Supporting information methods, figures and tables to:

Identification of potent and selective small molecule inhibitors of the cation channel TRPM4

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Methods:

Compound 5 selectivity profiling: Patch clamp studies on TRP family members.

TRPM7:

TRPM7 currents were evoked in HEK-293 cells stably expressing tetracycline-inducible, human TRPM7 protein described earlier (Schmitz et al., 2003). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), blasticidin (5 μg/ml) and zeocin (0.4 mg/ml). TRPM7 expression was induced 14 - 20hours before performing experiments by adding 1 μg/ml tetracycline to the culture medium. Whole cell currents were recorded using a 200 ms ramp from -100 to 120 mV every 2 sec from a holding potential of 0 mV. The currents were recorded with a HEKA patch clamp system (EPC-10, HEKA Elektronik Dr. Schutze GmbH, Rhein, Germany). Internal solution contained (in mM): 140 Cs-Glutamate, 8 NaCl, 10 HEPES, 5 EDTA and bath solution (in mM): 120 NaCl, 2.8 KCl, 2 MgCl2, 10 HEPES. Osmolarity was adjusted with glucose to 300 mosm and pH to 7.2 with CsOH. Inhibition of the current from TRPM7 by **5** was tested at 100 μM concentration. Stock solution of **5** was prepared in DMSO at 10 mM and diluted in bath solution to acquire final concentration. Compound **5** was bath-applied to the patched-cell using a custom made perfusion unit. Peak currents at +80 mV after application of either **5** or DMSO were normalized to peak current before application.

TRPM8:

TRPM8 currents were recorded from HEK-293 cells transiently transfected with plasmids (pcDNA5FRTTO Invitrogen) encoding the human isoform of TRPM8. Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 4 mM glutamine and 1% penicillin streptomycin. The cells were transfected and plated on 35 mm dishes and incubated 24 hours at 37 °C with 5% CO_2 prior to recording. Whole cell currents were recorded continuously from a holding potential

of -80 mV. The bath chamber was continuously perfused with a bath solution consisting of (mM) 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, HEPES 10 adjusted to pH 7.40 (NaOH). The pipette solution contained (mM) CsF 60, CsCl 50, NaCl 10, EGTA 20, HEPES 10 adjusted to pH 7.20 (CsOH). The channel was activated upon fast application of menthol (100 µM). Inhibition of the current from TRPM8 by **5** was tested at 10 µM concentration. All drug solutions were bath-applied to patch-clamped cell by means of a gravity driven rapid solution exchanger (2ms, RSC-200, Bio-Logic). Stock solutions of menthol (Sigma Aldrich, 63660-100g, BCBR7346V) and **5** were prepared in DMSO at 100mM and 10 mM respectively. Peak currents after application of either **5** or DMSO were normalized to peak current before application.

TRPV1:

TRPV1 currents were recorded from HEK-293 cells transiently transfected with plasmids (pcDNA5FRTTO Invitrogen) encoding the human isoform of TRPV1. Cells were cultured at 37 °C in DMEM supplemented with 10% FBS, 4 mM glutamine and 1% penicillin streptomycin. The cells were transfected and plated on 35 mm dishes and incubated 24 hours at 37 °C with 5% CO_2 prior to recording. Whole cell currents were recorded continuously from a holding potential of -80 mV. The bath chamber was continuously perfused with a bath solution consisting of (mM) 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, HEPES 10 adjusted to pH 7.40 (NaOH). The pipette solution contained (mM) CsF 60, CsCl 50, NaCl 10, EGTA 20, HEPES 10 adjusted to pH 7.20 (CsOH). The channel was activated upon fast application of capsaicin (10µM). Inhibition of the current from TRPV1 by **5** was tested at 10 µM concentration. All drug solutions were bath-applied to patch-clamped cell by means of a gravity driven rapid solution exchanger (2ms, RSC-200, Bio-Logic). Stock solution of capsaicin (Sigma Aldrich, M2028-50mg, 106K7060) and **5**

were prepared in DMSO at 10 mM. Peak currents after application of either **5** or DMSO were normalized to peak current before application.

TRPV6:

TRPV6 currents were evoked in HEK-293 cells stably expressing TRPV6 protein (provided by Prof. Matthias Hediger, University of Bern, Switzerland). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin and 0.5 mg/ml G418. Whole cell currents from TRPV6 expressing cells were performed as described for LNCaP. Internal solution contained 120 Cs-Glutamate, 10 HEPES, 3 $MgCl_2$, 10 BAPTA and bath solution contained 120 NaCl, 10 HEPES, 10 TEA-Cl, 2 $MgCl_2$, 10 $CaCl_2$. Osmolarity was adjusted with glucose to 300 mosm and pH to 7.2 with CsOH. Inhibition of the current from TRPV6 by **5** was tested at 100 µM concentration. Stock solution of **5** was prepared in DMSO at 10 mM and diluted in bath solution with 10 mM Ca^{2+} to acquire final concentration. Compound **5** was bath-applied to the patched-cell using a custom made perfusion unit. Peak currents at -80 mV after application of either **5** or DMSO were normalized to peak current before application.

All values are presented as mean ± SEM of atleast n = 4 independent cells. Statistically significant difference between means was determined using student *t* test for comparison.

Compound 5 selectivity profiling: Radioligand binding assay

For the radioligand binding assay, compound **5** binding was calculated as percentage inhibition of the binding of a radioactively labeled ligand specific for each target. For the cellular and nuclear functional assays, the cellular agonist effect of **5** was calculated as a percentage of control response to a known reference agonist for each target and cellular antagonist effect of **5** was calculated as percentage inhibition of control reference agonist response for each target.

