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TITLE PAGE

PHARMACOLOGY OF VANILLOID TRANSIENT RECEPTOR POTENTIAL CATION CHANNELS TRPV

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List of non-standard abbreviations: TRP, transient receptor potential; TRPV, transient receptor potential receptor vanilloid; AEA, *N*-arachidonylethanolamine; NADA, *N*-arachidonoyldopamine; RTX, resiniferatoxin; TG, trigeminal; DRG, dorsal root ganglia; 2-APB, 2-aminoethoxydiphenyl borate; TM domain, transmembrane domain; RR, Ruthenium Red;

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ABSTRACT

Depending on their primary structure, the 28 mammalian *T*ransient *R*eceptor *P*otential (TRP) cation channels identified so far can be sorted out into 6 subfamilies [TRPC ('Canonical'), TRPV ('Vanilloid'), TRPM ('Melastatin'), TRPP ('Polycystin'), TRPML ('Mucolipin'), and TRPA ('Ankyrin')]. The TRPV subfamily (vanilloid receptors) comprises channels critically involved in nociception and thermo-sensing (TRPV1, TRPV2, TRPV3, TRPV4), while TRPV5 and TRPV6 are involved in renal Ca^{2+} absorption/reabsorption. Apart from TRPV1, the pharmacology of these channels is still insufficiently known. Furthermore, only few small molecule ligands for non-TRPV1 vanilloid receptors have been identified, and little is known on their endogenous ligands, resulting in a substantial "orphan" state for these channels. In this review, we summarize the pharmacological properties of members of the TRPV subfamily, highlighting the critical issues and challenges facing their "deorphanization" and clinical exploitation.

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INTRODUCTION

"Transient Receptor Potential" (TRP), cation channels are unique cellular sensors characterized by a promiscuous activation mechanism (Nilius et al., 2007; Pedersen et al., 2005; Ramsey et al., 2006; Voets et al., 2005). Over 50 members of the TRP family have been characterized in yeast, worms, insects, fish, and mammals (Nilius and Voets, 2005; Vriens et al., 2004a), making them one of the largest group of ion channels. The 28 mammalian TRPs identified so far can be sorted out into 6 subfamilies: TRPC ('Canonical'), TRPM ('Melastatin'), TRPV ('Vanilloid'), TRPA ('Ankyrin'), TRPP ('Polycystin'), and TRPML ('Mucolipin'). TRPs are classified essentially according to their primary amino acid sequence rather than selectivity or ligand affinity, since their properties are heterogenous and their regulation complex. From a structural standpoint, TRP channels are membrane proteins with six putative transmembrane spans (TM) and a cation-permeable pore region formed by a short hydrophobic stretch between TM5 and TM6.The structural differences within the TRP subfamilies have been recently reviewed (Owsianik et al., 2006), and TRP proteins are essentially cation-permeable ion channels sensitive to a remarkable range of stimuli. Genetic approaches in worms, flies and mice have demonstrated the involvement of TRPs in a variety of sensory processes that includes thermosensation, osmosensation, olfaction, taste, mechanosensation, vision, and pain perception. Remarkably, mutations in different TRPs have also been linked to human diseases (Nilius et al., 2007).

Since TRPV1 is exquisively sensitive to chemical gating, it seems reasonable to assume that small molecules, selective ligands and endogenous ligands exist also for other TRPs, and in particular for those from the vanilloid subclass (TRPVs). However, the search for these compounds has so far been largely elusive, even for the TRPs more closely related to TRPV1. In this review, we will discuss the major reasons responsible for the orphan state of so many TRPs, and review the current state of the pharmacological modulation of TRPV and the clinical potential of their manipulation.

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REVIEW

TRPV1

The vanilloid receptor 1 or TRPV1, the founding member of the mammalian TRPV channels, was identified in expression cloning experiments using capsaicin (**1**) from hot pepper as a probe. Given the involvement of many TRPs in sensation and taste, it is not surprising that a compound of dietary origin was the first ligand to be discovered for this class of proteins. TRPV1 is a non-selective cation channel with a preference for calcium, activated by noxious stimuli, heat, protons (pH<5.9), and various, mostly obnoxious, natural products (Tominaga et al., 1998). TRPV1 is predominantly expressed in sensory neurons (Caterina et al., 1997), and is thought to play a crucial role in temperature sensing and nociception (Caterina et al., 2000), qualifying therefore as a molecular target for pain treatment. Thanks to the availability of a wealth of TRPV1 ligands, this ion channel is also the best-characterized member of the TRPV subfamily in terms of expression pattern, properties, and clinical translation of its manipulation (Vennekens et al., 2008).

(1) TRPV1 agonists

A distinction should be made between direct activators of TRPV1 and sensitizers. Strictly speaking, capsaicin and its biological analogues isolated from plants and animals are essentially sensitizers, since they act by lowering the thermal "physiological" activation threshold of TRPV1. Nevertheless, since these compound bind directly to TRPV1, they are conveniently considered as direct activators, in contrast to compounds that do not bind TRPV1, and affect its functioning indirectly, that are referred to as sensitizers. Owing to the large body of literature on TRPV1 ligands, only the most important chemotypes and the most recent additions will be discussed (for comprehensive review see (Appendino et al., 2008)), covering in a more exhaustive way the issue of the topology of the vanilloid binding site.

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Endogenous agonists

Good evidence has been presented on the existence of endogenous vanilloid agonists, a class of compounds referred to as endovanilloids, and on their capability to modulate the sensitivity of TRPV1 channels to thermal stimuli (Van Der Stelt and Di Marzo, 2004). Various lipids from the fatty acid pool have also been identified as TRPV1 activators. These compounds can be divided into conjugates of biogenic amines [e.g. *N*-arachidonylethanolamine (AEA, anandamide, **2a**), *N*-arachidonoyldopamine (NADA, **3a**), *N*-oleoylethanolamine (OLEA, **2b**), *N*-arachidonolylserine, and various *N*-acyltaurines and *N*-acylsalsolinols] (Appendino et al., 2008), and oxygenated eicosatetraenoic acids (Ahern, 2003; Wang et al., 2005) like the lipoxygenase products 5-, 12- and 15-hydroperoxyeicosatetraenoic acids (5S-, 12S-, 15S-HPETE, **4a-6a**) (Hwang et al., 2000), their reduced hydroxylic analogues (**4b-6b**), prostaglandins, and leukotriene B_4 (Huang et al., 2002). Also adenosine, ATP, and polyamines (such as spermine, spermidine and putrescine) activate TRPV1, as well as acidic conditions (pH<5.9) like those observed during inflammation (Ahern et al., 2006; Alexander et al., 2006; McNamara et al., 2005; Siemens et al., 2006; Szallasi and Di Marzo, 2000; Van Der Stelt and Di Marzo, 2004; Xu et al., 2005).

Endovanilloids of the fatty acid conjugate-type resemble capsaicinoids for the presence of a polar head and a lipophylic moiety linked by an amide group, but the polar head is generally aliphatic [ethanolamine in AEA (**2a**) and OLEA (**2b**)] or, if aromatic, as in NADA (*N*-arachidonoyldopamine, **3a**) (Hwang et al., 2000), is not of the guaiacyl, but of the catechol type (NADA, **3a**). Within this class of compounds, the term vanilloid is therefore devoid of structural meaning, and refer exclusively to their biological profile.

Endovanilloids are promiscuous agents. Thus, AEA (**2a**) and NADA (**3a**) bind not only TRPV1, but also cannabinoid receptors, while OLEA (**2b**) also bind PPARs. Within the many synthetic analogues of endovanilloids, of special interest are the methylated forms of *N*-oleoyldopamine (OLDA, **3b**), since reversal of activity from agonist to antagonist is observed upon *O*-methylation

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of the catechol system, with 3-methyl-*N*-oleoyldopamine (3-MOLDA, **3c**) acting as an agonist, and 4-methyl-*N*-oleoyldopamine (4-MOLDA, **3d**) as an antagonist (Almasi et al., 2008).

Polyamines are important endogenous regulators of TRPV1 channels, capable of modulating inflammation and nociception. Extracellular spermine, spermidine, and putrescine directly activate TRPV1 in a charge-dependent manner, both in heterologous expression systems and in sensory neurons. The threshold for activation by spermine is rather high (~ 500 μM at room temperature), but spermine can enhance capsaicin-evoked currents with an EC_{50} of approximately 5 μM. Experiments with TRPV1 mutants have identified extracellular acidic residues critical for polyamine regulation. Thus, neutralization of Asp646 abolished direct activation by spermine, whereas neutralization of this acidic residue or Glu648 inhibited spermine-induced sensitization. These data show that polyamines, by virtue of their cationic charge, can regulate the activity of TRPV1 at physiological concentrations (Ahern et al., 2006).

Exogenous agonists of natural, semi-synthetic and synthetic origin

TRPV1 can be activated by a heterogenous array of natural products that includes both dietary compounds [capsaicinoids (**7a**) and capsinoids (**7b**), piperine (**8**), eugenol (**9**), gingerol (**10**)], plant toxins [resiniferatoxin (RTX, **11a**)], and animal toxins (For review see (Vriens J, 2008)). Capsaicinoids and capsinoids occur in plants from the genus *Capsicum*, and are typical of hot- (chilli)- and non-pungent (bell) peppers, respectively. Over twelve pungent capsaicinoids have been characterized from hot peppers, and three major non-pungent capsinoids from bell peppers (Appendino, 2007). Capsaicinoids and capsinoids are characterized by an oxygenated vanillyl aromatic moiety bound via an amide (capsaicinoids) or ester (capsinoids) linker to a lipophilic acyl group. The vanillyl and the carbonyl linker contain polar groups capable of forming hydrogen bonds essential for activity, while the lipophylic moieity interacts with a corresponding cleft of the vanilloid binding site of TRPV1. Replacement of the medium-sized branched fatty acid of capsaicin with longer fatty acids is detrimental for activity (Morita et al., 2006), but remarkably, the presence

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of unsaturations restores and potentiates activity, as exemplified by oleoylvanillamine (**12a**, olvanil), a compound 10-fold more potent than capsaicin in TRPV1 activation assays (Appendino et al., 2002) (Table 1). A further increase of potency was obtained by the introduction of polar functions on the acyl moiety of olvanil, as discovered for phenylacetylrinvanil (**12b**), the most potent capsaicinoid reported to date (~ 500-fold more potent than capsaicin) (Appendino et al., 2005b). Capsiates are at least one order of magnitude less potent TRPV1 activators than capsaicin, but their lack of pungency is nevertheless puzzling. Unlike capsaicin, capsiates are hydrolytically unstable, and it has been suggested that they act essentially as prod-drugs of vanillic alcohol (Rosa et al., 2005).

Piperine (**8**) belongs to a group of alkaloid typical of plants from the *Piperaceae* family, like black pepper (*Piper nigrum* L.), a most popular spice. Piperine shows putative anti-inflammatory activity, may promote digestive processes, and might increase the absorpition of branched amino acids and several classes of small molecules of dietary and phytotherapy relevance (Srinivasan, 2007). The first evidence that piperine is a TRPV1 agonist was the demonstration of its ability to inhibit the binding sites of [^{3}H]-RTX in the dorsal horn of pig spinal cord (Szallasi et al., 1991). In addition, piperine activates inward currents in TG neurons, an action that was found to be sensitive to capsazepine (Liu and Simon, 1996). In a more recent study, Gunthorpe and co-workers concluded that piperine is not only similar to capsaicin in its effects at human TRPV1 expressed in HEK293 cells, but also exhibits a greater efficacy than capsaicin itself for both activation and desensitization of TRPV1 (McNamara et al., 2005). The reasons for the improved desensitization-to-excitation ratio of piperine compared to capsaicin are unclear. From a structural standpoint, there are three major differences between capsaicin and piperine, namely the replacement of the guaiacyl moiety with a methylendioxy group, a structural element associated to inhibition of cytochrome P450 metabolism (Srinivasan, 2007), the presence of the aromatic moiety on the acyl and not the amine

moiety, and the insertion of a four-carbon butadienyl linker between the amide and the aromatic moiety (Reen et al., 1996). An attractive hypothesis to explain the pharmacodynamic differences between capsaicin and piperine is that the latter might be a better inducer of dephosphorylative inactivation of TRPV1 (see below). Clearly, piperine is an attractive lead for the synthesis of improved TRPV1 agonists, but, surprisingly, very little is known on its structure-activity relationships.

