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High-throughput analysis of drug binding interactions for the human cardiac channel, Kv1.5

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ABSTRACT

The voltage-gated potassium channel Kv1.5 is one of the key regulators of membrane potential repolarization in human atrial myocytes and is considered a potential drug target to treat atrial fibrillation. In this study we sought to determine molecular mechanism of action of DPO-1, a diphenylphosphine oxide derivative recently shown to terminate experimental atrial arrhythmia without affecting ventricular refractory period. In addition, we provided similar analysis for additional two small molecule blockers, representing different structural classes: cyclohexanones (PAC) and nor-triterpenoids (correolide). To rapidly identify the residues within the Kv1.5 channel critical for blocking activity of these molecules, two functional high-throughput ion channel assays were employed together with site-directed mutagenesis. Our study revealed that the residues critical for blocking activity of for DPO-1 include T480, localized at the outer mouth of the pore, and two residues along S6 helix: V505 and I508. The overlapping site was identified for PAC and included residues T480 and V505. In contrast to DPO-1, the I508A mutation resulted in only a modest reduction in the block of Kv1.5 by PAC (9-fold). Correolide, the largest molecule examined, made widespread interactions along the entire length of the pore (from T480 to V516). In summary, we have identified multiple residues involved in forming high affinity binding site for Kv1.5 blockers. Similar approaches of high-throughput ion channel technologies, combined with site-directed mutagenesis, may allow for parallel, rapid and accurate analysis of ion channel interactions with multiple compounds and could facilitate the design of more potent and selective ion channel blockers.

1. Introduction

Voltage-gated potassium channels (Kv channels) are tetrameric integral membrane proteins that are found across all organisms from *C. elegans* to mammals [1,2], and play an important physiological role in many tissues including the cardiovascular system [3]. In the human heart, Kv1.5 is predominantly expressed in the atria [4] and is a primary component of the ultrarapid delayed rectifier current in atrial myocytes (I_{Kur}) regulating the action potential duration [5,6]. Due to its discrete expression in the atria and not the ventricle of cardiac muscle and its pivotal role in the regulation of atrial

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Abbreviations: Kv1.5, voltage-gated potassium channel, sub-type 1.5; DPO-1, 2-isopropyl-5-methyl-cyclohexyl diphenylphosphine oxide; PAC, 4-phenyl-4-cyclohexanone; CHO, Chinese hamster ovary cells; AAS, atomic absorption spectroscopy.

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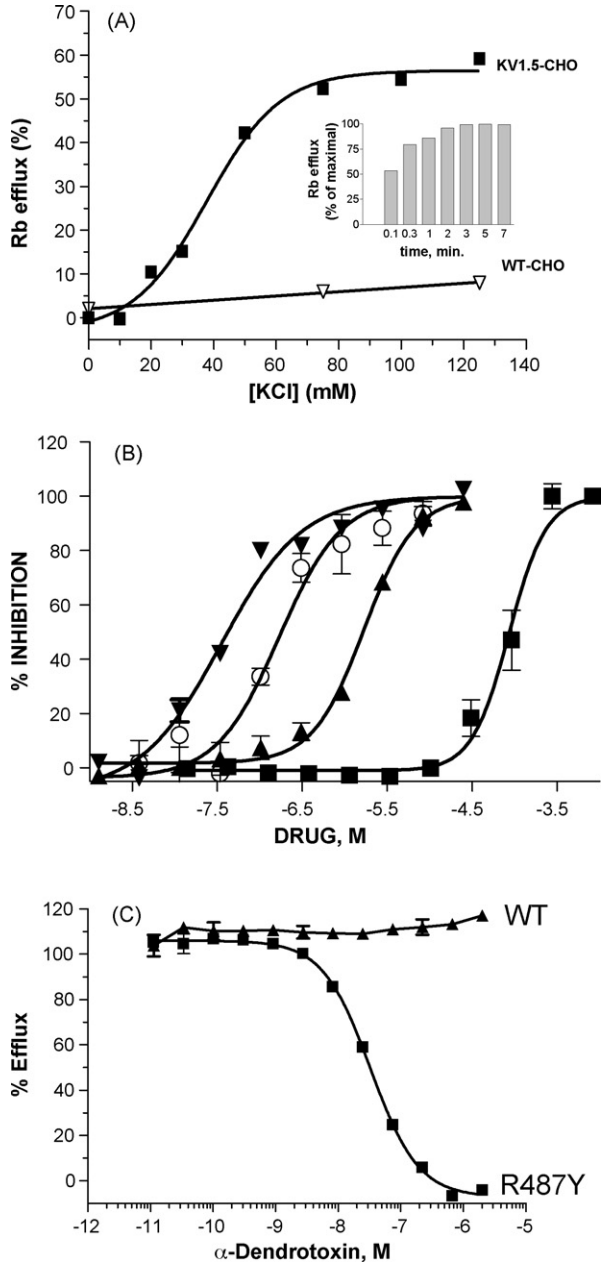


Fig. 1 – Analysis of KV1.5 channels expressed in CHO cells. (A) Activation of Kv1.5 by extracellular potassium. The Kv1.5 CHO cells or WT CHO cells were loaded with Rb^+ as described in Section 2. The activation of Kv1.5 was initiated by the addition of increasing concentrations of KCl. The sample of cell supernatant containing secreted Rb^+ was collected after 10 min and analyzed using atomic absorption spectroscopy (AAS). Rb^+ efflux was expressed as % of total Rb^+ in the well. Inset. Time course of Rb^+ efflux. The Kv1.5 cells were stimulated with 70 mM KCl, samples of supernatant collected at various times and secreted Rb^+ was analyzed by AAS. The data is expressed as percent of maximal response. (B) Concentration-dependent inhibition of the channel by Kv1.5 blockers. The cells were preincubated with increasing concentrations of tested compound for 5 min followed by stimulation with 70 mM KCl. The amount of secreted Rb^+ in the presence of PAC (filled triangles); DPO-1 (open circles), correolide (inverse triangles) or 4-AP (filled squares) was measured and expressed relative to the Rb^+ secretion in the presence of vehicle (0.25% of DMSO). The effect of 0.25% DMSO vehicle on Rb^+ efflux was negligible. Each data point is an average of three independent determinations. Error bars represent mean standard error. Data is fitted using 4 parameter logistic equation. Hill coefficient for all blockers was between 1.1 and 1.4. Calculated IC_{50}'s are summarized in Table 1. (C) The validation of Rb^+ assay to monitor properties of the mutant Kv1.5 channels. The single point mutation R487Y results in α-dendrotoxin (α-DTX) block of Kv1.5. WT KV1.5 or R487Y Kv1.5 cells were preincubated with increasing concentration of α-DTX for 5 min, depolarized with 70 mM KCl and Rb^+ efflux measured. The data is the average from two independent experiments.

