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Developing an Assay to Profile Upregulated TREK-1,

A Stretch-Activated Potassium Channel, in Prostate Cancer

A Thesis

Presented to the

College of Pharmacy and Health Sciences

&

The Honors Program

Of

Butler University

In Partial Fulfillment

Of the Requirements for Graduation Honors

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INTRODUCTION

Prostate cancer is the second leading cause of cancer death in American men. The disease prognosis is significantly limited by the finite therapeutic treatments. The limited treatment options are made worse by the significant reduction in the quality of life that often results from the severe side effects produced by these treatments.1 Therefore, any improvements of current treatments would be advantageous to the clinical setting. Recently, TREK-1, a tandem-pore domain (K_{2P}) potassium channel, has been shown to be upregulated in prostate cancer, but absent in normal prostate tissue.² The observation that TREK-1 expression is correlated with tumor malignancy suggests that TREK-1 may be a possible target for the development of novel drugs that target prostate cancer. Interestingly, other subtypes of the K_{2P} family also have upregulated expression in several other types of malignancies.^{2, 3, 4} These observations have prompted many research laboratories to characterize the role of TREK-1 in cancer cells. However, in order to explore the role of TREK-1 in cancer cells, pharmacological tools selective for TREK-1 need to be developed. Specifically, a large-scale screen is needed to identify molecules that selectively modulate TREK-1.

The K2P Family of Ion Channels

Potassium ion channels are large multi-subunit proteins that include: voltage-gated potassium channels (K_V) , inwardly rectifying potassium channels (K_{ir}) , and the tandem-pore domain potassium channels (K_{2P}) .⁵

The K_{2P} family was first identified in 1996, and since then, researchers have been working to characterize the distinction between the subtypes. 6 K_{2P} proteins possess two tandem P (pore-forming) domains. The P domain is a highly-conserved twenty amino acid motif that discriminates for potassium ions. 5 P domains are assembled in sets of four to create a functional potassium channel, and their arrangement is what distinguishes the different potassium channel families. Whereas the K_{2P} family members contain two P domains per subunit and form functional channels as dimers, other potassium channel families have only one P domain and function as tetramers. 5 Structural differences between the K_{2P} family and other potassium channels could potentially be exploited to develop molecules that selectively target members of the family, such as TREK-1.

Within the known fifteen subtypes of K_{2P}, TREK-1 is known as a polyunsaturated fatty acid (PUFA) and stretch-activated potassium channel.⁷ TREK-1 has low basal activity compared with other members of K_{2P} family.⁵ However, this receptor can be strongly activated upon application of arachidonic acid; this activation is reversible and concentration-dependent. The effect is specific to unsaturated fatty acids, including oleate, linoleate, arachidonate, eicosapentaenoate, and docosahexanenoate.⁵ In contrast to the long, lipophillic activators, the small molecule, riluzole, is also capable of channel activation.⁸ Other pharmacological agonists include inhaled anesthetics such as halothane and isoflurane.⁴ Activation of the channel can also be caused by environmental changes including internal acidification, shear stress, cell swelling, and negative

pressure and physical stretch to the cell membrane.³ Few inhibitors of the channel have been identified. Galladium and quinidine specifically inhibit TREK-1 activity.³

In addition to TREK-1, Other K_{2P} channels have been shown to be overexpressed in several malignancies. TASK-3, a close relative of TREK-1, is amplified in 10% of breast cancers and is overexpressed at a higher frequency in colon and metastatic prostate cancer.^{9, 10} One study suggests that TASK-3 overexpression in breast tissue may contribute to tumorgenesis by promoting cancer cell survival in poorly oxygenated areas of solid tumors.¹¹ Similarly, TREK-1 has the ability to become activated during an ischemic episode to protect neuronal function.¹² It is hypothesized that during brain ischemia, endogenous arachidonic acid is released, intracellular pH becomes more acidic, and neurons swell. These pathological alterations all contribute to activation of TREK-1, resulting in hyperpolarization, decreased excitotoxicity, and reduced neuronal damage.¹² It is possible that the gene that codes for TREK-1, *KCNK2*, may be a tumor survival gene and that TREK-1 upregulation is an adaptation for the cell or solid prostate cancer tumor to cope with a hypoxic environment.²

Drugs that Target Ion Channels

Several classes of ion channel agonists and antagonists have been developed, and these modulating drugs are used in therapeutic treatments of disease states such as epilepsy, hypertension, diabetes, and chronic pain.¹³ Of particular relevance to this study is the development of ion channel modulating compounds that have potential as cancer treatments.¹⁴ For example, the

compound, SKF96365, an inhibitor of a calcium release–activated Ca⁺² (CRAC) channel, has been shown to inhibit breast tumor metastasis in mouse models.¹⁵ Uncovering new treatments that target ion channels clearly have the potential to improve chemotherapy directly or by enhancing the efficacy of existing agents.

Identifying Selective Modulators of TREK-1

The discovery of specific activators/inhibitors of this receptor needs a highly efficient identification strategy because the current gold standard for modulator characterization, electrophysiology, is exceedingly costly and time inefficient. A method commonly employed to make drug discovery more efficient is known as High-Throughput Screening (HTS). The goal of HTS is to be able to take widely diverse chemical agents and analyze them in a designed assay both accurately and rapidly. The field of HTS greatly relies on automation to make the process more efficient, accurate, and manageable. Although there are several advantages and recent improvements made to HTS, the main limitation that still exists is data analysis and 'hit' determination. A 'hit' is best characterized as a lead candidate that gives a desired result from a screen. Further analyses of lead candidates are required to rule out the potential for false positives and negatives.

