DEPARTMENT OF CHEMISTRY

Randy Hamchand

How I Wrote My Prospectus

My initial thesis project was pitched to me by my research advisor. I had almost no experience with protein biochemistry and metabolomics, key skills needed for the project, at this point. Luckily, some preliminary work was already completed by a post-doc in the lab, so I had some mentorship to get data. While this project was later dropped, the process of doing this research helped me learn a plethora of tools and techniques that were indispensable in my later research.

Advice for Prospectus Writers

Don't overthink or worry too much about crafting the perfect prospectus. The "weight" of these milestones is very department dependent, so talk to peers in your department to determine how much you need to "stress" about this assignment.

Insights into Novel Metabolism of Human RECON Homologs

Thesis Proposal

Table of Contents

Insights into Novel Metabolism of Human RECON Homologs

In the intestinal tract, it is now widely recognized that the human microbiota can regulate host inflammation and genotoxicity. However, the molecular bases linking host-microbe interactions to local and systemic inflammation and DNA damage remain largely obscure. Recently, a murine non-canonical pattern recognition receptor (PRR) known as RECON (Reductase Controlling NF-κB) was implicated in modulating innate immunity in mice through the recognition of microbial cyclic-dinucleotides (cdNs) and subsequent regulation of NF-κB. RECON undoubtedly represents a key facet through which mouse-microbe interactions are monitored; thus, we reason that the discovery of a human RECON analog, which serves a related role to its mouse counterpart, would be of tantamount importance to the field of immunometabolism. The objective of this research is to elucidate the unique biochemical functions of human RECON homologs and to understand how their specific biochemistries facilitate host-microbe interactions.

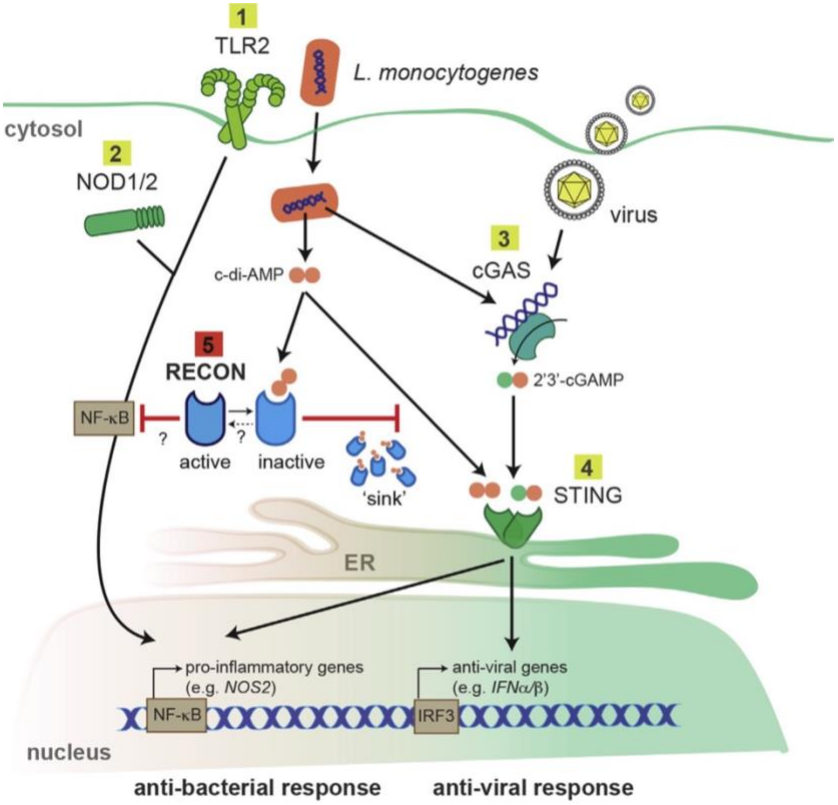

Figure 1. Interplay between RECON, STING, and cdNs in regulating innate immune responses. Graphic reprinted from Maelfait and Rehwinkel (46).

A. Specific Aims

Our hypothesis is that high-homology human homologs of RECON serve previously undescribed essential roles in activating innate immunity and that insights into their metabolomic profiles and unique biochemistries can lead to the discovery of a novel mechanism of inflammation. We will test this hypothesis through the following specific aims:

Aim 1: To deduce candidate human RECON analogs and characterize their enzyme-dependent metabolites and PAMP-binding spectra.

We will use bioinformatic analyses to determine the most probable human RECON analogs and will perform heterologous expression-based, *in vitro* enzyme-based, and mammalian-knock-out-based metabolomic experiments to identify enzyme-dependent small-molecules. Using preparative-scale HPLC, we will purify these metabolites on scales conducive for structural elucidation by multi-dimensional NMR and tandem-MS. Through biosynthetic retro-analysis, we will infer reasonable enzyme substrates for the compounds identified – to be either bought or synthetized – and will perform LC-MS-based and absorbance-based *in vitro* enzymatic experiments to determine the kinetic parameters, such as V_{max} and K_m, of the transformations. Furthermore, through a combination of *in vitro* assays and isothermal titration calorimetry, we will establish binding efficacies for candidate RECON analogs with known pathogen-associated molecular patterns (PAMPs).

