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14. ABSTRACT A dearth of truly high throughput mechanisms to screen ion channels has hampered the development of pharmacological tools and therapeutics for effectively treating diseases involving ion channels (channelopathies). Ion channels are drug targets for pain, cardiac function, and neurological disorders. We have developed a soluble assay for ion channels using a phosphatidylinositol 4,5-bisphosphate fluorescent probe (FL-PIP2) and detergent purified two pore domain potassium (K2P) and inward rectifying potassium (Kir) channels fused with a nano luciferase luminescent protein (n-Luc). The assay works by bioluminescence energy transfer (BRET). The objective of this grant is to establish a low-cost, soluble approach to screening broad families of ion channels and their many subtypes against libraries of >1 million compounds. We hypothesize that selective allosteric modulators of ion channels can be identified by observing the displacement of the ion channel regulatory lipid PIP2.					
15. SUBJECT TERMS Bioluminescence resonance energy transfer (BRET), nano-luciferase (nLuc), phosphatidic acid (PA), phosphatidylinositol 4,5 bisphosphate (PIP2), Twik related potassium channel K2P2.1 (TREK-1), high throughput screening (HTS), inward rectifier potassium (Kir) channel.					
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INTRODUCTION:

A lack of truly high throughput mechanisms to screen ion channels has hampered the development of medication for effectively treating pain in particular chronic pain. Ion channels are excellent targets but truly high throughput assays to purified proteins are lacking. We have developed a soluble assay an analgesic channel TREK-1 using a fluorescent lipid that binds to a regulatory site in the channel. The purpose of this 18-month grant is to show feasibility of lead compound identification for TREK-1 using automated high throughput equipment and to investigate the feasibility of adapting the assay to other pain channels. As part of the assay development an optimal protein and appropriate z'scores need to be established. The z'score is a measure of the assay's reliability. Its particularly important for HT-screening since the cost of doing replicates and the certainty of each hit compound need to be established.

KEYWORDS:

Bioluminescence resonance energy transfer (BRET), nano-luciferase (nLuc), phosphatidic acid (PA), phosphatidylinositol 4,5 biphosphate (PIP2), Twik related potassium channel K2P2.1 (TREK-1), high throughput screening (HTS), inward rectifier potassium (Kir) channel. Diacylglycerol pyrophosphate (DGPP)

ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1 Expand the BRET assay to additional channels, Kir 3.2, Kir2.3, and Kv7.2.

Aim2. Miniaturize human TREK-1 for full HTS automation and screen against a 25k subset of the Scripps's drug discovery chemical library.

Aim3 Determine the mechanism of action of hydroxychloroquine in SARS-CoV2 infection.

What was accomplished under these goals?

Aim 1: Expand the BRET assay to additional channels.

- 1) Major activities: We cloned Kir2.3, Kir1.1, Kir 3.2 with an nanoluciferase attached to the protein and expressed them in yeast expression. We used cKir2.2 as a positive control for Kir3.2.

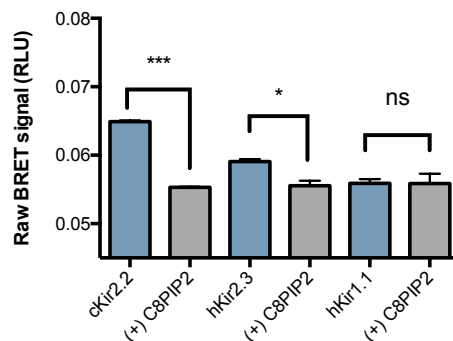


Figure 1. BRET assay for nLuc Kir2.3. from whole cell lysates. In blue is a signal with 500 nM TMR-PIP2 and in grey TMRPIP2 + 100 uM C8-PIP2. The non-fluorescent C8-PIP2 competes with the FL-PIP2 to reveal a specific PIP2 signal. Despite having a decent signal in the whole cell lysate, the Kir2.3 did not retain activity when purified.