Current assays for TREK-1 activity are incompatible with HTS.¹⁷ Thus it is the goal of this work to develop methods for screening TREK-1 channel activity modulators (agonist/antagonist).

HYPOTHESIS

It is hypothesized that TREK-1 potentiates tumor growth in prostate cancer. Developing a screen to identify molecules that modulate TREK-1 function

will provide tools for determining the role of TREK-1 in prostate caner and potentially lead to the development of novel prostate cancer therapy.

OBJECTIVE

The overall goal of this project is to develop a high throughput screening assay to identify molecules that either inhibit or activate TREK-1. Validation of TREK-1 expression and function in the selected cultured prostate cell culture model system is necessary prior to developing an HTS platform.

MATERIALS & METHODS

The expression of TREK-1 was quantified by Western blot analysis in the prostate cancer (PC3) cell line. The activity of TREK-1 was measured by the flux of potassium ions across the cell membrane using a fluorescence-based detection assay known as Fluorescence Imaging Plate Reader (FLIPR). Using both of these assays jointly allow for inferences concerning the activity of TREK-1 to be made.

Cell Culture

The PC3 human prostate cancer cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in Ham's F-12K medium (Sigma, St Louis, MO) with 10% fetal bovine serum (FBS) (Glibco). Cells were routinely passaged every week with 1x TripLE Select (Glibco). All cell lines were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Western Blot

Growth media was removed from cultured cells and then the cells trypsinized with TripLE Select. Growth media was added to the cell solution to

neutralize the trypsinization process when detachment of cells was complete. This cell solution was centrifuged to create a pellet and then the solution was removed. This pellet was washed with PBS in triplicate and stored at -80°C for later use. When used, pellets were dissolved with ice-cold homogenization buffer (Roche lysate product # 04719964001) with 1:100 dilution of Halt Protease Inhibitor Cocktail (Pierce). Sonification for 20 seconds in triplicate of the lysate was used for cell disruption. The membrane fraction was purified by centrifugation at 20,000 xg for 30 h at 4°C.

A protein assay protocol (Bio-Rad) was used to quantify protein content in lysate. This was a dye-binding assay in which a differential color change of a dye (Coomassie Brilliant Blue G-250) occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. A spectrophotometer was used to collect absorbance. Beer's law was applied for accurate quantification of protein concentration after development of a standard curve.

Proteins were separated by SDS-PAGE on a 10-14% gel (Lonza). Prestained protein standards (Bio-Rad) were used as a protein ladder. Samples were blotted onto nitrocellulose (Bio-Rad), and the membrane blocked with 1% bovine serum albumin in TBS/0.1% Tween 20. TREK-1 was detected with a polyclonal rabbit antibody raised against a portion of the NH2-terminal tail of the channel (Alomone Laboratories), followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz). The immunoreactive bands were

visualized by incubation with enhanced chemiluminescence reagent (Qiagen). Equal loading of samples was monitored by measuring the abundance of actin on the same membrane.

Blots were stripped using Abcam's medium stripping solution [1.5 g glycine, 100 mg SDS, 1ml Tween 20, pH 2.2, quantity to 100ml ultrapure water]. Membranes were incubated for two 10-minute washes of medium stripping solution, then two 10-minute washes of PBS, followed by one 5-minute wash of Tris buffer solution and 0.1% Tween 20 (TTBS), and one 5-minute TTBS. After the wash, the blot was prepared for re-blocking with primary and secondary antibodies.

Western Blot Analysis

Visualization of immunoreactive bands was performed using chemiluminescence and captured an AlphaInnotech digital imager. Quantification of the bands was completed with PDQuest software which determines the value of intensity of each band marked by TREK-1 antibody.

Fluorescence Imaging Plate Reader (FLIPR)

A membrane polarization assay from Molecular Devices was assessed for use as an indicator of TREK-1 activation in the PC3 prostate cancer cell line. This assay uses fluorescence detection in a fluorescence imaging plate reader (FLIPR, Molecular Devices). In this assay, changes in cell membrane potential control the partitioning of a fluorescent indicator dye into and out of the cell, with use of a cell impermeable fluorescence quencher. The properties of the two separate compounds in an *in vitro* experiment allow the indicator dye to escape

the inhibitor, thus more dye in the cell results in an increase in measured fluorescence.

Media used was phenol red-free DMEM/F12 (Sigma #D2906) that was made with 10% FBS (Gibco) to plate PC3 cell lines. Cells were plated 6.4 x 10⁴ cells/well in 100ul of media 14-hours before the experiment in 96-well flat-bottom black plates with clear bottoms (Costar #3603) and incubated in 5% CO₂ at 37°C overnight. FLIPR Membrane Potential Assay (R8042) was purchased from Molecular Devices which contains Blue Dye and Hank's BSS + 20mM Hepes. Before placing on plate reader, Blue Dye was reconstituted with 10 ml of Hank's BSS buffered with 20 mM Hepes and 100ul of solution was placed on top of media in each well. This was incubated at room temperature (22°C) for 1 hour. All compounds tested were stored as stock solutions in ethanol and were diluted in 10X PBS [1.37M NaCl, 101.6mM Na₂HPO₄, 17.6mM KH₂PO₄, pH 7.4] containing either 30 mM or 100 mM KCl prior to conducting experiments.

