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TITLE: Diacylglycerol Activation of T-Cell Receptor Signaling for Cancer Immunotherapy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have accomplished the goals and milestones outlined in this proposal, which has resulted in 8 peer-reviewed publications, 35 invited talks, and 9 conference abstracts to disseminate our research findings. As part of our research efforts, multiple graduate students received vital career development opportunities at prestigious national meetings. Our findings have impacted fields outside of chemistry and cancer immunology by showing the lipid biology field that chemical biology approaches can provide fundamental information on how lipid kinases impart metabolic and signaling specificity in cells. We generated preclinical data in a melanoma mouse model that ritanserin in combination with anticancer drugs can significantly increase survival compared with delivery of either agent alone. In a small cohort of mice, the combination showed complete resolution of their tumor beyond termination of therapy, and we observed increased CD8+ T cell infiltration in treated mice compared with controls. We discovered new lipid biomarkers for DAG kinase function in T cells. We also developed new chemistry to improve the potency of ritanserin by targeting novel regions of the DAG kinase-alpha active site through a covalent mechanism of inhibition. We believe our studies and findings have broader impacts in society by continuing to show our commitment to drug fat metabolism for immuno-oncology. Working with fats, especially as signaling molecules, is challenging and we are enthusiastic about educating the public and providing tools for the wider scientific community to study these metabolites.

15. SUBJECT TERMS

immunology; immunotherapy; lipid metabolism; T cell; melanoma; tumor; kinase; cancer; diacylglycerol kinase; phosphatidic acid; chemical proteomics

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TABLE OF CONTENTS

1. **INTRODUCTION:** The overall goal of this proposal is to understand how diacylglycerol kinase alpha (DGKA or DGKα) modulates diacylglycerol lipid signaling pathways to influence T cell function and related disorders. Proper immune response to infection and disease requires that T cells are active and responsive. Activation of T cells occurs by presentation of Ag-peptides to naïve T cells. Ag-specific T cells become activated and can specifically fight foreign material by rapid growth, moving to sites where the Ag is present, and producing molecules that serve as signals of cytotoxicity. Diacylglycerols (DAGs) play a prominent role in T cell activation by serving as messengers to recruit key proteins involved in signaling pathways that can control T cell function. Termination of DAG signaling is important because it prevents overactive immune response and the onset of autoimmunity. Diacylglycerol kinases (DGKS) are enzymes that prompt the conversion of DAG lipids to phosphatidic acid (PA). DGKA plays a critical role in immunity by regulating DAG signaling events. Strict balance between lipid, DAG, and enzyme, DGKA, is important in the regulation of proper T cell function. Excessive amounts of DGA can result in T cell anergy, where cells become only partially activated and unresponsive. This condition is highly noted in tumor microenvironments. We seek to identify the precise role of DGKA in T cells in order to translate DGK biology into novel therapies.

2. **KEYWORDS:** immunology; immunotherapy; lipid metabolism; T cell; melanoma; tumor; kinase; cancer; diacylglycerol kinase; phosphatidic acid; chemical proteomics

3. **ACCOMPLISHMENTS:**
Specific Aim 1: Test our hypothesis that ritanserin binds to a novel allosteric site(s) of DGKA to impart isoform specific inhibition.

Major Task 1: Mapping the ritanserin binding site of DGKA using competitive ABPP and quantitative LC-MS

- Subtask 1: Producing recombinant DGK isoforms (5 isoforms in total: alpha, kappa, epsilon, zeta, and theta plasmids have been validated in Hsu lab) in SILAC HEK293T cells (commercial source: ATCC)

  We completed this task in Year 1 and published our findings in References [1-3]

- Subtask 2: Preparing cell lysate treated +/- compounds for ABPP studies. Compounds to be tested: ritanserin (DGK inhibitor) and ketanserin (DGK-inactive negative control probe).

  We completed this task in Year 1 and published our findings in References [1-3]

- Subtask 3: Treating cell lysates with ATP acyl phosphate probe, enrichment, and preparation for nano LC-MS

  We completed this task in Year 1 and published our findings in References [1-3]

- Subtask 4: nanospray LC-MS analysis of samples prepared from ABPP analysis

  We completed this task in Year 1 and published our findings in References [1-3]

- Subtask 5: Data analysis of nanospray LC-MS results by IP2 bioinformatics software

Major Task 2: Evaluating selectivity of ritanserin against kinase superfamily

- Subtask 1: UVA IACUC & USAMRMC ACURO review for all animal studies proposed

  We received approval for the proposed animal studies from UVA IACUC in Year 1

- Subtask 2: Isolation and immunopurification (commercial source: Stemcell) of CD8+ T cells from spleens of C57BL/6J [10 mice X 7 groups = 70 mice total]

  Subtask 3: Culture and expansion of spleen CD8+ T cells using anti-CD3/CD28 beads (commercial source: ThermoFisher) in SILAC media

  We have successfully isolated CD8+ T cells from mouse spleens. We have also activated splenic CD8+ T cells using anti-CD3/CD28 and demonstrated these conditions can successfully expand these cells in culture. These unpublished data are shown in Figure 1.