Aim 2: To assess the immunoregulatory capabilities of human RECON homologs and to propose a tentative human RECON analog.

We will perform cell-based assays on mammalian cells either overexpressing human RECON homologs or lacking specific human RECON homologs to examine how regulation of these proteins affects production of immunomodulators, such as NF-κB, in the presence or absence of cdNs or other PAMPs. Findings from these studies will be supplemented with a $RECON^{-/-}$ mouse model of inflammation. Finally, by evaluating the differential regulation of immunomodulators between RECON and human RECON homologs – as well as by investigating their comparative metabolomic profiles and PAMP binding spectra – we will proffer, if possible, a human RECON analog.

B. Background and Significance

The innate immune system is the first line of defense against infections and other injuries. It is activated by pattern recognition receptors (PRRs) that broadly distinguish between foreign and host molecules (1). Through this discretion, PRRs initiate inflammatory responses and recruit macrophages – as well as other immune cells – to the site of injury. Dysregulation of these processes drives chronic inflammation and contributes to carcinogenesis (2,3). In the intestinal tract, the collection of microbes known as the microbiota contribute to host inflammation and have been shown to significantly affect human physiology, disease, and clinical response to drugs (4). Though the microbiota's influence on the innate immune system is evident, the molecular mechanisms connecting microbial signals to local and systemic inflammatory responses remain widely obscure.

Microbial cyclic-dinucleotides (cdNs) are recognized as activators of the Stimulator of the Interferon Genes (STING) pathway of innate immunity (5). In response to cytosolic DNA, mammals produce the cdN 2'3'-cyclic GMP-AMP (2'3'-cGAMP): the endogenous substrate of STING. In a process implicated in antiviral immune defenses and certain autoimmune responses *in vivo*, 2'3'-cGAMP binds to adaptor protein STING to induce an NF-κB and antiviral type I interferon response (5-6). In addition to 2'3'-cGAMP, STING also triggers an immune response upon binding the microbial cdNs 3'3'-cyclic di-GMP (c-di-GMP) and 3'-3' cyclic GMP-AMP (3'3'-cGAMP). However, while STING can recognize microbial cdNs, STING is not produced robustly throughout the body and microbial cdNs can induce a pro-inflammatory antibacterial state distinct from the STING pathway (7-9).

Recently, the murine liver enzyme RECON (reductase controlling NF-κB) – which represents a major PRR and enzyme of unknown function – was shown to antagonize the STING pathway and induce an alternative pro-inflammatory antibacterial state in response to microbial cdNs (7-9). Specifically, RECON recognizes microbial cdNs as microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) and regulates NF-κB via an unknown immunometabolic mechanism (**Fig. 1**). Intriguingly, cdNs bind to the NAD^+ site of RECON which would block its predicted oxidoreductase functionality (9).

RECON belongs to the aldo-keto reductase (AKR) superfamily of enzymes – also known as oxidoreductases (10). While oxidoreductases can classically be oxidases or dehydrogenases, AKRs are thought to predominately act as $NAD(P)^+$/NAD(P)H dehydrogenases that catalyze the reduction of aldehydes and ketones to primary and secondary alcohols, respectively (10). Additionally, some AKRs can also reduce double bonds and nitro groups (11-12). At present, there are 15 known human AKRs which serve diverse biological roles including metabolizing sugar and lipid aldehydes (such as advanced glycation end products and lipid peroxidation products) (13-15), chemical carcinogens (16), polycyclic aromatics hydrocarbons (PAHs) (17), steroids (18-21), and prostaglandins (22,23) (**Fig. 2**). *In vitro* studies have revealed that human AKRs exhibit substrate scopes much broader than their *in vivo* biochemistry may imply (24,25). Indeed, the ability of AKRs to recognize various classes of compounds with reactive carbonyl groups makes them key cytoprotective enzymes, especially in the mitigation of oxidative stress

and detoxification of xenobiotics (25). Dysfunction of human AKRs has been linked to a plethora of ailments including diabetes complications, bile acid deficiencies, and a multitude of cancers (26-32).

While RECON natively represses NF-κB activation through an unknown immunometabolic mechanism (9), little in known regarding human AKRs and NF-κB interactions. Though AKR1C3 is thought to regulate NF-κB through an indirect process involving prostaglandin metabolism (33), and AKR1B10 has been shown to promote the production of inflammatory cytokines via the NF-κB pathway