Binding of **5** was measured at a single concentration of 10 μM in accordance with CEREP's validation standard operating procedure (CEREP, Celle L'Evescault, France; www.cerep.fr). Reference and test compounds were tested in each experiment, and the data were compared with historical values determined at CEREP.

Binding of **5** was measured at 0.1, 1and 10 μM in duplicates on the hERG channel through automated whole-cell patch clamp, in accordance with CEREP's validation standard operating procedure (CEREP, St. Charles, USA; www.cerep.fr). Reference and test compound were tested in each experiment, and the data were compared with historical values determined at CEREP. An inhibition percentage of 2.0, 6.7 and 10.5 were obtained, respectively.

Cytosolic application of compound 5 and 4:

Cells were plated in 35 mm poly-D-lysine coated dishes (Corning, NY, USA) in induction medium (phenol red free DMEM medium (GIBCO-31053)) supplemented with 10 % FBS and 1 μg/mL tetracycline (Invitrogen, USA). After plating, cells were incubated for 48 hours at 37 °C in a 5% CO_2 incubator. Electrophysiological recordings were performed in the inside-out patch clamp configuration with patch pipettes (1-2 μm tip opening) pulled from 1.5 mm borosilicate glass capillaries (World Precision Instruments, Inc. Fl, USA) using DMZ Universal puller (Zeitz-Instruments, GmbH, München, Germany). Pipette tips were polished to have a pipette resistance of 2–4 M Ω in the bath solution. The pipette solution contained (in mM) 150 NaCl, 10 HEPES, 2 $CaCl_2$ (pH 7.4 with NaOH). The bath solution contained (in mM) 150 NaCl 10 HEPES, 2 HEDTA (pH 7.4 with NaOH) as 0 μM Ca^{2+} or 150 NaCl, 10 HEPES and 300 μM Ca^{2+}(pH 7.4 with NaOH) as 300 μM Ca^{2+} solution. Bath solutions with either 0 or 300 μM Ca^{2+} concentrations were applied to cytosolic side of the cells by a modified rapid solution exchanger

(Perfusion Fast-Step SF-77B; Warner Instruments Corp. CT, USA). Inhibitors in DMSO stock were diluted to appropriate concentrations in the 300 µM Ca^{2+}solution and applied on cytosolic side of the cells using the rapid solution exchanger. Membrane currents were recorded with a Multiclamp 700B amplifier (Molecular devices, CA, USA) controlled by Clampex 10 via a Digidata 1332A (Molecular devices, CA, USA) using a single voltage step protocol of 200 ms duration from -100 to +100 mV. Data were low-pass filtered at 5 kHz and sampled at 10 kHz. Peak currents were measured at the end of voltage step to + 100 mV. Experiments were performed at RT (20–25 °C).

Rescue of WT-TRPM4 with compound 5

HEK293 cells were transiently transfected with 300 ng (WT 100%), or 150 ng (WT 50%) of HA-TRPM4 WT plasmid, in a P100 dish (BD Falcon, Durham, North Carolina, USA) mixed with 30 µL of JetPEI (Polyplus transfection, Illkirch, France) and 250 µL of 150 mM NaCl. 24 hours after transfection, 50% WT cells were incubated with 50 uM compound 5 or incubated at 28C. TRPM4 expression was assessed using Western Blot technique as described previously.

Figure S1: *TRPM4 currents in excised membrane patches:* Representative current traces from excised membrane patches when exposed to either 0 (black) or 500 µM Ca^{2+} (blue) from TRPM4 expressing cells either induced with tetracycline or non-induced and TRPM4 CRISPR-Cas9 knocked down cell line (KD). Steady state IVs was constructed from currents at the end of 200 ms voltage steps (inset) (n = 3).

Figure S2: *Assay validation* **A)** Overlay of traces obtained on the FLIPR from TRPM4-expressing cells induced with ionomycin (red, signal) or without ionomycin (black, background). **B)** Z` prime factor and ratio of AUC from signal over background (S/B) calculated over 24 plates on different days of experiment were plotted on left and right Y-axis, respectively. **C)** Overlay of traces with different extracellular vehicle DMSO concentrations. **D)** Average AUC after incubation with varying DMSO concentrations (n = 3).

Figure S3: *Concentration-response studies.* Average current traces recorded from excised membrane patches in voltage clamp with either vehicle control or inhibitor at different concentrations. $n \geq 4$ for each concentrations of different inhibitors.

Figure S4: *Cytosolic application of [5] and [4]*. Representative current traces from excised membrane patches on TRPM4 expressing cells when exposed to either 0 or 300 µM Ca^{2+}. Furthermore, on the same cells, the currents were inhibited by cytosolic application of either 1 µM [5] **(A)** or 1 µM [4] (**B**) and subsequently washed out ($n \geq 6$).

Figure S5: *Compound **5** TRP selectivity.* **A)** *Left,* representative whole-cell current density trace at +80 mV from HEK cells stably expressing TRPM7 either induced with tetracyline or non-induced. After the currents were fully developed, either DMSO or 100 µM compound **5** were applied in bath solution. *Right*, the currents recorded in presence of DMSO (grey) or compound **5** (red) was normalized to the current before application on the same cell. **B)** *Left*, representative whole cell current density trace at –80 mV evoked by 100 µM menthol from HEK cells transiently transfected with TRPM8. The cells were later applied with either DMSO or 10 µM compound **5** . *Right,* the peak currents recorded in presence of DMSO (grey) or compound **5** (red) were normalized to the current before application on the same cell. **C)** *Left,*representative whole cell current density trace at – 80mV evoked by 10 µM capsaicin from HEK cells transiently transfected with TRPV1. *Right,* the peak currents recorded in presence of DMSO (grey) or compound **5** (red) were normalized to the current before application on the same cell. **D)** *Left*, representative whole-cell current density trace at -80 mV from HEK cells stably expressing TRPV6. Inward currents were observed with increase of Ca^{2+} to 10 mM in the bath solution. After the currents were fully developed, either DMSO or 100 µM compound **5** were applied in bath solution. *Right*, the currents recorded in presence of DMSO (grey) or compound **5** (red) was normalized to the current before application on the same cell.