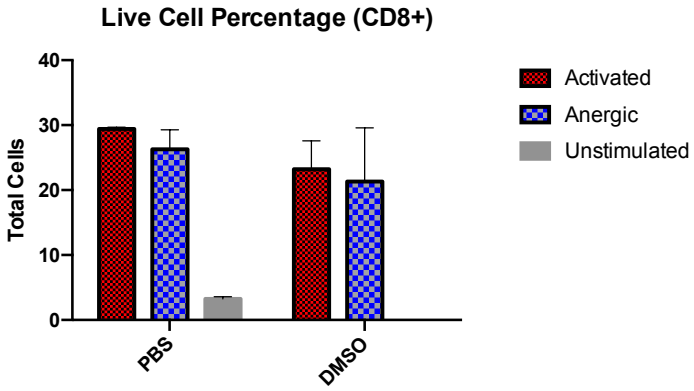


Figure 1. We successfully isolated, cultured, and expanded spleen CD8+ T cells from C57BL/6J mice. This data is work in progress and will be published in the future.

- Subtask 4: Preparing cell lysates treated with vehicle or ritanserin (100, 50, 10, 5, 1, 0.1 μM) for ABPP studies

  Subtask 5: ATP acyl phosphate probe labeling and sample preparation for nano LC-MS

  Subtask 6: nano LC-MS analysis

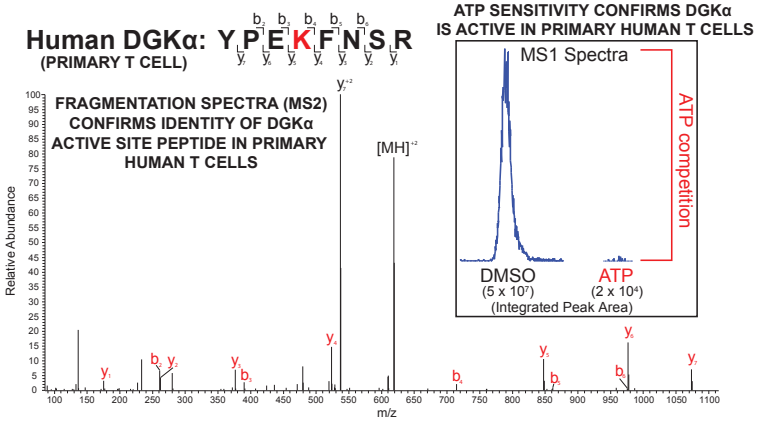


Figure 2. Detection of active DGKα in primary human T cells using chemical proteomics with ATP acyl phosphate probes.

We generated data showing that pretreatment of T cell proteomes with ATP (1 mM, 30 min, 25 °C) blocked DGKα active-site peptide (YPEKFNSR) enrichment indicating active enzyme in T cells. Interestingly, we did not detect other DGK

isoforms in primary human T cells using our chemical proteomics approach. This data support DGKα as a principal DGK isoform for targeting T cell biology (Figure 2).

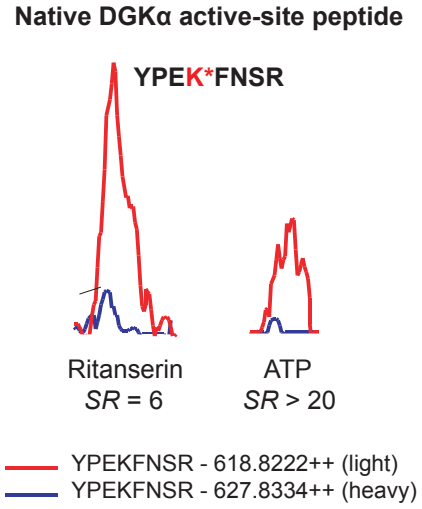


Figure 3. Detection and inhibition of native DGKα in cancer cell proteomes. MS1-extracted ion chromatograms of the probe labeled active-site peptide of DGKα. Pre-treatment of heavy A549 proteomes with ritanserin (100 μM) or ATP (1 mM) resulted in inhibition of DGKα active-site peptide probe labeling (ritanserin SR = 6; ATP SR > 20). All experiments were measured 3 times (technical replicates in LC-MS) using data from 3 independent biological replicates performed on separate days (n = 9). Peak images are a representative image from an individual injection.

We also detected native activity of DGKα but not other DGK isoforms in A549 proteomes and showed that treatment with ritanserin potently blocks probe labeling at the DGKα active site (Figure 3). Our findings that DGKα is expressed and active in A549 cancer cells is important because one of our future goals is to use DGKα inhibitors for activation of immunotherapy that can be synergistic with direct killing of tumor cells.

- Subtask 7: Data analysis by IP2 bioinformatics software

We completed this task in Year 1 and published our findings in Reference [4]

Specific Aim 2: Elucidate the mechanism of action and therapeutic potential of ritanserin for cancer immunotherapy.

Major Task 1: Determine endogenous substrates/products regulated by DGKA

- Subtask 1: Isolation and immunopurification of CD8+ T cells from spleens of C57BL/6J [20 mice X 3 groups = 60 mice total]

  Subtask 2: Lipid extraction from cells treated with vehicle, ritanserin, or ketanserin using Folch method

  Subtask 3: LC-MS analysis using PRM to measure endogenous 36:1, 40:5, and 40:6 DAG and PA species altered in ritanserin-treated T cells

  Subtask 4: Data analysis of PRM results

  We established a tandem liquid chromatography-mass spectrometry (LC-MS/MS) platform for comparing diacylglycerol (DAG) fatty acyl specificity across all 10 mammalian DGKs. These findings were recently published in the following References [5,6].

