Comparison of Potent Kv1.5 Potassium Channel Inhibitors Reveals the Molecular Basis for Blocking Kinetics and Binding Mode

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Key Words

Homology model • Binding site • Antiarrhythmic • S9947 • MSD-D • ICAGEN-4 • Block

Abstract

In this study, we analysed the inhibitory potency, blocking characteristics and putative binding sites of three structurally distinct Kv1.5 channel inhibitors on cloned human Kv1.5 channels. Obtained IC₅₀ values for S9947, MSD-D and ICAGEN-4 were 0.7 μ M, 0.5 μ M, and 1.6 μ M, respectively. The Hill-coefficients were close to 1 for S9947 and approximately 2 for MSD-D and ICAGEN-4. All three compounds inhibited Kv1.5 channels preferentially in the open state, with Kv1.5 block displaying positive frequency dependence, but no clear voltage and potassium dependence. In contrast to slow on- and off-rates of apparent binding of MSD-D and ICAGEN-4, S9947 had fast on- and off-rates resulting in faster adaptation to changes in pulse frequency. Utilizing Alanine-scanning and *in silico* modeling we suggest binding of the compounds to the central cavity with crucial residues Ile508 and Val512 in the S6-segment.

Residue Thr480 located at the base of the selectivity filter is important for ICAGEN-4 and S9947 inhibition, but less so for MSD-D binding. Our docking models suggest that the innermost potassium ion in the selectivity filter may form a tertiary complex with oxygens of S9947 and ICAGEN-4 and residue Thr480. This binding component is absent in the MSD-D block. As S9947 and ICAGEN-4 show faster block with proceeding channel openings, formation of this tertiary complex may increasingly stabilise binding of S9947 and ICAGEN-4, thereby explaining open channel block kinetics of these compounds.

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Introduction

Atrial fibrillation (AF) is the most common chronic arrhythmia in humans. It affects mainly the elderly and its prevalence is expected to further increase, as populations grow older in industrialised countries. Treatment of AF is still inadequate and presently used

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antiarrhythmic drugs are only weakly effective, may cause severe side effects, and even may provoke ventricular arrhythmia. Thus, for pharmaceutical companies one current strategy in the development of more effective and safer atrial antiarrhythmics, is to target channels that are only expressed in the atrium.

The human Kv1.5 channel generates the atrial ultrarapidly activating delayed rectifier K^+ current I_{Kur} or I_{Sus} [1-4], which mediates an outward potassium current with fast activation and slow inactivation kinetics [3, 5-7]. Due to its importance for repolarisation of the atrial action potential, its selective pharmacological inhibition is considered as a beneficial treatment for AF [8]. Selective I_{Kur} inhibitors by prolongation of the atrial action potential duration are expected to convert AF to sinus rhythm and to prevent recurrence of AF after cardioversion [9]. As I_{Kur} is not present in human ventricular myocytes [10, 11], its block should not affect ventricular repolarisation and consequently the QT interval of the ECG. This is in contrast to classical class III antiarrhythmics, such as I_{Kr} blockers, which as a result of lengthening the ventricular action potential duration, can provoke long QT syndrome and *torsades de pointes* arrhythmias [12, 13].

Structurally different Kv1.5-inhibitors like S9947, MSD-D and ICAGEN-4 are novel atrial-selective class III antiarrhythmics developed by Aventis Pharma, Merck, and ICAGEN (for a review see [14]). Here, we compared the general inhibiting and blocking kinetic properties of these Kv1.5-inhibiting compounds, and analysed their putative binding sites by an Alanine-scan of the Kv1.5 pore. Our results suggest that these structurally unrelated compounds preferentially block the open/activated state of the channel, which might result in superior blocking properties compared to existing class III antiarrhythmics.

Materials and Methods

Molecular biology

Kv1.5 was cloned from human heart and differs from the GenBank™ data base entry NM_002234 by two residues, K418R and K565E. The newer data base entry of the Kv1.5 sequence (NM_002234) has an N-Terminus with two additional residues. This results in a shift of the amino acid numbering of +2, compared to literature referring to the previous entry (M60451). Alanine-mutant Kv1.5 channels were described in detail elsewhere [15]. cDNA constructs in pSGEM vector [16] were linearised with *NheI* (Boehringer Mannheim, Mannheim, Germany), and capped cRNA was synthesised using the T7 mMessage mMachine mRNA synthesis kit (Ambion, Austin, TX, USA). Quality and quantity of cRNA was determined by gel electrophoresis and UV spectroscopy.

