Identification of novel and selective Kv2 channel inhibitors

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Novel small molecule Kv2 channel inhibitors

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Abbreviations
CNS, central nervous system; Kv channel, voltage-gated potassium channel; NaV channel, voltage-gated sodium channel; CaV channel, voltage-gated calcium channel; CHO, Chinese hamster ovary; MEM, Minimum Essential Media; FBS, fetal bovine serum; PPC, population patch clamp; DMSO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N',N''-tetraacetic acid; SAR, structure activity relationship.
Abstract

Identification of selective ion channel inhibitors represents a critical step for understanding the physiological role that these proteins play in native systems. In particular, voltage-gated potassium, Kv2, channels are widely expressed in tissues such as, CNS, pancreas, and smooth muscle, but their particular contributions to cell function are not well understood. Although potent and selective peptide inhibitors of Kv2 channels have been characterized, selective small molecule Kv2 inhibitors have not been reported. For this purpose, high-throughput automated electrophysiology (IonWorks Quattro) was used to screen a 200,000 compound mixture (10 compounds per sample) library for inhibitors of Kv2.1 channels. Following deconvolution of 190 active samples, two compounds (A1 and B1) were identified that potently inhibit Kv2.1 and the other member of the Kv2 family, Kv2.2 (IC50 0.1-0.2 μM), and that possess good selectivity over Kv1.2 (IC50 >10 μM). Modeling studies suggest that these compounds possess a similar three dimensional conformation. Compounds A1 and B1 are >10-fold selective over NaV channels and other Kv channels, and display weak activity (5-9 μM) on CaV channels. The biological activity of Compound A1 on native Kv2 channels was confirmed in electrophysiological recordings of rat insulinoma cells which are known to express Kv2 channels. Medicinal chemistry efforts revealed a defined structure-activity relationship and led to the identification of two compounds (RY785 and RY796) without significant CaV channel activity. Taken together, these newly identified channel inhibitors represent important tools for the study of Kv2 channels in biological systems.
Introduction

Voltage-gated potassium (K\textsubscript{V}) channels open in response to membrane depolarization and are present in many cell types. In excitable cells, K\textsubscript{V} channels serve as the primary mechanism of repolarization of action potentials whereas in non-excitable cells, K\textsubscript{V} channels control the cell resting potential. Given the role of K\textsubscript{V} channels, it is not surprising that they regulate many fundamental physiological processes and, therefore, are considered important therapeutic targets for treatment of autoimmune, metabolic, neurological and cardiovascular disorders, as well as cancer (Wulff et al., 2009). Despite these facts, there has been limited success in the clinical development of therapeutic agents that target K\textsubscript{V} channels. One reason for this is the result of many of the small molecules identified to date lacking true molecular selectivity across members of the K\textsubscript{V} and other ion channel families, which could significantly compromise their therapeutic index. The lack of ion channel selectivity appears to be due to binding of compounds to highly conserved regions across channels (Decher et al., 2006; Decher et al., 2004; Eldstrom et al., 2007; Hanner et al., 2001; Hanner et al., 1999; Karczewski et al., 2009; Rolf et al., 2000; Zimin et al., 2010). Another reason for the slow progress in drug development is the difficulty in screening large compound libraries with assays that measure channel function (i.e. K conduction) directly, although the development of automated electrophysiology platforms is beginning to address some of these issues (Dunlop et al., 2008).