Eugenol (**9**), an allyl chain-substituted guaiacol, is a member of the allylbenzene class of phenylpropanoids. It is the major constituent of clove oil form *Eugenia caryophyllata* L. Electrophysiological studies have shown that eugenol is able to activate inward currents in hTRPV1-HEK293 cells and TG neurons. This effect was completely prevented by the TRPV1 antagonist capsazepine, demonstrating the involvement of TRPV1 (Yang et al., 2003).

Within non-dietary naturally occurring vanilloids, a special position is held by resiniferatoxin (**11a**, RTX) from the dried latex of *Euphorbia resinifera*. The vanillyl group is critical for the activity of both capsaicin and RTX. This has led to the widespread use of the term “vanilloids” to refer to TRPV1 ligands. RTX is 3-4 orders of magnitude more potent than capsaicin both in terms of dose-response curve and effect on thermoregulation and neurogenic inflammation (Szallasi and Blumberg, 1989) (Table 1). RTX is structurally more complex than capsaicin, and its structure-activity relationships are still largely unknown. The methylene linker between the side chain of the aromatic ring and the carbonyl is critical, as are the C-3 carbonyl on ring A and the orthoester moiety in ring C of the diterpenoid core (Appendino et al., 2007). Also camphor (**13**), isolated from the wood of the camphor laurel tree (*Cinnamomum camphora* L.), can activate heterologously expressed TRPV1. Activation of rat TRPV1 by camphor is mediated by a distinct channel region from capsaicin, since it is insensitive to the competitive TRPV1 antagonist capsazepine. Camphor activates TRPV1 less effectively than capsaicin (Table 1), but in a more rapid and complete way. However, camphor is a nonselective TRPV1 agonist, since it also activates other TRP channels,

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including TRPV3 (Xu et al., 2005).

Spiders and jellyfish venoms are a major source of TRPV1 activators of animal origin. The presence of inhibitor cysteine knot peptides (ICK) is the hallmark of venoms from Indian tarantulas, and three of these peptides, cogently named vanillotoxins, have been shown to activate TRPV1 (EC_{50}~10^{-8}–10^{-4} M). TRPV1-mediated pain and inflammation are undoubtedly part of the mechanism by which animals avert predators (Siemens et al., 2006). From the marine front, the burning pain sensation caused by venom from the tentacles of certain cnidarians has been suggested to be mediated by removal (or inhibition) of TRPV1 desensitization, although the clear mechanism is still uncertain (Cuypers et al., 2006). However, the active principle(s) of these venoms is (are) still unknown (peptide vs bioactive small molecule), and their precise site of interaction remains to be elucidated (TRPV1 itself vs. its signal-transduction pathway). The study of offensive animals has surely the potential to provide new probes to investigate TRPV1, but the field has not yet been systematically investigated. Finally, TRPV1 is also activated by the synthetic agent 2-aminoethoxydiphenyl borate (2-APB) (EC_{50}~10^{-4}M), a blocker of store-operated Ca^{+2} entry and IP3 receptors (Alexander et al., 2006).

Also several synthetic drugs have been shown to interact with TRPV1. Thus, a surprising finding was the observation that the local anesthetic lidocaine (**14**) activates TRPV1, and, somewhat less efficiently, also TRPA1, both in heterologue systems and in native rodent dorsal root ganglion sensory neurons. Lidocaine also induced a TRPV1-dependent release of calcitonin gene-related peptide (CGRP) from isolated skin and peripheral nerve. Activation of TRPV1 by lidocaine required segments of the putative vanilloid-binding domain within- and adjacent to TM3, and was decreased by PIP2 depletion, an effect mimicked by a point mutation at residue Arg701 in the proximal C-terminal TRP domain. TRPV1 and TRPA1 are novel players in lidocaine-induced nociceptor excitation and CGRP release, and might also be involved in lidocaine-induced neurotoxicity (Leffler et al., 2008).

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The modulation of TRPV1 signalling by covalent modification of a cysteine residue in the *N*-terminus (Cys157) is complementary to the use of molecular probes to map the ligand-sensitive elements of TRPV channels. Thus, like TRPA1, also TRPV1 is activated by pungent extracts from garlic and onion and their active component allicin (**15**) and alliin (**16**). These agents activate TRPV1 by covalent modification of one single cysteine residues (Cys157) via Michael addition. Consistent with this mechanism, treatment with dithiothreitol (DTT), a disulphur exchange agent, produced a near-complete reversal of activation by allicin. MTSEA (2-aminoethylmethane thiosulfonate hydrobromide) sensitized the channel to capsaicin (Salazar et al., 2008), and it is therefore not surprising that mustard oil has been described as an activator of TRPV1 (~ mM range) (Ohta et al., 2007). Also a series of membrane-permeable oxidizing agents like diamide, chloramine-T, and copper (0) complexes can enhance the heat activation of TRPV1 by irreversible covalent modification of sulfydryl groups. Extracellular application of the membrane-impermeable reducing agent glutathione mimicked the effects of DTT in potentiating the heat TRPV1 responses, suggesting that redox-active substances can directly modulate channel activity at sites of both extracellular and cytoplasmic location (Susankova et al., 2006).

The burning and irritation induced by capsaicin and related vanilloids represent a major shortcoming for the therapeutic use of these compounds. In addition, loss of the homovanillyl ester group of RTX would generate the tumorigenicity diterpenoid ROPA (**17**), an issue that has been so far largely unadressed. The development of more potent, orally active vanilloids like olvanil (Alexander et al., 2006), SDZ-249482 (**18a**), and SDZ-249665 (**18b**) did not fully circumvent the discomfort of the side effects derived from irritation. In parallel to the identification of novel vanilloid agonists, research on the clinical translation of this class of compounds has also focused on innovative formulations for topical application as analgesics. In this context, the vanilloid analog capsavanil (**19**, DA-5018) and zucapsaicin (**20**) are currently being developed for this indication (Messeguer et al., 2006).

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The ligand-binding site on TRPV1

In sharp contrast to other ligand-gated channels that produce fast synaptic transmission, vanilloids show slow activation kinetics, in part because their binding site is located in the intracellular portion of the receptor. Capsaicinoids, resiniferonoids, and endovanilloids are highly lipophilic compounds, and must therefore cross the cell membrane to act on their intracellular binding site(s) on TRPV1. In fact, charged capsaicin analogs cannot cross the cell membrane, and are only effective when applied to the intracellular surface (Jung et al., 1999). The dynamic of this process is largely unknown, but its relevance is highlighted by comparison between olvanyl and its saturate analog (*N*-stearoylvanillamide, **12c**), that is at least five orders of magnitude less potent as a TRPV1 ligand, presumably because of a very slow kinetics of penetration into the cell (Appendino et al., 2002).

Recently, information on the ligand-binding site of TRPV1 has become to surface. Molecular modelling studies on the xenovanilloid capsaicin (**1**) and the endovanilloid 12-(S)-HPETE (**5a**) had suggested the sharing of specific amino acid residues in TRPV1 binding by these compounds (Hwang et al., 2000). A different approach was pursued by Julius and co-workers (Jordt and Julius, 2002), who capitalized on the observation that avian TRPV1 is heat- and proton sensitive, but capsaicin-insensitive, to get clues as to the location of the capsaicin-binding site. Thus, comparison of the primary structure of the human and the avian isoforms of TRPV1 and mutational studies revealed a critical role for Tyr511 and Ser512, located at the transition between the second intracellular loop and TM3, confirming that the vanilloid binding site is located intracellularly (Jordt and Julius, 2002). By analysing mammalian inter-species TRPV1 chimeras and site-directed TRPV1 mutants, a third critical residue in the putative TM4 segment (Met547 in rat, Leu547 in human and rabbit, Leu549 in guinea-pig) was identified. These studies have benefited on the availability of isoform-specific ligands, like phorbol 12-phenylacetate 13-acetate 20-homovanillate

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(PPAHV, **21**), an agonist selective for rat TRPV1, and of vanilloid antagonists like capsazepine (**22**) (Chou et al., 2004; Gavva et al., 2005). Tyr550 in rat and human also appears to contribute to vanilloid binding (Gavva et al., 2005), while Arg114 and Glu761 in the *N*- and *C*-termini, respectively, are involved in the different ligand-sensitivity of TRPV1 and TRPV2. The loss of proton activation in these mutants suggests that both residues may play a role in the general activation by agonists. The scattered distribution, in terms of primary structure, of the aminoacyl residues necessary for capsaicin binding suggests that in the tertiary and quaternary structure of the native channel these critical residues are relatively close to each other. Possibly other unidentified regions required for capsaicin binding might also exist.

The current helix-packing models of the voltage gated K^+-channels, as derived from helical periodicity analysis and chrystallographic approaches, show that the first, second and third TM domains are located on the lipid-facing periphery of the tetrameric channel complex, whereas the fifth and sixth TM domains are located closer to the pore-forming channel core. Assuming similar helix packing also for TRPV1, the lipophilic moiety of capsaicin may bind to the second and third TM domains on the channel –lipid interface, while the vanilloid moiety may interact with residues around Tyr511 in the cytosolic region, thus linking the two TM domains together with the cytosolic tail. The structural determinants involved in capsaicin binding are, overall, still poorly defined, but several molecular models for the vanilloid site have been proposed wich, upon refinement and validation, may facilitate the design of agonists with better therapeutic index than the current ligands.

Extracellular protons are believed to act primarily by increasing the probability of channel opening, rather than by altering unitary conductance or interacting directly with the vanilloid-binding site (Baumann and Martenson, 2000). Acid solutions evoke ionic currents with an EC_{50} ~ pH 5.4 when applied to outside-out, but not inside-out membrane patches excised from HEK293

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cells expressing TRPV1 (Baumann and Martenson, 2000). This observation suggests that protons act on amino acid(s) located in the extracellular domain of TRPV1, presumably causing protonation of one or more critical carboxylates. Surprisingly, TRPV1 is also activated by gadolinium (III) ions (EC_{50} ~ 72 μM at +40mV), an effect that requires the pore residues Glu600 and Glu648, two protonation sites close to the selectivity filter. Gd^{3+} also potentiates TRPV1 activation by capsaicin, and the two effects (activation and potentiation) might be related to the neutralization of specific proton binding sites (Tousova et al., 2005).

Sensitizers

One important aspect of TRPV1 regulation that has received considerable attention concerns the mechanisms by which the inflammatory mediators in damaged tissues sensitize TRPV1 to its chemical and physical stimuli. Whereas capsaicin acts directly on TRPV1, others influence TRPV1 indirectly, via receptors of intrinsic Tyr kinase pathways, G-protein-coupled receptors, or receptors coupled to the JAK/STAT signalling pathway. Like other ion channels, TRPV1 can be phosphorylated by kinases including PKA (Bhave et al., 2002), PKC (Bhave et al., 2003), Ca^{2+}/CaM-dependent kinase II (CaMKII) (Jung et al., 2004) or Src kinase (Jin et al., 2004). TRPV1 activity is also strongly modulated by phosphatidylinositol phosphates. Indeed, PIP2 appears to be associated constitutively with TRPV1, causing tonic channel inhibition (for detailed review see (Nilius et al., 2008)).

PKA plays a pivotal role in the development of hyperalgesia and inflammation by inflammatory mediators. Ser116 and Thr370 in the amino terminus of TRPV1 are reportedly phosphorylated by PKA and involved in desensitisation (Mohapatra et al., 2003), while phosphorylation of Ser116 by PKA inhibits dephosphorylation of TRPV1 caused by capsaicin exposure.

PKC-dependent phosphorylation of TRPV1 occurs downstream from activation of Gq-

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coupled receptors by several inflammatory mediators, including ATP, bradykinin, prostaglandins and trypsin or tryptase (Moriyama et al., 2003) (Sugiura et al., 2002) (Tominaga et al., 2001). PKC-dependent phosphorylation of TRPV1 not only potentiates capsaicin- or proton-evoked responses but also reduces temperature 'treshold' for TRPV1 activation, so that normally non-painful temperatures in the range of normal body temperature become capable of activating TRPV1, and generate a sensation of pain. Direct phosphorylation of TRPV1 by PKC has been located at Ser502 and Ser800 (Bhave et al., 2003), but the precise isoform of PKC responsible for TRPV1 sensitization in sensory neurons has not yet been identified.

