The 2024 Frontiers in Chemistry and Biology Interface Symposium

(FCBIS 2024)

May 4, 2024

University of Maryland, Baltimore County

Baltimore, Maryland

<u>Organizing Committee at UMBC (alphabetically)</u>

Dr. Songon "Song" An

Dr. Chengpeng Chen

Dr. Deepak Koirala

Dr. Herana Kamal Seneviratne

Dr. Aaron T. Smith

along with

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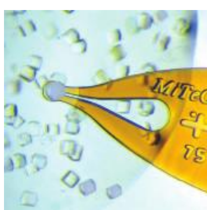

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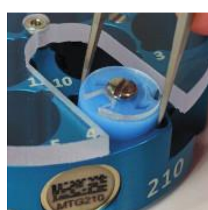

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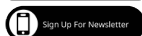

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Scientific Program (Saturday, May 4, 2024)

Engineering Building (ENG 027 & Atrium)

8:30 – 9:00 Registration & Light Breakfast

9:00 – 9:05 Opening Remark: Dr. Aaron T. Smith (UMBC)

9:05 – 9:55 Session 1: Moderator – Dr. Molly Sutherland (University of Delaware)

- *9:05 – 9:25 – Dr. Yanxin Liu (University of Maryland, College Park): Multidrug resistance-related calcium-binding protein Sorcin as a model system for investigating chaperone suppression of protein aggregation*
- *9:25 – 9:45 – Dr. Fernando Vonhoff (University of Maryland Baltimore County): The role of the Drosophila Amyloid Precursor Protein Like (APPL) in neurodevelopment and aging*
- *9:45 – 9:55 – Masha Kapitonova (University of Delaware – P.I. Dr. Sharon Rozovsky): Selenoprotien S: Deciphering the function in endoplasmic reticulum*

9:55 – 10:05 Morning Break

10:05 – 11:05 Session 2: Moderator – Dr. Sam Giannakoulias (SentauriAI.com)

- *10:05 – 10:25 – Dr. Jeffrey S. Mugridge (University of Delaware): Deciphering the structural and chemical mechanism for tRNA wobble base modification by a conserved radical SAM enzyme*
- *10:25 – 10:45 – Dr. Deepak Koirala (University of Maryland Baltimore County): Understanding RNA structure-based strategies of viral genome translation and replication*
- *10:45 – 10:55 – Juliet Obi (University of Maryland, School of Pharmacy – P.I. Dr. Daniel J. Deredge): A structural and dynamic basis for the interactions of the dengue nonstructural 5 protein with stem-loop A*
- *10:55 – 11:05 – Dr. Yuri Rafael de Oliveira Silva (Lehigh University – P.I. Dr. Oriana S. Fisher): Exploring the role of the ycn operon in copper acquisition by Bacillus subtilis*

11:05 – 11:50 Morning Poster Session I (Posters M1 – M54)

11:50 – 12:50 Lunch

12:50 – 1:35 Afternoon Poster Session II (Posters A1 – A54)

1:35 – 2:35 Session 3: Moderator – Dr. Mikell Paige (George Mason University)

- *1:35 – 1:55 – Dr. Euna Yoo (National Cancer Institute, Frederick): Chemoproteomic-enabled covalent ligand discovery*
- *1:55 – 2:15 – Dr. Xiongyi Huang (Johns Hopkins University): Expanding the reaction space of metalloenzymes*
- *2:15 – 2:25 – Amanda Vangieri (Saint Joseph's University – P.I. Dr. John Tomsho): Design, synthesis, and biological evaluation of novel leucyl-tRNA synthetase inhibitors with activity against common pathogenic bacteria*
- *2:25 – 2:35 – Mark Lee (University of Maryland Baltimore County – P.I. Dr. Aaron T. Smith): Structural insights to nucleotide promiscuity*

2:35 – 2:45 Afternoon Break

2:45 – 3:45 Session 4: Moderator – Dr. Cynthia "Cindy" Dowd (George Washington University)

- *2:45 – 3:05 – Dr. Amy M. Whitaker (Fox Chase Cancer Center): Interplay between oxidative damage repair in DNA G-quadruplexes and transcriptional regulation*
- *3:05 – 3:25 – Dr. Ozlem Dilek (George Mason University): Small molecule-click chemistry probes for fluorescence imaging in cellular systems*
- *3:25 – 3:45 – Dr. Kathryn Gunn (Stony Brook University): Cryo-electron tomography reveals lipoprotein lipase vesicular storage structure*

3:45 – 3:55 Afternoon Break

3:55 – 4:00 Keynote Introduction: Dr. Songon "Song" An (UMBC)

4:00 – 5:00 Keynote Speaker: Dr. Jennifer Lippincott-Schwartz (HHMI Janelia)

"Imaging protein synthesis at micron and atomic scales"

5:00 – 5:15 Poster Awards, Final Remark, and Announcement of the 2025 FCBIS

5:15 – 6:00 Social Hour and Departure

Keynote Speaker

Dr. JENNIFER LIPPINCOTT-SCHWARTZ

Senior Group Leader
Howard Hughes Medical Institute - Janelia Research Campus, Ashburn, VA 20147

EDUCATION

Ph.D. in Biochemistry, 1986
The Johns Hopkins University, Baltimore, Maryland

M.S. in Biology, 1979
Stanford University, Palo Alto, California

B.A. (with Honours) in Psychology and Philosophy, 1974
Swarthmore College, Swarthmore, Pennsylvania

MAJOR RESEARCH INTERESTS

Development and use of advanced imaging technologies (e.g., photoactivation, FRAP, single particle tracking, PALM, FIB-SEM, lattice light sheet microscopy and TIRF-SIM) to study the complexity of cell behaviors (e.g., cell crawling, polarity, cytokinesis, viral budding and intercellular transfer) and the dynamism/organization of subcellular organelles (e.g., ER, mitochondria, Golgi, endosomes, lysosomes, lipid droplets, actomyosin filaments, microtubules, autophagosomes, peroxisomes and cilia) under both healthy and pathological conditions.

PROFESSIONAL AWARDS/HONORS *(Selected)*

- **Dickson Prize 2024**, Carnegie Mellon University, PA
- **ABRF Award, 2022** Association of Biomedical Resource Facilities, USA.
- **E.B. Wilson Medal, 2020** American Society of Cell Biology
- **Named Fellow or Elected Member**
    - Elected to the Institute of Medicine of the National Academies, 2009
    - Elected to the National Academy of Sciences, Biochemistry Section, 2008
    - Elected AAAS Fellow, 2008, for "*Outstanding contributions to the field of fluorescent protein imaging, including the creation of photoactivable GFP and its use in new super-resolution imaging techniques*"
- **Over 88 Honorary Lectureships or Keynotes** in Universities and Conferences

PROFESSIONAL SERVICES

- **Associate Editor** *(Including Journal of Cell Science*, *Current Protocols in Cell Biology*, *Annual Review of Cell and Developmental Biology, and several more)*
- **Many Scientific Advisory/Review Board**
    - Chan Zuckerberg Imaging Institute (2023-present)
    - Allen Institute of Cell Science (2016-present)
    - Howard Hughes Medical Institute (2012-present)
- **President of the American Society of Cell Biology (2014)**
- **Meeting Organizer:** Cold Spring Harbor Meeting, EMBO Conference, FASEB Meeting, GRC Janelia Research Campus Meeting, ASCB and several more

Invited Presentation Abstracts

1. Gly-rich N-terminal domain regulates the calcium binding to the multidrug-resistant related protein Sorcin

Kathleen Joyce Carillo (1,2), Qiushi Ye (1,2), Angela Wu (2,3), Nicolas Delaeter (1,2), **Yanxin Liu** (1,2)
(1) Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20878
(2) Institute for Bioscience and Biotechnology Research, Rockville, MD 20850
(3) Montgomery Blair High School, Silver Spring, MD 20901

Sorcin is a penta-EF hand calcium-binding protein that confers multidrug resistance in cancer cells. It regulates cellular calcium homeostasis by interacting with calcium channels such as ryanodine receptor 2 (Ryr2) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) in a calcium-dependent manner. Using turbidity assay, we have characterized the kinetics of calcium binding-induced sorcin aggregation. Our results indicated that upon calcium binding, sorcin undergoes a conformational change that exposes its hydrophobic surface, resulting in sorcin aggregation. We found that the sorcin's sensitivity to calcium concentration is highly regulated by its Gly-rich N-terminal domain (NTD). We further conducted NMR studies on full-length sorcin and a truncated sorcin with the NTD deletion. Our backbone NMR assignments and the chemical shift perturbations reveal that the sorcin NTD unexpectedly enhanced the calcium-binding affinity (Kd) in the full-length sorcin. By mapping out the residues affected by calcium binding, we propose a mechanistic model in which the sorcin NTD directly interacts with the EF hands rather than participating in the sorcin conformational change upon calcium binding. Our results may also provide structural evidence for the interactions between mitochondrial chaperone TRAP1 and the recently discovered mitochondrial isoform of sorcin.

2. The role of the Drosophila Amyloid Precursor Protein Like (APPL) in neurodevelopment and aging

Fernando Vonhoff (University of Maryland, Baltimore County)

3. (M10) Selenoprotein S: Deciphering the function in endoplasmic reticulum

Masha Kapitonova, Fabio A. Gonzalez-Arias, Samiran Subedi, Sharon Rozovsky
Department of Chemistry and Biochemistry, University of Delaware

4. Deciphering the structural and chemical mechanism for tRNA wobble base modification by a conserved radical SAM enzyme

Jeffrey Mugridge (University of Delaware)

5. Understanding RNA structure-based strategies of viral genome translation and replication

Deepak Koirala
Department of Chemistry and Biochemistry, University of Maryland, Baltimore County

The extreme 5′ end of an enteroviral RNA genome contains a highly conserved replication-linked RNA (REPLR) domain that recruits multiple protein factors, such as viral 3CD and host PCBP2, to form an essential ribonucleoprotein (RNP) complex for synthesizing the (-)-stand RNA, an obligatory first step for the viral genome replication. However, the high-resolution structural and mechanistic studies of this critical virological process have been limited. Using Fab-assisted RNA crystallography, we determined the high-resolution crystal structures of the CVB3, RVB14, and RVC15 REPLRs. We revealed that enteroviral REPLRs exhibit a conserved H-shaped topology, including a unique RNA tertiary interaction between the two subdomains, consistent with a high degree of REPLR sequence similarity across the enteroviral species. The reoccurrence of common structural features in our crystal structures also allowed us to compute the structural models for the REPLRs from four additional enteroviral species, EV71, PV1, EVD68, and RVA2, using homology modeling that converged into a similar H-shaped topology. Furthermore, we revealed that the integrity of the H-shaped topology is critical for high-affinity binding of the viral 3CD and human PCBP2, suggesting enteroviral REPLR structures play crucial roles in recruiting these proteins. The H-shaped architecture perhaps facilitates the positioning of the 3CD and PCBP2 binding sites on the opposite ends of the REPLR, providing a pre-organized scaffold to bind these proteins separately, as no specific interaction between 3CD and PCBP2 has been observed. While our structural and binding study warrants further investigation, it has laid an initial foundation for understanding genome replication in enteroviruses and, in general, (+)-strand RNA viruses. Additionally, the DNA oligos complementary to the 3′ spacer, the high-affinity PCBP2 binding site, abrogated its interactions with enteroviral REPLRs, suggesting the critical roles of this single-stranded region in recruiting PCBP2 for enteroviral genome replication and illuminating the promising prospects of developing therapeutics against enteroviral infections targeting this replication platform.

6. (M6) Structural and dynamic basis for the interactions of the Dengue nonstructural 5 (NS5) protein with stem-loop A (SLA)

Juliet O. Obi, Kyle C. Kihn, Ally K. Smith, Linfah McQueen, and Daniel J. Deredge
University of Maryland School of Pharmacy

7. (M32) Exploring the role of the ycn operon in copper acquisition by Bacillus subtilis

Yuri Rafael de Oliveira Silva (1), Dia Zheng (1), Stephen C. Peters (2), Oriana S. Fisher (1)

(1) Department of Chemistry, Lehigh University, 6 E Packer Ave, Bethlehem, PA 18015, USA
(2) Department of Earth and Environmental Sciences, Lehigh University, 1 W Packer Ave, Bethlehem, PA 18015, USA

8. Chemoproteomic-enabled covalent ligand discovery

Euna Yoo (National Cancer Institute, Frederick)

9. Expanding the reaction space of metalloenzymes

Xiongyi Huang (Johns Hopkins University)

10. (M37) Design, Synthesis, and Biological Evaluation of novel Leucyl-tRNA Synthetase Inhibitors with Activity Against Common Pathogenic Bacteria

Amanda Vangieri, Olivia Pawlow, and Dr. John Tomsho

Department of Chemistry and Biochemistry, St. Joseph's University (Philadelphia)

11. Structural Insights to Nucleotide Promiscuity

Mark Lee and Aaron T. Smith

Department of Chemistry and Biochemistry, University of Maryland Baltimore County

12. Interplay between oxidative damage repair in DNA G-quadruplexes and transcriptional regulation

Amy M. Whitaker (Fox Chase Cancer Center)

13. Small molecule-click chemistry probes for fluorescence imaging in cellular systems

Ozlem Dilek (1,2), Eva-Maria Rudler (1), Amira T. Anwar (1), Abisoye O. Fafioye (1), Ewa Krawczyk (2), Komuraiah Myakala (2), Xiaoxin Wang (2), Moshe Levi (2)

(1) Department of Chemistry and Biochemistry, George Mason University, Institute for Advanced Biomedical Research, Manassas, VA, USA.
(2) School of Medicine, Department of Biochemistry and Molecular & Cellular Biology, Georgetown University, Washington, DC, USA.

Over the past decades, click chemistry has been used not only as a very powerful bioconjugation method, but also a fine-tuning reaction strategy in chemical biology. Using click chemistry in reactions is highly selective and biocompatible and can be performed in many complex cellular systems. The small-molecule fluorescent probes are of interest because of their high sensitivity and selectivity, as well as their potential for automated detection. For example, designed probes should have particular chemical and photophysical characteristics to achieve successful imaging: high stability, less toxicity, fast kinetics, good spectral properties such as large Stokes shifts, reasonable quantum yields, drastic changes on absorption and emission spectra. Therefore, expanding the probe toolset for click chemistry will provide precisely tuned reagents for manipulating bonds in distinct environments.

In this work, we developed several small-molecule probe systems that could act as turn off and on in biological systems. Our bioconjugation system includes either amine or hydrazine bond, which rapidly formed a fluorescent turn-on when it conjugates with the carbonyl functional groups in the biological systems. In our lab, we particularly focus on developing

small molecule- click chemistry probes for fluorescence imaging in several different biological systems. Use of imine/hydrazone bioorthogonal conjugation system has proven itself to be superior in satisfying many criteria (e.g., biocompatibility, selectivity, yield, stability, and so forth); our results will therefore provide a powerful probe technology that can be able to differentiate the normal and cancer cells in live cells or other relevant human diseases. We anticipate that development of new library of fluorophores will not only advance the discovery of future new therapeutics for human diseases, but also enable the development of better imaging agent technologies that have potential to diagnose early stages of selective human diseases.

14. Cryo-electron tomography reveals lipoprotein lipase vesicular storage structure

Kathryn Gunn (Stony Brook University)

Poster Abstracts

(Morning Session M1 – M54 & Afternoon Session A1 – A54)

M1. Proteome-Wide Assessment of Protein Structural Perturbations Under High Pressure

Haley Moran (1), Edgar Manriquez Sandoval (2), Piyoosh Sharma (3), Stephen Fried (4), and Richard Gillilan (5)

(1) Johns Hopkins University, Chemistry-Biology Interface Program

(2) Johns Hopkins University, Program for Molecular Biophysics

(3, 4) Johns Hopkins University, Department of Chemistry

(5) Cornell High Energy Synchrotron Source, Center for High Energy X-ray Science

One of the planet's more understudied ecosystems is the deep ocean, where organisms experience very high (30–110 MPa) hydrostatic pressures and little-to-no solar energy; yet, according to some estimates, these pelagic zones contain more biomass than all other ecosystems combined. The extent to which terrestrially relevant pressures up to 100 MPa deform most globular proteins – and which kinds – has not been established. Here, we report the invention of an experimental apparatus that enables structural proteomic methods to be carried out at high pressures for the first time. The method, called high-pressure limited proteolysis (Hi-P LiP), involves performing pulse proteolysis on whole cell extracts brought to high pressure. The resulting sites of proteolytic susceptibility induced by pressure are subsequently read out by sequencing the peptide fragments with tandem liquid chromatography-mass spectrometry. When applied to the thermophilic piezo-sensitive bacterium Thermus thermophilus, we find that ca. 40% of its soluble proteome is structurally perturbed at 100 MPa. Proteins with lower charge density are more resistant to pressure-induced deformation, as expected; however, contrary to expectations, proteins with lower packing density (i.e., more voids) are also more resistant to deformation. Furthermore, high pressure has previously been shown to preferentially alter conformations around active sites. Here, we show this is also observed in Hi-P LiP, suggesting that the method could provide a generic and unbiased modality to detect binding sites on a proteome scale. Hence, datasets of this kind could prove useful for training emerging AI models to predict cryptic binding sites with greater accuracy.

M2. Genomic context shapes DNA methylation & hydroxymethylation landscapes

Noa Erlitzki (1, 2) and Rahul M. Kohli (2, 3)

(1) Biochemistry & Molecular Biophysics Graduate Group, Perelman School of Medicine, University of Pennsylvania

(2) Department of Medicine, University of Pennsylvania

(3) Department of Biochemistry & Biophysics, University of Pennsylvania

DNA demethylation in the mammalian genome reverses the gene silencing program imposed by 5-methylcytosine (5mC) and is facilitated by Ten-Eleven Translocation (TET) enzymes, which catalyze the stepwise oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC). Oxidation of 5hmC to 5fC commits the modified cytosine to a pathway for active demethylation. By contrast, persistence as 5hmC allows, but does not necessitate, passive demethylation through an alternate pathway. 5hmC is thought to play key roles in various developmental pathways, including neuronal development, while its dysregulation is prominently associated with oncogenesis. Interestingly, it has been observed not only as a transient intermediate but also as a stable modification that likely has independent epigenetic functions. The in vitro kinetics of TET-mediated 5mC oxidation have been previously described and were found to be strongly influenced by DNA sequence context, with potential implications for the determinants of demethylation dynamics in vivo. However, prior methods have been unable to parse the generation and decay of 5hmC, a key fulcrum in progression through distinct demethylation pathways. We took a high-throughput enzymology approach utilizing dual-pipeline deep sequencing to observe, for the first time and at the base resolution, the dynamic generation and depletion of 5hmC in vitro. We newly report a sequence context dependence for 5hmC accumulation that is distinct from that of 5mC oxidation, establishing a molecular basis for the proposed unique biological roles for 5hmC as a stable epigenetic modification.

M3. Genetic Code Expansion for Site-Specific Dual Encoding of Fluorophore-Quencher Pairs

Yarra Venkatesh (1) Priyanda Giri (1), Moriah Mathis (2), Christina Hurley (1), Richard Cooley (2) Rahul Kohli (1) Ryan Mehl (2) E. James Petersson (1) *

(1) University of Pennsylvania, Philadelphia, PA, United States

(2) Oregon State University, Corvallis, OR, United States

The ability to precisely modify proteins at multiple locations in their natural environment represents an unprecedented opportunity for answering biological questions at the molecular and cellular levels. In this work, we introduce a versatile dual incorporation approach, which involves the site-specific incorporation of two distinct noncanonical amino acids with bioorthogonal properties into proteins in vitro. This innovative strategy has demonstrated remarkable efficacy in incorporating acridonylalanine (Acd) and m-4-methyltetrazinyl phenylalanine (Tet) bearing amino acids at respective TAG and TAA codons in a single protein using mutually orthogonal tRNA/synthetase sets. As proof-of-concept, we successfully applied the dual encoding approach to study two different proteins of interest: 1) α-synuclein, a protein that aggregates to form amyloid fibrils which play an important role in Parkinson's disease, 2) the LexA protein, a key regulator of the SOS response that enables acquired antibiotic resistance in bacteria. In this design, Tet can serve as an intrinsic quencher of Acd fluorescence or a biorthogonal reactive handle for attachment of FRET acceptors such as BODIPY through strained trans-cyclooctene (sTCO) labeling. Strategically introducing Acd/Tet modifications at specific positions in the α-synuclein or LexA protein enables 1) helps probe α-synuclein structural changes, amyloid fibril kinetics, and molecular interactions, yielding insights into disease mechanisms and 2) real-time monitoring of pH mediated or RecA*-dependent LexA cleavage kinetics through fluorescence activation and screening small-molecule inhibitors for their effects on both RecA*-stimulated cleavage and LexA autoproteolysis. These studies highlight the potential for Acd/Tet double labeling to be used in mechanistic analysis and drug discovery in a complex biochemical pathway with important implications for suppressing antibiotic resistance in pathogenic bacteria.

M4. The SARS-CoV-2 Fusion Domain Preferentially Initiates Fusion in an Environment akin to the Endocytic Pathway

Daniel Birtles, Jinwoo Lee

Department of Chemistry and Biochemistry, University of Maryland College Park

Membrane fusion is an integral component of the viral lifecycle that allows delivery of genetic material into a target cell and is facilitated through drastic conformational changes of viral glycoproteins. To gain a complete understanding of SARS-CoV-2 infectivity, the interactions between the spike proteins fusion domain (FD) and the target cells lipid membrane must be investigated in molecular detail. Utilizing a FRET based in vitro fusion assay, a preference for a low pH environment and the anionic lipid BMP was discovered for the FD, both of which are key characteristics of the endocytic pathway. From a structural perspective, a clear conformational rearrangement was witnessed when the FD embeds within a membrane environment via solution NMR. Intriguingly, only minor changes in the secondary structure were observed when the local pH was decreased despite the significant change in fusogenicity. When comparing the influence of different anionic lipids on the FD and the membrane through several biophysical techniques, it was found that BMP had a novel impact on lipid packing which was the primary reason for its specificity. A thorough mutagenesis analysis for residues of interest elucidated a key hydrophobic motif (821LLF823) essential for initiating fusion as well as the discovery of important electrostatic interactions between positive residues within the FD and anionic lipids. In conclusion, the SARS-CoV-2 FD initiates fusion more readily in a low pH environment with BMP present, due to minor structural changes within the protein allowing it to take advantage of the changing membrane properties imparted by BMP.

M5. D-amino acid-induced flexibility in peptides stapled by fluorine-thiol displacement reaction (FTDR) enhances membrane penetration and cellular uptake

Robert Maloney (1), Samuel Junod (1), Todd Lewis (1), Femil J. Shajan (1), Kyla Hagen (2), Thu Truong (2), Weidong Yang (1), Rongsheng (Ross) Wang (1)

(1) Temple University, Philadelphia, PA

(2) University of Minnesota Twin Cities, Minneapolis, MN.

Understanding how peptides enter cells is crucial for developing effective intracellular targeting strategies. Here, we demonstrate that our peptide stapling technique, fluorine-thiol displacement reaction (FTDR), produces flexibly constrained peptides with improved cellular and nuclear uptake. The FTDR platform enhances flexibility while maintaining significant α-helicity, yielding highly permeable peptides without additional cell-penetrating motifs. FTDR-stapling accommodates D-amino acids, amplifying flexibility in constructs like SRC2-LD, compared to purely L-amino acid containing peptides. Cellular uptake assays showed FTDR-stapled peptides had superior internalization (cytoplasmic and nuclear) compared to ring-closing metathesis (RCM)-stapled ones. Peptides targeting the ERα-coactivator interaction in ER+ breast cancer entered cells via energy-dependent transport processes including actin-mediated endocytosis and macropinocytosis. Despite less constrained secondary structure and weaker target binding, FTDR peptides exhibited enhanced anti-proliferative effects, emphasizing the importance of cellular uptake efficiency. Our findings challenge existing perceptions of cell permeability, providing insights into structural determinants crucial for cellular uptake of stapled peptides. Notably, α-helicity and lipophilicity alone do not determine high cell permeability, as shown by our less helical, more flexible, and less lipophilic FTDR-stapled peptides.

M6. A Structural and Dynamic Basis for the Interactions of the Dengue Nonstructural 5 (NS5) Protein with Stem Loop A (SLA)

Juliet O. Obi, Kyle C. Kihn, Ally K. Smith, Linfah McQueen, and Daniel J. Deredge

Department of Pharmaceutical Sciences, University of Maryland School of Pharmacy, Baltimore, MD 21201

Dengue virus is the most prevalent arthropod-borne virus and there are no clinically approved antivirals to date. The non-structural 5 (NS5) protein is the largest protein encoded by flaviviruses including dengue, with an N-terminal methyltransferase (MTase) domain responsible for 5' RNA capping, and a C-terminal RNA-dependent-RNA-polymerase (RdRp) domain responsible for de novo RNA synthesis. Stem Loop A (SLA) is an RNA element at the 5'-untranslated region which acts as a recognition motif for the initiation of RNA synthesis by NS5. We characterized the interaction of the full length and individual domains of NS5 from dengue serotype 2 with SLA using surface plasmon resonance (SPR) studies, differential scanning fluorimetry (DSF) and hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS). Results from our SPR and HDX-MS studies show that both MTase and RdRp domains of NS5 interact with SLA which is corroborated by a recently published Cryo-EM structure of DENV3 NS5 bound to SLA. We observed that SLA binding to NS5 transiently destabilizes the RdRp domain which coincides with deprotection seen in our HDX-MS data, and suggestive of an opening of the RdRp domain upon SLA binding as shown in the Cryo-EM structure. We modelled a DENV2 NS5-SLA complex and performed HDX-ensemble reweighting (HDXer) to identify upweighted ensembles which conform the most with our HDX-MS data. Ultimately, we aim to further explore and elucidate the dynamic interactions between NS5 and SLA for therapeutic development.

M7. Visualization of region-specific lipid alterations in the mouse brain in response to efavirenz treatment using MALDI mass spectrometry imaging

Nav Raj Phulara (1), Apurv Rege (2), Charles J Bieberich (2), and Herana Kamal Seneviratne (1)

(1) Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, MD 21250

(2) Department of Biological Sciences, University of Maryland, Baltimore County, Baltimore, MD 21250

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor used for human immunodeficiency virus (HIV) treatment in combination with other antiretrovirals such as tenofovir and emtricitabine. Notably, EFV has been listed in the World Health Organization's list of essential medicines. However, previous clinical studies have revealed that EFV has been associated with adverse effects on the brain. Since lipids play diverse physiological roles in the brain, their alterations can result in the onset of brain disorders. Of note, it has been reported that EFV-based antiretroviral regimens impact lipid profiles in plasma. To elucidate the association between EFV-based therapy and brain disorders, it is essential to understand the impact of EFV on brain lipid profiles. Moreover, changes in the brain lipid metabolism in response to EFV treatment are currently unknown. In this study, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry imaging was employed to generate region-specific spatial localization profiles of lipids in the mouse brain following EFV treatment. The area under the receiver operating characteristic curve was used to discriminate between the spatial localization profiles of lipids of interest in control and EFV-treated mouse brains. Additionally, mass spectrometry-based proteomics was performed to investigate changes in proteins in the mouse brain in response to the EFV treatment. Using MALDI mass spectrometry imaging, we discovered distinct region-specific localizations of phosphatidylcholine (PC), sphingomyelin (SM), ceramide phosphoinositol (PI-Cer), and hexosylceramide (HexCer) in mouse brain. Specifically, PC (32:0), PC (38:5), and SM (d18:1/18:0) exhibited their accumulation in the hippocampus region, while PI-Cer (38:8) showed its depletion in the same brain region in response to EFV treatment. In addition, we observed the depletion of PC (38:6), PC(40:6), and PI-Cer (40:3) in the thalamus region of the EFV-treated mice. Moreover, SM(d18:1/22:0), SM(d18:1/24:1), SM(d18:1/24:0), SM(d18:2/25:0), and SM(d18:1/25:0) exhibited high abundance, in the corpus callosum region in response to EFV treatment. In contrast, PI-Cer (38:1), PI-Cer (38:0), HexCer (t42:1), and HexCer (t42:2) exhibited their low abundance in the corpus callosum region of the EFV-treated mice as compared to control mice. From mass spectrometry-based proteomics analysis, we found the expression of lipid metabolizing enzymes, including ceramide synthase 2, acid sphingomyelinase, acid ceramidase, and calcium-independent phospholipase A2-gamma in the brain tissues. Further, the expression levels of twelve proteins were significantly decreased following EFV treatment. The above proteins include Na+/K+ transporting ATPase subunit alpha 1, tubulin beta-3 chain, neurofilament medium chain, and homer protein homolog 1. Taken together, these results provide insight into the EFV-induced alterations of lipid metabolism in the brain.

M8. Physics- and machine-learning-based method for identifying druggable binding sites with SILCS-Hotspots

Erik B. Nordquist, Mingtian Zhao, Anmol Kumar, Alexander D. MacKerell

Identifying druggable binding sites on proteins is an important and challenging problem, especially for so-called cryptic sites that are not obvious in x-ray, cryo-EM, or predicted structures. The Site-Identification by Ligand Competitive Saturation (SILCS) method addresses this challenge by accounting for the target protein's flexibility by using all-atom molecular simulations that include various small molecule solutes in aqueous solution. During the simulations, the combination of protein flexibility and comprehensive sampling of the water and solute spatial distributions identifies buried binding pockets. We are expanding the existing method for identifying binding sites, termed Hotspots, of small mono- or bi-cyclic compounds, some of which correspond to known binding sites of drug-like molecules. The new method uses a machine learning model to rank these Hotspots according to the likelihood they can accommodate drug-like molecules (e.g. molecular weight > 200 daltons). In the independent validation set consisting of various enzymes and receptors, our model identifies 59% and 89% of experimentally validated binding sites in the top 10 and 20 ranked Hotspots, respectively. This is a key advancement in the identification of orthosteric and allosteric binding sites for drug-like molecules to be used in combination with accurate docking of known binders and pharmacophore screening for new hits using SILCS methods.

M9. The LASV Stable Signal Peptide Undergoes a Conformational Change During Viral Fusion

Shane Collins, Jinwoo Lee

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Lassa virus (LASV), the predominant member of the arenavirus family, has been identified by the World Health Organization as a top-priority research target due to its current public health burden and potential to cause future pandemics. A pivotal event in the LASV life cycle is membrane fusion, a process initiated by the surface glycoprotein complex (GPC) upon exposure to endosomal pH. A distinctive feature of the GPC is the presence of the stable signal peptide (SSP), which is positioned adjacently to the transmembrane region of the main fusion protein, GP2. Previous research has demonstrated SSP has a crucial, yet currently undefined role in fusion. This study is aimed to address two main objectives: first, to comprehend the structural changes experienced by SSP during the fusion process; and second, to establish a correlation between the structural changes of SSP and membrane fusion. NMR analysis revealed that SSP is predominantly alpha-helical and undergoes conformational changes when the pH is lowered to 4 to mimic endosomal pH. A comprehensive array of fluorescence experiments demonstrated that the induced conformational change is likely a result of an increased association of SSP with the membrane. This conformational change was validated through CD, indicating that the structural shift occurs exclusively at pH4. Furthermore, employing an in vitro fusion assay, we illustrated that SSP interactions with GP2 directly enhance fusion and lead to increased fusion efficiency at a lower pH. Our conclusion is that SSP is essential for initiating LASV fusion at a precise location within the endocytic pathway, thereby enhancing fusion efficiency. Ultimately, this contributes to our understanding of the intricate LASV fusion mechanism involving the unique SSP.

M10. Selenoprotein S: Deciphering the function in endoplasmic reticulum

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Selenoprotein S (selenos), a small transmembrane protein, has been implicated in various diseases, including cancer, cardiovascular issues, diabetes, and thyroid disorders. To delve deeper into its functionalities, our focus lies on its involvement in alleviating endoplasmic reticulum (ER) stress. This stress condition arises from the accumulation of misfolded proteins within the ER membrane and lumen. Selenos plays an active role in endoplasmic reticulum-associated degradation (ERAD), a process crucial for identifying misfolded proteins, extracting them from the ER, and degrading them in the cytosol. Specifically, selenos acts as an adapter between derlin, presumed to initiate the translocation of substrates through the ER membrane, and AAA+ ATPase p97, which is responsible for extracting these substrates from the ER into the cytosol. Our hypothesis suggests that selenos may assist in substrate recognition by derlin or regulate derlin's ability to dislocate substrates from the ER. To shed light on the role of selenos in ERAD, we expressed and purified the selenos/derlin 2 complex using an insect cell expression system. The complex co-purified in a two-step affinity chromatography process, indicating a robust protein association. AAA+ ATPase p97 was purified from bacterial cells, and a low-resolution Cryo-EM model was resolved to aim for further reconstitution of selenos, derlin 2, and p97. Further refinement of the purification process for structural studies is necessary.

M11. Identifying Cognition-Associated Protein Structural Changes in Aged Rodent Hippocampi Using Limited-Proteolysis Mass Spectrometry

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Cognitive decline in aging is a major issue, causing both personal and economic hardship in an increasingly aging society. There are several known misfolded proteins, such as amyloid beta and alpha synuclein, that are associated with neurodegenerative disease. However, many studies have found that the proteostasis network, which works to keep proteins properly folded, is impaired with age, suggesting that there may be more global protein structural changes. Our study utilizes limited-proteolysis mass spectrometry (LiP-MS) to investigate protein structural changes proteome-wide in a rodent model of aging. LiP-MS identifies structural changes, such as conformational changes, via pulse proteolysis with proteinase K, a protease that cuts preferentially at solvent-exposed and flexible areas, encoding structural information. These cut sites can be compared using label free quantification. We compared hippocampi from aged rodents with normal cognition to hippocampi from aged rodents with impaired cognition. Over 3000 proteins were identified in our study, with several hundred being characterized as structurally different between the aged unimpaired and aged impaired populations. We explored trends in the characteristics of these proteins, such as number of domains, isoelectric point, and percent disorder. We also found correlations between these trends and those of protein refoldability, a separate measure of how well a protein can independently refold to its native state after complete denaturation. Protein hits in our initial study were enriched with nonrefoldable proteins. Potential confounding factors of our study such as LiP reproducibility and post-translational modifications were assessed and found not to be of concern. Our study overall suggests that neuronal protein structural changes are global in nature and are more often intrinsically nonrefoldable, which may partially explain their susceptibility to structural change due to proteostasis network breakdown in age.

M12. RNA-based strategy of enteroviral genome replication

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Enteroviruses comprise a significant class of pathogens that cause various human diseases ranging from the common cold to poliomyelitis, acute flaccid paralysis, and myocarditis. The enteroviral (+)-strand RNA genome replication, essential for viral proliferation, has been proposed to depend on RNA structures at the genome's extreme 5′ end. Such replication-linked RNAs (REPLRs) recruit essential proteins, the host poly-C binding protein 2 (PCBP2) and viral 3CD protein (precursor of the viral protease 3C and RNA-dependent RNA polymerase D) during genome replication, but the tertiary structures and mechanisms of the enteroviral REPLRs are mainly unknown. Recently, we have determined the crystal structures of CVB3, RVB14, and RVC15 REPLRs, revealing their highly conserved H-type four-way junction folds with co-axially stacked subdomains. The sA helix stacks on the sD helix, the sB helix on the sC helix, and the structure forms a unique long-range A•C•U base-triple between the sC-loop and the sD-helix. These conserved features enabled us to perform the structural prediction of additional enteroviral REPLRs through a homology modeling approach. The structure-guided binding studies with viral 3C revealed its primary binding site being the sD tetra-loop and a dinucleotide bulge. Moreover, the human PCBP2 binding studies revealed two binding sites for this protein – the sB loop and 3′ spacer, which collectively bind a single PCBP2 cooperatively. We also showed that the A•C•U base-triple disruption did not affect the 3C binding but did abrogate the PCBP2 interactions with the REPLR, suggesting a crucial role of this tertiary interaction in positioning the 3C and PCBP2 binding sites within the enteroviral REPLRs. Furthermore, oligonucleotides complementary to the spacer region diminished REPLR-PCBP2 interactions, highlighting the crucial function of this single-stranded segment in recruiting PCBP2. This insight sheds light on the potential for developing therapeutics to combat enteroviral infections by targeting this replication platform.

M13. Covalent-modification of Tyrosine 204 on Tyrosyl-DNA Phosphodiesterase 1 Catalytic Groove Using SuFEx-mediated Access to Quinolone Fluorosulfates

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Covalent modification of proteins has been found to be a very useful technique in many biological and therapeutic settings. Site-selective protein modification by small molecules can provide invaluable insights into mechanisms of biological function. Sulfur (VI) fluoride exchange (SuFEx) has emerged as a new genre of biocompatible “Click Chemistry” reactions. Fluorosulfate-containing agents, which are easily prepared, can react with multiple natural amino acids, in particular, with the sidechain hydroxyls of Tyr residues. SuFEx-derived molecules are currently being widely applied to rationally design covalent ligands that target specific residues in a diverse array of enzymes. Recently, using an X-ray crystallographic fragment screen, we identified quinolone “Hot Spot”-binding moieties within the active site of tyrosyl-DNA phosphodiesterase 1 (TDP1). Quinolones are important pharmacophore structures that have been widely used in antibiotics. X-ray crystal structures of TDP1 in complex with the quinolone fragments reveal that the oxygen and carboxylic acid on the quinolone forms hydrogen bonds with the key catalytic His-Lys-Asn residues (HKN motifs) at the TDP1 catalytic site. Substituents at the 8-position of the quinolones are directed toward the relatively narrow and positively charged DNA-substrate-binding pocket. The evolutionary conserved residues, Tyr204 and Phe259, are located proximately in the catalytic groove. Our current presentation will detail the development of a series of substituted quinolones that have sulfonyl fluoride moieties tethered at the 8-position of the quinolone scaffold. Importantly, the X-ray crystal structures of a subset of these quinolones in complex with TDP1, confirm the formation of sulfonyl adducts to the Tyr204 residue within the catalytic pocket. In certain covalent complexes with TDP1, the tethered aromatic functionality projects toward the key Phe259 residue. The structural information obtained from these crystal structures may be helpful to inform the optimization of these covalent inhibitors to transform them into more potent agents.

M14. GS-441524 is a potent inhibitor of the ADP-ribosyl hydrolase activity of the SARS-CoV-2 macrodomain

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Macrodomains are conserved protein folds that can remove ADP-ribose residues from ADP-ribosylated protein substrates. In coronaviruses and alphaviruses, the macrodomain protein plays a crucial role in viral replication and pathogenesis, making it an attractive target for drug development. Despite its importance, there are currently no cell-permeable inhibitors for this target. Recent studies have shown that GS-441524, a metabolite of remdesivir (a potent antiviral against viral polymerases) binds strongly to the SARS-CoV-2 macrodomain, but its ability to inhibit the hydrolase activity of the viral macrodomain is not known. We used an in-house assay to demonstrate that GS-441524 is a potent inhibitor of the SARS-CoV-2 macrodomain ADP-ribosyl (ADPr) hydrolase activity, with an IC50 of 14.4 µM. In addition, it could also inhibit the Chikungunya virus (IC50: 218 µM; alphavirus) and SARS-CoV (IC50:109 µM; coronavirus) macrodomain, albeit at a higher concentration, indicating the potential for developing broad-spectrum antivirals. Importantly, GS-441524 is highly selective for the viral protein and did not inhibit the phylogenetically closest human homolog, MacroD2. Inside cells, GS-441524 is phosphorylated for bioactivation. Interestingly, the addition of 5' diphosphate to GS-441524 increased its potency against the SARS-CoV-2 macrodomain. Furthermore, we tested the effect of GS-441524 in inhibiting the SARS-CoV-2 macrodomain inside cells using an IFN-γ-based in-cell assay. In this immunofluorescence-based assay, IFN-γ treatment results in increased ADP ribosylation, forming cytoplasmic ADPr punctate structures. Upon overexpression of the SARS-CoV-2 macrodomain in IFN-γ-treated cells, a decrease in ADP-ribosylation occurs, resulting in fewer ADPr puncta. Our findings indicate that GS-441524 can restore ADPr puncta formation in the presence of the SARS-CoV-2 macrodomain, suggesting inhibition of the ADPr-hydrolase activity of the SARS-CoV-2 macrodomain within cells. In summary, our study identifies GS-441524 as a selective inhibitor of the SARS-CoV-2 macrodomain both in vitro and in cells.

M15. Thermotolerance of Thermus thermophilus proteins arises from kinetic stability, not thermodynamic stability, thereby explaining its nonrefoldability

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The folding problem has been tried to be addressed for decades, now. We understand now the forces that, drive the proteins to its native structure and that, the sequence encodes the structure. However, our understanding of proteins with low folding efficiency, is still little. Here, we have studied the unfolding of a protein from a thermophile that is 'non-refoldable', at a physiological time scale. Phosphoglycerate kinase (PGK) from Thermus thermophilus is a 43 kDa protein involved in the glycolytic pathway. From our proteome-wide refolding studies using limited proteolysis followed by mass spectrometry as probe, the protein was found not to refold in a physiologically relevant time scale. This had led us to think that once the protein unfolds, there is a huge barrier encountered by the protein to traverse back to its native state. Here, we determine the thermodynamic and kinetic stabilities associated with unfolding, using far UV-CD spectroscopy as our probe, to understand the 'non-refolding' behaviour of T. thermophilus PGK. We find that the N and U states are indeed separated by an enormous energy barrier (~ 70 kcal/mol, at the optimal growth temperature), whereas the thermodynamic stability, under the identical conditions, was only ~ 4 kcal/mol. In conclusion, it can be said that 'non-refoldability' arises from extreme kinetic stability.

M16. Development of a Bacterial Surface Display System for High Throughput Screening of Bacterial Biofilm Exopolysaccharide Hydrolase Enzymes

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Biofilm formation is characterized by bacterial clusters embedded within a self-assembled extracellular polymeric substance (EPS) matrix. Biofilms pose a formidable challenge in healthcare settings by impeding the host immune response, blocking decontamination efforts, and inhibiting conventional antibiotic treatments. Hydrolysis of biofilm exopolysaccharides by glycoside hydrolase enzymes is an established strategy for biofilm disruption. Hydrolase enzymes targeting distinct biofilm polysaccharides are invaluable tools for understanding the functional roles of exopolysaccharides in biofilm assembly. Dispersin B (DspB) catalyzes the hydrolysis of poly-β-(1→6)-N-acetyl-D-glucosamine (PNAG), a fundamental exopolysaccharide in both Gram-positive and Gram-negative bacteria, has garnered attention as a potential therapeutic agent for biofilm dispersal. Despite promising outcomes, its application is hindered by lower catalytic activity compared to other glycosyl hydrolase enzymes. Our laboratory has explored the improvement of DspB activity by enhancing substrate recognition at sites distal from the catalytic pocket. Hence, to enhance the catalytic activity of the DspB protein by improving substrate binding, requires a high-throughput screening platform for protein engineering. Here, we have developed a bacterial surface display platform that allows us to simultaneously assess the concentration of protein and protein activity in a single assay using intact cells. In this approach, ice nucleation protein (InaP) is used as a surface anchoring motif to enable the display of diverse proteins on the outer membrane of E. coli cells. As well, a eGFP tag enables quantification of the protein concentration. This eliminates the need for cell lysis and subsequent protein purification processes, as the protein activity can be directly monitored using whole cells. Hence, this system presents an ideal platform for efficiently screening a vast library of variants for their activity, while minimizing the need for extensive protein preparation efforts.

M17. Structure of saguaro cactus virus 3′ translational enhancer mimics 5′cap for eIF4E binding

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The genomes of several plant viruses contain RNA structures at their 3′ends called cap-independent translation elements (CITEs) that bind the host protein factors such as mRNA 5′cap-binding protein eIF4E for promoting cap-independent genome translation. However, the structural basis of such 5′cap-binding protein recognition by the uncapped RNA domains remains largely unknown. Here, we have determined the crystal structure of a 3′CITE RNA, panicum mosaic virus-like translation enhancer (PTE), from the saguaro cactus virus (SCV), made possible by a Fab crystallization chaperone. The PTE RNA folds into a three-way junction architecture with a pseudoknot between the purine-rich R domain and pyrimidine-rich Y domain, which organizes the overall RNA structure to protrude out a specific guanine nucleotide, G18, from the R domain that comprises a major interaction site for the eIF4E binding. The superimposable crystal structures of the wild-type, G18A, G18C, and G18U mutants suggest that the PTE scaffold is preorganized with the flipped-out G18 ready to dock into the eIF4E cap-binding pocket. The binding studies with recombinantly expressed wheat and human eIF4Es using gel electrophoresis and isothermal titration calorimetry, and molecular docking computation for the PTE-eIF4E complex formation demonstrated that this SCV PTE structure essentially mimics the mRNA 5′cap for eIF4E binding. Such 5′cap mimicry by the uncapped and structured viral RNA domains highlights how viruses can exploit RNA structures to mimic the host protein-binding partners and bypass the canonical mechanisms for their genome translation, providing opportunities for a better understanding of virus-host interactions and non-canonical translation mechanisms found in many pathogenic RNA viruses.

M18. Biophysical characterization of Mus musculus ATE1-1

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Post-translational modifications are polypeptide alterations that affect protein function, solubility, and activity, among other characteristics. Some post-translational modifications involve the addition and/or removal of an amino acid. One such modification is arginylation, a post-translational modification that uses the enzyme arginyltransferase 1 (ATE1) to catalyze the post-translational addition of the amino acid Arg to a eukaryotic protein. Arginylation has been shown to be essential to eukaryotic cellular homeostasis and overall human health. Currently there are structures for yeast ATE1s, but there are no known structures of higher-order eukaryotic ATE1s. In this work, we have cloned, overproduced, and purified mouse (Mus musculus) ATE1 isoform 1 (ATE1-1) for structural and biophysical studies. Purified mouse ATE1-1 produced high yields of monomeric protein. Crystallization trials of this protein are currently ongoing, and small-angle X-ray scattering (SAXS) was performed. The experimental SAXS data suggest that mouse ATE1-1 may be composed of two separate, ordered domains with a flexible linker, similar to the AlphaFold model. Hydrogen Deuterium Exchange Mass Spectrometry was performed on the protein which corroborates the SAXS data.

M19. Genetic and enzymatic characterization of Amy13E from Cellvibrio japonicus reclassifies it as a cyclodextrinase also capable of α-diglucoside degradation

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Cyclodextrinases are carbohydrate-active enzymes involved in the linearization of circular amylose oligosaccharides. Primarily thought to function as part of starch metabolism, there have been previous reports of bacterial cyclodextrina ses also having additional enzymatic activities on linear malto-oligosaccharides. This substrate class also includes environmentally rare α-diglucosides such as kojibiose (α−1,2), nigerose (α−1,3), and isomaltose (α−1,6), all of which have valuable properties as prebiotics or low-glycemic index sweeteners. Previous genome sequencing of three Cellvibrio japonicus strains adapted to utilize these α-diglucosides identified multiple, but uncharacterized, mutations in each strain. One of the mutations identified was in the amy13E gene, which was annotated to encode a neopullulanase. In this report, we functionally characterized this gene and determined that it in fact encodes a cyclodex trinase with additional activities on α-diglucosides. Deletion analysis of amy13E found that this gene was essential for kojibiose and isomaltose metabolism in C. japonicus. Interestingly, a Δamy13E mutant was not deficient for cyclodextrin or pullulan utilization in C. japonicus; however, heterologous expression of the gene in E. coli was sufficient for cyclodextrin-dependent growth. Biochemical analyses found that CjAmy13E cleaved multiple substrates but preferred cyclodextrins and maltose but had no activity on pullulan. Our characterization of the CjAmy13E cyclodextrinase is useful for refining functional enzyme predictions in related bacteria and for engineering enzymes for biotechnology or biomedical applications.

M20. Optimization of Polarizable Drude Nucleic Acid Force Field using Parameter Reweighing via Machine Learning

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Molecular Dynamics (MD) simulations play an essential role in determining the structural and dynamical properties of biomolecular systems. The underlying force field in MD simulations helps to determine the accuracy of the simulations. The CHARMM classical Drude oscillator polarizable force field has been implemented and developed to more accurately model the electronic response in MD simulations. To improve the MD studies of nucleic acids additional optimization of the force field has been undertaken to more accurately treat the equilibrium between the folded and unfolded states of RNA and DNA hairpins along the canonical DNA and RNA structures and quantum mechanical (QM) data on small model compounds. Trajectories from MD simulations of the RNA and DNA hairpin structures are first generated and utilized as inputs along with user selected order parameters that are clustered and selected to generate a set of reaction coordinates (RC) using machine learning to sample folding/unfolding transitions. These RCs act as the basis of enhanced sampling simulations to characterize the ensembles of conformations associated with folding and unfolding dynamics of the RNA and DNA hairpins. The distribution of conformations are then used to optimize selected dihedral parameters to produce equal populations of folded and unfolded states at the respective melting temperatures along with structural properties of canonical RNA and DNA and potential energy scans from the QM calculations. The final force field is anticipated to be of utility for simulation studies of a range of nucleic acids in different environments, including in the presence of various ions.

M21. Further Optimization and Validation of Classic Drude Polarizable Protein Force Field Targeting the Equilibrium between the Folded and Unfolded States of Intrinsically Disordered Peptides

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This work aims to improve the accuracy of the Drude polarizable protein force field (FF), with respect to treating the equilibrium between the folded and unfolded states of intrinsically disordered peptides (IDP) while maintaining the accuracy of treating globular and transmembrane proteins. To obtain the needed conformational sampling metadynamics will be applied in conjunction with deep learning (DL) to facilitate identification of appropriate order parameters (OP) and reaction coordinates (RC). The Automatic Mutual Information Noise Omission (AMINO) approach is employed to maximize the possibility of selecting optimal OPs, increasing the number of systems for which comprehensive sampling can be achieved. OPs selected by AMINO will then be applied in an iterative strategy that uses variational autoencoders in a DL framework: the Reweighted Autoencoded Variational Bayes for Enhanced Sampling (RAVE) method. RAVE captures essential aspects of the molecular simulation trajectory by combining deep learning iterations with input OPs identified by AMINO to generate an increasingly precise probability distribution in a low-dimensional latent space representing the RCs. Iterative cycles of metadynamics followed by AMINO/RAVE re-evaluation of the RCs are then applied to maximize sampling in simulations performed at the melting temperatures of the respective IDPs from which the equilibrium between the folded and unfolded states is calculated. Parameter optimization, emphasizing the dihedrals and CMAP, targets the balance between the two states for a collection of IDPs for which Tm values are available. Additional target data includes QM data on relevant dipeptides and larger model compounds, along with a range of condensed phase data on additional peptides and proteins.

M22. Synthetic anti-RNA antibody derivatives for RNA visualization in living cells

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Cellular RNA visualization provides critical information about its dynamics, transport, and localization, which is crucial for understanding gene expression and regulatory processes in normal and disease cellular conditions. While antibody derivatives such as Fabs and scFvs against peptides and proteins have revolutionized cellular protein imaging, quantification, and tracking, antibody-based tools and analogous strategies are unavailable for RNA visualization. We have developed the first-of-a-kind synthetic anti-RNA scFv (called sarabody) probes and their GFP fusions and demonstrated their great potential to visualize RNA in living mammalian cells. We showed that our sarabodies could be recombinantly expressed and purified as soluble proteins, which allowed us to characterize their kinetic and steady-state binding affinities using native gel electrophoresis and biolayer interferometry (BLI) measurements. As a proof-of-concept, we also developed the sarabody-GFP modules for multiple sarabodies, characterized their binding with target RNAs and then used one of the modules to visualize a target RNA in live mammalian cells. Our RNA imaging strategy is analogous to the existing MCP-MS2 system; however, our sarabody probes provide robust flexibility to develop target RNA-specific modules to visualize unmodified RNAs in cells, as these probes can be selected from a library through approaches such as phage display selection. While detecting a single-molecule fluorescence by conventional microscopy is complicated, with the advancement of microscopy and brighter fluorophores in the future, sarabody probes are readily amenable to visualize the single unmodified RNAs in living cells. Therefore, we envision that, like the protein visualization strategies, our sarabody approach will allow the tracking of mature RNAs and visualizing and quantifying the co-transcriptional folding and dynamics of nascent RNA, conformational changes, and spatiotemporal dynamics of RNA molecules in living cells.

M23. Structural and biophysical characterization of the prokaryotic ferrous iron transport (Feo) system

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The acquisition of ferrous iron (Fe2+) is essential for colonization and to establish infection in pathogenic bacterial species. Bacteria may employ multiple dedicated high-affinity transport systems specific to the complexation and oxidation states of iron in the environment, however, across the prokaryotic domain, the ferrous iron transport (Feo) system is the most widely conserved system employed for Fe2+ uptake with strong implications is bacterial pathogenesis. The canonical Feo system consists of three proteins: FeoA, FeoB, and FeoC. FeoA and FeoC are both small (ca. 8 kDa), cytoplasmic proteins, while FeoB is a large (ca. 85 kDa) polytopic transmembrane protein with a soluble, N-terminal G-protein domain (NFeoB), that has been shown to possess GTP hydrolysis activity for the transport of Fe2+ across a lipid bilayer into the cytosol. FeoA and FeoC are both known to interact with NFeoB in vivo based on work that examined the Feo system within Vibrio cholerae. Importantly, no study has determined a structure of FeoA, NFeoB, and FeoC complexed together, and no work has functionally characterized the tripartite system using intact FeoB, limiting our understanding of this important system. In this work, I have developed a purification protocol for the expression and purification of each soluble protein/construct to deliver mg quantities of homogeneous proteins of the V. cholerae Feo system (VcFeoA, VcNFeoB, and VcFeoC) individually. Using gel filtration/size-exclusion studies and SDS-PAGE analyses, I have shown that VcFeoA and VcNFeoB interact to form a complex as do VcFeoC and VcNFeoB. Additional biophysical and structural analyses such as isothermal titration calorimetry (ITC) and X-ray crystallography are used to characterize the tripartite complex further. The results from this work will provide the first atomic-level information demonstrating how FeoA and FeoC interact in concert with NFeoB to regulate Feo function, which could be leveraged in the design of novel therapeutics to treat diseases caused by these increasingly resistant pathogens.

M24. Design, Synthesis and Evaluation of N-Substituted Bicyclic Carbamoyl Pyridones (BiCAPs) as Potent Integrase Strand Transfer Inhibitors (INSTIs) Against Cabotegravir-resistant HIV-1 Integrase Mutants

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HIV-1 enzyme integrase (IN) catalyzes the insertion of the double-stranded viral DNA generated by reverse transcriptase into the host genome via two-strand transfer (ST) reactions. Integrase strand transfer inhibitors (INSTIs) block the ST reactions by binding in the active site and chelating the two Mg2+ catalytic cofactors. The recently FDA-approved INSTI cabotegravir (CAB, 2021) is being used in long-duration formulations; however, missed doses can result in the persistence of unacceptably low levels of CAB. Low levels of the drug can select for resistant mutant forms of IN and subsequent virological failure in people living with HIV. Mutations near the IN active site can affect the positioning of the Mg2+ ions, impacting INSTI binding and reducing inhibitory potency. Our goal is to develop compounds that retain the ability to bind tightly to the active sites of resistant mutant forms of IN. We envisioned that the metal-chelating triad of heteroatoms contained within the bicyclic carbamoyl pyridone (BiCAP) of CAB could serve as the basis for simplified analogs. We removed the third ring of CAB and replaced it with simpler N-substituted modifications. This should allow the C—N bond with a greater rotational flexibility, which may allow the metal-chelating triad of compounds to bind to drug-resistant IN mutants with changes in the active site arising from mutations in the IN protein. We designed N-substituted BiCAPs and developed a four-step one-pot synthetic protocol for their synthesis that gave ≥ 99% HPLC purity with only a single purification at the last step. We found that many of these new compounds potently inhibit WT and resistant mutant forms of HIV-1 IN in single-round replication assays without causing measurable cytotoxicity in cultured cells. Among the new BiCAPs, 7 exhibits the best antiviral profile against a panel of CAB-resistant mutants. Compound 7 is more potent than CAB against the clinically important drug-resistant mutants E138K/Q148K (>12-fold) and G140S/Q148R (> 36-fold). Some of the new BiCAP-based compounds show greater than two-fold better efficacies than CAB against the important R263K and E138K/G140C/Q148R mutants. The antiviral data supports our original design hypothesis and provides information that can be used in the continued development of BiCAPs that are broadly effective against drug-resistant variants. Such molecules could potentially be used in long-acting formulations.

M25. Characterization of the metal-binding properties of arginyltransferase 1 (ATE1)

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Arginylation is a critically important eukaryotic post-translational modification that modulates protein stability and regulates protein half-life thereby affecting global cellular homeostasis. Protein arginylation is catalyzed by arginyltransferase 1 (ATE1), an enzyme nearly conserved across the eukaryotic domain that transfers an Arg moiety from an aminoacylated Arg-tRNAArg to an acceptor protein in a non-ribosomal, ATP-independent process. We have recently determined the X-ray crystal structure of Saccharomyces cerevisiae ATE1 (ScATE1), which revealed the presence of a bilobed structure and the GCN5-related N-acetyltransferase fold (GNAT) enzymatic active site. A cryo-EM structure of ScATE1 in complex with uncharged tRNA confirmed these results and revealed that the 3' hydroxyl end of the tRNA molecule buries itself inside this polar GNAT fold. Adjacent to this region in three-dimensional space is the presence of the conserved N-terminal regulatory domain of ATE1 that contains four critical Cys residues. We have shown that this regulatory domain binds an O2-sensitive [Fe-S] cluster that regulates arginylation both in vitro as well as in vivo. However, the cryo-EM structure as well as a homologous ATE1 structure suggests the presence of a Zn2+ ion in this location when ATE1 is purified from recombinant sources. To date, no evidence has shown the relevance of this Zn2+ ion to the function of ATE1, and we hypothesize that the metal-binding domain of ATE1 may be promiscuous. To test this hypothesis, I have performed in vitro metal-binding experiments in which several thiophilic metal ions (Co2+, Zn2+, and Cd2+) were titrated into purified apo ScATE1. The presence of any of these metals generally disrupts the fold of ScATE1, with Cd2+ showing the most notable destabilization. For the protein that remains stable, the presence of Zn2+ or Cd2+ appears to results in a compaction of structure based on changes in size-exclusion elution profiles of metal-bound ScATE1. Enzymatic assays and structural studies are currently underway to understand the effects (if any) that these non-native metals illicit on the function and the structure of ScATE1. The results of these studies will help define the metal-binding properties of ATE1 and will determine how the presence of these metals regulates the structure and the function of this enzyme that is critical to eukaryotic homeostasis.

M26. Development of Chemical Tools for Studying Hydropersulfides (RSSH) and Exploring Their Therapeutic Potential

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Hydrogen sulfide (H2S) is recognized as a key bioactive signaling molecule that regulates a range of physiological processes, including cell differentiation, development, and immune responses. However, emerging evidence suggests that many biological effects attributed to H2S may instead be mediated by other reactive sulfur species (RSS), particularly hydropersulfides (RSSH) and other high-order polysulfur species (RSSnH, RSSnR, HSnH, n > 1). These RSS are widely prevalent in biological systems and possess unique biochemical properties, such as the ability to modify protein cysteine residues and scavenge reactive oxygen species and electrophiles. Nevertheless, the investigation of RSSH species is hindered by their inherent instability. In this presentation, I will introduce new chemical tools designed to investigate the chemistry and biology of RSSH. I will showcase the development of several classes of stable organic compounds capable of controlled RSSH release under physiological conditions: (a) S-Substituted Thioisothioureas (STI) with half-lives ranging from 5 to 18 minutes at pH 7.4 and 37 °C; (b) Alkylamine-Substituted Perthiocarbamates (APT) exhibiting half-lives from 1.4 to 484 minutes; and (c) Alkyl Sulfenyl Thiocarbonates (AST) with half-lives between 28 and 129 minutes. The potential therapeutic applications of these donor molecules will be highlighted, emphasizing the efficacy of RSSH over other reactive sulfur species in reducing myocardial ischemia-reperfusion injury and in effectively mitigating doxorubicin-induced cardiotoxicity, a severe and life-threatening chemotherapy side effect.

M27. Secretory routing determines the maturation of a coronavirus spike vaccine candidate

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The post-translational modifications (PTMs) that accompany the secretion of viral proteins are key modulators of virus-host interactions. Hence, an in-depth understanding of PTMs is crucial for designing effective genetic vaccines. Here, we focus on the SARS-CoV-2 spike protein, which is pivotal in viral assembly, transmission, and immune recognition. Despite its successful application in COVID-19 mRNA vaccines, the fundamental principles governing the PTMs of the spike protein during bidirectional secretory trafficking remain poorly understood. We address this gap by investigating the structure-function of spike protein constructs arrested at different secretory trafficking stages using mass spectrometry, single particle cryo-electron microscopy (cryoEM), molecular dynamics simulations, and binding assays. Our study reveals clusters of N-glycans whose remodeling is intimately linked to spike recycling and secretory routing. Remarkably, these spike constructs, despite differing N-glycans and trafficking routes, exhibit robust binding to neutralizing antibodies. Leveraging these insights, we engineer a novel spike vaccine candidate with enhanced secretion to the cell surface, and minimal interference in the endogenous trafficking pathways, which otherwise cause stress, autoimmunity, and inflammation. A single particle cryoEM analysis of this vaccine candidate reveals the conformational details of key immunogenic epitopes. This research provides a foundation for the development of next-generation genetic vaccines, offering enhanced secretion and immune display for effective viral control.

M28. The Histidine Kinase NahK regulates Tyrosine Metabolism through RsmA in Pseudomonas aeruginosa

Saigoutham Paturu and Elizabeth M. Boon

Pseudomonas aeruginosa is a Gram-negative bacterium that form biofilms, which aids in their pathogenicity and is the causative agent for hospital-acquired infections such as ventilator-associated pneumonia. Our lab studies Nitric Oxide (NO) mediated regulation of biofilms and has shown that the heme protein NosP (Nitric Oxide Sensing Protein) recognizes NO and leads to biofilm dispersal, through inhibition of co-cistronic associated histidine kinase (NahK) in P. aeruginosa. We have also established that a deletion strain of nahK (ΔnahK) leads to an overproduction of pyocyanin, by decreasing the level of the RNA binding protein RsmA and increasing the level of the quorum sensing molecule PQS. I now aim to understand the molecular mechanism behind the increased PQS production in ΔnahK. Studies have shown that phe/tyr induce PQS production, and my preliminary qRT-PCR data shows that the gene phhC (induced by tyr) is upregulated. Also, supplementing M9 glucose minimal media with tyr/phe increases the pyocyanin in ΔnahK and ΔrsmA. These data implicate that NahK influences phe/tyr production through RsmA.

M29. Creating Biomedical Fluorophores Using Click Chemistry: A Small Molecule Development Approach

Amira Anwar, Eva-Maria Rudler, Abisoye Fafioye, Ozlem Dilek, Kinga Ulloa

Disease detection in biological systems remains a critical challenge in healthcare and biomedical research. The development of highly sensitive and specific probes for the early and accurate diagnosis of diseases is a crucial area of investigation. Oxidative stress-induced carbonylation is a very well-known diagnostic biomarker which is reported to associated with the development of cellular damage, cancer, and several age-related disorders. Oxidative stress often induces carbonylation of biomolecules in cellular systems. Detection of carbonyl-containing biomolecules in live cells with novel fluorophores using a click chemistry strategy provides a fast, selective, and stable approach for imaging. To introduce fluorophores into the cellular systems, these fluorophores should have distinct chemical and photophysical characteristics to achieve successful imaging: high stability, less toxicity, fast kinetics, good spectral properties (e.g., large Stokes shifts, reasonable quantum yields, drastic changes on absorption and emission spectra). In this work, we developed small molecule-based fluorophores that have a reactive functional group, which rapidly formed a fluorescent turn-on product upon reacting with the carbonyl groups in biological system—is called bioorthogonal click labeling so that we were able to visualize carbonylation process in various cancer cell lines. Our spectroscopic and confocal microscopy results showed that newly synthesized fluorophores can be labeled successfully in human dermal fibroblasts along with several different cancer cell lines. Use of click chemistry method to monitor carbonylation with a fluorophore has proven itself to be superior in satisfying many criteria (e.g., biocompatibility, selectivity, yield, stability, and so forth); our results will therefore provide a powerful probe technology that can label carbonyl moieties in live cells. We anticipate that these fluorescent probes stand as indispensable instruments, enabling the visualization and quantification of biomolecules and cellular processes, thus contributing significantly to our understanding of disease mechanisms and offering promising prospects for enhancing diagnostics and drug developments.

M30. Influence of Physiologically Relevant Anionic Lipids on the Initiation of Fusion by the Lassa Virus Fusion Domain

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Lassa virus (LASV) is an emerging arenavirus endemic to West Africa that has no FDA-approved vaccines or antivirals despite an increased incidence of direct, human-to-human transmission and high mortality rates. Delivery of LASV's genetic material into the host cell is accomplished via membrane fusion – a process widely accepted to be initiated by the fusion domain (FD). However, the initial interaction and structure of the LASV FD with the target host cell membrane, namely the lysosome, has not been discerned. Here, we sought to investigate the influence of membrane composition on LASV FD-initiated fusion, particularly anionic lipids, which are a key constituent of the lysosomal membrane. Through a FRET-based lipid mixing assay, we witnessed a positive, exponential relationship between FD-initiated fusion and increased anionic lipid concentrations. This was affirmed via ITC where there was a direct correlation between the binding affinity of the LASV FD to the lipid bilayer and increased anionic lipids. Additionally, we found that this functionality of the LASV FD was dependent on the type of anionic lipid present, specifically BMP and not PS or PE. In conclusion, our findings suggest that anionic lipids, particularly BMP, have a specific method of action to modulate LASV FD-initiated fusion, providing critical insights toward the potential development of therapeutic interventions.

M31. Steric-Free Bioorthogonal Profiling of Cellular Acetylation via Fluorine-Displacement Reactions

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Lysine acetylation is one of the most well-studied protein post-translational modifications (PTMs), which plays a critical role in the regulation of various cellular processes. However, traditional immunoaffinity-based methods and previous bioorthogonal chemical tagging approaches are limited by their inherent constraints. In this project, we introduce a novel steric-free strategy utilizing bioorthogonal fluorine-displacement reactions to overcome these limitations in the efficient detection of protein acetylation in cells. Combined with stable isotope labeling by amino acids in cell culture (SILAC), we conducted a quantitative proteomic study of the prostate cancer cell line PC-3 with fluorinated pro-metabolite treatment, globally profiling both known and many previously unreported acetylation substrate proteins. This not only expanded our understanding of the acetylation landscape of prostate cancer cells, but also shed light on the discovery of new regulatory roles of specific acetylation events in biological processes.

M32. Exploring the role of the ycn operon in copper acquisition by Bacillus subtilis

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Copper participates in processes such as electron transport and oxidative respiration, which are essential for bacterial survival, and is also an important cofactor for cuproenzymes (1). However, excess copper is toxic to cells, and can lead to the production of highly reactive radical oxygen species and mismetallation of metalloproteins (2). The Gram-positive bacterium Bacillus subtilis uses the Ycn machinery to facilitate copper acquisition. This system consists of three proteins, all encoded by a single operon: a transcriptional regulator (YcnK) in the cytoplasm, a proposed transmembrane copper importer (YcnJ), and a protein of unknown function (YcnI) in the extracellular milieu (3). We have previously determined the crystal structure of YcnI, revealing a novel copper-binding site and a potential role for YcnI as an extracellular metallochaperone (4). Now, using X-ray crystallography, BCA assays, ICPMS, and ITC, we investigate the copper-binding properties of YcnI and how copper can be transferred between it and YcnJ. We demonstrate that YcnI binds copper preferentially in its Cu(II) state in a 1:1 stoichiometry, and that a conserved tryptophan stabilizes the Cu-binding residues through weak interactions. We also show that Cu transfer can occur between YcnI and the CopC domain of YcnJ. We hypothesize a regulatory role for YcnI in controlling the availability of copper for YcnJ.

