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Screening for Non-Pore Binding Modulators of EAG K+ Channels

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Abstract

Members of the EAG family of voltage gated K^+ channels are involved in several pathophysiological diseases and there has been a great interest in screening for drugs that modulate the activity of these channels. Many drugs have been shown to bind in the pore of these channels blocking ion flux and causing disease pathology. In this report we present two independent screening campaigns in which we wanted to identify small molecules that bind to either the intracellular cytoplasmic amino terminal PAS domain from the human ERG channel or bind to the amino or carboxy terminal globular domains from the mouse EAG1 channel affecting their interaction. We report that in both cases compounds were identified that showed weak, non-specific binding. We suggest alternative routes should be pursued in future efforts to identify specific, high affinity binders to these cytoplasmic domains.

Keywords

hERG; EAG1; CNBh; PAS; ion channel; drug screening

Introduction

The EAG family of voltage gated K^+ channels cluster by sequence similarity into three subfamilies: *ether-à-go-go* (EAG), eag-related gene (ERG) and eag-like K^+ Channels (ELK). They are encoded by the KCNH1–8 genes[1]. These channels are involved in several human pathophysiological diseases. Loss of human ERG (hERG) K^+ channel function either by inherited mutations or by secondary pharmacological effects is associated with long QT syndrome (LQTS), which can lead to ventricular arrhythmias and sudden cardiac death. In addition, expression of both hERG and human EAG1 is increased in a number of cancer cell types where they are associated with proliferation and invasiveness phenotypes[2, 3]. Over the years, the majority of studies on potassium channels have focused on the inhibitory effects of various compounds on channel gating. However, in an effort to understand the molecular mechanisms underlying channel dysfunction and associated rescue strategies, an increasing number of studies are shifting the focus from channel blockage to other aspects of channel regulation[4].

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Channels in the EAG family share a characteristic architecture with four subunits arranged to form a central K^+ conducting pore. Each subunit has large cytoplasmic amino and carboxy terminal regions (Figure 1). The amino terminal region contains the Per-Arnt-Sim (PAS) domain that in other proteins has been shown to bind small molecules and modulate protein activity[5]. Crystal structures have been reported for the PAS domains from hERG and mouse EAG (mEAG) channels; they show that these domains have cavities similar to where other PAS domains bind small molecules; however, no ligands have been reported thus far[6]. The carboxy terminal region contains a cyclic nucleotide-binding homology (CNBh) domain, however, the EAG family of channels are not regulated by direct binding of cyclic nucleotides. Rather it has been shown that an intrinsic ligand occupies this pocket; it has also been suggested that flavonoids could compete within this binding pocket to potentiate channel function[7, 8]. Additionally, biochemical and functional data has shown that the PAS domain associates with the CNBh domain in solution. A recent X-ray crystal structure of a complex of the mEAG1 PAS domain and CNBh domain shows that this protein interface is a hot spot for many of the known mutations that are associated with human disease pathology[9]. It is therefore likely that the interaction between the PAS domain and the CNBh domain plays a key role in modulation of channel function.

In this study we present our conclusions from two independent screening campaigns, each of ~5,000 compounds, to identify small molecules that bind specifically and with high affinity to the cytoplasmic domains of EAG channels. These high affinity binding molecules could potentially be used as novel modulators of channel activity. Firstly we screened for small molecules which affect the stability of the hERG PAS domain using a fluorescence based thermal shift assay and concurrently we screened for small molecules which could modulate the association between the mEAG1 PAS and CNBh domains (which are 98.5% and 100% identical to the human EAG1 proteins respectively) using a fluorescence polarization assay.

Material and Methods

Protein Production and Labeling

The hERG PAS wild-type domain (residues 1–135) was expressed and purified as previously described[10]. The mouse EAG1 PAS protein (residues 1–137) was cloned, using sequence specific primers, expressed and purified following the procedure described previously for a construct spanning residues 27–136[6]. The mouse CNBh domain (residues 552–724) was expressed and purified as described[7]. KtrA from *Bacillus subtilis* was purified as previously described[11]

To label mEAG1 PAS on accessible cysteine residues with fluorescein, the purified protein was loaded on a HiTrap Desalting column (GE Healthcare) to remove DTT, using 50 mM Tris pH8.0, 150 mM NaCl as the buffer. Protein was incubated with a 5 or 10 fold molar excess of fluorescein 5-maleimide (Thermo Fisher Scientific) for 1 h at room temperature protected from light. The reaction was stopped by adding DTT to 5 mM. Free fluorescein was removed by dialysis overnight at 4°C against 20 mM Tris pH8.0, 150 mM NaCl, 5mM DTT. The fluorescein to protein molar ratio of fluorescein-labeled PAS (F-PAS) was calculated as indicated by the manufacturer and was typically found to be 3 or 4.

