



From Pan-Reactive K_V7 Channel Opener to Subtype Selective Opener/Inhibitor by Addition of a Methyl Group

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Abstract

The voltage-gated potassium channels of the K_V7 family ($K_V7.1-5$) play important roles in controlling neuronal excitability and are therefore attractive targets for treatment of CNS disorders linked to hyperexcitability. One of the main challenges in developing K_V7 channel active drugs has been to identify compounds capable of discriminating between the neuronally expressed subtypes ($K_V7.2-5$), aiding the identification of the subunit composition of K_V7 currents in various tissues, and possessing better therapeutic potential for particular indications. By taking advantage of the structure-activity relationship of acrylamide K_V7 channel openers and the effects of these compounds on mutant K_V7 channels, we have designed and synthesized a novel K_V7 channel modulator with a unique profile. The compound, named SMB-1, is an inhibitor of $K_V7.2$ and an activator of $K_V7.4$. SMB-1 inhibits $K_V7.2$ by reducing the current amplitude and increasing the time constant for the slow component of the activation kinetics. The activation of $K_V7.4$ is seen as an increase in the current amplitude and a slowing of the deactivation kinetics. Experiments studying mutant channels with a compromised binding site for the $K_V7.2-5$ opener retigabine indicate that SMB-1 binds within the same pocket as retigabine for both inhibition of $K_V7.2$ and activation of $K_V7.4$. SMB-1 may serve as a valuable tool for K_V7 channel research and may be used as a template for further design of better subtype selective K_V7 channel modulators. A compound with this profile could hold novel therapeutic potential such as the treatment of both positive and cognitive symptoms in schizophrenia.

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Introduction

The neuronally expressed members of the K_V7 family ($K_V7.2-5$) are the molecular correlates of the M-current, a slowly activating and slowly deactivating potassium current [1,2,3]. K_V7 channels open at voltages below the threshold required for generation of an action potential and play a fundamental role in the control of neuronal excitability. Accordingly, mutations in the genes encoding $K_V7.2$ and $K_V7.3$ result in a form of neonatal epilepsy [4,5,6]. Hence, drugs that increase the activity of K_V7 channels have a great therapeutic potential for the treatment of diseases characterized by hyperexcitability, such as epilepsy and neuropathic pain. Retigabine (Trobal/Potiga), which activates $K_V7.2-5$, was approved as an add-on treatment for partial onset seizures in 2011 and has proven effective in preclinical models for a wide variety of diseases [7,8,9,10].

Based on mutation studies, retigabine has been shown to bind to a hydrophobic pocket between transmembrane segments 5 and 6 of the channel proteins. Specifically, channel activation by

retigabine is critically dependent on a tryptophan residue (W236 in $K_V7.2$) in the cytoplasmic part of S5 [11,12]. The cardiac $K_V7.1$ channel has a leucine at this position explaining its resistance to retigabine-induced enhancement. It appears that the tryptophan residue constitutes a structural element of a promiscuous binding site in the channels, since compounds which are structurally different from retigabine, like BMS-204352, (S)-1 and (S)-2 also lose their activating effects when the tryptophan is substituted for a leucine [13,14]. Yet, compounds like ZnPy [15], ICA-27243 [16,17] and fenamic acids [18] are not dependent on its integrity, suggesting that other activator binding sites exist. A leucine within the pore loop and a leucine extending from S6 of the adjacent subunit have also been found to be important residues for retigabine sensitivity [19]. These residues are conserved between the neuronal subtypes whereas $K_V7.1$ carries valines in both positions. Introduction of the three critical residues in $K_V7.1$ confers full retigabine sensitivity [19].

We have previously shown that the acrylamide (S)-2 (fig. 1A) activates K_v7.2–5 [14]. For K_v7.4 and K_v7.5 the effect of (S)-2 is purely positive, while the compound has a bimodal effect on homomeric K_v7.2 and heteromeric K_v7.2/3 channels. For K_v7.2, the positive effects of (S)-2 are a hyperpolarizing shift in the voltage-dependence of activation, a slowing of the deactivation kinetics (τ_{deact}) and an acceleration of the fast component of the activation kinetics ($\tau_{\text{act_fast}}$). At voltages below -10 mV the compound also increases the current amplitude and accelerates the slow component of the activation kinetics ($\tau_{\text{act_slow}}$). However, at voltages above -10 mV the compound has a secondary inhibitory effect. At these depolarized voltages the effect on the current amplitude and $\tau_{\text{act_slow}}$ crosses over and becomes inhibitory. When we tested (S)-2 on a mutated K_v7.2 channel where the tryptophan residue in S5 (which is essential for the effect of retigabine) was substituted for a leucine (K_v7.2-W236L) all activating effects of (S)-2 were lost and the compound became purely inhibitory. Based on these observations we proposed that the mechanism behind the inhibitory effect on K_v7.2-W236L and the secondary inhibitory action at the WT K_v7.2 may be the same and that the effect of (S)-2 on K_v7.2 can be divided into two entities: 1) an activating part visible as a shift in the voltage-dependence of activation and 2) an inhibitory part visible as a decrease in the current amplitude and an increase in $\tau_{\text{act_slow}}$. According to this proposal, the interpretation of the experimental data is that the W236L mutation disables only the (S)-2 induced activation and thereby unmasks the inhibitory component of the drug effect. Consistent with this proposal is the observation that for K_v7.4, where the effect of (S)-2 is purely positive, the retigabine insensitive mutant (K_v7.4-W242L) is fully insensitive to (S)-2.

This raised the question of whether it would be possible to design a compound that mimics the effect of the W-L mutation, resulting in an inhibitor of K_v7.2 that would not affect K_v7.4. Interestingly, it is described in the structure activity-relationship of the acrylamide class of compounds that methylation of the amide nitrogen of a related acrylamide K_v7.2 opener (S)-1 results in a compound that inhibits K_v7.2 [20]. In our hands (S)-1 is also an inhibitor of K_v7.2-W236L (unpublished observation) and has been reported to be an inhibitor of K_v7.4-W242L [13]. We hypothesized that this chemical modification of the acrylamide may have the same effect as the W-L mutation where the methylated version of (S)-1 would be an inhibitor of both K_v7.2 and K_v7.4, while the methylated version of (S)-2 would inhibit K_v7.2 but not K_v7.4. Here we describe the synthesis and characterization of an analogue of (S)-2, termed SMB-1, where the amide nitrogen is methylated (fig. 1B). We show that SMB-1 is indeed an inhibitor of K_v7.2. In contrast, K_v7.4 is activated by SMB-1. However, SMB-1 does not have sufficient penetration into the rodent brain to allow examination of its *in vivo* CNS profile.

Materials and Methods

Molecular biology

Point mutations were introduced using mutated oligonucleotide extension (PfuTurbo Polymerase, Stratagene, La Jolla, CA, USA) from a plasmid template harboring the cDNA of interest, digested with DpnI (Fermentas, St. Leon-Roth, Germany) and transformed into *E. coli* XL1 Blue cells. The construct was verified by complete DNA sequencing of the cDNA insert. cRNA was prepared from linearized human wild-type (WT) and mutant K_v7 channels in the pGEM-HE vector using the T7 m-Message Machine kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Electrophysiology

Extraction of *Xenopus laevis* oocytes and injection of cRNA was performed as described previously [14]. Oocytes were kept in Modified Barth's Saline (in mM: 88 NaCl, 1 KCl, 2.4 NaHCO₃, 0.41 CaCl₂, 0.82 MgSO₄, 0.3 Ca(NO₃)₂, 15 HEPES, pH 7.4 supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin) at 18°C, and currents were recorded after 2–7 days. The care of *Xenopus laevis* and the oocyte extraction procedure were performed according to national guidelines and approved by the Danish Animal Experiments Inspectorate.