  We also generated unpublished findings on the role of DGKs in regulation of DAG and PA metabolism in human T cells. Using Jurkat cells where DGKα has been genetically knocked out by CRISPR-Cas9[7], we compared phosphatidic acid (PA) abundances in Jurkat T-cells activated under anergic conditions to reveal a predominant role of DGKα in mediating regulation of this signaling lipid. The results show that these genetic DGKα/ζ KO cells produce less PA lipids following induction of anergy with DGKζ having less of an effect overall. This downward trend in lipid abundance is reflected in other lipid species, particularly with phosphatidylserine (PS), which is a known signal

for apoptosis. The degree to which this phospholipid experiences changes in metabolism is related to the expression of DGKα with its KO and the double-KO experiencing the most prominent shifts. Conversely, full stimulation of the TCR complex results in T-cell activation and Jurkat cells in this state accumulate PA species in a DGKα/ζ expression-dependent manner. Again, DGKα regulates these PA signaling lipids more so than DGKζ as the removal of the type 1 DGK isoform leads to higher dysregulation of their abundances. Additionally, activation of DGKα or ζ KO Jurkats influences PS levels but the double-KO results in accumulation of this phospholipid to a greater degree.

We plan to use these newly identified DGK lipid biomarkers from our HEK293T and Jurkat T cell studies to target these lipids in primary mouse and human T cells to understand the substrate specificity of this enzyme in T cell biology. These data are part of works in progress that we plan to publish in the future.

Major Task 2: Test whether ritanserin modulates T cell biology and enhances anti- tumor immunity

- Subtask 1: Establishing preclinical melanoma models by injecting PmeI CD8+ T cell transferred-C57BL/6J mice with B16F1 melanoma cells

  Subtask 2: Treatment of tumor-bearing mice with vehicle, ritanserin, or ketanserin (50 mg kg^{-1}, once daily)

  Subtask 3: Isolate TIL and effector CD8+ T cells from vehicle/compound-treated mice by flow cytometry

  Subtask 9: Measure primary tumor size, lung metastases, and overall survival of ritanserin-treated tumor-bearing mice [12 mice X 3 groups = 36 mice total]

We have generated data showing promising efficacy for ritanserin in combination with the anticancer drug gefitinib in an established $NRAS^{Q61R}$-driven melanoma mouse model developed by our collaborator Sheri Holmen[8]. We found that the combination of

oral ritanserin (100 mg/kg) and gefitinib (200 mg/kg) significantly increased survival compared with delivery of either agent alone.

Importantly, 2 of 6 mice tested with the combination showed complete resolution of their tumor beyond termination of therapy, and we observed increased CD8 T cell infiltration in treated mice compared with controls. These studies were performed with collaborators here at UVA including Tim Bullock, Michael Weber, Dan Gioeli, BJ Purow, and Thurl Harris as well as Sheri Holmen at the University of Utah. Additional replicate studies are needed to determine if this effect is reproducible across different cohorts of mice. These data are part of works in progress and will be published in the future.

- Subtask 4: Assay proliferation of TILs and effector CD8+ T cells by CFSE and flow cytometry [12 mice X 3 groups = 36 mice total]

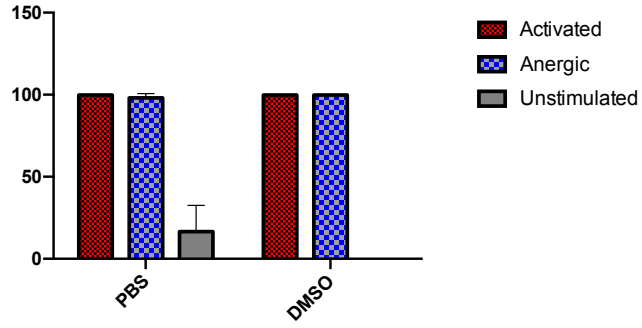
  Subtask 5: Assay IL-2 and IFN-gamma production by TILs and effector CD8+ T cells by intracellular cytokine staining [12 mice X 3 groups = 36 mice total]

  Subtask 6: Assay production of perforin and granzyme B by TILs and effector CD8+ T cells [12 mice X 3 groups = 36 mice total]

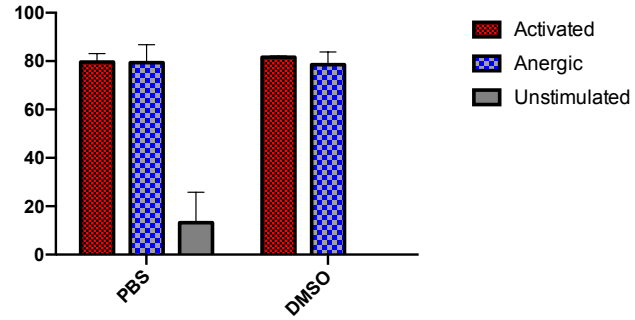
  Subtask 7: Assay viability of TILs and effector CD8+ T cells by CellTiter-Glo [12 mice X 3 groups = 36 mice total]

  Subtask 8: Assay trafficking by TILs and effector CD8+ T cells by flow cytometry [12 mice X 3 groups = 36 mice total]