repolarization, Kv1.5 has been proposed as an important target for the development of drugs to treat atrial fibrillation. DPO-1 is a novel diphenyl phosphine oxide compound recently identified as a potent inhibitor of human Kv1.5 [7]. DPO-1 was shown to potently block recombinant hKv1.5 as well as native I_{Kur} in isolated human atrial myocytes with 15-fold selectivity over Kv3.1. In two separate studies DPO-1 was shown to markedly increase atrial refractory period in dogs [8], rats and non-human primates [9]. No change in ventricular refractory period, ECG intervals or blood pressure was observed. Taken together, these findings suggest that Kv1.5-selective blockers may be effective for the treatment of atrial fibrillation. In order to further optimize the potency and selectivity of Kv1.5 blockers like DPO-1, we investigated the interactions of this compound within the pore of Kv1.5 relative to blockers from other structural chemotypes. In order to determine the residues critical for blocking activity of Kv1.5 inhibitors, we have used atomic absorption spectroscopy (AAS) and high-throughput (HT) electrophysiology. The combination of these two techniques allowed for rapid and accurate analysis of the Kv1.5 channel activity under varied experimental conditions. We have examined the interaction of each blocker with Kv1.5 and its mutants and mapped a unique binding footprint for each molecule.

2. Materials and methods

2.1. Materials

The blockers used in this study: (2-isopropyl-5-methyl-cyclohexyl) diphenylphosphine oxide (DPO-1), 4-phenyl-4-cyclohexanone (PAC), nor-triterpenoid (correolide) and dendrotoxin were prepared at Merck Research Laboratories. All other chemicals were obtained from Sigma–Aldrich Corp. (St. Louis, MO).

2.2. Molecular biology

Human Kv1.5 was cloned into pCDNA5/FRT (Invitrogen, Carlsbad, Ca) vector using *Nhe I* and *Not I* sites. PCR-based site-directed mutagenesis was performed using methylated Kv1.5/pCI according to the modified GeneTailor method

(Invitrogen, Carlsbad, Ca). Briefly, after PCR amplification using a pair of mutagenic oligonucleotides, mutated unmethylated product was selected in DH5α *E. coli* cells (Invitrogen, Carlsbad, Ca). The 396 bp cassette between *Stu I* and *Bam HI* containing the mutated site was swapped into wild-type Kv1.5 in pCDNA5/FRT. The identity of all mutant constructs was confirmed by restriction mapping and DNA sequencing.

2.3. *Expression of Kv1.5 and mutants in Flp-In CHO cells*

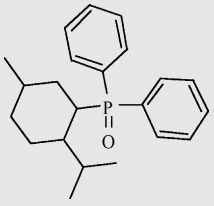
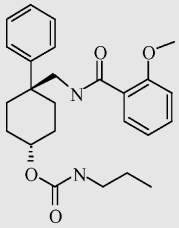
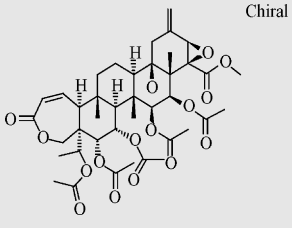
Kv1.5 and the mutants were expressed in CHO cells using the Flp-In system (Invitrogen, Carlsbad, CA). Flp-In host CHO cells containing a single integrated Flp Recombination Target (FRT) site were transfected with KV1.5 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the Flp-In system manual. Briefly, 0.4 μg Kv1.5/pCDNA5/FRT (or mutant Kv1.5/pCDNA5/FRT), 3.6 μg pOG44 (for transient expression of Flp recombinase) and 0.25% Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was incubated with Flp-In CHO cells for 5 h in Opti-MEM I serum-free medium (Invitrogen, Carlsbad, CA). Hygromycin-resistant cells were selected by culturing in F-12 medium containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml hygromycin (complete medium) at 37 °C with 5% CO_2. The expression of Kv1.5 and mutant channels was confirmed by Western Blot analysis using a rabbit anti-Kv1.5 antibody (A50) developed internally. In order to improve surface expression of the Kv1.5 mutants I508A and V512A, cells were grown at 30 °C.

2.4. *HT electrophysiology*

Cells expressing wild-type or mutant Kv1.5 were grown to 90–100% confluence. Cells were harvested using Versene buffer (Invitrogen, Carlsbad, CA) then washed in PBS and suspended at the final concentration of 2×10^6 cells/ml of PBS. A 3.5 ml aliquot of cell suspension was placed in the IonWorks HT instrument (Molecular Devices, Sunnyvale, CA).

