Physiology and Pharmacology of the Vanilloid Receptor

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Abstract: The identification and cloning of the vanilloid receptor 1 (TRPV1) represented a significant step for the understanding of the molecular mechanisms underlying the transduction of noxious chemical and thermal stimuli by peripheral nociceptors. TRPV1 is a non-selective cation channel gated by noxious heat, vanilloids and extracellular protons. TRPV1 channel activity is remarkably potentiated by pro-inflammatory agents, a phenomenon that is thought to underlie the peripheral sensitisation of nociceptors that leads to thermal hyperalgesia. Cumulative evidence is building a strong case for the involvement of this receptor in the etiology of both peripheral and visceral inflammatory pain, such as inflammatory bowel disease, bladder inflammation and cancer pain. The validation of TRPV1 receptor as a key therapeutic target for pain management has thrust intensive drug discovery programs aimed at developing orally active antagonists of the receptor protein. Nonetheless, the real challenge of these drug discovery platforms is to develop antagonists that preserve the physiological activity of TRPV1 receptors while correcting over-active channels. This is a condition to ensure normal pro-prioceptive and nociceptive responses that represent a safety mechanism to prevent tissue injury. Recent and exciting advances in the function, dysfunction and modulation of this receptor will be the focus of this review.

THE TRP RECEPTOR SUPERFAMILY

The Transient Receptor Potential (TRP) mammalian gene superfamily consists of 28 different gene products encoding non-selective cation channels that play a wide diversity of physiological functions [24,79]. These channels are considered molecular gateways in sensory systems, since several of these channels transduce chemical and physical stimuli into neuronal activity, *i.e.* action potentials. Thus, TRP channels have emerged as major transducers of chemically and physically evoked sensations.

Based upon their sequence homology, mammalian TRP channels are divided into six subfamilies, namely TRPC, TRPV, TRPP, TRPM, TRPA and TRPML [24,79,82]. An additional family, TRPN, has been described in *Drosophila*. These proteins have a common topology of six trans-membrane segments (S1-S6) with a pore region between the fifth and sixth segment, and cytoplasmic N- and C-termini. TRPV and TRPC contain two-to-four ankyrin domains that are thought to interact with the cytoskeleton, as well as with other cytosolic proteins [24]. TRP proteins are remarkable channels because of the diversity of their activation mechanisms, cation selectivity and biological function. Some members are activated through the phospholipase C-inositol trisphosphate pathway, although the specific mechanism of activation is still elusive [24,77]. Others, such as some TRPV members, are ionotropic receptors activated by chemical and physical stimuli [24,61]. The molecular diversity of TRP proteins correlates with their wide number of biological functions, ranging from fertility to vision, taste, olfaction, osmo/mechanosensation, and nociception [61,77, 79]. Therefore, TRP channels constitute a family of sensory receptors.

THE TRPV1 CHANNEL

Among the TRP channel superfamily, TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1 are thermoreceptors [80]. These receptors are designed to detect a wide range of temperatures from hot (TRPV1 and TRPV2) and cold (TRPA1) noxious temperatures to innocuous thermal stimuli (TRPV3, TRPV4 and TRPM8) [24,80]. In addition, these channels are activated by chemical agents such as capsaicin (TRPV1), menthol (TRPM8), camphor (TRPV3) and mustard (TRPA1) [19,60,74,81,80,100]. Furthermore, TRPV1 is also gated by protons and endocannabinoids [19,61]. Notably, the physiological properties of the receptor have signalled it out as a key thermosensory transducer in the nervous system [19,80]. In support of this tenet, analysis of vanilloid receptor null mice has substantiated the involvement of this channel in pain sensation [18,30].

At the molecular level, TRPV1 is a calcium permeable non-selective cation channel. The functional receptor is a tetrameric membrane protein with four identical subunits assembled around a central aqueous pore, although hetero-meric association with TRPV3 has been reported [98]. Structurally, each TRPV1 subunit protein shows a membrane domain composed of six transmembrane segments (S1-S6), with an amphipathic region between the fifth and sixth segment that forms the channel conductive pore. This region contains glutamic acids that are involved in the pH-dependent gating of the receptor [19]. The protein also has a cytoplasmic N- and C-termini (Fig. **1**). In the N-terminus, TRPV1 channels exhibit three ankyrin domains that mediate protein-protein interactions with cytosolic proteins, and show consensus sequences for protein kinases. The protein displays a cytosolic C-terminus domain containing phospho-inositide, and calmodulin binding (CAM) domains, as well

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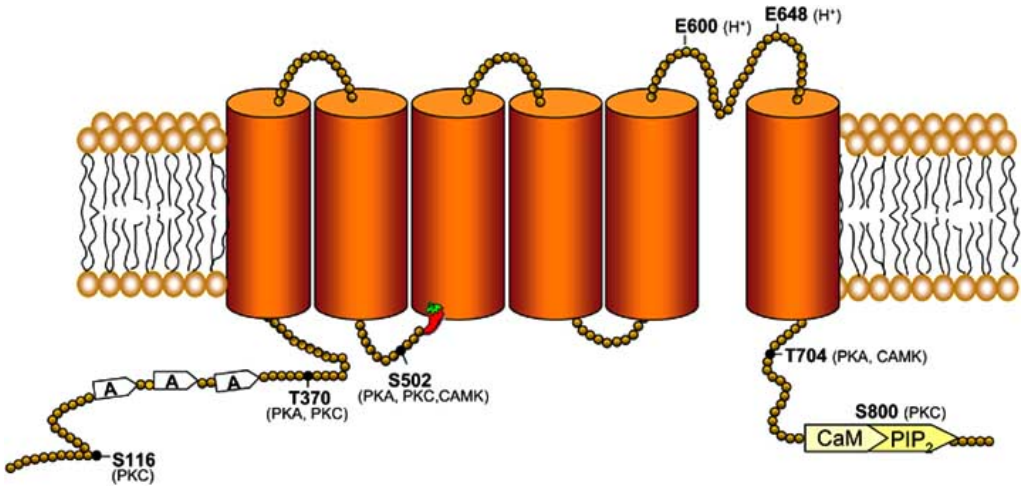

Fig. (1). Proposed model of the topology of TRPV1 subunits. Each subunit consists of six putative transmembrane segments and intracellular N- and C- domains. Functional regulatory domains of the receptors and phosphorylation sites are indicated. The capsaicin binding site is labelled withe the chili icon.

as phosphorylation sites [24]. In addition, the C-end has a TRP-like motif that functions as an association domain of receptor subunits [39].

TRPV1 is an integrator of noxious stimuli that gates in response to heat (≥ 42ºC), vanilloids, protons, and pro-algesic substances [19,61]. Temperature sensing seems related to voltage dependent gating, as evidenced by the shift in TRPV1 voltage-dependent activation in response to changes in temperature [111]. Chemical activators of TRPV1 channels can be grouped into those agents that directly gate the channel, and those that allosterically modulate the protein by post-translational modifications. Furthermore, agonists of the receptor act as gating modifiers reducing the temperature threshold of the channel gating [111]. Although TRPV1 is a temperature-gated channel with a temperature threshold of activation of 42ºC, it was recently shown that TRPV1 may not be required for the detection of noxious heat in intact C-fiber afferents [117]. However, the channel becomes a pivotal heat sensor under pathological/ inflammatory states as concluded from genetic and pharmacological TRPV1 knock-out studies [18,30,38]. The receptor has two abundant single nucleotide polymorphisms that produce amino acid substitutions, one in codon 315 (Met^{315} Ile) at the N-terminus domain, and the other at amino acid 585 (Ile^{585} Val) located in the fifth transmembrane segment. Interestingly, gender, ethnicity and temperament seem to contribute to individual variation in thermal and cold pain sensitivity by interactions in part with these TRPV1 single nucleotide polymorphisms [66].