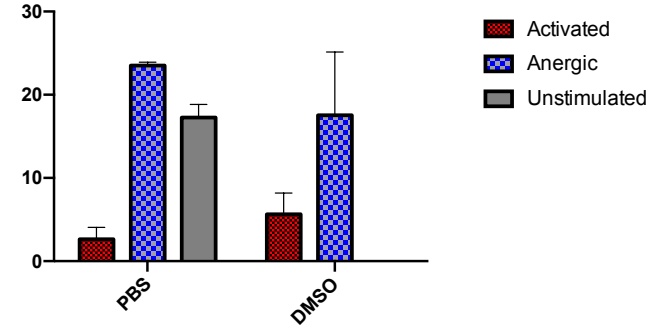
IFNg+ Percentage (CD44+/CD25+, Live, CD8+)



TNFa+ Percentage (CD44+/CD25+, Live, CD8+)



IL2+ Percentage (CD44+/CD25+, Live, CD8+)



Activated Ki67+ Percentage (Live, CD8+)

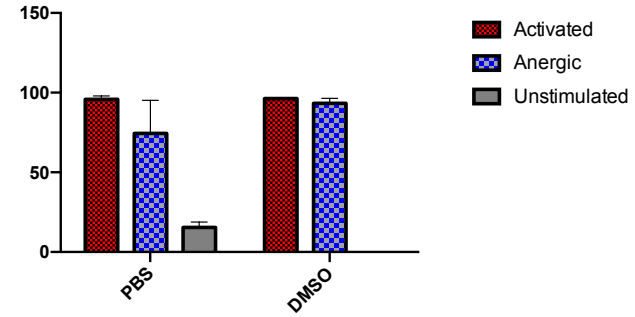


Figure 4. We established flow cytometry assays for evaluating proliferation (Ki67), cytokine production (IFNγ, IL-2, TNFα), and activation (CD25, CD44) of primary CD8+ T cells. Primary CD8+ T cells were isolated from mouse spleens using commercial immunopurification kits and activated using standard (CD3+CD28) or anergic (CD3 only) conditions and subjected to flow cytometry analyses.

In collaboration with Tim Bullock's group, we have established the proposed flow cytometry assays to evaluate effects of ritanserin treatments on primary CD8+ T cell function (Figure 4). The ability to assess activation, proliferation, and cytokine response of T cells using flow cytometry will provide an important tool to understand ritanserin pharmacology with high resolution at the single cell level. These data are part of works in progress that will be published in the future.

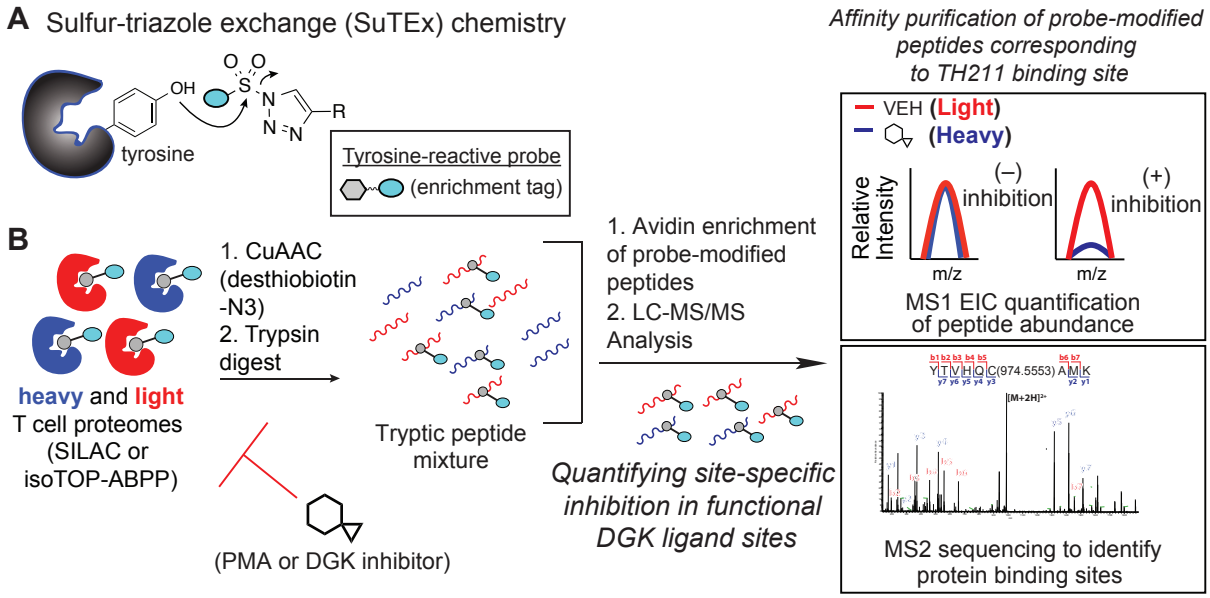


Figure 5. Chemical proteomic platform for activity-based profiling of native DGKs in T cells. (A) Sulfur-triazole exchange (SuTEx) chemistry for driving innovations in developing DGK activity-based probes and inhibitors. (B) LC-MS chemical proteomics to identify site of binding (peptide sequencing by MS2) for ligands (DGK inhibitor) and substrates (PMA is a DAG mimetic) by competition with TH211 probe labeling, which reduces probe-modified peptide signals (+ inhibition, MS1) that can be quantitated by SILAC or isoTOP-ABPP (for primary T cells) peptide ratios. Light (vehicle) and heavy (+competitor) proteomes from lysate or live cell studies are mixed prior to protease digestion, enrichment of probe-modified peptides, and LC-MS analyses.