The K\textsubscript{V}2 channel family consists of two members, K\textsubscript{V}2.1 and K\textsubscript{V}2.2. K\textsubscript{V}2.1 is prominently expressed in the brain, notably pyramidal neurons of the hippocampus and cortex, where it regulates excitability (Misonou et al., 2005). In rodents, K\textsubscript{V}2.1 channels are present in cardiac ventricular myocytes (Nerbonne and Kass, 2005). K\textsubscript{V}2.1 also regulates insulin secretion from the pancreatic \(\beta\)-cell (Jacobson et al., 2007). K\textsubscript{V}2.2 is expressed in brain (Hwang...
et al., 1992), smooth muscle (Schmalz et al., 1998) and somatostatin secreting δ-cells of the pancreatic islet (Wolf-Goldberg et al., 2006; Yan et al., 2004), however, little is known about the role of Kv2.2 channels in these tissues. The assessment of the roles of Kv2.1 and Kv2.2 channels in tissues where they are expressed, and the consequences of channel modulation in vivo, has been hampered by the lack of selective pharmacological tools. Gating modifier peptides highly selective for Kv2 channels have been identified in the venoms of tarantulas (reviewed in (Swartz, 2007)). However, the limited availability of these peptides has often hampered their use in the study of physiological systems. Highly selective, small molecule inhibitors of Kv2 channels would be useful in this regard, but the reported number of these molecules is quite limited. For example, although the antiarrhythmics propafenone and flecanide appear to block Kv2.1 channels more potently than Kv1 channels (Rolf et al., 2000), these compounds have actions on other channels as well. C-1, a besipirdine derivative, has been shown to have some selectivity for Kv2.1 channels over other Kv channels (MacDonald et al., 2002). Thus, there is a need for identifying novel and selective Kv2 inhibitors with which to investigate the role of these channels and develop their pharmacology.

The IonWorks Quattro automated electrophysiology instrument functions in a 384-well format that is well suited for screening compound libraries for activity on Kv channels, and in a previous work we reported on the development of a robust assay for Kv2.1 channels using this platform (Ratliff et al., 2008). In this study, we apply this assay to screen a 200,000 compound library for inhibitors of Kv2.1 channels. We report the discovery and optimization of two series of compounds with striking selectivity for Kv2 channels over other Kv, and CaV and NaV channels.
Materials and Methods

Materials. CHO cells stably expressing human KV2.1 were obtained from Dr. O. Pongs (Institut fuer Neurale Signalverarbeitung, Hamburg, Germany). CHO cells stably expressing human KV1.2 were prepared at Merck Research Laboratories (Rahway, NJ). INS-1 cells (clone 832/13) were supplied by Dr. C. Newgard (Duke University, Durham, NC). Compounds were synthesized by the Department of Medicinal Chemistry, Merck Research Laboratories, Rahway, NJ. All tissue culture media and additives were purchased from Life Technologies (Carlsbad, CA), unless otherwise noted. Chemicals were from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell culture. hKV2.1.CHO cells were maintained in MEM Alpha media with nucleosides supplemented with 10% certified FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine, and 2 µg/ml blasticidin S HCl. hKV1.2.CHO cells were maintained in Iscove's Modified Eagle Medium supplemented with 10% certified FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine, 1X HT supplement, and 0.5 mg/ml G418. hKV2.1.CHO were grown in the presence of 10% CO₂; whereas hKV1.2.CHO cells were grown at 5% CO₂. INS-1 cells were cultured as described (Hohmeier et al., 2000).

Automated 384-well electrophysiology. KV2.1 currents were recorded using the IonWorks® Quattro™ system (Molecular Devices, Sunnyvale, CA) in Population Patch Clamp™ (PPC) mode as described previously (Ratliff et al., 2008). The standard voltage pulse protocol was a series of forty 100 msec pulses at a frequency of 5 Hz. The pre-pulse holding potential was -80 mV and the steps were to +50 mV. Currents were sampled at a rate of 1.25 kHz. Following an initial read, compound (or vehicle) was added for ~3 min and a second voltage train was applied. Ten point concentration dilution series were created by serial diluting a 2 mM DMSO stock 1:3
in DMSO. The upper final concentration applied to cells was 20 μM. The final concentration of DMSO (1%) had no effect on control current recordings.

