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Screening Metabolites of Host Microbial Origin that Activate Ligand-Dependent Transcription Factors

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ABSTRACT

The microbiome is a complex symbiosis of microorganisms that inhabit the human body. Numbering in the trillions, these microorganisms play essential roles in human health and maintaining overall homeostasis. Gut microbiota work to metabolize a large number of natural products in concert with host metabolism. Many of these metabolites produce their physiological effects through the binding of transcription factors that mediate expression of genes important in metabolism. One such transcription factor is the pregnane X receptor (PXR), a type II nuclear receptor important in regulating the expression of proteins that function in host metabolism. To gain a better understanding of how gut microbiota metabolism affects expression of genes important in host metabolism, we screened 200 metabolites of microbial origin for their potential to activate PXR. We performed a firefly luciferase reporter gene assay utilizing human hepatocyte cells engineered to express a modified form of PXR that binds to and initiates transcription of the firefly luciferase gene. Expression of luciferase leads to quantifiable bioluminescence that acted as a proxy for PXR activation. After screening each microbial metabolite for its ability to activate PXR, we chose the most active metabolites and generated dose-response curves for PXR activation. Our results furthered the paradigm that the microbiome is highly involved in human physiology, especially the metabolism of xenobiotic materials.

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Chapter 1

Introduction

The Microbiome

Human life as we know it would not be possible without the help of the microbes that make up the microbiome. Defined as the community of microorganisms that may exist in a specified environment, the microbiome comprises trillions of living organisms that are not of host origin.[1] These microbes can be anything from different species of bacteria to fungi, archaea, and viruses. Bacteria present in the microbiome are important regulators of digestion along the gastrointestinal (GI) tract.[2] The bacterial phyla most dominant include Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Fusobacteria, and Verrucomicrobia, although nearly 90 percent of gut microbiota are represented by Firmicutes and Bacteroidetes.[2] Interestingly, every individual on Earth has a completely unique microbial signature that develops at birth. This is due to factors over one's lifetime, such as lifestyle, diet, social environment, exposure to xenobiotics, and use of antibiotic treatments.[3] The fact that there are an estimated 500 to 1,000 different species of bacteria within the human body at any one time also alludes to individual microbial uniqueness.[4] Due to the many roles it plays in human health as well as human disease, the microbiome has attracted significant interest.

The field of microbiome research has made great strides over the past two decades as a result of technological developments, but its history spans over hundreds of years. Its origins began in the field of microbiology. The innovation of microscopy in the 17th century led to the discovery of

a previously unknown world of microorganisms through the work of Anthony van Luewenhook.[5] This paved the way for Ferdinand Cohn to separate bacteria into the taxonomic classifications still in use today.[5] Eventually, environmental microbiology work in the late 19th century showed that these microbes are everywhere in the natural environment and provide benefits for the hosts they comprise. This changed the widely accepted idea that microorganisms are only associated with disease and should be eliminated.[5] Discovery of deoxyribonucleic acid (DNA) in the 1940s as the molecular basis of genetic information eventually allowed for the development of technologies that would further advance the field of microbiology, including sequencing techniques for elucidating specific DNA sequences in organisms and polymerase chain reaction for amplifying DNA sequences in order to study them closer.[5] It was around this time too that researchers laid the foundation for the idea that microorganisms affect metabolism in mammalian hosts. Danielsson and Gustafsson found that germ-free rats, or rats isolated from microorganism exposure, had increased levels of serum-cholesterol compared to control rats, partly due to their inability to metabolize cholesterol into coprostanol.[6] Further advances in technology, such as observing bacterial 16S ribosomal ribonucleic acid which allows for more accurate identification of bacterial taxa, have created a wealth of available microbial data.[5] These historical landmarks, coupled with the increasing range of data, have led researchers to the current paradigm that microbes are ubiquitous and critical to the health of both animals and plants.[5]

The microbiome extends beyond the GI tract, populating a large portion of body tissues and surfaces.[7] Aerobic organisms, or those reliant on oxygen, are generally prevalent on more exterior surfaces of the host, like the skin, due to oxygen availability. Larger proportions of microorganisms inhabit occluded areas of the skin, like the perineum and between the toes, as a

result of more moisture and ideal temperatures.[7] As one travels deeper into the host along the GI tract, an increasing abundance of anaerobic organisms is present as oxygen availability decreases. Microorganisms within the host are mainly located along the lining of the large intestine. It is estimated that the floral concentration in the colon ranges between 10^9 and 10^{11} bacteria per gram, with large concentrations of anaerobic bacteria such as Bacteroides and Bifidobacterium.[7] The exception to this large concentration of microorganisms in the GI tract is within the stomach, where bacteria concentrations are usually between 10^3 and 10^6 after meals and significantly lower after digestion.[7] The stomach's highly acidic environment makes it difficult to inhabit, and microbiota here are often transient, considering the level of acidity at different times of digestion.[7] Microbiota within the colon produce metabolic products as a result of reacting with endogenous host chemicals.[7] These reactions mediate many of the beneficial effects conferred by the microbiome to its host.

Advances in technological development throughout history have allowed researchers to understand that host microorganisms perform a multitude of functions crucial for human health. These functions include protection against pathogens, immune support, biotransformation of foreign materials, and food digestion.[3] Human digestive enzymes are incapable of breaking down many complex carbohydrates and plant polysaccharides, so microbes in the gut work to ferment these dietary biomolecules to facilitate nutrient absorption.[8] Nondigestible carbohydrates, including dietary fibers that pass through the small intestine into the large intestine intact, are one of the primary groups that are fermented by the microbiome.[9] These dietary fibers can be separated into soluble and insoluble, where physicochemical differences allow for different degrees of fermentation by gut microbiota. Even though insoluble fibers help with bowel movements and can relieve constipation, soluble fibers

are generally degraded by microbial enzymes due to greater accessibility from their increased water solubility.[10] These soluble fibers are often metabolized into short chain fatty acids, which promote a wealth of beneficial functions for humans including anti-inflammatory effects in the gut and promoting intestinal absorption.[10] This functionality highlights that metabolites generated by microbial metabolism support human health, but disruption in this homeostasis often results in detrimental effects.

The human microbiome plays essential roles in maintaining homeostasis, and disruptions in its functioning can contribute to various human diseases. The gut microbiome profile is observed to be altered in conditions like type II diabetes, obesity, and cardiovascular disease.[11] An example of a disease directly linked to dysregulation within the microbiome is an infection with *Clostridioides difficile* (*C. diff*). Normally, a healthy microbiome offers significant protection against *C. diff* infections. However, the administration of antibiotics causes an extreme disruption in patients' microbiomes, leading to an observed decrease in microbial diversity within days of first use.[12] This abrupt shift in the composition of the microbiome creates an opportunity for the colonization of pathogenic microbes, including *C. diff*.[12] Research in rodent models and *in vitro* models showed that exposure to clindamycin, an antibiotic, reduced populations of obligate anaerobic bacteria in the gut and resulted in an overabundance of Enterobacteriaceae bacterial families.[13] Also, tigecycline exposure led to lower Bacteroidetes populations and higher Proteobacteria populations.[13] Both of these specific microbial effects are linked to an increased susceptibility to *C. diff* Infection.[13] *C. diff* infection causes inflammation of the intestinal lining, or colitis, leading to water loss through diarrhea and often severe abdominal pain.[14] Treatment often involves administering different antibiotics to disrupt *C. diff*,

but a common outcome is recurrent *C. diff* infections due to continuous microbial dysregulation.[13]

The microbiome provides the host with an abundance of benefits essential for survival. The understanding of its role in human health is the culmination of a long history of biochemical research and technological advancements. Normal microbial operation is so crucial that dysregulation in its functioning can lead to deviations from host homeostasis and creates opportunities for pathogens to invade. The significant concentration of anaerobic microorganisms in the host's colon participates in metabolic reactions, many of which confer nutrients that the host cannot produce through endogenous mechanisms. How metabolites from these reactions interact with the body is crucial to the microbiome's significance to human life. The mechanisms driving the beneficial effects that the microbiome provides for the host, as well as how these may interact with endogenous reactions in host cells, were further investigated.

Microbial Biotransformation

The role of the microbiome in facilitating biotransformation reactions was a focus of this research. Gut microbiota secrete enzymes that catalyze transformation reactions within the host, representing a key mechanism through which the microbiome mediates its beneficial effects.[15] These enzymes play a crucial role in transforming xenobiotic substances as well as endobiotic products from host metabolic reactions (Figure 1). The microbial enzymes involved in these biotransformation reactions essentially add or subtract functional groups, often resulting in a more physiologically active biomolecule. An example of a microbially catalyzed bioactivation reaction is the metabolism of tryptophan, an essential amino acid in the human

diet.[16] While the majority of dietary tryptophan is absorbed through the small intestine, a significant portion reaches the large intestine where it is degraded by gut microbes into various bioactive metabolites, including tryptamine and 3-indolepropionic acid.[16] These metabolites produce different effects that may not have occurred without microbial metabolism, including the prevention of reactive oxygen species damage to the brain (Figure 2).[16]

Many of the biomolecules processed by gut microbiota cannot be digested by the host themselves, including the aforementioned nondigestible carbohydrates such as dietary fibers. The metabolism of these dietary molecules often yields essential nutrients that the body utilizes in physiological processes. The microbiome's ability to perform certain biotransformation reactions, when the host cannot, is due to the extensive gene pool that trillions of microbes within the microbiome offer.[3] The microbial gene pool in the human gut is 100 times larger than the host genome in total.[3] Such a large genetic collection is diverse and could encode for a wide range of proteins capable of catalyzing reactions that the host would be unable to perform. This is especially true with respect to anaerobic species within the colon. An example is the anaerobic microbial species *Bacteroides thetaiotaomicron*, primarily found in the colon.[11] *Bacteroides thetaiotaomicron* encodes a wealth of metabolic enzymes that catalyze reactions in the anaerobic fermentation of dietary fibers.[11] The presence of microbes producing proteins important in anaerobic fermentation significantly benefits the host, enabling the absorption of essential nutrients even though the host cannot digest dietary fiber itself.

Bifidobacterium bifidum and *Bifidobacterium longum* produce enzymes important in folate, or vitamin B9, synthesis, further highlighting the microbiome's diverse genome. Vitamins are essential nutrients that act as precursors for important reactions in the body, supporting

physiological systems such as the immune system and the nervous system while also aiding in the absorption of dietary energy and blood clotting.[17] Humans are incapable of producing vitamins through endogenous mechanisms, so vitamins must be absorbed from exogenous sources. While the primary means of acquiring most natural vitamins is through the diet, gut microbes are also able to synthesize vitamins that host cells utilize to their advantage.[18] Microbes in the gut synthesize vitamin K, a fat-soluble vitamin crucial in the synthesis of blood-clotting proteins like prothrombin.[18] Different species of bacteria in the phylums of Firmicutes, Bacteroidetes, and Proteobacteria possess genomes capable of producing the necessary proteins for the vitamin K molecular synthesis pathway.[19] In addition to folate, gut microbiota also synthesize other water-soluble B vitamins, such as biotin, cobalamin, riboflavin, and thiamine.[17] Folate plays a crucial role in processes such as DNA replication by synthesizing nucleotides and contributing to amino acid synthesis.[17] Microbially synthesized vitamins are generally absorbed through the colon of the large intestine.[17]

Microbial biotransformation can result in the inactivation of xenobiotic and endobiotic chemicals rather than increasing their physiological activities. In this case, enzymatic reactions result in a decreased capacity to interact with biological systems, resulting in lower bioactivity. While this inactivation reduces the efficacy of beneficial drugs, it can also decrease the toxicity of adverse compounds.[20] An example of a microbial reaction leading to the decreased efficacy of a drug is digoxin. Digoxin is a cardiac glycoside used in treating heart failure and heart arrhythmias through increasing the force of contraction of the heart.[21] It has been discovered that the Actinobacterium *Eggerthella lenta* is responsible for a portion of digoxin metabolism through reducing enzymes that convert digoxin to dihydrodigoxin, a metabolite of digoxin.[22] This metabolism can lead to poor efficacy of digoxin in heart disease patients due to the generation of

this inactive metabolite.[22] Mechanisms through which drug efficacy for the host is decreased by the microbiome emphasizes the importance of both the microbial genome and the host genome in metabolism.

