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Pharmacology of TRPC Channels and its Potential in Cardiovascular and Metabolic Medicine

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Abstract

Transient Receptor Potential Canonical (TRPC) proteins assemble to form homo- or heterotetrameric, non-selective cation channels permeable to Na^+ and Ca^{2+}. TRPC channels are thought to act as complex integrators of physical and chemical environmental stimuli. Although the understanding of essential physiological roles of TRPC channels is incomplete, their implication in various pathological mechanisms and conditions of the nervous system, kidney and cardiovascular system in combination with the lack of major adverse effects of TRPC knockout or TRPC channel inhibition are driving the search of TRPC channel modulators as potential therapeutics. Here, we review the most promising small-molecule TRPC channel modulators, the understanding of their mode-of-action, and their potential in the study and treatment of cardiovascular and metabolic disease.

INTRODUCTION

Following identification of the gene underlying the photoreceptor Transient Receptor Potential (TRP) phenotype of mutant *D melanogaster*, related genes were identified in mammals and first reported in the literature a quarter of a century ago (1, 2). The first mammalian TRPs recognised were those with the closest sequence similarity to *D melanogaster* TRP, which became known as TRPCs (with the C indicating Classical or Canonical) (3, 4). There are seven such genes encoding proteins in mammals. In *H sapiens* and closely related primates, *TRPC2* is a pseudogene (5), so there are considered to be six human TRPC proteins (TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7) (3, 4, 6). In some instances there are splice variants, notably for TRPC4, further increasing complexity (7).

As with *D melanogaster* TRP, the TRPCs assemble as tetramers around a single central ion pore that is gated and non-selectively permeable to cations. When the gate is closed (i.e., the channel is not activated), the tetramers are non-permeable. Activation stimuli lead to opening of the gate. In physiology, the gated permeability results in influx of Na^+ and Ca^{2+} across the plasma membrane and efflux of K^+. The entry of Ca^{2+}, the special signalling ion, usually leads to an elevated cytoplasmic Ca^{2+} concentration, which stimulates normal cellular activity unless the Ca^{2+} elevation is too high, which may be detrimental to cell survival (8). The non-selective flux of cations has a depolarising influence on the membrane potential, driving it from a negative value towards zero. In cells that fire action potentials, such as neurones, depolarisation usually increases action potential firing and excitability; in cells that do not, such as endothelial cells, quiescence may be promoted because of decreased electrical driving force on Ca^{2+} entry. Na^+ entry can be important in its own right by elevating the intracellular Na^+ concentration: stimulating Na^+/K^+-ATPase activity, driving Na^+ entry into mitochondria and reducing the transmembrane Na^+ gradient, thereby decreasing the energy available for Ca^{2+} extrusion on Na^+-Ca^{2+} exchangers (9).

Voltage sensitivity of TRPC channels

TRPC channels are broadly similar in structure to voltage-gated K^+ channels, such as those of the K_V1 type. However, TRPC channels are not K^+-selective and not considered to be voltage-gated. The activation mechanisms for TRPC channels are not as clear-cut as they are for many other types of ion channel. The K_V1s, for example, are activated by depolarisations of specific magnitude, without which they do not usually open; ligand-gated ion channels, such as P2X receptors, are activated specifically by exposure to one chemical or a related set of chemicals. The concepts are vaguer and often multifactorial for TRPCs (4, 9, 10). Although not considered to be voltage-gated, these channels exhibit variable voltage-dependence, especially when over-expressed in cell lines. Their activity may increase substantially as membrane voltage becomes positive and conversely decrease in activity or conductance as voltage becomes negative; hence the channels show signature double-rectifying current-voltage relationships in voltage-clamp experiments (11–14). Such voltage-dependence may occur on a background of constitutive activity or chemically stimulated activity (3, 15). The physiological significance of the voltage-dependence is unclear. Instead, direct and indirect chemical activation mechanisms are thought to be most important (4, 9, 13).

Physiological activation of TRPC channels

The chemical activators of TRPC channels are numerous and there are reasonable cases to be made for the channels being coincidence detectors of multiple chemical signals, sensors of cocktails of chemicals and perhaps broad sensors of the chemical melees of the

extracellular and intracellular environments (4, 9, 16). A common theme of TRPCs is their association with G protein-coupled receptors and their downstream signalling pathways (4, 11, 17). There is ample evidence for activation by agonists (e.g., acetylcholine, ATP, histamine, sphingosine-1-phosphate) that act via G protein-coupled receptors and downstream Gαq/11 or Gαi proteins to stimulate TRPC channel activity (4, 11, 17, 18). The channels are associated with receptors and G proteins linked to phospholipase C activation and the lipid substrates and products of phospholipase Cs (4, 10, 19). Such activity usually promotes channel opening, but may also drive subsequent channel desensitisation (20). Elevation of the intracellular Ca^{2+} concentration is often associated with these mechanisms, for example via IP_3-evoked Ca^{2+} release, which can be a powerful enhancer of TRPC channel activity in conjunction with other stimulators. The channels are probably not simply Ca^{2+}-activated (contrasting, for example, with the Ca^{2+}-activated K^+ channels) because Ca^{2+} elevation alone does not activate or is a poor activator; it seems that co-factors are needed and so the channels might best be considered as Ca^{2+}-facilitated (10, 11, 21, 22). There are also lipid and redox stimulators of the channels, some of which may act directly, such as diacylglycerol (23–25), oxidised phospholipids (26) and reduced thioredoxin (13). Other factors to consider are protons (27, 28) and temperature (29). The latter is an important regulator of other mammalian TRP channels such as TRPV1 and TRPM8 (30). However, while there is evidence for TRPC5 channels being activated by noxious cold (29, 31), temperature change is not generally considered to be a major stimulant for TRPCs.

TRPC heteromerisation

TRPC1 stands out amongst the TRPCs because it generates little or no channel activity when expressed alone in a host cell line and does not usually reach the surface membrane (18, 32, 33). In contrast, when co-expressed with TRPC4 or TRPC5, it readily forms heteromers with them, impacting the overall voltage-dependence, pore conductance and ion selectivity (12, 13, 33, 34). TRPC1 is in some ways comparable to electrically-silent Kv channel subunits (35): a γ-subunit that is similar in structure to α-subunits, such as TRPC4, but unable to function on its own, yet able to incorporate with α-subunits and function with them as an assembly (32, 33). The other TRPCs are all capable of forming functional homomers when expressed in cell lines and some of them may do so natively in physiology – particularly TRPC3 and TRPC6. TRPC4 and TRPC5 seem more likely to exist physiologically as functional heteromers with TRPC1, which is widely expressed. In general, native TRPC channel compositions are technically difficult to determine and so remain uncertain in most situations (36). The biophysical characteristics and activation mechanisms of the channels often make it challenging to convincingly distinguish TRPC channel activity from other channel activity or background signals in native cells. Advances in TRPC channel pharmacology, as we describe in this article, increasingly enable better delineation of native activation mechanisms and determination of the relative importance of these channels in physiology and disease.