FLIPR Analysis

FlexStation II (Molecular Devices) was used to read the fluorescence output. Methodology for instrument setup included setting SoftMax Pro 4.8 Software to do a "Flex" kinetic assay. Fluorescence detection parameters included: excitation wavelength 530 nm, Emission wavelength 565 nm, and Emission cut-off 550 nm. A MatLab analysis program was used to plot the average of sample replicates versus time. The program then determines the lowest point (minFL) between points 3-54 seconds (provided drug is added at point =20 seconds) and picks the highest point (maxFL) between points 3-54

seconds of the plotted averages. The difference between maxFL and minFL becomes the new mean difference in fluorescence. The standard deviation was also calculated. The program then performs a t-test on the all of the mean differences in fluorescence of each sample. This was compared to the controls to determine significance.

RESULTS & DISCUSSION

Protein Analysis of TREK-1

The Western blot method was chosen for its ability to quantify TREK-1 protein expression. This method has the ability to measure proteins that are actively being maintained in the cell membrane. The use of a Western blot is a gold standard for protein quantification and an appropriate tool for TREK-1 detection. The Figure 1 shows an immune blot for TREK-1 and actin in the PC3 cell line.

The only antibody manufactured to target TREK-1 protein for Western blot analysis has non-specific binding to several other proteins in PC3 cells, as apparent in Figure 1A. In response to the non-specific binding of the antibody, the manufacturer created a negative control antigen to provide evidence that the antibody was targeting TREK-1. This antigen was incubated with the primary antibody before application to the membrane. The antigen is designed to prevent binding of TREK-1 antibody to TREK-1 protein. If the imaged band corresponding to TREK-1 was absent in the incubation with antigen (compared to antibody incubation alone) it can be inferred that TREK-1 is present and interacting with the antibody, confirming TREK-1 expression in PC3 cells. However, as shown in

Figure 1C, the absence of a band after incubating with the primary antibody with the antigen was not observed.

Another concern when performing a Western blot to probe for TREK-1 protein was determining an appropriate loading control for comparison. loading control is a ubiquitous protein that is measured on the same blot as the studied proteins to verify that equivalent amounts of protein are loaded in each lane. 19 Based on the molecular weight of available loading controls, two different loading controls could have been used for TREK-1 quantification: actin (43 kDa) and GAPDH (37 kDa). Due to TREK-1 protein's size (45 kDa), it was important to allow sufficient band separation during SDS-PAGE for distinguishing TREK-1 from the loading controls. Although GAPDH would be better resolved from TREK-1 by molecular weight, it has been shown to vary widely in expression levels across different cell lines and was therefore not ideal for comparison.²¹ Therefore, actin was used as a loading control for the purpose of quantification. The blot shown in Figure ID was re-probed for actin and imaged, after stripping TREK-1 primary antibody. This figure shows that there is less actin protein compared to TREK-1 in Figure 1A, although it is possible that some protein was lost during the stripping process.

Because actin and TREK-1 proteins are too similar in size and TREK-1 has non-specific binding, the two proteins cannot be differentiated conclusively from one another. It cannot be determined when looking at Figure 1A, if the TREK-1 antibody is non-specifically binding to similar sized proteins such as

actin or appear to be expressed at a higher concentration than what exists within the cell membrane.

The factor limiting the detection of TREK-1 is the antibodies' lack of specificity for the TREK-1 receptor. The antibody used for the purpose of TREK-1 quantification in this study failed to be sufficiently selective. Therefore, it is necessary to use additional methods to better confirm TREK-1 expression in PC3 cell line.

TREK-1 Activation

The FLIPR assay was selected to measure TREK-1 activity based on its ease of use, the ability to conduct several experiments in a short time frame, and HTS compatibility. Other accepted methods to measure channel activation are extremely limited due to time and training requirements. The most common alternative method to measure membrane potential involves electrophysiology and often requires several months (or years) to master its use. Patch-clamp electrophysiology in mammalian cells is still the gold-standard assay for measurements of ion channels.²² However, electrophysiological measurements are not compatible with HTS, since only a few compounds can be screened on an individual cell at any given time.

The FLIPR assay is unique in its mechanism of action. The indicator dye used in this assay is a derivative of the potential-sensitive dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Dibac₄(3) dye, or bis-oxonol).²³ Until FLIPR, Dibac dyes had been limited by the excessive time necessary for incubation which ultimately leads to cell toxicity.²³ Molecular Devices, Inc.

created two separate formulations for the Membrane-Potential Assay that require less incubation time which helps maintain the viability of the cells and helps produce higher quality of data. The systems combine the indicator dye with a dye quencher that inhibits the indicator dye from fluorescing when not inside the cell. The mixture of dyes helps to increase the possibility of a successful assay with a specific cell line/ion channel.²⁴

The fluorescent indicator dye in the kit is a lipophilic, anionic dye that can partition across the cytoplasmic membrane of live cells dependent upon the potential across the plasma membrane. The quenching dye does not enter the cell but masks all extracellular fluorescence of the indicator dye. Therefore, during application of these compounds to the cells, baseline fluorescence was measured and no significant change in fluorescence occurred unless the electrical potential equilibrium is disrupted. This could occur by either mechanical and/or pharmacological means.

