EXPERT OPINION

Advances in modulating thermosensory TRP channels

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Introduction: Thermosensory channels are a subfamily of the transient receptor potential (TRP) channel family that are activated by changes in the environmental temperature. These channels, known as thermoTRPs, cover the entire spectrum of temperatures, from noxious cold (< 15°C) to injurious heat (> 42°C). In addition, dysfunction of these channels contributes to the thermal hypersensitivity that accompanies painful conditions. Moreover, because of their wide tissue and cellular distribution, thermoTRPs are also involved in the pathophysiology of several diseases, from inflammation to cancer.

Areas covered: Although the number of thermoTRPs is increasing with the identification of novel members such as TRPM3, we will cover the recent advances in the pharmacology of the classical thermosensory channels, namely TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1. This review will focus on the therapeutic progress carried out for all these channels and will highlight the tenet that TRPV1, TRPM8 and TRPA1 are the most exploited channels, and that the interest on TRPV3 and TRPV4 is growing with the first TRPV3 antagonist that moves into Phase-II clinical trials. In contrast, the pharmacology of TRPV2 is yet in its infancy.

Expert opinion: Despite the tremendous academic and industrial investment to develop therapeutic modulators of thermoTRPs, it apparently seems that we are still far from the first successful product, although hope is maintained high for all compounds currently in clinical trials. A major concern has been the appearance of side effects. A better knowledge of the thermosensory protein networks (signal-plexes), along with the application of system biology approaches may provide novel strategies to modulate thermoTRPs activity with improved therapeutic index. A case in point is TRPV1, where acting on interacting proteins is providing new therapeutic opportunities.

Keywords: cancer, inflammation, ion channel, nociception, pain, sensory

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1. Introduction

Thermal sensing is achieved thanks to the existence of specialized ion channels that are capable of transducing environmental temperature changes into neuronal action potentials that are conveyed to the brain. The discovery of the superfamily of TRP channels and, in particular, of TRPV1 shed light on the molecular and cellular mechanisms in temperature sensing. TRP channels respond to a variety of sensory cues and are considered as true cellular sensors. Indeed, these channels respond to several sensory stimuli such as temperature, pain, osmolarity, touch, pheromones, taste, and others [1]. This family of ion channels amounts to 28 members grouped in 8 subfamilies, namely TRPC, TRPV, TRPM, TRPN, TRPA, TRPML, TRPP, and the recently identified TRPY in yeast [1]. Members of the TRPV (V1-V4),

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Article highlights.

- ThermoTRP channels are becoming pivotal therapeutic targets for the treatment of several human pathologies.
- The initial clinical development was hampered by the unexpected side effects.
- Novel scaffolds along with new pharmacological approaches are providing new opportunities for a successful clinical candidate.
- With several clinical trials ongoing, the hopes for thermoTRP-based drug are high.

This box summarizes key points contained in the article.

TRPM (M2, M3, M5 and M8) and TRPA (A1) that are gated by temperature changes, extending from noxious cold (< 15°C) to injurious heat (> 42°C), are known as thermoTRPs. Furthermore, these channels may respond to chemical compounds, voltage, and some of them are activated by mechanical stimuli [1-3]. Notably, several thermoTRPs have been implicated in abnormal temperature and mechanical sensing under pathological conditions such as inflammation, where mild noxious or even nonnoxious physical stimuli may be sensed as extremely unpleasant and painful [2,3], two pathological phenomena referred to as hyperalgesia and allodynia, respectively.

ThermoTRP are cation selective ion channels that permeate Na^+, K^+, and some of them display a notable Ca^{2+} permeability. These channels act as integrators of several environmental cues and signalling pathways, including those mediated by cell surface expressed receptors. At the molecular level, these ion channels are homotetrameric integral membrane proteins [1], although the formation of heterotetramers has also been proposed [1-3]. The basic channel subunit displays a modular organization having N- and C-terminal cytosolic domains and a six-transmembrane region that contains a large extracellular domain between the fifth and sixth transmembrane segments (**Figure 1**). The cytosolic N-terminus of the thermoTRPs may contain ankyrin repeats or TRPM homology regions, and phosphorylation consensus sites for serine/threonine and tyrosine kinases [1]. Ankyrin repeats are known for their role in mediating protein–protein interactions [1-4]. The C-terminus of thermoTRPs is also a modular domain that contains a multimerization motif (known as the TRP domain), calmodulin and tubulin binding regions, and consensus sequences for protein kinases [4]. Furthermore, it may have molecular determinants of the channel temperature sensor [4]. The TRP domain appears to be critical for functional coupling stimuli sensing to pore opening [4].

ThermoTRP dysfunction has been implicated in several pathologies, including diverse kinds of pain, inflammation, pulmonary disorders, gastrointestinal hypersensitivity, bladder dysfunction, cardiac hypertrophy, skin sensitivity, ischaemic cell-death, and neurodegeneration [2,3]. These pathological processes either display an increase of channel activity or an augmented channel expression or both [4]. Furthermore, gene association studies have reported that some of the thermoTRP members display gain-of-function mutations in human inheritated diseases [3]. Taken together, it is widely accepted that thermoTRPs are pivotal drug targets, and the development of therapeutic compounds for pharmacological intervention is actively pursued by the academy and the industry.

Here, we review the current status of this exciting topic, focusing on recent advances for the TRPV1-V4, TRPA1, and TRPM8. We particularly report recent advances in the identification of compounds that inhibit TRPA1 and TRPM8, as well as the progress of TRPV3 antagonists into clinical trials. In contrast, inhibition of TRPV2 and TRPV4 is still in its infancy. Because excellent reviews have been recently published for TRPV1 [2-7], we will only highlight new approaches for this channel that may be expanded to the other thermoTRPs.

2. TRPV1

TRPV1, also known as the capsaicin receptor, was cloned from rat dorsal root ganglion (DRG) neurons using an expression-cloning screening strategy [1]. To date, TRPV1 orthologs have been identified in eukaryotes including human, rat, guinea pig, rabbit, mouse, dog, and porcine tissues, but not in prokaryotes [1]. In mammals, TRPV1 displays a wide tissue and cellular expression, including the peripheral and central nervous systems, the gastrointestinal tract, the uroepithelium of the urinary bladder, the inner ear, the dermis and epidermis, and the human hair follicle [2-7].

The channel is activated by vanilloid compounds such as capsaicin and resiniferatoxin (RTX), voltage, noxious temperatures ≥ 42°C, and low pH (< 5.9). Other compounds such as anandamide, eicosanoids or ethanol have also been proven to activate the receptor. TRPV1 undergoes desensitization in the continuous presence of an activating stimulus. This phenomenon can occur rapidly after a prolonged single application of an agonist, or slowly following repeated agonist applications (also known as tachyphylaxia). Receptor desensitization is Ca^{2+}-dependent and implies phosphorylation of the receptor and its endocytosis and degradation in lysosomes [8].

The ability of TRPV1 to respond to noxious stimuli and to be functionally sensitized by pro-inflammatory mediators has implicated it as a "pathological" receptor, having a significant role in the pain transduction pathway, and in the maintenance of inflammatory conditions in a variety of diseases and injury states. The validation of the 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. All this effort has resulted in a plethora of patent applications protecting a highly diverse family of receptor agonists and antagonists (reviewed by [3] and [7]), as well as different formulations of capsaicin [3]. However, and despite all this tremendous excitement, the number of new molecules, apart from novel capsaicin formulations, that have progressed into clinical trials has been disappointing (**Table 1**).

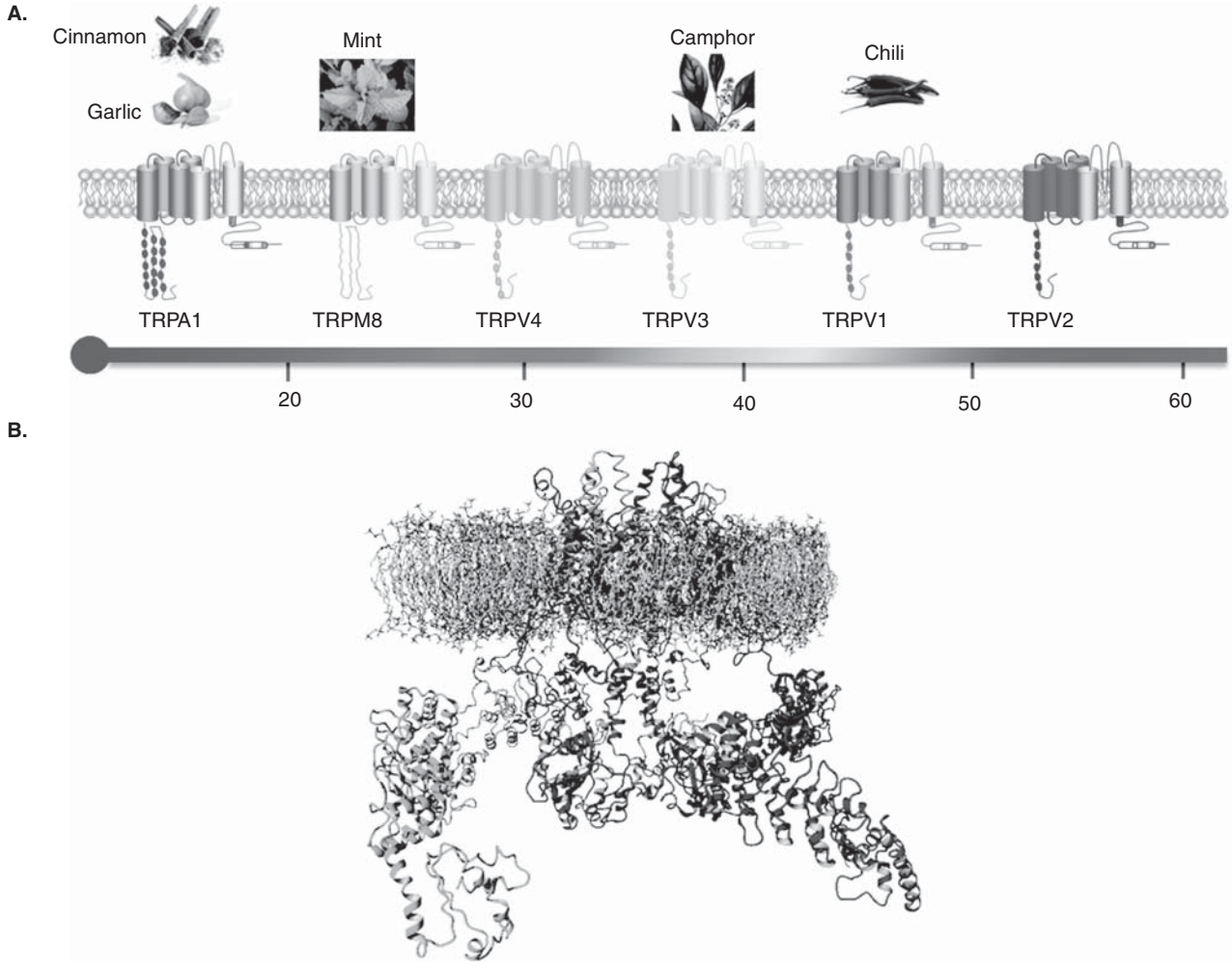


Figure 1. A. Schematic representation of the six mammalian thermoTRP channels. Each subunit consists of six transmembrane domains (S1-S6), a hydrophobic pore loop linking transmembrane segments five (S5) and six (S6), and large cytoplasmic N- and C- terminals. All thermoTRPs have a variable number of ankyrin repeat domains in the N-terminus (except TRPM8 which has none). ThermoTRPs display distinct thermal thresholds from very hot (TRPV2) to cold (TRPA1) and can be also activated by specific natural compounds and by synthetic substances, which are also known to induce the relevant thermal and pain sensations in humans. **B.** Side view of the ribbon structural model of a typical tetramer arrangement of TRP channels inserted into the lipid bilayer, after molecular dynamic simulation.

A major therapeutic drawback was the unexpected hyperthermia produced by most of the TRPV1 antagonists tested in clinical trials. Hyperthermia has been related with the potency of the compounds blocking the activation of TRPV1 by acidic pH [2,3,6], although compounds that abrogate this mode of activation and display modest hyperthermia in rodents have been reported [9], suggesting that the hyperthermic effect may be more complex than expected.

Recent progress on TRPV1 pharmacology includes three approaches aimed at developing inhibitors with alternative blockade mechanisms as classical competitive antagonists (**Figure 2**). Firstly, activity-dependent blockers such as uncompetitive antagonists that preferentially recognize the overactive receptors has led to the identification of a triazine-based compound **1** (triazine Aa) that blocks TRPV1 with nanomolar affinity and displays *in vivo* anti-nociceptive activity [10]. At variance with other uncompetitive antagonists, this triazine does not activate the channel at low concentration, suggesting an increased therapeutic index. Nonetheless, pre-clinical safety pharmacology studies are necessary to unveil its potential side effects. Secondly, a class of allosteric modulators that target the TRP domain of the channel has also been reported (**2**, TRPducin TRP-p5) [11]. These are short peptides, named as TRPducins, which mimic the sequence of the N-end region of the TRP domain and block selectively the channel by interacting with a cytosolic binding side. Palmitoylation anchored de active sequences to the plasma membrane facilitating the access to their binding site. Notably, compound **2** displayed

Table 1. Developmental status of thermoTRPs modulators.

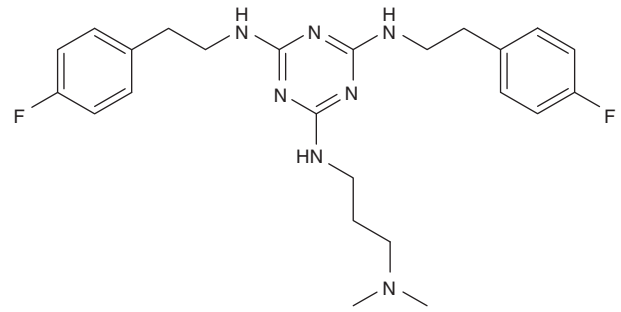
Target	Compound	Therapeutic application	Development status	Ref.
TRPV1				
	DD04107 (neuronal exocytosis blocker)‡	Pain	Pre-clinical	[3]
	Capsaicin oral gel	Burning mouth syndrome	Phase-0. Completed	NCT00875537
	PAC-1028	Pain	Phase-I. Recruting	NCT01264224
	Piperine	Oropharyngeal dysphagia	Phase-I. Recruting	NCT01383694
	PHE337	Neuropathic pain	Phase-I. Completed	[3]
	DWP-05195	Neuropathic pain	Phase-I. Completed	[3]
	ABT-102	Pain	Phase-I. Completed	NCT00854659
	JNJ-39439335	Relief of pain using termal-grill experimental model	Phase-I. Completed	NCT01006304
	JTS-653	Pain	Phase-II. Ongoning	[3]
	SB-705498	Migraine	Phase-II. Completed	NCT00269022
	SB-705498	Dental pain	Phase-II. Completed	NCT00281684
	SB-705498	Rectal pain study	Phase-II. Completed	NCT00461682
	SB-705498	Non-allergic Rhinitis	Phase-II. Completed	NCT01424514
	MK-2295	Tooth extraction	Phase-II. Completed	NCT00387140
	SB-681323 (p38 inhibitor)	Neuropathic pain	Phase-II. Completed	NCT00390845
	Civamide nasal solution	Episodic Cluster headache	Phase-III. Not yet recruiting	NCT01341548
	ALGRX-4975	Analgesia after knee replacement surgery and bunionectomy	Phase-III. Ongoing	NCT00130962
	WL-1001	Cluster headache, osteoarthritis	Phase-III. Completed	NCT00077935
	NGX-4010 (Qutenza)*	Postherpetic neuralgia; neuroalgia post-hepatic	Phase-IV.	NCT01416116
TRPV3	GRC 15300	Pain	Phase-I. Completed	Glenmark´s press release
TRPA1	CB-625	Pain	Pre-clinical. Completed	Cubist Pharmaceuticals/ Hydrabiosciences
	GRC 17536	Diabetic neuropathy	Phase-II. Ongoing	Glenmark´s press release
TRPM8	D3263	Solid tumours	Phase-I. Completed	NCT00839631
	Biofreeze	Knee osteoarthritis	Phase-III. Not yet recruiting	NCT01565070
		Neck pain		NCT01542827
	Menthol	Hypertension	Phase-III. Ongoing	NCT01408446

*40 clinical trials with capsaicin formulations listed in Clinicaltrials.gov.
‡60 clinical trials with Botulinum for treating pain conditions in Clinicaltrials.gov.