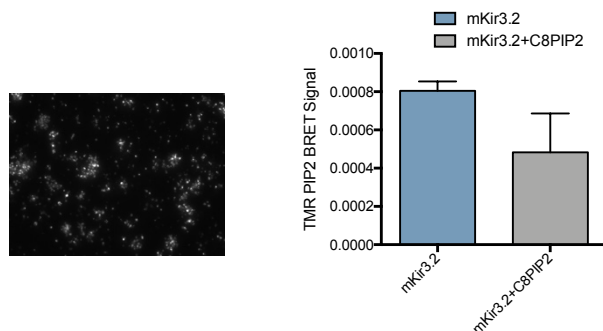


Figure 2 (left) shows expression of GFP tagged Kir3.2 in yeast cells. (right) Purified nLuc Kir3.2 in a BRET assay. 1 μ M FL-PIP2 bound weakly to Kir3.2. The protein is stable for a HTS assay.

2) Conclusions:

Kir3.2 appears suitable for screening for allosteric agonists directly with purified protein in a HTS format. The PIP2 is barely bound without the agonist, so any increase in FL-PIP2 affinity should increase the signal. The assay is not suitable for finding direct competitors, but since Kir3.2 is a potassium channel and decreases neuronal firing, an allosteric agonist is ideal.

Kir2.3 was selected for specificity. The ability to screen for ligands to Kir2.3 would be nice but not necessary. Drugs that are found binding to Kir2.2 or Kir3.2 could be screened against the Kir2.3 prep we have developed. Future work could likely improve the biochemical stability of Kir2.3.

We synthesized a fluorescent pyro-phosphatidic acid (FL-PPA) probe similar to our fluorescent PIP2 probe. The PPA probe would be less expensive for screening >100K compounds if the affinity were tighter than PIP2. However, the affinity wasn't sufficiently high to justify the change. We will likely develop a secondary screen with the FL-PPA.

Aim2. Miniaturize human TREK-1 for full HTS automation and screen against a 25k subset of the Scripps's drug discovery chemical library.

1) Major activities.

Preparing the TREK-1 protein for automated 25K pilot compound screening and establishing z'scores on automation equipment. Characterizing ML335 TREK small molecule agonists and its potential as a positive control in our assay. We also synthesized and tested a new BRET probe. Rhodamine pyrophosphate.

2) Specific Objectives.

Stabilizing the human TREK-1 protein. Our preliminary data was all with a zebrafish TREK-1 that had been stabilized for x-ray crystallography studies. The human TREK-1 is a better target, so we tested it for protein expression, stability, and sensitivity in our soluble BRET assay.

Establishing appropriate z' scores for our TREK-1 BRET assay on automated equipment.

We first tested a full-length human TREK-1 construct. A nLuc tag was added to the C-terminus of full-length human TREK-1. We expressed and purified the protein from yeast and tested its ability to detect FL-PIP2 binding in our BRET assay. The constructed tested fresh was sufficient for the BRET assay in the Hansen laboratory, but when the protein was handed off to the drug screening core their significant variability compared to the zebra fish protein. The protein didn't seem to give the same signal after a day or two. This is a problem for drug screening since a screening campaign can last several weeks. We spent another few months

truncating the human protein this helped substantially but it still wasn't quite as good for a single measurement screen. However, we did determine that the throughput of the assay was sufficient to run in triplicate with the current z' scores and

In an effort to improve the z-score we characterized a ML335 a TREK-1 agonist discovered in yeast screen by the laboratory of Dan Minor at UCSF. Concentration of ML335 in the micromolar range activate TREK-1 in mammalian cells. We found the drug can activate TREK-1 through phospholipase D which is a protein conserved in yeast and likely an off target effector of of their TREK-1 screen. While this suggest the compound is unsuitable as a positive control for our direct assay, it did highlight the need for direct assays for TREK-1 drug development. First, we tested ML335 in a flux assay to confirm its agonist like properties. The flux assay is a purified system and measures the ions the pass through the channel showing function. Compared to our control (a gain of function mutant), the ML335 compound had no activity. PLD2 is known to bind to and activate the channel by producing PA locally. For years anesthetics were also thought to agonize TREK-1 but we showed that inhaled anesthetics indirectly activated TREK-1 through PLD (Pavel PNAS 2020). We tested ML335 and found similar to inhaled anesthetic it activates PLD2. This finding is extremely important since it demonstrates that our assay that screens for direct activators will avoid being misled by a compound that doesn't appear to exert its effect directly through the channel. ML335 was the subject of a Nature paper and a science paper with the claim that this compound could be developed into an agonist for TREK-1. Our findings suggest this conclusion may be incorrect and direct mechanism of detection are needed.