Isolation and electrophysiological recordings from *Xenopus* oocytes

Methods for isolation and injection of oocytes were as described elsewhere [15]. The oocytes were stored at 18°C in standard recording solution ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4), with additional sodium pyruvate (275 mg l⁻¹), theophylline (90 mg l⁻¹) and gentamycin (50 mg l⁻¹). 5 ng wild-type or mutant Kv1.5 cRNA was injected into each oocyte. Standard two-electrode voltage clamp technique was used to perform recordings 3 - 5 days after injection. Current and voltage electrodes were filled with 3 M KCl and had resistances of 0.5 - 1.5 MΩ.

Blockers were added to recording solutions from 10 mM stock solutions in DMSO. The modified solutions ND92, ND78, and ND38 (containing 6, 20, and 60 mM KCl, respectively, with a corresponding substitution in sodium concentration) were used to analyse the effect of extracellular K^+ on the binding of the compounds to Kv1.5.

Standard two-electrode voltage clamp recordings were performed with a Turbo Tec 10CD (NPI, Tamm, Germany) amplifier and an ITC-16 interface combined with Pulse software (HEKA Elektronik, Lambrecht, Germany). Alternatively, Digidata 1322A interface combined with Clamp/Ex software (Axon Inc., USA) was used for data acquisition.

Data Analysis

PulseFit (HEKA Elektronik), Clamp/Fit software (Axon Inc., USA), Igor Pro software (Wavematrix Inc., Lake Oswego, OR) and Origin 6.0 software (Microcal) were used for data analysis. Exponential fits of curves were made with the following formulae:

$Y = Y_0 + A \exp^{-t/\tau}$ for a single exponential decay

$Y = Y_0 + A_1 \exp^{-t/\tau_1} + A_2 \exp^{-t/\tau_2}$ for a two exponential decay

$Y = Y_0 + A \exp^{t/\tau}$ for a single exponential increase

where τ_1 and τ_2 are the time constants, A_1 and A_2 are the amplitudes of each component and Y_0 is the baseline value.

To calculate IC_{50} values, the current amplitudes were measured at the end of test pulses and subsequently applied to the formula:

$$I/I_{\max} = 1 / \{1 + (IC_{50}/c)^H\}$$

where I is the measured current, $I_{\max}$ is the maximum amplitude of measured current, c is the blocker concentration, H is the Hill-coefficient and IC_{50} is the concentration at half-maximal block.

Molecular modeling

The molecular modeling strategy applied was used before to determine the binding sites of a blocker and a potassium channel activator [17, 18]. In brief, three dimensional structural models of the S5-S6 domains of Kv1.5 were constructed based on the crystal structure of the corresponding domains of Kv1.2. Docking of energy-optimised blocker conformers (IsisDraw, MDL; ArgusLab31, Planaria, Seattle and ACD/ChemsSketch, Advanced Chemistry Development, Inc., Ontario, Canada) into the Kv1.5 pore homology model was performed using a combination of a hydrophobic docking approach with the Global Range Molecular Matching program (Gramm v1.03 program) and manual docking [19]. The Gramm program uses

Fig. 1. Chemical structures of S9947 (2'-(Benzyloxycarbonylaminomethyl)biphenyl-2-carboxylic acid 2-(2-pyridyl) ethylamide), ICAGEN-4 (N-[3-(4-Ethyl-benzene-sulfonylamino)-2-hydroxy-indan-5-yl]-3-methoxy-benzamide) and MSD-D (2-(4-Methoxy-phenyl)-5-methyl-3-oxo-6-p-tolylsulfanyl-2,3-dihydro-pyridazine-4-carbonitrile).

an exhaustive 6 dimensional search for possible binding conformations and ranks the putatively best conformations.