When cells are depolarized, more dye enters the cells, causing an increase in fluorescence signal.²³ The dye crosses the cell membrane passively because the fluorescent dye is anionic and is not believed to be carried via a transporter. Conversely, when the cells are hyperpolarized, dye exits the cells, resulting in a decrease in fluorescence signal.²³

Due to the nature of TREK-1, if potassium is at higher concentration outside the cell and the protein is activated, the channel will attempt to equilibrate the potassium concentrations by pulling potassium into the cell. The influx of potassium will increase the membrane potential resulting in an increase of dye in

the cell, with a concomitant increase in fluorescence measured. Since potassium flux could be affected by the activity of other potassium channels, the assay was optimized to establish specific activation of TREK-1 using known agonists and antagonists. The assay was calibrated through the use of carefully controlled, high concentration doses of potassium to induce potassium influx in addition to either TREK-1 activators (expected to further increase potassium influx) or inhibitors (expected to result in diminished potassium influx).

It is important that the activators and the inhibitors used in this assay were relatively selective for TREK-1 so that TREK-1 activation could be discriminated. Known TREK-1-specific activators, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were used to limit interactions with other potassium receptors.³ EPA and DHA are both long chain fatty acids and can potentially increase membrane permeability to potassium. In addition to PUFAs, riluzole, another known activator of TREK-1, is a much smaller compound without the capability of inserting itself into the membrane. For this reason, riluzole was used as a TREK-1 activator in the fluorescence assay. Quinidine, a known non-specific inhibitor of TREK-1, was used as the antagonist when conducting dose-response curves.³

TREK-1 Activation – Preliminary Results

The FLIPR assay was optimized for assessing TREK-1 channel activity. Preliminary testing using known agonists DHA and EPA was necessary to determine whether the FLIPR platform could be used to measure the membrane potential activity for the prostate cancer PC3 cell line (Figure 2).

Preliminary findings, as shown in Figure 2, help validate the hypothesis. Within seconds of addition of an agonist and potassium ions, an instantaneous depolarization occurred. In the presence of a TREK-1 activator, the increase in fluorescence was significant when compared to the control. An increase in fluorescence was also observed in the agonist-free controls; however, this fluorescence signal change was not significant when compared to lower drug concentrations (Table 1). There are many factors contributing to why an increase in signal baseline was observed for the controls in the absence of an agonist. Stress on the cells from additional pressure changes during pipette application of control solution could have potentially resulted in activation of TREK-1 since pressure and shear stress opens the channel. Another explanation for this observation could be from a cellular response to an increase in ethanol concentration. Reported observations demonstrate cellular activity changes in ethanol concentrations less than 0.05%.25 It is also possible that there was a cellular response to equilibrate potassium concentrations. Due to the presence of these potential factors, controls were performed in triplicate.

A confounding issue observed upon analysis of the preliminary results came from a decreased fluorescence signal at higher drug concentrations. At 20 μ M concentrations of both DHA and EPA, the signal was determined to be equivalent to that of the control. However, it has been reported that concentrations of up to 100 μ M of DHA lead to activation of TREK-1.²⁶ Although not previously reported, it was hypothesized that the drug might act upon off-target sites, thus mimicking an "antagonist" effect at high concentrations. This

was an interesting observation, and it was a consistent trend which appeared to be reproduced in other unrelated cell lines tested including MCF-7 and MDA-MB-231 breast cancer cell lines. Therefore, it was determined to be due to another factor. It is possible that the receptors were saturated with compounds at these higher concentrations but with inadequate extracellular potassium to elicit a response required to produce the level of depolarization necessary for an influx of the dye. If this was true, there are two adjustments that should be made to the assay: either increase the potassium content or decrease the drug concentration.

When accounting for the amount of potassium in the current drug design, there was not a significant difference in ion concentration at baseline compared to post-drug addition. Potassium accounted for approximately 5.6 mM potassium from the cell media (calculated from the buffer composition) and the FLIPR dye. After the drug addition, there was only an overall 7.8 mM potassium difference. Most electrophysiological studies with the FLIPR assay recommend a difference of 15 mM potassium.²³ Therefore, an adjustment of the potassium content was necessary to produce greater signal. An increase of potassium was made to ensure that after application of the drug, there would be a 15 mM potassium concentration difference. A reduction in drug concentrations also led to a decrease in observed fluorescence. Therefore, this procedural optimization eliminated the anomaly observed at high drug concentrations.

TREK-1 Activation – DHA, EPA, & Riluzole

After accounting for the potassium concentration, results improved for EPA (Figure 4), but still persisted when DHA was used (Figure 3). It is possible

that there is still not enough of a potassium gradient post-drug application of DHA. However, because the structure of DHA is closer to arachidonic acid (AA) than EPA and AA is known for its potent activation of TREK-1 (Figure 7), ²⁷ it is likely that DHA is a more potent activator of TREK-1 than EPA. This indicates that DHA requires more available potassium at test concentrations to see anticipated effects. It is also possible that DHA is more capable inserting itself into the cell membrane than EPA due to having a longer carbon chain, which would increase its lipophilicity. If DHA were inserting itself rather than acting on the receptor, this could disrupt the cell membrane and result in either TREK-1 activation or potentially another compensating mechanism that resists membrane depolarization.