Expression of TRPC channels

D melanogaster TRP is specifically associated with the fly's photo-transduction but in mammals, photo-transduction occurs via other mechanisms involving different ion channels. TRPC expression, at least at mRNA level, is broadly detected across many, if not all, mammalian cell types, and there may be no mammalian cell type that completely lacks TRPC expression (4, 37). However, TRPC proteins and functional TRPC channels may not be expressed and important in all cell types. Instead, there seems to be differential expression and functional importance in different cell types and contexts, depending for example on whether the system is stressed by inflammation or disease. Perhaps unsurprisingly – because

TRPCs form ion channels with potentially major impact on cell function – their abundance is quite low and often at the limits of reliable detection by biochemical or functional assays.

TRPC channel in physiology and disease

Genetic knockouts of TRPCs, either alone or in combination, are not lethal for mice (38). This suggests that TRPCs are not critical for life, or at least life of laboratory mice. This does not mean that TRPCs lack importance. Experiments on animal models of disease and studies of cells and tissues from humans with disease suggest TRPC channels or their excessive activation may cause or exacerbate disease; proposed functions of TRPCs are often linked to disease or a model of disease (37), including CNS disorders, kidney disease, cancer and cardiovascular and metabolic disease. Such findings support the idea that inhibitors of TRPCs may be beneficial against certain types of disease and have relatively mild or no adverse effects.

Here we provide an overview of the most promising chemical TRPC channel modulators that can be used to investigate TRPC channels in cells, tissues and animals. We also discuss recent progress with structural studies of TRPC channels that are starting to unveil the modes of action of chemical modulators, and we present key findings of the role of TRPC channels in cardiovascular and metabolic physiology and pathology.

SMALL-MOLECULE TRPC MODULATORS

Unravelling the roles of TRPC channels in physiology and pathology benefits from carefully designed combinations of genetic and pharmacological approaches. Since our last review of the field in 2013 (37), academic and industrial groups have reported many high-quality small-molecule TRPC channel modulators, some of which have been used to discover new biological functions of TRPC channels. Wang et al. recently published a comprehensive review of the TRPC channels and their small-molecule modulators (39), and several recent, more focussed reviews are available (36, 40–42). Here, we focus on TRPC modulators that: 1) are the most promising for use as potent and selective chemical probes; 2) have been used to discover biological functions of specific TRPC channels; and 3) provide new insights into TRPC channel regulation (for example through structural studies). We advise on TRPC1/4/5 pharmacology from direct experience. For TRPC3/6/7 pharmacology, we describe observations reported by other groups.

TRPC channel activators (Table S1)

TRPC1/4/5 channel activators

The most potent, efficaceous and selective TRPC1/4/5 activator is the natural product (-)-englerin A (EA) (43–45). It activates TRPC1/4/5 currents at low nanomolar concentrations; so far, no other targets have been found that are modulated at such concentrations. The toxicity of EA to certain human cancer cells correlates with expression of TRPC4 and/or TRPC5, and its cytotoxic effect on A498 renal cancer cells and SW982 synovial sarcoma cells has been demonstrated to result from increased Na^+ influx mediated by heteromeric TRPC1:C4 channels (44, 46). EA has been used to activate endogenous TRPC1/4/5 channels in cells (43, 44, 46), tissues (47) and animals (48). However, its (on-target) toxicity (45, 49) and instability in (rodent) plasma and the GI tract (45) need to be considered when using EA for in vivo studies.

The xanthine AM237, a close analogue of Pico145 (see below), is a potent partial agonist of homomeric TRPC5:C5 channels that inhibits TRPC4:C4 channels and heteromeric TRPC1/4/5 channels (50). AM237 activation of TRPC5:C5 is apparently competitively inhibited by Pico145. AM237 is selective with respect to TRPC3, TRPC6, TRPV4 and

TRPM2 channels. The xanthine-based photoaffinity probes Pico145-DA and Pico145-DAAlk mimic the functional effects of AM237 on TRPC1/4/5 channels, and have been used to demonstrate direct interactions between xanthine-based TRPC1/4/5 modulators and TRPC5 protein in cells (51). These studies highlight AM237 as a potentially useful tool in distinguishing TRPC5:C5 channels from other TRPC1/4/5 tetramers, and provide insights into the mode-of-action of xanthines as TRPC1/4/5 modulators (see below).

The marketed drug riluzole has been reported to activate TRPC5:C5 (EC_{50} 9.2 μM) and TRPC1:C5 channels, but not TRPC4 channels (52). Its suitability for oral dosing has led to the use of riluzole as a TRPC5 activator for in vivo studies (53). Although its activity is thought to be relatively direct, based on activities in excised patch recordings and reversibility on washout, riluzole modulates a large number of targets, including many ion channels (54). This needs to be considered when using riluzole in functional studies, for example by including controls in which riluzole's effect on TRPC5 channels is inhibited using a selective TRPC5 inhibitor.

TRPC3/6/7 channel activators

A high throughput screen followed by structure-activity relationship studies resulted in the discovery of pyrazolopyrimidines (including the highly potent pyrazolopyrimidine 4n) as TRPC3/6/7 channel activators that do not activate TRPC4, TRPC5 or several other TRP channels (55). Close analogues were subsequently reported as potent TRPC6 inhibitors (56).

Researchers at GSK developed the potent and selective TRPC3/6 activator GSK1702934A, which was used in studies with murine Langendorff hearts, in which it enhanced contractility and evoked arrhythmia (57, 58). Tiapko et al. developed a photoswitchable analogue of GSK1702934A, called OptoBI-1 (59), which displayed faster kinetics than the previously developed OptoDArG (60) and allowed optical control of endothelial and neuronal TRPC3 channels.

Recently, researchers at Amgen reported the discovery of the potent TRPC6 channel activator AM-0883, and the identification of its binding site by cryo-EM (see below) (61).

