# Validation of an ASIC1a ligand-gated assay on an automated patch clamp platform and it's use for novel ligand screening

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## Introduction

There is growing interest in automated patch clamp (APC) assays for ligand-gated targets which are expressed throughout the peripheral and central nervous system. The Acid-Sensing Ion Channel (ASIC) family comprises combinations of ASIC1-4 proteins that form acid-activated cation-selective channels.[1] ASIC channels underlie complex neurological processes and diseases such as cognition, synaptic plasticity, pain, ischemia and epilepsy.[2] ASIC channels are subject to evolutionary predator-prey arms races as shown by potent snake and spider toxins such as Mambalgin-1 and Psalmotoxin-1.[3,4]

Owing to the widespread expression of ASIC channels and their role in key neurological processes they are an emerging class of ion channel drug discovery targets, and several approaches are being developed to find specific modulators. Discovery of new natural toxins and optimisation of known peptide scaffolds is one method but may not easily deliver drug-like hits or lead molecules owing to toxin instability and impermeability. Several small molecules programs have delivered lead compounds (e.g. Abbott[5]), whilst the lack of novel tractable and novel small molecule have led other groups to screen for low molecular weight fragments that bind to ASIC channels (e.g. Merck[6,7]).

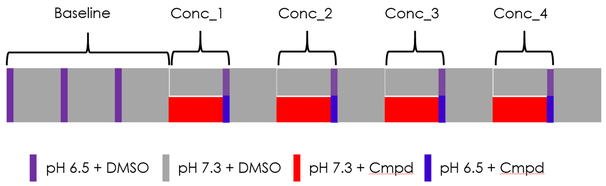
We therefore set out to demonstrate the following:

1. Set-up and optimise a ligand-gated assay for ASIC1a on an APC platform
2. Pharmacologically validate the ASIC1a APC assay with a toolbox of small molecules, animal peptide toxins and previously published compound fragments
3. Use the APC electrophysiology to screen a proprietary fragment library to identify novel scaffolds necessary for the design of new ASIC1a modulators.

## Materials and Methods

**ASIC1a APC recordings:** A CHO cell line stably expressing the human ASIC1a protein was cultured according to vendor instructions (b-Sys GmbH) and harvested using optimised protocols to obtain stable, moderate amplitude currents on the gigaseal QPatch48 APC system (Sophion). Internal solution contained (in mM) 90 K-gluconate, 40 KCl, 10 NaCl, 5 HEPES, 3.2 $MgCl_2$ and 3.2 EGTA; pH 7.3. External solution contained (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$ and 11.1 glucose; pH was controlled using 10 HEPES (pH ≥7.0) or MES (<7.0). Whole-cell currents were elicited from a holding potential of -60 mV.

**Ligand-gated application protocol:** ASIC1a currents were elicited by rapid application (and wash-off) of saline or compound-containing solutions of varied pH (typically 7.0. 6.8, 6.5, 6.0 and 5.5 to enable full range of activation around the $EC_{50}$ of ~ 6.6). A schematic of the ligand application protocol is shown below:




Briefly, cells were washed three times with external solution (5 µl) between applications of activating external solution to establish a stable baseline. Cells were then stimulated with repeated 3 sec applications (5 µl) of either pH 6.5 (screening mode) or varied pH ($EC_{50}$). Increasing concentrations of reference compounds were applied before and during each pH stimulus ($IC_{50}$ mode), or a single concentration of different test fragments (200 µM) were applied as Conc_1 and Conc_3 with wash-off during Conc_2 and Conc_4 application periods in single point screening mode. Cells were allowed at least 2 min between applications of each external solution to aid recovery from desensitization.

**Reference compounds and toxins:** A selection of published reference compounds and peptide toxins were purchased from commercial sources.

