# Expanding the Toolbox: Novel Modulators of Endolysosomal Cation Channels

Susanne Rautenberg, Marco Keller, Charlotte Leser, Cheng-Chang Chen, Franz Bracher, and Christian Grimm

## Contents



**Abstract**

Functional characterization of endolysosomal ion channels is challenging due to their intracellular location. With recent advances in endolysosomal patch clamp technology, it has become possible to directly measure ion channel currents across endolysosomal membranes. Members of the transient receptor potential (TRP) cation channel family, namely the endolysosomal TRPML channels (TRPML1-3), also called mucolipins, as well as the distantly related two-pore channels (TPCs) have recently been characterized in more detail with endolysosomal patch clamp techniques. However, answers to many physiological questions require work in intact cells or animal models. One major obstacle thereby is that the known endogenous ligands of TRPMLs and TPCs are anionic in nature and thus impermeable for cell membranes. Microinjection, on the other

Susanne Rautenberg and Marco Keller contributed equally to this work.

S. Rautenberg · M. Keller · C. Leser · C.-C. Chen · F. Bracher (✉)
Department of Pharmacy – Center for Drug Research, Ludwig-Maximilians-University, Munich, Germany
e-mail: franz.bracher@cup.uni-muenchen.de

C. Grimm (✉)
Department of Pharmacology and Toxicology, Medical Faculty, Ludwig-Maximilians-University, Munich, Germany
e-mail: christian.grimm@med.uni-muenchen.de



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hand, is technically demanding. There is also a risk of losing essential co-factors for channel activation or inhibition in isolated preparations. Therefore, lipophilic, membrane-permeable small-molecule activators and inhibitors for TRPMLs and TPCs are urgently needed. Here, we describe and discuss the currently available small-molecule modulators of TRPMLs and TPCs.

**Keywords**

Lysosome · Small-molecule activator · Small-molecule inhibitor · TPC · TRPML

## 1 Introduction

Transient receptor potential (TRP) channels represent potential targets for the treatment of various diseases. Several members of the TRP channel family have been investigated as potential targets for the treatment of neuropathic and other pain conditions and more than 20 drugs targeting TRPV1, TRPV2, TRPM8, or TRPA1 are still under active clinical development (Weyer-Menkhoff and Lotsch 2018; Dietrich 2019). Other disease areas with TRP channels as potential drug targets are: bladder dysfunctions (Birder 2007), inflammatory bowel disease (Zhang and Li 2014), pulmonary oedema (TRPV4) (Thorneloe et al. 2012), hyperkeratosis, inflammatory skin disorders with itch and/or pain (TRPV3) (Imura et al. 2009; Yoshioka et al. 2009; Lin et al. 2012), prostate cancer (TRPM8) (Zhang and Barritt 2006), inflammation, infection (Parenti et al. 2016), ischaemia reperfusion (Ma et al. 2017), Alzheimer's disease (TRPM2) (Jiang et al. 2018a), chronic cough and asthma (TRPA1) (Belvisi and Birrell 2017), lysosomal storage disorders and neurodegenerative diseases (TRPML1; (Huang and Szallasi 2017)).

In the past decade, remarkably many highly potent and efficacious agonists and antagonists for TRP channels have been developed by the pharmaceutical industry. Meanwhile, many of these compounds have become commercially available. Some of them, e.g. PF-4840154 (TRPA1 agonist) (Ryckmans et al. 2011), GSK1016790A (TRPV4 agonist) (Thorneloe et al. 2008), WS-12 (TRPM8 agonist) (Sherkheli et al. 2008), AMG9810 and AMG517 (TRPV1 antagonists) (Gavva et al. 2005, 2008), GSK2193874 (TRPV4 antagonist) (Cheung et al. 2017), ML204 and HC-070 (TRPC4/5 antagonists) (Miller et al. 2011; Just et al. 2018) are presented in Fig. 1.

For the endolysosomal TRPML channels (TRPML1-3; mucolipins 1-3) and the distantly related two-pore channels (TPCs) no agonists/antagonists, albeit under development have been released by the pharmaceutical industry so far. Several compounds are however available as a result of high-throughput screening (HTS) efforts by academic institutions. Here, we describe and discuss in the first part the currently available TRPML channel agonists and antagonists and give an overview of the general characteristics of the three TRPML channel subfamily members. In the second part, we focus on general characteristics of the endolysosomal TPCs (TPC1

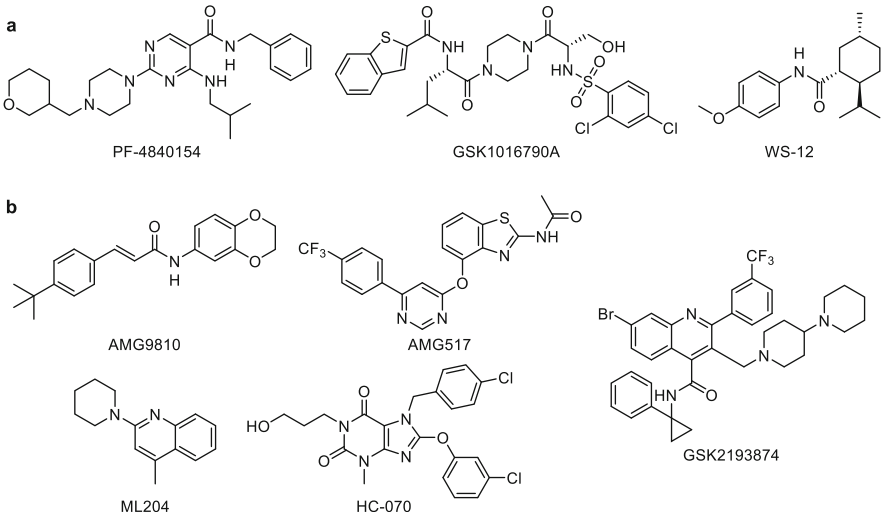


**Fig. 1** Small-molecule modulators of TRP ion channels. (**a**) Agonists of TRPA1 (PF-4840154), TRPV1 (GSK1016790A), and TRPM8 (WS-12). (**b**) Antagonists of TRPV1 (AMG9810, AMG517), TRPV4 (GSK2193874), and TRPC4/5 (ML204, HC-070)

and TPC2) and present small molecules, which are currently available for their activation or inhibition.

## 2 Small-Molecule Tools for the Modulation of TRPMLs

The TRPML subfamily of TRP channels comprises three members in mammals. Loss or mutation of TRPML1 in humans and mice results in severe neurodegeneration as well as corneal clouding and retinal degeneration, which eventually leads to blindness (mucolipidosis type IV phenotype; (Bargal et al. 2000)). Gain-of-function mutations in TRPML3 cause deafness and circling behaviour in mice (varitint-waddler phenotype; (Di Palma et al. 2002; Xu et al. 2007; Nagata et al. 2008; Kim et al. 2007; Grimm et al. 2007, 2009)). TRPML2 enhances viral entry, viral trafficking, and thus infection with viruses such as yellow fever virus, Dengue virus, influenza A virus, or equine arteritis virus. The mutation K370Q within TRPML2 is found at higher frequencies in African populations compared to other geographic populations and leads to a loss of viral enhancement (Rinkenberger and Schoggins 2018).

The phosphoinositide phosphatidylinositol 3,5-bisphosphate (PI(3,5)$P_2$; Fig. 2), a major constituent of endolysosomal membranes has been described in 2010 as the first endogenous activator of TRPML channels (Dong et al. 2010). In contrast, phosphatidylinositol 4,5-bisphosphate (PI(4,5)$P_2$; Fig. 2), which mainly occurs in the plasma membrane, was identified as an inhibitor of TRPML channels (Zhang et al. 2012); Tables 1, 2, and 3.

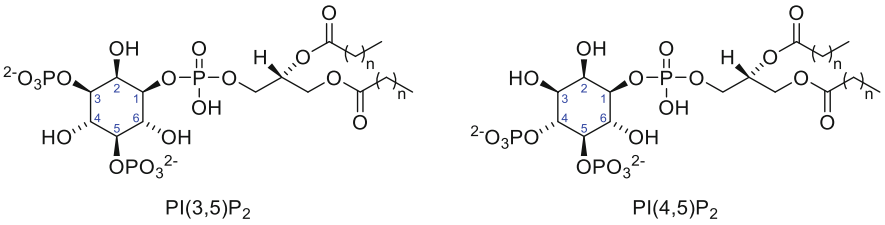


**Fig. 2** Phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$, left) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$, right). Inositols are substituted with two phosphate residues in 3,5 or 4,5 positions and carry phosphate glycerol ester and various fatty acids in 1 position (e.g. $n = 16$ or 18)

$PI(3,5)P_2$ is present in both Rab5 positive early endosomes (EE) as well as Rab7 positive late endosomes (LE) and lysosomes (LY; (Takatori et al. 2016)). Defects in $PI(3,5)P_2$ signalling are linked to human diseases such as Charcot–Marie–Tooth disease and amyotrophic lateral sclerosis. Cryo electron microscopy (Cryo-EM) revealed that $PI(3,5)P_2$ and $PI(4,5)P_2$ bind to the extended helices of transmembrane domains S1, S2, and S3 of TRPML1. The phosphate group of $PI(3,5)P_2$ induces amino acid Y355 to form a π–cation interaction with R403, moving the S4–S5 linker, thus allosterically activating the channel (Fine et al. 2018). This is different from the $PI(3,5)P_2$ binding observed in TPC1. Here $PI(3,5)P_2$ binds to the first S6 domain (She et al. 2018).

In addition to phosphoinositides in the membrane of endo-lysosomes, the proton concentration in the lumen of endo-lysosomes controls TRPML channel activity. TRPML1 activity is enhanced by protons (acidic pH; (Dong et al. 2008; Chen et al. 2014)), while TRPML2 and TRPML3 activity is reduced by protons (Kim et al. 2008; Grimm et al. 2012; Miao et al. 2015; Lev et al. 2010; Plesch et al. 2018). Further regulators of TRPML1 channel activity are sphingomyelin, which inhibits TRPML1 activity in NPC1 (Niemann Pick type C1) cells (Shen et al. 2012), adenosine (Zhong et al. 2017), reactive oxygen species (ROS; (Zhang et al. 2016)), and TOR kinase via phosphorylation of the TRPML1 channel (Onyenwoke et al. 2015).

Several synthetic ligands have become available in recent years for TRPML1, 2, and 3 as a result of high-throughput screening (HTS) and medicinal chemistry efforts, which have been discussed in detail before (Grimm et al. 2010, 2012, 2014a; Yamaguchi and Muallem 2010; Saldanha et al. 2011; Shen et al. 2012; Cuajungco et al. 2014; Chen et al. 2014; Kilpatrick et al. 2016). TRPML channel activators identified in the HTS belong to different substance families including (hetero)-arylsulfonamides (e.g. SN-1, SF-11, SF-21, SF-22, and MK6-83; (Chen et al. 2014)), phthalimidoacetamides (SF-51 and ML-SA1; (Shen et al. 2012)), isoxazol (in)es (e.g. SN-2, ML2-SA1, EVP-21), and others (Grimm et al. 2010, 2012, 2014a; Saldanha et al. 2011, 2013; Cuajungco et al. 2014; Kilpatrick et al. 2016); Fig. 3).

Besides their structural differences, these activators also differ in activity and selectivity. While the tetrahydroquinoline ML-SA1 activates all human TRPML

**Table 1** Summary of the characteristics of TRPML1

| TRPML1 (*MCOLN1*) | |
|---|---|
| Associated phenotypes | *Loss of function*: Mucolipidosis type IV (MLIV), neurodegenerative lysosomal storage disease in humans. Knockout mouse model shows pathology similar to human mucolipidosis IV phenotype (Micsenyi et al. 2009; Grishchuk et al. 2014, 2015, 2016) |
| Expression pattern | Ubiquitous; highest in brain, kidney, spleen, liver, and heart |
| Subcellular localization | Lysosomal, by virtue of dileucine sorting motif |
| Endogenous regulation | *Channel activation*: PI(3,5)$P_2$ (direct); luminal $H^+$; ROS; TFEB (transcriptional)<br>*Channel inhibition*: PI(4,5)$P_2$; sphingomyelin; adenosine; mTOR (debated) |
| Functions | • Non-selective cation channel<br>• Lysosomal ion homeostasis<br>– Cation and heavy metal (iron/zinc) homeostasis<br>– Lysosomal *pH* regulation (debated)<br>• Lysosomal trafficking<br>– Calcium-dependent lysosomal recruitment of motor proteins<br>– Lysosomal exocytosis<br>– Lysosomal lipid and cholesterol trafficking<br>• Phagocytosis |
| Available agonists | • ML-SA1 (commercially available); not isoform selective<br>• MK6-83 (commercially available); not isoform selective<br>• SF-22, SF-51 (>30 μM); not isoform selective<br>• ML-SA3; isoform selectivity unclear<br>• ML-SA5; isoform selectivity unclear<br>• ML1-SA1 (EVP-169); isoform selective (TRPML1 selective) (Spix et al. 2022) |
| Available antagonists | • ML-SI1; not isoform selective; stereochemistry of the active isomer not yet elucidated; dependent on activator<br>• ML-SI2 (structure not published)<br>• ML-SI3 (racemic *trans*-isomer commercially available; both enantiomers available by enantioselective synthesis (Kriegler et al. 2022); not isoform selective<br>• EDME (17β-estradiol methyl ether); isoform selective (Rühl et al. 2021)<br>• PRU-10; isoform selective (Rühl et al. 2021)<br>• PRU-12; isoform selective (Rühl et al. 2021) |

channel isoforms, and TRPML1 and TRPML3 in mouse, its dehydro analogue SF-51 preferentially activates TRPML3 (Grimm et al. 2010). A small modification of SF-22 (from 2-chlorothiophene in SF-22 into 2-methylthiophene in MK6-83) results in strongly increased activity. Nevertheless, both SF-22 and MK6-83 activate mouse and human TRPML1 and TRPML3 (Chen et al. 2014).

Although the structural differences between SN-2 and ML2-SA1 are rather small, they result in this case in very different selectivities within the TRPML family. SN-2, however, is preferentially activating TRPML3, while ML2-SA1 (EVP-22) is a selective agonist of TRPML2. All modifications are a result of systematic analysis

**Table 2** Summary of the characteristics of TRPML2

| TRPML2 (*MCOLN2*) | |
|---|---|
| Associated phenotypes | No identified phenotypes in human nor mice beyond impaired chemokine secretion (Gerndt et al. 2020a) |
| Expression pattern | Restricted to myeloid and lymphoid organs (thymus, spleen, lymph nodes) and kidney |
| Subcellular localization | Recycling endosomal and lysosomal |
| Endogenous regulation | *Channel activation*: PI(3,5)$P_2$ (direct); LPS endotoxin (macrophages, transcriptional)<br>*Channel inhibition*: Luminal $H^+$ |
| Functions | • Non-selective cation channel<br>• Chemokine secretion (various chemokines; CCL2 best characterized)<br>• Enhances viral infection, viral entry, virus trafficking (yellow fever virus, dengue virus, influenza A virus, equine arteritis virus)<br>• Acceleration of endosomal trafficking<br>– Endocytic transferrin processing<br>– ARF6-dependent endocytosis of CD59<br>– Recycling of GPI-APs |
| Available agonists | • ML-SA1 (commercially available); not isoform selective<br>• ML2-SA1 = EVP-22 (published (Plesch et al. 2018), not commercially available); isoform selective |
| Available antagonists | • ML-SI1 and 3; not isoform selective |

of structure–activity relationships (Plesch et al. 2018; Grimm et al. 2010; Chen et al. 2014). MK6-83 has an $EC_{50}$ of 0.1 μM for hTRPML1 in calcium imaging experiments (i.e. in intact cells), while ML2-SA1 has an $EC_{50}$ of 1.2 μM for hTRPML2, and SN-2 showed an $EC_{50}$ of 1.8 μM for hTRPML3 (Grimm et al. 2010, 2014a; Chen et al. 2014). A further modified congener of the isoxazoline SN-2, which selectively activates hTRPML3, is the isoxazole EVP-21, an aromatic analogue of SN-2 with an annulated cyclohexane moiety instead of a bicycloheptane moiety (Plesch et al. 2018). Although EVP-21 has a higher $EC_{50}$ (4.3 μM in calcium imaging experiments), it shows an increased efficacy and selectivity on human TRPML3 over human TRPML1 and human TRPML2 compared to SN-2. EVP-21 can elicit TRPML3 currents in both LE/LY and EE (Fig. 4).

Very recently, new selective agonists for TRPML1 and mouse TRPML3 were published (Spix et al. 2022). Thus, EVP-169 (ML1-SA1) selectively activates human and mouse TRPML1. EVP-77 (ML3-SA1) selectively activates mouse TRPML3 (Spix et al. 2022) (Fig. 3).

Wang et al. (2015) further published a TRPML activator, the aryl-bis-sulfonamide ML-SA3 with increased potency compared to ML-SA1. However, the stereochemistry of this compound on the cyclohexane ring was not further specified. More recently, another TRPML activator, ML-SA5, another aryl-bis-sulfonamide, was described ((Yu et al. 2020), Fig. 5).

Besides TRPML channel agonists, synthetic small molecules for TRPML channel inhibition named ML-SI1, ML-SI2, and ML-SI3 were reported (Samie et al.

**Table 3** Summary of the characteristics of TRPML3

| TRPML3 (*MCOLN3*) | |
|---|---|
| Associated phenotypes | No identified phenotypes in man, but TRPML3 *gain-of-function* mutations (A419P; I362T) cause the varitint-waddler (Va and VaJ) phenotype in mice, characterized by deafness, circling behaviour, head bobbing, and coat colour dilution (Di Palma et al. 2002; Nagata et al. 2008; Xu et al. 2007; Kim et al. 2007; Grimm et al. 2007, 2009) |
| Expression pattern | Hair cells of the inner ear, organ of Corti, utricle, stria vascularis, skin melanocytes, kidney, bladder, lung, liver, olfactory bulb, nasal cavity, thymus, colon, trachea, brain, and thymus |
| Subcellular localization | Early endosomal and lysosomal |
| Endogenous regulation | *Channel activation*: $PI(3,5)P_2$ (direct)<br>*Channel inhibition*: $PI(4,5)P_2$ (direct); luminal $H^+$, $Na^+$ |
| Functions | • Non-selective cation channel<br>• Endosomal maturation<br>• Endosomal trafficking<br>– EGF/EGFR trafficking<br>• Lysosomal trafficking<br>– Expulsion of pathogen-infected vesicles |
| Available agonists | • ML-SA1 (commercially available); not isoform selective<br>• MK6-83 (commercially available); not isoform selective<br>• SN-2 (commercially available); isoform selective<br>• EVP-21 (not commercially available); isoform selective (human TRPML3 selective)<br>• ML3-SA1 (EVP-77); isoform selective (mouse TRPML3 selective) (Spix et al. 2022) |
| Available antagonists | • None |

2013; Chen et al. 2014; Wang et al. 2015; Zhang et al. 2016; Kilpatrick et al. 2016). Only for two of these three compounds (ML-SI1 and ML-SI3) the chemical structures have been released (Wang et al. 2015). Unfortunately, it remained unclear from the original publication which of the stereoisomers of ML-SI1 and ML-SI3 are functionally active. Possible diastereomers would have *cis* or *trans* configuration and each of them can further occur in two enantiomeric forms (*R*,*R* or *S*,*S* for *trans*; *R*,*S* or *S*,*R* for *cis*) for its absolute configuration. We have synthesized both compounds, compared them with commercially available variants and analysed their activity on the TRPML isoforms.

ML-SI1, as published by Wang et al. (2015), is based on an indoline moiety and cannot be purchased from commercial providers. In contrast, commercially available GW405833 (CAS number: 18002-83-9), which was formerly (erroneously) offered as ML-SI1, is based on a fully aromatic indole framework (Fig. 6a). To elucidate which one of the two structures is able to block TRPML channels, we synthesized the indoline version of ML-SI1 and purchased the indole version (GW405833) of ML-SI1. ML-SI1 was synthesized as a racemic mixture of diastereomers, containing four different stereoisomers (*cis*/*trans* and enantiomers of each). Calcium imaging

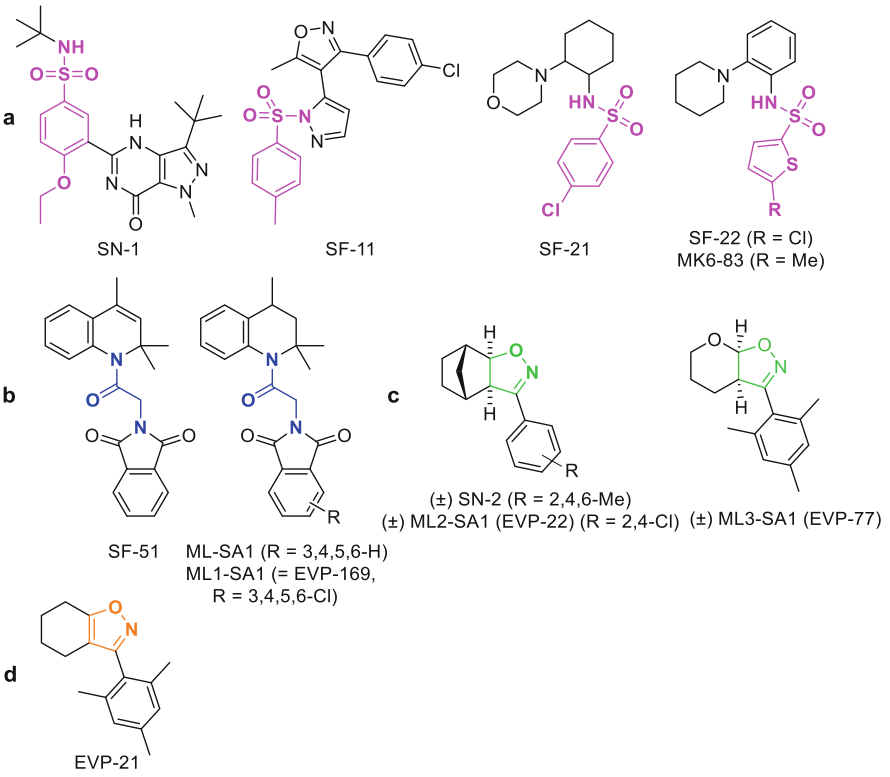


**Fig. 3** Small-molecule TRPML activators. (**a**) TRPML3 activators with the structural motif of (hetero)arylsulfonamides (highlighted in pink). SN-1, SF-11, and SF-21 only share an aryl sulfonamide moiety, whereas SF-22 and MK6-83 only differ in the substituent at the thiophene residue. (**b**) The dihydroquinoline SF-51 and the tetrahydroquinoline ML-SA1 both sustain phthalimidoacetamide moieties (blue) and only differ in one double bond. ML1-SA1 (= EVP-169) contains a tetrachlorophthalimidoacetamide moiety instead of a phthalimidoacetamide moiety in SF-51 and ML-SA1. ML-SA1 activates all human TRPML isoforms while SF-51 preferentially activates TRPML3 and ML1-SA1 selectively TRPML1. (**c**) The isoxazoline (green) SN-2 and selective TRPML2 activator ML2-SA1 (= EVP-22) differ merely in the substitution pattern of the phenyl ring. ML3-SA1 (= EVP-77) is a selective activator for mouse TRPML3 and contains a fused oxane ring instead of a fused norbornane ring in SN-2. (**d**) Selective TRPML3 activator EVP-21 with an isoxazole (orange) as structural motif (selective for human TRPML3)

experiments confirmed blocking effect of indoline ML-SI1 on TRPML1 (32% inhibition) and TRPML2 (33% inhibition) after activation with ML-SA1, whereas TRPML3 could not be blocked. Further experiments showed different blocking activities after activation with MK6-83. While ML-SI1 can block 45% of TRPML1 activity after stimulation with ML-SA1, the blocking effect after activation with MK6-83 is only around 20%. These results indicate that the antagonistic effect of ML-SI1 depends on the mode of activation. The commercially available indole analogue GW405833 showed no effect in patch clamp experiments on TRPML1

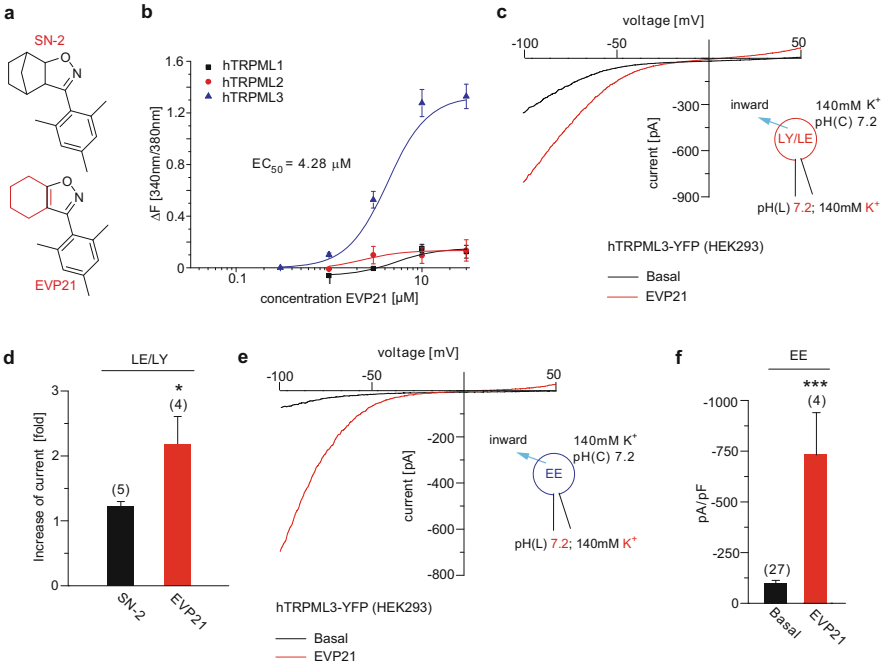


**Fig. 4** TRPML3 agonist EVP-21. Characteristics assessed in calcium imaging as well as in endolysosomal patch clamp experiments. (**a**) Structures of SN-2 and its analogous compound EVP-21. (**b**) Concentration-effect curves (CEC) obtained from Fura-2-AM calcium imaging experiments after application of EVP-21 at different concentrations. CEC shows the effect of EVP-21 on hTRPML1, 2, and 3. (**c**, **d**) Results of whole-LE/LY patch clamp recordings and (**e**, **f**) whole-EE patch clamp recordings using ramp protocols (−100 to +100 mV in 500 ms, every 5 s, holding potential 0 mV). For measurement, organelles (LE/LY (YM201636-enlarged) and EE (wortmannin/latrunculin B-enlarged)) were isolated from hTRPML3-YFP stably expressing HEK293 cells. Shown are representative EVP-21 stimulated TRPML3 currents in LE/LY (**c**) and EE (**e**). Pipette (luminal) solution contained 140 mM K-MSA, 5 mM Na-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, and 10 mM HEPES. Bath (cytoplasmic) solution contained 140 mM K-MSA, 5 mM KOH, 4 mM NaCl, 0.39 mM $CaCl_2$, 1 mM EGTA, and 10 mM HEPES. The current amplitudes at −100 mV were extracted from individual ramp current recordings with and without agonist. In (**d**) currents were normalized to those obtained without agonists. (**f**) Statistical analysis of currents as shown in (**e**). In all statistical analysis of endo-lysosome recordings, mean values of n (in parentheses) independent experiments are shown as indicated. $***p < 0.001$, $*p < 0.05$, Student's t test, unpaired

after activation with ML-SA1 (Fig. 6). There are, up to now, no data for the four single stereoisomers of the active indoline ML-SI1 available.

The second TRPML inhibitor published by Wang et al. (2015) is the aryl-sulfonamide ML-SI3. Commercially available ML-SI3 (CAS No.: 891016-02-7) was identified as racemic mixture of *trans*-isomers by NMR spectrometry. Further confirmation of the stereochemistry was provided by independent synthesis of (±)-

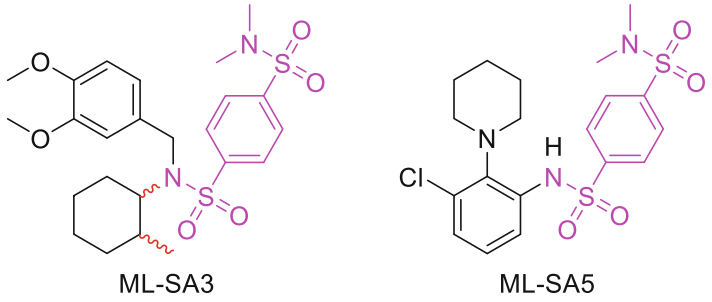


**Fig. 5** Structure of the aryl-bis-sulfonamides ML-SA3 and ML-SA5. Both compounds are TRPML1 activators. Unidentified stereochemistry of ML-SA3 is marked in red and the aryl-bis-sulfonamide moieties are marked in pink

*trans*-ML-SI3 in our laboratory (Leser et al. 2021). Inhibitory effects of ML-SI3 were analysed via calcium imaging and endolysosomal patch clamp experiments. Like ML-SI1, ML-SI3 is inhibiting ML-SA1 activated TRPML1 and 2 channels (55% and 66% inhibition), but not TRPML3. Also, in endolysosomal patch clamp experiments, ML-SI3 showed strong inhibitory effect on hTRPML2 after activation with the TRPML2 specific agonist ML2-SA1. Furthermore, no difference in the inhibitory effect was observed after activation with different TRPML activators. ML-SI3 was able to block hTRPML1 after activation with both ML-SA1 and MK6-83 (Fig. 7). Furthermore, an activating effect of the racemic mixture of *trans*-ML-SI3 on hTRPML2 could be observed. Separation of the enantiomers of *trans*-ML-SI3 by chiral HPLC showed that exclusively the (+)-*trans*-ML-SI3 is responsible for this activation, which in enantiopure form activates hTRPML2 ($EC_{50}$: 2.8 μM) and hTRPML3 ($EC_{50}$: 11 μM) whereas it inhibits hTRPML1 ($IC_{50}$: 5.6 μM). Very recently, we assigned the (*R*,*R*)-configuration to the active (+)-*trans* enantiomer by unambiguous chiral synthesis and single-crystal X-ray structure analysis (Kriegler et al. 2022). The (−)-*trans*-ML-SI3 has, however, a pure inhibitory effect on all three subtypes ($IC_{50}$ (hTRPML1):1.4 μM; $IC_{50}$ (hTRPML2): 2.2 μM; $IC_{50}$ (hTRPML3):11 μM) and is also the eutomer on TRPML1 ($IC_{50}$ of the racemic mixture: 2.7 μM). The racemic mixture of *cis*-ML-SI3 has a weaker inhibitory effect on hTRPML1 ($IC_{50}$: 19 μM) and activates TRPML2 ($EC_{50}$: 8.9 μM) and TRPML3 ($EC_{50}$: 27 μM). All $EC_{50}$ and $IC_{50}$ values were determined by a Fluo-4 calcium-imaging based FLIPR (Fluorescence Imaging Plate Reader) system using ML-SA1 as activator (Leser et al. 2021).

Comparing the two TRPML inhibitors, ML-SI3 seems more potent, as its blocking effect on hTRPML1 is about 55% while the blocking effect of ML-SI1 on hTRPML1 is around 32%. Drawbacks of these compounds are their lack of selectivity and the activator-dependent effects.

Ou et al. (2020) have used a not further specified TRPML(1) inhibitor named "130" without disclosing its structure or source, and without any information on isoform selectivity.

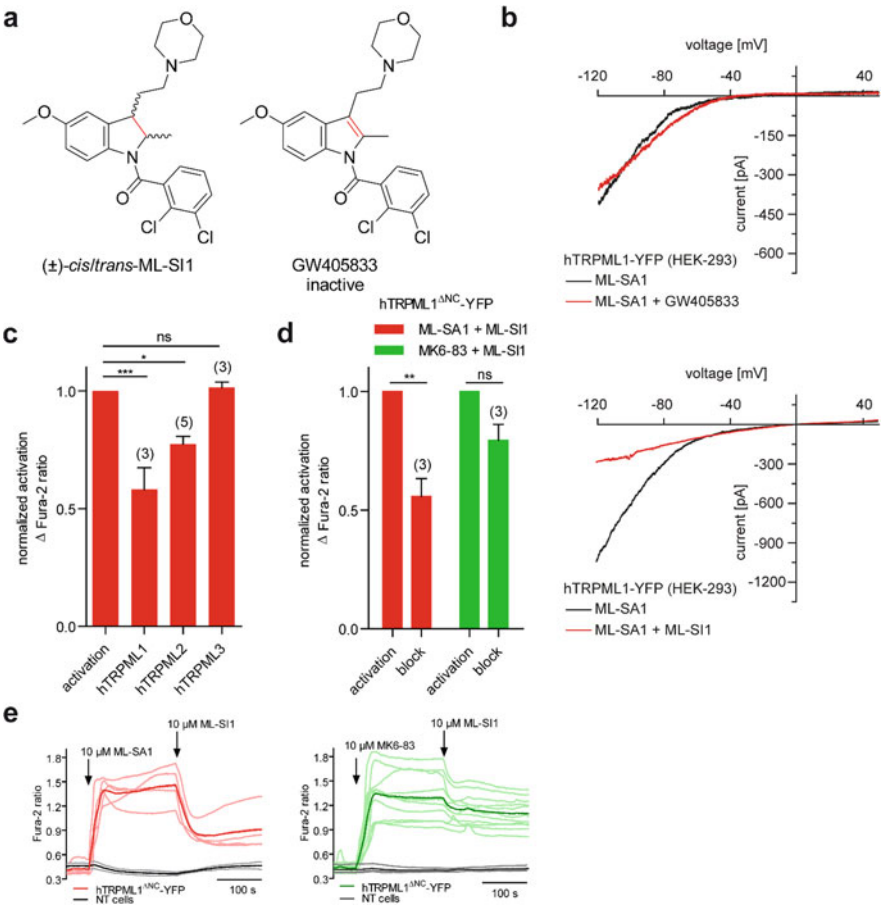


**Fig. 6** Small-molecule TRPML inhibitor ML-SI1 and its indole-type analogue GW405833. Characteristics assessed in calcium imaging as well as in endolysosomal patch clamp experiments. (**a**) Structure of racemic *cis*/*trans*-ML-SI1, with an indoline scaffold and commercially available, achiral indole GW405833. (**b**) In contrast to ML-SI1 GW405833 (10 μM) has no effect on hTRPML1-YFP transfected HEK-293 cells after activation with ML-SA1 (10 μM) in patch clamp experiments. Experimental conditions were applied as in Fig. 4c with the exception that pipette (luminal) solution contained 140 mM Na-MSA (Chen et al. 2017), 5 mM K-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM MES at pH 4.6 (Chen et al. 2017). (**c**) Statistical analysis of the inhibitory effect on TRPMLs in Fura-2-AM calcium imaging experiments (normalized activation). Experiments were carried out as previously described (Plesch et al. 2018) on a Polychrome IV monochromator (for hTRPML1) or a Leica DMi8 live cell microscope (for TRPML2 and 3). After stimulation with ML-SA1 (10 μM, activation) for 200 s, the inhibitor ML-SI1 (10 μM) was applied for further 200 s. For measurements HEK-293 cells stably expressing hTRPML2-YFP or hTRPML3-YFP, and transiently transfected hTRPML1-YFP cells were used (Grimm et al. 2010). Stably expressing hTRPML2-YFP cells were generated as previously described (Chen et al. 2014). (**d**) Statistical analysis as in (**c**), using ML-SA1 (10 μM, red) or MK6-83 (10 μM, green) for activation of hTRPML1$^{\Delta NC}$-YFP (plasma membrane variant of TRPML1) transiently transfected HEK-293 cells, followed by inhibition using ML-SI1 (10 μM). (**e**) Representative $Ca^{2+}$ signals recorded from hTRPML1-YFP transiently transfected HEK-293 cells, loaded with Fura-2-AM and stimulated with ML-SA1 (10 μM, red) or MK6-83 (10 μM, green), followed by addition of the inhibitor ML-SI1 (10 μM). Highlighted lines represent means,

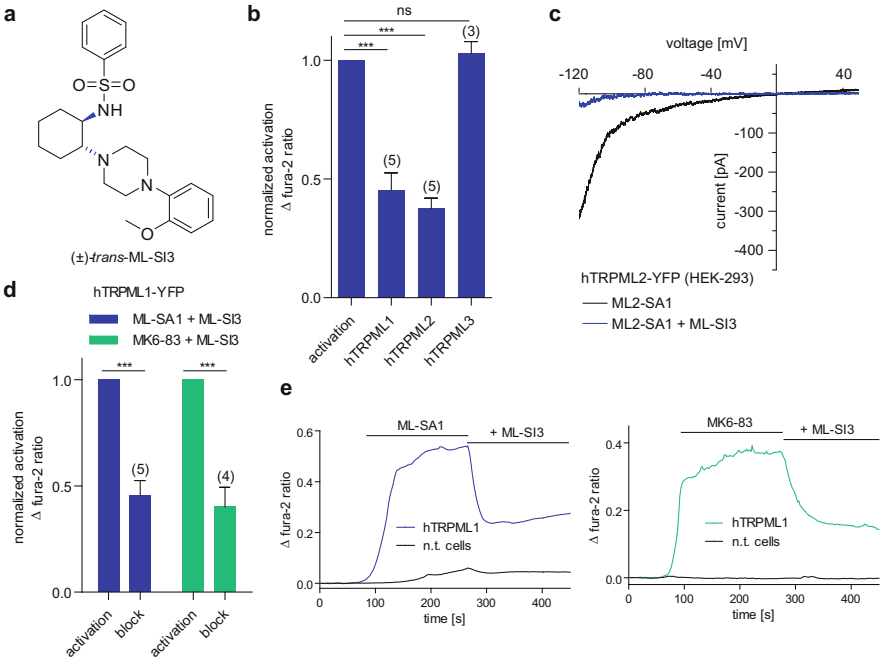


**Fig. 7** Small-molecule TRPML inhibitor ML-SI3. Characteristics assessed in calcium imaging as well as in endolysosomal patch clamp experiments. (**a**) Racemic mixture of *trans*-isomers of ML-SI3 identified as an active species. (**b**) Patch clamp experiments show block of hTRPML2-YFP transiently transfected HEK-293 cells with ML-SI3 (10 μM) after activation with the TRPML2 selective agonist ML2-SA1 (10 μM) as described in Fig. 4c. Pipette (luminal) solution contained 140 mM Na-MSA, 5 mM K-MSA, 2 mM Ca-MSA, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM MES at pH 7.2. (**c**) Statistical analysis of ML-SI3 (10 μM) after activation of hTRPML1, 2, or 3 with ML-SA1 (10 μM, activation), as described in Fig. 5c. (**d**) Statistical analysis as in (**c**), using ML-SA1 (10 μM, blue) or MK6-83 (10 μM, green) for activation of hTRPML1-YFP transiently transfected HEK-293 cells, followed by inhibition using ML-SI3 (10 μM). (**e**) Representative $Ca^{2+}$ signals recorded from hTRPML1-YFP transiently transfected HEK-293 cells, loaded with Fura-2-AM and stimulated with ML-SA1 (10 μM, blue) or MK6–83 (green, 10 μM), followed by addition of the inhibitor ML-SI3 (10 μM). Highlighted lines represent means, shaded lines single cell trances. In all statistical analyses of calcium imaging experiments, mean values of n (in parentheses) independent experiments are shown as indicated. *** indicates $p < 0.001$, ** indicates $p < 0.01$, ns = not significant, one-way ANOVA test followed by Tukey's post-hoc test

**Fig. 6** (continued) shaded lines single cell traces. In all statistical analyses of calcium imaging experiments, mean values of n (in parentheses) independent experiments are shown as indicated. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$, ns = not significant, one-way ANOVA test followed by Tukey's post-hoc test

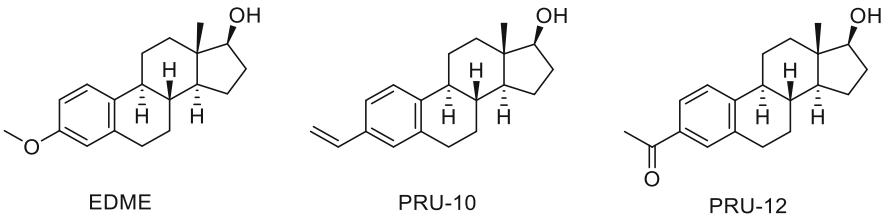


**Fig. 8** Chemical structures of the TRPML1 isoform selective blockers EDME, PRU-10, and PRU-12

Very recently (Rühl et al. 2021), we have presented novel TRPML1 isoform selective blockers which are based on the identification of a steroidal lead structure (EDME, 17$\beta$-estradiol methyl ether) in a high-throughput screening of a library of drug-like small-molecule compounds containing numerous FDA-approved drugs. Based on this screening hit we developed, by systematic structure variations, the advanced analogues PRU-10 and PRU-12 (Fig. 8). In whole-cell patch clamp experiments using EDME and the plasma membrane variant of TRPML1 (TRPML1$^{\Delta NC}$) the $IC_{50}$ measured for TRPML1 was 0.22 μM. No block for TRPML3 was found with this compound. TRPML2 was blocked with an $IC_{50}$ of 3.8 μM. For comparison, in analogous patch clamp experiments ML-SI3 blocked TRPML1 with an $IC_{50}$ of 4.7 μM and TRPML2 with an $IC_{50}$ of 1.7 μM, suggesting that ML-SI3 has an almost threefold stronger effect on TRPML2 compared to TRPML1 and is >20-fold weaker on TRPML1 than EDME. The synthetic analogues PRU-10 and PRU-12 showed a further improved selectivity profile compared to EDME (Rühl et al. 2021).

In the following, we present a summary of the characteristics of the three TRPML isoforms and the presently available modulators.

## 3 Small-Molecule Tools for the Modulation of TPCs

Two-pore channels (TPCs, TPCNs) are only distantly related to the TRPML channels in terms of sequence similarities, but both channel families share a number of functional features. Both TRPMLs and TPCs are non-selective cation channels in endo-lysosomes, they are permeable to sodium and calcium, they are activated by PI(3,5)$P_2$, and both are involved in endolysosomal trafficking, autophagy, TFEB (Transcription Factor EB) and mTOR signalling (Grimm et al. 2018). TPCs have been shown to be activated by NAADP (nicotinic acid adenine dinucleotide phosphate; (Brailoiu et al. 2010; Calcraft et al. 2009; Ogunbayo et al. 2011; Grimm et al. 2014b; Jha et al. 2014; Pitt et al. 2014; Ruas et al. 2015a)), although it remained unclear until recently whether activation is mediated directly or indirectly, e.g. via an auxiliary subunit (Walseth et al. 2012; Lin-Moshier et al. 2012; Morgan and Galione 2014; Morgan et al. 2015; Ruas et al. 2015b; Gerasimenko et al. 2015; Pitt et al. 2016; Grimm et al. 2017; Nguyen et al. 2017; Jiang et al. 2018b). In 2021, two

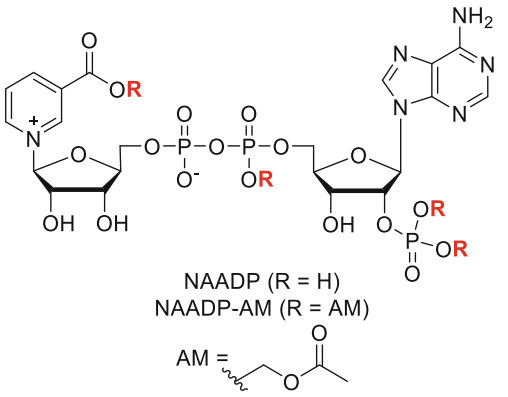


**Fig. 9** NAADP and its membrane-permeable acetoxymethyl (AM) ester prodrug variant NAADP-AM

groups have published independently neurological expressed 1-like protein (HN1L) or Jupiter microtubule-associated homologue 2 (JPT2) as NAADP binding protein. However, interaction was only confirmed for TPC1 but not TPC2, leaving open the question how NAADP acts on TPC2 (Gunaratne et al. 2021; Roggenkamp et al. 2021). Both activators $PI(3,5)P_2$ and NAADP are not plasma membrane permeable. A commercially available variant of NAADP, NAADP-AM (a lipophilic acetoxymethyl (AM) ester prodrug, Fig. 9) is plasma membrane permeable (Parkesh et al. 2008; Galione et al. 2014), but due to its instability very limited in use. Hence, there was an urgent need for lipophilic, plasma membrane-permeable small-molecule activators of TPCs.

Recently, small-molecule activators for TPCs have been identified by two independent groups. Zhang et al. (2019) identified tricyclic antidepressants (TCAs: clomipramine, desipramine, imipramine, amitriptyline, and nortriptyline), phenothiazines (chlorpromazine, trifluprom azine), and the benzothiazole riluzole by screening Sigma's LOPAC library (Fig. 10). All of these compounds are registered drugs and are able to activate TPC2 with $EC_{50}$ values between 43 and 112 μM in whole-cell patch clamp experiments. While clomipramine and desipramine can additionally activate TPC1, chlorpromazine and riluzole inhibit TPC1. Furthermore, currents evoked with the TCAs and phenothiazines were strongly voltage-dependent while the activation using riluzole was voltage-independent. This suggests different agonist-specific gating mechanisms within one ion channel. TCAs were introduced to treat depression, bipolar and panic disorder, chronic pain, and insomnia. Additionally, they block monoamine (dopamine, norepinephrine, and serotonin) reuptake and inhibit cholinergic, histaminic, and alpha-adrenergic transmission. Due to the broad range of adverse effects TCAs were meanwhile mostly replaced as antidepressants by the selective serotonin reuptake inhibitors (Trindade et al. 1998; Shelton 2019). For amitriptyline, imipramine, and clomipramine it is reported that they are potent CYP450 inhibitors, that block CYP450 2C19 and 1A2, which raises the risk of undesired drug–drug interactions (Gillman 2007; Gerndt et al. 2020b). Riluzole is an FDA-approved drug, which is used for the treatment of

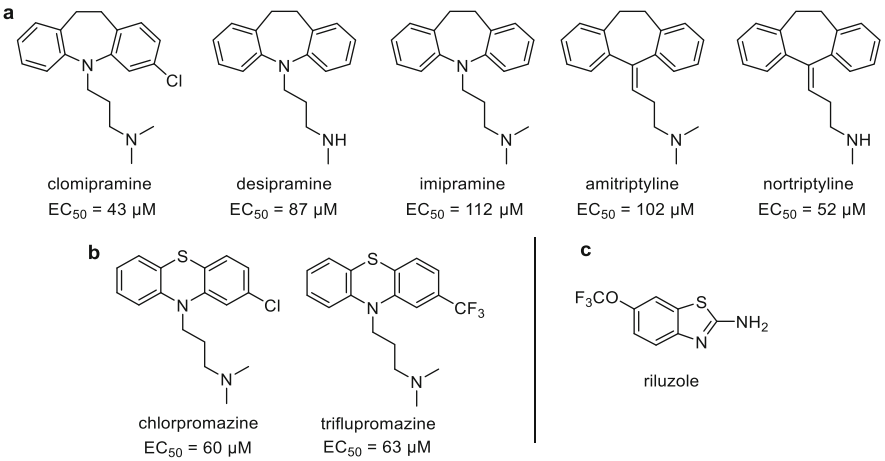


**Fig. 10** hTPC2 agonists published by Zhang et al. (2019). (**a**) Structures of the TCAs as hTPC2 activators and $EC_{50}$ values. (**b**) Structures of the phenothiazines and their $EC_{50}$ values as hTPC2 activators. (**c**) Structure of the hTPC2 agonist riluzole

amyotrophic lateral sclerosis (ALS) (Bissaro and Moro 2019; Liu and Wang 2018) and furthermore blocks tetrodotoxin-sensitive $Na^+$ channels (Song et al. 1997), kainite and NMDA (*N*-methyl-*D*-aspartate) receptors (Hubert et al. 1994; Debono et al. 1993; Malgouris et al. 1994).

Another HTS was performed by our consortium (Gerndt et al. 2020a), screening the 80.000 compound-strong Roche Explore libraries. Two differentially acting lipophilic small-molecule agonists of TPC2, namely TPC2-A1-N and TPC2-A1-P (Fig. 11a, b) were identified in calcium imaging experiments using the calcium indicator dye Fluo-4-AM, followed by re-evaluation in single cell calcium imaging using Fura-2-AM (Fig. 11 a, b). $EC_{50}$ values in fluorescence-based calcium imaging experiments were 7.8 μM (TPC2-A1-N) and 10.5 μM (TPC2-A1-P), while $EC_{50}$ values in electrophysiological endolysosomal patch clamp experiments were both 0.6 μM. Both compounds neither activated TPC1 nor activated TRPML1, 2, and 3 (Fig. 11c–f). In addition, it was shown that the activators mimicked the activation of NAADP (TPC2-A1-N) and $PI(3,5)P_2$ (TPC2-A1-P), respectively, rendering the channel either more calcium-(TPC2-A1-N) or more sodium-(TPC2-A1-P) permeable. TPC2-A1-N itself is known as anthelmintic agent (Sjogren et al. 1991), while there is no previous report on TPC2-A1-P in literature.

While there is comprehensive knowledge on the pharmacological profiles of TCAs, phenothiazines, and riluzole, due to their long-term application in therapy, the newly identified activators TPC2-A1-N and TPC2-A1-P (Fig. 11) require an in-depth pharmacokinetic and pharmacological characterization. On the other hand, TCAs are known for their unwanted side effects and also seem to be less potent activators of TPC2 with much higher $EC_{50}$ values compared to TPC2-A1-N and TPC2-A1-P.

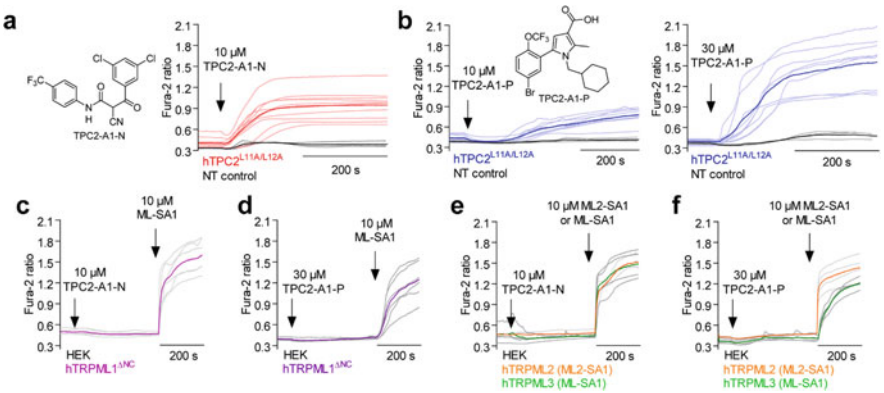


**Fig. 11** Confirmation of TPC2-A1-N and TPC2-A1-P as TPC2 activators (Gerndt et al. 2020a). (**a**, **b**) Structures of the activators and representative calcium signals, recorded from HEK-293 cells transiently transfected with plasma membrane targeted human TPC2 (hTPC2$^{L11A/L12A}$) and loaded with Fura-2-AM. Cells were activated with TPC2-A1-N (10 µM) or TPC2-A1-P (10 or 30 µM). Highlighted lines represent the mean response from a population of cells. Shaded traces represent responses of single cells. (**c**, **d**) Experiments as in a-b but cells were transiently transfected with human hTRPML1$^{\Delta NC}$-YFP (plasma membrane variant of TRPML1) and sequentially stimulated with TPC2-A1-N (10 µM) or TPC2-A1-P (30 µM) and the TRPML agonist ML-SA1 (10 µM). (**e**, **f**) Experiments as in (**c**, **d**), but cells were transiently transfected with human TRPML2, or TRPML3. Cells were sequentially stimulated with TPC2-A1-N (10 µM) or TPC2-A1-P (30 µM) and the TRPML agonist ML-SA1 (10 µM) or the TRPML2 selective agonist ML2-SA1 (10 µM)

Several TPC inhibitors have been proposed in the past. TPC activity can be blocked by commercially available *trans*-Ned-19 ((Naylor et al. 2009; Kelu et al. 2015; Nguyen et al. 2017); Fig. 12). In 2016, Kintzer and Stroud presented an X-ray structure of *trans*-Ned-19 bound to TPC1 from *Arabidopsis thaliana* and claimed direct interaction with the ligand involving F229 in S5, W232 in S5, L255 in P1, F444 in S7, and W647 in S12, thus clamping the pore domains and VSD2 (voltage-sensing domain 2) together (Kintzer and Stroud 2016). This results in an allosteric block of channel activation. The validity of these data has however been a matter of debate since then.

Besides *trans*-Ned-19, several other compounds have been shown to block TPCs: ATP (via mTOR (Cang et al. 2013)), the bisbenzylisoquinoline alkaloid tetrandrine (Sakurai et al. 2015; Nguyen et al. 2017), the flavonoids naringenin (Pafumi et al. 2017), pratensein (MT-8), and duartin (UM-9) (Netcharoensirisuk et al. 2021) as well as the marketed drugs fluphenazine and raloxifene ((Penny et al. 2019); Fig. 12).

The herbal alkaloid tetrandrine (isolated from *Stephania tetrandra*, Menispermaceae) is known to also block voltage-gated $Ca^{2+}$ channels, large-conductance $Ca^{2+}$-activated $K^+$ (BK) channels, and intracellular $Ca^{2+}$ pumps (sarcoplasmic reticulum $Ca^{2+}$-ATPase pumps; (Wang et al. 2004)). For naringenin it has been shown that it also blocks TRPM3 (Straub et al. 2013), voltage-gated sodium channels (Gumushan Aktas and Akgun 2018), cardiac HERG (human

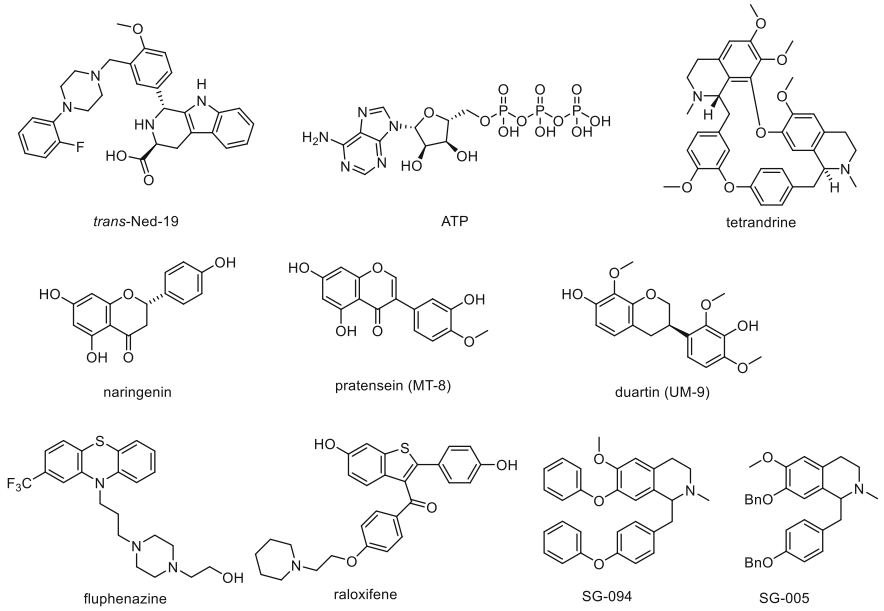


**Fig. 12** TPC2 blockers described in literature: *trans*-Ned-19, ATP, alkaloid tetrandrine, flavonoids naringenin, pratensein, and duartin, the drugs fluphenazine and raloxifene as well as benzylisoquinolines SG-094 and SG-005

Ether-à-go-go-Related Gene; Kv11.1) channels (Scholz et al. 2005), HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase, and ACAT (acetyl-CoA-acetyltransferase; (Lee et al. 1999)), and it enhances the activity of large-conductance $Ca^{2+}$-activated $K^+$ (BK) channels (Hsu et al. 2014). Likewise, the flavonoids pratensein and duartin may have other channel and non-channel targets. However, in contrast to naringenin ($IC_{50}$ = 74 μM for hTPC2), the $IC_{50}$s for pratensein and duartin are much lower, 2.6 μM and 9.5 μM, respectively (Netcharoensirisuk et al. 2021). Fluphenazine is an anti-psychotic drug used to treat psychotic disorders such as schizophrenia. Fluphenazine, like chlorpromazine and haloperidol, belongs to the first generation of antipsychotics and has a number of severe adverse effects, in particular extrapyramidal effects including acute dystonia, akathisia, Parkinsonism, and tardive dyskinesia (Divac et al. 2014). Fluphenazine blocks postsynaptic mesolimbic dopaminergic D1 and D2 receptors in brain. It also blocks neuronal voltage-gated sodium channels (Zhou et al. 2006) and the ATP-sensitive $K^+$ channel (Müller et al. 1991). Penny et al. 2019 reported an $IC_{50}$ of 8.2 μM in patch clamp experiments after TPC2 stimulation with $PI(3,5)P_2$. Raloxifene has an $IC_{50}$ of 0.63 μM in patch clamp experiments after TPC2 stimulation with $PI(3,5)P_2$ (Penny et al. 2019). Raloxifene belongs to the class of selective oestrogen receptor modulators (SERM) and is used for the treatment and prevention of osteoporosis in postmenopausal women, for reduction in risk of invasive breast cancer in postmenopausal women with osteoporosis and those at high risk for breast

**Table 4** Summary of the characteristics of TPC1

| TPC1 (*TPCN1*) | |
|---|---|
| Associated phenotypes | No identified phenotypes in man; mouse knockouts show delayed endosomal trafficking (Ruas et al. 2014; Castonguay et al. 2017), mature-onset obesity (Lear et al. 2015), and augmented systemic anaphylaxis and mast cell activity (Arlt et al. 2020) |
| Expression pattern | Broad, highest in heart and kidney |
| Subcellular localization | Throughout the endosomal system (EE to LE) |
| Endogenous regulation | *Channel activation*: PI(3,5)$P_2$ (direct); cytosolic $Ca^{2+}$; depolarization; sphingosine; NAADP?<br>*Channel inhibition*: Luminal $H^+$; luminal $Ca^{2+}$; ATP and mTORC1 (direct) |
| Functions | • Non-selective cation channel<br>• Endosomal ion homeostasis<br>– Endosomal *pH* regulation<br>– Endosomal $Ca^{2+}$ regulation<br>• Endosomal trafficking<br>– Virus (Ebola, SARS-CoV, and MERS-CoV) trafficking<br>Bacterial toxin trafficking through EE/RE (RE = recycling endosomes) |
| Available agonists | • Clomipramine; not isoform selective, other targets known<br>• Desipramine; not isoform selective, other targets known |
| Available antagonists | • *Trans*-Ned-19<br>• Tetrandrine; not isoform selective<br>• BZ194?; PPADS? (no endolysosomal patch clamp data available)<br>• Chlorpromazine, riluzole (both activate TPC2)<br>• SG-094, SG-005 (Müller et al. 2021); more potent and less toxic than tetrandrine |

cancer. Raloxifene also inhibits L-type and T-type voltage-sensitive $Ca^{2+}$ channels (Tsang et al. 2004; Wang et al. 2011) as well as Kv4.3 channels (Chae et al. 2015).

Other reported compounds which interfere with NAADP-mediated calcium signalling are BZ194, an *N*-alkylated nicotinic acid derivative (Dammermann et al. 2009), and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; (Singaravelu and Deitmer 2006; Billington and Genazzani 2007)). For both compounds, no direct measurements of TPC inhibition are published, in particular no endolysosomal patch clamp recordings are currently available.

In 2021, Müller et al. reported about novel TPC2 blockers SG-005 and SG-094, which represent truncated analogues of the bisbenzylisoquinoline alkaloid tetrandrine. These quite easily accessible compounds block TPC2 with an increased potency compared to tetrandrine and at the same time show less toxicity. SG-005 additionally blocked the activation of TRPML1 and TPC1, whereas SG-094 had no considerable inhibitory effect on TRPML1, only on TPC1.

In sum, for none of the currently available TPC antagonists isoform selectivity has been demonstrated and target selectivity needs to be further improved.

In the following, we present a summary of the characteristics of the two TPC isoforms (Tables 4 and 5).

**Table 5** Summary of the characteristics of TPC2

| TPC2 (*TPCN2*) | |
|---|---|
| Associated phenotypes | *Gain of function*: Associated with blond hair colour in man, either through pore dilation (SNP M484L) or decreased ATP inhibition/mTORC1 interaction (SNP G734E; (Sulem et al. 2008; Chao et al. 2017; Böck et al. 2021))<br>*Loss of function*: Mouse knockouts show reduced exercise endurance after fasting (Cang et al. 2013), delayed endolysosomal degradation/trafficking (Grimm et al. 2014b; Ruas et al. 2014), increased susceptibility to fatty liver disease (Grimm et al. 2014b), VEGF-induced vessel formation failure (Favia et al. 2014), decreased susceptibility to infectious diseases, e.g. Ebola (Sakurai et al. 2015), mature-onset obesity (Lear et al. 2015), and decreased tumour growth (Müller et al. 2021) |
| Expression pattern | Ubiquitous |
| Subcellular localization | Predominantly lysosomal by virtue of dileucine sorting motif |
| Endogenous regulation | *Channel activation*: $PI(3,5)P_2$ (direct); NAADP (not determined if direct or through unidentified accessory subunit), luminal $H^+$<br>*Channel inhibition*: ATP, mTORC1 (direct interaction), cytoplasmic/lysosomal $Mg^{2+}$<br>*Channel modulation:* By protein kinases (MAPKs, JNK, P38) |
| Functions | • Non-selective cation channel<br>• Melanosome maturation<br>• Intracellular NAADP signalling<br>• Endolysosomal trafficking<br>– Virus (Ebola, SARS-CoV, and MERS-CoV) trafficking<br>– LDLR/LDL trafficking<br>– EGFR/EGF trafficking |
| Available agonists | • Clomipramine, desipramine, imipramine, amitriptyline, nortriptyline, chlorpromazine, triflupromazine, riluzole (other targets known, commercially available)<br>• TPC2-A1-N and TPC2-A1-P (Gerndt et al. 2020a); isoform selective (TPC2 selective) |
| Available antagonists | • *Trans*-Ned-19; tetrandrine; raloxifene; fluphenazine<br>• ATP<br>• Naringenin<br>• Naringenin related flavonoids: pratensein (MT-8), duartin (UM-9) (Netcharoensirisuk et al. 2021; lower $IC_{50}$s compared to naringenin)<br>• BZ194?; PPADS? (no endolysosomal patch clamp data available)<br>• SG-094, SG-005 (Müller et al. 2021); more potent and less toxic than tetrandrine |

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