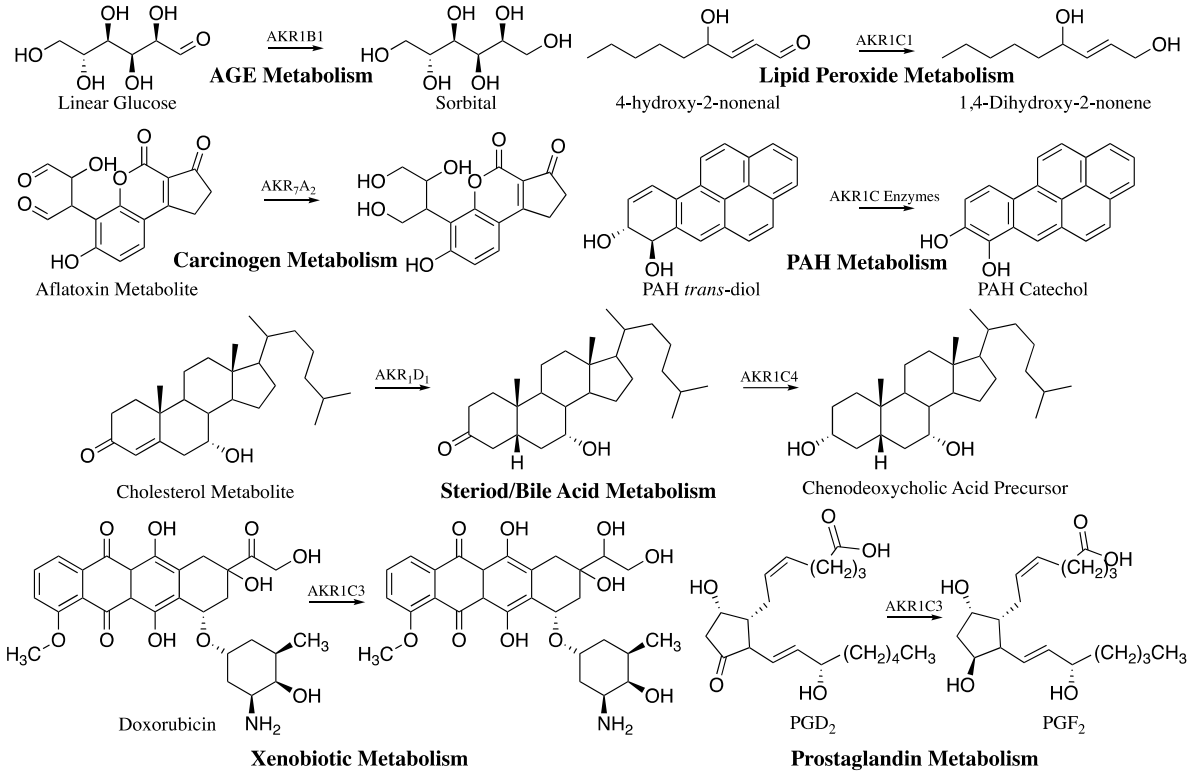

Figure 2. Overview of Metabolic Activity of human AKRs.

(34), none of the proposed routes resemble that of RECON's. Moreover, it is unknown if human AKRs can bind cdNs – though AKR1C1 has been purified from cdN-based pulldowns of mammalian cell lysates (9). Interestingly, AKR1C1 – along with seven other AKRs – is strongly induced by Nrf2: a nuclear factor which upregulates expression of certain genes in response to oxidative stress. Nrf2 and NF-κB are known to modulate oxidative stress and inflammation, respectively, however there is substantial interplay between the two pathways (35). Specifically, Nrf2 seems to be important in regulating expression of anti-inflammatory genes (36).

Currently, there is a dearth of knowledge concerning the interactions of human AKRs with PAMPS and their resulting effects on human immune responses. By examining high-homology human homologs of RECON, we hope to discover previously undescribed biochemistries of these redox enzymes which may implicate them, mechanistically, in immune and oxidative stress responses. Furthermore, by comparing the metabolomic profiles and immunoregulatory capabilities of these enzymes with RECON, we hope to deduce a possible human RECON analog.

C. Preliminary Results

C.1 Bioinformatic Analysis of Human AKRs

a.) Homology Analysis

To determine which AKRs should be prioritized in our pursuit to identify a human RECON analog, we performed comparative protein sequence BLAST (Basic Local Alignment Search Tool) queries between the 15 human AKRs and mouse RECON. A representative phylogenetic tree for this analysis is shown in **Fig. 3**.

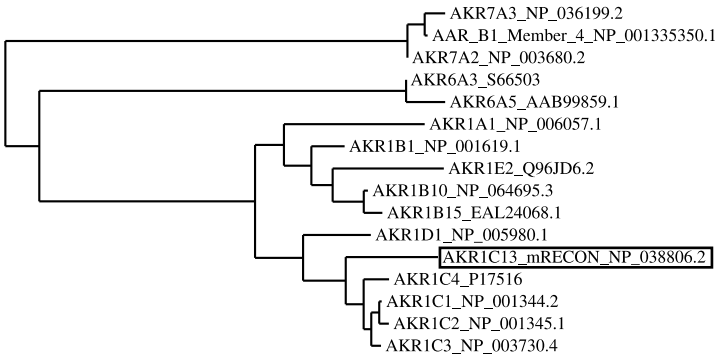

Figure 3. Phylogenetic relationship between RECON and human AKRs.

The BLAST survey revealed all Family 1 AKRs had at least one isoform which covered at ≥95% of the RECON amino acid sequence. Moreover, of the Family 1 AKRs, only the C and D subfamilies had ≥50% identity (i.e. the extent to which two amino acid sequences have the same residues at the same positions in an alignment). Specifically, AKRs 1C1, 1C2, 1C3, 1C4 and 1D1 had 67%, 66%, 65%, 66%, and 52% homology with RECON, respectively. When similarity is considered as a function of percent positive substitutions (i.e. identity but with conservative substitutions – substitutions that preserve the physico-chemical properties of the original residue – marked as identical), AKRs 1C1, 1C2, 1C3, 1C4, and 1D1 had 80%, 79%, 78%, 82%, and 68% homology with RECON, respectively. RECON, along with all of the AKR1C enzymes, had a 323 amino acid protein sequence; AKR1D1 had a 326 amino acid sequence.