**Conventional patch clamp electrophysiology.** Membrane currents were recorded at room temperature (23-25°C) using standard dialyzed, whole-cell voltage clamp techniques as described previously (Herrington et al., 2005). The internal solution was (in mM): 100 K-Aspartate, 40 KCl, 10 EGTA, 10 HEPES, 4 mM MgATP, pH 7.2 with KOH. The external solution was (in mM): 150 NaCl, 4 KCl, 1.8 CaCl2, 0.5 MgCl2, 10 HEPES, 3 glucose, pH 7.4 with NaOH. Compounds were diluted in external solution from 10-20 mM stocks in DMSO. The final concentration of DMSO did not exceed 0.1%.
Results

A ~200,000 compound library was screened on hKv2.1 channels stably expressed in CHO cells using the IonWorks Quattro 384-well automated electrophysiology platform. The details of this assay have been described previously (Ratliff et al., 2008). Briefly, a 40 pulse train of voltage steps was applied at 5 Hz. This protocol is designed to detect use-dependent block (i.e. greater inhibition of current at the 40th pulse compared to the 1st pulse). To maximize the throughput of the screen and to contain the cost of consumables, each compound well contained a mixture of 10 compounds. Initial studies revealed that a screening concentration of 1 μM per compound (10 μM total in the well) was optimal for achieving a modest hit rate. Higher screening concentrations produced too many active wells, presumably due to the additive effects of 10 compounds in each well. Figure 1A shows the number of active wells for the 56 384-well plates used in the screen. Using a cutoff of 40% inhibition at pulse 40, 7.8 ± 0.6 active wells were detected per plate. From the primary screen of the library, 190 wells were selected for deconvolution, yielding 1894 compounds. These compounds were tested in isolation in two different Kv2.1 paradigms: IonWorks and a fluorescence assay measuring changes in membrane potential. Results from testing in IonWorks Quattro (in triplicate) and the resulting histogram are shown in Fig. 1B. Based on the 40% inhibition cutoff at pulse 40, 180 compounds were confirmed as active, yielding an overall hit rate for the IonWorks Quattro screen of approximately 0.1%.

Evaluation of the 1894 compounds at 4 μM in the membrane potential assay (data not shown) identified 31 compounds that were chosen for retesting on Kv2.1 in the IonWorks assay. As an initial test for selectivity, the 31 compounds were tested in parallel on Kv1.2 using the same IonWorks assay protocol. Data from these experiments are illustrated in Figure 1C where
the percent inhibition of Kv2.1 (40\textsuperscript{th} pulse) is plotted versus percent inhibition of Kv1.2 (40\textsuperscript{th} pulse). Notably, two compounds, identified by arrows, showed apparent selectivity for Kv2.1 over the Kv1.2 channel. The structures of these compounds (Compounds A1 and B1) are shown in Fig. 1D. Superposition of 3D conformations of A1 and B1 predicted reasonable overlap. Good overall overlap can be maintained by superimposing the center benzene ring of both A1 and B1, with the imidazole ring of A1 mapping onto the aniline amide of B1. This overlay also places the thiazole ring of A1 on top of the cyclopentane of B1.

The activities of A1 and B1 on Kv2.1 were re-confirmed by purification or re-synthesis of the compounds. Inspection of the recordings from the IonWorks assay revealed that the compounds are use-dependent inhibitors of Kv2.1 (Fig. 2A, top). For Compound A1, the potency was 10-fold higher at pulse 40 versus pulse 1 (Pulse 40 IC\textsubscript{50} = 0.20 μM; Pulse 1 IC\textsubscript{50} = 2.0 μM, n = 4). The potency of Compound B1 shifted similarly (Pulse 40 IC\textsubscript{50} = 0.15 μM; Pulse 1 IC\textsubscript{50} = 2.1 μM, n = 4). Compound B1 was further resolved to its enantiomers by chiral HPLC. The S enantiomer (Compound B1 (S)) and the R enantiomer (Compound B1 (R)) had similar potency on Kv2.1 (IC\textsubscript{50}S 0.15 μM and 0.20 μM, respectively). Further profiling showed that these compounds are equipotent inhibitors of Kv2.1 and Kv2.2 channels (Table 1).