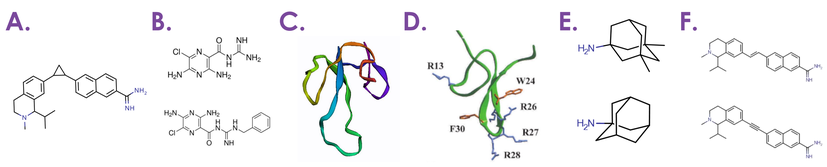



**Figure 1: Structures of ASIC reference compounds and fragments.**
**A:** Abbott A-317567[5] **B:** Amiloride and Benzamil. **C:** Mambalgin-3[3]. **D.** Psalmotoxin-1 (PcTX)[4]. **E.** Memantine and Amantadine[8]. **F.** Merck ASIC3 compounds 10a and 10b[7]

## 1. Creating a stable ligand-gated ASIC1a assay

The first challenge for any APC assay is to ensure optimum current expression, such that a high fraction of recordings deliver current amplitudes above a suitable minimum amplitude but avoid large currents that cannot be adequately clamped. In single cell/single hole mode it was possible to optimise expression to achieve the desired current range, and these treatments did not affect ASIC1a biophysics or pharmacology (e.g. pH $EC_{50}$).

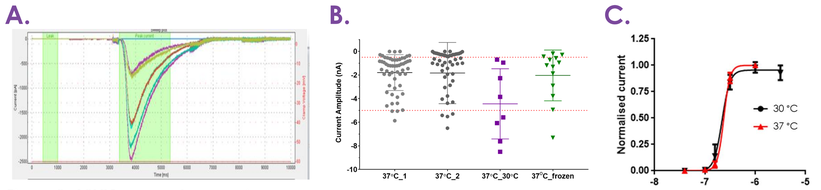



**Figure 2: ASIC1a current expression.**
**A.** Single cell / single hole gigaseal recording of ASIC1a currents on QPatch, showing rapid pH-dependent activation followed by desensitisation and wash-out back to baseline. **B.** Current amplitude distribution under indicated cell culture conditions. **C.** No change in pH $EC_{50}$ under different culture conditions.

The second target was to optimise the ligand application protocol to minimise channel desensitisation and current rundown to allow for stable, repeated pH-activated responses that would enable design of a successful screening assay.

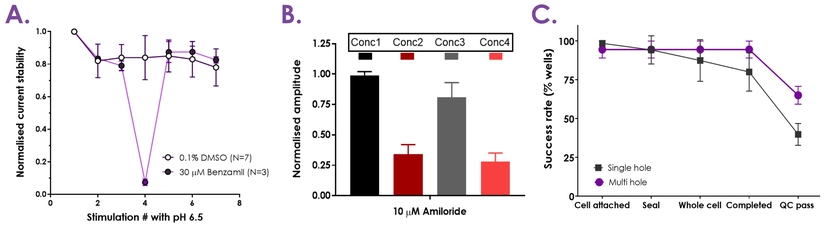



**Figure 3: Stable ASIC1a current responses and success rate suitable for a compound screening assay.**
**A.** Current amplitude in response to repeated application of pH 6.5 in 0.1% DMSO (open) and after pre-incubation and co-application of Benzamil during 4th stimulus (closed). **B.** Sequential fragment screening mode of liquid application protocol delivers reliable repeat inhibition data with Amiloride after intervening compound washoff. **C.** 'Patchability' and overall QC pass success rate of single and multi-hole assay format.

## 2. Pharmacological validation of ASIC1a assay

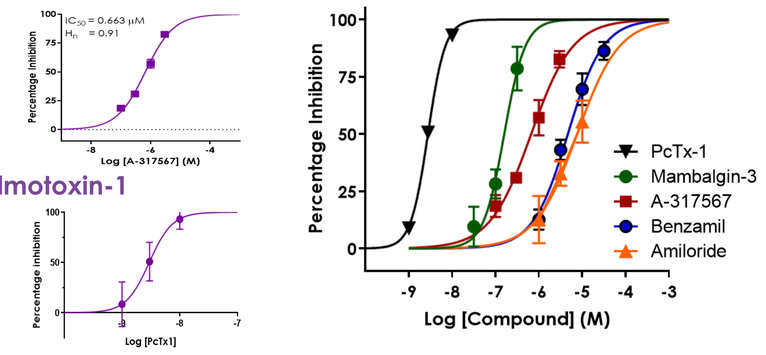



**Figure 4: Reference small molecule and toxin pharmacology of ASIC1a assay.**
**A.** Examples of inhibition of pH-activated currents by exemplar literature compounds A-317567[5] and Psalmotoxin.[4] **B.** Summary of concentration-response data for reference compound toolbox, illustrating the wide range of potencies detected for different ligand types.

