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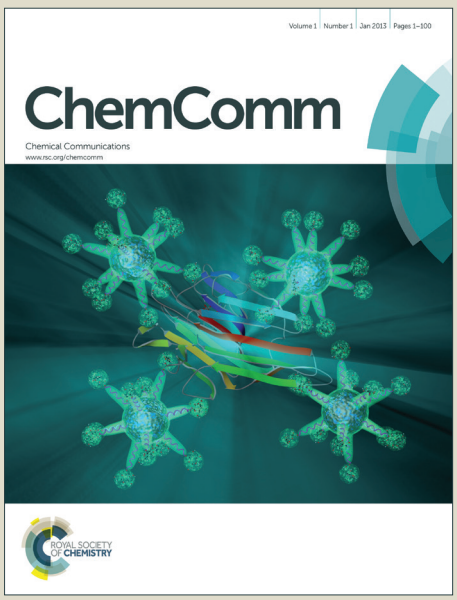

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Porphyrin derivatives as potent and selective blockers of neuronal Kv1 channels

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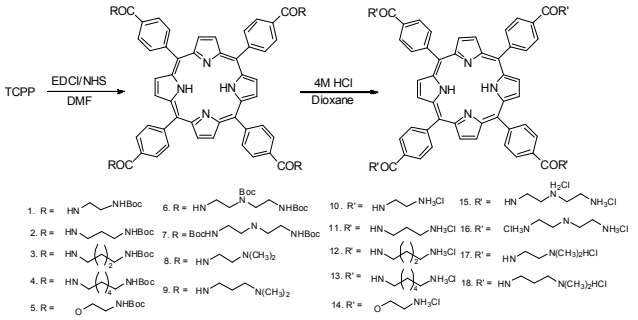
Selective inhibitors of voltage-activated K+ channels are needed for the treaetment of multiple sclerosis. In this work it was discovered that porphyrins bearing 2-4 carbon alkyl ammonium side chains predominantly blocked the Kv1.1 current whilest Kv1.2 was susceptible to a porphyrin bearing polyamine side chains.

Voltage-gated K^+ channels (Kv1) control neuron excitability and synaptic transmission; their alteration (e.g. mutation or expression levels) underlies human diseases.[2] Kv1 channels are membrane-spanning oligomeric sialoglycoproteins [3,4] (Mr ~400 k) consisting of 4 pore-forming α subunits and 4 cytoplasmically-associated auxiliary β proteins.[5,6] When expressed *in vitro*, each of the major α subunit genes [Kv1.1-1.7] yields a homo-tetrameric channel with distinct biophysical and pharmacological profiles.[7] Kv1 members, exposed on demyelinated axons in patients suffering from multiple sclerosis (MS), contribute to abnormal propagation of nerve signals with resultant debilitating muscle weakness.[8] Although aminopyridines can inhibit these culprit channels, such therapy results in serious off-target effects including seizures, due to blockade of other K^+ channel types.[8] Recently, Kv1.1- and 1.2-containing channels were found to be abnormally expressed in optic nerve demyelinated axons from a cuprizone-induced mouse model that resembles MS. Thus, finding smaller extracellular inhibitors for such K^+ channels should aid progress towards developing new drugs to alloriate MS-related symptoms. It is notable that symmetrically-substituted porphyrins bearing alkyl amino groups (cationic-charged at physiological pH) tightly bind a KcsA-Kv1.3 channel.[1,10] This involves docking into a distinctive part of the pore (e.g. outer turret region) of the Kv1.3 channel, as determined by solid state NMR.[10] Therefore, porphyrins can provide the 4-fold symmetry required for interaction with Kv1 channels, but a tetra-phenyl-porphyrin with alkyl ammonium side chains blocks several different Kv1 members.[11] Hence, the aim of the present study was to carryout a structure activity study with various substituted prophyrins to determine their selectivity for subtypes of Kv1 channels. This task was complemented using a model of the rat homologous Kv1.1 channel, derived from the crystallographic structure of Kv1.2,[12] to perform molecular modelling of its interaction with the porphyrins.. The prophyrins examined in this (**10-18**) study are shown in Scheme 1. Of particular interest was to determine the effects on Kv1 selectivity of porphyrin substituent chain length (**10-13**), amide linkage (**14**), primary amine groups (**17**, **18**) and multiple charged groups (**15**, **16**). Four of the compounds purified were shown electrophysiologically to inhibit Kv1.1, 1.2 or both homo-tetrameric channels expressed in mammalian cells.



Scheme 1. Synthesis of porphyrins **10 – 18** from precursor TCPP.

Porphyrins 10-18 were prepared by the introduction of mono N-Boc alkyl diamines using carbodiimide coupling as outlined in Scheme 1. Porphyrins **1-4** were prepared by treating TCPP with EDCI and the respective mono N-Boc-protected alkyldiamines in the presence N-hydroxysuccinimide.[14] Final yields of the Boc protected prophyrins after silica gel chromatography ranged from 14-70 %. Porphyrin **5** was prepared using a modified Steglich esterification by reacting N-Boc aminoethanol with TCPP in the presence of EDCI and 4-dimethylaminopyridine (DMAP) as catalyst in DMF for 24 hrs.; it was isolated in 80% yield after silica gel chromatography. Preparation of porphyrins **6** and **7** was complicated by the need to selectively protect primary over secondary amines. In the case of **6**, the required diprotected triamine was prepared according to the procedure of Leonor et al.[15] and then treated with TCPP using the EDCI procedure described above; this resulted in a yield of 35% after silica gel chromatography. For porphyrin **7**, the required diprotected triamine was prepared[16] and coupled to TCPP using the EDCI protocol to give **7** in 66% yield after silica gel

chromatography. The same EDCI coupling was employed for making porphyrins **8** and **9** from the corresponding amines (N,N-ethylenediamine and N,N-dimethyl-1,3-diaminopropane) in 62% and 59% yield, respectively. Conversion of all of the Boc protected porphyrins **1**-**7** to their respective amine hydrochloride salts, **10**-**16**, was achieved using 4M HCl in dioxane (Scheme 1); this afforded pure products in quantitative yields after washing. Likewise, the amine hydrochloride salts of **8** and **9** were generated to give **17** and **18** in quantitative yields. Compounds **10**-**18** were characterised by ^{1}H NMR and high-resolution MALDI-TOF mass spectrometry.†

The reactivities of **10**-**18** were tested on homomeric (Kv1.1, 1.2, 1.3, 1.4 and 1.6) channels stably expressed in HEK-293 cells, using whole-cell patch clamp recording.† Alignments of the pore amino acid sequences of 5 neuronal Kv1 channels (Fig. 1) revealed mainly conserved residues except at the outer and inner turret regions.[17,18] Hence, it was anticipated that these Kv1 channels might show

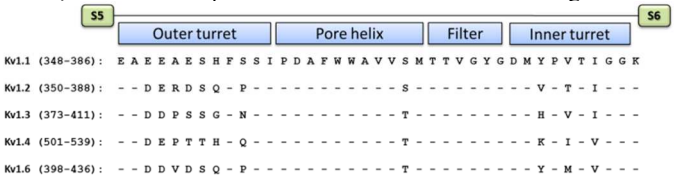


Fig. 1 Alignment of the pore sequence in neuronal Kv1 subunits. Letters highlight the residue differences at the outer / inner turret and pore helix between each channel.

