# VICB Student Research 2017 Poster Abstracts

## A. Systems Analysis –

### A1. Exploration of Gain-Of-Function p53 Protein Mutations in Triple Negative Breast Cancer

Spencer Lea, Jennifer Pietenpol, Timothy Shaver, Hailing Jin

Previous research efforts have shown that, generally, cases of Triple-Negative Breast Cancer (TNBC) lack common oncogenic drivers, thus leading to worse outcomes on average for patients with TNBC than for those with breast cancer as a whole. Without known oncogenic drivers it is difficult to develop therapies that target specific mutations. However, research efforts have also shown that a majority of TNBC cases do include mutations in the p53 tumor suppressor (TP53) gene, resulting in the production of a protein with a single amino acid difference. The Pietenpol lab and others have identified special "gain of function" tumor-promoting properties conferred by missense mutant forms of p53, including increased cellular growth, genomic instability, and resistance to chemotherapeutic agents. Despite such properties having been described, their underlying mechanistic causes remain poorly understood. Prior experimentation has demonstrated that in vitro cell lines containing p53 point missense mutations have increased cell size and exhibit aneuploidy at an increased frequency as compared to wild type and null point mutation cell lines. To further elucidate these "gain-of-function" properties I will perform in-vitro clonal competition assays between wild-type, null, and missense p53 mutants. These assays will provide insight as to whether previously observed p53 missense phenotypes pertaining to metabolism will translate into increased survivability when in direct competition with wild-type and null cells.

### A2. Correlating Resolving Power, Resolution, and Collision Cross Section: Broadscale depiction of Separation Efficiency in Ion Mobility Spectrometry

James N. Dodds; Jody C. May; John. A. McLean

In mass spectrometry (MS) the separation capability of a specific instrument configuration (*e.g.* quadrupole, TOF, sector) is defined as mass resolving power ($R_P$, synonymous with mass resolution). Although there is some variability in how this value is calculated, mass spectrometry $R_P$ is generally accepted as a benchmark for overall MS instrument performance. Here, we generate a relationship between percent difference in collision cross section (CCS), instrumental resolving power ($R_p$), and overall separation efficiency (two-peak Resolution $R_{p\text{-}p}$). We then determined that other instrument platforms (*e.g.* TWIMS, TIMS, FAIMS) can also be characterized in this fashion to standardize the field under a common descriptor of separation efficiency. Overall, 21 ion mobility separations published in the literature were studied to determine if the various IM instrument configurations (FAIMS, TIMS, TWIMS) could be

described by a similar separation parameter based on CCS, which is comparable to mass based RP in MS. Each published spectra was fitted with a Gaussian distribution to determine if a normal distribution could model IM behavior across different IM platforms and the resolving power was calculated. The percent difference in CCS was then calculated from the presented cross section value or a standard value determined in our own laboratory, and the overall experimental resolution was examined to determine if each IM technique was able to be characterized by our previously developed equation for drift tube IM. Briefly, results from our study showed that most ion mobility instruments can be characterized by our definition of resolving power, and we hope that the findings developed here will help to standardize future instrument developments and characterization.

## A3. *Acinetobacter baumannii* employs a zinc binding carboxypeptidase to overcome host-imposed nutrient and envelope stress

Zachery R. Lonergan, Brittany L. Nairn, Walter J. Chazin, and Eric P. Skaar

*Acinetobacter baumannii* has emerged as an important nosocomial pathogen that is capable of causing a range of diseases, including respiratory infections and bacteremia. Treatment for these infections is limited due to increasing rates of antibiotic resistance, underscoring the importance of identifying new targets for antimicrobial drug development. During infection, *A. baumannii* must acquire nutrient metals in order to survive and colonize the host. Vertebrates have developed mechanisms to sequester these metals from invading pathogens by a process termed "nutritional immunity." The vertebrate protein calprotectin (CP) inhibits *A. baumannii* growth through zinc (Zn) sequestration. We have found that one of the most upregulated genes during Zn starvation encodes a Zn-binding lipoprotein that localizes to the *A. baumannii* inner membrane and is a putative carboxypeptidase involved in peptidoglycan remodeling. Based on these findings, we have named this gene *zrlA* (Zur-regulated Lipoprotein A). Genetic inactivation of *zrlA* results in diminished growth in the presence of CP. The *zrlA* mutant also displays increased cellular envelope permeability, decreased outer membrane barrier function, and increased antibiotic susceptibility. Finally, a mouse model of *A. baumannii* pneumonia revealed the *zrlA* mutant is defective in colonizing the lungs and disseminating to the liver. Taken together, these results show that *A. baumannii* utilizes ZrlA to overcome Zn limitation and maintain cell envelope integrity and suggests that ZrlA is an important bacterial component to survive the host immune response.

## A4. Method Development for the Metabolomic Profile of Cyanobacteria with Altered Circadian Rhythms

Berkley Ellis, Chi Zao, Yao Xu, Randi Gant-Branum, Carl H. Johnson, John A McLean

Cyanobacteria have shown utility through applications in biosynthesis, bioremediation, and alternative fuel sources. The underlying mechanism to this organism's productivity lies in its

unique prokaryotic circadian system. Using an untargeted metabolomics approach, we aim to determine the functionality of the genes and enzymes responsible for the rhythm's oscillation. To characterize small molecules within the breadth of the metabolome, we measure separation (retention time and drift time) and identification features (mass to charge ratio and fragmentation pattern) via Liquid Chromatography-Ion Mobility Mass Spectrometry (LC-IM-MS). We acquired data in an untargeted manner to provide unbiased sampling of these features to identify and annotate metabolites of cyanobacteria with modified circadian rhythms.

## A5. Development of a Ozonolysis Flow-Cell Coupled to IM-MS for Determining Double Bond Position in Lipids

Rachel A. Harris, Craig A. Stinson, Yu Xia, Jody C. May, John A. McLean

The increasing focus on lipid metabolism has revealed a need for accurate structural characterization of lipid species. Ozonolysis reactions coupled to mass spectrometry have previously been examined by several labs as a means for establishing double bond position in lipid samples. This work explores the utility of a flow-cell based ozonolysis device coupled online with ion mobility-mass spectrometry (IM-MS) for determining the degree of unsaturation and double-bond positions in lipids. Here we describe an online, solution-phase reaction with ozone produced using a low-pressure mercury lamp which generated aldehyde products diagnostic of cleavage at a particular double bond position. The reaction was shown to be effective for multiple lipid species in both positive and negative ionization modes, and the conversion efficiency from precursor to product ions was optimized by varying the flow rate through the ozonolysis device. Ion mobility separation of the ozonolysis products generated additional structural information and revealed the presence of saturated species in a complex mixture. The method presented here is readily coupled to existing instrument platforms. For these reasons, application to standard lipidomic workflows is possible and aids in complete structural characterization of a myriad of lipid species.

## A6. Visualizing and Conceptualizing Multidimensional Metabolomics Data from LC-IM-MS/MS Analysis

Jaqueline A. Picache, Charles M. Nichols, John C. Fjeldsted, Jody C. May, Stacy D. Sherrod[1], and John A. McLean

Technological advancements in mass spectrometry (MS) have enabled acquisition of larger, more detailed data sets. These multidimensional capabilities have high utility in untargeted multi-omics studies. While these data sets are rich with information, they are accompanied by the challenges of analyzing and representing complex data in a meaningful way. Currently, there is no consensus on how to analyze and represent multidimensional data generated with multi-stage analytical instrumentation. We address some of the challenges of elucidating significance from large data sets and visually representing multidimensional data. The data was generated with a liquid chromatography system coupled to an ion mobility–mass spectrometer with tandem capabilities (LC-IM-MS/MS). Here, we represent LC-IM-MS/MS data in terms of

chromatographic retention time, ion mobility drift time, analyte mass-to-charge, pre-cursor mass intensity, and fragmentation spectra of extracted metabolites from a time course study of MV-4-11 human macrophages treated with Janus kinase (JAK) inhibitor and all-trans retinoic acid (ATRA). Lists for statistically significant features were curated. We demonstrate schemes to visualize highly dimensional data that incorporate identifying details of analytes which are often overlooked in data representation. To the best of our knowledge, this work presents the first figure where six analytical dimensions of identifying characteristics are represented.

## A7. Assessing the Imaging Compatibility and Performance of Tissue Fixation Strategies Using MALDI FTICR MS with Imaging Mass Spectrometry

Marissa Jones, Jeffrey Spraggins, N. Heath Patterson, William J. Perry, Boone M. Prentice, Richard Caprioli

Imaging Mass Spectrometry (IMS) enables the unlabeled mapping of molecular distributions from tissue. IMS lipidomic workflows provide information about cell structure and can give insight into diseased phenotypes through the study of metabolic molecular products. There is increased interest in developing IMS methods for analyzing formalin-fixed, paraffin-embedded (FFPE) samples due to the vast collections of annotated clinical specimens stored in tissue banks. However, formaldehyde fixation heavily distorts IMS lipid profiles, and paraffin embedding introduces incompatible chemical interferences that requires harsh organic washes prior to MS analysis, often eliminating endogenous metabolites and lipids. Fresh frozen tissue samples are more compatible with IMS analysis; however, fresh frozen tissue is prone to fracturing during freezing, and limits the effectiveness of certain fluorescent antibodies for multimodal imaging studies. The combined effect of tissue fixing, freezing, and embedding on lipids is an important consideration when selecting an IMS sample preparation strategy, but it has yet to be systematically investigated. Here, we examine the effect of various tissue preparation strategies on both image quality and global lipidomic profiles using high resolution MALDI-TOF IMS, and accurate mass Fourier transform ion cyclotron resonance (FTICR) MALDI IMS. As manual interpretation and comparison of mass spectra used for analysis can introduce bias and error, we have developed a custom statistical analysis pipeline using SCiLS software (Bruker Daltonics), the LIPID MAPS database (Lipidomics Gateway), and a custom-built sorting and visualization program in MATLAB. These programs allow for the automated identification of lipids based on accurate mass and receiver operating characteristic (ROC) analyses to compare the various conditions' effects on the intensity of identified lipids when compared to fresh frozen tissues. These experiments led to the observation that formalin fixation without paraffin embedding contributes to tissue fragility and decreased slide adhesion that is combatted by paraffin embedding in FFPE samples. In general, formaldehyde fixed tissues not embedded in paraffin show a decrease in ion intensities for phosphatidylethanolamines. On the contrary, glycerophosphates displayed an increase in ion intensity when compared to fresh frozen tissues. FFPE samples were found to show nominal lipidomic expression. In all sample preparations, ammonium formate washes directly correlated to increased lipid signal intensity for all lipid classes.