Comparisons between the human AKRs uncovered further insights. In particular, AKRs 1C1 and 1C2 differ by only seven residues (five if positive substitutions are considered) and the similarity of all 1C AKRs, especially when positive substitutions are considered, is remarkably high (≥90%).

From this analysis, we believe the most likely human RECON candidates reside in the AKR1C subfamily. Moreover, based on their high homologies, it is possible that multiple AKR 1C enzymes serve similar roles to RECON.

b.) Tissue Distribution Analysis

In addition to homology analysis, we also examined the comparative expression of family 1 AKRs and RECON through a tissue distribution analysis (**Fig. 4**). The human data was compiled through RNA-seq on normal human tissues (37) and the RECON expression data was compiled through RNA profiling of the mouse genome (38).

The heatmap highlights that RECON is predominately expressed in the liver and colon of mice. This finding is consistent with the original identification of RECON which entailed a cdN-based lysate pulldown of mouse liver cells (9). Subfamilies A, B, and E AKRs have expression profiles distinct from RECON's: AKR1A1 is expressed ubiquitously, though upregulated in the kidney; AKR1B1 is almost exclusively expressed in the adrenal; AKRs 1B10 and 1B15 are predominately expressed in the duodenum and stomach; and AKR 1E2 is almost exclusively expressed in the testis. Conversely, the 1C and 1D families of human AKRs share vastly more similar expression profiles to RECON: AKRs 1C4 and

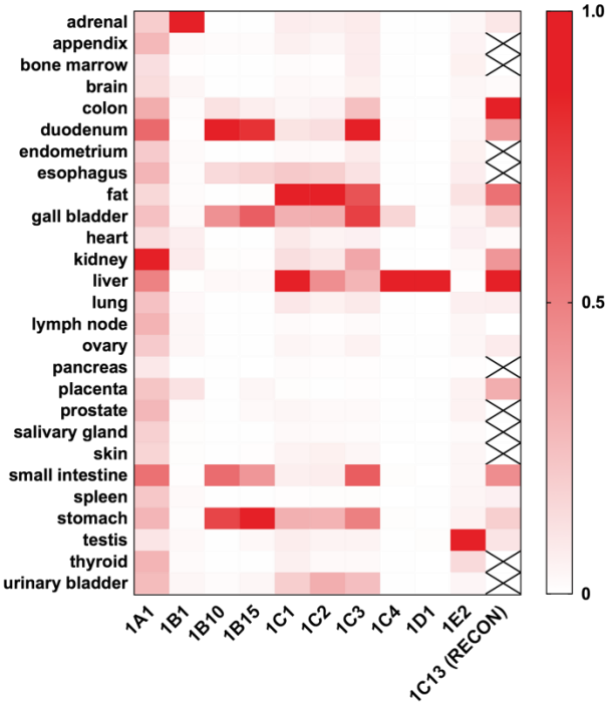

Figure 4. Normalized tissue distributions of RECON and human family 1 AKRs. "X" indicates a lack of comparable data.

1D1 are almost completely liver specific; AKRs 1C1, 1C2, and 1C3 all have somewhat ubiquitous distributions, though there is some preference for the liver and fat. Interestingly, none of the AKRs, other than perhaps AKRs 1C3 and 1A1, exhibit pronounced colon expression. This analysis, together with the homology analysis, leads us to believe that AKRs 1C1, 1C2, 1C3, 1C4, and 1D1 are the most likely candidates for a human RECON analog.

C.2 Heterologous-Expression Metabolomics of Human AKRs

a.) Metabolomic Media Profiling

Based on insights from the bioinformatic analysis, AKR 1C1, 1C2, 1C3, 1C4, and 1D1 genes were codon optimized for *E. coli* and cloned into the pBad18(amp) plasmid vector; gene insertion was validated through Sanger sequencing (Note: AKR1C3 is being worked on by another member of the group). The proteins were heterologously expressed in *E. coli* in either LB, DMEM + 10% FBS, BHI, 10 g/L horse liver (HL) extract in M9 + 0.4% glucose, or 10 g/L HL extract in M9 + 0.4% glycerol media.

By comparing the untargeted metabolomes of cell cultures overexpressing or lacking a human AKR, we identified dozens of enzyme-dependent compounds in each medium; some compounds were medium specific while others were present in multiple media. When sorting the "hits" by m/z ion intensity, it became apparent that cultivating *E. coli* in 10 g/L HL extract in M9 + 0.4% glycerol media is the most potent method of producing enzyme-dependent ions of intensity $\geq 10^5$ counts (10^5 counts is an empirical minimum intensity needed for a compound to be reasonable isolated through large-scale cultivation). Moreover, AKR1C2 has far more 10^5 hits than the other enzymes, thus, it was chosen first for large-scale cultivation.

b.) Large-Scale Cultivation of AKR1C2

18L cultivation of AKR1C2 resulted in sub milligram-scale purification of a single enzyme-dependent metabolite. The compound was observed in both AKR1C1 and AKR1C2, though the ion intensity is several-fold stronger in 1C2 (**Fig. 5A**). The metabolite had an experimental mass of 231.1126 m/z corresponding to a molecular formula of $C_{13}H_{14}N_2O_2$ ([M+H] mass of 231.1128 m/z). Through a combination of tandem MS, UV-Vis spectrometry, and 2-D NMR (COSY, HSQC, and HMBC) the compound was identified as 3-phenyl-5-(2-hydroxypropyl)-pyrazinone (**Fig. 5B**).