distinct susceptibilities to the above-noted porphyrins bearing different alky-ammonium side chains. Representative K^+ current traces from expressed Kv1.1 channel were blocked by 10 μM compound **10** but not by the same concentration of **15**, while the opposite effect was observed for Kv1.2 channel (Fig. 2A). Only compounds **10**-**12** and **15** inhibited certain Kv1 channels at 10 μM, with **13** and **14** (Fig. 2B) and **16**-**18** (not shown) proving ineffective. Notably, **10** predominantly blocks Kv1.1 and to a lesser extent Kv1.4 channel, while **11** preferentially inhibits Kv1.1 and 1.2 K^+ currents. On the other hand, the inhibitory effect of **12** was much more pronounced on Kv1.1 than Kv1.2, 1.3 or 1.4, with Kv1.6 being virtually non-susceptible (Fig. 2B). It is notable that porphyrins containing 2, 3 or 4 carbon (**10**-**12**) displayed inhibition whereas **13** with 6 carbons was devoid of activity (Fig. 2B). Also, the importance of an amide linking the alkylamine substituents to the porphyrin became evident from **10** being active but not **14**, in which the amide was replaced with an ester. Another significant finding is the loss of activity when the primary amines were replaced with tertiary amines, revealed on relating the inhibition by compound **10** and **11** (Fig. 2B) to the inactive **17** and **18** (not shown). This lack of blockade might be due to the absence of hydrogen bond (HB) donors or size constraints. Moreover, increasing the number of terminal amine groups, from one as in the case of **10** to two in **16**, eliminated activity, although this could also arise from the lack of a HB donor at the amide linkage.

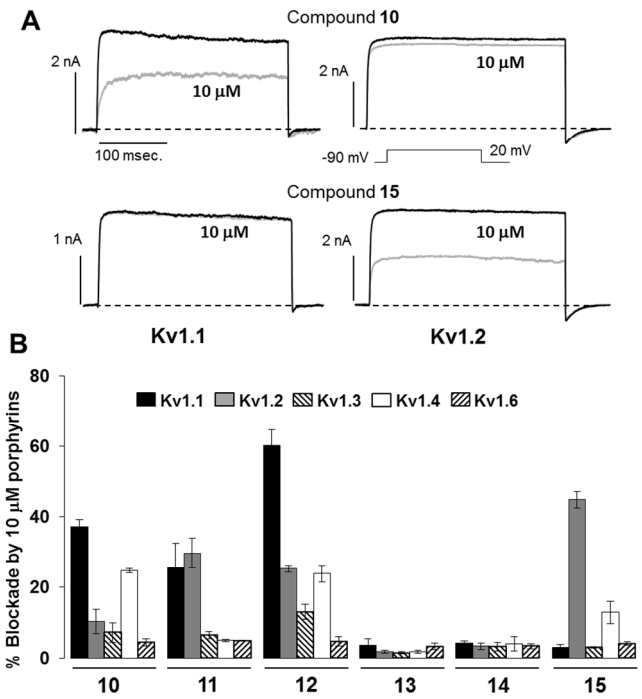


Fig. 2 Differential inhibition by compounds **10**-**12** and **15** of currents mediated by various Kv1 channels expressed in HEK-293 cells. (A) Representative current traces from Kv1.1 and Kv1.2 homomeric channels in the absence (black) and presence of 10 μM compound **10** or **15**, generated by a step voltage from the holding potential to 20 mV, as shown. (B) Summary of the dissimilar pharmacological profiles of five Kv1 channels shown for six compounds.

Interestingly, inserting an amino group instead of a methylene moiety into **13**, as is the case for **15**, restores inhibition. This subtle modification of placing both a charged group and HB donor in the centre of the chain proved to be of great significance, because **15** almost exclusively inhibits Kv1.2 (Fig. 2A, B).

To quantify the potencies of compounds **10**, **12** and **15** for inhibiting Kv1.1, 1.2 and 1.4 channels, their IC_{50} values were calculated from dose-response curves, obtained from Qpatch recordings (Fig. 3). For Kv1.1 and 1.4, **10** gave IC_{50}s of 13 ± 0.4 μM and 21 ± 1 μM [n = 7], respectively, with Kv1.2 exhibiting negligible sensitivity. On the other hand, Kv1.1 was somewhat more