The intracellular solution contained (mM): K-gluconate 100, KCl 40, MgCl2 3.2, EGTA 3, N-2-hydroxylethylpiperazine-N1-2-ethanesulphonic acid (HEPES) 5, adjusted to pH 7.3 and 0.1 mg/ml of amphotericin (Sigma, St. Louis, MO). The external solution was Dulbecco's Phosphate-Buffered Saline (Invitrogen, Carlsbad, CA) and contained (mM): $CaCl_2$ 0.90, KCl 2.67, KPO_4 1.47, $MgCl_2$ 0.50, NaCl 138, $NaPO_4$ 8.10, pH 7.4. All compounds were prepared as 10 mM stock solutions in DMSO and diluted such that the final concentration of DMSO was <0.33%. Currents were recorded at room temperature (21–23 °C). Membrane currents were amplified (RMS ~10pA) and sampled at 10 kHz. The leak subtraction was performed in all experiments by applying a 160 ms hyperpolarizing (10 mV) pre-pulses 200 ms before the test pulses to measure leak conductance. In order to measure drug effect, the Patchplate™ (Molecular Devices, Sunnyvale, CA) wells were loaded with 3.5 μl of external buffer, then the planar micropipette hole resistances (Rp) were determined by applying a 10 mV, 160 ms potential difference across each hole (Hole test). Cells were added into the Patchplate™ and allowed to form high resistance seals with the 1–2 μm holes at the bottom of each Patchplate™ well and a seal test scan was performed. In order to gain electrical access to the cells, intracellular solution containing amphotericin was circulated for 4 min on the bottom side of the Patchplate™. A pre-compound addition test pulse was applied to each well on the Patchplate™. Cells were voltage-clamped at a membrane holding potential of −80 mV for 15 s. This was followed by application of a 5 Hz stimulus train (27 × 150 ms depolarizations to +40 mV). The membrane potential steps to +40 mV evoked outward (positive) ionic currents. Compounds were added to each well of the Patchplate™ and allowed to incubate for 5 min. A post-compound addition test pulse protocol was then applied. Cells were voltage-clamped at a membrane holding potential of −80 mV for 15 s. This was followed by application of a 5 Hz stimulus train (27 × 150 ms depolarizations to +40 mV). Paired comparisons between pre-blocker and post-blocker additions were used to determine the inhibitory effect of each compound. Percent inhibition of the peak control current during the 27th depolarization to +40 mV (in the 5 Hz train) was plotted as

Table 1 – The summary of potency of KV1.5 blockers measured using Rb^+ efflux and HT electrophysiology.

Chemical name	DPO-1	PAC	Correolide
Structure			 Chiral
AAS (nM)	170	29	1771
HT-EP (nM)	266	389	958
Manual EP (nM)	30-160(a)	300(b)	67% inhibition at 1 μM (c)

IC_{50}'s were calculated 10 point titration curves. The maximum concentration of DMSO was 0.33% and did not significantly affect the recordings. The IC_{50}s are the average of two independent experiments; SD < 10%.
(a) From Lagrutta et al. [7].
(b) Tested on Kv1.3 channel, from Schmalhofer et al. [31].
(c) From Remillard et al. [36].

a function of concentration of tested blocker. The concentration of inhibitor required to block the current by 50% (IC_{50}) was determined by fitting of the Hill equation to the concentration-response data.

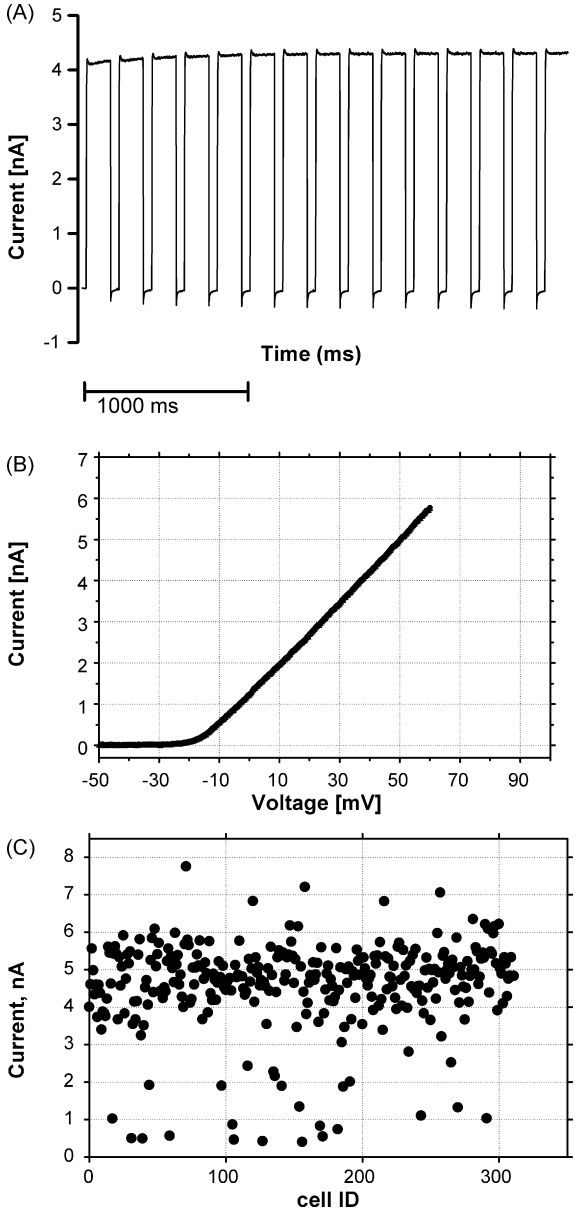


Fig. 2 – Electrophysiological characterization of Kv1.5 expressed in CHO cells. (A) Voltage-clamp protocol for stimulation of Kv1.5 stably expressed in CHO FLP-IN cells. The depolarizing step from −70 to +40 mV at 5 Hz was applied and the current recorded. The recordings were from a single cell, representative for Kv1.5 cells. (B) Current voltage curve. The voltage is applied with the linear gradient from −60 to +60 mV and resulting current is recorded. The recording is from a single cell, representative for 10 other Kv1.5 cells. (C) The peak current distribution measured in 310 individual kv1.5 cells. The voltage-clamp protocol as in (A) is applied and the peak current at the end of the last pulse is measured. The currents were recorded from 310 individual cells expressing Kv1.5 and the current distribution plotted.

