Fluorescence-based drug repurposing screen of the potassium channel, $K_V3.1$ with V434L mutation

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Introduction

$K_V3.1$ is a voltage-gated potassium channel encoded by the KCNC1 gene. Mutations in the $K_V3.1$ protein can manifest as a variety of neurological disorders including myoclonic epilepsy and ataxia due to K^+ channel mutation (MEAK), developmental epileptic encephalopathy (DEE), or hypotonia.

The KCNC1 Foundation[1] was founded by the parents of Eliana, a child from Canada who was diagnosed with an ultra-rare *de novo* mutation (V434L) in the KCNC1 gene, which encodes the $K_V3.1$ ion channel in humans, at age 9 months. Eliana does not display typical DEE, but exhibits significant hypotonia, cortical-visual impairment, vertical nystagmus, and global delays. The KCNC1 Foundation has registered 36 patients affected by 14 different genetic variants in the KCNC1 gene. Of these patients, 25% share the A421V variant, 12.5% have MEAK caused by the R320H variant, a few exhibit the V432M variant, and the remaining variants are seen in 1 - 3 patients.

Materials and Methods

Cell lines

DNA sequence encoding KCNC1 wild type (WT) or KCNC1 V434L in an expression vector was generated, and sequence verified (GenScript). CHO-K1 cells were transfected using Lipofectamine 3000 (ThermoFisher) and monoclonal cells obtained using dilution cloning. Putative clones were initially screened using a thallium flux assay, before more detailed assessment of the most promising clones by thallium flux and Qube automated patch clamp assays.

Electrophysiology assays

CHO-K1 cells were transiently transfected with 0.5 - 2 µg KCNC1 WT or V434L DNA. Whole-cell patch clamp recordings were performed using the voltage protocol below (**Figure 1**) with an extracellular solution consisting of (in mM) 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.4, and intracellular solution of 125 KCl, 25 KOH, 1 $CaCl_2$, 2 $MgCl_2$, 5 Na_2ATP, 10 EGTA, 10 HEPES, pH 7.2. A sampling rate of 20 kHz and inter-sweep interval of 15 seconds was used.

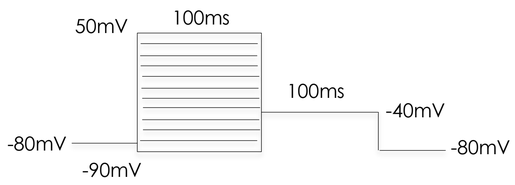

Figure 1: Voltage protocol used in manual patch recordings.

The automated patch clamp assay was performed using single hole QChips on Qube 384 (Sophion) using the same voltage protocol as the manual patch clamp assay. The extracellular solution consisted of (in mM) 140 NaCl, 2 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 Glucose, pH 7.4, and intracellular solution of 120 KF, 20 KCl, 10 EGTA, 10 HEPES, pH 7.2.

Thallium flux assay

CHO-K1 cells expressing $K_V3.1$ V434L were seeded into black/clear 384 well plates (Greiner) at 10,000 cells/well, incubated for 18 - 20 hr at 37°C, 5% CO_2. Media overthrown and cells loaded with Potassium Assay dye (Molecular Devices) prepared in buffer (in mM): 140 Na Gluconate, 2.5 K Gluconate, 6 Ca Gluconate, 2 Mg_2SO_4, 5 Glucose, 10 HEPES, pH 7.3, and 1 mM probenecid. Test and control compounds prepared at 5x assay concentration and added to cells on FLIPR® Penta. After 20 minutes incubation at 37 °C, 1 mM Tl_2SO_4 and 10 mM K_2SO_4 was added at 6x final concentration and ΔRFU (Ex 470 - 495nm, Em 515 - 575 nm) monitored for 3 minutes. Data analysed using the rate of Tl^+ influx over the first 35 sec after addition of Tl_2SO_4 and K_2SO_4.

Results

Biophysical characterisation of V434L variant

Whole-cell patch clamp experiments were performed on CHO-K1 cells transiently transfected with WT or V434L KCNC1 (**Figure 2**). Biophysical data of the WT and variant channels was consistent with published results[2], with a clear leftward shift in the voltage dependence of activation (**Figure 2B**) confirming V434L as a gain-of-function mutation.

