21st edition of the
Pacific Coast Protease Workshop

Palm Canyon Resort
Borrego Springs, California

April 27th – 29th 2025

Organized by Matt Bogyo, Olivier Julien, Anthony O'Donoghue, Antoine Dufour and Kasia Groborz

ABOUT THE WORKSHOP

This workshop is designed to provide a stimulating expert environment for the discussion of new information on proteolytic enzymes, their substrates, their inhibitors, and their function.

The objective is to provide trainees (junior faculty, post-docs, and graduate students) with experience in presenting their work to an expert audience, chairing scientific sessions, and to foster collaborations. Expert external faculty will give guest presentations and stimulate discussions.

The work presented here is state-of-the-art and much is yet to be published. Consequently, professional use of the data and ideas learned at the workshop is prohibited without the expressed consent of the respective presenters and their supervisors.

There will be awards at the end of the workshop, including the coveted "Half Moon" for the best presentation.

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PRESENTATIONS AND ABSTRACTS

Trainee talks are 15 min (12 min + 3 min discussion).

Guest faculty talks are 30 min (25 min + 5 min discussion).

Speaker abstracts are listed in order of presentation.

Sunday, April 27th

3:00 pm	Matt Bogyo, Olivier Julien, Anthony O'Donoghue, Antoine Dufour	**Welcome!**	Location: Conference Room

Chairs: Kolden van Baar and Althea Hansel

Time	Speaker	Title	Lab
3:20 pm	Amy Weeks	New enzyme chemistry to decipher post-translational modification function	Outside Expert (University of Wisconsin Madison)
3:50 pm	Alyssa Carter	Utilizing Oxadiazolone-Based Probes to Define the Role of FabH in C. difficile Sporulation and Germination	Bogyo
4:05 pm	Kasia Groborz	Grasping life from the throes of death: Lazarus effect of tailored caspase inhibitors	Unaffiliated
4:20 pm	Taylor Bader	Defining the Role of the Protease HTRA1 on Spinal Deformities Using Proteomic Profiling of Intervertebral Disc Components	Dufour
4:35 pm	Isabella Ruud	Engineered BoNT/E protease variants cleave neurodegenerative disease targets	O'Donoghue/ Chavez
4:50 pm		***10 min Discussion***	
5:00 pm	Coffee break	Location: Conference Room	

Chairs: Rachell Martinez-Ramirez and Shiyu Chen

Time	Speaker	Title	Lab
5:20 pm	Amit Bhavsar	Cutting through the noise: uncovering the role of matrix metalloproteinases in cisplatin-induced hearing loss	Outside Expert (University of Alberta)
5:50 pm	Tulsi Upadhyay	Covalent-fragment screening identifies selective inhibitors of multiple Staphylococcus aureus serine hydrolases important for virulence and biofilm formation	Bogyo
6:05 pm	Alessandra Riccio	Unraveling the catalytic machinery of Bfp1: a structure-based computational study	Forli
6:20 pm	Jiapeng Li	Targeting Viral Protease with Optimized Activity and Bioavailability: AVI-4773, A Main Protease (Mpro) Inhibitor with Broad-Spectrum Activity Against Multiple Coronaviruses and High Drug Exposure in the Lung and Brain	Craik
6:35 pm	Jehad Almaliti	Development of Macrocyclic Peptide-Based Proteasome Inhibitors with Enhanced Blood-Brain Barrier Penetration for Treating Brain Neoplasms	O'Donoghue/ Gerwick
6:50 pm	Isabella Orchard	Thrombin fuels pathogenic behavior of gut microbiota biofilms in Crohn's disease: a proteomic/N-terminomic approach	Dufour
7:30 pm	Dinner	Big Horn Burgers & Shakes (Palm Canyon Hotel & RV Resort)	

Monday, April 28th

Chairs: Alyssa Carter and Duno Dantis

Time	Speaker	Title	Lab
8:30 am	Breakfast	Location: Conference Room	
9:00 am	John Widen	Working in Biotech from an Early Career Perspective	Outside Expert (Tenvie Therapeutics)
9:30 am	Cody Loy	Immunoproteasome-Mediated Release of a Monomethyl Auristatin E Prodrug	Trader
9:45 am	Eric Jordahl	Characterizing the role of the rhomboid protease RHBDL4 in pancreatic cancer	Neal
10:00 am	Kolden van Baar	The inflammatory caspases and their substrates	Julien
10:15 am		***15 min Discussion***	
10:30 am	Coffee break	Location: Conference Room	

Chairs: Isabella Orchard and Jiapeng Li

Time	Speaker	Title	Lab
11:00 am	Shiyu Chen	Sequential AND-gate Fluorescently Quenched Activity-based Probe for Selective Imaging of Cysteine Cathepsin Activity	Bogyo
11:15 pm	Jainilkumar Patel	Identifying caspase-8 and caspase-10 substrates using N-terminomics	Julien
11:30 am	Shih-Po Su	Development of a caspace1-activated SWIR fluorescent probe for tumor detection and therapeutic monitoring	Bogyo
11:45 am	Kyle Lesack	PCPSV: Profiling Caenorhabditis elegans Proteases under Structural Variation	Dufour
12:00 pm	Lunch	Location: Conference Room (salads & sandwiches)	
1:00 pm	Borrego Springs	Hike (Slot Canyon Trail)	
7:00 pm	Dinner	Pablito's Mexican Bar & Grill	
8:00 pm		Trivia Night	

Tuesday, April 29th

Chairs: Tulsi Upadhyay and Cody Loy

Time	Speaker	Title	Lab
8:30 am	Breakfast	Location: Conference Room	
9:00 am	Stefano Forli	Cosolvent Molecular Dynamics for discovery and characterization of protein binding sites	Outside Expert (Scripps)
9:30 am	Jeyun Jo	Oxadiazolone-based Probes for Selective Detection of Implant Biofilms in Chronic Staphylococcus aureus Infections	Bogyo
9:45 am	Althea Hansel	Ab initio computational modeling of covalent macrocyclic FphB serine hydrolase inhibitors	Forli
10:00 am	Justin Zabos	Proteomic Study of H1N1 Influenza Infection in Mammalian Cells	Julien
10:15 am	Vic Hempstead	Covalent PSMA-based probe library for targeted prostate cancer therapeutics	Bogyo
10:30 am	Coffee break	Location: Conference Room	

Chairs: Jainilkumar Patel and Eric Jordahl

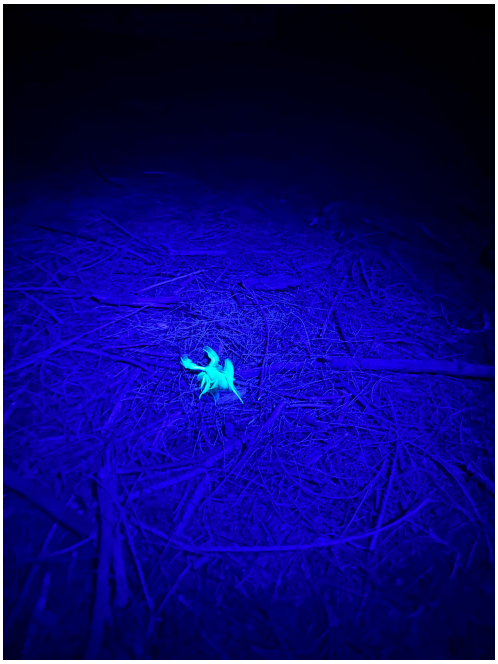
Time	Speaker	Title	Lab
11:00 am	Jiyun Zhu	Design, Synthesis and Characterization of Chemi-luminescent Probes for non-invasive Diagnostic Imaging	Bogyo
11:15 am	Diego Trujillo	Evaluating the 20S proteasome of the early-branching eukaryote Giardia lamblia as a valuable drug target	O'Donoghue
11:30 pm	Kristyna Blazkova	Identification of microbial proteases that regulate Protease-activated receptor 2 to control barrier function, pain and inflammation in the gut	Bogyo
11:45 pm		***15 min Discussion***	
12:00 pm	Lunch	Location: Conference Room (sandwiches & salad)	