For comparison with the PUFAs, the small molecule riluzole (Figure 7) was tested to compare fluorescence responses. Riluzole demonstrated a consistent dose-dependence and exhibited significant responses at all concentrations tested when compared to control (Figure 5). It is hypothesized that the effects observed are not necessarily due to cell activity but due to a chemical interaction with the dye and/or media upon treatment.

TREK-1 Activation – Negative Control

When using fluorescence drug assays, it is important to verify that the drugs do not interact with the fluorescent dyes in the absence of cells. If an interaction occurs, this can indicate either a false-positive or negative response. Molecules fluoresce based upon energy state relaxation and is dependent upon conformation of the structure. DHA could be interacting with the FLIPR dye

resulting in decreased fluorescence when there are higher drug concentrations present. Figure 6 shows that there is potentially an interaction occurring between DHA and the dye. It is also possible that the observed decrease in fluorescence after drug addition is a dilution effect on the dye rather than a quenching of the fluorescent molecule. However, the change in fluorescence is very significant and lends itself to being attributed to a physical interaction.

In-depth chemical analysis to determine the DHA's interaction (Figure 6) with the dye is difficult to perform. Because the fluorescent dyes are premixed, there is no way to determine if the dye interaction with DHA is due to the associated complex of the quencher and indicator or by a single component. It is possible that a decrease in fluorescence is due to promotion of the ionic association between the indicator and quencher, or that DHA aids in the quenching of the indicator. However, it is plausible that a combination of the two mechanisms exists.

TREK-1 Activation – Antagonist Assay: Riluzole & Quinidine

Riluzole produced the most reliable data for this platform (Figure 5). The smaller compound, when tested, lacked the support for other underlying mechanisms that explain observations noted with the PUFAs. This molecule produced cleaner data; therefore, future studies of the FLIPR can be performed using riluzole to produce an appropriate HTS for TREK-1.

To sufficiently determine that riluzole is acting on TREK-1, an inhibitor of the receptor is necessary to block the actions of the drug so that its activity can be verified as ligand-mediated. As stated earlier, there are no specific antagonists for TREK-1, but quinidine at high concentrations ($IC_{50} = 100\mu M$) results in TREK-1 activation.¹ Table 4 lists riluzole's change in fluorescence, observed in PC3 cells at specific concentrations as well as in the varying concentrations of quinidine. Unfortunately, no reversible inhibition was observed for riluzole in the presence of quinidine.

Dose-response curves are extremely difficult to conduct when known agonists and antagonists lack specificity for the target. This makes interpretation challenging to determine which parameters to adjust to produce more reliable data. From this data, riluzole concentration should be increased in the assay. Further analysis should be performed to assess the validity of the results gathered.

CONCLUSION AND FUTURE DIRECTIONS

The utility of an HTS assay is limited by the selectivity of the assay. In the case of TREK-1, there are no known selective inhibitors of the channel, and therefore, an HTS assay for TREK-1 cannot be produced with the assurance of differentiating the individual channel from other related potassium receptors. However, using the FLIPR assay to create a platform that is selective for potassium channels can still assist in the discovery of activators and inhibitors of TREK-1. Screening compounds through this method can then produce a more finite list of molecules to be tested in electrophysiology studies to confirm or deny TREK-1 activation. Future discoveries from this methodology can then be integrated into the FLIPR assay to improve its selectivity for the channel.

For the purposes of developing an HTS, there are several challenges associated with conducting the FLIPR assay. First, to ensure that only potassium channel activation is occurring, the assay must be designed to limit other channel involvement. One potential way to solve this would be to remove or limit uninvolved ions such as magnesium and calcium in assay solutions. The solutions applied during these experiments contained the activating and inhibitory compounds in diluted potassium ion solutions. However, not all ions can be completely removed from the solutions. This is because a balance of ions needs to exist inside and outside the cell to maintain a proper electrochemical gradient. Without the minimum required ions, a cell will not be permitted to perform the necessary biological activities, and TREK-1 activity will not be accurately depicted.

The second challenge with the FLIPR assay was how to measure specifically for TREK-1 ion channel activity, because this assay measures the activity of all potassium channels. One method to discriminate between particular ion channel activities is through the use of pharmacological agents. The expectation was that by measuring a dose response with highly-selective TREK-1 agonists and antagonists, the fractional potassium flux activity due to TREK-1 could be measured. However, it is still possible that the selective agents could be facilitating undiscovered reactions with other ion channels and thus, interfering with measurements.

However to facilitate solutions to these problems, future plans could involve using genetically modified cell lines to create cells with and without

TREK-1 receptors. A comparison of response between the two cell lines could help differentiate TREK-1 specific activities and modulators when screening libraries. There are two commonly practiced methods to genetically modify the cell lines: insertion of a gene for human TREK-1 to up-regulate its protein expression or the use of short interfering RNA (siRNA), a gene silencer, directed at knocking down TREK-1 expression. Depending on the cell line enlisted for the foundation of the screen, one method may be more optimal to use than the other. For example, in the case of PC3 cells, TREK-1 expression has been documented, 2 so the use of commercially available siRNA may be more appropriate. However, this method may be only partially effective at eliminating gene expression and is highly dependent on the cell line used.²⁸ Depending upon the amount of overexpressed TREK-1 in PC3 cells and the efficiency of siRNA in this particular cell line, gene silencing could produce a poor model to be used for comparison. This is why it is beneficial to produce better tools to quantify TREK-1 in specific cells lines so that data can be quantitative in nature. That notwithstanding, until these new tools are produced, siRNA presents a future opportunity and a new direction for this project.