Results and Discussion

Several class Ic and class III antiarrhythmics inhibit the cardiac I_{Kur} current [20-22], and are presently also used for treatment of AF [23]. The biphenylic compound S9947 was previously described as a highly potent blocker of Kv1.5 [24]. In this study, we analysed the kinetics of inhibition of Kv1.5 currents by S9947 and compared it with those of two other blockers, namely MSD-D and ICAGEN-4. For our functional studies we used the *Xenopus* oocyte expression system which already demonstrated a good concurrence between the effects of compounds blocking currents of native I_{Kur} and of cloned Kv1.5 [22, 25, 26].

Expression of human Kv1.5 channels in *Xenopus* oocytes yielded characteristic potassium currents in response to depolarising voltage steps. The IC_{50} values, which were calculated at the end of a 1.5 s test pulse to a voltage of +40 mV, and the Hill-coefficients of current block mediated by the different inhibitors are summarised in Table 1. The obtained rank order of block was MSD-D > S9947 > ICAGEN-4 with IC_{50} values of 0.5, 0.7 and 1.6 μ M, respectively. The Hill-coefficients were approximately 2 for MSD-D and ICAGEN-4, and 1 for S9947. Figures 2a-c show that all three substances produced a concentration-dependent decrease of Kv1.5 potassium currents. However, the three compounds showed different blocking kinetics. While all three compounds effectively suppressed the steady-state current, ICAGEN-4 and MSD-D also caused a strong reduction of peak currents. Of these two compounds

compound	IC_{50} (μ M)	Hill-coefficient
S9947	0.7 ± 0.1	1.1 ± 0.05
ICAGEN-4	1.6 ± 0.1	1.9 ± 0.10
MSD-D	0.5 ± 0.1	2.0 ± 0.05

Table 1. Kv1.5 block characteristics of compounds. Data are expressed as mean $\pm$ S.E.M..

Fig. 2. Concentration dependence and on-rate of block of different Kv1.5 channel blockers. Representative Kv1.5 currents recorded before and after perfusion of oocytes with increasing concentrations (0.03, 0.1, 0.3, 1, 3, and 10 μ M; only 3 concentrations are shown) of (a) S9947, (b) ICAGEN-4, and (c) MSD-D. Currents were recorded by stepping the membrane potential to +40 mV for a 1.5 s pulse from a holding potential of -80 mV. IC_{50} values and Hill-coefficients ($n = 3 - 7$) were calculated at the end of the pulses and are summarised in Table 1. (d) Time constants of apparent binding (on-rates) were determined by fitting the current traces in a-c to a single exponential function. Mean on-rates ($n = 5$) were calculated from these values and plotted against the compound concentrations. (e) Onset of current block. The ratio of current in the presence of blocker (2 μ M) divided by the current before blocking was determined to calculate the fraction of unblocked current as a function of time.

Fig. 3. Time-dependent recovery from block of Kv1.5 channels. Compounds were perfused at their approximate IC_{50} values ($1 \mu M$). An interpulse protocol was used consisting of two 1 s pulses to +40 mV from a holding potential of -80 mV, separated by a pulse to -80 mV of increasing duration. The difference in peak currents between the first and second pulse was measured and plotted as the normalised current ($n = 4 - 5$) against the interpulse time.

MSD-D achieved the strongest effect, almost immediately reducing the current to its steady-state level of inhibition. In contrast, even at $1 \mu M$ S9947 only had a minimal effect on peak outward currents, suggesting that block only occurs once the channel is in the open state. Only at much higher concentrations a small decrease in peak current amplitude was observed. These results point to a strong preference of S9947 and to a lesser extent also for ICAGEN-4 for open and/or inactivated channel states.