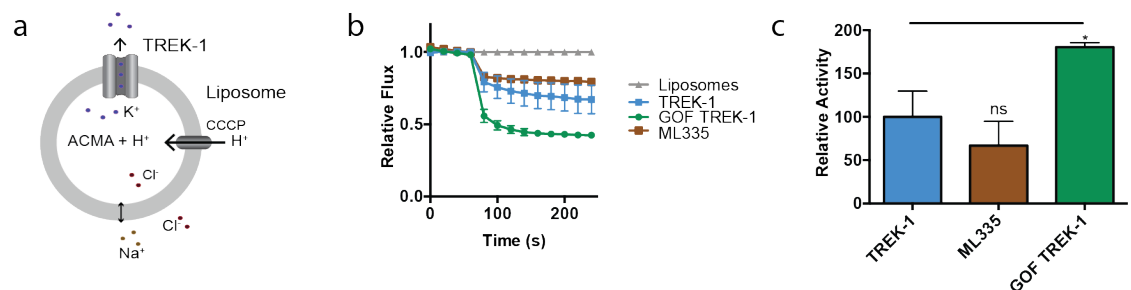


Figure 3. Ion flux assay with ML335. A setup of ion flux assay. When TREK-1 is activated it allows potassium ions to flow out. The outflow of potassium is couple to a fluorescent readout. (b) Flux correlates with a decrease in fluorescent. (c) the activity is quantified in. The gain of function mutant (double cystine that locks TREK-1 in the open conformation) shows an increase in Flux (green) compared to partially activated TREK-1 (blue). The agonist ML335 has no effect on the channel.

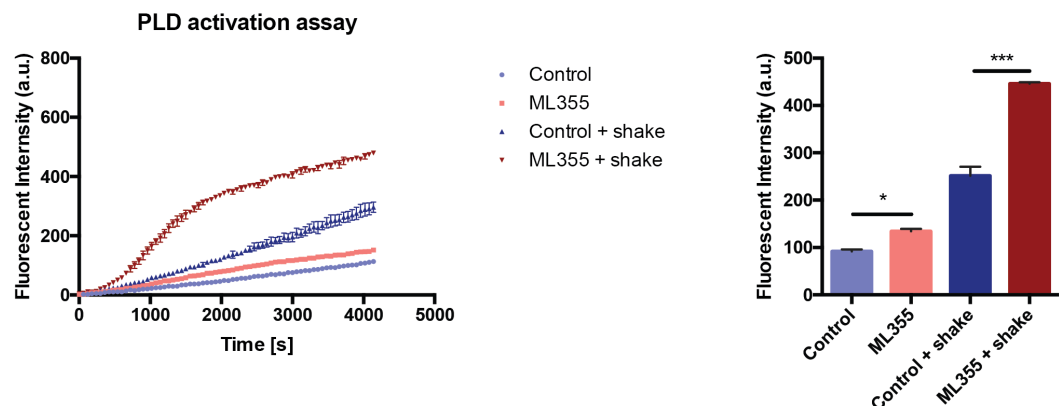


Figure 4. ML335 activates phospholipase D. A live cell fluorescent PLD product release assay is shown TREK-1 agonist ML335. ML335 is thought to directly activate TREK-1 and was intended to be a control for our BRET assay. But as can be seen in the data The drug is activating PLD. PLD is also mechanosensitive and the adding mechanical shear greatly enhances ML335 activation of PLD.

Future experiments will need to be done to confirm our finding by transfecting HEK cells with TREK-1 and a catalytically dead mutant of PLD2 and showing ML335's effect is all through PLD2 and not through direct activation. We had planned to do similar experiments on novel compounds identified from the 25K screen. We

still plan to do these experiments but its important that we characterize the control. If the control isn't what the literature says it is, then we can't use it as our control.

