Phenotyping Drug Polypharmacology via Eicosanoid Profiling of Blood

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Running title: lipidomics for drug profiling
Abstract

It is widely accepted that small molecule drugs, despite their selectivity at primary targets, exert pharmacological effects (and safety liabilities) through a multiplicity of pathways. As such, it has proven extremely difficult to experimentally assess polypharmacology in an agnostic fashion. Profiling of metabolites produced as part of physiological responses to pharmacological stimuli provides a unique opportunity to explore drug pharmacology. 122 eicosanoid lipids in human whole blood were monitored from 10 different donors upon stimulation with several inducers of immunological responses and treatment with modulators of prostaglandin (PG) and leukotriene (LT) biosynthesis, including clinical and investigational molecules. Such analysis revealed differentiation between drugs nominally targeting different eicosanoid biosynthetic enzymes, or even those designed to target the same enzyme. Profiled agents—some of them marketed products—affect eicosanoid biosynthesis in ways that cannot be predicted from information on their intended targets. As an example, we employed this platform to discriminate drugs based on their ability to silence PG biosynthesis in response to bacterial lipopolysaccharide (LPS), resulting in differential pharmacological activity in an in vivo model of endotoxemia. Some of the observed effects are subject to variability among individuals, indicating a potential application of this methodology to the patient stratification, based on their responses to bench mark drugs and experimental compounds read on the eicosanome via a simple blood test.

Supplementary key words

Eicosanoid biosynthesis, metabolic phenotyping, drug differentiation, polypharmacology, patient profiling, LC/MS
Introduction

The era of personalized and precision medicine for drug development has been heralded, with the lofty goal of utilizing a patient’s unique genetic profile and disease status to find the right drug match. Several pharmaceutical and biotechnology companies are placing substantial effort in the development of such tailored therapeutics, with the prospects of significantly improved clinical outcomes: efficacy associated with reductions in adverse events (1, 2).

While precision medicine shows already high potential for the discovery of highly efficacious, safer medicines, targeted drug development requires in-depth understanding of the mechanism of action (MOA) of experimental compounds, as well as robust systems for accurate profiling (and prediction) of therapeutic response. The systematic analysis of drugs that interact with more than one molecular target offers an opportunity to dissect the complexities of drug action, and constitutes the first step for the construction of computational models to predict polypharmacology (3, 4). Advances in omics platforms over the past decade have enabled early exploration of global physiological perturbations (genome, proteome, metabolome, epigenome and lipidome) in health and disease, thus ushering the era of systems biology. Several approaches drawing from this emerging discipline have been utilized to build drug-target networks for complex diseases, involving multiple drugs acting on distinct targets. Those efforts have aided in the formulation of combinatorial therapy hypotheses (5, 6). A less desirable, yet a central aspect to polypharmacology is ever-present off-target effects. One major reason for de-prioritization of candidate drugs is the discovery of toxicities, often during late pre-clinical profiling and clinical development stages. Emerging computational methodologies are starting to provide more efficient means to assess compound attributes and “drug-likeness” (7). With the increasing availability of large pharmacological databases (8, 9), in silico prediction algorithms are starting to provide a foundation for the analysis of interaction networks involving drugs, targets and anti-targets. Complementing in silico approaches, advances in analytical chemistry, particularly in the field of mass spectrometry, are enabling larger scale quantification of multiple analytes with unprecedented specificity, sensitivity and accuracy. In spite of
this progress, methodologies applicable to clinical studies are still lacking the simplicity and throughput to become widely used. Once these hurdles are overcome, efficient, disease-focused platforms applicable for patient phenotyping will become essential for stratification based on biochemical differences in their responses to inducers of disease, and drug action.