Chairs: Kristyna Blazkova and Jehad Almaliti

Time	Speaker	Title	Lab
1:00 pm	Jim Janetka	Aspartic proteases as novel therapeutic targets in parasitic worms	Outside Expert (Washington University)
1:30 pm	Rachell Martinez-Ramirez	Allosteric Role of Heparin on Human β-Tryptase Structure and Activity	Lazarus
1:45 pm	Duno Dantis	Development of Immunoproteasome Substrate Labeling Assays (iSLAy)	Trader
2:00 pm		***15 min coffee break***	
2:15 pm	Ifeanyichukwu Eke	Phenotypic screening of covalent fragment libraries for growth inhibitors of Staphylococcus aureus	Bogyo
2:30 pm	Kyle Anderson	Structure-guided Identification of Serine Protease Inhibitors from Biased Fab Phage-display Libraries	Craik
2:45 pm	Xilin Gu	Structure-Based Design of Inhibitors of the Mycobacterium tuberculosis 20S Proteasome Suppress Persistence of the Bacterium in Infected Macrophages	Sello
3:00 pm	Laney Flanagan	Investigation of cysteine proteases in Bacteroides cellulosilyticus for desensitization of Protease-activated receptor 2	Bogyo
3:15 pm		***15 min Discussion***	
		Free time	
7:00 pm	Dinner	Carlee's Bar & Grill	
	Awards	Palm Canyon Hotel & RV Resort	

Wednesday, April 30th

9:00 am		Departure



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Utilizing Oxadiazolone-Based Probes to Define the Role of FabH in *C. difficile* Sporulation and Germination

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Clostridioides difficile (*C. difficile*) is a gram-positive, spore-forming bacterium that releases toxins, damages the gut epithelium, and induces diarrheal disease upon the depletion of commensal gut microbiota after use of broad-spectrum antibiotics. Once formed, *C. difficile* spores are metabolically dormant and resist antibiotic treatment, which allows *C. difficile* to persist in the gut and creates an urgent need for new therapeutics. When incubated with *C. difficile*, oxadiazolone-based covalent probes inhibit vegetative growth as well as spore formation and subsequent outgrowth. Chemoproteomic analysis with the oxaziazolone-probe reveals that ß-ketoacyl-ACP synthase III (FabH), the enzyme that catalyzes the first step in the bacterial fatty acid synthesis pathway, is enriched by these probes (**Fig 1**). While many bacteria can subvert the use of FabH by trafficking exogenous lipids, *C. difficile* utilizes the FapR regulatory system, which does not allow for complete subversion of the pathway, suggesting *C. difficile* FabH (CdFabH) is a promising target for a therapeutic that selectively disrupts *C. difficile* spore formation and outgrowth while leaving commensal gut microbiota intact, which could be a key strategy for preventing recurrent infection of *C. difficile*. Thus, we aim to 1) develop potent covalent inhibitors specific to CdFabH, 2) validate CdFabH as a therapeutic target to selectively disrupt spore formation and subsequent outgrowth of the *C. difficile* spore, and 3) define the role of FabH in *C. difficile* spore formation and germination.

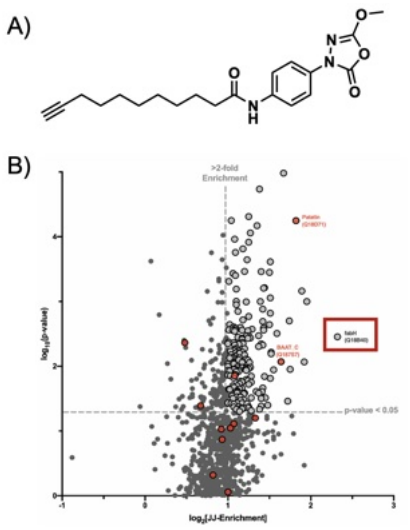

Fig 1: Target identification of covalent probe. A) Structure of oxadiazolone-based probe, JJ-OX-004. B) Chemoproteomic enrichment of JJ-OX-004 with *C. difficile* cell lysate. The most enriched protein is FabH (red box).

Grasping life from the throes of death: Lazarus effect of tailored caspase inhibitors

Kasia Groborz[1, 2], Melissa Truong[1], Irma Stowe[1], Bettina Lee[1], Marcin Poreba[2], Kim Newton[1], Vishva Dixit[1]

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Programmed cell death is a tightly regulated process orchestrated by cysteine proteases known as caspases. While apoptotic caspases selectively cleave intracellular substrates to drive controlled cell dismantling, inflammatory caspases initiate the maturation of proinflammatory cytokines and promote innate immune responses to infection. Caspases mediate several forms of programmed cell death, including apoptosis and pyroptosis. Although both processes share some morphological features, a key distinction lies in their immunological consequences: apoptosis is largely immunologically silent, whereas pyroptosis results in plasma membrane rupture and the release of proinflammatory intracellular contents.

Over the years, numerous caspase inhibitors have been developed and evaluated in preclinical models. Despite initial promise, many of these compounds failed in clinical trials, often due to off-target toxicity, including liver damage. In this study, we introduce a new approach that seeks to selectively manipulate cell death pathways by targeting cells already undergoing inflammatory demise.

We generated a focused library of over 100 tetrapeptide-based caspase inhibitors bearing a reactive acyloxymethyl ketone (AOMK) warhead. Using comprehensive kinetic profiling across all human caspases, we identified several compounds exhibiting improved selectivity toward individual members of caspase family. These inhibitors were subsequently tested in cell-based models of cell death to evaluate their functional selectivity and biological activity in complex cellular environments. Our results indicate that applying a dual selectivity strategy—leveraging membrane changes during pyroptosis alongside structurally optimized caspase inhibitors—can significantly alter the biological outcomes of inflammatory insult and opens new therapeutic avenues for targeted immune modulation.

Defining the Role of the Protease HTRA1 on Spinal Deformities Using Proteomic Profiling of Intervertebral Disc Components

Taylor J. Bader[1], Paul Salo[1,2], David A. Hart[1], Holly Sparks[1], Ganesh Swamy[1,2], Antoine Dufour[1]

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As individuals age their intervertebral discs (IVD), the dominant stabilizers of the spine, degenerate. As escalation of this breakdown is known as degenerative disc disorders (DDD), which can lead to debilitating pain through the structural failure of the IVD and spinal deformities (SD). The etiology of these conditions is not well understood. Previous studies found reductions in the shear stiffness in DDD and SD patients IVD tissue collected from the operating theatre when compared to healthy donor tissue. Pilot proteomics analysis found increased amounts of HTRA1, a serine protease associated with DDD, was upregulated in these same tissues. To test if HTRA1 is a key upstream driver of the development of structural instability, we aim to; 1. further refine and characterize the proteomes of the AF using shotgun and extracellular matrix enrichment proteomics protocols, 2. identify which proteins are degraded by HTRA1 and other proteases using degradomics, and 3. investigated changes in degeneration of ex-vivo lab models using HTRA1 inhibitors. Understanding the differences in proteins, how they are being cleaved between conditions, and how HTRA1 impacts them will not only provide insight into knowing how these painful conditions are progressing but also help establish new ways to potentially inhibit disease progression.