2.5. *Rubidium efflux assay*

200 μl aliquots of CHO cells (2×10^5 cells/ml) in complete culture medium were added per well to a 96-well V-bottom microtiter plates and allowed to grow for 24–48 h. The medium was then aspirated, Rb-Load Buffer (Aurora Biomed, Vancouver, Canada) containing 5.4 mM RbCl added and cells were incubated at 37 °C for 3 h. Extracellular Rb was removed by washing cells five times with Hanks' Balanced Salt Solution (HBSS). 100 μl of HBSS containing 0.25% DMSO or tested compound diluted in DMSO was added and incubated with cells for 10 min prior to the addition of 100 μl of 2× high-K^+ depolarization buffer (modified-HBSS: replacement of 140 mM NaCl with 140 mM KCl). After 5 min incubation at room temperature, the supernatant was transferred into a clean 96-well plate and cell lysis buffer (200 μl/well) was added to the assay plate to release intracellular Rb^+ (Aurora Biomed, Vancouver, Canada). The Rb^+ concentration was measured in both the supernatant and the cell lysate (100 μl sample of each) using an automated atomic absorption spectrophotometer (Model: ICR-8000; Aurora Biomed, Vancouver, Canada). All liquid handling was performed using a Biomek FX Laboratory Workstation equipped with 96-well pipetting head (Beckman Coulter Inc., Fullerton, CA). Rb^+ efflux was calculated using Eq. (1):

$$\%\ \text{Efflux} = 100 \times \frac{Rb^+_{sup}}{Rb^+_{sup} + Rb^+_{lys}} \quad (1)$$

In order to quantify the effect of blockers, the data was converted into % inhibition calculated using Eq. (2):

$$\%\ \text{Inhibition} = 100 - \left(\frac{\text{Efflux}_{blocker} - \text{Efflux}_{min}}{\text{Efflux}_{max} - \text{Efflux}_{min}}\right) \quad (2)$$

where $\text{Efflux}_{blocker}$ is Rb^+ efflux in the presence of tested compound, Efflux_{min} is Rb^+ efflux in the presence of 25 μM of tested compound, Efflux_{max} is Rb^+ efflux in the presence of 0.25% DMSO.

Non-linear regression analysis was used to calculate the IC_{50} values using the program Prism (GraphPad Software, San Diego, CA).

In case of weakly active compounds, if Rb^+ efflux was blocked less than 50% at the highest concentration tested (25 μM), IC_{50}'s were reported as >25 μM.

3. Results

3.1. *Analysis of Kv1.5 blockers by atomic absorption spectroscopy*

In order to rapidly identify structure–function relationships, an assay based on the measurement of rubidium efflux by atomic absorption spectroscopy (AAS) was developed using a stable CHO cell line transfected with human Kv1.5 (see Section 2). Rb^+ efflux following the addition of high-K^+ buffer was quantified by AAS and was essentially proportional to the concentration of extracellular K^+ (EC_{50} = 40 mM). Background Rb^+ efflux from parental CHO cells was negligible and was not dependent on extracellular K+ concentration, similar to

previous reports from studies employing $^{86}Rb^+$ [10]. The efflux of Rb^+ was rapid and reached a plateau within 1 min following the addition of high-K^+ buffer (Fig. 1A, Inset). DPO-1 inhibited Rb^+ efflux in concentration-dependent manner and its potency determined by AAS (IC_{50} = 170 nM) was consistent with the value determined by manual patch clamp electrophysiology (30–160 nM) [7]. 4-Aminopyridine (4-AP), a non-selective potassium channel blocker was included in this analysis as a reference compound. The potency for 4-AP to block Rb^+ efflux (IC_{50} = 86 μM) was in a good agreement with published results obtained using whole-cell recordings from Kv1.5 cells (IC_{50} = 50 μM) [11]. The concentration-response curves for all tested compounds are shown in Fig. 1B and their blocking potencies summarized in Table 1.

3.2. Analysis of Kv1.5 blockers by electrophysiological recordings

The pharmacology of Kv1.5 blockers was also explored using HT patch clamp electrophysiology (IonWorks^{TM} HT). Cells were voltage-clamped at a holding potential of −80 mV. Depolarization was elicited by the application of a 5 Hz stimulus train (27 × 150 ms depolarizations to +40 mV) that evoked outward (positive) ionic currents (Fig. 2A). The analysis of current–voltage curves from HT electrophysiology revealed the activation potential threshold V_{act} of ∼ −20 mV (Fig. 2B), consistent with the value reported for human Kv1.5 [12]. The average peak current calculated from the entire 384-well Patchplate was 4.4 ± 1 nA. The channel expression was very robust and uniform with >90% of the cells displaying peak currents greater than 3 nA (Fig. 2C). To determine the potency of investigated compounds, the current amplitude was measured in the presence of compound and compared with current amplitude before addition of the compound (see Section 2). Preincubation of cells with each of the three tested inhibitors resulted in concentration-dependent inhibition of Kv1.5 current. The reference compound (4-AP) effectively blocked Kv1.5 current with IC_{50}∼400 μM (data not shown). DPO-1, PAC, and correolide all produced concentration-dependent block of Kv1.5 current with an IC_{50} of 389, 312, and 958 nM, respectively. The results are summarized in Table 1.

3.3. Rapid structure–function analysis of Kv1.5 using Rb^+ efflux assay

Structure–function studies have been widely applied for identifying the interactions of peptides and small molecules with ion channels. However, for researchers who want to work with mammalian cells and/or have limited quantities of blocker, the electrophysiological single cell recordings traditionally used for this purpose can be labor-intensive and time-consuming. Rb^+ efflux assays are an alternative method that can be employed in parallel with multiple mutants expressed in mammalian cells to rapidly identify mutations in ion channels that significantly alter the inhibitory activity of tested blockers. The initial validation of this approach using AAS was achieved by introducing a mutation that enables dendrotoxin (DTX) binding to Kv1.5. Kv1.5 is resistant to DTX whereas other channels of the Kv1.x family, such as Kv1.1, are

Table 2 – Effect of mutations on the potency of Kv1.5 blockers determined in Rb^+ efflux assay.

