et al., 2011)

#Abbreviations: urine (U), feces (F), kidney (K), plasma (P), liver (L), heart (H), duodenum (D), jejunum (J), ileum (I) and colon (C).

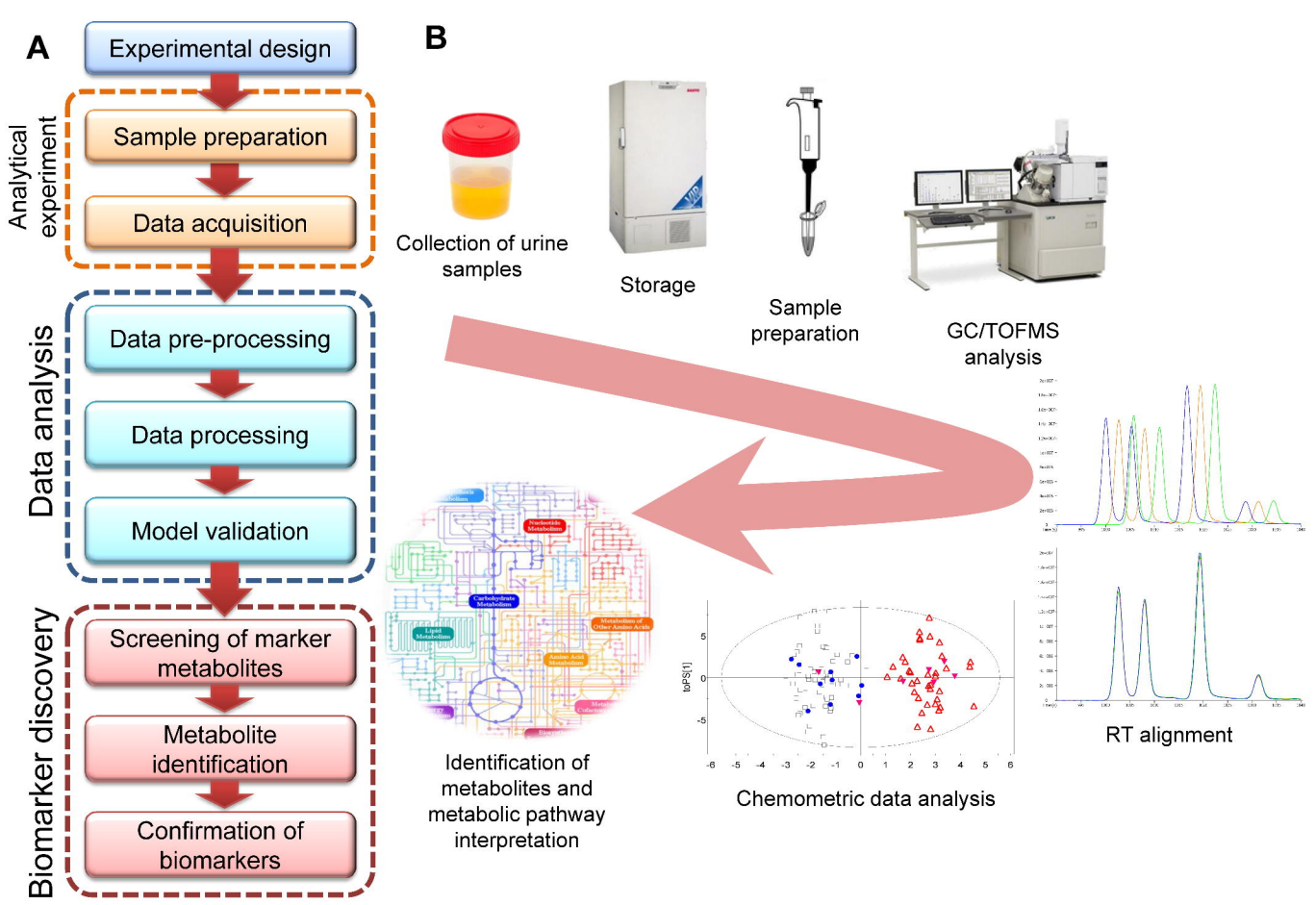
A
Experimental design
Analytical experiment
Sample preparation
Data acquisition
Data analysis
Data pre-processing
Data processing
Model validation
Biomarker discovery
Screening of marker metabolites
Metabolite identification
Confirmation of biomarkers
B
Collection of urine samples
Storage
Sample preparation
GC/TOFMS analysis
RT alignment
Identification of metabolites and metabolic pathway interpretation
Chemometric data analysis

Figure 1.

