



A “Target Class” Screen to Identify Activators of Two-Pore Domain Potassium (K2P) Channels

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Abstract

Two-pore domain potassium (K2P) channels carry background (or leak) potassium current and play a key role in regulating resting membrane potential and cellular excitability. Accumulating evidence points to a role for K2Ps in human pathophysiology, most notably in pain and migraine, making them attractive targets for therapeutic intervention. However, there remains a lack of selective pharmacological tools. The aim of this work was to apply a “target class” approach to investigate the K2P superfamily and identify novel activators across all the described subclasses of K2P channels. Target class drug discovery allows for the leveraging of accumulated knowledge and maximizing synergies across a family of targets and serves as an additional approach to standard target-based screening. A common assay platform using baculovirus (BacMam) to transiently express K2P channels in mammalian cells and a thallium flux assay to determine channel activity was developed, allowing the simultaneous screening of multiple targets. Importantly, this system, by allowing precise titration of channel function, allows optimization to facilitate the identification of activators. A representative set of channels (THIK-1, TWIK-1, TREK-2, TASK-3, and TASK-2) were screened against a library of Food and Drug Administration (FDA)-approved compounds and the LifeArc Index Set. Activators were then analyzed in concentration–response format across all channels to assess selectivity. Using the target class approach to investigate the K2P channels has enabled us to determine which of the K2Ps are amenable to small-molecule activation, de-risk multiple channels from a technical point of view, and identify a diverse range of previously undescribed pharmacology.

Keywords

ion channel, K2P, screening, potassium channel, assay

Introduction

Ion channels are pore-forming membrane proteins, generally classified by their primary physiologically permeant ion. Despite diverse and important roles, ion channels remain relatively underexploited as drug targets. Two-pore domain potassium (K2P) channels function to carry background (or leak) potassium current in a variety of cell types and primarily act to maintain the resting membrane potential and cellular excitability. The mammalian K2P superfamily is made up of 15 members, which can be further divided into six subfamilies (TWIK, TASK, TREK, TRESK, THIK, and TALK) and share a characteristic topology consisting of four transmembrane domains and two pore-forming loops, with subunits dimerizing to form the functional channel.¹ The activity of K2P channels can be up- or downregulated by a diverse range of modulators, including small molecules, temperature, mechanical stretch, pH, signaling

pathways (e.g., G-protein coupled receptors and kinases), and other interacting proteins (e.g., 14-3-3 and calcineurin), allowing them to integrate diverse cellular signals into changes in cellular excitability.¹ K2P channels are implicated in a range of pathophysiology, including, but not limited to, pain (TREK-1, TREK-2, and TRAAK) and

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migraine (TRESK and TASK-2),² Birk-Barel syndrome (TASK-3),³ Balkan endemic nephropathy (TASK-2),⁴ deafness (TASK-2),⁵ pulmonary arterial hypertension (TASK-1),⁶ and cancer (TASK-2, TASK-3, and TWIK-1).^{7,8} However, a lack of potent and/or selective pharmacological tools has hindered efforts to understand the precise roles of K2Ps in pathophysiological conditions.

An increasing body of genetic and functional evidence points to K2P channels having important roles in the perception and transmission of pain. TREK-1 knockout mice are more sensitive to a variety of painful stimuli, including heat and mechanical and osmotic stimuli,⁹ and small-molecule TREK-1 activators have been reported to display antinociceptive properties in vivo.¹⁰ TREK-2 channels are selectively expressed in IB4⁺ binding C-nociceptors, and siRNA knockdown of TREK-2 has been shown to depolarize these cells, suggesting that TREK-2 contributes to the resting membrane potential. Furthermore, siRNA knockdown of TREK-2 in vivo increased spontaneous pain behaviors following Complete Freund's Adjuvant (CFA)-induced inflammation.¹¹ TREK-2 knockout mice are more sensitive to mechanical stimulation, are not sensitized to osmotic pain by the pro-inflammatory mediator PGE2 in the same way as wild-type mice, and demonstrate increased sensitivity to nonnoxious raised and lowered temperatures, indicating roles for TREK-2 in mechanical pain perception, osmotic pain, and heat perception.^{12,13} TRAAK (the third member of the TREK subfamily) knockout mice also display pain phenotypes, demonstrating increased sensitivity to mechanical stimulation and nonnoxious raised and lowered temperatures.¹² Furthermore, a study of 561 patients suggested an association between TRAAK and postsurgical neuropathic pain.¹⁴ TASK-2, a member of the TALK subfamily of channels, demonstrated an association with migraines in a meta-analysis of genome-wide association studies.¹⁵ TASK-2 has been shown to be highly expressed in the dorsal root ganglion (DRG) and trigeminal ganglion (TG) and is suggested to likely play a greater role than TRESK or TREK-1 in setting the resting membrane potential of sensory neurons.^{16–18} TASK-3, a member of the TASK subfamily of K2P channels, has also been shown to be associated with breast pain in preoperative breast cancer patients.¹⁹ Elevated TASK-3 mRNA expression in rat DRGs was similarly shown to correlate with increased spontaneous foot lifting frequency in a CFA-induced inflammation model,²⁰ and TASK-3 activators have been shown to have analgesic effects in a number of in vivo pain models.^{21,22} A loss of function frameshift mutation in the TRESK gene has been associated with typical migraine with aura.²³ TRESK has also been shown to be highly expressed in both DRG and TG neurons.²⁴ Taken together, these findings suggest that K2Ps could be attractive targets for the development of novel therapeutics for the treatment of pain and migraine.