hERG PAS Thermal Shift Screen

A screening campaign was conducted using the ApexScreen Library from TimTec Inc. The ApexScreen is a collection of 5,080 compounds that were selected to represent the diversity within the TimTec stock of over 160,000 compounds. The library was provided in a 96 well format as 100 μL of a 3 mM stock in 100% DMSO. The 1st and 12th column were empty and used as positive and negative controls, therefore each plate contained 80 compounds. 25 μL of stock compounds were transferred to an intermediate plate containing 275 μL of buffer (50 mM Hepes pH7.5, 150 mM NaCl, 5 mM DTT and 4% DMSO), to produce an intermediate plate contained 250 μM of compound and 12% DMSO.

A thermal shift assay that reports the stability of the hERG PAS domain in solution was essentially as previously described[10] but adapted for compound library plate based screening. In brief, 20 μL of purified hERG PAS protein (7.5 μM) was combined with 20 μL of Sypro Orange Dye (6.25×) (Sypro Orange solution from Sigma-Aldrich comes as a 5000× stock) in a 96 well Bio-Rad Multiplate white PCR plate. To this assay plate 10 μL of intermediate compound plate was added resulting in a 50 μL assay volume with final concentrations of 3 μM hERG PAS, 2.5× Sypro Orange Dye and 50 μM compound in 50 mM Hepes pH7.5, 150 mM NaCl, 5 mM DTT, 2% DMSO. The final protein to compound molar ratio was 0.06. Importantly, we verified that the thermal profile of the PAS protein was not affected by the presence of 2% DMSO. The plates were heated from 25°C to 80°C in an iQ5 Real Time Detection system (Bio-Rad). Unfolding of hERG PAS protein was monitored using the excitation and emission filters 545/30 and 585/20 respectively.

Thermal Shift Assay for Retest

Compounds from both screening assays were tested in a thermal shift format at a final concentration of 50 μM or 100 μM. Purified mEAG1 PAS, CNBh, or hERG PAS were added to a 96-well plate at 3 μM concentration in 50 mM HEPES pH 7.5, 150 mM NaCl, 5 mM DTT, 2 % DMSO. Data were recorded as mentioned above. When thermal stability of PAS was tested against compound concentration, DMSO was kept at 2%. Change in T_m was plotted against compound concentration and the K_d was determined from fitting the experimental data with Eq. (1) or with similar equation described in[12]:

$$Y = B\max^{*} X/[\mathrm{Kd}+X] \quad \text{Eq. (1)}$$

In this equation Y represents the change in T_m on binding; X represents the concentration of compound and B_{max} represents the maximal binding.

mEAG1 PAS and CNBh Domain Fluorescence Anisotropy Screen

A screening campaign was conducted using a compound library purchased from the University of Wisconsin Small Molecule and Screening Facility. The facility possesses a compound library from the ChemBridge DIVERSet, a collection of "universally" diverse, pre-designed drug-like small molecules. The compounds have been rationally selected based on 3D pharmacophore analysis to cover the broadest part of biologically relevant pharmacophore diversity space. The library was provided in 384 well format as 50 μL of a

10 mM stock in 100% DMSO. Using a Liquidator 96 Manual Pipetting System (Rainin) 5 μL of stock compounds (5,760 compounds) were transferred to an intermediate plate containing 95 μL of buffer (20 mM Tris pH8.0, 150 mM NaCl, 5 mM DTT and 15.8% DMSO), to produce an intermediate plate contained 500 μM of compound and 20% DMSO.

A fluorescence anisotropy assay that reports the interaction of fluorescein-labeled mEAG1 PAS protein with the CNBh domain was adapted for plate based screening from a previously described assay[9]. The assay is based upon an increase of F-PAS fluorescence anisotropy (r value) when the F-PAS and CNBh complex is formed. In brief, F-PAS (0.11 μM) and CNBh (11 μM) or F-PAS (0.11 μM) alone, were added in a volume of 90 μl assay buffer in a 96 well, black, flat bottomed microplate (Greiner Bio-One). In each fourth plate, rows A to D of column 1 contained no compound and were used as positive controls, and rows E to H of the same column contained F-PAS only and were used as negative controls. To this assay plate 10 μL of intermediate diluted compound was added resulting in a 100 μl assay volume with final concentrations of 0.1 μM F-PAS, 10 μM CNBh and 50 μM compound in 20 mM Tris pH8, 150 mM NaCl, 5 mM DTT, 2 % DMSO. The final protein to compound molar ratios were 0.002 and 0.2, for F-PAS and CNBh, respectively. We verified that the interaction between F-PAS and CNBh protein is not altered by the presence of 2% DMSO. F-PAS (0.1 μM) and CNBh (10 μM) concentrations were chosen such that under these conditions the measured r value is 60 % of saturation, allowing for either an increase or decrease of r value upon compound addition. The plates were incubated for 70 mins at room temperature in the dark before fluorescence anisotropy, r value, was read on a BioTek Synergy 2 plate reader, using excitation and emission filters 485/20 and 528/20 nm, respectively.