TRPC channel inhibitors (Table S2)

The potency and efficacy of TRPC channel inhibitors can be activator-dependent. The choice of activator used for inhibitor discovery can be pragmatic, and may depend on the assay type and cell lines used. For example, EA (for TRPC1/4/5) and OAG (for TRPC3/6/7) give robust responses in both fluorometric assays and electrophysiology, while responses to specific G-protein coupled receptor-activation may be more relevant to physiology, but can be more difficult to distinguish from background signals. Therefore, we recommended (where possible) profiling of new TRPC inhibitors against multiple activators and considering activity against specific activators when combining activators and inhibitors in functional studies.

TRPC1/4/5 channel inhibitors

Xanthine derivatives were claimed as TRPC5 channel inhibitors in a patent by Hydra Biosciences describing >600 examples (62). One of these xanthines, Pico145 (also called HC-608), is the most potent TRPC1/4/5 inhibitor reported to date (63, 64), with picomolar potencies against heteromeric channels. Bauer et al. used a competitive photoaffinity labelling approach to demonstrate that the effect of xanthines such as Pico145 on TRPC5 channels is mediated by a direct binding interaction (65), and subsequent cryo-EM studies revealed the TRPC5 binding site and mode of Pico145 (see below) (66). In addition, Yu et al. reported the development of a Pico145-based ^{11}C and ^{125}I radiotracers (67, 68). Just et al. reported the anxiolytic and antidepressant effects in mice of a close analogue of Pico145,

named HC-070 (64). They confirmed HC-070 and Pico145 as potent inhibitors of human, mouse and rat TRPC1/4/5 channels. In addition, both compounds were >400-fold selective against a large set of ion channels, receptors, enzymes, kinases and transporters (>2000-fold for most), and orally bioavailable. HC-070 has been reported to bind to the same site of TRPC5:C5 as Pico145 (69). Overall, Pico145 and HC-070 are considered valuable chemical probes for functional studies of TRPC1/4/5 channels, with demonstrated use for inhibition of endogenous TRPC1/4/5 channels in cells (46, 63, 70–72), tissues (71, 73, 74) and animals (49, 62, 64, 74, 75).

A team at Goldfinch Bio discovered GFB-8438 as a potent inhibitor of rat and human TRPC4:C4 and TRPC5:C5 channels, with favourable physicochemical properties and good selectivity against TRPC3/6/7, other TRP channels, and cardiac channels (76). So far, its activities against heteromeric TRPC1/4/5 channels have not been reported. The TRPC4 binding site and mode of GFB8438 was recently determined by cryo-EM (see below) (77).

In 2011, Miller et al. reported ML204 as a low micromolar inhibitor of TRPC4:C4 and TRPC5:C5 channels with high selectivity with respect to other channels, receptors and transporters (78). ML204 has been used for in vivo studies of TRPC5 (53). It should be noted that its activity on heteromeric TRPC1/4/5 channels may be activator-dependent (53, 79).

TRPC3/6/7 channel inhibitors

One of the first sub-micromolar TRPC3 channel inhibitors was Pyr3, which showed selectivity with respect to TRPC5 channels and was suggested to bind directly to TRPC3 based on photoaffinity labelling (80). The compound suppressed cardiac hypertrophy in mice. Although Pyr3 also targets calcium-release-activated calcium channel (ORAI1) activity with similar potency, further analogues have been developed that show different selectivities between TRPC and ORAI channels (81).

GSK2833503 is a member of a class of potent, selective TRPC3/6 inhibitors based on a 2-aminothiazole core, some of which were used to inhibit pathological cardiac hypertrophy in mice (82, 83). The enantiomer of GSK2833503 is 10-fold less potent against TRPC3 and 100-fold less potent against TRPC6, providing a potential control compound with well-matched physicochemical properties. The analogue BTDM was used to determine the location of a small molecule binding site of the TRPC6:C6 channel by cryo-EM (see below) (84).

The TRPC3/6/7 channel inhibitor BI749327 is most potent against TRPC6, and does not inhibit TRPC5, other TRP channels and cardiac channels. The compound is suitable for oral dosing and was used for studies in mouse models of heart and kidney disease (85).

Derivatives of the natural product (+)-larixol have been described as inhibitors of TRPC3/6/7 channels (86, 87), with the methyl carbamate derivative SH045 being the most potent one (87). SH045 is selective with respect to TRPC4, TRPC5 and other TRP channels, and was shown to decrease edema in explanted mouse lungs.

The indane derivative SAR7334 was reported as a nanomolar TRPC3/6/7 inhibitor with selectivity against TRPC4/5 and TRPC5 channels. SAR7334 is orally bioavailable and suppresses acute pulmonary vasoconstriction in mice (88). The most potent TRPC6 inhibitor reported so far is its close analogue, AM-1473, which was used to determine the location of a an indane binding site of TRPC6:C6 channel by cryo-EM (see below) (61). DS88790512 is another analogue that, while lacking the aromatic indane core, retains high potency against TRPC6 channels as well as oral bioavailability (89).

STRUCTURAL INSIGHT INTO TRPC CHANNEL PHARMACOLOGY

Determination of 3-dimensional structures of proteins can aid the understanding of their molecular interactions and function. TRPC proteins form large, membrane spanning,

flexible and structurally heterogeneous channels, which may explain why, so far, crystallographic approaches have been unsuccessful. However, cryo-EM structures of multiple TRPC channels have been determined to resolutions sufficient to observe amino acid side chains as well as bound small molecules and lipids (39, 90, 91).

TRPC channel structures

Sub-4 Å structures have been reported for several homomeric TRPC channels, including human TRPC3:C3 (84, 92), human TRPC6:C6 (61, 84), zebrafish (77, 93) and mouse (94) TRPC4:C4, mouse TRPC5:C5 (95) and human TRPC5:C5 (66) (**Table 1**). Each structure shows the same overall fold, consisting of a homotetramer with each monomer providing six transmembrane helices (**Figure 1**). The first four transmembrane helices (S1-S4) of each monomer independently fold into a voltage-sensor-like domain (VSLD) followed by two transmembrane helices (S5 and S6) with a re-entrant P loop in-between that forms the ion pore of the channels. The channels have internal N- and C-termini that fold into a large intracellular domain, and several, relatively short external (E) loops. Although the overall structures of TRPC channels are similar, TRPC1/4/5 channels contain additional residues in the E3 loop, which have been implicated in their differential response to lanthanides when compared to TRPC3/6/7 channels (96). In contrast, TRPC3/6/7 channels contain additional residues in the E1 and E2 loops, further from the central pore. It should be noted that the largest differences between TRPC proteins are in the intracellular N- and C-terminal domains of TRPC channels, which contain intrinsically disordered regions (IDRs). In structural studies, these domains are often truncated or not modelled. (97)