Here we describe a molecular phenotyping study of human whole blood, by analysis of eicosanoid lipids derived from the conversion of membrane phospholipids to arachidonic acid (AA), an early and critical event in the initiation and establishment of multiple inflammatory responses. The biosynthesis of eicosanoids occurs well within an hour upon exposure of cells to a host of stimuli, providing an opportunity to take quick “snapshots” of metabolic pathways driving the production of lipid messengers. We employed a high-throughput platform including an informatics workflow that allows unsupervised, simultaneous quantification of 122 eicosanoids in human whole blood, tissues, and other matrices (10). Several researchers have reported previously on the systematic measurement of eicosanoid production in whole blood upon stimulation (11, 12), yet the platform that we describe here offers higher resolution, sensitivity and throughput, making its application to clinical sample analysis, and compound screening or profiling for drug discovery, more practical. Covered lipids include PGs, TXs, LTs, eoxins, and lipoxins, shown to be sensitive immunological markers (13-15). When used in survey mode, this platform can discern human donors based on their responses to stimuli, and silencing with clinically relevant compounds. Distinct responses against compounds with the same nominal target protein allow visualization of global pharmacology of individuals with unprecedented resolution, including off-target responses.
Methods

Compound treatments. Fresh heparinized human whole blood samples from donors that met the predefined election criteria (no anti-inflammatory treatments) were obtained from the Normal Blood Donor Service at the Scripps Research Institute on the day of experiment, and used immediately within 30min. HWB samples were processed as previously described (10). Briefly, HWB samples were diluted 1:1 with RPMI-1640 medium and dispensed to 96-well plates at 200 μL/well. Each well was then pretreated with or without clinical and experimental molecules targeting PG and LT biosynthesis (Supplemental Table 3), in concentration-response mode (0.03, 0.3, 3, 30 μM) at 37°C for 15 min, followed by a 30-min stimulation with calcium ionophore, A23187 (final concentration 30 μM) or LPS from E. coli 0111:B4 (Sigma, St Louis, MO, final concentration 1 μg/ml) at 37 °C. Plates were then centrifuged at 1300 rpm at 4°C for 10 min and 100 μl supernatants were immediately transferred to 96-well plate containing 1ml 10% MeOH in water containing a mixture of deuterated internal standards (1ng each).

Mouse endotoxemia model. Female, Balb/C mice (n=5/group, 6-8 weeks, ~20 g; Charles River) acclimated for at least 7 days with free access to food and water with 12 h light/dark cycle. Indomethacin and rofecoxib were prepared in 20% HPβCD at concentrations of 1 and 0.1 mg/mL each and administered at doses of 1 and 10 mg/Kg, (po) 30 min before ip injection of LPS (20 μg/mouse). Blood samples were collected 2 h after LPS dose and processed for serum.

Lipidomics profiling. An in-house developed UPLC-MS based lipidomics platform has been described previously(10). Briefly, human whole blood samples from healthy donors subject to different treatments were processed employing 96-well format solid phase extraction. Extracted lipids were then subjected to simultaneous quantitative UPLC-MS analysis in scheduled MRM (sMRM) mode.
**TNFα quantification in mouse blood.** Mice were pre-dosed with compounds followed by intraperitoneal administration of LPS. Blood samples were drawn into after the animals were sacrificed. Levels of TNFα were then quantified employing ELISA kits (R&D Systems, Minneapolis, MN).

**Data analysis and visualization.** The absolute concentrations (pg/μL) of 122 eicosanoid lipids in individual sample were quantified, based on a calibration curve generated from serial diluted standard samples in each experiment, using our customized R-based quantification software. Data analysis, including principal component analyses (PCA)-based quality control (QC) process to detect outlier samples, differential production analysis of eicosanoid lipids in response to various biological perturbations and hierarchical clustering, were conducted using ArrayStudio V7.0 (www.omicssoft.com). For visualization purposes, both the absolute concentrations of lipids under various biological conditions, and the ratios of lipids in response to various biological perturbations were log2 transformed, and presented in heat maps to reflect the expression profiles of multiple lipids under different conditions.
Results

**Metabolic Phenotyping.** Human whole blood samples from healthy donors were stimulated with the calcium ionophore A23187 to induce activation of cPLA2, a cellular phospholipase that converts membrane phospholipids to AA and other fatty acids in response to intracellular calcium mobilization. Depending on the cell type, AA and other long fatty acyl chain lipids can be converted through enzymatic and non-enzymatic reactions into multiple species that can be grouped into two main classes: (1) PGs derived from the initial production of PGA by cyclooxygenases 1 & 2 (COX1/2) and (2) LTs derived from the initial intermediate LTA4, produced through the combined action of 5-lipoxygenase (5-LO) and its accessory protein, 5-LO activating protein (FLAP). The heat map views that we present provide an efficient, facile way to survey individual variations in lipid profiles derived from COX, LOX as well as non-enzymatic pathways. The absolute amount of each lipid was transformed into log2 values in order to accommodate a larger dynamic range, and the colors (from blue to red) represent the changes in log2 transformed values. A23187 stimulation produced dramatic increases in multiple eicosanoids (Supplemental Figure 1). Consistent with previous observations, approximately half of the lipids in the panel showed significant increase in concentration upon A23187 stimulation for 30 min (10) and many of them remained elevated above the baseline levels for up to 24 hr. These findings contrast with short half-lives, typically in the order of minutes, reported for exogenously added eicosanoids in cellular systems. This remarkable persistency allows testing of serum samples to capture polypharmacological effects of drugs without undue demands on sample collection and preservation.