A large body of evidence indicates that post-translational modification of TRPV1 increases the channel activity of the receptor [11,32]. Indeed, TRPV1 has several consensus sequences for protein kinases A (PKA), C (PKC), calmodulin kinase II (CAMKII) and the tyrosine specific kinase Src (Fig. **1**). PKA phosphorylation potentiates TRPV1 responses by reducing agonist-induced desensitisation. Desensitisation of the receptor depends on the presence of extracellular Ca^{2+}, and it is characterised by a profound run-down of its channel activity upon repetitive agonist stimulation. PKA exerts its action through partial rescue of desensitised receptors by direct phosphorylation of amino acids S116 and T370 at the N-terminus domain. Similarly, CaMKII-mediated phosphorylation, along with calcineurin-induced dephosphorylation of the receptor, notably contributes to determine its activation and desensitisation state [63,78]. Likewise, in colonic dorsal root ganglion neurons, the channel activity of TRPV1 is enhanced by tyrosine phosphorylation with the nonreceptor tyrosine kinase Src [58]. Tyrosine phosphorylation of the protein may underlie in part to the IL-1β-induced potentiation of heat-activated currents in rat sensory neurons [86].

PKC-dependent phosphorylation of TRPV1 can be primarily induced either by extracellular ATP released from injured cells, IL-1β, proteases, neurotrophins and bradykinin [86,102,110]. *In vitro* studies have identified S502, T704 and S800 as PKC phosphorylation sites (Fig. **1**). Different PKC isoforms have been involved in TRPV1 sensitisation. For instance, phorbol esters primarily activate PKCα [87], although they can also act as a direct ligands of TRPV1 [10,83]. In contrast, stimulation of bradykinin B1 and B2 receptors leads to PKCε activation [20,102]. More recently, PKCμ, a kinase related to, but distinct of the PKC family has been shown to phosphorylate and sensitise both heterologously expressed and native TRPV1 channels [115]. PKC phosphorylation of the vanilloid receptor notably enhances its responsiveness by augmenting the channel open probability [93]. Akin to vanilloids and pH, TRPV1 phosphorylation by PKC decreases the receptor heat threshold, thus leading to activation of the channel at body temperature. In addition, PKC activation promotes the rapid recruitment of vesicular receptors to the cell surface [83]. The occurrence of both events provides a molecular mechanism for the pain experienced in response to a warm stimulus, such as showering sunburnt skin.

EXPRESSION OF TRPV1 CHANNELS

In situ hybridisation, immunocytochemical analysis, and drug binding assays have shown TRPV1 expression in ≈50% of dorsal root and trigeminal ganglion neurons, in dorsal

horn of spinal cord and caudal nucleus of spinal trigeminal complex [19,107]. The majority of TRPV1 positive neurons also colocalise with the nerve growth factor (NGF) receptor *trkA*, the lectin IB4, and the neuropeptides involved in nociceptive transmission such as substance P (SP) and calcitonin gene related peptide (CGRP) [19,61]. Vanilloid sensitive nociceptors are peptidergic, small diameter neurons that give rise to unmyelinated C fibers, although some Aδ fibers are responsive to vanilloid derivatives [49]. Somatic and visceral primary afferents express TRPV1 at both the spinal and peripheral terminals. Furthermore, TRPV1 expression has been reported in vagal afferents in jugular and nodose ganglion neurons [54,84].

In addition to a subset of nociceptors, TRPV1 is present in neurons of the central nervous system and non-neuronal cells. For instance, TRPV1 mRNA or protein is widely expressed in brain regions such as the olfactory nuclei, cerebral cortex, dentate gyrus, central amygdala, striatum, centromedian and paraventricular thalamic nuclei, hypothalamus, substantia nigra, reticular formation, locus coeruleus, inferior olive and cerebellar cortex [see 84 and references therein]. The role of TRPV1 in the central nervous system is still elusive, although it may mediate endovanilloid signalling promoting the release of excitatory neurotransmitter such as L-glutamate, noradrenaline and dopamine [71,72].

In the skin, TRPV1 positive cells have been found in the dermis and epidermis [46], primarily in Meissner corpuscles and keratinocytes [56,88]. Moreover, this receptor is also expressed in mast cells, where its activation could release mast cell pro-algesic mediators that bind to histamine and proteinase-activated receptors on sensory terminals [99]. More recently, functional TRPV1 channels were found in the human hair follicle [15]. Activation of this receptor in cultured keratinocytes inhibited cellular proliferation, induced apoptosis, up regulated known endogenous hair growth inhibitors, and down regulated hair growth promoters. Thus, these results suggest an important role of TRPV1 in epithelial growth disorders [15].

It is also worth mentioning that TRPV1 receptors appear to be highly expressed in visceral afferents [53], and are present in the gastrointestinal (GI) tract within the myenteric and submucous plexus and some enteric intrinsic neurons [116]. Although capsaicin does not desensitise gastric epithelial cells, activation of TRPV1 expressing primary afferents has been shown to thicken the protective barrier in the stomach and duodenum [84], and sensitisation of this receptor plays a role in GI inflammation and function [41]. In pathological conditions, TRPV1 has been implicated in the hypermotility of the GI tract in abdominal pain associated to functional bowel disorders, and in the neurogenic component of pancreatitis [1,49].

TRPV1 expression is also prominent in other tissues. In the urinary bladder, functional channels are expressed in the uroepithelium in both the superficial and basal layers [4], where they are implicated in the development of the micturition reflex in both normal and pathological conditions [12]. In the lungs, capsaicin stimulates airway specific C-fibers and may play a role in the generation of non-productive cough [65]. Neurogenic inflammation of mucosal nociceptors also seems to be a key factor in the pathogenesis of asthma [44]. Under normal conditions, these fibers do not express SP but begin to produce it after allergic inflammation and viral infection [17,51]. In the vascular system, TRPV1-expressing fibers accompany blood vessels in all layers of the viscera. These receptors appear involved in Bayliss myogenic constriction which leads to hypertension [97]. Furthermore, TRPV1 seems to contribute to myocardial protection by releasing CGRP and nitric oxide from capsaicin-sensitive afferents in the ischemic heart [101]. Another tissue that expresses TRPV1 channels is the inner ear, where the protein is present in inner and outer hair cells, inner and outer pillar cells, Hensen cells and satellite cells [8,121]. These channels appear to be involved in hearing, as evidenced by the vanilloid-induced alteration of cochlear sensitivity [104].

Taken together, all these findings illustrate that TRPV1 channels are widely expressed in neuronal and non-neuronal cells of both endodermal and mesodermal origin, and suggest that the receptor is involved in diverse physiological functions. These include thermosensory transduction, as well as chemical signalling presumably mediated by endovanilloid compounds. In addition, they hint that dysfunction of the channel may underlie the etiology of pathological sensory transduction such as that occurring in inflammation. Taken together, all these observations underscore the notion that TRPV1 is a widely expressed protein whose function may be critical for diverse physiological conditions.

TRPV1 A THERAPEUTIC TARGET FOR PAIN MANAGEMENT

The pivotal role of TRPV1 in physiology has suggested a contribution of the channel to the mechanism of diverse human diseases. In particular, cumulative evidence is substantiating the tenet that nociceptor sensitisation by inflammatory agents is primarily achieved by TRPV1. This receptor is the endpoint target of intracellular signalling pathways triggered by inflammatory mediators that lead to potentiation of its channel activity which, in turn, promotes the hyperexcitability of nociceptors. Enhancement of TRPV1 function by pro-algesic agents may be accomplished either by direct activation of the channel or by its post-translational modification mediated by intracellular metabolic cascades [24,80]. Direct activation of TRPV1 responses has been reported for lipid mediators such as arachidonic acid metabolites including anandamide, N-arachidonyl-dopamine (NADA), N-oleyldopamine and, 12-(hyperoxy)eicosatetrenoic acid (12-HPETE). These compounds act as weak agonists, but notably increase TRPV1-mediated $[Ca^{2+}]_i$ in both heterologous expression systems, and in primary sensory neurons [22,50,52]. In addition, the acidosis that develops in inflamed tissues is also a direct activator of the TRPV1 channel activity [19]. The potency and efficacy of each singular mediator is quite low but in inflammatory conditions, several of these modulators are simultaneously released and act synergistically. Noteworthy, most of these TRPV1 ligands act by reducing the heat threshold of channel activation from 42ºC to body temperature (≈35ºC) [11]. Therefore, direct gating of TRPV1 responses by inflammatory agents acting as channel agonists notably increases the

excitability of nociceptors resulting in a hyperalgesic condition. In addition, the TRPV1-mediated $[Ca^{2+}]_i$ rise, in turn, triggers the release of pro-inflammatory agents at peripheral terminals, thus further increasing the excitability of the nociceptors. This feedback circuit notably contributes to enhance the hypersensitivity of inflamed tissue.