Also calcineurin inhibits desensitisation of TRPV1, indicating that a phosphorylation/dephosphorylation process is important for TRPV1 activity. Indeed, CaMKII reportedly controls TRPV1 activity upon phosphorylation of TRPV1 at Ser502 and Thr704 by regulating capsaicin binding (Jung et al., 2004). Phosphorylation of TRPV1 by three different kinases seems therefore to control channel activity, dynamically balancing phosphorylation and dephosphorylation.

Sensitization by PKC appears to involve increased exocytotic delivery of TRPV1 to the plasma membrane (Morenilla-Palao et al., 2004). Similarly, insulin and IGF-I increase translocation of TRPV1 to the plasma membrane *via* activation of receptor tyrosine kinases, which, in turn, leads to PI(3)kinase and PKC activation (Van Buren et al., 2005). Neurotrophic factors, such as nerve growth factor (NFG), glia-derived growth factor (GDGF) and neurotropin 3 (NT3), also increase the number of TRPV1 expressing neurons in sensory ganglia, elevate TRPV1, and increase the response to capsaicin in single DRG neurons. This "NTF-induced gain of TRPV1 function" may relate to the pathophysiological significance of TRPV1, generating pain during tissue repair and growth (Anand et al., 2006).

Activation of TRPV1 is potentiated by stimulation of the metabotropic 5-hydroxytryptamine (5HT) receptors $5HT_{2A}R$ and $5HT_7$. 5HT is released in injured and inflamed tissues and causes

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hyperalgesia. This potentiation is due to 5HT receptor-mediated activation of PKA and PKC (Ohta et al., 2006). NGF potentiates TRPV1 activation via binding to its TrkA receptor. This effect is attenuated by wortmannin, a PI(3) kinase inhibitor. PI(3) kinase is directly bound to TRPV1 via its p85 binding, as shown elegantly by total internal reflection fluorescence. An increased plasma membrane insertion of functional TRPV1 is induced, leading to a potentiation of currents through these channels and ultimately causing thermal hyperalgesia by proalgesic agents as NGF (Stein et al., 2006). However, PI(3)K binding to TRPV1 does not seem to be promoted by NGF, and does not result in TRPV1 tyrosine phosphorylation (as shown for Tyr199 (Zhang and McNaughton, 2006)).

Within eicosanoids, PGE_2 and PGI_2 sensitize TRPV1 through EP_I or IP receptors and PKC activation. Furthermore, a PKA sensitive pathway for TRPV1 activation via EP4 and IP receptors (PGE_2 and PGI_2) has also been postulated (Moriyama et al., 2005).

TRPV1 is sensitized by activation of the protease-activated receptor 2 (PAR2) (Amadesi et al., 2004). PAR2 colocalizes with PKCε and PKA, and, upon stimulation, causes PKCε and PKA dependent activation of TRPV1 (Amadesi et al., 2006), ultimately triggering the thermal hyperalgesia that underlies inflammatory pain (Elitt et al., 2006; Surprenant, 2007).

The cyclin-dependent kinase, CdK5 plays an important role in pain transduction and nociceptive signalling (Pareek et al., 2006; Pareek and Kulkarni, 2006). Thr407 in TRPV1 can be directly phosphorylated by CdK5, while inhibition of CdK5 activity decreases TRPV1 function and Ca^{2+} influx. Interestingly, the CdK5 conditional knockout mouse shows hypoalgesia (Pareek et al., 2007).

Recently, a variety of 1,4-dihydropyridines (DHP) were developed as novel 'enhancers' of TRPV1 activity. These compounds could increase the maximal capsaicin effect on $^{45}Ca^{2+}$-uptake in DRGs. The DHP enhancers contain a 6-aryl substitution, alkyl groups at the 1 and 4 positions, and a 3-phenylalkylthioester (Roh et al., 2008).

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General anesthetics (Gas, e.g. isoflurane, sevoflurane, enflurane and desflurane) sensitize TRPV1 at clinically relevant concentrations, in a process that can exacerbate post-surgical pain and inflammation (Cornett et al., 2008; Harrison and Nau, 2008). Also tramadol activates TRPV1, and it has been suggested that the "unexpected" local side effects of this analgesic (initiation of burning pain and erythema) are due to TRPV1-mediated local release of vasoactive neuropeptides (Marincsák et al., 2008).

(2) TRPV1 antagonists:

The most advanced knowledge on the pharmacological modulation of a TRP channel has been obtained for TRPV1, owing to the relevance of this protein for the management of chronic pain, migraine, and gastrointestinal disorders (for review see (Szallasi et al., 2007)). As a result, numerous companies have initiated programmes to discover TRPV1 modulators, identifying many novel and potent antagonists. Only the best-documented groups of synthetic antagonists will be discussed, dividing the various chemotypes into two major classes. The classic antagonists are characterized by the presence of a carbonyl group of the ester-, amide-, urea-, or thiourea type that links two moieties, generally with different polarity. In the non-classic antagonists, the carbonyl group is either present as part of a heterocyclic ring (es: 4-aminoquinazolines) or is unrecognizable.

Despite the structural heterogenicity of TRPV1 antagonists, a general model for their binding interaction with TRPV1 antagonists has been proposed (Szallasi et al., 2007). Shortly, the unifying structural feature of TRPV1 antagonists is the presence of a central hydrogen-bond acceptor/donor motif flanked by a lipophilic side chain on one side, and a more polar aromatic group that incorporates a hydrogen-bond acceptor on the other. A hydrogen-bonding motif is present in most known TRPV1 antagonist structures. In the classic antagonists, the central core can act as a H-bonding donor and acceptor, while in some non-classic antagonists, it can only act as a H-bonding acceptor. In the polar arm, mono- or bicyclic-aryl and heteroaryl rings with a properly positioned

hydrogen-bond acceptor (HBA) are critical to both potency and drug-likeness. In the apolar arm, the placement of lipophilic substituents like a *para*-trifluoromethyl or a *para*-*t*-butyl is crucial for potency, while the relevance of the aromatic ring in both arms is presumably related to the possibility of π–π interaction with the critical tyrosine group present in the vanilloid binding site.

2.1. Competitive antagonists

2.1.1. Naturally-occurring, endogenous and classic TRPV1 antagonists

Naturally-occurring TRPV1 antagonists

TRPV1 antagonists are rare within natural products, in sharp contrast with the variety of agonists reported from the pool of secondary metabolites. This observation is consistent with an ecological role for the production of TRPV1 agonists in nature, where these compounds seemingly act as feeding deterrent. The naturally occurring TRPV1 antagonists discovered so fare are structurally unrelated to the synthetic inhibitors, and show only modest potency. The sesquiterpene lactone thapsigargin (**23**) is the irritant principle of *Thapsia garganica* L., a plant used in traditional and folk medicine as an anti-inflammatory agent. Thapsigargin behaves not only as an ultrapotent (subnanomolar) SERCA inhibitor, but also, paradoxically, as a TRPV1 antagonist (Table 2). No attempt has been done so far to dissect these activities and to assess the value of thapsigargin as a template to design TRPV1 antagonists (Toth et al., 2002). Yohimbine (**24**), an indole alkaloid from the tree *Pausinystalia yohimbe* Pierre (formerly known as *Corynanthe yohimbe* K. inhibits TRPV1 and the firing activity of DRGs exposed to capsaicin (Dessaint et al., 2004).

Endogenous TRPV1 antagonists

Dynorphins, natural arginine-rich brain peptides that bind to κ-opioid receptors, are potent blockers of TRPV1, an activity that might contribute to their analgesic profile (Dessaint et al., 2004). Adenosine has also been proposed as an endogenous direct antagonist of TRPV1

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(Puntambekar et al., 2004), while various dietary omega-3 fatty acids like eicosapentaenoic- and linolenic acids inhibit TRPV1, and might be beneficial for the treatment of pain (Matta et al., 2007). More recently, the endogenous fatty acid amide hydrolase (FAAH) inhibitor *N*-arachidonoyl serotonin (**25**) has been shown to cause a direct block of TRPV1 (IC_{50}~40 nM) and to inhibit the generation of anandamide. This dual effect represents a promising treatment against acute and chronic peripheral pain (Maione et al., 2007). Finally, also polyamines have been reported to block TRPV1 channels. Thus, putrescine, spermidine, and spermine permeate TRPV1 with P_x/P_{Na} values between 3 and 16, and spermine reduced the single channel conductance from 96 to 49 pS (Ahern et al., 2006).

1,3-Di(arylalkyl)thioureas

The first identified competitive antagonist for TRPV1 was capsazepine (**22**, Table 2), a thiourea structurally related to capsaicin. Capsazepine competes for the capsaicin-binding site on TRPV1, inhibits capsaicin –mediated channel activation, and can displace RTX from its binding site in radioligand bindings (Tominaga et al., 1998). Capsazepine emerged in the course of the Sandoz program aiming at the discovery of new vanilloid agonists, and its discovery was therefore serendipitous. This program, to date the most comprehensive one done on capsaicin, was based on the recognition that the structure of this natural alkaloid can be divided into three moieties: the aromatic and hydrogen bonding A ring, the lipophilic C tail, and the carbonyl-containing B-linker (Suh and Oh, 2005). In principle, capsazepine can be considered as a conformationally restricted capsaicin analog where the amide bond of the natural product is replaced by a thiourea moiety, and where the presence of a tether between the aromatic A ring and the B-linker forces the catecholic aromatic moiety into an orthogonal orientation with respect to the thiourea bond. Pharmacological studies showed that capsazepine exhibited low metabolic stability and poor pharmacokinetic properties in rodents, thus preventing its clinical development (Walker et al., 2003). The tether was

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not critical for activity, since powerful capsaicinoid antagonists devoid of this structural feature were developed, with 1,3-di(arylalkyl)thioureas emerging as one of the most promising non-vanilloid class of TRPV1 antagonists. These compounds are endowed with excellent therapeutic potential in pain regulation, with an IC_{50} values generally lower than 100 nM (Suh et al., 2005; Suh and Oh, 2005) (Chung et al., 2007). Within these compounds, the replaced of the guaiacyl moiety of capsaicinoids with a 3-fluoro-4-sulfonylamido group was critical to revert biological activity. This, coupled to the design of C-region moiety mimicked on RTX, led eventually o compound **26** (Table 2), a high affinity TRPV1 antagonist wich showed excellent analgesic activity in mice (Lee et al., 2003) (Lee et al., 2005). An alternative optimisation of the lipophilic C region, led to JYL1421 (**27**, Table 2), another promising clinical candidate (Wang et al., 2002).

Iodinated vanillyl derivatives

Though RTX is an ultrapotent agonist for TRPV1, the introduction of an iodine atom *ortho* to the phenolic hydroxyl of the homovanillyl moiety reverts its biological activity, generating the powerful antagonist 5-iodoRTX (**11b**, Table 2) (Wahl et al., 2001). However, enthusiasm for 5-iodoRTX as a TRPV1 antagonist was later tempered by the observation that 5-iodoRTX can still activate TRPV1, possibly due to partial agonism, *in vivo* de-iodination to RTX (Shimizu et al., 2005), or the presence 6-iodoRTX (**11c**) as an impurity (Ech-Chahad, 2006). Interestingly, 6-iodoRTX is a partial agonist, while aromatic iodination either in 5 or in 6 of capsaicin and in 6 of phenylacetylrinvanil led to a reversal of activity, generating TRPV1 antagonists (Appendino et al., 2005a). These observations show that the introduction of iodine at the 6-position is a general maneuver to revert the activity of capsaicinoids, although the molecular details of this remarkable effect are unknown.

Di(arylalkyl)- and aryl(arylakyl)ureas

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This structural template has been intensively investigated as a source of TRPV1 antagonists, and only the most advanced compounds in the class will be discussed. A series of capsaicin analogues of the urea type were developed by acylation of homovanillylamine and related amines with different 4-(α-pyridyl)piperidine-1-acyl chlorides (Bakthavatchalam, 2002). The presence of a polar amino moiety in the hydrophobic C region of capsacinoids was crucial to couple potency and hydrophilicity, mimicking similar observations that led to the discovery of phenylacetylrinvanil from olvanil. Several other ureas emerged as remarkably active TRPV1 antagonists. Compared to capsazepine, the piperazinyl urea (**28** (BCTC) (Table 2)) showed a higher selectivity profile against a wide variety of enzymes and channels (Pomonis et al., 2003), while the related very potent and specific TRPV1 antagonist A-425619 (**29**) (Table 2) could attenuate pain associated with inflammation and tissue injury in rats, showing two-digit nanomolar IC_{50} values in dorsal root ganglia and trigeminal ganglia, and high efficiency in blocking capsaicin- and NADA-evoked secretion of calcitonin gene-related peptide (CGRP) (McDonald et al., 2008).