| Compound | Metrion $IC_{50}$ (µM) | Literature |
|---|---|---|
| **Psalmotoxin-1** | 0.003 | 0.001 ([4]) |
| **Mambalgin** | 0.16 | 0.06 ([8]) |
| **A-317567** | 0.66 | 2.0 ([5]) |
| **Benzamil** | 4.66 | 3.5 ([2]) |
| **Amiloride** | 7.62 | 10 ([4]) |

**Table 1: Summary of ASIC1a assay pharmacology for literature small molecules and peptide toxins.**
Good correlation between $IC_{50}$ values derived from QPatch ASIC1a assay concentration-response fits in Fig. 4 and representative values obtained from the literature for reference toxins and various small molecules.

## 3. Fragment screening

The ability of the pharmacologically validated ASIC1a QPatch APC assay to detect low potency ASIC1a inhibition by known low molecular weight fragments and small molecule analogues was determined to expand the palette of scaffolds that could be used in the final fragment library screen:

- We confirmed a previous study showing that Memantine (215 MW) and Amantadine (151 MW) had low µM potency (315 and 510 µM) for hASIC1a[8]
- We extended work by Merck on ASIC3 to show that analogues of A-317567 are also relatively potent inhibitors of hASIC1a[7]

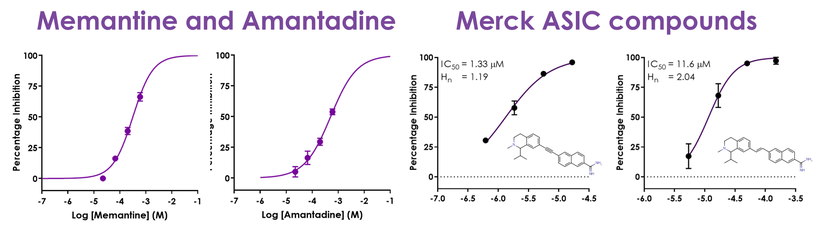



**Figure 5: Inhibition of ASIC1a by low molecular weight small molecules and compound analogues.**
**A.** Confirmation that small molecules Memantine and Anandamide inhibit ASIC1a with low µM potency. **B.** Extension of Merck's work showing that A-317567 analogues inhibit ASIC1a with moderate potency.[7]

Finally, we tested a range of low MW fragments derived from A-317567 (Fig. 6) as well as a proprietary library of >1,000 fragment compounds (Table 2), revealing a mix of activity (at 200 µM) including a collection of hits with promising SAR and potential as building blocks to design and optimise novel ASIC1a modulators.

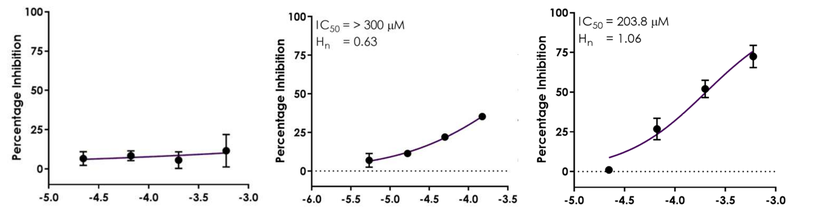



**Figure 6: Inhibition of ASIC1a by low molecular weight small molecule fragments.**
Low MW fragments derived from A-317567 and a proprietary library show a range of inhibitory activity against human ASIC1a.

| | Hit cut-off based on SD | |
|---|---|---|
| | 2 x SD | 3 x SD |
| **% inhibition** | 44.6 | 66.9 |
| **# hits** | 68 | **40** |

**Table 2: Summary of ASIC1a fragment library screen hit identification.**
Mean inhibition cut-off determined from either 2x or 3x standard deviation (SD) for all tested compounds vs DMSO vehicle yields 68 and 40 hit compounds, respectively.

## Conclusions

- A stable and reproducible ligand-gated APC assay for ASIC1a channel was created using optimised cell culture and pipette-based liquid application methods that enable rapid activation and wash-off without desensitisation.
- The gigaseal QPatch APC platform can replicate the nM – µM potency of literature and reference peptide toxins and small molecules.
- Sequential single point applications and cumulative concentration-response $IC_{50}$ testing allows for flexible and accurate drug discovery screening.
- A fragment library screen identified novel ASIC1a ligand building blocks.

## References

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