A range of high-throughput screening strategies have been employed to address the lack of selective K2P modulators. A high-throughput, yeast-based screening assay was used²⁵ to screen a library of 106,000 small molecules. This assay used a growth-based functional readout and identified two new inhibitors and three new activators of TREK-1. ML67-33 was developed from one of the activators and is reported to selectively activate members of the TREK subfamily. High-throughput, fluorescence-based thallium flux assays have also been used to identify channel modulators. 11-Deoxyprostaglandin F2 α was identified as an activator of TREK-2 in a screen of a bioactive lipid library.²⁶ 11-Deoxyprostaglandin F2 α was used to optimize a TREK-2 thallium flux assay ahead of a larger screen of 76,000 small molecules, which identified further selective TREK-2 activators. Interestingly, 11-deoxyprostaglandin F2 α and other selective TREK-2 activators identified in this screen were reported to inhibit TREK-1, and these molecules have been used to investigate which regions of TREK channels are involved in either activation or inhibition of the channel. *N*-(2-((4-nitro-2-(trifluoromethyl)phenyl)amino)ethyl)benzamide (NPBA) was identified as a selective activator of TASK-3 in a thallium flux screen of 300,000 compounds.²⁷ We have previously reported using thallium flux to screen a library of 1000 Food and Drug Administration (FDA)-approved compounds and identified cloxyquin,²⁸ terbinafine,²⁹ and pranlukast³⁰ as selective activators of TRESK, TASK-3, and TREK-2, respectively. Recent work²¹ has since demonstrated that terbinafine has antinociceptive effects in models of neuropathic and inflammatory pain. Liao et al.²² used structure-based drug design to identify a biguanide compound, CHET-3, as a selective allosteric activator for TASK-3. CHET-3 was shown to decrease excitability in a limited population of DRG neurons and demonstrated analgesic effects in a range of acute and chronic pain models. A related approach is the use of rubidium efflux, where the movement of ⁸⁶Rb through the channel is measured. GI-530159 was identified as a selective activator of TREK-1 and TREK-2 channels³¹ using this methodology. This compound was also reported to reduce excitability in rat DRG neurons. Further K2P tool molecules that have been identified include ML-335 and ML-402,³² which activate TREK-1 and TREK-2 by binding to a pocket adjacent to the selectivity filter, termed the K2P modulator pocket. Fenamates, such as flufenamic acid, are nonsteroidal anti-inflammatory compounds and have been shown to activate several K2P channels, including TREK-1, TREK-2 and TRAAK,³³ TRESK,³⁴ and TASK-3.³⁵ BL-1249 is another fenamate-like compound and was identified as a putative activator of TREK-1 currents in cultured human bladder myocytes.³⁶ BL-1249 has been shown to activate members of the TREK subfamily.^{37,38} However, in common with other fenamates, BL-1249 is not selective for K2P channels.³⁹

“Target class discovery,” as described by Barnash et al.,⁴⁰ involves the simultaneous prosecution of multiple targets from a target family. This is complementary to the commonly used single-target approaches in drug discovery and also offers an alternative to disease centered approaches. The authors suggest that this approach is advantageous as the expertise and assets (molecules and tools) accumulated from one program or target can be leveraged or used across multiple targets. This in turn diminishes the chance of technical failure, that is, reduces the likelihood a ligand will not be found that modulates a single target. This is achieved through multiple synergies, including shared knowledge of structure and “ligandable pockets,” while also addressing selectivity at an early stage of the preclinical discovery process. Importantly, a target class approach also drives innovation across a group of targets by delivering high-quality probes and ligands for targets that may lack current disease validation. Often the drug discovery industry is not willing or able to use large screening and medicinal chemistry resources to investigate targets that lack validation. The paradox here is of course that high-quality pharmacological tools are one of the best ways to validate a target in pathophysiology.

We sought to use a target class approach to investigate the K2P channels. Our work aimed to identify activators across the family, as we believe these will likely be of more therapeutic benefit. Our aim was to define which of the K2P channels were assayable (i.e., we could easily develop reagents and conditions suitable for high-throughput screening) and therefore, in turn, which K2P channels were ligandable (i.e., activators could be developed). We used a common assay platform, thallium flux, as a surrogate measure of channel activity and a common screening library (the LifeArc Index Set⁴¹). We were able to develop a system that identified multiple novel and selective K2P activators.

Materials and Methods

Cell Culture/BacMam Transduction

U-2 OS cells (ATCC, Manassas, VA) were maintained in MEM (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA). BacMam solutions (SB Drug Discovery, Glasgow, UK) were used to generate transiently expressing cells. Typically, U-2 OS cells from -150°C storage were thawed at 37°C for 5 min and resuspended in fresh media. Cells were centrifuged at 300g for 5 min before resuspending in media and performing a cell count. Cells were then diluted to the desired concentration in fresh media containing the desired concentration of BacMam. For each channel, the volume of BacMam added was as follows (% v/v): TREK-2 (1%), TASK-2 (1%), TASK-3 (5%), TWIK-1 (10%), and THIK-1 (0.05%). Cells were plated on black,

clear-bottom, TC-treated plates (Greiner Bio-One, Kremsmunster, Austria) at 5000 cells per well and incubated overnight at 37°C , 5% CO_2 . For TWIK-1, the [I293A, I294A] mutant form of the channel was used to improve membrane trafficking. Cells were prepared as described above but were added to a T175 flask and incubated with BacMam for 24 h at 37°C , 5% CO_2 before being plating at the desired cell density and the incubation continued at 37°C , 5% CO_2 for another 24 h. Initial matrix experiments were carried out using a range of cell densities and BacMam concentrations to determine the optimal number of cells and BacMam concentration for each target.