K_v7 currents in *Xenopus laevis* oocytes were recorded using two-electrode voltage-clamp at room temperature in Ringer buffer (in mM: 115 NaCl, 2.5 KCl, 1.8 CaCl₂, 0.1 MgCl₂, 10 HEPES, pH 7.4) as described previously [14]. Data was acquired using pCLAMP 10.2 software (Molecular Devices, CA, USA) and analyzed using pCLAMP 10.2 and GraphPad Prism 4.0 (GraphPad Software Inc., CA, USA). Currents were elicited from a holding potential of -80 mV by 5 s steps to potentials between -100 and $+50$ mV in 10 mV increments, followed by a 2 s step to -120 mV.

SMB-1 was dissolved in dimethyl sulfoxide (DMSO) to obtain a concentrated stock solution. On the day of experiments the stock solution was thawed and diluted in Ringer buffer to the final concentrations. A concentration of 10 µM SMB-1 was used unless indicated otherwise. The final DMSO concentration never exceeded 0.1%.

Curve fitting and statistical analysis

Current – voltage (*I*–*V*) relationship curves were generated by normalizing the steady-state peak current measured at potentials between -100 and $+50$ mV to the current measured at $+50$ mV in control recordings and plotting the values against the test potential.

The voltage-dependence of activation was determined from tail current analysis using the current measured immediately after the transient capacitive current after stepping to -120 mV from potentials between -100 mV and $+50$ mV. Data was normalized to extend from 0–1 and the tail current-voltage relationship was fitted to the Boltzmann equation:

$$I(V) = \frac{I_{\text{max}} - I_{\text{min}}}{1 + \exp[(V - V_{0.5})/k]} + I_{\text{min}} \quad (\text{Equation 1})$$

where I_{max} is the maximum tail current, I_{min} is the minimum tail current, $V_{0.5}$ is the potential for half maximal activation and k is the slope factor. A $V_{0.5}$ was calculated for each individual experiment and statistical significance was estimated by paired two-tailed Student's *t*-test.

Activation kinetics for K_v7.2 and K_v7.2-W236L was determined by fitting the traces recorded at potentials between -40 and $+40$ mV to a double exponential function:

$$I(t) = I_{\text{fast}} \times \left(1 - \exp\left(-\frac{t}{\tau_{\text{fast}}}\right) \right) + I_{\text{slow}} \times \left(1 - \exp\left(-\frac{t}{\tau_{\text{slow}}}\right) \right) \quad (\text{Equation 2})$$

where $I(t)$ is the current at time t , I_{fast} and I_{slow} are the current amplitudes at infinite times, and τ_{fast} and τ_{slow} are the time constants of the fast and slow components, respectively.

Activation kinetics for K_v7.4 and K_v7.4-W242L was determined by fitting the traces recorded at potentials between -30

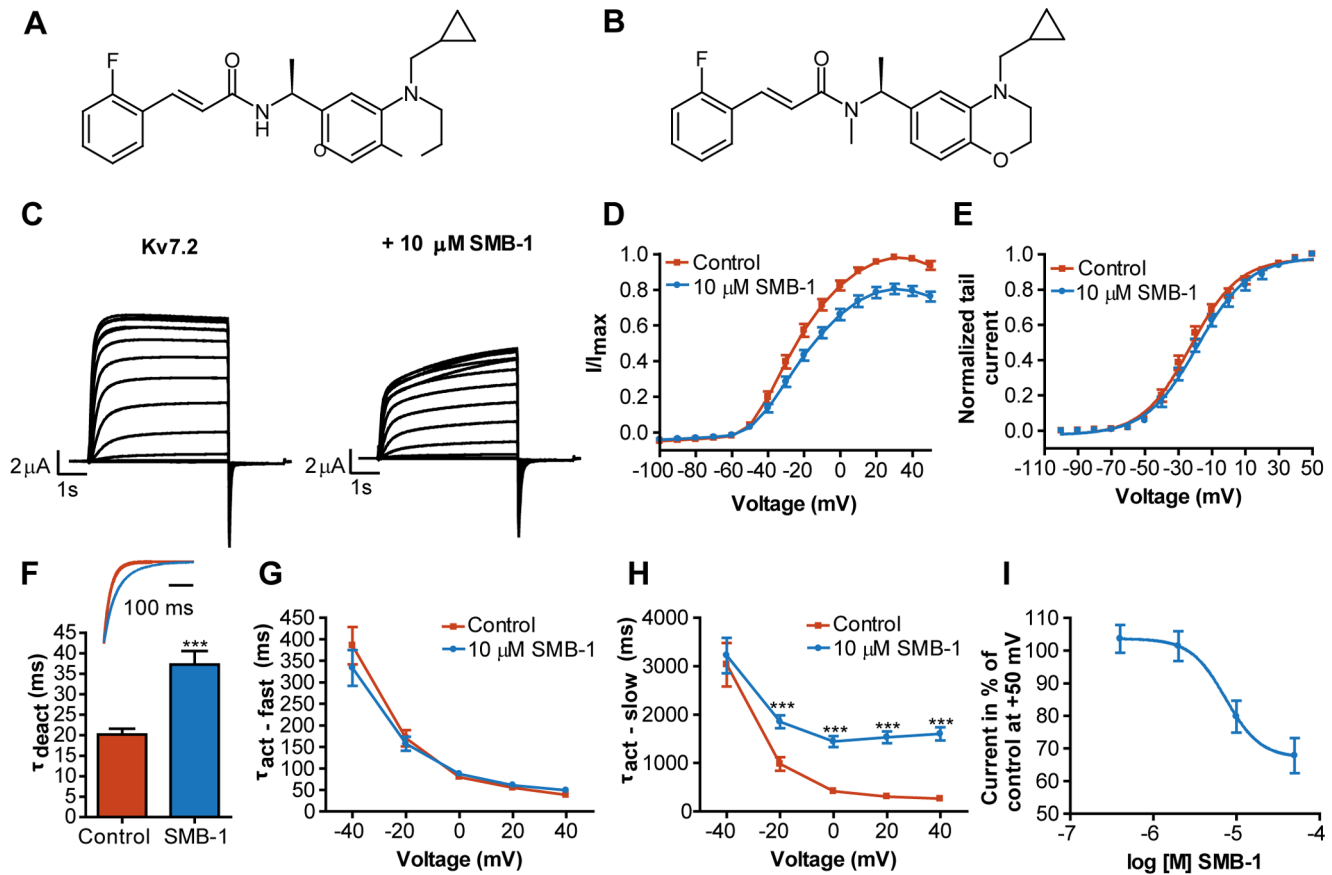


Figure 1. Inhibition of K_v7.2 by SMB-1. Chemical structure of (S)-2 (A) and SMB-1 (B). (C) Representative current traces for K_v7.2 in the absence and presence of 10 μM SMB-1 (D) Effect of SMB-1 on current-voltage relationship. (E) Effect of SMB-1 on voltage-dependence of activation. (F) Effect of SMB-1 on deactivation kinetics. Statistical significance was determined by paired, two-tailed Student's *t*-test. Representative tail current traces in the absence and presence of 10 μM SMB-1 are shown in the inset. Effect of SMB-1 on the fast (G) and slow (H) component of the activation kinetics. Statistical significance was determined by two-way repeated measurements ANOVA followed by Bonferroni post-test. Y-values were log-transformed before the statistical analysis to meet the assumption of normality. (I) Dose-response relationship for the effect of SMB-1 on K_v7.2. *** *p*<0.001. Bars represent S.E.M and *n*=5–9. Note that the error bars in some instances are too small to be visible. doi:10.1371/journal.pone.0100209.g001

and +50 mV to a single exponential function:

$$I(t) = I_0 \times \left(1 - \exp\left(-\frac{t}{\tau}\right)\right) \quad (\text{Equation 3})$$

where $I(t)$ is the current at time t , I_0 is the peak current and τ is the activation time constant.