Further research has led to a variety of small molecul antagonists of TRPV1, including the ureas SB-705498 (**30**), SB-452533 (**31**) (Rami et al., 2006; Rami et al., 2004), and ABT-102 (**32**, see for details Table 2), a compound that has entered clinical trials. Finally, replacement of substituted benzyl groups with an indan rigid moiety in a previously described *N*-indazole- *N*'-benzyl urea antagonists has led to compounds with significantly increased in vitro potency and enhanced drug-like properties (Surowy et al., 2008)

Cinnamides

N-Arylcinnamides have emerged as potent as an important class of TRPV1 antagonists, endowed with high potency ($IC_{50} \leq 10^{-6}$ M for SB-366791, **33**) (Table 2), competitive and specific activity in both human and rat TRPV1 receptors, and an overall profile of receptor selectivity much better than that of capsazepine (Patwardhan et al., 2006) (Gunthorpe et al., 2004). Within this series of

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compounds, AMG-9810 (**34**) exhibited high antagonist potency (IC_{50} ~ 10^{-5} M), and was assayed in a series of in vivo experiments, showing good oral bioavailability in rats, as well as a promising pharmacokinetic profile (Doherty et al., 2005), boding well for clinical efficacy. Another potent blocker from this group is AMG0347 (**35**) (Wu et al., 2008), that was shown in a postoperative pain trial to be able to decrease capsaicin-induced heat- and mechanical hyperalgesia and to block central TRPV1 receptors.

Carboxamides

Several TRPV1 antagonists of the carboxamide type have been discovered. These compounds show IC_{50} in the 10-100 nM range, and are structurally quite heterogenous, as exemplified by comparison of the nicotinamide derivative SB-782443 (**36**) the thiazolylcarboxamide (**37)**, and the tetrahydropyridylcarboxamide (**38)** (Westaway et al., 2008a) (Table 2)**.** SB-782443 **(36)** showed excellent potency at human, guinea pig and rat TRPV1, a favourable in vitro DMPK profile, and remarkable in vivo activity in an inflammatory pain model (Brown et al., 2008; Westaway et al., 2006). Based on their in vitro profile, several compounds of this class qualified for pre-clinical development (Westaway et al., 2008a).

2.1.2. Non classic TRPV1 antagonists

Non classic antagonists lack the urea, thiourea or amide groups typical of the classic TRPV1 ligands. Two major structural types of non classic antagonists have been discovered.

Imidazole derivatives

Starting from a 4,6-disubstituted benzimidazole lead structure, a series of 4,5-biarylimidazoles capable to block both capsaicin- and acid-induced calcium influx in TRPV1-expressing CHO cells were obtained. Imidazole **39** was identified as a highly potent (IC_{50} ~ 10^{-9} M)

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and orally bioavailable TRPV1 antagonist worth further development (Gore et al., 2007).

Diaryl ethers and amines

Compounds from the quinazoline series can be considered as conformationally restricted analogues of a biarylamide series (Table 2). A series of heterocyclic cores was investigated, ranked in terms of activity in this sequence: 5-isoquinoline > 8-quinoline = 8-quinazoline > 8-isoquinoline > or = cinnoline ≈ phthalazine ≈ quinoxaline ≈ 5-quinoline (Blum et al., 2008). Of particular relevance is AMG517 (**40**), a compound that lacks any recognizable carbonyl motif. This compound potently blocks capsaicin, proton, and heat activation of TRPV1 in vitro, inhibits capsaicin-induced flinch in rats in vivo, and shows, apart from hyperthermia, a good tolerability profile (Gavva et al., 2007).

2.2. Non-competitive antagonists (Pore blockers)

Ruthenium Red (RR) is a non-competitive pan-inhibitor of all TRP channels. This compound presumably interacts not only with the ligand binding site of TRPs, but apparently blocks also its aqueous pore. RR binds TRPV1 with high potency (Table 2) in a voltage dependent manner, i.e. inward currents are efficiently blocked but not outward currents (Garcia-Martinez et al., 2000). Commercial RR is a mixture of various ruthenium derivatives but surprisingly, its active component has not yet been characterized. Tetrabutylammonium (TBA) blocks the conductions pore of TRPV1. TBA belongs to a family of classic potassium channel blockers that have been widely used as tools for determining the localization of the activation gate and the properties of the pore of several ion channels. TBA blocks TRPV1 in a voltage-dependent fashion, consistent with being an open-state blocker and interfering with closing of an activation gate located cytoplasmically (Oseguera et al., 2007). Indeed, TRPV1 is permeable to organic cationic dyes and aminoglycoside antibiotics, suggesting the existence of a large pore (Meyers et al., 2003; Myrdal and Steyger, 2005). The pore block and the divalent permeability of TRPV1 depend on the negatively charged

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residues decribed above within the pore region of the protein (Garcia-Martinez et al., 2000).

Robust TRPV1 inhibitory activity was found in the venom from the North American funnel web spider, *Agelenopsis aperta*. Fractionation of the venom resulted in the purification of two acylpolyamine toxins, AG489 and AG505 (**41a** and **41b**, respectively), which both inhibit TRPV1 channels from the extracellular side of the membrane. AG489 (Table 2) was found to inhibit TRPV1 in a voltage-dependent way, with relief from inhibition being observed at positive voltages, an observation consistent with a model where the toxin inhibits the channel through a pore-blocking mechanism (IC_{50} ~ 33nM at –40 mV). Negatively charged residues in the pore vestibule have indeed been identified as the AG489 binding site. Mutations of these site (Glu636, Asp646, Glu651, Asn628) results in weakening of the block, whereas mutations of Tyr627 and Cys636 in the pore region enhance the toxin affinity (Kitaguchi and Swartz, 2005).

Since arginine-rich hexapeptides like RRRRWW-NH2 block TRPV1 channels expressed in *Xenopus* oocytes in a non-selective manner (Himmel et al., 2002), various polymethylene tetraamines were investigated as TRPV1 channel blockers. These studies identified methoctramine (**42**), a muscarinic M2 receptor antagonist, as a non-competitive capsaicin antagonist with an IC_{50} of 2 μM, and notable voltage dependent block (Mellor et al., 2004). Finally, screening of a library of tripeptoids *N*-alkylglycines, identified DD161515 (**43**, Table 2) and DD191515 as TRPV1-blockers with micromolar potency and moderate voltage-dependency (Garcia-Martinez et al., 2002).

An interesting mechanism of analgesia has been developed capitalizing on TRPV1 pore permeability. Thus, the membrane-impermeable Na^+ channel blocker QX-314 can permeate cells through capsaicin-activated TRPV1. Under these conditions, it can block from the inner site the Na^+ channels in DRG neurons, preventing the generation of an action potential, and behaving as a long-lasting analgesic (Binshtok et al., 2007).

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TRPV2:

TRPV2 has been proposed as a potential pain target, both for its sequence similarity to TRPV1 (50% identical) and for its activation by noxious high temperatures (>52°C) (Caterina et al., 1999). This view is supported by the finding that TRPV2 is widely expressed in medium – to large –diameter Aδ mechanosensitive and thermosensitive neurons in the rat dorsal root ganglia (Caterina et al., 1999), trigeminal ganglia (Ichikawa and Sugimoto, 2000), and the spinal cord (Lewinter et al., 2004).

(1) **TRPV2 Agonists**

TRPV2 is activated by noxious heat, with an activation threshold above 52°C (Caterina et al., 1999), and by a number of exogenous chemical ligands, but ligand- and thermal activation shows species specificity. For example, whilst noxious heat (up to 53°C) and the non-selective agonist 2-aminoethoxydiphenyl borate (2-APB) activate rat and mouse TRPV2, human TRPV2 does not respond to 2-APB (up to 1 mM) or to elevated temperatures (up to 53°C; (Neeper et al., 2007). Analysis of chimeric constructs of mouse and human TRPV2 channels showed that the molecular determinants of 2-APB sensitivity are localized in the intracellular *N*- and *C*-termini. Further experiments showed that human TRPV2 exerts, with an unknown mechanism, a dominant-negative effect on 2-APB activation of native rodent TRPV2 channels (Juvin et al., 2007). Another phenylborate, DPBA, is also able to activate mouse TRPV2 with similar EC_{50} values (Juvin et al., 2007). Rat TRPV2 is activated by the uricosuric agent probenecid (**44**, Table 3), and five other sensory thermoTRPs-(TRPV1, TRPV3, TRPV4, TRPM8 and TRPA1) failed to show a response to this drug (Bang et al., 2007). Very recently, several novel cannabinoid rat TRPV2 modulators were identified. Cannabidiol (**45**), a non-psychotropic compound, was found to be a more robust and potent agonist (EC_{50} ~ 4 μM) than the psychotropic agent Δ^9-tetrahydrocannabinol (**46**, THC), the main psychoactive substance found in *Cannabis sativa* L. (EC_{50} ~ 14μM), and cannabinol (**47**, EC_{50} ~ 78μM) (Table 3) (Qin et al., 2008). TRPV2 has been proposed as a potential pain target, but very

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little is known on its activation mechanism or on possible candidates for specific or endogenous TRPV2 activators.

Sensitizers

While no selective exogenous or endogenous activators of TRPV2 have been identified to date, evidence has been growing that translocation may be an important mechanism for TRPV2 functional activation. Growth factors up-regulate TRPV2 expression and function by promoting its translocation from the intracellular pools to the plasma membrane, where it demonstrates constitutive activity (e.g. IGF-1) (Boels et al., 2001; Iwata et al., 2003; Kanzaki et al., 1999). Heat and phosphatidylinositol-3-kinase (PI3-K) also promote TRPV2 membrane insertion, whereas PI3-K inhibition reduces TRPV2 membrane insertion (Penna et al., 2006). In accordance with this scenario, the PI3 kinase inhibitors LY2934001 could block the translocation of TRPV2 to the plasma membrane (Nagasawa et al., 2007).

(2) TRPV2 antagonists

Despite the possible role of TRPV2 as a potential pain target, few specific blockers have been identified yet. Some general blockers like ruthenium red (RR) and trivalent cations, such as La^{3+} and Gd^{3+} have been described as blockers of TRPV2 (Leffler et al., 2007). SKF96365 (**48**) and the diuretic amiloride (**49**) also behave as efficient blockers (Table 3). In addition, the potassium channel blockers tetraethylammonium (TEA), 4-aminopyridine (4-AP), and 1-(2-(trifluoromethyl)phenyl) imidazole (TRIM), were all found to inhibit TRPV2 activation (Juvin et al., 2007). Also the monoterpene aldehyde citral (**50**) inhibited, in a voltage-independent way, the 2-APB-evoked activity of TRPV2 (K_d ~ 534µM) (Stotz et al., 2008).

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TRPV3

TRPV3 was cloned by using its sequence homology to other heat-activated TRP channels, and shares 40% identity with TRPV1. TRPV3 shows threshold in the physiological temperatures range of 32 to 39°C, and can also be activated by 2-APB and camphor (**13**) (for a review see (Nilius and Mahieu, 2006). In humans, TRPV3 has been reported to be present in pain pathways including DRG and TG neurons, spinal cord, keratinocytes and brain (Xu et al., 2002). However, in mouse and rat, the distribution of TRPV3 is more controversial (Peier et al., 2002).

(1) TRPV3 Agonists

TRPV3 is highly expressed in the skin, tongue and nose (Xu et al., 2002), and is the target for several plant-derived aromatic agents as well as for skin sensitization. TRPV3 is activated by a number of exogenous ligands, including natural irritants (eg. camphor (**13**) (Moqrich et al., 2005), thymol (**51**) from thyme, and eugenol (**9**) from clove, and might be involved in the anesthetic, analgesic and antipruritic properties of these compounds (Vogt-Eisele et al., 2007) (Table 4). Vanillin (**52a**), the active ingredient of vanilla (*Vanilla planifolia* Jacks ex Andrews), weakly activates TRPV3, whereas the synthetic flavor ethylvanillin (**52b**), the homologue of the natural product, activates TRPV3 much more potently (Xu et al., 2006). Surprisingly, a recent report showed that menthol (**53**) from peppermint (*Mentha piperita* L.), a popular cooling agent and TRPM8 activator, also binds to and activates TRPV3 (Vogt-Eisele et al., 2007). The disinfectant cresol (methylphenol) also activates TRPV3. Solutions of this simple phenol are used as household cleaners and disinfectants, perhaps most famously under the trade name lysol. Cresol was also promoted as a disinfecting vaginal douche in mid-twentieth century America. It also occurs in foods, wood and tobacco smoke, crude oil, and coal tar (Vogt-Eisele et al., 2007).