Thallium Flux Assay/Screening

Cells were plated as described above. The following day, channel activity was measured using the FLIPR Potassium Assay Kit and a FLIPR Tetra (Molecular Devices, San Jose, CA). Media was removed and replaced with 40 μL thallium-sensitive fluorescent dye using a BlueWasher (BlueCatBio, Neudrossenfeld, Germany). Cell plates were then incubated with dye for 2 h at room temperature. Compounds were prepared in 100% DMSO and diluted in Hanks' balanced salt solution, containing 20 mM HEPES, on either a Biomek FX or ECHO (Beckman Coulter, Brea, CA). Inhibitor controls (0%) were added to columns 1 and 2 and DMSO controls (100%) to columns 23 and 24. For TASK-2, TWIK-1, and THIK-1, the inhibitor control was 30 μM TPA; for TREK-2, it was 100 nM PMA; and for TASK-3, 10 μM PK-THPP, all final assay concentration (fac). Compounds were preincubated with cells for 30 min prior to thallium addition (2 mM Tl^+ fac) and reading on the FLIPR Tetra. Preaddition baselines were established, and channel activity was calculated as the rate of fluorescence increase following thallium addition. Exemplar raw FLIPR data for each target are presented in the supplementary material (Suppl. Fig. S1). The time points used in the rate calculation for each target were as follows: TREK-2 (13–19 s), TASK-2 (15–28 s), TASK-3 (14–24 s), TWIK-1 (18–36 s), and THIK-1 (18–36 s). Each time point was chosen based on maximizing the signal window of known activators and therefore assay performance. Control activators were available for TASK-2 (Pyr-6), TASK-3 (terbinafine), and TREK-2 (BL-1249). Where no control activators were available (TWIK-1 and THIK-1), a standard condition of 18–36 s was used. The LifeArc FDA Set (1000 compounds) and Index Set (~11,000 compounds) were screened against all five targets at 10 μM fac. For hit confirmation, all compounds were screened at five concentrations against four targets (TREK-2, TASK-2, TASK-3, and TWIK-1) and nontransduced cells, using a through-plate dilution method (10 μM top fac; 1:10 dilutions). Due to the absence of activators observed during development, compounds were not routinely screened against THIK-1. Following chemistry

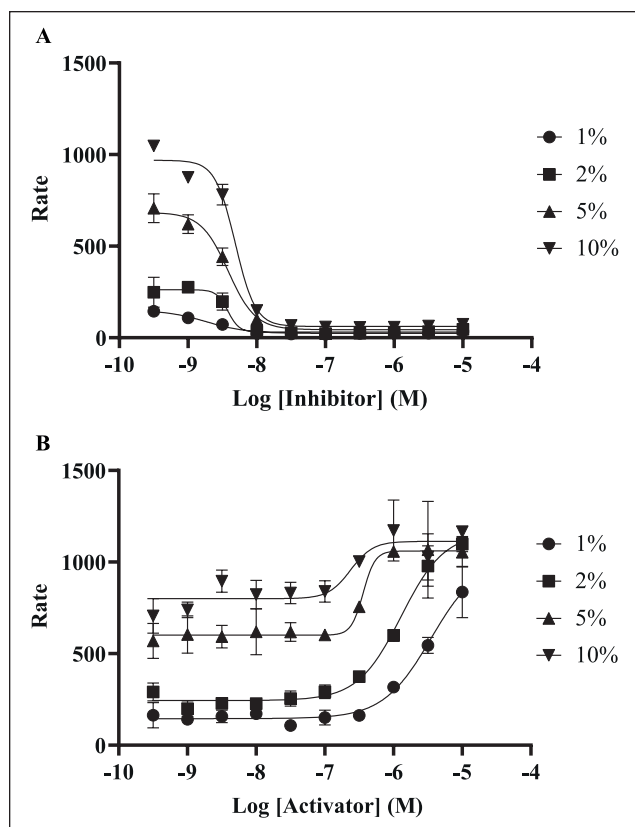


Figure 1. Exemplar concentration–response curves for TREK-2 demonstrating the effect of expression level on activator and inhibitor assay window. Cells were transduced with four different levels of BacMam and then exposed to a known (A) inhibitor or (B) activator. The asymptote maxima and minima clearly demonstrate that the highest level of transduction gives the best inhibitor window, while the lower levels of transduction give a better activator window. Rate denotes the rate of thallium flux (fluorescence intensity units/second) between 13 and 19 s.

trriage and selectivity analysis, preferred compounds were then screened using 10-point concentration–response curves (10 μ M top fac; half-log dilutions).

Data Analysis

Data represent mean \pm standard deviation, where n is the number of independent experiments. Activity was defined as the rate of fluorescence increase using 470–495 nm excitation light-emitting diodes and a 515–575 nm emission filter over the predetermined time period. All data were acquired using ScreenWorks 4.0 software (Molecular Devices). Percent activity was calculated relative to inhibitor control (0%) and DMSO control (100%). The number of compounds chosen for progression from each Index Set screen was set at \sim 1% of the total compounds screened, resulting in a variable activity cutoff for the different targets.

Compounds with the desired activation and selectivity profiles were taken forward for further analysis. Ten-point concentration–response curves were iteratively fitted to a four-parameter logistic model using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA).

Automation

The use of a consistent assay platform across each of the K2P targets has enabled the automation of large parts of the assay process. Cells were plated using a Multidrop Combi (Thermo Fisher Scientific). Media was removed from the cell plates and replaced with thallium-sensitive fluorescent dye on a BlueWasher, and either a Biomek FX or ECHO was used for compound handling. The automation of liquid handling processes has increased the throughput and flexibility of the system, allowing large numbers of compounds to be screened at a single target or smaller numbers of compounds to be screened at multiple targets side by side.