For investigation of deactivation kinetics the tail current traces recorded at -120 mV when stepping from the channels' respective $\sim V_{0.5}$ were fitted to a single exponential function (Eq. 3).

Dose-response curves from electrophysiological experiments were made by plotting the increase in steady state current at +50 mV expressed in percentages as a function of drug concentration. The data were then analyzed by non-linear regression and fitted to the equation for sigmoidal dose-response with variable slope:

$$Y = R_1 + \frac{R_2 - R_1}{1 + 10^{-(\log EC_{50} - X) \times nH}} \quad (\text{Equation 4})$$

where R_1 is the initial response, R_2 is the maximum response, X is

the logarithm of the drug concentration and nH is the slope (Hill coefficient) of the curve.

For data concerning activation kinetics statistical significance was determined by two-way ANOVA followed by Bonferroni post-tests (if the overall P-value of the drug factor was less than 0.05). Before the statistical analyses of the fast component of the activation for K_v7.2 and K_v7.2-W236L the measured time constants were log-transformed to meet the assumption of normality. For traces fitted to double exponential functions only those where the sum of I_{fast} and I_{slow} equaled the peak current were included in the analysis.

For analysis of the remaining data statistical significance was determined by two-tailed Student's *t*-test if single comparisons were made and by two-way ANOVA followed by Bonferroni post-tests if multiple comparisons were made. Statistical analyses were carried out using GraphPad Prism 4.0. *p*<0.05 was accepted for statistical difference. All values are shown as mean \pm S.E.M.

Rodent exposure studies

Ethical permission for the *in vivo* rat procedures used in these studies was granted by the animal welfare committee, appointed by the Danish Ministry of Justice and all animal procedures were

carried out in compliance with EC Directive 86/609/EEC and with the Danish law regulating experiments on animals.

Brain and plasma exposure of SMB-1 was evaluated in male Sprague-Dawley rats (225–250 g, Charles River Lab., UK). SMB-1 was administered subcutaneously in solution at a dose of 20 mg/kg in 25% solutol (dose volume 5 ml/kg) and exposure was assessed after 30 and 120 min ($n = 3$). Under isoflurane anesthesia, cardiac blood was obtained in EDTA-coated tubes and centrifuged for 10 min at 4°C, after which plasma was harvested. While still under anesthesia, the animal was decapitated, the brain was removed and brain homogenate was prepared by homogenizing the whole brain with 70% acetonitrile (1:4 v/v) followed by centrifugation and collection of the supernatant. Concentrations of SMB-1 were determined using turboflow chromatography (dual column, focus mode; Thermo Fisher Scientific, Waltham, MA) followed by tandem mass spectrometry detection (Sciex API-3000 MS; Applied Biosystems, Foster City, CA). The limit of detection was 1 ng/ml in plasma and 5 ng/g in brain.

Membrane permeability and free fraction determination

The bidirectional permeability (P_{app}) of SMB-1 was examined in an MDCK cell system expressing human MDR1 [ABCB1, P-glycoprotein (P-gp)] as described previously [21]. Permeation of the test compound from apical (A) to basal (B) direction or B to A direction was determined in triplicate over a 150-min incubation at 37°C. The efflux ratio was calculated as the ratio between P_{app} (B to A) and P_{app} (A to B). Free fraction of SMB-1 was determined using a standard equilibrium dialysis methodology with freshly isolated rat brain homogenate or plasma [22]. Equilibrium dialysis was performed by incubating at 37°C for 5 h in triplicates.

Chemistry

(S)-2 was prepared according to a literature procedure [23]. Synthesis of SMB-1 ((E)-N-[(S)-1-(4-Cyclopropylmethyl-3,4-dihydro-2H-1,4-benzoxazin-6-yl)-ethyl]-3-(2-fluoro-phenyl)-N-methylacrylamide): To a solution of (S)-2 (100 mg, 262.8 μ mol) in a mixture of anhydrous tetrahydrofuran (THF, 1.5 mL) and dimethylformamide (DMF, 0.3 mL) at 0°C was added NaH (16 mg, 394 μ mol, 60% dispersion in mineral oil) and MeI (17.8 μ L, 289 μ mol). The mixture was stirred at 0°C for four hours and at room temperature overnight. The mixture was cooled to 0°C, and diluted with ethylacetate (EtOAc, 10 mL). Water (10 mL) was added carefully, the organic phase was separated, and the aqueous layer was extracted by EtOAc. The combined organic layers were washed by brine, dried (NaSO₄), and concentrated to give SMB-1 (104 mg, yield: 100%) as an oil. ¹H NMR (CDCl₃ 400 MHz TMS): δ 7.63–7.53 (m, 1 H), 7.35–7.25 (m, 1 H), 7.15–7.05 (m, 1 H), 7.01–6.78 (m, 3 H), 6.58–6.30 (m, 3 H), 5.90–5.82 (m, 0.58 H), 5.12–5.02 (m, 0.33 H), 4.06 (t, $J = 4.4$ Hz, 2 H), 3.22 (t, $J = 4.4$ Hz, 2 H), 2.95–2.86 (m, 2 H), 2.61 (s, 3 H), 1.45–1.36 (m, 1 H), 1.28 (d, $J = 6.8$ Hz, 2 H), 0.84–0.74 (m, 1 H), 0.38–0.30 (m, 2 H), 0.50–0.00 (m, 2 H).

Results

SMB-1 is an inhibitor of K_v7.2

We heterologously expressed K_v7 channels in *Xenopus laevis* oocytes and assessed their biophysical properties by two-electrode voltage-clamp. Upon activating voltage-steps, K_v7.2 channels displayed slowly activating and deactivating currents as expected [1]. Representative current traces are shown in Figure 1C. Application of 10 μ M SMB-1 led to a reduction of current amplitudes at all activating potentials (fig. 1C and D). At +50 mV the current amplitude was 81.7 \pm 3.1% ($n = 9$) of control. A small,

but significant, shift to more positive potentials was observed in the voltage-dependence of activation ($V_{0.5}$: control: -22.5 ± 2.2 mV; SMB-1: -18.0 ± 2.7 mV, $n = 8$, $p < 0.05$, fig. 1E). We also assessed kinetic parameters by determining the time constants of deactivation (τ_{deact}) and activation (τ_{act_fast} and τ_{act_slow}). Deactivation kinetics were significantly slowed in the presence of SMB-1 (control: 20.2 ± 1.4 ms, SMB-1: 37.3 ± 3.3 ms, $n = 7$, $p < 0.001$, fig. 1F). SMB-1 did not affect τ_{act_fast} (fig. 1G) but increased τ_{act_slow} at -20 mV and above (fig. 1H). These effects of SMB-1 are remarkably similar to the effect of (S)-2 on K_v7.2-W236L [14].