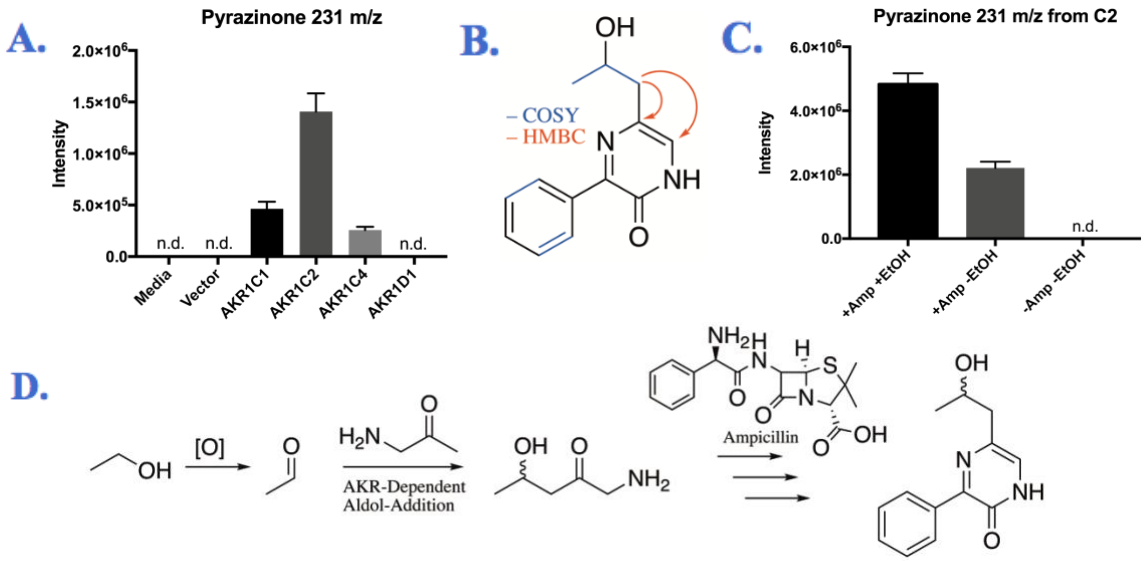

Figure 5. A.) Production screen of metabolite 231 by enzyme. B.) Chemical structure of metabolite 231. C.) Ampicillin/EtOH dependence screen of metabolite 231. D.) Proposed biosynthesis of metabolite 231.

To determine AKR1C2's role in synthesizing the pyrazinone, the metabolomic experiment was repeated under three conditions: with ampicillin dissolved in 1:1 water:ethanol, with ampicillin in water, and without ampicillin. The pyrazinone was determined to be ampicillin-dependent and upregulated in the

presence of ethanol (**Fig. 5C**). Tentatively, we believe this study supports the claim that AKR1C2 and AKR1C1 facilitate carbon-carbon bond formations through a stereospecific aldol addition mechanism. The ability for AKRs to catalyze aldol additions has been shown, *in vitro*, through AKR1A1 (39). We posit that excess ethanol is endogenously oxidized to acetaldehyde which subsequently undergoes an AKR1C2-catalyzed aldol addition with amino acetone – a common bacterial metabolite. This is intriguing because the reactive carbonyl is neutralized through an aldol reaction as opposed to hydride reduction, which is thought to be the dominant mechanism in cells. The resulting product cyclizes with ampicillin and spontaneously oxidizes to yield the functionalized pyrazinone (**Fig. 5D**). The discovery of AKR1C2 and 1C1s' ability to catalyze aldol additions may be of biological significance; however, since our proposed mechanism utilizes a bacterial metabolite (amino acetone), this specific pathway is likely not applied by mammalian cells. Nevertheless, we will evaluate mammalian aldol reactions in future studies.

c.) Shared RECON-AKR Metabolites

Through comparative analysis of RECON and human AKR metabolomes, we discerned several shared metabolites. One such compound that has been structurally characterized in the **xxx** lab is a 14-carbon lipid attached to an N-acetyl-glucosamine (GlcNAc) (**Fig. 6A**). This lipomonosaccharide metabolite, herein referred to as LMS, has the lipid moiety attached at either the C3 or C6 position in a 1:1 ratio. LMS was preferentially upregulated in AKR1C1 (**Fig. 6B**). Intriguingly, the C3-functionalized LMS resembles intermediates in Gram-negative lipopolysaccharide (LPS) biosynthesis (*e.g.*, Lipid IV_A) (**Fig. 7**). Indeed, these newly identified metabolites are secreted at basal levels during bacterial cultivation, which likely represents spontaneous hydrolysis products of early lipid IV_A intermediates. Because LPS intermediate lipid IV_A, which is largely cell associated like LPS, is the minimal structural unit for toll-like receptor 4 (TLR4) binding and signaling (40), these results suggest that RECON could be a new sensor for the early diffusible intermediates of LPS biosynthesis.