## A8. Global lipidomic changes associated with MALDI matrices using MALDI FTICR imaging mass spectrometry

William J. Perry; Jeffrey M. Spraggins; N. Heath Patterson; Marissa Jones; Boone M. Prentice; Raf Van de Plas; Richard M. Caprioli

Matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI IMS) is a technology used to visualize complex molecular distributions from thin tissue sections. In MALDI IMS, the tissue is first coated with a chemical matrix, typically a small organic compound that contains aromatic rings to facilitate absorption of the MALDI laser energy. Irradiation of the sample with a MALDI laser induces analyte desorption and ionization, allowing the acquisition of mass spectra at discrete x, y positions across the sample surface. Improving MALDI IMS lipidomic analyses is in part dependent upon maximizing the sensitivity of a matrix for targeted classes of analytes as well as ensuring the stability of prepared samples during storage, image acquisition, and between sequential experiments. Optimization of this matrix choice allows for more targeted, reproducible approaches in assessing lipidome modulations associated with biological processes. Much of the development in improving IMS lipid analysis has focused on determining matrices optimized for specific lipid classes (*e.g.* 4-chloro-α-cyanocinnamic acid for phosphatidylethanolamine chloramines) or improving overall signal for specific tissues. However, little work has been done to systematically compare the global lipidomic changes associated with common conventional matrices. A thorough assessment of sensitivity for different lipid classes and overall matrix stability once tissue is prepared is critical for the development of reproducible, longitudinal IMS lipidomic methods. Here, we systematically assess differences in IMS lipid signatures and sample stabilities for a range of common and specialty matrices. Using high resolution MALDI Fourier transform ion cyclotron resonance (FTICR), we have performed IMS analyses of liver tissue using 2,6-dihydroxyacetphenone (DHA), 1,5-diaminonaphthalene (DAN), 5-chloro-2-mercaptobenzothiazole (CMBT), 2,5-dihydroxybenzoic acid (DHB) and 9-aminoacridine (9AA) matrices. These experiments resulted in the detection of numerous lipid classes, including phosphatidylcholines (PC), phosphatidylethanolamines (PE), sterols (ST), phosphatidylglycerols (PG), phosphatidylinositols (PI), and phosphatidylserines (PS). Triplicate analyses showed clear differences in observed lipid profiles between the various matrices. DHA resulted in IMS data with a relatively even distribution of the number of lipid species for many of the phospholipid classes (*e.g.*, 26 PIs, 39 PEs and 27 PSs). By comparison, CMBT was less sensitive towards phospholipid classes, detecting fewer PIs (25), PEs (14), and PSs (16). While all other matrices tested produced stable results (*i.e.*, similar signal intensity and lipids detected) over the three-day sequential analyses, 9AA was found to degrade rapidly, providing minimal signal two days after matrix deposition. Currently, lipid identifications are made using accurate mass measurements and tandem mass spectrometry (MS/MS) using MALDI FTICR MS. In order to statistically evaluate these results, data analysis pipelines have been developed to enable in-depth differential lipid analysis between MALDI matrices (*e.g.*, lipid class, acyl chain length, and acyl chain unsaturation).

## A9. Conformational and Configurational Equilibra of a 2'-Deoxyribosylurea Adduct in Single Strand and Duplex DNA

Andrew H. Kellum Jr., Vijay Jasti, Ashis Basu, and Michael P. Stone

2-Deoxyribosylurea (urea) lesions form within DNA as a result of the cleavage of thymine from hydroxyl radicals resulting from ionizing radiation. In addition, urea lesions are able to form from 8-oxoguanine. Previous NMR studies of the urea lesion on the nucleoside level showed that the 2′-deoxyribose ring equilibrated between the alpha (α) and beta (β) configurations (acknowledge who did this study/person's lab who have this finding). In this study we examined the urea lesion in the single strand oligodeoxyribonucleotide 5′-(CTXA)-3' and in duplex DNA 5'-GCGXGCG-3' (X=urea). Reverse phased HPLC revealed a ratio of 1:1 between two equilibrating species in single stranded DNA. In addition, the presence of two equilibrating species was present in duplex DNA. Trapping the different species in basic environment slowed the equilibration supporting the hypothesis that the species are anomers. NMR spectroscopy was used to definitely identify the two different species in single strand DNA. NOESY and TOCSY spectra were used to assign the resonances of the different nucleotides. NOESY spectra revealed the identity of the two different species as α and β anomers of the urea lesion. TOCSY and one-dimensional NMR spectroscopy showed that the urea lesion was in the anti conformation regardless of the configuration the 2′-deoxyribose ring. The presence of the α anomer is a possible explanation as to why the lesion acts a strong block to replication. Future studies will isolate the individual anomers and study the structural effects of each anomer in duplex DNA by NMR spectroscopy.

## A10. Multiplexed Flow Cytometric Quantification of Cell Cycle Phase and DNA damage response allows for High Throughput Assessment of Cell Cycle Specificity of Anti-Leukemic Natural Products

Benjamin J. Reisman, David E. Earl, Jonathan H. Boyce, John A. Proco, Jonathan M. Irish, P. Brent Ferrell, Brian O. Bachmann

Phenotypic screening represents one strategy for identifying compounds with novel mechanisms of action by prioritizing compounds, which elicit a phenotype of interest without regard to their molecular target. Cell based systems are ideal for phenotypic screening as they have the potential to faithfully reproduce the molecular physiology of disease state. However, unlike target based screens which use purified protein targets, not every cell in a well is identical and the cellular response to chemical perturbation may differ between subpopulations of cells. One of the most common forms of cellular heterogeneity arises from the cell cycle – at a given moment some cells are quiescent, others are synthesizing new DNA, while yet others are undergoing mitosis. Simultaneous assessment of cell cycle phase and intracellular processes is especially would be

especially useful for identifying and characterizing novel anti-cancer compounds, many of which act at specific points in the cell cycle.
Multiparametric flow-cytometry is a technique which allows for simultaneous assessment of cell identity and cellular state by assaying surface markers and intracellular markers on a single cell basis, thereby allowing the effect of chemical perturbations to be quantified on each cell subtype present in heterogenous samples. Cell cycle phase can similarly be delineated on flow-cytometry using DNA stains such as Hoechst or Propidium Iodide, however these traditional stains have poor spectral properties which severely limit the total number of markers which can be measured simultaneously.
In this work we combined DNA staining using YO-Pro-1, an oxazole yellow derivative, with Fluorescent Cell Barcoding, a technique for multiplexing flow-cytometry which allows upwards of 48 wells to be combined, stained, and acquired simultaneously. By staining all samples in a single tube, technical variation and antibody consumption was greatly reduced while sample throughput increased significantly, allowing entire chemical libraries to be analyzed in a single tube. The relatively narrow excitation and emission profiles of YO-Pro-1 also allowed for simultaneous quantification of additional cell cycle markers to delineate all phases of the cell cycle, as well as functional markers such as apoptosis and elements of the DNA damage response. We validated this approach using a set of compounds with known cell cycle specificity and applied our panel to a library of natural products and synthetic, natural product-like compounds.

## A11. Tracking Single Quantum Dots to Unravel Membrane Diffusion Dynamics of the ADHD/Autism/Bipolar Disorder-Associated Ala559Val Dopamine Transporter Coding Variant

Lucas B. Thal, Lauren Harris, Ian D. Tomlinson, Oleg Kovtun, Randy D. Blakely, Sandra J. Rosenthal

The dopamine transporter (DAT) is a transmembrane protein that terminates dopamine (DA) signaling in the brain by driving rapid DA reuptake into the presynaptic nerve terminals. Several lines of evidence indicate that DAT dysfunction is linked to neuropsychiatric disorders such as attention-deficit/hyperactivity disorder (ADHD), bipolar disorder, and autism spectrum disorder (ASD). Since membrane trafficking is an important mechanism of DAT regulation, our group pioneered the use of antagonist-conjugated quantum dots (QDs) to monitor individual membrane DAT molecules in live cells. In a new research effort, we implemented our QD labeling approach and total internal reflection fluorescence (TIRF) microscopy to characterize the membrane diffusion dynamics of a recently discovered ADHD/ASD/bipolar disorder-associated hDAT A559V coding variant. Published studies have demonstrated that the A559V mutant is hyper-phosphorylated at N-terminus serine residues and demonstrates anomalous DAT-mediated DA efflux. We hypothesize that the A559V substitution and the consequent DAT hyper-phosphorylation will destabilize the transient interactions between the transporter and its binding partners provoking an increase in DAT membrane mobility. Our mean square displacement analysis of WT DAT and DAT Val559 transiently transfected HEK-293 cells shows the mutant coding variant exhibits significantly higher diffusion coefficients compared to the wild type, supporting the empirical hypothesis. The altered diffusion dynamics therefore suggest a possible

relationship between lateral membrane mobility and hDAT A559V dysfunction. Subsequent investigation on the effect of various stimuli on DAT diffusion will also be discussed.

## A12. Illuminating Molecular Mechanisms of Serotonin Transporter Regulation with Single Quantum Dot Tracking

Danielle M. Bailey; Ian D. Tomlinson; Oleg Kovtun; Qi Zhang; Sandra J. Rosenthal.

Serotonin transporter protein (SERT) is the critical target for treating numerous mental illnesses as it is the primary site of action for the class of antidepressants known as selective serotonin reuptake inhibitors (SSRIs). Research over the past few decades has shown that SERT function and expression is under tight regulation through a variety of mechanisms, but illuminating SERT structure and function at the single protein level remains a challenge. Membrane microenvironment, specifically membrane cholesterol, plays a key role in SERT regulation and has been found to affect SERT lateral mobility and conformational state. Cholesterol may also play a critical role in posttranslational SERT modification, specifically in phosphorylation of threonine 276 (Thr276), the phosphorylation site of the cGMP-stimulated PKG pathway. Additionally, a gain-of-function SERT coding variant, Gly56Ala (G56A) SERT, has been identified in autism spectrum disorder (ASD) patients and is carried by about three million Americans. G56A SERT exists in a hyperphosphorylated state and is resistant to 8-Br-cGMP stimulation, thus enabling the investigation of the direct link between the extent of phosphorylation and SERT lateral mobility.

Our studies are utilizing specifically targeted quantum dots (QDs), nanometer-sized semiconductor nanocrystals, to analyze SERT lateral mobility at the single molecule level. QDs are excellent probes for single molecule imaging due to their photostability, high brightness, and size-dependent, narrow emission spectra. After QD labeling, the hyperphosphorylated G56A SERT exhibited a significantly higher rate of diffusion compared to wild type SERT, potentially due to higher phosphorylation levels and subsequent disrupted interactions with binding partners. Additionally, cholesterol depletion also increased mobility of SERT, for both wild type SERT and G56A SERT. Endogenous rat neuronal SERT also showed similar increases in mobility upon cholesterol depletion, as well as after 8-Br-cGMP stimulation. To further correlate mobility with phosphorylation status and address the effect cholesterol depletion has on Thr276 phosphorylation, we are utilizing phospho-flow cytometry which provides insights at the single cell level. This research will provide new insights about SERT mobility, its associations with membrane cholesterol, and SERT phosphorylation status. I also anticipate that information gleaned from these studies will result in improved therapeutic strategies aimed at treating mental disorders in the future.

## B. Molecular Discovery –

### B1. Improving performance of Molprint2D descriptor using QSAR models trained on ANNs with dropout

Oanh Vu, Jeffrey Mendenhall, Jens Meiler

Artificial neural network (ANN) models have been used extensively to model complex non-linear functions, including bioactivity prediction from chemical properties, also known as quantitative structure-activity relationship (QSAR) models (Butkiewicz et al., 2013). Fragment-based similarity searching is a simple and yet robust computer assisted drug discovery (CADD) technique for database searching and quantitative structure–activity relationship (QSAR) analysis. Recently, a large benchmark was performed to identify broadly applicable fragment-based fingerprint methods (Chen et al., 2002), and identified Molprint2D (Bender et al., 2004) as among the leading fingerprint methods, especially when combined with the Buser metric (Baroni-Ubani & Buser, 1975). In this project, ANNs were trained with Molprint2D fingerprints, and tested in combination with a previously published descriptor set (Mendenhall & Meiler, 2016). The performance of ANN-QSAR models with Molprint2D descriptors was compared to that of a traditional similarity search. Our preliminary data suggests that using MolPrint2D fingerprints to train a highly-regularized dropout ANN consistently yields higher AUCs on 9 large (60k-380k molecules) benchmark datasets than a direct similarity search using a traditional Tanimoto or Buser fingerprint metric. However, our results are not yet able to compete with descriptors based on the 3D structure of molecules, nor do the MolPrint2D fingerprints used in conjunction with 3D-based descriptors further improve the results of these neural networks. We conclude that despite the limitations of single-conformation QSAR models, the most critical features that can be learned from state-of-the-art fingerprint methods are already adequately encoded in the simpler 3D-descriptors.