**Effect of Disease Relevant Stimuli on Eicosanoids.** Stimulation of the AA pathway with A23187 offers a consistent way of profiling multiple donor samples (and drugs) with a standard, yet non-physiological stimulus. Correlations between eicosanome signatures and their causal physiology can only be found from examination of putative inducers of inflammation, and their modulation by experimental and marketed compounds. We employed a set of agents that trigger inflammatory responses through the activation of neutrophils, macrophages, mast cells and other cell types involved in innate, early immune responses,
including bacterial LPS, LTE4 and PGE2. LTE4 was chosen as it is a more stable Cys-LT with known immunomodulatory effects (16) and can drive neutrophilia in humans (17). The changes in eicosanome in 2 donors induced by A23187 (included as a comparator), LPS, PGE2 and LTE4 are summarized in Figure 1. In contrast to A23187, the LPS-induced eicosanome occurred with a delayed onset.

Upon application of LTE4 (up to 0.25 μg/mL) and PGE2 (up to 1 μg/ml) to whole blood, in addition to the added lipids, metabolites of both (such as EXE4, 11t-LTE4 of LTE4 and PGD2, PGJ2, PGE1 of PGE2) were detected throughout the time course study indicating metabolic conversion of the added eicosanoid. Surprisingly, LTE4 and PGE2 application also led to detectable changes in eicosanome perhaps consistent with their immunomodulatory activities (16). Noteworthy are marked increases in PGD2 following application of PGE2. While no literature precedence exists for the conversion of PGE2 to PGD2, or induction of production of PGD2 by increased PGE2, our evaluation of experimental procedures has ruled out analytical artifacts (Supplemental Figure 2). When we spiked d4-PGE2 (1μg/ml) in HWB, no d4-PGD2 was observed. But we noticed a significant increase in the concentrations of PGE2 metabolites previously detected such as PGD2, PGJ2, LXB4 and 11b-PGE2. Additional studies are needed to elucidate mechanisms of PGE2 dependent PGD2 production in human whole blood.

**Human Drug Response Differentiation.** We monitored changes in the lipidomics signature of A23187 in human blood with or without pre-incubation with several marketed or experimental COX and LOX pathway modulators developed for the treatment of multiple inflammatory conditions (Figure 2A; signature changes for all individuals are shown on Supplemental Figure 3). A23187 stimulation alone induced changes in 33 lipids relative to untreated condition (Figure 2A, leftmost column). Depending on inhibition of COX or LOX pathway, observed effects on the lipidome were biased towards the PGs or LTs, respectively (Figure 2A-C). In addition, off-pathway activities were also observed. The first generation anti-inflammatory drug discovered over half a century ago, Indomethacin, a dual COX1/2 inhibitor, potently blocked the synthesis of multiple PGs, with modest effects on other branches of the eicosanome. We observed two distinct differences, one between Indomethacin vs COX-2 inhibitors and
the second between celecoxib/valdecoxib vs rofecoxib, all known COX-2 inhibitors (Figure 2B). The selective COX2 inhibitors celecoxib (Celebrex™) and valdecoxib (Bextra™; (18)) displayed inhibitory effects on certain LTs on top of their expected PG pharmacology (19, 20). In susceptible individuals, aspirin can trigger asthma, a clinical phenotype shared by several COX-inhibiting NSAIDs. The flux of AA through COX/LOX pathway generates mediators that have either bronchoconstrictive (CysLTs, PGD2) or bronchodilatory (PGE2, PGI2) functions. While the exact etiology of aspirin-sensitive asthma is unknown, shifting of AA metabolism into preponderance of bronchoconstrictor eicosanoids may contribute to the bronchospasm. Only recently, the safety of COX-2 inhibitors in asthmatic subjects was established (19, 21) implicating a role for COX-1 pathway/pathway-derived mediators in aspirin-sensitive asthma. Equally surprising is our observation of only tenuous pharmacological activity across the surveyed eicosanome for Rofecoxib (Vioxx™(22); Figures 2B and 3B-D), a drug now retired from the market because of cardiovascular safety liability and mortality (23). It has been suggested that this liability may stem from inhibition of PGI2 biosynthesis (24). Based on the lack of strong pharmacological activity across the eicosanome at concentrations well above its Ki, we suggest that alternative mechanisms may be operative. Consistent with this, it has been suggested that Rofecoxib conversion to an ionized species under physiological conditions mediates accumulation of maleic anhydride, which in turn may promote formation of oxidized lipids and increases in low-density lipoprotein (LDL). LDL could serve as the trigger for the cardiac liabilities seen with this drug. Other COX1/2 modulators are not ionizable in a similar way to Rofecoxib, and do not trigger the resulting adverse effect (25-27).