Mutant	% Control efflux	DPO-1 (nM)	PAC (nM)	Correolide (nM)
WT	100	170	29	1771
T480A	99	>25000	>25000	>25000
R487Y	96	125	66	1456
A501G	56	268	10	547
V505A	120	>25000	17124	>25000
T507A	134	70	24	2624
I508A	81	>25000	272	22025
A509S	54	14	57	826
V512A	49	210	99	3199
V514A	108	178	41	2400
V516A	56	109	162	14535
N520A	39	49	32	1682

For each mutant Kv1.5, efflux is expressed as a percent of efflux measured in WT KV1.5 cells (column 1). The IC_{50}'s for PAC, DPO-1 and correolide were calculated from 10 point titration curves, from two independent experiments, SD < 10%. IC_{50}'s were reported as >25 μM if Rb^+ efflux was blocked by less than 50% at the highest concentration tested (25 μM).

Table 3 – Effect of key S6 mutations determined by HT-Electrophysiology.

	Peak current (nA)	DPO-1 (nM)	PAC (nM)	Correolide (nM)
WT	4.4	266	389	958
T480A	0.9	>33000	>33000	>33000
I508A	2.1	>33000	503	7148
V516A	1.9	218	580	4134

The IC_{50}s derived from 10 point titration curves, average of two independent experiments, SD < 10%.

potently blocked by DTX. In Kv1.1 the major binding site for DTX, Y379 is located within the S5–S6 loop of the channel [13]. In case of Kv1.5, the corresponding position is arginine R487. It has been previously shown, using electrophysiology recordings, that Kv1.5 becomes sensitive to blocking by DTX when R487 is mutated to Y [14]. We were able to demonstrate that DTX potently blocked Rb^+ efflux from R487Y mutant channel (IC_{50} = 29 nM; Fig. 1C) whereas WT Kv1.5 was completely insensitive to DTX at the concentration up to 2 μM. The observed effect of R487Y mutation measured by Rb^+ efflux confirmed results obtained in classical electrophysiology recordings [14] and suggested that the AAS Rb^+ efflux assay could be utilized to reliably and rapidly monitor structure–function analysis of various drug interactions and effects of site-directed mutagenesis.

In order to identify residues critical for blocking activity of selected compounds, mutations along the S6 segment of Kv1.5 as well as residues near the pore entrance were introduced. Residues of interest were replaced with alanine or, if alanine in the native channel, to glycine. The mutant Kv1.5 formed the functionally active channels, as assessed by Rb^+ efflux. The Rb^+ efflux from the mutant channels relative to WT channels ranged from 39% to 134% (Table 2). The full concentration-response curves for DPO-1, PAC, and correolide covering 5-log concentration range (1–25,000 nM) were generated and IC_{50}'s calculated using non-linear regression analysis. The results are summarized in Table 2. Blocking activity of all three compounds was differentially affected by each mutation, ranging from 2- to 1000-fold reduction of blocking potency. In order to better visualize the effect of specific mutations, the potency to block the mutant channel was calculated relative to potency against the wild-type Kv1.5 channel (Fig. 3). The analysis revealed that Kv1.5 mutants T480A, V505A and I508A became completely insensitive to block by DPO-1, even at concentrations 100-fold higher than the IC_{50} for WT Kv1.5. The lack of blocking activity was not related to significant changes in the function of mutant channels, since similar extent of activation, relative to WT channel, was observed. In contrast to these mutations, the A509S mutation resulted in marked (12-fold) gain of potency for DPO-1. The remaining mutant channels examined, including R487Y, A501G, V512A, V514A, V516A and N520A were blocked by DPO-1 with the relative potency ranging between 125 nM (R487Y) and 268 nM (A501G). Similar to DPO-1, mutant channels T480A and V505A were completely insensitive to the block by PAC. However, in contrast to DPO-1, the I508A mutation had a much more subtle effect and resulted only in 9-fold reduction in potency. In addition, the A509S substitution resulted in a 2-fold reduction in potency, as compared to marked gain of potency observed for DPO-1. Finally for correolide, block potency was significantly reduced by mutations T480A, V505A, I508A, as well as V516A.

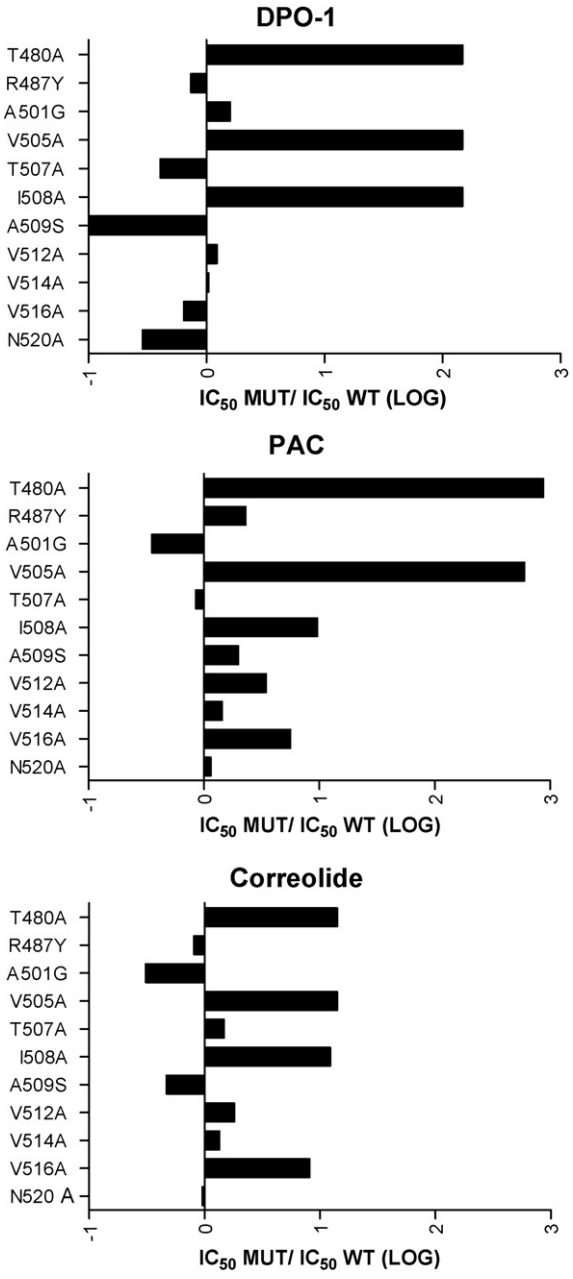


Fig. 3 – The histogram of the drug potency on Kv1.5 mutants relative to the wild-type Kv1.5. The IC_{50}'s from Table 2 were expressed as log($IC_{50\ mutant}/IC_{50\ WT}$). Standard error was <10%.