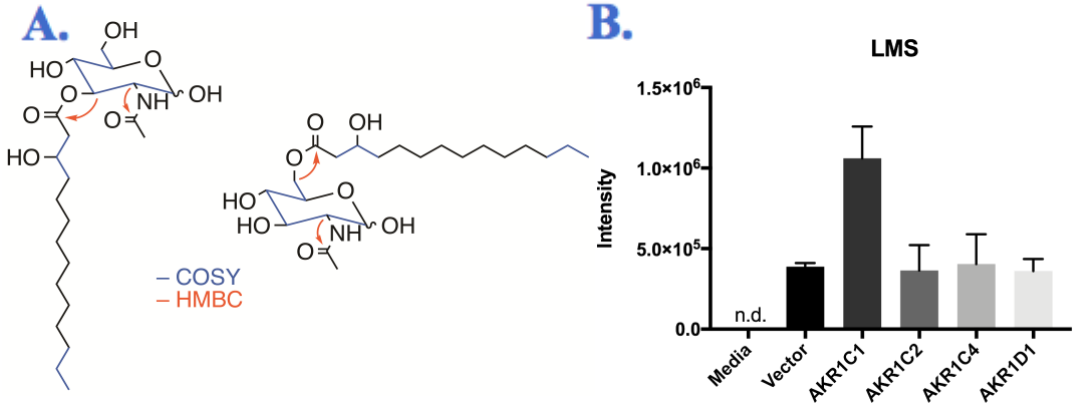

Figure 6. A.) Chemical structures of LMS. B.) Production screen of LMS by enzyme.

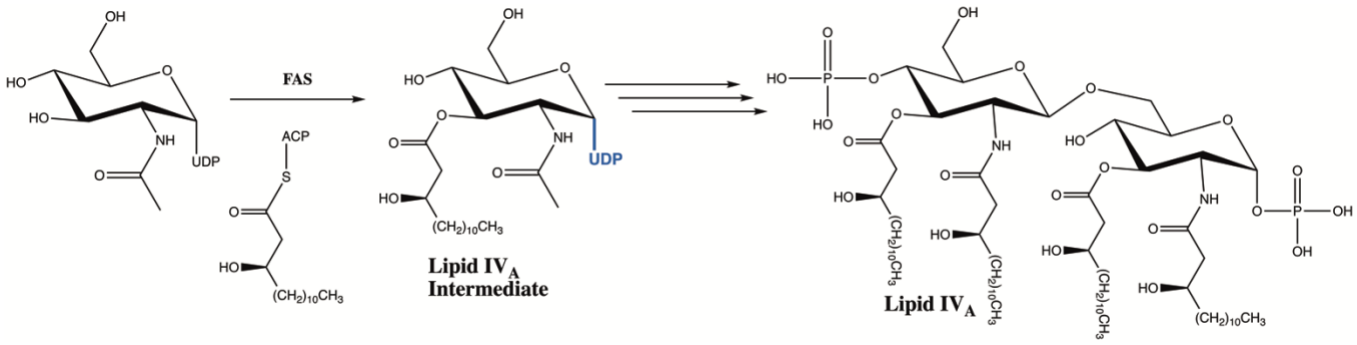

Figure 7. Biosynthesis of LPS Lipid IV_A through an LMS like intermediate.

Additionally, an alkyl pyrone metabolite was also detected as a RECON/AKR1C3 associated metabolite (**Fig. 8**). Structurally related pyrones have recently been implicated in gammaproteobacterial quorum sensing (41), and this molecule may serve a similar yet-to-be supported functional role in *E. coli*.

We have established that heterologously-expressed RECON sequestered this pyrone and structurally-related pyrones in the cytosol of *E. coli*. Thus, these studies suggest that bacterial pyrones can serve as RECON PAMPs and that similar binding interactions occur in related human homologs.

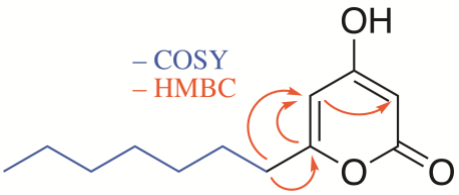

Figure 8. Chemical structure of pyrone.

D. Proposed Research

D.1 Continued Metabolomic Profiling and Metabolite Elucidation

In order to accurately detail the metabolomic profiles of the human AKRs and RECON, it is necessary to conduct a variety of metabolomic experiments. So far, we have completed heterologous expression metabolomics which has revealed the presence of several PAMPs (LMS and pyrone) – which may selectively bind to RECON and human AKR homologs – and a novel aldol addition functionality within AKRs 1C1 and 1C2. In future studies, we aim supplement these results with *in vitro*, oxidative stress, and mammalian metabolomic screens.

a.) In vitro Metabolomics

In vitro metabolomics offers a simple and robust method through which we can identify media substrates for our enzymes as well as bacterial PAMPs (**Fig. 9**). Briefly, during synthesis of heterologously expressed AKRs, the enzymes naturally bind endogenous cofactors and bacterial PAMPs. During protein purification, some cofactors and PAMPs may remain associated with their enzymes, resulting in small molecules within the pure protein extract. These small molecules can be detected through MS and ultimately identified through NMR spectroscopy. Additionally, the pure protein extract can be incubated in choice media to determine unique media-based substrates of AKRs in the absence of bacteria.