To determine the potency of SMB-1 on K_v7.2 the compound was tested at four concentrations (0.4, 2, 10 and 50 μ M). Visual inspection of the solution indicated that the drug precipitated at concentrations above 50 μ M. The current amplitude in percentage of control at +50 mV was plotted against the logarithm of the concentration. Fitting the data to the equation for sigmoidal dose-response with variable slope determined the IC₅₀ value of SMB-1 to ~ 7.4 μ M (pIC₅₀: 5.1 ± 0.2 M, $n = 5$, fig. 1I). The compound was not able to fully inhibit the current within the concentration range tested, reaching a maximal inhibition of 67.6 \pm 5.3% of control ($n = 5$) at +50 mV.

SMB-1 is an activator of K_v7.4

Next, we addressed the effect of SMB-1 on K_v7.4. Submitting *Xenopus laevis* oocytes expressing K_v7.4 channels to a voltage-step protocol elicited slowly activating currents. A representative experiment is shown in Figure 2A. Application of SMB-1 resulted in increased current amplitudes at -40 mV and above (fig. 2A and B). The current amplitude was 184.5 \pm 12.4% of control ($n = 8$) at +50 mV in the presence of 10 μ M SMB-1, which is low compared to the efficacy of (S)-2 on K_v7.4 (1927% of control at +40 mV) [14]. SMB-1 did not affect the voltage-dependence of activation of K_v7.4 (fig. 2C, $V_{0.5}$: control: -0.2 ± 3.6 mV; 10 μ M SMB-1: -1.4 ± 3.4 mV, $n = 8$, $p = 0.59$). Yet, 10 μ M SMB-1 significantly increased τ_{deact} (control: 10.3 ± 0.6 ms; SMB-1: 16.4 ± 1.2 ms, $n = 8$, $p < 0.001$, fig. 2D), and τ_{act} at all potentials tested (fig. 2E). The EC₅₀ value was determined to 5.7 μ M (pEC₅₀: 5.2 ± 0.4 M, $n = 5$) and the maximal efficacy to 212.7 \pm 33.1% ($n = 5$) at +50 mV (fig. 2F).

Dependency on the critical tryptophan residue for inhibition/activation by SMB-1

To determine whether SMB-1 was dependent on the critical tryptophan residue in the 'retigabine binding-site' for its inhibitory/activating effects, the compound was applied to mutant channel proteins K_v7.2-W236L and K_v7.4-W242L (fig. 3 and 4). K_v7.2-W236L was inhibited by SMB-1 similar to wild-type (WT) K_v7.2 (fig. 3A and B). The current amplitude at +50 mV was 68.8 \pm 3.9% of control ($n = 6$). We observed a trend towards an increase in the half-maximal value for voltage-dependence of activation $V_{0.5}$ (control: -27.1 ± 2.4 mV; SMB-1: -15.0 ± 10.1 mV, $n = 4$, $p = 0.2$, fig. 3C). There was a small but significant increase in τ_{deact} (control: 23.3 ± 2.9 ms; SMB-1: 27.0 ± 3.0 ms, $n = 6$, $p < 0.05$, fig. 3D). Like for WT K_v7.2, there was no significant effect of SMB-1 on τ_{act_fast} (fig. 4E), while τ_{act_slow} was significantly increased at potentials above -20 mV (fig. 3F).

In contrast, the activating effect of SMB-1 on K_v7.4 was abolished in the W242L mutant channel; the mutant channel was inhibited by the compound (fig. 4A and B). The current amplitude was 74.8 \pm 4.2% of control ($n = 5$) at +50 mV. At 10 μ M, SMB-1 did not affect the voltage-dependence of activation (control: $V_{0.5} = 5.5 \pm 4.5$ mV; SMB-1: $V_{0.5} = 4.3 \pm 4.9$ mV, $n = 5$, $p = 0.2$, fig. 4C) or the deactivation kinetics of K_v7.4-W242L (control:

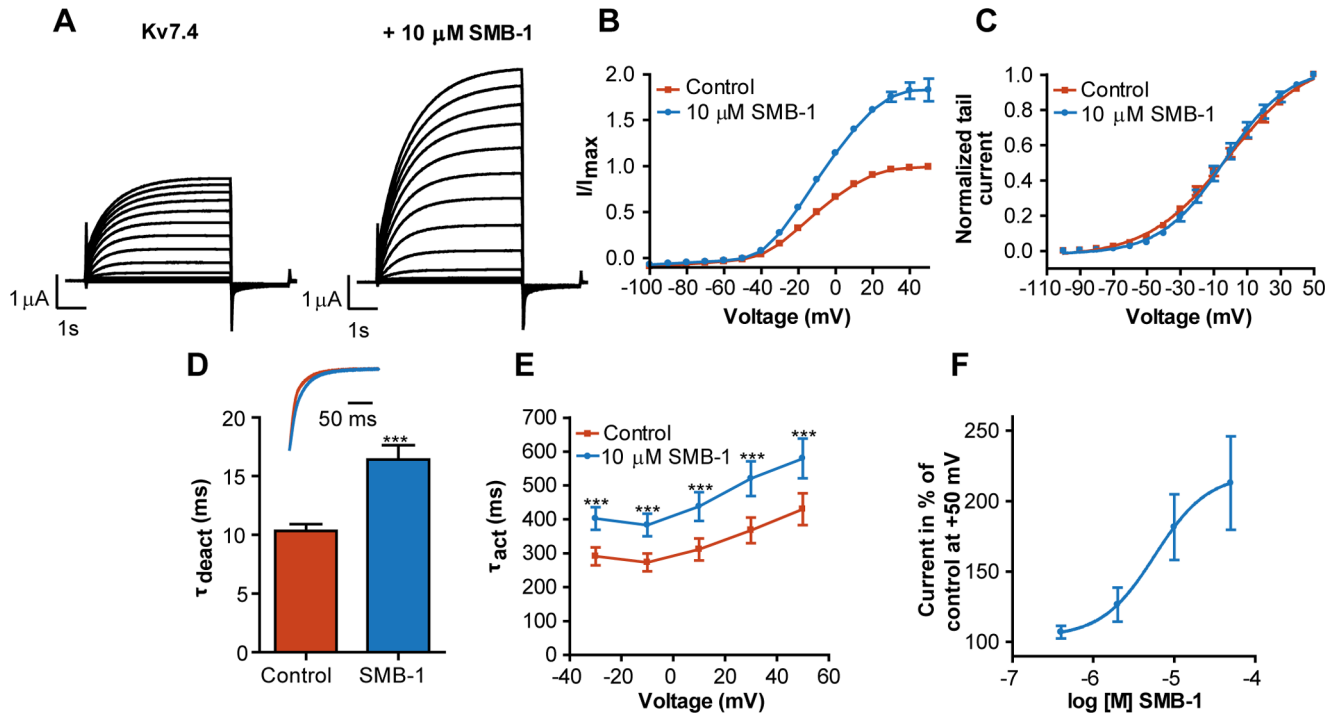


Figure 2. Activation of $K_v7.4$ by SMB-1. (A) Representative current traces for $K_v7.4$ in the absence and presence of 10 μM SMB-1. (B) Effect of SMB-1 on current-voltage relationship. (C) Effect of SMB-1 on voltage-dependence of activation. (D) Effect of SMB-1 on deactivation kinetics. Statistical significance was determined by paired, two-tailed Student's *t*-test. Representative tail current traces in the absence and presence of 10 μM SMB-1 are shown in the inset. (E) Effect of SMB-1 on activation kinetics. Statistical significance was determined by two-way repeated measurements ANOVA followed by Bonferroni post-test. (F) Dose-response relationship of SMB-1 on $K_v7.4$. *** $p < 0.001$. Bars represent S.E.M and $n = 5-8$. doi:10.1371/journal.pone.0100209.g002

$\tau_{\text{deact}} = 12.5 \pm 2.2$ ms; SMB-1: $\tau_{\text{deact}} = 12.3 \pm 2.2$ ms, $n = 5$, $p = 0.5$, fig. 4D), and there was neither any significant effect on activation kinetics (fig. 4E).