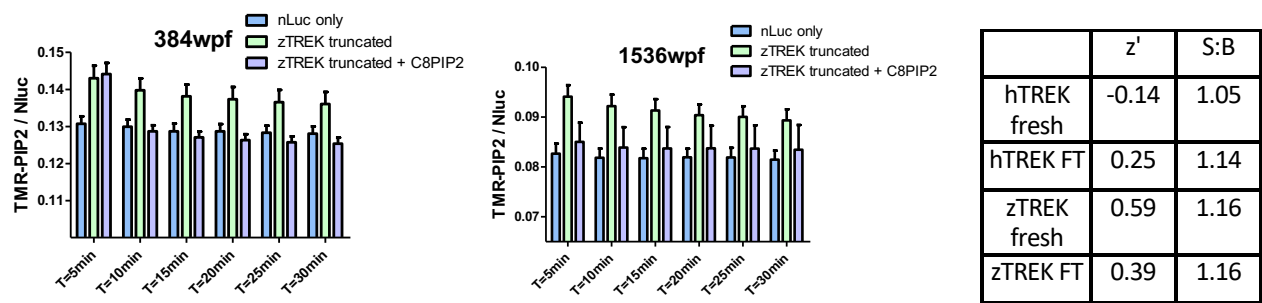


Figure 5 implementation screening core. (left) Cell free TREK-1 BRET assay optimized for 384-well plate and run on implementation equipment in the drug screening core. nLuc tagged zTREK is compared to an nLuc only control (blue) and with non-fluorescent C8-PIP2 (dioctanoyl). C8-PIP2 clearly competes out TMR-PIP2. (middle) An unoptimized replicate run in 1536-well plate (only run one time). (right) A table compares human to zebra fish freeze-thawed (FT) and 4 degrees C for 4 days (fresh)..

As shown in Figure 3 we have reasonable z' scores for running the assay. Using freshly prepared zTREK. We have the assay reasonably worked out on 1536 plates.

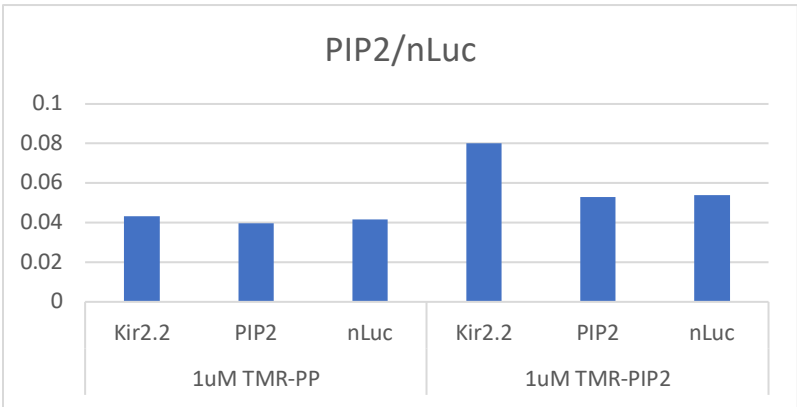


Figure 5 (left) Tetramethylrhodamine (TMR) Diacylglycerol pyrophosphatidic acid (TMR-PP) was synthesized and tested for binding to Kir2.2 ion channel using a BRET assay using nLuc tagged inward rectifier 2.2. (Kir2.2). No specific signal was seen compared to (right) TMR PIP2 .

We have determined that the screen can be completed in triplicate with fresh protein. The Hansen lab will prepare protein twice a week during the screening. The screen should be completed within a week. The reagents for the 25K screen will be ordered as follows.

One of the goals not met is the development of additional BRET assays for Kir7.2. In our review the reviewers commented that the scope of the grant was very ambitious for an 18-month grant. We have made advancements in aim one as outlined in the proposed work, including showing our BRET assay works for Kir2.3 and finding an agonist for Kir3.2. The new BRET probe, rhodamine diacyl glycerolpyrophosphate (TMR-PP) will likely be valuable for other proteins that binding glycerol phosphates with high affinity. The non-fluorescent DGPP is a full agonist for TREK-1 and bound slightly better than phosphatidic acid to TREK-1. We hoped the fluorescent (TMR-PP) would bind tight enough for our 25K screen. We tested first in Kir2.2 since it had the highest affinity. But this was not the case.