The cembrane diterpenoid incensole acetate (IA, **54**), a constituent of Frankincense, is a potent TRPV3 agonist that causes anxiolytic-like and antidepressive-like behavioral effects in wild-type mice (Table 4). These behavioural effects were not evidenced in TRPV3$^{-/-}$ mice. IA activates

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TRPV3 channels also in native keratinocytes but only modest effects were observed on TRPV1, TRPV2, and TRPV4 (Moussaieff et al., 2008). The synthetic compounds 2-APB and DPBA can also activate TRPV3. In single-channel analysis of inside-out membrane patches excised from TRPV3-expressing cells, 1 μM 2-APB evoked single-channel openings (Chung et al., 2004). The 2-APB analog diphenylboronic anhydride (DPBA) and 2,2-diphenyltetrahydrofuran (DPTHF) also influences TRPV3. DPBA activates TRPV3 in a similar manner as 2-APB (Table 4), while DPTHF has an opposite action, blocking TRPV3. Apparently, a structural feature important for activation of TRPV3 lacks in DPTHF, resulting in inhibition even though it binds to a 'stimulatory site' (Chung et al., 2005).

Very recently, evidence was found that 2-APB activation of TRPV3 is separable from other activation mechanisms, and depends on two cytoplasmic residues (Hu et al., 2009). Thus, Hu and co-workers found two residues (His426 and Arg696) specifically required for sensitivity of TRPV3 to 2-APB, but not to camphor or voltage (Hu et al., 2009). Chicken TRPV3 is weakly sensitive to 2-APB, and the equivalent residue at 426 is an asparagine. Mutating this residue to histidine induced 2-APB sensitivity of chicken TRPV3 to levels comparable for other TRPV3 orthologs. The cytoplasmic C-terminal mutation R696K in the TRP box displayed 2-APB specific deficits only in the presence of extracellular calcium, suggesting involvement in gating. Mutating these two residues in TRPV4, a 2-APB insensitive channel, to TRPV3 sequences, was sufficient to induce TRPV3-like 2-APB sensitivity (Hu et al., 2009).

Sensitizers

TRPV3 is also sensitized and/or directly activated by endogenous ligands, including downstream elements of the inflammatory cascade, such as unsaturated fatty acids (eg. arachidonic acid) and protein kinases (eg. PKC) (Hu et al., 2006). TRPV3 would be expected to be basally active at body temperature. However, TRPV3 activity may be further enhanced under inflammatory conditions.

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(2) TRPV3 antagonists

There are very few potent and selective inhibitors of TRPV3. Recently, the TRPV3 antagonist HC-001403 was reported to be active in animal models of pain, like the rat CFA inflammatory hyperalgesia, the formalin induced flinching assay, and the thermal injury pain (Moran M, 2007). Two other antagonists were recently reported. These compounds (GRC 15133 and GRC 17173) displayed potency in the low hundred nanomolar range, with excellent selectivity in terms of TRPs binding, and efficacy in inflammatory and nerve injury models (Khairatkar-Joshi, 2008).

TRPV4

TRPV4 functions as a Ca^{2+} entry channel that exhibits a surprising gating promiscuity. The channel can be activated by physical stimuli (cell swelling and innocuous warmth (~ 27-35°C)), and by chemical ligands. Endogenous chemical ligands (e.g. endocannabinoids and arachidonic acid metabolites) as well as small molecule exogenous ligand from the pool of natural products (e.g. bisandrographolide A, **55**), natural products derivatives (e.g. α-phorbol esters, **56a-d**), and fully synthetic compounds (e.g. GSK1016790A, **57**) have been identified. TRPV4 is widely expressed in brain, CNS, kidney, bladder, vascular endothelium, keratinocytes and multiple excitable and non-excitable pheripheral cell types (for review see (Nilius et al., 2004)).

(1) TRPV4 Agonists

TRPV4 was originally identified as a channel activated by hypotonic cell swelling (Liedtke et al., 2000; Nilius et al., 2001b; Strotmann et al., 2000; Wissenbach et al., 2000). Activation of TRPV4 by cell swelling is caused by PLA2 activation (Vriens et al., 2005; Watanabe et al., 2003). PLA2-mediated release of arachidonic acid (AA) from membrane lipids and subsequent metabolisation of arachidonic acid by cytochrome P450 (CYP) epoxygenase activity leads to the formation of epoxyeicosatrienoic acids (EETs), which activate TRPV4 in a membrane limited-

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manner. Given the high ligand-sensitivity, it does not seem unreasonable to assume that TRPV4 has also endogenous ligands, but none of them has so far been identified (Nilius et al., 2004; Nilius et al., 2003; Vriens et al., 2005; Watanabe et al., 2003).

Phorbol esters, like 4α-PDD (**56a**), bind TRPV4 in a very specific (lack of response in *TRPV4* deficient mice (Vriens et al., 2005)) and membrane-delimited manner (Vriens et al., 2004b; Watanabe et al., 2002). Activation by 4α-PDD is slow, suggesting that diffusion in the cell might be rate limiting. In line with an intracellular mode of action, application of 4α-PDD from the cytoplasmic side of inside-out patches activates TRPV4. A tyrosine-serine motif located in the intracellular loop between the second and third transmembrane domain determines the responsiveness to 4α-PDD, but not to hypotonic solutions or arachidonic acid. Furthermore, mutations of two hydrophobic residues in the central part of TM4 (Leu584 and Trp586) caused a severe reduction of the sensitivity of the channel to 4α-PDD and heat, whereas responses to cell swelling, arachidonic acid, and 5,6-EET remained unaffected. Conversely, the mutation of two residues in the *C*-terminal region of TM4 (Tyr591 and Arg594) affected channel activation of TRPV4 by all stimuli, suggesting an involvement in channel gating rather than in interaction with ligands. Comparison of the responses of wild type- and mutant TRPV4 to a series of 4α-phorbol diesters established a critical role for the length of the acyl moiety, that is optimal for 6- and 10 carbons (Vriens et al., 2007) (Table 5). The 4α-configuration is apparently not essential for channel activation, since 4β-PDD (**56b**) could activate TRPV4 with potency similar to 4α-PDD (Nilius et al., 2004), while also phorbol-12-myristate-13-acetate (PMA, **58**), the archetypal tumor-promoting PKC activator, could activate TRPV4, albeit with a 10-50 fold lower potency than 4α-PDD. In sharp contrast to the structure-activity relationships for PKC activation by phorbol esters, removal of the 4-hydroxyl and conversion to cage-compounds from the lumi-series were not critical for the activity of 4α-phorbol diesters. Conversely, two ester groups of similar length were necessary for

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activity (Vriens et al., 2007).

TRPV4 is also activated by the dimeric diterpenoid bisandrographolide A (BAA, **55**) (Table 5) from the Indian medicinal plant *Andrographys paniculata*). BAA functions also in cell-free inside out patches, indicating a membrane-delimited action (Smith et al., 2006). Evidence was provided that the TM3-TM4 region of TRPV4 forms an important site for channel activation by BAA. In particular, mutations at positions Leu584 and Trp586 in TM4 strongly affect channel activation by BAA (Vriens et al., 2007).

Recently, a novel very potent activator for TRPV4 has been reported (GSK1016790A, **57**) (Thorneloe et al., 2008). This compound produced dramatic and complex cardiovascular effects associated with endothelial barrier failure, eventually leading to circulatory collapse, in mouse, rat and dog (Willette et al., 2008). TRPV4 is highly expressed in the endothelium, where it is involved in intracellular Ca^{2+} homeostasis and the regulation of cell volume (Vriens et al., 2005). The discovery that its inappropriate activation leads to massive disruption of microvascular permeability suggests that TRPV4 migt be associated with oedema and microvascular congestion.

Sensitizers

TRPV4 can be sensitized to activation by proteases generated under inflammation conditions, like the protease-activated receptor 2 (PAR_2). PAR_2 agonists sensitize activation of TRPV4 by 4α–PDD and hypotonic cell swelling probably via PLCß, PKA, PKC, and PKD (Grant et al., 2007) (Surprenant, 2007).

In native ciliated epithelial cells, the PLA_2-EET primary pathway and the PLC pathway, both activated by ATP, cooperate and support TRPV4 gating, while IP_3, per se not an agonist, sensitizes TRPV4 to EET. Co-immunoprecipitation assays indicated a physical interaction between TRPV4 and IP_3R3. TRPV4 channels and intracellular store Ca^{2+} channels are both required to initiate and maintain the oscillatory Ca^{2+} signal triggered by high viscosity and hypotonic stimuli that do not

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reach a threshold level of PLA_2 activation (Fernandes et al., 2008).

(2) TRPV4 antagonists

Besides the classic TRP inhibitors ruthenium red (RR) that reversibly inhibits inward but not outward TRPV4 currents (Watanabe et al., 2002), Gd^{3+} and La^{3+}, two new TRPV4 antagonists with micromolar (RN-1734) and nanomolar (RN-9893) potency with selectivity versus other thermo-TRPs, were described. RN-9893 was also reported to possess good pharmacokinetic properties, but no *in vivo* data have been published (Wei ZL, 2007). Citral (**50**) from lemongrass oil, a compound commonly used to repell insects, inhibits TRPV4 activity in a voltage independent way (IC_{50} ~ 32μM) (Stotz et al., 2008).

TRPV5 and TRPV6

Compared to TRPV1-V4, TRPV5 and its highly homologous TRPV6 are only distantly related, as evident from the phylogenic tree depicted in Figure 1. Unsurprisingly, they also show somewhat different functional properties, whose hallmark is the high Ca^{2+} selectivity. Expression profiling of TRPV5 and TRPV6 showed expression in kidney, small intestine, placenta, prostate, pancreas, salivary gland, brain, colon and rectum (Hoenderop et al., 1999). In general, TRPV5 expression seems to be typical of kidney, whereas TRPV6 is predominantly expressed in small intestine and prostate (Hoenderop et al., 2000). Currents through TRPV5 and TRPV6 are strongly inwardly rectified and highly Ca^{2+} selective ($P_{Ca}/P_{Na} > 100$).

(1) Agonists of TRPV5 and TRPV6

No direct activator of TRPV5 and TRPV6 has so far been identified. TRPV5 and TRPV6 channels are constitutive active when inserted into the plasma membrane. Very likely, regulation occurs by variation of the channel density on the plasma membrane. Expression of TRPV5 is influenced by 1,25-dihydroxy vitamine D3 (Yamauchi et al., 2005), the Ca^{2+} binding protein

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S100A10, and annexin II (van de Graaf et al., 2006). Calmodulin binds to both TRPV5 and TRPV6 in a Ca^{2+} -dependent fashion (Lambers et al., 2004) and supports, at least, the activation of TRPV6. Extracellular alkalisation causes TRPV5 accumulation in subplasmalemmal vesicles. From this pool, TRPV5 can be rapidly recruited into the plasma membrane (Lambers et al., 2006).

An important regulatory pathway comprises the tissue serine protease kallikrein (TK). TK activates the bradykinin receptor-2, which in turn activates PKC dependently on DAG via PLC_{β}. Phosphorylation of TRPV5 increases membrane insertion of the channel and delays retrieval (Gkika et al., 2006). The protein kinase C (PKC) activator 1-oleoyl-acetyl-*sn*-glycerol (OAG) also increased TRPV5 surface abundance. The OAG-mediated increase of TRPV5 was prevented by preincubation with specific PKC inhibitors. Knockdown of caveolin-1 also prevented the increase of TRPV5 by OAG. Parathyroid hormone (PTH) increased TRPV5 current density in cells coexpressing TRPV5 and type 1 PTH receptor. The increase caused by PTH was prevented by PKC inhibitor, mutation of Ser299/Ser654, or by knockdown of caveolin-1. This mechanism of regulation by PKC may contribute to the acute stimulation of TRPV5 and renal Ca^{2+} reabsorption by PTH (Cha et al., 2008).