Engineered BoNT/E protease variants cleave neurodegenerative disease targets

Bruce Culbertson[1]*, Isabella Ruud[2]*, Andrea Pence[2,3], Reema Apte[2], Farhad Forouhar[4], Pavla Fajtova[3], Anthony J. O'Donoghue[3], Alejandro Chavez[5], *equal contribution

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The targeted degradation of pathogenic, aggregation-prone proteins is a promising mechanism to treat neurodegenerative diseases, but the difficulty of dictating protease-substrate interactions limits the development of such therapeutics. Here, we engineer the botulinum neurotoxin type E (BoNT/E) protease using random and structure-guided mutagenesis to generate protease variants that are selected for activity on a desired substrate using a circuit that links substrate cleavage to *Saccharomyces cerevisiae* growth. Using this platform, we first targeted a substrate in ataxin3 (ATXN3), a protein whose aggregation causes spinocerebellar ataxia type 3. We evolved BoNT/E towards the ATXN3 substrate using a series of intermediate substrates that incorporate increasing numbers of residues from the ATXN3 substrate into the canonical BoNT/E substrate, SNAP25. We were able to demonstrate robust BoNT/E activity on the ATXN3 substrate, effectively re-programming 16/21 of the substrate residues implicated in its recognition. Substrate profiling of BoNT/E variants that emerged during the ATXN3 engineering campaign revealed patterns that could direct towards other clinically relevant targets. From these patterns, we identified a cleavable motif in TAR DNA-binding protein 43 (TDP43), a protein whose mislocalization and subsequent cytoplasmic aggregation is seen in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar disease (FTLD). Using structure-guided mutagenesis, we rapidly engineered a BoNT/E variant capable of cleaving the TDP43 substrate in a single step, despite changing 19/21 of the residues in the substrate recognition motif. Further engineering led to a BoNT/E variant with activity on full-length TDP43 in mammalian cells and no activity on SNAP25. There is evidence that cleavage at this site ameliorates TDP43-induced toxicity in yeast and mammalian cell models, making it an attractive therapeutic target for further development.

Covalent-fragment screening identifies selective inhibitors of multiple *Staphylococcus aureus* serine hydrolases important for virulence and biofilm formation

Tulsi Upadhyay[1], Emily C. Woods[1], Stephen D. Ahator[3], Kjersti Julin[3], Franco F. Faucher[2], Md Jalal Uddin[3], Marijn J. Hollander[1], Nichole J. Pedowitz[1], Daniel Abegg[4], Isabella Hammond[1], Ifeanyichukwu E Eke[1], Sijie Wang[1], Shiyu Chen[1], John M. Bennett[2], Jeyun Jo[1], Christian S. Lentz[3], Alexander Adibekian[4], Matthias Fellner[5] and Matthew Bogyo[1,6]

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Staphylococcus aureus is a leading cause of bacteria-associated mortality worldwide. This is largely because infection sites are often difficult to localize and the bacteria forms biofilms which are not effectively cleared using classical antibiotics. Therefore, there is a need for new tools to both image and treat *S. aureus* infections. We previously identified a group of *S. aureus* serine hydrolases known as fluorophosphonate-binding hydrolases (Fphs), which regulate aspects of virulence and lipid metabolism. However, because their structures are similar and their functions overlap, it remains challenging to distinguish the specific roles of individual members of this family. In this study, we applied a high-throughput screening approach using a library of covalent electrophiles to identify inhibitors for FphB, FphE, and FphH. We identified inhibitors that irreversibly bind to the active-site serine residue of each enzyme with high potency and selectivity without requiring extensive medicinal chemistry optimization. Structural and biochemical analysis identified novel binding modes for several of the inhibitors. Selective inhibitors of FphH impaired both bacterial growth and biofilm formation, while inhibitors of FphB and FphE significantly affected bacterial virulence. These results suggest that all three hydrolases likely play functional, but distinct roles in biofilm formation and virulence. Overall, we demonstrate that focused covalent fragment screening can be used to rapidly identify highly potent and selective electrophiles targeting bacterial serine hydrolases. This approach could be applied to other classes of lipid hydrolases in diverse pathogens or higher eukaryotes.

Unraveling the catalytic machinery of Bfp1: a structure-based computational study

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Protease-activated receptor-2 (PAR2) is a G protein coupled receptor (GPCR) related to several physiopatological conditions including pain, cancer, metabolic diseases and inflammation. In this context, it has been shown that an excessive proteolysis in the gut and subsequent PAR2-signaling have been linked to inflammatory-bowel diesease (IBD) and irritable bowl syndrome (IBS) severity and related intestinal pain. Bfp1, a member of the S41 family of serine proteases, has been identified as a gut-secreted enzyme capable of activating PAR2 by cleaving the N-terminal RSLIGKD sequence, thereby triggering receptor activation. While it is known that cleavage occurs between the arginine (R) and serine (S) residues within the PAR2 substrate sequence, the catalytic mechanism of Bfp1 remains uncharacterized due to a lack of structural data. Since S41 proteases could use either the dyad (Ser-His/Lys) or tetrad (Ser-His-Ser-Glu) of residues present in its binding site in a catalytic arrangement, we investigated the molecular basis of Bfp1 functions using structure-based approaches. First, a structural homology search using Foldseek against a curated dataset of 74 crystallized serine proteases identified Ser449 as a conserved nucleophile and subsequent structural analysis of Bfp1 revealed His124 in close proximity to Ser449, suggesting a catalytic dyad arrangement. AlphaFold3 aided in modeling the interaction between Bfp1 and the PAR2-derived N-terminal motif, yielding a high-confidence structural prediction score (pTM = 0.84). The predicted binding site was further validated by aligning the Bfp1–PAR2 complex with the crystal structure of a homologous protease in complex with a peptidomimetic ligand. The observed match between the experimentally validated ligand-binding pocket and the predicted PAR2 interface supports a shared recognition site, suggesting a conserved functional role for this region and further reinforcing the structural plausibility of the modeled substrate-binding pocket. The MD-based method Cosolvkit was then employed to analyse the Bfp1 binding site using a mix of fragments selected to reflect the physicochemical properties of the PAR2 peptide sequence, especially mapping polar and charged residues as well as key hydrophobic features in the RSLIGKD motif. As a result, elevated hydrophobic interactions were observed surrounding Ser449, matching the hypothesized position of the leucine-isoleucine-glycine (LIG) motif in the AlphaFold3 model of the Bfp1–PAR2 complex.Altogether, our results suggest that Bfp1 cleaves PAR2 via a Ser-His dyad, with substrate recognition driven by a specific pocket that accommodates hydrophobic residues. This study provides the first structural characterization of the Bfp1 catalytic mechanism and lays the groundwork for the rational design of novel inhibitors targeting Bfp1-mediated PAR2 activation, offering a promising therapeutic strategy for the treatment of IBD and IBS.

Targeting Viral Protease with Optimized Activity and Bioavailability: AVI-4773, A Main Protease (M^{pro}) Inhibitor with Broad-Spectrum Activity Against Multiple Coronaviruses and High Drug Exposure in the Lung and Brain.

Jiapeng Li,[1] Luca Lizzadro,[1] Gilles Degotte,[1] Taha Y. Taha,[2] Tyler C. Detomasi,[1] Kris White,[3] Briana McGovern,[3] Francisco J. Zapatero-Belinchon,[2] Eric R. Hantz,[1] Sijie Huang,[1] Amy Diallo,[4] Nevan J. Krogan,[5] Kliment A. Verba,[4] Brian K. Shoichet,[1] Melanie Ott,[2] Adam R. Renslo,[1] Charles Craik[1,*]

[1]Department of Pharmaceutical Chemistry, University of California, San Francisco (UCSF), San Francisco, CA, USA. [2] Gladstone Institute of Virology, Gladstone Institutes, San Francisco, CA, USA. [3] Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA. [4] Department of Cellular and Molecular Pharmacology, University of California, San Francisco (UCSF), San Francisco, CA, USA. [5] Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco (UCSF), San Francisco, USA.

The main protease (M^{pro}) is responsible for the proteolytic processing of the viral polyprotein essential for viral replication. M^{pro} is conserved across various coronaviruses, including SARS-CoV, SARS-CoV-2, and MERS-CoV. Coronaviruses are often highly contagious and quickly spread among animals and human communities. It is important to have an effective oral drug that can be conveniently used. Despite the SARS-CoV-2 virus becoming endemic, challenges remain: First, many antivirals have limited drug exposure in the lungs and brain. In particular, the brain can be employed by SARS-CoV-2 as a reservoir due to the blood-brain barrier (BBB). Available antivirals, such as remdesivir, nirmatrelvir, and ensitrelvir, cannot achieve adequate concentrations in the brain. Additional concerns are emergent drug-resistance mutations and other coronaviruses that could avoid current treatments.