On the LT branch (Figure 2C), the 5-LO inhibitor Zileuton (Zyflo™ (28)) displayed a relatively broad profile at the two higher concentrations, with moderate pharmacological effects across the eicosanome. Two experimental drugs, MK591 (Quiflapon (29)) and its predecessor MK886 (30), targeting the 5-LO activating protein FLAP, showed a cleaner profile, blocking only the production of LTs and leaving the COX pathway intact. Montelukast (Singulair™ (31)), a selective antagonist of the LTD4 receptor CysLT1,
had a relatively circumscribed pharmacological footprint on the eicosanome, affecting exclusively cysteinyl LT levels. This is somewhat surprising and may indicate the existence of feedback loops connecting signaling from LT-sensing G protein-coupled receptors to their biosynthetic enzymes. These observations are consistent with the inhibition of CysLT production in ragweed and mite-stimulated peripheral blood mononuclear cells from patients with asthma (32) and correlation of clinical response to Montelukast treatment in asthmatics with decreases in urinary LTE4 levels, a marker for systemic CysLT tone (33). These findings also raise the question on the relative merits of targeting LTC4 synthase pathway as an alternative to CysLT receptor antagonism for treating CysLT driven pulmonary conditions.

We also performed a comparison of two 5-LO inhibitors, Zileuton (Zyflo™), a marketed drug that is efficacious in severe asthma, yet is not widely prescribed because of idiosyncratic elevations in liver enzymes (34), and MK-0633 (35, 36), an experimental molecule developed by Merck that did not show efficacy in asthma and chronic obstructive pulmonary disease (COPD) trials (40). We used these two molecules to illustrate the remarkable differences that can be revealed between two inhibitory molecules targeting the same enzyme, 5-LO. MK-0633 has much higher potency relative to Zileuton in blocking the production of LOX pathway eicosanoids, and is more selective as well: Zileuton's off-target effects on the COX pathway lipids are more significant at the higher concentration range (Figure 2C). Given that MK-0633, a more selective 5-LOX inhibitor molecule with a superior physiochemical profile relative to Zileuton, did not meet efficacy end points in proof of concept studies in asthma and COPD asthma trial (36), the data reveals that mechanisms in addition to 5-LOX inhibition, such a COX inhibition, contributes to the efficacy of Zileuton.