Activation of intracellular protein networks during inflammation results in TRPV1 phosphorylation [11], release of tonically-inhibited receptors [23], and an increment of the surface expression of functional channels [83], all being major events underlying the nociceptor activation and sensitisation that leads to hyperalgesia. Indeed, TRPV1 expression is upregulated in tissue samples from patients with inflammatory bowel disease and Crohn´s disease [118], and also in patients with rectal hypersensitivity [21], as well as those affected of vulvodynia [108]. Thus, TRPV1 receptors are strong candidates for an important role in the sensitisation of primary afferents after injury or inflammation. The involvement of TRPV1 in heat hyperalgesia is underscored by the reduced thermal hypersensitivity of mice lacking TRPV1 [18,30]. Similarly, non-competitive antagonists of the TRPV1 channel notably attenuate the heat hyperalgesia triggered by inflammation *in vivo* [38]. The important contribution of TRPV1 receptor to the onset and maintenance of neurogenic inflammation has validated it as a therapeutic target for inflammatory pain management.

In addition to the contribution of the vanilloid receptor as a target of the neurogenic inflammation underlying different diseases, TRPV1 is gaining interest for the treatment of neuropathic, postoperative and chronic pain and, recently, for the therapy of epithelial disorders. Thus, for instance, topical capsaicin or resiniferotoxin have been used in postherpetic neuralgia, diabetic neuropathy, postmastectomy pain and arthritis [64,103]. Recently, TRPV1 has been clearly validated as a key target for management of chronic pain in bone cancer [42]. As a result, the development of specific TRPV1 antagonists is a central focus of current drug discovery programs.

PHARMACOLOGY OF TRPV1 RECEPTORS

The TRPV1 receptor is a molecular entity with diverse drug binding sites. Structure-function studies have unmasked the molecular determinants of the capsaicin binding site in an intracellular domain of the receptor (Fig. **1**) [40,59], and those involved in the interaction with the non-competitive antagonist ruthenium red at the extracellular vestibule of the pore domain [37]. The existence of these binding sites has prompted the discovery of novel vanilloid-like agonists, as well as competitive and non-competitive antagonists with the hope that they will be of clinical use to treat human TRPV1 receptor dysfunction. Further summarized are the efforts undertaken to develop these three classes of TRPV1 modulators.

TRPV1 AGONISTS

Agonists are molecules that directly gate the channel by a mechanism that involves the reduction of the heat threshold of activation. Prolonged exposure of the receptor to the agonist in the presence of Ca^{2+} induces channel closure by desensitisation and tachyphylaxia [103], by a mechanism that involves phosphorylation of a key residue at the C-terminus of the protein [11]. Agonist-induced desensitisation is thought to underlie the analgesic activity of capsaicin. However, capsaicin analgesia may also arise from selective deletion of TRPV1-expressing nociceptors due to the Ca^{2+} overload that may induce the ligand [64]. Thus, high-affinity agonists that promote receptor tachyphylaxia and/or nociceptor ablation could be used as efficacious pain relievers.

The identification of several natural products that act as TRPV1 ligands prompted the use of these molecules as pharmacological tools to study this receptor even before its molecular identity was known. In addition, these studies opened different medicinal chemistry programs based on structure-activity relationships (SAR) directed to improve the therapeutic profile of the initial hits.

Vanilloids are the best known kind of TRPV1 agonists. Capsaicin (**1**, Fig. **2**) is the representative example of this family. This molecule bears the 4-hydroxy-3-methoxybenzyl moiety characteristic for homovanillin derivatives, an amide linker and a lipophilic region. The homovanillyl motif and the amide linker contain polar groups capable of forming hydrogen bonds. The SAR studies performed on capsaicin analoges suggest that these polar moieties are essential for exciting sensory neurons. In contrast, the hydrophobic region, with an optimal chain length of 8-15 carbon atoms, would interact with a hydrophobic region in the receptor. Unlike other ligand-gated channels that produce fast synaptic transmission, vanilloids show a slow activation kinetics, in part because their binding site is located in the intracellular portion of the receptor (Fig. **1**) [31,40,59,62]. Indeed, a cytosolic region spanning the third transmembrane domain in TRPV1 appears to be essential for capsaicin binding, presumably through hydrophobic interactions with the vanilloid carbon chain [40,59]. The structural determinants involved in capsaicin binding are being unravelled and molecular models for the vanilloid site have been proposed which, upon refinement, may facilitate the design of agonists with higher therapeutic index [40].

There are other known classes of naturally occurring vanilloids, such as Resiniferatoxin (RTX, **2**), a compound isolated from *Euphorbia resinifera*, that exhibits a TRPV1 agonistic activity much more potently than capsaicin (IC_{50}= 10 pM). As shown in Fig. **2**, RTX is a rather complex molecule and the pharmacophoric groups have not been clearly defined yet. SAR studies suggest that the homovanillic moiety of RTX, the C3-keto group and the orthoester phenyl moiety in ring C are essential structural elements for eliciting its extremely high potency desensitising TRPV1 channel activity. As a consequence, RTX is being developed as a sensory neuron desensitising agent for the treatment of urinary urge incontinence and pain associated with diabetic neuropathy [105], two human pathologies mediated by TRPV1 dysfunction. In spite of these results, the limited availability of RTX from natural sources and its difficult chemical synthesis hamper its therapeutic use.

The efficacy of both capsaicin and RTX for treating overactive bladder and neuropathic pain has stimulated the efforts for identifying orally active TRPV1 agonists. A major shortcoming for the therapeutic use of capsaicin and related