We discovered an innovative antiviral M^{pro} inhibitor compound AVI-4773-P1 that adopts a weakly electrophilic alkyne warhead covalently binding to the active site (Cys145) of SARS-CoV-2 M^{pro} as a latent electrophile. AVI-4773-P1 showed an IC_{50} of 2.5 nM against *in vitro* SARS-CoV-2 M^{pro} protease, stronger than nirmatrelvir (IC_{50}= 3.3 nM) and ensitrelvir (IC_{50}= 4 nM). AVI-4773-P1 also exerted strong antiviral efficacy against MERS-CoV and SARS-CoV. Meanwhile, AVI-4773 achieves outstanding oral bioavailability and drug exposure in the lungs and brain, with five-fold higher concentrations in the brain than ensitrelvir. As a result, AVI-4773-P1 dramatically reduced the viral titers of SARS-CoV-2 and MERS-CoV by one-million- and ten-thousand-fold, respectively, in mice within two days, significantly stronger than nirmatrelvir and ensitrelvir that reduced viral titers by only one hundred-fold or even less.

Development of Macrocyclic Peptide-Based Proteasome Inhibitors with Enhanced Blood-Brain Barrier Penetration for Treating Brain Neoplasms

Jehad Almaliti[1,2*], Momen Al-Hindy[1], Pavla Fajtová[3], Evgenia Glukhov[1], Theodore Berger[1], Yan Graf[1], Cesar Emiliano Hoffmann da Silva[3], Chandler Huang[1], Narek Azizyan[1], Yuxian He[1], Anthony J. O'Donoghue[3], and William H. Gerwick[1,3]

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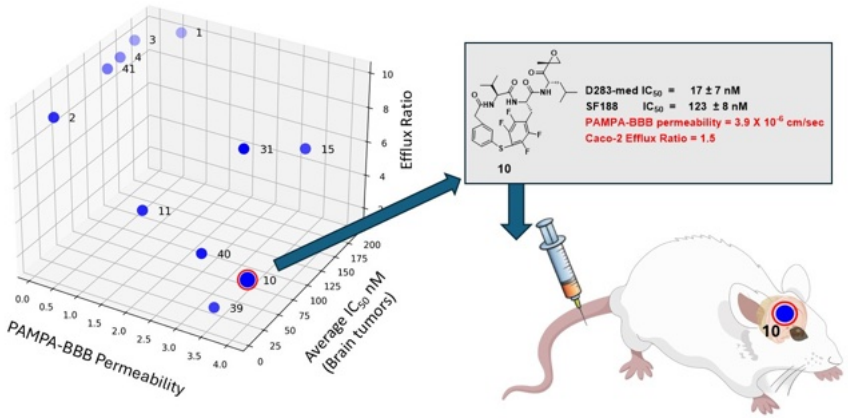
Proteasome inhibitors have transformed the treatment of hematologic malignancies; however, their efficacy against solid tumors—especially brain cancers—remains limited due to poor cellular permeability, low chemical stability, and restricted penetration across the blood–brain barrier (BBB). In this study, we employed a multilayered screening approach to design and evaluate novel macrocyclic peptide epoxyketone-based proteasome inhibitors with improved drug-like characteristics. We began by synthesizing a series of new analogs, which were then screened for cytotoxicity against both brain and non-brain cancer cell lines. Several compounds demonstrated potent activity, with IC_{50} values below 100 nM. The most promising candidates were further assessed using the PAMPA-BBB assay to predict *in vivo* BBB permeability, alongside evaluations for microsomal and plasma stability, as well as Caco-2 cell permeability. Among the compounds tested, fluorinated macrocyclic analogs—particularly compound **10** —exhibited enhanced cell permeability, selective inhibition of the β5 proteasome subunit, and strong *in vitro* cytotoxic effects. *In vivo* pharmacokinetic studies in mice confirmed that compound **10** possesses favorable plasma stability and efficiently crosses the BBB. These findings highlight compound **10** as a promising candidate for the treatment of malignant brain tumors and demonstrate how macrocyclic scaffolds can overcome the limitations associated with conventional linear peptide inhibitors. Additionally, I will briefly discuss novel, orally bioavailable proteasome inhibitors that exhibit selective activity against the *Plasmodium falciparum* proteasome over the human constitutive and immunoproteasome.



Thrombin fuels pathogenic behavior of gut microbiota biofilms in Crohn's disease: a proteomic/N-terminomic approach

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Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by severe inflammation of the gastrointestinal tract. Studies to further elucidate the etiology of CD have started to focus on gut microbiome changes that occur in CD, and the potential contribution of host factors in these changes. One host factor, the human protease thrombin, is produced by the human gut epithelium and is upregulated in the colons of CD patients. Thrombin has been shown to modulate bacterial biofilms and increase biofilm pathogenicity, though the mechanisms behind thrombin's impact on biofilms remain poorly understood. Thus, the aim of this project is to investigate the effects that human thrombin released from the gut epithelium has on microbial proteins of gut-derived bacteria grown as biofilms. In this project, I tested a variety of N-terminomics methods to develop an optimized protocol to analyze biofilms and determined that a hydrophobic tagging N-termini enrichment method was the best available method for this purpose. I performed this protocol, as well as an established proteomics protocol, on mono-species biofilms of Bacteroides thetaiotaomicron, Enterococcus faecalis, Escherichia coli LF82 and Escherichia coli NRG857c grown with or without thrombin. I then tested a carboxylate-modified sp3 bead cleanup method for removing contaminants to follow the optimized N-terminomic technique and the established proteomics protocol. To determine if this cleanup method is effective, further troubleshooting of the bioinformatics analysis step is required. In the future, this cleanup method could be used on the *N*-terminomics and proteomics samples of the five bacterial strains of interest prior to liquid chromatography and tandem mass spectrometry analysis (LC-MS/MS). Following LC-MS/MS analysis of these samples, I aim to determine the bacterial substrates of thrombin and the impact of thrombin on the proteomic profiles of bacterial biofilms. This information will help to elucidate the mechanism by which thrombin modulates biofilms in CD and may provide candidate targets for future CD therapeutics.

Working in Biotech from an Early Career Perspective

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Not so long ago, I was a graduate student and postdoc in the sciences trying to decide between academia or industry, biotech or large pharma, primary undergraduate institution or research professor appointment. I won't read Tarot cards and tell anyone what to do, but I will discuss my career path and experiences working at small to medium sized biotech companies. I will cover common misconceptions about the pharma industry, how we spend hundreds of millions of dollars in our pursuit of discovering therapeutics, current trends in hiring, networking, what to put on CVs and resumes, and how to handle the interview process. I hope my talk will help with the career-related decision-making process and why working at a biotech company is the best option. I also hope to salt and pepper my talk with some science. Time allowing, I will discuss medicinal chemistry principles for developing CNS penetrant small molecule therapeutics, which is the focus of my current biotech company.

