Supplemental Material

A thallium based screening procedure to identify molecules that modulate the activity of Ca^{2+}-activated monovalent cation selective channels.

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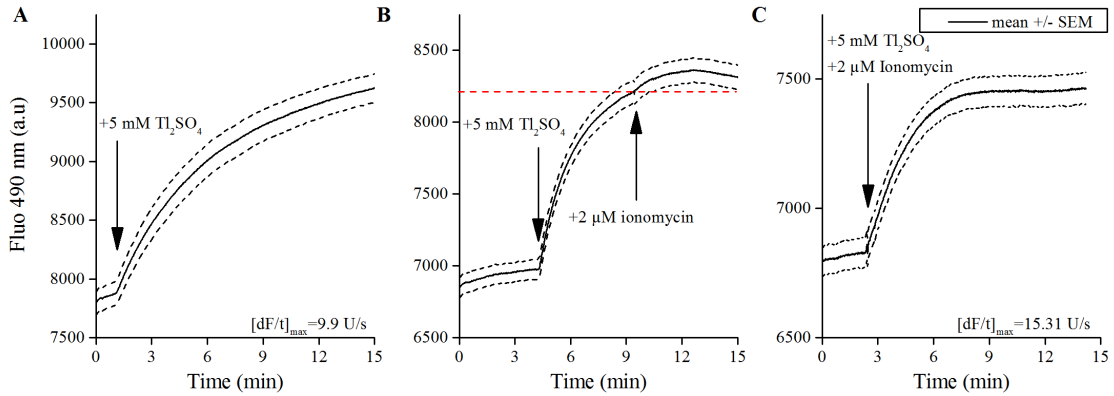
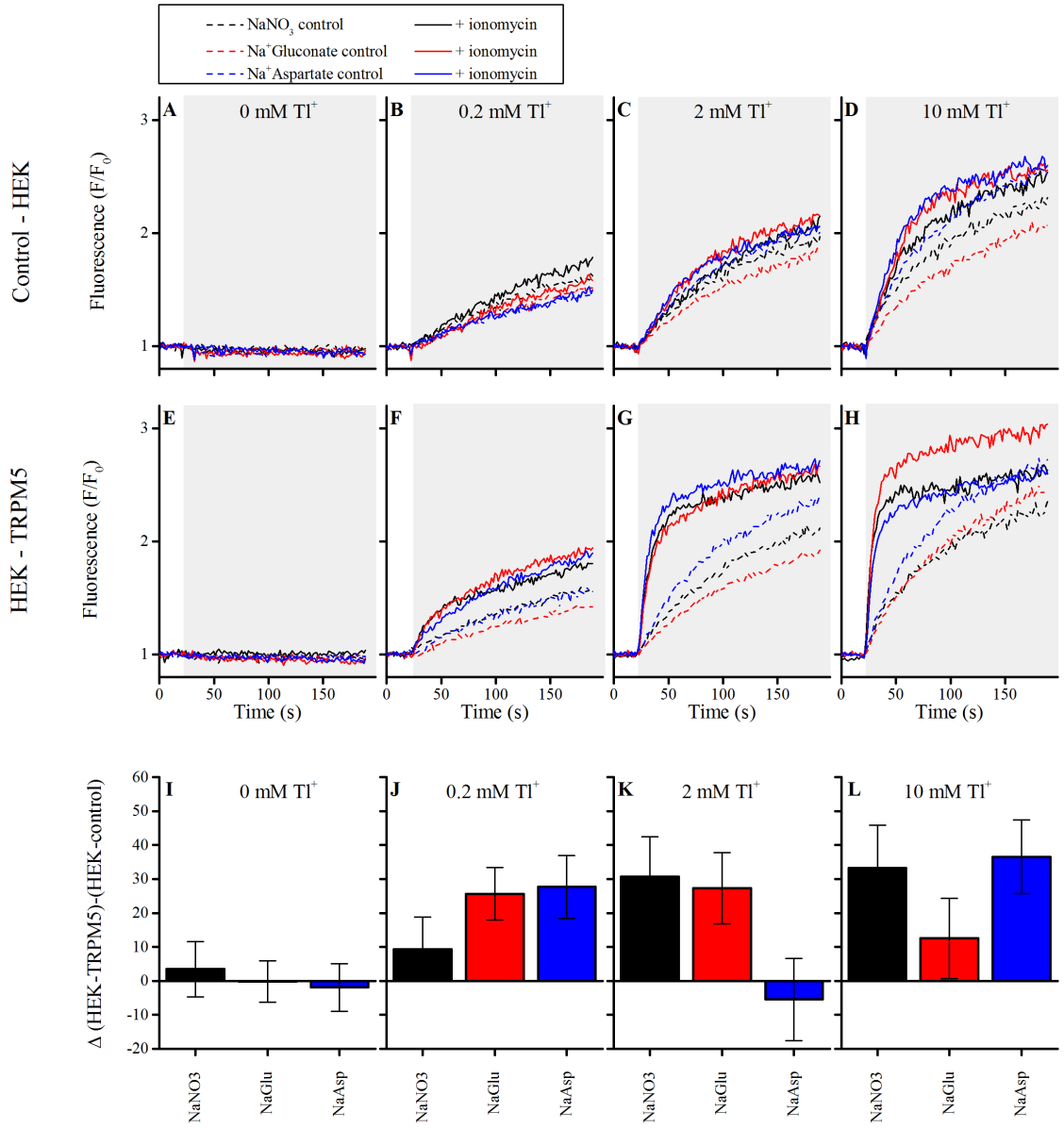
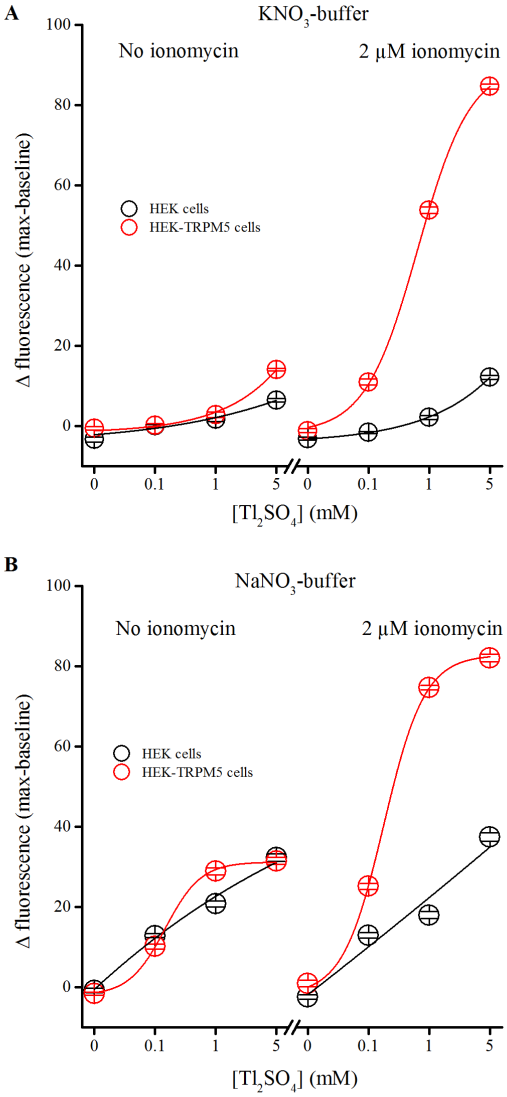
Keywords: Fluorescence methods, Cell-based assays, High content screening, Ion channels, Natural products screening



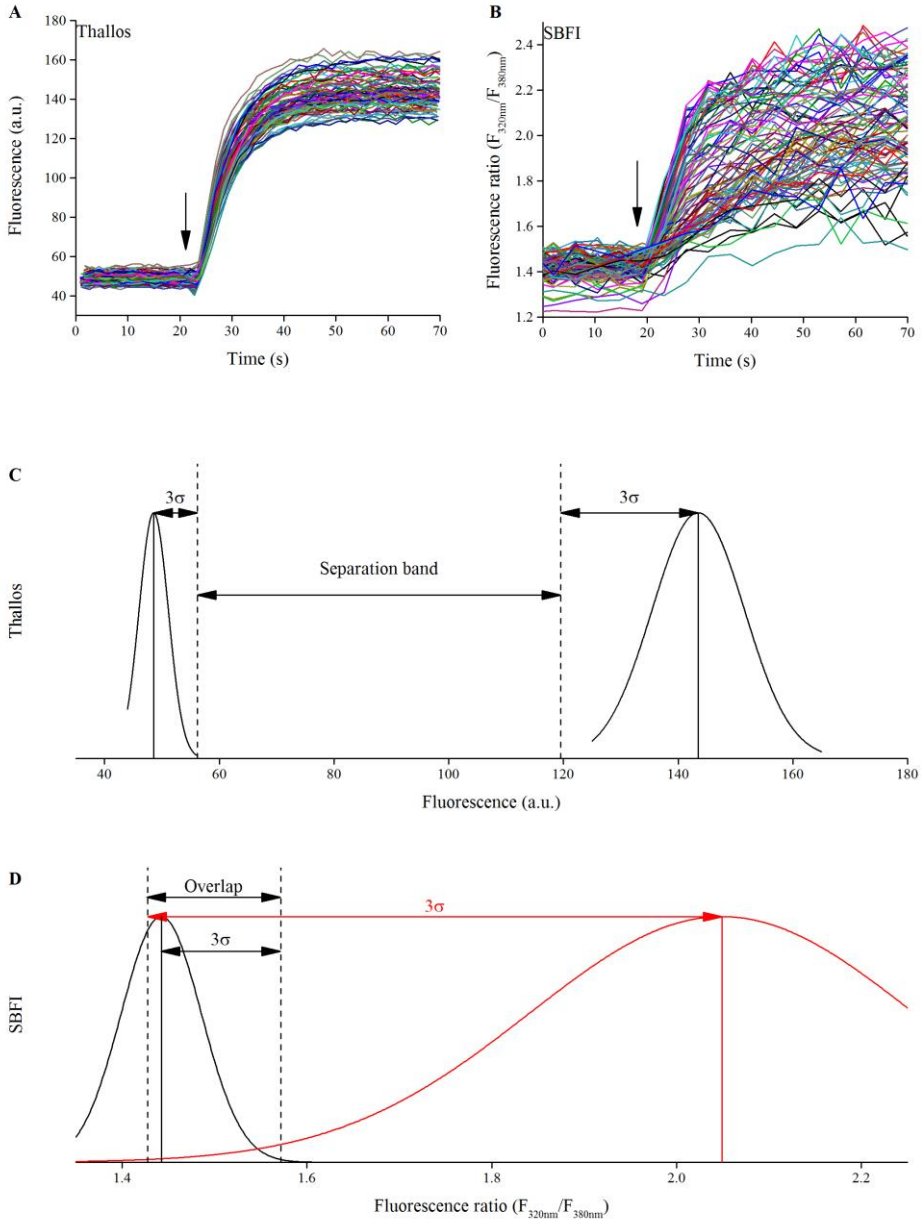
Supplemental Figure 1: Thallium flux through TRPM5. (A) Intracellular thallium fluorescence increases upon addition of 5 mM Tl_2SO_4 in the extracellular medium of HEK cells expressing TRPM5 (n = 23). (B) 2 µM ionomycin increases the thallium fluorescence (n = 49). (C) Simultaneous addition of ionomycin and thallium shows a faster fluorescence increase than thallium alone (n = 48).



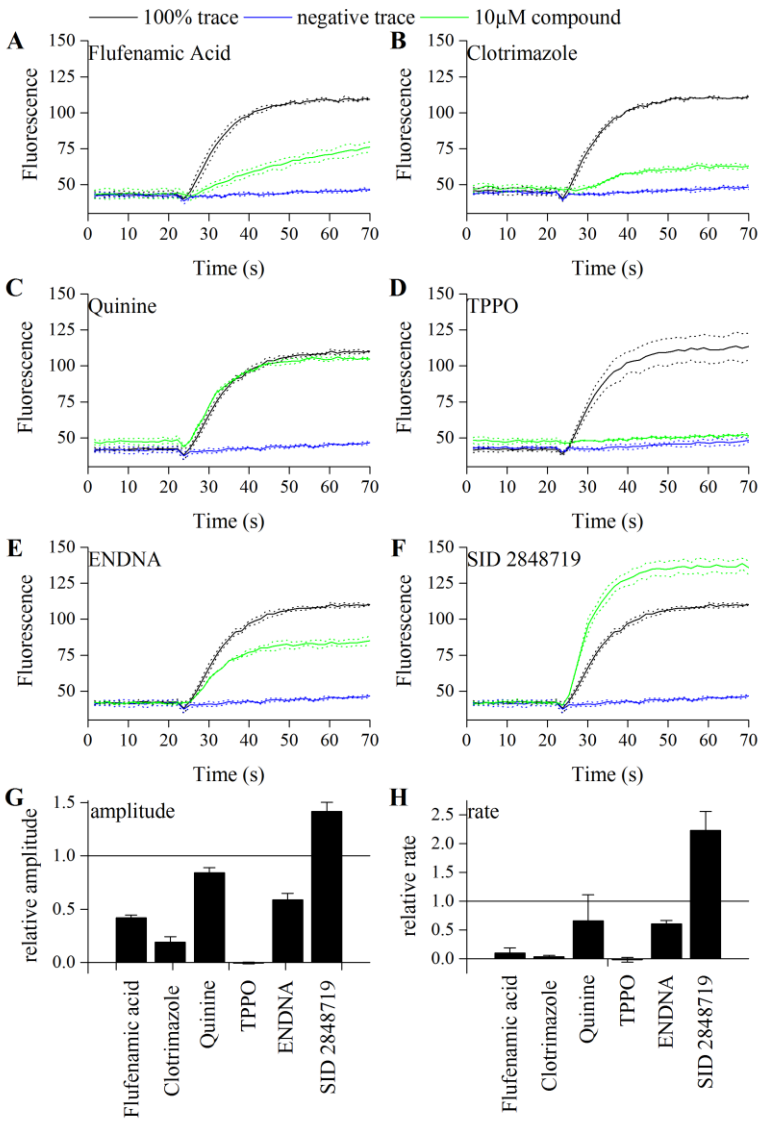
Supplemental figure 2: Thallium fluorescence increase in Na⁺ buffers with different anions. The thallium fluorescence measured in the FlexStation3, in each panel, after 25 s baseline measurement the appropriate amount of thallium sulphate (0 mM, 0.1 mM, 1 mM and 5 mM) and ionomycin (0 µM or 2 µM) is added to the well (grey background) and fluorescence responses were measured for 170 s. (A, B, C, D) Traces from wells containing control HEK cells, showing the TRPM5 independent fluorescence increase. (E, F, G, H) Traces in the same conditions in HEK cells overexpressing TRPM5. The dashed lines represent control conditions, in the absence of ionomycin. The solid lines represent the conditions with ionomycin, where TRPM5 is activated. (I, J, K, L) The differences in fluorescence amplitude of the ionomycin-induced effect between control HEK cells and HEK-TRPM5 cells. A positive value indicates a larger effect of ionomycin in HEK-TRPM5 cells.



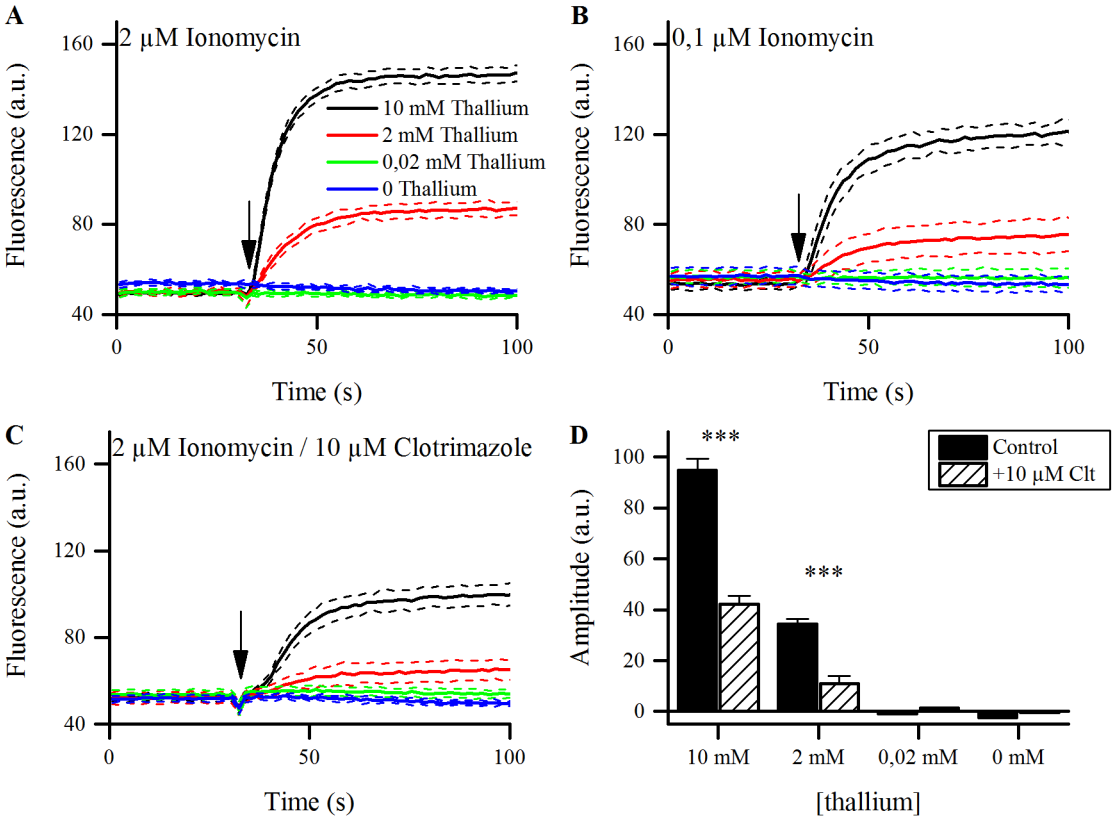
Supplemental Figure 3: Fluorescence signals with nitrate buffers. Data extracted from the traces in Fig. 2 A-H and Suppl. Fig. 2 A-H. (A) The absolute increase of fluorescence in KNO_3-buffer in the presence of different thallium concentrations on control HEK cells (black) and HEK-TRPM5 cells (red) without ionomycin (left) and with ionomycin (right). Note the substantial effect of ionomycin in HEK-TRPM5 cells and almost no effect in the control HEK cells. (B) As A but in $NaNO_3$ buffer. Note the higher signals in HEK-TRPM5 cells with ionomycin but also the substantial aspecific fluorescence increase. In the absence of ionomycin, the fluorescence signal with 1 mM Tl_2SO_4 is almost tenfold higher than in the KNO_3 condition.



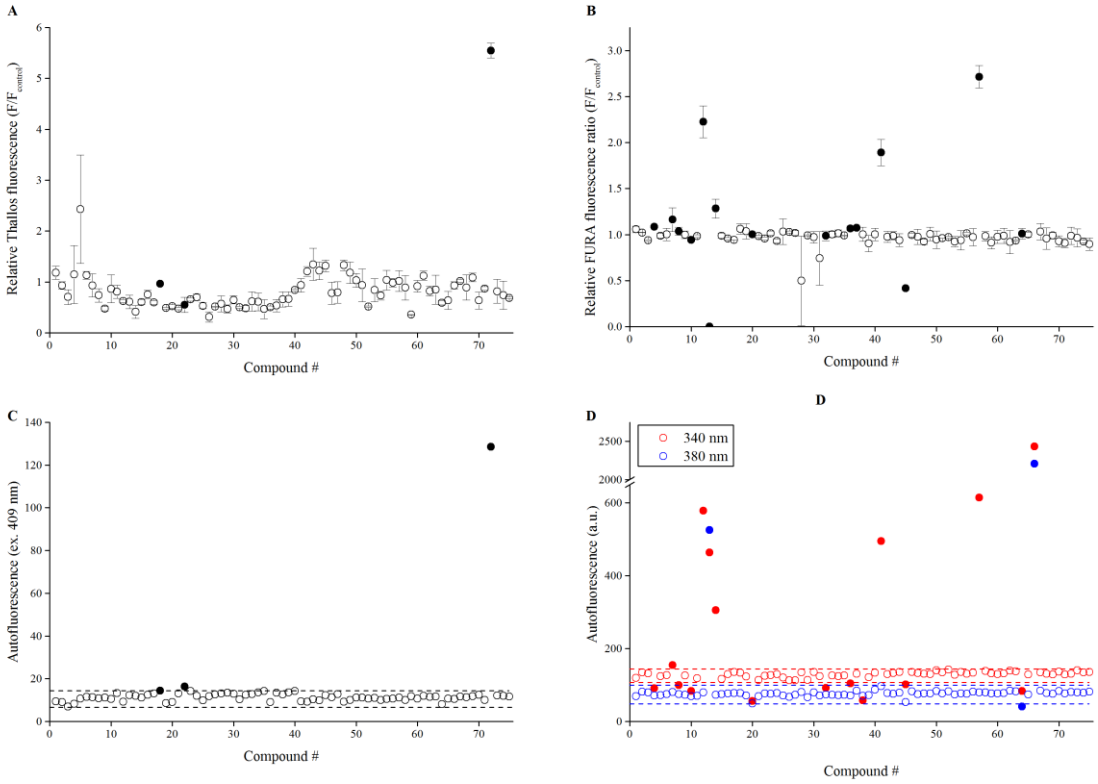
Supplemental figure 4: Comparison of the Na^+ sensitive dye SBFI and the Tl^+ sensitive dye Thallos. (A) The results of a typical plate in control conditions measured according to the protocol indicated in figure 3 with Thallos. Each trace represents one well of a 96-well plate. The arrow indicates the addition of 2 µM ionomycin to the well to initiate Ca^{2+} influx and TRPM5 activity. (B) Results of a plate in control conditions measured with SBFI dye. The arrow indicates the addition of 2 µM ionomycin to the well to initiate Ca^{2+} influx and TRPM5 activity. (C) The normal distributions of the Thallos fluorescence signal of 96 wells before ionomycin addition (left) and after ionomycin addition (right). The separation band is indicated as the difference between the means subtracted with 3 times the standard deviations. (D) As (C) but with the SBFI dye. We observe there is no separation band and a complete overlap of the 3σ intervals.



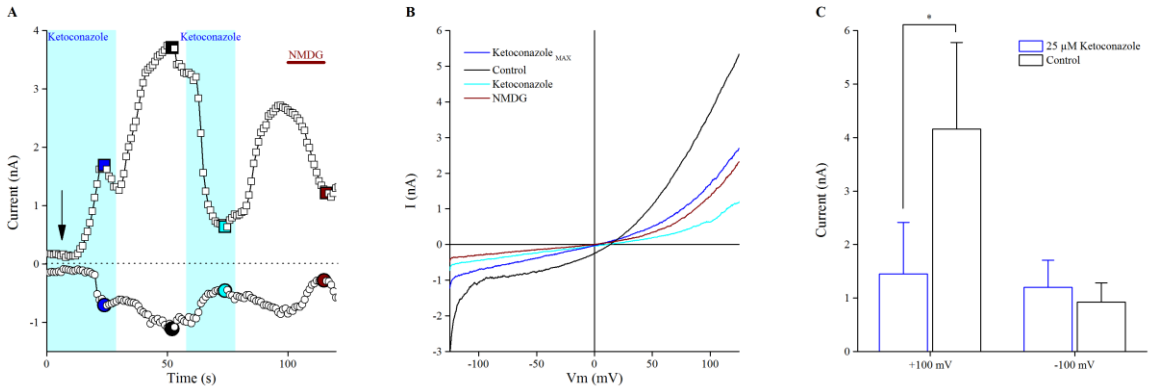
Supplemental Figure 5: Test of the assay with TRPM5 modulators. (A) Fluorescence increase upon addition of 10 µM FA to TRPM5 expressing HEK cells loaded with Thallos dye (green) compared to the control conditions (black) with ionomycin alone and the negative control condition (blue) without ionomycin. (B, C, D, E, F) As in (A) but with Clotrimazole (B), Quinine (C), TPPO (D), a TRPM5 inhibitor (E) and a TRPM5 activator (F). (G) Relative increase in the amplitude of the fluorescence signal in the presence of the compound compared to the control conditions. (H) Relative increase of the thallium flux as the rate of fluorescence increase in the presence of the compound compared to the control conditions.



Supplemental Figure 6: Modulating TRPM4 changes Thallos fluorescence. (A) Activating TRPM4 by increasing $[Ca^{2+}]_i$ with 2 µM ionomycin (application time indicated with the arrow) leads to an increase in the fluorescence signal. The amplitude and rate of fluorescence increase is dependent on the extracellular thallium concentration. (B) 0.1 µM ionomycin leads to a lower TRPM4 activity and a lower fluorescence signal. (C) Inhibiting TRPM4 with 10 µM clotrimazole leads to a decreased fluorescence signal. (D) Statistics of the amplitude change of the traces in A and C. (Clt: Clotrimazole)



Supplemental figure 7: Control experiments on untransfected cells and autofluorescence. (A) Relative Thallos fluorescence changes measured in untransfected HEK cells of the compounds as numbered in supplemental table 1 with the same measurement protocol as in figure 3. Compounds with autofluorescence at the used excitation wavelength (490 nm) are solid circles (average ± SD of duplicate measurements). (B) The effect of the compounds in supplemental table 1 on the intracellular calcium of untransfected HEK cells displayed as relative FURA-2 fluorescence changes compared to control (average ± SD of duplicate measurements). The solid circles are compounds with autofluorescent or quenching properties at the used excitation wavelengths (340 nm or 380 nm). Cells were seeded as in figure 3 and incubated with 1 µM FURA-2 AM supplemented with 0.6 mg/µL pluronic F-127 for 30 min before the experiment. Assay buffer was (mM) 150 NaCl, 6 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES and 10 glucose at pH 7.4 (NaOH). Fluorescence emission above 480 nm was followed after excitation at 340 nm and 380 nm. Compound transfer occurred as in figure 3. (C) Autofluorescence of the compounds at 490 nm, the excitation wavelength during the Thallos experiments. The compounds are dissolved at 100 µM in the same KNO_3-based buffer conditions as used during the Thallos screening. Compounds deviating > 3*SD of the average compound free control (n = 12) are considered to exhibit significant autofluorescence and are displayed as solid circles. The dashed lines represent the 3*SD interval. (D) Autofluorescence of the compounds at the excitation wavelengths used during FURA-2 Ca^{2+} measurements, 340 nm (red) or 380 nm (blue). Note the break in the y-axis. Compounds are dissolved at 100 µM in the same NaCl-based buffer conditions as during the FURA-2 screening in (B). The solid circles indicate compounds with significant (> 3*SD) deviation of the average fluorescence in compound-free control conditions, the dashed lines represent the 3*SD interval.