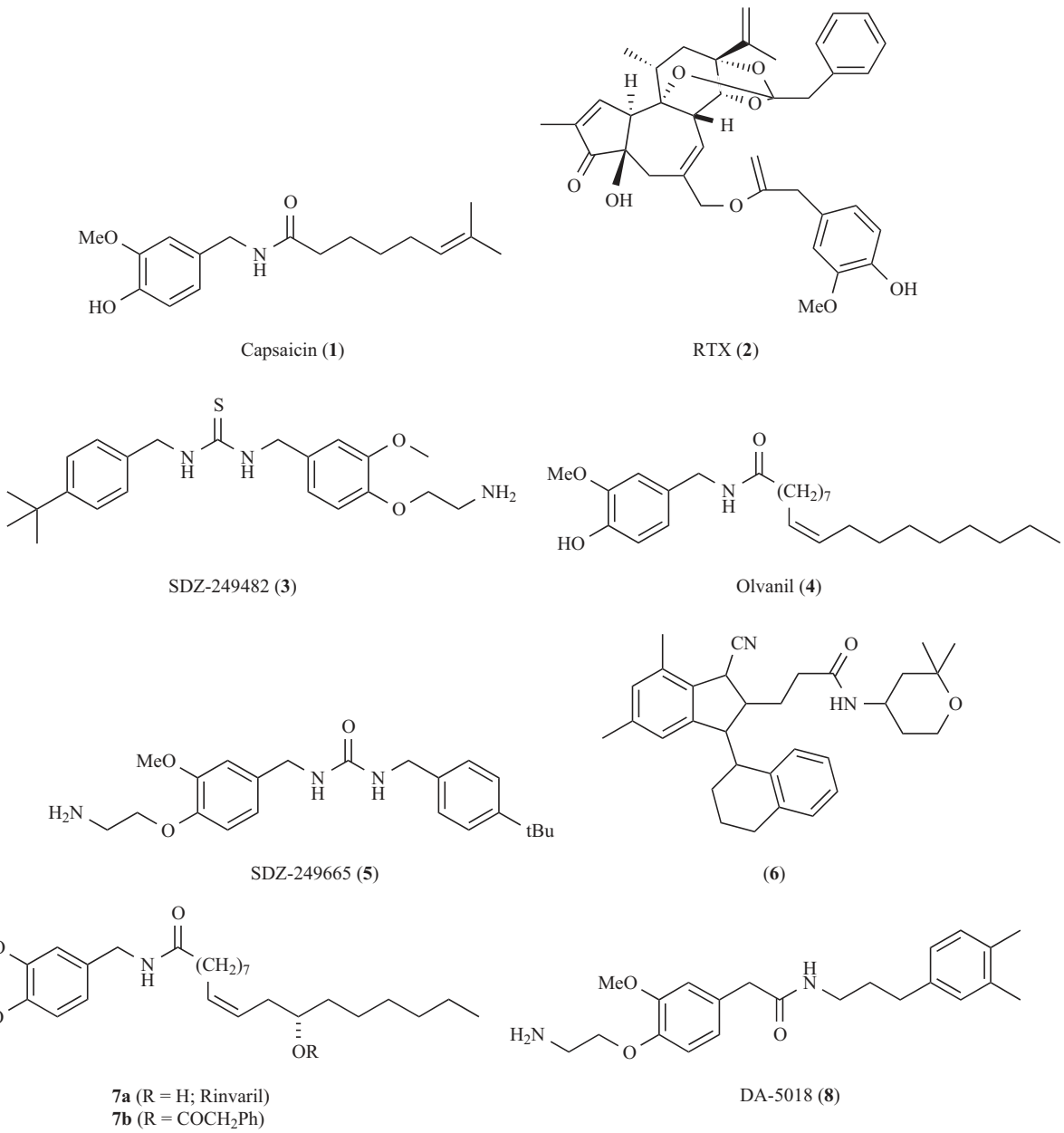

Fig. (2). Structures of a selection of natural and synthetic TRPV1 agonists.

vanilloids is the burning sensation and irritation that they provoke. In addition, the phorboid group of RTX has raised concerns of tumorigenicity. The development of more potent, orally active vanilloid derivatives (See Fig. **2**) such as SDZ-249482 (**3**, Fig. **2**), olvanil (**4**, Procter and Gamble), SDZ-249665 (**5**, Novartis) or the complex capsaicin analogue reported by Takeda (**6**) did not fully circumvent the discomfort of the side effects derived from irritation. Recently, homoallylic hydroxylation and further acylation of the fatty acyl chain of olvanil has led to the identification of phenylacetylrinvanil (**7b**, Fig. **2**), an ultra-potent capsaicinoid (EC_{50}=11 pM) that reduces bladder detrusor overactivity *in vivo* in a rat model of urinary incontinence with a potency similar to resiniferatoxin [3].

In parallel to the identification of vanilloid-like agonists, the development of new formulations for capsaicin such as dermal patches (NGX-4010 from NeurogesX) or localised injections (ALGRX-4975 by AlgoRx) are currently evaluated as analgesic strategies. The vanilloid analog DA-5018 (**8**, Dong A Pharmaceuticals) is also under development as a topical analgesic. Thus far, clinical data gathered indicate that the use of TRPV1 agonists as analgesics has clear cut advantages such as long lasting effects, broad mechanism of action, and high potency for the case of RTX. However, these molecules exhibit side effects such as pungency and pain that limit their clinical application. Therefore, the identification of non-pungent vanilloid-like drugs that induce a long-lasting desensitisation of the receptor will be of great clinical value.

There is good evidence on the occurrence of endogenous vanilloid receptor agonists, coined with the term endo-vanilloids, and that these agents modulate the sensitivity of TRPV1 channels to thermal stimuli [109]. For instance, it has been described that a polyunsaturated compound derived from arachidonic acid, anandamide (**9**, Fig. **3**) activates both native and recombinant TRPV1 receptors [122]. The structure of the molecule does not give evidence that it is a vanilloid analog. This fact caused some confusion about the term "vanilloid" depending on whether it is used chemically or pharmacologically. Moreover, anandamide is also considered as an endocannabinoid, since it activates cannabinoid (CB) receptors CB1 and CB2 at concentrations lower than those required to gate TRPV1 [122]. Similarly, several eicosanoids, particularly those derived from the enzymatic action of 5-lipoxygenase or 12-lipoxygenase, are capable of activating rat TRPV1. In particular, 12-HPETE (**10**, Fig. **3**) and leukotriene B_4 (**11**, LTB_4, Fig. **3**) have exhibited the most potent agonistic activity [50]. It is believed that eicosanoids function as intracellular vanilloids of TRPV1 receptors in the cells where they are synthesised. Recently, NADA (**12**, Fig. **3**) has been identified as a brain endovanilloid [52]. NADA is an endogenous anandamide analog several times more potent than anandamide on TRPV1, although it still activates CB1 receptors.

Another related approach to modulate TRPV1 signalling is the identification of the so-called hybrid cannabinoids/ TRPV1 ligands. Since TRPV1 colocalises with CB1 receptors in brain, and both proteins have overlapping ligand recognition properties, it is possible to conceive compounds capable of activating both receptor types simultaneously even by different mechanisms. Arvanil (*N*-[3'-methoxy-4'-hydroxybenzyl]arachidonamide, **13**, Fig. **3**) is one of these agents. Arvanil has affinity for CB1 receptors and activates TRPV1 receptors more potently than capsaicin or anandamide [75]. This compound is an antiproliferative agent for human breast cancer cells sensitive to both CB1 and TRPV1 receptor antagonists, as well as a spinal analgesic and, a relaxant of mouse vas deferens. Similarly, arachidonyl ethanolamine, an endogenous cannabinoid that also acts on the TRPV1 channel, induced strong apoptosis of uterine cancer cells that aberrantly express the vanilloid receptor [24]. Accordingly, it is of great interest for the future development of improved hybrid CB1/TRPV1 agonists.

TRPV1 ANTAGONISTS

Intense efforts have been carried out to design TRPV1 competitive and non-competitive antagonists [91,106]. The competitive antagonists bind to the agonist binding site, and lock the channel in the closed, nonconductive state. In contrast, non-competitive antagonists interact with additional binding sites on the receptor structure preventing receptor opening by the agonist or blocking its aqueous pore [91]. Non-competitive antagonists acting as open channel blockers are therapeutically attractive because they preferentially recognise the population of over-activated TRPV1 channels with a marginal recognition of physiologically working receptors. Thus, this kind of drugs target pathologically-activated receptors, which can reduce the potential unwanted side effects.

TRPV1 COMPETITIVE ANTAGONISTS

The first competitive TRPV1 antagonist, capsazepine (**14**, Fig. **4**), was identified by Sandoz (now Novartis) and

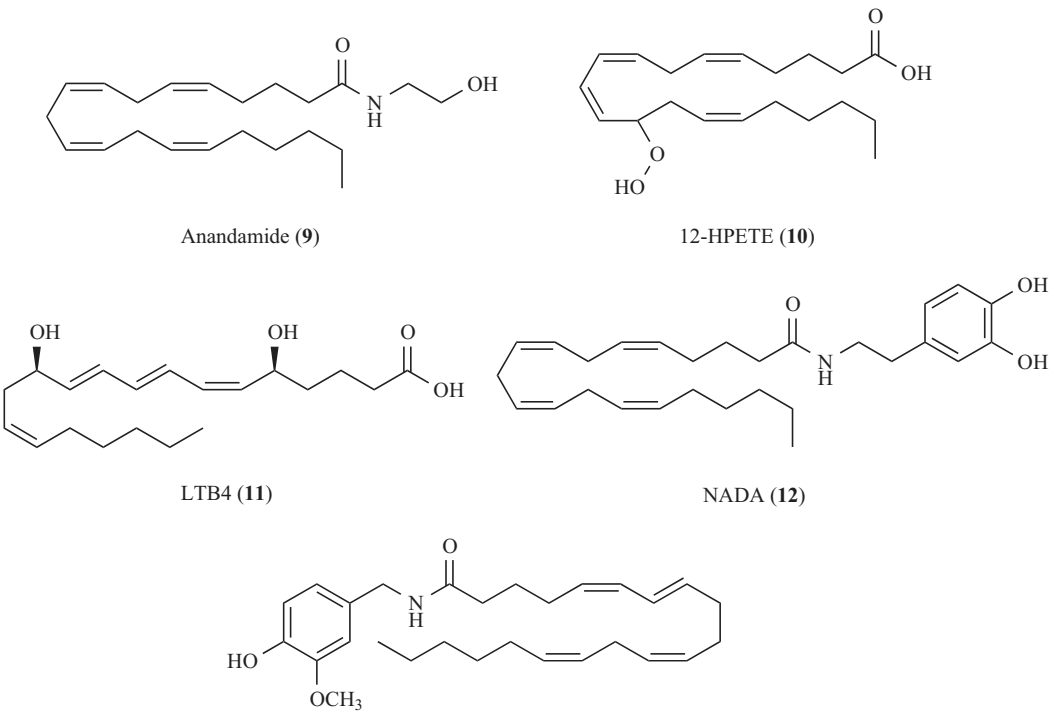

Fig. (3). Structures of TRPV1 endogenous ligands and hybrid canabinoid-TRPV1 modulators.

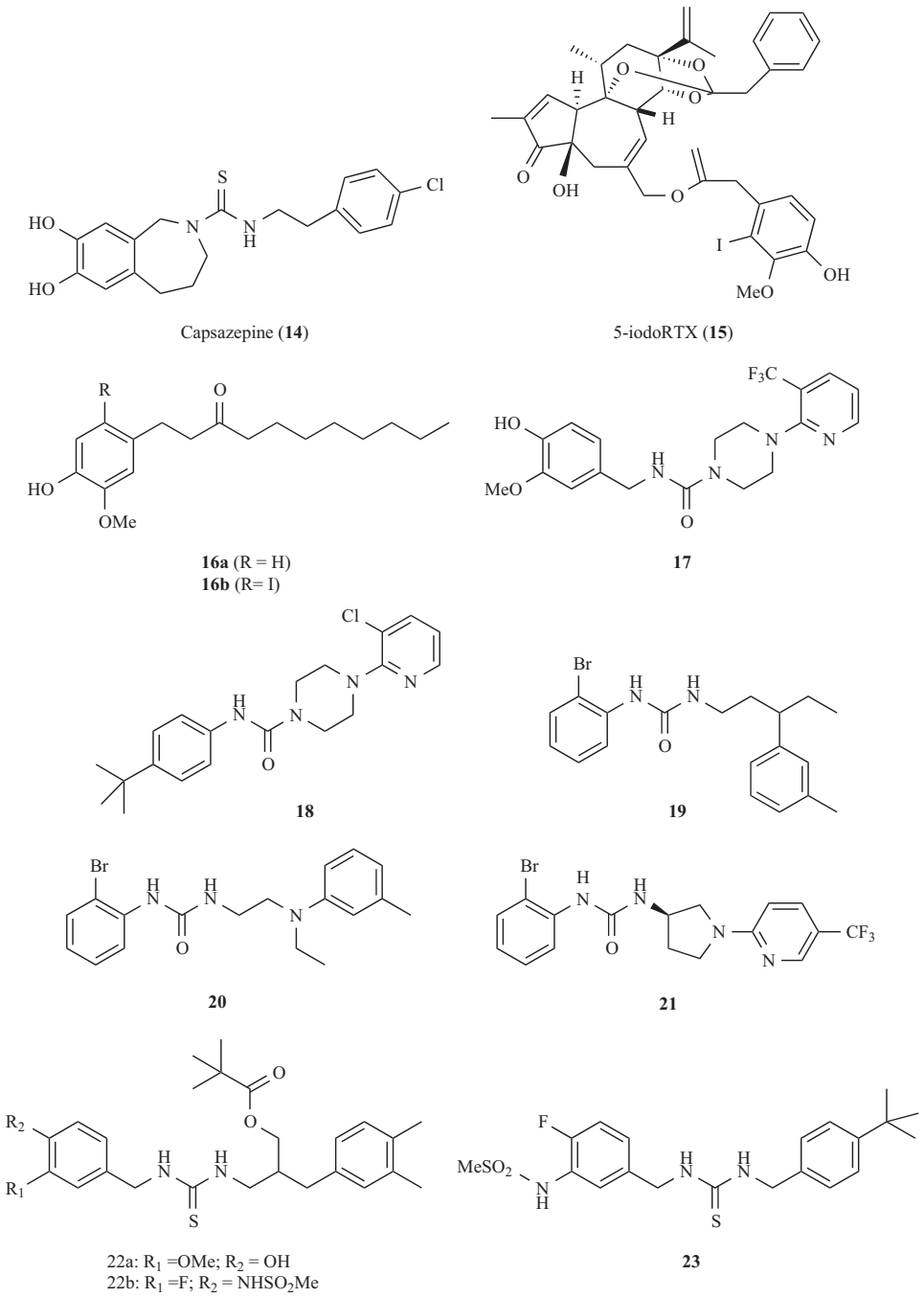

Fig. (4). A selection of TRPV1 competitive antagonists.

was used as a valuable pharmacological tool to understand the capsaicin-induced effects on nociceptors *in vivo* before the TRPV1 channel was cloned. This compound can be considered as a conformationally-restricted capsaicin analog bearing a thiourea moiety instead of the urea present in capsaicin. The propylidene linker of the seven-membered ring forces the dihydroxylated aromatic moiety to adopt an orthogonal orientation with respect to the thiourea bond. This steric constraint was considered essential for eliciting the receptor antagonistic activity, although development of acyclic vanilloid-based derivatives lacking this spatial disposition and eliciting higher inhibitory potency than capsazepine questioned the relevance of this SAR statement. Pharmacological studies showed that capsazepine exhibited low metabolic stability and poor pharmacokinetic properties in rodents, thus preventing its clinical development. Furthermore, pre-clinical studies on animal models of pain have provided conflicting results. For instance, the compound shows poor anti-inflammatory and analgesic activity in rats, although it remarkably attenuates thermal and mechanical hyperalgesia in guinea pigs [113].

The discovery that the introduction of an iodine atom on the vanillyl moiety of RTX agonist modulates its pharmacological activity depending on the position of the halogen ended up with the identification of 5-iodoRTX (**15**, Fig. **4**) as a potent antagonist of TRPV1 (IC_{50} = 3.9 nM) [73,112]. This compound produced analgesic activity *in vivo* and it is currently in clinical studies. Similar to RTX, a major drawback of compound **15** is its complex chemical structure and, the cost of its synthesis from RTX itself. In addition, 5-iodoRTX exhibits low oral bioavailability probably due to its poor pharmacokinetic properties. Nevertheless, the discovery of compound **15** stimulated the SAR studies on the halogenation of the vanillyl moiety present in simple capsaicin derivatives. For instance, taking nonivamide as a model (**16a**, Fig. **4**), the substitution at C-6 afforded more potent inhibitors than at C-5 and the inhibitory potency correlated with the size of the halogen (I > Br > Cl) [2]. The most potent derivative identified was that bearing the iodine atom at C-6 (**16b**). Similarly, iodination of phenylacetylrinvanil (**7b**) produced a potent TRPV1 antagonist (IC_{50} = 0.8 nM) [3]. These results showed that halogenation could be an appropriate strategy for reversing the activity of vanilloids. This observation, together with the aim of synthesising simpler derivatives of RTX that preserve the inhibitory potency of the parent compound, would constitute an attractive field for developing orally active, competitive TRPV1 antagonists with improved pharmaceutical profiles for clinical use.

A related family of capsaicin analogues was developed by Neurogen through the formal amidation of the homovanillylamino moiety with different 4-(α-pyridyl)piperidine-1-acyl residues. The urea **17** (Fig. **4**) is a representative example of this family [6]. The presence of an amino moiety at the hydrophobic region of the capsacinoid appeared to be crucial for obtaining a potent inhibitor that was, at the same time, a highly improved drug lead in terms of hydrophilicity. This observation was also important to recognise that the C-region of capsaicin should be considered as a domain that plays a key role in the interaction with the receptor. Another piperazinyl urea (BCTC, **18**) was reported by Purdue-Pharma to be more selective than capsazepine against a wide variety of enzymes and channels. This compound exhibited *in vivo* oral activity in animal models of inflammatory and neuropathic pain [92]. Less conformationally-restricted analogs of BCTC such as compounds **19** and **20,** along with the recently reported pyrrolidine urea **21** (Fig. **4**), also elicit a good antagonistic TRPV1 potency maintaining the receptor selectivity. The ongoing pre-clinical characterization of these compounds will uncover whether they have a higher therapeutic index than BCTC [94]. It is worth noting that most of these molecules were identified from the screening of chemical combinatorial libraries, thus showing the usefulness of this strategy in drug discovery programs.

Structurally connected to capsaicin related compounds, thiourea **22a** (Fig. **4**) elicited a potent agonist activity (Ki < 10 nM). Interestingly, the presence of fluorine at C-3' and the methanesulfonamido moiety at C-4', replacing the methoxy and hydroxy groups, respectively, afforded compound **22b**, a molecule exhibiting an antagonistic TRPV1 activity [68]. Further modification of these derivatives, now at the lipophilic region, led AmorePacific to obtain optimized compound **23**, bearing a *ter*t-butyl group at the aromatic ring [114].

The continuous efforts to improve the pharmacological properties of TRPV1 antagonists have resulted in the development of novel templates (See Fig. **5**). In this regard, several non-symmetric ureas have been reported by Janssen [25], Abbott [69] and Merck [16], where the 5-aminoisoquinoline moiety appears as a common feature (cf. **24**-**26**). Alternatively, Bayer has reported the 4-aminoindole **27** [119] and the hydroxylated tetrahydronaphthalene **28** [119] urea derivatives. All these compounds show an inhibitory potency ≤ 10 nM. Taken together, these results emphasise the relevance for antagonistic activity of a functionalised lipophilic moiety, preferentially with a basic group, for interacting with the capsaicin binding site on TRPV1.

The replacement of the urea moiety by other isosteric groups has also been explored. Euroceltique reported

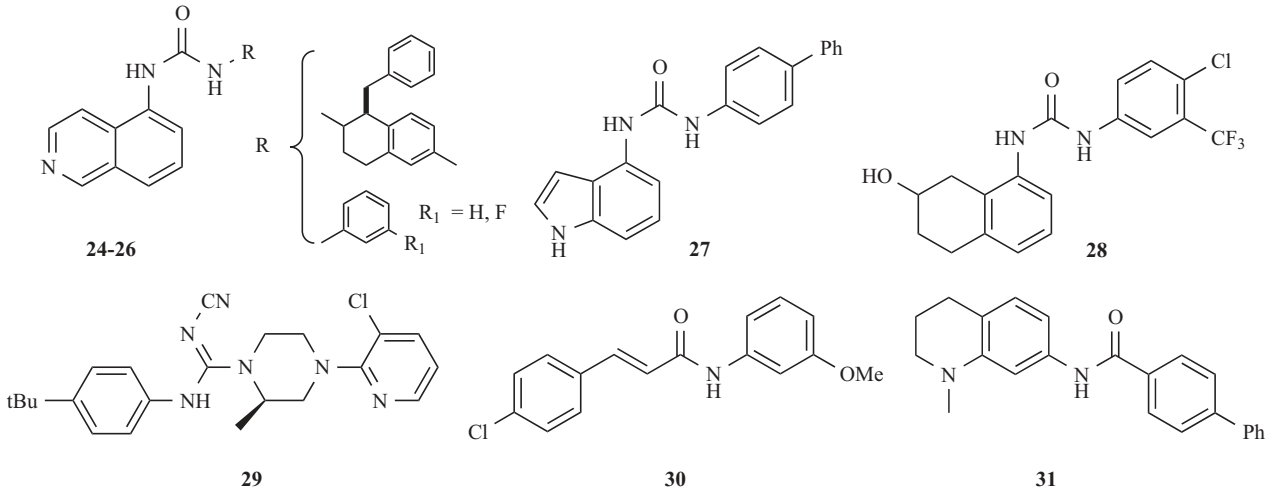

Fig. (5). TRPV1 antagonists based on novel templates.

compound **29** (Fig. **5**), based on the piperidine templates reported earlier (cf. **17** and **18**), where the cyanoguanidine group substituted the urea [67]. A further alternative to the ureas are the amides explored by GlaxoSmithKline. Compound SB-366791 (**29**) exhibits a potent competitive antagonistic activity ($IC_{50} \leq 5$ nM) in both the human and rat TRPV1 receptors and it shows a better selectivity profile than capsazepine [45]. Further studies led to the identification of compound **30**, which also exhibits a good *in vitro* inhibitory activity. In addition, this molecule is readily amenable for structural optimisation, as shown by the synthesis of the tetrahydroquinoline **31.**

A further approach for the development of TRPV1 efficient antagonists is characterised by those derivatives lacking the urea, thiourea or amide groups (see Fig. **6**). Compounds **32**-**37** are examples of this approach that have been reported from different companies. The pyridine derivative **32** [28], the quinazolin-4-one **33** [29] from Novartis and the quinazoline-4-ylamine **34** [7] from Neurogen were the first compounds disclosed. A second derivative from Neurogen **35** [8] containing a residue of propionic acid (or phosphate or phosphonate) attached to the quinazoline moiety followed. In addition, two more derivatives, compounds **36** [14] and **37** [9], from Amgen containing heterocyclic moieties at the central regions have been also reported. More recently, the group of Abbott has described a collection of urea derivatives from which an analogue of compound **24** displaying a trifluoromethyl substituent at the *meta* position of the phenyl residue exhibited an IC_{50} = 4 nM, a 46% of oral bioavailability and, *in vivo* analgesic activity in animal models of visceral and inflammatory pain [43]. Overall, the strength of the heterocyclic pharmacophores studied was 5-isoquinoline > 8-quinoline = 8-quinazoline > 8-isoquinoline ≥ cinnoline ≈ phtalazine ≈ quinoxaline ≈ 5-quinoline. Lastly, a group from Amgen has published a study on different analogs based on its acrylamide antagonist **38**. From the *N*-aryl cinnamides synthesised, optimised compounds **39** and **40** exhibited high antagonist potency and good oral bioavailability in rats, as well as a favourable pharmacokinetic profile [33]. Although much of these compounds display *in vivo* anti-inflammatory and analgesic activity in animal models, further studies are required to assess the efficacy in the clinic. These exciting results are eagerly awaited.

Collectively, all these findings illustrate that development of potent competitive vanilloid antagonists of TRPV1 activity is a hot field of current neuropharmacology. Analysis of the structures shows the use of scaffolds that preserve little resemblance to the original vanilloid group or even to the capsacinoid family. On the other hand, even in the absence of detailed knowledge of the topology of the TRPV1 receptor, the wide variety of active structures reported thus far provide a significant database for initiating molecular modelling and SAR approaches aimed at the