The initial observation concerning the selectivity of these compounds for Kv2.1 over Kv1.2 channels was confirmed in detailed concentration-response measurements (Fig. 2B). Both compounds displayed weak activity as inhibitors of Kv1.2 at either Pulse 40 or Pulse 1 (Figure 2, Table 1). For example, Compound A1 inhibited Kv1.2 at Pulse 40 with an IC\textsubscript{50} of 12.1 μM (n = 3), which is 50-fold higher than the IC\textsubscript{50} for inhibition of Kv2.1.

The two Kv2.1 inhibitors were also tested on a variety of voltage-gated channels, using primarily functional assays. For comparison to other Kv channels, the identical IonWorks
electrophysiology assay of Kv2.1 was employed to allow direct comparison with Kv2 channel data. Both compounds displayed weak activity on the Kv channels, Kv1.5 and Kv3.2. The compounds also displayed weak activity on hERG (Kv11.1) based on a radioligand binding assay as well as, on NaV and CaV channels in functional assays. In general, compound B1 showed greater selectivity for Kv2 channels over other channels (average = 75-fold) compared to Compound A1 (average = 35-fold). Compound B1 was screened on 163 additional targets in a panel of enzyme and radioligand binding assays (performed by MDS Pharma Services, King of Prussia, PA). This panel included 10 additional ion channel targets. At 10 μM, Compound B1 displayed significant activity (>50% inhibition) on only three targets: adenosine receptor A3 (IC\textsubscript{50} 0.84 μM, radioligand binding), 5-lipoxygenase (IC\textsubscript{50} 2.0 μM, enzyme activity), and serotonin receptor 2B (IC\textsubscript{50} 6.5 μM, radioligand binding).

Despite the selectivity of A1 and B1 for many ion channels, both compounds, however, display moderate activity on CaV1.2 and CaV2.3 channels (Table 1). Since functional block of CaV channels will limit the utility of these compounds in the evaluation of certain physiological systems, medicinal chemistry efforts were aimed to identify analogs of these compounds with reduced activity on CaV channels. Two analogs were found that retained potency on Kv2 channels, but had much reduced activity on CaV2 channels. These compounds were termed RY785 and RY796 (Table 1).

In initial structure activity relationship (SAR) studies, analogs of both A and B series were found to display similar SAR at the corresponding overlapping regions, as shown in Table 2. It is thus likely that the two compound series bind at overlapping sites on Kv2 channels. A stereochemical preference for binding to Kv2.1 was present in some analogs in the B series. For compound B2, the S enantiomer (RY796) is 5-fold more potent than the R enantiomer. For the
A series of compounds, a stereochemical preference for Kv2.1 inhibition did not appear to exist. For example, RY785 is the first (fast) eluting enantiomer (IC$_{50}$ 0.05 μM) from the chiral column separation of a racemic mixture. The slow eluting enantiomer was equally active as an inhibitor of Kv2.1 (IC$_{50}$ 0.07 μM). Similar results were observed when the m-MeO group in RY785 was replaced with o-Cl (IC$_{50}$’s of 0.12 and 0.14 μM for inhibition of Kv2.1 by the enantiomers).

Pancreatic β-cells are known to express Kv2 channels (reviewed in (MacDonald and Wheeler, 2003)). The rat insulinoma cell line, INS-1, expresses both Kv2.1 and Kv2.2 channels (Su et al., 2001). The majority of Kv current in INS-1 cells likely arises from Kv2 channels based on its sensitivity to the Kv2 gating modifier peptide GxTX-1E (Herrington, 2007). Thus, we tested the newly identified Kv2 inhibitors on the Kv current in INS-1 cells. Compound A1 inhibited the majority of current in these cells (Fig. 3), and inhibition appears to be reversible upon washing out the compound. Based on recordings from three cells, Compound A1 blocked INS-1 Kv current an average of 71% at 0.3 μM and 84% at 3 μM (n=2 per concentration), providing further evidence that the compounds identified by the automated electrophysiology screen are indeed inhibitors of native Kv2 channels.
Discussion