Results

Designing Specifically Engineered Systems for the Identification of K2P Activators

We believe that identification of channel activators is highly dependent on the use of appropriately designed assay reagents specifically optimized for this purpose. As described previously,^{28–30} we developed cellular systems in which a level of channel expression is chosen, based on function, to allow activation to be observed. To achieve this, baculovirus (BacMam) is used to deliver the channel of interest into cells. The primary reason for choosing BacMam is that it allows the precise titration of the gene of interest, allowing the development of cell systems with a range of levels of expression and therefore measurable function (Fig. 1)—in this particular case, a range of expression levels of a particular K2P channel. To measure channel function, we utilized thallium flux. Thallium flows through the potassium channel into cells preloaded with a thallium-sensitive fluorescent dye. Increases in fluorescence are therefore proportional to channel activity. To fully quantify channel activity, we used rate of fluorescence increase as the primary metric. As shown in Figure 2A, using TASK-2 as an example, increases in the amount of BacMam added to cells lead to an expected increase in channel activity as determined by rate of fluorescence. Exemplar raw FLIPR data at each concentration of BacMam are presented in the supplementary material (Suppl. Fig. S1A).

Typically, when designing stable cell lines, clones are chosen based on magnitude of signal, this being analogous to the cells transduced with the highest volume of BacMam reagent. These systems are often confirmed as expressing the channel of interest by their response to small-molecule

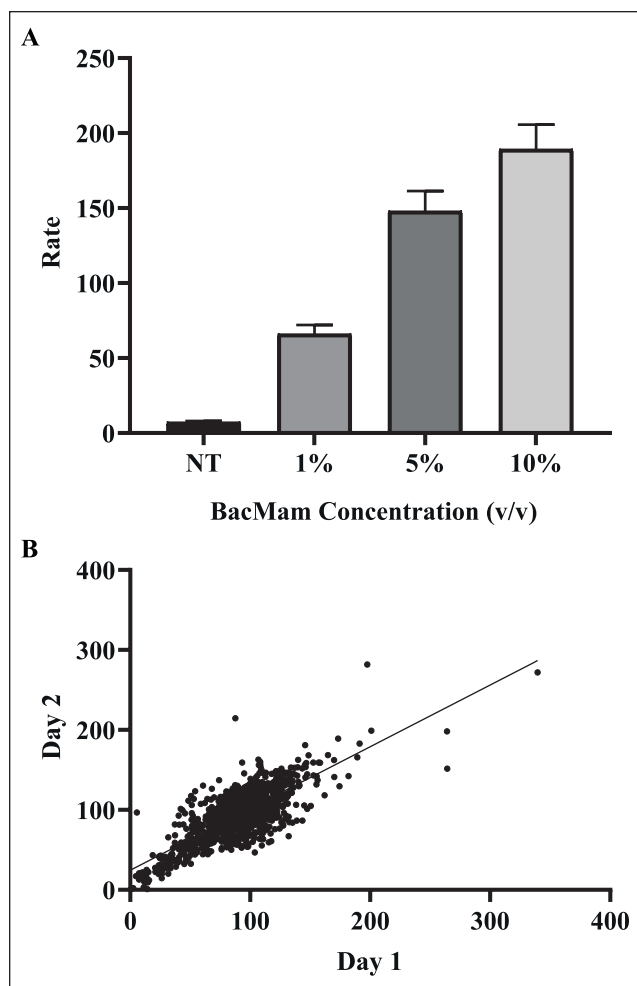


Figure 2. (A) Titration of BacMam correlates with channel activity. Exemplar data for TASK-2 demonstrating that increasing amounts of TASK-2 BacMam (v/v) cause an increase in the rate of thallium flux into the cells, indicative of channel activity. (B) Interday reproducibility of the thallium flux assay. Exemplar data for TASK-2 demonstrate the activity of approximately 1000 druglike molecules in two independent assays conducted on different days ($R^2 = 0.58$, slope = 0.77). Rate denotes the rate of thallium flux (fluorescence intensity units/second) between 15 and 28 s.

inhibitors. **Figure 1** demonstrates that high-expressing systems are less effective for the identification of K2P activators. **Figure 1A** shows four batches of cells transduced with differing levels of BacMam and therefore expressing differing levels of K2P channel. Each cell system is then exposed to differing concentrations of a known channel inhibitor. The compound response curves clearly show that the highest level of transduction gives the best “inhibitor window” in terms of the difference between the control (basal fluorescence activity) and maximum inhibitor concentration. The magnitude of this inhibitor window decreases as the BacMam level decreases. **Figure 1B** shows the same

batches of cells, transduced with differing levels of BacMam, and the response to differing concentrations of a known channel activator. While at the highest concentrations of BacMam activators show a more potent response, the “activator window” is significantly reduced. This is primarily due to the assay having a maximal measurable response and higher levels of target giving a higher basal response. Therefore, lower concentrations of BacMam show a significantly increased signal window, enabling the development of better assays for screening and the subsequent identification of compounds that may show a lower magnitude of activation at screening concentrations. This work therefore highlights the use of appropriate cellular systems, but also the use of appropriate pharmacological tools (i.e., activators) when optimizing reagents for subsequent screening. It should also be noted that neither the activator nor the inhibitor showed an effect in nontransduced cells.

While thallium flux offers many advantages in terms of throughput and cost, it is noted that it is a surrogate of channel activity. Manual whole-cell patch-clamp electrophysiology still represents the gold standard in terms of measuring ion channels and their activity; however, the low throughput negates its use in screening efforts. To ensure our thallium flux was representative of electrophysiology, an exemplar group of activators identified through the BacMam/thallium flux system (i.e., not literature activators) were screened using manual whole-cell patch clamp. Previously described examples of this are cloxyquin, terbinafine, and pranlukast.^{28–30} In each case, whole-cell patch-clamp electrophysiology confirmed activation of compounds. These data suggest that our thallium flux assays for K2P channels are predictive of activity in electrophysiology. Therefore, this system is a good starting point for identifying activators that will translate in downstream assays.