3.4. *Analysis of compound pharmacology in mutant Kv1.5 by electrophysiological recordings*

In order to rapidly confirm the key findings from Rb^+ efflux assay, we performed a series of experiments based on high-throughput electrophysiology using IonWorks™ instrument. Since most of the interaction sites identified for DPO-1 as well as PAC, and correolide by Rb^+ efflux assay were consistent with the overall topology of the channel pore and overlapped with binding sites previously reported for other classes of Kv1.5 blockers, this confirmatory analysis was limited to the residues T480A, I508A and V516A, identified as critical for binding of all blockers identified using the Rb^+ efflux assay. As assessed by HT electrophysiology, T480A, I508A and V516A mutants formed functionally active channels (Table 3). The average single cell current recording revealed the average peak currents of 4.4 nA (n = 313) for WT Kv1.5, 0.9 nA (n = 10) for T480A, 2.1 nA for I508A and 1.9 nA (n = 10) for V516A. In

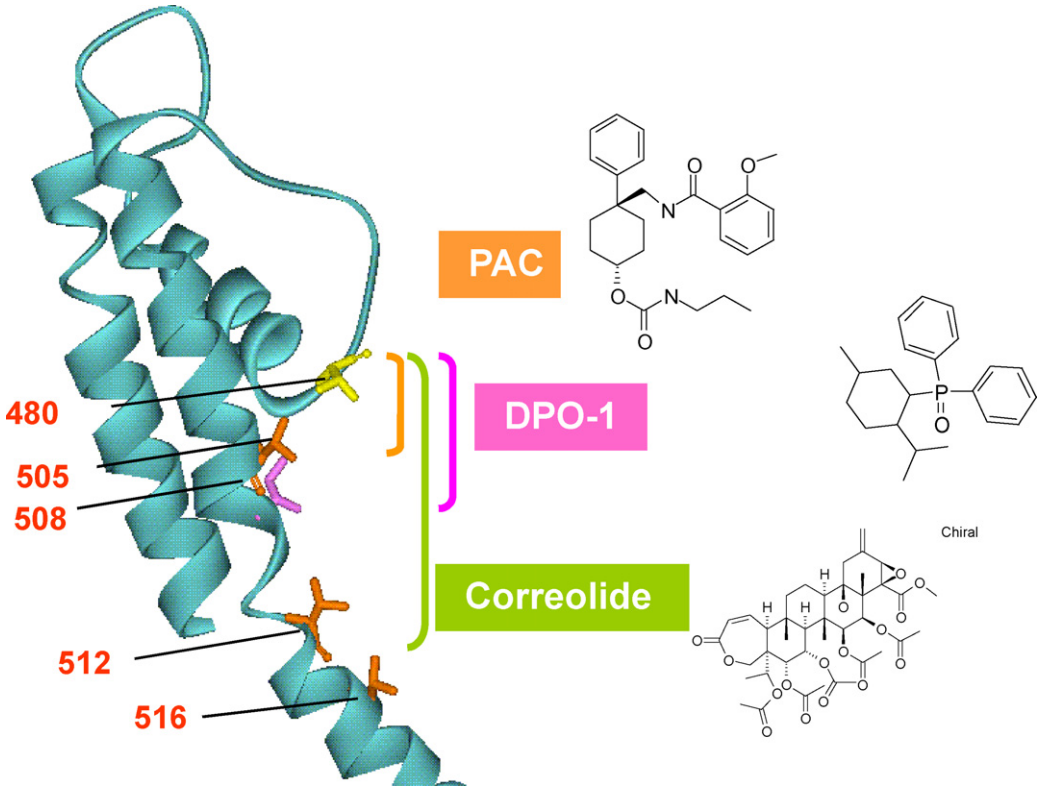


Fig. 4 – The binding zones for Kv1.5 blockers representing different structural classes. The residues along the S6 are forming the primary binding site for the Kv1.5 blockers. Unique contacts are observed depending on compound structure forming discreet "binding zones". The decrease of potency for PAC is much smaller with mutations at V516 (5-fold) versus T480 (154-fold) or V505 (105-fold). Residues T480 and V505 are therefore likely acting as major anchor points for PAC.

agreement with the Rb^+ efflux results, DPO-1 did not significantly block the I508A Kv1.5 mutant, even at compound concentrations 100-fold above the IC_{50} for the native channel. More subtle changes in blocking potency to block I508A were observed for PAC, again confirming results from Rb^+ efflux assay.