As part of our exploration of DGKα inhibitors, we developed sulfur-triazole exchange (SuTEx) chemistry as a new electrophile for covalent modification of tyrosine residues across thousands of functional protein binding sites in lysates and live cells (Figure 5A). Importantly, we could modify the structure of SuTEx probes to achieve high selectivity for tyrosine over other amino acids modified in the proteome (>5-fold). We believe this new chemistry combined with our chemical proteomics technology (Figure 5B) can be applied to improve the inhibitory activity of ritanserin (e.g. through development of a covalent ritanserin analog) against DGKα. These findings are published in the following References [9,10]

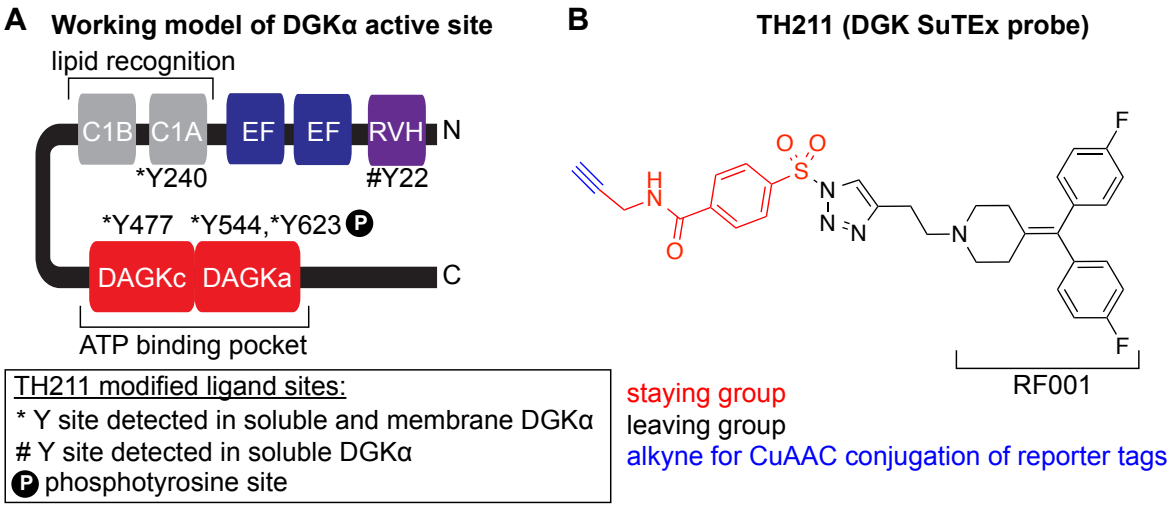


Figure 6. Proposed interdomain architecture and corresponding activity-based probes of the DGKa active site. (A) The DGKa active site is composed of the C1, DAGKa, and DAGKa domains based on our chemical proteomic and metabolomics studies. (B) TH211 is a DGK activity-based probe that binds and covalently modifies tyrosine residues in the active site of native human DGKa in live T cells. TH211-modified sites on DGKa from live T cell treatments (50 μM TH211, 2 hours) are depicted in A and detected using the platform shown in Figure 4.

Building on these studies, we developed a new DGK-directed SuTEx probe TH211 for chemical proteomic evaluation of native DGKα active site through covalent binding to tyrosine sites in the C1 (Y240), catalytic (Y477), and accessory domain (Y544, Y623) of native DGKα (Figure 6A). Our rationale for developing tyrosine-reactive probes is the capability for liganding phosphotyrosine sites (e.g. Y623 on DGKα, Figure 6B), which presents an exciting opportunity for targeting DGK function through a post-translational modification (PTM) site. These findings will be reported in a future publication.

Subtask 10: Measure primary tumor size, lung metastases, and overall survival of ritanserin treatment in immunodeficient mice [12 mice X 3 groups = 36 mice total]

We did not complete this task because we are still finishing analysis of ritanserin effects in immunocompetent mice (see findings from above). We plan to complete this task in future studies.

References:

1. Franks, C.E., Campbell, S.T., Purow, B.W., Harris, T.E. & Hsu, K.L. The Ligand Binding Landscape of Diacylglycerol Kinases. *Cell Chem Biol* **24**, 870-880 e875 (2017). pmcid:**PMC5551460**
2. McCloud, R.L., Franks, C.E., Campbell, S.T., Purow, B.W., Harris, T.E. & Hsu, K.L. Deconstructing Lipid Kinase Inhibitors by Chemical Proteomics. *Biochemistry* **57**, 231-236 (2018). pmcid:**PMC5771882**
3. Campbell, S.T., Franks, C.E., Borne, A.L., Shin, M., Zhang, L. & Hsu, K.L. Chemoproteomic Discovery of a Ritanserin-Targeted Kinase Network Mediating Apoptotic Cell Death of Lung Tumor Cells. *Mol Pharmacol* **94**, 1246-1255 (2018). pmcid:**PMC6160665**
4. Franks, C.E. & Hsu, K.L. Activity-Based Kinome Profiling Using Chemical Proteomics and ATP Acyl Phosphates. *Curr Protoc Chem Biol* **11**, e72 (2019). 10.1002/cpch.72
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6. Ware, T.B., Shin, M. & Hsu, K.-L. Chapter Seventeen - Metabolomics analysis of lipid metabolizing enzyme activity. in *Methods in Enzymology*, Vol. 626 (ed. Garcia, B.A.) 407-428 (Academic Press, 2019).
7. Knott, G.J. & Doudna, J.A. CRISPR-Cas guides the future of genetic engineering. *Science* **361**, 866-869 (2018). 10.1126/science.aat5011
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9. Hahm, H.S., Toroitich, E.K., Borne, A.L., Brulet, J.W., Libby, A.H., Yuan, K., Ware, T.B., McCloud, R.L., Ciancone, A.M. & Hsu, K.L. Global targeting of functional tyrosines using sulfur-triazole exchange chemistry. *Nat Chem Biol* **16**, 150-159 (2020). pmcid:**PMC6982592**
10. Brulet, J.W., Borne, A.L., Yuan, K., Libby, A.H. & Hsu, K.L. Liganding Functional Tyrosine Sites on Proteins Using Sulfur-Triazole Exchange Chemistry. *J Am Chem Soc* **142**, 8270-8280 (2020). 10.1021/jacs.0c00648