Immunoproteasome-Mediated Release of a Monomethyl Auristatin E Prodrug

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Developing therapeutics that are effective in diseased cells, while remaining nontoxic to the surrounding healthy tissue remains a challenge in drug discovery. One technique that has been applied broadly is to cage the toxic molecule with a pro-moiety sequence that can be removed by an enzyme or external source to release the active compound in the desired location. Antibody-drug conjugates (ADC) have shown great efficacy in selectively delivering a toxic compound to the diseased site, typically cancerous cells. Although this method is effective at treating tumors with known antigens that can allow for the development of selective ADCs, other tumors that are more evasive remain a challenge to treat using this method. An approach that still relies on caging the compound from eliciting toxicity to healthy cells but can be liberated by an enzyme that is only being expressed in the cancerous cells, could alleviate the need for antibody recognition. The immunoproteasome (iCP) is a disease specific isoform that is expressed under conditions of inflammation such as interferon-γ exposure. The iCP incorporates different catalytic subunits than the standard CP (sCP), allowing for the same substrate to be degraded into different peptide products between the two. Previously, we have identified a 4-mer peptide recognition sequence through a one-bead-one-compound library screen that is selective for the iCP. With this we have reported the design of a fluorescent probe that can monitor iCP activity in cells called TBZ-1. Here, we demonstrate swapping the fluorescent reporter for a chemotherapy agent, MMAE, leads to selective release and toxicity in cancerous cells expressing iCP, while healthy cells remain viable. This prodrug was effective at killing cancerous cells at low nM concentrations and was successful in reducing tumor volume *in vivo.* This provides the first therapeutically relevant use of an immunoproteasome prodrug targeting cancerous cells.

Characterizing the role of the rhomboid protease RHBDL4 in pancreatic cancer

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Pancreatic cancer is an aggressive disease with a survival rate of only 10%, 5 years post-diagnosis due to a lack of effective treatments. The predominant form (~95%) of pancreatic cancer is Pancreatic Ductal Adenocarcinoma (PDAC). PDAC is largely driven by the mutation of the oncogene, KRAS, a common hallmark of many cancers. Recent studies demonstrate that the endoplasmic reticulum (ER), an essential organelle where many proteins are folded, is important for promoting cancer growth and survival in KRAS mutant cancers, like PDAC. Patient sample databases show that an important ER protein, the rhomboid protease RHBDL4, is upregulated in pancreatic cancer. RHBDL4's canonical role in cells is cleaving misfolded proteins from the ER and targeting them for degradation by the proteasome, mitigating the toxic stress associated with accumulated misfolded proteins. This stress, when at high levels, can trigger apoptosis (programmed cell death), which cancer cells aim to avoid. In human PDAC cell lines, we made an RHBDL4 knockout (KO) and found that there is a drastic decrease in cell growth and increase in apoptosis compared to WT cells. However, role of RHBDL4 in PDAC cell health has yet to be elucidated. We aim to identify the role of RHBDL4 in PDAC cells and how this pathway drives tumor progression. We hypothesize that PDAC cells upregulate RHBDL4 to compensate for high levels of misfolded proteins, exploiting RHBDL4's quality control function to avoid ER stress driven apoptosis. This work aims to elucidate potential therapeutic avenues for PDAC targeting ER stress relieving machinery.

The inflammatory caspases and their substrates

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Caspase-1, -4, -5 are human cysteine proteases that play a role in inflammatory cell death, also known as pyroptosis. In response to bacterial and viral insults, caspase-1 is activated via the inflammasome in the canonical pyroptotic pathway, while caspase-4 and -5 are key activators of the noncanonical pyroptotic pathway. Pyroptosis has been linked to the inflammation and neuronal demyelination observed in multiple sclerosis (McKenzie, 2020). However, the substrates of these inflammatory caspases are largely unknown. Previously, Agard *et al.* identified 82 caspase-1 substrates, four caspase-4 substrates and no caspase-5 substrates using N-terminal labelling and mass spectrometry (LC-MS/MS). Importantly, this study reported that caspase-1 cleaves gasdermin D at residue Asp276, which results in the generation of N- and C-terminal fragments. The N-terminal fragments of gasdermin D can re-localize from the cytoplasm to the cell membrane, where they oligomerize and form pores at the cell surface, resulting in the uptake of water and release of inflammatory cytokines. Since the previous study only identified a handful of caspase-4/-5 substrates and given the recent advances in N-terminal labeling and mass spectrometry methods, we are re-visiting these two proteases and aim to identify novel caspase-4/-5 substrates.

We first expressed and purified active human caspase-4/-5. After the addition of the purified caspase to a THP-1 monocyte cell lysate, we labeled and captured the newly formed N-termini generated by caspase proteolysis and identified these cleavage sites using LC-MS/MS. Using this approach, we identify hundreds of caspase substrates. By studying these newly discovered caspase substrates, we hope to better define the biology of these two inflammatory caspases.

Sequential AND-gate Fluorescently Quenched Activity-based Probe for Selective Imaging of Cysteine Cathepsin Activity

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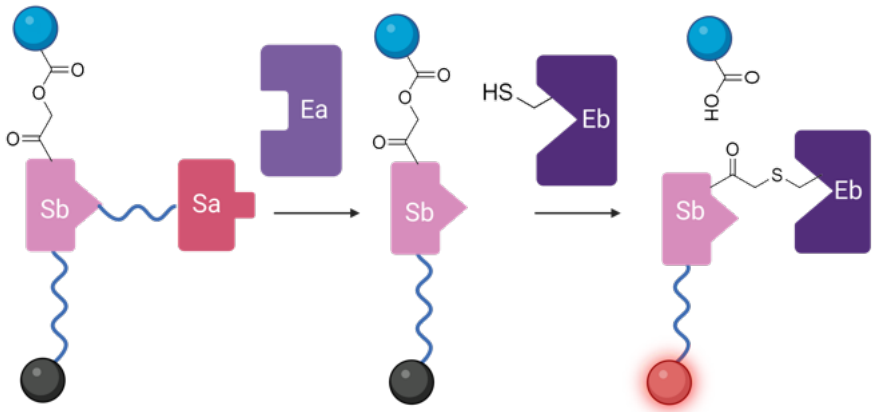
Cysteine cathepsins are a family of proteases shown to be involved in multiple stages of tumorigenesis. Here we report the design and synthesis of a new class of sequential AND-gate (SAND-gate) quenched fluorescent activity-based probes (qABPs) containing an acyloxymethylketone (AOMK) electrophile. These SAND-gate probes require the processing of a tumor specific enzyme fibroblast activation protein (FAP) before they covalently modified cathepsins and emit a fluorescent signal with significantly improved selectivity and sensitivity to tumor tissue. We demonstrated these probes in mouse models of mammary tumors and ex vivo biochemical profiling.



Identifying caspase-8 and caspase-10 substrates using N-terminomics

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Caspases are cysteine-aspartic proteases that are involved in many cellular processes such as programmed cell death, apoptosis, cell differentiation and inflammatory cell death[1]. These proteases are generally classified into one of three categories: initiator (caspase-2, -8, -9, and -10), executioner (caspase-3, −6, and −7), or inflammatory (caspase-1, −4, −5, and −12). Initiation of apoptosis through the extrinsic pathway typically involves the activation of caspase-8 and -10, resulting in the cleavage of specific substrates, including caspase-3. In addition to its role in apoptosis, caspase-8 has also been identified to have several non-apoptotic roles including, proliferation, and cell differentiation[2]. While 62 protein substrates have been identified for caspase-8[3], only a handful protein substrates have been reported for caspase-10[4,5], highlighting a potential gap in our understanding of these protease biological functions. The aim of this project is to acquire a comprehensive dataset of caspase-8 and -10's substrates by employing N-terminal labeling of the cleaved substrates and identification using mass spectrometry. To achieve this, I am expressing and purifying recombinant caspase-8 and 10 in *E. coli* and characterizing their enzymatic activity in vitro. I will then add the purified caspases in mammalian cell lysate to identify caspase-8 or -10 cleaved substrates. These cleaved proteins will then be labelled at their N-termini using a subtiligase-based N-terminomic approach, allowing for their identification using mass spectrometry. We hope our results will allow us to identify new biological roles for caspase-8 and -10, both in apoptotic and non-apoptotic functions.