### B2. Spatially Resolved Proteomics to Improve Protein Identification in Imaging Mass Spectrometry

Daniel Ryan, David Nei, Boone Prentice, Jeffrey Spraggins, Richard Caprioli

Abstract: One of the more difficult steps in an IMS experiment is the unambiguous chemical identification of an analyte of interest. To provide biological context to molecular images, structural identification is key; and large, singly-charged proteins can make this process a challenge. To overcome the challenges associated with protein identifications in a MALDI IMS experiment, a number of orthogonal approaches have been established to produce complimentary downstream bottom-up or top-down LC-MS/MS; including tissue homogenizations and liquid surface extraction methodologies from serial sections of the tissue of interest. Liquid extraction surface analysis uses small volumes of solvent dispensed from a robotic mandrel to generate small, liquid micro-junctions between the tissue, liquid, and mandrel; allowing for the diffusion of analytes into the solvent. Top-down LC-MS/MS of LESA extractions from tissue sections has

been shown to detect approximately 50-100 proteins with minimal sample preparation from manually pipetted extracts. Herein we describe the use of an enhanced LESA plus LC extraction with a new glass capillary that is coupled directly to HPLC. Using a 150 $\mu$m i.d. capillary we are able to generate an increased droplet resolution on tissue using various solvent compositions, as well as generate data that is both robust and reproducible across a given experiment; showing a great improvement from our previous methods using manual pipetting. Lastly, the extraction is injected online to LC-MS in order to provide a new means to generate a higher-throughput means to gather spatially relevant protein identifications.

## B3. Chemical Genetics of Cation Chloride Cotransporter Activation and Oligomerization

Francis J. Prael III, Eric Delpire, C. David Weaver

An estimated 1 in 26 people are predicted to develop epilepsy at some point in their life. Epilepsy, a chronic neurological condition defined by recurrent seizures, is a tremendous burden on those afflicted with the disease. With nearly one-third of epilepsy patients being refectory to all treatments, novel approaches for the treatment of epilepsy are urgently needed. In the C. David Weaver laboratory, I work on discovering and developing chemical tools targeting transporters that are dysfunctional in epilepsy. We will use these chemical tools to evaluate the therapeutic potential of correcting dysfunction of these transporters in epilepsy and to further characterize fundamental characteristics of these proteins relevant to their dysfunctional state in epilepsy. If successful, our work could lay the foundation for novel approaches to the treatment of epilepsy.

## B4. Target-Based Screen to Identify Small Molecule Inhibitors of the Mitotic Kinesin Kif15

Megan Dumas, Nicole Kendrick, Alex Waterson, Gary Sulikowski, and Ryoma Ohi

The mitotic spindle is microtubule (MT)-based machine that segregates a replicated set of chromosomes during cell division. Many chemotherapeutics target the spindle by altering or disrupting microtubules, the polymer that forms the spindle. While these drugs are efficacious, microtubules are a major component of all cells and their disruption can have deleterious effects on cell types that rely on MTs for function, such as neurons. In addition to tubulin, MT-dependent motors that function during mitosis are logical targets for drug development. Eg5 (Kinesin-5) and Kif15 (Kinesin-12), in particular, is an attractive pair of motor proteins to pharmacologically target since they work in concert to drive centrosome separation and promote spindle bipolarity. Kinesin 5 inhibitors (K5Is) have been extensively studied since their discovery, with many advancing to both Phase 1 and 2 clinical trials. Despite the initial excitement for K5Is due to their promising results in cell and mouse tumor models, they have largely failed in the clinic. Since Kif15 over expression has been shown to overcome K5I treatment in tissue culture cells, a potential explanation for K5I clinical failure may be due to the

cell's ability to utilize a Kif15 dependent spindle assembly pathway. Recently, our laboratory discovered that the emergence of K5I resistance, a phenomenon commonly observed in tissue culture cells, depends on the expression of Kif15. This result underscores the hypothesis that a combinatorial drug approach to target spindle assembly, by inhibiting both Eg5 and Kif15, will cripple rapidly dividing cancer cells. Therefore, we set out to perform a small molecule screen on a focused group of known kinase inhibitors, with the goal of identifying lead chemical scaffolds that inhibit Kif15. Using an *in-vitro* ATPase assay, the Published Kinase Inhibitor Set (distributed by GSK) was screened in duplicate and two compounds, both containing oxindole cores, significantly inhibited Kif15's MT stimulated ATPase activity. The activities of both compounds were confirmed in a MT gliding assay as well as a second ATPase assay. Concentration response curves were performed in triplicate and IC50s were calculated for each. VU0482674 became our lead compound, exhibiting an IC50 of 800nM. Similarly, VU0482674's IC50 in the MT gliding assay was calculated to be 734nM. Furthermore, treatment with VU0482674 on K5I resistant cells (KIRCs), whose ability to form bipolar spindles relies on Kif15, results in nearly 100% monopolar spindles. VU0482674 has no effect on mitotic progression in normal RPE-1 cells, suggesting that the compound primarily inhibits Kif15 during cell division. Structure Activity Relationship (SAR) analysis of VU0482674 is currently underway.

## B5. Database of Secondary Metabolites: Conformational mapping using IM-MS for natural product discovery

Andrzej Balinski, John A. McLean, Jody C. May, Brian O. Bachmann, Sarah M. Stow

Hypogean, or cave-dwelling, bacteria thrive in resource-scarce environments and thus are a rich source of new secondary metabolite species. These metabolites are structurally-unique molecules which possess interesting bioactivity. Previously, we have used bioactivity, genomics, mass spectrometry, and bioinformatics to prioritize target species for isolation and characterization as potentially new drug candidates. Here, we evaluate the use of ion mobility-mass spectrometry (IM-MS) and collision cross section (CCS) structural measurements as a proxy for elucidating structural uniqueness, which is a hallmark of effective bioactive agents.

Both helium and nitrogen drift gases as utilized. Nitrogen CCS values ($^{DT}CCS_{N2}$) have become the standard measurement for commercially-available IM-MS instruments, whereas helium CCS values ($^{DT}CCS_{He}$) can be better correlated to theory. A total of 45 natural products representing different molecular scaffolds (e.g., polyketides, glycoconjugates, cyclic peptides, etc.) were measured in both helium and nitrogen drift gases. All measurements were obtained using a commercial uniform field IM-MS (6560 IM-QTOF-MS, Agilent Technologies). This instrument was outfitted with a mass flow regulator to accurately control and characterize the gas pressures, which is critical for the high precision, absolute CCS measurements conducted in this work. High purity helium and nitrogen were further purified through passive filtration. To expand this initial suite of molecules, a variety of small molecule mixtures (metabolites, peptides, carbohydrates, 50 to 3200 Da) were analyzed, resulting in a database of 346 different species for comparison and quantification of structural uniqueness. An additional 180 CCS values were obtained for tryptic peptides in both gases, yielding a total of 526 CCS values for unique ions in

each gas. To assemble a canonical and directly comparable database, we have obtained measurements on the instrument mentioned previously which integrates both helium and nitrogen absolute CCS values. Preliminary work has also expanded the initial study to higher order charge states and various charged adducts. This comprehensive natural product database allows us to interpret the effects of drift gas on this unique suite of molecules, where long-range interaction potentials can convolute data interpretation. Specifically, natural products contain heteroatoms, stereochemistry, and heterofunctional groups in comparison with more conventional biomolecules such as proteins, carbohydrates, and lipids.

## B6. Molecular Basis for Base Excision Repair of Bulky DNA Adducts and Interstrand DNA Crosslinks

Elwood A. Mullins, Rongxin Shi, Garrett M. Warren, Noah P. Bradley, and Brandt F. Eichman

DNA glycosylases are important editing enzymes that protect genomic stability by excising chemically modified nucleobases, thereby initiating the base excision repair (BER) pathway. Previous studies have shown that a hallmark of these and other DNA repair enzymes is their use of base flipping to pull modified nucleotides from the DNA helix and into an active site pocket. Consequently, base flipping is generally regarded as an essential aspect of lesion recognition and a necessary precursor to base excision. We recently described the first DNA glycosylase mechanism that does not require base flipping for either binding or catalysis. The homologous bacterial DNA glycosylases YtkR2 and AlkD catalyze excision of methylpurine lesions solely through contacts with the DNA backbone, while the modified nucleobase remains stacked in the duplex. We now report the means by which YtkR2 and AlkD excise bulky lesions formed by yatakemycin (YTM), an extraordinarily toxic DNA alkylating agent with potent antimicrobial and antitumor properties and the most recent addition to the CC-1065 and duocarmycin family of natural products. We also report the structure of AlkZ, the defining member of the HTH_42 superfamily of uncharacterized winged helix-containing proteins and an eighth structural class of DNA glycosylase. We present a molecular docking model for how AlkZ can unhook either or both sides of an azinomycin B (AZB)-derived interstrand crosslink (ICL) without needing to pull the modified nucleotides from the DNA duplex. These unique non-base-flipping mechanisms allow YtkR2 and AlkZ to provide self-resistance against YTM and AZB to the natural product-producing strains *Streptomyces* sp. TP-A0356 and *Streptomyces sahachiroi*. We believe the continued identification of novel DNA glycosylases with non-traditional substrates is likely to further expand the known role of BER in genomic maintenance.

## B7. Selective base excision repair of DNA damage by the non-base-flipping DNA glycosylase AlkC

Rongxin Shi, Elwood A. Mullins, Xing-Xing Shen, Kori T. Lay, Philip K. Yuen, Sheila S. David, Antonis Rokas, and Brandt F. Eichman

DNA glycosylases help preserve genome integrity by removing chemically modified nucleobases from the phosphoribose backbone as a first step in the base excision repair (BER) pathway. These enzymes define BER specificity for discreet, small modifications, and thus the mechanisms by which they locate damage within the genome is of particular interest. The bacterial AlkC and AlkD enzymes represent a glycosylase superfamily specific for cationic alkylated nucleobases and comprised of a HEAT-like repeat (HLR) fold. We recently discovered that AlkD uses a unique non-base flipping mechanism that enables excision of bulky major and minor groove lesions normally processed by nucleotide excision repair. In contrast, AlkC has a much narrower specificity for small lesions, principally N3-methyladenine (3mA). Here, we describe how AlkC selects for and excises 3mA using a non-base-flipping strategy similar to but distinct from that of AlkD. The crystal structure of an AlkC-DNA catalytic intermediate shows that the enzyme uses its HLR domain and a unique immunoglobulin-like fold to induce a sharp kink in the DNA duplex, exposing the damaged nucleobase and allowing active site residues to project into the helix and contact 3mA. We also show how this unique active site can accommodate and excise N3-methylcytosine (3mC) and N1-methyladenine (1mA) nucleobases normally demethylated by AlkB, providing a possible alternative mechanism for repair of these lesions in bacteria.