What opportunities for training and professional development has the project provided?

In year 1, two of my senior graduate students were provided the opportunity to share and present their research via poster presentations at the Spring 2018 255th American Chemical Society National Meeting in New Orleans, LA. Both students were also provided the opportunity to present posters of their research and one student gave an oral presentation at the highly competitive 2018 Bioorganic Gordon Research Seminar and Bioorganic Gordon Research Conference in Andover, NH. In year 2, three of my senior graduate students were provided the opportunity to share and present their research via poster presentations at the highly competitive 2019 Gordon Research Conference on High Throughput Chemistry & Chemical Biology in New London, NH. In 2020, travel for students was not possible to the COVID-19 pandemic.

How were the results disseminated to communities of interest?

Results from our studies were disseminated to the chemistry and cancer community in the form of peer-reviewed manuscripts and conference abstracts both at the American Chemical Society,

Bioorganic Gordon Research Seminar, and the American Association for Cancer Research. In addition, I was invited to present a seminar at 30+ universities and institutions in the past 3 years to discuss my DGK research. We have also listed our publications on the lab website (hsulab.com), which provides additional opportunities for the local, national, and international general public to see the outcomes from our proposed studies.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

Our studies have impacted the chemistry and cancer immunology field by revealing that DGKα is active in primary human T cells and blockade of this enzyme has profound effects on T cell metabolism. We have also shown that ritanserin can kill tumor cells at concentrations that are non-toxic to noncancerous primary immune cells and demonstrates the synergistic potential of this drug for chemo- and immuno-therapy. In this last year, we also have generated preclinical data in mouse models showing the promising efficacy of ritanserin for activating CD8+ T cell responses to control tumor outgrowth and a cohort of mice. We also introduced new chemistry to further improve the inhibitory activity of ritanserin by converting into a compound that works through a covalent mechanism. Collectively, our studies continue to explore the druggability of DGKα and how blockade of this enzyme can be exploited for activating T cells in the tumor environment.

What was the impact on other disciplines?

Our findings have impacted fields outside of chemistry and cancer immunology by showing the lipid biology field that chemical biology approaches can provide fundamental information on how lipid kinases select lipid substrates in cells. We continue to pave the way for innovative chemical approaches to tackle lipid kinases.

What was the impact on technology transfer?

We introduced new covalent chemistry that can impact therapeutic and drug discovery by our group and the wider academic and pharmaceutical industry. We discovered new lipid biomarkers for DGKα function in T cells. We believe these findings will not only enable drug discovery efforts in our group but also allow other research group and pharmaceutical companies to explore this target.

What was the impact on society beyond science and technology?

We believe our studies and findings have broader impacts in society by continuing to show our commitment to drug fat metabolism for immuno-oncology. Working with fats, especially as signaling molecules, is challenging and we are enthusiastic about educating the public and providing tools for the wider scientific community to study these metabolites.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

1. Franks, C.E., Campbell, S.T., Purow, B.W., Harris, T.E. & Hsu, K.L. The Ligand Binding Landscape of Diacylglycerol Kinases. *Cell Chem Biol* **24**, 870-880 e875 (2017). pmcid:**PMC5551460**
2. McCloud, R.L., Franks, C.E., Campbell, S.T., Purow, B.W., Harris, T.E. & Hsu, K.L. Deconstructing Lipid Kinase Inhibitors by Chemical Proteomics. *Biochemistry* **57**, 231-236 (2018). pmcid:**PMC5771882**

3. Campbell, S.T., Franks, C.E., Borne, A.L., Shin, M., Zhang, L. & Hsu, K.L. Chemoproteomic Discovery of a Ritanserin-Targeted Kinase Network Mediating Apoptotic Cell Death of Lung Tumor Cells. *Mol Pharmacol* **94**, 1246-1255 (2018). pmcid:**PMC6160665**
4. Franks, C.E. & Hsu, K.L. Activity-Based Kinome Profiling Using Chemical Proteomics and ATP Acyl Phosphates. *Curr Protoc Chem Biol* **11**, e72 (2019). 10.1002/cpch.72
5. Ware, T.B., Franks, C.E., Granade, M.E., Zhang, M., Kim, K.B., Park, K.S., Gahlmann, A., Harris, T.E. & Hsu, K.L. Reprogramming fatty acyl specificity of lipid kinases via C1 domain engineering. *Nat Chem Biol* **16**, 170-178 (2020). 10.1038/s41589-019-0445-9
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8. Brulet, J.W., Borne, A.L., Yuan, K., Libby, A.H. & Hsu, K.L. Liganding Functional Tyrosine Sites on Proteins Using Sulfur-Triazole Exchange Chemistry. *J Am Chem Soc* **142**, 8270-8280 (2020). 10.1021/jacs.0c00648

Presentations.