Development of a caspace1-activated SWIR fluorescent probe for tumor detection and therapeutic monitoring

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Short-wave infrared (SWIR) imaging (1000–1700 nm) is particularly effective for non-invasive tumor visualization in deep tissues, offering clear advantages over traditional visible-light fluorescence imaging. For example, the liver is challenging to visualize with visible light fluorescence due to significant light scattering. In this study, we investigated the caspace1-activated fluorescent probe SP309 as a potential imaging biomarker. Our fluorescence-guided surgery experiments demonstrated that this approach is both feasible and effective at detecting tumor lesions, assisting in surgical decision-making. The results confirm the biocompatibility of SP309 and highlight its potential for in vivo imaging and fluorescence-guided surgery (FGS) in liver treatment.

PCPSV: Profiling *Caenorhabditis elegans* Proteases under Structural Variation

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Structural variants (SVs) describe large alterations in chromosome structure with crucial roles in genome evolution and adaptation. Notably, deletions and duplications are key mechanisms of genetic diversification, with the latter being the primary source of novel genes. As a result, valuable evolutionary insights can be gained by characterizing SVs in natural populations, especially for those that impact genes or regulatory regions. Despite being widespread in natural populations, most SV research has been limited to laboratory adapted strains and experimental evolution. As a result, comprehensive profiles of the genes under structural variation in natural populations are lacking for most species.

In this study, we used Sniffles2 (v2.0.7) to call SVs in 14 wild *Caenorhabditis elegans* strains using PacBio DNA sequencing data. Functional annotation was then performed using Variant Effect Predictor (v.109.3), which identified high-impact SVs that overlapped with 1,485 deletions, 214 duplications, and three inversions (collectively affecting 1,258 genes). To determine which functions were likely impacted by SVs, an overrepresentation analysis was performed using the annotated gene sets from WormCat (V2). Notably, proteolysis featured prominently among the overrepresented functional categories. To further characterize the relationship between SVs and proteolysis in *C. elegans*, the genes associated with high-impact variants were then queried against the *MEROPS* database (release 12.5) to identify those representing proteases and their inhibitors. A total of 17 proteases were affected by SVs (15 deletions and 2 duplications) and an additional five protease inhibitors harbored high-impact deletions.

These results highlight the importance of proteolysis in the evolution of *C. elegans* and suggest that SVs in proteases may facilitate how these worms adapt to diverse habitats around the world. This study also demonstrates the feasibility of genome-wide, population-scale SV profiling and could serve as a first step towards more comprehensive surveys of proteases under structural variation in other species.

Oxadiazolone-based Probes for Selective Detection of Implant Biofilms in Chronic *Staphylococcus aureus* Infections

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Staphylococcus aureus (*S. aureus*) is a major bacterial human pathogen responsible for a wide range of infections, from skin and soft tissue infections (SSTIs) to life-threatening sepsis. *S. aureus* can form biofilms on the surface of prosthetics, often leading to chronic infections associated with implant biofilms. However, the identification of implant infections usually involves invasive surgical procedures to collect samples directly from the implant. Therefore, there is urgent need for novel, non-invasive method to detect surgical implant infections. We previously designed FphE selective probe JJ-OX-007 and demonstrated its ability to selectively image *S. aureus*. In this study, we modified the fluorophore of JJ-OX-007 to develop a Cy5-labeled version, JJ-OX-012, suitable for *in vivo* application. JJ-OX-012 showed strong *in vitro* labeling of *S. aureus*, with little to no labeling of other species such as *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Furthermore, JJ-OX-012 enabled non-invasive fluorescent imaging for selective detection of *S. aureus* biofilms *in vivo* in a mouse surgical implant infection model. These results highlight the potential of FphE-targeted probes as powerful tools for selective detection of *S. aureus* biofilms *in vivo* and suggest their future application in the diagnosis and monitoring of implant-associated infections.

Ab initio computational modeling of covalent macrocyclic FphB serine hydrolase inhibitors

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Antibiotic resistance in Staphylococcus aureus is a growing global threat, demanding new therapeutic targets. The serine hydrolase FphB has been identified as a key virulence factor, with the Bogyo Laboratory recently discovering two macrocyclic inhibitors (FphB-OX-5 and FphB-OX-14) via mRNA display. However, the lack of an FphB crystal structure limits experimental insights into inhibitor binding. To address this, we used an ab initio computational approach, combining AlphaFold modeling, cosolvent molecular dynamics, and reactive AutoDock-GPU docking with pre-sampled macrocycle conformations. Further molecular dynamics simulations refined the binding poses and revealed stable inhibitor complexes that align with experimental mutagenesis data. Our findings demonstrate that ab initio computational modeling can provide critical insights into drug binding, informing structure-activity relationships for novel antibiotic strategies.

Proteomic Study of H1N1 Influenza Infection in Mammalian Cells

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Influenza A viruses (IAV) are a group of single-stranded RNA viruses from the family *Orthomyxoviridae* that are partially responsible for seasonal flu outbreaks in humans. The H1N1 subtype of IAV also has pandemic potential, sparking the 1918 Spanish flu and 2009 swine flu pandemics. Over the years, various transcriptomic and proteomic methods have been used to study proteome changes in influenza-infected cells. However, recent advances in the sensitivity and resolution of mass spectrometers have enabled deeper proteome coverage and the identification of low-abundance proteins, many of which may be involved in viral replication or host response mechanisms. Here, we employed label-free quantitative proteomics to determine proteome changes in bulk A549 human lung epithelial cell cultures infected with A/PR/8/34 (H1N1) at 8, 24, and 48 hours post-infection, compared to uninfected controls. Using an Orbitrap Exploris 480 (Thermo Scientific) in data-independent acquisition (DIA) mode, we quantified 6,497 unique protein groups. Of these, 288 host protein groups were significantly increased and 439 were significantly decreased at least one time point during the infection time course. Proteins involved in innate and adaptive immunity, proteolysis, and peroxidase activity increased in abundance during infection, while proteins involved in lipid metabolism, lysosomal function, and aldo-keto reductase activity decreased in abundance. To investigate the heterogeneity of these responses among single infected cells, we also developed and optimized a label-free single-cell proteomics workflow for mammalian cells. Our optimized single-cell proteomics workflow demonstrates reproducible proteome coverage and throughput with minimal sample loss, quantifying over 2000 proteins per cell. Taken together, these studies enhance knowledge of H1N1 infection mechanisms and propose a novel experimental protocol to study these mechanisms at single cell resolution.

Covalent PSMA-based probe library for targeted prostate cancer therapeutics

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Prostate-specific membrane antigen (PSMA) is a well-established and highly specific prostate epithelia cell membrane antigen that has been identified as a valuable target in the treatment of prostate cancer. Since PSMA is overexpressed in prostate cancer cells, therapeutics that target PSMA with high specificity have previously been leveraged to improve surgical margins with probes and radioligand therapies. But current highly specific PSMA probes bind non-covalently which limits their therapeutic potential due to transient interactions. Conversely, existing covalently binding PSMA radioligands are not specific enough and show significant off target effects and potential damage to kidneys and salivary glands.

Prostate cancer is the second leading cause of cancer in men worldwide, but current treatments lack potency and specific targeting. To date, radical prostatectomies are the most effective treatment for prostate cancer but can lead to lifelong incontinence and impotence. The development of advanced covalent PSMA probes will help avoid complications and the need for radical surgery. Covalent probes will meaningfully impact treatment outcomes by allowing long retention of potent treatment in affected areas that non-covalent chemistries lack.

Here, we plan to synthesize a library of high affinity PSMA ligands containing a reactive electrophile to target and bind covalently to prostate tumors with high enough selectivity to avoid off-target effects. The library will systematically explore how the position of the covalent warhead impacts covalent binding specificity and affinity for PSMA-positive cells. This will be used to identify the best covalent probe candidates which can further be functionalized as imaging and therapeutic agents via a chemical handle. With this, we plan to generate a comprehensive and modular library of optimized covalent ligands with various fluorescent agents and therapeutics. Ideally, these probes will have the potential to improve outcomes for prostate cancer patients and provide a modular platform to explore PSMA targeting therapies.