To compare the apparent binding rates of the substances to Kv1.5, exponential fits of the current curves shown in Figures 2a-c were used to calculate the time constants (τ) of apparent binding at 1, 3, and $10 \mu M$ of each inhibitor. Due to the very slow time-dependent inactivation rate of Kv1.5 (Fig. 2a), only a minor fraction of the channels are inactivated at the end of the test pulse, and therefore contribution of inactivation to overall block could be neglected [3, 27, 28]. When the calculated apparent on-rates of block were plotted against the compound concentrations (Fig. 2d), the resulting curves demonstrate a strong dependence of the rate of binding of S9947 on the concentration upon channel opening, only

Fig. 4. Analysis of closed channel block. Kv1.5 currents were activated by repetitive 1.5 s pulses from a holding potential of -80 mV to 0 mV (0.067 Hz) until they reached steady-state. Then pulsing was stopped and oocytes were clamped to -120 mV to accumulate channels in closed states. Compounds were perfused at their approximate IC_{50} values ($1 \mu M$) for 300 s. After that time pulsing was started again. Steady-state current amplitudes were measured and normalised to the current amplitudes before inhibitor application ($n = 4$).

weak concentration dependence for the on-rate of ICAGEN-4, while that of MSD-D was concentration independent. Similarly, the time-dependent decay of unblocked current is most prominent for S9947 (Fig. 2e). These results again underline that S9947, and to a lesser extent also ICAGEN-4, act as classical open channel blockers.

Open channel blockers usually display a reduced affinity for closed channel states, resulting in decreasing potency of block with longer membrane hyperpolarisation, which favours channel closing. We therefore analysed the unblock of the inhibitors upon Kv1.5 channel closure using a double pulse protocol while compounds were perfused at a concentration of $1 \mu M$. The current amplitude of the second pulse was normalised to that of the corresponding first pulse. Figure 3 shows the normalised peak current amplitudes plotted against the increasing interval time between paired pulses. Unblocking of the Kv1.5 channel was time-dependent for all three substances. The curves were then fitted using an equation for a mono-exponential increase in order to determine the rates of recovery from block (unblock, off-

Fig. 5. Frequency-dependent block of Kv1.5 channels. Oocytes were perfused with compounds at 1 μ M (about their individual IC₅₀ value concentrations), and currents were elicited by repetitive 1.5 s test pulses to +40 mV at a frequency of 0.067 Hz until a steady-state of block was attained. Subsequently, currents were elicited by repetitive 0.5 s test pulses to +40 mV at pulse frequencies of 0.5 and 1 Hz in the presence of (a) S9947, (b) ICAGEN-4 and (c) MSD-D, at 1 μ M. The peak currents were measured (n = 4 – 5) and normalised to the peak amplitudes of the currents elicited by the first pulse and plotted against the time. Time constants of block were determined by fitting the resulting data by double-exponential functions. The obtained values are shown in Table 2.

rate). S9947 showed the fastest rate of unblock with a value of 0.652 1/s, followed by ICAGEN-4 with 0.182 1/s, and MSD-D with 0.159 1/s (Fig. 3). This analysis is slightly affected by the recovery from inactivation of the channels under control conditions in the absence of inhibitors (Fig. 3a). However, the size of increase of current amplitude due to recovery from inactivation is much smaller than the increase of current due to recovery of blockers and thus was neglected in the analysis of off-rates of block. The observed recovery of current amplitudes demonstrated a closed channel-dependent unbinding for all three blockers. These unblocking characteristics do not provide any evidence for a trapping of the blockers in the closed channel as it was reported for block of HERG channels by methanesulfonanilide [30], but are rather consistent with a preferential block of the open channel.

To investigate, whether blockers can access their binding sites in closed channels, oocytes were held for 5 minutes at -120 mV while perfusing the compounds at 1 μ M (~ IC₅₀ values) before channels were activated by repetitive pulses to 0 mV. Block was analysed at the end of the test pulse. S9947 blocked steady-state currents already maximally at the first pulse, while block by MSD-D and ICAGEN-4 increased with time of pulsing (Fig. 4). These results demonstrate that also MSD-D and ICAGEN-4 are open channel blockers. It therefore appears that the highly rapid open channel block by S9947 is a result of very fast access to its binding site, resulting in steady-state block during the first pulse. This finding is consistent with the fast on- and off-rates determined for S9947. Similar fast blocking properties were recently reported for the structurally-related Kv1.5 blocker AVE0118 by Decher et al [29].