1. *Sulfur-Triazole Exchange Chemistry: Applications for Chemical Proteomics and Protein Ligand Discovery*. Department of Pharmaceutical Sciences, UC Irvine, Irvine, CA (2020).

2. *Chemical Biology and Chemistry for Translational Lipid Biology and Beyond*. Department of Chemistry, Virginia Tech, Blacksburg, VA (2019).
3. *Chemical Biology and Chemistry for Translational Lipid Biology and Beyond*. Department of Chemistry and Department of Molecular and Structural Biochemistry, NC State, Raleigh, NC (2019).
4. *Chemical Biology and Chemistry for Translational Lipid Biology*. Department of Chemistry and Biochemistry, UNC Wilmington, Wilmington, NC (2019).
5. *Chemical Biology and Chemistry for Translational Lipid Biology and Beyond*. Department of Chemistry, University of Virginia, Charlottesville, VA (2019).
6. *Tunable Chemistry for Global Discovery of Protein Function and Ligands*. Southeast Regional Meeting of the American Chemical Society (SERMACS), Savannah, GA (2019).
7. *Targeting Endocannabinoid Biosynthetic Pathways for Inflammation and Pain*. Chemistry and Pharmacology of Drug Abuse Conference, Boston, MA (2019).
8. *Tunable Chemistry for Global Discovery of Protein Function and Ligands*. Gordon Research Conference: Enzymes, Coenzymes and Metabolic Pathways, Waterville Valley, NH (2019).
9. *Mapping Diacylglycerol Pathways for Immunomodulation*. Department of Biochemistry and Molecular Pharmacology, UMASS Medical School, Worcester, MA (2019).
10. *Mapping Diacylglycerol Pathways for Immunomodulation*. Chemistry Department, Boston College, Chestnut Hill, MA (2019).
11. *Mapping Diacylglycerol Pathways for Immunomodulation*. Department of Chemistry, The Scripps Research Institute, La Jolla, CA (2019).

12. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC (2019).
13. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Department of Chemistry, Duke, Durham, NC (2019).
14. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Department of Chemistry, New York University, New York, NY (2019).
15. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Chemical Biology Program, Memorial Sloan Kettering Cancer Center, New York NY (2019).
16. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Center for Cancer Research, National Cancer Institute, Bethesda, MD (2019).
17. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Department of Chemistry, The Scripps Research Institute, Jupiter, FL (2019).
18. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Vanderbilt Institute of Chemical Biology, Nashville, TN (2019).
19. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Department of Chemistry, Temple University, Philadelphia, PA (2019).
20. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Pharmacology and Chemical Biology Seminar Series, Baylor College of Medicine, Houston, TX (2019).
21. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Chemical Biology & Therapeutics Department, St. Jude Children's Research Hospital, Memphis, TN (2019).
22. *Exploring Ligand Binding Space to Discover New Lipid Biology*. College of Chemistry, UC Berkeley, Berkeley, CA (2019).

23. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Molecular Physiology and Biological Physics Seminar Series, UVa, Charlottesville, VA (2018).
24. *Exploring Ligand Binding Space to Discover New Lipid Biology*. Thermo Fisher Scientific / METRIC symposium, North Carolina State University, Raleigh, NC (2018).
25. *Using mass spectrometry to exploit a single isoleucine/leucine difference in ERK substrate binding sites for activity-based profiling of MAPK signaling*. Advances in Mass Spectrometry session, 256th American Chemical Society National Meeting, Boston, MA (2018).
26. *Chemical proteomic discovery of new agents that induce apoptotic cell death of lung tumor cells*. Early Career Investigators in Biological Chemistry session, 256th American Chemical Society National Meeting, Boston, MA (2018).
27. *Chemoproteomic discovery of ligand binding hotspots in the lipid kinome*. Molecular Basis of Signaling Spotlight Session, American Society for Biochemistry and Molecular Biology Annual Meeting, San Diego, CA (2018).
28. *Chemoproteomic Strategies to Decode Metabolic Regulation of Lipid Signaling*. Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA (2018).
29. *Chemoproteomic discovery of lipid kinase inhibitors for immuno-oncology*. Early Career Investigators in Biological Chemistry session, 255th American Chemical Society National Meeting, New Orleans, LA (2018).
30. *Chemoproteomic Strategies to Decode Metabolic Regulation of Lipid Signaling*. Department of Biochemistry, Virginia Tech, Blacksburg, VA (2018).

31. *Using Mass Spectrometry to Understand and Target Diacylglycerol Metabolism and Signaling*. Thermo Scientific Mass Spec Users' Meeting, Bethesda, MD (2017).
32. *Targeting the lipid kinome for cancer immunotherapy*. Southeast Regional Meeting of the American Chemical Society (SERMACS), Charlotte, NC (2017).
33. *Fighting melanoma through the body's immune system*. Heritage University in partnership with the Leadership Alliance, Toppenish, WA (2017).
34. *Liposomal DAGLB inhibitors - towards targeted NSAIDs for treatment of chronic inflammation*. Virginia Nanomedicine Symposium, UVA, Charlottesville, VA (2017).
35. *Chemical Proteomic Profiling of Diacylglycerol Kinases*. Southeastern Chemical Biology Symposium, University of Georgia, Athens, GA, (2017).