4. Discussion

Selective blockers of voltage-gated ion channels have been implicated as therapeutically important in the treatment of a variety of conditions such as epilepsy, diabetes, pain or cardiac arrhythmias. Human Kv1.5 (*KCNA5*) channel is highly expressed in atrial tissue, underlying the ultrarapid delayed rectifier potassium current in atria and is a key element of atrial repolarization. These characteristics suggest that selective blockers of Kv1.5 could provide an effective way to manage patients with atrial fibrillation (AF). Several small molecule blockers of Kv1.5 have been identified, including quinidine [15–17], 4-aminopyridine [18], nifedipine [19], benzocaine [20], AG-1478 [21], bisaryl compounds [22], benzamides (S0100176) [23] and AVE0118 [24–26]. Several Kv1.5 blocking agents have been studied *in vivo* [27,28], AVE1231 [29] and DPO-1 [8,9] are just a few examples. DPO-1, a novel diphenylphosphine oxide, is a potent state-dependent inhibitor of Kv1.5 [7]. DPO-1 exhibits selectivity for Kv1.5 over Kv3.1 in the recombinant systems [7] and prolongs action potentials in isolated human atrial but not ventricular myocytes [7]. Recent *in vivo* studies revealed that DPO-1 terminated atrial arrhythmia in dogs [8], and elicited increases in atrial refractoriness in African Green Monkeys [9]. Our data indicate that the high affinity binding site of DPO-1 is comprised of three residues: T480, V505 and I508 localized inside the channel pore. The binding zone for DPO-1 (Fig. 4) partially overlaps with binding zones for two other compounds included in our study, PAC and correolide, as well as with a number of other blockers studied elsewhere [20,23,26,30]. The di-substituted cyclohexyl derivative PAC has been originally identified as a potent immunosuppressant acting on Kv1.3 [31]. It was shown to potently and reversibly block Kv1.3 ion channels in human T-lymphocytes and inhibit $^{86}Rb^+$ efflux in Kv1.3-CHO cells with K_i of ~300 nM [31]. In contrast to DPO-1, mutations at I508 have limited effect on blocking potency of PAC, suggesting that this residue does not play a critical role in formation of high affinity binding site for this class of compounds, but is essential for binding of DPO-1, the compound shown to be 15-fold more selective for Kv1.5 versus Kv3.1 [7]. AVE0118 is another potent Kv1.5 blocker and antiarrhythmic drug [25]. Several residues along the S6 have been identified as critical for binding of this molecule, including V505, I508, V512 and V516, as well as T480 [26]. The "foot in the door" mode of interaction, typical for elongated molecules such as AVE0118 results in slowing the

rate of channel activation. Our data indicate that correolide shares a similar ''foot in the door'' molecular footprint and is likely to display characteristics similar to AVE0118 including unique mode Kv1.5 inactivation as well as lack of selectivity against other members of Kv1.x family [32]. Site-directed mutagenesis and molecular modelling was used to establish the binding site for another inhibitor of Kv1.5, anthranilic acid derivative, S0100176 [23]. The residues critical for inhibitory activity of S0100176 overlap with DPO-1, but extend down to residue V512. It is worth noting that V512 is located at the position corresponding to F656 of hERG, which is a key residue for drug interactions with the hERG channel [33,34]. It could be speculated that compounds interacting with this residue may display higher degree of cross reactivity with hERG, a highly undesired effect which could in turn result in serious side effects related to blockade of hERG. The unique molecular footprint of DPO-1 could provide useful clues for the development of novel, high affinity Kv1.5 inhibitors with improved safety profile. In particular, designing molecules which bind to I508 and extend their interactions either to the upper or lower part of the channel pore could result in the discovery of inhibitors with novel blocking properties. The potential limitations of presented screening paradigm, common to most high-throughput methods, are related to precision of measured potency and influence of expression levels for mutant channels on IC_{50}'s estimates. In addition, it is not known if more subtle changes in potency could be reliably measured using high-throughput techniques. Some of these limitations could be addressed by the analysis of other mutations at critical residues to amino acids of varying charge and size, including unnatural amino acids [35]. Another approach is to study structure–activity relationship for a series of very close analogs of the active compound (Spencer et al., manuscript in preparation). While the conventional electrophysiology is still ''the golden standard'' in ion channel research, the approach presented in this paper which combines low-cost Rb^+ flux assay with high-throughput electrophysiology may provide a cost-effective and rapid platform which could be applied to such expanded analysis, and also be adapted to other potassium channels thus greatly facilitate the drug discovery process.

REFERENCES

[1] Miller C. An overview of the potassium channel family. Genome Biol 2000;1. REVIEWS0004.

[2] Moulton G, Attwood TK, Parry-Smith DJ, Packer JC. Phylogenomic analysis and evolution of the potassium channel gene family. Recept Channels 2003;9:363–77.

[3] Gopalakrishnan M, Char-Chang Shieh C, Chen J. Potassium channels. In: Mannhold RK, Hugo/Folkers Gerd, editors. Voltage-gated ion channels as drug targets. Wiley-VCH; 2006.

[4] Gaborit N, Le Bouter S, Szuts V, Varro A, Escande D, Nattel S, et al. Regional and tissue specific transcript signatures of ion channel genes in the non-diseased human heart. J Physiol 2007;582:675–93.

[5] Wang Z, Fermini B, Nattel S. Sustained depolarization-induced outward current in human atrial myocytes. Evidence for a novel delayed rectifier K+ current similar to Kv1.5 cloned channel currents. Circ Res 1993;73:1061–76.

[6] Fedida D, Wible B, Wang Z, Fermini B, Faust F, Nattel S, et al. Identity of a novel delayed rectifier current from human heart with a cloned K+ channel current. Circ Res 1993;73:210–6.

[7] Lagrutta A, Wang J, Fermini B, Salata JJ. Novel, potent inhibitors of human Kv1.5 K+ channels and ultrarapidly activating delayed rectifier potassium current. J Pharmacol Exp Ther 2006;317:1054–63.

[8] Stump GL, Wallace AA, Regan CP, Lynch Jr JJ. In vivo antiarrhythmic and cardiac electrophysiologic effects of a novel diphenylphosphine oxide IKur blocker (2-isopropyl-5-methylcyclohexyl) diphenylphosphine oxide. J Pharmacol Exp Ther 2005;315:1362–7.

[9] Regan CP, Wallace AA, Cresswell HK, Atkins CL, Lynch Jr JJ. In vivo cardiac electrophysiologic effects of a novel diphenylphosphine oxide IKur blocker (2-Isopropyl-5-methylcyclohexyl) diphenylphosphine oxide, in rat and nonhuman primate. J Pharmacol Exp Ther 2006;316:727–32.

[10] Hu W, Toral J, Cervoni P, Ziai MR, Sokol PT. Depolarization-induced 86Rb+ efflux in CHO cells expressing a recombinant potassium channel. J Pharmacol Toxicol Methods 1995;34:1–7.

[11] Bouchard R, Fedida D. Closed- and open-state binding of 4-aminopyridine to the cloned human potassium channel Kv1.5. J Pharmacol Exp Ther 1995;275:864–76.