Screening for Activators of K2P Channels

The aim of this work was to use the described thallium flux assay principle to develop a target class approach to K2P screening. Essentially, we wished to develop an understanding of which targets were ligandable and what level of selectivity was achievable across all K2Ps and between particular subfamilies. To achieve this, we sought to develop assays and screen for activators at a representative member of each of the six subfamilies of the K2P superfamily. A pragmatic approach was taken by choosing channels in which an activator may represent some potential therapeutic benefit. We initially sought to study TREK-2, TASK-2, THIK-1, TWIK-1, and TASK-3. For each of these channels, all assay development and optimization were carried out in parallel. Our previous work has studied the identification of activators of TRESK,²⁸ and the work on that channel was therefore not included in this target class piece.

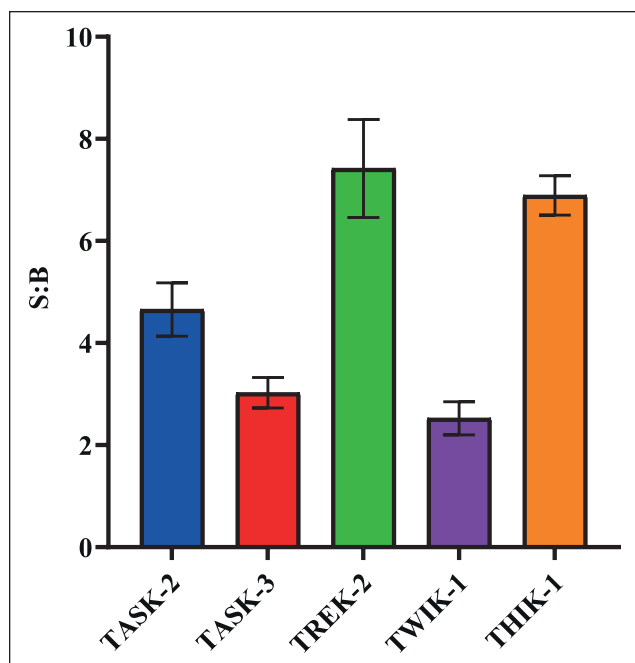


Figure 3. The signal/background ratio for each target in the Index Set screen. The signal/background ratio was calculated relative to the DMSO (100%) and inhibitor (0%) controls and was used to confirm channel expression. Note that as the assays were designed to identify activators, the signal/background values, which were calculated based on the inhibitor window, could appear low for some targets.

In addition to the previously described functional advantages of BacMam, it also offers a significant practical advantage in terms of assay development conditions. We used the same host cell line for each channel transduced. U-2 OS cells were chosen because of their ability to be transduced at high levels, and they are also strongly adherent in plate-based assays. For method development (and during screening), we used frozen U-2 OS cells, which were transduced “in plate.” Cells were mixed with BacMam solution and then put directly into 384-well microplates. This offers significant time savings in terms of cell line generation and gives a standardized set of conditions to optimize. Furthermore, as the host cell remains consistent, automation and liquid handling techniques remained consistent for all targets. This allowed the possibility of screening different numbers of plates for different targets simultaneously.

For each target, the first parameter to optimize was the volume of BacMam to be added. It was important to observe that as BacMam concentration increased, so did channel activity, as defined by the increase in fluorescence. As described in the previous sections, BacMam concentrations were subsequently chosen that were below the maximum signal. An initial matrix was then carried out using several

BacMam concentrations and cell numbers. Because of the high-throughput nature of the FLIPR/thallium flux assay and the adaptability of BacMam, this assay optimization was easily carried out in one screening. The conditions that gave the best signal over baseline (nontransduced) and lowest variability (percent coefficient of variation [CV]) were then chosen. Once these conditions were defined, we would then typically assess the pharmacology of activators or inhibitors, where available. This confirmed that the response we were seeing was mediated by the channel we expected and also allowed us to confirm that the channel could be “activated” by small molecules in this thallium flux system. Additionally, it also allowed us to modify the time parameters used for analysis. We chose to use rate of fluorescence increase as our measure of channel activity. This is the rate of fluorescence increase after the addition of thallium. For some channels, in the presence of an activator, the maximum was reached very quickly; therefore, a shorter time period over which we measured the fluorescence increase was used. Exemplar raw FLIPR data for each target, in the presence of activator (where available) DMSO and inhibitor, are presented in the supplementary material (**Suppl. Fig. S1B**). It should be noted here that tools were not available for all the K2P channels being studied. We then moved on to investigate robustness. A library of approximately 1000 drug or druglike molecules was initially used to assess assay suitability for high-throughput screening and also to measure inter- and intraday variability. Each assay showed good reproducibility (**Fig. 2B**). Importantly, where tool molecules were not previously known, this library provided novel activators that could be used to examine pharmacology and were subsequently used to examine translation into downstream assays (as described above).

To probe each channel in detail, we used the LifeArc Index Set to screen for activators. This library is an approximately 11,000-compound set that is representative of the larger LifeArc collection. For the screen, each part of the process was automated if possible. This allowed subsequent transition to larger screening libraries without further optimization and ensured consistency across targets. For each channel, we defined activity relative to the DMSO (vehicle) wells (100%) and a small-molecule inhibitor (0%). This was chosen as small-molecule activators were not available for each channel. The signal/background ratio, calculated relative to the DMSO and inhibitor controls, for each target is presented in **Figure 3**. It should be noted that due to the assay being designed to be sensitive to activators, but the signal/background ratio being defined by an inhibitor, the assay window can appear small. As **Figure 3** shows, for TASK-3 this was often in the region of 3:1. Given that the percent CVs for all assays were 10.39% (TASK-2), 10.00% (TASK-3), 13.27% (TREK-2), 6.32% (TWIK-1), and 7.34% (THIK-1), this was deemed acceptable, as it falls well below the hit cutoffs subsequently used. **Figure 4**