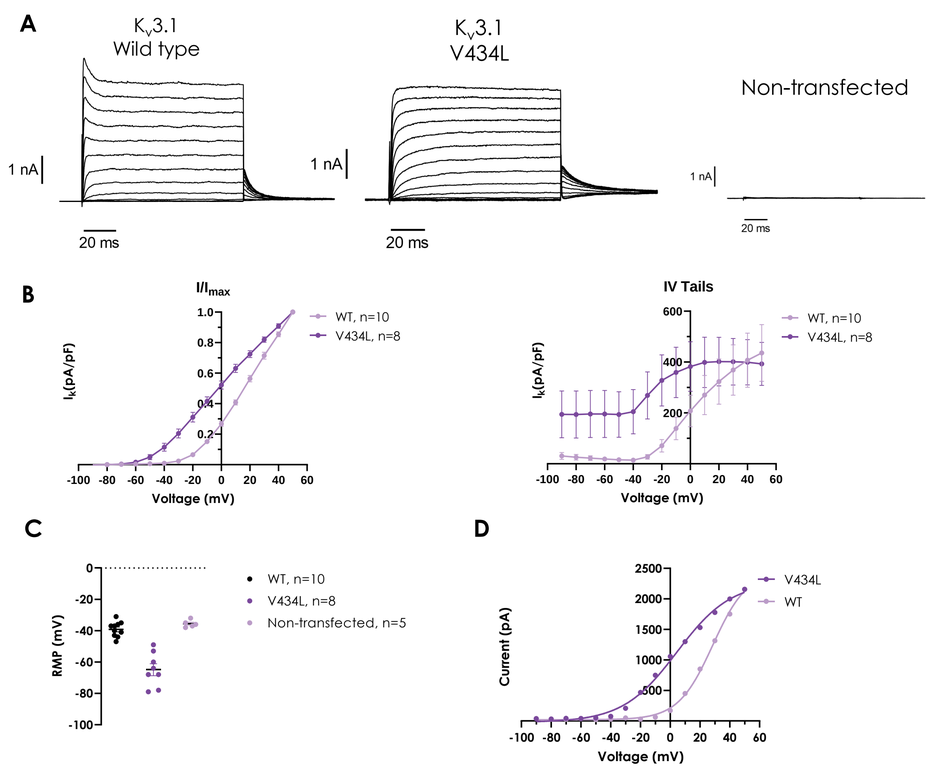

Figure 2: Representative current traces for WT and V434L-variant $K_V3.1$ channels, and non-transfected CHO-K1 cells (A), current density plots of voltage-dependent activation and tail currents, mean ± SEM (B), comparison of resting membrane potential (C). Example IV plots from Qube automated patch clamp recordings performed using same voltage protocol shown for comparison (D).

Pharmacological validation of WT and V434L variant

Two known $K_V3.1$ channel modulators, 4-aminopyridine (4-AP; Sigma Aldrich) and AUT1 (Cayman Chemicals), were selected for the initial pharmacological validation of WT and V434L $K_V3.1$ transiently expressed in CHO-K1 cells using whole-cell patch clamp technique **(Figure 3**).

The pharmacological data revealed clear differences between WT and the V434L $K_V3.1$ variants, with 3 mM 4-AP almost fully inhibiting WT $K_V3.1$ at +40 mV, but only partially inhibiting the V434L variant. For AUT1, there was inhibition of the current in V434L at +40 mV, but an increase in current amplitude evoked under the same conditions in the WT $K_V3.1$.