[12] Snyders DJ, Tamkun MM, Bennett PB. A rapidly activating and slowly inactivating potassium channel cloned from human heart. Functional analysis after stable mammalian cell culture expression. J Gen Physiol 1993;101:513–43.

[13] Hurst RS, Busch AE, Kavanaugh MP, Osborne PB, North RA, Adelman JP. Identification of amino acid residues involved in dendrotoxin block of rat voltage-dependent potassium channels. Mol Pharmacol 1991;40:572–6.

[14] Liang AH, Santarelli VP, Bennett P, Imredy JP. Effect of neutralization of lysine clusters flanking the binding domain of dendrotoxin on its affinity for a Kv1.5 mutant. Biophys J 2002;82:584.

[15] Zhang H, Zhu B, Yao JA, Tseng GN. Differential effects of S6 mutations on binding of quinidine and 4-aminopyridine to rat isoform of Kv1.4: common site but different factors in determining blockers' binding affinity. J Pharmacol Exp Ther 1998;287:332–43.

[16] Yeola SW, Rich TC, Uebele VN, Tamkun MM, Snyders DJ. Molecular analysis of a binding site for quinidine in a human cardiac delayed rectifier K+ channel. Role of S6 in antiarrhythmic drug binding. Circ Res 1996;78:1105–14.

[17] Fedida D. Gating charge and ionic currents associated with quinidine block of human Kv1.5 delayed rectifier channels. J Physiol 1997;499(Pt 3):661–75.

[18] Grissmer S, Nguyen AN, Aiyar J, Hanson DC, Mather RJ, Gutman GA, et al. Pharmacological characterization of five cloned voltage-gated K+ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. Mol Pharmacol 1994;45:1227–34.

[19] Lin S, Wang Z, Fedida D. Influence of permeating ions on Kv1.5 channel block by nifedipine. Am J Physiol Heart Circ Physiol 2001;280:H1160–72.

[20] Caballero R, Moreno I, Gonzalez T, Valenzuela C, Tamargo J, Delpon E. Putative binding sites for benzocaine on a human cardiac cloned channel (Kv1.5). Cardiovasc Res 2002;56:104–17.

[21] Choi BH, Choi JS, Rhie DJ, Yoon SH, Min DS, Jo YH, et al. Direct inhibition of the cloned Kv1.5 channel by AG-1478, a tyrosine kinase inhibitor. Am J Physiol Cell Physiol 2002;282:C1461–8.

[22] Peukert S, Brendel J, Pirard B, Bruggemann A, Below P, Kleemann HW, et al. Identification, synthesis, and activity

of novel blockers of the voltage-gated potassium channel Kv1.5. J Med Chem 2003;46:486–98.
[23] Decher N, Pirard B, Bundis F, Peukert S, Baringhaus KH, Busch AE, et al. Molecular basis for Kv1.5 channel block: conservation of drug binding sites among voltage-gated K+ channels. J Biol Chem 2004;279:394–400.
[24] Wirth KJ, Paehler T, Rosenstein B, Knobloch K, Maier T, Frenzel J, et al. Atrial effects of the novel K(+)-channel-blocker AVE0118 in anesthetized pigs. Cardiovasc Res 2003;60:298–306.
[25] Gogelein H, Brendel J, Steinmeyer K, Strubing C, Picard N, Rampe D, et al. Effects of the atrial antiarrhythmic drug AVE0118 on cardiac ion channels. Naunyn Schmiedebergs Arch Pharmacol 2004;370:183–92.
[26] Decher N, Kumar P, Gonzalez T, Pirard B, Sanguinetti MC. Binding site of a novel Kv1.5 blocker: a ''foot in the door'' against atrial fibrillation. Mol Pharmacol 2006;70:1204–11.
[27] Naccarelli GV, Wolbrette DL, Samii S, Banchs JE, Penny-Peterson E, Stevenson R, et al. Vernakalant—a promising therapy for conversion of recent-onset atrial fibrillation. Expert Opin Investig Drugs 2008;17:805–10.
[28] Fedida D. Vernakalant (RSD1235): a novel, atrial-selective antifibrillatory agent. Expert Opin Investig Drugs 2007;16:519–32.
[29] Wirth KJ, Brendel J, Steinmeyer K, Linz DK, Rutten H, Gogelein H. In vitro and in vivo effects of the atrial selective antiarrhythmic compound AVE1231. J Cardiovasc Pharmacol 2007;49:197–206.
[30] Eldstrom J, Wang Z, Xu H, Pourrier M, Ezrin A, Gibson K, et al. The molecular basis of high-affinity binding of the antiarrhythmic compound vernakalant (RSD1235) to Kv1.5 channels. Mol Pharmacol 2007;72: 1522–34.
[31] Schmalhofer WA, Bao J, McManus OB, Green B, Matyskiela M, Wunderler D, et al. Identification of a new class of inhibitors of the voltage-gated potassium channel, Kv1.3, with immunosuppressant properties. Biochemistry 2002;41:7781–94.
[32] Hanner M, Schmalhofer WA, Green B, Bordallo C, Liu J, Slaughter RS, et al. Binding of correolide to K(v)1 family potassium channels. Mapping the domains of high affinity interaction. J Biol Chem 1999;274:25237–44.
[33] Thai KM, Ecker GF. A binary QSAR model for classification of hERG potassium channel blockers. Bioorg Med Chem 2008;16:4107–19.
[34] Thai KM, Ecker GF. Predictive models for HERG channel blockers: ligand-based and structure-based approaches. Curr Med Chem 2007;14:3003–26.
[35] Beene DL, Dougherty DA, Lester HA. Unnatural amino acid mutagenesis in mapping ion channel function. Curr Opin Neurobiol 2003;13:264–70.
[36] Remillard CV, Tigno DD, Platoshyn O, Burg ED, Brevnova EE, Conger D, et al. Function of Kv1.5 channels and genetic variations of KCNA5 in patients with idiopathic pulmonary arterial hypertension. Am J Physiol Cell Physiol 2007;292:C1837–53.