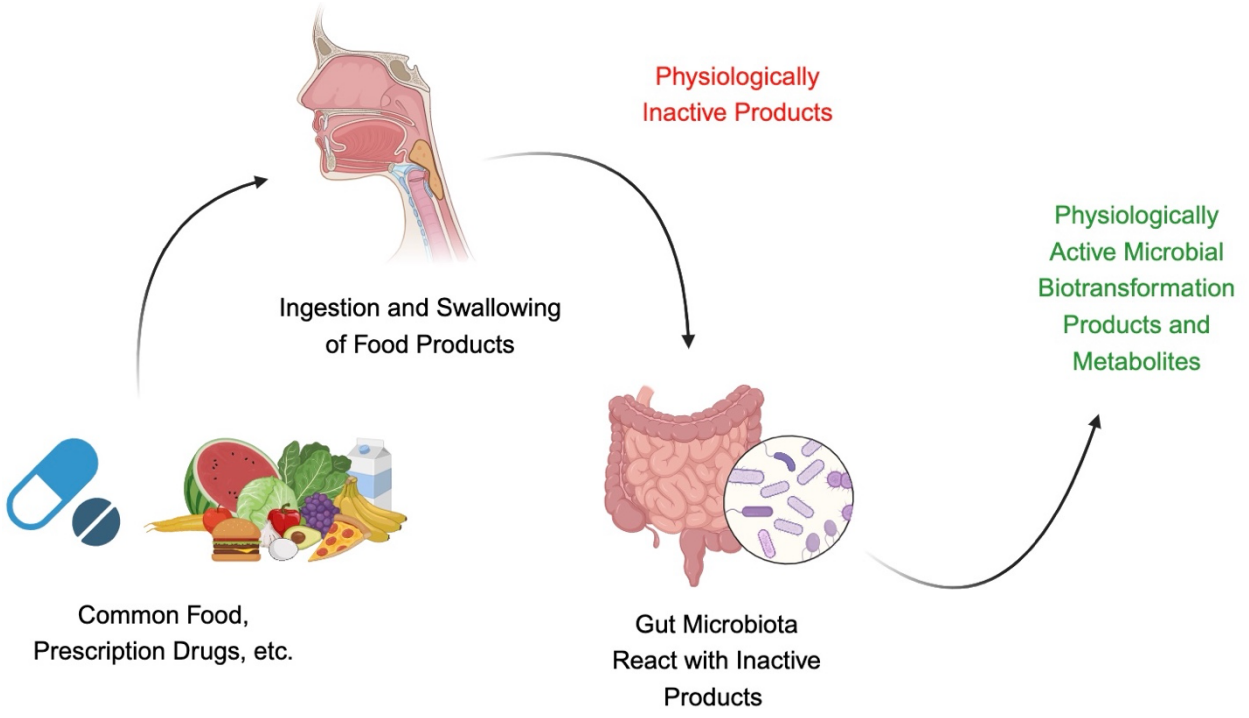


Figure 1: General illustration of the biologically inactive products pathway to microbial biotransformation and subsequent production of physiologically active metabolites with increased bioavailability to react with the body.

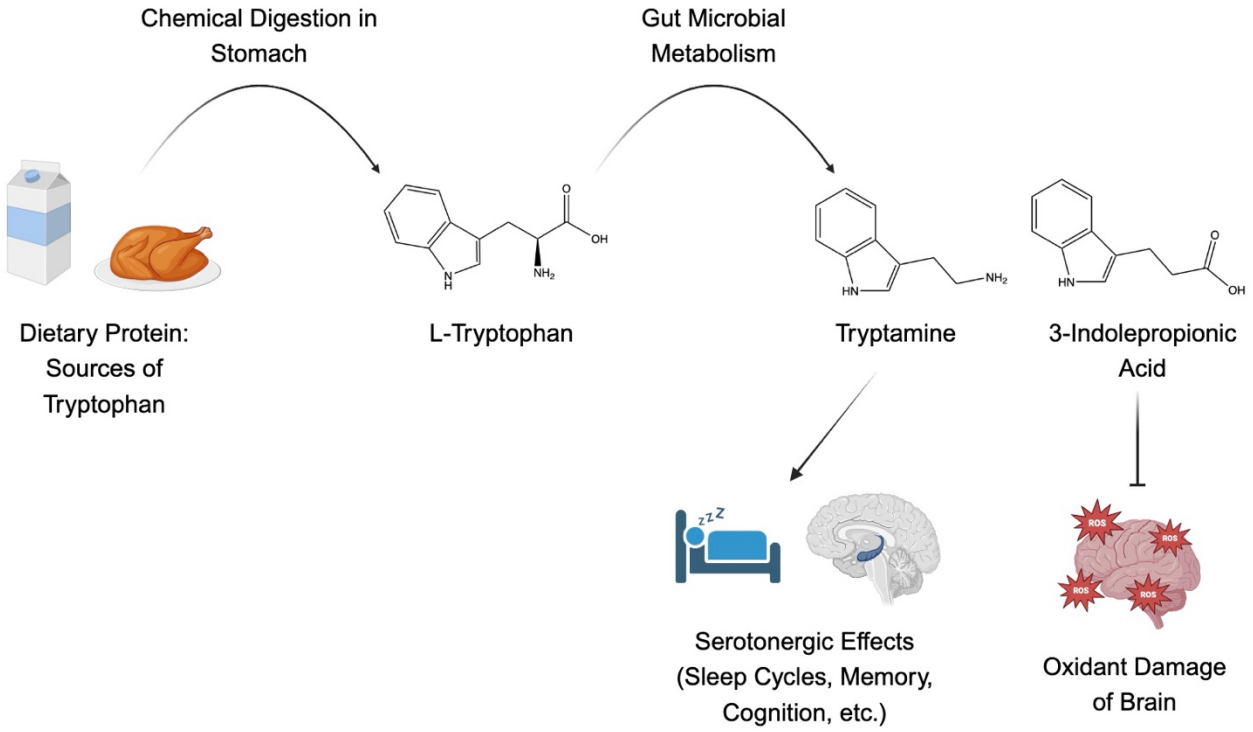


Figure 2: A common microbial bioactivation reaction involves metabolizing tryptophan into Tryptamine and 3-Indolepropionic Acid (3-IPA) through gut microbial catalysts. Tryptamine is a regulator of different neurotransmitter pathways, including serotonin, while 3-IPA is a potent antioxidant in the brain that may be implicated in the gut-brain axis (adapted from Gao et al., 2020).[16]

Host Metabolism

Human cells perform biotransformation reactions of chemicals from dietary, pharmaceutical, or toxic sources alongside microbial biotransformation. The parameters of how the body affects exogenous substances through absorption, distribution, metabolism, and excretion are termed pharmacokinetics.[23] Substances introduced to the body from these sources are first absorbed into the circulatory system. Absorption into the bloodstream can occur through oral, inhalational, and dermal routes as well as through direct administration with an injection.[23] Aside from direct

administration, these substances are absorbed through membranes such as the GI tract after digestion into macro-chemical products.[23] Absorption into the circulatory system allows the chemicals to distribute throughout the body where they can interact with body tissues.[23] In order for these chemicals to be suitable for excretion, they are often processed into different forms through metabolic reactions.

The main site of host metabolism is the liver. Venous blood flow from organs throughout the body is shunted to the liver via the hepatic portal system. It is here that most host metabolic enzymes catalyze biotransformation reactions in order to make chemicals easier to excrete. These transformations make the chemicals more water soluble so that they can be removed through urination or defecation. Host metabolism can be differentiated into phase I, and phase II metabolism.[24] Phase I metabolism consists mainly of oxidation, reduction, and hydrolysis reactions that add functional groups that make the drug more polar, or hydrophilic.[24] These reactions are generally catalyzed by a group of metabolizing enzymes termed cytochrome P450s located mainly in hepatocytes, or liver cells. Reactions that occur during phase II metabolism utilize these hydrophilic functional groups to conjugate the chemicals with endogenous molecules to further increase water solubility and thus excretability.[24] Conjugation reactions are catalyzed by a large number of enzymes, but important phase II enzymes include uridine 5'-diphospho-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs).[25] UGTs work through transferring a glucuronic acid group to the chemical and SULTs work by transferring a sulfate group, both of which increase the water-solubility of the compound for excretion.[25] Phase III metabolism can ultimately occur where the

metabolized chemical is essentially transported out of the cell where phase I and phase II occurred.[24]

Chemicals are more suited for excretion after being metabolized into more water soluble forms. Elimination of chemicals from the body can be dependent upon the given concentration or independent of the given concentration. Most drug excretion is dependent on its concentration, although one example of where elimination is independent of concentration is alcohol. Ethanol is excreted from the body at a constant rate of around 15 milliliters per hour regardless of how much is consumed.[24] Clinicians use these principles of drug elimination to provide specific doses to patients based on factors such as their ability to metabolize the drug and their size.[24] Not all chemicals are completely excreted from the body after consumption, though. Compounds that are highly lipophilic are often not completely excreted and have the ability to bioaccumulate in the body.[23] Adverse effects can occur when drugs bioaccumulate to a large degree. An example of this is the bioaccumulation of psychiatric drugs, such as antidepressants and anti-anxiety drugs.[26] Bioaccumulation of these psychiatric drugs has been associated with endocrine disorders and deficiencies in reproductive, growth, and metabolic functioning.[26]

The activity of pharmaceutical drugs and adverse chemicals often decreases through the effects of host biotransformation reactions. Similarly to those discussed with microbial biotransformation, host metabolic reactions can lead to bioactivation of chemicals too. Through this mechanism, drugs can be designed to stay inactive until host metabolism, usually during phase I, transforms it into its active state.[24] These pharmaceuticals are termed prodrugs.[24] One of the main reasons why it may be beneficial to design a prodrug rather than just administer the

parent, bioactive drug itself is because the parent drug is often unstable when administered, resulting in lower pharmaceutical efficacy.[27] A specific example of host metabolism leading to activation of a prodrug is valacyclovir.[27] Valacyclovir is a prodrug that is metabolized to the active pharmaceutical acyclovir, which is a treatment for herpes virus.[27] Valacyclovir is administered over its parent drug molecule because it is absorbed to a greater extent through the host intestinal lumen due to its greater affinity for the human peptide transporter 1.[27] Once valacyclovir is distributed to different tissues in the body, it is quickly metabolized to acyclovir intracellularly catalyzed by the enzyme serine hydrolase.[27] This highlights the important functionality of host metabolism in pharmaceutical efficacy, paralleling that of microbial metabolism.

Microbial biotransformation occurs simultaneously in the body with host biotransformation, a process integral to metabolism.[28] It is common for substances to undergo transformation by the microbiome directly upon ingestion without prior host metabolism.[3] However, products of hepatic metabolic reactions performed by the host often return to the intestinal lumen through bile released by the liver.[3] This makes the metabolites available for further metabolism by gut microbiota. Gut microbiota reprocess bile acids through this process. Bile acids are cholesterol-based macromolecules produced by host liver cells and possess both hydrophilic and hydrophobic components.[29] These primary bile acids are stored and released by the gallbladder into the small intestine where hydrophilic and hydrophobic parts are crucial to aiding the host in lipid absorption. Bile acids that are not absorbed in the small intestine through this process are converted to secondary bile acids, like deoxycholic acid (DCA) and lithocholic acid (LCA), by gut microbiota in the large intestine.[29] Secondary bile acids can be absorbed passively through the colon where they can facilitate beneficial effects for the host such as glucose homeostasis and

immune system modulation.[29] The concerted work of host cells and microbial cells in cometabolizing substances is essential to homeostasis.

Considering host reactions and microbial reactions are occurring simultaneously, it is possible that host metabolism and microbial metabolism could compete for similar pathways. An example of this host-microbiome cometabolism is the common non-prescription drug acetaminophen, or Tylenol.[30] Sulfonation of acetaminophen during phase II is crucial for its excretion, so chemicals that compete for this pathway would influence acetaminophen metabolism. Data suggests that microbial production of *p*-cresol competes for this metabolic pathway as *p*-cresol is sulfonated to *p*-cresol sulfate.[30] While Clayton et al. did not explore the exact effects, it is thought that *p*-cresol sulfonation competing with acetaminophen sulfonation could lead to increased acetaminophen hepatotoxicity.[30] This interaction highlights the important symbiotic relationship that the microbiome has developed with the host in metabolism. We further investigated how microbial metabolic products interact with the host.

Nuclear Receptors

Metabolites generated by gut microbes can produce their effects through the binding of host receptors that initiate the response. An important class of biological receptors that is focused upon in this work is nuclear receptors (NRs). NRs are notable for being activated by hormonal and metabolic ligands that readily pass through the plasma membranes of cells due to their lipophilic natures.[31] Ligands that bind and activate biological receptors such as NRs are considered agonists. The large number of NRs endogenous to humans play many roles in

biological processes, including metabolism, inflammation, and reproduction.[32] They mediate this through regulating expression of genes important in performing these processes, thus why they are considered transcription factors. Common to almost all NRs is a domain that binds to their natural ligand and a domain that binds to the promoter region of the gene it regulates, which are termed the ligand binding domain (LBD) and the DNA binding domain (DBD), respectively.[31] They then fall into categories based on the way that they interact with their ligands as well as how they interact with DNA.