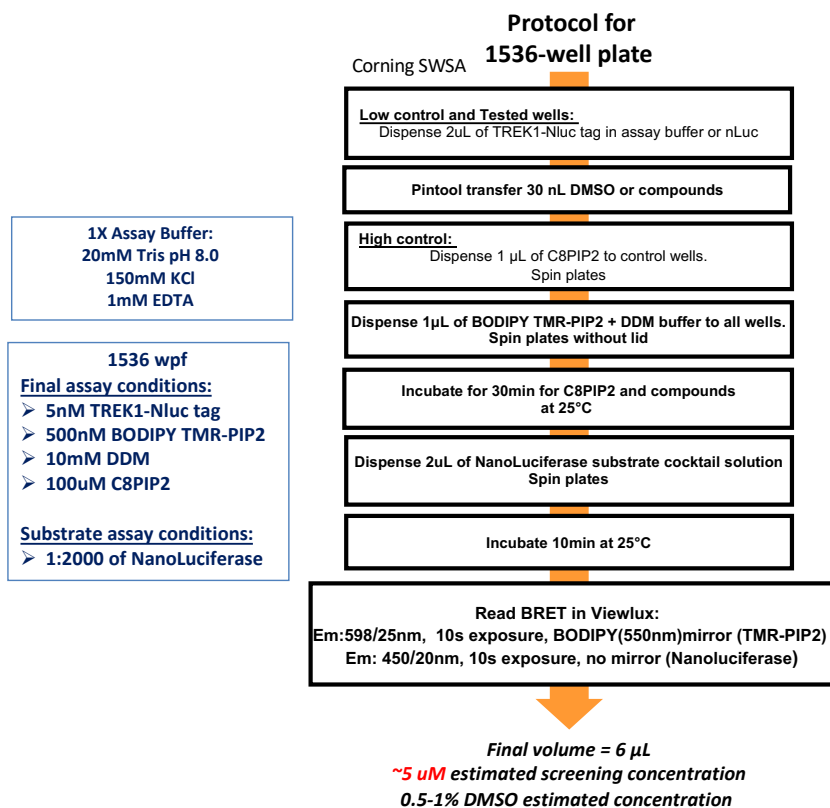


Figure 3. Final protocol for 25K HTS screen with purified protein zTREK-1 validated on HTS equipment in the scripps drug screening core.

PRIMARY	Final Assay conditions	Amount needed for 25K in 3X
TREK1 protein	5 nM	7 nMoles
BODIPY TMR-PIP2	500 nM	700 nMoles
n-Dodecyl-β-D-Maltopyranosid, Sol Grade (DDM)	7 mM	40 mMoles
NanoGlo-substrate	1/2000 Dilution	1 mL
C8PIP2 Positive Control	100 uM	7.7 uMoles
Nluc only protein	5 nM	0.1 mg (2 fresh preps)

Figure 4 Estimated reagents for a 25K screen.

AIM 3: Determine the mechanism of action of hydroxychloroquine in SARS-CoV2 infection.

The following was accomplished during a 6 week period during the COVID19 shutdown. Due to local and state regulations the Scripps Research Institute was not able to continue research except for in the case of COVID19 research. Since the mechanism we had been studying related to this grant could be applied to COVID19 research we added a COVID19 related aim which was completed in part with this grant with staff working part time in the lab.

Two major findings. 1) that cholesterol regulates uptake of the virus. This explained the severity of COVID19 in obese and smokers.

COVID19 cholesterol: To show the importance of cholesterol in Viral entry, we loaded kidney cells HEK and VeroE6 with cholesterol and tested entry of a pseudo virus. Cholesterol dramatically increased viral entry. A cholesterol lowering drug blocked entry both in veroE6 and in calu3 human lung cells.

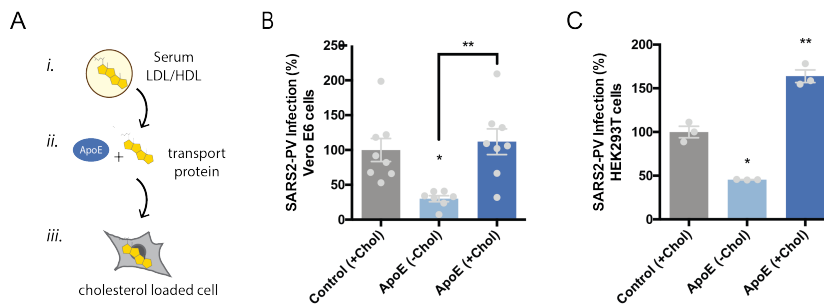
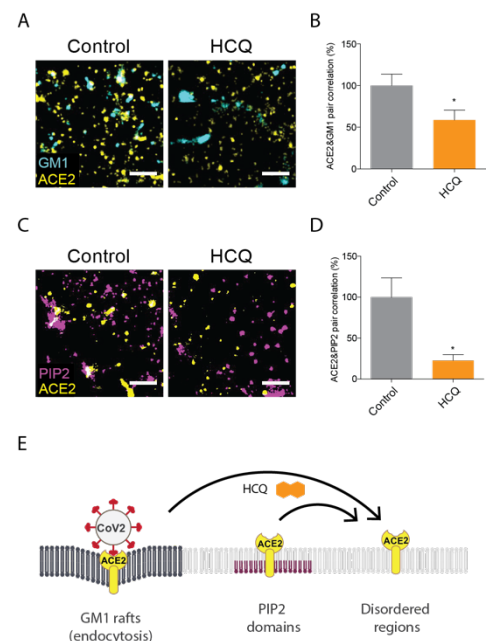