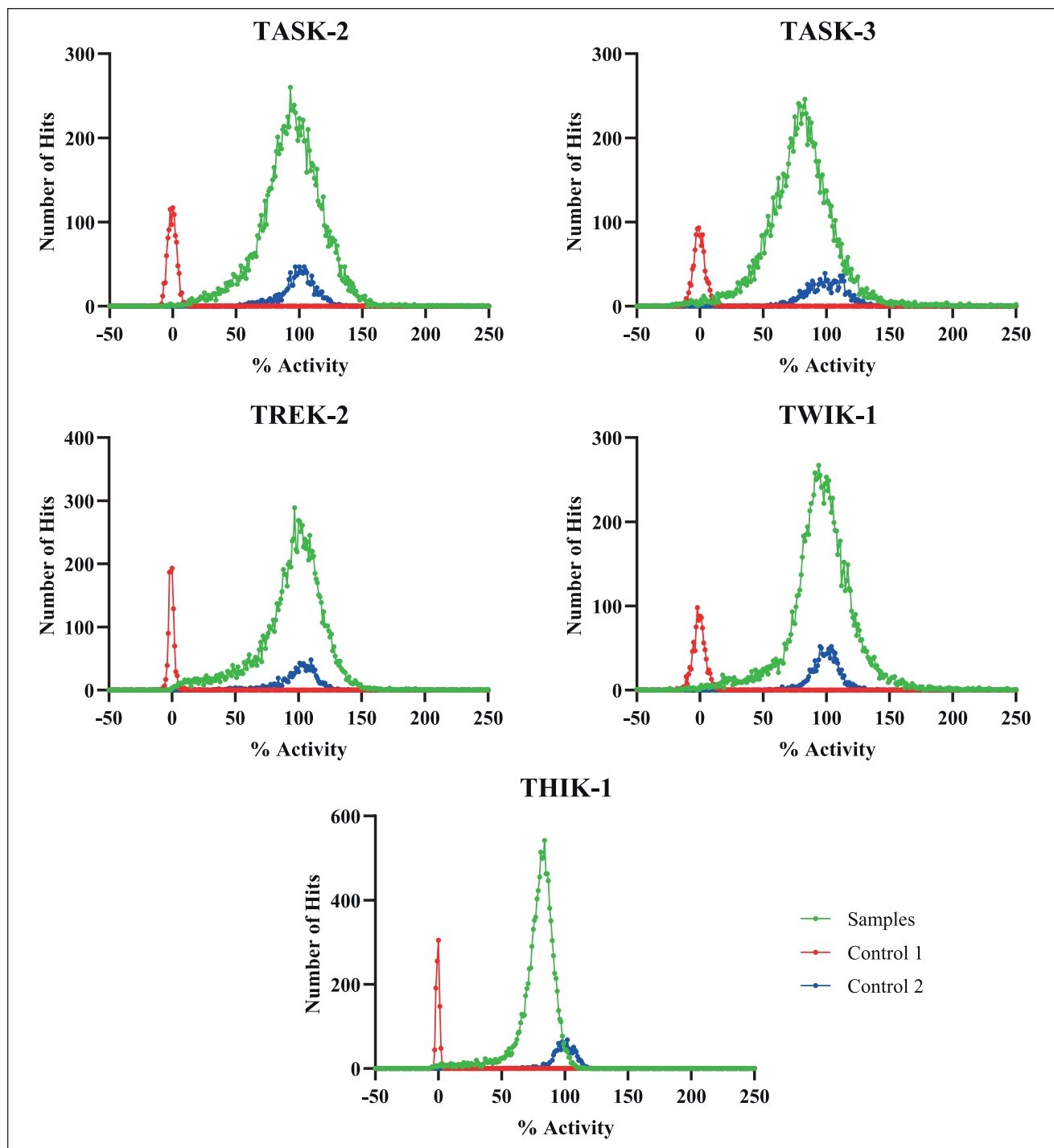


Figure 4. Cumulative frequency plots of test compound and control well activities. Included are all 11,000 compounds in the LifeArc Index Set plotted alongside high (DMSO) and low (inhibitor) control well data for each target. The frequency and distribution of each plot demonstrate the median compound/control activity and the variation within. The median activity, and particularly (when present) the notable skew in each test compound distribution, indicates an increased likelihood of detecting either inhibitors or activators depending on the direction of skew.

shows the distribution of activities observed for all 11,000 compounds screened and the high (DMSO) and low (inhibitor) controls. Of note is the distribution plot for THIK-1. The sample data are clearly skewed below 100%, and this is

indicative of the observation that we were unable to identify activators for this channel. The remaining channels all show a symmetrical distribution, and for TASK-2, TWIK-1, and TREK-2, distribution centered around 100% DMSO activity.

For TASK-3 activity centers below 100% (as is also observed for THIK-1), this suggests that for TASK-3, although activators can be identified, we were more readily able to observe inhibition with small molecules in our screening set.

Hit Selection and Follow-Up

To follow up on molecules identified in the primary screen, we took a pragmatic approach to hit selection. For each target, we used a hit cutoff that equated to approximately a 1% hit rate (i.e., approximately 110 compounds for the 11,000-compound screen). This in turn meant that the activity used to define a hit was slightly different for each channel. For TASK-2, TASK-3, TWIK-1, and TREK-2, the hit cutoffs used were 149%, 160%, 169%, and 220%, respectively. These numbers allowed us to also make observations to the degree by which a channel could be activated by small molecules in our thallium flux system. These data suggest that the magnitude by which TREK-2 can be activated is larger than that for the other channels studied. Hit confirmation rates were ~70% with the exception of TASK-2; this is likely reflective of the hit cutoff being closer to 3 standard deviations of the control than the other assays. As previously described, no activators were progressed for THIK-1.