With regard to the work presented here, future tool developments could easily improve the methodology of this study. For instance, it would be beneficial to improve TREK-1 immunodetection for Western blot analysis. One way to accomplish this would involve producing a protein-tag-fused recombinant TREK-1 to serve as a more specific epitope, and then using an antibody against that epitope for detection rather than TREK-1 for enhanced specificity. If the

constructed epitope was sufficiently orthogonal, it would result in highly specific visualization of only epitope-fused TREK-1 protein.²⁹ Another alternative for quantification of TREK-1 is reverse transcriptase-polymerase chain reaction (RT-PCR), which measures mRNA levels rather than expressed protein.

The Western blot method was specifically chosen for the detection and quantification of TREK-1 expression in these cells due to its ability to measure the current protein that is actively being maintained in the cell membrane. Alternatively, for the purpose of quantifying TREK-1 expression in PC3 cells, another assay is available that measures gene transcript levels, encoding TREK-1 protein. This method, RT-PCR, can show that TREK-1 protein has the capability of being expressed, but does not confirm protein expression. Therefore, it may not be reliable for validating TREK-1 expression because determining the presence of coding material does not verify that the code is translated into an active protein channel on the cell surface. Therefore, the use of a Western blot is usually the ideal method for protein visualization, but in regards to TREK-1, until another antibody is developed, RT-PCR may provide more conclusive evidence.

APPENDIX

Figures & Tables

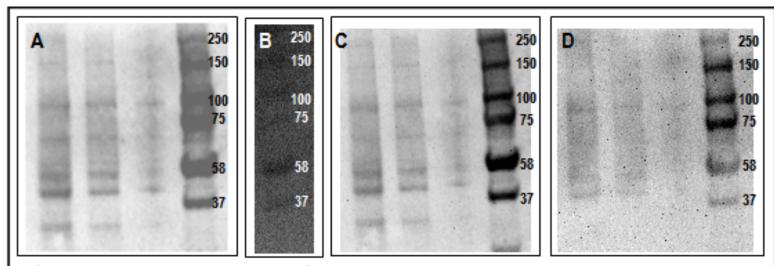


Figure 1 - Western Blots of Protein from PC3 Lysate: PC3 lysate was loaded in three different concentrations for Western blot analysis. Lane 1 contained 35 μ g, Lane 2, 8 μ g, and Lane 3, 3 μ g total protein. Each image (except figure B) was exposed for 1 minute exposure time to collect light emission. TREK-1 is 45kD in size and β-Actin is 43kD in size.

Figure 1A: Western Blot of TREK-1 antibody

Figure 1B: Image of colored ladder before exposure of Chemiluminescence solution

Figure 1C: Western Blot of TREK-1 antibody + Antigen (incubation for 1 hour prior to application on blot)

Figure 1D: Western Blot of β-Actin antibody

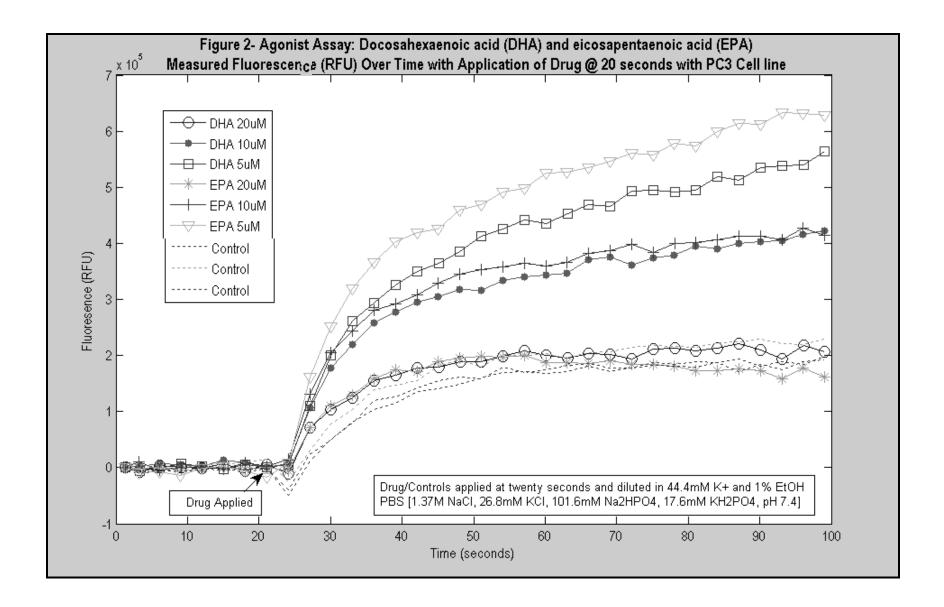


Table 1 – Agonist Assay: DHA and EPA <i>t</i> -Test Analysis				
Drug	Concentration	Mean [‡] Difference in FL (RFU)§	Mean [‡] Difference in FL of control (RFU)§	P -value
DHA	20 µM	227395	231427	0.86
	10 µM	360476	231427	**
	5 µM	465987	231427	**
EPA	20 µM	230357	231427	0.92
	10 µM	388085	231427	**
	5 µM	538153	231427	**

< 0.01 (**) < 0.05 (*)