Figure 5. (B-C) SARS-CoV-2 pseudovirus (SARS2-PV) entry assay in Vero E6 cells (B) and HEK293T cells (C). Cells were treated with a luciferase expressing retrovirus pseudotyped with the SARS-CoV-2 spike protein that recapitulates viral entry. Infectivity was monitored by a luciferase activity in cells treated with or without apoE.

COVID19 Hydroxychloroquine (HCQ): In a separate study we looked at the mechanism of hydroxychloroquine's potential as a drug to treat COVID19 patients. Hydroxychloroquine had been identified early as a potential treatment based on its many years of use as an antimalarial compound and its use in treating autoimmune diseases.

In a mechanism very similar to ML335 activation of TREK-1, we showed that HCQ caused ACE2 to translocation from GM1 cluster. Furthermore, HCQ caused ACE2 to dissociate from PIP2 clusters, likely due to a positive charge in ACE2. See figure 8.

Fig. 3. Hydroxychloroquine moves ACE2 from GM1 rafts and PIP₂ domains. (A) Representative dSTORM super resolution images showing the effect of HCQ (50 μ M) on the nanoscale localization of ACE2 (yellow) with GM1 rafts (cyan) after loading HEK293T cells with cholesterol (scale bars = 1 μ m). (B) Percent of pair correlation (Fig. S3A) calculated at short distances (0-5 nm). HCQ decreased the pair correlation between ACE2 and GM1 rafts indicating a decrease in association between PLD and GM1 rafts. Data are expressed as mean $\pm$ s.e.m., * $P \leq 0.05$, unpaired t-test, $n=6$. (C) Representative dSTORM super resolution images of ACE2 (yellow) and PIP₂ domain (magenta) in HEK293T cells at normal cholesterol level after the treatment of HCQ (50 μ M) (scale bars = 1 μ m). (D) HCQ decreased the pair correlation between ACE2 and PIP₂ domains indicating a decrease in association between PLD and PIP₂ domains. Data are expressed as mean $\pm$ s.e.m., * $P \leq 0.05$, unpaired t-test, $n=5$. (E) Model showing HCQ (orange hexagon) inducing translocation of ACE2 (yellow receptor) from GM1 rafts (dark grey lipids) in high cholesterol. HCQ disrupts ACE2 interaction with PIP₂ domains causing ACE2 to translocate to the disordered region.



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

The main driver of this project is Hao Wang, a new graduate student in the lab. Hao has learned how to purify protein, run BRET and live cell PLD assays, and setup an HT drug screening campaign.

Arif Pavel, a postdoctoral researcher in the lab conducted the electrophysiology on the ML335 test compound. Dr. Pavel learned to reconstitute TREK-1 and perform ion flux assays with purified TREK-1 channel. Dr. Pavel already had experience with electrophysiology, but he also applied these techniques for testing ML335. Dr.

Pavel obtained a faculty position (research assistant professor) at the University of Illinois at Chicago and his training on this grant was in part responsible for obtaining his position.

How were the results disseminated to communities of interest?

Hao Wang reported the initial findings of the project titled “A Soluble Ion Channel Binding Assay for Allosteric Modulator Screening”, presented at the Experimental Biology (EB) meeting and published in the journal FASEB. EB is the main annual forum for the American Society for Pharmacology and Experimental Therapeutics (ASPET) with more than 10,000 scientists in attendance.