## B8. *Staphylococcus aureus* HemX modulates glutamyl tRNA reductase abundance to regulate heme biosynthesis

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*Staphylococcus aureus* is responsible for a significant amount of devastating disease. Its ability to colonize the host and cause infection is supported by a variety of proteins that are dependent on the cofactor heme. Heme is a porphyrin cofactor that is used broadly across Kingdoms and is synthesized *de novo* from simple precursors. While heme is critical to bacterial physiology, it is also toxic in high concentrations, requiring organisms to encode elaborate regulatory processes to control heme homeostasis. In this work, we describe a regulatory step in *S. aureus* heme biosynthesis. The first committed enzyme in the *S. aureus* heme biosynthesis pathway, GtrR, is a glutamyl-tRNA reductase that is regulated by heme abundance and the integral membrane protein HemX. GtrR abundance increases dramatically in response to heme deficiency, suggesting a mechanism by which *S. aureus* responds to the need to increase heme synthesis. Additionally, HemX is required to maintain low levels of GtrR in heme-proficient cells, and inactivation of *hemX* leads to increased heme synthesis. Excess heme synthesis in Δ*hemX* is sufficient to activate the staphylococcal heme stress response, suggesting that regulation of heme synthesis is critical to reduce self-imposed heme toxicity. Analysis of diverse organisms has found that HemX is broadly conserved among heme synthesizing bacteria, establishing HemX as a common factor involved in the regulation of GtrR abundance. Together, this work demonstrates that *S. aureus* regulates heme synthesis by modulating GtrR abundance in response to heme deficiency and under the control of the broadly conserved HemX.

## B9. Characterization of the 2,6-Diamino-4-hydroxy-$N^5$-(methyl)-formamidopyrimidine DNA Lesion

Stephanie N. Bamberger, Chanchal K. Malik, Summer K. Brown, Carmelo J. Rizzo, and Michael P. Stone

The N7 imidazole nitrogen of guanine is the most susceptible site for DNA methylation, resulting in the formation of the cationic adduct 7-methylguanine (7MeG). Opening of the imidazole ring occurs upon exposure to hydroxide ion, producing the 2,6-diamino-4-hydroxy-$N^5$-(methyl)-formamidopyrimidine (MeFapy-dG) lesion. This lesion is known to convert between α and β anomers, but the possibility of additional conformational isomers cannot be ruled out. We report progress towards characterizing this DNA adduct using a 3mer oligodeoxynucleotide sequence (5′-TXT-3′; X=MeFapy). An isotopically labeled sample (5′-TXT-3′; X=$^{13}$C-MeFapy) in which the methyl group of MeFapy was $^{13}$C-labeled was used to determine whether rotation of the formyl group of the lesion occurs. An HSQC experiment revealed that the lesion exists as eight major and 2 minor species, split into two groups of five peaks each. Based on the $^3J_{CH}$ between the methyl carbon and formyl proton of MeFapy it was determined that the cause of the chemical shift difference between the two groups was rotation of the formyl group; the upfield group was identified as the *trans* isomers while the downfield group was identified as the *cis* isomers. The *trans* isomer was the major species. NOESY experiments revealed the eight major species were comprised of four α anomers and four β anomers; H3′-H1′ and H4′-H1′ NOE cross peaks were used for the assignment of anomers. The α:β ratio was found to be ~35:65. NOE cross peaks between the methyl group and $N^6$H of the eight major species established that the *cis* and *trans* isomers are distributed between the anomers such that there are two *cis* α anomers, two *trans* α anomers, two *cis* β anomers, and two *trans* β anomers.

## B10. Charge Studies of Group I Adduct Effect on Polymeric Polyester's: Ion Mobility-Mass Spectrometry

Tiffany M. Crescentini, David M. Hercules, & John A. McLean

Polyurethanes are versatile polymers contributing to our society through the development of medical devices, automobiles, and many other consumer products. Polyurethanes are comprised of hard and soft block units that contribute to their broad range of applications. Polyesters are commonly used soft blocks, aiding in polyurethane's elasticity. Characterizing the structure and gas phase mobility of polyester polymers is essential to an understanding the heterogenetic complexity within a polyurethane sample. Ion mobility-mass spectrometry (IM-MS) aids in resolving gas phase ions based on their shape and mass-to-charge ratio. In this study, we characterized the effects of Group I metal ion adducts on an industrial grade polyester sample. Using Group I metal ion adducts, we compared the collision cross sections generated for the multiply charged species (+1, +2, and +3) using $Li^+$, $Na^+$, $K^+$, $Rb^+$, and $Cs^+$ adducts. As the charge state was increased for each polyester oligomer, we observed an increase in collisional cross section. Unique features observed led us to believe that polymer folding only occurs when the polymer ionizes with more than one cation (i.e. +2, +3, etc.). Folding was observed for +2

and +3 adducts, but not for charge state of +1. This data suggest that the number of charged metal ions increases as the oligomer length increases. Further characterization of Group 2 metal ion adducts using IM-MS may aid in the understanding of how multiply charged ions affect increasing polyester oligomer units.

## B11. Analysis of dichloroisoeverninic acid biosynthesis in pursuit of novel everninomicin analogs

Audrey E. Yñigez-Gutierrez, Emilianne M. Limbrick, and Brian O. Bachmann

The growing threat from antibiotic resistant infections has renewed interest in revitalizing previously described natural products with known antimicrobial properties. One such class of molecules are the everninomicins, complex oligosaccharides produced by *Micromonospora carbonacea* with broad activity against Gram-positive pathogens. Previous development of the everninomicins was hindered by toxicity issues and an inability to easily access analogs. However, interest in this potent class of molecules continued due to their apparently novel inhibition of protein translation. Recent crystal and cryo-EM structures of everninomicin A bound to the bacterial ribosome provide a detailed map of the vital interactions responsible for the everninomicins' activity. Specifically, the structures reveal crucial interactions between the 50S subunit and the aromatic dichloroisoeverninic acid (DCE) moiety of everninomicin. All natural everninomicin analogs contain at least one iterative type I polyketide synthase (iPKS)-derived DCE moiety. However, this conserved functionality's biosynthesis is poorly understood. We propose to investigate the biosynthesis of the DCE moiety to access everninomicins with derivatized aromatic moieties with increased antibacterial potency. In order to gain a better understanding of DCE biosynthesis, we first deleted the four putatively associated genes: an iPKS, an O-methyltransferase (O-MT), a flavin-dependent halogenase (FDH), and a *trans* acyltransferase (AT). A functional analysis of these four genes confirmed their assignment and provided seven novel everninomicin metabolites, which were analyzed via liquid chromatography-mass spectrometry. These results demonstrate that the iPKS EvdD3 is responsible for the biosynthesis of the DCE core scaffold, orsellinic acid. The orsellinic acid is transferred to the terminal D-olivose sugar residue by AT EvdD1and subsequently tailored by O-MT EvdM5 and FDH EvdD2 to yield DCE. Unexpectedly, we also revealed two other steps of everninomicin biosynthesis with this work. Based on the metabolites isolated, we discovered that the transfer of the evernitrose sugar to the terminal D-olivose requires the presence of the nearly complete DCE ring. We also confirmed the function of the O-MT EvdM7, which was previously assigned based only on homology data. Through genetic deletion of the four genes putatively associated with DCE biosynthesis, we generated seven novel everninomicin metabolites and clarified crucial steps of everninomicin biosynthesis. Current work is focused on *in vitro* analysis of the DCE-associated enzymes to identify preferred substrates and to evaluate incorporation of non-natural substrates. This work will further elucidate the biosynthesis of the everninomicins and provide novel analogs in an attempt to revitalize this potent class of antibiotics.

## B12. Progress Towards the Elucidation of Eurakenate Biosynthesis

Callie Dulin, and Dr. Brian Bachmann

With bacterial infections becoming more resistant to current treatments, the need for new, potent antibiotics has never been greater. Without antibiotic development, the risk of entering a period equivalent to the pre-antibiotic era is significant. The orthosomycins are a class of highly potent antibiotics, including avilamycin and everninomicin, which are characterized by orthoester bonds between sugar residues. The orthoester bonds are both unique and important for antibiotic activity; when these bonds are reduced the antibiotic activity of the orthosomycins is significantly limited, if not completely abolished. Recently, cryo-EM structures of everninomicin and avilamycin bound to the ribosome have shown the unique binding site of the orthosomycins, highlighting the absence of cross-resistance with other classes of antibiotics. The orthosomycins represent an interesting class of antibiotics that warrant further study due to the potential they provide for the field of antibiotics. Between the avilamycins, their derivatives the gavibamycins, and the everninomicins, the central core of the larger orthosomycins remains relatively unchanged. However, at either end of the molecules variation does occur. With everninomicin specifically, derivatization could occur on the aromatic ring, the evernitrose sugar, as well as the eurakenate sugar. The eurakenate sugar is a branching sugar with an orthoester bond to the neighboring lyxose, along with a methylenedioxy bridge. Studying the biosynthesis of this sugar could provide valuable insight into the biosynthesis of everninomicin as a whole. Currently, genetic deletion experiments of the enzymes hypothesized to create the branching point of the eurakenate sugar are underway. This will hopefully confirm the function of these enzymes, as well as point to when the sugar tailoring occurs in the total biosynthesis. Future work will explore the orthoester bond formation between the eurakenate and lyxose, along with the formation of the methylenedioxy bridge. The everninomicin gene cluster contains three α-ketogluterate oxygenase enzymes with the oxidative capability to form the two orthoester bonds and the methylenedioxy bridge. Previous work in our lab has shown that deletion of these genes results in the complete loss of everninomicin production. As an alternative method to studying these enzymes, a feeding study of C1-deuterium labeled glucose is underway. This could provide information about the role of hydrogen or deuterium abstraction in the mechanism of the orthoester bond formation. A greater understanding of these oxygenase enzymes, including their substrates, and possibly their mechanism, could provide valuable insight into the everninomicin biosynthesis. Additionally, understanding the oxygenase enzymes and the unique bonds they form could provide much needed insight into the orthosomycin class of antibiotics that rely on these bonds for their potent activity.

## B13. High throughput screening for the modulators of N-acyl phosphatidylethanolamine phospholipase D

Geetika Aggarwal, Paige Vinson, David Weaver, and Sean S. Davies

Obesity has become a global epidemic disease. Around 25-30% population in United States are obese. Current therapeutic drugs have had limited success, so novel approaches are needed. *N*-acyl-ethanol amides (NAEs) are biologically active lipids present in many mammalian tissues

that contribute to control of energy balance. *N*-acyl-phosphatidylethanolamine hydrolyzing phospholipase D (NAPE-PLD) is final enzyme in NAE biosynthesis. NAPE-PLD is mechanistically very distinct from classical PLDs like PLD1 and PLD2, which cannot hydrolyze NAPE. Deletion of NAPE-PLD from adipose tissue results in increased obesity. NAE levels are reduced in obese individuals, so increasing NAPE-PLD activity either through small molecule activators of NAPE-PLD or by reducing levels of endogenous inhibitors of NAPE-PLD may be useful therapeutic approach for obesity. Recently, two endogenous bile acids were reported to modulate NAPE-PLD activity. Deoxycholic acid (DCA) increased NAPE-PLD activity, while lithocholic acid (LCA) markedly inhibited activity. These results raise the possibility that many bile acids act as NAPE-PLD modulators and that the specific bile acid profile of an individual may determine their levels of NAPE-PLD activity. To test our hypotheses, we needed to develop methods for rapidly measuring NAPE-PLD activity, both in isolated tissues and in vitro so that we could screen compound libraries that included endogenous compounds like bile acids, as well as other small molecule for their effects on NAPE-PLD activity. Using a fluorescent NAPE analog (PED-A1) and recombinant NAPE-PLD, we developed a robust in vitro high throughput screening assay (Z factor >0.5). We then screened a library of 24 bile acids for their effects on NAPE-PLD activity. We found that in addition to lithocholic acid, several other bile acids such as chenodeoxycholic acid, glycol-lithocholic acid, tauro-lithocholic acid, alpha-muricholic acid, and beta-muricholic acid are also the potent NAPE-PLD inhibitors. None of the bile acid tested enhanced NAPE-PLD activity. Future studies will screen the VU small molecule libraries for NAPE-PLD modulators. We are also working to adapt the in vitro assay for measurement of tissue NAPE-PLD activity, but non-specific background hydrolysis of the fluorogenic NAPE analog in NAPE-PLD$^{-/-}$ mice remains a major challenge.

## C. Therapeutics and Translation –

### C1. The IsdG family of heme oxygenases is conserved across Kingdoms.

Lisa J. Lojek, Allison J. Farrand, Jennifer H. Wisecaver, Crysten E. Blaby-Haas, Brian W. Michel, Sabeeha S. Merchant, Antonis Rokas, Eric P. Skaar

Heme is essential for respiration across all domains of life. However, heme accumulation can lead to toxicity if cells are unable to either degrade or export heme, or its toxic by-products. Under aerobic conditions, heme degradation is performed by heme oxygenases, enzymes, which utilize oxygen to cleave the tetrapyrrole ring of heme. The HO-1 family of heme oxygenases has been identified in both bacterial and eukaryotic cells, whereas the IsdG family has thus far only been described in bacteria. We identified a hypothetical protein in the eukaryotic green alga, *Chlamydomonas reinhardtii*, which encodes a protein containing an Antibiotic Biosynthesis Monooxygenase (ABM) domain consistent with IsdG family members. This protein, which we have named cMO, degrades heme, contains similarities in predicted secondary structure to IsdG family members, and retains the functionally conserved catalytic residues found in all IsdG family heme oxygenases. These data establish cMO as an IsdG family member, and extend the distribution of IsdG family members beyond bacteria. To gain further insight into the distribution

of the IsdG family, we used the cMO sequence to identify 866 IsdG family members, including representatives from all domains of life. These results indicate that the distribution of IsdG family heme oxygenases is more expansive than previously thought, underscoring the broad relevance of this enzyme family.

## C2. The application of microvirin to lipoarabinomannan-based tuberculosis diagnostics

Megan van der Horst, Westley S. Bauer, Leshern Karamchand, Jonathan Blackburn, David Wright

Tuberculosis (TB) is an infectious disease primarily affecting the lungs caused by *Mycobacterium tuberculosis*. TB remains a major global health problem due to the challenge of diagnosis in low-resource settings, where the disease is widespread. One TB biomarker, lipoarabinomannan (LAM), is a carbohydrate that is the principle component of mycobacterial cell walls. The capping motif of LAM varies depending on the mycobacterial species. Most anti-LAM antibodies bind to the polysaccharide backbone of LAM and thus are not specific for *M. tuberculosis* LAM versus LAM of other mycobacteria. This makes it difficult to accurately diagnose TB with traditional antibody based diagnostics. Microvirin (MVN) is a lectin with high specificity for Manα(1-2)Manα moieties which are present in the mannose caps of *M. tuberculosis* LAM (ManLAM). Using bio-layer interferometry, MVN was evaluated as a novel molecular recognition element specific for ManLAM and *M. tuberculosis*. MVN was found to bind only to ManLAM, not to LAM of *M. smegmatis*. Furthermore, MVN was found to have a lower dissociation constant ($K_D$) than anti-LAM antibodies. In order to address inadequacies in the current diagnostic landscape, such as low specificity, low sensitivity, and high resource requirements, an enzyme-linked immunosorbent assay (ELISA) and a rapid diagnostic test (RDT) that utilize MVN as the molecular recognition element are being developed. To develop assays with maximum specificity and sensitivity, a number of variables will be optimized, including the molecular recognition elements for capture and detection and blocking reagent to minimize non-specific signal. For the ELISA, preliminary results have shown success with MVN (1.3 μg/mL) as the capture element and a murine monoclonal antibody specific for LAM, clone 25 (0.5 μg/mL) as the detection element. Bovine serum albumin and casein are being investigated as blocking agents. For the RDT, the molecular recognition elements being studied are MVN and murine monoclonal antibody specific for LAM, clones 25 and 170. Denhardt's reagent and Pierce protein-free blocking solution will be explored as the blocking reagent in addition to bovine serum albumin and casein.

## C3. Progress toward the Development of Selective Positive Allosteric Modulators of the Metabotropic Glutamate Receptor 2/4 Heterodimer ($mGlu_{2/4}$)

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The metabotropic glutamate receptors (mGlus) were previously thought to exist only as homodimers, but several groups have elucidated their ability to form heterodimeric complexes both *in vitro* and in native tissues. Group I mGlus have been shown to form heteromers exclusively with each other, while group II and III mGlus are able to dimerize both within and between groups. The $mGlu_{2/4}$ heterodimer has been of particular interest due to the ability to modulate each monomer with specific orthosteric and allosteric modulators. Interestingly, certain $mGlu_4$ positive allosteric modulators (PAMs) (i.e. - VU0155041) were shown to be active at the heterodimer, while other PAMs (i.e. - PHCCC) exhibited reduced activity, suggesting conformational changes induced by heterodimerization that may allow for selective modulation. The $mGlu_{2/4}$ heterodimer has also been shown to be localized presynaptically to corticostriatal synapses, where cortical signaling is converted into changes in plasticity across the striatum, having impacts on motor learning, habit formation, and cognition. However, the lack of a selective $mGlu_{2/4}$ PAM has prevented more detailed studies into the specific roles that these heterodimeric complexes play in modulating neuronal signaling. Initially, a screen of ~2,000 internally synthesized $mGlu_4$ PAMs revealed numerous compounds active at the heterodimer. Initial medicinal chemistry efforts around the VU0155041 scaffold proved difficult, revealing challenges unique to targeting a heterodimeric protein. Recently, the Vanderbilt collection of ~150,000 compounds was screened against the heterodimer, resulting in several exciting hits, including a novel chemotype. Here, we demonstrate our medicinal chemistry efforts towards these new scaffolds, with the goal of synthesizing a PAM that is fully selective for the heterodimer and with a favorable pharmacokinetic profile for *in vivo* testing.

## C4. Developing Positive Allosteric Modulators for the Metabotropic Glutamate Receptor 7

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The metabotropic glutamate receptor 7 ($mGlu_7$) is one of eight subtypes of metabotropic glutamate receptors in the body. $mGlu_7$ is widely expressed throughout the brain- specifically, on the presynaptic terminals of neurons. There, $mGlu_7$ is believed to act as a synaptic regulator to prevent overstimulation, reducing the amount of glutamate and GABA released from the synapse upon its activation. This function of $mGlu_7$ is important for the maintenance of proper neurotransmission. For example, polymorphisms in the gene *GRM7*, which encodes $mGlu_7$, have been linked to many different neurological disorders including anxiety disorder, depression, schizophrenia, epilepsy, and autism.

The biology of metabotropic glutamate receptors ($mGlu_{4,}$ $mGlu_8$), including $mGlu_7$, have been probed in these disorders, but the specific study of $mGlu_7$'s role has been difficult, in part, due to a lack of specific tool compounds. To date, there have been no fully selective $mGlu_7$ positive allosteric modulators with favorable pharmacokinetic properties available for *in vivo* studies. Currently available tool compounds are non-selective and/or metabolized rapidly making them unamenable for animal studies.

My project aims to develop and validate a selective, brain-penetrant, $mGlu_7$ PAM with favorable pharmacokinetic properties for *in vivo* proof-of-concept studies modeling neurological disorders.

## C5. Clinical Translation of [11C]L-Glutamine PET for a First-In-Human Clinical Trial (VICC GI 1781)

Adam J. Rosenberg, Michael L. Nickels, H. Charles Manning

*L*-Glutamine (Gln) is essential for cell growth and proliferation. In addition to glucose, cancer cells utilize Gln as a carbon source for ATP production, biosynthesis, and as a defense against reactive oxygen species. Laboratory-based radiochemical methods leading to carbon-11-labeled glutamine ([11C]Gln) have been previously reported by others but no suitable cGMP methods have been reported to facilitate clinical translation of this PET tracer. Here we report a novel cGMP-ready radiochemical production of [11C]-Gln to enable the first-in-man clinical study of this tracer at Vanderbilt University Medical Center.

Prior reports utilized a simple solid phase extraction method to purify the labeled intermediate. However, the process was never designed to remove the unreacted precursor from the labeled intermediate. The presence of the unreacted precursor has the undesired consequence of lowering of the overall chemical purity of the final drug product solution. In addition to the purity concerns, all methodologies reported for the preparation of [11C]Gln have been accomplished using non-commercialized, custom built, reaction platforms. In an effort to allow for fast transfer of technology from production site to production site, development of this product on commercially available platforms is needed. Here, we report the development and utilization of methodology for the automated production of [11C]Gln that meets all criteria for human use, as approved by Vanderbilt's Radioactive Drug Research Committee.

We are opening a clinical trial (VICC GI 1781) that will explore the first-in-human use of [11C]Gln for quantitative measurement of glutamine uptake in colorectal cancer (CRC) subjects. This trial will utilize [11C]Gln in combination with another positron emission tomography (PET) tracer, [18F]FSPG, to quantitatively determine the tumor metabolic profile, which in turn can lead to a personalized approach for treatment of the specific types of cancer in a given patient. As an innovative molecular imaging approach, this study will evaluate in stepwise fashion [11C]-Gln PET/CT prior to [18F]FSPG PET/CT within the context of WT *RAS* and WT *RAF*, stage 4 CRC. Like L-Gln itself, [11]Gln is transported into cells by ASCT2, the sodium-dependent neutral amino acid transporter encoded by gene *SLC1A5*. ASCT2 is over-expressed in CRC tumors, which has been linked to poor survival in CRC patients. The use of [11C]Gln and

[18F]FSPG in this clinical trial will allow for determination of a gene signature for differing genotypes of colorectal cancer.

## C6. VU590 Inhibits Kir1.1 and Kir7.1 Potassium Channels Through Distinct Molecular Mechanisms

Sujay V. Kharade, Jonathan H. Sheehan, Eric E. Figueroa, Jens Meiler, and Jerod S. Denton

VU590 was the first publicly disclosed, sub-micromolar-affinity (IC50=0.2 $\mu$M), small-molecule inhibitor of the inward rectifier potassium (Kir) channel, Kir1.1, an emerging diuretic target for the treatment of hypertension. VU590 also inhibits Kir7.1, albeit with 40-fold lower potency (IC50~8 $\mu$M), and has been used as a Kir7.1 tool compound to uncover new roles of the channel in regulation of myometrial contractility and melanocortin signaling in the brain. Here, we employed molecular modeling, site-directed mutagenesis, and patch clamp electrophysiology to elucidate the molecular mechanisms underlying inhibition of Kir1.1 and Kir7.1 by VU590. Block of Kir1.1 and Kir7.1 is voltage dependent and reduced by increasing the electrochemical driving force for K+ influx, suggesting the VU590 binding site is located within the pore of both channels. Scanning mutagenesis analysis in Kir1.1 revealed that asparagine 171 (N171) is the only pore-lining residue required for high-affinity block, and that substituting negatively charged residues (N171D, N171E) at this position dramatically weakens VU590 block. In striking contrast, substituting a negatively charged residue at the equivalent position in Kir7.1 enhances block by VU590, suggesting the VU590 binding mode is different in Kir1.1 and Kir7.1. Interestingly, mutations of threonine 153 (T153) in Kir7.1 that reduce constrained polarity at this site (T153C, T153V, T153S) make wild type and binding site mutants (E149Q, A150S) more sensitive to block by VU590. The Kir7.1-T153C mutation enhances block by the structurally unrelated inhibitor VU714, but not by a higher-affinity analog ML418, suggesting that the polar side chain of T153 creates a barrier to low-affinity ligands that interact with E149 and A150 in Kir7.1. Reverse mutations in Kir1.1 suggest that this mechanism is conserved in other Kir channels. This study reveals a previously unappreciated role of membrane pore polarity in determination of Kir channel inhibitor pharmacology.

## C7. Therapeutic Drug Monitoring of 14 Antiepileptic Drugs using LC-MS/MS

Don E. Davis, Jr., R. L. Gant-Branum, Stacy D. Sherrod, Jennifer Colby and John A. McLean

Epilepsy is a neurological disorder characterized by a long-term risk of recurrent seizures. Antiepileptic drugs (AEDs) are effective at controlling seizures in only 70% of epileptic patients. Side effects such as toxicity, nausea and fatigue are associated with AED usage. Varying absorption, distribution, metabolism and excretion (ADME) determine how patients administer AEDs. Therefore, a factor to consider is that AED absorption is variable due to: metabolism, genetic factors, liver/kidney function, co-ingested medications, time of day medication is taken and whether it's taken with food or not. To minimize side effects, point of care testing must

routinely monitor the drug concentration in the blood of patients to ensure the concentration falls within a therapeutic range. Hence, therapeutic drug monitoring (TDM) for AEDs is useful for studying the variation of ADME, optimizing treatment for the individual and explaining adverse effects. Routine clinical TDM is typically performed using immunoassays but these analyses are not direct measurements because they need to be linked through an antibody or antigen to be detected. This limit immunoassays to single drug detection, a narrow dynamic measuring range and susceptibility to high false-positive rates/interferences. Separation followed by detection with ultraviolet light is currently the technique for quantifying AEDs in the clinic. However, these analyses are subject to issues such as co-elutions and matrix interferences resulting from isobaric species. This work reports an analytical method to quantify 14 AEDs in human serum using LC – MS/MS. Calibration curves for the AEDs extracted from serum were generated to assess quantitative linearity in the analytical method, while the experimentally determined limit of detection and upper/lower limits of quantification were evaluated to determine the sensitivity of the method. The optimal extraction of these AEDs from serum, as well as the recovery, matrix effect, precision and carry-over is reported. These analyses will benefit both clinicians and epileptic patients by providing a method that quantitates AEDs in serum; these analyses will also allow clinicians to make decisions toward optimizing medication dosage at the individual level and toward the goal of reducing side effects in patients.

## C8. Targeting metabotropic glutamate receptor subtype 5 for treatment of Alzheimer's disease

Deepa H. Rajan, Jonathan W. Dickerson, Daniel H. Remke, Ayan Ghoshal, Sean P. Moran, Gregory N. Roop, Mohammed Noor Tantawy, Jerri M. Rook

Alzheimer's disease (AD) is the most frequently observed cause of dementia and current therapies lack efficacy or provide only modest improvement in cognitive function. Glutamate is the primary excitatory neurotransmitter of the central nervous system (CNS) and glutamatergic transmission is severely disrupted in AD. Among the principle cognitive deficits associated with AD are deficiencies in hippocampal-mediated learning and memory, which rely on glutamatergic transmission. Thus, enhancement of glutamatergic neurotransmission has been proposed as a potential treatment for the cognitive deficits associated with AD. The metabotropic glutamate receptor subtype 5 (mGlu5) plays critical roles in multiple forms of synaptic plasticity that are thought to underlie learning and memory. Interestingly, recent studies suggest that proteins important for mGlu5 function are lost and that mGlu5 signaling is impaired in tissue from AD patients. Furthermore, activation of mGlu5 can induce favorable non-pathologic processing of amyloid precursor protein to reduce brain levels of pathogenic amyloid beta (Aβ). Over the past decade, highly selective positive allosteric modulators (PAMs) of mGlu5 have emerged as a promising new approach for improving cognitive function in schizophrenia and other non-degenerative CNS disorders. As opposed to direct activation of mGlu5, PAMs dramatically potentiate the response of the receptor to its endogenous ligand, glutamate, and offer high selectivity while avoiding unwanted side-effects seen with direct activation of the receptor. These new compounds provide an unprecedented opportunity to evaluate the potential of mGlu5 as a novel target for the treatment of symptoms associated with AD and other neurodegenerative disorders. We performed a series of electrophysiology, behavioral, and imaging studies to test

the hypothesis that acute administration of mGlu5 PAM, VU0092273, will provide efficacy in improving impaired cognitive function and reverse changes in cerebral metabolic activity in CK-p25 mice. The CK-p25 pre-clinical mouse model, which demonstrates impaired learning and memory as well as decreased brain mass, decreased neuronal density, neurodegeneration, increased tau phosphorylation, as well as elevated levels of Aβ, resembles the AD human condition. Our data demonstrate that mGlu5 PAMs are able to reverse deficits in hippocampal synaptic plasticity, as well as multiple models of cognitive function in this mouse model of neurodegeneration. Additionally, using [$^{18}$F]FDG PET imaging, we show that selective mGlu5 activation restores normal glucose metabolism in CK-p25 mice. These data provide exciting new evidence that mGlu5 PAMs can alleviate the diminished neural and cognitive function in a preclinical model of AD.

## C9. Catch-and-Release Flow-Through Strategy for Enhancement of Multiplex Malaria Diagnostics

Carson P. Moore, Lauren E. Gibson, Westley S. Bauer, David W. Wright

Malaria is a mosquito-borne disease caused by parasites that belong to the genus *Plasmodium*. This disease is globally endemic, particularly in low- and middle- income countries where state-of-the-art diagnostics are prohibitively expensive. The rapid diagnostic test (RDT) is a lateral flow assay, which is widely used due to its low cost, low resource requirements, and simplicity for untrained users. However, in regions moving towards control and elimination, RDTs are not sensitive enough to detect low levels of infection. Therefore, infected patients who are asymptomatic or who have submicroscopic parasite burdens may appear disease free. Malaria RDTs test for a variety of biomarkers, including the *Plasmodium falciparum* specific histidine-rich protein II (HRPII) and the pan-*Plasmodium* enzyme *plasmodium* lactate dehydrogenase (pLDH). In order to improve the sensitivity of existing RDTs, a simple sample preparation method has been proposed which utilizes zinc-iminodiacetic acid (Zn-IDA) functionalized cellulose membranes to concentrate and purify both biomarkers from blood samples. HRPII has been shown to bind to the Zn-IDA membrane with 99% capture efficiency, due to the high affinity of $Zn^{2+}$ for histidine. Subsequent elution releases approximately 60% of the total protein. Additionally, the test will utilize free binding sites on the Zn-IDA substrate to capture and elute $His_6$-tagged anti-pLDH antibodies which bind free pLDH in a sample. Although pLDH occurs in significantly lower levels than HRPII, it is a more accurate indicator of active infection as the concentration of pLDH rises and falls concurrent with parasitemia. Thus, tests incorporating pLDH are ideal for use in monitoring the efficacy of treatment, as well as diagnosing active infection. Once fully optimized for application with malaria biomarkers, the catch-and-release membrane system could easily be altered for use with a broad spectrum of biomarkers for other devastating diseases.

## C10. Rapid Sample Isolation of Malarial Antigen HRPII Using a Flow-Through Pipette Tip Format

Kelly A. Richardson, Westley S. Bauer, Nicholas M. Adams, Keersten M. Ricks, David J. Gasperino, Simon J. Ghionea, Mathew Rosen, Kevin P. Nichols, Bernhard H. Weigl, Frederick R. Haselton, David W. Wright

According the World Health Organization, nearly half of the world's population remains at risk for malaria. Full elimination efforts will require point of care procedures designed to function in a low-resource setting. Rapid diagnostic tests (RDTs) such as lateral flow assays are becoming more prevalent in malaria testing because of their low cost and simplicity. While many of these tests function effectively with high parasite density samples, their poor sensitivity can often lead to misdiagnosis when parasitemia falls below 100 parasites/μL. In this study, a flow-through pipette-based separation column was explored as a cost-effective means to increase RDT sensitivity by concentrating the *Plasmodium falciparum* histidine rich protein II (HRPII) antigen available in large-volume whole blood samples into smaller, RDT-compatible volumes. By systematically investigating immobilized metal affinity chromatography (IMAC) divalent metal species and solid phase supports, we established the optimal design parameters necessary to create a flow-through column incorporated into a standard pipette tip. These findings were used to produce a manufactured commercial prototype, which served as a platform to finalize the sample preparation method. Results show this bidirectional flow format optimizes mixing efficiency, minimizes sample processing time, and increases signal intensity up to a factor of twelve from HRPII concentrations as low as 25 pM. In addition, the limit of detection per sample was decreased by a factor of five when compared to the RDT manufacturer's suggested protocol. Both the development process and commercial viability of this application are examined, serving as a potential model for future applications.

## C11. Leveraging the Vanderbilt HTS resources to identify oxygen-dependent factors in bacterial biofilms

Allison Eberly, Tomas Bermudez, Madison Fitzgerald, Maria Hadjifrangiskou

Urinary tract infections (UTIs) account for a large portion of the antibiotics prescribed in the United States. The primary causative agent of UTIs is uropathogenic *Escherichia coli* (UPEC). UPEC strains form biofilms on biotic and abiotic surfaces such as catheters, urothelial tissues and within bladder epithelial cells. Adhesive pili, primarily type 1 pili (*fim*), mediate UPEC adherence to these diverse niches. Previous studies indicated that within a biomass, Fim-expressing UPEC subpopulations localize to the most oxygenated regions, suggesting the presence of an oxygen-responsive mechanism that regulates piliation and contributes to biofilm heterogeneity. We have previously reported that gradually decreasing the oxygen concentration corresponds to decreased levels of UPEC biofilm formation, except at 4% oxygen. Interestingly, bladder oxygen concentration is reported to be between 4-5.5% in healthy individuals. To identify oxygen-dependent regulators of biofilm formation, we created a saturated transposon library in cystitis isolate UTI89. We are screening for mutants that no longer exhibit wild-type

biofilm phenotypes at the following oxygen concentrations: ambient (21% $O_2$), hypoxic (4% $O_2$), and anoxic (0% $O_2$). To analyze the biofilm outputs, we are utilizing the Vanderbilt High-Throughput Screening core data analysis tools. Hit-picking data analyses were designed in WaveGuide in collaboration with the Vanderbilt High-Throughput Screening Core. The parameters used to identify biologically significant changes were established as follows: mutants that formed at least 50% more biofilm than the wild-type or 50% less biofilm than the wild-type under ambient and/or hypoxic conditions are considered a "hit". Mutant biofilm formed at 21% oxygen is also compared to its biofilm formed at 4% oxygen for differences greater than 50%. After hits are identified, secondary screens confirm phenotypes, followed by transposon mapping, to identify the disrupted gene(s) and characterization for roles in biofilm formation. These data will identify targets that inhibit biofilm formation in infection-relevant conditions.

## C12. **Exogenous fatty acids control *N*-acyl-phosphatidylethanolamines (NAPE) profile of therapeutically-modified colonized gut bacteria**

Noura S. Dosoky, Lilu Guo, Zhongyi Chen, Andrew V. Feigley, and Sean S. Davies

Engineering the gut microbiota to produce specific beneficial metabolites represents an important new potential strategy for treating chronic diseases. We previously engineered *E. coli Nissle 1917* (*EcN*) to produce *N*-acyl-phosphatidylethanolamines (NAPEs), a family of lipids that are the immediate precursors of the lipid satiety factors *N*-acyl-ethanolamides (NAEs), by heterologous expression of *A. thaliana* NAPE acyltransferase in the *EcN* (*pNAPE-EcN*). Administration of *pNAPE-EcN* to C57BL/6 mice in their drinking water inhibited their development of obesity when fed a high fat diet. This anti-obesity effect persisted for at least four weeks after ending administration due to colonization of the intestinal tract by *pNAPE-EcN*. While these studies demonstrated the potential of engineering the gut microbiota to treat obesity, they used conditions limiting their applicability to humans such as pretreatment with antibiotics and continuous administration of *pNAPE-EcN* for eight weeks. We therefore sought to determine if shorter periods of administration without antibiotic pretreatment would yield sustained colonization and anti-obesity effects. We also sought to determine if heterologous expression of the human NAPE acyltransferase PLAAT2 in *EcN* (*pPLAAT2-EcN*) or the BL21 lab strain of *E. coli* (*pPLAAT2-BL21*) would be as effective as *pNAPE-EcN*, since use of human genes and lab strain *E. coli* might be more palatable to regulatory agencies overseeing approval of new therapies. We found that administration of *pNAPE-EcN* in drinking water for two weeks generated anti-obesity effects that were sustained for at least four weeks after ending administration. Surprisingly, pretreatment with antibiotic did not enhance the long-term efficacy of *pNAPE-EcN* treatment. In contrast, treatment with antibiotics immediately following the two weeks of *pNAPE-EcN* treatment completely ablated the long term anti-obesity effects of the *pNAPE-EcN* treatment, consist with the notion that colonization by *pNAPE-EcN* was required for these sustained effects. Two week administration of *pPLAAT2-EcN* or *pPLAAT2-BL21* also exerted similar sustained anti-obesity effects as *pNAPE-EcN*. Comparison of the profile of NAPE species biosynthesized by these strains showed that even though the human and *A.*

*thaliana* NAPE acyltransferases use very different mechanisms for NAPE biosynthesis, they biosynthesize nearly identical NAPE species because both are highly responsive to exogenous fatty acids. This accounts for the high level of C16:0NAPE and C18:0NAPE formation when *pNAPE-EcN* or *pPLAAT2-EcN* colonize mice fed a high fat diet which is rich in palmitic acid (C16:0) and stearic acid (C18:0). Together, these results suggest the feasibility of engineering the gut microbiota to express NAPE as a sustainable treatment for obesity as well as a dietary method to fine tune the specific NAPE species produced by this engineered microbiota.

## C13. **Isolevuglandin, a highly reactive lipid dicarbonyl, causes deleterious consequences to high-density lipoprotein structure and function**

Linda S. Zhang, Huan Tao, Tiffany Pleasent, Mark S. Borja, Michael N. Oda, W. Gray Jerome, Patricia G. Yancey, L. Jackson Roberts II, MacRae F. Linton, Sean S. Davies

Cardiovascular disease (CVD) risk depends on levels of functional HDL particles, not HDL-cholesterol. In CVD, increased oxidative stress generates reactive lipid species that alter HDL function. Isolevuglandins (IsoLGs), generated in parallel to isoprostanes, are extremely reactive lipid dicarbonyls that react with lysine residues of proteins and headgroups of phosphatidylethanolamine (PE). Importantly, IsoLG protein and PE adducts are elevated in atherosclerosis. Recently, our group observed a 42% reduction of atherosclerotic lesion size when salicylamine, a small molecule scavenger of dicarbonyls including IsoLG, was administered to $LDLr^{-/-}$ mice. Little is known about the consequences of IsoLG to HDL function. The aim of this study is to compare effects of IsoLG on apolipoprotein crosslinking, morphology and size of HDL to three HDL functions: cholesterol efflux, HDL-apoA-I exchange, and anti-inflammation. Purified human HDL was incubated overnight at 37ºC with IsoLG. Thioglycolate-induced intraperitoneal macrophages were harvested from $apoE^{-/-}$ mice. IsoLG crosslinked HDL structural apolipoproteins, apoA-I and apoA-II, starting at 0.3 mol IsoLG per mol apoA-I (0.3 eq). HDL modified with 3 eq IsoLG formed subpopulations of two distinct sizes, 6-13 nm and 16-23 nm. A 40.6±0.04% decrease in $^{3}$H-cholesterol efflux from macrophages was observed at 1 eq IsoLG compared to unmodified control HDL. At this IsoLG concentration, HDL-ApoA-I exchange was reduced ($P<0.01$, n=4), from 47.4±2.8% with control HDL to only 24.8±5.8%, suggesting that IsoLG inhibited apoA-I from disassociating from HDL to interact with ABCA1. Intriguingly, IsoLG inhibited HDL's protection against LPS-stimulated inflammatory response in macrophages at 0.03 eq as shown by IL-1β and TNFα mRNA expression comparable to LPS alone. At 0.1 eq IsoLG, HDL becomes pro-inflammatory, as indicated by a 927±309% increase in IL-1β mRNA expression ($P<0.001$). Unlike cholesterol efflux, these effects occurred independent of HDL apolipoprotein crosslinking. We report a novel pathway by which HDL becomes dysfunctional, by mechanisms involving IsoLG-mediated alterations of HDL proteins and structure. Upcoming studies will test the efficacy of small-molecule scavengers of IsoLGs and related lipid dicarbonyls in preventing HDL dysfunction.

## C14. **Comparing 4-oxo-2-nonenal with other reactive aldehydes in modifying high density lipoprotein**

Linda S. Zhang, Mark S. Borja, Tiffany Pleasant, Amarnath Venkataraman, Patricia G. Yancey, MacRae F. Linton, Sean S. Davies

The Framingham study established an inverse relationship between levels of high density lipoprotein (HDL) cholesterol and cardiovascular disease risk, and numerous population and animal studies have confirmed the anti-atherogenic properties of HDL. Despite these evidence, high levels of HDL are not always protective in patients, which suggests that not all HDL are functional in preventing atherosclerosis. Recent studies support the notion that some HDL is dysfunctional or even proinflammatory. One mechanism that may cause HDL dysfunction is its modifications by reactive lipid aldehydes generated during oxidative stress. We have developed small molecule therapeutics that target reactive lipid aldehydes to prevent their reaction with proteins and other biomolecules. Recently, we discovered that these scavengers can significantly reduce atherosclerosis in animal models, suggesting a major role of lipid aldehydes in atherogenesis. Hydroxy-2-nonenal (HNE) is a well-studied product of lipid peroxidation that causes HDL dysfunction. Generated in parallel to HNE is 4-oxo-2-nonenal (ONE), which is less studied potentially due to its far greater reactivity. The potential consequences of ONE modification to HDL function is not known. We *hypothesize* that ONE modifies HDL to a greater extent and with a greater consequence for its function than HNE. In this study, we examine the effects of ONE on crosslinking HDL proteins and altering HDL functions. We compare these effects to another γ-ketoaldehyde, isolevuglandin (IsoLG or isoketal), which we recently found to potently crosslink HDL proteins and render HDL pro-inflammatory (*Zhang 2017, Abstract*). In addition, we examine the scavenging ability of small molecules in preventing HDL from modification. We find that ONE crosslinked apolipoprotein (apo) A-I on HDL at a concentration of only 3 ONE molecules for every 10 apoA-I proteins. This concentration is 100 fold lower than HNE but comparable to IsoLG. ONE-mediated crosslinking of HDL proteins preferentially produces a 39 kDa band on SDS-PAGE, likely an apoA-I/apoA-II heterodimer. Similar to IsoLG, ONE dramatically decreases the ability of apoA-I to exchange among HDLs, from 46.5±5.6% to only 18.4±3.1%. In contrast, ONE-modified HDL only partially inhibits the ability of HDL to protect against the inflammatory response of macrophages (as shown in TNF and IL-1b mRNA expression), but do not seem to render HDL pro-inflammatory. Effects of ONE-modification on HDL-mediated cholesterol efflux are currently being investigated. While small molecules including salicylamine (SAM) and pentylpyridoxamine (PPM) are shown to partially prevent IsoLG-induced HDL crosslinking, we find that that PPM nearly completely blocks ONE induced crosslinking, while SAM is less efficacious. Our study is first to show that ONE causes HDL dysfunction, and demonstrate that not all modified HDLs result in the same "dysfunction". We also demonstrate the use of PPM in preferentially scavenging ONE in biological systems.

## D. Synthesis –

### D1. Desymmetrization of a carboxylic acid by chiral Brønsted base activation

Matthew T. Knowe, Michael W. Danneman, Sarah Sun and Jeffrey N. Johnston

Carboxylic acids are widely valued for their availability and stability. Despite this, methods to activate carboxylic acids as chiral nucleophiles have evolved at a slow pace, making them relatively underutilized nucleophiles in enantioselective reactions. For this reason, a challenging desymmetrizing lactonization was investigated that provided mechanistic insight into Brønsted base activation of carboxylic acids, establishes three stereocenters concomitantly (one being an all-carbon quaternary center) with high enantioselectivity (up to 94% ee), and produces unique bicyclic lactone products. Through multiple catalyst-substrate cocrystal structures, a novel triple hydrogen bonding motif was identified, which is hypothesized to desymmetrize the carboxylate and generate a chiral carboxylate. This hypothesis drove optimization of catalyst structure to achieve high reactivity and enantioselectivity. The bicyclic lactone products are easily derivatized and provide facile access to carbocyclic nucleoside motifs.

### D2. Ion Templated Synthesis of Macrocyclic Oligomeric Depsipeptides

Suzanne M. Batiste and Jeffrey N. Johnston

Structurally complex and chemically diverse cyclic peptide and depsipeptide natural products are attractive scaffolds in the development of sensors, new materials, and therapeutics. Although synthetic methods using coupling reagents have been developed to produce these molecules and their derivatives on commercial scale, the waste generated by their use has long been recognized as a necessary evil. Additionally, the synthesis of macrocyclic peptides and depsipeptides typically suffers from the disadvantages associated with the preparation of their linear precursors. We have developed a rapid synthesis to collections of natural product-like cyclodepsipeptides which includes a platform for control over complex structural elements through a hybridization of enantioselective synthesis, Umpolung Amide Synthesis (UmAS), and an ion-templated macrocyclooligomerization (MCO). Alkali salt additives are used as tools to bias macrocycle formation during the Mitsunobu-enabled MCO. With the addition of a straightforward analytical technique, Isothermal Titration Calorimetry (ITC), we have shown that the size-distribution of MCO products formed in the presence of different alkali metal salts can be explained through analysis of trends in dissociation constants ($K_d$), enthalpy of binding ($\Delta H$), and stoichiometry of complexation (N). ITC is a very powerful tool for studying the thermodynamics of a binding event, unlike many commonly used analytical methods which are not always applicable in the analysis of complexes that are disorderly, fragile, insoluble, paramagnetic, and/or have multiple binding modes. The salt effects in the MCO reactions provide a rare example of organic solvent-based, precise and consistent thermodynamic data on less than 1 mg of isolated macrocycles from an MCO experiment. This work provides a 6-step

synthesis of the complex cyclodepsipeptide natural product verticilide, in addition to several other analogs varying in structure, stereochemistry (D/L), and ring-size.

## D3. Progress Towards the Total Synthesis of the Siderophore Coelichelin

Jade Williams, Brendan Dutter, Hunter Imlay, Matthew Draelos, Gary Sulikowski

The emergence of antibiotic-resistant strains of bacteria has been clearly documented as a significant public health concern necessitating the discovery of novel antibiotics. Ideally, these novel compounds would utilize modes of action dissimilar from those currently available. It has been well-documented that bacteria require metals for metabolism and pathogenesis, rendering metal acquisition pathways a potential target for such antibiotics. The production and secretion of siderophores is among the methods bacteria use to acquire metals from their environments. The siderophore, coelichelin, is produced by the bacterium *Streptomyces coelicolor* in iron-deficient conditions. Coelichelin chelates ferric iron, and the bacterium reuptake the siderophore-metal complex. Coelichelin could potentially serve as a tool compound in the study of metal acquisition pathways and bacterial responses to iron depletion. However, coelichelin can only be isolated in small quantities, necessitating the development of a concise and robust synthesis. This synthesis would provide sufficient quantities of coelichelin and derivatives to enable biological study.

## D4. Progress towards the Total Synthesis of Chrysophaentin A

Christopher Fullenkamp, Somnath Jana, Kwangho Kim, Jade Williams, Gary A. Sulikowski

Antibiotic resistance is now known for every clinically prescribed antibiotic, which means new antibiotics with novel mechanisms of action are needed. Chrysophaentin A, a marine natural product isolated from the alga *Chrysophaeum taylori,* exhibits micromolar activity against gram positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). The mechanism of action of this natural antimicrobial agent is believed to be the inhibition of FtsZ, which results in the direct inhibition of cellular division. Unfortunately further study of Chrysophaentin A has been inhibited due to its limited supply by isolation from its marine source. Total synthesis of Chrysophaentin A will not only allow further study of its novel mechanism of action but also access to other analogs for further development. To date our lab has been able to synthesize a derivative of Chrysophaentin A, which showed significant antimicrobial activity. Our immediate goal remains the total synthesis of Chrysophaentin A.

## D5. **Progress toward a Unified Chemical Synthesis of Hemiketals D2 and E2**

Alexander Allweil, Robert Davis, Juan Antonio, Claus Schneider, Gary Sulikowski

Hemiketals $D_2$ and $E_2$ ($HKE_2$/$HKD_2$) are unusual eicosanoids produced from arachadonic acid (AA), identified by the Schneider group as a product of an enzymatic cross-over pathway between 5-LOX and COX-2. Like many metabolites from the AA cascade it is hypothesized that Hemiketals may play an important role in various inflammatory processes. Many diseases result in a chronic inflamed state, such as cancer, diabetes, and some neurodegenerative diseases. With this in mind it is important to fully understand physiological consequences of Hemiketal production in relation to the inflammation process. However, to date only nano gram quantities of both hemiketals have been produced using an in vitro enzymatic procedure. In order to study its biological study a rigorous and reproducible chemical synthesis route to both hemiketals must be developed.

## D6. **Total Synthesis of the Isofurans in Support of Biological Studies**

Zachary Austin, Joshua Fessel, Gary Sulikowski

Pulmonary arterial hypertension (PAH) is a fatal vascular disease within the lungs marked by oxidative stress and endothelial cell proliferation. The free radicals generated in the oxidative stress process initiate non-enzymatic metabolism of arachidonic acid (AA) leading to a family of compounds known as the isofurans (IsoF's). This metabolism is non-stereospecific leading to 8 constitutional isomers each consisting of 32 stereoisomers. The role of these 256 IsoF's in PAH remains unclear and their biological production is minimal (nanogram quantities). Therefore, a stereodivergent synthesis from readily available starting materials is being employed to produce these IsoF's in sufficient amounts for biological studies.

## D7. **Synthetic and Biological Studies of Bielschowskysin and Pleiogenone A**

Jason Hudlicky, Jordan Froese, Cameron Overbeeke, Tomas Hudlicky, and Gary Sulikowski

Natural products have a long history of serving as therapeutic leads (e.g. taxol and compactin) and biological tools (e.g. rapamycin and oligomcyin). Our lab seeks to develop synthetic routes to rare natural products to provide material for biological study as well as provide significant training and education in the practice of chemical synthesis. Bielschowskysin, a rare and structurally complex marine diterpene, was isolated in 2004 from the extracts of the gorgonian coral, *Pseudopterogorgia kallos*. Initial biological assays indicated moderate antiplasmodial activity against *Plasmodium falciparum* ($IC_{50}$ = 10 $\mu$g/mL) and cytotoxicity against EKVC non-small cell lung cancer ($GI_{50}$ < 0.01 $\mu$M) and CAKI-1 renal cancer ($GI_{50}$ = 0.51 $\mu$M).[1] Presented

here are the recent efforts toward the total synthesis of bielschowskysin.[2,3] Another molecule that has piqued our interest due to its biological activity is pleiogenone A, recently isolated by Kingston[4] and synthesized by Hudlicky.[5] In a collaboration with the Hudlicky lab at Brock University (St. Catharines, Ontario, Canada) the synthetic sample of pleiogenone A has demonstrated activity against MV411 cells ($GI_{50}$ = 310 nM) and further studies are ongoing. Future work will focus on preparing synthetic analogs of pleiogenone A to improve activity.

### D8. Chemical Syntheses of Tetranor PGE-1 and PGD-M

Jennifer Benoy, Somnath Jana, Kwangho Kim, Cal Larson, Ginger Milne, Gary Sulikowski

Prostaglandin $D_2$ ($PGD_2$) and Prostaglandin $E_2$ ($PGE_2$) are both cyclic, oxygenated arachidonic acid metabolites produced in almost all mammalian cells. Prostaglandins are further metabolized to a number of different biomolecules, including tetranor PGE-1 as a metabolite of $PGE_2$ and PGD-M as a major metabolite of $PGD_2$. Both PGD-M and tetranor PGE-1 are important mediators of inflammation and therefore can be used as biomarkers to study different physiological and pathophysiological conditions in humans. To perform necessary assays, an isotopically labeled standard of each metabolite is required for accurate quantification. We present a completed synthesis for tetranor PGE-1 and recent progress towards the synthesis of PGD-M.

## E. Faculty Posters –

### E1. Carbon Direct Detect Backbone Assignment by NMR for Novel Peptides

M. Voehler, A. Maddur, I.M. Verhamme, and P. E. Bock

Two complimentary NMR strategies were utilized to achieve sequential backbone assignments for a peptide of interest and assignment of its $^{15}$N-HSQC spectrum. The first strategy involved two novel carbon direct detect backbone assignment methods as described by Pantoja-Uceda and Santoro [Santoro, JBNMR, 2013/14], which were compared to the conventional amide detected backbone assignment strategy. Complete assignment with the carbon-detected strategy was achieved with a set of only two 3D, one 2D, and a HNCO measurement, which was necessary to translate the information to the HSQC. Detection of the carbon nuclei is possible with the cold carbon channel of newer cryoprobes, higher fields and sufficiently long relaxation times on low- or non-structured peptides. The measurements were done on a 900 MHz Bruker spectrometer with CPTCI cryoprobe. Circumventing amide proton detection provides more uniform signal intensity and better chemical shift dispersion for the individual amino acid resonances, which are often problematic in unstructured peptides. It further allows the assignment of the proline backbone residues. In one set of the carbon detect experiments, the amides were used as the starting point of the magnetization transfer pathway, leading to a disruption of the backbone

assignment at each proline residue. Alternatively, another set of carbon detect experiments was utilized using Ha as starting point. This enables full sequential assignments, including the proline residues and has the advantage of distinct recognition of the glycine residues by their opposite phase. All three methods were used and will be presented. With these assignments in hand, interaction studies were readily possible and site-specific analysis of the results yielded novel insight into the mode of interaction with this peptide's partner.

## E2. High-Throughput Screening Core

Paige Vinson

The goal of the High-throughput Screening (HTS) Core Facility is to provide screening-based services to aid research investigators in the identification and investigation of new compounds for basic research and pharmacological discovery. We have developed a highly dynamic environment that utilizes industry standard practices and novel technologies for biological screening. The HTS facility is equipped to guide investigators through the drug discovery process from assay conception to lead optimization. In the HTS facility, we serve the basic research needs of scientific investigators by providing walk-up or full-service access to state-of-the-art instrumentation, distribution of compound libraries, and informatics solutions.

## E3. VICB Chemical Synthesis Core: Broadly Supporting Biomedical Investigator Needs

Plamen Christov, Benjamin Guttentag, Somnath Jana, Kwangho Kim, Ian Romaine, Shaun Stauffer, Gary Sulikowski, Jianhua Tian, Alex Waterson

The VICB chemical synthesis (CS) core is a unique resource, with expert capabilities in a broad number of chemical synthesis-based areas of technology and research. Currently, the CS core supports several medicinal chemistry programs in cancer therapeutic discovery within the NCI Experimental Therapeutics program (Vanderbilt Center for Cancer Drug Discovery). The core also provides support for many Vanderbilt investigators working in both basic and translational areas of research. The CS core typically engages in over 30 projects annually and has accumulated knowledge in the synthesis of small molecule tools for chemical biology, unnatural/natural nucleotide and peptide synthesis, natural products, and small molecule lead development. This poster will provide specific examples of areas of expertise and compounds currently available in the core's growing library of chemical probes and other compounds.

# E4. $^{1}$H NMR Analysis of Urinary Metabolome in Ischemia-Repurfusion Acute Kidney Injury

Donald F. Stec, Jacob Summers, Allyson Puckett, Nataliya Skrypnyk, Mark DeCaestecker, and Paul Voziyan

Acute kidney injury (AKI) is a common and independent risk factor for chronic kidney disease (CKD). With cardiac surgeries becoming increasingly common, more people are at an increased risk for ischemia-reperfusion acute kidney injury (IR-AKI) as renal blood supply is temporarily cut off during surgery. Currently, there is no effective treatment of AKI due, in part, to the lack of reliable biomarkers of this disease. Urinary metabolite levels can potentially serve as disease biomarkers as they reflect metabolic status of the kidney.

In this study, IR-AKI was induced in 10 to 12 week-old male BALB/c mice by left renal pedicle clamping for 31 minutes followed by contralateral nephrectomy after 8 days. Urine samples were collected before the induction of IR-AKI (day 0) and on days 9, 14, 21 and 28 after the initial IR-AKI induction (1).

Proton ($^{1}$H) nuclear magnetic resonance (NMR) spectroscopy in combination with statistical methods specifically developed for NMR metabolomics (2) were employed to analyze concentrations and identities of small molecule metabolites in mouse urine. Concentrations of 9 urinary metabolites were found to be significantly different between the control and the IR-AKI groups, i.e. trigonelline, allantoin, taurine, trimethylamine, citrate, acetoacetate, acetate, urea, and 2-oxoglutarate. These metabolites have potential as biomarkers of AKI initiation and/or progression to CKD.