The aim of the present study was to identify novel inhibitors of Kv2 channels with improved selectivity over other available small molecule tools. We chose to utilize automated electrophysiology as the primary assay because it provides a direct measurement of channel activity and is the method best suited to identify compounds that interact with Kv channels in a state-dependent manner. The IonWorks Quattro 384-well platform allowed sufficient throughput to screen a library of about 200,000 compounds. Despite the higher density format of IonWorks as compared to other automated electrophysiology devices, we needed to test compounds in mixtures to maximize throughput and minimize consumable costs. This approach, however, did not allow a screening concentration higher than 1 μM per compound (10 μM total) in order to achieve manageable hit rates. Nevertheless, our data provide convincing evidence that this approach has utility for the screening of large compound libraries by automated electrophysiology. Importantly, the use of a single instrument and voltage protocol allowed the unbiased measurement of selectivity of the newly identified Kv2 inhibitors across Kv channel subtypes.

The successful identification of small molecules that specifically target Kv2 channels using automated electrophysiology suggests areas for future work. In addition, further studies with the Kv2 inhibitors concerning their site of interaction with the channel need to be pursued. These compounds may in fact represent a novel pharmacophore in Kv2 channels that should be exploited for designing new molecule ion channel modulators. Few mapping studies of other Kv2 channel inhibitors exist. Flecainide and propafenone, two antiarrhythmics with broad ion channel activity, are micromolar Kv2.1 inhibitors with weaker potency on Kv1.2 (Rolf et al., 2000). Flecainide and propafenone are thought to interact with residues at the interface of the P-helix of one subunit and the inner S6 helix of an adjacent subunit while blocking ion permeation.
from within the central cavity (Madeja et al., 2003; Madeja et al., 2010). Since the residues at the subunit interface are less conserved than those that line the inside of the central cavity, such a binding site may provide a degree of selectivity across families of channels. Interestingly, the molecules identified in this study are electroneutral whereas flecainide and propafenone possess cationic groups. Recently, a mechanism for block of \( \kappa \) channels by electroneutral molecules has been proposed (Zimin et al., 2010). Thus, it will be interesting to determine the binding site(s) and mechanism of block of the \( \kappa \)2 inhibitors identified in this study.

The future identification of novel molecules specifically targeting distinct \( \kappa \) channels holds considerable promise for the discovery of therapeutics to treat a spectrum of diseases. Such agents, in addition to their selectivity for other ion channels, will need to be optimized for favorable pharmacokinetic and drug metabolism profiles, as well as other parameters, before they can be considered to enter clinical development. Medicinal chemistry efforts will be needed to determine whether or not the new classes of \( \kappa \)2 inhibitors described in the present study can be modified to accomplish such a goal. In the meantime, these agents may prove to be useful to evaluate the role that \( \kappa \)2 channels play in native tissues.
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Authorship Contributions

Participated in research design: J. Herrington, K. Solly, N. Li, Y.-P. Zhou, A. Howard, L. Kiss, M.L. Garcia, O.B. McManus, G.J. Kaczorowski, R. Desai, Y. Xiong

Conducted experiments: J. Herrington, K. Solly, K.S. Ratliff, N. Li, R. Desai

Contributed new reagents or analytic tools: R. Desai

Performed data analysis: J. Herrington, K. Solly, K.S. Ratliff, M.L. Garcia, Q. Deng, Y. Xiong

Wrote or contributed to the writing of the manuscript: J. Herrington, Y.-P. Zhou, M.L. Garcia, O.B. McManus, G.J. Kaczorowski, Q. Deng, Y. Xiong
References