Conference abstracts.

1. Borne AL, Brulet JW, Hahm HS, Toroitich EK, Libby AH, Yuan K, and Hsu KL. Identification, Characterization and Applications of a Global Chemical Probe. Poster presentation delivered at the Gordon Research Conference – High Throughput Chemistry & Chemical Biology, New London, NH (2019).
2. Ware TB, Franks CE, Granade M, Harris TE, and Hsu KL. Diacylglycerol kinase fatty acyl specificity is encoded by C1 domains. Poster presentation delivered at the Gordon Research Conference – High Throughput Chemistry & Chemical Biology, New London, NH (2019).
3. Brulet JW, Borne AL, and Hsu KL. C1-Tailored Activity Based Probes Function as Membrane Sensors of Protein Activation. Poster presentation delivered at the Gordon Research Conference – High Throughput Chemistry & Chemical Biology, New London, NH (2019).

4. Franks CE, McCloud RL, Campbell ST, Purow BW, Harris TE, Hsu KL. Quantitative chemical proteomics to evaluate lipid kinase inhibitor binding profiles. Oral presentation delivered at the Bioorganic Chemistry Gordon Research Seminar, Andover, NH, June 2018.
5. Franks CE, McCloud RL, Campbell ST, Purow BW, Harris TE, Hsu KL. Quantitative chemical proteomics to evaluate lipid kinase inhibitor binding profiles. Poster presentation delivered at the Bioorganic Chemistry Gordon Research Conference, Andover, NH, June 2018.
6. Campbell ST, Franks CE, Borne AL, McCloud RL, Hsu KL. Using Activity-Based Protein Profiling Proteomics to Determine Novel Pathways and Therapeutic Monitoring Targets. Oral presentation delivered at the ACLPS Annual Meeting, Houston, TX, June 2018.
7. Hsu, KL. Targeting diacylglycerol kinases for immuno-oncology. Poster presentation delivered at the American Association for Cancer Research Annual Meeting, Chicago, IL, April 2018.
8. Franks CE, Campbell ST, Purow BW, Harris TE, Hsu KL. Development of a chemical proteomic strategy to target diacylglycerol kinases. Poster presentation delivered at the 255th ACS National Meeting, New Orleans, LA, March 2018.
9. Campbell ST, Franks CE, Borne AL, McCloud RL, Hsu KL. Using Activity-Based Protein Profiling Proteomics to Determine Novel Pathways and Therapeutic Monitoring Targets. Poster presentation delivered at the MSACL - Annual Congress is Clinical Mass Spectrometry, Palm Springs, CA, January 2018.

- **Website(s) or other Internet site(s)**
  https://www.youtube.com/watch?v=nJULdCGgduc&feature=youtu.be
- **Inventions, patent applications, and/or licenses**

Title: Global targeting of functional tyrosines in proteomes using sulfur-triazole exchange chemistry Inventors: Ku-Lung Hsu, Heung Sik Hahm, Emmanuel Toroitich, Jeffrey W. Brulet, and Adam L. Borne Invention Report Date: 10/30/2019 Federal funding: NIH grants DA035864 and DA043571; ARMY/MRMC grant W81XWH-17-1-0487 Provisional patent: 62/929,473 filed on 11/1/2019 PCT patent: PCT/US2020/024286 filed on 3/23/2020

- **Other Products**

  Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project? (Person months are total for 3 years)

Name:	*Ku-Lung Hsu, PhD*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-5620-3972*
Nearest person month worked:	11
Contribution to Project:	*Prof. Hsu is responsible for planning and direction of the project, for data analyses, and for scientific reports and publications. He is also responsible for training and mentoring of graduate students and postdoctoral fellows.*
Funding Support:	This award and departmental funding

Name:	*Caroline Franks*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-1728-1526*
Nearest person month worked:	*4*
Contribution to Project:	*Ms. Franks performed work in the area of chemical proteomics and mass spectrometry experimentation, and data analysis.*
Funding Support:	Departmental fellowship

Name:	*Sean Campbell, PhD*
Project Role:	*Postdoctoral Fellow*

Researcher Identifier (e.g. ORCID ID):	*0000-0001-5465-5107*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Campbell was responsible for executing standard molecular biology, biochemistry, cell biology, and pharmacology experiments.*
Funding Support:	*The National Cancer Institute (training grant)*

Name:	*Jeffrey Brulet*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	0000-0003-1959-2475
Nearest person month worked:	13
Contribution to Project:	*Mr. Brulet performed work in the area of design, synthesis, and evaluation of small molecules.*
Funding Support:	This award

Name:	*Tao Huang, PhD*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	16
Contribution to Project:	*Mr. Huang performed work in the area of design, synthesis, and evaluation of small molecules.*
Funding Support:	This award

Name:	*Roberto Mendez*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	*Mr. Mendez performed work to annotate lipid substrate specificity of DGKs*

Funding Support:	This award

Other individuals who were paid from the award, but worked less than one-month:
Timothy Bullock, PhD, Collaborator
Elizabeth Lee, laboratory Technician

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable.

QUAD CHARTS: Not applicable.

9. APPENDICES: Not applicable