Screening Statistics

The Z´ value for both screening campaigns was on average 0.9 showing that both assay formats were of excellent quality[13] to screen at a high final compound concentration of 50 μM. During the screening campaign any plate which had a Z value less than 0.5 was individually analyzed for “outlying high hitting” wells which generally correlated with highly colored compounds. For the thermal shift screen, all compounds that gave a T_m shift of either ≥ 1°C or ≤ 1°C from the no compound control wells were retest confirmed in duplicate at 50 μM. For the fluorescence anisotropy screen, all compounds that gave a change in anisotropy of 3× standard deviations from the mean value were retest confirmed in triplicate at 50 μM with the inclusion of 6 positive and 6 negative controls per plate. To eliminate false hits due to intrinsic compound fluorescence contributing to the measured r value, a control without F-PAS was included for each selected compound. Individual fluorescence intensities from the vertical polarized light, $I_{\parallel}$ and horizontal polarized light, $I_{\perp}$ of the control were subtracted from those of the complete assay, and fluorescence anisotropy was calculated using the equation $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$.

Retest Confirmation

Compounds that retest confirmed at a single dose of 50 μM from both screening campaigns were reordered as powders and tested for dose response. Commercial analogues of these compounds were also obtained to evaluate structure activity relationships.

Affinity Measurements using Fluorescence Anisotropy

The fluorescence anisotropy assay was performed as described above, but with increasing concentrations of CNBh protein, from 2 to 80 μM, in the presence or absence of compounds at 100 μM in triplicate. The dependence of r with CNBh concentration was plotted and the dissociation constant K_d was determined from the fitting of the experimental data with Eq. (2) as previously described[9]:

$$r=\alpha\left[\frac{(\mathrm{R}_t+K_d+L_t)-\sqrt{(-\mathrm{R}_t-K_d-L_t)^2-4\times\mathrm{R}_t\times L_t}}{2}\right]+\beta \quad \text{Eq. (2)}$$

where R_t and L_t are total receptor (F-PAS) and ligand (CNBh) concentrations, and α and β are a scaling factor and an offset factor, respectively.

Affinity Measurements using Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed in a VP-ITC instrument (MicroCal) at 15 °C with a first injection of 2 μl, followed by twenty eight injections of 10 μL. Purified mEAG1 PAS and CNBh proteins were dialyzed overnight against 50 mM HEPES pH7.5, 150 mM NaCl, 1 mM TCEP. DMSO was added to the protein to be injected, and DMSO or a selected compound dissolved in DMSO was added to the protein to be titrated; in all situations the final concentration of DMSO was 2%. The injected protein was placed in the syringe at 200 to 300 μM concentration, while the titrated protein was 10 times less concentrated in the cell. Titrations were performed with mEAG1 PAS into CNBh and CNBh into mEAG1 PAS, in the presence or absence of 50 or 100 μM of the selected compounds. Data were analyzed using Origin 7 (MicroCal). The dissociation constant of interaction was obtained by fitting the data to a single site binding model.

Results and Discussion

Initial Screening and Retest Confirmation

Two independent *in vitro* screening campaigns for small molecules that bind specifically and with high affinity to the cytoplasmic domains of EAG channels were executed. Firstly, in order to identify novel small molecules that bound to the hERG PAS protein, the ApexScreen Library from TimTec comprising 5,080 chemically diverse compounds was screened at 50 μM using a thermal shift assay format. From the initial screen 83 compounds were identified as “hits” that gave a change in T_m of hERG PAS protein of at least 1°C with a 1.6% hit rate (Table 1). Initial hits were retest confirmed in duplicate from liquid at 50 μM resulting in 30 compounds: 18 compounds that gave a T_m shift ≥ 1°C (stabilizers) and 12 compounds that gave a T_m shift ≤ 1°C (destabilizers) resulting in a 36% retest confirmation rate (Table 1).

Secondly, in order to identify small molecules that could modulate the interaction between the mEAG1 PAS and CNBh domains 5,760 compounds from the Chembridge DIVERset were screened at 50 μM using fluorescence anisotropy as an assay format. From the initial screen 92 compounds were identified as “hits” that showed a change of fluorescence

anisotropy greater or equal to 3× standard deviations from the mean value with a 1.6% hit rate (Table 1). Initial hits were retest confirmed in triplicate from liquid at 50 μM resulting in 28 compounds: 12 compounds showed an increase in anisotropy and 16 compounds showed a decrease in anisotropy, a 30% retest confirmation rate (Table 1).