Type I NRs are initially bound to chaperone proteins in the cytoplasm of cells and freed upon ligand binding. Ligand binding causes homodimerization, or complexing with another identical protein sequence, and subsequent translocation to the nucleus of the cell where it recruits coactivator proteins to bind to and regulate transcription of their target genes.[32] Type I NRs also typically bind to DNA sequences that are organized as inverted repeats.[33] Type II NRs are initially located in the nucleus bound to their DNA response elements, generally as a heterodimer with the retinoid X receptor (RXR), even in the absence of their ligands and exert repressive activity on the genes that they regulate.[32] Upon ligand binding, the corepressors are replaced by coactivators, which allow for transcription of their target genes with the help of the coactivator enzymatic activity. Type III NRs are functionally similar to type II NRs in that they exist in the nucleus and ligand binding causes displacement of corepressors for coactivators, except that they bind to DNA as a homodimer rather than a heterodimer with RXR.[32] Type IV NRs are also similar to type II NRs except that they bind to DNA as a monomer rather than a heterodimer like type II or even a homodimer like type III.[32] This classification scheme can be found in Table 1.

Microbially reprocessed bile acids mediate many of their physiological effects through binding host NRs.[29] The farnesoid X receptor (FXR) represents a NR activated by both primary bile acids and secondary bile acids.[34] FXR is a type II NR, where it binds to DNA and regulates gene expression as a heterodimer with RXR.[32] It is considered a nuclear bile acid sensor since it induces the expression of small heterodimer partner, a NR that represses the function of transcription factors important in inducing bile acid synthesis enzymes like cytochrome P450 7A.[29] Thus, one of FXR's main functions is to regulate the production of bile acids endogenously. FXR has a large number of bile acid ligands, including secondary bile acids like DCA and LCA.[34] Bile acid activation of FXR is implicated in a number of physiological functions, including glucose metabolism.[29] FXR increases insulin secretion through a genomic interaction with Krueppel-like factor 11, a transcription factor that induces expression of insulin through mediation by glucose.[35] FXR also affects glucose homeostasis non-genomically. Activation of FXR was shown to increase translocation of glucose transporter 2 to the plasma membrane of cells which increases glucose uptake.[35]

Another example of metabolites originating from microbial biotransformation reactions activating host transcription factors involves the aryl hydrocarbon receptor (AHR). AHR is unique from most transcription factors in that it is part of the basic helix-loop-helix/Per-ARNT-SIM family of proteins and does not fall neatly into any of the aforementioned NR subtypes.[36] AHR is normally found in the cytoplasm of the cell bound to chaperone proteins that keep it in an inactive state.[36] Upon ligand binding in the cytoplasm, AHR translocates into the nucleus of the cell where it complexes with the AHR Nuclear Translocator (ARNT).[37] AHR has a large number of ligands, such as indole based compounds from vegetables, as well as

xenobiotic ligands like carcinogenic compounds such as polyaromatic hydrocarbons.[36] The AHR-ARNT complex associates with the promoter of their target genes to affect transcription of proteins involved in functions such as immune homeostasis and metabolism.[37] Metabolites of L-tryptophan digestion by the microbiome can act as ligands that activate AHR (Figure 3).[37] A specific example of this is indole-3-aldehyde, a metabolite of dietary L-tryptophan metabolism by Lactobacilli microorganisms in the gut.[38] Indole-3-aldehyde agonizes AHR to induce transcription of IL-22, an immune protein important in mediating inflammatory responses.[38] A major focus of this work was to observe the effects of metabolites originating from the microbiome acting as natural ligands to activate ligand-activated transcription factors of interest similarly to the mechanism of AHR.

Subtype	Bioactivity and Functionality	Examples
Type I	Anchored in the cytoplasm by chaperone proteins. Ligand binding frees them from chaperones which allows for homodimerization and translocation to the nucleus. Recruitment of coactivator proteins in the nucleus causes DNA binding along the DBD at inverted DNA sequences and transcription of target genes.	Androgen Receptor, Estrogen Receptor, Progesterone Receptor, Glucocorticoid Receptor
Type II	Present in the nucleus bound to specific DNA response elements and generally form heterodimers with RXR. Ligand binding halts repressor activity as it replaces corepressors for coactivators. Enzymatic activity of coactivators allows for transcription of the NR target genes.	Thyroid Hormone Receptor, Pregnane X Receptor, Farnesoid X Receptor,
Type III	Present in the nucleus bound to DNA response elements as a homodimer. Ligand binding causes displacement of corepressors for coactivators which allows for transcription of target genes.	Vitamin D Receptor
Type IV	Present in the nucleus bound to DNA response elements as a monomer. Ligand binding replaces corepressors with coactivators and allows for transcription of target genes.	Liver Receptor Homolog - 1, Steroidogenic Factor 1

Table 1: Subtypes of nuclear receptors along with their general functioning and examples of each.

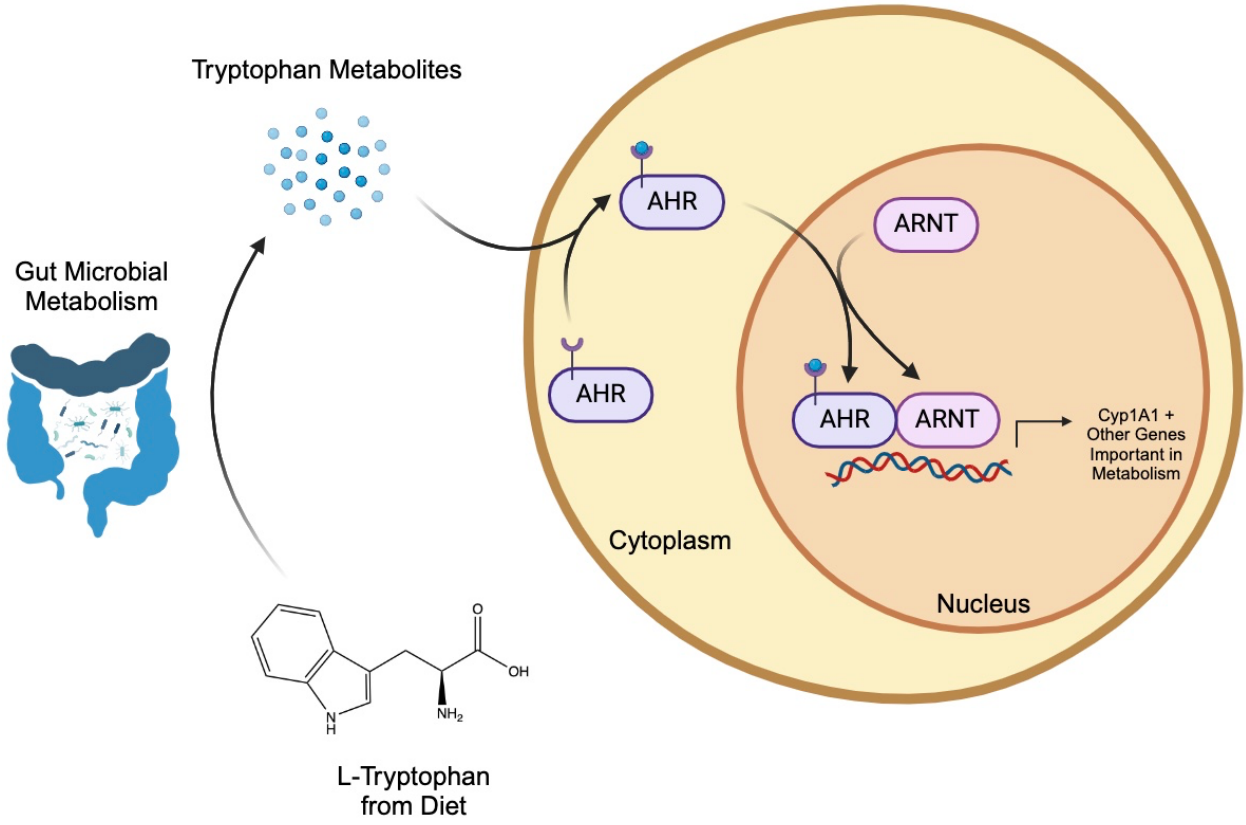


Figure 3: Example of metabolites derived from gut microbial metabolism acting as ligands for the aryl hydrocarbon receptor, an endogenous transcription factor (adapted from Patel et al., 2023).[37]

Nuclear Receptor of Interest – Pregnane X Receptor

The NR that we were interested in for this study was the pregnane X receptor. PXR is a mammalian transcription factor whose name is derived from its activation by pregnanes, a class of steroids.[39] It is agonized by a large number of ligands endogenous to humans, like steroid hormones and bile acids.[40] It has also been shown that many clinical drugs, including statins and

antidepressants, as well as environmental pollutants are also ligands of PXR.[41] What partly explains the large number of compounds that act as ligands for PXR is its relatively large and flexible LBD compared to other NRs that is lined with mainly hydrophobic amino acid residues permitting binding to a wide range of lipophilic ligands.[40] PXR falls under the category of type II NRs and is located in the nucleus as a heterodimer with RXR. Binding of ligands to PXR displaces corepressor proteins, allowing for coactivator proteins to associate with the PXR-RXR heterodimer complex bound to the PXR response elements of promoter regions in DNA. The enzymatic activity of coactivator proteins, which associate with the PXR-RXR complex, drives transcription of PXR's large number of target genes.

PXR has been demonstrated to induce the expression of genes important in a number of different functions, including metabolism of xenobiotic and endobiotic substances, drug detoxification, and drug transport.[40] This functioning alludes to the fact that PXR is primarily expressed in the liver where much of chemical metabolism occurs.[40] The proteins produced by these target genes include those involved in phase one metabolism, such as cytochrome P450s that catalyze phase one oxidation reactions.[39] Among these cytochrome P450s that PXR regulates is the cytochrome P450 3A (CYP3A) family, which is especially meaningful because CYP3A4 is involved in around 60 percent of drug metabolism and drug transport.[41] PXR also regulates the expression of UGTs and SULTs, both important in phase two metabolism.[41] These enzymes function to make metabolites more hydrophilic for easier excretion through transferring polar groups to them. UGTs work through glucuronidation and SULTs work through sulfonation.

PXR affecting the expression of these enzymes culminates in the metabolism of various substances, both natural and foreign to the body. Due to the numerous drug metabolizing

enzymes that PXR regulates in response to xenobiotic exposure, it is considered a master xenobiotic sensor.[42] PXR's action to regulate drug metabolizing enzymes works in an autoregulation mechanism.[40] Drug ligands induce PXR to express genes whose function works to metabolize these same drugs in a feedback fashion.[40] These mechanisms often lead to an easily excretable, detoxified form of the substance. An example of this is rifampicin, an antibiotic used to treat mycobacterial infection that causes CYP3A4 expression through activating PXR.[43] One of the most significant clinical implications of PXR is drug-drug interactions.[44, 45] PXR activation and induction of CYP3A4 by certain drugs leads to the increased ability for CYP3A4 metabolism of other drugs administered to the body.[45] The effects of these PXR mediated drug-drug interactions is generally the decreased therapeutic efficacy of the administered drugs.[46] It is because of this large role in metabolism that we were interested in its connection to microbial biotransformation in the body. Discovery of novel metabolites from the microbiome that activate PXR could be important in elucidating a greater connection between gut microbiota and expression of genes important in metabolism.

Reporter Gene Assay

An effective experiment to observe if a certain compound activates a nuclear receptor of interest is a reporter gene assay. These experiments provide a way to quantify activation of a transcription factor through an easily detectable signal. In the assay, cells are engineered in such a way that the DBD for a transcription factor of interest is swapped for another DBD. This new DBD associates with a promoter linked to a gene that expresses proteins with detectable functionality.[47] A popular reporter gene used in many studies expresses the luciferase protein,

which catalyzes a reaction that produces an easily quantifiable luminescent signal.[48] The luciferase enzyme is what allows for certain species, such as fireflies, to emit light through biological processes.[48] Thus, the transcription factor associates with the promoter linked to this luciferase gene leading to luciferase expression after being bound to an agonist. In our reporter gene assay, the LBD of PXR was linked to the DBD of Gal4, a protein important in galactose-mediated yeast growth.[49] The Gal4-DBD associates with the Gal4 DNA binding sequence, an enhancer region engineered in our assay to be connected with the luciferase gene in the mammalian cells we utilized (Figure 4). A higher amount of detected luminescence is correlated with ligands that activate the transcription factor to a greater extent. Other reporter gene assays link PXR's DNA response element directly to the luciferase gene so that PXR activation leads to luciferase expression. However, the reporter gene assay we utilized was of greater use to our research goal because other nuclear receptors often associate with PXR's DNA response element, making that type of assay less reliable for observing PXR activation. Luciferase reporter gene assays are also sensitive, allowing for the quantification of small levels of transcription.[50] We used these benefits to our advantage to observe if metabolites generated by microbial biotransformation agonized PXR and led to greater luminescence.