TRPV5 is highly regulated by WNK4, a protein serine/threonine kinase whose gene mutations cause familial hyperkalemic hypertension. Expression of WNK4 increases surface expression of TRPV5 and therefore modulates calcium reabsorption mediated by this channel (Jiang et al., 2007).

The pro-urine hormone klotho can stimulate TRPV5 and TRPV6 by *N*-oligosaccharide hydrolysis. Klotho is a beta-glucuronidase that hydrolyzes extracellular sugar residues on TRPV5, entrapping the channel in the plasma membrane. Klotho effects are primarily restricted to the epithelial Ca^{2+} channels TRPV5 and TRPV6 (Chang et al., 2005; Lu et al., 2008; Schoeber et al., 2007).

(2) TRPV5 and TRPV6 antagonists

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The most effective inhibitors of currents through TRPV5 described so fare are ruthenium red (RR) and the antifungal azole econazole (**59**), with IC_{50} values between 0.1 and 1 μM. The divalent cation current block profile for TRPV5 is Pb^{2+}=Cu^{2+} >Zn^{2+} >Co^{2+} >Fe^{2+}, with IC_{50} values between 1 and approximately 10 μM (Nilius et al., 2001a). Importantly, also TRPV6 can be blocked by RR, but has a 100-fold lower affinity for RR (IC_{50} ~ 9μM) than TRPV5 (Nilius et al., 2001a). Ca^{2+} - induced inactivation occurs at hyperpolarized potentials in the presence of extracellular Ca^{2+}. Half maximal inactivation by $[Ca^{2+}]_i$ occurs at a concentration of ~ 100 nM. TRPV6 is Ca^{2+} dependently inactivated, a process triggerd by Ca^{2+} dependent PLC activation and depletion of PIP2. U/3122 and edelfosine induces sustained TRPV6 activation in a duodenal sac assay. Thus, PLC modulators can be used as novel TRPV6 Ca^{2+} reabsorption modulators (Thyagarajan et al., 2009).

Blockade of TRPV5 and TRPV6 by extracellular Mg^{2+} is voltage-dependent, and critically depends on a single aspartate residue in the selectivity filter of the TRPV5/6 pore. Intracellular Mg^{2+} also exerts a voltage dependent block that is alleviated by hyperpolarization and contributes to the time-dependent activation and deactivation of TRPV6-mediated monovalent cation currents (Voets et al., 2003; Voets et al., 2001).

TRPV5 is inhibited by binding of protons to the extracellular Glu522. In addition, mutation of the pore helix residue E535Q further enhances inhibition by H^+ by shifting the pKa value for inhibition towards more alkaline values. This mechanism may contribute to hypercalciuria with high acid load (Yeh et al., 2006).

TRPV6 interacts with the protein tyrosine phosphatase 1B (PTP1B), and is probably inhibited by Ca^{2+} via an intermediate reactive oxygene species. Inhibition of tyrosine dephosphorylation by PTP1B inhibition causes a maintained Ca^{2+} influx via TRPV6, thus, inducing a positive feedback (Bogeski et al., 2006; Sternfeld et al., 2005).

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CONCLUDING REMARKS

Ion channels of the TRPV subfamily form an important aspect of the sensory mechansism of animals. Indeed, it has been shown that one part of the TRPV subfamily, namely TRPV1 –TRPV4, react to thermal, mechanical and /or painful stimuli. The properties as nociceptors qualify TRPV1-TRPV4 as important tools for the traitment of pain. TRPV1, perhaps the most important signal integrator in sensory nociceptors, is well established as an intriguing novel target for the treatment of pain. TRPV1 antagonism represents one of several novel mechanistic approaches to pain relief *en route* to the next-generation analgesic, and preclinical data indicate that TRPV1 antagonists might also provide a useful therapeutic option for urinary incontinence, pancreatitis, cough, and migraine (Szallasi et al., 2006). Most anti-inflammatory drugs under development target the propagation and transmission of signals to the spinal cord, while TRPV1 antagonists target the generation of the nociceptive stimulus.

In this review, we clearly illustrate that TRPV channels are sensitive to modulation by exogenous small molecules. However, our limited knowlege of their endogenous ligands make them still substantially ‘orphan’ receptors. Furthermore, except for TRPV1, the pharmacology of these channels is sparse and selective molecular tools (ligands, blockers, sensitizers) are missing but urgently required. In addition, information on the regions and amino acids involved in TRPV function is still missing. Since members of the TRPV family are critical players in pheripheral nociception, a better knowledge of the ligand binding region could be used to rationally design novel anti-nociceptive or anti-inflammatory agents, and chrystallographic analysis could shed light on the structural determinants of TRPV functionality. Finally, as our knowledge on the physiology and regulation of vanilloid receptors increases, so does the clinical relevance of the pharmacological manipulation of these ion channels.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Phylogenetic relations of the mammalian vanilloid transient receptor potential (TRPV) channels and their related other TRP subfamilies (polycystin TRPPs, canonical TRPCs, melastatin, TRPMs, ankyrin, TRPA and mucolipin, TRPMLs). This consensus tree was obtained as ascribed in detail in (Yu and Catterall, 2004) from an analysis of the pore regions. The scale bar corresponds to the number of changes in amino acids needed to explain the differences in the protein sequences. Note the distance between the highly Ca^{2+} permeable TRPV5, TRPV6 and the thermoTRPs. TRPV1-4. Figure adapted from (Yu and Catterall, 2004), for more details see also (Montell, 2005; Nilius et al., 2007; Vennekens et al., 2008).

Figure 2: Formulas of compounds 1-7a,b (for a detailed description of all compounds in figures 2-7 see text and tables)

Figure 3: Formulas of compounds 8-20

Figure 4: Formulas of compounds 21-32

Figure 5: Formulas of compounds 33-40

Figure 6. Formulas of compounds 41-43

Figure 7. Formulas of compounds 44-59

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TABLES

Table 1. TRPV1 agonists. Ref n° is the reference number given in the manuscript.

Group	Compound	Ref n°	EC_{50}	Species	Reference
Endogenous agonists (Fatty Acids)	Anandamide	**2a**	10^{-5} M	Rat	(Ahern, 2003)
	NADA	**3a**	10^{-8} - 10^{-7} M	Human & rat	(Huang et al., 2002)
	12 HPETE	**5a**	10^{-5} M	Human & rat	(Hwang et al., 2000)
Exogenous agonists of natural, semi-synthetic and synthetic origine	Capsaicin	**1**	10^{-8} – 10^{-6} M	Rodent	(Caterina et al., 1997)
	Piperine	**8**	10^{-5} – 10^{-4} M	Human	(Liu and Simon, 1996)
	Eugenol	**9**	10^{-3} - 10^{-2} M	Human	(Yang et al., 2003)
	Resiniferatoxin	**11a**	10^{-10} - 10^{-8} M	Rat	(Szallasi and Blumberg, 1989)
	Olvanil	**12a**	10^{-10} - 10^{-9} M	Human	(Appendino et al., 2005b)
	Phar	**12b**	10^{-11} - 10^{-10} M	Human	(Appendino et al., 2005b)
	Camphor	**13**	10^{-3} - 10^{-2} M	Rat	(Xu et al., 2005)

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Table 2. TRPV1 antagonists. Ref n° is the reference number given in the manuscript

Group	Compound	Ref n°	IC_{50}	Species	Reference
Naturally -occuring	Thapsigargin	**23**	10^{-6} - 10^{-5} M	Rat	(Toth et al., 2002)
	Yohimbine	**24**	10^{-5} - 10^{-4} M	Rat	(Dessaint et al., 2004)
1,3-Di(arylalkyl)thioureas	Capsazepine	**22**	10^{-7} - 10^{-6} M	Rodent	(Caterina et al., 1997)
	Compound	**26**	10^{-5} M	Rat	(Lee et al., 2003)
	JYL1421	**27**	10^{-8} M	Rat	(Wang et al., 2002)
Iodinatede vanillyl derivatives	5-iodoRTX	**11b**	10^{-9} M	Rat	(Wahl et al., 2001)
Di(arylalkyl)- and	BCTC	**28**	10^{-9} M	Rat	(Pomonis et al., 2003)
aryl(arylakyl)ureas	A-425619	**29a**	10^{-9} M	Rat	(McDonald et al., 2008)
	SB-452533	**31**	10^{-8} - 10^{-7} M	Human	(Rami et al., 2004)
	ABT-102	**32**	10^{-9} M	Human	(Surowy et al., 2008)
Cinnamides	SB-366791	**33**	10^{-9} M	Human & rat	(Patwardhan et al., 2006)
	AMG-9810	**34**	10^{-8} M	Human & rat	(Doherty et al., 2005)
Carboxamides	SB-782443	**36**	10^{-8} - 10^{-7} M	Human & rat	(Westaway et al., 2008b)
	Compound	**38**	10^{-8} - 10^{-7} M	Human & rat	(Westaway et al., 2008b)
Non classic antagonists					
Imidazole derivatives	Compound	**39**	10^{-9} M	Rat	(Gore et al., 2007)
	AMG 517	**40**	10^{-10} M	Rat	(Gavva et al., 2007)
Non competitive	Ruthenium Red		10^{-7} M	Rat	(Garcia-Martinez et al., 2000)
	AG 489	**41a**	10^{-8} M	Rat	(Kitaguchi and Swartz, 2005)
	DD161515	**43**	10^{-7} – 10^{-6} M	Rat	(Garcia-Martinez et al., 2002)

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Table 3. TRPV2 activators and inhibitors. Ref n° is the reference number given in the manuscript. EC_{50} and IC_{50} values are given in column with concentration range.

Group	Compound	Ref n°	Conc. R	Species	Reference
Activators	Probenecid	**44**	10^{-5} M	Rat	(Bang et al., 2007)
	Cannabidiol	**45**	10^{-6} M	Human & rat	(Qin et al., 2008)
	THC	**46**	10^{-5} M	Human & rat	(Qin et al., 2008)
	Cannabidiol	**47**	10^{-4} M	Human & rat	(Qin et al., 2008)
Inhibitors	SKF96365	**48**	10^{-5} M	Mouse	(Juvin et al., 2007)

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Table 4. TRPV3 activators. Ref n° is the reference number given in the manuscript.

Group	Compound	Ref n°	EC_{50}	Species	Reference
Activators	Camphor	**13**	10^{-4} - 10^{-2} M	Human & mouse	(Moqrich et al., 2005)
	Thymol	**51**	10^{-4} M	Human & mouse	(Xu et al., 2006)
	Menthol	**53**	10^{-4} - 10^{-3} M	Murine	(Macpherson et al., 2006)
	Eugenol	**9**	10^{-5} - 10^{-3} M	Human & mouse	(Xu et al., 2006)
	Vanillin	**52a**	10^{-2} - 10^{-1} M	Human & mouse	(Xu et al., 2006)
	Incensole acetate	**54**	10^{-5} M	Mouse	(Moussaieff et al., 2008)
	2-APB		10^{-6} M	Mouse	(Chung et al., 2004)

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Table 5. TRPV4 activators. Ref n° is the reference number given in the manuscript.

