Structure-based identification of novel KNa1.1 inhibitors:
a stratified target for *KCNT1*-related epilepsies

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1. Background

- Missense, heterozygous, gain-of-function mutations of KNa1.1, a Na^+-activated K^+ channel encoded by *KCNT1* are associated with severe, pharmacoresistant epilepsies in children that are accompanied by psychomotor and intellectual disabilities. Malignant migrating partial seizures in infancy (MMPSI) and autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE) are two examples, but a number of other early-onset epileptic encephalopathies (EOEE) have also been identified [1,2,3]. There is a wide phenotypic spectrum associated with *KCNT1* mutations, and no specific inhibitors acting on the channel, making both prediction of disease outcome and treatment difficult [4]. The channel is widely distributed in the nervous system, and is thought to be involved in generation of the slow afterhyperpolarisation following a single action potential or trains of action potential firing [5].
- There are currently three known inhibitors of the channel: the antiarrhythmic drugs quinidine, bepridil, and clofilium [6,7]. All are non-selective and inhibit cardiac K^+ and Na^+ channels. Clinically, quinidine has had poor results, likely due to its non-selectivity and lack of potency. It is therefore important to develop a novel, selective inhibitor of KNa1.1.
- The structure of the chicken KNa1.1 channel was recently resolved using cryo-EM [8]. We hypothesised that this structure could be used to identify novel inhibitors *in silico* by docking a library of compounds into the pore-forming region of the channel. We have identified six novel compounds, all of which are more potent inhibitors of KNa1.1 than quinidine *in vitro.*

2. Methods

- Molecular docking was conducted using the cryo-EM structure of chicken KNa1.1, using SwissDock and GLIDE. Firstly, quinidine and bepridil were docked into the pore domain to identify potential binding sites, which were then used for virtual high-throughput screening using a Chembridge library of 100,000 commercially-available compounds. 17 high-scoring compounds were selected, ordered, and dissolved in DMSO (10 mM).
- HEK293-MSR cells were transiently transfected with wild-type (WT) or mutant human KNa1.1 constructs, or hERG.
- Currents were recorded by whole-cell patch clamp electrophysiology, using both voltage pulse and ramp protocols. Physiological solutions were used, with 10 mM Na^+ included in the pipette (intracellular) solution for KNa1.1 recordings. Compound stock solutions were diluted with extracellular solution, and perfused onto cells serially or in increasing concentrations. Concentration-response curves were fitted using a Hill function, from which IC_{50} values could be derived.
- Cell viability was assessed using a WST-1 assay of non-transfected (NT) HEK293 cells cultured in 96-well plates and measuring absorbance at 450 nm. Cell viability was determined using the equation:
  % cell viability = $((A_{450}\text{-}A_{650})$ experiment well/$(A_{450}\text{-}A_{650})$ control well) x100.

3. Results I

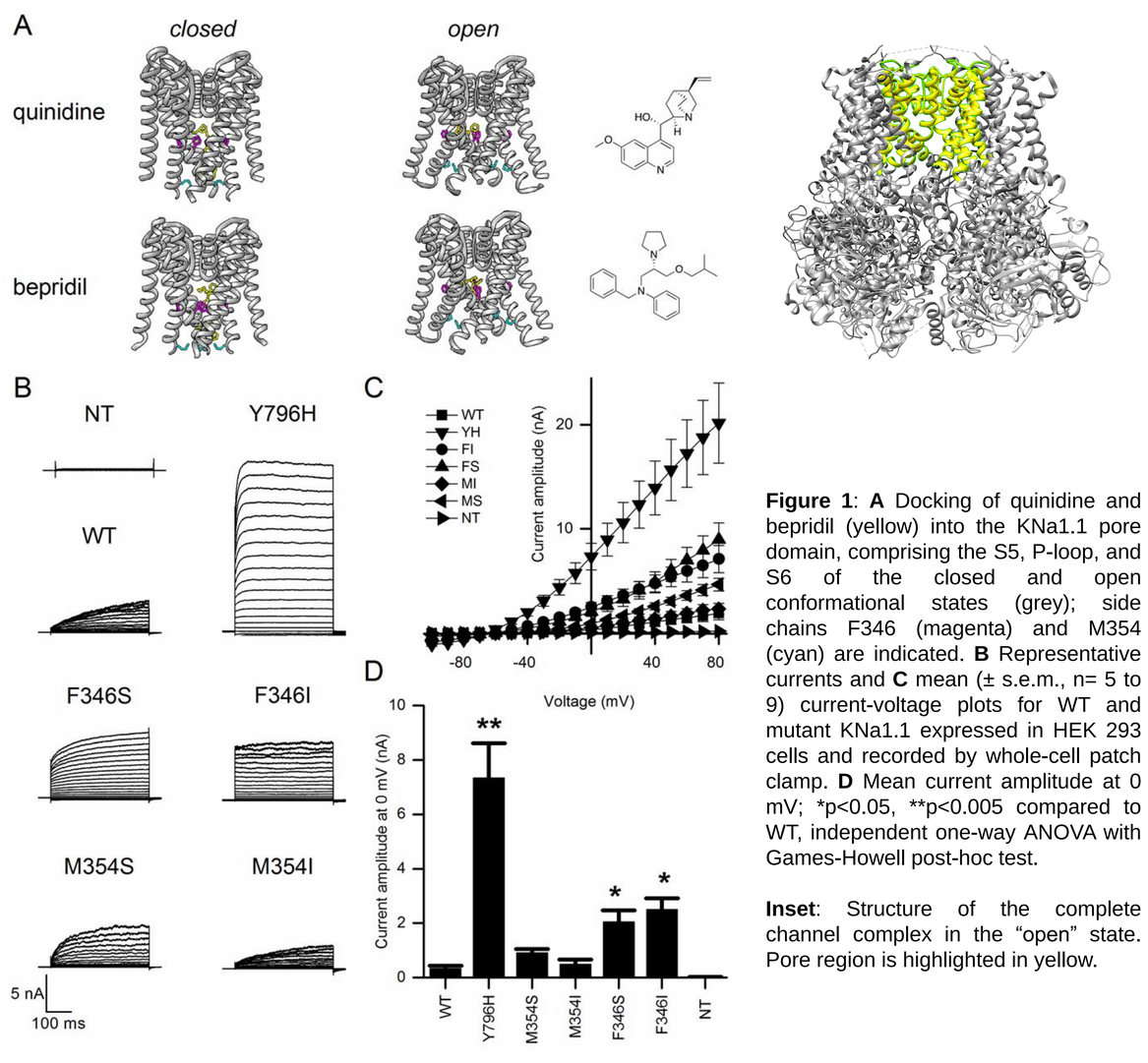


Figure 1: **A** Docking of quinidine and bepridil (yellow) into the KNa1.1 pore domain, comprising the S5, P-loop, and S6 of the closed and open conformational states (grey); side chains F346 (magenta) and M354 (cyan) are indicated. **B** Representative currents and **C** mean (± s.e.m., n= 5 to 9) current-voltage plots for WT and mutant KNa1.1 expressed in HEK 293 cells and recorded by whole-cell patch clamp. **D** Mean current amplitude at 0 mV; *p<0.05, **p<0.005 compared to WT, independent one-way ANOVA with Games-Howell post-hoc test.

Inset: Structure of the complete channel complex in the "open" state. Pore region is highlighted in yellow.

