Supporting Information Dataset 2

Development and validation of a potent and specific inhibitor for the CLC-2 chloride channel

Anna K. Koster[a,b], Austin L. Reese[c], Yuri Kuryshev[d], Xianlan Wen[b], Keri A. McKiernan[a], Erin E. Gray[a], Caiyun Wu[d], John R. Huguenard[c]*, Merritt Maduke[b]*, J. Du Bois[a]*

[a]*Department of Chemistry, Stanford University, Stanford, CA 94305, USA*
[b]*Department of Molecular & Cellular Physiology, Stanford University School of Medicine, Stanford, CA 94305, USA*
[c]*Department of Neurology & Neurological Sciences, Stanford University School of Medicine, Stanford, CA, USA*
[d]*Charles River Laboratories, Cleveland, OH 44128, USA*

***Corresponding Authors:** Justin Du Bois, jdubois@stanford.edu**;** Merritt Maduke, maduke@stanford.edu**;** John R. Huguenard john.huguenard@stanford.edu

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Dataset 2, Table 1. PDSP primary binding assay screening results

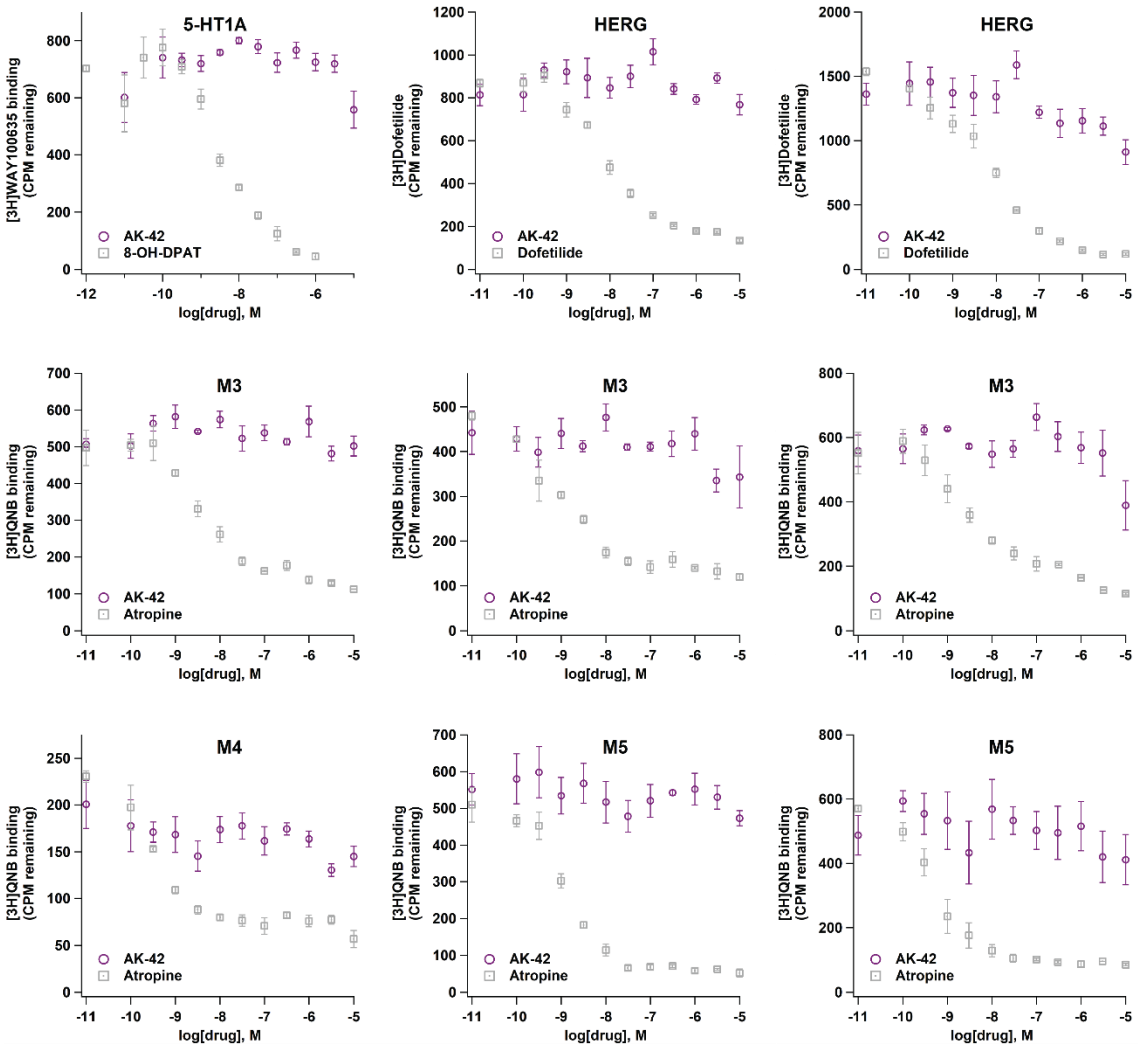
To examine the specificity of AK-42 for CLC-2 in the brain, this compound was screened against a panel of CNS receptors, transporters, and ion channels. The primary screen involved either a comprehensive binding assay (55 of 58 targets) or a functional assay (3 targets for which no binding assays were available). Results are summarized in **Dataset 2**, **Table 1**. Data for functional assays and for secondary binding assays are shown in **Dataset 2**, **Figure 1**.