**Inter-subject Variability.** Blood samples from 23 human donors were analyzed to understand inter-individual variability in A23187-induced eicosanoids (Supplemental Figure 3). The results show remarkable variability in the levels of lipid production. The observed qualitative and quantitative differences in levels of specific PGs and LTs both in basal and stimulated conditions indicate that there is an individual “inflammatory response potential”, which is readable through the eicosanome. To rule out
the possible variations caused by cellular activations or sample handling artifacts, we also demonstrated that eicosanoid generation is not dependent upon time of sample processing after the blood was collected from donor. Over two hours of incubation at 37 °C, the primary eicosanoid levels remain relatively unchanged and no new metabolite was observed in the blood spiked with deuterated AA (Supplemental Figure 4). Additionally, we took into account the donors’ smoking status (37, 38) and dietary supplements intake in order to avoid the external inference with the eicosanoid levels. With 2 smokers and 1 person taking fish oil out of our 23 donors, the data showed no bias due to these two factors. The lower detection limits (LLODs) and upper detection limits (ULODs) of all the lipids in 10 donors were summarized in the Supplemental Table 1, as well as their quantification ranges (pg/μL). The measured amounts of eicosanoids under stimulated conditions in human whole blood are consistent with reported levels (39). Most of the lipids exhibited inter-donor variation at the baseline measurements, which is intrinsic to the human blood studies. Because of the interest in baseline eicosanoid quantification, the standard curves were prepared in RPMI medium rather than blood; this could be a potential source of error/variation due to differential suppression from media and human blood. Even though no two individuals were identical in their eicosanome signatures in response to calcium ionophore stimulation and modulations of the stimulation by various compounds, there are clear evidences for coordinate changes in clusters of eicosanoids, suggesting the possibility of phenotypic profiling of targeted populations via their eicosanoid signatures.

**Predicting in vivo Pharmacology from Lipidomics Signatures.** LPS is a bacterial product and a TLR4 agonist, and LPS activation of monocytes/macrophages leads to elaboration of various cytokines such as tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-10, and pro-inflammatory lipid mediators such as PGs, LTs and platelet-activating factor (PAF). Under the conditions of the experiment, LPS induced the production of mainly COX pathway metabolites (Figure 3A), including PGE2, TXB2, and 12-HHT (40, 41) with delayed kinetics relative to the calcium ionophore control, starting at 4 h post-addition. The overall LPS induction profiles of the lipids are summarized in Supplemental Table 2,
illustrating donor variability. We examined the effects COX1/2 and selective COX-2 inhibitors on LPS induced lipidomics signature in human whole blood as a model of inflammation. Average changes on this signature from eight donors are shown on Figure 3B (changes for all individuals are shown on Supplemental Figure 5). Indomethacin (42) displayed higher potency relative to Celecoxib (43) and Rofecoxib (22) in inhibiting the production of COX pathway eicosanoids, Rofecoxib exhibited an inhibitory effect only at the top concentrations tested (3 and 30 μM).

PG metabolites such as TXB2 and PGE2 have modulatory effects on TNFα synthesis and release. Inhibition of thromboxane (TX) biosynthesis attenuates endotoxin-induced TNFα production (44) whereas PGE2 suppresses TNFα release, and blockade of PGE2 biosynthesis not only stimulates TNFα release, but prevents its breakdown (45, 46). Therefore, it would not be possible to predict a priori the outcome of inhibiting COX pathway. The effect of combinatorial inhibition TXB2 and PGE2, on LPS induced release of cytokines such as TNFα: would need to be addressed in vivo. We set out to investigate the outcome of combinatorial inhibition of TXB2 and PGE2 on TNFα release in an endotoxemia model in vivo. We examined the effects of Indomethacin and Rofecoxib in LPS induced inflammation model in mouse with serum levels of TNFα as a biomarker of inflammatory response and, PGE2 and TXB2 levels as measures of COX pathway inhibition. For this, a sub-lethal dose of LPS was given to mice pre-dosed with Indomethacin, Rofecoxib or saline solution. Figure 4A shows that a limited lipidomics signature resembling the one detected with human whole blood was observable in blood samples from those animals, including elevations in TXB2 and PGE2. Again something that could not be predicted a priori, Indomethacin, but not Rofecoxib, induced a dramatic elevation in TNFα levels (Figure 4B). The serum levels of PGE2 (Figure 4C) showed strong negative correlation (r = -0.727) to those of TNFα, with a p value of 0.00006 (Figure 4D). This observation indicated that, in the mouse, up-regulation of TNFα production in response to LPS by inhibition of PGE2 biosynthesis may over-ride down-regulation expected from silencing of TXB2 biosynthesis. While NSAIDs are successfully used to treat several inflammatory conditions in clinical practice, the observations from the present study shed light on
potential side effects induced by NSAIDs and selective COX-2 inhibitors. For example, literature reports indicate that NSAIDs such as indomethacin increased LPS induced elaboration of IL-6 and TNFα in human whole blood, and exogenously added PGE2 attenuated LPS-induced cytokine response and reversed the effects of Indomethacin. Interestingly, selective COX-2 inhibition (NS398), but not selective COX-1 inhibition (SC-560), exerted a stimulatory effect on the expression of pro-inflammatory cytokines (47). In a more translational study, Endres and coworkers showed that short term use of either aspirin or ibuprofen results in a ‘rebound’ increase in cytokine (eg., IL-1β) -induced cytokine synthesis (TNFα) in humans (48). These results collectively support homoeostatic functions of arachidonic metabolites. The lipidomics platform combined with protein arrays to interrogate cytokine pathways may help identify novel, differentiated anti-inflammatory agents. Our observations provide a plausible hypothesis to explain seemingly contradictory outcomes from the use of NSAIDs for the treatment of inflammatory bowel disease (IBD). NSAIDs have been inconsistently implicated in cases of de novo IBD and disease exacerbations (49). While there is conflicting data regarding NSAID use and IBD exacerbations, with subsets of patients in which NSAIDs use worsens disease activity and others in which selective COX-2 inhibitors lower disease activity (50). The identification of those distinct patient populations may impact optimal clinical management. Our work that shows that indomethacin dramatically increased TNFα levels, while the selective COX-2 inhibitor, Rofecoxib did not. This may explain instances of de novo IBD and disease exacerbation in certain patients exposed to non-selective NSAIDs. Reductions in TNFα levels in IBD patients exposed to some COX-2 inhibitors may also explain the better tolerance of selective COX-2 inhibitors versus NSAIDs, and the occasional improvement in disease activity (51). Interestingly both NSAIDs and TNFα antagonists are mainstays of therapy for many inflammatory and rheumatologic diseases. Our findings call for comprehensive clinical pharmacology studies to explore potential unintended antagonism as a result of dual therapy, if the NSAID is not chosen carefully.