Hence, the activating effect of SMB-1 on $K_v7.4$ is critically dependent on the tryptophan residue while the inhibitory effect on $K_v7.2$ is not.

Effect of SMB-1 on channels with mutations in the refined retigabine binding site

To test if SMB-1 depends on other residues important for the action of retigabine, we generated channels where the two leucine residues (L275 and L299 in $K_v7.2$, L281 and L305 in $K_v7.4$) shown to be part of the binding site in $K_v7.3$ [19] were substituted with valines, the corresponding residues in $K_v7.1$. The resulting channels $K_v7.2$ -L275V, $K_v7.2$ -L299V, $K_v7.4$ -L281V and $K_v7.4$ -L305V were expressed in *Xenopus laevis* oocytes. We determined their *I-V* relationship in the absence and presence of 10 μM SMB-1 (fig. 5). All channels behaved similar to the WT channels but displayed a voltage-dependence of activation that was shifted in the positive ($K_v7.2$ -L275V: $V_{0.5} = -7.7 \pm 1.1$ mV, $n = 36$ and $K_v7.2$ -L299V: $V_{0.5} = -8.7 \pm 1.3$ mV, $n = 30$) or negative ($K_v7.4$ -L305V: $V_{0.5} = -10.7 \pm 2.2$ mV, $n = 37$) direction. The L281V mutation in $K_v7.4$ resulted in a non-functional channel. A mutation affecting this residue (L281S) has been found in patients with DFNA2 [24], indicating that it may be very critical for $K_v7.4$ channel function.

The L275V mutation in $K_v7.2$ affected the efficacy of SMB-1 (fig. 5A). The current amplitude was $90.4 \pm 4.1\%$ of control at +50 mV ($n = 4$). $K_v7.2$ -L299V was inhibited to a similar degree as the WT channel (current amplitude $69.3 \pm 5.6\%$ of control at +50 mV, $n = 4$, fig. 5B). The L305V mutation in $K_v7.4$ abolished

the effect of the compound (current amplitude $100.8 \pm 10.3\%$ of control at +50 mV, $n = 6$, fig. 5C).

Exposure of SMB-1 in rats

Following subcutaneous administration of 20 mg/kg, SMB-1 was found to be rapidly absorbed systemically, and high plasma concentrations were observed (Table 1). In contrast, we found the brain exposure to be very low relative to plasma with corresponding brain/plasma partition coefficients in the range of 0.04–0.07. Considering the contribution of residual blood in the brain tissue to the brain partition coefficient, these results suggest that SMB-1 possesses limited brain penetration capabilities. In line with these findings, *in vitro* transport studies in MDCK cells showed that SMB-1 exhibited low membrane permeability of $2.5 \pm 0.3 \times 10^{-6}$ cm/s with an efflux ratio of 0.7. In addition, SMB-1 was found to be highly bound in plasma (free fraction $0.6 \pm 0.4\%$) and in brain homogenate (free fraction $0.1 \pm 0.1\%$) (mean \pm S.E.M., $n = 3$).

Discussion

Although an increasing number of compounds has been found to activate or inhibit K_v7 channels there is still a lack of truly subtype specific drugs that can discriminate between $K_v7.2-5$. This has hampered the determination of the individual contribution of each subtype to the *in vivo* effects of e.g. retigabine and made it difficult to establish which subtype to target for each of the many indications where K_v7 channels have been suggested as attractive therapeutic targets.

In an attempt to develop subtype-specific K_v7 active drugs, we took advantage of previous findings that had established the so-

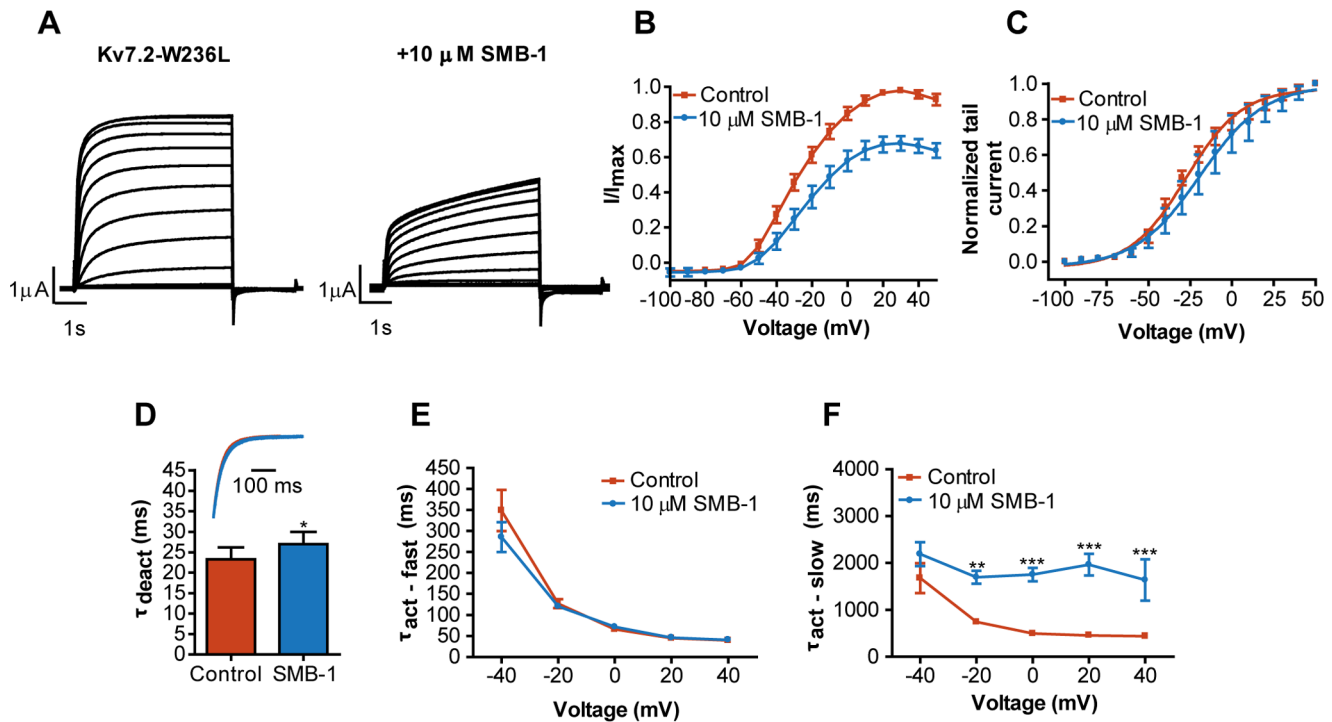


Figure 3. Effect of SMB-1 on K_v7.2-W236L. (A) Representative current traces for K_v7.2-W236L in the absence and presence of 10 μM SMB-1 (B) Effect of SMB-1 on current-voltage relationship. (C) Effect of SMB-1 on voltage-dependence of activation. (D) Effect of SMB-1 on deactivation kinetics. Statistical significance was determined by paired, two-tailed Student's *t*-test. Representative tail current traces in the absence and presence of 10 μM SMB-1 are shown in the inset. Effect of SMB-1 on the fast (E) and slow (F) component of the activation kinetics. Statistical significance was determined by two-way repeated measurements ANOVA followed by Bonferroni post-test. Y-values were log-transformed before the statistical analysis to meet the assumption of normality. * *p*<0.05, ** *p*<0.01, *** *p*<0.001. Bars represent S.E.M and *n*=4–6. doi:10.1371/journal.pone.0100209.g003

called retigabine binding site and the SAR of acrylamide K_v7 channel compounds [11,12,19,20]. Trying to mimic the effect of a tryptophan substitution, we designed and synthesized a methylated version of (S)-2, termed SMB-1. This compound oppositely affects K_v7.2 and K_v7.4 channels.

SMB-1 inhibits K_v7.2 by reducing the current amplitude and increasing the time constant for the slow component of the activation kinetics remarkably similar to the effects of (S)-2 on K_v7.2-W236L [14]. The potency of (S)-2 on K_v7.2-W236L (IC₅₀=8.1 μM) and SMB-1 on K_v7.2 (IC₅₀=7.4 μM) is similar [14]. Hence, our data suggests that methylation of the amide nitrogen disables the activating component of (S)-2's effect on K_v7.2 while preserving the inhibitory component, and thereby recapitulates the pharmacological consequence of the W236L mutation.

The increase in K_v7.4 current levels by SMB-1 was weak compared to the high efficacy of (S)-2 [14]. As K_v7.4-W242L was inhibited by SMB-1, we speculate that the methyl-group in SMB-1 promotes an inhibitory binding mode and thereby tips the activation/inhibition equilibrium towards reduced efficacy relative to the effects of (S)-2 on K_v7.4. The positive effect of SMB-1 on K_v7.4 was critically dependent on the tryptophan residue in S5. Furthermore, K_v7.4-L305V was SMB-1 insensitive, leading us to conclude that SMB-1 binds to the promiscuous retigabine binding site for activation of K_v7.4.

In contrast, the inhibitory action of SMB-1 on K_v7.2 was not critically dependent on the tryptophan residue. However, the L275V mutation in K_v7.2 reduced the inhibitory efficacy of 10 μM SMB-1, indicating that its binding to K_v7.2 also occurs within the border of this binding pocket. Since this site

accommodates pan-reactive compounds, it has been suggested that it would not allow for discrimination between neuronal K_v7 channel subtypes [25]. These findings demonstrate that this binding site can indeed accommodate compounds that oppositely affect neuronal K_v7 channels thereby giving rise to subtype selectivity. To further examine the basis of the opposite effect of SMB-1 on K_v7.2 and K_v7.4 in more detail, a cross-mutational approach may be applied where non-conserved residues within the binding pocket are swapped between the two subtypes.

What may this compound be used for? Foremost, determining the contribution of different K_v7 channel subtypes to currents in various regions may assign particular physiological functions to particular subtypes.

Compounds modulating K_v7 channels may have a therapeutic potential in relation to schizophrenia. This disease is characterized by psychotic or positive symptoms such as hallucinations, delusions, thought disorder, disorganized speech and paranoia; negative symptoms, which include lack of mental activities such as thoughts and motivation; and cognitive symptoms comprising failure of working memory, impairments of learning and attentional dysfunction [26]. According to the *dopamine hypothesis of schizophrenia* the positive symptoms result from over-active neurotransmission in the mesolimbic dopamine pathway [27,28]. This hypothesis is based on the fact that antipsychotic drugs work by blocking dopamine D₂ receptors, while compounds that increase dopamine levels can induce psychosis or exacerbate schizophrenia [27,29]. On the other hand, a hypodopaminergic state in the frontal cortical terminal fields of the mesocortical dopamine neurons is thought to underlie the negative and cognitive symptoms [28]. The antipsychotic drugs available on

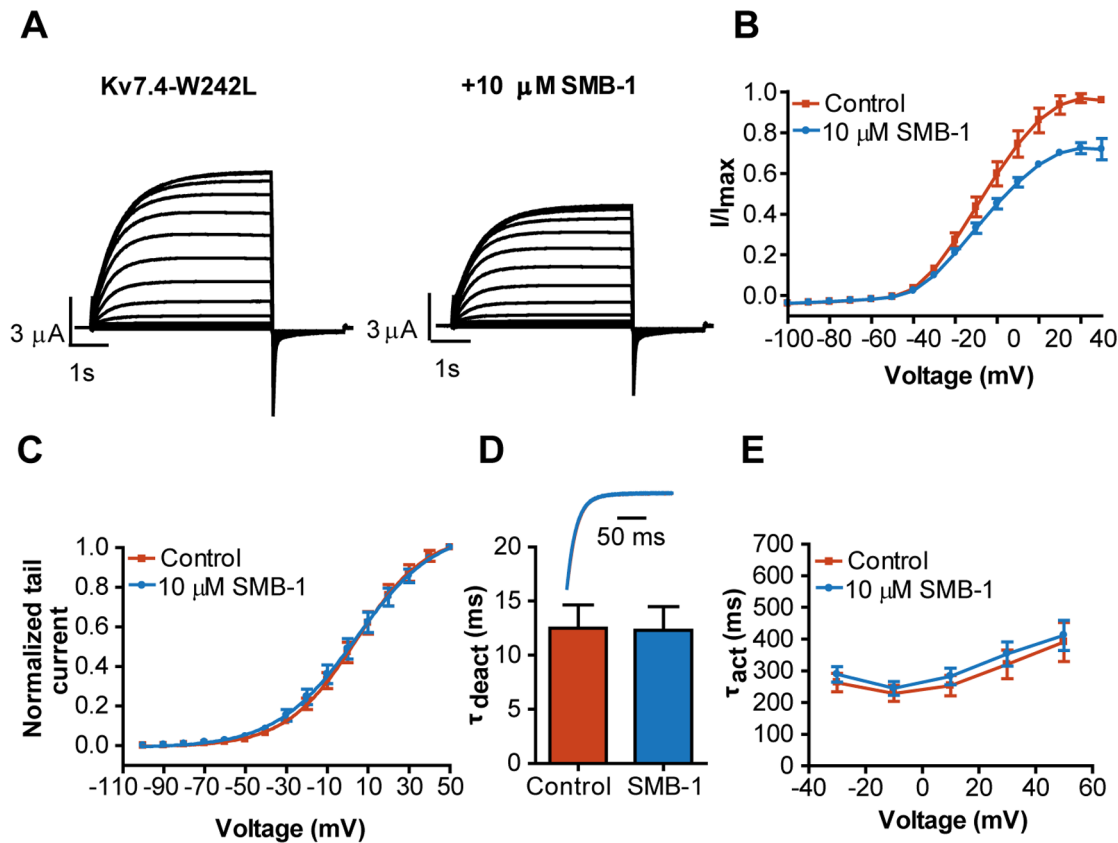


Figure 4. Effect of SMB-1 on $K_V7.4$ -W242L. (A) Representative current traces for $K_V7.4$ -W242L in the absence and presence of 10 μ M SMB-1. (B) Effect of SMB-1 on current-voltage relationship. (C) Effect of SMB-1 on voltage-dependence of activation. (D) Effect of SMB-1 on deactivation kinetics. Statistical significance was determined by paired, two-tailed Student's *t*-test. Representative tail current traces in the absence and presence of 10 μ M SMB-1 are shown in the inset (note that the traces are completely overlapping). (E) Effect of SMB-1 on activation kinetics. Statistical significance was determined by two-way repeated measurements ANOVA followed by Bonferroni post-test. Bars represent S.E.M and $n=5$. doi:10.1371/journal.pone.0100209.g004

the market today are more or less efficient in ameliorating the positive symptoms but have little or no effect on the negative and cognitive symptoms. Compounds with differential modulatory action across the K_V7 channel family could aid in determining the most optimal subtype profile.

K_V7 channel inhibitors were initially developed as cognition enhancers based on their ability to increase acetylcholine release [30,31,32]. This effect probably stems from inhibition of $K_V7.2$

and $K_V7.2/3$ channels as the reference inhibitor XE-991 has been shown to more potently inhibit $K_V7.2$ and $K_V7.2/3$ than $K_V7.4$ channels albeit the data are reported from different laboratories [1,33]. K_V7 channels also play a role in the dopaminergic system and retigabine has anti-dopaminergic effects in the basal ganglia [9,34,35,36]. $K_V7.4$ has been suggested as the subtype mediating the efficacy of pan-reactive K_V7 channel openers in modulation of the dopaminergic system [9,35,36]. Hence, we speculate that a

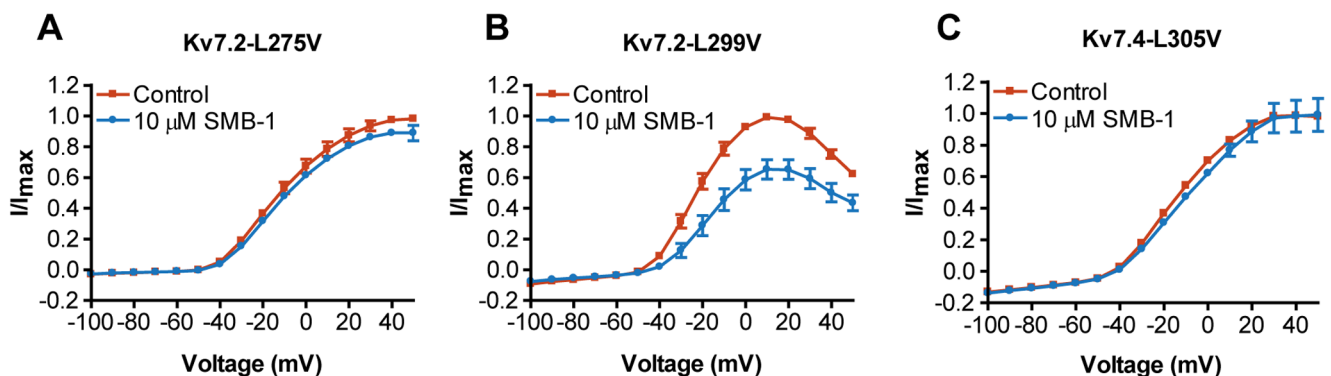


Figure 5. Effect of SMB-1 on channels with mutations in the refined retigabine binding site. Effect of 10 μ M SMB-1 on the current-voltage relationship of (A) $K_V7.2$ -L275V, (B) $K_V7.2$ -L299V and (C) $K_V7.4$ -L305V. Bars represent S.E.M. and $n=4-6$. doi:10.1371/journal.pone.0100209.g005

Table 1. Plasma and brain exposure of SMB-1 in rats following subcutaneous administration of 20 mg/kg.

Time (min)	Plasma (μM)	Brain (μM)
30	14±1.5	0.55±0.04
120	6.4±0.70	0.44±0.07

Data shown as mean total concentrations ± S.E.M (n=3).
doi:10.1371/journal.pone.0100209.t001

balanced inhibition of K_V7.2 and K_V7.2/3 in combination with enhancement of K_V7.4 current may be efficacious with respect to targeting of both positive and cognitive symptoms in patients suffering from schizophrenia. As this is hypothetical, proof of this concept necessitates an evaluation of SMB-1 in animal models of psychosis and cognition. Unfortunately, even though SMB-1 was not identified as a substrate for the efflux transporter P-gp judged from the efflux ratio in MDR1-MDCK cells, we found it to have low membrane permeability with poor CNS distribution in rats limiting its *in vivo* pharmacological potential after systemic administration in the CNS. However, the recent years have provided evidence for a role of the so-called neuronal K_V7 channels in the vasculature [37,38,39]. K_V7.4 channels in particular have been involved in the regulation of vascular tone in e.g. renal, coronary, and cerebral arteries [40,41,42]. Hence, further studies are warranted to investigate whether SMB-1 might

have vasorelaxant effects which could in turn have an impact on cerebral circulation.

In conclusion, we have identified a novel K_V7 channel active compound with a unique characteristic in that it inhibits K_V7.2 but activates K_V7.4. SMB-1 provides valuable insight into the pharmacology of K_V7 channels and may be used as a template for the further design of better subtype selective K_V7 channel modulators.

Author Contributions

Conceived and designed the experiments: SMB MR JK CB HSJ. Performed the experiments: SMB MR JK CB. Analyzed the data: SMB CB. Contributed reagents/materials/analysis tools: NS. Wrote the paper: SMB MR JK CB NS HSJ.