Dose Response Confirmation

From the screening campaigns we have identified compounds which either alter the protein stability of hERG PAS protein or alter the mEAG1 PAS/CNBh complex. Since both compound libraries were obtained as frozen liquid stock solutions we re-ordered all compounds for retest confirmation as powders to analyze in each assay format by dose response through a range of compound concentrations (0 – 200 μM). For hERG PAS all compounds that stabilized or destabilized the apparent T_m in the thermal shift assay were assessed. However, it became clear that the 18 compounds that apparently had stabilized the T_m profile all showed a decrease in maximum fluorescence response as the compound concentration increased, suggesting that the PAS protein was aggregating or precipitating in the well resulting in a false shift in T_m. We had previously noticed this effect during assay development in that as the concentration of hERG PAS protein decreased the apparent T_m increased. In contrast, compounds that had destabilized the T_m of hERG PAS protein at a single dose showed compound titration up to 200 μM without affecting the maximal fluorescence response and we focused on hit compounds ST020986, for further hit expansion (Figure 2A), and ST057196, a phenol based compound.

From our second screen, 28 compounds were tested for dose response, of which 14 compounds were selected for retest from powders. On testing these compounds from 0 to 200 μM, 7 compounds showed an increase in anisotropy suggesting that they affected the mEAG1 PAS interaction with CNBh. From these retest compounds we focused on three compounds: 6024883, 6044866 and 6044806 (the latter included hit expansion as shown in Figure 2B).

Chemical Series Expansion and Proposed Mechanism of Action

The compound ST020986 has the familiar chemical name of Bisphenol AF and two analogues were commercially available, Bisphenol A and F (Figure 2A). Interestingly, compounds within this series have previously been shown to bind to the human PAS Kinase (hPASK) domain with varying affinities[14]. The structure activity relationship of binding of this compound series and hydrophobic nature of these compounds suggested that they were ideal candidates for binding in the hydrophobic environment of the PAS protein cavity. We also noticed that ST057196, another phenol like compound, also destabilized hERG PAS protein in the thermal shift assay and so it was tested in dose response also. As shown in Figure 3A, titration of 3 μM PAS protein with 0–200 μM Bisphenol AF resulted in a significant dose dependent decrease in T_m of the hERG PAS protein. At 200 μM compound addition T_m decreased at least 4.5°C. Saturation in the assay was not achieved, due to difficulties in compound solubilization at higher concentrations. Fitting these data with two binding equations (see Material and Methods: Eq. (1) and equation in[12]) revealed a K_d of 388 μM or 310 μM, respectively, indicating a low affinity interaction between protein and compound. No shift in T_m was observed for Bisphenol A and F suggesting weak to no

binding of these compounds; however, compound solubility issues limited this study. This rank order of potency for the hERG PAS protein of Bisphenol AF>Bisphenol A or F is consistent with that observed previously for the hPASK PAS domain. It is generally thought that the extra interactions established between a receptor and its ligand result in increased temperature stability of the complex relative to the apo-receptor. However, the decrease in temperature stability observed for the PAS protein after exposure to Bisphenol AF probably results from interaction of the compound with an unfolded state of the protein[15]. In addition, we verified that the destabilizing effect of this compound was non-specific, since when titrated against an unrelated protein, KtrA from *Bacillus subtilis*[11], it also decreased the T_m in a dose dependent manner and the data could be fitted as above (K_d of 31 μM) Altogether, this led us to stop pursuing this series of compounds. Relative to the single phenol derivative ST057196, titration of PAS with 0–200 μM compound resulted in a steep concentration dependence of T_m, where on addition of 50 μM of compound the protein T_m is already decreased by more than 18°C (Figure 3B). We noticed that ST057196 contains a sulfhydryl group that could potentially modify any of the 8 cysteine residues present in the hERG PAS domain and cause destabilization of the protein. In fact, as shown in Figure 3B inset, titration of KtrA[11] with ST057196 also decreased the T_m in a dose dependent manner which could be fitted as above with a K_d of 25 μM. These results show that the effect of ST057196 is non-specific and is consistent with a covalent modification of the protein. This led us to stop pursuing this series of compounds.

From our second screening campaign we wanted to know whether compounds 6024883, 6044866 and 6044806 induced changes in the K_d of binding of mEAG1 PAS with CNBh protein. To do this, compound was present at 100 μM during ITC titrations performed between the PAS and CNBh proteins. Solubility issues prevented analysis of compound 6044806 in this assay format. The interaction between mEAG1 PAS and CNBh protein is an endothermic reaction with a K_d of 2.1 ± 0.8 μM and N=1.3 ± 0.27. Presence of 100 μM of compounds 6044866 (K_d of 1.1 μM;N=1.2) and 6024883 (K_d of 3.3 μM;N=0.77) had no effect on this interaction (Figure 3C). Compound 6044806 and three other compounds from this series were ordered as powders (5273899, 574288 and 6048300) and all four were tested in the fluorescence anisotropy assay at 100 μM with F-PAS at 0.1 μM and CNBh protein from 2–80 μM to see whether they would affect the interaction. As shown in Figure 3D after fitting the data with Eq. (2), none of the compounds affected the K_d of interaction (No compound control K_d of 3.1 ± 1.1 μM; 6044806 K_d of 1.8 ± 0.56 μM; 5273899 K_d of 3.6 ± 1.2 μM; 6048300 K_d of 2.3 ± 0.93 μM and 5274288 K_d of 2.4 ± 0.67 μM). However, we noticed that three of the compounds (6044806, 574288 and 6048300) increased the maximal anisotropy signal compared with the no compound control and compound 5273899. Using the thermal shift fluorescence assay we individually added each compound to either mEAG1 PAS or CNBh domain alone and interestingly saw that these same three compounds had varying destabilizing effects on the CNBh protein compared to minor effects on the mEAG1 PAS protein (Figure 3E). In order to see whether this was a true binding effect we further tested compound 6048300 for binding to CNBh using ITC, as this compound had the best solubility profile. Unfortunately no signal above heat of dilution could be obtained (data not shown) and therefore we cannot find evidence for a specific interaction with CNBh protein.

Future Prospects for Non-Pore Modulation of EAG K+ Channels

Although members of the EAG voltage gated K+ channels have been under extensive study over the years there is still a lot that is unknown about their molecular mechanisms of modulation. Small molecules that specifically bind to the channel´s cytosolic domains and alter the functional properties of the channel would be of great utility for understanding those properties. In addition, novel small molecules that modulate channel activity would be of great interest due to the role of EAG channels in LQT syndrome or cancer.

We have executed two independent screening campaigns to identify small molecule ligands for EAG channel regions that have not been extensively explored in terms of their small-molecule binding potential. We searched for compounds that could either bind to the N terminal PAS domain of the hERG K^+ channel, or that interfere with the interaction between mEAG1 PAS and CNBh domains. We recognize that both of the screening libraries used in these campaigns were of limited size, however, they contained a good chemical diversity and both assays were robust and of excellent quality. High resolution crystal and NMR structures of PAS domains from hERG, mouse EAG and Drosophila Elk channels have been reported[6, 16] and although these structures did not contain ligands they have reasonably large hydrophobic cavities that for other PAS domains have been suggested to bind ligands. In fact in our first screen we focused on phenol based compounds of which the bisphenol series been identified as binding to the hPASK PAS domain with varying affinities. However, we showed that in both cases these compounds had solubility issues and caused protein destabilization, a non-specific effect. Similarly, for our second screening effort we identified a series of compounds in which some seemed to increase the apparent maximal anisotropy signal of mEAG1 PAS binding to CNBh protein, however they turned out to not have an effect on the affinity of this interaction.

In conclusion both screening campaigns resulted in compounds that have weak *in vitro* effects and challenging solubility issues. Our results strongly suggest that it will be challenging to identify small molecules that recognize these cytoplasmic regions (PAS domain or PAS:CNBh interface) and modulate channel function. As a consequence it could be argued that modulation of EAG channels through these domains is not feasible. However, considering the functional impact of mutations within these channel regions we believe that alternative strategies should be pursued. One such strategy would be the use of antibodies or antibody fragments. These molecules have the advantage of being highly specific and can be re-engineered in terms of potency and toxicity. They also have great potential for interfering with protein-protein interfaces like the one between the PAS and CNBh domains. We are aware that this strategy raises the specific problem of targeting a biological molecule to the cytoplasmic environment. However, this is a common challenge to many different therapeutic fields and is one that is being addressed by many research groups[17].

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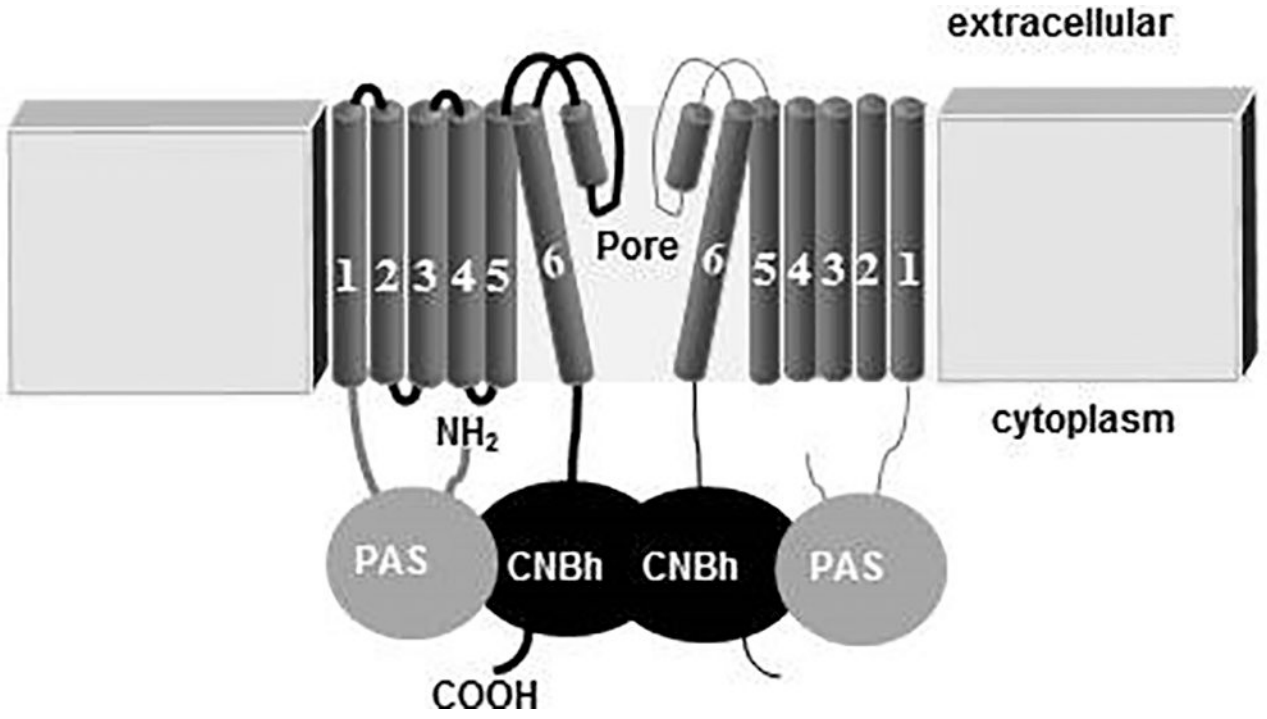

Figure 1.

Schematic diagram showing two subunits of a tetrameric EAG K^+channel. Each subunit consists of six transmembrane spanning domains. S1–S4 represent the voltage sensing domain and S5-helix-S6 represent the pore domain. Each subunit contains a PAS domain in the amino terminus and a CNBh domain in the carboxy terminus.

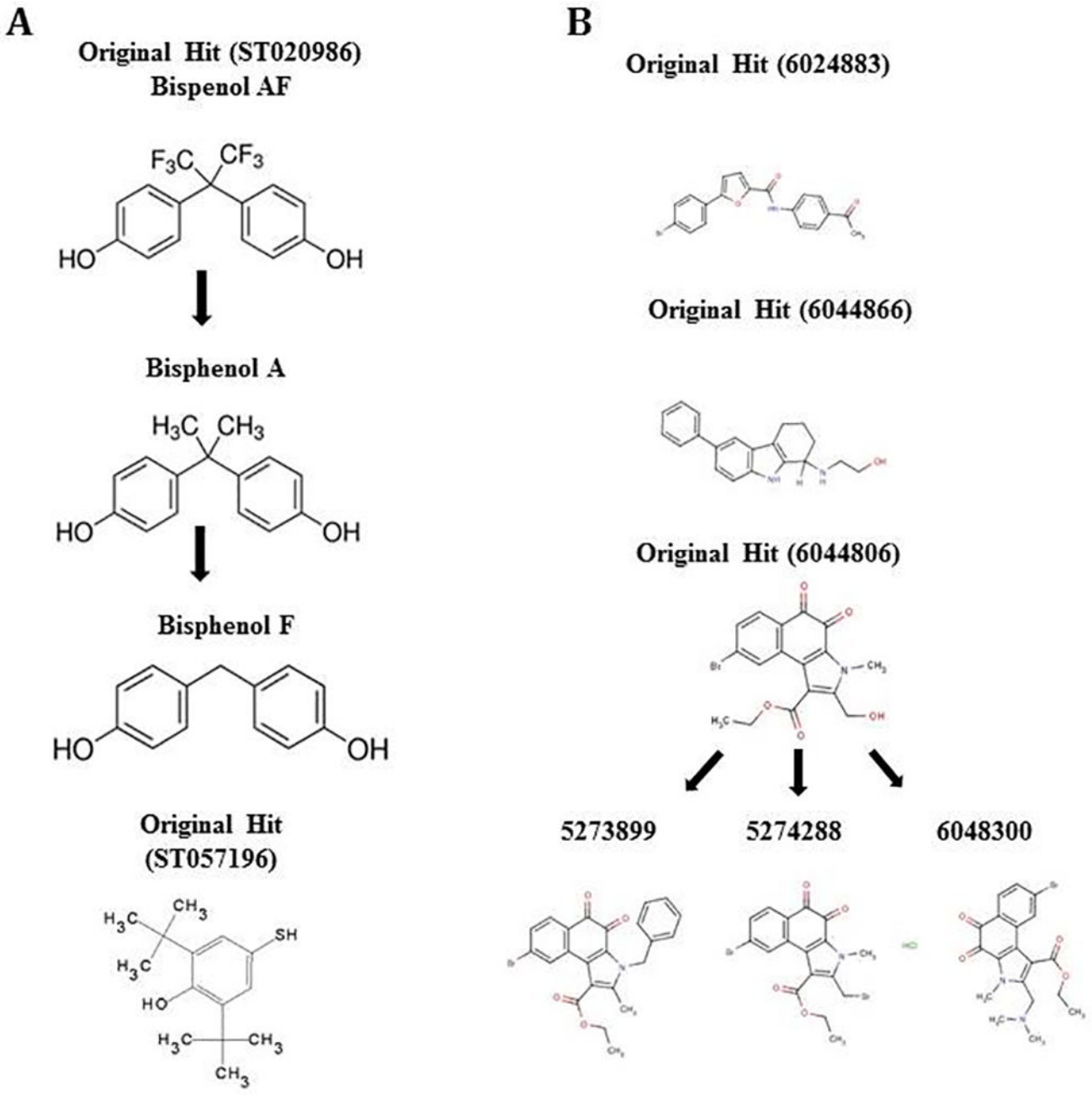

Figure 2.

A Thermal Shift Screen. Chemical structure of original hit ST020986 and two related commercially available compounds and ST057196. **B** Fluorescence Anisotropy Screen. Chemical structure of original hit 6044806 and three related compounds plus 6024883 and 6044866.

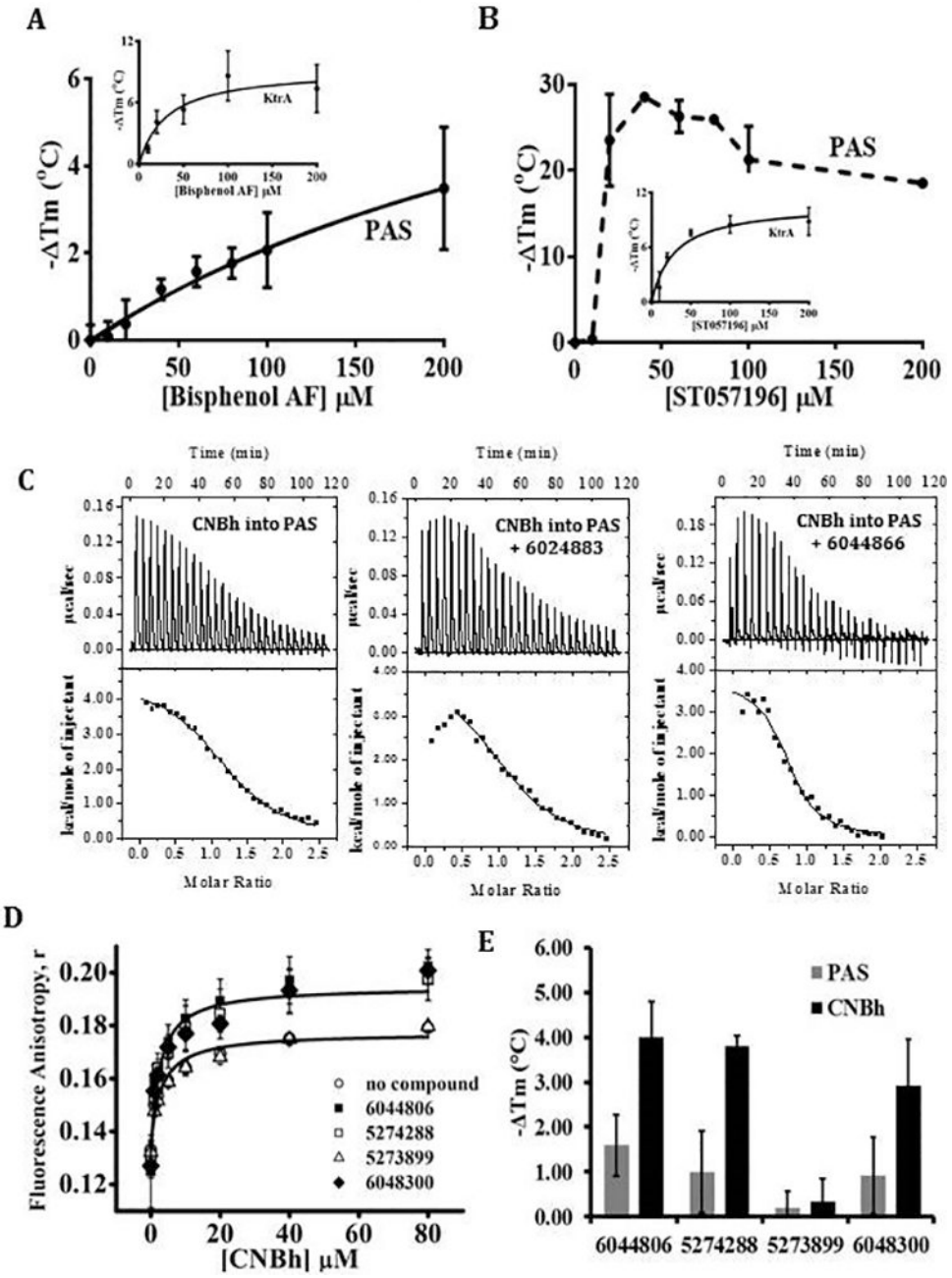

Figure 3.

A Change in T_m of hERG PAS (and in inset, of KtrA from *Bacillus subtilis*) on addition of 0 to 200 μM of compound Bisphenol AF. **B** Change in T_m of hERG PAS (and in inset, of KtrA from *Bacillus subtilis*) on addition of 0 to 200 μM of compound ST057196. Plotted data is the mean and standard deviation of 2–3 independent experiments each having at least 4 replicates per compound concentration. **C** Representative ITC profiles of mEAG1 CNBh into PAS in the absence or in the presence of 100 μM of compound 6024883 or 50 μM of 6044866. In each case, the upper panel shows the raw data collected during titrations, and the bottom panel shows the integrated heat change for each injection together with the fit of

the binding isotherm. Titration in the absence of compound (CNBh into PAS) was performed in 3 independent experiments resulting in a K_d of 2.1 ± 0.8 μM; N=1.3 ± 0.27. Titrations in the presence of compound 6044866 (K_d of 1.1 μM;N=1.2) and 6024883 (K_d of 3.3 μM;N=0.77) were single experiments. **D** Change in fluorescence anisotropy of mEAG1 F-PAS with increasing concentrations of CNBh in the absence or presence of 100 μM of compound 5273899, 6044806, 5274288, or 6048300. For clarity only twofitted curves, with Eq. (2), are shown. The curves correspond to titrations in the absence of compound (K_d = 3.1 ± 1.1 μM) and in the presence of compound 5274288 (K_d = 2.4 ± 0.67 μM). Identical fits for the other compounds gave the following K_d: 6044806 K_d of 1.8 ± 0.56 μM; 5273899 K_d of 3.6 ± 1.2 μM; 6048300 K_d of 2.3 ± 0.93 μM. Plotted data is the mean and standard deviation of 3–4 independent experiments each having 2–3 replicates. **E** Change in T_m of mEAG1 PAS and CNBh upon addition of each of the indicated compounds at 100 μM, using the thermal shift assay. Plotted data is the average change in T_m and standard deviation of the mean from 2 independent experiments each having 6 replicates.

Table 1

Summary of High Throughput Screening and Lead Follow-Up Strategies

	HTS Screening Statistics	
	(A) hERG PAS Domain HTS	**(B) mEAG PAS:CNBh Domain HTS**
96 Well Assay format	Thermal Shift - Sypro Orange	Binding - Fluorescence Anisotropy
Number of compounds Screened	5,080 compounds - TimTec Apexscreen Library	5,760 compounds - University of Wisconsin
Compound Concentration	50 μM	50 μM
Initial Hits	83 compounds showed ΔTm ≥1°C from control	92 compounds showed Δanisotropy ≥3 stdev from mean value
Retest Confirmation n=2 at 50 μM	18 compounds showed an increase in Tm ≥1°C	10 compounds showed an increase in r
	12 compounds showed a decrease in Tm ≥1°C	4 compounds showed a decrease in r
Dose Response (powder)	0 –200 μM in duplicate	0 –200 μM in duplicate
ITC Binding	n.d.	no effect