Supplemental figure 8: Ketoconazole inhibits TRPM5 currents. (A) Currents extracted at +100 mV and -100 mV of a patch clamp experiment on HEK293 cells overexpressing TRPM5. Ketoconazole (25 µM) is applied during the cell-attached phase. Upon establishing the whole cell configuration (indicated with the arrow), Ca^{2+}-induced TRPM5 dependent currents are partially attenuated until the washout of ketoconazole. (B) Current/Voltage relationships from the time points indicated in (A) in the respective conditions. (C) Ketoconazole inhibits a significant portion of the Ca^{2+}-induced TRPM5-dependent outward currents when applied before calcium loading of the cell (one-sided paired sample t-test, n = 3).

#	Compound name	CAS #	TRPM5				Untransfected HEK				Autofl.
			Rate	SD	Amp.	SD	Amp.	SD	Rate	SD	490 nm
1	Triphenylphosphine Oxide	791-28-6	8%	0.01	5%	0.08	258%	0..48	118%	0..	9..371
2	Clotrimazole	23593-75-1	9%	0.14	23%	0.12	69%	0..04	93%	0..	8..982
3	Ketoconazole	65277-42-1	11%	0.02	17%	0.04	73%	0..06	70%	0.1	6.995
4	Flufenamic Acid	530-78-9	12%	0.09	41%	0.02	146%	0.13	115%	0.5	8.325
5	Econazole	27220-47-9	14%	0.27	37%	0.5	708%	1.25	243%	1.0	10.658
6	(+\-)Miconazole Nitrate Salt	75319-48-1	26%	0.41	76%	0.24	148%	0.40	113%	0.0	11.477
7	Chlorpromazine	69-09-0	29%	0.14	32%	0.01	96%	0.17	93%	0.2	11.367
8	ENDNA	/	49%	0.2	66%	0.17	92%	0.13	74%	0.1	10.886
9	Campher 96%	76-22-2	63%	0.09	93%	0.09	94%	0.06	47%	0.0	11.190
10	Indomethacin	53-86-1	66%	0.25	90%	0.17	83%	0.01	86%	0.2	10.457
11	L+ Ascorbic Acid	50-81-7	67%	0.06	95%	0.27	109%	0.10	81%	0.1	13.328
12	Quinidine	6151-40-2	71%	0.06	96%	0.21	89%	0.09	62%	0.0	9.235
13	7,12-dimethyl-Benz-*a*-	57-97-6	73%	0.03	86%	0.1	124%	0.19	61%	0.1	12.332
14	Triphenylamine	603-34-9	73%	0.18	90%	0.06	104%	0.14	41%	0.1	12.010
15	Papaverine	61-25-6	74%	0.27	77%	0.1	78%	0.12	61%	0.0	11.154
16	Cinnamyl Alcohol	104-54-1	78%	0.29	81%	0.25	122%	0.10	75%	0.0	12.439
17	3-Isobutyl-1-Methylxanthin	28822-58-4	81%	0.06	91%	0.04	97%	0.00	60%	0.0	13.077
18	L-Phenylephrine	61-76-7	84%	0.1	90%	0.02	101%	0.05	96%	0.0	14.447
19	L-Glutamic Acid	617-65-2	86%	0.26	101%	0.19	119%	0.20	49%	0.0	8.610
20	Colchicine 95%	64-86-8	87%	0.01	103%	0.32	104%	0.14	52%	0.0	9.031
21	Ouabain octahydrate	11018-89-6	88%	0.22	87%	0.04	100%	0.01	48%	0.0	13.036
22	N-Acetyl-L-Cysteine	616-91-1	88%	0.02	93%	0.18	126%	0.13	55%	0.1	16.356
23	Urethane	51-79-6	89%	0.19	90%	0.09	102%	0.05	66%	0.0	14.264
24	6-Propyl-2-Thiouracil	51-52-5	90%	0.06	89%	0.02	87%	0.00	70%	0.0	11.979
25	Choline chloride	67-48-1	90%	0.13	102%	0.17	82%	0.24	53%	0.0	9.868
26	Acetylcholine chloride	60-31-1	92%	0.31	90%	0.15	108%	0.16	31%	0.1	11.837
27	Ketotifen fumarate salt	34580-14-8	92%	0.19	92%	0.01	100%	0.11	51%	0.0	12.568
28	Atropine	5908-99-6	92%	0.25	96%	0.11	104%	0.13	57%	0.1	13.100
29	Theophylline	58-55-9	93%	0.17	93%	0.08	92%	0.02	47%	0.0	13.411
30	Theobromine 99%	83-67-0	93%	0.02	108%	0.02	88%	0.01	65%	0.0	12.972
31	Oxalic Acid	144-62-7	93%	0.07	85%	0.11	123%	0.12	50%	0.0	10.325
32	Niflumic Acid	4394-00-7	93%	0.15	88%	0.09	100%	0.04	48%	0.0	12.524
33	Histamine	56-92-8	94%	0.72	97%	0.13	109%	0.14	62%	0.1	12.782
34	1,1-Dimethylguanidine	598-65-2	94%	0.06	95%	0.01	110%	0.13	61%	0.1	13.502
35	Picrotoxin	124-87-8	95%	0.18	89%	0.08	86%	0.10	47%	0.1	14.221