[‡] Denotes mean of eight replicates
§ Represents the lowest point (minFL) between points 3-54 seconds (provided drug is added at point =20seconds) and selects the highest point (maxFL) between points 3-54 seconds of the plotted averages. The difference between maxFL and minFL becomes the new mean difference in fluorescence

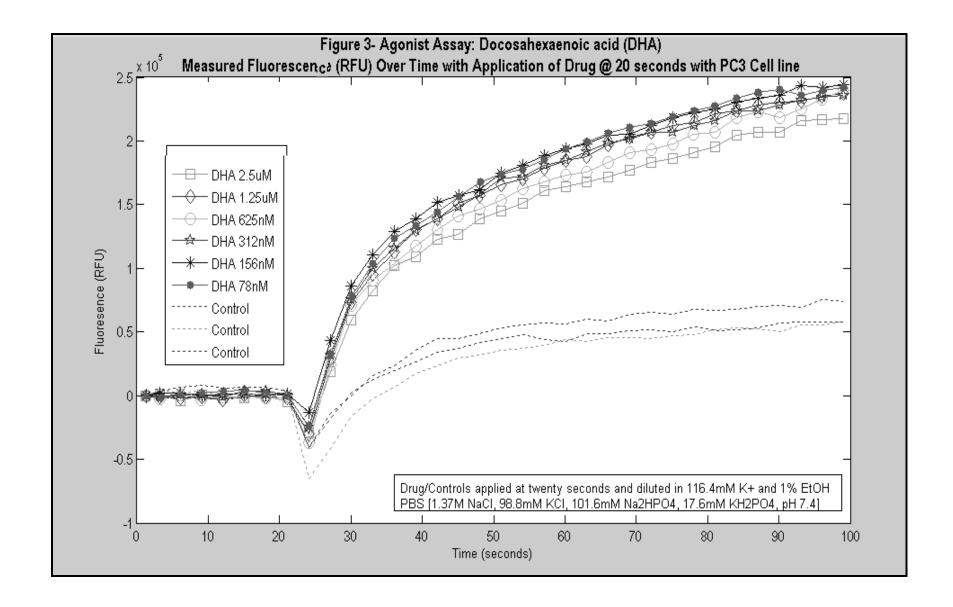


Table 2 – Agonist Assay: DHA <i>t</i> -Test Analysis				
Drug	Concentration	Mean [‡] Difference in FL (RFU)§	Mean [‡] Difference in FL of control (RFU)§	P -value
DHA	2.50 µM	193335	101771	**
	1.25 µM	210946	101771	**
	625 nM	205901	101771	**
	312 nM	209364	101771	**
	156 nM	205772	101771	**
	78 nM	213207	101771	**

< 0.01 (**) < 0.05 (*)

<sup>Denotes mean of eight replicates
Represents the lowest point (minFL) between points 3-54 seconds (provided drug is added at point =20seconds) and selects the highest point (maxFL)</sup> between points 3-54 seconds of the plotted averages. The difference between maxFL and minFL becomes the new mean difference in fluorescence

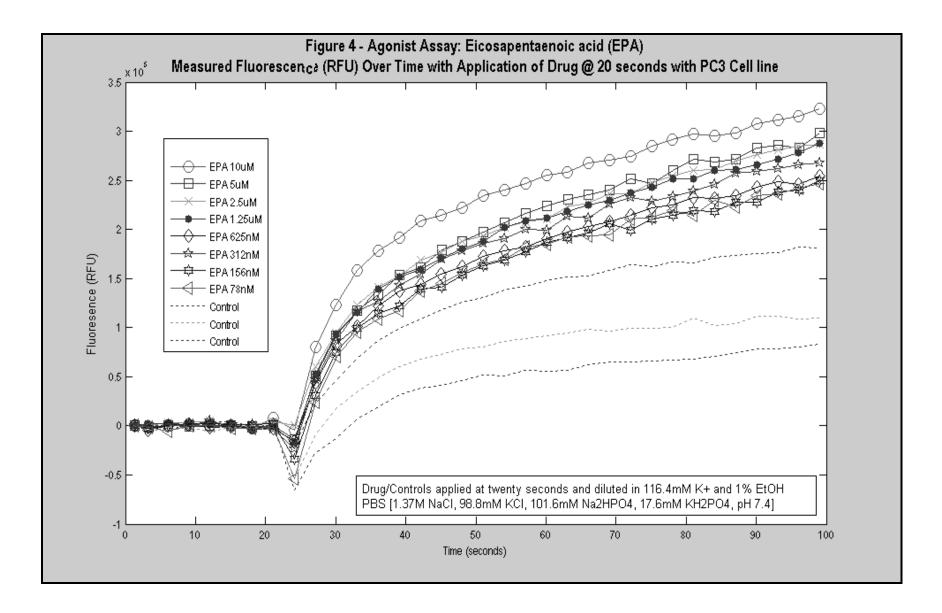


Table 3 – Agonist Assay: EPA <i>t</i> -Test Analysis				
Drug	Concentration	Mean [‡] Difference in FL (RFU)§	Mean [‡] Difference in FL of control (RFU)§	P -value
EPA	10.00 µM	236894	123392	**
	2.50 µM	220832	123392	**
	1.25 µM	232165	123392	**
	625 nM	212318	123392	**
	312 nM	227373	123392	**
	156 nM	213578	123392	**
0.04 (78 nM	237785	123392	**