While luciferase reporter gene assays are a common method of measuring promoter activity, their limitations must be noted. The most important limitation is that the enzymatic activity of luciferase, which allows for quantifiable bioluminescence, is dependent upon adenosine triphosphate. This means that the metabolic state of the mammalian cells in use can affect the enzymatic activity of luciferase and thus could influence the overall results of the experiment.[50] The methods used to create a reporter assay construct greatly affect the reliability of the results too. Transfection involving the amount of reporter construct entering the cells,

DNA purity, and growth history of host cells are only some of the factors that could influence the results to be less reliable across experiments.[51] The stability of the chemical of interest in its stock solution could also produce variability in results. Considering these limitations and how they could affect our results, we deemed it necessary to utilize this assay to screen how metabolites originating from the microbiome affect PXR.

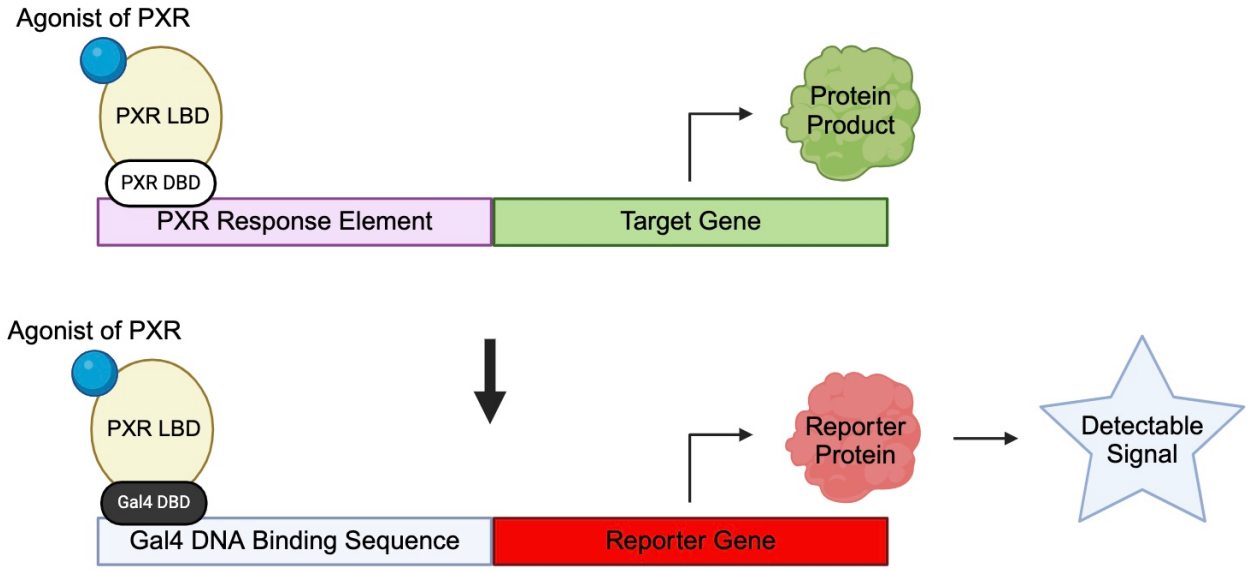


Figure 4: Basic workflow for engineering a reporter gene assay useful in observing induced luminescence by a chemical treatment as a proxy for activation of a transcription factor of interest (visually adapted from Indigo Biosciences).[47]

Research Goal

The microbiome is a complex interaction between trillions of different microorganisms that are essential to human life. Gut microbiota secrete enzymes that catalyze the biotransformation of substances entering the body. Certain organisms here produce enzymes capable of catalyzing the metabolism of substances that host cells cannot. This allows the host to absorb important nutrients that it otherwise would not obtain. The microbiome also aids in the metabolism of pharmaceutical drugs and toxic chemicals alongside host cell mechanisms. It is often the case that substances biotransformed by gut microbiota were already metabolized by host cells, although it is possible for substances to first undergo microbial metabolism. Considering the number of biotransformation reactions the microbiome catalyzes as well as the broad number of metabolites it produces, it is important to recognize how these compounds interact with the body. Metabolites from microbial biotransformation reactions can mediate their effects through binding host transcription factors. This was a central paradigm behind the goal of our study.

The goal of this research was to discover if metabolites of microbial biotransformation reactions agonize PXR to induce transcription of its target genes. This was performed by utilizing a library of 200 compounds that are metabolites of microbial reactions originating in the microbiome (Table 2). We screened the compounds in this library for their ability to agonize PXR through a luciferase reporter gene assay. The reporter gene assay that we used possessed HepG2 cells, a human hepatocyte carcinoma cell line, containing PXR expressing a DBD that binded to a DNA promoter region functionally linked to the firefly luciferase gene (Figure 4). If a metabolite agonized PXR, then this would lead to expression of firefly luciferase and thus detectable luminescence. Luminescence could be quantified where a higher relative

luminescence was associated with greater agonizing ability of the metabolite for PXR. Quantification of luminescence when observing the metabolites was compared to rifampicin, a reference ligand of PXR that agonizes it to a large degree. Top metabolites that activated PXR from the initial screen were used in a dose-response assay. Using increasing doses and observing the induced luminescence, we sought to discover if these top metabolites agonized PXR in a dose-dependent manner.

Discovering microbial metabolites that activate PXR would provide a great deal of information in understanding how what we consume, whether it be everyday food or pharmaceutical drugs, affects the microbiome and leads to important downstream physiological effects. A stronger connection could be made between the microbiome and host metabolism considering that PXR affects the expression of genes important in catalyzing metabolic reactions. Finding novel metabolites of microbial biotransformation reactions that agonize PXR would also increase the known collection of functional PXR ligands. This work would be important in future research seeking to further the understanding of microbial metabolism affecting the expression of host metabolic genes. Overall, we hope to further the field of microbiome research by elucidating a greater connection between microbial metabolism and PXR activation.

Chapter 2

Materials and Methods

Materials

A library of 200 compounds representing metabolites originating from the microbiome was obtained from the Cleveland Clinic. The human PXR luciferase reporter assay kit as well as the cytotoxicity assay kit were both acquired from Indigo Biosciences. These reporter assay kits possessed HepG2 cells, which is a human hepatocyte carcinoma cell line commonly used in drug metabolism studies. HepG2 cells in these kits were engineered to constitutively express a PXR-reporter gene construct, thus we deemed them reporter cells. Along with these reporter assay kits, materials to perform the experiments were obtained from Indigo Biosciences as well. These materials included cell recovery media (CRM) and compound screening media (CSM) for upkeep of *in vitro* assay cells, as well as a reference agonist for PXR. For analyzing luciferase luminescence, luciferase detection reagent (LDR) was made by mixing luciferase detection substrate with luciferase detection buffer again acquired from Indigo Biosciences. Luminescence was measured using a Glomax Multi-Detection System Luminometer. Finally, different software was used to generate instructional figures and results graphs, including Biorender, Chemdraw by Revvity Signals, GraphPad Prism, and Microsoft Excel.

Methods – Cytotoxicity Assay

To ensure that a lethal dose was not used for the widespread metabolite screen and to prevent false negatives, a cytotoxicity assay was performed. In this assay, reporter cells were rapidly thawed by addition of CRM and placed at 37°C for 5-10 minutes. Cell suspensions were then transferred to a 96-well plate and incubated for 2-6 hours in an atmosphere of 37°C and 5% CO_2. While cell suspensions were incubating, test metabolites were diluted to 1:500 in CSM from their stock solutions of 10 millimolar in a DMSO (vehicle) solvent. After 2-6 hours, the CRM was thoroughly discarded and replaced with the metabolite-treated CSM and incubated for 16-24 hours. After 16-24 hours, culture media was removed and LDR was added. The suspension was allowed to rest for at least 5 minutes and luminescence was then quantified using the Glomax Multi-Detection System Luminometer.

Methods – Human Luciferase Reporter Gene Assay for Library Screen

Agonist activity was observed by utilizing a firefly luciferase reporter gene assay (Indigo Biosciences) for PXR. The assay was performed by first rapid thawing the HepG2 cells constitutively expressing the Human PXR reporter gene construct by adding CRM and placing them in a water bath at 37°C for 5-10 minutes. Cells were then transferred to a 96-well assay plate and incubated for 2-6 hours in a 37°C, 5% CO_2 environment. While the cells were incubating, each compound of interest was diluted in CSM within another 96-well plate based upon the results from the cytotoxicity assay. Compounds that were above 70% cell viability from the cytotoxicity assay were diluted to 1:500 while compounds that caused lower than 70% cell viability were further diluted to 1:1000. The reference compound for PXR was also diluted

to 1:500 in CSM. After the cells had incubated for 2-6 hours, the culture media was discarded, and the cells were then suspended in the metabolite-treated CSM solutions and incubated again for 16-24 hours. After 16-24 hours, all CSM was discarded thoroughly and LDR was added to each well. The wells were allowed to rest for at least 5 minutes. Luminescence was then quantified using the Glomax Multi-Detection System Luminometer.

Methods – Dose-Response Assay

A dose-response experiment was performed to understand how top metabolites activated PXR. A firefly luciferase reporter gene assay was used with a similar protocol as previously described. However, differing from the previous experiment, each top metabolite was diluted in a 2-fold dilution series with eight final concentrations starting at 30 micromolar. 30 micromolar concentration was used because it was the same starting concentration as the PXR reference compound, rifampicin. After each metabolite-treated CSM solution was added to the cell mixture and allowed to incubate for 16-24 hours, luminescence was again quantified using the Glomax Multi-Detection System Luminometer.

Methods – Statistical Analysis

Statistical analyses were performed using several different methods. After quantifying luminescence for the cytotoxicity assay, the luciferase reporter gene assay, and the dose-response assay using the Glomax Multi-Detection System Luminometer, data were generated and analyzed using GraphPad Prism software and Microsoft Excel. Quantification of luminescence from respective experiments was expressed in relative luminescence units (RLU), where

luminescence for each metabolite treatment was divided by the average background luminescence for cells that were not treated with any metabolites, or untreated cells. All data was graphed alongside a reference compound for PXR, rifampicin, that acted as a positive control. Significance values were generated using an analysis of variance (ANOVA) test compared to vehicle-treated cells that acted as a negative control.