One of the key advantages of a target class approach is the ability to assess selectivity at an early stage of screening. Once we had established “hit lists” for each target, we then sought to screen each molecule versus each channel. Also of note is that K2P channel activators can show inhibition at high concentrations, giving rise to biphasic curves. This can mean that activity can be underestimated when using single concentrations for screening. To address this, we screened each activator at five concentrations. This therefore enabled us to generate compound response curves against multiple channels. To reduce liquid handling time, a through-plate dilution protocol was developed. This coupled with the BacMam system meant we were able to efficiently screen multiple targets and concentrations simultaneously. Visualization of all compound response curves for each compound allowed us to progress only compounds that showed a preferred profile. Initially, this focused on compounds that were only activators at a single channel, that is, showed no activation at any other K2P screened, but also did not show inhibition at another K2P. Our previous work, and that of others, has shown that compounds within a chemical series can easily move between an activator or an inhibitor at a particular channel, even with relatively minor changes.⁴² This was subsequently expanded to compounds that showed a clear (>30-fold) window between activity at alternative K2Ps. It was also clear from our screening data that the majority of activators were highly selective in terms of activation. Of the 464 compounds selected as hits from the four

targets, only 15 were discarded because they were activators at multiple K2P channels. This is also highly suggestive that selectivity for activators is readily achievable.

Compounds that showed the desired profile were subsequently screened using full 10-point dose–response curves to confirm pharmacology. As **Figure 5** shows, we were able to identify multiple potent activators at each channel. In the case of TASK-2, the most potent activators observed were in the low nanomolar range. Furthermore, we had already established that these activators were selective. It is worth noting that the system we used was able to provide detailed comparison to known standards in terms of both potency and efficacy. The high-throughput nature of the assay, as compared with electrophysiology, allowed detailed quantification of full dose–response curves. **Figure 5** shows that the small molecules identified showed a diverse range of efficacies and potencies relative to previously published and used activators. However, for TREK-2 and TASK-2, the compounds identified were clearly more potent than the standards (BL-1249 and Pyr-6) used. A summary of the key physicochemical properties for the hit compounds at each target is provided in the supplementary material (**Suppl. Table S1**). The LifeArc Index Set⁴¹ provides the opportunity to rapidly identify close analogs from the larger LifeArc compound collection and make observations on whether these compounds are showing any early structure–activity relationships and therefore represent interesting starting points for medicinal chemistry programs.

Discussion

K2P channels represent an attractive group of targets for the development of novel analgesics. However, understanding of these channels and improving their utility as therapeutics have, in part, been hindered by a lack of pharmacological tools. We describe here a system to allow the rapid identification of ligands across the K2P family. Our target class approach allowed the simultaneous screening of multiple channels, using screening synergies and building on the described advantages of target class drug discovery to deliver selective small-molecule activators of K2P channels. We developed a cellular assay system and subsequent screening cascade specifically optimized for the identification of activators. We believe the specific design of assays driven toward the identification of activators, achieved by maximizing the assay window by engineering lower levels of channel expression, to be critical to the success of this approach.

The target class paradigm in drug discovery, described by Barnash et al.,⁴⁰ suggests that using this approach fundamentally reduces the technical risk in drug discovery and therefore helps reduce late-stage attrition. This was achieved in this particular work by simultaneous assay development and screening, showing which targets could not be assayed