Group	Compound	ref n°	EC_{50}	Species	Reference
Activators	Arachidonic Acid		10^{-5} M	Mouse	(Watanabe et al., 2003)
	5,6 EET		10^{-6} M	Mouse	(Watanabe et al., 2003)
	BAA	**55**	10^{-7} - 10^{-6} M	Mouse	(Smith et al., 2006)
	4α-PDD	**56a**	10^{-6} M	Mouse	(Watanabe et al., 2002)
	GSK10116790A	**57**	10^{-9} - 10^{-8} M	Human & mouse	(Thorneloe et al., 2008)

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FIGURES

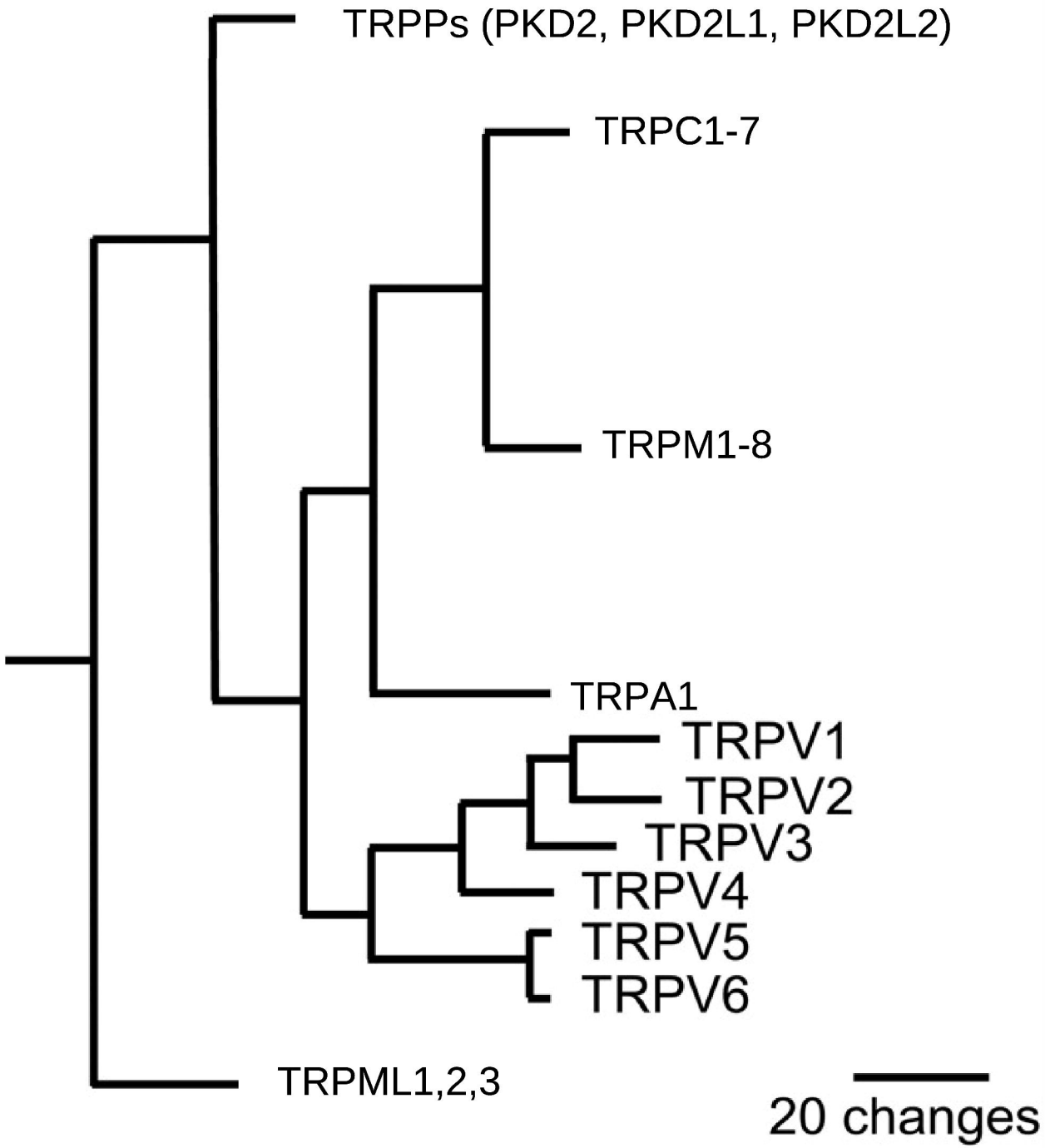
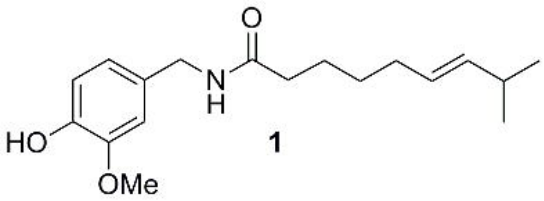


Figure 1



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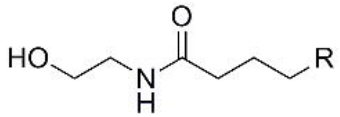

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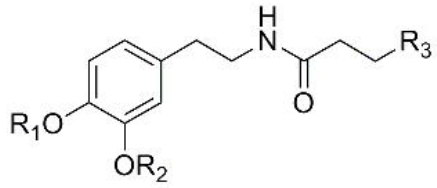

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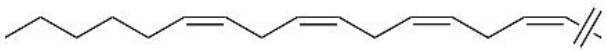
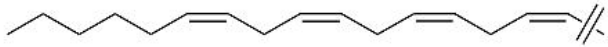
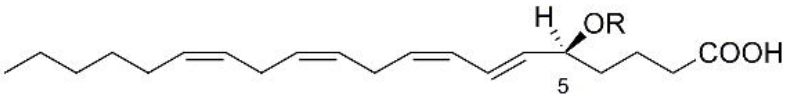
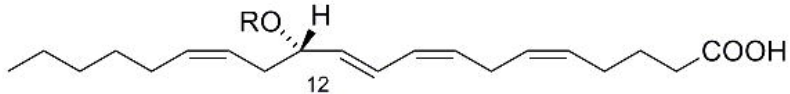


2b

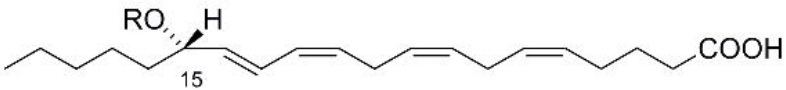
	R_1	R_2	R_3
3a	H	H	
3b	H	H	
3c	Me	H	
3d	H	Me	



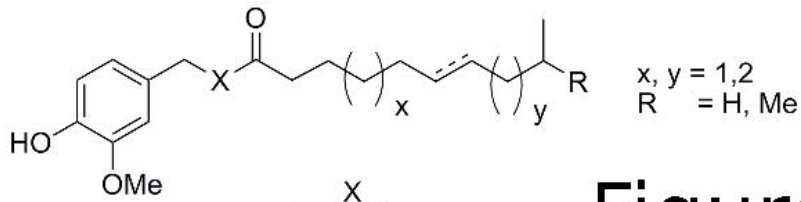
	R
4a	OH
4b	H



	R
5a	OH
5b	H

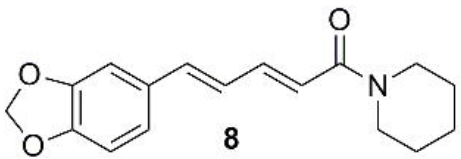


	R
6a	OH
6b	H



	X
7a	NH
7b	O

Figure 2



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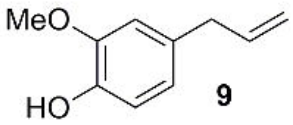

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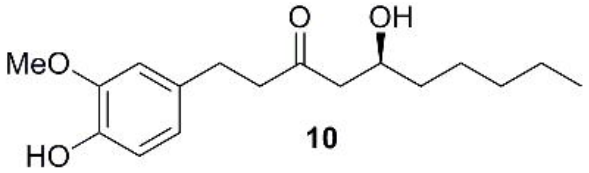

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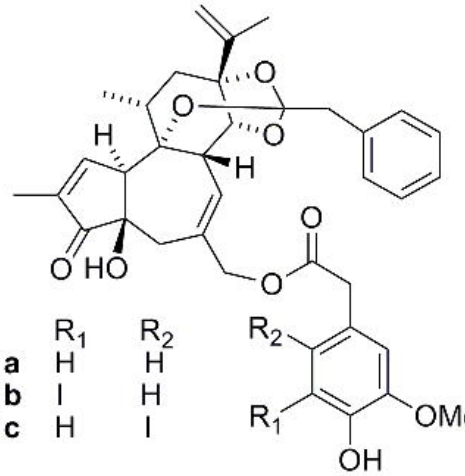
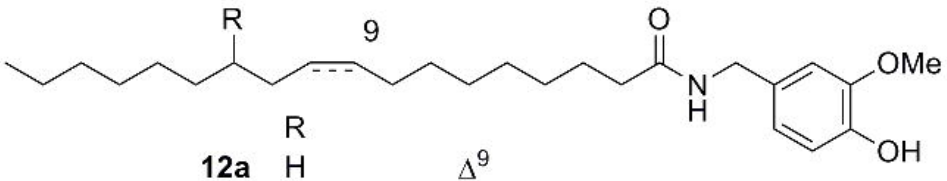
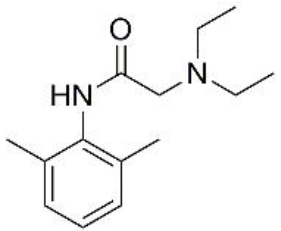


	R_1	R_2
11a	H	H
11b	I	H
11c	H	I



	R	
12a	H	Δ^9
12b	$COCH_2Ph$	Δ^9
12c	H	

13



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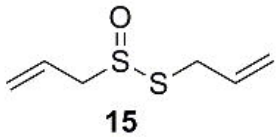

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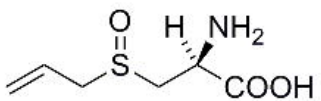

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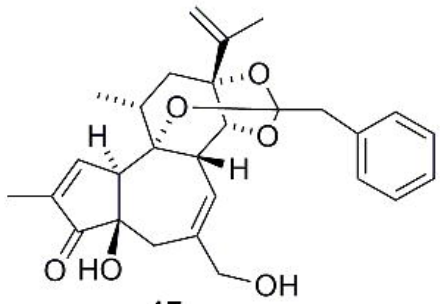

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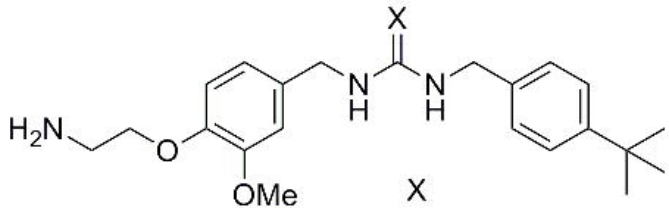
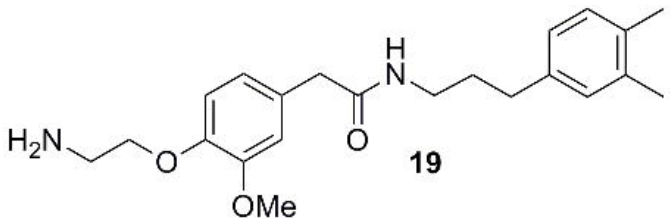


	X
18a	S
18b	O



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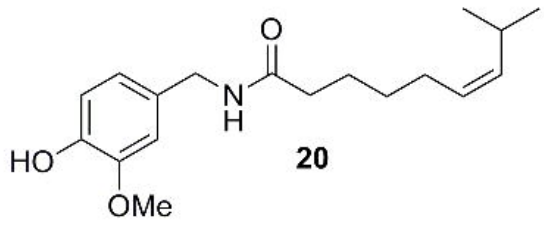