Lipid binding sites of TRPC channels

Membrane proteins often have essential and specific interactions with membrane lipids, which can have stabilising or regulatory roles. Because lipids play a role in regulation of TRPC channel activity, the observation of lipids in some of the TRPC cryo-EM structures is of interest. Although several lipid-like groups are usually visible in EM maps, there are two main sites with well-defined lipids or lipid-like molecules (**Figure 1**). Lipid site 1 is found in the inner leaflet in the VSLD, whereas lipid 2 is observed in the outer leaflet bound to the P-loop and S6 helix of adjacent subunits. In the absence of small-molecule modulators, the TRPC5:C5 (66, 95) and TRPC4:C4 (93, 94) structures contain in site 2 a well-defined lipid (thought to be ceramide-1-phosphate or phosphatidic acid), which interacts with phenylalanine and tryptophan residues conserved within the LFW motif of the TRPC family. In addition, density was observed in site 1, which was attributed to cholesterol hemisuccinate (CHS; added during purification). The 3.3 Å TRPC3:C3 structure and 3.1 Å structures of TRPC6:C6 also show two additional non-protein densities in these sites (61, 92). The density observed in site 1 in these TRPC6:C6 structures (PDB 6uza and PDB 6uz8) is also modelled as CHS. In TRPC3:C3, this density was modelled as a phospholipid, as it is not CHS-shaped and no CHS was added during purification. This suggests that lipid site 1 might bind endogenous lipid in all TRPC channels. The TRPC6:C6 structures also contain additional modelled lipids in the inner (PE) and outer (CHS) leaflets of the membrane. Lipid 2 in TRPC6:C6 occupies an overlapping site with the lipid observed in TRPC4:C4 and TRPC5:C5, again making interactions with phenylalanine and tryptophan residues of the LFW motif. However, the lipid is shifted towards the extracellular side and rotated close to perpendicular compared to lipid 2 in TRPC4/5 structures,

Small molecule binding sites of TRPC channels (Table 1)

Recently, structures of TRPC4, TRPC5 and TRPC6 channels have unambiguously revealed binding sites and binding modes of small-molecule modulators (**Figure 1**).

Structures of hTRPC5:C5 in the presence of the potent inhibitor Pico145 showed that Pico145 binds to lipid site 2 and replaces the phospholipid observed in this site in TRPC4/5 structures determined in the absence of small-molecule modulators (66). A similar replacement of lipid 2 was observed in the structure of TRPC6:C6 in the presence of the TRPC6 agonist AM-0883 (61). Structures of drTRPC4:C4 in the presence of the closely related inhibitors GFB-8438, GFB-8749 and GFB-9289 show that these molecules bind to a region close to the modelled cation in the VSLD (77). This binding site is also observed in the structure of TRPC6:C6 in the presence of the inhibitor AM-1473 (61), suggesting that this is a common site for modulation of TRPC channels. The structure of TRPC6:C6 was also determined in the presence of the inhibitor BTDM (84), which showed additional unmodelled density between the VSLD and the pore (an interaction between S4, the S4-S5 linker and S5) in a distinct site, at an equivalent position to resiniferatoxin in the structure of TRPV1:V1 (98).

Although structures have been solved in the presence of activators and inhibitors, structures of TRPC channels in the open state have remained elusive. Additionally, the native state of many TRPC channels may not be a homotetramer; various heteromeric states (of unknown stoichiometries) may be present in many tissues, especially for TRPC1/4/5 channels.

PHYSIOLOGY AND PATHOPHYSIOLOGY

Most TRPC channels are ubiquitously expressed in cardiovascular cells including vascular smooth muscle cells (VSMCs), endothelial cells (ECs), and cardiac pacemaker cells and myocytes. Significant evidence exists for roles of TRPCs in cardiac electrical activity, excitation-contraction coupling, and vascular tone (99–101). TRPC1-, TRPC3- and TRPC4-mediated Ca^{2+} influx in VSMCs and ECs can regulate vasoreactivity and thus vascular tone (102). Although a role for TRPC5 was suggested in baroreceptor mechanosensors (103), this finding has been challenged (104–106). TRPC1/3/4/5 channels have also been shown to regulate angiogenesis by controlling different endothelial functions such as proliferation, migration and tube formation (107). Recently Zhu and colleagues showed that pharmacological activation of endothelial TRPC5 improves recovery from hypoxic injury through NFATc3-ANGPT1 signalling pathway using hind limb ischemia model (108). While the physiological contributions of TRPC channels to cardiovascular cellular function are still debatable, unequivocal evidence exists for their involvement in models of cardiovascular disease (see (100, 102, 109–113) for reviews). By and large, inhibiting channel activity or decreasing expression reduce such cardiovascular disease-like pathologies. Here, we summarise current evidence on the participation of TRPC channels in the major cardiac, vascular and metabolic pathologies.

Cardiac Disorders

TRPC-mediated Ca^{2+} influx and downstream events involving calcineurin and NFAT are implicated in murine models of cardiac hypertrophy induced by neurohormonal agents such as angiotensin II (82, 114), and by ischemia/reperfusion injury (115, 116), aortic constriction (82, 114) and pulmonary hypertension (112). TRPC channels may act as receptor- and store-operated Ca^{2+} entry channels in cardiac cells and there is some evidence for their role in stretch-activated Ca^{2+} entry too (109, 117). Gene knockout and dominant-negative expression studies showed that TRPC1/4 and TRPC3/6 are involved in hypertrophic remodelling (113, 118). Both in vitro and murine data suggest that stimulation of GPCRs by hypertrophic agents and mechanical stress leads to TRPC3/6/7 activation and thus cardiac hypertrophy (119). Londono et al. showed that reduction of background Ca^{2+} entry into

cardiac myocytes (through constitutively active TRPC1/4) in TRPC1/4 double knockout mice ameliorated cardiac hypertrophy induced by neurohormonal and mechanical stimulation, and suggested no role for TRPC3/6 (114). However, the TRPC3 inhibitor Pyr3 inhibited hypertrophic growth in rat neonatal cardiomyocytes and in pressure overload-induced cardiac hypertrophy in mice (80). Moreover, using genetic and chemical approaches, Seo et al. showed that TRPC3/6 inhibition reduces cardiac hypertrophy (82). Such discrepancy may be explained by different genetic background (C57Bl/6J vs mixed), or compensatory changes in the expression of other TRPCs or related genes/pathways. The TRPC3/6/7 inhibitor BI749327 improved left heart function by reducing interstitial fibrosis in a pressure overload mouse model (85). Upregulation of TRPC channels in myocytes of murine and human hypertrophic hearts has been observed, which may indicate that their over-expression could be part of the pathogenesis (113, 120). Activation of the mineralocorticoid pathway has been suggested as the molecular mechanism of this upregulation (121). In addition, TRPC1/3/4/6 were upregulated in post-MI heart, while knockout of TRPC3/6/7 reduced the infarct size and tissue damage (115). Triple knockout of TRPC3/6/7 reduced ischemia/reperfusion (I/R) injury and thus these channels were proposed as specific targets for I/R injury. Interestingly, a well-known cardioprotective action of urocortin-2 in I/R injury was suggested to act through reduced expression of ORAI1 and TRPC5 (116). Notably TRPC3/6 channels can physically interact with and activate ROS-producing NADPH oxidase (Nox) enzymes, and thus induce oxidative stress in cardiomyocytes and cardiac fibroblast leading to cardiac remodelling and fibrosis (122–124). Given the central role of ROS in cardiac failure, these findings suggest that TRPC channels are a potential target for heart failure. In support of this, increased expression of TRPC5 and TRPC6 was observed in failing human hearts (125, 126). Because sarcoplasmic reticulum Ca^{2+} is a key regulator of action potential generation/propagation and cardiac contraction, and roles of TRPC channels have been suggested, it is not surprising that investigators have proposed that dysregulation of TRPC expression and function can lead to arrhythmia (127). TRPC1/4/5 channels (upregulated by aldosterone-induced mineralocorticoid signalling (128)) and TRPC3/6 channels (activated by angiotensin II (129) or the TRPC3/6 agonist GSK1702934A (58)) have been associated with arrhythmias that are sensitive to the non-selective TRPC channel inhibitor SKF-96365 (128, 129). TRPC channels may play key roles in electromechanical conduction in developing hearts (99). Stretch-dependent modulation of TRPC6 expression in atrial endocardium has been suggested to regulate endothelin-1 (ET-1) production and thus play a key role in the development of myocardial calcium transients and arrhythmia (130). For detailed reviews of these topics, see (109, 131).

Vascular Disorders

While physiological functions of TRPC channels in the vasculature remain under-explored, we and others have conducted extensive work elucidating their roles in vascular pathophysiological processes and disorders including angiogenesis, atherosclerosis, neointimal hyperplasia, inflammation and systemic and pulmonary hypertension. Phenotypic transition of contractile VSMC to a synthetic type is one of the key pathological processes regulated by TRPC channels, and underlies many of these vascular diseases (132–134). A summary of salient findings on the role of TRPC channels in major vascular disorders is given below.

Atherosclerosis and vascular inflammation

Intracellular Ca^{2+} is well-established as a key signalling ion in fundamental pathophysiological processes – including endothelial dysfunction (135), leukocyte extravasation and adhesion (136), smooth muscle migration and proliferation (137), and oxidative stress (138) – that contribute and lead to vascular inflammation and atherosclerosis (139). Hence Ca^{2+} channel inhibitors have long been advocated for treating atherosclerosis (140). TRPC1 induces smooth muscle migration and proliferation and its expression was enhanced in pig models of vascular injury and in vitro human vein culture models (134). Importantly inhibition of TRPC1 reduced neointima formation in those models. Vazquez and colleagues found that Ca^{2+} influx through TRPC3 channels is essential for cell adhesion molecule expression and activity in endothelial cells, and the TRPC3 inhibitor Pyr10 (81) reduced ER stress-induced apoptosis in endothelial cells. These findings suggest TRPC3 as a potential target for atherosclerosis management (141, 142). Moreover, activity of TRPC3 in macrophages can protect them from apoptosis, which can enhance atherosclerotic lesion progression (143). TRPC1/5 and TRPC3/4 are redox-sensitive (13, 144). As described in cardiac disorders, also in endothelial cells TRPC3 interacts with Nox enzymes and contributes to ROS generation (141). Knockout studies showed that TRPC5 and TRPC6 activity impairs endothelial healing in vitro and in vivo after endothelial injury (145, 146).

Systemic and pulmonary hypertension

Given the significant role of TRPC1/3/6 in VSMC phenotypic switching and proliferation, NO signalling and regulation of vascular tone, it is not surprising that multiple studies have shown a link between TRPC channels and primary systemic hypertension (100, 112). Defective Ca^{2+} homeostasis and increased expression of TRPC1, TRPC3 and TRPC5 have been described in the vasculature and peripheral blood cells of hypertensive humans and animals (100). In contrast, TRPC6 knockout mice exhibited hypertension, which could – at least partly – be explained by compensatory increase in TRPC3 expression (147). However, mesenteric vessels of 11-deoxycorticosterone acetate-treated hypertensive rats showed increased TRPC6 expression and activity (148). These studies suggest that the role of specific TRPC channels in contraction and resultant blood pressure regulation is complex, and dependent on both the partner channels and the pathophysiological context. Increased pulmonary vascular contractility and resistance led to pulmonary remodelling, resulting in pulmonary arterial hypertension (PAH) (149). TRPC1/4/6 are commonly implicated in pulmonary artery smooth muscle (PASMC) and endothelial cell (PAEC) Ca^{2+} influx and proliferation, hypoxic vasoconstriction, and subsequent vascular remodelling and PAH (150, 151). Importantly treatment with β-carboline derivative or larixyl acetate, probably through inhibition of TRPC6-containing channels, can reduce the hypoxic pulmonary vasoconstriction, suggesting TRPC channels as promising targets for treatment of PAH (86, 152, 153). Similarly the TRPC3/6/7 inhibitor SAR7334 suppressed acute hypoxic pulmonary vasoconstriction in mice, but mean arterial pressure was unaltered in spontaneously hypertensive rats (88).

Other cardio-metabolic disorders

Cardiovascular complications of diabetes

Physiologically, TRPC3 channels in hypothalamic neurons are essential for sensing glucose and thus insulin secretion and glucose regulation (154). Leptin-induced TRPC4 activation leads to trafficking of K_{ATP} channels to the plasma membrane of pancreatic β-cells during fasting, and thus dampens insulin secretion (155). While expression of TRPCs has

been described in the islet cells, their roles remain largely unknown. However, TRPC channels have been implicated in development of diabetic complications such as nephropathy (156), neuropathy (157, 158), retinopathy (159) and vasculopathy (160, 161). Diabetes upregulates the expression of various TRPC channels in EC and VSMC and thus it is conceivable that TRPC-mediated pathological signalling leading to vascular disorders could be exacerbated in diabetes (160). Involvement of TRPC5 and TRPC6 has been extensively studied in kidney disease (see (162) for a review). Recent knockout studies have shown that TRPC6 signalling may have mixed effects on diabetic nephropathy, which highlights the complex roles on TRPC channels in different tissues and conditions (163). Quadruple knockout (TRPC1/4/5/6) mice were protected from hyperglycaemia-induced retinal changes through the preservation of Muller and microglial functions (159). Involvement of TRPC6 has been described in peripheral neuropathy using a streptozotocin-induced rat model of diabetes (158).

Adipose tissue and obesity

Major functions of adipocytes – including metabolism, insulin signalling and adipokine secretion – require Ca^{2+}-mediated signals. The physiological functions of TRPC channels in adipose tissue and their pathophysiological importance in obesity-associated metabolic diseases are not yet completely understood. In an in vitro adipocyte model cell line, 3T3-L1, adipocyte differentiation induces the expression of TRPC1 and TRPC5 (15). In addition, murine and human adipocytes express constitutively active TRPC1 and TRPC5 channels that sense ω-3 fatty acids; inhibiting the channels leads to increased secretion of the cardioprotective adipokine adiponectin both in vitro and in vivo (15). In agreement with this study, a transgenic mouse model with TRPC5 pore mutation that prevents ion permeation through the channels showed reduced weight gain and favourable adipose phenotype upon high fat feeding (164). Using global TRPC1 knockout mice, it was shown that TRPC1 channels have a significant role in the regulation of cellular energy metabolism, and that loss of TRPC1 results in increased fat mass and insulin resistance upon high-fat feeding compared to wild type litter mates (165). It has also been reported that TRPC1 regulation of adiposity is through its contrasting effects on autophagy and apoptosis of adipocytes (165). These studies suggest that inhibition of TRPC1:C5 channels could be a potential therapeutic strategy in metabolic disorders, and may improve the beneficial effects of exercise. In contrast, disruption of *Trpc1* increased weight gain and adversely affected metabolic profile by downregulating thermogenic genes expression in brown adipose tissue (166). Furthermore, a study using a neuronal and pro-opiomelanocortin (Pomc)-specific *Trpc5* knockout model showed increased weight gain in knockout animals, leading to the hypothesis that TRPC5 is essential for leptin regulation of hunger/satiety and energy homeostasis (167). Further work is needed to clearly elucidate the roles of TRPC channels in adipose tissue and obesity.

CONCLUSIONS

TRPC channels remain a fascinating class of ion channels, especially because of their ability to assemble as various distinct tetramers and integrate a wide range of physical and chemical signals. Essential physiological roles of TRPC channels are still incompletely understood. However, involvement of TRPCs in diverse cardiovascular and metabolic diseases, in combination with the lack of major adverse effects of TRPC knockout or TRPC channel inhibition, render TRPC channels attractive therapeutic targets. For many cardiovascular and metabolic diseases, neither the exact composition of the relevant TRPC channels nor the implication of potential redundancy and compensatory upregulation of other channels is known. In addition, in only very few studies of TRPC channels in the cardiovascular system, specific TRPC channel modulators were used. Recent advances in the

development of potent and selective chemical probes of specific TRPC channel subtypes will enable the design of studies that carefully combine targeted genetic approaches (e.g., conditional/site-specific knockouts or gene editing) with high-quality pharmacological approaches. In addition, rapidly developing insights into the mode-of-action of small-molecule modulators, through structural biology and detailed pharmacological studies, will underpin the design of the next generation of chemical probes and drug candidates.

DISCLOSURE STATEMENT

David J. Beech is an inventor on the following patent applications: 1) PCT/GB2018/050369. TRPC ion channel inhibitors for use in therapy. Filing date: 9th February 2018. 2) 62/529,063. Englerin derivatives for the treatment of cancer. Filing date: 6th July 2017.

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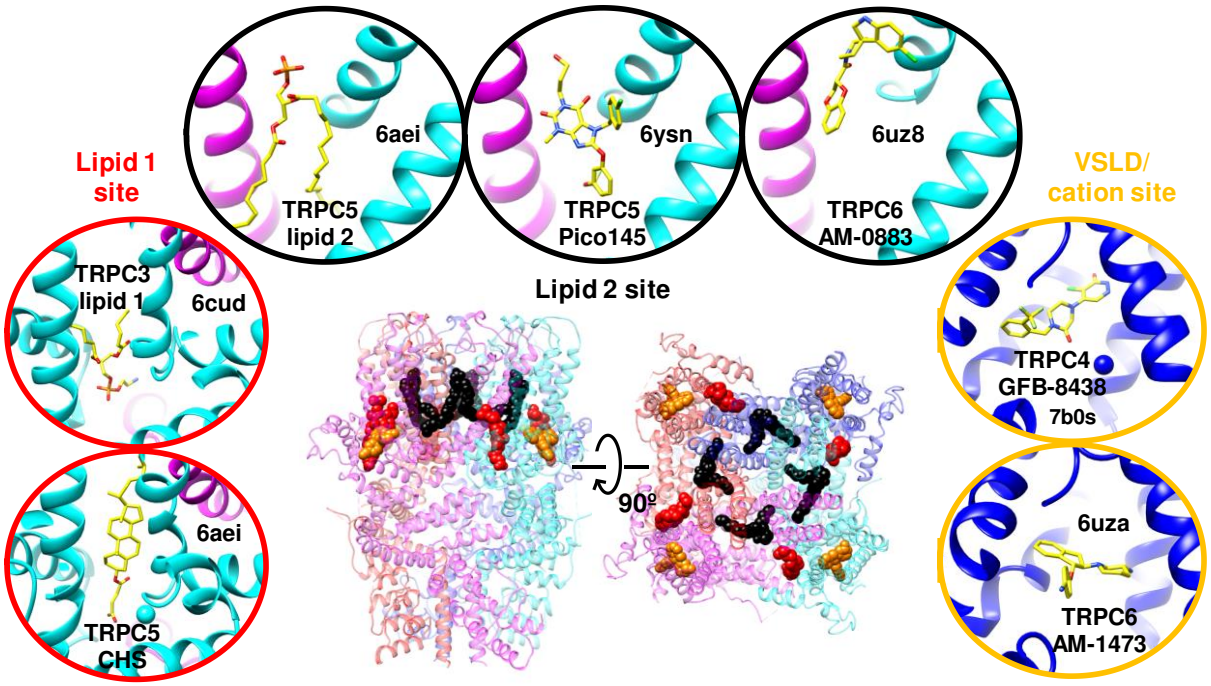