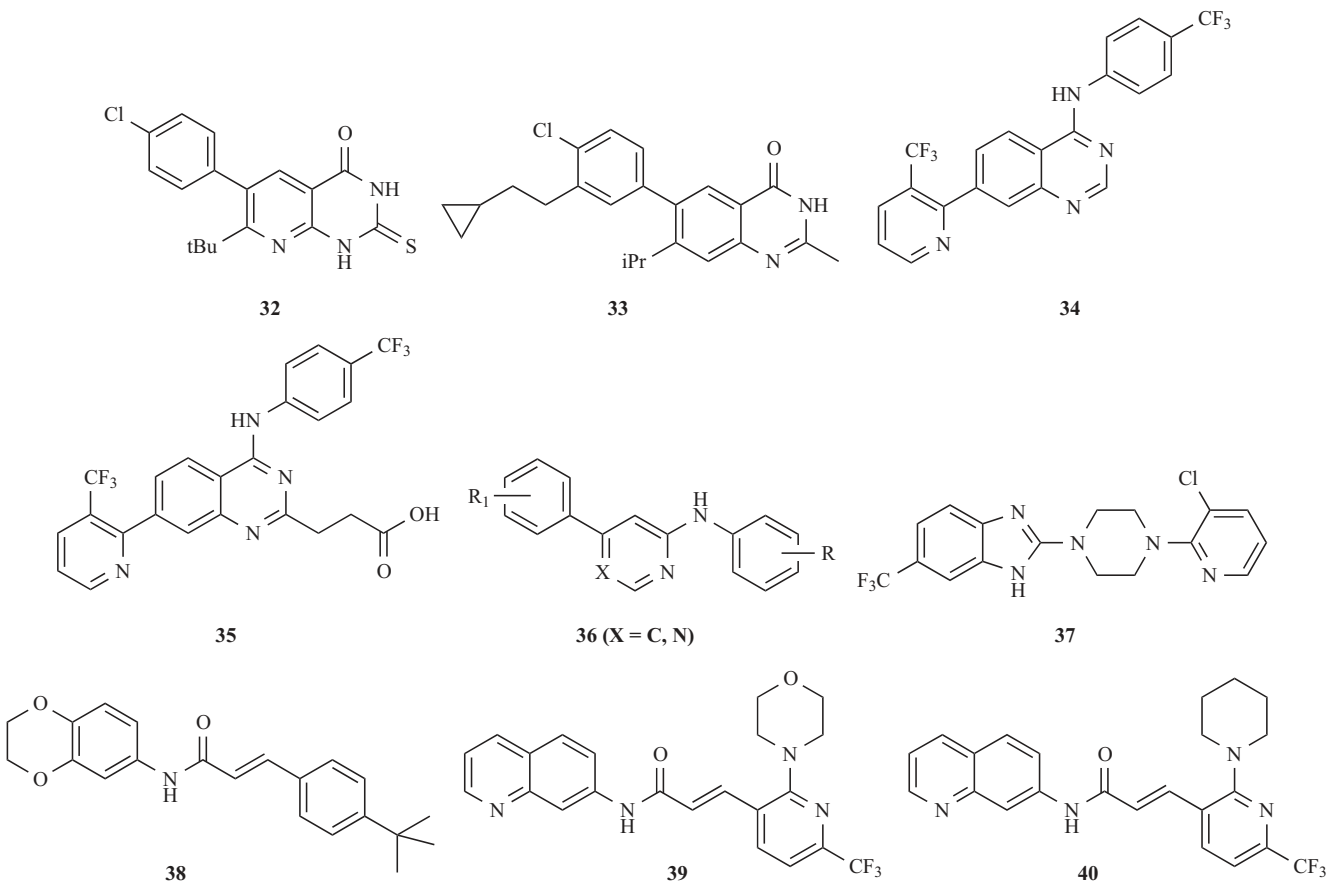

Fig. (6). Novel TRPV1 antagonists lacking the urea or thiourea groups (**31**-**36**), or based on the *N*-arylcinnamide moiety (**37**-**39**).

identification of the pharmacophoric features required for orally active, potent antagonistic activity. These studies are also necessary to clarify whether the above structural variety is due to the interaction with sames of different drug binding sites on the receptor.

TRPV1 NON-COMPETITIVE ANTAGONISTS

The first non-competitive TRPV1 antagonist, and the only molecule available for several years as vanilloid receptor blocker was the trinuclear polyamine complex, Ruthenium red (**41**, Fig. **7**). This compound binds to the pore region of the channel with high potency (IC_{50}, 100 nM) and weak voltage dependency by a mechanism that involves interaction with negatively-charged residues present at the outer vestibule of the ionic pore [37]. The poor receptor selectivity of this compound seems to underlie its proconvulsive activity in animal models that preclude the clinical development. Arginine-rich hexapeptides such as RRRRWW-NH_2 block recombinant TRPV1 channels expressed in *Xenopus* oocytes in a non-competitive manner with submicromolar potency (**42**, Fig. **7**) [90]. However, it was later suggested that these derivatives might partially act as competitive antagonists [48]. Nonetheless, similar to ruthenium red, their lack of receptor selectivity resulted in severe side effects and toxicity, thus preventing further their development as analgesics.

A parallel effort was carried out to evaluate poly-methylene tetraamines as TRPV1 channel blockers [76]. These studies identified methoctramine (**43**) as a non-competitive capsaicin antagonist with an IC_{50} of 2 μM, and notable voltage-dependent blockade (Fig. **7**). This molecule

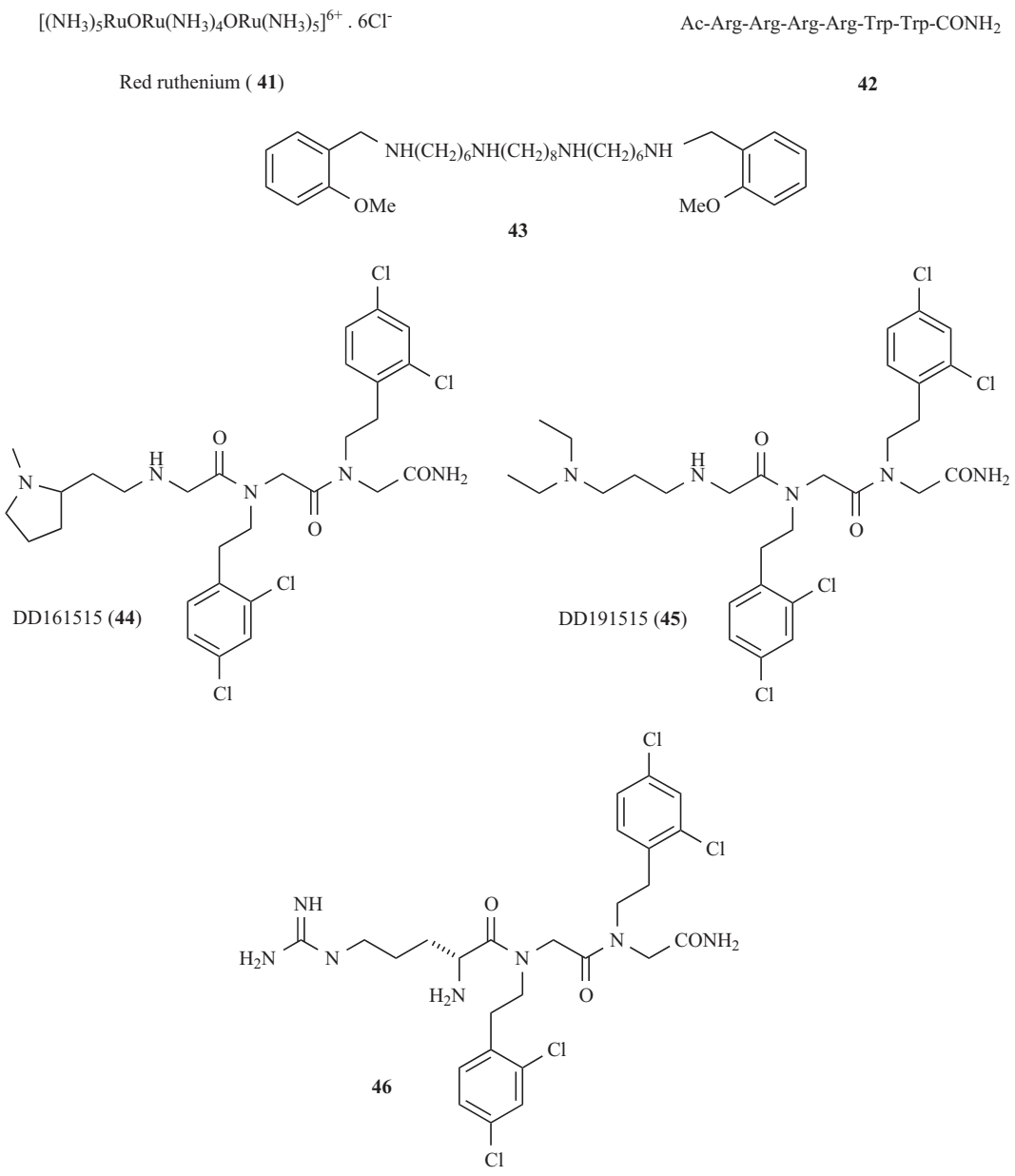

Fig. (7). TRPV1 non-competitive antagonists.

antagonised native TRPV1 receptors in dorsal root ganglion neurons activated by either capsaicin or protons. Therefore, the inhibitory activity of methoctramine analogs has provided a new pharmacophoric scaffold for non-competitive antagonists of TRPV1. Thus far, the lack of receptor selectivity has restrained the use of these compounds *in vivo*.