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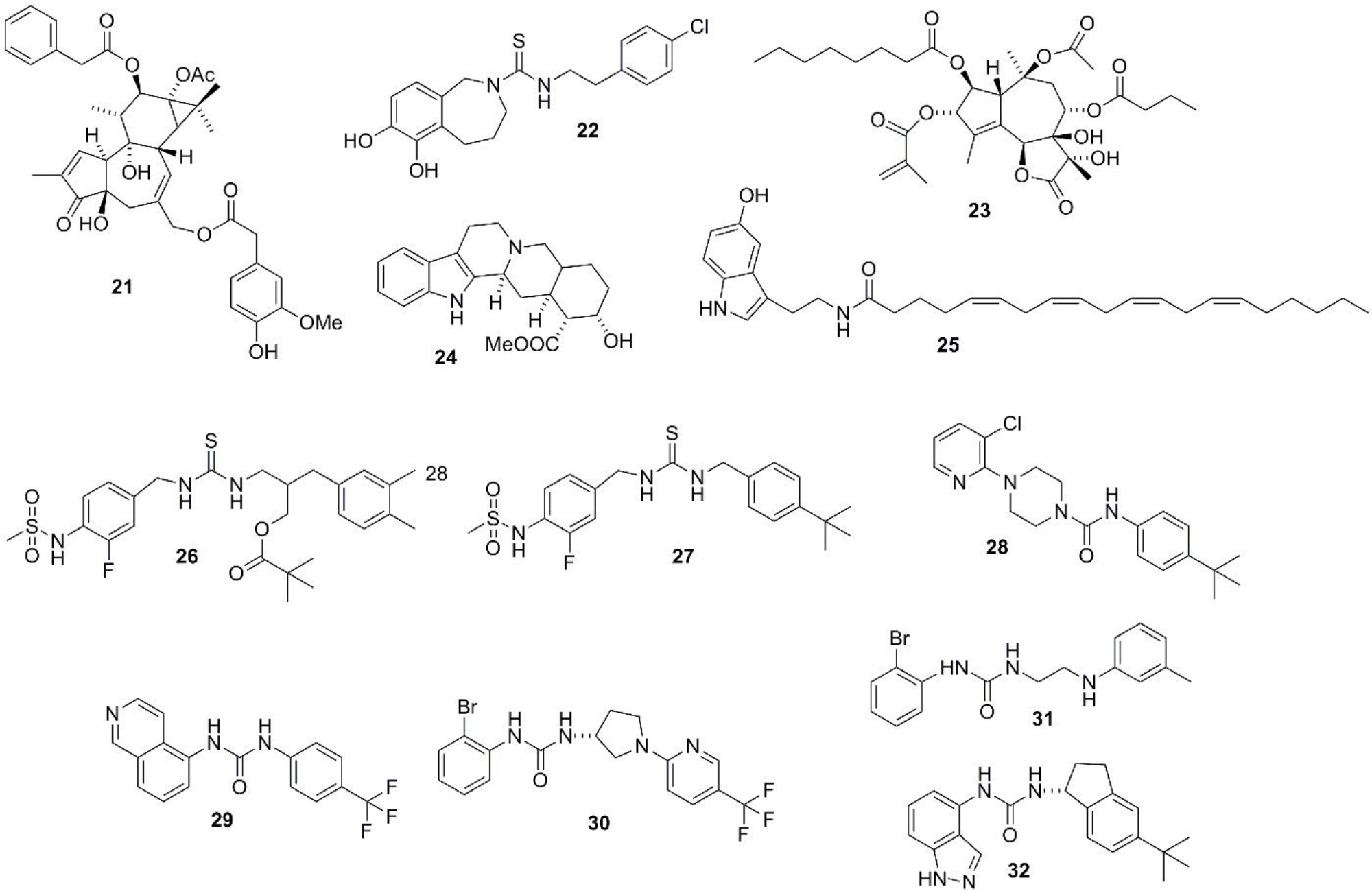
Figure 3



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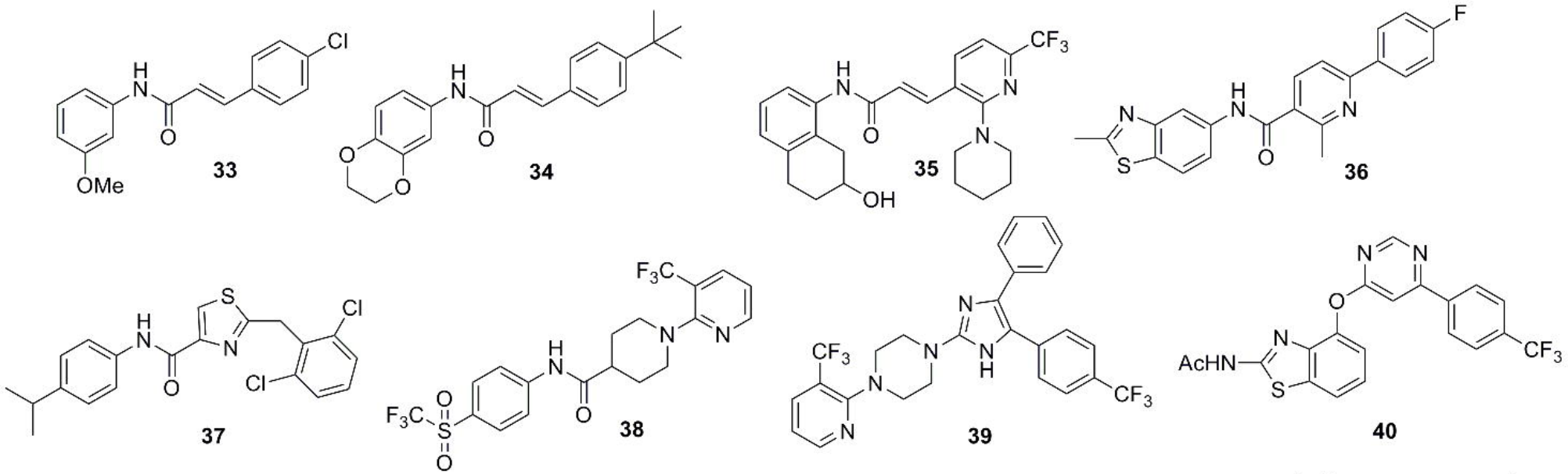
Cl
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CF3
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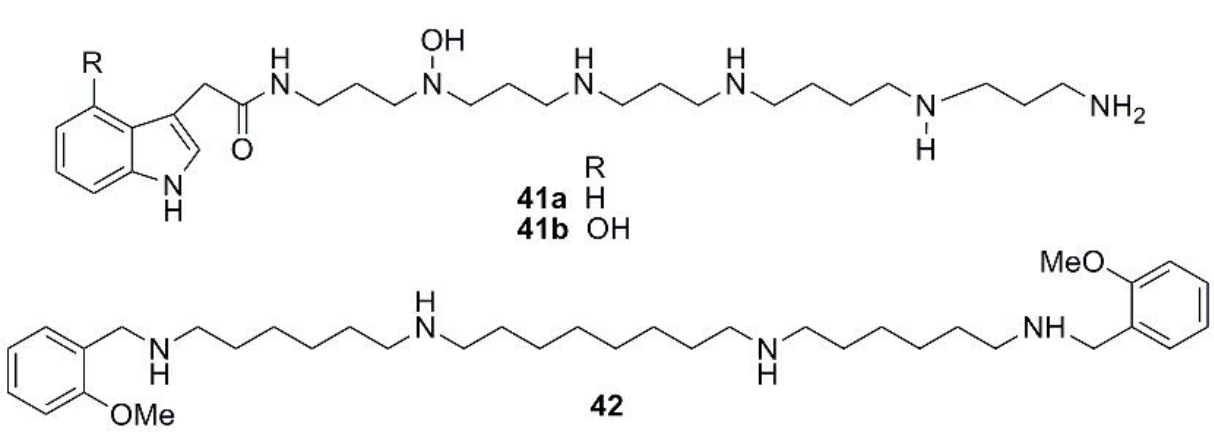
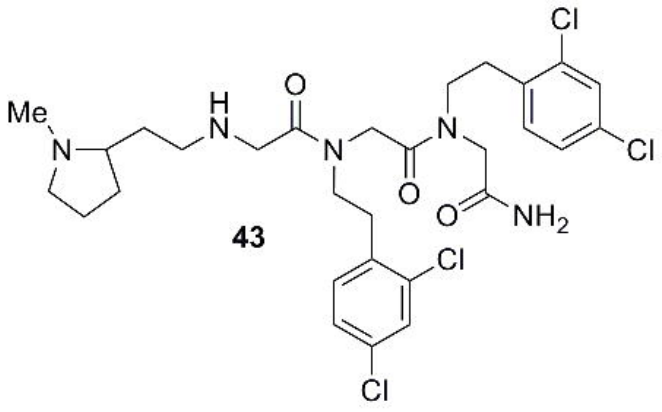
Figure 5





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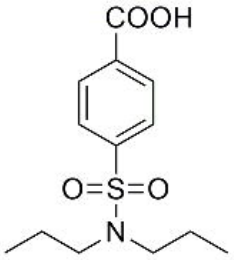

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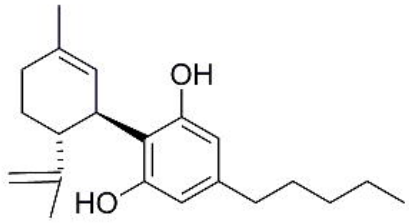

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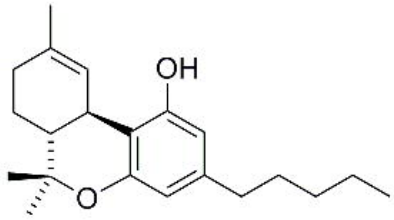

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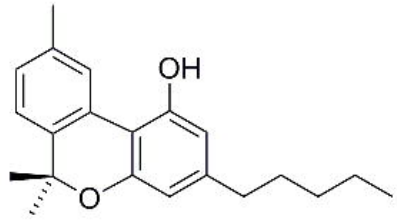

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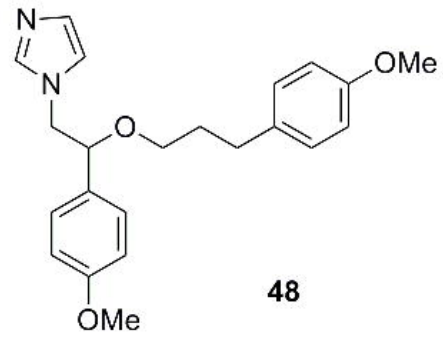

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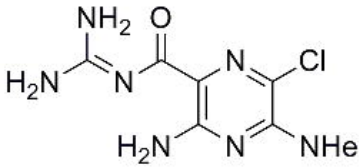

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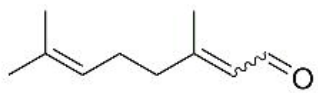

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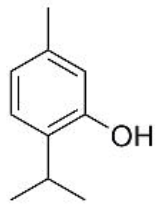

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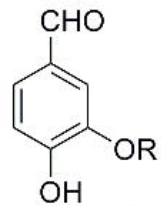
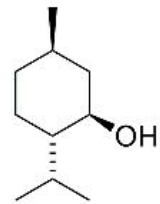


R
52a Me
52b Et



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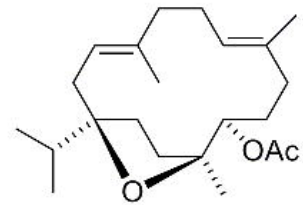

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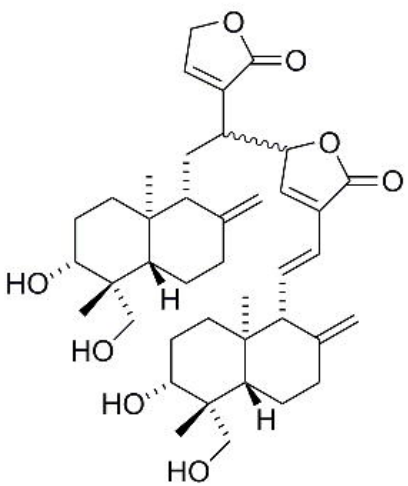

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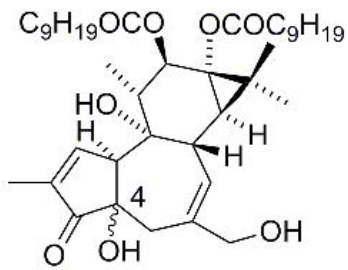
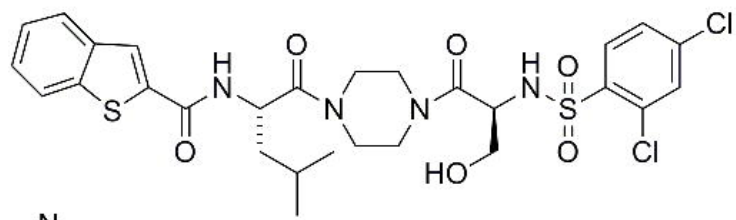


56 a 4α-OH
56b 4β-OH



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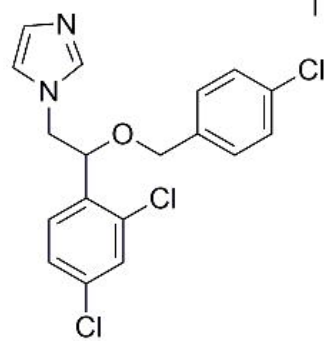
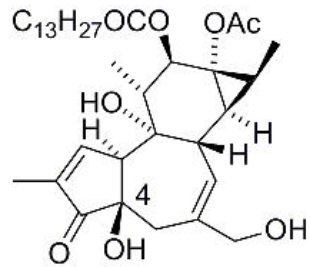


59



58

Figure 7