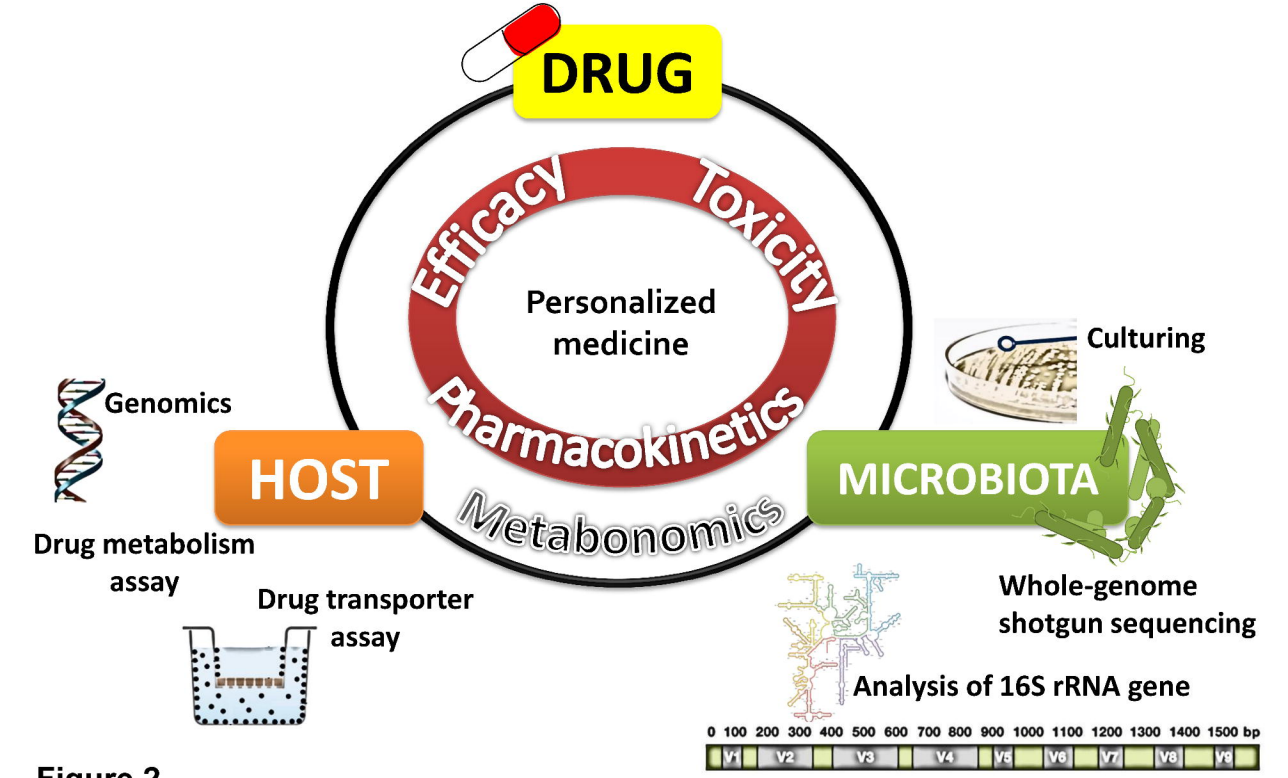
DRUG
Efficacy
Toxicity
Pharmacokinetics
Metabonomics
Personalized medicine
HOST
MICROBIOTA
Genomics
Drug metabolism assay
Drug transporter assay
Culturing
Whole-genome shotgun sequencing
Analysis of 16S rRNA gene
0 100 200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 bp
V1
V2
V3
V4
V5
V6
V7
V8
V9

Figure 2.