Dataset 2, **Table 1.** Primary specificity-screening assay data. Compounds eliciting >50% mean effect at 10 µM (n = 4, highlighted in gray) were subjected to additional assays (raw data shown in **Dataset 2**, **Figure 1**). Entries marked with * denote targets that were tested via functional assays instead of binding assays.

Entry	Receptor	Mean % inhibition (10 µM AK-42)
1	5-HT1A	53.99
2	5-HT1B	49.89
3	5-HT1D	44.06
4	5-HT1E	10.43
5	5-HT2A	18.91
6	5-HT2B	45.16
7	5-HT2C	–13.39
8	5-HT3	28.75
9	5-HT5A	28.19
10	5-HT6	18.68
11	5-HT7	17.51
12	A2A	–8.70
13	Alpha1A	12.29
14	Alpha1B	4.90
15	Alpha1D	–10.12
16	Alpha2A	–2.55
17	Alpha2B	–28.62
18	Alpha2C	3.41
19	AMPA	28.20
20	Beta1	22.04
21	Beta2	4.77
22	Beta3	24.76
23	BZP rat brain site	29.32
24	Calcium channel	23.69
25	D1	–9.96
26	D2	7.69
27	D3	–4.66
28	D4	–0.65
29	D5	31.58
30	DAT	9.23
31	DOR	16.59
32	$GABA_A$	–4.29
33	H1	15.48

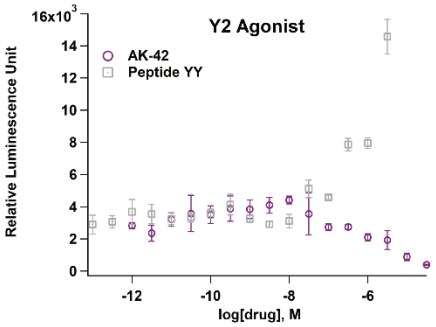
34	H2	5.35
35	H3	–0.92
36	H4	2.23
37	HERG	–43.87
38	KA	36.60
39	KOR	4.11
40	M1	11.29
41	M2	–8.25
42	M3	54.64
43	M4	56.16
44	M5	136.55
45	mGluR1*	--
46	mGluR5*	--
47	MOR	–0.18
48	NET	17.36
49	NMDA	24.18
50	NOP	21.14
51	Oxytocin	–1.41
52	PBR	8.22
53	SERT	30.25
54	Sigma 1	38.52
55	Sigma 2	–8.14
56	V1A	23.96
57	V1B	40.53
58	Y2*	--

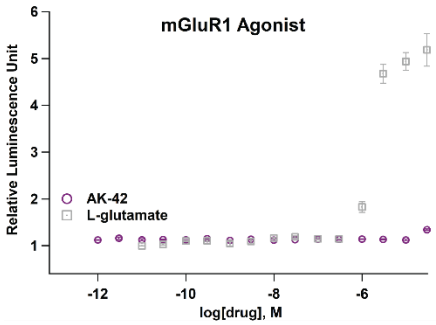
Dataset 2, Figure 1. PDSP secondary binding assays

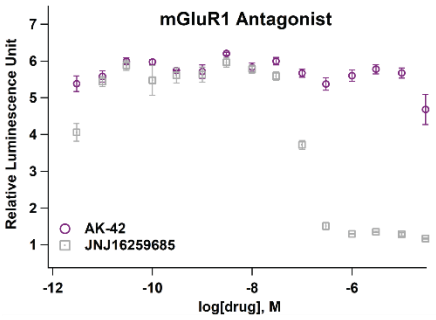


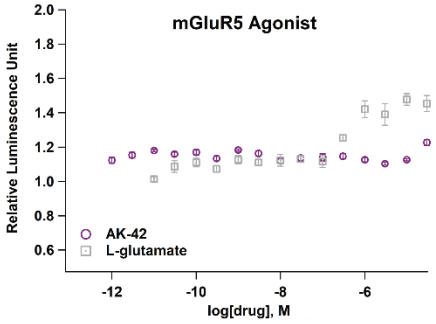
PDSP secondary binding assays. Screen results from functional secondary binding assays (5-HT1A, HERG, M3, M4, and M5) and from functional assays (mGluR1, mGluR5, and Y2). Secondary binding assays were performed in a 96-well plate format in which 12 concentrations of the test compound, ranging from 0.1 nM to 10 µM (n = 3), were evaluated relative to the same concentrations of an appropriate known receptor agonist or antagonist as a positive control (n = 3). Competitive binding of each applied compound was measured as the remaining binding of a radiolabeled ligand appropriate to the receptor of interest in counts per million (CPM). Points represent the average CPM for each compound concentration ± SEM. The radioligands and control compounds for each assay are indicated in each plot. Each plot represents data from a given day. Assays that were repeated on subsequent days are shown as replicates in individual plots (HERG, M3, and M5) as a measure of reproducibility. Functional assays were performed by measuring changes in fluorescence upon Ca^{2+} flux (mGluR1 and mGluR5) or transcriptional activation of a β-lactamase reporter construct (Y2, Tango GPCR assay) relative to a known chemical modulator. (See https://pdspdb.unc.edu/pdspWeb/ for detailed protocols.)

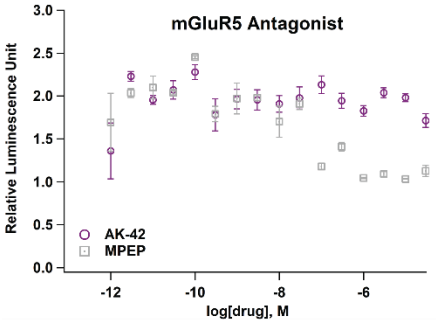
Dataset 2, Figure 1, *continued*

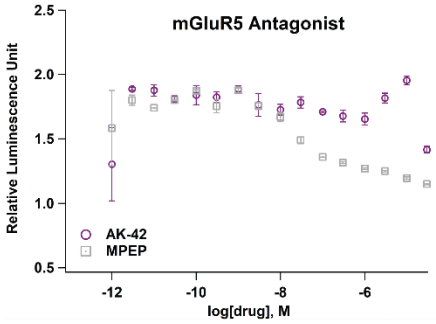






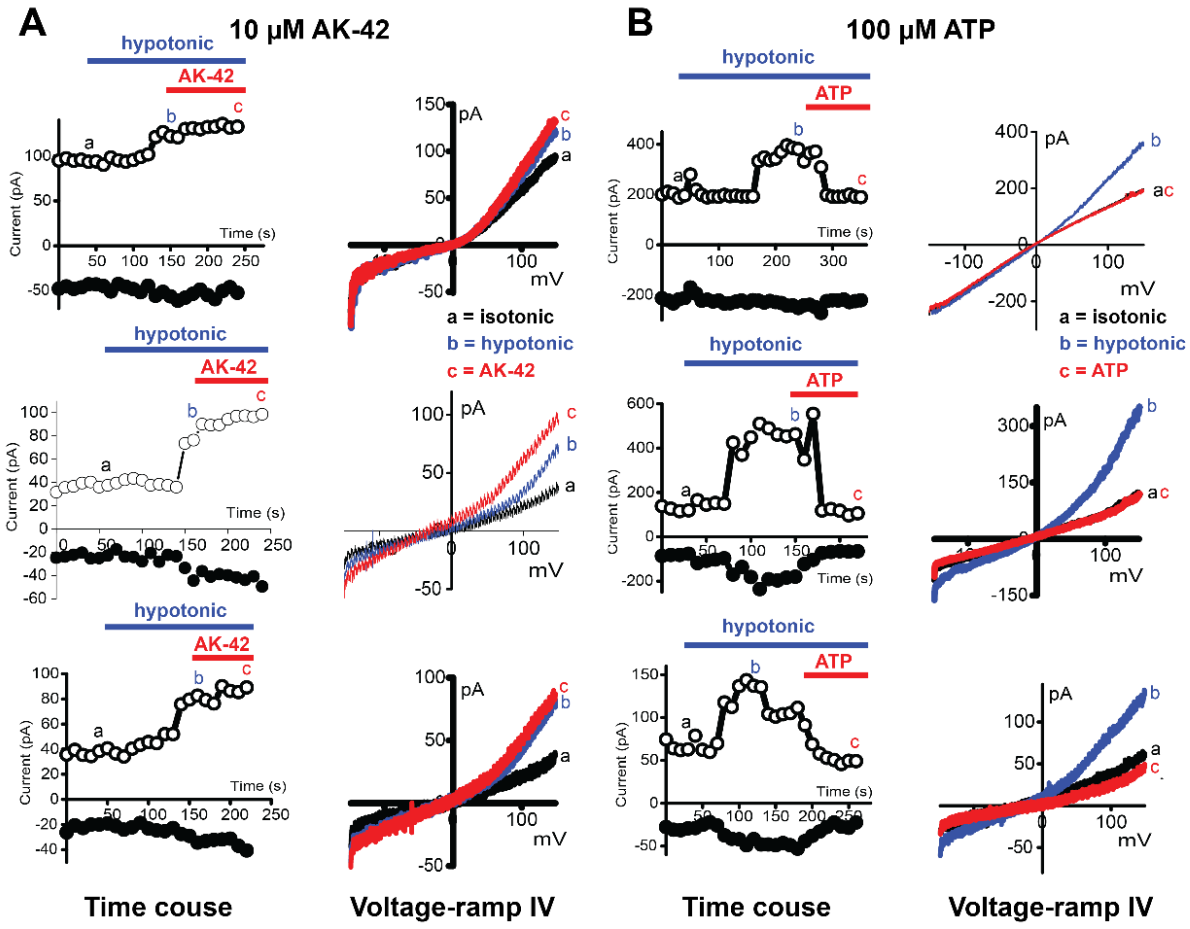






Dataset 2, Figure 2. Testing AK-42 against the VRAC anion channel.

Methods: Endogenous VRAC (volume-regulated anion channel, human *LRRC8A* gene) currents from HEK293 (ATCC® CRL-1573) cells were recorded by whole-cell patch-clamp at room temperature (20–22 ºC) according to previously reported protocols[1]. The extracellular solution contained 88 mM NaCl and 10 mM Na-HEPES with either 110 mM mannitol (isotonic, 300 m Osm/kg) or 30 mM mannitol (hypotonic, 230 mOsm/kg), pH 7.4. Internal solution contained 130 mM CsCl, 10 mM Na-HEPES, and 4 mM Mg-ATP, pH 7.3.



A) Response of VRAC currents to 10 µM of AK-42. Currents were measured in response to voltage ramps from −150 to 150 mV over 1 s, with an inter-ramp interval of 10 s and a holding potential of 0 mV. Each data point represents the current at −150 mV (closed circles) or +150 mV (open circles) at the beginning and end of each 1-second voltage ramp cycle over the time course of a 250-second experiment. Isotonic solution was applied first, followed by application of hypotonic solution at the time indicated by the

[1] a) Qui, Z. Dubin, A.E., Mather, J., Tu, B., Reddy, K. Miraglia, L.J., Reinhardt, J., Orth, A.P., Patapoutian, A. Swell1, a plasma membrane protein, is an essential component of volume-regulated anion channel. *Cell.* **2015**. 157 (2), 447-458.; b) Kefauver, J.M., Saotome, K., Dubin, A.E., Pallesen, J., Cottrell, C.A., Cahalan, S.M., Qui, Z., Hong, G., Crowley, C.S., Whitwam, T., Lee, W., Ward, A.B., Patapoutian, A. Structure of the human volume regulated anion channel. *eLife.* **2018**. 7:e38461.

Dataset 2, Figure 2, *continued*

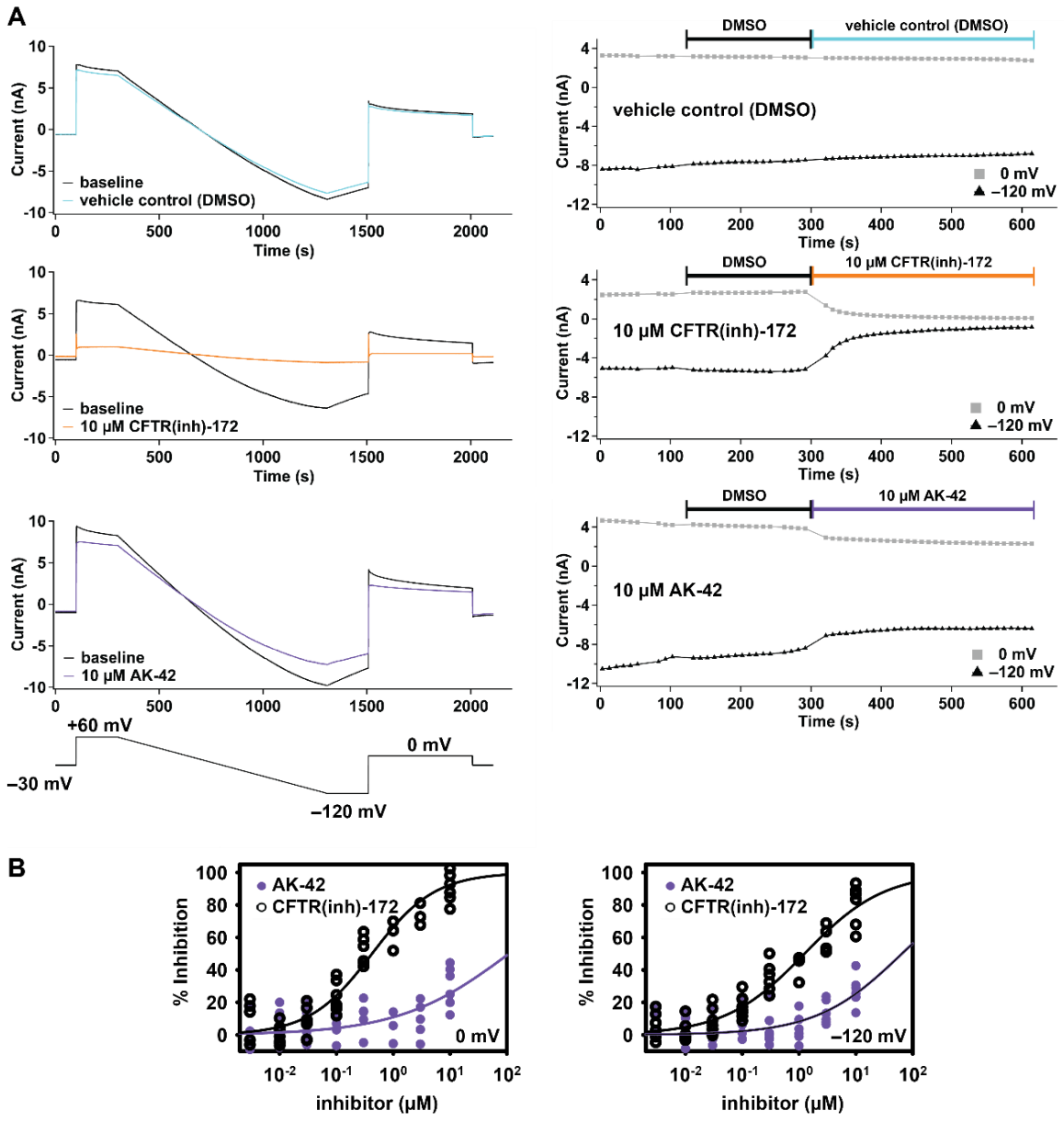
blue bar above the plot (VRAC channels are activated upon cell swelling in the presence of hypotonic solution, seen as an increase in outward current at positive +150 mV). Currents at −150 mV (closed circles) were plotted to ensure that the recording was stable throughout the experiment. At the time point indicated by the red bar above the plot, 10 µM AK-42 was perfused over the cells. Representative I-V plots for from the voltage-ramp protocol are shown (right) at the time points indicated by a (black, isotonic), b (blue, hypotonic), and c (red, AK-42) on the corresponding time course plot (left) for each of three individual cells. **B) Response of VRAC currents to 100 µM ATP.** Experiments were performed as described in (**A**) with ATP in place of AK-42. High concentrations of ATP inhibit VRAC currents and were used as a control for each batch of cells. The time courses for three individual cells are shown. These data show that 10 µM AK-42 does not inhibit VRAC currents, while the control (100 µM ATP) yields the expected inhibition. Experiments were performed on three separate batches of HEK293 cells on three separate days. Each day, one experiment was done with AK-42 and one with ATP. Several attempts were made to obtain recordings in which AK-42 and ATP were tested on the same cell (with washout in between); however, the cell swelling required for activation of VRAC channels reduces stability of the patch-clamp seal, making it challenging to record on one cell for prolonged periods of time.

Dataset 2, Figure 3. Testing AK-42 against the CFTR chloride channel.

Methods: A cell line stably expressing CFTR (CHO; human *ABCC7* gene; CRL Catalog #CT6113; GenBank accession number NM_000492.3) was constructed as described previously[2]. The cells were maintained in 100-mm cell culture dishes. The absence of mycoplasma species in these cell lines were confirmed with the MycoAlert Kit (Lonza Rockland, Inc.). Before testing, cells in culture dishes were washed twice with Hank's Balanced Salt Solution (HBSS, Life Technologies, Grand Island, NY) and treated with Accutase™ (Innovative Cell Technologies, San Diego, CA) for approximately 20 minutes. Immediately before use in SP384PE, the cells were washed in HBSS to remove the Accutase™ and re-suspended in HEPES-Buffered Physiological Saline (HB-PS); extracellular solution (composition is indicated below). All experiments were performed at ambient temperature. Cells were recorded using the APC system - SyncroPatch™ 384PE (SP384PE; Nanion Technologies, Livingston, NJ) with the following intracellular (in mM: CsCl, 10; CsF, 110; NaCl, 10; $MgCl_2$, 2; EGTA, 2.5; HEPES, 10; pH adjusted to 7.2 with CsOH) and extracellular (in mM: HB-PS (composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 3.8; $MgCl_2$, 1; HEPES, 10; glucose, 10; pH adjusted to 7.4 with NaOH, refrigerated until use) buffers. For each experiment, HEPES-buffered intracellular solution for the whole cell recording was loaded into the intracellular compartment of Nanion 384-well Patch Clamp chip (NPC), and extracellular buffer (HB-PS) was loaded into NPC wells (60 µL per well). The cell suspension was pipetted into the wells of the NPC (20 µL per well). After establishment of the whole-cell configuration, membrane currents were recorded using patch-clamp amplifiers of SP384PE system. Either AK-42 or a control inhibitor (CFTR(inh)-172) was applied to naïve cells (n = 8, where n = replicates/ concentration). Each application consisted of addition of 40 µL of 2× concentrated inhibitor solution to the total 80 µL of final volume of the extracellular well of the NPC electrode. Duration of exposure to each compound concentration was 5 minutes.

[2] Wible B.A., Kuryshev Y.A., Smith S.S., Liu Z., Brown A.M.: An ion channel library for drug discovery and safety screening on automated platforms. *Assay Drug Dev Technol*. **2008**, 6:765–780.

Dataset 2, Figure 3, *continued*

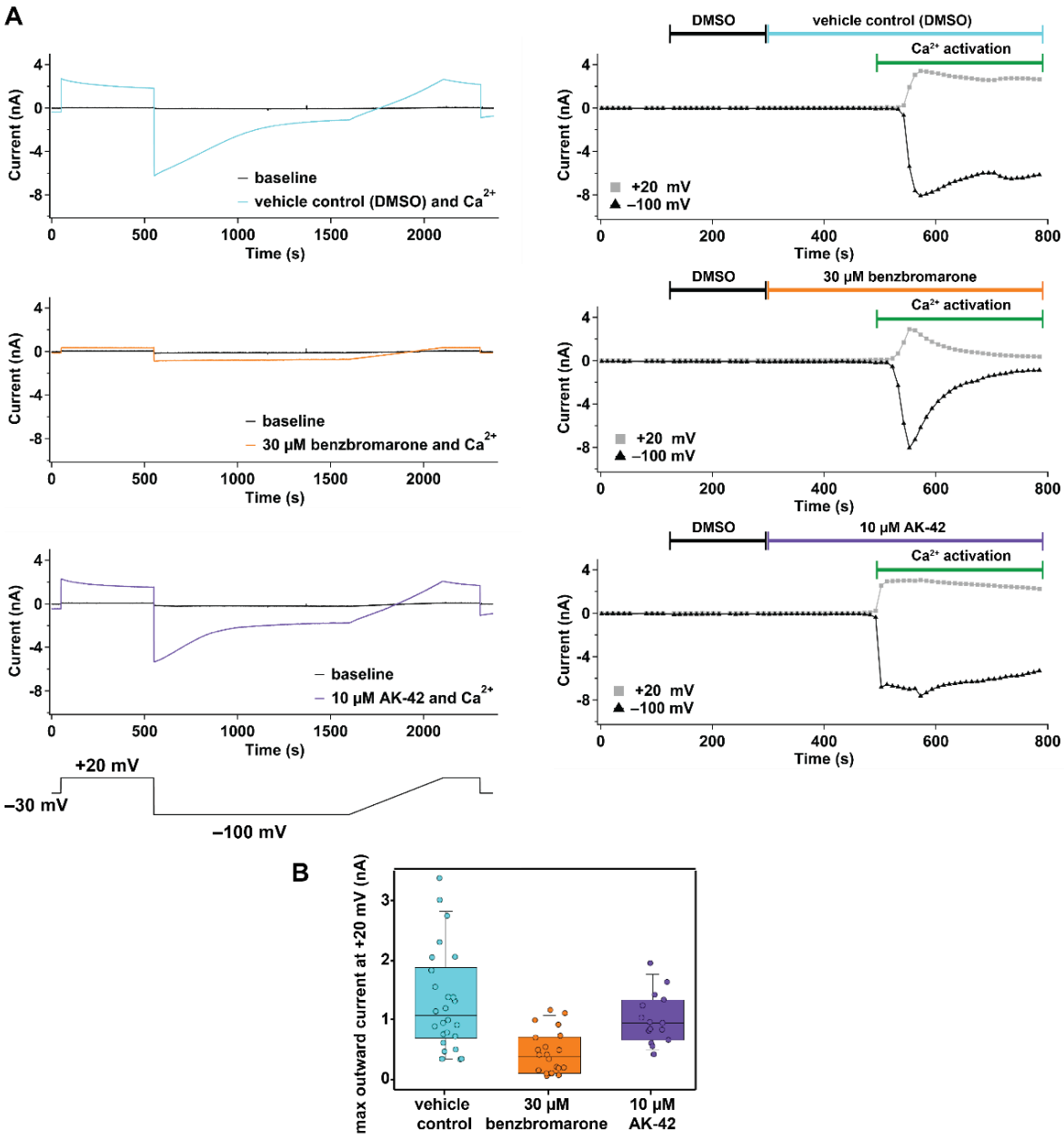


A) Evaluation of AK-42 effects on CFTR currents. *Left:* Representative CFTR current traces, elicited with the voltage ramp illustrated below, before (black) and after application of vehicle control, CFTR-inhibitor control, or AK-42 (cyan, orange, or purple, respectively). *Right:* Maximum currents at 0 and –120 mV recorded as the ramp protocol was repeated with a stimulation frequency of 0.1 Hz. DMSO (0.3%, baseline) was applied at the time indicated by the black bar, followed by application of the vehicle control (0.3% DMSO, cyan), positive control (10 μM CFTR(inh)-172, orange), or AK-42 (10 μM, purple). **B) Dose-response summary data for AK-42 against CFTR.** Inhibition determined based on peak current at 0 mV (*left*) or –120 mV (*right*) after 5-minute application of AK-42 or control inhibitor (n = 5–8, individual points shown). Inhibition with 10 μM of AK-42 (purple, closed circle) has weak effects (<30% inhibition) on CFTR currents at 0 and –120 mV relative to the control inhibitor CFTR(inh)-172 (black, open circles).

Dataset 2, Figure 4. Testing AK-42 against the TMEM16A anion channel.

Methods: Cell line stably expressing TMEM16A(abc) (HEK293; human *ANO1* gene; CRL Catalog #CT6612; GenBank accession number XM_006718598) ion channels was constructed according to methods described previously[2]. The cells were maintained in 100-mm cell culture dishes. The absence of mycoplasma species in these cell lines were confirmed with the MycoAlert Kit (Lonza Rockland, Inc.). Before testing, cells in culture dishes were washed twice with Hank's Balanced Salt Solution (HBSS, Life Technologies, Grand Island, NY) and treated with Accutase™ (Innovative Cell Technologies, San Diego, CA) for approximately 20 minutes. Immediately before use in SP384PE, the cells were washed in HBSS to remove the Accutase™ and re-suspended HEPES-Buffered Physiological Saline (HB-PS); extracellular solution, composition is indicated below. All experiments were performed at ambient temperature. Cells were recorded using the APC system - SyncroPatch™ 384PE (SP384PE; Nanion Technologies, Livingston, NJ) with the following solutions: baseline intracellular (in mM: KCl, 50; KF, 40; K_2SO_4, 50; $MgCl_2$, 5; EGTA, 2.5; HEPES, 10; pH adjusted to 7.2 with KOH; nominally Ca^{2+} free (<0.1 µM). In preparation for a recording session, this intracellular solution was loaded into the intracellular compartment of NPC-384 chip); activating intracellular (in mM: KCl, 50; KF, 40; K_2SO_4, 50; $MgCl_2$, 4.7; $CaCl_2$, 0.575; EDTA, 1.0; HEPES, 10; pH adjusted to 7.2 with KOH; ~0.05 mM free Ca^{2+}. After baseline recording, the activating intracellular solution was applied to the cells); extracellular HB-PS (in mM: NaCl, 137; KCl, 4.0; $CaCl_2$, 3.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH). HEPES-buffered baseline intracellular solution for the whole-cell recording was loaded into the intracellular compartment of Nanion 384-well Patch Clamp chip (NPC). Extracellular buffer (HB-PS) was loaded into NPC wells (60 µL per well). The cell suspension was pipetted into the wells of the NPC (20 µL per well). After establishment of the whole-cell configuration, membrane currents were recorded using patch clamp amplifiers of SP384PE system. Either AK-42 or a control inhibitor, benzbromarone, was applied to naïve cells (n = 32, where n = replicates/ concentration). Each application consisted of addition of 40 µL of 2× concentrated inhibitor solution to the total 80 µL of final volume of the extracellular well of the NPC electrode. Duration of pre-treatment with each compound was 3 minutes.

Dataset 2, Figure 4, *continued*



A) Evaluation of AK-42 effects on TMEM16A currents. *Left:* Representative TMEM16A current traces following Ca^{2+} activation, elicited using the voltage protocol shown in the bottom panel. *Right:* Peak currents at +20 and –100 mV recorded as the ramp protocol was repeated with a stimulation frequency of 0.1 Hz. DMSO (0.3%, baseline) was applied at the time indicated by the black bar, immediately followed by application of the vehicle control (0.3% DMSO, cyan bar), positive control (30 µM benzbromarone, orange bar), or AK-42 (10 µM, purple bar). TMEM16A was then activated for 3 minutes by application of intracellular Ca^{2+} (green bar). **B) Summary Data.** TMEM16A current amplitudes at +20 mV following treatment with vehicle control (0.3% DMSO, cyan, n = 26), positive control (30 µM benzbromarone, orange, n = 22), and AK-42 (10 µM, purple, n = 15) and Ca^{2+} activation (as in (**A**)). Individual data points and box plots are shown. P-values (P = 0.000028 for benzbromarone and 0.33 for AK-42 vs. vehicle control) were determined using One-Way ANOVA, Tukey *post hoc* test.