1.	4'-acetylchrysomycin B	2.	naringenin-7-O-glucuronide
3.	cyanidin-3-glucoside	4.	cyanidin-3-rutinoside
5.	formononetin	6.	urdamycin B
7.	skatole	8.	salicylic acid
9.	2,3,4-trihydroxybenzoic acid	10.	3-hydroxyphenylacetic acid
11.	3-hydroxybenzoic acid	12.	phenylacrylic acid
13.	urolithin A	14.	urolithin B
15.	xanthuneric acid	16.	serotonin
17.	hydrocinnamic acid	18.	pterostilbene
19.	indole-3-propionic acid	20.	phloroglucinol
21.	2-(4-hydroxyphenyl)propionic acid	22.	picolinic acid
23.	indole	24.	oxindole
25.	deoxycholic acid	26.	indole-3-acetic acid
27.	cinnamtannin (B-1)	28.	indole-3-carboxaldehyde
29.	indole-3-carboxylic acid	30.	4-fluoro-α-pyrrolidinobutiophenone

31.	GYY 4137	32.	*p*-coumaric acid
33.	ursocholanic acid	34.	3-phenylpropionic acid
35.	syringic acid	36.	gamma-aminobutyric acid
37.	α-aminobutyric acid	38.	phenylethylamine
39.	putrescine	40.	cadaverine
41.	1,3-diaminopropane	42.	3-(4-hydroxyphenyl)propionic acid
43.	sodium 3-methyl-2-oxobutyrate	44.	2,4,6-trihydroxybenzaldehyde
45.	imidazole propionic acid	46.	3-(3-hydroxyphenyl)propanoic acid
47.	3,4-dihydroxybenzoic acid	48.	geranin
49.	dihydrotetrodecamycin	50.	hyocholic acid
51.	4',7-di-O-methylnaringenin	52.	7,10-dihydroxy-8(E)-octadecenoic acid
53.	hirsutide	54.	tryptamine
55.	3-hydroxyphenylpropionic acid	56.	tricarballylic acid
57.	3-methylindole	58.	indole-3-butyric acid
59.	3,4-dihydroxyphenylacetic acid	60.	3-phenyllactic acid
61.	anthranilic acid	62.	neopterin (D-+-neopterin)
63.	4-hydroxybenzoic acid	64.	hyodeoxycholic acid
65.	tryptophol	66.	isoorientin
67.	4-hydroxyhippuric acid	68.	biochanin A
69.	lithocholic acid	70.	lumichrome
71.	ursodeoxycholic acid	72.	gastrodin
73.	2-hydroxyhippuric acid	74.	indole-3-acetamide
75.	benzoic acid	76.	homogentisic acid
77.	vanillic acid	78.	4-hydroxyphenylacetic acid
79.	trans-cinnamic acid	80.	D-quinic acid

81.	N-cinnamylglycine	82.	β-aminobutyric acid
83.	spermidine	84.	spermine
85.	(+)-fenchone	86.	mycolic acid
87.	3,3'-diindolylmethane	88.	2-nonanone
89.	phenylpyruvic acid	90.	urolithin C
91.	N-(3-indoleacetyl)-L-alanine	92.	β-rubromycin
93.	p-cresol sulfate	94.	vanillic acid 4-B-D-glucopyranoside
95.	acetoin	96.	tridecane
97.	hexadecane	98.	furfural
99.	5-hydroxy-methyl-furfural	100.	β-caryphyllene
101.	2-tridecanone	102.	2-undecanone
103.	acetophenone	104.	benzaldehyde
105.	β-humulene	106.	(+)-ledene
107.	1H-pyrole	108.	α-humulene
109.	(S)-(-)-limonene	110.	(-)-fenchone
111.	ocimene	112.	ethanethoic acid
113.	undecane	114.	valencene
115.	zingiberene	116.	farnesene
117.	sativene	118.	jasmonic acid
119.	sodium oxalate	120.	sodium glyoxylate monohydrate
121.	2,4-dimethoxy-6-methylbenzoic acid	122.	trimethylamine N-Oxide
123.	oleuropein	124.	serinol
125.	TMA-HCl	126.	1-arachidonyl serinol
127.	heronapyrrole A	128.	prostaglandin E2
129.	nigericin	130.	monosodium urate

131.	p-hydroxybenzaldehyde	132.	methyl-4-hydroxybenzoate
133.	3-(2-hydroxyphenyl)propionate	134.	vitexin
135.	isovitexin	136.	casticin
137.	fulvic acid	138.	mulberroside A
139.	arctiin	140.	matairesinol
141.	enterolactone	142.	aranorosin
143.	geosmin	144.	2-methyl-isobomeol
145.	1-octen-3-ol	146.	α-pinene
147.	camphene	148.	camphor
149.	s-methyl thioacetate	150.	2-methyl propanol
151.	3-methyl-2-butanol	152.	myrcene
153.	α-terpinene	154.	methyl salicylate
155.	β-phenylethanol	156.	(R)-(+)-limonene
157.	aromadendrene	158.	p-cymene
159.	guaiene	160.	carveol
161.	nobiletin	162.	trigonelline
163.	cafestol	164.	caffeic acid
165.	phenylpropionoylglycine	166.	tyramine
167.	12-methyltetradecanoic acid	168.	cis-9,10-methylenehexadecanoic acid
169.	nicotinic acid	170.	norspermine
171.	commendamide	172.	indole-3-acetic acid
173.	sodium acetate	174.	sodium proprionate
175.	valeric acid	176.	isovaleric acid
177.	sodium butyrate	178.	vitexin 2-O-rhamnoside
179.	celastrol	180.	cryptochlorogenic acid

181.	3,5-dicaffeoylquinic acid	182.	thaxtomin A
183.	oxychlororaphine	184.	terrein
185.	usnic acid	186.	violacein
187.	luteoreticulin	188.	6-prenylindole
189.	calpinactam	190.	chebulic acid
191.	glycolithocholate sulfate	192.	acetomycin
193.	norstictic acid	194.	urdamycin A
195.	pyrrolnitrin	196.	sparsomycin
197.	roccellic acid	198.	phenylacetyl-glycine
199.	hippuric acid	200.	2,4,6-trihydroxybenzoic acid

Table 2: Comprehensive list of compounds used in the luciferase reporter gene assay screen for PXR activation. Compounds represent products of biotransformation reactions catalyzed by gut microbiota.

Chapter 3

Results

Cytotoxicity Evaluation of Metabolite Library

This experiment observed the cytotoxicity of a standard working concentration for a widespread reporter gene assay screen. Cell viability was measured after treating cells with a 10 millimolar stock solution diluted to 1:500 in CSM. Metabolites that caused a lower than 60% cell viability were diluted further so that a lethal dose was not used in future experiments. Metabolites that were further diluted to 1:1000 in CSM are represented by asterisks (***) in the widespread metabolite library screen observing agonist activity against PXR (Figure 5).

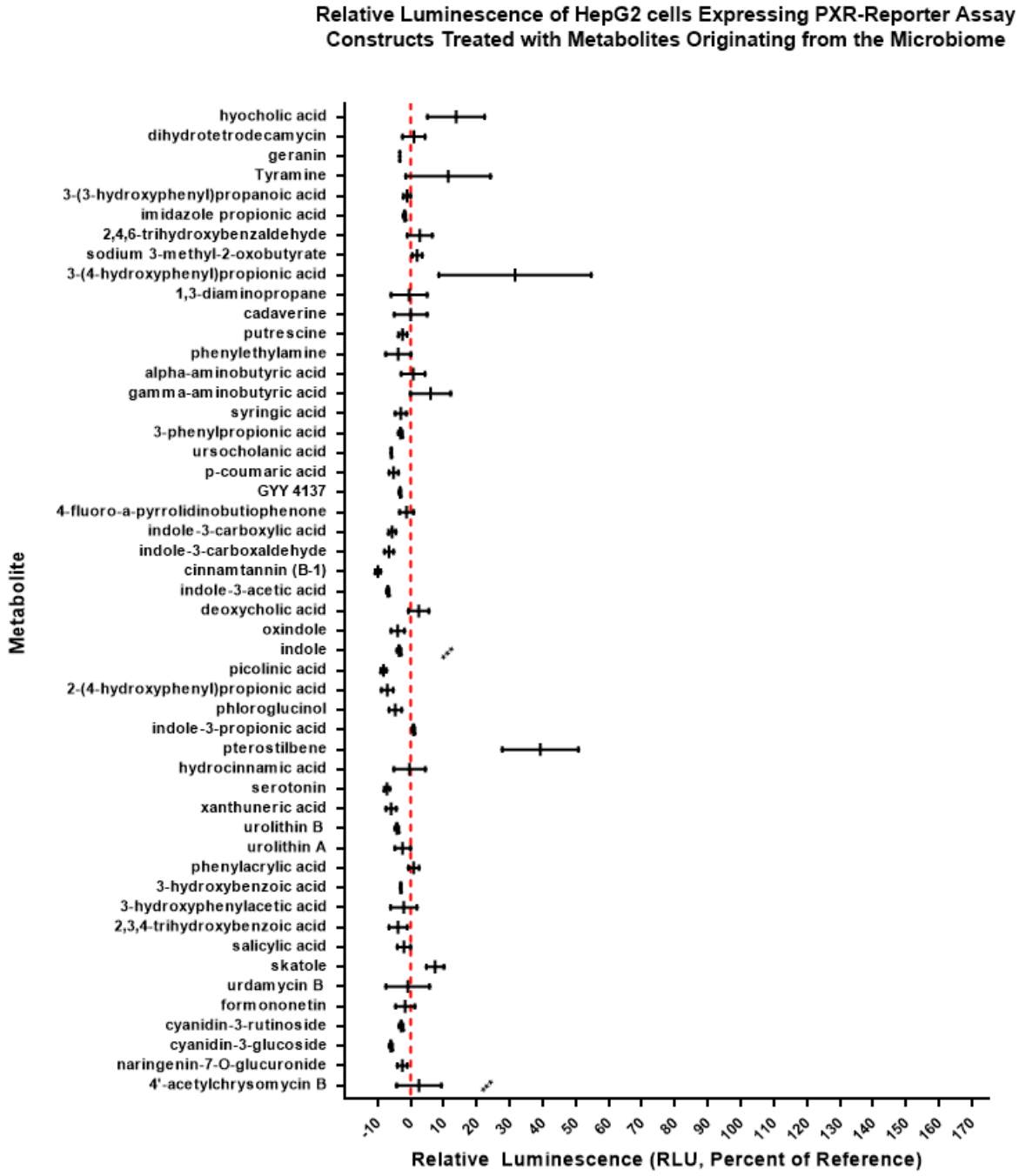
Metabolite Library Screen Against PXR

After performing the cytotoxicity assay and determining which metabolites needed further dilution to be non-toxic, we screened the entire library of 200 metabolites for their ability to induce luminescence in PXR reporter cells. This screen again utilized a luciferase reporter gene assay, and luminescence was quantified in RLU after treatment of cells with each metabolite. Figure 5 shows the induced luminescence of cells treated with each metabolite expressed as a percentage of the induced luminescence for cells treated with rifampicin (Figure 5A-D). Results were the average of duplicated (n = 2) experiments.

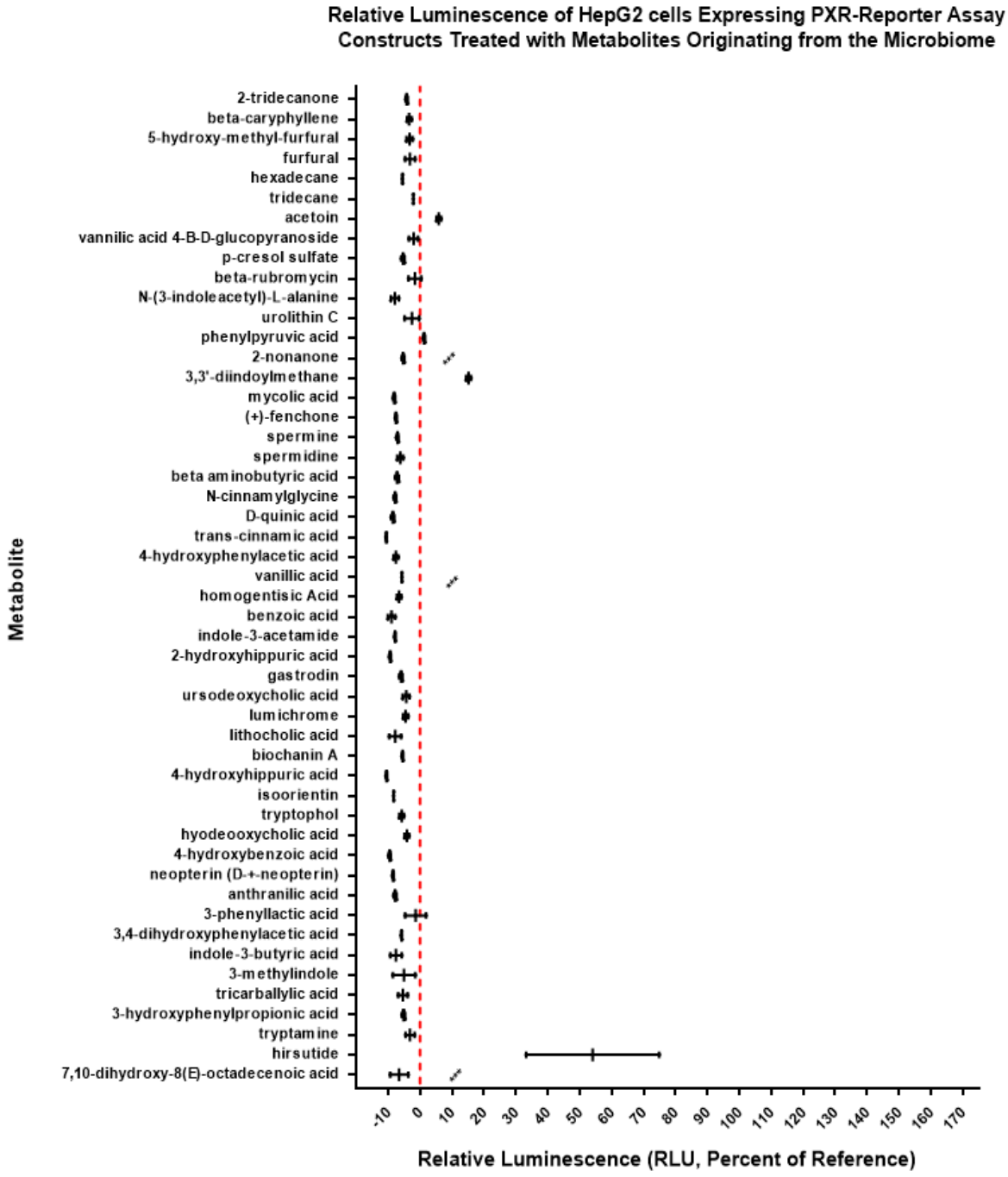
Metabolites that induced the greatest amount of relative luminescence compared to the reference agonist were elucidated and deemed, “top metabolites.” The top metabolites we identified for further experimentation were hyocholic acid, 3,4-dihydroxybenzoic acid, 3-(4-

hydroxyphenyl)propionic acid, pterostilbene, pyrrolnitrin, 3,3'-diindolylmethane, hirsutide, geosmin, enterolactone, matairesinol, arctiin, nigericin, β-humulene, calpinactam, and isovaleric acid (Table 3). We continued to observe the luminescence that these metabolites induced in PXR reporter cells by examining this response at different concentrations of metabolite treatment.