< 0.01 (**) < 0.05 (*)

‡ Denotes mean of eight replicates

§ Represents the lowest point (minFL) between points 3-54 seconds (provided drug is added at point =20seconds) and selects the highest point (maxFL) between points 3-54 seconds of the plotted averages. The difference between maxFL and minFL becomes the new mean difference in fluorescence

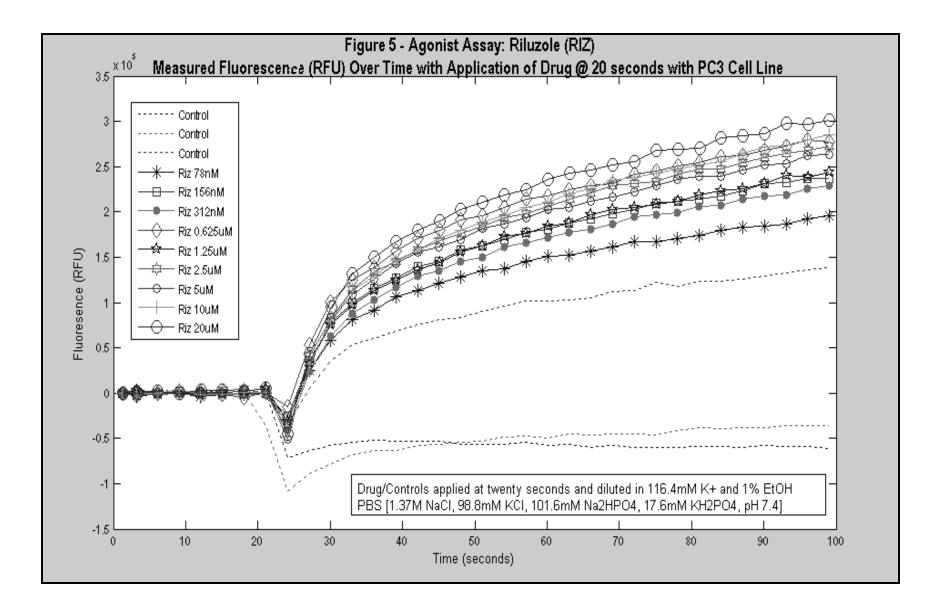
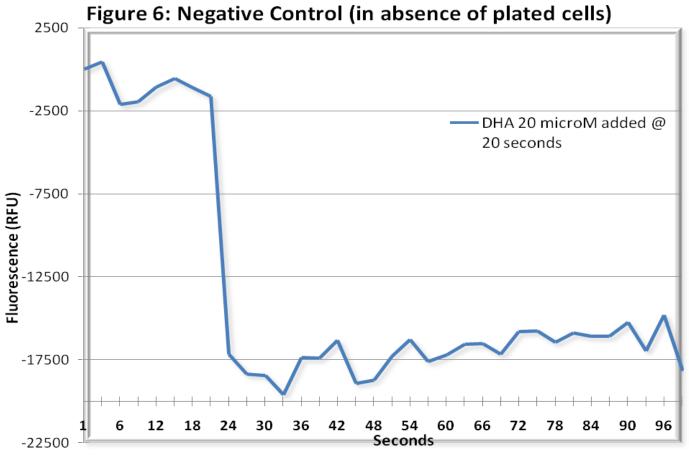


Table 4 – Antagonist Assay: Riluzole +/- Quinidine <i>t</i> -Test Analysis					
Drug	Concentration	Presence of Quinidine	Mean [‡] Difference in FL (RFU)§	Mean [‡] Difference in FL of control (RFU)§	P -value
Riluzole	20 μM	N/A	270682	113523	**
	10 μM	N/A	254453	113523	**
	5 µM	N/A	246234	113523	**
	2.5 µM	N/A	233408	113523	**
	1.25 µM	N/A	209144	113523	**
	626 nM	N/A	232773	113523	**
	313 nM	N/A	206306	113523	0.07
	156 nM	N/A	203628	113523	0.33
	78 nM	N/A	175393	113523	*
	2.5 µM	800 µM	231177	211162	0.11
	2.5 µM	400 µM	200883	211162	0.57
	2.5 µM	200 μM	232180	211162	0.27
	2.5 µM	100 μM	207157	211162	0.85
	2.5 µM	50 µM	314819	211162	**
	2.5 µM	25 µM	200766	211162	0.55
	2.5 µM	12.5 µM	213871	211162	0.82
	2.5 µM	6.5 µM	213945	211162	0.82
	2.5 µM	3.2 µM	203339	211162	0.52

< 0.01 (**) < 0.05 (*)

[‡] Denotes mean of eight replicates

§ Represents the lowest point (minFL) between points 3-54 seconds (provided drug is added at point = 20 seconds) and selects the highest point (maxFL) between points 3-54 seconds of the plotted averages. The difference between maxFL and minFL becomes the new mean difference in fluorescence



Drug/Controls applied at twenty seconds and diluted in 116.4mM K+ and 1% EtOH in absence of cells 10X PBS [1.37M NaCl, 98.8mM KCl, 101.6mM Na₂HPO₄, 17.6mM KH₂PO₄, pH 7.4]

Figure 7: TREK-1 Modulators

Arachidonic Acid (AA)	
Docosahexaenoic Acid (DHA)	
Eicosapentaenoic Acid (EPA)	
Riluzole (RIZ)	Quinidine

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