3. Results II

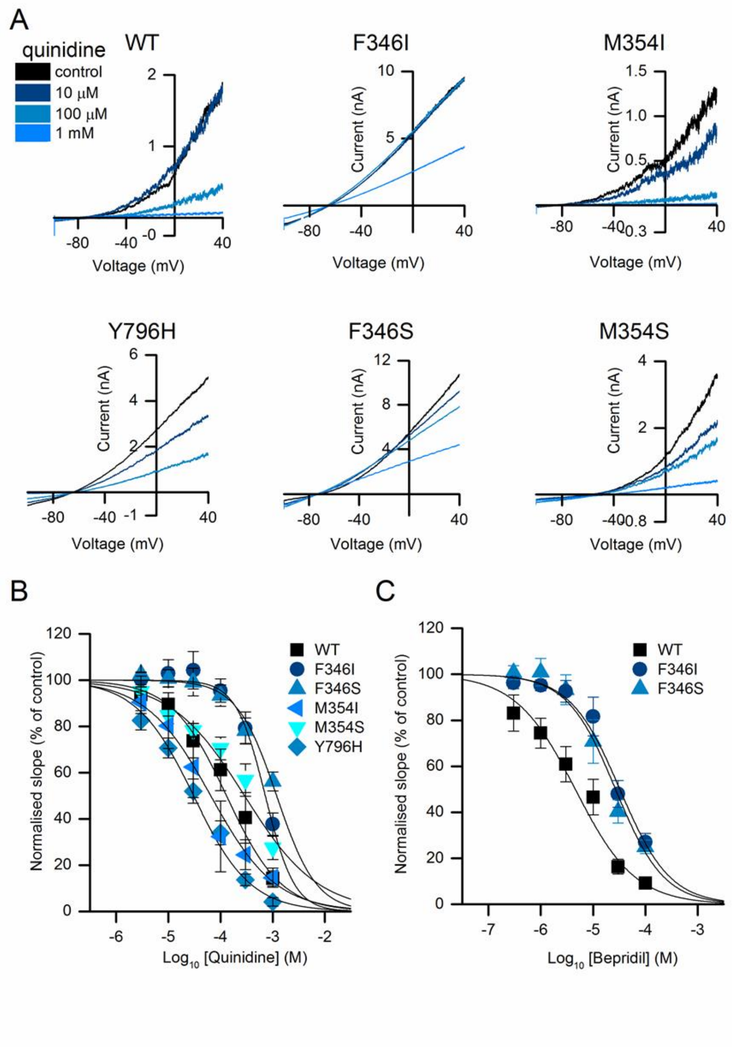
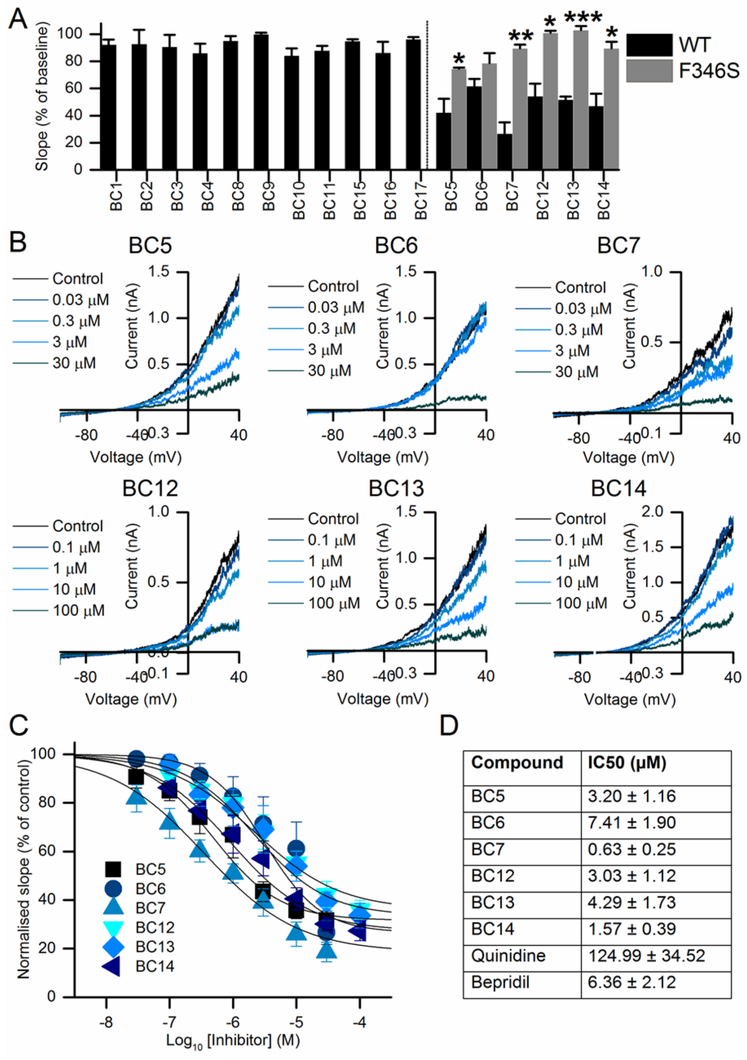


Figure 2: **A** Representative currents from voltage ramps from cells expressing WT or mutant KNa1.1 with increasing concentrations of quinidine as indicated. **B** Concentration-inhibition plots for wildtype and mutant KNa1.1 channels in response to 3μM-1mM quinidine. IC50 for WT, 124.99 ± 34.52 μM (n=5); F346I, 736.08 ± 94.09 μM (n=5); F346S, 1.23 ± 0.19 mM (n=4); M354I, 99.23 ± 49.61 (n=5); M354S, 247.16 ± 19.96 μM (n=5); Y796H, 38.00 ± 12.89 μM (n=5). **C** Concentration-inhibition plots for wildtype and mutant KNa1.1 channels in response to 0.3μM-100μM bepridil. IC50 for WT, 6.36 ± 2.12 μM (n=5); F346I, 35.91 ± 11.01μM (n=4); F346S, 23.43 ± 5.17 μM (n=5).