Positive frequency dependence may be advantageous for a drug effective at fast action potential firing

compound	frequency (Hz)	τ_1 (s)	τ_2 (s)	normalised amplitude
S9947	0.5	1.0 ± 0.04	85 ± 2.4	0.87
	1	0.5 ± 0.01	67 ± 2.0	0.58
ICAGEN-4	0.5	8.0 ± 0.03	142 ± 6.2	0.42
	1	5.0 ± 0.05	56 ± 3.9	0.23
MSD-D	0.5	9.5 ± 0.16	105 ± 3.8	0.46
	1	8.2 ± 0.17	48 ± 1.2	0.24

Table 2. Frequency-dependence of Kv1.5 block. Time constants τ_1 and τ_2 of Kv1.5 block by inhibitors as determined by the protocol described in legend to Fig. 5 and materials and methods. Data are expressed as mean $\pm$ S.E.M.

rates characteristic for AF. To analyse the steady-state frequency dependence of the blockers test pulses to +40 mV at a frequency of 0.067 Hz (one 1.5 sec pulse every 15 sec) were applied and the substances were washed in for 5 min at a concentration around their IC₅₀ values. Under these conditions block induced by the respective substance reached steady-state. We then applied pulses to +40 mV at 0.5 Hz and 1 Hz (one 0.5 sec pulse every 2 or 1 sec, respectively). As shown in Figure 5, in all cases, block of currents increased with the higher stimulation frequency. The initial response to the increase in pulse frequency was fastest for S9947. While the overall reduction of steady-state current amplitude by S9947 was weaker than that seen for ICAGEN-4 and MSD-D, the difference in magnitude of current inhibition at both frequencies was largest with S9947 (see Table 2). Compared to partially inhibited control currents at 0.067 Hz,

Fig. 6. Effects of membrane voltage and external K^+ concentration on block characteristics. Oocytes were perfused with different concentrations (0.1, 1, 10 μM) of (a) S9947, (b) ICAGEN-4, and (c) MSD-D in solutions containing different amounts of K^+ (2, 6, 20, and 60 mM) until steady-state block was achieved. Currents were elicited by 1.5 s pulses in 10 mV increments to potentials between -10 and +50 mV from a holding potential of -80 mV at 0.1 Hz. The peak currents were measured ($n = 3-5$) and normalised to peak values under control conditions. IC_{50} values at different K^+ -concentrations were determined and plotted against the test voltages. Data for ICAGEN-4 in 20 mM K^+ at 0 mV and -10 mV were highly variable and are not shown.

S9947 further reduced currents by $61 \pm 0.7\%$ at 0.5 Hz, and $74 \pm 2.3\%$ at 1 Hz. Thus, at higher test frequency, block by S9947 reached its steady-state already after a single pulse. This was also the case with the closely related biphenyl derivative AVE0118 [29], and similar with Kv1.5 block by the anthranilic-acid derivative S0100176 [15]. MSD-D showed a slower response to increased frequency, while that of ICAGEN-4 was intermediate (Fig. 5a-c). Time constants of block and final steady-state current amplitudes (at maximal block) are summarised in Table 2, and were calculated by double exponential fits of the curves resulting from plotting the maximal current amplitudes against time. By application of the exponential fit, we were able to calculate the final steady-state amplitudes of the curves (Table 2). S9947 demonstrated the largest difference ($\Delta_{\text{mean}} = 0.29$ units) between the steady-state amplitudes at the two frequencies, indicating strong positive frequency dependence. Slightly lower frequency dependence was observed with ICAGEN-4 and MSD-D ($\Delta_{\text{mean}} = 0.19$ and 0.22 units,

respectively) even though their overall inhibition of currents was much stronger at higher frequencies compared to S9947. The determined frequency dependence correlates well with the kinetics for the onset of block of the three compounds. Although both MSD-D and ICAGEN-4 demonstrated a frequency-dependent block, a preferential open channel block and a closed channel unblock similar to S9947, our results show that the kinetics of their effects are considerably slower. Therefore, the different blocking properties observed for MSD-D (Fig. 2c), which does not show the typical characteristics of an open channel blocker, can be explained by its slow rates of block and unblock.