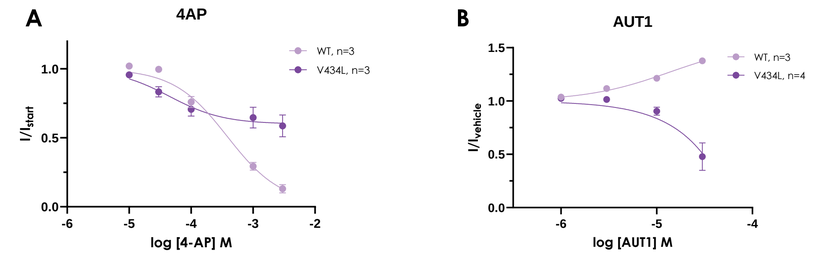

Figure 3: Concentration-response data for 4-AP (A) and AUT1 (B) in WT and V434L $K_V3.1$ variants (mean ± SEM).

Development of clonal cell line

Initial assessment of the polyclonal V434L $K_V3.1$ cell population in single hole QChips on the Qube indicated a low percentage (~5%) of cells expressing >400 pA current at +40 mV. 384 distinct cell populations derived by dilution cloning of the polyclonal cell line were subsequently tested in the thallium flux assay on the FLIPR. Ten clones were selected for further assessment in the fluorescence assay before final assessment of four clones by automated patch clamp assays on the Qube **(Figure 4**), which led to Clone 2G6 being selected for screening activities.

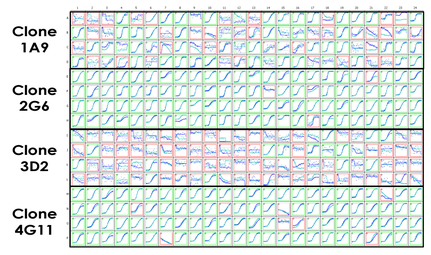

Figure 4: Assessment of four putative CHO-K1 monoclonal cell lines expressing the $K_V3.1$ V434L variant using Qube single hole QChip. Percentage of cells achieving acceptable QC parameters (input resistance ≥ 200 MΩ and current amplitude ≥ 400 pA; highlighted with green outline) was 95.8% for clone 2G6.

Thallium flux assay validation and repurposing library screen

The thallium flux assay was optimised for high throughput screening and the protocol validated using mock screening plates consisting of randomly spiked wells containing two known $K_V3.1$ modulators, AUT1 and fluoxetine **(Figure 5A**). For the screen, The Broad Institute Repurposing Library of 6,718 compounds was tested in duplicate at a final concentration of 10 µM. Percent inhibition in the test wells was calculated from in-plate controls consisting of 0.5% DMSO (0% inhibition) and 10 mM TEA-Cl (100% inhibition). Example data and a summary of the screening plate statistics is shown in **Figure 5B and C**.

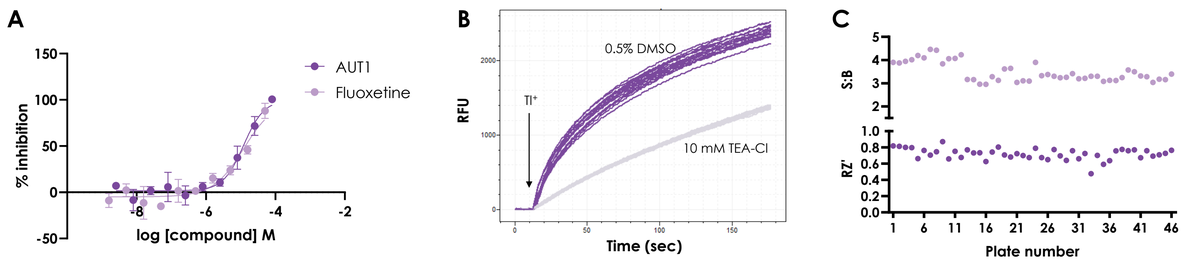

Figure 5: Concentration-response data for AUT1 (12.1 µM) and fluoxetine (12.3 µM) in spiked plate test (A). Example kinetic data showing response to the addition of 1 mM Tl_2SO_4 and 10 mM K_2SO_4 in the control wells (B) and summary of plate statistics for the 46 screening plates (C), robust Z' = 0.72 ± 0.07, signal-to-background = 3.48 ± 0.42 (mean ± SD).

Hit analysis

The duplicate percent inhibition data showed good correlation between the n=1 and n=2 determinations (**Figure 6**). 320 compounds (4.8%) inhibited the thallium response by 50% or more, with 34 compounds (0.5%) inhibiting above 90%.

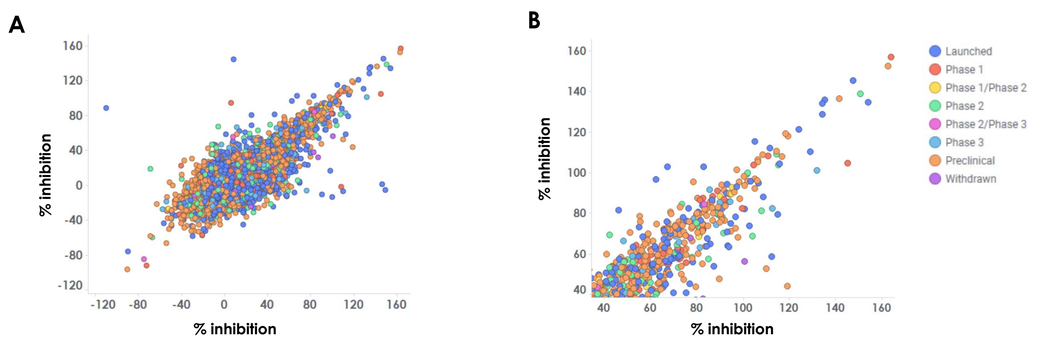

Figure 6: Correlation of duplicate percent inhibition data (A) with compounds inhibiting greater than 50% annotated with phase in drug development (B).

Using a combination of activity in the thallium flux assay and the annotations in The Broad Library registry such as clinical phase and known target class, eighty compounds were subsequently selected for concentration-responding testing in the thallium flux assay. Compounds were screened as ten-point curves in duplicate with each plate also containing TEA-Cl and AUT concentration-response curves to verify the assay performance.

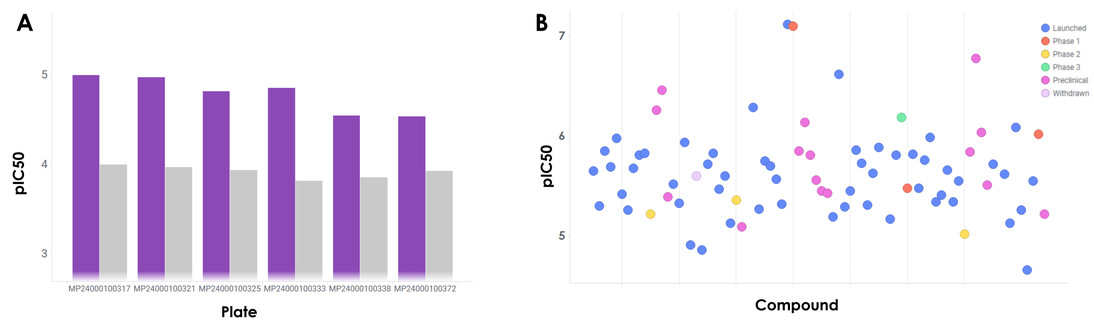

Figure 7: pIC_{50} values of AUT1 (purple bars) and TEA-Cl (grey) control compounds in each screening plate (A), and pIC_{50} values for the 80 test compounds (B). 49/80 compounds have pIC_{50} > 5.5, and 16/80 have pIC_{50} > 6.0.

Conclusions

- Stable cell line expressing $K_V3.1$ V434L variant developed and characterised, confirming published data describing V434L as a gain-of-function mutation.
- 6,718 compounds from The Broad Institute Repurposing Library screened with a high number of confirmed hits identified.
- 16 compounds identified with sub-micromolar IC_{50}, including 5 compounds launched for other indications
- Identification of functional inhibitors of $K_V3.1$ V434L validates the strategy to screen the repurposing library as a fast and cost-effective approach to discover potential new therapeutics for patients with this mutation.

References

1. www.kcnc1.org.
2. Clatot J, Ginn N, Costain G, Goldberg EM. A KCNC1-related neurological disorder due to gain of Kv3.1 function. *Ann Clin Transl Neurol.* 2023 Jan;**10**(1):111-117.

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