Figure 1. **TRPC channel cryo-EM structures revealing bound lipids and small-molecule modulators.** Centre: structure of mTRPC5:C5 (PDB 6aei), side view and top view, showing the four TRPC5 subunits (magenta, blue, cyan and salmon) and the binding sites of lipid 1 (CHS; red) and lipid 2 (modelled as PA; black). In addition, a molecule of GFB-8438 (from the drTRPC4:C4 structure PDB 7b0s; orange) was superposed onto the VSLD/cation binding site. Surrounding the mTRPC5:C5 structure: examples of lipid and small-molecule binding sites from different TRPC cryo-EM structures (PDB codes shown), with colours of the surrounding ring matching the binding sites displayed on the full mTRPC5:C5 structure. For a full overview of sub-4 Å TRPC channel structures, see Table 1.

Table 1. Overview of sub-4 Å TRPC channel structures

Channel (construct[1])	PDB (EMDB)	Resolution (Å)	Bound molecules (site)	Ref
hTRPC3 (full length)	6cud (7820)	3.30	Two unidentified lipids, modelled as a PE (lipid 1) and a diglyceride (lipid 2)	(92)
mTRPC4 (1-758 of 974)	5z96 (6901)	3.28	CHS (lipid 1); PA or C1P (lipid 2)	(94)
drTRPC4 (full length)	6g1k (4339)	3.60	CHS (lipid 1); PA or C1P (lipid 2)	(93)
drTRPC4 (full length)	7b0s (11970)	3.60	CHS (lipid 1); PA or C1P (lipid 2); GFB-8438 (VSLD/cation)	(77)
drTRPC4 (full length)	7b05 (11957)	3.80	CHS (lipid 1); PA or C1P (lipid 2); GFB-8749 (VSLD/cation)	(77)
drTRPC4 (full length)	7b16 (11979)	3.15	CHS (lipid 1); PA or C1P (lipid 2); GFB-9289 (VSLD/cation)	(77)
mTRPC5 (1-765 of 975)	6aei (9515)	2.8	CHS (lipid 1); PA or C1P (lipid 2)	(95)
hTRPC5 (1-765 of 973)	6ysn (10903)	3.00	Unmodelled density (lipid 1); Pico145/HC-608 (lipid 2)	(66)
hTRPC6 (full length)	5yx9 (6856)	3.80	BTDM (between VSLD and pore); weak density for lipids (lipid 1; lipid 2)	(84)
hTRPC6 (73-end)	6uza (20954)	3.08	AM-1473 (VSLD/cation); CHS (lipid 1); PC (lipid 2); CHS (outer leaflet), PC (inner leaflet)	(61)
hTRPC6 (73-end; V867T/ L868T)	6uz8 (20953)	2.84	CHS (lipid 1); AM-0883 (lipid 2); CHS (outer leaflet), PC (inner leaflet)	(61)

[1] Note that not all residues and domains of the used constructs could be observed/modelled. CHS = cholesteryl hemisuccinate; PA = phosphatidic acid; C1P = ceramide-1-phosphate; PC = phosphatidyl choline.

Table S1. Selected TRPC channel activators

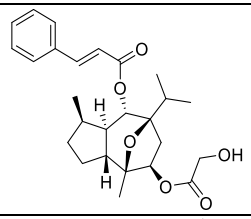
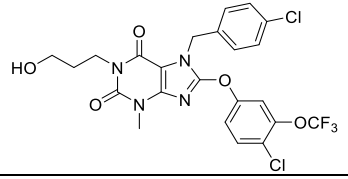
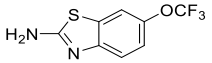
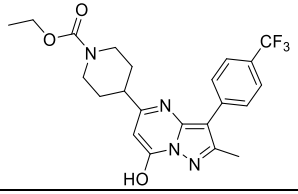
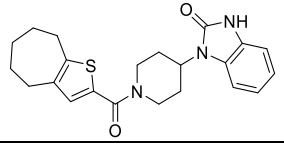
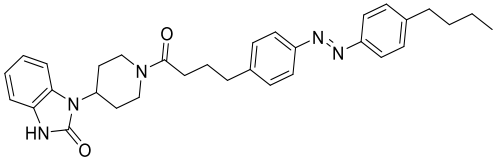
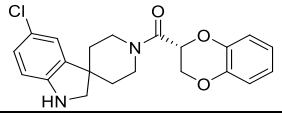
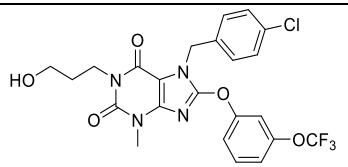
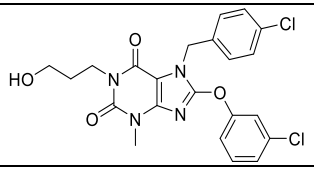
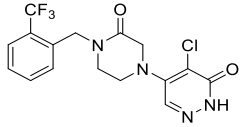
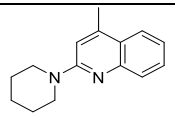
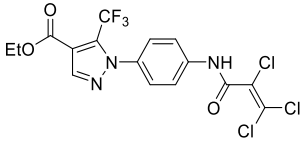
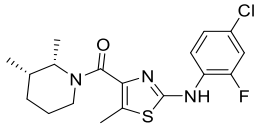
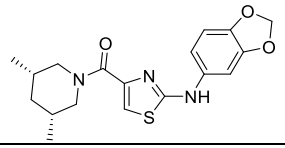
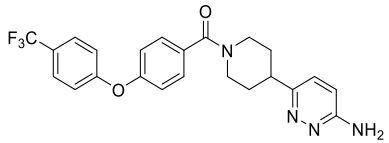
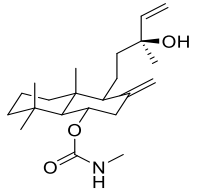
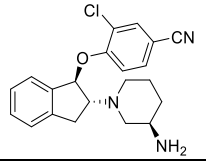
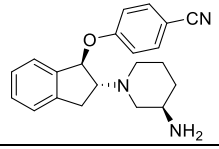
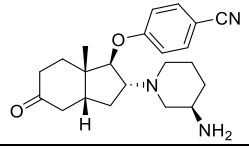
Name (alias)	Chemical structure	Targets (EC_{50})	Comments	Ref
(-)-englerin A (EA)		**TRPC4** (11 nM) **TRPC5** (7.6 nM) **TRPC1:C4** (10 nM) **TRPC1:C5** **TRPC4-C1** **TRPC5-C1**	Highly selective; kills cancer cells expressing TRPC4 or TRPC5; rapidly metabolised; on-target toxicity in mice is inhibited by Pico145	(43–47, 49)
AM237		**TRPC5** (15-20 nM)	nM inhibitor of other TRPC1/4/5 channels but not TRPC3/6, TRPV4 and TRPM2; Pico145 is a competitive antagonist of AM237; analogous photoaffinity probes available	(50, 62, 65)
riluzole		**TRPC5** (9.2 μM)	Approved drug; no TRPC4 activation; many other targets; no effects on heteromeric TRPC1/4/5 channels reported	(52–54)
pyrazolo-pyrimidine 4n		**TRPC3** (19 nM) **TRPC6** (1.4 nM) **TRPC7** (90 nM)	Selective with respect to TRPC4, TRPC5, and other TRP channels; close analogues are TRPC6 inhibitors(56)	(55)
GSK1702934A		**TRPC3** (80 nM) **TRPC6** (440 nM)	Selective with respect to ion channels and receptors; enhances contractility and evokes arrhythmia in Langendorff hearts of mice over-expressing TRPC3	(57, 58)
OptoBI-1		**TRPC3** **TRPC6** **TRPC7**	Photoswitchable activator; chimera of GSK1702934A(57, 58) and photoswitchable lipid OptoDArG(60); tested at 10-20 μM	(59)
AM-0883		**TRPC6** (46 nM)	Binding site and pose identified by cryo-EM	(61)