Figure S6: *Specificity of compound* **5** *in rescuing A432T loss-of-expression variant.* **A)** Western blot analysis showing total expression rescue of A432T variant after pre-incubation at 28 °C. **B)** Western blot analysis shows that compound **5** failed to rescue membrane expression of Dupl hERG variant, whereas the variant was successfully rescued by pre-incubation at 28 °C. **C)** Western blot analysis showing membrane expression rescue of WT TRPM4 after pre-incubation at 28 °C. **D)** Western blot analysis showing total expression rescue of WT TRPM4 after pre-incubation with 50 μM [**5**]. The protein band intensity at each concentration was quantified from 3 individual experiments. * $P \leq 0.05$, Student *t* test.

Figure S7: *Cytotoxicity study of the most potent inhibitors*. An MTT viability assay was performed for **4**, **5** and **6** on HeLa cells. The data represent the means of three values and are normalized against DMSO control.

Supplement table S1. Anthranilic acid variations: from top to bottom, isosteric substitution of the chlorine group and variation of the carboxylic acid's position. a) Racemic mixture. b) Activity on TRPM4 given as Normalized counts of Na^+ influx at 5 μM. Values above 0 are active.

Supplement table S2. From top to bottom, substitution variations and bicyclic variations of the phenoxy moiety. a) Racemic mixture. b) Activity on TRPM4 given as normalized counts of Na^+ influx at 5 μM. Values above 0 are active.

Supplement table S3. Acetyl linker modifications. a) Racemic mixture. b) Activity on TRPM4 given as Normalized counts of Na^+ influx at 5 μM. c) The enantiomerically pure isomers of the racemic **4** were separated by chiral HPLC. d) Activity measured at 10 μM.

Supplement table S4: *In vitro pharmacology profiling of* **5** *on several ion channels.* a) Compound **5** was tested on radio ligand binding assay at 10 μM. b) Compound **5** was tested on cellular and nuclear functional assays at 10 μM. The hERG channel inhibition was also tested on automated whole-cell patch clamp at 10 μM. Inhibition below 50% is considered not significant. The data represent the mean of two experiments. No significant inhibition (NSI).

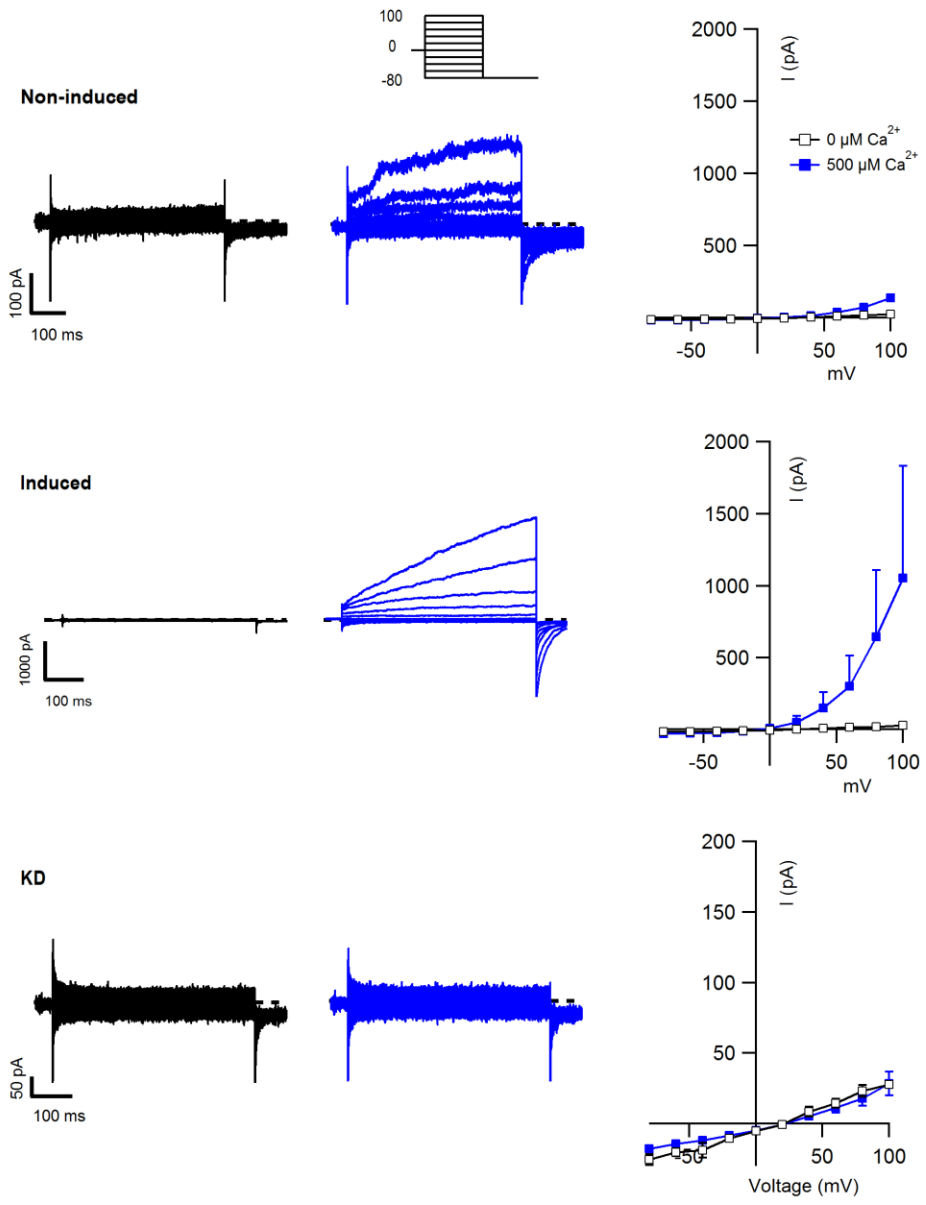

Non-induced
100
0
-80
100 pA
100 ms
2000
1500
1000
500
I (pA)
0 µM Ca2+
500 µM Ca2+
-50
50
100
mV
Induced
1000 pA
100 ms
2000
1500
1000
500
I (pA)
-50
50
100
mV
KD
50 pA
100 ms
200
150
100
50
I (pA)
-50
50
100
Voltage (mV)