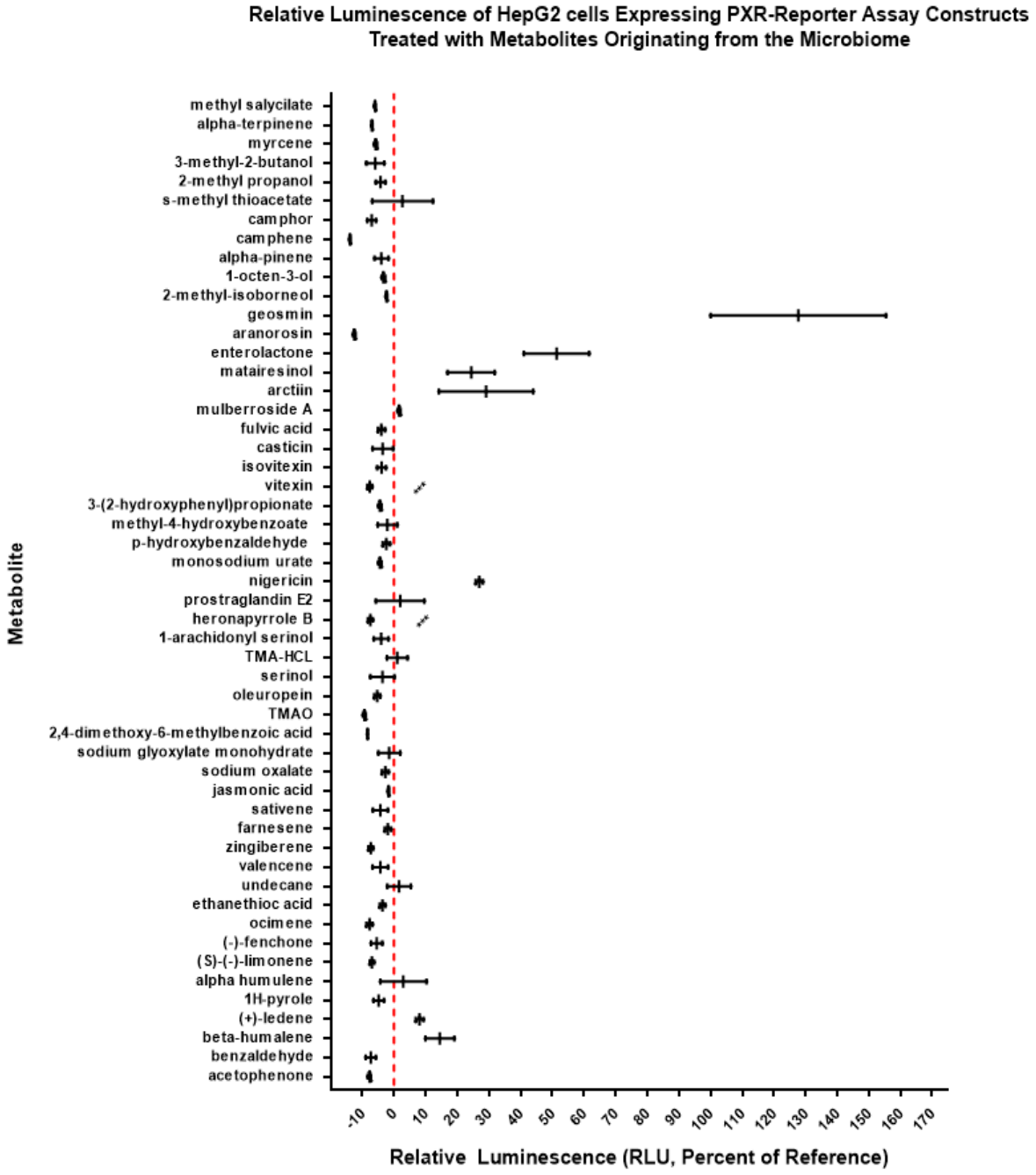
A


Relative Luminescence of HepG2 cells Expressing PXR-Reporter Assay Constructs Treated with Metabolites Originating from the Microbiome
Metabolite
hyocholic acid
dihydrotetrodecamycin
geranin
Tyramine
3-(3-hydroxyphenyl)propanoic acid
imidazole propionic acid
2,4,6-trihydroxybenzaldehyde
sodium 3-methyl-2-oxobutyrate
3-(4-hydroxyphenyl)propionic acid
1,3-diaminopropane
cadaverine
putrescine
phenylethylamine
alpha-aminobutyric acid
gamma-aminobutyric acid
syringic acid
3-phenylpropionic acid
ursocholanic acid
p-coumaric acid
GYY 4137
4-fluoro-a-pyrrolidinobutiophenone
indole-3-carboxylic acid
indole-3-carboxaldehyde
cinnamtannin (B-1)
indole-3-acetic acid
deoxycholic acid
oxindole
indole
picolinic acid
2-(4-hydroxyphenyl)propionic acid
phloroglucinol
indole-3-propionic acid
pterostilbene
hydrocinnamic acid
serotonin
xanthuneric acid
urolithin B
urolithin A
phenylacrylic acid
3-hydroxybenzoic acid
3-hydroxyphenylacetic acid
2,3,4-trihydroxybenzoic acid
salicylic acid
skatole
urdamycin B
formononetin
cyanidin-3-rutinoside
cyanidin-3-glucoside
naringenin-7-O-glucuronide
4'-acetylchrysomycin B
***
***
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
Relative Luminescence (RLU, Percent of Reference)

B


Relative Luminescence of HepG2 cells Expressing PXR-Reporter Assay Constructs Treated with Metabolites Originating from the Microbiome
2-tridecanone
beta-caryphyllene
5-hydroxy-methyl-furfural
furfural
hexadecane
tridecane
acetoin
vannilic acid 4-B-D-glucopyranoside
p-cresol sulfate
beta-rubromycin
N-(3-indoleacetyl)-L-alanine
urolithin C
phenylpyruvic acid
2-nonanone
3,3'-diindoylmethane
mycolic acid
(+)-fenchone
spermine
spermidine
beta aminobutyric acid
N-cinnamylglycine
D-quinic acid
trans-cinnamic acid
4-hydroxyphenylacetic acid
vanillic acid
homogentisic Acid
benzoic acid
indole-3-acetamide
2-hydroxyhippuric acid
gastrodin
ursodeoxycholic acid
lumichrome
lithocholic acid
biochanin A
4-hydroxyhippuric acid
isoorientin
tryptophol
hyodeooxycholic acid
4-hydroxybenzoic acid
neopterin (D-+-neopterin)
anthranilic acid
3-phenyllactic acid
3,4-dihydroxyphenylacetic acid
indole-3-butyric acid
3-methylindole
tricarballylic acid
3-hydroxyphenylpropionic acid
tryptamine
hirsutide
7,10-dihydroxy-8(E)-octadecenoic acid
***
***
***
Metabolite
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
Relative Luminescence (RLU, Percent of Reference)

C


Relative Luminescence of HepG2 cells Expressing PXR-Reporter Assay Constructs
Treated with Metabolites Originating from the Microbiome
Metabolite
methyl salycilate
alpha-terpinene
myrcene
3-methyl-2-butanol
2-methyl propanol
s-methyl thioacetate
camphor
camphene
alpha-pinene
1-octen-3-ol
2-methyl-isoborneol
geosmin
aranorosin
enterolactone
matairesinol
arctiin
mulberroside A
fulvic acid
casticin
isovitexin
vitexin
3-(2-hydroxyphenyl)propionate
methyl-4-hydroxybenzoate
p-hydroxybenzaldehyde
monosodium urate
nigericin
prostraglandin E2
heronapyrrole B
1-arachidonyl serinol
TMA-HCL
serinol
oleuropein
TMAO
2,4-dimethoxy-6-methylbenzoic acid
sodium glyoxylate monohydrate
sodium oxalate
jasmonic acid
sativene
farnesene
zingiberene
valencene
undecane
ethanethioc acid
ocimene
(-)-fenchone
(S)-(-)-limonene
alpha humulene
1H-pyrole
(+)-ledene
beta-humalene
benzaldehyde
acetophenone
-10
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
Relative Luminescence (RLU, Percent of Reference)

D

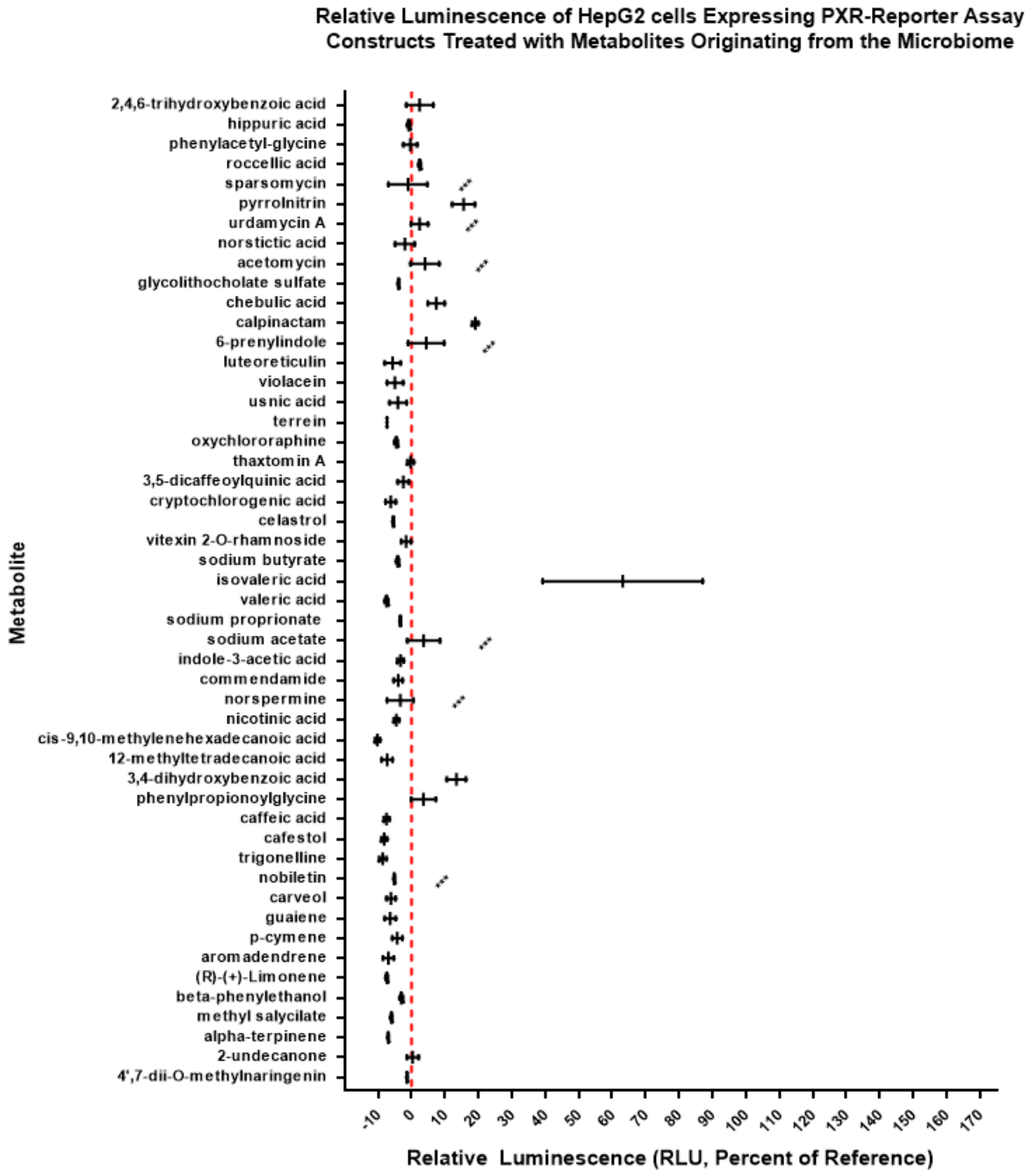


Figure 5: (A-D) Luminescence of PXR reporter cells after treatment with chemicals from microbial metabolite library for 24 hours at 20 μM. Results presented in RLU as a percentage of the max luminescence induced by rifampicin, the positive control and reference agonist for PXR. Data represents mean ± range (n=2). Asterisks represent metabolites diluted further due to cytotoxicity (***).