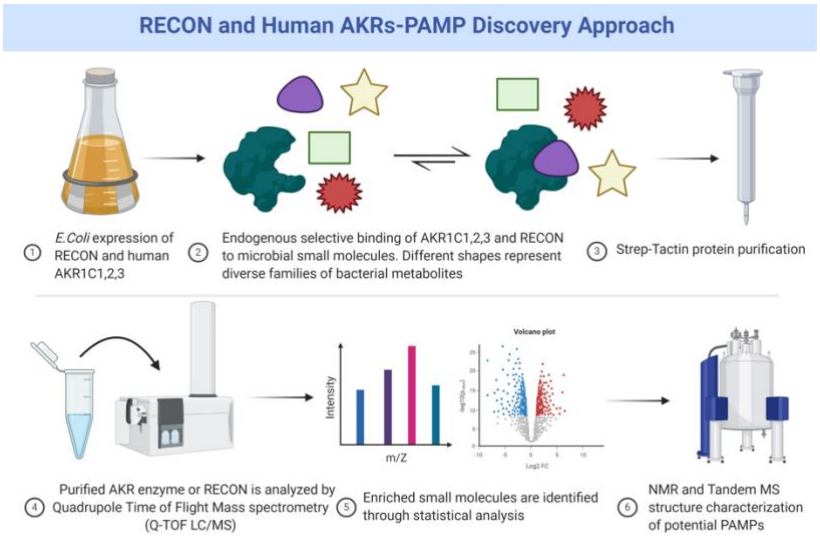

Figure 9. *In vitro* metabolomics workflow for discovery of RECON/AKR associated PAMPs.

b.) Oxidative Stress Metabolomics

The expression of human AKRs – specifically AKRs 1C1 and 1C2 – is heavily induced under oxidative stress through the Keap1-Nrf2 signaling pathway (42-44). Given their role in remediating oxidative stress *in vivo*, we believe performing oxidative stress metabolomics may be crucial in determining the biologically relevant substrates of AKRs. We hypothesize that some natural AKR substrates may only be produced under oxidative stress and thus we may not identify them through normal heterologous expression metabolomics. Utilizing methods previously published by our lab (45), we propose to challenge bacteria heterologously overexpressing AKRs with sub-inhibitor levels of paraquat and to determine their resulting metabolomic profiles.

c.) Mammalian Metabolomics

RECON has been shown to be an immunoregulatory PRR that – to our knowledge – specifically recognizes bacterial PAMPs (9). Therefore, a bacterial expression system may not be an optimal strategy to gauge the metabolomic profiles of AKRs with similar activity to RECON's. Hence, we propose to

complement our current studies with metabolomic experiments performed on human (liver) cells. By generating mammalian metabolomic profiles, we aim to cross-reference the enzyme-dependent "hits" with various culture media extracts to check if the metabolites can be purified and characterized.

We plan to perform the metabolomic experiments through two approaches: 1.) by transfection of HepG2 liver cells with a plasmid containing a specific AKR and 2.) by generating specific AKR knockouts in HepG2 liver cells through CRISPR-Cas9 gene editing. The results of both approaches will be compared to control HepG2 liver cells to deduce reproducible metabolomic hits.

d.) Characterization and Synthesis of Novel Metabolites

In addition to continued profiling of the AKR metabolomes, we will also continue our efforts to purify and characterize AKR-dependent metabolites. As we structurally elucidate these compounds, we will begin efforts to acquire them – through either synthesis or commercial vendors – for *in vitro* study. Currently, the lab is working on the synthesis of the LMS (**Fig. 10A**) and pyrone (**Fig. 10B**) metabolites.