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

As this is a final report there is not a next reporting period. Nonetheless, we do plan to submit an R61 grant for a full screen using the assay developed and validated by this work. We now have the data to confirm that an HT-screen is feasible. The screen will benefit from identification of a probe PAM. The z-scores would likely be better if we were comparing no signal to a full PAM signal. We are forced to estimate z scores from partially activated to inhibited. This is not ideal for validating a z'score but has no impact on identifying a PAM. Nonetheless, the z' score during automation was still good enough to move forward by doing the assay in triplicate. This will allow us to propose a full screen.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The impact on TREK-1 research is twofold. We showed the assay worked in automated form. Without the test PAM the z' scores will require doing the assay in triplicate, but this is still plenty good for a screen. The second finding was that ML335 activates PLD. This will be impactful to the field and very significant for the TREK-1 field and drug screening in general. Since its discovery ML335 has been assumed to exert all its effects through direct binding to the channel. We did not see any direct activation suggesting the effect of direct binding is likely working through the lipid binding site and PLD production of phosphatidic acid. Our data explains why development using structure activity has not yielded a high affinity ligand.

The data highlights the importance of having an assay with purified protein and identifying selective ligands that directly bind to the channel.

Our impact on the viral community is also significant. The preprint of the cholesterol paper has been cited almost 40 times in less than a year.

What was the impact on other disciplines?

Most disciplines assume that if a compound activates a channel in a cellular environment that it must be through direct binding (e.g., fields of anesthesia and depression). But many conserved proteins exist in all cell types and their role in activating a channel cannot be ruled out with cellular assays alone. This will be important to the fields of anesthesia and psychiatry where many hydrophobic drugs have been developed but interact with many channel targets.

Our application of the TREK-1 mechanism to viral entry showed a completely new way to think about virus research. We introduced techniques to study viral entry proteins moving between nanoscopic lipid compartments in the membrane.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

The public now understand that cholesterol and high fat diets are important for avoiding SARS-COV-2 infections. Eating better diets have no doubt helps to avoid the disease. More than 25,000 people read the preprint and 5 newspapers reported on our findings. An article on the paper in the Pittsburgh Post-Gazette trended number 1 for more than a day and with a circulation of 317,000 was seen by thousands of people.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

The grant was extended 6 months with the hope of completed the screen. But the screening project was put on hold due to COVID. The grant was only a 18th month grant and letting the staff go without pay would preclude the completion of the grant. Extending the time without doing the final screen depleted the funds for the final screen. Were possible with little to no impact on budget additional experiments in COVID19 research was added.

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

Due to COVID19 the final 25K screen has not completed. This limited the testing the assay at full rate.

Changes that had a significant impact on expenditures

Rather than let personnel go, the funds for salaries were continued. Some of the effort was switched to COVID19 research but only when there was no ability to complete work on the drug screening project. But after realizing the virus was going to continue to impede this progress, we completed the COVID19 studies on cholesterol and HCQ and prepared the manuscripts for publication.

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.

PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

1. Wang H, Yuan Z, Pavel MA, Hansen S. The role of high cholesterol in aged related COVID19 lethality. bioRxiv. 2020; 2020.05.09.086249. doi:10.1101/2020.05.09.086249
2. Yuan Z, Pavel MA, Wang H, Hansen S. Hydroxychloroquine: mechanism of action inhibiting SARS-CoV2 entry. bioRxiv. 2020; 2020.08.13.250217. doi:10.1101/2020.08.13.250217

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Hao Wang, Scott Hansen "A Soluble Ion Channel Binding Assay for Allosteric Modulator Screening"
FASEB April 1, 2019 (33) 1_supplement p811.3-811.3

Website(s) or other Internet site(s)**Technologies or techniques**

Nothing to report.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

Nothing to report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name:	<i>Hao Wang</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Mr. Wang has performed the work in the area of expression and purification of all the proteins nLuc tagged probes described
Funding Support:	NIH, DOD & Institutional
Name:	Scott B. Hansen
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	291307
Nearest person month worked:	2

Contribution to Project:	Dr. Hansen helped with cloning, designing constructs, and teaching Mr. Wang how to purify TREK-1 proteins, purification of all the proteins nLuc tagged probes described
Name:	Arif Pavel
Project Role:	Postdoc Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	
Funding Support:	NIH, DOD & Institutional
Name:	Virnaliz Fernandez Vega
Project Role:	Scientific associate in the HTS core Facility
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Virnaliz is responsible for miniaturizing the BRET assay and adapting the assay to run on HTS equipment.
Funding Support:	Institutional

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.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report.

QUAD CHARTS:

See attached quad chart.

APPENDICES:

Nothing to report