Discussion
Two compelling lessons emerge from our analysis. (1) Lipidome-focused profiling provides a unique scope for observation of the biochemical mechanisms of action of experimental and physiological inducers of inflammatory responses and anti-inflammatory mechanisms impinging directly or indirectly on arachidonic pathway. This is not surprising, as the metabolome is the spoken language of proteins as they interact with each other directly through physical contact, or indirectly through modulatory effects mediated by biosynthetic intermediaries and second messengers. (2) Even for the best characterized physiological agents and pharmacological tools, responses are complex. Thus, traditional approaches that rely on target proximal or distal signaling events to assess dose effect relationships (target biology) seem inappropriate to capture the true (poly) pharmacology of essentially all therapeutics. We need to depart from traditional target-by-target selectivity panels common in pharmaceutical industry, in favor of more comprehensive analytical readouts and finger prints for unbiased exploration target pharmacology. The highly efficient platform for the profiling of drug polypharmacology described here exemplifies such a platform.

Our studies offer a great example of applying lipidomics profiling to drug discovery. Firstly we have a comprehensive, highly efficient eicosanoid panel that enables large-scale compound screening. Secondly, this is the first time to employ this eicosanoid panel to differentiate clinical molecules and demonstrate their distinct poly-pharmacology. Last but not least, this is also the first in the field to employ the platform to reveal TNFα pharmacology and investigate the individual differences in immune responses. We envision the broad utility of the agonistic lipidomics profiling platform described here to complex biological problems, for example to defining lipidomics networks in health and disease, pharmacogenomics, hypotheses generation, pathway based drug discovery and re-purposing existing pharmacopoeia. In turn, we expect that information obtained from such profiling work will provide much needed complementary data to enable building more efficient networks that are today dominated by expression array data, lacking predictive value with regard to the actual production of proteins and
modulation of their specific functions. These lessons, taken as a whole, provide an illustration for how precision medicine may become a reality, hopefully in the not-so-distant future.
Acknowledgements

We thank Tatiana Koudriakova for generously sharing Janssen Discovery Sciences-La Jolla mass spectrometry instrumentation that are essential for this work. We also thank Xiaohua Xue, Alec Lebsack and Jim Karras from Janssen Immunology Discovery for helpful discussions and additions to the manuscript, and the laboratory of Prof. Ed Dennis at UCSD for help in method development and troubleshooting. We also wish to acknowledge Drs. Philip E. Needleman, Peter C. Isakson, Henry P. Halushka, James P. Edwards and Anne M. Fourie, for their extensive review of draft versions of the manuscript, with many insightful critiques, and Laura Pace for thoughtfully pointing out the relevance of our findings to the identification of IBD patient subpopulations with differential responses to NSAIDs.
References