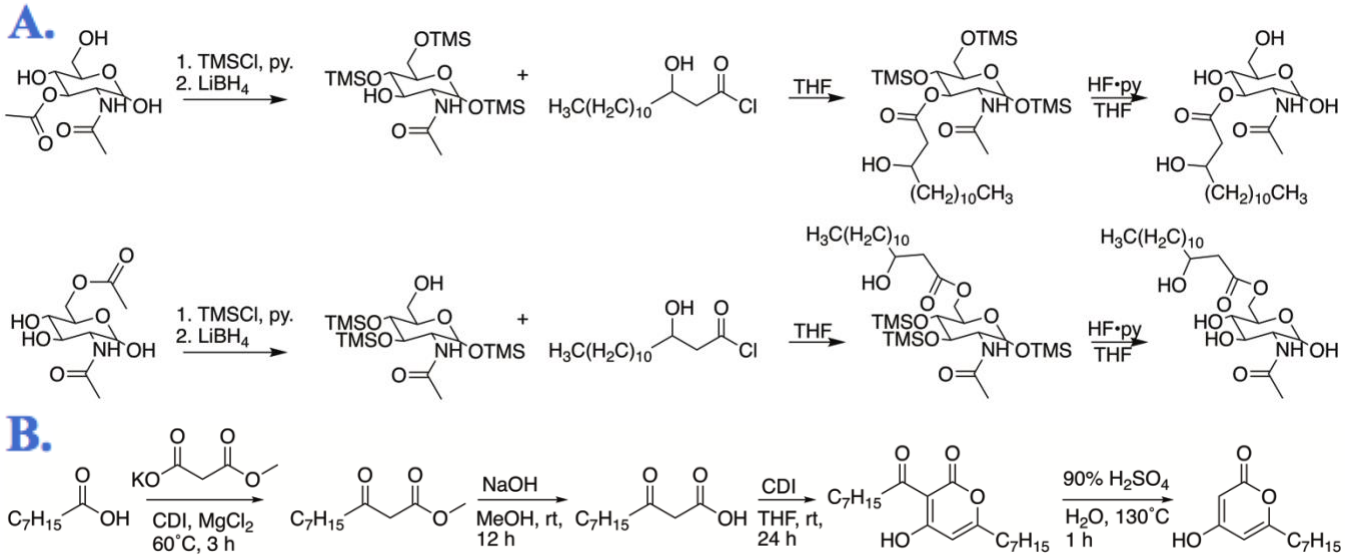

Figure 10. A.) Proposed synthesis of LMSs. B.) Proposed synthesis of pyrone.

D.2 Enzyme Kinetics Analyses and PAMP Binding Assays

a.) Enzyme Kinetics Analyses

A key facet of our AKR studies is the determination of the enzyme kinetics governing biologically relevant metabolic transformations. Therefore, we aim to deduce reasonable AKR substrates – based on biosynthetic retroanalysis of the enzyme products – and conduct LC-MS-based and absorbance-based kinetic analyses. Through LC-MS, we plan to chart the change in product concentration as a function of substrate concentration under Michaelis-Menten conditions to determine the K_m and V_{max} of the transformations. Moreover, we will also chart the change in NAD^+/NADH concentration, as a function of substrate concentration, through absorbance spectroscopy to rapidly obtain kinetic parameters and be able to evaluate point mutants and alternative substrates.

b.) PAMP Binding Assays

Though RECON was originally determined to only bind cdNs, our preliminary results indicate that RECON and its human homologs may recognize a broader spectrum of PAMPs, including LMS, pyrone, and other fatty acid-type metabolites. The ability of these enzymes to bind a variety of PAMPs could be significant in determining the immunometabolic mechanisms through which they regulate innate immunity. Thus, we intend to utilize isothermal titration calorimetry to measure the binding affinities (K_a) and thermodynamic parameters (ΔH, ΔS, ΔG) between human AKRs and LMS, pyrone (and its derivatives), microbial cdNs, and additional PAMPs we elucidate in future studies.

D.3 Study of Immunoregulatory Roles of AKRs

a.) PAMP Effects on Immunomodulators

In order to evaluate the NF-κB regulatory activities of our AKRs, we plan to conduct *in vitro* transfection assays on a human THP-1 reporter cell line expressing plasmid (pcDNA3) containing AKR genes. The NF-κB levels of these cells will be measured in the presence and absence of pyrone, LMS, and any additional PAMP we discover, in order to determine the regulatory effects of AKRs and these PAMPs on immune response. Furthermore, we can also investigate differential cytokine production between HepG2 cells and AKR-KO HepG2 cells, in the presence of PAMPs, as an orthogonal approach to deduce the role(s) of AKRs in modulating immune responses.

b.) Biological Validation of Immunoregulation through in vivo Mouse Models.

Through a collaboration with Flavell group at Yale School of Medicine, we have developed RECON$^{-/-}$ mice. To place the AKR structural and biochemical studies into an *in vivo* inflammatory context, we will utilize our RECON$^{-/-}$ mouse model in two intestinal inflammation models. Because RECON has been implicated in antibacterial inflammation in cell line studies, we will evaluate wildtype and RECON$^{-/-}$ mice in a *Salmonella enterica* serovar Typhimurium intestinal infection model and in a DSS-colitis model. We will measure relative intestinal pro-inflammatory cytokine and chemokine signaling in RECON$^{-/-}$ versus wildtype control (C57BL/6) mice at the level of secretion (TNFα, IL-6, IL-12b; ELISA-based secretion assays) and expression (IL-1ß, MCP-1, IL-6, IL-12b, TNFα; qRT-PCR assays). Additionally, we will measure fecal microbiome profiles before and after treatments (**t** = 0, 1 week, 2 weeks) by 16S rRNA sequencing and quantify RECON products in feces, luminal contents, gastrointestinal mucosa and in homogenized mouse tissues (bone marrow-derived macrophages and lamina propria lymphocytes) and organs spleen, inguinal lymph nodes, bone marrow, thymus, liver, kidney, duodenum, jejunum, ileum, cecum, and colon by targeted metabolomics, untargeted metabolomics, and molecular networking.

E. Conclusion

Here we describe a proposal to detail the unique metabolomic profiles of human aldo-keto reductases closely linked to the immunoregulatory enzyme RECON. We propose to identify and characterize AKR-dependent metabolites and AKR-associated PAMPs and to determine how these compounds, in conjunction with their associated enzymes, modulate immune responses. Collectively, these studies will advance our understanding of the molecular roles of human RECON homologs in regulating host-microbe interactions. Elucidation of such novel immunometabolic pathways at the host-bacteria interface could illuminate new drug targets, identify unique small-molecule immunomodulators, and close major gaps in our knowledge regarding the general molecular mechanisms of microbe-induced immune responses.

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