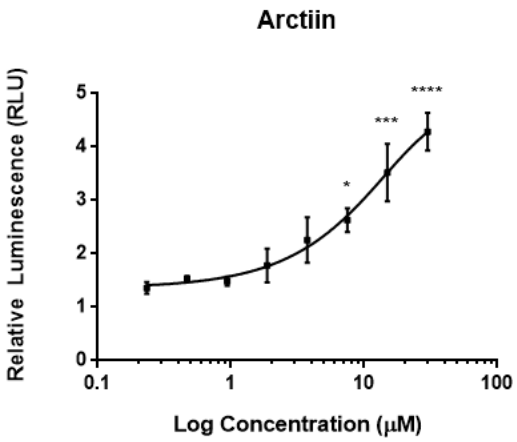
Metabolite	Induced Luminescence (RLU, % of Max Reference)
Geosmin	127.7
Isovaleric Acid	63.2
Hirsutide	54.1
Enterolactone	51.4
Pterostilbene	39.3
3-(4-Hydroxyphenyl)propionic acid	31.6
Arctiin	29.1
Nigericin	27.0
Matairesinol	24.4
Calpinactam	19.1
Pyrrolnitrin	15.6
3,3'-diindolylmethane	15.2
β-Humulene	14.5
Hyocholic Acid	13.8
3,4-dihydroxybenzoic acid	13.4

Table 3: Metabolites that induced the greatest luminescence in HepG2 cells expressing PXR-luciferase reporter gene constructs. Luminescence reported in RLU as a percentage of the max luminescence induced by rifampicin. Top metabolites represented in descending order from largest to smallest induced luminescence.

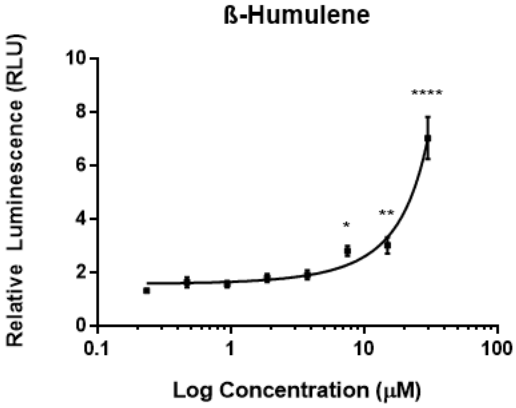
Dose-Response Assay of Top Metabolites

Top metabolites that agonized PXR from the widespread library screen were used in a full dose-response experiment to further explore their degree of activation of PXR in PXR reporter cells. A summary of the findings from these experiments can be seen in Table 4 (Table 4). All luminescence values were compared to the average induced luminescence values for vehicle-treated reporter cells in an ANOVA test (Figure 6 A-P). Rifampicin-treated reporter cells produced a maximum luminescence of 29.6 RLU at 30 micromolar concentration with an EC50 value of 6.8 (Figure 6P). Hirsutide and nigericin induced similar luminescence in reporter cells as rifampicin at concentrations of 30 micromolar (Figure 6G and Figure 6M). The maximum luminescence induced by hirsutide was 24.3 with an EC50 value of 6.4, and the maximum luminescence induced by nigericin was 23.2 with an EC50 value of 7.9 (Table 4). The only metabolites that did not cause a statistically significant increase in luminescence compared to the vehicle-treated cells at any metabolite concentrations were 3,4-dihydroxybenzoic acid, 3-(4-hydroxyphenyl)propionic acid and hyocholic acid (Figure 6H-J).

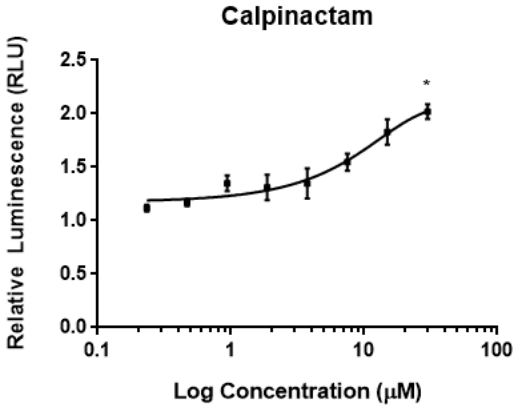
A


Arctiin
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0
1
2
3
4
5
*
***
****

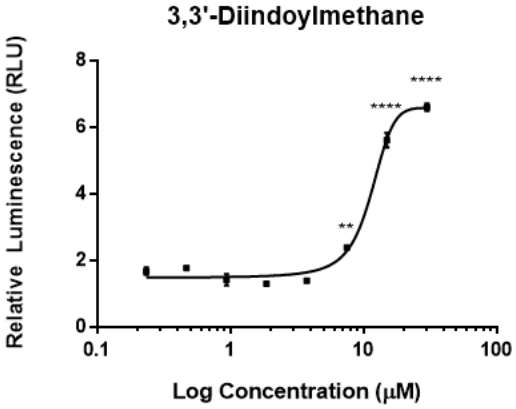
B


ß-Humulene
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0
2
4
6
8
10
*
**
****

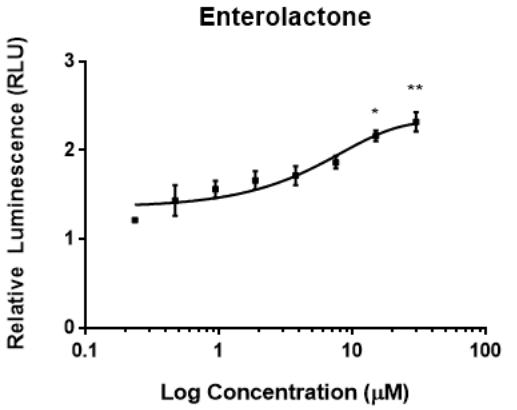
C


Calpinactam
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0.0
0.5
1.0
1.5
2.0
2.5
*

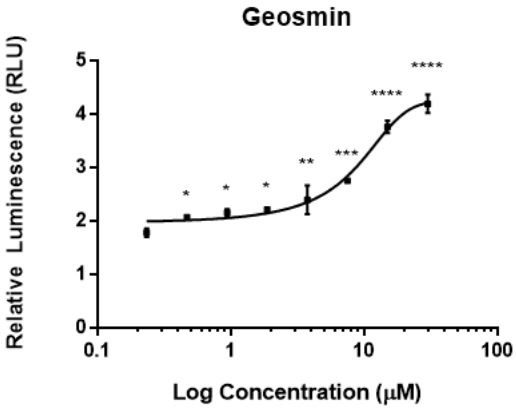
D


3,3'-Diindoylmethane
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0
2
4
6
8
**
****
****

E


Enterolactone
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0
1
2
3
*
**

F


Geosmin
Relative Luminescence (RLU)
Log Concentration (μM)
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1
10
100
0
1
2
3
4
5
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*
*
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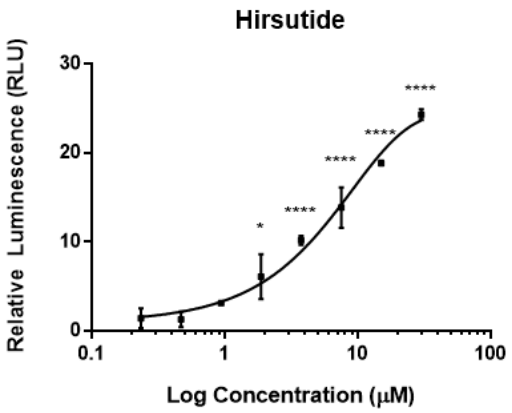
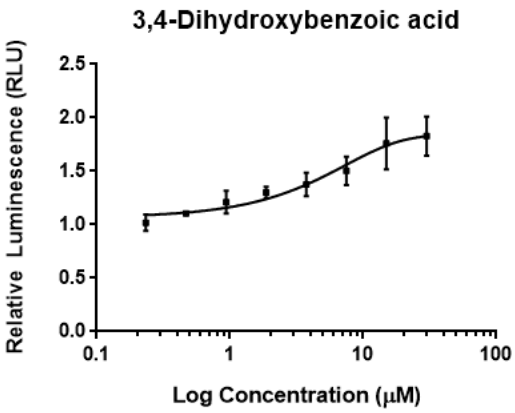
****

G


Hirsutide
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0
10
20
30
*
****
****
****
****

H


3,4-Dihydroxybenzoic acid
Relative Luminescence (RLU)
Log Concentration (μM)
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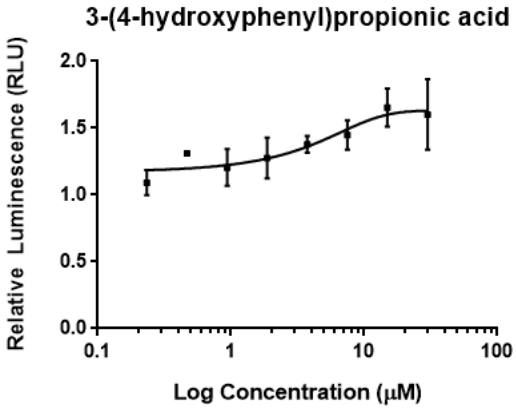
1
10
100
0.0
0.5
1.0
1.5
2.0
2.5

I


3-(4-hydroxyphenyl)propionic acid
Relative Luminescence (RLU)
Log Concentration (μM)
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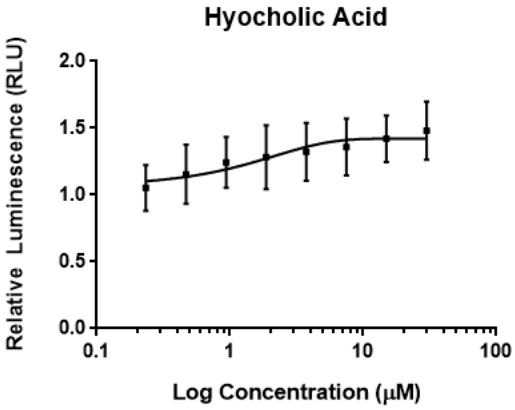
1
10
100
0.0
0.5
1.0
1.5
2.0

J


Hyocholic Acid
Relative Luminescence (RLU)
Log Concentration (μM)
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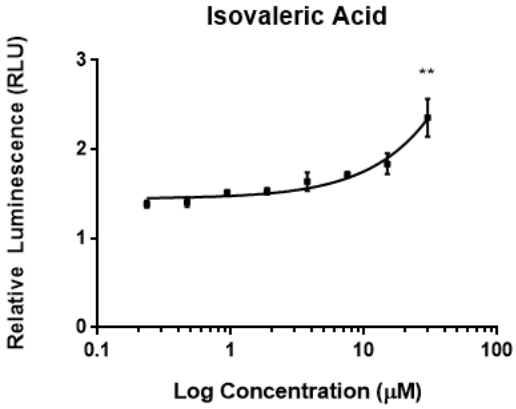
10
100
0.0
0.5
1.0
1.5
2.0

K


Isovaleric Acid
Relative Luminescence (RLU)
Log Concentration (μM)
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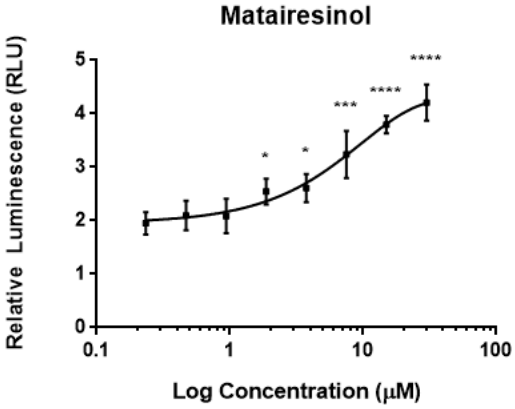
1
10
100
0
1
2
3
**