36	Amiodarone	1951-25-3	96%	0.26	102%	0.01	99%	0.02	50%	0.0	9.011
37	Taurine	107-35-7	96%	0.12	88%	0.1	110%	0.00	54%	0.1	13.484
38	Vanillin	121-33-5	96%	0.09	88%	0.03	120%	0.03	66%	0.1	12.540
39	Estrone	53-16-7	96%	0.14	94%	0.07	120%	0.03	67%	0.1	13.559
40	Aconitine	302-27-2	97%	0.2	96%	0.1	104%	0.00	84%	0.0	14.185
41	9-Phenanthrol	484-17-3	100%	0.12	107%	0.02	94%	0.09	94%	0.1	9.392
42	Levamisole	16595-80-5	100%	0.11	94%	0.12	102%	0.11	121%	0.1	9.160
43	Clonidine	4205-91-8	101%	0.16	97%	0.04	102%	0.05	134%	0.3	10.284
44	Dobutamine	49745-95-1	103%	0.19	93%	0.08	93%	0.03	122%	0.1	9.941
45	Dantrolene	14663-23-1	104%	1.01	103%	0.06	95%	0.09	132%	0.1	12.236
46	Caffeine	58-08-2	104%	0.14	95%	0.11	94%	0.07	78%	0.2	11.257
47	Pregnonolone Sulphate	1852-38-6	104%	0.57	104%	0.23	108%	0.16	79%	0.2	12.770
48	Tolbutamide	64-77-7	104%	0.14	95%	0.07	97%	0.05	133%	0.1	9.152
49	trans-Cinnamic Acid	140-10-3	105%	0.31	106%	0.15	93%	0.03	118%	0.2	9.991
50	Capsaicin	404-86-4	106%	0.19	87%	0.1	76%	0.07	103%	0.1	11.153
51	Neomycin trisulphate	1405-10-3	107%	0.17	93%	0.09	94%	0.28	93%	0.3	11.229
52	Guanidine hydrochloride	50-01-1	107%	0.48	100%	0.07	96%	0.11	52%	0.0	10.659
53	Allyl Isothiocyanate	57-06-7	108%	0.18	97%	0.1	95%	0.17	84%	0.2	11.036
54	Aspirin	50-78-2	108%	0	106%	0.14	80%	0.03	73%	0.0	9.965
55	NMDA	6384-92-5	109%	0.03	109%	0.27	88%	0.08	104%	0.1	10.468
56	Putrescine	333-93-7	111%	0.1	95%	0	90%	0.01	98%	0.0	10.663
57	Quinine	6119-47-7	112%	0.64	91%	0.17	93%	0.05	102%	0.2	11.149
58	Diazoxide	364-98-7	112%	0.24	105%	0.11	98%	0.09	89%	0.2	10.044
59	(1S,2R,5S)-(+)-Menthol	15356-60-2	113%	0.12	95%	0.14	51%	0.35	36%	0.0	11.756
60	Lidocaine	6108-05-0	114%	0.31	108%	0.35	85%	0.06	92%	0.1	10.600
61	Stevioside	57817-89-7	115%	0.24	115%	0.17	83%	0.08	112%	0.0	11.921
62	gamma-Aminubutyric Acid	56-12-2	116%	0.58	104%	0.04	86%	0.04	82%	0.1	11.514
63	Terbutaline hemisulphate	23031-32-5	117%	0.07	107%	0.07	92%	0.11	85%	0.2	11.623
64	Xylene Cyanole FF	220-167-5	117%	0.41	99%	0.03	35%	0.04	59%	0.0	8.139
65	Iodoacetamide	144-48-9	118%	0.12	96%	0.01	90%	0.10	64%	0.1	10.680
66	Hydrocortisone	50-23-7	119%	1.04	105%	0.17	69%	0.02	93%	0.0	10.437
67	Urea	57-13-6	119%	0.01	114%	0.19	98%	0.07	102%	0.0	11.685
68	Estriol	50-27-1	121%	0.18	96%	0.02	74%	0.06	89%	0.2	11.390
69	Deoxycholic acid	302-95-4	122%	1.04	107%	0.19	97%	0.24	109%	0.0	11.813
70	D-Tartaric Acid	147-71-7	122%	0.05	92%	0.03	81%	0.05	64%	0.1	12.513
71	(-)Menthol	2216-51-5	133%	0.08	106%	0.01	97%	0.07	87%	0.0	9.947
72	Quinacrine	69-05-6	147%	0.54	123%	0.06	333%	0.12	555%	0.1	128.559
73	Eugenol	97-53-0	184%	2.36	134%	0.51	94%	0.11	82%	0.2	12.065
74	Rebaudioside A	58543-16-1	200%	0.51	130%	0.18	100%	0.19	74%	0.2	11.958
75	SID 2848719	/	201%	0.76	151%	0.33	183%	0.41	69%	0.0	11.653

Supplemental table 1: Library with 75 compounds and their relative activity on TRPM5, untransfected cells and autofluorescence. SD = standard deviation, Amp. = maximal amplitude of fluorescence increase, Autofl. = Autofluorescence, Fw = Molecular mass of the compound. The compounds indicated in red have an autofluorescence deviating > 3*SD from the average. The compounds in green show a significant Thallos fluorescence increase in TRPM5 negative cells.

Product Name	Untransfected HEK cells				Autofl.	