**Figure Legends**

**Figure 1.** Identification of Kv2.1 inhibitors by high throughput automated electrophysiology screening. A. Plot of the number of active wells per screening plate versus plate number for the screen. Wells with >40% inhibition of pulse 40 current were considered active. B. Histogram of percent inhibition at pulse 1 (open bars) and pulse 40 (solid bars) for 1894 compounds from the deconvolution of the original 190 active mixture wells. C. Plot of the percent inhibition of Kv2.1 versus percent inhibition of Kv1.2 for 31 compounds when tested at 3 μM. The arrows point to two compounds (Compound A1 and Compound B1) with apparent selectivity for Kv2.1 over Kv1.2. D. Overlay of 3D conformations of Compounds A1 and B1. The color scheme for various atoms is shown in the inset.

**Figure 2.** Compounds A1 and B1 are use-dependent inhibitors of Kv2.1 with selectivity over Kv1.2. A. Representative IonWorks recordings of Kv2.1 (top) and Kv1.2 (bottom) prior to (control, solid lines) and after addition of A1 (left) or B1 (right) (gray filled traces). Note the differences in concentrations (0.7 μM for Kv2.1; 6.7 μM for Kv1.2) and the use-dependent inhibition of Kv2.1. B. Concentration-response relationships for A1 (left) and B1 (right). Pulse 40 current expressed as percent of control is plotted versus compound concentration for Kv2.1 (○) and Kv1.2 (△). The solid lines are fits of the Hill equation to the data. For Compound A1, parameters of the fits are: Kv2.1 IC₅₀ = 0.16 μM, nH = 1.2; Kv1.2 IC₅₀ = 13.4 μM, nH = 1.7. For Compound B1, parameters of the fits are: Kv2.1 IC₅₀ = 0.13 μM, nH = 0.9; Kv1.2 IC₅₀ = 19.6 μM, nH = 1.3.
Figure 3. Inhibition of Kv current in rat insulinoma INS-1 cells by Compound A1. A. Currents activated in response to a 500 msec step to +20 mV from a holding potential of -80 mV are shown. Currents prior to (control) and following application of 0.3 μM, 1 μM, and 3 μM Compound A1 are shown. B. Plot of the peak current versus time for the recording in A. The period of application of Compound A1 is denoted by the solid bars.
Table 1. Summary of activity on selected voltage-gated ion channels

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<th>B1&lt;sup&gt;f&lt;/sup&gt; (IC&lt;sub&gt;50&lt;/sub&gt; μM)</th>
<th>RY785 (IC&lt;sub&gt;50&lt;/sub&gt; μM)</th>
<th>RY796 (IC&lt;sub&gt;50&lt;/sub&gt; μM)</th>
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a) Reported IC<sub>50</sub> values were measured by automated electrophysiology (IonWorks Quattro) at pulse 40 of a 5 Hz train.

b) Kv7.1/KCNE1 (IK<sub>s</sub>) values determined from an automated electrophysiology (PatchXpress) assay.

c) Values determined from a <sup>35</sup>S-MK-499 radioligand displacement assay.

d) Values from fluorescence-based functional FLIPR assays (Dai et al., 2008).

e) Values from fluorescence membrane potential-based assay (Felix et al., 2004).

f) Data for Compound B1 on Kv<sub>2.1</sub>, Kv<sub>1.2</sub>, Kv<sub>1.5</sub> and Kv<sub>3.2</sub> were obtained using the racemic mixture; all other data were obtained using the pure S enantiomer. Pure S and R enantiomers of Compound B1 had equal potency on Kv<sub>2.1</sub>.
Table 2. Kv2.1 activities of analogs of Compound A1 and B1

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</tr>
<tr>
<td></td>
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</tr>
</tbody>
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Figure 1

A. Histogram showing the distribution of active wells across different plates.

B. Bar graph depicting the number of compounds with different percentages of inhibition, comparing Pulse 1 and Pulse 40.

C. Scatter plot illustrating the relationship between Kv1.2 and Kv2.1 percent blockage, indicated by arrows.

D. Structural diagrams of compounds with their respective molecular interactions highlighted.