References

- Wang HS, Pan Z, Shi W, Brown BS, Wymore RS, et al. (1998) KCNQ2 and KCNQ3 Potassium Channel Subunits: Molecular Correlates of the M-Channel. *Science* 282: 1890–1893.
- Jentsch TJ (2000) Neuronal KCNQ potassium channels: physiology and role in disease. *Nat Rev Neurosci* 1: 21–30.
- Brown DA, Adams PR (1980) Muscarinic suppression of a novel voltage-sensitive K⁺ current in a vertebrate neurone. *Nature* 283: 673–676.
- Singh NA, Charlier C, Stauffer D, DuPont BR, Leach RJ, et al. (1998) A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nat Genet* 18: 25–29.
- Biervert C, Schroeder BC, Kubisch C, Berkovic SF, Propping P, et al. (1998) A Potassium Channel Mutation in Neonatal Human Epilepsy. *Science* 279: 403–406.
- Charlier C, Singh NA, Ryan SG, Lewis TB, Reus BE, et al. (1998) A pore mutation in a novel KQT-like potassium channel gene in an idiopathic epilepsy family. *Nat Genet* 18: 53–55.
- Blackburn-Munro G, Jensen BS (2003) The anticonvulsant retigabine attenuates nociceptive behaviours in rat models of persistent and neuropathic pain. *Eur J Pharmacol* 460: 109–116.
- Wu YJ, Boissard CG, Greco C, Gribkoff VK, Harden DG, et al. (2003) (S)-N-[1-(3-Morpholin-4-ylphenyl)ethyl]-3-phenylacrylamide: An Orally Bioavailable KCNQ2 Opener with Significant Activity in a Cortical Spreading Depression Model of Migraine. *J Med Chem* 46: 3197–3200.
- Hansen HH, Ebbesen C, Mathiesen C, Weikop P, Ronn LC, et al. (2006) The KCNQ Channel Opener Retigabine Inhibits the Activity of Mesencephalic Dopaminergic Systems of the Rat. *J Pharmacol Exp Ther* 318: 1006–1019.
- Korsgaard MPG, Hartz BP, Brown WD, Ahring PK, Strobaek D, et al. (2005) Anxiolytic Effects of Maxipost (BMS-204352) and Retigabine via Activation of Neuronal Kv7 Channels. *J Pharmacol Exp Ther* 314: 282–292.
- Wuttke TV, Seeborn G, Bail S, Maljevic S, Lerche H (2005) The New Anticonvulsant Retigabine Favors Voltage-Dependent Opening of the Kv7.2 (KCNQ2) Channel by Binding to Its Activation Gate. *Mol Pharmacol* 67: 1009–1017.
- Schenzer A, Friedrich T, Pusch M, Saffig P, Jentsch TJ, et al. (2005) Molecular Determinants of KCNQ (Kv7) K⁺ Channel Sensitivity to the Anticonvulsant Retigabine. *J Neurosci* 25: 5051–5060.
- Bentzen BH, Schmitt N, Calloe K, Dalby-Brown W, Grunnet M, et al. (2006) The acrylamide (S)-1 differentially affects Kv7 (KCNQ) potassium channels. *Neuropharmacology* 51: 1068–1077.
- Blom SM, Schmitt N, Jensen HS (2009) The acrylamide (S)-2 as a positive and negative modulator of Kv7 channels expressed in *Xenopus laevis* oocytes. *PLoS ONE* 4: e8251.
- Xiong Q, Sun H, Zhang Y, Nan F, Li M (2008) Combinatorial augmentation of voltage-gated KCNQ potassium channels by chemical openers. *Proc Natl Acad Sci U S A* 105: 3128–3133.
- Padilla K, Wickenden AD, Gerlach AC, McCormack K (2009) The KCNQ2/3 selective channel opener ICA-27243 binds to a novel voltage-sensor domain site. *Neurosci Lett* 465: 138–142.
- Blom SM, Schmitt N, Jensen HS (2010) Differential effects of ICA-27243 on cloned K(V)7 channels. *Pharmacology* 86: 174–181.
- Miceli F, Soldovieri MV, Martire M, Tagliatela M (2008) Molecular pharmacology and therapeutic potential of neuronal Kv7-modulating drugs. *Curr Opin Pharmacol* 8: 65–74.
- Lange W, Geissendorfer J, Schenzer A, Grotzinger J, Seeborn G, et al. (2009) Refinement of the Binding Site and Mode of Action of the Anticonvulsant Retigabine on KCNQ K⁺ Channels. *Mol Pharmacol* 75: 272–280.
- Wu YJ, He H, Sun LQ, L'Heureux A, Chen J, et al. (2004) Synthesis and Structure-Activity Relationship of Acrylamides as KCNQ2 Potassium Channel Openers. *J Med Chem* 47: 2887–2896.
- Risgaard R, Etrup A, Balle T, Dyssegaard A, Hansen HD, et al. (2013) Radiolabelling and PET brain imaging of the α₁-adrenoceptor antagonist Lu AE43936. *Nuclear medicine and biology* 40: 135–140.
- Redrobe JP, Elster L, Frederiksen K, Bundgaard C, Jong IEM, et al. (2011) Negative modulation of GABA_A α5 receptors by RO4938581 attenuates discrete sub-chronic and early postnatal phencyclidine (PCP)-induced cognitive deficits in rats. *Psychopharmacology* 221: 451–468.
- Wu YJ, Boissard CG, Chen J, Fitzpatrick W, Gao Q, et al. (2004) (S)-N-[1-(4-Cyclopropylmethyl-3,4-dihydro-2H-benzo[]oxazin-6-yl)-ethyl]-3-(2-fluorophenyl)-acrylamide is a potent and efficacious KCNQ2 opener which inhibits induced hyperexcitability of rat hippocampal neurons. *Bioorg Med Chem Lett* 14: 1991–1995.
- Talebizadeh Z, Kelley PM, Askew JW, Beisel KW, Smith SD (1999) Novel mutation in the KCNQ4 gene in a large kindred with dominant progressive hearing loss. *Hum Mutat* 14: 493–501.
- Gribkoff VK (2008) The therapeutic potential of neuronal Kv7 (KCNQ) channel modulators: an update. *Expert Opin Ther Tar* 12: 565–581.
- Mueser KT, McGurk SR (2004) Schizophrenia. *Lancet* 363: 2063–2072.
- Seeman P (1987) Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse* 1: 133–152.
- Duncan GE, Sheitman BB, Lieberman JA (1999) An integrated view of pathophysiological models of schizophrenia. *Brain Res Brain Res Rev* 29: 250–264.
- Lieberman JA, Kane JM, Alvir J (1987) Provocative tests with psychostimulant drugs in schizophrenia. *Psychopharmacology (Berl)* 91: 415–433.
- Nickolson VJ, Tam SW, Myers MJ, Cook L (1990) Du P 996 (3,3-Bis(4-Pyridinylmethyl)-1-Phenylindolin-2-One) Enhances the Stimulus induced Release of Acetylcholine From Rat Brain In Vitro and In Vivo. *Drug Dev Res* 19: 285–300.
- Cook L, Nickolson VJ, Steinfels GF, Rohrbach KW, Denoble VJ (1990) Cognition enhancement by the acetylcholine releaser DuP 996. *Drug Dev Res* 19: 301–314.

32. Aiken SP, Lampe BJ, Murphy PA, Brown BS (1995) Reduction of spike frequency adaptation and blockade of M-current in rat CA1 pyramidal neurones by linopirdine (DuP 996), a neurotransmitter release enhancer. *Br J Pharmacol* 115: 1163–1168.
33. Sogaard R, Ljungstrom T, Pedersen KA, Olesen SP, Jensen BS (2001) KCNQ4 channels expressed in mammalian cells: functional characteristics and pharmacology. *Am J Physiol Cell Physiol* 280: C859–866.
34. Sotty F, Damgaard T, Montezinho LP, Mork A, Olsen CK, et al. (2009) Antipsychotic-like effect of retigabine [N-(2-Amino-4-(fluorobenzylamino)-phenyl)carbamic acid ester], a KCNQ potassium channel opener, via modulation of mesolimbic dopaminergic neurotransmission. *J Pharmacol Exp Ther* 328: 951–962.
35. Hansen HH, Andreassen JT, Weikop P, Mirza N, Scheel-Kruger J, et al. (2007) The neuronal KCNQ channel opener retigabine inhibits locomotor activity and reduces forebrain excitatory responses to the psychostimulants cocaine, methylphenidate and phencyclidine. *Eur J Pharmacol* 570: 77–88.
36. Hansen HH, Waroux O, Seutin V, Jentsch TJ, Aznar S, et al. (2008) Kv7 channels: interaction with dopaminergic and serotonergic neurotransmission in the CNS. *J Physiol* 586: 1823–1832.
37. Mackie AR, Byron KL (2008) Cardiovascular KCNQ (Kv7) Potassium Channels: Physiological Regulators and New Targets for Therapeutic Intervention. *Molecular Pharmacology* 74: 1171–1179.
38. Greenwood IA, Ohya S (2009) New tricks for old dogs: KCNQ expression and role in smooth muscle. *British Journal of Pharmacology* 156: 1196–1203.
39. Stott JB, Jepps TA, Greenwood IA (2013) Kv7 potassium channels: a new therapeutic target in smooth muscle disorders. *Drug Discov Today*.
40. Chadha PS, Jepps TA, Carr G, Stott JB, Zhu H-L, et al. (2014) Contribution of Kv7.4/Kv7.5 Heteromers to Intrinsic and Calcitonin Gene-Related Peptide-Induced Cerebral Reactivity. *Arteriosclerosis, Thrombosis, and Vascular Biology* 34: 887–893.
41. Khanamiri S, Soltysinska E, Jepps TA, Bentzen BH, Chadha PS, et al. (2013) Contribution of Kv7 Channels to Basal Coronary Flow and Active Response to Ischemia. *Hypertension* 62: 1090–1097.
42. Chadha PS, Zunke F, Zhu H-L, Davis AJ, Jepps TA, et al. (2012) Reduced KCNQ4-Encoded Voltage-Dependent Potassium Channel Activity Underlies Impaired β -Adrenoceptor-Mediated Relaxation of Renal Arteries in Hypertension. *Hypertension* 59: 877–884.