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Review

Beyond Hot and Spicy: TRPV Channels and their Pharmacological Modulation

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Abstract
Transient receptor potential vanilloid (TRPV) channels are part of the TRP channel superfamily and named after the first identified member TRPV1, that is sensitive to the vanillylamide capsaicin. Their overall structure is similar to the structure of voltage gated potassium channels (K_v) built up as homotetramers from subunits with six transmembrane helices (S1-S6). Six TRPV channel subtypes (TRPV1-6) are known, that can be subdivided into the thermoTRPV (TRPV1-4) and the Ca^{2+}-selective TRPV channels (TRPV5, TRPV6). Contrary to K_v channels, TRPV channels are not primary voltage gated. All six channels have distinct properties and react to several endogenous ligands as well as different gating stimuli such as heat, pH, mechanical stress, or osmotic changes. Their physiological functions are highly diverse and subtype as well as tissue specific. In many tissues they serve as sensors for different pain stimuli (heat, pressure, pH) and contribute to the homeostasis of electrolytes, the maintenance of barrier functions and the development of macrophages. Due to their fundamental role in manifold physiological and pathophysiological processes, TRPV channels are promising targets for drug development. However, drugs targeting specific TRPV channels, that are suitable for drug therapy, are rare. Moreover, selective and potent compounds for further research at TRPV channels are often lacking. In this review different aspects of the structure, the different gating stimuli, the expression pattern, the physiological and pathophysiological roles as well as the modulating mechanisms of synthetic, natural and endogenous ligands are summarized.

The structure of TRPV channels

In 1969 Cosens and Manning described a mutated *Drosophila melanogaster* strain, that showed abnormal electroretinogram (ERG) and impaired phototransduction. The ERG was characterized by a transient receptor potential (TRP) instead of a rather sustained potential in the wildtype [1]. Later, this mutated strain was associated with the first invertebrate TRP

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cation channel, which was cloned in 1989 [2]. Finally, in 1995 the first mammalian homologue of the transient receptor potential canonical (TRPC) family, was cloned and characterized [3, 4]. From then to now 28 members of the TRP cation channel superfamily were discovered in mammals, that can be subdivided by sequence homology into two groups and 6 families: Group I contains the families transient potential receptor canonical (TRPC1-7), vanilloid (TRPV1-6), ankyrin (TRPA1) and melastatin (TRPM1-8), while the families polycystin (TRPP2, TRPP3, TRPP5) and mucolipin (TRPML1-3) belong to Group II (Fig. 1A) [5, 6]. The TRPV channels are named after the first discovered member TRPV1, that is sensitive to stimulation by the vanillylamide capsaicin [7]. TRPV1 is formed by assembling of four TRPV1 subunits, that have a similar transmembrane structure like voltage gated K^+ channels (Fig. 1B-D) [8, 9]. Later on, the vanilloid subfamily was extended by TRPV2 (previously VRL1), TRPV3 (OLMS, VRL3), TRPV4 (VRL2, Trp12, VROAC, OTRPC4), TRPV5 (ECaC1, CaT2) and TRPV6 (ECaC2, CaT1) [10–13].

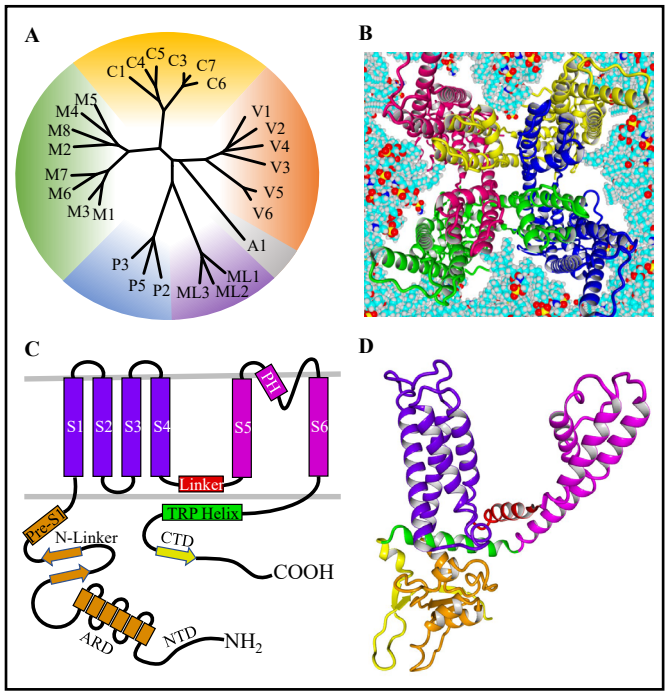

Fig. 1. A. Phylogenetic tree of TRP channels found in human including the subfamilies canonical (C1, C3-C7), vanilloid (V1-6), ankyrin (A1), melastatin (M1-8), polycystin (P2, P3, P5) and mucolipin (ML1-3). B. Homotetrameric TRPV1 channel (PDB ID 5IRX) embedded into cell membrane [129]. Single subunits are colored in magenta, green, blue, and yellow. C and D. Schematic depiction (C) and corresponding Cryo-EM structure (D; PDB ID 5IRX) of a single TRPV1 subunit including the N-terminal domain (NTD, orange), the ankyrin repeat domain (ARD, orange), the N-terminal linker (N-linker, orange), the pre-S1 helix (Pre-S1, orange), the voltage-sensing-like domain (S1-S4; purple), the S4S5-linker (Linker, red), the inner pore domain (S5-S6, magenta), the pore helix (PH, magenta), the TRP helix (green) and the C-terminal domain (CTD, yellow) [129].

All TRPV channels possess a large cytosolic N-terminal and a smaller C-terminal region [14]. The N-terminal region forms an ankyrin repeat domain (ARD) with six ankyrin repeats, two β-sheets (N-Linker) and a pre-S1 helix (Fig. 1C) [15]. The cytosolic C-terminal region encompasses several amino acids forming one β-sheet, that together with the two N-terminal β-sheets and the ARD of an adjacent subunit allow for efficient subunit assembling [15]. The intracellular regions are also binding sites for modulating proteins and second messengers. The N-terminus contains binding sites for calmodulin (CaM) and ATP, that both can modulate channel activity [16]. The shorter C-terminal region also binds CaM and other regulating proteins including A-kinase anchor protein (AKAP) [17, 18]. Moreover, TRPV channels are strongly regulated by phosphorylation at different sites of the N- and C-terminal regions performed by protein kinase A (PKA) as well as protein kinase C (PKC) [19, 20].

The transmembrane region consists of six helices (S1-S6) forming the voltage sensor-like domain (VSLD, S1-S4) and an inner pore region (S5-S6) connected by the S4S5-linker [21]. The ion channel pore is formed by the selectivity filter (SF) and the pore helix (PH; also called pore turret) between the S5 and S6 helix [22]. Further, residues from the lower part of the S6 helix form an activation gate [23]. In comparison to voltage gated potassium channels, the upper gate formed by the PH / SF is shorter and the pore radius differs within

the different TRPV subtypes influencing the selectivity of the channels [24]. Based on the ion selectivity the six subtypes are subdivided into two groups: TRPV1-4 form polyselective cation channels, that conduct monovalent as well as divalent cations with a preference for Ca^{2+} and conductivity ratios for Ca^{2+} over Na^+ ranging from $P_{Ca/Na}$ 3 (TRPV2) over 6 (TRPV4) up to 10 (TRPV1, TRPV3) [25]. In contrast, TRPV5 and TRPV6 are nearly Ca^{2+} selective channels with $P_{Ca/Na}$ >100, while monovalent cations only permeate in absence of divalent cations [26, 27].

Gating stimuli of TRPV channels

Although the structure of the transmembrane region is similar to the structure of voltage gated potassium channels, the voltage dependency of TRPV channels is rather low [28]. Together with another activation stimulus (agonist, pH or heat), TRPV1 as well as TRPV4 show a prominent outward rectification at highly positive voltages and slight inward rectification at negative voltages [28, 29]. Only TRPV1 exerts weak voltage dependent opening without any other stimulus [28]. TRPV3 does not show voltage dependent activation up to highly positive voltages without additional stimulation by heat or ligands [30]. However, activation of TRPV3 by 2-APB leads to similar voltage dependency as observed for TRPV1 [31]. TRPV2 channels exert also dual rectification with slightly dominant outward currents under heat activation [10]. In contrast to the polyselective TRPV channels TRPV5 and TRPV6 show strong inward rectification due to voltage-dependent intracellular Mg^{2+}-block at positive potentials [32, 33]. The ion channels of the two TRPV groups differ additionally in heat-sensitive gating.

While TRPV1-4 are sensitive to heat showing Q_{10} values >10, TRPV5 and TRPV6 show no altered gating at different temperatures [34]. Thus, TRPV1-4 are also called thermoTRPV channels contributing to temperature sensing *in vivo* [24]. While TRPV4 (threshold 24-33 °C) and TRPV3 (31-39 °C) are activated by warm temperatures, TRPV1 (>43 °C) and rTRPV2 (>53 °C) are activated by noxious heat [34]. Interestingly, the human orthologue of TRPV2 does not respond to heat [35]. Beside the heat response, species dependent differences for TRPV channels occur frequently. In this review, we specify the generic term TRPV with the exact species if the given information in the literature cannot guarantee identical findings for other species.

The vanilloid channels also respond differently to H^+. TRPV1 and TRPV4 are known for activation by a high extracellular concentration of H^+ (low pH) [36, 37]. For TRPV1, residues in the S3S4 linker contribute to the recognition of extracellular H^+ [36]. Moreover, low extracellular pH values also increase the sensitivity of TRPV1 for capsaicin and heat [38, 39]. Contrary to TRPV1 and TRPV4, TRPV3 is activated by a high intracellular concentration of H^+ [40]. The amino acid H426 at the N-terminal region was identified to contribute to H^+-gating by increased H^+ concentrations [40]. TRPV2 channels have only low sensitivity for protons, while TRPV5 is inhibited by high extracellular as well as high intracellular H^+ concentrations [36, 41].

Some TRPV channels also react to mechanical or osmotic stimuli. Most prominent for such gating is TRPV4, which is activated under extracellular hypotonic conditions [42]. This activation results in cell swelling and subsequent production of arachidonic acid (AA) and different epoxyeicosatrienoic acids (EETs) and their metabolites [43]. Also TRPV2 is known to be activated by hypotonic solutions and cell stretching [44]. In addition to membrane stretching, membrane lipids like phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) modulate the TRPV channel activity. Depletion of $PI(4,5)P_2$ together with calmodulin contributes to the strong Ca^{2+}-induced inactivation of TRPV5 and TRPV6, while elevated concentrations activate these channels [32, 45]. Similar effects by $PI(4,5)P_2$ depletion were observed for Ca^{2+}-dependent desensitization of TRPV2 without contribution of calmodulin [46]. On the other hand, hydrolysis of $PI(4,5)P_2$ was associated with enhanced activity of TRPV3 [47]. For TRPV1 and TRPV4 activating as well as inhibitory effects were described leading to the conclusion that these channels are regulated in a very complex way by $PI(4,5)P_2$ [48–51].

Expression pattern, physiological function and pathophysiology

TRPVs are widely expressed in all kinds of tissues and organs contributing to a plethora of physiological as well as pathophysiological effects. Therefore, TRPVs are interesting pharmacological targets even if the risk of side effects is possibly high due to their broad expression pattern. The best characterized vanilloid channel for pharmacological targeting is TRPV1. The channel is expressed in neuronal as well as non-neuronal cells. High expression rates are detected in peripheral sensory neurons (C- and Aδ-fibres), dorsal root ganglia (DRG), trigeminal ganglia and vagal ganglia [52, 53]. Non-neuronal expression can be found in arteriolar smooth muscles of skin and trachea [54, 55]. Knockout mice TRPV1$^{-/-}$ show reduced thermal hyperalgesia after inflammation and impaired nociception [56]. Combining these findings with the gating stimuli by noxious heat as well as elevated H^+ concentration make TRPV1 a promising target for the treatment of pain. Interestingly, TRPV1 agonists as well as antagonists are efficient as analgesics especially in neuropathic pain. While blocking of TRPV1 channels directly prevents Ca^{2+} influx and subsequent signal transduction from sensory neurons, overactivation by agonists lead to internalization and subsequent desensitization of afferent nerve endings or degeneration of neurons by Ca^{2+}-induced neurotoxicity [57]. Today, this therapeutic concept is used in form of transdermal application of high dose capsaicin for the treatment of neuropathic pain in the periphery [58]. Also, the local application of the highly potent agonist resiniferatoxin (RTX) as a "molecular scalpel" in the treatment of extensive pain (cancer) is under clinical trial [57]. It should be noted, that some TRPV1 antagonists failed in clinical trials due to the induction of hyperthermia as well as the increment of sensation threshold for noxious heat in human increasing the risk for severe injuries [59]. However, these side effects are compound specific and can be reduced by chemical structure optimization [59].

TRPV2 is ubiquitously expressed in almost all types of cells, but especially high expression levels were found in the brain, lung and spleen [60]. In particular, macrophages show high expression rates of TRPV2 [61]. It is also found in vascular smooth muscles as well as cardiomyocytes, where it contributes significantly to the Ca^{2+} homeostasis [62, 63]. Under non-stimulating conditions TRPV2 channels are predominantly localized in endosomes [61]. Stimulation of TRPV2 positive cells by growth factors like IGF-I or mechanical cell stimulation lead to translocation of the channels to the plasma membrane [60]. TRPV2 knockout in mice does not lead to an abnormal phenotype. However, impaired macrophage activation was observed in TRPV2 deficient mice leading to increased vulnerability upon bacterial infections [64]. Overstimulation of TRPV2 is associated with muscular dystrophy, myocardial fibrosis and cardiomyopathy due to Ca^{2+} overload of cells [65, 66]. Overexpression of TRPV2 was found in different types of cancers [67]. Thus, targeting TRPV2 by selective agonists and/or antagonists could be an innovative strategy for treatment of these pathophysiological processes.

TRPV3 is distributed in the brain, sensory neurons, DRG, spinal cord and skin [11]. It is prominently expressed in epithelial cells like keratinocytes [68]. TRPV3$^{-/-}$ knockout mice exhibit phenotypes with wavy hear coat, curly whiskers and an altered temperature preference [69, 70]. Moreover, TRPV3 is needed for formation of normal skin barrier function [70]. On the other hand, elevated TRPV3 activity can cause skin lesions and increase the risk for dermatitis [71]. TRPV3 activity can be increased by inflammatory messengers like histamine [72]. Gain-of-function mutations of the *TRPV3* gene can cause the Olmsted syndrome, which is characterized by progressing hyperkeratosis leading to mutilation of hands and feet, inflammatory dermatitis infiltration, abnormal hair growth, itching and pain [73]. Patients with these gain-of-function mutations would benefit from a selective antagonist for TRPV3 channels.

In contrast to the other thermoTRPVs, TRPV4 is linked to many channelopathies indicating a broad expression pattern and versatile physiological functions. High TRPV4 expression is found in the brain, sensory neurons, kidney, urinary tract, skin, musculoskeletal tissues, epithelia and vasculature [74–80]. Since TRPV4 reacts to moderate heat, it may

represent an important temperature sensor [81]. However, TRPV4$^{-/-}$ mice showed only slightly altered temperature response, while other physiological functions were clearly more affected [81]. The knockout mice had impaired bladder function and increased bladder volume, increased bone mass, impaired vascular endothelial function as well as impaired pressure and pain sensing [37, 76, 82, 83]. In contrast to mildly altered phenotypes in mice, more than 50 mutations of the *TRPV4* gene were detected that cause a variety of diseases in human patients such as different forms of skeletal dysplasia, neuropathies and muscle atrophies [84]. These diseases have highly variable phenotypes and can be caused by gain-of-function, alteration-of-function as well as loss-of-function mutations. This fact underlines the importance of activation as well as inhibition of TRPV4 channels by selective compounds [84].

The expression pattern of Ca^{2+}-selective TRPV5 and TRPV6 is rather restricted. In particular, TRPV5 is almost exclusively expressed in kidney [85, 86]. Lower amounts of mRNA for TRPV5 were found in human placenta, osteoclasts and lymphocytes [87–89]. In contrast, TRPV6 was found in the placenta, pancreas, gastrointestinal tract (GIT), testis and prostate [88, 90]. Human prostate cancer and most other common cancers clearly overexpress TRPV6 [90–92]. Both channels are key players in the Ca^{2+} homeostasis, since TRPV5 is involved in Ca^{2+} reabsorption in kidney, while TRPV6 contributes to the Ca^{2+} absorption in the GIT [93]. Therefore, results from TRPV5 and TRPV6 knockout mice are not surprising. TRPV5$^{-/-}$ mice showed strong urinary excretion of Ca^{2+} and subsequent elevated concentrations of calcitriol and parathormone (PHT) as compensation mechanisms [94–96]. As a consequence of elevated hormone serum concentrations, reduced bone density and increased Ca^{2+} reabsorption in the GIT were observed [97]. Reduced bone density and elevated Ca^{2+} concentration in serum were also detected in TRPV6 knockout mice [98]. Additionally, fertility defects in male and female mice were reported [99]. Since overexpression of TRPV6 is observed in many common cancers, drug discovery aims selective inhibition of TRPV6 [100]. First promising drug candidates are the soricidin-derived peptides SOR-C13 and SOR-C27 displaying effective inhibition of ovarian and breast cancer growth [101, 102]. Consequently, SOR-C13 entered clinical phase I studies for the treatment of cancer patients. However, the exact mechanism and binding site is not known yet.

Challenges in TRPV channel modulation

Today many synthetic, natural, or endogenous compounds targeting vanilloid channels are known. However, modulation of TRPV channels *in vitro* as well as *in vivo* is challenging. *In vitro* modulation of TRPV channels by different compounds often depends on assay conditions as well as different target aspects. Some compounds (e.g. 2-APB, capsaicin) can act synergistically with different gating stimuli (heat, H^+ concentration). Also, extracellular ions can have an influence on TRPV pharmacology, since increased osmolarity (TRPV2, TRPV4) or inactivation (TRPV5, TRPV6) can occur. On the other hand, the experimental expression system and the TRPV species influences the determination of EC_{50}/IC_{50} values. Low amounts of TRPV2, TRPV5 and TRPV6 are found in the plasma membrane, while high amounts are located intracellular. In case of TRPV2 mechanical stress and media supplements can increase the translocation to the membrane. Also, the used expression system can influence the surface expression of TRPV2. Species specific effects of TRPV modulating compounds is also frequently observed. Thus, assay conditions must be highly controlled and well documented, otherwise resulting EC_{50}/IC_{50} values are not comparable. The following passages summarize structural and mechanistical data for the most commonly used TRPV channel modulators. EC_{50}- and IC_{50}-values for species specific TRPV channel modulation are summarized in Table 1, while binding sites detected by Cryo-EM and crystal structures are summarized in Fig. 2A.

Table 1. Prominent TRPV channel modulatorsSummary of prominent TRPV modulators and their corresponding EC_{50} / IC_{50} values at different TRPV channels from human (h), mouse (m), rat (r) or rabbit (rb). Values in μM as well as the used assay and reference [ref.] is given. If compound did not show activity, abbreviation n.a. (not active) is mentioned

ligand	channel	EC_{50} / IC_{50} [μM]		assay	ref.
2-APB	mTRPV1	EC_{50}	114	Ca^{2+} imaging (HEK293)	[117]
	hTRPV2	-	n.a.	Ca^{2+} imaging (HEK293)	[118,163]
	mTRPV2	EC_{50}	129	Ca^{2+} imaging (HEK293)	[117]
	hTRPV3	EC_{50}	78	Ca^{2+} imaging (HEK293)	[119]
	hTRPV4	-	n.a.	Ca^{2+} imaging (HEK293)	[117]
	hTRPV5	-	n.a.	$^{45}Ca^{2+}$ uptake (HEK293)	[116,117]
	hTRPV6	IC_{50}	71	$^{45}Ca^{2+}$ uptake (HEK293)	[116]
4α-PDD	mTRPV4	EC_{50}	0.37-0.72	Ca^{2+} imaging (HEK293)	[149,150]
4α-PDH	mTRPV4	EC_{50}	0.07	Ca^{2+} imaging (HEK293)	[150]
4α-PMA	mTRPV4	EC_{50}	3	Ca^{2+} imaging (HEK293)	[149]
5,6-EET	mTRPV4	EC_{50}	0.13	patch clamp (HEK293)	[148]
6'-iodononivamide	hTRPV1	IC_{50}	0.01	Ca^{2+} imaging (HEK293)	[132]
6-tert-butyl-m-cresol	mTRPV3	EC_{50}	370	TEVC (Xenopus laevis)	[146]
anandamide	hTRPV1	EC_{50}	1.14	Ca^{2+} imaging (HEK293)	[136]
arachidonic acid	mTRPV4	EC_{50}	2	patch clamp (HEK293)	[148]
(+)-borneol	mTRPV3	EC_{50}	3450	TEVC (Xenopus laevis)	[146]
camphor	mTRPV3	EC_{50}	6030	TEVC (Xenopus laevis)	[146]
cannabidiol	hTRPV2	EC_{50}	32	Ca^{2+} influx (HEK293)	[142]
	rTRPV2	EC_{50}	3.7	Ca^{2+} influx (HEK293)	[142]
cannabinol	rTRPV2	EC_{50}	78	Ca^{2+} influx (HEK293)	[142]
capsaicin	hTRPV1	EC_{50}	0.038	$^{45}Ca^{2+}$ uptake (CHO)	[127]
capsazepine	hTRPV1	IC_{50}	0.053 – 0.069	$^{45}Ca^{2+}$ uptake (CHO)	[127]
carvacrol	mTRPV3	EC_{50}	490	TEVC (Xenopus laevis)	[146]
cis-22a	hTRPV6	IC_{50}	0.08	patch clamp (HEK293)	[161]
drofenine	hTRPV3	EC_{50}	207	Ca^{2+} imaging (HEK293)	[119]
econazole	rbTRPV5	IC_{50}	1.3	patch clamp (HEK293)	[103]
farnesyl pyrophosphate	hTRPV3	EC_{50}	0.13	Ca^{2+} imaging (HEK293)	[144]
GSK1016790A	mTRPV4	EC_{50}	0.018	Ca^{2+} influx (HEK293)	[152]
	hTRPV4	EC_{50}	0.002	Ca^{2+} influx (HEK293)	[152]
GSK2193874	hTRPV4	IC_{50}	0.05	Ca^{2+} influx (HEK293)	[154]
	mTRPV4	IC_{50}	0.005	Ca^{2+} influx (HEK293)	[154]
	rTRPV4	IC_{50}	0.002	Ca^{2+} influx (HEK293)	[154]
HC-067047	hTRPV4	IC_{50}	0.048	patch clamp (HEK293)	[156]
	mTRPV4	IC_{50}	0.017	patch clamp (HEK293)	[156]
	rTRPV4	IC_{50}	0.133	patch clamp (HEK293)	[156]
I-RTX	hTRPV1	IC_{50}	0.0005 – 0.0054	patch clamp (CHO)	[131]
miconazole	rbTRPV5	IC_{50}	1.8	patch clamp (HEK293)	[103]
NADA	hTRPV1	EC_{50}	0.063	Ca^{2+} imaging (HEK293)	[135]
OLDA	hTRPV1	EC_{50}	0.036	Ca^{2+} imaging (HEK293)	[135]
PPAHV	rTRPV1	EC_{50}	0.034	$^{45}Ca^{2+}$ uptake (CHO)	[127]
	hTRPV1	EC_{50}	15.5	$^{45}Ca^{2+}$ uptake (CHO)	[127]
probenecid	rTRPV2	EC_{50}	32	Ca^{2+} influx (HEK293)	[138]
resiniferatoxin (RTX)	hTRPV1	EC_{50}	0.005	$^{45}Ca^{2+}$ uptake (CHO)	[127]
RN-1734	hTRPV4	IC_{50}	2.3	$^{45}Ca^{2+}$ uptake (CHO)	[155]
	mTRPV4	IC_{50}	5.9	$^{45}Ca^{2+}$ uptake (CHO)	[155]
	rTRPV4	IC_{50}	3.2	$^{45}Ca^{2+}$ uptake (CHO)	[155]
RN-1747	hTRPV4	EC_{50}	0.77	$^{45}Ca^{2+}$ uptake (CHO)	[155]
	mTRPV4	EC_{50}	4.0	$^{45}Ca^{2+}$ uptake (CHO)	[155]
	rTRPV4	EC_{50}	4.1	$^{45}Ca^{2+}$ uptake (CHO)	[155]
ruthenium red	hTRPV1	IC_{50}	0.9	Ca^{2+} imaging (CHO)	[104]
	rTRPV1	IC_{50}	0.2	Ca^{2+} imaging (CHO)	[104]
	rTRPV2	IC_{50}	0.6	TEVC (Xenopus laevis)	[10]
	mTRPV3	IC_{50}	<1	patch clamp (CHO)	[105]
	hTRPV4	IC_{50}	<1	patch clamp (HEK293)	[106]
	rbTRPV5	IC_{50}	0.1-0.4	patch clamp (HEK293)	[103]
	mTRPV6	IC_{50}	9	patch clamp (HEK293)	[107]
SOR-C27	hTRPV6	IC_{50}	0.064	patch clamp (HEK293)	[101]
TH-1177	rTRPV5	IC_{50}	456	$^{45}Ca^{2+}$ uptake (X. laevis)	[158]
	rTRPV6	IC_{50}	675	$^{45}Ca^{2+}$ uptake (X. laevis)	[158]
thymol	mTRPV3	EC_{50}	860	TEVC (Xenopus laevis)	[146]
vanillotoxin I	rTRPV1	EC_{50}	9.9	Ca^{2+} imaging (HEK293)	[134]
vanillotoxin II	rTRPV1	EC_{50}	1.4	Ca^{2+} imaging (HEK293)	[134]
vanillotoxin III	rTRPV1	EC_{50}	0.45	Ca^{2+} imaging (HEK293)	[134]
ZINC17988990	rbTRPV5	IC_{50}	0.1	patch clamp (HEK293)	[160]
	hTRPV6	IC_{50}	n.a.	patch clamp (HEK293)	[160]
ZINC9155420	rbTRPV5	IC_{50}	2.9	patch clamp (HEK293)	[160]
	hTRPV6	IC_{50}	4.1	patch clamp (HEK293)	[160]
Δ^9-THC	rTRPV2	IC_{50}	15.5	Ca^{2+} influx (HEK293)	[142]

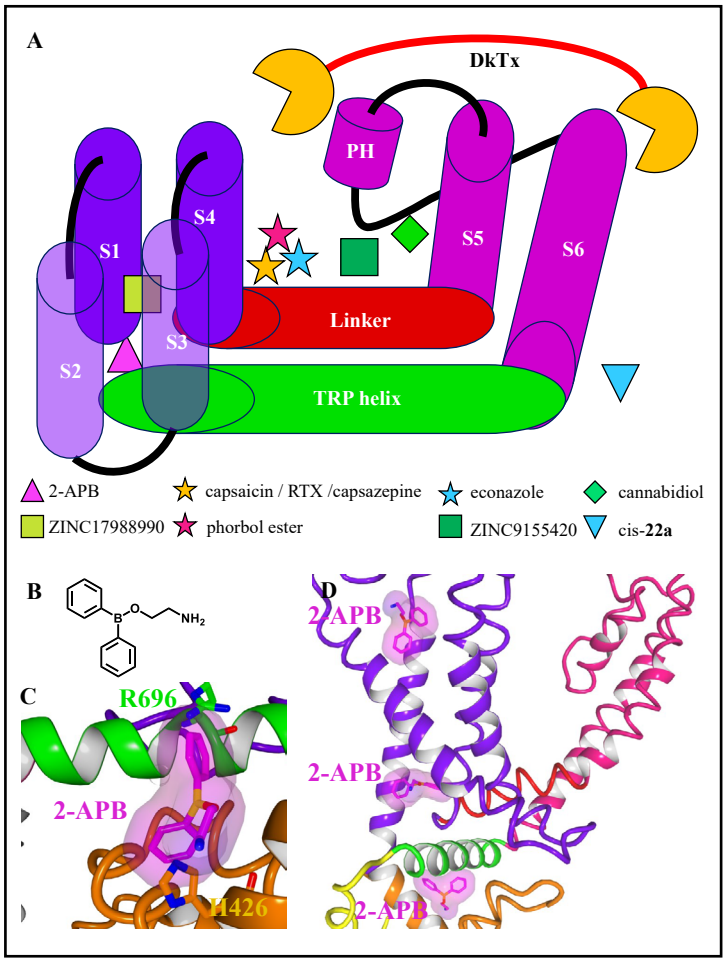

Fig. 2. A. Schematic depiction of TRPV subunit with binding sites of TRPV modulators. B. Structure of 2-APB. C. Depiction of 2-ATB (magenta) binding site at mTRPV3 based on cryo-EM structure PDB 6DVY [121]. Colors of secondary structure are defined in Fig. 2A. D. Depiction of 2-ATB (magenta) binding sites at mTRPV3 based on cryo-EM structure PDB 6DVZ [121].

Unspecific TRPV ligands and ion channel blockers

Compounds targeting TRPV channels often exert subunit specificity, however, some compounds and ions act similar on the different TRPV channels. Ruthenium red is a complex salt, that is known as universal TRPV ion channel pore blocker [10, 103–107]. It also blocks TRPA1 and different CatSper channels as well as ryanodine receptors [108–110]. The IC_{50} value for TRPV1-5 is in the submicromolar range, while TRPV6 is inhibited at higher concentrations (Table 1). Since the IC_{50} value for TRPV6 is almost 100 times higher than for TRPV5, ruthenium red can be useful in differentiation of these two channels. Furthermore, Gd^{3+} and La^{3+} ions inhibit TRPV2, TRPV4, TRPV5 and TRPV6 channels at low concentrations [111–113]. Recently, Ag^{+} was identified as a pore blocker for rTRPV1 and rTRPV2, but not mTRPV3 [114]. However, a mutation in the selectivity filter could introduce pore blocking properties for Ag^{+} in mTRPV3 I674C [114]. Other polyvalent metal ions can also affect conductivity of TRPVs, however, the inhibitory effects are less consistent and more individual for the different subtypes.

A synthetic modulator with different actions at TRP channels is (2-aminoethyl) diphenylboronate (2-APB, Fig. 2B). Originally, it was denoted as a general channel blocker of TRP channels, since TRPM2, TRPM3, TRPM6 and almost all TRPC channels are inhibited by 2-APB [115]. hTRPV6 is also inhibited by 2-APB, but only at high micromolar concentrations [116]. Surprisingly, high concentrations of 2-APB activate TRPV1, TRPV3 and mTRPV2, while hTRPV2, hTRPV4 and hTRPV5 are completely or nearly insensitive [35, 117–119]. The presence of 2-APB also enhances the potency of capsaicin at TRPV1 and protons at

TRPV1-3 [117]. The mechanisms of action as well as the reason for specific modulations are still not completely understood. Since activity as well as affinity of 2-APB is quite low, the detection of a possible binding site by crystallization or cryo-EM studies is difficult. Early studies showed that 2-APB insensitive mTRPV4 channel can be transferred into a 2-APB sensitive mTRPV4 channel by exchange of two amino acids to their corresponding amino acids in TRPV3 [120]. These residues are located in the pre-S1 helix (mTRPV4 N426H; H426 in mTRPV3) as well as in the TRP helix (mTRPV4 W737R; R696 in mTRPV3) forming a binding site at the interface of inner membrane and cytosol (Fig. 2C). On the other hand, mutation of the chicken TRPV3 containing an asparagine (N) at position 426 with only weak response to 2-APB to histidine (N426H) increases the 2-APB response to comparable levels found in mTRPV3 [120]. As mentioned above, H426 contributes to the sensing of intracellular rising H^+ concentrations. Further cryo-EM analysis of mTRPV3 confirmed binding of 2-APB at the postulated site. However, two additional binding pockets in the lower as well as upper VSLD bundle were observed (Fig. 2D) [121]. The contribution of these two binding sites to the agonistic effect of 2-APB is still under discussion since further studies at hTRPV3 did not reveal 2-APB specific densities in the VSLD bundle region [122]. Moreover, clear electrophysiological data for these two binding sites are missing for TRPV3 [122]. Cryo-EM studies with 2-APB were also performed with the hTRPV6 channel [123]. Since TRPV6 is inhibited instead of activated, the mechanism of action could be different. Indeed, cryo-EM structures showed clear evidence for binding of 2-APB and bromo-2-APB at the lower VSLD bundle previously postulated for the TRPV3 [123]. Electrophysiological analysis of mutants from residues of the lower S2, the lower S4 and the TRP helix revealed clear alteration of 2-APB activity supporting the cryo-EM observations.

TRPV1 targeting compounds

The subfamily of vanilloid receptors is named after the most prominent TRPV1 agonist capsaicin. This vanillylamide specifically activates TRPV1 channels [7]. The effects of capsaicin and the even more potent natural product resiniferatoxin (RTX; found in *Euphorbia resinifera*) on neuronal tissues were known before TRPV1 was identified as an ion channel [124]. Indeed, both compounds together with the first synthetic antagonist capsazepine were used to characterize the vanilloid receptor (Fig. 3A) [124, 125]. After cloning of the TRPV1 channel, site directed mutagenesis provided essential amino acids for the agonistic as well as antagonistic activity of capsaicin, RTX and capsazepine. For example, mutation of the lower S3 residues tyrosine 511 (rTRPV1 Y511A) or serine 512 (rTRPV1 S512Y) abolished capsaicin sensitivity of rTRPV1 [126]. However, the interaction with these two amino acids could not explain the observed species differences in capsaicin response. Although rabbit and chicken orthologous ion channels containing the same amino acids, they are almost insensitive to capsaicin, RTX and capsazepine [127]. These observations were used to search for more amino acids responsible for ligand activity. The results identified further essential residues from the S4 segment (rTRPV1 M547, rTRPV1 T550), that are different in the capsaicin-insensitive species. Therefore, it was postulated that the binding site is located between the VLSD and inner region [127]. Cryo-EM studies confirmed the postulated binding site for capsaicin, RTX and capsazepine (Fig. 3B). The binding pocket is formed by the lower transmembrane segments S3 and S4 from the VSLD together with the S4S5-linker and the lower S5 and S6 segments from the adjacent subunit [128]. Later on, the mechanism of action was postulated based on further cryo-EM studies [129]. The identified binding site is known for interactions with membrane lipids with their polar head groups pointing to the S4S5-linker [129]. Capsaicin and RTX displace the membrane lipid and promote a salt bridge between the amino acids R557 (S4) and E570 (S4S5-linker) [129]. Consequently, the S4S5-linker is pulled to the VSLD leading to opening of the channel gate [129]. In contrast, capsazepine displace the membrane lipid without promoting this salt bridge [129]. Combining the results of binding site analysis also explain why TRPV2

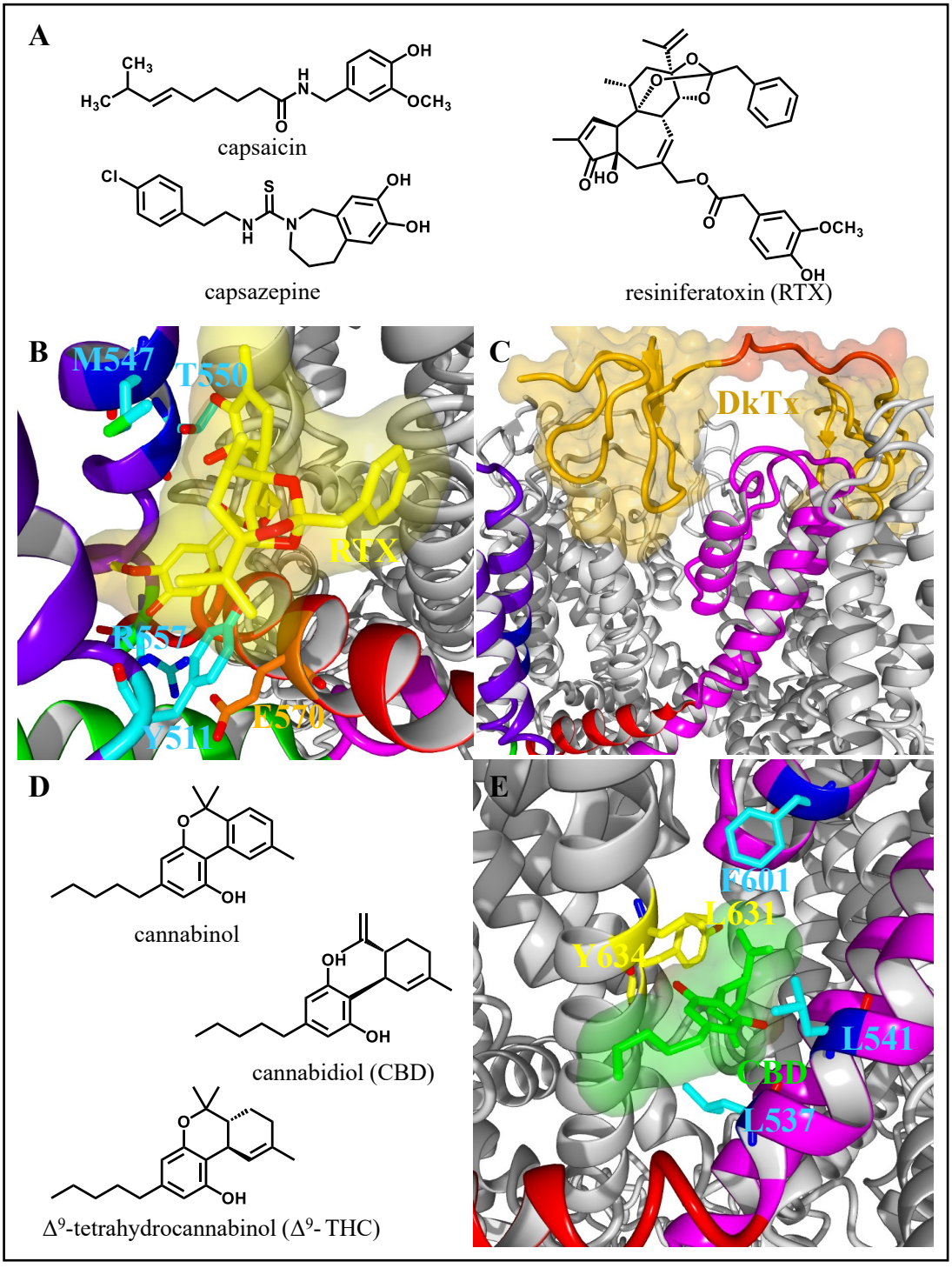

Fig. 3. A. Structures of capsaicin, resiniferatoxin (RTX) and capsazepine. B. Depiction of RTX (yellow) binding site at rTRPV1 based on cryo-EM structure PDB 5IRX [129]. Colors of secondary structures are defined in Fig. 1. C. Depiction of DkTx (orange/red) binding site at rTRPV1 based on PDB 5IRX. D. Structures of cannabinol, cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC). E. Depiction of CDB (green) binding site at rTRPV2 based on cryo-EM structure PDB 6U88 [143].

channels are not affected by RTX. Changing the corresponding amino acids of RTX-insensitive TRPV2 to S512, M547, T550 and E570 produces a RTX-sensitive TRPV2 channel [130]. The agonistic effect of RTX, capsaicin and its synthetical analogue nonivamide can be reversed to an antagonistic effect by halogenation in 5- or 6-position of the vanillylamide substructure [131, 132]. 5'-Iodoresiniferatoxin (I-RTX) as well as 6'-iodonordihydrocapsaicin represent potent TRPV1 antagonists with IC_{50} values in the low nanomolar range (Table 1).

The cryo-EM studies also revealed a possible mechanism of action for several animal toxins especially for the double knot toxin (DkTx) from the chinese bird spider *Ornithoctonus huwena*, a known activator of TRPV1 channels [129]. The peptide consists of two lobes (knots), that are formed by altered β-sheets connected by cysteine bridges building up the typical inhibitory cysteine knot motive (Fig. 3C) [133]. The lobes are connected by a short linker with low flexibility. Two DkTx molecules can bind to one TRPV1 channel. Each lobe of one molecule binds on the extracellular site to the upper S6 segment and the upper S5-PH-linker of two adjacent subunits. As a consequence of subunit connection it is postulated, that the ion channel is trapped in an open, but sub conductive state [129]. Further prominent venomous peptides activating TRPV1 are the single knot vanillotoxins VaTx I-III from the tarantula *Psalmopoeus cambridgei* and the single knot toxin found in the Chinese red-headed centipede (RhTx) [133, 134]. Endogenous modulators of TRPV1 are metabolites of arachidonic acid (AA) including anandamide, N-arachidonoyldopamine (NADA) and the dopamine conjugate N-oleoyldopamine (OLDA) [135, 136]. All three fatty acid amides are structurally related to capsaicin. Anandamide and NADA also activate cannabinoid receptors rendering them as dual agonists. This dual modulation at TRPV channels and CB receptors is frequently observed also for further ligands [135, 137].

TRPV2 targeting compounds

In contrast to TRPV1, only a few compounds are known to activate or inhibit the highly homologous TRPV2 channel. Probenecid, an inhibitor of the organic anion transporter 1 (OAT1) used for the treatment of gout, is able to activate the TRPV2 channel at higher micromolar concentrations [138]. The exact mechanism of action is not known. Even though activation of TRPV2 by probenecid needs high concentration, the selectivity profile towards TRPV1, TRPV3, TRPV4 and TRPM8 is sufficient for *in vitro* discrimination [138]. Although probenecid activates TRPV2, several studies suggest positive outcomes in models of cardiomyopathy [139, 140]. The authors suggest that increased influx of Ca^{2+} by activation of TRPV2 leads to increased contractility and increased ejection fraction [139, 140]. For inhibition of TRPV2 channels the histamine releasing inhibitor tranilast can be used [141]. However, detailed characterization for this inhibitory effect is missing. More potent activators of the rTRPV2 channels are the cannabinoids cannabidiol (CBD), cannabinol (CBN) and Δ^9-tetrahydrocannabinol (Δ^9-THC) with CBD being the most and CBN being the least potent compound (Fig. 3D) [142]. Again, species differences were observed, since hTRPV2 required 10-fold higher concentrations of CBD than rTRPV2 [142]. Recent cryo-EM studies revealed the binding site of CBD at rTRPV2 channels, which is formed by mostly hydrophobic and aromatic residues from the S6 helix (L631, Y634), the S5 helix (L537, L541) and the lower part of PH (F601) from the adjacent subunit. Thus, CBD is positioned above the S4S5 linker (Fig. 3E) [143]. Although the CBD-bound structure showed a displacement of the S4S5-linker similar to those observed for capsaicin-provoked gating of TRPV1, the structures did not provide a CBD-provoked gating mechanism in detail [143]. Further studies are needed.

TRPV3 targeting compounds

Pharmacological TRPV3 studies are challenging, since only a few compounds targeting this channel are available. Moreover, most of these compounds need high concentrations up to millimolar range. An exception is farnesyl pyrophosphate (FPP), an endogenous intermediate from the mevalonate pathway (Fig. 4A), which was found to activate hTRPV3 channels in submicromolar concentrations without side effects at TRPV1. TRPV1 is often colocalized with other TRPV channels including TRPV3 [144]. FPP shifted the voltage-dependent activation of TRPV3 and increased heat sensitivity. Co-culture experiments showed that FPP-evoked activation in keratinocytes was transmitted to sensory neurons resulting in nociceptive response [144]. Later, isopentenyl pyrophosphate (IPP), a precursor of FPP, was found to inhibit hTRPV3 with an IC_{50} value of 239 nM [145]. However, IPP also influenced other TRPV and TRPA channels [145]. Monoterpenes like camphor, carvacrol and thymol can activate TRPV3 (Fig. 4B) [72, 146]. In comparison to FPP, these monoterpenes activate TRPV3 only at significantly higher concentrations. Whereas bicyclic monoterpenes like camphor and (+)-borneol activate TRPV3 channels with EC_{50} values in the micromolar range, aromatic monoterpenes are more potent possessing EC_{50} values ranging from 370 µM to 860 µM (Table 1) [146].

The antimuscarinic drug drofenine was identified as an activator of TRPV3 channels based on structural similarity (Fig. 4C) [119]. The activity of drofenine is comparable to the activity of 2-APB and carvacrol at hTRPV3 channels. However, selectivity of drofenine over TRPA1, TRPV1, TRPV2, TRPV4, and TRPM8 was significantly improved [119]. Recently, the first potent and selective TRPV3 antagonist 74a was synthesized and preclinically evaluated (Fig. 4C) [147]. 74a has a K_B value of 0.54 µM at hTRPV3 channels and shows positive outcome in models for neuropathic and central pain [147]. Although a few studies provide some indirect structural information for FPP/IPP, the monoterpenes and drofenine, the exact binding sites were not yet determined.

TRPV4 targeting compounds

Similar to TRPV1, TRPV4 is modulated by several endogenous metabolites of the anandamide metabolism [148]. Anandamide is metabolized by the fatty acid amide hydrolase (FAAH) to provide arachidonic acid (AA) that directly activates mTRPV4 (Fig. 4D) [148]. An even more potent activator of mTRPV4 is the AA metabolite 5',6'-epoxyeicosatrienoic acid (5',6'-EET) (Table 1). The activation of TRPV4 by AA and its metabolites contribute significantly to the cell-swelling induced activation of TRPV4, since cell swelling leads to activation of phospholipase A_2 (PLA_2) and subsequently elevated concentrations of AA [43]. Another similarity between TRPV1 and TRPV4 is the modulation by phorbol esters, although, the TRPV4-activating phorbol esters differ from those activating TRPV1. While TRPV1 is activated by phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) and RTX, TRPV4 is activated by the phorbol esters 4α-phorbol 12,13-didecanote (4α-PDD), 4α-phorbol 12,13-dihexanoate (4α-PDH) and 4α-phorbol 12-myristate 13-acetate (4α-PMA) with 4α-PDH being the most and 4α-PMA being the least potent activator (Fig. 4D) [149, 150]. Although crystal- and cryo-EM-structures for these TRPV4 modulating compounds are not available, structural similarity as well as site directed mutagenesis indicate that the binding site is located between S3 and S4 similar to the observed binding site in TRPV1 for RTX [149]. Especially the mutation W586A (upper S4) as well as Y556A (lower S3) generated strong right-shifted dose response curves indicating a strong contribution of these amino acids to the agonistic mechanism [149]. While these phorbol esters are useful for *in vitro* characterization of TRPV4 the *in vivo* application is not feasible due to their high toxicity induced by strong activation of protein kinase C (PKC) [151].

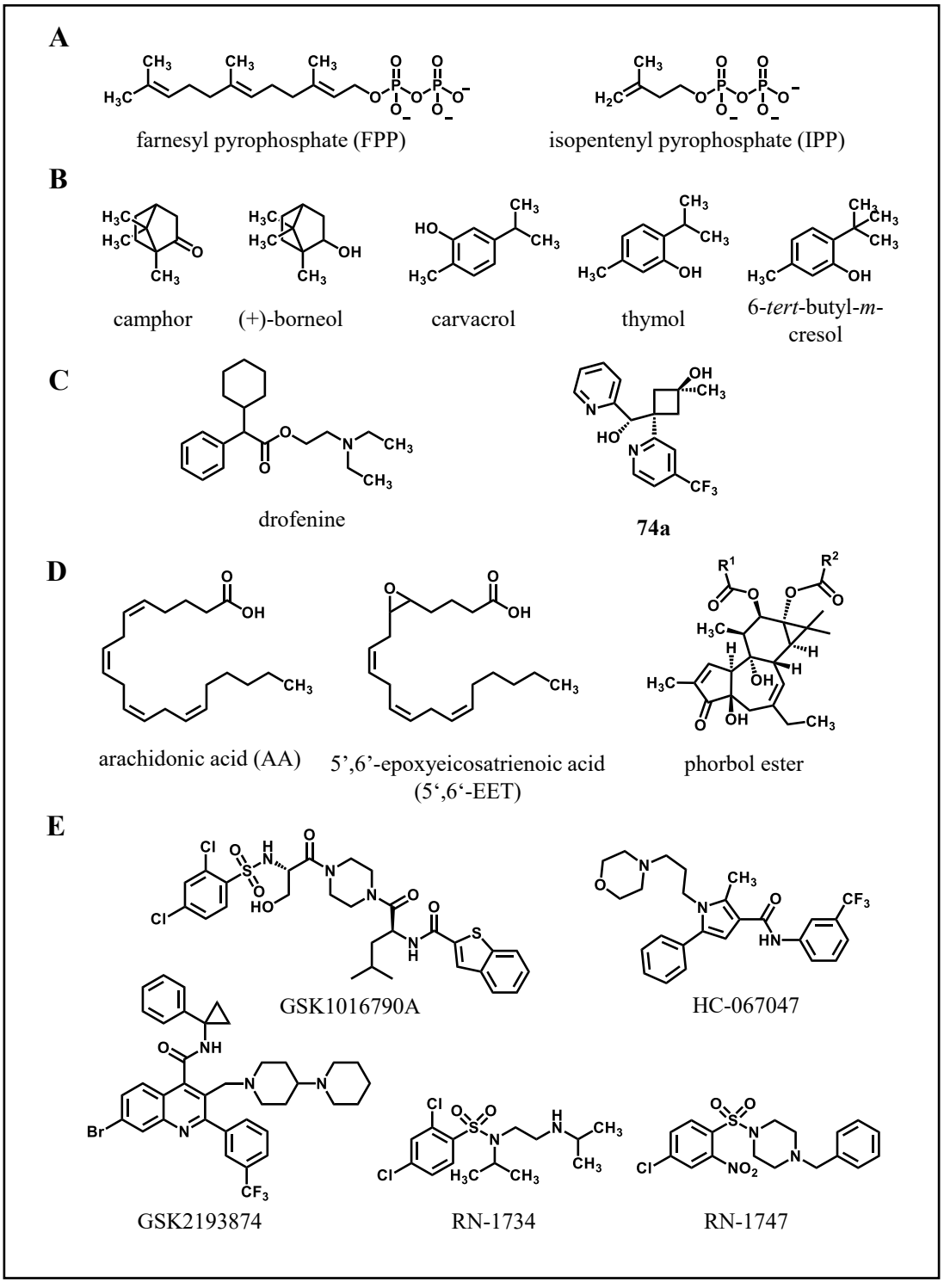

Fig. 4. A-C. Structures of TRPV3 agonists and antagonists. D-E. Structures of TRPV4 agonists and antagonists. Several phorbol esters with different side chains at 12-position (R^1) and 13-position (R^2) are known to activate TRPV4 channels.

Therefore, pharmaceutical companies tried to develop suitable TRPV4 agonists and antagonists with improved *in vivo* properties (Fig. 4E). Based on thorough SAR studies, GlaxoSmithKline developed several compounds targeting the TRPV4 channel including the strong TRPV4 agonist GSK1016790A [152]. GSK1016790A displayed high activity in assays including TRPV4-expressing chondrocytes from different species with EC_{50} values ranging from 2 nM (hTRPV4) up to 18 nM (mTRPV4) [152]. First local *in vivo* application in bladder of mice led to bladder overactivation, which was absent in TRPV4-knockout mice [152]. However, systemic administration to mice, rats and dogs caused dose-dependent hypotonic crisis up to circulatory collapse and death [153]. Experimental evaluation led to the conclusion that observed circulatory collapse was a result of high microvascular permeability resulting in disrupted barrier function [153]. Further investigations led to GSK2193874, a potent and selective antagonist of TRPV4 antagonizing the effect of GSK1016790A *in vitro* [154]. Moreover, the compound showed a positive outcome in preclinical tests for pulmonary edema caused by heart failure [154].

Screening of commercially available libraries revealed the antagonist RN-1734 and the agonist RN-1747 [155]. Both compounds show moderate to high TRPV4 activity and high selectivity against TRPV1, TRPV3 and TRPM8 [155]. More detailed selectivity and *in vivo* examination were performed for HC-067047 showing comparable inhibition of hTRPV4 as GSK2193874 and improved bladder function in mice and rats suffering from cystitis [156].

TRPV5 and TRPV6 targeting compounds

TRPV5 and TRPV6 share almost 70 % sequence identity, which makes it difficult to find selective compounds useful for pharmacological differentiation. One of the first discovered compounds targeting TRPV5 and TRPV6 is TH-1177 (Fig. 5A) [157]. Initial studies characterized the compound as inhibitor of Ca^{2+} entry resulting in reduced human prostate cancer proliferation *in vitro* and in a mouse model. However, the exact target of this Ca^{2+} entry block was not known [157]. Later, Landowski et al. showed the inhibition of rTRPV5 and rTRPV6 by TH-1177 with a slight preference for rTRPV5 [158]. They also tested the antimycotics econazole and miconazole previously described as inhibitors of rbTRPV5 [103]. Surprisingly, both antimycotics revealed 1.5-2.2 higher activity at rTRPV6 [158]. The recently published cryo-EM structures of the rbTRPV5 channel allowed the identification of the hydrophobic binding pocket for econazole, which is analogously occupied by capsaicin or RTX in the TRPV1 channel (Fig. 5B) [159]. The cryo-EM structure was supported by site directed mutagenesis of the S3 segment using TEVC measurements at rbTRPV5 F425A. Decreased activity of econazole at rbTRPV5 F425A indicated a contribution of the S3 segment to the inhibitory mechanism. Contrary to the vanilloids at TRPV1, econazole repositions the S1-S4 domains and the S4S5-linker promoting closure of the lower gate [159].

Based on these findings, further TRPV5 inhibitors were searched by structure-based virtual screening followed by electrophysiological testing and cryo-EM imaging. These investigations led to the identification of ZINC9155420 and ZINC17988990 (Fig. 5C) [160]. Although both compounds show high structural similarity, their interactions with TRPV5 and TRPV6 as well as their binding sites at TRPV5 and TRPV6 differ considerably. ZINC9155420 binds above the S4S5-linker and to the S6 domain from the adjacent subunit next to a binding site of membrane lipids (Fig. 5D). The localization found in cryo-EM structure was functionally addressed by mutation of an amino acid at the end of the S4S5-linker. rbTRPV5 M491A produced a slight right shift in the dose response curve. However, ZINC9155420 was not selective and inhibited rbTRPV5 and hTRPV6 at almost the same concentrations. On the other hand, ZINC17988990 was found to inhibit rbTRPV5 selectively, since almost no activity at hTRPV6, mTRPV1, mTRPV3, rTRPV4 and rTRPM8 was detected. Evaluation of the cryo-EM structures and subsequent mutational analysis revealed binding of ZINC17988990 to a different binding site located in the S1-S4 bundle next to the TRP helix overlapping with one of the postulated binding sites for 2-APB in the mTRPV3 structure [121, 160]. Especially

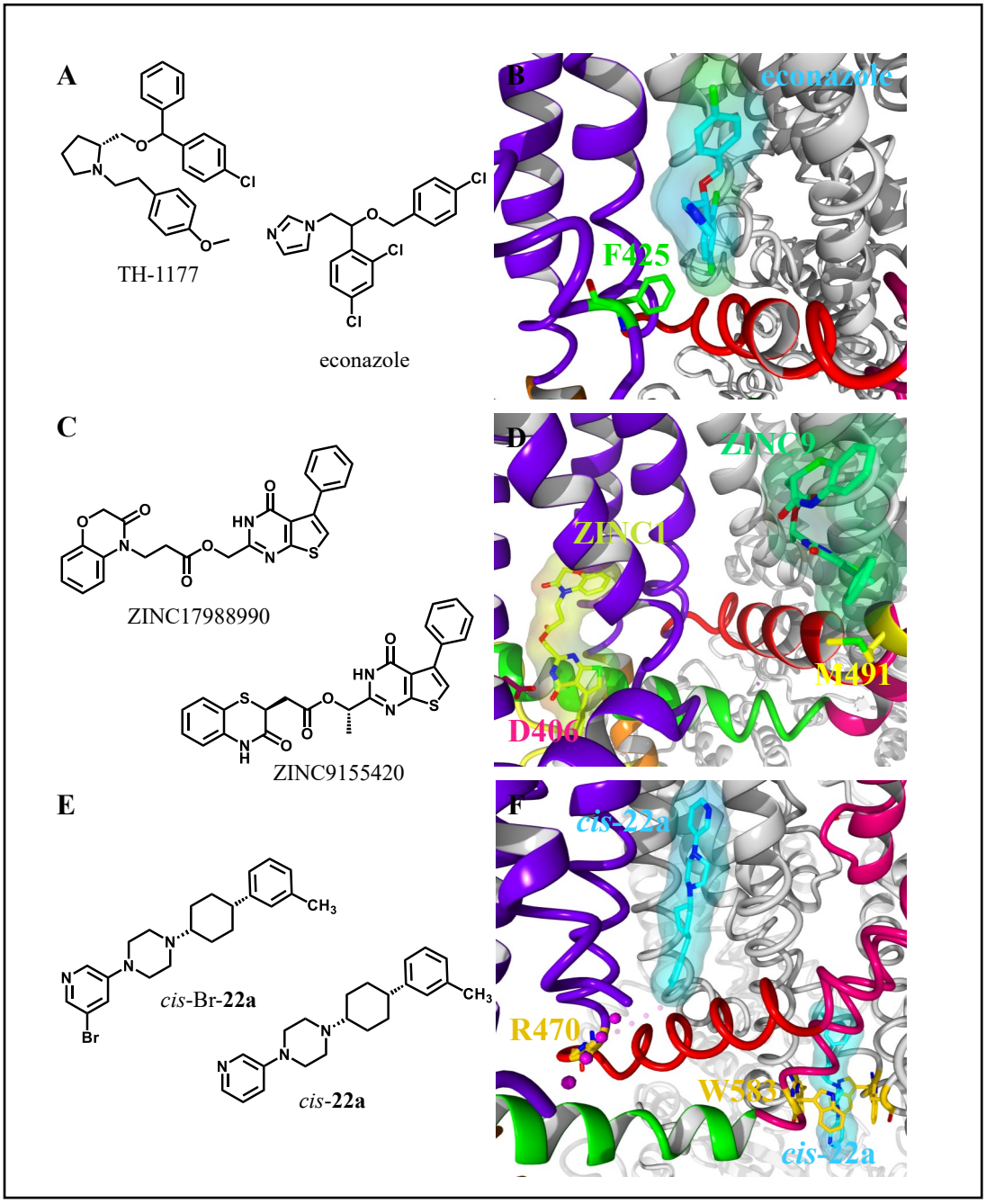

Fig. 5. A, C, E. Structures of TH-1177 and econazole (A), ZINC17988990 and ZINC9155420 (B) and cis-Br-22a and cis-22a (E). B. Cryo-EM structure of econazole (cyan) bound to rbTRPV5 (PDB 6B5V) [159]. Colors of secondary structures are defined in Fig. 1. D. Overlay of cryo-EM structures of ZINC17988990 (ZINC1, lime) and ZINC9155420 (ZINC9, green) bound to rbTRPV5 (PDB 6PBF and 6PBE) [160]. F. Cryo-EM structure of cis-22a (cyan) bound to hTRPV6 (PDB 7K4B) [161].

the mutant rbTRPV5 D406A, that is located at the lower S2 helix, was found to be crucial for the compound activity. Even though more functional data are needed, ZINC17988990 represents a promising starting point for the development of TRPV5 selective inhibitors [160].

Very recently, *cis*-configured 1-(4-phenylcyclohexyl)-4-(pyridin-3-yl)piperazine 22a was described as TRPV6-selective antagonists allowing structural and mechanistic studies at this ion channel [161]. Structure optimization led to compound *cis*-22a, that shows a good selectivity profile towards TRPV1, TRPV3, TRPM8 and TRPV5 and antiproliferative effects on TRPV6 positive breast cancer cells [162]. Preparation of cryo-EM structures of *cis*-22a and its brominated derivative (*cis*-Br-22a) together with hTRPV6 and a truncated rTRPV6 revealed two binding sites of the compound (Fig. 5E-5F) [161]. The first binding site is located at the typical lipid binding site, which corresponds to the capsaicin/RTX-binding site in TRPV1. Unexpectedly, the second binding site was detected within the ion pore domain below the activation gate. Mutational analysis allowed the identification of crucial amino acids involved in the inhibitory mechanism at both binding sites. Mutation of the most effecting amino acid W583 to alanine located in the lower S6 domain produced a remarkable right shift of the dose-response curve resulting in a 75-fold higher IC_{50} value compared to the wildtype ion channel, while the most effecting mutant R470A located in the lower part of the S4 domain of the second binding site shifted the IC_{50} value only by factor 6. Thus, the inhibitory effect of *cis*-22a results predominantly from interaction with the lower gate. The authors postulated, that *cis*-22a mimics the natural inactivation by calmodulin by blocking the ion channel pore [161].

Conclusion

In conclusion, TRPV channels are involved in a plethora of physiological and pathophysiological processes rendering them promising targets for pharmacological intervention. Nevertheless, *in vivo* function of many TRPV channels as well as the molecular gating mechanisms by different stimuli are still not evaluated in detail. *In vitro* and *in vivo* administration of highly selective TRPV modulators could enhance the understanding of the contribution to physiology and pathophysiology. However, highly selective compounds are often not available or not applicable *in vivo*. Moreover, frequently observed species differences complicate the drug development process. Thus, further research for highly selective TRPV agonists and antagonists useful for *in vivo* application is needed. In future research, pharmacological investigations with newly designed selective compounds can form the basis for the development of new drugs with analgesic (TRPV1) or immune modulating (TRPV2, TRPV3) effects as well as for drugs, that can be used for the treatment of overactive bladder (TRPV4) or different cancer types (TRPV5, TRPV6).

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Disclosure Statement

The authors declare no conflict of interests exist.

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