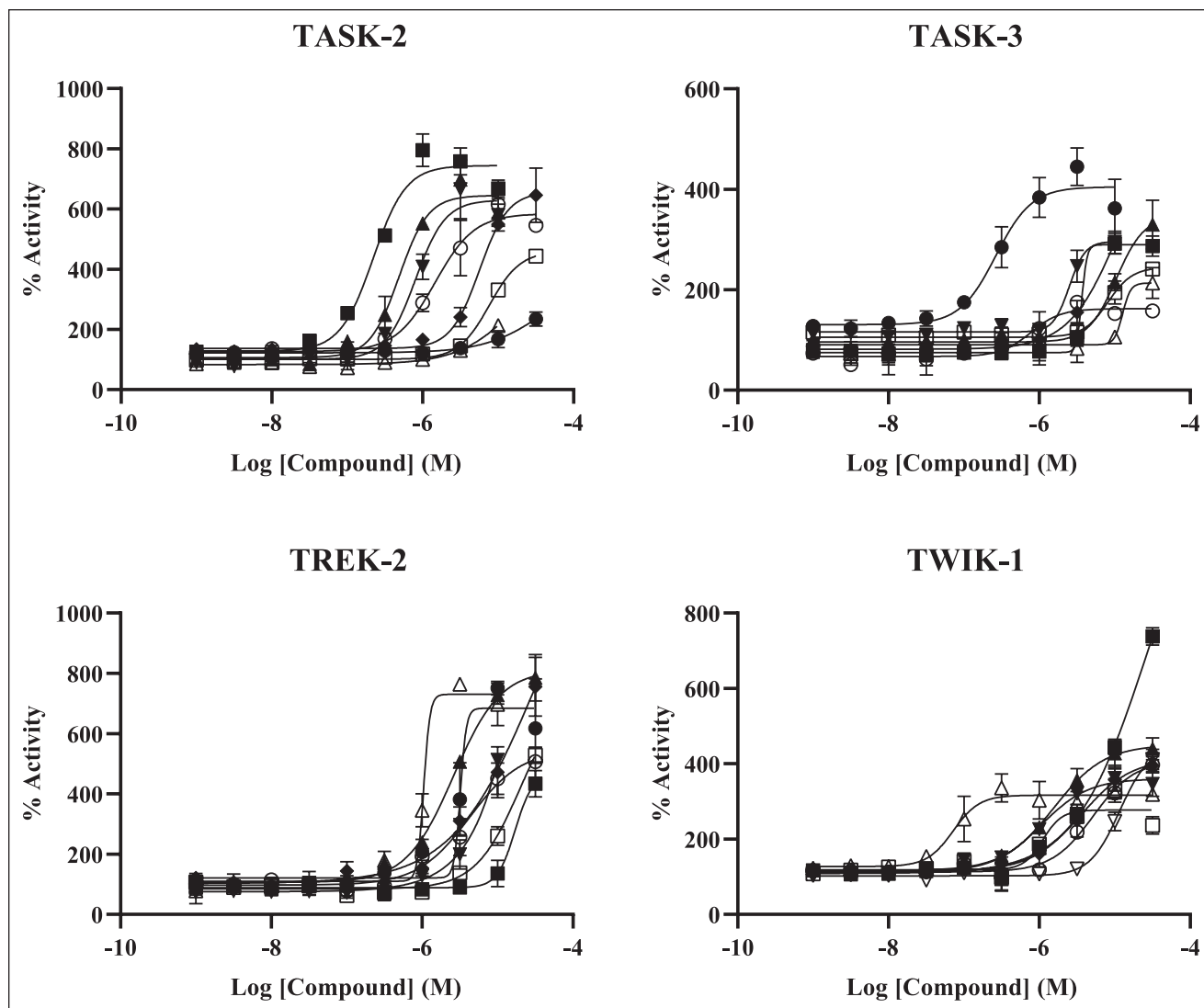


Figure 5. Exemplar concentration–response curves for activators in the TASK-2, TASK-3, TREK-2, and TWIK-1 thallium flux assays. Where possible, activity was compared with that of a control activator (+), TASK-2 (Pyr-6), TASK-3 (terbinafine), and TREK-2 (BL-1249). Note that the figures represent a distinct set of compounds at each target. Compounds that displayed steep slopes or had undesirable structural features were removed from follow-up lists. All graphs show percent activity relative to DMSO and the inhibitor control. Data represent the mean \pm standard deviation of two replicate measurements.

or activated by small molecules, and subsequently early quantification of selectivity across the family. Shared knowledge of downstream cascades and translational approaches was also significantly advantageous. By increasing our chances of success, we should of course contribute to reduced costs of developing novel therapeutics. This is pertinent in the most likely area of therapeutic intervention for K2Ps, which is pain. Pain portfolios across the drug discovery industry are characterized by a lack of potential first-in-class therapeutics. This is driven by the complexity of developing drugs and the lack of translation in a number of pain models and targets. By adopting this approach, we hope to lower the risk associated with analgesic development by identifying high-quality preclinical

molecules across a range of targets. Barnash et al. describe how target class drug discovery drives innovation by using the power of drug discovery companies and medicinal chemistry efforts to develop high-quality probes to less validated targets. Our work has allowed the publication of multiple novel K2P activators.^{28–30} These have subsequently been used to build upon the role of targets within pain and contribute to the validation of K2Ps as targets for analgesia.

While there are clear operational advantages for target class discovery in small- to medium-sized companies (significantly less effort required to prosecute five related targets than five discrete targets), another advantage is the ability to build research networks. The accumulation of

knowledge and unique research tools has allowed the development of multiple collaborations, across diverse areas of the life sciences sector. It offers organizations the potential to become a central component of a particular field, attracting high-quality collaborators.

It is, however, also worth noting that there are some potential disadvantages to pursuing a target class approach. In terms of hit identification, there is the increased cost compared with a single target. While the cost savings are significant compared with multiple nonrelated targets, there is still an increase. Similarly, there is a requirement for increased throughput and capacity. Automation and liquid handling techniques need to be managed efficiently to minimize this. After hit identification, a target class approach may also not be as efficient if selective molecules have been identified. In simple terms, this could mean that multiple targets are screened to show no activity. To mitigate this, we have moved the initial target class approach to a more focused target approach once chemical optimization is initiated, typically screening compounds routinely versus two or three targets.

An important component of designing a system specifically optimized for activators has been the ability to quantify the pharmacology of known activators in addition to identifying novel molecules. Similarly, once “hits” have been identified, they can be readily “benchmarked” against previously described molecules. The increased sensitivity of the assay to activation allows us to readily quantify differences in both efficacy (magnitude of effect) and potency (EC_{50} in the thallium flux assay). As described in previous publications,^{29,30} we have been able to not only compare the selectivity of activators across multiple targets, but also identify which activators are most active relative to each other at a single target. We believe this provides critical information about the pharmacology of activators, as comparison of activators and their selectivity is complex. The data generated using thallium flux can be compared with other systems (automated patch clamp/manual patch clamp) to allow more informed decisions around which compounds are the most appropriate for the analysis of particular K2P channels. Similarly, it can allow comparative quantification of system differences (e.g., thallium flux vs automated patch clamp).

It is hoped that the small molecules identified within these screens can become starting points for medicinal chemistry programs, aimed at developing therapeutically useful small-molecule K2P modulators. The approach we have taken has confirmed which K2Ps are ligandable and for which channels high-quality, high-throughput assays can be developed. The activators identified have also been shown to be selective versus the K2Ps screened. The ability to develop these molecules has been enhanced by recent developments in elucidating structures of K2P channels. This includes a description on the gating mechanisms of K2P channels and the description of the “cryptic binding

site,” a potential shared pharmacophore across the K2P family.³² Furthermore, we are beginning to understand further which subsets of cells express particular K2Ps,²² helping our development of translational assays, and the likely role individual channels play in excitability.

Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: D.M., E.O., J.M.L., D.C.T., J.J., and P.D.W. are employed by LifeArc, and their research and authorship of this article were completed within the scope of their employment with LifeArc. Professor Mathie reports grants from LifeArc, during the conduct of the study; grants from Leverhulme Trust, grants from Bayer, grants from Cardiovascular Medical Research and Education Fund, outside the submitted work.

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