Table S2. Selected TRPC channel inhibitors

Name (alias)	Structure	Targets (EC_{50}; activator)	Mode-of-action/comments	Ref
Pico145 (HC-608; c31)		**TRPC4** (0.49-11 nM; EA, S1P or CCh/M2R) **TRPC5** (0.32-7.6 nM; EA, S1P, La^{3+} or CCh/M1R) **TRPC4-C1** (9-481 pM; S1P or EA) **TRPC5-C1** (199 pM; EA) **TRPC1:C4** (1.3 nM; CCh/M2R) **TRPC1:C5** (1.4-4.4 nM; La^{3+} or CCh/M1R)	Active on human, rat and mouse channels; minimal inhibition of >300 other targets up to 1-2 µM; direct binder according to photoaffinity labelling; inhibits cytotoxicity of EA; efficacy demonstrated in cells, tissues and animals; developed into PET tracer; high plasma protein binding; binding site and pose in TRPC5 revealed by cryo-EM; analogous photoaffinity probes available	(49, 62, 64–68, 70–72, 74, 79)
HC-070		**TRPC4** (0.49-1.8 nM; CCh/M2R) **TRPC5** (0.32-3.4 nM; La^{3+} or CCh/M1R) **TRPC1:C4** (1.3 nM; CCh/M2R) **TRPC1:C5** (1.4-4.4 nM; La^{3+} or CCh/M1R)	Active on human, rat and mouse channels; minimal inhibition of >300 other targets up to 1-2 µM; efficacy demonstrated in cells, tissues and animals; binding site and pose in TRPC5 revealed by cryo-EM	(62, 64, 69, 73)
GFB-8438		**TRPC5** (0.18-0.28 µM; riluzole) **TRPC4** (0.29 µM; EA) **hERG** (8.7 µM)	Good selectivity; favourable physicochemical properties and PK data; no data for heteromeric channels reported; efficacy demonstrated in FSGS model; binding site and pose in TRPC4:C4 identified by cryo-EM	(76, 77)
ML204		**TRPC4** (0.96-2.6 µM; CCh+M2R/µOR) **TRPC4-C1** (58 µM; EA) **TRPC5** (inhibits at 10 µM; DAMGO+µOR) **TRPC6** (18.4 µM, ACh)	Selective with respect to other TRP and non-TRP channels; profiled against 68 membrane proteins and in 397 further assays; used in multiple in vivo studies; inhibition of heteromeric TRPC1/4/5 channels is activator-dependent; effect on TRPC6 may be through ACh receptor inhibition	(78, 79)
Pyr3		**TRPC3** (0.5-0.8 µM, OAG, UTP, ATP) **CRAC** (0.54 µM)	Selective with respect to TRPC1:5; photoaffinity labelling suggests direct interaction with TRPC3; suppresses cardiac hypertrophy in mice	(80, 81)

GSK2833503A (GSK503A)		**TRPC3** (21-100 nM; CCh) **TRPC6** (3-16 nM; CCh)	Selective with respect to other TRP channels and cardiac channels; enantiomer is 10-fold less potent against TRPC3 and 100-fold less potent against TRPC6; inhibits pathological cardiac hypertrophy	(82, 83)
BTDM		**TRPC3** (11 nM; OAG) **TRPC6** (10 nM; OAG)	TRPC6 binding site identified by cryo-EM	(84)
BI749327		**TRPC3** (940-1100 nM; OAG) **TRPC6** (13-19 nM; OAG) **TRPC7** (550-580 nM; OAG)	Selective with respect to TRPC5, other TRP channels, $Na_V1.5$ and hERG; suitable for oral dosing; suppresses interstitial fibrosis and associated signalling in mouse models of heart disease and kidney disease	(85)
SH045		**TRPC3** (440-634 nM; OAG) **TRPC6** (5.2-5.8 nM; OAG) **TRPC7** (18-22 nM; OAG)	Selective with respect to TRPC4, TRPC5 and other TRP channels; decreased edema formation in explanted mouse lungs	(87)
SAR7334		**TRPC3** (282 nM; OAG) **TRPC6** (7.9-9.5 nM; OAG) **TRPC7** (226 nM; OAG)	Selective with respect to TRPC4, TRPC5; suitable for chronic oral dosing; suppresses acute hypoxic pulmonary vasoconstriction in mice but does not change mean arterial pressure in spontaneously hypertensive rats	(88)
AM-1473		**TRPC6** (0.22 nM, OAG)	Close analogue of SAR7334; used to identify TRPC6 binding site and pose by cryo-EM	(61)
DS88790512		**TRPC6** (11 nM; OAG)	Selective with respect to hERG and $hNa_V1.5$; orally bioavailable	(89)