	Rate	SD	Amp.	SD	Ex. 340	Ex. 380
Triphenylphosphine Oxide	104%	0.06	106%	0.03	119.584	69.689
Clotrimazole	99%	0.03	102%	0.00	134.936	81.834
Ketoconazole	89%	0.03	94%	0.00	131.389	79.703
Flufenamic Acid	106%	0.01	109%	0.01	91.202	71.457
Econazole	91%	0.00	99%	0.02	124.537	72.397
(+\-)Miconazole Nitrate Salt	90%	0.07	100%	0.06	127.213	74.455
Chlorpromazine	112%	0.09	116%	0.13	154.301	80.725
ENDNA	103%	0.02	104%	0.04	99.432	74.971
Campher 96%	100%	0.06	100%	0.04	126.223	72.677
Indomethacin	96%	0.03	94%	0.01	83.845	69.677
L+ Ascorbic Acid	100%	0.00	98%	0.01	118.574	70.409
Quinidine	180%	0.04	223%	0.17	578.981	79.952
7,12-dimethyl-Benz-*a*-Antracene	2%	0.01	0%	0.01	464.215	525.146
Triphenylamine	121%	0.16	128%	0.10	305.188	72.959
Papaverine	99%	0.04	99%	0.02	116.566	74.728
Cinnamyl Alcohol	98%	0.07	96%	0.01	129.499	76.773
3-Isobutyl-1-Methylxanthin	96%	0.00	94%	0.00	135.853	77.389
L-Phenylephrine Hydrochloride	111%	0.06	106%	0.05	133.721	77.961
L-Glutamic Acid	99%	0.09	104%	0.08	122.821	71.419
Colchicine 95%	102%	0.05	100%	0.02	56.048	48.932
Ouabain octahydrate	93%	0.01	98%	0.01	114.016	69.227
N-Acetyl-L-Cysteine	99%	0.02	96%	0.01	125.045	77.027
Urethane	105%	0.00	101%	0.02	127.423	76.011
6-Propyl-2-Thiouracil	92%	0.00	93%	0.02	129.895	77.651
Choline chloride	102%	0.14	103%	0.13	120.554	70.916
Acetylcholine chloride	106%	0.02	103%	0.01	112.686	67.512
Ketotifen fumarate salt	97%	0.01	102%	0.02	113.264	73.354
Atropine	52%	0.48	50%	0.48	135.101	80.656
Theophylline	98%	0.02	99%	0.00	114.05	66.705
Theobromine 99%	96%	0.10	97%	0.06	135.602	80.057
Oxalic Acid	80%	0.34	74%	0.28	123.889	71.098
Niflumic Acid	97%	0.04	99%	0.05	92.293	74.27
Histamine	104%	0.04	100%	0.03	126.079	72.71
1,1-Dimethylguanidine Sulphate	103%	0.05	101%	0.03	123.823	72.037
Picrotoxin	102%	0.05	99%	0.00	126.16	72.738
Amiodarone	102%	0.07	107%	0.02	103.975	71.125
Taurine	99%	0.01	107%	0.01	130.494	84.219
Vanillin	100%	0.08	100%	0.07	57.506	69.934
Estrone	91%	0.08	90%	0.08	121.611	72.492
Aconitine	95%	0.03	100%	0.06	133.574	87.388
9-Phenanthrol	164%	0.23	189%	0.14	495.204	96.884
Levamisole	99%	0.22	98%	0.05	130.337	77.574
Clonidine	100%	0.13	99%	0.04	133.122	75.758
Dobutamine	97%	0.06	94%	0.06	135.922	78.835
Dantrolene	57%	0.00	42%	0.01	101.697	53.192
Caffeine	102%	0.10	100%	0.02	135.571	82.314
Pregnonolone Sulphate	102%	0.06	98%	0.08	132.815	74.577
Tolbutamide	101%	0.09	92%	0.02	130.434	76.832
trans-Cinnamic Acid	114%	0.26	101%	0.07	130.072	76.531
Capsaicin	103%	0.05	94%	0.09	140.823	83.439
Neomycin trisulphate	96%	0.04	96%	0.00	134.331	77.171
Guanidine hydrochloride	102%	0.00	97%	0.01	142.062	82.959
Allyl Isothiocyanate	94%	0.15	92%	0.05	128.098	74.065
Aspirin	101%	0.26	94%	0.10	135.156	76.847
NMDA	110%	0.12	101%	0.01	130.709	76.206
Putrescine	99%	0.08	97%	0.09	133.765	81.24
Quinine	231%	0.18	271%	0.12	614.488	79.861
Diazoxide	93%	0.07	98%	0.05	138.2	80.027
(1S,2R,5S)-(+)-Menthol	99%	0.11	91%	0.06	131.505	76.54
Lidocaine	110%	0.24	98%	0.07	129.694	76.421
Stevioside	104%	0.13	99%	0.08	132.266	77.991
gamma-Aminubutyric Acid 99%	106%	0.18	92%	0.12	139.442	84.33
Terbutaline hemisulphate	89%	0.02	94%	0.00	137.264	82.615
Xylene Cyanole FF	99%	0.01	101%	0.05	83.449	40.771
Iodoacetamide	101%	0.06	100%	0.02	128.682	74.427
Hydrocortisone	SAT.		SAT.		2433.854	2208.57
Urea	102%	0.15	103%	0.09	135.061	84.613
Estriol	110%	0.10	96%	0.11	131.25	77.465
Deoxycholic acid	100%	0.00	99%	0.03	128.661	75.746
D-Tartaric Acid	95%	0.18	93%	0.05	136.881	83.221
(-)Menthol	90%	0.24	91%	0.04	129.35	77.161
Quinacrine	109%	0.20	99%	0.09	131.281	80.19
Eugenol	111%	0.08	96%	0.09	140.455	79.691
Rebaudioside A	91%	0.07	92%	0.01	134.335	78.339
SID 2848719	105%	0.13	90%	0.06	135.996	81.236

Supplemental table 2: Control experiments on the compound library using FURA-2 Ca^{2+} imaging. Compounds indicated in red, exhibit autofluorescence at the used wavelengths for FURA-2 compared to Thallos (supplemental table 1). (SAT.: Saturated fluorescence signal)