Design, Synthesis and Characterization of Chemi-luminescent Probes for non-invasive Diagnostic Imaging

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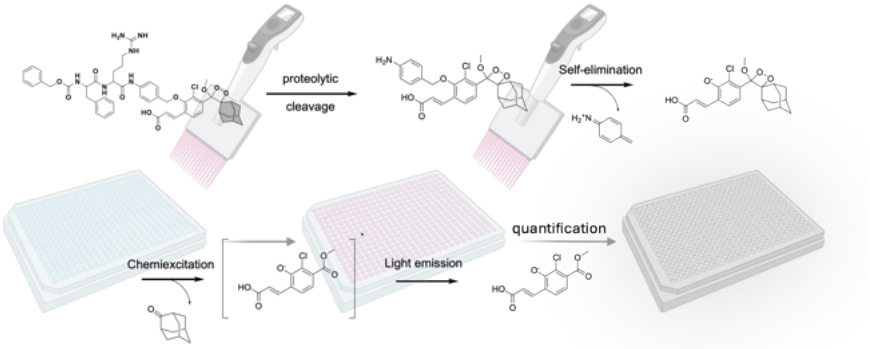
Chemiluminescence has emerged as a powerful biosensing strategy for detecting bacterial infections and other biological targets, due to its inherently high signal intensity and low background noise. However, the diagnostic potential of chemiluminescent probes remains underexplored.

Traditional chemiluminescent systems, such as those based on luminol, often lacks specificity. Inspired by the substrate preference, we developed a series of chemiluminescent probes incorporating the substrate specificity of human cathepsin L (hCatL), a highly expressed cysteine protease in macrophages present in a wide range of diseases. Upon enzymatic cleavage by hCatL, these probes generate a high-intensity signal with a favorable signal-to-noise ratio.

The probes were evaluated using steady-state kinetic assays, which revealed classic Michaelis-Menten behavior, confirming both their specificity and catalytic efficiency toward hCatL. Furthermore, enzymatic and cellular assays demonstrated a dose-dependent relationship between luminescent signal intensity and enzyme or cell concentration, as measured by both plate-reader and imaging-based platforms.

Together, these findings demonstrated the potential of our chemiluminescent probes as sensitive, non-invasive diagnostic tools for diseases marked by elevated cathepsin L activity.



Evaluating the 20S proteasome of the early-branching eukaryote *Giardia lamblia* as a valuable drug target

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Giardia lamblia (*G. lamblia*) is a protozoan parasite that colonizes the intestinal tract and causes diarrheal disease with >300 million annual cases worldwide. Giardiasis, the disease caused by *G. lamblia*, is primarily treated with oral nitro-heterocyclic drugs. These drugs can have significant adverse effects, and resistance occurs in a significant fraction of patients. The proteasome is an essential large multi-subunit protease complex found in all eukaryotes. In our preliminary studies, we have validated the *Giardia* proteasome (Gl20S) as a druggable target and have shown that the Gl20S is significantly different structurally and evolutionarily from its human counterpart (c20S). We hypothesize that inhibitors can be developed to selectively target the Gl20S, which could yield more effective and safer drug candidates.

In this project, I will evaluate the substrate specificity and structural differences between the Gl20s and the c20S. I will use Multiplex Substrate Profiling by Mass Spectrometry to uncover substrate cleavage preferences for each subunit. The substrate specificity profile of the Gl20S will be directly compared to profile of the c20S. Cryo-EM structural and docking studies will be done in parallel to understand how specific substrates and currently available inhibitors are fitting into the Gl20S. Leveraging insights from structural and substrate specificity differences, we aim to design ≈30 inhibitors tailored to selectively target the parasite proteasome. These inhibitors will be evaluated in cellular and biochemical assays, followed by structural optimization guided by structure-activity relationships. Our long-term goal is to develop an oral proteasome inhibitor as a treatment for *Giardia* infections.

Identification of microbial proteases that regulate Protease-activated receptor 2 to control barrier function, pain and inflammation in the gut

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Protease-activated receptor 2 (PAR2) has been implicated in inflammatory bowel disease (IBD). Yet, clear molecular connections between gut microbiota and PAR2 activation remain elusive. PAR2 is activated by the proteolytic cleavage of its extracellular domain by a range of proteases. PAR2 regulates barrier integrity, inflammation, and pain in the gut and, therefore, represents a potential checkpoint in disease onset and progression. Notably, protease activity is greatly increased in the gut lumen of IBD patients.

We screened conditioned media of a library of 140 gut commensal bacterial strains using a substrate corresponding to the N-terminal domain of PAR2. Using chemoproteomics we identified several candidate proteases and prepared knock-out strains. Using multicellular and *in vivo* models we characterized the effects of microbial proteases on barrier function (human intestinal organoids), inflammation and pain signaling (mouse models) through PAR2-depenedent regulation.

We found that 19% of strains, mainly from *Bacteroides*, cleaved the N-terminal domain of PAR2. We investigated *B. fragilis* and identified a novel protease, Bfp1, that activates PAR2 through proteolytic cleavage and triggers downstream signaling. Using human ileal organoids from healthy donors, we found that Bfp1-containing supernatants disrupt barrier function. In a mouse model, we observed both increased expression of pro-inflammatory cytokines as well as increased pain signaling that was dependent on both Bfp1 and PAR2.

PAR2 regulation by microbial-derived proteases is relatively common in gut commensal bacteria. In particular, we identified a novel microbial protease Bfp1 that directly cleaves and activates PAR2 signaling. PAR2 activation by Bfp1 triggers impaired barrier function, inflammation and pain as shown in multicellular, ex vivo and in vivo models suggesting it is a viable target to alleviate IBD symptoms.

Our study provides a molecular connection between secreted microbial proteases and IBD symptoms with a particular focus on one such protease – Bfp1 in *B. fragilis*.

Allosteric Role of Heparin on Human β-Tryptase Structure and Activity

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Human β-tryptase is a trypsin-like serine protease, highly abundant in mast cell secretory granules, and part of an inflammatory response upon mast cell activation. It is active as a donut-shaped homotetramer bound to heparin with 4 active sites inside the pore. Dissociation of active tetramers results in inactive monomers. While it is known that heparin stabilizes the tetramer, we found that heparin also allosterically activates β-tryptase, although the precise mechanism remains elusive. Here, we present the first crystal structure of β-tryptase bound to a pentameric heparin oligosaccharide solved at 2 Å, offering insights into its allosteric mechanism. We identified 5 key positively charged surface-exposed residues in relatively close proximity (R187, R188, K159, K26, K202), which constitute the heparin binding site. We assessed their importance in tetramer formation and enzymatic activity by mutation to alanine or glutamate to remove or disrupt interactions of β-tryptase with heparin. Tetramerization was evaluated by size exclusion chromatography, while activity was measured using the chromogenic substrate S-2288. The R187A and R188A mutants reduced tetramer formation by ~21% while enzymatic activity was moderately reduced. With charge reversal mutations (R188E, K159E, and K26E), tetramer formation was dramatically reduced by ~77%. Notably, tetramers R188E and R187E/R188E showed 89% and 100% loss in activity, respectively despite being distal to the active site. Since R188 is next to D189 at the bottom of the S1 specificity pocket, we hypothesize that R188 provides an allosteric link between heparin binding and enzyme activity, as well as tetramer formation required for fully active β-tryptase.

Development of Immunoproteasome Substrate Labeling Assays (iSLAy)

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The immunoproteasome (iCP) is a isoform of the proteasome that is expressed constitutively in immune cells and can be induced upon inflammatory insults in others. The iCP differs from the standard proteasome (sCP) in its catalytically active subunits, LMP2 (β1i), MECL-1 (β2i) and LMP7 (β5i), attenuating its cleavage preferences, producing peptides that are more amenable for MHC-I binding, rendering it vital to antigen presentation. Compared to the sCP, little is known of the iCP's interactors, including its substrate profile. At present, no proteomic platform is selective to profiling the iCP. Herein, we describe a novel technique we have named Immunoproteasome Substrate Labeling Assays (iSLAy). We expand the capabilities of a mature proximity labeling strategy, APEX2-MS, a platform demonstrated to be adept at profiling transient protein interactions such as between enzymes and their substrates.1 With our discovery of an iCP-specific peptide recognition sequence (ATMW), we introduce the concept of Caged Proximity Substrate Labels (CaPSuLs), comprised of a proximity label that is liberated only upon the iCP-mediated hydrolysis of the sequence. This allows for subsequent activation of the label by APEX2 fused to iCP regulator, PA28, and labeling only the proteome proximal to the iCP. With the development of this technology, we hope to isolate iCP substrates and glean insights on the properties of the iCP and its role on the cellular phenotype.