Figure S1:

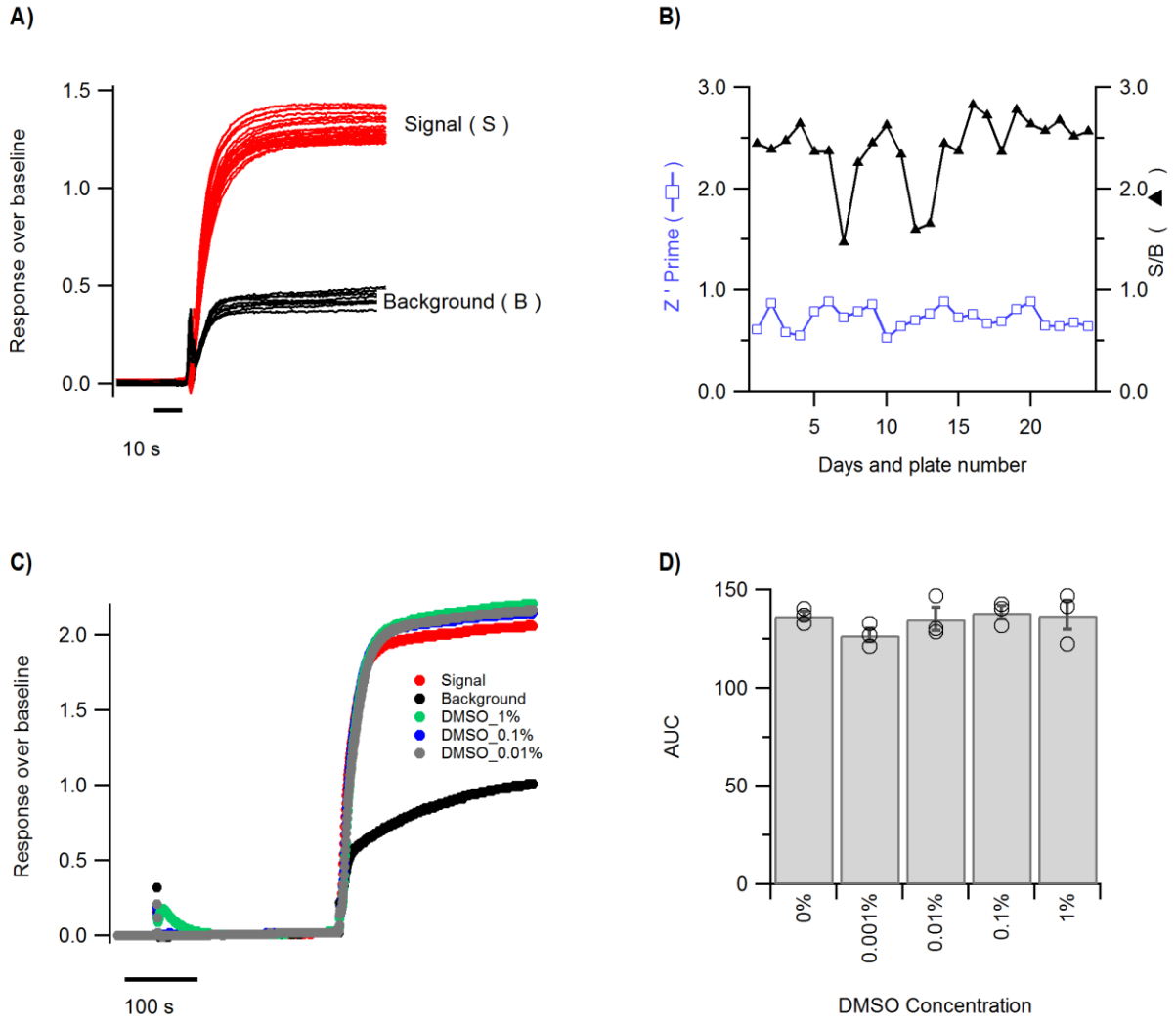

A)
Response over baseline
1.5
1.0
0.5
0.0
Signal (S)
Background (B)
10 s
B)
Z ' Prime ( )
S/B ( )
3.0
2.0
1.0
0.0
5
10
15
20
Days and plate number
C)
Response over baseline
2.0
1.5
1.0
0.5
0.0
Signal
Background
DMSO_1%
DMSO_0.1%
DMSO_0.01%
100 s
D)
AUC
150
100
50
0
0%
0.001%
0.01%
0.1%
1%
DMSO Concentration

Figure S2:

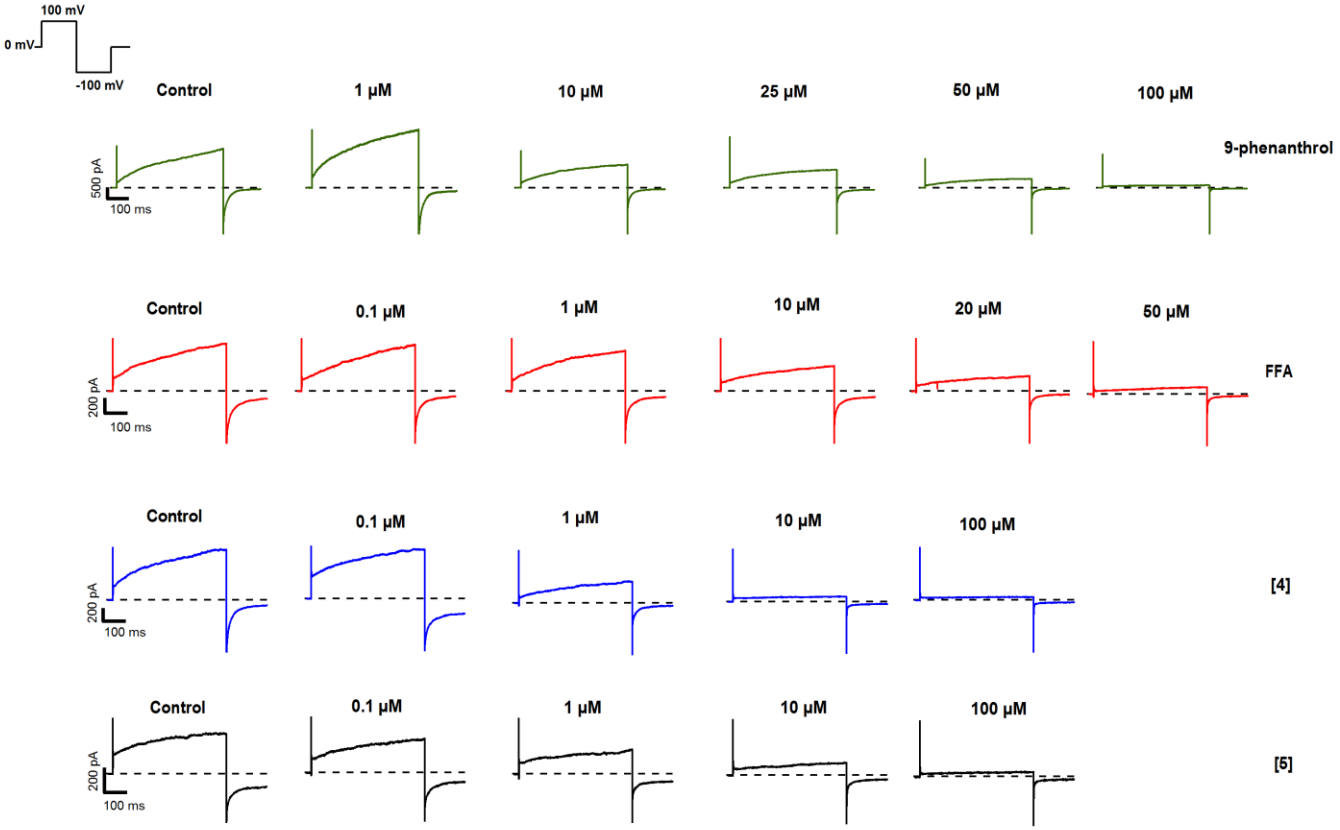

100 mV
0 mV
-100 mV
Control
1 µM
10 µM
25 µM
50 µM
100 µM
9-phenanthrol
500 pA
100 ms
Control
0.1 µM
1 µM
10 µM
20 µM
50 µM
FFA
200 pA
100 ms
Control
0.1 µM
1 µM
10 µM
100 µM
[4]
200 pA
100 ms
Control
0.1 µM
1 µM
10 µM
100 µM
[5]
200 pA
100 ms

Figure S3:

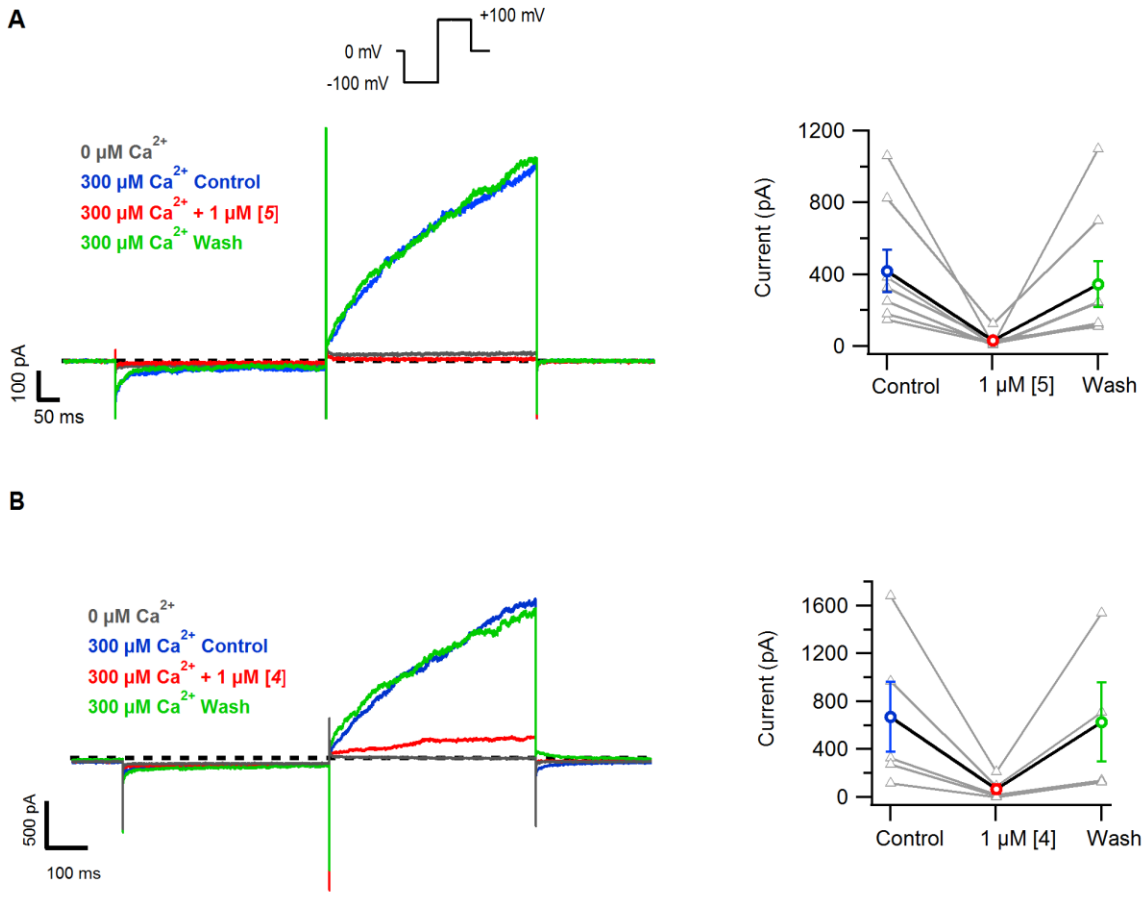

A
+100 mV
0 mV
-100 mV
0 µM Ca2+
300 µM Ca2+ Control
300 µM Ca2+ + 1 µM [5]
300 µM Ca2+ Wash
100 pA
50 ms
Current (pA)
1200
800
400
0
Control
1 µM [5]
Wash
B
0 µM Ca2+
300 µM Ca2+ Control
300 µM Ca2+ + 1 µM [4]
300 µM Ca2+ Wash
500 pA
100 ms
Current (pA)
1600
1200
800
400
0
Control
1 µM [4]
Wash

Figure S4:

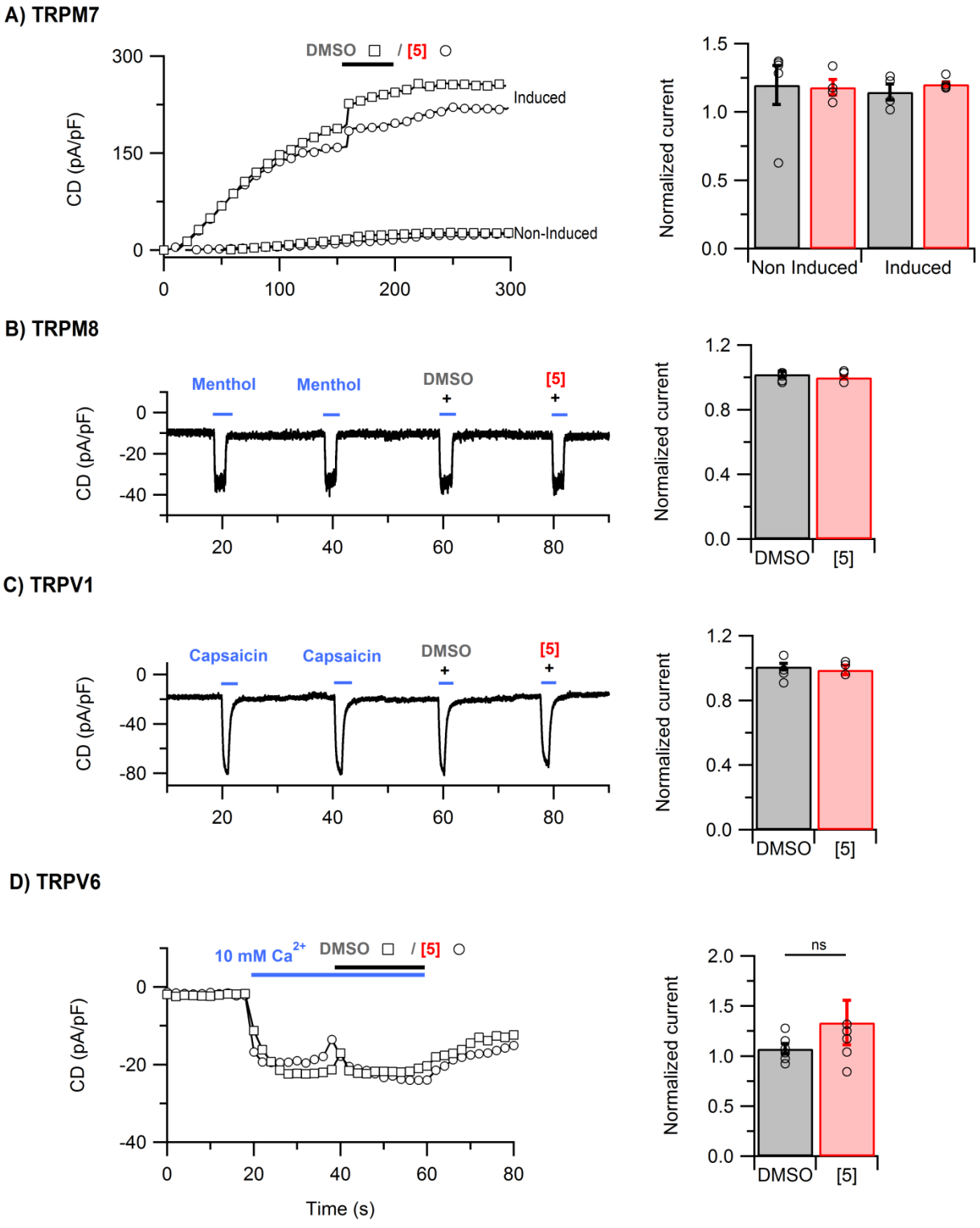

A) TRPM7
DMSO □ / [5] ○
Induced
Non-Induced
CD (pA/pF)
Normalized current
Non Induced
Induced
B) TRPM8
Menthol
Menthol
DMSO
[5]
C) TRPV1
Capsaicin
Capsaicin
D) TRPV6
10 mM Ca2+
ns
Time (s)