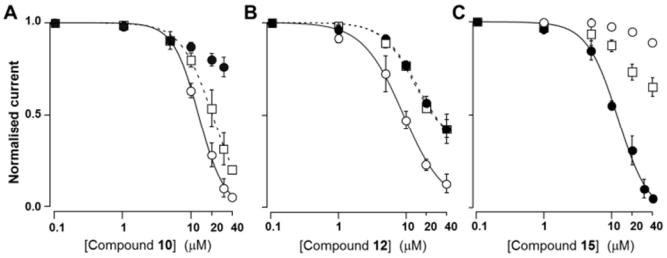


Fig. 3 Distinct inhibitory potencies of 10, 12 and 15 on Kv1.1, 1.2 and 1.4 channels. Dose-response curves, obtained from Qpatch recordings, for Kv1.1 (○), Kv1.4 (□) and Kv1.2 (●). Some of the error bars fall within the data points. The IC_{50} values are given in the text.

susceptible to **12** (IC_{50} = 9 ± 1 μM [n = 7]) but Kv1.2 and 1.4 displayed similar albeit lower sensitivities (IC_{50} = 21 ± 2 μM and 19 ± 3 μM [n = 5]) (Fig. 3B). However, compound **15** is selective for Kv1.2 having IC_{50}= 12 ± 1 μM [n = 5] with greatly diminished inhibition of Kv1.4 > 1.1 (Fig. 3C). Notice the steeper concentration dependence for **10** and **15** than **12**, reflected by the observed Hill coefficients of 1.9, 2.5, and 1.5. These values indicate cooperative interactions of both compounds with Kv1.1 and 1.2 channels similar to the behaviour reported for **10** with Kv1.3.[11] In the latter study which used *Xenopus* expression system, compound **10** inhibited Kv1.1 and 1.6 with nM IC_{50} values, and displayed lower potency for Kv1.2, 1.3, 1.4 and 1.5. Our stably transfected mammalian cells tolerated >1 μM of **10** (a concentration that made *Xenopus* cells leaky) and showed distinct pharmacological profiles. Notwithstanding the lower potencies of the compounds tested herein, these exhibited greater selectivity for Kv1.1 (as in case of **10** and **12**), Kv1.2 (**15**) or both (**11**) with little blockade of Kv1.3 or 1.6 channels. These differences in potency and selectivity are probably attributable to the use of amphibian and human-derived cells. Our study employed the latter because of the focus on generating Kv1 channels relevant to those over expressed in demyelinated neurons, specifically Kv1.1 and to a lesser extent 1.2, as potential targets for MS.[9]

Some of the compounds tested also acted as gating modifiers. An example is shown in Fig. 2A where inhibition of Kv1.1 current by 10 μM **10** is associated with slowing of its activation time course. The time constant (τ) calculated by fitting the current traces at +20 mV potential with a single exponential function† indicated a ~5-fold difference [$\tau_{control}$ = 1.74 ± 0.4 ms vs. $\tau_{compound\ 10}$ = 8.4 ± 2.3 ms, p = 0.029, n = 4, each]. Alteration of Kv1 channel gating by **10** accords with that reported recently using Kv1 channels expressed in *Xenopus* oocytes.[11]

A possible mode of interaction between the porphyrins and the Kv1.1 channel was derived from homology modelling (HM),[19] using the crystal structure of Kv1.2 as a template[12] (Fig. 4A). The geometric quality of the backbone conformation, residue interactions and contact plus energy profile of the structure fall well within the restrictions established for reliable structures (e.g. 94.2 % in core and 5.7 % in allowed regions) and comparable to the template used.[20, 21] Autodock4[22] was used to refine the side chains from the outer and inner turrets in the docking models for **12** and **10**. Predicted interactions between **12** and the channel included HBs with: chain A (Glu353Gly374, Tyr375), chain B (Gly374), chain C (Glu351, Ala352, GLu353, Gly374and Tyr375) and chain D (Glu353; Gly374).