Through variation of the extracellular potassium concentration and the membrane voltage hints to the location of a compound binding site can be obtained. In case of an internal pore blocker an increase in $[K^+]_{\text{out}}$ can reduce inhibition [31, 32]. This effect may be weaker and even absent for substances, which bind to sites distant to the selectivity filter and which are uncharged and/or bind with high affinity. We then determined IC_{50} values at different external potassium concentrations ($[K^+]_{\text{out}} = 2, 6, 20$ or 60 mM). All three blockers did not show a significant voltage dependence of block between -10 mV and +50 mV (Fig. 6), while a mild reduction in potency was seen at this potential. At -10 mV channels are mainly in early open states but also tend to close, which may result in the mildly increased IC_{50} values of the open channel blockers at -10 mV. The block by the compounds was not dependent on the extracellular potassium concentration. Since extracellular K^+ -concentration modulates slow inactivation of Kv1.5, the lack of modulation of block may indicate that inhibitors do not discriminate between open and inactivated states. Though block by the inhibitors is not sensitive to changes in extracellular potassium and membrane potential, block of the open pore near the selectivity filter inside the pore cavity is still possible, since all three structures are uncharged and show high affinity. Indeed, a pore plugging mechanism is suggested by the finding that voltage dependence of channel activation seems not to be significantly modified by any of the substances as would be the case with a gating modifier. In addition, complete inhibition of Kv1.5 currents could be achieved at high positive voltages. If the blockers inhibited the Kv1.5 currents by stabilizing closed states, a much less pronounced block would be expected at more positive voltages where open states are favoured. This has previously been shown with gating modifying toxins [33]. On the other hand, stabilization of the inactivated state

Fig. 7. Localisation of the putative blocker binding sites. (a) Wild-type and mutant Kv1.5 channels were activated by repetitive 2 s pulses to 0 mV every 20 seconds. The blockers were perfused at 2 μ M for 5 min and the unblocked current was determined. (b) 3D structural models of S5-S6 domains of three channel subunits were generated based on homology to the open state Kv1.2 crystal structure using Swiss model, and obtained pore models were then energy optimised (gromos96 force field parameters). Compounds were designed and energy optimised using Isis/Draw, ACD/Chemsketch and ArgusLab31. Several energy favourable conformations were docked either manually or automatically (Gramm v1.03) into the channel pore model. Energy optimised models with compounds in favoured docking positions are shown as viewed from the side. Channel subunits are shown in ribbon representation and residues identified as most important for inhibitor molecule interaction are shown as coloured sticks (CPK code: T480; orange: I508; green: V512). The blocker molecules are shown in CPK-colour code and space fill representation. To the right: magnified stereo views of the region of the binding sites with compounds, interacting channel residues and K⁺ ions shown in space fill representation (CPK code: T480; orange: I508; green: V512; gray: K⁺ ions). n.f., non-functional mutant channel.

Fig. 8. Hypothetical access pathways to the putative common drug binding site in the central cavity of Kv1.5 channels. Two possible major access pathways may exist in Kv1.5 channels, one through a cytosolic entrance way and the other through a crevice between S5 and S6. The long access pathway via the cytosol may include an additional binding step before the compounds bind to the pore resulting in a Hill-coefficient of about 2. S9947 can access Kv1.5 channels in the open state much faster and shows a Hill-coefficient of about 1 indicating that this compound may enter the central cavity binding site by a different route without intermediate high affinity binding events.

would also favour block at more depolarised voltages. However, this was not observed here for either blocker.

The observed differences in blocking properties also suggest different binding modes of the compounds. Recently, residues in the Kv1.5 pore helix and in the S6 transmembrane segment were identified as important components of the binding site of Kv1.5 blockers S010076 and AVE0118 [16, 29]. To further investigate this possibility, we studied mutant Kv1.5 channels harbouring individual alanine-substitutions of residues in the lower selectivity filter signature sequence and S6 segment for their blocker sensitivity. We identified 3 mutants with strongly reduced sensitivity to S9947 and ICAGEN-4, namely T480A, I508A, and V512A. Similarly, MSD-D also was sensitive to mutations in S6 (I508A and V512A) but to a lesser extent to the T480A exchange (Fig. 7a). All three inhibitors blocked the S6-mutant channels (I508A and V512A) with considerably lower potency, indicating that they bind in the central pore cavity, and interact with residues at the bottom of the pore helix and the S6-segment. Thus, this site constitutes a conserved K⁺ channel binding motif for small lipophilic blockers [16–18, 29, 34, 35]. The location of the binding site further supports an open channel block mechanism consistent with the experimental data.

We performed unbiased automated docking into the Kv1.5 homology model. The conformation of the three compounds was generated *in silico* (at least 8 different energy optimised conformers) and docked to a Kv1.2-based open channel homology model of Kv1.5. This docking model suggests hydrophobic interactions of the blocker molecules with residues Ile508A and Val512A and electrostatic interactions of the compounds oxygen atoms with the innermost potassium ion in the selectivity filter (Fig. 7b). Formation of such a tertiary complex between channel residues, a permeating potassium ion in the selectivity filter and the inhibitor molecule was recently proposed to occur in chromanol 293B-block of KCNQ1 channels and in correolide-block of Kv1.3 channels [36, 37]. It is noteworthy that all three compounds probably bind to the central cavity to overlapping binding sites but exhibit marked differences in blocking kinetics. Of the compounds mostly S9947 showed the characteristics typical for a fast acting open channel blocker, namely a clear time- and frequency-dependent block. In the S9947-docking model, and this is also true to a certain degree for ICAGEN-4, the central potassium ion and the oxygen atoms of the compounds are in close proximity to each other therefore enabling electrostatic interactions that would significantly contribute to the compound binding

energy [36, 37]. Hence, accumulation of these tertiary compound-channel complexes may explain the strong positive use-dependence of block by S9947 and ICAGEN-4.

It is difficult to explain the different Hill-coefficients (Table 1) and the slower on- and off-rates of channel block (Figs. 2, 3) by MSD-D and ICAGEN-4. A Hill-coefficient of 2, as found for MSD-D and ICAGEN-4, could indicate either cooperativity of binding, i.e. binding of more than one blocker molecule, or a successive interaction with two different binding sites. Alternatively, indication of cooperativity or interaction with two binding sites was not found for S9947, which has a Hill-coefficient of 1. Our molecular docking of the substances into the channel does not support binding of two molecules in the central cavity and therefore does not explain a Hill-coefficient of 2. A possible explanation would be different access pathways of MSD-D and ICAGEN-4 with an additional binding site (Fig. 8). Alternative access routes to the central pore were suggested for block of voltage-gated sodium channels by local anesthetics [38]. A binding model with successive binding to several binding sites was also proposed for guidance of substrates via three distinct active binding sites in proteins like tryptophan synthase or carbamoyl phosphate synthase [39]. Occupancy of two or more high affinity binding sites would result in slower on- and off-rates, which we actually demonstrate for MSD-D and ICAGEN-4. Clearly, a model of successive binding to sites along a characteristic access route for MSD-D and ICAGEN-4 is currently speculative and further investigations to prove it are necessary.

The exact mechanism of action of an atrial-selective Kv1.5 blocker might be crucial for its efficacy in treatment of AF. Since AF leads to accumulation of open and inactivated channels, inhibitors blocking channels preferentially in these states might be the most effective ones [3]. Here, we demonstrate that S9947 and ICAGEN-4 possess these favourable properties, namely a fast onset and a positive use-dependency of block. Clinical studies finally will have to demonstrate that Kv1.5 blockers with open channel block properties are indeed a successful pharmacological approach to treat AF.

Abbreviations

τ (time constants of block or unblock); I_{Kr} (rapid delayed rectifier K⁺ current); I_{Kur} (ultrarapid delayed rectifier K⁺ current); I_{Sus} (sustained outward current);

[K⁺]_{out} (extracellular potassium concentration); c (concentration); S9947 (2'-(Benzyloxycarbonylaminomethyl)biphenyl-2-carboxylic acid 2-(2-pyridyl)ethylamide); ICAGEN-4 (N-[3-(4-Ethylbenzenesulfonylamino)-2-hydroxy-indan-5-yl]-3-methoxy-benzamide); MSD-D (2-(4-Methoxy-phenyl)-5-methyl-3-oxo-6-p-tolylsulfanyl-2,3-dihydro-pyridazine-4-carbonitrile).

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