Figure Legends

Figure 1. Human whole blood eicosanoid signatures in response to stimulations of LPS, LTE4 and PGE2. Heat map of averaged log2 ratios (from two donors) of 26 eicosanoids in human whole blood, in response to stimulation of calcium ionophore (A23187; Calon), LPA, LPS, and LTE4, relative to untreated samples. These 26 eicosanoids were induced by either one of the three stimulations (LPS, LTE4, and PGE2), with fold change greater than 2 and false discovery rate less than 0.05, in both donors in at least two combinations of time and concentrations. The color index in heatmap reflects the magnitude of induction (or reduction), with a range from -4 (reflecting 16 fold reduction) to +8 (reflecting 256 fold induction).

Figure 2. Effect of COX inhibitors, FLAPs, and Montelukast on A23187 induced eicosanoids in human whole blood. A. Heat map of averaged log2 ratios of 33 eicosanoids in human whole blood, in response to stimulation of calcium ionophore (A23187, 30 uM), and modulations of the stimulation by four COX inhibitors (Celecoxib, Indomethacin, Rofecoxib, and Valdecoxib), four 5-LOX/FLAP Inhibitors (MK-0633, MK591, MK886, and Zileuton), and Montelukast, a LTD4 receptor antagonist, at four concentrations (0.03 – 30 uM) each. These 33 eicosanoids were induced by calcium ionophore (Calon), with fold change greater than 1.5 and false discovery rate less than 0.05, in 23 donors collectively; log2 ratios for each compound treatment were averaged among at least 10 donors. Data for individual donor were presented in Supplement Figure 2. B. Heat map of averaged log2 ratios of 6 eicosanoids in COX pathway, in response to calcium ionophore stimulation and its modulation by COX inhibitors. B. Heat map of averaged log2 ratios of 21 eicosanoids in LOX pathway, in response to calcium ionophore stimulation and its modulation by FLAPs and Montelukast. Condition of CaIon: calcium ionophore vs. untreated; Condition of individual compound: Calon + Compound vs. Calon + Vehicle.

Figure 3. Effect of COX inhibitors on human whole blood LPS eicosanoid signature. A. Heat map of averaged log2 ratios (from two donors) of five eicosanoids in human whole blood, in response to stimulation of calcium ionophore (A23187, 30 uM; Calon), or LPS (0.01 – 1.25 ug/mL). These five eicosanoids were induced by LPS stimulation, with fold change greater than 2 and false discovery rate less than 0.05, in both donors in at least two combinations of time and concentrations. B. Heat map of log2 ratios (averaged among 4-8 donors) of the LPS eicosanoid signature, in human whole blood in response to LPS stimulation and COX inhibitors after 4 hours. Data for individual donor were presented in Supplement Figure 3. C and D. Box plots of log2 ratios of the TXB2 and PGE2 in individual human whole blood samples, in response to LPS stimulation and COX inhibitors. Condition of LPS: LPS (1 ug/mL) vs. untreated; Condition of COX inhibitors: LPS + COX inhibitor vs. LPS + Vehicle.

Figure 4. Effect of LPS and COX inhibitors on human LPS eicosanoid signature in mouse. A. Heat map of human LPS eicosanoid signature in mouse whole blood, after in vivo LPS stimulation, with or without treatment of COX inhibitors. Log2 transformed lipid levels in mice from six treatment groups were normalized by the median value of each lipid in all animals. B. TNFα levels in mouse whole blood measured by ELISA. C. PGE2 levels in mouse whole blood. D. The correlation between the concentrations of PGE2 and TNFα in whole blood from mice with LPS stimulation. G1: Saline, G2: LPS (1 mg/Kg) + vehicle, G3: LPS (1 mg/Kg) + 1 mg/Kg Indomethacin, G4: LPS + 10 mg/Kg Indomethacin, G5: LPS + 1 mg/Kg Rofecoxib, and G6: LPS + 10 mg/Kg Rofecoxib.
Figure 2

A

B

C

Pathway

Condition

Cmpd Conc

Starting Molecules
COX
CYP
LOX
Non-Enzymatic
Figure 3