3. Results III



Compound	IC50 (μM)
BC5	3.20 ± 1.16
BC6	7.41 ± 1.90
BC7	0.63 ± 0.25
BC12	3.03 ± 1.12
BC13	4.29 ± 1.73
BC14	1.57 ± 0.39
Quinidine	124.99 ± 34.52
Bepridil	6.36 ± 2.12

Figure 3: Functional evaluation of top-scoring molecules from *in silico* docking. **A** WT KNa1.1 conductance, relative to baseline, in the presence of 10 μM test compound; with those that were active (right of dashed line) counter-tested with F346S KNa1.1 pore mutant (*p<0.05, **p<0.005, ***p<0.0005, T-test.). **B** Representative traces and **C** mean (± s.e.m., n= 5 to 7) concentration-inhibition plots for active inhibitors. **D** Summary table with mean potencies of inhibitors, including quinidine and bepridil.

3. Results IV

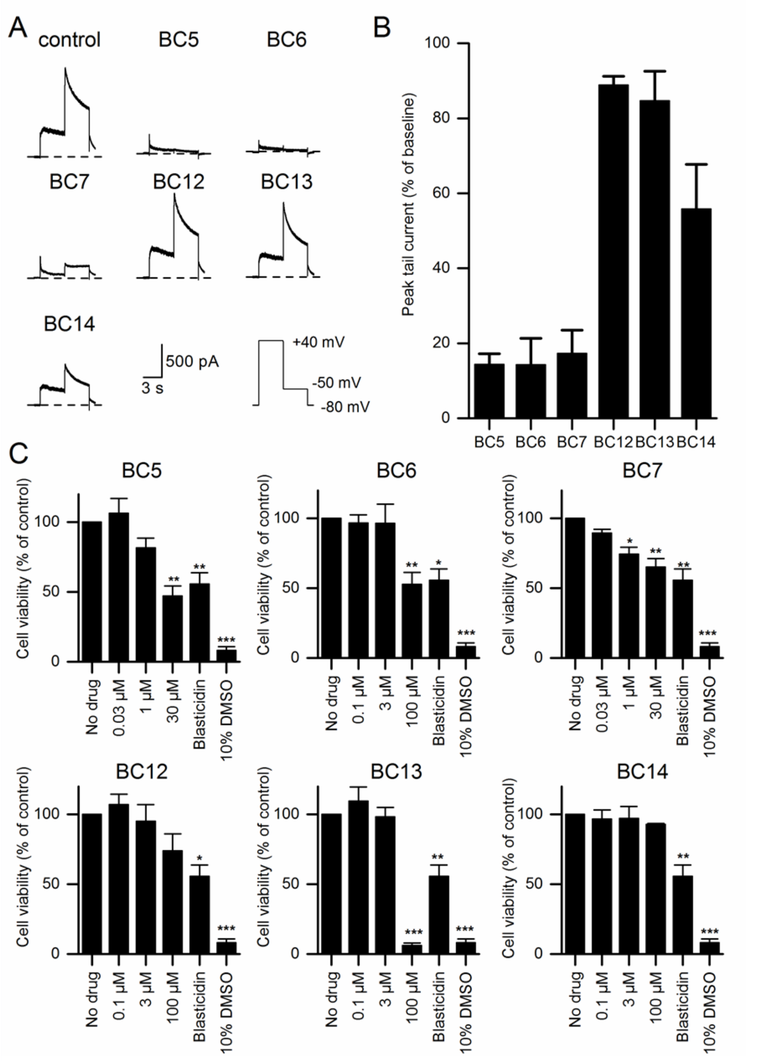


Figure 4: Preliminary toxicological data. **A** Representative hERG whole-cell currents in the absence (control) and presence of 10 μM inhibitor, as indicated. **B** Mean (± s.e.m., n=3) tail current at -50 mV remaining in the presence of each inhibitor. **C** Cytotoxicity assays indicating mean (± s.e.m., n=3) viability of HEK 293 cells using WST-1 reagent following overnight exposure to inhibitor at the indicated concentrations, with 5 μM blasticidin and 10% v/v DMSO as positive control; *p<0.05, **p<0.005, ***p<0.0005, independent one-way ANOVA with Dunnett's post-hoc test.

4. Conclusions

- A phenylalanine residue, F346, in the pore-forming region of KNa1.1 is important for inhibition of the channel by quinidine and bepridil.
- Epilepsy-causing mutation Y796H increases quinidine sensitivity of the channel.
- The structure of the chicken KNa1.1 channel resolved by cryo-EM was successfully used to identify novel inhibitors of the channel using computer-aided methods.
- Reduced efficacy of the six compounds with F346S suggests they are specifically inhibiting the pore-forming region of KNa1.1.
- These are potential tool compounds or novel starting points for developing KNa1.1-specific inhibitors, though some may have toxic effects.

5. References/ acknowledgements

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