Figure S5:

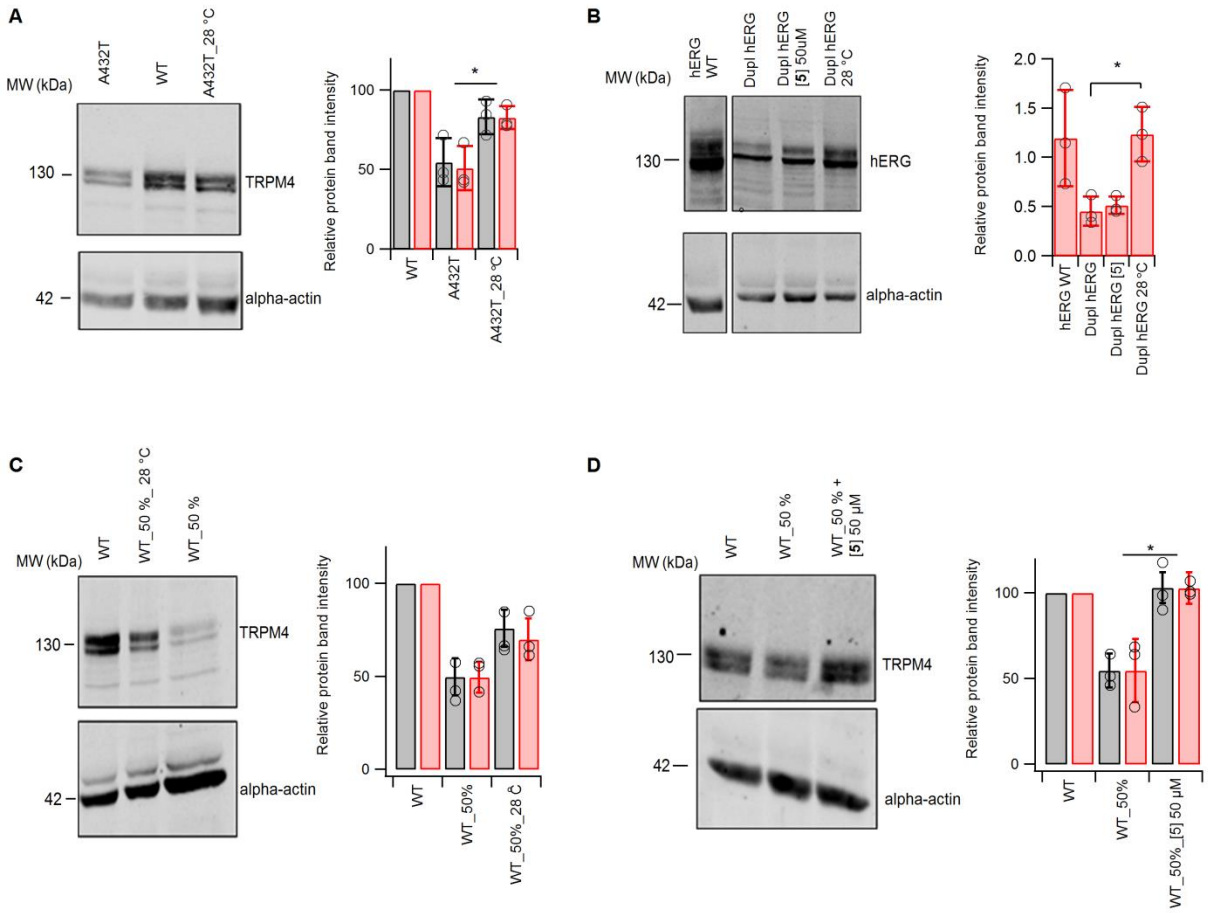

A
MW (kDa)
A432T
WT
A432T_28 °C
130
TRPM4
42
alpha-actin
Relative protein band intensity
100
50
0
*
WT
A432T
A432T_28 °C
B
MW (kDa)
hERG WT
Dupl hERG
Dupl hERG [5] 50uM
Dupl hERG 28 °C
130
hERG
42
alpha-actin
Relative protein band intensity
2.0
1.5
1.0
0.5
0.0
*
hERG WT
Dupl hERG
Dupl hERG [5]
Dupl hERG 28 °C
C
MW (kDa)
WT
WT_50 %_ 28 °C
WT_50 %
130
TRPM4
42
alpha-actin
Relative protein band intensity
100
50
0
WT
WT_50%
WT_50%_28 C
D
MW (kDa)
WT
WT_50 %
WT_50 % + [5] 50 µM
130
TRPM4
42
alpha-actin
Relative protein band intensity
100
50
0
*
WT
WT_50%
WT_50%_[5] 50 µM

Figure S6:

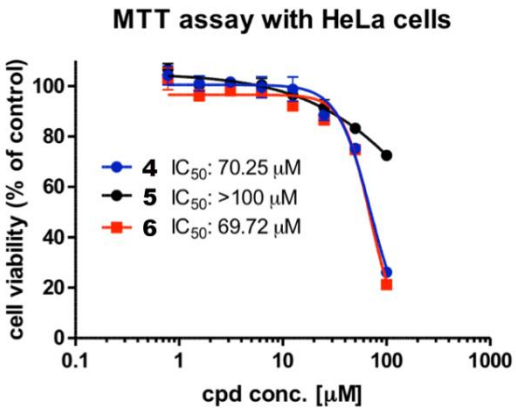
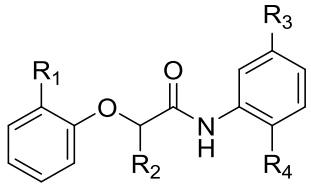
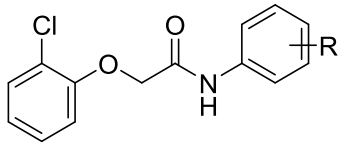