These residues are largely in the inner turret region (Fig. 4B). A flexible docked Kv1.1 channel conformation was then used to predict interactions with **10**. Predicted interactions between **10** and Kv1.1 channel include: chain A (Glu353, Gly374, Tyr375), chain B (Gly374, Tyr375) chain C (Phe356, Asp361, Gly374, Tyr375) and chain D (Val373, Gly374, Gly376); these residues are again largely in the inner turret region (Fig. 4C). Our predicted binding pose for **10**, with one substituent of the porphyrin derivative inserted into the inner turret, is similar to that described by Gradl et al.[1]; this showed the bound porphyrin penetrating into the selectivity filter of a Kv1.3 model, where the protonated amine favourably interacts with the K^+ binding site. This docking model indicates that compound **10** when compared with **12** has broader interactive sites and a shallower interaction in the inner turret region, consistent with its steep concentration dependence and Hill coefficients (Fig. 3A).

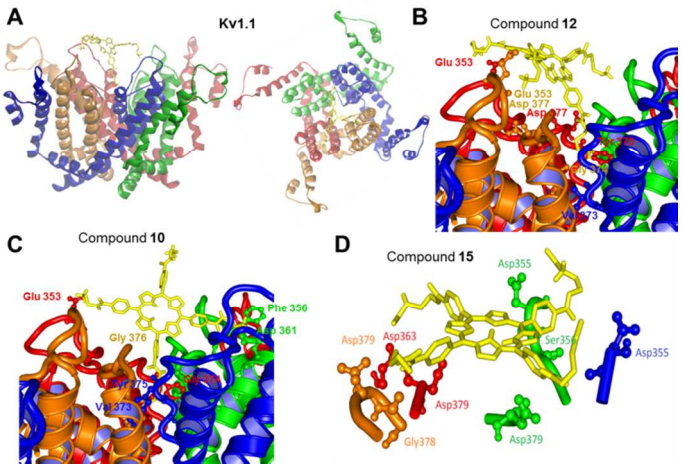


Fig. 4 Porphyrin derivatives docking into a Kv1.1 channel. (A) Side (left) and top (right) views of a representative compound **12**, (yellow), interacting with the Kv1.1 channel structure (derived from Kv1.2), with its four α subunits represented in red (chain A), blue (chain B), green (chain C) and orange (chain D). (B) A closer side-view of Kv1.1 subunits interacting via residues from the outer turret region and those lining the deep pore, with the side chains of compound **12**. For clarity, the interacting residues of chain A (red) are not labelled. (C) A typical flexibly docked side-view shows residues from the outer and inner turret of Kv1.1 subunits interacting with the side chains of compound **10** (in yellow). (D) A side-view of the flexible docking reveals residues from the outer and inner turret regions of all Kv1.2 subunits interacting with the side chains of compound **15** (in yellow).

Interestingly, **15** was found to exhibit selectivity for the Kv1.2 channel over the other subtypes. Following a similar docking procedure, **15** was flexibly docked into the mammalian Kv1.2 complex (PDB: 2A79).[12] Here, docked **15** lies across the top of the Kv1.2 tetramer, making contacts with each chain, and blocking the entrance to the inner turret. Key interactions are predicted to occur with residues Asp355 and Ser356 of the outer turret; Asp363 of the pore helix, and Gly378 and Asp379 of the inner turret region. These interactions could explain the absolute specificity of compound **15** for the Kv1.2 channel.

Slowing the activation kinetics, in addition to abilities of **10**-**12** and **15** to inhibit selective Kv1 channels would be advantageous, for example, in de-accelerating the activation of unwanted Kv1 channels over-expressed in demyelinated axons.[9] For therapeutic applications in future, the more selective porphyrin-based inhibitors could be improved by retaining the 4-fold scaffold bearing the appropriate side chains that exhibit selectivity for members of Kv1 channels but with a non-photo-reactive core.

In Summary, this work provides a proof of principle for the feasibility of developing small inhibitors for particular subtypes of neuronal Kv1 channels.

Notes and references

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†Electronic Supplementary Information (ESI) available: DNA constructs and expression of Kv1 channels, Electrophysiological recordings and data analysis, Computational methods, Homology modelling, Molecular docking, Synthesis of compounds 10-18 and their precursors and NMR and mass spectra for compounds 10-18. See DOI: 10.1039/c000000x/

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