L


Matairesinol
Relative Luminescence (RLU)
Log Concentration (μM)
0.1
1
10
100
0
1
2
3
4
5
*
*
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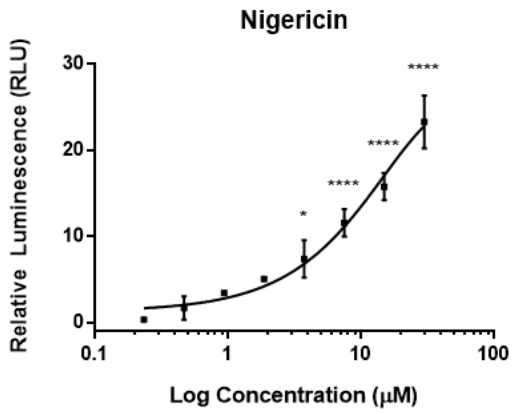
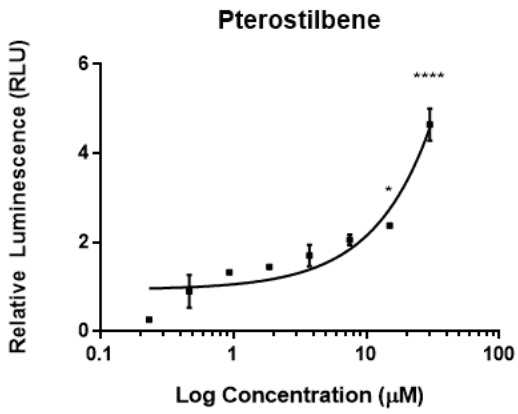
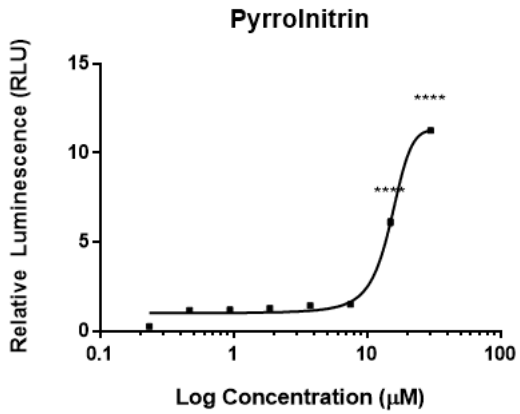
M



N



O



P

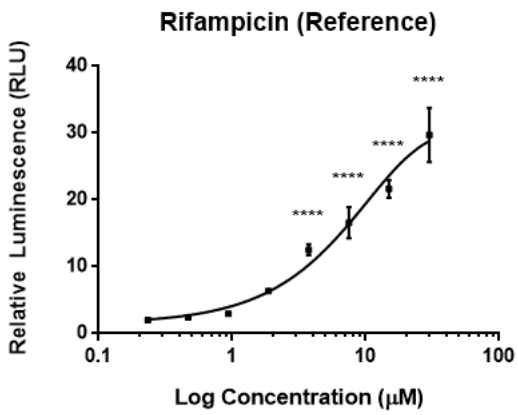


Figure 6: (A-P) Induced luminescence of HepG2 cells expressing PXR-reporter gene constructs treated with each of the top metabolites elucidated from a widespread library screen. Response reported as luminescence relative to background, untreated cells. Data represents mean ± range (n=2). Significance compared to vehicle-treated control by ANOVA test (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001)

Top Metabolite	EC50 (μM)	Average Maximum Induced Luminescence (RLU)
Rifampicin (Reference)	6.8	29.6
Arctiin	9.1	4.3
β-Humulene	19.3	7.0
Calpinactam	8.1	2.0
3,3'-Diindolylmethane	11.1	6.6
Enterolactone	5.0	2.3
Geosmin	9.3	4.2
Hirsutide	6.4	24.3
3,4-Dihydroxybenzoic acid	5.1	1.8
3,4-Dihydroxyphenylpropionic acid	3.7	1.6
Hyocholic acid	1.5	1.5
Isovaleric acid	16.0	2.4
Matairesinol	6.5	4.2
Nigericin	7.9	23.2
Pterostilbene	15.5	4.6
Pyrrolnitrin	14.4	11.3

Table 4: Comparison of EC50 and maximum luminescence values for top metabolites from dose-response assays compared to rifampicin.

Chapter 4

Discussion

We screened a library of metabolites originating from microbial biotransformation reactions in the microbiome for their ability to activate PXR *in vitro.* This was accomplished by using a firefly luciferase reporter gene assay that functionally linked PXR activation to an easily detectable response. In doing this, we elucidated a subset of chemicals that activated PXR within a certain percentage of the PXR reference agonist, rifampicin. We observed these top compounds further through a dose-response assay that showed us the relative luminescence of PXR reporter cells treated with each top compound at certain doses alongside rifampicin. Through statistical analysis of these results by comparing the luminescence produced by metabolite treated cells to vehicle-treated control cells, we found that many of these top compounds induced statistically significant luminescence. Certain top metabolites lead to a level of luminescence in the dose-response assay like the reference, where the statistical significance rivaled that of rifampicin.

Luminescence produced by the HepG2 luciferase reporter cells after treatment with each microbial metabolite was a proxy for activation of PXR. This is due to the design of the HepG2 cells in the Indigo Biosciences kits we utilized. In these reporter cells, PXR was engineered to contain the Gal4-DBD, which binds to its DNA response element functionally linked to the firefly luciferase gene rather than its endogenous DBD (Figure 4). Ligands of PXR will recruit coactivator proteins to induce expression of the firefly luciferase enzyme in these cells, which produced the natural bioluminescence that we quantified. Thus, we can deduce that the top compounds we found from our widespread compound library screen activate PXR considering

the significant relative luminescence they induced in the PXR reporter cells (Figure 5A-D). Also, expressing luminescence results from the widespread compound library screen as a percentage of the known reference agonist, rifampicin, allowed for easily observable results to understand which metabolites activate PXR similarly to the positive control (Figure 5A-D).

Performing a targeted dose-response assay for top metabolites allowed us to further explore their agonist relationship with PXR. Observing the relative luminescence induced by the top metabolites at increasing concentrations gave us information regarding their potency and their maximum response. EC50 values for our experiment represented the concentration of metabolite that induced a response in 50 percent of PXR reporter cells and provided us with an idea of how potent the metabolites were in inducing luminescence (Table 4). The maximum response was the largest amount of relative luminescence induced by top metabolites in PXR reporter cells. It was the case in almost all top metabolites that the largest concentration we utilized, 30 micromolar, produced the greatest luminescence in PXR reporter cells (Figure 6A-P). However, one exception was 3,4-Dihydroxyphenylpropionic acid, which induced its largest amount of luminescence at 15 micromolar rather than 30 micromolar (Figure 6I). Interestingly, certain top metabolites were probably able to induce an even larger amount of luminescence in PXR reporter cells at concentrations greater than 30 micromolar. An example is pterostilbene, where the slope of its dose-response curve is continuously increasing even at the highest concentration of 30 micromolar (Figure 6N). This suggests that pterostilbene may agonize PXR to a greater degree at higher concentrations and should be explored in future experiments.

The compound library we utilized was representative of metabolites originating from reactions in microorganisms generally found within the human microbiome. Hirsutide, the top metabolite that induced the largest luminescence in PXR reporter cells, is a specific metabolite of fungi in the genus of *Hirsutella*.[52] Interestingly, species of fungi within the genus of *Hirsutella* are often pathogenic to certain insects such as butterflies and mites as well as many arachnids.[53] Hirsutide's structure is arranged cyclically with aromatic regions facing its exterior.[52] Nigericin was the top metabolite that induced the second greatest amount of luminescence in PXR reporter cells. Nigericin is a toxic metabolite with antibiotic activity derived from *Streptomyces hygroscopicus,* a species of bacteria.[54] Its antibiotic activity stems from its effects on potassium efflux across cell membranes which activates the NLRP3 protein to mediate an inflammatory immune response against pathogenic bacteria.[54] Nigericin's structure is a large, linear compound consisting of oxygen-containing ring structures.[55] The large, ring-containing natures of the compounds we found to activate PXR to a large degree may allude to their binding affinity for PXR's LBD, although it must be noted that PXR has a large hydrophobic binding domain that allows it to be bound by a structurally diverse set of compounds.[40]

Previous research has already identified certain compounds in our top metabolites to be agonists of PXR. For example, Dring et al found that pterostilbene partially activated PXR when compared to rifampicin, the reference PXR agonist they used, when utilizing a similar luciferase reporter assay.[56] They found that other stilbenes, compounds that generally act as antifungal agents and the chemical classification pterostilbene belongs to, also activated PXR to a similar degree.[56] Our findings agree with these results, where pterostilbene induced a statistically significant luminescence value at the two highest concentrations in our targeted dose-response

assay (Figure 6N). 3,3'-diindolylmethane has also been found to be an agonist of PXR.[57] Pondugula et al discovered that 3,3'-diindolylmethane induces CYP3A4 gene expression in a PXR-dependent manner through observing that CYP3A4 expression due to 3,3'-diindoylmethane treatment was significantly decreased in PXR knockdown cells.[57] Results from this experiment showed that 3,3'-diindolylmethane induced a significantly increased relative luminescence in PXR reporter cells, indicating its agonism of PXR and thus agreeing with previous findings.

Certain results we obtained did not align with our expectations. DCA and LCA, both secondary bile acids present in the metabolite library, did not produce significant luminescence in the widespread screen we performed. This contradicts previous data that suggests many secondary bile acids, including DCA and LCA, activate PXR and are considered ligands.[58] While this was indeed surprising, a possible explanation was that the standard working concentration that we used for the metabolite library screen was not ideal for PXR activation by these secondary bile acids. Another possibility was that some compounds were subjected to degradation during the time that their stock solutions were frozen. The freeze-thaw cycle often puts compounds at great risk to degrade in their stock solutions. Degradation of chemicals in stock solutions could have limited all obtained results too. To mitigate the effects of degradation in the dose-response assay, we diluted top metabolites in stock solutions and immediately treated PXR-reporter gene HepG2 with the metabolites. This, along with other steps such as keeping thaw-refreeze cycles to a minimum and making fresh stock solutions often, should be used in future studies to limit stock solution degradation.

Furthermore, our results were obtained *in vitro* from a mammalian hepatocarcinoma line containing PXR specifically engineered to express a DBD that associated with a DNA response

element linked to the firefly luciferase enzyme. This information limits the external validity of the results we obtained since we did not observe the effects of these metabolites on PXR that still possesses its endogenous functionality. Other limitations were present within our study that could have influenced the results too. We deemed it necessary to use a small number of replicates (n = 2) considering we were screening from a large pool of compounds that were metabolites of microbial biotransformation reactions. Using a small number of replicates such as this could influence the interpretation of our results. This also decreases the statistical power of significance results we obtained from the ANOVA tests we conducted for the dose-response assays.

Results from these experiments implicate several future directions to explore. The next step is to quantify PXR expression from a human cell line treated with the top metabolites we elucidated from these findings. This would allow us to observe if top metabolites are causing a transcriptional change in the level of PXR within the cell and establish a stronger understanding that top metabolites are acting as PXR agonists. Results from this experiment could increase the validity of our results since we would be utilizing a cell line that possesses endogenous PXR functioning rather than cells engineered to constitutively express the PXR-reporter gene construct. Another future direction for this work would be to explore the connection between the activation of PXR by these top metabolites and induction of CYP3A4 gene expression. This would be beneficial in understanding if top metabolites from our finding are affecting the expression of metabolic enzymes that PXR is responsible for mediating since CYP3A4 is responsible for nearly 60% of drug metabolism by the host. It would also allow us to understand

if top metabolites are activating PXR with its endogenous functionality to induce gene expression, rather than in a reporter gene construct.

These findings have practical implications. They contribute to the current paradigm that the microbiome mediates beneficial effects for the host through effecting its physiology. We have uncovered novel compounds to be added to the functional PXR ligand collection. To our knowledge, no previous research has found that many of our top metabolites, including those that activated PXR similarly to rifampicin, agonize PXR. Considering these compounds are metabolites of microbial reactions, these results uncover a greater understanding of the interaction between the microbiome and host physiology. Specifically, the connection between microbial metabolites and expression of genes important in both endobiotic metabolism and xenobiotic metabolism through inducing PXR. We hope that these findings will prove useful in future experiments exploring the affects that these top metabolites have on gene expression mediated by PXR.

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