MTT assay with HeLa cells
cell viability (% of control)
100
80
60
40
20
0
0.1
1
10
100
1000
cpd conc. [µM]
4 IC50: 70.25 µM
5 IC50: >100 µM
6 IC50: 69.72 µM

Figure S7:

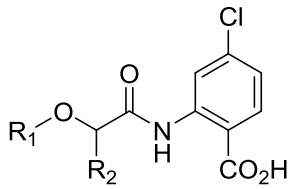


ID	R_1	R_2	R_3	R_4	Normalized counts[b]
17	Cl	H	Cl	CN	1
18	Cl	H	Cl	$CONHCH_3$	-7
19	Cl	H	Cl	NO_2	-2
20	Cl	H	Cl	NH_2	7
21	Cl	H	H	SO_2OH	1
22	Cl	H	H	SO_2NH_2	14
23	Cl	H	Cl	1H-tetrazol-5-yl	-20
24	Cl	H	Cl	5-methyl-1,2,4-oxadiazol-3-yl	-3
25	Cl	H	F	COOH	68
11	Cl	H	Br	COOH	67
26[a]	H	CH_3	OH	COOH	2
27	Cl	H	CH_3	COOH	-3
28	Cl	H	CF_3	COOH	37
29	Cl	H	COOH	COOH	4



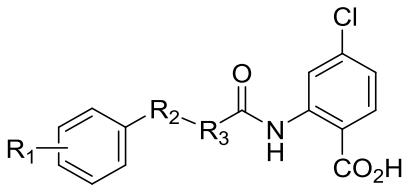
ID	R	Normalized counts[b]
30	4-COOH	29
31	3-COOH	10
32	3-Cl-4-COOH	14

Table S1:



ID	R_1	R_2	Normalized counts[b]
33[a]	phenyl	CH_3	42
12[a]	2,4-diCl-phenyl	CH_3	84
13	2,4-diBr-phenyl	H	97
14	2,4-diF-phenyl	H	92
34	2,5-diCl-phenyl	H	91
35	2,6-diCl-phenyl	H	64
15	2-Cl-5-NO_2-phenyl	H	70
36	4-NH_2-phenyl	H	-7
37	4-*t*Bu-phenyl	H	84
38	2-phenylphenyl	H	86
16	3-phenylphenyl	H	81
39	4-phenylphenyl	H	55
6	naphthalen-1-yl	H	92
7	4-Cl-naphthalen-1-yl	H	84
10	5,6,7,8-tetrohydronaphthalen-1-yl	H	93
40	isoquinilin-5-yl	H	-7
41	quinolin-8-yl	H	11
42	quinolin-5-yl	H	43
43	quinolin-4-yl	H	-10
44	quinolin-2-yl	H	-1
45	quinazolin-4-yl	H	16
46	5-bromoquinolin-8-yl	H	0
47	2-oxo-1,2-dihydroquinolin-8-yl	H	2
48	phenantren-9-yl	H	31
49	1*H*-indazol-5-yl	H	44

Table S2:



ID	R_1	R_2	R_3	Normalized counts[b]
50[a]	2-Cl	O	$CHCH_3$	40
51	2-CH_3-4-Cl	O	CH_2	54
52[c]	2-CH_3-4-Cl	O	$CHCH_3$	92
53[c]	2-CH_3-4-Cl	O	$CHCH_3$	85
54	2-Cl	CH_2	CH_2	55
55	2-Cl	S	CH_2	48
56	2-Cl	CH_2	NH	-12[d]
57	2-Cl	CH_2	O	14[d]
58	H	CH_2	O	58[d]

Table S3:

Channel	% Inhibition of control specific binding at 10 µM[a]
GABAA1 (agonist radioligand) (h)	NSI
NMDA (antagonist radioligand)	8.0 ± 13
N neuronal α4β2 (agonist radioligand) (h)	-11.8 ± 0.8
P2X (agonist radioligand)	-5.0 ± 9.3
Ca^{2+} channel, L-type, dihydropyridine site (antagonist radioligand)	-13.3 ± 7.1
Ca^{2+} channel, L-type, diltiazem site (antagonist radioligand)	-8.2 ± 11.6
Ca^{2+} channel, L-type, verapamil site (antagonist radioligand)	-0.1 ± 11
Ca^{2+} channel, N-type (antagonist radioligand)	-27.9 ± 4.7
hERG (h)	-5.1 ± 3.5
hERG (CHO)	10.5 ± 4.0
Kv channel (antagonist radioligand)	-12.3 ± 16
Na^{+} channel, site 2 (antagonist radioligand)	18.1 ± 1.6

Channel	% of control agonist response at 10 µM[b]
LXRα (agonist effect) (h)	0 ± 0
PPARγ (agonist effect) (h)	-0.2 ± 0.07
PXR (agonist effect) (h)	35 ± 3.3
TRPM8 (agonist effect) (h)	-3.5 ± 5.9
TRPV3 (agonist effect) (h)	0.1 ± 0.07
TRPV1 (VR1) (agonist effect) (h)	-5.1 ± 2.1

Channel	% inhibition of control agonist response at 10 µM[b]
LXRα (antagonist effect) (h)	3.8 ± 0.3
PPARγ (antagonist effect) (h)	22.8 ± 5.6
PXR (antagonist effect) (h)	-7.8 ± 5.9
TRPM8 (antagonist effect) (h)	60.6 ± 16
TRPV3 (antagonist effect) (h)	-9.3 ± 4.9
TRPV1 (VR1) (antagonist effect) (h)	-4.2 ± 12

Table S4: