High-throughput electrophysiology and fluorescence-based screening platforms for identification of activators of the lysosomal ion channel TMEM175

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Discovery from Charles River, Chesterford Research Park, Saffron Walden, UK

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1 Abstract

TMEM175 is a cation permeable ion channel located on the lysosomal and endosomal membrane which functions as a proton-activated proton channel and a potassium ion leak channel. This channel maintains lysosomal luminal pH through direct efflux of protons and as a counter-ion flux of potassium ions to balance influx of cytosolic protons by the V-ATPase (Cang et al., 2015). This has a significant role in maintaining lysosomal function that becomes dysregulated in disease leading to compromised proteolytic processing.

Loss of function variants, such as the M393T point mutation, are risk factors for earlier onset and increased severity of neurodegenerative diseases including Parkinson's disease (Wie et al., 2021; Nalls et al., 2015). Unstable lysosomal pH leads to decreased lysosomal catalytic activity, decreased glucocerebrosidase activity, impaired autophagosome clearance, e.g. of accumulated α-synuclein, by the lysosome and decreased mitochondrial respiration.

Small molecule activators of TMEM175 may have therapeutic potential to normalise luminal pH and restore lysosomal activity to patients suffering neurodegeneration or those who are at risk of an early onset. Development of screening capabilities are therefore the first step in identifying novel chemical starting points for drug discovery.

Here we show assay data from Charles River Laboratories comparing cell lines constitutively expressing the WT and M393T variants of human TMEM175 and WT mouse TMEM175 channels. Activation of TMEM175 was observed in automated patch-clamp (Sophion Qube) for both outward Cs^+ and inward H^+ currents, and FLIPR Thallium flux assay after addition of the tool activator DCPIB.

2 Methods

Cell lines cultured in complete growth media (DMEM/F12 + 10% HI-FBS + 1x MEM NEAA) with selection for constitutive expression of WT human/mouse TMEM175 (250 µg/mL G418) or M393T TMEM175 (200 µg/mL Hygromycin B)

Electrophysiology was carried out on the Sophion Qube 384 well automated patch clamp platform.

For both outward (Cs^+) and inward (H^+) currents, intracellular solutions contained (in mM): CsF (120), CsCl (20), HEPES (10), EGTA (1); pH 7.2 (CsOH); 310 mOsM and extracellular solutions contained NaCl (145), KCl (4), $MgCl_2$ (1), HEPES (10), $CaCl_2$ (2), glucose (10); pH 7.4 (NaOH); 315 mOsm. All experiments used multi-hole Qchips.

Outward Cs^+ currents:

Cs^+-based internal solution was used to isolate TMEM175 from other K^+ channels. A voltage-ramp protocol was applied from -100 to +100 mV over 1 s from a holding potential of -80 mV. 2 vehicle (0.5 % DMSO) applications preceded 3 applications of test compound, with 2 applications of full activation (100 µM DCPIB) and 1 final full block application (1 mM 4-AP). Currents were measured at +50 mV.

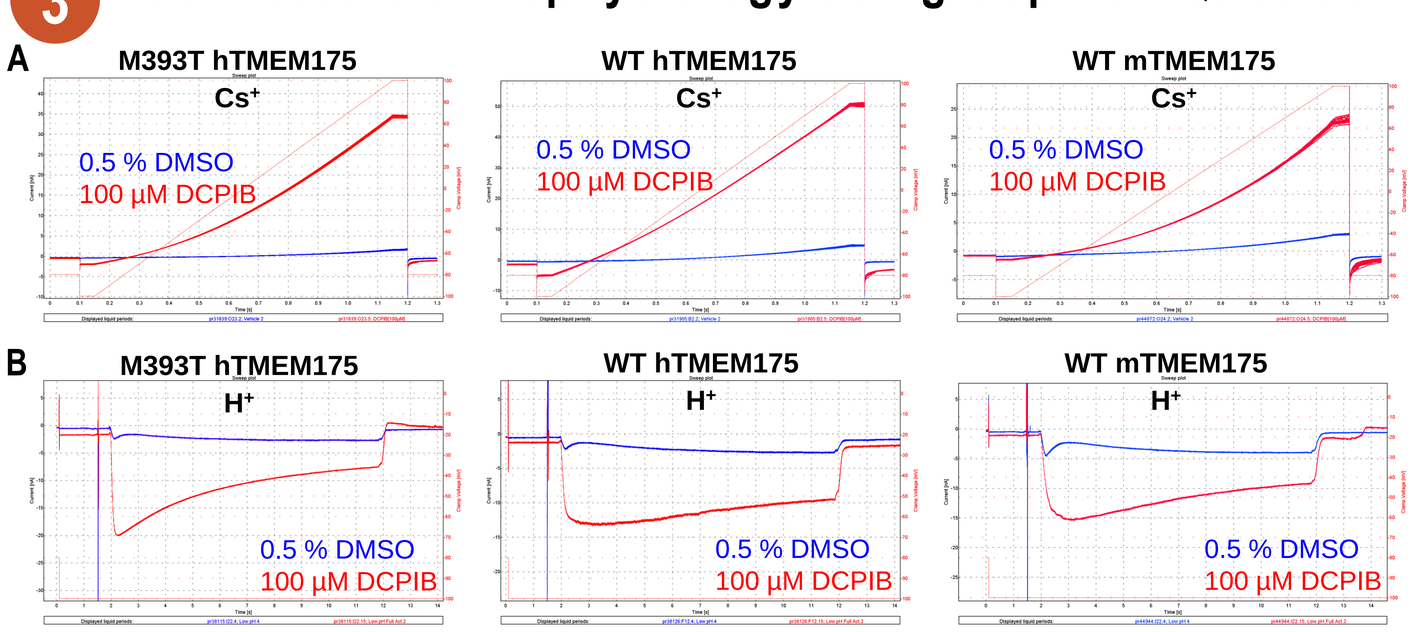
Inward H^+ currents:

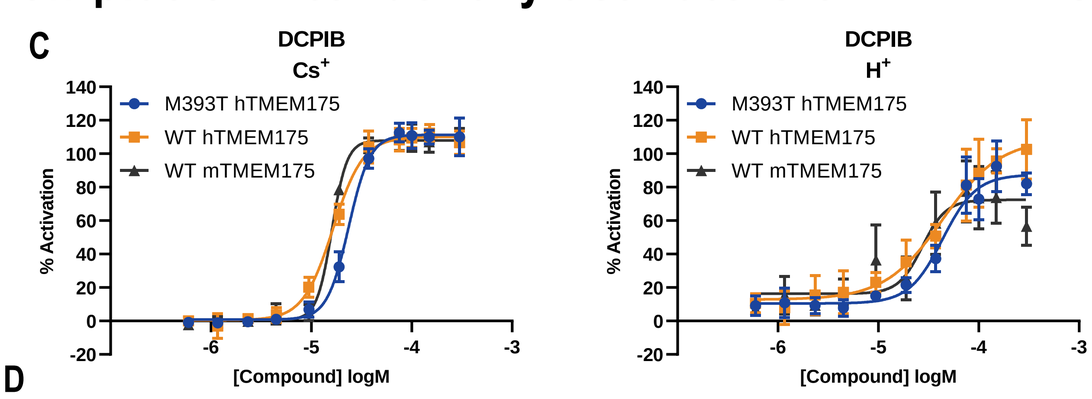
Low pH challenge liquid periods consisted of voltage step from -80 to -100 mV with pH 4.6 external application, followed by pH 7.4 solution washout after 10 s. Compounds were applied 3 times, and full activation (100 µM DCPIB) applied twice, in pH 7.4 solution at V_{Hold}, with low pH challenge after each application. Current was measured 2 s after voltage step to -100 mV

FLIPR Thallium Flux Assay:

Cells were plated at either 20k cells/well (HEK parental) or 25k cells/well (WT & M393T hTMEM175 and WT mTMEM175) on PDL-coated 384 well plates 24 hr prior to assay. Cells were dye loaded with Tl^+-sensitive dye for 90 min at room temperature in the dark. Test compounds were added in buffer containing 0.01% F-127 Pluronic acid without thallium and incubated at room temperature for 30 min. 833 µM thallium was added after compound incubation and fluorescence read for 3 minutes. The slope of the fluorescence rise was analysed between reads 15-30, for 15 s immediately after thallium addition.

3 Automated electrophysiology using Sophion Qube 384 well platform to identify activators of TMEM175





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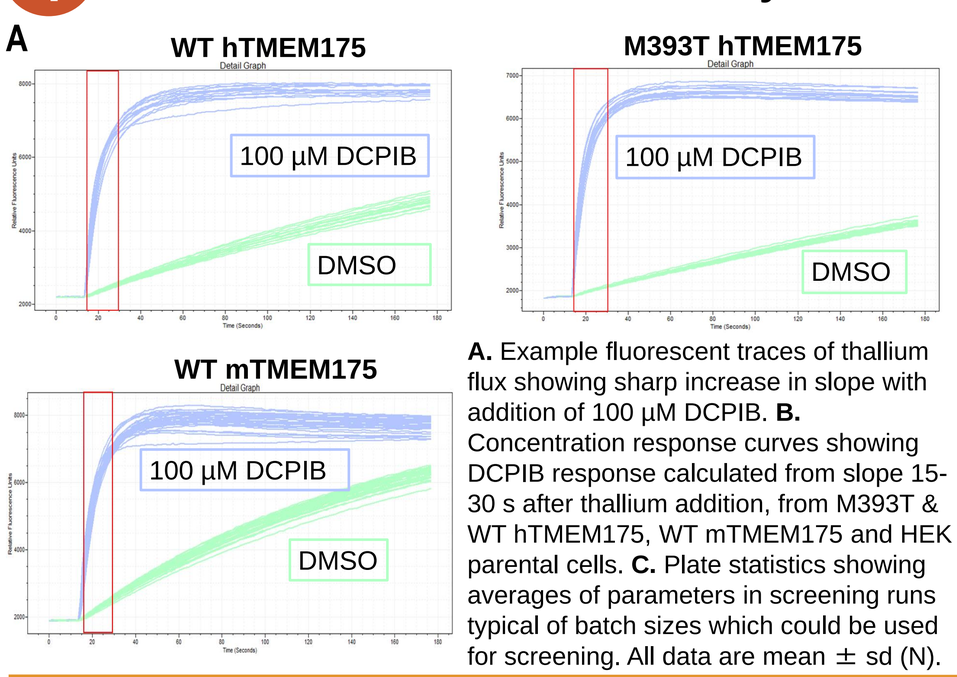
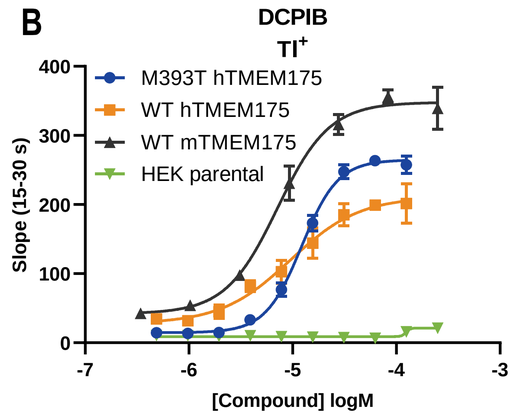
Cs^+ assay parameter	M393T hTMEM175		WT hTMEM175		WT mTMEM175	
	Plate 1	Plate 6	Plate 1	Plate 6	Plate 1	Plate 6
Current with 100 µM DCPIB (nA)	25.1 ± 5.2	31.9 ± 6.1	21.1 ± 5.8	23.2 ± 5.8	19.5± 5.1	18.6± 4.5
Success rate (%)	87.0	68.5	90.1	82.3	80.9	81.5
DCPIB EC_{50} (µM)	23.5	18.5	16.5	16.9	16.0	17.1

H^+ assay parameter	M393T hTMEM175		WT hTMEM175		WT mTMEM175	
	Plate 1	Plate 6	Plate 1	Plate 6	Plate 1	Plate 4*
Current with 100 µM DCPIB (nA)	16.2 ± 3.6	17.0 ± 3.3	17.3 ± 4.4	15.3 ± 3.7	17.1 ± 3.1	15.6 ± 3.9
Success rate (%)	92.7	85.4	92.7	88.8	81.0	78.1
DCPIB EC_{50} (µM)	43.7	71.4	43.9	39.6	27.4	20.8

A. Example outward Cs^+ currents showing 1 s voltage ramp between -100 and +100 mV applied from a holding potential of -80 mV, activated upon incubation with 100 µM DCPIB. **B.** Example inward H^+ currents showing voltage step from -80 to -100 mV alongside pH 4.6 solution application, activated upon pre-incubation with 100 µM DCPIB. **C.** Representative concentration response curves showing DCPIB response calculated from outward currents measured at +50 mV during voltage ramps (Cs^+) or current density at -100 mV step (H^+) from the first plate of a longevity test stacker run. **D.** Plate statistics showing averages of parameters across first and last plate in a longevity test. All data are mean ± sd.
**mTMEM175 only showed longevity for up to 4 hrs.*

4 A fluorescence-based assay to identify activators of TMEM175





A. Example fluorescent traces of thallium flux showing sharp increase in slope with addition of 100 µM DCPIB. **B.** Concentration response curves showing DCPIB response calculated from slope 15-30 s after thallium addition, from M393T & WT hTMEM175, WT mTMEM175 and HEK parental cells. **C.** Plate statistics showing averages of parameters in screening runs typical of batch sizes which could be used for screening. All data are mean ± sd (N).

C

Assay parameter	M393T hTMEM175	WT hTMEM175	WT mTMEM175
S:B	21.2 ± 2.0 (34)	22.2 ± 1.3 (12)	8.3 ± 0.3 (6)
Z'	0.72 ± 0.11 (34)	0.84 ± 0.05 (12)	0.70 ± 0.08 (6)
DCPIB EC_{50} (µM)	5.08 ± 0.1 (32)	5.87 ± 0.76 (6)	6.03 ± 1.19 (6)

5 Conclusions

Here we have shown methods for detection of activators of TMEM175 (WT & M393T human and WT mouse variants), through development of automated electrophysiology and fluorescence-based screening assays.

Compounds can be assessed for activation of potassium conductance, through assessment of Cs^+ flux on the Sophion Qube platform and Tl^+ flux on the FLIPR platform. A separate assay on the Sophion Qube platform can be used to assess activation of inward H^+ conductance.

The high quality and stability of both Qube and FLIPR assays for typical screening batch sizes allow for high-throughput testing of compounds to discover modulators or direct activators of TMEM175, which have the potential to modulate lysosomal pH *in vivo*.

Future work will examine the effects of modulating TMEM175 function on lysosome morphology and function.

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