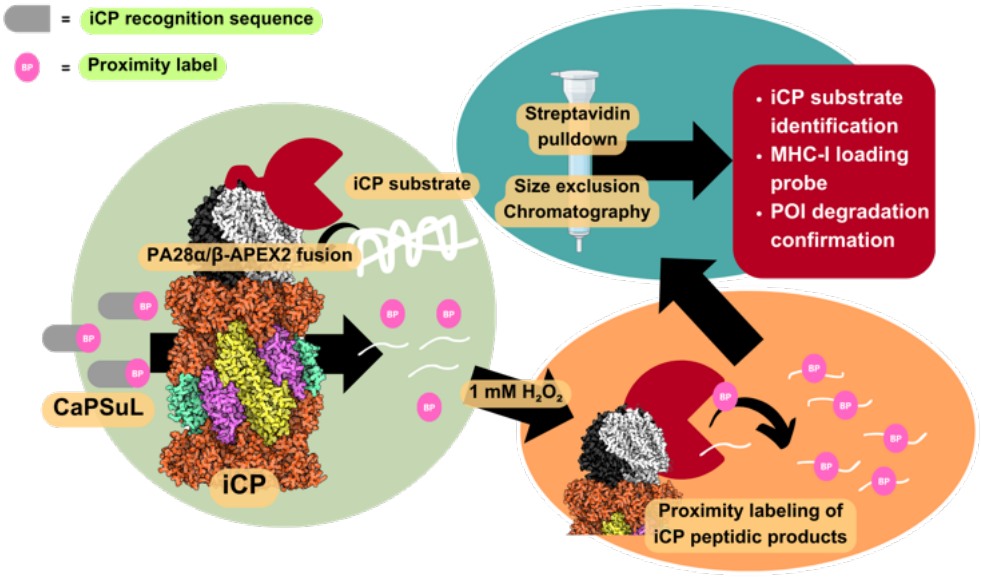

Figure 1. Overview of iSLAy technology. This new platform that is activity and proximity-based will be able to tag and enrich iCP protein substrates, allowing for their identification.

Phenotypic screening of covalent fragment libraries for growth inhibitors of *Staphylococcus aureus*

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The increasing emergence of antibiotic resistance in *Staphylococcus aureus* calls for the need to develop new chemical matter or therapeutic targets for the infection. Given the ability of electrophilic fragments to target residues that are essential for the activity of a bacterial enzyme, the evolution of target-based resistance against the fragments is usually associated with fitness defects in the resistant bacteria. Therefore, electrophilic fragments represent interesting chemical matter that can be further developed as potential antibiotics for *S. aureus*. In this study, we screened the Enamine 1600-serine and 3200-cysteine fragment libraries for growth inhibition of *S. aureus* and identified 96 compounds that inhibited the growth of the bacteria. This represents a hit rate of 1.31% for the serine library and 2.34% for the cysteine library. Chloromethyl ketones (63/75) and cyanoacrylamides (8/75) were the major compound classes in the cysteine hits, while nitroethyl benzenes were common in the serine hits. Given the over-representation of chloromethyl ketones fragments in the cysteine hits, we conducted an activity cliff analysis and uncovered the structure-activity landscape of the series. Overall, this screen represents a good starting point for the characterization of bacterial growth inhibitors from covalent fragment libraries that can be further developed as potential antibiotics.

Structure-guided Identification of Serine Protease Inhibitors from Biased Fab Phage-display Libraries

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We designed a biased Fab phage display library capable of efficiently identifying antibody-based serine protease inhibitors. Using key inhibitory CDR H3 motifs derived from potent matriptase-targeting antibodies as a foundation, we constructed six synthetic libraries with a total diversity of ~10^{10}. From selections against matriptase, we identified sixteen Fab inhibitors with K_i values below 100 nM—demonstrating over 100,000-fold improvement in potency relative to the circularized peptide motif alone. Selections against TMPRSS2, a homologous serine protease implicated in viral entry and aggressive prostate cancers, yielded several selective inhibitory antibodies. While these initially exhibited modest inhibitory potency and binding affinity, an affinity maturation library guided by structure-based predictions improved both properties. AlphaFold models of these complexes provided a structural framework to interpret the binding mode of the antibodies and to rationalize, from a structural perspective, the merits of our biased library design in enabling discovery of serine protease inhibitors. These models further enabled high-throughput binding energy predictions across interface residues, guiding the design of improved mutants. The predictive accuracy of these models was supported by results from the affinity maturation library and validated through targeted mutagenesis. This work establishes a promising framework that integrates biased Fab libraries with structure-based computational analysis, enabling the accelerated development of selective inhibitory antibodies to investigate serine protease structure, function, and roles in disease.

Structure-Based Design of Inhibitors of the *Mycobacterium tuberculosis* 20S Proteasome Suppress Persistence of the Bacterium in Infected Macrophages

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The remarkable and problematic capacity of *Mycobacterium tuberculosis* to survive within macrophages is dependent on a functional 20S proteasome that is structurally and functionally analogous to its mammalian counterpart. It has been proposed that this enzyme is critical for the bacterium's response to nitrosative stress. Here, we report the structure of a rationally designed and species-selective inhibitor of the *Mtb* 20S proteasome in complex with the enzyme. The inhibitor was inspired by the syringolin natural products that are covalent proteasome inhibitors that mimic proteasome substrates. In this case, the structure revealed that the inhibitor also mimics a proteasome substrate but engages the active site in a completely different fashion than the syringolin natural products. The conformation of the designed inhibitor in the active site is strikingly reminiscent of that of an acyclic proteasome inhibitor in its complex with the *Mtb* enzyme. The structure was used to guide the design of a more potent and selective inhibitor, as evidenced in kinetic assays for enzyme inhibition. The first and second-generation inhibitors are permeable to and active in *M. tuberculosis*. They also sensitize the bacterium chemically and pharmacologically-induced nitrosative stress *in vitro*. Importantly, they suppress the persistence of live mycobacteria in murine and human macrophages.

Investigation of cysteine proteases in *Bacteroides cellulosilyticus* for desensitization of Protease-activated receptor 2

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Protease-activated receptor 2 (PAR2) is a G-protein coupled receptor that is activated by proteolytic cleavage of its N-terminal extracellular domain. Due to its role in inflammation, pain, and barrier integrity in intestinal epithelial cells, PAR2 is implicated in inflammatory bowel disease (IBD) and has been shown to be modulated by several gut commensal microbial proteases. One bacterial strain identified in a screen for PAR2 cleavage is *Bacteroides cellulosilyticus*, a common gut commensal which has been associated with the microbiota of healthy patients. Screening and chemoproteomics of *B. cellulosilyticus* with the cysteine protease inhibitor E64 identified two putative cysteine proteases that could be responsible for its PAR2 cleavage activity. Using a subset of PAR2 N-terminal extracellular domain substrates, we determined that the *B. cellulosilyticus* supernatant cleaves further C-terminal than the canonical activation site and could potentially be desensitizing PAR2 to cleavage by pro-inflammatory microbial proteases. Here, I plan to create a knockout strain to confirm the function and identity of these proteases, then explore effects of PAR2 desensitization in gut colonoids. This raises the possibility of probiotic development for IBD patients and will give greater insight into the relationship between microbial proteases in the gut microbiome.