The first small organic molecules identified as non-competitive TRPV1 emerged from the screening of a peptidomimetic combinatorial library. *N*-Alkylglycines (also known as peptoids), constitute a class of compounds of synthetic origin that exhibit interesting biological properties derived from their structural features, *i.e*, the absence of CO-NH bonds and then of the hydrogen bond interactions that they induce, an improved stability against proteases and a high degree of conformational freedom. In addition, the structural simplicity of peptoids makes these peptidomimetic compounds amenable to structural manipulation, thus facilitating the optimisation of identified hits for improving their drug-like properties [47]. This is important if it is considered that the high conformational mobility of these molecules can generate selectivity problems due to undesired interactions with non-target receptors.

The screening of the library of trimers of *N*-alkylglycines led to the identification of two compounds referred to as DD161515 (**44**) and DD191515 (**45**) that preferentially block TRPV1 channel activity with micromolar potency and moderate voltage-dependency (Fig **7**) [38]. These peptoids appear to recognise a receptor site different from that of capsaicin. Intraperitoneal administration of both trialkyl-glycines into mice significantly attenuated pain and neurogenic inflammation induced by injection of capsaicin into the hindpaws and, reduced thermal hyperlalgesia due to mustard oil-evoked tissue irritation. In contrast, these peptoids did not affect capsaicin triggered-mechanical hyper-sensitivity [38]. These findings suggested that TRPV1 active trialkylglycines may be developed into analgesics to treat inflammatory pain. However, the *in vivo* doses required for analgesic and anti-inflammatory activity were too high (≥25 mg/kg), thus preventing their development into useful drugs. Therefore, the design of more potent, non-competitive antagonists of TRPV1 is a yet unmet goal of drug discovery platforms. Recent progress in this arena, along with molecular modelling of the TRPV1 pore domain [35], is providing this kind of compounds. For instance, by combining the chemical features of arginine-rich peptides and peptoid DD161515, a potent non-competitive antagonist (H-Arg-15-15C, **46**) of TRPV1 was discovered (Fig. **7**) [34]. Compound H-Arg-15-15C blocked capsaicin-induced TRPV1 activity with 10-fold higher potency than the original peptoid DD161515, without compromising the receptor selectivity. Noteworthy, at variance with the parental peptoids, compound H-Arg-15-15C potently abrogated pH-activated TRPV1 responses. As for DD161515, the blockade mechanism was non-competitive acting as a channel blocker. Thus, introduction of a strong positive charge in the pharmacophore provided by peptoid DD161515 results in a significant increase in the binding efficacy of the molecule to the receptor. Noteworthy, compound **46** notably attenuated the inflammatory discomfort exerted by intraplantar injection of capsaicin. The i.p. dose required to produce analgesia was 10-fold lower than that of peptoid DD161515. In addition, the compound showed activity on the Phase II of the formalin model, although at doses relatively high, suggesting a modest activity of the antagonist in neuropathic pain models [96]. Indeed, H-Arg-1515C did not mitigate mechanical allodynia in the partially ligated sciatic nerve model of neuropathic pain. In contrast, **46** completely reversed the complete Freund Adjuvant (CFA)-induced thermal hyperalgesia at therapeutic doses (5 mg/kg, i.p.). Collectively, these findings show that compound H-Arg-15-15C is a potent non-competitive antagonist of the receptor that exhibits notable anti-inflammatory and analgesic activity in pre-clinical models of acute and chronic pain. However, compound **46** displays limited oral and i.p. bioavailability *in vivo*, presumably because of its lack of compliance with the Lipinski rule of 5 [70]. Thus, chemical modification on the H-Arg-15-15C structure will be necessary to increase its therapeutic potential. All these results demonstrate that TRPV1 antagonists that act on a site different from the vanilloid binding site are also promising candidates for analgesic/anti-inflammatory drug development.

THE TRPV1 RECEPTOR COMPLEX AS A THERAPEUTIC TARGET

Similar to other TRP channels, TRPV1 is arranged in major molecular complexes establishing high order signalling networks that notably determine the response to external stimuli. These protein networks are in turn the target of intracellular signaling pathways that modulate their composition, structure and function. Thus, an emerging notion is that ion channels are not isolated entities in cell membranes but pivotal components of these macromolecular assemblies. These complexes are composed of a plethora of diverse polypeptides including scaffolding proteins, receptors, enzymes and cytoskeletal proteins. The molecular components of the TRPV1 complex identified thus far include the high affinity neurotrophic receptor (TrkA), phospholipase C (PLC), and calmodulin [85,95]. The composition of these assemblies appears important for the regulation of the receptor activity. Activation of trkA and PLC, along with translocation of PKC, decrease the tonic inhibition that phosphatidylinositol bisphosphate exerts on TRPV1, thus promoting channel activity [23]. Similarly, activation of PKA and CAMKII gives rise to a decrease in Ca^{2+}-induced receptor desensitisation and tachyphylaxia. Cumulative evidence indicates that modulation of receptor complexes underlie the TRPV1 potentiation by inflammatory mediators.

A yeast-two hybrid screen of a rat brain library using the N-terminus of TRPV1 as a bait identified two synaptic vesicle proteins, Snapin and Synaptotagmin IX, as interacting partners of TRPV1 [83]. Noteworthy, these proteins bind to SNARE proteins and participate in neuronal exocytosis [36,55], suggesting that surface delivery of TRPV1 channels is a highly regulated, Ca^{2+}-dependent exocytotic process. Indeed, the interaction of both vesicular proteins with TRPV1 appears temporal and seems not involved in the formation of the molecular complexes at the cell surface. However, these interactions are pivotal for the trafficking and surface expression of TRPV1 channels. In support of

this tenet, PKC-induced TRPV1 trafficking to the plasma membrane was blocked by botulinum neurotoxins, thus indicating that PKC- sensitisation of TRPV1 receptors is due at least in part to the regulated exocytosis of channels located in a reserve pool of cytosolic vesicles [83]. A recent observation that botulinum neurotoxin attenuates heat hyperalgesia substantiates this hypothesis [27], although additional *in vivo* experiments are needed to determine the precise role of receptor exocytosis to the onset and maintenance of neurogenic inflammation. Therefore, regulation of TRPV1 surface density in nociceptor peripheral terminals appears to be an important mechanism for both the development and preservation of inflammatory hyperalgesia. CFA-induced inflammation triggers an increased translation and trafficking of TRPV1 channels to the peripheral terminals [57]. These findings imply that modulation of TRPV1 trafficking may be a therapeutic approach for pain management. Accordingly, botulinomimetic compounds such as small peptides that inhibit SNARE-dependent exocytosis could be developed as anti-inflammatory and analgesic compounds [13].

CONCLUSION

The recognition of the important role the TRPV1 in the etiology of neurogenic inflammation and pain transduction has thrust the development of down regulators of its channel activity as analgesic drugs. Several of these compounds are initiating clinical studies in humans and their results are awaited. However, a note of caution must be sound because of the pivotal and diverse physiological functions played by the TRPV1 channel. Its widespread distribution in different tissues should be taken into account since undiscriminate pharmacological block of the receptor may lead to severe side effects that may complicate the use of antagonists of TRPV1 activity. Thus, the challenge of molecular neuropharmacology would be to develop receptor-selective drugs that preserve the physiological activity of TRPV1 while down regulating the function of overactive receptors. This kind of compound would not compromise the normal sensory signaling that is essential for survival.

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ABBREVIATIONS

TRPV1 = transient receptor potential vanilloid subunit 1

PKA = protein kinase A

PKC = protein kinase C

NGF = nerve growth factor

RTX = resiniferatoxin

SP = substance P

CGRP = calcitonin gene related peptide

NADA = N-arachidonyl-dopamine

SAR = structure-activity relationships

CFA = complete Freund adjuvant

CAMK = calmodulin kinase

PLC = phospholipase C

GI = gastrointestinal

TrkA = high affinity neurotrophic receptor.

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