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M33. Bacterial cytochrome c biogenesis: elucidation of protein-protein interactions in System I, CcmABCDEFGH

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Cytochromes c are highly conserved proteins which play an important role in various cellular functions such as respiration and photosynthesis via electron transport chains in eukaryotes and prokaryotes. Cytochrome c requires covalent attachment of heme to a CXXCH motif for proper folding and function. This heme attachment process is known as cytochrome c biogenesis. Different cytochromes c get heme attached by one of the three pathways: System I (prokaryotes), II (prokaryotes) and III (eukaryotes). Here we focus on System I which is a complex pathway composed of eight membrane proteins, CcmABCDEFGH. Putative functions have been described in the literature for each System I proteins: CcmABCD form a complex that attaches heme to CcmE. HoloCcmE is released from CcmABCD via ATP hydrolysis driven by CcmAB. HoloCcmE delivers heme to CcmFH for attachment to apocytochrome c. CcmG functions as a thioredoxin to reduce the CXXCH cysteines. While it is known that System I proteins are required for cytochrome c biogenesis, how these proteins interact remains elusive. Two models have been proposed in the literature: A two-step model and maturase supercomplex model. According to the two-step model, there are two protein subcomplexes (Complex I – CcmABCD and Complex II – CcmFH) linked by CcmE as there is no evidence of direct interaction between the two protein complexes. According to supercomplex model, System I functions as a single multi subunit supercomplex known as a Ccm machine. I hypothesize that System I functions in two steps, and to test this the System I pathway was engineered to encode three affinity tags (GST:CcmA, MBP:E, CcmF:His). A well-developed Escherichia coli recombinant expression system will be used for expression and affinity purification of the differentially tagged System I pathways using 3 nonionic detergents n-Dodecyl-beta-D-Maltoside (DDM – preserves membrane protein structure), Triton X-100 (high efficiency for membrane solubilization) and digitonin (preserves supercomplexes) followed by SDS-PAGE and western blot analysis. The objective of this experimental approach is based on stoichiometric copurification of the interacting proteins during affinity purification. Preliminary results using DDM detergent indicate that CcmABCDE and CcmEFGH co-purify and support the two-step model. Future work will include affinity purifications in different detergents, determination of complex stoichiometry and imaging studies to support the preliminary results. These studies will elucidate the model of System I cytochrome c biogenesis pathway protein-protein interactions.

M34. Quantitative Analysis of Solutions and Drug Products Using Water Proton NMR (wNMR)

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Water proton NMR (wNMR) is a non-invasive analytical technique demonstrated its capability to analyze many critical quality attributes of vaccines and other therapeutic drugs and their long-term stability. The drug formulations always consist of inactive ingredients, excipients, cryoprotectants, etc., along with the active pharmaceutical ingredient which contribute to the physical properties such as solubility and viscosity of the overall drug product and prolong the stability of drug products. In this work, we used wNMR to quantify the components of binary mixtures of water with sugars such as glucose, maltose, sucrose, α-cyclodextrins,γ-cyclodextrin, used as excipients in many drug product as well as other solutes. We also quantified the oil and water composition in marketed emulsion drug products such as oral supplement Microlipid® and short-term anesthetic Diprivan®. wNMR was also used to study the stability of Monoferric® which is used for the treatment of iron deficiency anemia in adults.

M35. Metabolic Labeling of Glycoproteins Using Fluorinated Monosaccharides and Bioorthogonal Fluorine Displacement Probes

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Glycans play crucial roles in various biological processes, and their interactions with other biomolecules are essential for sustaining diverse cellular functions. Biochemical tools that study these interactions offer a lens to better understand the biological roles of glycans and provide opportunities for new diagnostic and therapeutic applications. A major tool being implemented to investigate the biochemical interplay of glycans is metabolic glycoengineering (MGE), which enzymatically incorporates carbohydrate-based analogs that insert bioorthogonal handles onto proteins. These handles allow for selective chemical ligation with complementary probes, enabling the visualization and analysis of glycans within their native environment. Crucial to the success of a synthetic metabolite precursor is the ability to effectively hijack cellular enzymatic machinery. Many metabolic analogs have demonstrated great success in MGE, particularly derivatives that bear click handles such as N-Azidoacetylmannosamine (ManNAz) and NAzidoacetylgalactosamine (GalNAz). However, some glucosamine derivatives necessary to probe O-GlcNAcylation have been reported to exhibit poor enzymatic uptake, thus bottlenecking related enzymatic pathways, and necessitating the need for artificial cellular/protein engineering. In consideration of this, we began to explore the MGE potential of monosaccharide analogs that bear a steric-free fluorine handle. Uniquely, the C-F bond structurally mimics the native C-H bond, enabling more efficient metabolic incorporation of the related analogs into enzymatic machinery. Respective proteins tagged with the fluorine handle can then be ligated with designed bioorthogonal fluorine displacement probes as reported in our previous publications (J. Am. Chem. Soc. 2021, 143, 1341–1347 & bioRxiv 2022.09.13.507737). These novel fluorine displacement probes are equipped with a nucleophilic selenol warhead which selectively displaces fluorine and are functionalized with fluorescent or affinity moieties that enable direct visualization or pulldown experiments. From this, O-GlcNAcylated modified proteins have been demonstrated to be successfully imaged in various cancer cell lines. With the development of fluorinated monosaccharide metabolites and novel fluorine displacement probes, we hereby provide a set of powerful tools in MGE that can contribute to a deeper understanding of glycan function in disease and pave the way for novel diagnostics and therapeutics targeting glycan-mediated processes.

M36. Investigating the active site of CcsBA, the bacterial holocytochrome c synthase

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Cytochromes are one of the metalloprotein family that uses heme as a cofactor and cytochrome c is an important member of this protein family operating in diverse electron transport chains, for critical cellular functions like respiration, apoptosis, photosynthesis, and detoxification. The ability of microorganisms to survive and thrive under adverse conditions largely comes from the plasticity of their electron transport chains, in which cytochromes c play a critical role. Cytochrome c is unique among all other cytochromes due to its requirement for covalently attached heme for proper folding and function, a process known as cytochrome c biogenesis. Nearly all living organisms contain cytochromes c where the heme attachment occurs within the cysteine thiol groups of the conserved CXXCH motif in apocytochrome c and the vinyl groups of heme (both of which needs to be in a reduced state to form the thioether bond), where the histidine of this CXXCH motif acts as the axial heme ligand. Three different pathways: System I (prokaryotes), System II (prokaryotes), and System III (eukaryotes), have developed throughout the evolutionary process to accomplish this heme ligation. In this study, we test the hypothesis that System II pathway (composed of two proteins CcsB & CcsA) has conserved heme handling mechanisms. Sequence alignment of CcsBA proteins from different organisms revealed that despite low sequence similarity, key heme handling residues are conserved across CcsBA's. Previous studies have shown that the conserved transmembrane histidines (TM-His1, TM-His2) act as an axial ligand to heme, and periplasmic histidines (P-His1, P-His2) act as an axial ligand to heme bound in the conserved WWD domain. To study the similarities in heme binding mechanisms in CcsBA, a comprehensive structure-function analysis of CcsBA from human pathogens Helicobacter pylori and Campylobacter jejuni was undertaken. Biochemical and holocytochrome c synthase functional assays revealed three heme-interaction residues that are necessary in the three CcsBAs, providing insights into the general mechanisms of heme interaction in cytochrome c biogenesis. The conservation of heme binding in the active site, combined with the fact the CcsBA is essential in some pathogens, suggests that the WWD domain could represent a novel therapeutic target.

M37. Design, Synthesis, and Biological Evaluation of novel Leucyl-tRNA Synthetase Inhibitors with Activity Against Common Pathogenic Bacteria

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The progression of antibiotic resistance necessitates new treatment options to combat the nearly 3 million yearly cases of treatment resistant infections in the United States. Previous work performed by our lab produced a lead compound, 6-[(N-hydroxybenzoylamido)methyl] benzoxaborole, shown to have antibacterial activity against Staphylococcus aureus (S. aureus) and methicillin-resistant S. aureus (MRSA). Here, we will investigate the structure activity relationships (SAR) of this lead compound by creating a library of related compounds and evaluating their antibacterial activity. The mechanism of action for the antifungal activity of tavaborole is the oxaborole tRNA trapping (OBORT) of leucyl-tRNA synthetase (LeuRS) within the pathogen. The compound library will be rationally designed and evaluated by in silico molecular docking to inform our SAR study and synthetic pursuits. Compounds will be synthesized through reasonable synthetic routes. The biological activity of our library will be established using minimum inhibitory concentration (MIC) assays against several bacterial strains and isothermal titration calorimetry (ITC) assays against the isolated LeuRS protein. The information provided by these in silico and in vitro assays will be applied to our SAR study to enable the refinement of our boron-containing compound library, with the intent of identifying potent and selective antibacterial agents.

M38. Glucosomes alter physicochemical properties to adapt to hyperosmotic stress

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Glucose metabolism is one of the nexuses connecting both energy metabolism and building block biosynthesis in living cells. In our previous study, human enzymes in glucose metabolism, such as PFKL and PKM2, are found to be spatially organized into membraneless compartments named glucosomes. However, how glucosomes respond to environmental changes remains to be investigated. We observed that enzymes rapidly change their efficiency of partitioning into glucosomes as NaCl concentration in the media varies. As a result of it, the enzyme composition ratio inside glucosomes is altered. These results suggest that hyperosmotic pressure may affect the metabolic activity of the glucosomes.

M39. Mechanism of PNAG biosynthesis by the Gram-negative PgaCD complex

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Bacterial biofilms can be found in diverse medical environments ranging from dental, prosthetic, and various medical instruments resulting in a prominent issue in modern medicine. Biofilm formation occurs when planktonic bacterial cells adhere to a biotic or abiotic surface, where they transition into a sessile multicellular community encased in a self-produced extracellular polymeric substance (EPS). The architecture of the biofilms is heavily reliant on the components of EPS, which consist of proteins, extracellular DNA, and most notably exopolysaccharides. A common exopolysaccharide found in bacterial biofilms is Poly-β-1,6-N-acetyl-D-glucosamine (PNAG). Although this polysaccharide has been studied extensively, it is still unclear how polymerization is initiated.

Our goal is to identify the initiator substrate for PNAG using the protein complex PgaCD, a PNAG glycosyl transferase found in gram-negative bacteria, as our model system. We were able to express and isolate the enzyme to perform PNAG polymerization in vitro. Subsequent solid state 13C NMR confirmed PNAG presence when compared to literature spectra. Additionally, solid state 31P NMR displayed 2 distinct phosphorus signals which could be evident of phospholipids, but further experiments are required. The goal is to perform additional experiments to confirm whether the signals are from contamination or association of a phosphate compound (such as phospholipids). The polymer was then truncated using acid/enzymatic hydrolysis and will be subjected to ESI-MS studies. In addition to identifying the initiator, we are attempting to visualize the polysaccharides present in the biofilms. As previously mentioned, PNAG is a prominent polysaccharide found in biofilms, however other glycosyl-transferases are also encoded within the genome of the same bacteria. For example, a common lab E. coli strain MG1655 encodes both pgaCD (produces PNAG) and NfrB (unknown poly-ManNAc polysaccharide). It is not known whether this ManNAc polysaccharide is incorporated in the biofilm's EPS. Additional tools are required to possibly identify novel polysaccharide incorporated in bacterial biofilms.

Recently, the Poulin lab has been interested in creating a toolbox comprised of specific inactive polysaccharide hydrolase enzymes coupled with fluorescent proteins to label and visualize polysaccharides in live biofilm models. However, we lack the proper platform to fully utilize these probes. The current method involves growing bacterial biofilms on the air-liquid interface of a glass coverslip. However, all subsequent labelling experiments resulted in the disruption of the biofilms. Thus, an ongoing project within my studies includes designing and creating a microfluidic device to harbor the biofilms for labelling experiments. This device will allow us to introduce the hydrolase-fluorescent conjugates to live biofilms without disturbing the existing biofilm. In addition to labelling live biofilms, advanced confocal microscopy will allow for detailed quantification of these polysaccharides present in the biofilm's EPS. Our hope is to further elucidate the roles of these polysaccharides within biofilms and eventually create or improve upon biofilm disruption strategies.

M40. Design, Syntheses and Comparative QSAR Analyses of Aryl Adamantylamines and Bicyclo[2.2.2]octylamines as Uncompetitive NMDAR Antagonists

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The N-Methyl-D-Aspartate Receptor (NMDAR) is an ionotropic glutamate receptor that is hypothesized to play a critical role in the pathophysiology of many Central Nervous System (CNS) disorders, including neurodegenerative diseases such as Alzheimer's Disease (AD), psychological disorders such as depression, and neuropathy. Overactive NMDARs occur through excessive glutamate binding, leading to cytotoxic effects and neurodegeneration. To address this issue, uncompetitive NMDAR antagonism continues to be a worthwhile pursuit, as demonstrated by FDA approval of three compounds in that class – memantine for the treatment of AD, amantadine for the treatment of Parkinson's Disease (PD), and the recent approval of Ketamine for treatment-resistant major depressive disorder. However, the potencies of the compounds in this class are known to be correlated with undesirable side effects - such as hallucination, dissociation, and elevated levels of aggression. To address the need for a predictive model of NMDAR uncompetitive antagonist binding, a novel series of aryl adamantylamines and arylbicyclo[2.2.2]octylamines were designed to probe the effects of conformational restriction and steric bulk, i.e., a quantitative structure-activity relationships (QSAR) study was designed. To date, 14 target aryl adamantylamines from our proposed series have been synthesized. Characterization of each target compound includes GC/MS, high-resolution MS, NMR, and melting point analysis. Binding affinities for the NMDAR will be determined via competitive radioligand binding. In silico molecular docking is also being explored to study the binding mode of a range of compounds with desirable affinity for the phencyclidine (PCP) site of the NMDAR. These probes will ultimately aid in the generation of a predictive pharmacophore and QSAR model for uncompetitive NMDAR binding.

M41. Live Cell Proximity Labeling of PNAG Interacting Proteins in Staphylococcus epidermidis Biofilms

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The ability of bacteria to form biofilms has posed a challenge for the medical field for many years due to the increased resistance to antibiotics conferred to cells within the biofilm. Biofilms consist of bacterial cells embedded in an extracellular matrix that is composed of exopolysaccharides, proteins, and extracellular DNA, but little is known about how these biomolecules interact or how these interactions contribute to biofilm formation. Understanding the binding interactions between proteins and exopolysaccharides in the biofilm will lead to new potential mechanisms to treat biofilm infections through the disruption of these binding interactions. One of the most common structural exopolysaccharides responsible for the formation of biofilms in both Gram-positive and Gram-negative bacteria is poly-□-(1□6)-N-acetyl-D-glucosamine (PNAG). The aim of this research project is to identify and study proteins present in the biofilm that bind to and interact with PNAG using a live cell proximity labelling approach. The engineered Ascorbate Peroxidase – Dispersin B (APEX2–DspBE184Q) enzyme is recruited for the labeling process and the labeled samples are subjected to mass spectrometry for proteomic studying. Identified protein, elastin binding protein (Ebps) in this case, is subjected to co-expression with green fluorescence protein (GFP) for binding affinity test with S. epidermidis biofilm.

M42. Using Cryo-Electron Microscopy to understand the physiological role of selenoprotein S

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Selenoprotein S (selenos) is a membrane protein involved in cellular stress management and various signaling pathways. It assists protein homeostasis through the endoplasmic reticulum (ER)-associated degradation (ERAD) pathway, a mechanism from the stress response machinery to degrade misfolded proteins and maintain cellular homeostasis. In the ERAD pathway, selenos recognizes the AAA ATPase p97. This p97 ATPase is the main energy source to extract misfolded proteins for degradation. Selenos is a crucial adapter for p97 function under stress conditions. How these proteins associate remains unclear, limiting the understanding of the role of selenos in assisting p97 functions. To investigate this question, we use cryo-electron microscopy (CryoEM) to reconstruct 3D structures of the selenos/p97 complex. The 3D reconstructions of the selenos/p97 complex reveal insights into the assembly mechanism of protein complexes in the ERAD. Unveiling how these proteins associate will depict the molecular principles governing protein degradation in the ER.

M43. Understanding biomolecular behavior with mass photometry

Refeyn Ltd

Biophysical characterization describes a wide variety of scientific problems, whether it is solving a crystal structure, investigating the interaction between two proteins, or simply optimizing a protein purification process. There are many techniques available for each of these problems, but they can be very time and resource consuming or too specialized to be applicable to multiple questions. Refeyn is the pioneer of an exciting new technology known a mass photometry. Mass photometry allows for accurate mass measurements of single molecules in solution in only a few minutes. The masses of these molecules are obtained by measuring the amount of light scattered by the molecules as they bind to a glass surface. This technique enables its users to characterize both proteins and nucleic acids in a label-free environment, in their native state. The ability to measure the mass of single molecules in a bulk sample provides access to subpopulations, which makes it possible to determine complex stoichiometry and oligomeric state. Furthermore, the single molecule counting over a wide mass range that mass photometry provides enables the detection of low abundance species and the characterization of sample heterogeneity. We believe that mass photometry can help accelerate scientific discovery by making sample characterization more straightforward and accessible, and enabling analysis of complexes, assembly, and interactions in new ways.

M44. Developing an MS-based Proteomics Approach to Study the Forms of ADP-ribosylation

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Introduction: ADP-ribosylation involves the addition of one or more ADP-ribose units onto proteins to regulate essential cellular processes like DNA repair. ADP-ribosylation comes in two forms: the monomeric form (MARylation) induces conformational changes to modulate protein function, while the polymeric form (PARylation) acts as a scaffold for the assembly of protein complexes and biomolecular condensates. Balancing the addition and removal of ADP-ribosylation—and maintaining the correct form—is essential for proper cellular regulation. Studying ADPribosylation is challenging because of its dynamic, reversible, and low abundant nature. All previous MS-based proteomics approaches can identify ADP-ribosylation sites but cannot determine the associated ADP-ribosylation form. Our workflow can fill this gap to better understand the functional differences of the ADP-ribosylation forms.

Methods: Proof-of-principle assays and enrichment workflows were performed on synthetic MARylated peptide standards. The location of the MARylated peptide and protein-free PAR chain during the enzymatic assays and preliminary enrichment workflow was confirmed by MALDI-MS analysis. The localization and mass shift of the modification on the MARylated peptide standard after the enrichment workflow was validated LC-MS/MS analysis.

Preliminary Data: Previously published proteomics methods typically involve processing down ADP-ribosylation to a unique mass shift to facilitate site identification. However, these methods cannot distinguish between the forms of ADP-ribosylation because there is no molecular signature to discern sites that were MARylated from those that were PARylated. We are utilizing an enzyme that specifically targets the linkage between the terminal ADP-ribose on modified peptides and the enrichment tag used to pull them down. Notably, a molecular mark, confirmed after in vitro enzymatic assays by MALDI-MS analysis, is left at the site of de-conjugation and has a mass shift distinct from that of ADP-ribosylation alone. By targeting the linkage between the terminal ADP-ribose unit and the enrichment tag, we aim to deconjugate modified peptides with their ADP-ribosylation intact. By adding a third enzyme to the workflow, we hypothesize that this de-conjugation signature can be used as a unique mass tag to distinguish between MARylation and PARylation. Preliminary data of in vitro biochemical assays confirmed that the de-conjugation signature is retained on MARylated but not PARylated substrates after de-PARylation. As a proof-or-principle of applying this triple enzyme workflow to our enrichment protocol, we spiked the synthetic MARylated peptide into H2O2-treated HeLa cell lysate peptides. We were able to recover the internal standard peptide with the de-conjugation signature for a MARylation site that is also localized to the correct residu e. Currently, we are optimizing the workflow to identify endogenous ADP-ribosylation sites and their associated forms in one MS experiment.

Novel Aspect: Our workflow can potentially further characterize the ADP-ribosylome, providing insight into functions of ADP-ribosylation forms in physiological and disease states.

M45. Detection of Ceramides, Sphingomyelins, and Phosphatidylcholines in the Mouse Brain Corpus Callosum Region

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Brain disorders such as Schizophrenia affect females and males differently and are known to be associated with altered lipid levels in the brain. Specifically, the corpus callosum (CC) region of the brain has been reported to be affected in Schizophrenia. However, sex-specific localizations of lipid molecules in the brain are poorly understood. To investigate sex-specific alterations in lipid localizations in the CC, matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI MSI) was employed. MALDI MSI experiments were performed on brain tissue sections obtained from adult female and male mice utilizing 2,5-dihydroxybenzoic acid as a chemical matrix. Data acquisition was performed using a rapifleX MALDI mass spectrometer in positive ion mode. MALDI MSI analysis enabled the detection of a range of ceramides (Cer), sphingomyelins (SM), and phosphatidylcholines (PC) in the CC. Interestingly, Cer(d18:1/20:0) exhibited high abundance in the male CC compared with the female CC. In contrast, HexosylCer(d42:2), SM(d18:1/20:0), SM(d43:2), PC(36:1), and PC(38:6) showed high abundance in the female CC compared with the male CC. Notably, MALDI MSI analysis enabled the visualization of lipid precursors and metabolites, including SM(d18:1/20:0) and Cer(d18:1/20:0). These preliminary findings form a foundation for elucidating the sex-specific lipid localizations in the brain.

M46. Biophysical Characterization of Inhibitor-Bound *Escherichia coli* Metallo-β-Lactamases (MBLs)

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Antibiotic resistance is one of the most serious global threats to treating microbial infections, has been attributed to over 5 million deaths globally, and poses a threat to human, animal, and plant life. Pathogens have developed various ways to become antibiotic resistant, and one specific class of enzymes that confers antibiotic resistance through antibiotic inactivation is metallo-β-lactamases (MBLs). MBLs can cleave lactam rings, a common chemical moiety present in many prescribed antibiotics that is critical to antibiotic function. Thus, there is an important and pressing need to develop and to assess inhibitors of MBLs to preserve antibiotic function. To meet this need, we have expressed and purified to homogeneity three MBLs from *Escherichia coli* (*E. coli*): *Ec*VIM-2, *Ec*IMP-1, and *Ec*CTX-M-1. Interestingly, though *Ec*VIM-2 and *Ec*IMP-1 are known to utilize a di-zinc active site in order to function, the biophysical properties of our purified MBLs suggest mismetallation may occur during *E. coli* overexpression. We are currently determining the presence of metals in our proteins through inductively coupled plasma mass spectrometry (ICP-MS). In collaboration, several putative inhibitors of *Ec*VIM-2, *Ec*IMP-1, and *Ec*CTX-M-1 have been synthesized, and we have attempted crystallization of these MBLs in the presence of the purified inhibitors. In the future, we plan to zinc-load our proteins and to perform isothermal titration calorimetry (ITC) with the synthesized inhibitors to test their affinities, while we also crystallize MBL-inhibitor complexes. Additionally, to test if enzymatic function is preserved with non-native metals, these experiments will be performed in the presence of various divalent metals. Ultimately, this work will shed light on the metal promiscuity of these MBLs and provide structural information on antimicrobial inhibitors that bind MBLs with high affinity, thereby providing novel therapeutics to combat this method of antibiotic resistance.

M47. Decoding Glucosome: Revealing the Function-Dependent Composition of Glucosomes

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Glucosome is a multi-enzyme assembly that regulates glucose flux between glycolysis and building block biosynthesis (i.e., the pentose phosphate pathway and serine biosynthesis) in human cells. Initially, glucosome was discovered to be composed of at least four rate-determining enzymes from glucose metabolism, including phosphofructokinase, fructose bisphosphatase, pyruvate kinase, and phosphoenolpyruvate carboxykinase. Subsequent high-content imaging assays and mathematical modeling approaches have revealed that glucose flux is regulated by glucosomes in an assembly size-dependent manner. However, the current understanding of the glucosome composition does not explain the mechanism behind the size-dependent functions of glucosomes. We hypothesize that the rest of enzymes in glucose metabolism also play an important role in controlling glucose flux through glucosome assemblies in human cells. Here, we have constructed fusion proteins of phosphoglucose isomerase, aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and enolase with a monomeric enhanced green fluorescent protein. Fluorescent live-cell imaging has been then used to determine if the reversible enzymes present any spatial relationship with the rate-determining enzymes in glucosomes. Collectively, we envision that this study would advance our understanding of how glucose metabolism is regulated inside living human cells, thereby leading therapeutic intervention toward the treatment of human metabolic diseases.

M48. Probing alpha-synuclein aggregation and protein interactome with μMap photo proximity labeling

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One million Americans are estimated to live with Parkinson’s disease (PD), with a direct healthcare cost of 14 billion dollars annually. A hallmark of PD is the misfolding and aggregation of αSynuclein (αS) into insoluble Lewy Bodies (LBs). Misaggregation is linked to other neurodegenerative disorders, but not all synucleinopathies are the same. Distinct αS fibril polymorphs have been isolated in PD, Lewy Body Dementia (LBD), and Multiple System Atrophy (MSA). As a result, it is possible that different biological mechanisms underly αS nucleation and aggregation. Furthermore, interactions with other amyloid proteins like tau have been associated with, but not well characterized in different disease contexts. As such, this proposal aims to develop a novel, unbiased proximity-labeling tool for mapping αS interactomes in a site-dependent, disease-relevant manner. Recently, μMap has emerged as a next-generation photo-proximity labeling system. μMap utilizes Iridium photocatalysts to activate diazirine-containing compounds under blue light to form carbenes that non-discriminately insert into neighboring proteins and residues. The short half-life of the carbene intermediate in solution results in a tighter labeling radius and improved spatiotemporal control than either APEX or BioID and can be functionalized with fluorescent moieties or biotin pulldown tags. We will employ this technology to look at site-specific αS interactomes in three chemically distinct regions: the positively charged N-terminus, nonpolar non-amyloid-β component (NAC) region, and negatively charged C-terminus.

M49. Zinc Finger Proteins Persulfidation and Inflammation

Haoju Li, Thibaut Vignane, Andrew T. Stoltzfus, Madison M. Worth, Maureen A. Kane, Milos R. Filipovic, and Sarah L. J. Michel

Zinc finger proteins (ZFs) are ubiquitous in cells and rely on zinc for folding and function. ZFs bind zinc through cysteine and/or histidine residues in a tetrahedral geometry. These proteins play crucial roles in various biological processes, including the regulation of gene expression by directly binding with DNA or RNA, and facilitating protein-protein interactions. Conventionally, zinc's function within ZFs has been considered primarily structural, due to its d(10) electronic configuration. However, recent findings from our lab challenge this perspective. We have discovered that the zinc finger protein tristetraprolin (TTP) can interact with the gaseous signaling molecule hydrogen sulfide (H_2S), leading to a post-translational modification (PTM) known as persulfidation. This modification causes the loss of both zinc and RNA binding activities of TTP. A series of spectroscopic techniques revealed that this reaction relies on the presence of Zn-bound protein and O_2, indicating Zn’s role as a conduit for electron transfer. Considering ZFs represent 3-10% of the human proteome, our observations on TTP raise a broader question: Is persulfidation of ZFs a general PTM? To address this question, we employed a meta-analysis approach to existing persulfidation-specific proteomic data, followed by a targeted chemoselective proteomics study using THP-1 cells. Our research establishes ZF persulfidation as a general PTM and has characterized the types of ZFs that are persulfidated. Our analysis also highlights that ZFs involved in the NFκB inflammation pathway can be persulfidated. To investigate the potential signaling role of persulfidation in this pathway, a THP-1 cell model with the CSE enzyme (a primary source of cellular H_2S production) knocked out was utilized. This presentation will outline our findings and ongoing research into the biological significance of persulfidation in zinc finger proteins.

M50. High-throughput, quantitative targeted lipidomic method for large-scale measurement of lipids in human plasma samples

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Introduction: Lipidomics involves the characterization of the lipid content within a biological system. There are two main mass spectrometry (MS) strategies for lipidomics: untargeted high-resolution MS and targeted low-resolution MS. The selection of which strategy to use is dependent on the research question. Liquid chromatography (LC) is commonly interfaced to mass spectrometry for lipidomics. The two widely used LC-MS/MS separations are reversed-phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC). The primary mode of separation for RPLC is based on the fatty acyl/alkyl chain hydrophobicity. Conversely, HILIC separations are based on head group polarity leading to co-elution of lipid classes. Here, we highlight the advantages of targeted LC-MS/MS lipidomics and offer examples on when RP or HILIC are most appropriate.

Methods: EquiSPLASH and UltimateSPLASH were used as internal standards. Additional lipid standards were used for optimizing chromatography and MS parameters including retention times, collision energy and RF lens using machine learning to predict the endogenous lipid parameters to achieve the highest response. Plasma total lipid extracts were used following MTBE lipid extraction. The LC-MS/MS experiments were performed on an Ultimate 3000 Ultra High-Performance Liquid Chromatograph coupled to a Thermo TSQ Altis Tandem Quadrupole Mass Spectrometer. Chromatographic separation was achieved with ACQUITY Premier CSH C18 (1.7 µm, 2.1 x 100 mm) column within 15 minutes and ACQUITY Amide BEH column (1.7 µm, 2.1 × 100 mm) within 8 minutes. MS/MS detection was achieved using selected reaction monitoring (SRM).

Preliminary Data: SRM library set up. Lipid transitions are selected from a database. The RF lens and collision energy were predicted from machine learning. To maximize the coverage, six methods totaling >1000 lipids were initially screened. The lipid list was filtered based on 1) lipid present in 80% of QC samples, 2) relative standard deviation (RSD) of t_R <5%, and 3) peak area RSD <30%. The final lipid list consisted of 153 lipids in ESI- and 291 lipids in ESI+.

Chromatography: Nonpolar lipids (e.g., cholesteryl ester (CE) and triglycerides (TG)) retained poorly on HILIC. Acidic lipids like phosphatidylserines (PS) and lysophosphatidylserines (LPS) had better peak shapes on C18 separations.

Isotopic correction: To achieve accuracy of quantitation, type II isotopic correction was applied on the HILIC method. One such example is the isotopic overlap of PC 16:0_20:1 and the [M+2] of PC 16:0_20:2. Before the correction, PC 16:0_20:1 had a calculated value of 0.95 nmol/mL and after correction was determined to be 0.44 nmol/mL.

Internal standard (IS) t_R on quantitative values: We explored whether the t_R distribution of the IS affected quantification in C18 and HILIC separations. HILIC separations allow for co-elution of IS with each lipid class. Whereas C18 separations provide varied retention of IS and endogenous lipids. Our data suggested co-elution of the IS with endogenous lipids is optimal but can vary for lipid classes and individual lipids.

NIST standard reference material 1950: Using the one IS per lipid class approach, we quantified endogenous lipids from NIST 1950 plasma and compared with previously reported consensus mean estimate values from literature. Of note, the fatty acid quantification results on HILIC were close to the consensus concentrations, but the C18 values were not. Also, the ceramide quantification results were consistent for both HILIC and C18.

Novel Aspect: A comparative analysis between HILIC and RPLC methods to assess the quantitative advantages for high-throughput, targeted lipidomics.

M51. (Poster Presentation)

Nicholas Pizzi
St. Joseph's University (Philadelphia)

M52. (Poster Presentation)

Magdalena Kaminska
St. Joseph's University (Philadelphia)

M53. Deciphering the structural and chemical mechanism for tRNA wobble base modification by a conserved radical SAM enzyme

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Eukaryotic RNAs are modified co-transcriptionally by the addition of a 5´ 7-methyl-guanisine cap (m7G). This “canonical” m7G cap is responsible for determining the fate and function of most eukaryotic mRNAs by directing nuclear export, translation initiation and preventing degradation by exoribonucleases. Prokaryotic mRNAs [MJ1] do not utilize the canonical m7G cap, and until recently it was thought that these contained only 5´ triphosphate ends. However, a novel class of chemically diverse 5´ cap species have recently been identified both on prokaryotic and eukaryotic RNAs. These novel 5´ caps include endogenous metabolites such as NAD(H), dpCoA, FAD(H), UDP-Glucose and UDP-N-Acetyl-Glucosamine (UDP-GlcNAc) and possibly UDP-N-AcetylMuramicAcid (UDP-MurNAc). While the biological functions and regulatory effects of metabolite caps are largely unknown, it has recently been demonstrated that RNA polymerase incorporates the metabolites as non-canonical initiating nucleotides (NCINs). [MJ2] The ability for organisms to use metabolites to initiate transcription provides a new mechanism by which gene expression can be directly regulated by cellular metabolism. This project aims to elucidate the functions of Glyco capped RNAs, specifically those capped with UDP-GlcNAc and UDP-MurNAc in E. coli. The first critical step toward understanding the functional and molecular impacts of these novel metabolite caps is sequence identification of 5´ UDP-GlcNAc and UDP-MurNAc capped RNAs. Therefore, we have developed a novel sequencing technique called “ClickNAc-Tag seq”, which bioorthogonally tags and identifies Glyco-RNAs by using bacterial engineering, click chemistry and single molecule nanopore sequencing.

M54. (Poster Presentation)

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Poster Abstracts

(Morning Session M1 – M54 & Afternoon Session A1 – A54)

A1. Investigating the Mechanism of Inhibition by Trihydroxybenzaldoximes, Towards Mechanistic Probes of DXPS

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JHMI Pharmacology and Molecular Sciences

Outside of controlled laboratory conditions, bacteria encounter rapidly changing environments which require equally fast responses for survival. In order to achieve this, bacteria utilize a litany of tools to immediately respond to environmental cues. We believe 1-deoxy-D-xylulose-5- phosphate synthase (DXPS) plays an important role in bacterial adaptation. DXPS utilizes thiamine diphosphate (ThDP) as a cofactor to catalyze the decarboxylative condensation of pyruvate and D-glyceraldehyde-3-phosphate (GAP) to form 1-deoxy-D-xylulose-5-phosphate (DXP), a precursor for isoprenoid and B vitamin biosynthesis (1). Unlike other ThDP dependent enzymes which engage in ping-pong kinetics, DXPS engages in a unique preferred order random sequential mechanism (1,2). In this mechanism, pyruvate reacts with ThDP to form a long-lived lactyl-ThDP (LThDP) adduct which is coordinated by an active-site network of residues (2,3). GAP then binds to this E-LThDP complex and induces a conformational change which ostensibly disrupts this network to activate LThDP for decarboxylation (4). Our lab previously produced a series of trihydroxybenzaldoxime inhibitors which are competitive with GAP, but uncompetitive with pyruvate, suggesting oximes bind after E-LThDP complex formation (5). To gain a deeper understanding of the uncompetitive mechanism, our lab is exploring two mechanistic models of inhibition: 1) inhibitor binding stabilizes the LThDP complex to prevent decarboxylation, or 2) inhibitor binding either stabilizes the resulting E-carbanion complex or promotes decarboxylation and unproductive side product formation in a substrate wasting mechanism. Here we present our discovery that these inhibitors are capable of promoting the non-enzymatic decarboxylation of pyruvate to form bicarbonate and acetate in a pH- and oxygen-dependent manner. Understanding the underlying reactivity of oximes is critical for mechanistic studies of this inhibitor class, toward the development of functional probes to investigate the role of DXPS in bacterial adaptation.

1. Brammer et al. Org. Lett. 2009, 11 (20), 4748-4751
2. Bartee et al. Acc. Chem. Res. 2018, 51, 2546-2555
3. Toci et al. Biochemistry. 2024, 63 (5), 671-687
4. Zhou et al. Proc. Natl. Acad. Sci. U. S. A. 2017, 114, 9355−9360
5. Bartee et al. ChemBioChem 2015, 16, 1771-1781

A2. Examining the Compatibility between Phosphopantetheinyl Transferases and Acyl Carrier Proteins

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Humans have repurposed microbial secondary metabolites that confer a competitive advantage to host organisms as antibiotics and anti-cancer agents. Type II polyketides are structurally complex secondary metabolites with particularly strong track records in the clinic. These polyaromatic molecules are produced within the organism by a protein assembly called the polyketide synthase (PKS). Ketosynthases (KSs), chain length factors (CLFs), and acyl-carrier proteins (ACPs) serve as three critical domains of type II PKSs. ACPs transport the malonyl-based building blocks and beta-keto intermediates to enzymatic partners throughout the biosynthetic process. To do this, ACPs are first activated by phosphopantetheinyl transferase (PPTase) by attaching a 4' phosphopantetheinyl (PPant) arm, which acts like a tether between the ACP and its molecular cargo. This modification is what transforms apo-ACP (inactive) to its active state (holo-ACP). In the current work, we explore the ability of two prototypical PPTases (AcpS and Sfp), as well as a newly discovered PPTase (vulcPPTase from Dictyobacter vulcani) to activate a diverse set of nine ACPs from actinomycete and non-actinomycete type II PKSs. Trends on which PPTases could activate which ACPs are currently being analyzed to determine the molecular ground rules for ACP-PPTase compatibility. This work helps us expand our understanding of type II PKS machinery and contributes to a biosynthetic toolbox that can be used to access new molecular diversity.

A3. Leveraging HDX-MS Experiments to Drive Molecular Dynamics Simulations

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Conventional molecular dynamics (cMD) simulations depict the structure and dynamics of biomolecules with atom-level resolution. Due to timescale limitations, forcefield inaccuracies, and crystallographic inherent biases in high-resolution structure determination techniques, however, cMD may inaccurately reflect protein behavior in solution. This discrepancy can beis further complicated by the conformational heterogeneity of the molecule. For example, calcium-free calmodulin retains a compact, well-ordered structure throughout long cMD simulations despite experiments illustrating the C-terminal domain's flexibility in solution. Enhanced sampling methods for molecular dynamics (eMD) bias the system to facilitate exploration of the protein's conformational landscape. Certain eMD approaches incorporate experimental data as a restraint to limit spurious conformations, but this requires a well-defined experimental observable. Hydrogen-deuterium exchange mass spectrometry (HDX-MS) is a solution-based technique that labels protein backbone amide hydrogens across a range of time points. Compared to other biophysical methods, HDX-MS offers unique insights into native protein structure and dynamics. produces a wealth of data about native protein structure and dynamics at fairly high spatial and temporal resolution. Yet, the low spatial resolution of HDX-MS, coupled with a poor understanding of how various structural features influence the rate of H/D exchange, limits its compatibility with these experiment-directed modes of eMD. Here, we develop an HDX-MS-guided adaptive sampling workflow to resemble model solution-based ensembles more closely. Post-equilibration, five unbiased replicas of apo-calmodulin were initiated using the CHARMM36m forcefield. After averaging the predicted HDX rates for every 200 ns trajectory, the simulations were scored by their RMSE to the experimental data, and the two highest-ranking iterations were selected to seed three 100 ns simulations each. This algorithm was repeated until reaching a 3 μs trajectory. Whereas cMD simulations diverged from the in-solution ensemble, HDX-MS-guided adaptive sampling consistently improved. Moreover, adaptive sampling captured the C-terminal domain motion of apo-calmodulin more accurately than cMD, as demonstrated by Rgyr and secondary structure comparisons. As a proof of concept, we demonstrate the ability of HDX-MS-steered adaptive sampling to model the native state ensemble of apo-calmodulin.

A4. Exploring Multimeric Protein Signaling Through a Single-Molecule Study of PKA

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Protein kinases are ubiquitous enzyme complexes consisting of a highly conserved catalytic core and diverse regulatory domains. The diversity of the regulatory domains makes them key targets for drug development. Consequently, it is important to understand how they interact with various binding partners and signaling molecules and how these communication channels are disrupted by mutations. During the activation cycle of the Protein Kinase A (PKA) holoenzyme tetramer, the regulatory subunits adopt distinct conformations depending on whether they are bound to catalytic subunits or to cyclic adenosine monophosphate (cAMP). Single-molecule optical tweezers allow us to selectively probe and manipulate one element in a complex and track how that element responds to binding interactions and mutations through conformational and energetic changes. The full-length regulatory subunit dimer of PKA unfolds in five steps, where the first four correspond to the unfolding of the four cyclic nucleotide binding (CNB) domains (two per subunit). The fifth unfolding event occurs at a much higher force, and its change in extension is consistent with the partial unfolding of the highly stable dimerization and docking (D/D) domain of the regulatory subunits via the breakage of one of its disulfide bonds. Our results indicate a stabilization effect for the cAMP-bound and the catalytic subunit-bound regulatory subunits compared to the apo state. Interestingly, besides the highly stable D/D domain we don't observe a significant interaction between the two subunits in the regulatory subunit dimer, suggesting that any other interaction between them is dynamic and/or very unstable.

A5. A Novel Polar Linker Undergoes Efficient Two-Step Cleavage and Improves the Properties of Antibody Conjugates

Xiaoyi Li

NCI/NIH

Antibody-drug conjugates (ADCs) have become an important class of therapeutics. Although considerable efforts have been made in the areas of antibody engineering and labeling methodology, improving the overall physicochemical properties of the linker/payload combination remains an important challenge. Here we propose an approach to create an intrinsically polar linker domain using a tandem 1,6–1,2 elimination sequence to release secondary amines. Using a fluorogenic, hydrophobic hemicyanine as a model payload component, we show that the zwitterionic linker improves the degree of labeling and reduces antibody aggregation when compared to a conventional linker approach. Cellular and in vivo fluorescence imaging studies demonstrate that the model payload is released in antigen-expressing cells and tumors with high specificity. Broadly, this strategy may provide a general approach to mask the hydrophobicity of payload molecules.

A6. Mechanistic Exploration of Bacterial SOS Response using Site-Specific Dual Encoding and labeling

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Antibacterial resistance is one of the greatest current threats to human health. The urgency of this threat makes it important to understand the adaptive mechanisms that permit the bacteria to evade antibiotic stress. Bacteria respond and adapt to antibiotic stress through different ways (such as by acquiring mutations, transferring resistance genes, and inducing protective states) which gets turned on as part of the bacterial SOS response. This SOS response acts as a switch whose activation is largely controlled by two proteins: the repressor-protease LexA and the DNA damage sensor RecA. In this work, we developed a LexA(Acd/Tet) assay containing a full-length and minimally perturbed LexA with a directly encoded fluorophore/quencher pair (Acridone/Tetrazine) which provides real-time fluorescence readout of LexA auto-proteolysis. This dual construct helps in monitoring the RecA-independent LexA cleavage under different pH conditions and RecA* induced LexA cleavage at different concentrations of wt-RecA protein (acts as a co-protease and accelerates LexA auto-proteolysis) and ss-DNA. Moreover, using additional dual labeled constructs of LexA we were able to capture the function of RecA* in inducing a conformational change to an energetically unfavorable cleavable conformation of the peptide loop containing the cleavage site (Ala84-Gly85). Additionally, we also explored the possibility of using this dual labeled LexA for developing a High-throughput assay to screen for inhibitors of auto-proteolysis. Altogether, this work highlights the potential for leveraging the fluorophore/quencher-based assay in acquiring mechanistic insights within the bacterial SOS response and improving the existing leads for SOS inhibition and subsequently aid in developing novel therapeutics targeting a pathway essential for escape from antibiotics.

A7. Detection of lipid alterations in mouse heart and liver tissues in response to efavirenz treatment using mass spectrometry imaging

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Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor of human immunodeficiency virus and is widely used in antiretroviral therapy. It is known that EFV exerts adverse effects, including cardiotoxicity and hepatotoxicity. Notably, it has been reported that EFV treatments are associated with aberrant lipid levels in plasma. In organs such as the heart and liver, lipids have crucial roles through their involvement in energy storage, signaling, and structural roles. Previous studies have shown that there is an association between the changes in lipid levels and an increased risk of adverse effects in the heart and liver. However, the alterations in the localization of individual lipid molecules in response to EFV treatment in heart and liver tissues are yet to be elucidated. Thus, we employed a matrix-assisted laser desorption/ ionization mass spectrometry imaging (MALDI MSI) approach to determine lipid alterations in murine heart and liver tissues in response to EFV treatment. Additionally, the changes in serum proteins in response to EFV treatment were investigated using mass-spectrometry-based proteomics. From this work, MALDI MSI results revealed distinct spatial localization patterns of phosphatidylcholine (PC), ceramide (Cer), lysophosphatidylcholine (LPC), and sphingomyelin (SM) molecules in both heart and liver tissues. Specifically, hexosylceramide (HexCer) (d42:2) exhibited high abundance in the left ventricle region of the EFV-treated heart tissues as compared to control heart tissues. Moreover, PC(34:1) showed low abundance in the EFV-treated liver tissues compared to control liver tissues. From mass spectrometry-based proteomics analysis, lipid metabolizing enzymes such as hepatic triacylglycerol lipases were detected. Further, proteomics results revealed that 17 mouse serum proteins were significantly altered following EFV treatment. More specifically, heterogenous nuclear ribonucleoprotein, nucleophosmin, and alpha-synuclein exhibited high expression levels in response to EFV treatment. Taken together, the results from this work reveal the localized patterns of alterations in lipid molecules in murine heart and liver tissues following EFV treatment.

A8. Design, synthesis, and biological evaluation of β-L-thymidine, β-L-2’-deoxycytidine, and other L-nucleoside reverse fleximer analogues

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Both β-L-thymidine (LdT) and β-L-2’-deoxycytidine (LdC) have potent anti-HBV activity, with LdT later becoming the pharmaceutical drug Telbivudine. However, like all current anti-HBV drugs, LdT is unable to fully clear HBV from the body and is susceptible to resistance caused by common HBV point mutations. Additionally, co-infections with other viruses is not uncommon, including Ebola and Dengue. Other viruses, such as Epstein-Barr Virus (EBV), have been linked to causing HBV reactivation.

Fleximers are a shape-modified purine nucleoside analogues with a split ring system, endowing the molecule with increased flexibility. They have been shown to have increased, broad-spectrum activity compared to their parent compounds and proven to be able to overcome point mutations. Similarly, reverse fleximers have the connectivity reversed where the glycosidic bond is to the 6-membered ring. By applying this technology to nucleosides like LdT and LdC, there is the potential to impart broad spectrum antiviral activity as well as decrease susceptibility to point mutations.

Two reverse fleximer analogue series were synthesized, with parent compounds LdT and LdC. Additional series are also being worked on, including β-L-ribose and another based on the HBV drug lamivudine. Initial data has shown several LdC analogues with broad-spectrum antiviral activity as well as one LdT analogue with activity against Epstein-Barr virus which has been associated with HBV reactivation. Additionally, some anti-SARS-CoV-2 activity was observed with a β-L-uridine reverse fleximer analogue. Further optimization is currently underway with a preliminary SAR study and exploration of prodrugs.

A9. The Systematic Characterization of Linker-Quencher Molecules for FLIM-based RNA Visualization

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RNAs take many forms in cells and accomplish a similarly large number of cellular tasks, including protein translation. Among the tools that can be used to visualize RNA in living cells is Riboglow-FLIM, a platform we recently established. Riboglow-FLIM utilizes fluorescence lifetime imaging microscopy (FLIM) to detect RNAs of interest using a fluorescent molecule, called the Riboglow probe. The Riboglow probe consists of a fluorophore covalently bound to a quencher molecule, cobalamin (Cbl). When Cbl binds to a short riboswitch-derived RNA sequence, the quenching of the probe is reduced and the change in fluorescence lifetime is detectable. The dequenching efficiency of the probe is an important characteristic of this system and is directly related to the degree of RNA multiplexing that can be accomplished using FLIM. Previous research has demonstrated that changing the chemical properties of the probe linker segment, which connects the fluorophore and Cbl, has a significant effect on the dequenching efficiency of the probe upon binding to RNA. To characterize this relationship for rational probe design efforts, we designed the glycine linker series to systematically vary the length of the linker segment while maintaining linker rigidity and electronic properties. New probes are characterized through fluorescence assays, binding assays, and nuclear Overhauser effect spectroscopy (NOESY) to investigate interactions of the quencher and fluorophore. This work serves to educate future optimization of the Riboglow-FLIM system to illuminate complex RNA dynamics in live cells.

A10. The Role of Lipid Packing on the Fusion Domain for SARS-CoV-2 Fusion

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SARS-CoV-2 and its spike glycoprotein are responsible for the devastation during the COVID-19 pandemic. Using its spike glycoprotein, the virus delivers its genetic information into the targeted cells by membrane fusion in a process initiated by its fusion domain (FD). It is understood that the FD preferentially prompts fusion in an environment resembling the late endosomal membrane due to the presence of the anionic lipid, BMP, and a low pH as well as an environment with decreased lipid packing. How this would translate to the interactions between the protein and organ-relevant lipid membranes would increase insight of viral tropism. First, the WT FD was purified via denaturing conditions, Ni-NTA affinity and finally, by size exclusion chromatography (SEC). Next, using a FRET-based lipid mixing assay, we monitored fusion activity for the WT with different cell line lipid extracts like the brain, heart, and liver to understand the interactions between the proteins and complex lipid compositions which determined the liver cell type with the greatest fusion. Interestingly, the brain lipid compositions displayed less membrane fusion than the liver despite a higher anionic lipid concentration. Then, using C-Laurdan as a fluorescent probe, the lipid packing of the cell lines were monitored, which determined the liver cell type with the least amount of packing and the brain and heart cell types with increased lipid packing. It was shown that the liver lipid composition displayed the most fusion and the lowest amount of lipid picking. Additionally, lowering the pH to the endosomal pH served to increase lipid packing. In conclusion, there is an inverse relationship between lipid packing and membrane fusion mediated by the FD with a preference for decreased lipid packing more than anionic lipid concentration for membrane fusion. This has implications for which systems in the body are more at risk and for greater caution in preventing future infection.

A11. Bacterial cytochrome c biogenesis: heme reduction in CcmFH, the holocytochrome c synthase

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Most life on Earth encodes a protein called cytochrome c that is well-known for its role in cellular energy production via electron transport chains. All cytochromes c require the covalent attachment of heme to a conserved CXXCH motif to properly fold and function. Despite the diversity of cytochromes c, all cytochrome c biogenesis (i.e. covalent heme attachment) is performed by one of three pathways called System I (bacteria), System II (bacteria and chloroplasts) or System III (eukaryotic mitochondria). System I is composed of 8 integral membrane proteins (CcmABCDEFGH) that function in two steps to both transport heme across the bacterial inner membrane and attach it to cytochrome c in the periplasm. CcmF is the holocytochrome c synthase and has been shown to require two hemes for function: a stable b-heme in its transmembrane domain and a heme in the periplasmic active site for attachment to cytochrome c. The active site heme is received by CcmE on the periplasmic side of the membrane. The literature has hypothesized that the b-heme is involved with reduction of heme in the WWD domain, thus facilitating its attachment to apocytochrome c. To test this hypothesis, I will be using Quikchange II sitedirected mutagenesis to mutate two highly conserved Tryptophan residues to Alanine within CcmF. These residues are proposed to be required for reduction of the active site heme. Subsequent work with these constructs will include functional assays for CcmF synthase activity. Future work will include affinity purification and subsequent biochemical analysis such as UV-vis spectroscopy and redox potential. Together, these experiments will investigate whether these residues are crucial for proper synthase function and further elucidate the mechanism of CcmF.

A12. Not Provided (Poster Presentation)

Ailing Li and Peter Nemes

Normal embryonic development requires the establishment of different cell lineages to form tissues and organs. Xenopus laevis, the South African Clawed Frog, is an excellent model to study molecular mechanisms of cell differentiation due to large cell size, reproducible and stereotypical tissue fates, and egg laying all year round. Despite decades of research, we know little about the proteome activity state of single cells during tissue specification when all the critical, early-state cell fate decisions are made to pattern the embryonic body. Our goal here is to characterize the evolution of cell-to-cell proteome heterogeneity during early patterning of the chordate X. laevis embryo as critical tissue lineages emerge with development. Although cells in the early embryo are large for dissection, rapidly shrinking cell size makes this approach difficult to scale to every cell in the embryo. As an alternative, we microfabricated capillaries that we mounted on a microinjection station to collect cell contents from identified cells in live embryos. With ~30 s/cell sampling, this approach provided sufficient throughput and accuracy to systematically aspirate the contents of every cell in the 16-cell embryo. Traditional steps of the bottom-up proteomics were scaled to the resulting limited amounts of proteomes. For deeper proteome detection, we reduced abundant yolk proteins that hinder protein identifications in X. laevis by depleting yolk platelets prior to analysis. Offline high-pH fractionation helped simplify the chemical complexity of the proteome. At present, we are applying this approach to measure the proteome of every cell in the 2-to-16-cell X. laevis embryo. Beside novel approaches, this work will provide previously unprecedented information on the single-cell proteome evolution of tissue heterogeneity during early embryonic development.

A13. Investigation of RNA Binding and H2S Reactivity of ZRANB2, a Zinc Finger Protein Involved in Alternate Splicing

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Zinc Finger (ZF) proteins are a large class of eukaryotic proteins that require zinc as a structural cofactor to fold and function. ZFs contain domains in which cysteine (C) and histidine (H) residues serve as ligands for zinc, and their function is typically gene regulation via DNA or RNA recognition. In some cases, the mechanism for metal mediated DNA/RNA recognition is not well understood. Additionally, there is emerging evidence that H2S can target ZFs and persulfidate cysteine residues. My research is focused on zinc finger ran-binding domain-containing 2 (ZRANB2), a CCCC-type ZF known for its role in RNA binding and alternate splicing. ZRANB2 has been identified in persulfide-specific proteomics screens as being persulfidated. Utilizing single finger peptides and a two-domain expressed construct of ZRANB2, we have demonstrated that the ZF domains readily bind zinc(II) and adopt a folded conformation. To understand the protein's RNA-binding properties, we have also characterized the binding footprints of several RNA targets including a physiological splicing target of ZRANB2, the Tra2β mini-gene exon 3, via FA and HDX-MS. In addition, to decipher if and how persulfidation, a proposed post-translational modification, can affect ZRANB2 and impact its RNA binding abilities, we have employed a variety of spectroscopic techniques. We have obtained evidence for persulfidation of ZRANB2 by H2S and discovered that persulfidation abrogates Tra2β RNA binding.

A14. Generation of Monoclonal Antibodies Specific for Peptidoglycan Fragments

Min Liu, Rachel Putnik, Klare Bersch, Luc Teyton, MG Finn, and Catherine Grimes

Among the 13 trillion bacteria that live on/in the human body, opportunistic bacteria Staphylococcus aureus (Staph) poses a serious threat, resulting in nosocomial infections. With the rise of antibiotic-resistant Staph strains (V/MRSA, i.e., Vancomycin/Methicillin-resistant bacteria), new mechanisms to target these infections are urgently needed. Monoclonal antibodies (mAbs) are promising therapeutics to combat V/MRSAs. Our lab is developing peptidoglycan (PG) probes that can be used to click onto viral-like nanoparticles (VLP) to produce potent adjuvant for generating highly specific and selective mAbs. Initial data suggest that our mAbs exhibit nanomolar binding affinity with PG fragment: muramyl dipeptide (MDP) and show significant potential for treating Staph infection in mice. We aim to generate novel mAbs towards various fragments in our PG library, including mono- to tetra-saccharides containing wall teichoic acid (WTAs), a critical modification that contains 1,6-phosphodiester linkage, which contributes to the pathogenesis of V/MRSAs. We focus on generating 6-phosphorylated PGs as 6-phosphorylated MDP has been shown to activate a NOD2-dependent NF-kB immune response. With these fragments in hand, biological and binding assays can provide insights into the mechanism of how PGs interact with the human’s innate immune system. Further, generating robust mAbs from these fragments can lead to developing therapeutics against V/MRSAs.

A15. Ubiquitin K27M Mutation Demonstrates the Limits of Static Protein Models and the Need for Dynamic Insight in Protein Research

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Ubiquitin (Ub) can form isopeptide bonds between its C-terminal glycine and any of seven lysines of another ubiquitin. Lysine-27 is uniquely positioned deep into the hydrophobic core and is less than 5% solvent accessible. The buried charge is neutralized by the aspartate-52 side chain, creating a salt bridge believed to be vital for ubiquitin stability. The K27M Ub mutant, frequently utilized in structural research and cellular assays, has lower stability than WT Ub. However, the changes in structure and stability imparted by the K27M mutation have not been characterized at the atomic level. Herein, K27M Ub is investigated using solution NMR to better understand the stability, structure and dynamics imparted by residue lysine-27 on WT Ub monomer and how this applies to K27-Ub2 dynamics. Initial K27M Ub Alphafold prediction and subsequent NMR-determined structure indicate high resemblance (<1.0Å and <1.5Å RMSD, respectively) to WT Ub structure. However, titration studies show decreased affinity of K27M Ub for known WT Ub ligands RPN10 and Ubiquillin-1. Further NMR characterization via 15N relaxation and Hydrogen-Deuterium exchange indicate that K27M Ub exhibits increased dynamics compared to WT Ub. NMR Relaxation Dispersion and Chemical Exchange Saturation Transfer (CEST) experiments confirmed that local conformational exchange is occurring on a sub-millisecond timescale. The strongest structural perturbations are found in the β-grasp region of the protein and not the region surrounding the native salt bridge. This finding refines the current narrative regarding salt bridge and hydrophobic interactions influence on WT Ub structure and stability. Despite a similar structure to WT Ub, K27M Ub exhibits modified dynamics that impair physiological function. K27M Ub highlights the limitations of static protein structures and emphasizes the need for dynamic information in protein investigation.

A16. Assessing the Biological Significance of the Anomalous Arrhenius Behavior of Thermolysin with Ionic Strength and Macromolecular Crowding

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The Arrhenius equation models the typically linear relationship between temperature and enzymatic rate; however, a growing number of both eukaryotic and prokaryotic enzymes demonstrate deviations from this model. These deviations manifest as biphasic kinetic temperature dependences, suggesting sudden conformational or dynamic changes in an enzyme at the temperature at which the Arrhenius breakpoint exists. Thermolysin, a thermophilic metalloprotease derived from Bacillus thermoproteolyticus, exhibits one such kinetic temperature dependence consisting of two individually linear phases which meet at a breakpoint of 26 °C. Since this biphasic behavior of thermolysin originates nearly 50 °C lower than the optimal temperature of the enzyme, it was hypothesized that this behavior could be the result of studying thermolysin in dilute buffer solutions which do not adequately mirror native, in vivo conditions. To evaluate the biological significance of its anomalous Arrhenius behavior, thermolysin was kinetically characterized using a full protein substrate under conditions of ionic strength and macromolecular crowding. Elevated concentrations of both of these biophysical factors resulted in the severity of the breakpoint being greatly diminished, with a loss of biphasic character across the breakpoint occurring with macromolecular crowding. The effect of ionic strength and macromolecular crowding on the kinetics and underlying energetics of activation of thermolysin will be presented.

A17. Targeting Protein-Protein Interactions within the MAPK Pathway for Cancer Therapy

Shrhea Banerjee, Alison Yu, Tarah Trebino, and Zhihong Wang

The Mitogen Activated Protein Kinase (MAPK) cascade, comprising RAS, RAF, MEK, and ERK, regulates essential cellular functions like cell proliferation and survival. Notably, mutations in RAF kinase and RAS proteins often hyperactivate this pathway, a common occurrence in various human cancers. We conduct comprehensive biophysical and biochemical characterization of Braftide, a peptide developed in our lab to allosterically target the RAF dimer interface. Our findings suggest that Braftide also holds promise in targeting RAF signaling by disrupting the CDC37-RAF kinase interface, presenting a novel proof-of-concept strategy. This approach offers a route to intervene in the molecular chaperone network involving Heat Shock Protein 90 (HSP90) and its co-chaperone, CDC37, both regulators pivotal in kinase-specific protein folding, stabilization, and activity. In alignment with our research focus, we also study the interaction between RAS and RAF proteins. This interaction triggers significant conformational changes in RAF, however, the specific order and mechanistic details of these interactions remain elusive. Critical to this process are the interplays among RAF regulatory domains (RAS Binding Domain (RBD), Cystine Rich Domain (CRD), Kinase Domain (KD)), governing the interaction with RAS and the consequential conformational shifts that drive RAF activation. Our research aims to elucidate these key details using in vitro biophysical methods such as Open Surface Plasmon Resonance (OpenSPR) and deuterium exchange mass spectrometry (HDX). We study the RAS-RAF interactions by accessing alterations in binding affinities and conformations within the regulatory domains upon exposure to different RAS isoforms (HRAS, KRAS, and NRAS), along with pan-RAS inhibitors currently under clinical development. Collectively, our efforts enhance understanding of the biochemical mechanisms governing RAF activation and regulation. Furthermore, our exploration of innovative approaches for targeting RAF and RAS mutations holds promise for advancing therapeutic interventions in these malignancies.

A18. Design and Synthesis of Linker-expanded Fleximer Nucleoside Analogues

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Nucleoside analogues have been widely used in the development of antiviral and anticancer therapeutics due to their ability to target key biological pathways in the replication cycle of numerous diseases. The analogues of naturally occurring nucleosides are exploited by making changes in the sugar, nucleobase, and phosphate group. The Seley-Radtke group developed novel "fleximers", which are flexible nucleoside analogues characterized by the "split" in the purine ring, connecting the imidazole and pyrimidine ring via a single bond. My project expands on the connectivity between these two rings to potentially change the flexibility, bond distance, and spatial geometry of the nucleobase group by the addition of linker groups such as an alkene, alkyne, and amine. The change in bond distance and sterics within the nucleobase may alter its affinity towards an enzyme's binding site altering its antiviral activity. These expanded fleximers can provide significant information towards drug design on whether the flexibility or rigidity of a nucleoside scaffold can improve the biological activity of the compound.

A19. Structure-Property Relationships of Near-Infrared Fluorphores

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Near-infrared (NIR) fluorophores are highly important for their biological imaging properties. NIR-1 (700-900 nm) and NIR-II (1000-1700 nm) possess decreased photon scattering, tissue autofluorescence, and greater tissue penetration. We have designed and synthesized an array of benzobisthiadiazole (BBTD) and thiadiazoloquinoxaline (TDQ) dyes which are part of the Donor-Acceptor-Donor (D-A-D) class of NIR-I/II fluorophores. The photophysical properties of these dyes are greatly affected by the donors attached to the central core. Electron-dense donor groups are connected to the central electron-deficient core to produce long-wavelength fluorophores. This research focuses on the effects of changing the heteroatom of the typical thiophene ring to oxygen (furan) and nitrogen (pyrrole) along with Ring-locking aniline derivatives to probe rotational impact on fluorescence.

A20. Importance of Proximal Domain and the C-terminal Tail of ISG15 and K48-linked Polyubiquitin for Recognition and Processing by Papain-like Protease from SARS-CoV 2 Virus

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Papain-like protease (PLpro) is essential for coronavirus infections. PLpro also removes posttranslational conjugates of poly-ubiquitin and ISG15 from target proteins. ISG15 has two ubiquitin-like domains connected by a flexible linker; di-ubiquitin consists of two ubiquitins connected via isopeptide bond. Despite these proteins having similar tertiary folds, our previous work revealed different binding modes for K48-linked di-ubiquitin and ISG15. To explore how components of these proteins contribute to PLpro recognition, we engineered ISG15-ubiquitin chimeras, where the proximal or distal domain of ISG15 was substituted with ubiquitin. Our NMR studies revealed that PLpro binds to only the proximal domain for the proximal ubiquitin-containing chimera, while universal perturbation across both domains occurs for the distal ubiquitin chimera. Further exploration revealed that truncation or extension of the C-terminal tail (LRGG) of the proximal domain of either ISG15 or di-ubiquitin impeded PLpro binding. Interestingly, despite the tail extension resulting in weaker binding, PLpro can still remove the extension; these results highlight the importance of the proximal domain for PLpro recognition and processing of substrates. Our results provided structural insight into potential drug designs that can target PLpro.

A21. Synthesis of Dendronized Gold Nanoparticles Bearing Docetaxel and an Antibody Fragment for Targeted Chemotherapy of Metastatic Prostate Cancer

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Prostate cancer is a highly prevalent malignancy in men that results in diminished survivability as the disease progresses, which is especially true at metastatic stages. Treating metastatic prostate cancer involves a combination of radiotherapy, surgery, hormone therapy, and chemotherapy. Docetaxel, an antineoplastic taxane, is a first-line chemotherapy drug used in the treatment of prostate cancer, but it suffers from adverse effects due to promiscuous interactions with cells throughout the body. To alleviate docetaxel's adverse effects, dendronized gold nanoparticles offer a stable drug delivery system that can carry docetaxel to disease sites in a more selective manner. Through synthetic modification, docetaxel can undergo conjugation to dendrons using acid-labile hydrazone bond chemistry, which will induce drug release at acidic pH that are present within the lysosomes of tumor cells. Additionally, using strain promoted azide-alkyne cycloaddition, a 1C1 fragment antibody (Fab) specific for targeting metastatic prostate cancer can be conjugated to dendronized gold nanoparticles to enable active targeting. In this study, the design and synthesis of a dendronized gold nanoparticle bearing both 1C1 Fab and hydrazone-linked docetaxel is explored.

A22. Revealing the roles of conserved aromatic residues in the EcDXPS spoon and fork motifs on intermediate formation and persistence

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The essential bacterial metabolic enzyme 1-deoxy-D-xylulose 5-phospphate (DXPS) is absent from humans making the enzyme an attractive antimicrobial target. The product DXPS sits at a branchpoint leading to the biosynthesis of pyridoxal phosphate (PLP), ThDP, and isoprenoids DXP is formed by the decarboxylation of pyruvate and subsequent carboligation with D-glyceradehyde-3-phosephate thiamin diphosphate (ThDP) dependent manner. Unique among ThDP-dependent enzymes, DXPS undergoes a gated mechanism where pyruvate binds forming a C2α-lactyl-ThDP intermediate which persists on the enzyme until binding of a second substrate such as D-GAP. The LThDP adduct formation coincides with a shift in conformation to the closed form where a flexible loop termed the fork motif dramatically repositions over the active site, bringing the key reside H299 in contact with the newly formed intermediate. However, the mechanism in which the fork motif remains in the closed conformation to allow for H299's presence in the active site of the stable LThDP complex remains unknown. We propose the conserved aromatic residues Y288, F298, and F304 in the spoon and fork motifs function to maintain this form until binding of D-GAP as a trigger molecule. Binding in turn induces a shift to the open conformation resulting in activation of LThDP and decarboxylation. Y288, F298, and F304 were mutated to a nonaromatic residue that maintains similar sterics to elucidate each residue's role in conformational cycling and intermediate formation. As predicted, these DXPS variants showed a shift to the open conformation resulting in a slower rate of of intermediate formation presumably due to the inability to sustain histidine presence in the active site. While the flexible spoon and fork motifs exhibit sequence heterogeneity among DXPS homologs, this work highlights the roles of conserved aromatic residues within these structural motifs in the unique LThDP persistence on the DXPS.

A23. Systematic Expansion of Riboglow-FLIM for Color Multiplexing

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RNA is an essential aspect of biology with subcellular localizations critical for RNA function. Visualization tools by genetically tagging fluorescent RNA reporters to an RNA of interest are needed to investigate RNA localizations and gain insights into associated processes. We have developed a fluorescence lifetime imaging microscopy (FLIM)-based tagging platform, Riboglow-FLIM. Here, a genetically encoded RNA tag binds a fluorescent probe, causing an increase in fluorescence intensity and a substantial increase in fluorescence lifetime. Importantly, the Riboglow platform is derived from a bacterial riboswitch family, resulting in a large series of different RNA tags that may be the basis for developing multiplexing capabilities. We observed fluorescence lifetime differences of Riboglow-FLIM for multiple tags both in vitro and in vivo in mammalian cells, however, in vitro was the focus here. To evaluate multiplexing capabilities, multiple methods of expanding the genetic tag were investigated, primarily phylogenic- and mutation-based. A phylogenic tree of riboswitch-derived tags was constructed, in which tags from the different branches were chosen. Mutations were conducted using previously developed Riboglow-tags. Using different tags yielded a wide range of lifetimes, serving as the basis for visualizing different RNAs simultaneously by FLIM. With the selection of multiple Riboglow-RNA tags, binding affinities will be determined using methods such as isothermal titration calorimetry (ITC), fluorescent induction binding (FIB) assay, and surface plasmon resonance (SPR). The binding of the fluorescent probe is crucial for the unquenching mechanism which allows the probe to fluoresce. Ongoing work is underway to expand the analysis to allow separation of lifetimes close in values. This analysis would be central to expanding multiplexing capabilities.

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A24. Physico-chemical Characterization of the Iron Nanoparticle Drug Monoferric

Alex E. Sestok, Matthew S. Hursey, and Sarah L.J. Michel

Iron is an essential nutrient for nearly every living organism and is required for numerous biological processes such as oxygen transport and DNA biosynthesis. Maintaining a delicate balance of iron stores in the body is crucial as both iron overload and iron deficiency can cause a variety of debilitating conditions, such as iron deficiency anemia (IDA). IDA can lead to impaired cognitive function and immune deficiencies, necessitating drugs to treat such diseases. Iron replacement therapy can be achieved using either oral or intravenous (IV) iron drugs. While oral medications are effective, accessible, and inexpensive, adverse gastrointestinal events can lead to discontinued use. Additionally, oral treatments rely on intestinal absorption, which is impaired in some patients. Therefore, IV iron supplementation is preferred. Though IV iron drugs are typically more costly and can be more time-consuming, they have increased bioavailability. Currently, seven IV iron drugs have been approved with only two generics. The pace of approving generics of IV iron products has been slow because it is not always clear which components of the drug make up the active pharmaceutical ingredient, adding to the complexity of synthesizing new drugs. Therefore, we set out to characterize the physico-chemical properties of a newly approved IV iron drug, Monoferric. Using a combination of UV-Vis spectroscopy, DLS, GPC, XAS, and ICP-MS, we characterized the iron core of Monoferric, it’s in vitro half-life, particle size, and Fe and Na content to better understand which components of the drug make up the API. This information should lend itself to the production of more generic IV iron drugs.

A25. Development and use of fluorescent unnatural amino acids for biophysical studies

Kyle D. Shaffer, Chloe M. Jones, Venkatesh Yarra, Vinayak V. Pagar, Richard B. Cooley, Ryan A. Mehl, and E. James Petersson

Fluorescent unnatural amino acids (Uaa's) can be powerful biophysical tools, as they provide a site specific and minimally perturbing way to monitor protein folding and/or aggregation. The Petersson lab has previously shown that the Uaa acridonylalanine (Acd) can site specifically label proteins and be used as a Förster resonance energy transfer (FRET) partner or a fluorescence polarization (FP) probe. Acd has impressive fluorescent properties such as 0.95 quantum yield (QY) in water, a fluorescence lifetime of 15 nanoseconds, and high photostability. Previously, Acd incorporation was limited to E. coli due to the limited orthogonality of its synthetase. We have demonstrated successful incorporation of Acd into proteins in both HEK293 cells and E. coli via a newly evolved pyrrolysyl tRNA synthetase and demonstrated Acd's utility as a fluorescence lifetime probe. Currently, we are working towards the synthesis of red-shifted amino acids in order to produce a genetically incorporable FRET acceptor for Acd. We have also investigated Acd's effect on in vitro α-synuclein (αS) aggregation/disaggregation dynamics to validate its use for studying the effects of small molecules on αS, and we are working to express Acd-labeled αS in a neuronal cell type in order to carry out these studies in live cells.

A26. Thioamides: Incorporation & Utilization to Study Protein Systems

Denver Y. Francis, Evan Yanagawa, Kristen E. Fiore, Marie Shimogawa, and E. James Petersson

The thioamide moiety, a single-atom substituted molecular isostere of the peptide backbone, offers unique properties ideal for studying peptides and proteins with minimal perturbation. Native Chemical Ligation (NCL) is commonly used to integrate thioamide and other amino acids into proteins, involving fragment syntheses and ligation-desulfurization strategy. Thioamides can effectively probe protein secondary structures, like those implicated in Parkinson's disease (PD), characterized by amyloid fibril formation from alpha-synuclein. We propose leveraging the thioamide's unique properties to disrupt the hydrogen bonding network involved in beta-sheet formation and amyloid aggregation. Additionally, we explore Spycatcher-Spytag technology as an alternative to NCL, utilizing the spontaneous isopeptide bond formation between reactive lysine and aspartate residues. By employing enhanced versions of the technology, Spycatcher003 and Spytag003, we investigate the thioamide's positional effects on protein structure and association kinetics. These efforts not only advance our understanding of PD, but they also offer a streamlined method for generating thioproteins, facilitating future structural and mechanistic studies.

A27. A Pseudomonas aeruginosa biliverdin IXβ-dependent transcriptional regulator (BdrB) integrates extracellular heme metabolism with Type IV pili-dependent motilities.

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Pseudomonas aeruginosa, a multidrug-resistant Gram-negative opportunistic pathogen, displays remarkable adaptability in chronic infections by exploiting heme as its primary iron source at the expense of siderophore uptake. This uptake is facilitated by two distinct heme uptake systems within the pathogen, namely, the Pseudomonas heme uptake (Phu) system and the heme assimilation system (has), each fulfilling specific roles in heme transport and sensing. Within P. aeruginosa, heme undergoes enzymatic cleavage by the heme oxygenase HemO, resulting in the release of iron, carbon monoxide (CO), and extracellular heme metabolites, biliverdin β/δ (BVIX β/δ). Our previous investigations have underscored the pivotal role of BVIX β/δ metabolites as signaling molecules that intricately regulate the Has heme uptake system. Additionally, we have elucidated the unique functions of BVIX β/δ in Type IV pili (TFP) associated motilities including twitching motility and biofilm formation, thereby orchestrating the transition of Pseudomonas from a planktonic to a sessile state commonly observed in chronic infections. Despite these insights, the precise mechanisms underlying these regulatory processes have remained elusive. In this study, through a combination of thermal proteome profiling (TPP) followed by quantitative mass spectrometry (MS), we identified and characterized a novel BVIXβbinding protein termed bdrB (biliverdin-dependent regulator responsive to BVIX), that exhibits significant sequence homology to helix-turn-helix transcriptional regulators. Deletion of the gene displayed a marked reduction in Type IV pili (TFP) associated motilities including twitching. Moreover, similar to a hemOa allelic strain that produces only BVIXa and is deficient in twitching, the DbdrB strain showed reduced pili by TEM and Western blot. This is the first report of a BVIXdependent transcriptional factor in a bacterial pathogen and provides a regulatory link between iron utilization and lifestyle adaptation within the host. By elucidating the molecular mechanisms underlying the action of BdrB, we hope to uncover new therapeutic strategies for combating P. aeruginosa chronic infections, particularly those characterized by multidrug resistance.

A28. Tools to study the role of exopolysaccharide fragments in the production of bacterial biofilm.

Kwaku Obeng, Olivia Vincent, Steven Hong, Myles Poulin

Biofilms are a consortium of bacteria encased in a self-produced, self-sustained matrix called the extracellular polymeric substances (EPS) that is composed of carbohydrates, proteins, and extracellular DNA (eDNA). Biofilm associated bacterial infections account for most bacterial infections approximately 80% of chronic infections, and 60% of all human bacterial infections. Biofilms form as a response to environmental stress and serve to protect the bacteria. Aside from acting as a survival “shell” for bacteria during harsh conditions, bacterial biofilms serve as a major virulence factor and contribute to antibiotic resistance. Formation begins with planktonic cells adhering to each other or to a surface. As cells multiply, cell-cell communication triggers the secretion of EPS components such as exopolysaccharides, proteins, and nucleic acids. These components interact to form a robust matrix, which tends to make bacteria impervious to biocides and antibiotics. Biofilm exopolysaccharides are known structural components of the biofilm EPS of numerous bacterial pathogens, but there is limited information on how they contribute to biofilm production. There is limited evidence that the interaction of biofilm exopolysaccharides with bacterial cells can upregulate biofilm production, but the mechanism of this regulation is not understood. Here, we have developed new probes to explore the role of exopolysaccharides in Escherichia coli biofilm production.

A29. Transformation of venetoclax (VEN) into PROTACs affords recovery of anti-leukemic activity in VEN-resistant AML cells

Goodis C., Chan A., Eberly C., Civin C. Fletcher S.

A standard-of-care treatment for acute myeloid leukemia (AML) is the FDA-approved selective BCL-2 inhibitor Venetoclax (VEN). Contained within the binding groove of BCL-2 are shallow hydrophobic pockets (p1-p4) in which pro-apoptotic protein recognize and bind, preventing cell death from occurring. VEN works by recognizing pockets p2-p4 and replaces the sequestered pro-apoptotic proteins, restoring apoptosis in cancer cells. However, VEN is only effective for up to ~17 months, at which point patients develop resistance mechanisms. One such resistance manifests as drug-induced mutations on the BCL-2 protein that hampers the binding affinity of VEN. G101V is the most common point mutation found in patients located in the p2 pocket of the protein. This mutation is attributed to a ~180-fold decrease in VEN binding affinity. Mutation studies conducted in mice have also identified additional VEN resistant point mutations. Specifically, F104L and F104C mutations contribute to a ~10-fold and ~1500-fold decrease in binding. Crucially, these mutations have little impact on BCL-2 function. The most straightforward solution to this issue requires a decade long pursuit of lead optimization to redesign the original FDA-approved drug that can overcome each point mutation, which might result in multiple drugs having to be synthesized and administered. We propose an alternative approach that will allow us to leverage the original therapeutic VEN by converting it into a Proteolysis-Targeting Chimera (PROTACs). PROTACs are heterobifunctional compounds created by linking a protein of interest (POI) inhibitor to a ligand that recognizes E3 ligases. The interaction between the PROTAC, POI, and E3 ligase ultimately results in polyubiquitination and subsequent degradation of the POI. Advantages of PROTAC to traditional drug design are twofold: (1) PROTACs have shown to be more robust to point mutations as seen with the recovery of ibrutinib activity towards mutated BTK via PROTAC design, and (2) PROTACs are recycled after POI degradation, meaning their activity is catalytic and requires less drug per dose. These factors culminate in PROTACs being an effective treatment strategy to counter upregulation-mediated resistance while also allowing for a diverse use of chemical motifs for safe combination treatment. Specifically, we will modify the solvent-exposed tetrahydropyran of VEN and graft on various linkers that are also anchored to different E3 ligase ligands. PROTACs will be tested against wild-type and G101V BCL-2 proteins at which point, they will also be tested in VEN-sensitive and resistant AML cells.

A30. Design, Synthesis, and Biological Evaluation of Novel, Selective Histone Deacetylase 8 Inhibitors for Treatment of Neuroblastoma

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Neuroblastoma is an adolescent cancer of the sympathetic nervous system. It affects the neuroblast cells primarily of the adrenal gland during development. Despite making up only 7-8% of childhood cancer cases, Neuroblastoma accounts for 15% of childhood cancer deaths. Prior research has suggested that Neuroblastoma develops through an evasion of tumor suppressor proteins such as p53. This evasion likely occurs via an upregulation of histone deacetylase proteins, which regulate gene expression by deacetylating lysine residues on histone proteins. This deacetylation condenses the chromatin and prevents the genes within from being expressed, including p53. Histone deacetylase 8 (HDAC8) has been identified as being overexpressed in Neuroblastoma cells with subsequent knockdown giving evidence towards slower proliferation. Current treatments involving histone deacetylase inhibition have been developed for pan-inhibition leading to 3 FDA-approved inhibitors for various cancer types. The pan-inhibition exhibited by these inhibitors has been shown to induce toxic/off-target side effects (FDA approval of Panobinostat as a multiple myeloma treatment was withdrawn in 2021). With HDAC8 specifically overexpressed in neuroblastoma cells, there is a need to develop inhibitors selective for HDAC8 over the other isoforms. Prior inhibitors have shown selectivity in vitro utilizing a hydroxamic acid functionality to chelate to the Zn2+ ion in the HDAC8 active site and an “L-shaped” overall molecular geometry. However, due to the very strong Zinc (and other metal) chelation by the hydroxamic acid, toxicity has prevented these from progressing into clinic. Keeping these binding pocket features in mind, along with the utilization of computational modeling via Site Identification by Ligand Competitive Saturation, a first generation of selective HDAC8 inhibitors has been developed.

A31. RNA co-transcriptional folding and processing: an extra level of complexity for the regulation of the Pseudomonas aeruginosa Heme Assimilation System

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The opportunistic pathogen Pseudomonas aeruginosa, classified by the WHO as one of the six highly virulent and antibiotic resistant ESKAPE pathogens, is a frequent cause of nosocomial and life-long infections in cystic fibrosis (CF) patients. As most bacterial pathogens, Pseudomonas requires iron for its survival and virulence. To overcome the limitation of this essential nutrient enforced by the host during infection, Pseudomonas has evolved multiple mechanism to acquire iron, such as the synthesis of siderophores or the use of heme. The two heme uptake systems encoded by Pseudomonas aeruginosa (the Pseudomonas heme uptake system (Phu) and the Heme Assimilation System (Has)) serve nonredundant functions during infection, with the Has system involved in extracellular heme sensing during acute infection and the Phu system being as the major heme transport system in chronic infection (1). The expression of the Has system is tightly regulated by both transcriptional and post-transcriptional mechanisms. Previous findings have shown that the genes encoded within the operon are co-transcribed resulting in the polycistronic hasRADEF mRNA. The polycistronic mRNA is processed yielding the individual hasR, hasAp and hasDEF transcripts (2). Furthermore, the extracellular heme metabolites biliverdin IX (BVIX)β and BVIXδ from the catalytic activity of the heme oxygenase HemO, play a key role in the translation of the hemophore HasAp (3). In the present work, we show that the activity of this BVIXdependent regulation relies on the co-transcriptional folding of hasAp and the subsequent processing of the polycistronic hasRADEF transcript. Utilizing an RNAse E mutant (rneΔ50bp) (4) we further show the contribution of this specific RNAse in the BVIX-dependent regulation of HasAp. Finally, based on these results we propose a model of regulation for HasAp translation, where the co-transcriptional folding as well as the processing of the hasRADEF mRNA drives the formation of a novel BVIX-dependent riboswitch. This complex post-transcriptional regulatory mechanism allows Pseudomonas aeruginosa to rapidly respond to fluctuating extracellular heme levels as a function of heme metabolism and iron release.

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A32. Fletcher Lab: Medicinal Chemistry the Discovery of Novel Oncotherapeutics

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Cancer cells' ability to rapidly proliferate and evade apoptosis results from overexpressed proteins and dysregulated protein-protein interactions. We will present a compendium of our laboratory's current strategies towards the discovery of novel oncotherapeutics. Traditional small molecule therapeutics often succumb to drug-resistance mutations. Two methods to overcome this limitation are covalent drug design and targeted protein degradation. Covalent drug design grafts an electrophilic warhead onto an inhibitor which allows it to irreversibly bind to an accessible nucleophilic residue, most often a cysteine. Targeted protein degradation with the use of proteolysis-targeting chimeras (PROTACs) connects a ligand that binds a protein of interest and an E3 ligase ligand together by a synthetic linker. The BCL-2 family of proteins consists of pro-apoptotic and anti-apoptotic proteins that are sequestered to one another in healthy cells. In cancer cells anti-apoptotic proteins within the BCL-2 family, such as BFL-1 and BCL-2 itself, are upregulated. Building off validated non-selective BCL-2 inhibitors, we are generating BFL-1 selective inhibitors by grafting on electrophilic warheads predicted by site identification by ligand competitive saturation (SILCS) to bind Cys55. Furthermore, we will synthesize Bcl-2 PROTACs by grafting on various linkers and E3 ligase ligands to venetoclax, a Bcl-2 selective inhibitor that is prone to resistant mutations. Histone deacetylases (HDACs) are involved in gene regulation and their overexpression in cancer leads to the silencing of tumor suppressor genes. Pan-HDAC inhibitors in the clinic are limited due to toxic effects, therefore selective inhibition of specific HDAC enzymes is being investigated. We will optimize validated selective HDAC6 and HDAC8 inhibitors using electrophilic warheads and chelating groups.

A33. Pseudomonas aeruginosa heme metabolites biliverdin IXβ and IXδ are integral to lifestyle adaptations associated with chronic infection

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Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen requires iron for its survival and virulence. Due to the scarcity of iron during infection, Pseudomonas has evolved several strategies to overcome this limitation including two non-redundant heme acquisition systems, the Pseudomonas heme utilization (Phu), the major heme transport system, and the hemophore-dependent heme assimilation system (Has), required for sensing extracellular heme. PhuS, a cytoplasmic heme chaperone to the iron-regulated heme oxygenase (HemO) controls the flux of heme into the cell, where it is degraded to biliverdin IXβ (BVIXβ) and BVIXδ with the release of iron and CO. Our laboratory has previously shown that BVIXβ and/or BVIXδ acts as a positive post-transcriptional regulator of the extracellular hemophore HasAp. To further understand the role of these unique BVIXβ and BVIXδ metabolites in cell signaling and regulation we performed comparative phenotypic as well as comparative proteomic analysis of the Pseudomonas aeruginosa PAO1 strain and two allelic strains either lacking HemO enzymatic activity (hemOin), or one with a re-engineered HemO that oxidatively cleaves heme to produce BVIXα (hemOα). Our results showed significant heme dependent growth defects and quorum sensing (QS) dysregulation in the hemOin and hemOα allelic strains when compared to the wild type PAO1. Additionally, the hemO allelic strains were compromised in both swarming and twitching and showed reduced biofilm mass. Interestingly, these defects were partially recovered when exogenous BVIXβ/δ was supplemented. Furthermore, a comparative proteomics analysis of PAO1 versus hemOin and hemOα allelic strains in static conditions showed significant repression of chemosensory pathways including Type IV pili, several chemoreceptors, and secreted virulence factors. Additionally, increases in several phosphodiesterase's associated with biofilm dispersal are consistent with a defect in the switch from a motile to sessile lifestyle. Taken together we propose BVIXβ and/or BVIXδ function as signaling or chemotactic molecules linking the switch to heme utilization as an iron source with cooperative behaviors associated with adaptation to a biofilm lifestyle.

A34. C–H/O and N–H/N Interactions within Protein Structure and Conformational Dynamics

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University of Delaware

Proline residues are ideal models for studying interactions within proteins that would otherwise be difficult to isolate when in competition with hydrogen bonding. This is due to proline's lack of an amide hydrogen. Without a hydrogen bonding source of structure stabilization, two noncovalent interactions become significant driving forces for complex assembly at proline residues in proteins, N–H/N and C–H/O interactions. Synthetic proline derivatives were identified due to previously reported 4S-proline preferences for the endo and the δ conformations. With a C-terminal amide hydrogen donor, these derivatives should prefer conformations which may facilitate N–H/N interactions. Several 4-hydroxyproline derivatives with i+1 amides were synthesized and crystallized for X-ray analysis. In the small molecule crystal structure of Boc-(2S,4S)-(4- iodophenyl)-4-hydroxyproline cyclohexyl amide, an i/i+1 N–H/N interaction was observed with a H•••N distance of 2.31 Å and nitrogen pyramidalization of -12°, indicative of lone pair localization about the amide N. This N–H/N interaction stabilizes the δ conformation in this residue [$(\phi, \psi) \approx (-80^{\circ}, 0^{\circ})$]. This conformation is found in β-turns and provides the appropriate molecular geometry required. Computational studies further demonstrated the energetic favorability of the N–H/N interaction, as structures containing this interaction were energy minima among the conformations tested. These studies have shown that the δ conformation is promoted by N–H/N interactions within peptides. The absence of backbone hydrogen bond interaction competition at proline residues also promotes the formation of C–H/O interactions as a means of structural stabilization. The polarized nature of the C–H bonds on proline's pyrrolidine ring introduce a second stabilizing force, forming noncovalent interactions with electron-rich groups within proteins. To investigate the role of C–H/O interactions in stabilizing proline assembly 27 crystal structures from the group were analyzed. Subsequent DFT calculation allowed for hydrogen optimization of these models to provide a more accurate representation of these interactions within the crystals. Each of these 27 structures were found to contain at least one significant C–H/O interaction within their assembly. The closest H•••O distance was 2.27 Å and typical distances were ~2.5 Å with most frequent interactions occurring at Cδ . This hypothesis was further examined with a search of the Cambridge Crystallographic Database. This search identified 2,238 proline-containing structures exhibiting at least one C–H/O interaction with a distance below the sum of the van der Waals radii for hydrogen and oxygen. This search identified C–H/O interactions in protein structures, short peptides, and in single residue crystal structures, demonstrating the widespread occurrence of these interactions beyond the derivatives synthesized in this study. These investigations highlight the ubiquity of C– H/O interactions and their significance in driving assembly.

A35. Looped translation for the secretory production of long repetitive peptide materials

Qi Xie, Stephen D. Fried

Synthetic circular RNAs, generated from in vitro reactions using the permuted intron-exon splicing strategy or RNA ligases, have recently become a promising platform in the context of RNA therapeutics, with broad potential applications. Here we sought to optimize a circular RNA system for efficient production and further secretion of repetitive protein-based biomaterials from microbial biofactories. Using the permuted group I self-splicing intron, we have previously improved loopable translation with minimal scar sequence from the internal guide sequences. In parallel, we have developed an orthogonal signal peptide/peptidase secretion apparatus that can efficiently secrete silk peptides materials from Bacillus subtilis and autonomously process them into materials. To enable the synthesis, secretion, and assembly of long repetitive peptide chains into biomaterials, we have combined these two systems and removed the stop codons so that the ribosomes are enforced to iteratively translate the coding sequence many times into a single repetitive peptide chain. The construct for mRNA circularization and biomaterial production has been characterized with a wide range of in vitro and in vivo assays that will be described. In summary, this work lays the foundation for the autonomous assembly of protein-based biomaterials from bacteria.

A36. Developing Saccharomyces cerevisiae RAS G19C for Modeling Human KRAS G12C Cancers

Katrina William, Samuel Barnett, Idrees Chaudry, Mentor: Joseph C. Sparenberg

Cancer is characterized by uncontrolled cell growth due to mutations and is often caught in later stages. One such mutation in humans is in the oncogene KRAS. The protein translated from KRAS regulates cell growth, and mutations are implicated in cancer. GTP is the substrate for the KRAS protein, which activates the protein leading to cell replication. When GTP is converted to GDP, KRAS is inactivated. Mutations in KRAS can prevent GTP to GDP conversion leading to unregulated proliferation. We are using Saccharomyces cerevisiae (baker's yeast) cells, due to their similarities to human cells (30-40% genetically identical) and transferable methods. We propose a method to model a commonly studied mutation, the glycine to cysteine substitution at the 12th amino acid in KRAS-based cancer (KRASG12C) in humans using S. cerevisiae by modifying RAS1 (an analog to KRAS). Modifications of the RAS1 gene leading to a mutation in the subsequent protein may disrupt signaling by restricting the RAS1 protein from being deactivated, leading to rapid cell proliferation (analogous to KRAS tumorigenesis). Electrophoresis results indicated a custom plasmid is required. The RAS1 and MS2 encoding regions within the custom plasmid can lead to direct transfection and visualization with a complementary tagging plasmid. Our model could visualize a live cell to observe pathways and mimic tumor growth in real-time. Using S. cerevisiae cells as a carcinogenesis model may elucidate inhibitors to potentially create an on/off switch for replication related to the KRAS G12C mutation.

A37. Uncovering the Mechanism of Aggregation of a PKA RIβ Mutant Linked to Dementia

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Protein Kinase A (PKA) is a crucial enzyme in neurons involved in learning, memory and synaptic plasticity. PKA RIβ functions as PKA's regulatory subunit within neurons. PKA RIβ is composed of a dimerization and docking (D/D) domain found at the N terminus, followed by an inhibitory sequence, and then two cyclic-nucleotide binding domains which bind cAMP to activate the enzyme. The D/D domain functions as the site at which PKA RIβ subunits dimerize with one another. Recently, a mutation in the D/D domain of PKA RIβ resulting in an Arg residue replacing a Leu residue (RIβ L50R) was identified as the causative mutation in patients displaying a neurodegenerative disorder. This mutation was found to trigger an aggregation cascade within neurons, however, the mechanism by which the L50R mutation induces aggregation is unknown. Further, while protein aggregation is a pathological hallmark of numerous well known neurodegenerative disorders, such as Parkinson's and Alzheimer's, the exact underlying mechanisms by which protein aggregation occurs, in general, is still poorly understood. We have revealed the L50R mutation destabilizes the helical structure of the D/D domain seen in the wild-type protein, prevents stable dimers from forming and promotes both aggregation as well as monomerization. We hypothesize this loss of helical structure and subsequent lack of dimerization exposes two candidate sites that induce aggregation. One of these sites is a hydrophobic pocket buried within the dimerization interface of the wild-type dimer and the other are Cys residues (C18 and C39) involved in disulfide bonds between subunits in the wild-type dimer. Interestingly, textbook helical propensities suggest Leu and Arg are equally likely to occur in α-helices which highlights the importance of considering neighboring residues when determining whether introduction of a specific residue will impact helicity.

A38. Synthesis of Azoreductase-Triggered Hydropersulfide Precursors

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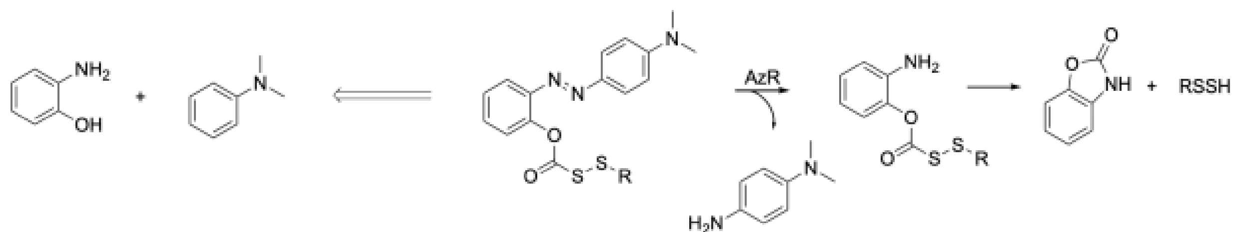

Hydropersulfides (RSSH) are unique chemical compounds found in nature usually in the form of Cys-SSH or Glutathione-SSH in proteins and have biological utility as species that can interact with toxic reactive oxygen species (ROS). Hydropersulfides work like natural antioxidants by scavenging and subsequently neutralizing them by stopping the propagation of radical species (1). Recently, they have been found as an effective treatment for ROS-caused oxidative stress conditions like myocardial ischemia-reperfusion injury (2) and inhibiting ferroptosis by preventing lipid peroxidation due to ROS (3). Furthermore, the persulfidation of cysteine residues, Cys-SH, in proteins to Cys-SSH can serve as a protective, reversible protein modification. These reversible modifications can prevent the irreversible oxidation or electrophilic damage of cysteine residues and proteins as a whole (4). Previous work in the Toscano lab has shown success in developing pH-sensitive and esterase-sensitive hydropersulfide precursors that release RSSH (5). We are exploring an Azoreductase-triggered release that reduces azo (N=N) bonds, converting the azo bond to two terminal amines. To form the azo-precursor, we have been exploring diazonium coupling and a Baeyer-Mills reaction, but the presence of the phenolic OH on our starting molecule has proven to be difficult. As such, we have utilized protecting groups, specifically benzyl ether, and have been successful in our synthesis. As such we have been exploring different protecting groups starting with o-aminophenol including benzyl ether (Bn-OR), trimethyl silyl ether (TMS-OR), t-butyldiphenylsilyl ether (TBDPS-OR), and tosyl (Ts) protecting groups. We have been successful with the synthesis of benzyl-protected o-aminophenol. Currently, we are working on the deprotection of the benzyl group, investigating other protecting groups like tosyl, and looking into using other starting materials, like o-nitroaniline. According to literature, diazonium coupling has never been successfully completed when first forming a phenol-containing diazonium salt. Therefore, future work is needed to investigate an efficient synthesis to access azoreductase-triggered hydropersulfide donors for potential prodrug development.

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A39. Glycosite-Specific Antibody Conjugations: Design and Development for Antibody-Drug Conjugates (ADCs)

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Antibody-drug conjugates (ADCs) are one of the fastest-growing anticancer therapies. Site-specific homogenous ADCs are considered superior to their heterogenous counterparts obtained from random conjugation from the aspect of stability, pharmacokinetics, and safety profiles. In this study, we have developed a glycoside-specific antibody functionalization in a one-pot, one-enzyme manner and demonstrated its application in the preparation of homogeneous ADCs.

A40. Modulating the size distribution of glucosomes to understand their functional contributions to cellular redox homeostasis

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Glucosomes are liquid-liquid phase separated condensates formed by phosphofructokinase liver type (PFKL) and other rate-determining enzymes of glycolysis and gluconeogenesis in human cells. Spatially, glucosomes are assembled into three different sizes at subcellular levels (i.e., small-, medium-, and large-sized glucosomes). Functionally, they are active in regulating glucose flux between energy metabolism and building block biosynthesis in a size-specific manner. However, the heterogeneous distribution of different glucosome sizes in both single-cell and population levels complicates our efforts to mechanistically investigate their size specific functional contribution in live cells. In this work, we are aiming to understand the functional association of medium-sized glucosomes with the pentose phosphate pathway in response to redox perturbation. To date, we have observed population-wide upregulation of medium-sized glucosomes with the treatment of hydrogen peroxide (H_2O_2) and oxidized glutathione (GSSG), respectively. In addition, using an optogenetic glucosome that is fused with a light-dependent oligomerizing domain, *Arabidopsis thaliana* cryptochrome 2 (Cry2), we are capable of reversibly modulating the formation of medium-sized glucosome with and without light in living cells. Taking together, we have developed strategies to modulate medium-sized glucosomes in human cells, which will allow us to gain mechanistic insights of the functional role of medium-sized glucosomes in the glucose metabolism.

Keywords: Glucosome, glucose metabolism, redox homeostasis, optogenetics, pentose phosphate pathway

A41. Synthesis and Evaluation of a Series of Ribose and 2'-Deoxy-2'-Fluoro-2'-Methyl Ribose Fleximers

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For over two decades, the Seley-Radtke group has developed flexible nucleos(t)ide analogues, known as fleximers, and explored their potential as antiviral therapeutics. The 2020-2023 SARS-CoV-2 pandemic has highlighted the necessity of such compounds as well as the dearth of novel antiviral therapeutics ready to be tested against emerging and re-emerging infectious diseases. Herein, we report the synthesis and evaluation of a series of fleximer nucleos(t)ide analogues based off of the ribose and 2'deoxy-2'fluoro-2'-methyl ribose sugar scaffolds with various modifications to the purine nucleobase moiety. In vitro testing of these compounds has been performed in numerous viruses from various viral families of pandemic concern, including flaviviruses, filoviruses, coronaviruses, bunyaviruses, and more with select data being shown below. Moderate activity has been shown in some of these analogues which highlights the need for further research and development through a structure activity relationship study (SAR) to find various combinations of functional group modifications will produce a more potent analogue. The activity shown also highlights the necessity of prodrug modifications like the McGuigan ProTide.

A42. Maca-derived lipid nanoparticle traps pro-inflammatory cytokines to attenuate inflammation and improve sepsis survival

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Plant-derived nanoparticles (PDNPs) are recently discovered nano-sized particles that may be isolated from various edible plants, encompassing root plants such as curcumin and ginger, as well as fruits like grapefruit, grape, and lemon. These natural sources contain bioactive components involved in regulating cellular immune responses against pathogenic intrusion and inflammation. The present study describes the isolation, characterization, and development of a novel PDNPs that possesses potent anti-inflammatory characteristics. PDNPs were isolated by density gradient ultracentrifugation. Lipidomic analysis revealed triacylglycerols and phytoceramides as major constituents comprising PDNPs. *In vitro* studies showed that PDNPs were non-toxic and effectively sequestered pro-inflammatory cytokines to reduce NF-κB activity. In a therapeutic lipopolysaccharide (LPS)-induced endotoxemia mouse model, PDNP-treated mice experienced an accelerated recovery, as indicated by a significant reduction in systemic pro-inflammatory cytokines, improved histological index, and increased survival. In summary, this research presents PDNPs as a novel anti-inflammatory platform with potent pro-inflammatory cytokine sequestration abilities that holds promise for broadly treating inflammatory diseases.

A43. Chemoenzymatic Synthesis of Site-Selectively Fluorinated N-Glycans and Fluorinated Antibodies for Probing Enzymatic Substrate Specificity and Fc Receptor Recognition

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Glycosylation, particularly conserved N-linked at residue N297, plays a crucial role in modulating the effector functions of various antibody types. There has been a significant increase in the development of methods used for synthesizing antibodies with homogeneous glycoforms, known as antibody glycoengineering, and they have subsequently been employed for detailed structure-activity relationship on interactions between IgG N-glycans and FcγR receptors. Fluorination is one of the most common modification strategies used in the study of glycans functionality. In this study, fluorinated ManGlcNAc and Man3GlcNAc oxazolines with varied number and location of fluorine were chemically synthesized and evaluated as substrates for Endo-S2 WT or Endo-S2 D184M catalyzed transglycosylation. We found that complementarily using Endo-S2 WT and Endo-S2 D184M could efficiently transfer the synthetic fluorinated oxazolines to form homogeneous antibodies. ELISA binding analysis indicated that C2-fluorination of internal mannose enhanced the affinity between the antibody and the receptor, while C2 or C6-fluorination of the terminal mannose has the opposite effect on affinity. Using fluorinated N-glycans as fluorine source, we report for the first time the use of 19F NMR to investigate the interaction between antibody Fc and FcγR receptors. We believe that this technique will serve as a powerful tool for further research on antibody effector functions and various protein-protein interactions.

A44. Comparative Analysis of Drug-like EP300/CREBBP Acetyltransferase Inhibitors

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The human acetyltransferase paralogs EP300 and CREBBP are master regulators of lysine acetylation whose activity has been implicated in various cancers. In the half-decade since the first drug-like inhibitors of these proteins were reported, three unique molecular scaffolds have taken precedent: an indane spiro-oxazolidinedione (A-485), a spiro-hydantoin (iP300w), and an aminopyridine (CPI-1612). Despite increasing use of these molecules to study lysine acetylation, the dearth of data regarding their relative biochemical and biological potencies makes their application as chemical probes a challenge. To address this gap, here we present a comparative study of drug-like EP300/CREBBP acetyltransferase inhibitors. First, we determine the biochemical and biological potencies of A-485, iP300w, and CPI-1612, highlighting the increased potency of the latter two compounds at physiological acetyl-CoA concentrations. Cellular evaluation shows that inhibition of histone acetylation and cell growth closely aligns with the biochemical potencies of these molecules, consistent with an on-target mechanism. Finally, we demonstrate the utility of comparative pharmacology by using it to investigate the hypothesis that increased CoA synthesis caused by knockout of PANK4 can competitively antagonize the binding of EP300/CREBBP inhibitors and demonstrate proof-of-concept photorelease of a potent inhibitor molecule. Overall, our study demonstrates how knowledge of relative inhibitor potency can guide the study of EP300/CREBBP-dependent mechanisms and suggests new approaches to target delivery, thus broadening the therapeutic window of these preclinical epigenetic drug candidates. This sets the stage for future applications in proximity-based therapeutics and targeted protein degradation.

A45. Developing RNA Modifications to Enable Neo-Antigen Discovery

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Synthetic messenger RNA (mRNA) is an emerging therapeutic platform with important applications in oncology and infectious disease. Effective mRNA medicines must be translated by the ribosome but not trigger a strong immune response. To expand the medicinal chemistry toolbox for these agents, we have investigated the properties of the naturally occurring nucleobase N4-acetylcytidine (ac4C) in synthetic mRNAs. Replacement of cytidine with ac4C diminishes inflammatory gene expression in immune cells. Synthetic mRNA can also be used as a platform for HLA-based neoantigen discovery and screening without relying on MHC binding algorithms. We are investigating the effects of RNA modifications, such as homogenous incorporation of ac4C and N1-methyl pseudouridine, on the production of neoantigens capable of stimulating the T-cell response. Our studies hope to improve the general use of RNA modifications as a component of next-generation neo-antigen discovery platforms.

A46. Targeting human oncogene PKCι in various cancers through PROTAC targeting degradation

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The protein kinase C (PKC) family, a subsect of serine/threonine kinases, were thought to be involved in tumor promotion, however in a landmark study by Antal and Colleagues, they found a majority of mutations in novel and conventional PKCs are LOF, indicating PKCs are tumor suppressors. Studies found that PKCi, an atypical isoform, was an amplified driver kinase in many cancers with amplifications of its gene PRKCI in lung squamous cell carcinoma (SCC) (36%), ovarian serous carcinoma (23%), esophageal adenocarcinoma (19%), and head and neck SCC (14%). Phenotypes often implicated in cancer have been shown to be regulated by PKCi, including tumor growth, cellular development, and cell survival. PKCi has also been shown to have a regulatory role in tumorigenesis via protein-protein interactions with MEK5, zip/p62, and par6. Therefore, a PKCi degrader targeting both the catalytically dependent and independent functions of PKCi could serve as a potential novel therapeutic against cancers of unmet need. We aim to develop PROTACs that target PKCi, characterize the downstream effects of PKCi inhibition and degradation, and understand the efficacy of PROTACs in in vivo studies.

A47. Chemoenzymatic synthesis and application of 15N site-specifically labeled nucleotides

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While nuclear magnetic resonance (NMR) spectroscopy is a powerful tool capable of atomic level structural determination of RNAs, it suffers from line broadening and signal overlap as the molecular weight of the RNA increases. However, given that 15N has intrinsically sharp linewidths due to its low gyromagnetic ratio, we have developed some chemoenzymatic routes to site specifically label RNAs with 15N. This takes away the need to use selective pulses when restricted to uniformly labeled 15N RNAs. Furthermore, these site-specific labels are particularly useful when different positional 15N isotopes have very similar chemical shift windows they resonate in. In addition, this chemoenzymatic approach affords us the ability to further sharpen the 15N linewidths by removing some dipole-dipole relaxation factors. Taken together, we showcase the versatility of our labeling schemes in structural assignment, dynamics measurements, and detection of binding to cognate ligands. We demonstrate the application of these 15N site-specific labels on a variety of nucleic acids, ranging from 9 kDa to 44 kDa in size. Finally, we propose the amenability of our 15N site-specifically labeled nucleotides for segmental labeling using newly proposed SegModTex strategy (1).

(1) R. Haslecker, V. V. Pham, D. Glänzer, C. Kreutz, T.K. Dayie, V.M. D’Souza, Extending the toolbox for RNA biology with SegModTeX: a polymerase-driven method for site-specific and segmental labeling of RNA, Nat Commun 14 (2023).

A48. Development and Evaluation of Novel Targeted Near-infrared Imaging Agent for Neuroendocrine Tumors

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Targeted fluorescence guided surgery has emerged as an advantageous tool for physicians by enabling precise and real-time visualization of tumors. However, the application of the technique is limited by the absence of FDA-approved imaging agents specifically targeting commonly occurring neuroendocrine tumors (NETs), a limitation that underscores the need for innovative solutions. In response to this, our study introduces a novel near-infrared (NIR) targeted imaging agent for NETs, designed to specifically bind to the frequently overexpressed Somatostatin Receptor 2 (SSTR2) in NETs, thereby offering unprecedented specificity and diagnostic capabilities. Recognizing the challenge of off-target binding, multiple NIR fluorophores were evaluated, resulting in the selection of a fluorophore that displayed significantly minimized non-specific interactions. The targeted imaging agent was synthesized through a comprehensive multi-step process, utilizing a blend of traditional and optimized synthetic methodologies. Its targeting efficacy was validated in vitro using various cancer cell lines, with fluorescence intensity and specificity assessed through fluorescence microscopy and flow cytometry. Results demonstrated the imaging agent's notable specificity and affinity for SSTR2 in cancer models. This development not only paves the way for more precise surgical interventions but also holds the potential of significantly improving NET patient outcomes.

A49. Investigating the aggregation kinetics of calcium-binding protein sorcin by stopped-flow

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Sorcin is a soluble resistance-related calcium-binding protein and is involved in multidrug resistance in cancer and neurodegenerative diseases. It functions as a sensor and a modulator to regulate cellular calcium homeostasis by interacting with calcium channels in a calcium-dependent manner. In the absence of interacting partners such as under in vitro conditions, sorcin aggregates through its exposed hydrophobic surface induced by calcium binding. Here we established sorcin as a model system to study protein aggregation. The second timescale fast kinetics of calcium binding induced sorcin aggregation requires the utilization of a stopped-flow apparatus. Our results show that at the micromolar concentration region, the aggregation kinetics can be described by a double exponential function. The relation between aggregation half-time and the initial sorcin concentration follows a power-law function with a scaling exponent of -3. We show that the aggregation kinetics including the lag phase, half-time, and plateau can be further tuned by calcium concentration, salt concentration, pH, divalent ion competition, and point mutations. The calcium-induced sorcin aggregation is completely reversible by removing calcium with the chelating agent EGTA, indicating a unique amorphous aggregation system that does not involve protein misfolding. In summary, the model system we established here paved the way for the mechanistic investigation of aggregation kinetics using NMR and cryoEM at atomic resolution in the future.

A50. A Heme-Dependent Conformational Switch in PhuS in Pseudomonas Aeruginosa Dictates Function from Heme Chaperone to Transcriptional Regulator

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Pseudomonas aeruginosa (*Pa*) is a pathogenic bacterium that requires iron for survival and virulence. Within the host heme is the preferred source of iron particularly in chronic infection where Pa switches to utilize heme at the expense of iron-siderophores. Heme can enter the cell through one of two non-redundant heme uptake systems, the heme assimilation system (Has) and the *Pseudomonas* heme uptake (Phu) system. Once in the cytoplasm, heme is bound by PhuS, which selectively transfers heme to heme oxygenase (HemO) for metabolism and release of iron and the biliverdin (BVIX)β and BVIXδ metabolites. The transfer of heme to HemO is thought to occur via a conformational rearrangement following coordination of the axial ligand, H209, to the iron in heme which allows for a protein-protein interaction. This protein-protein interaction is thought to trigger a His-switch from H209 to H212 which positions the heme towards the binding interface and facilitates transfer to HemO. Additionally, we have previously shown that in its apo-form PhuS also binds to the promoter upstream of the *prrF1, F2* operon regulating the expression of the read through transcript PrrH in a heme dependent manner. Previous studies using hydrogen deuterium exchange mass spectrometry (HDX-MS) also showed significant differences in the conformational states of apo- and holo-PhuS, particularly following heme coordination which provided a stabilizing effect to the C-terminal helices. This provides significant insight into the mutual exclusivity of the apo- and holo- forms and their respective functions. Herein we utilize biophysical and bioanalytical approaches to show the PhuS H209A variant (where heme is coordinated to H212) exhibits altered kinetics and conformational rearrangement following coordination of heme, as well as a loss in mutual exclusivity of heme and DNA binding. *In vivo* this translated to a de-coupling of heme transfer to HemO resulting in an overall increase in all BVIX metabolites, including α-BVIX which is produced by the other heme oxygenase in *Pa*, BphO. Data will be presented to offer explanations for the loss of mutual exclusivity over heme and DNA binding, and show the *in vitro* observations are consistent with an increase BVIX metabolites and a loss of heme dependent regulation over PrrH in the *phuSH209A* allelic strain.

A51. Activatable SWIR Imaging Probes to Tract the In Vivo Fate of Protein Therapeutics

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Altered pH homeostasis is a hallmark of cancer. The ability to visualize changes in pH in complex organisms could enable new fluorescence-guided surgical strategies that will allow precise tumor margin delineation, facilitating thorough resection while sparing healthy tissue. Such approaches might also help track the fate and activation of targeted-drug delivery strategies, such as antibody drug conjugates. To realize this goal, we hypothesize that responsive, protein-targeted probes in the short-wave infrared (SWIR) region (1000 nm to 1400 nm) would be enabling technology. SWIR imaging enables improved resolution and tissue penetration, though existing probes are unsuitable for our goals. We designed Benz-NorCy7-pH, a cyanine with absorption maxima at 980 nm and an emission maxima of 1000 nm, which shows remarkable pH sensitivity. When conjugated with Panitumumab (an anti-EGFR antibody), Benz-NorCy7-pH revealed an excellent tumor-to-background ratio of approximately 23-fold. SWIR imaging also allows for the multiplexing abilities where two different dyes can be associated with two distinct biochemical processes. A mixture of two mAb probe dyes: FNIR-766 (785 nm excitation) representing antibody internalization and Benz-NorCy7-pH (890 nm excitation) as lysosomal degradation can be used to calculate their respective half-life. In addition, Benz-NorCy7-pH can compare activity of protease cleavable linker like Val-Cit with noncleavable linkers. Conjugation of Benz-NorCy7-pH with albumin lead to rapid tumor accumulation, but also rapid clearance, providing insight in the biological fate of this common drug delivery vehicle. Overall, these novel probes provide a new strategy to visualize the fate and local molecular environment of biomolecules.

A52. (Poster Presentation)

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