in vivo anti-nociceptive and anti-pruritus activity in a model of chronic liver failure. As for uncompetitive antagonists, we await the preclinical studies that will characterize its pharmacological safety. And, thirdly, modulation of TRPV1 recruitment in inflammatory conditions, by blocking its exocytotic incorporation to the plasma membrane using a palmitoylated peptide patterned after the SNAP25 protein of the SNARE complex (3, DD04107), has an important and long-lasting anti-nociceptive activity in models of chronic neuropathic and inflammatory pain [12]. Notably, this therapeutic activity was largely devoid of secondary effects, including the hyperthermia, implying a promising therapeutic index. This compound is currently in development. Taken together, although we eagerly await positive clinical results using TRPV1 antagonists, the diversity of compounds, along with the distinct mechanisms of action, increase the probability of finding relevant therapeutic compounds for this thermoTRP.

3. TRPV2

Transient receptor potential vanilloid 2 (TRPV2) was isolated as a channel sensitive to temperatures above 52°C [13], and a channel that translocated into the plasma membrane in response to IGF-1 to modulate the entry of Ca^{2+} [14]. In addition, TRPV2 expression and function is upregulated by heat, phosphatidylinositol 3-kinase (PI3-kinase), and by association with the recombinase gene activator (RGA) chaperone protein [15-17]. Furthermore, it has been shown that TRPV2 is also activated by hypotonicity, membrane stretching and a number of exogenous chemical ligands such as 2-aminoethoxydiphenyl borate (2-APB) and probenecid, lysophospholipids [18,19] and cannabinoids [20]. The response of TRPV2 to these ligands is low and variable and quite dependent of the species [21], which has prevented a detailed pharmacological characterization of the channel [22,23].



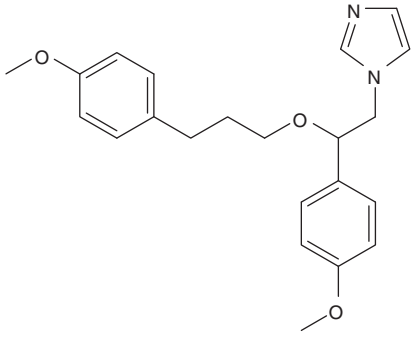
1 (Aa)
IQAC-UMH

Pal-MGETVNKIAQES-NH_2

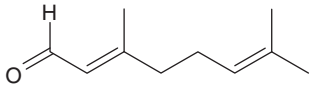
2
(TRP-p5) UMH

Pal-EEMQRR-NH_2

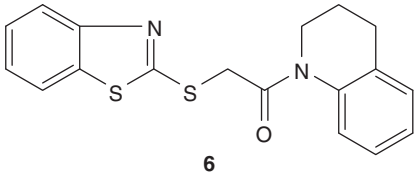
3
(DD04107)
BCN-Peptides



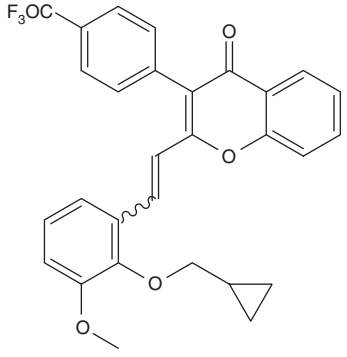
4
(SKF96365)



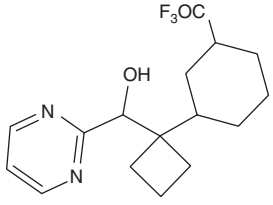
5
(Citral)



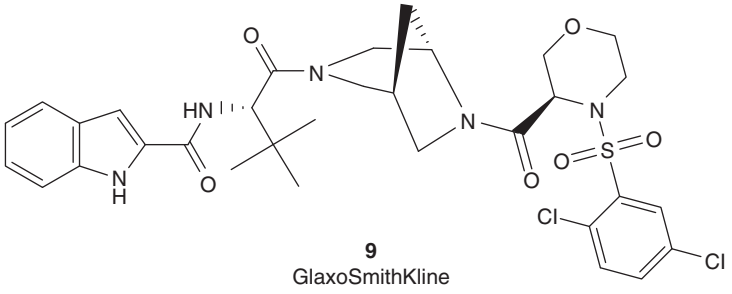
6
Hydra



7
Glenmark



8
Abbott



9
GlaxoSmithKline

Figure 2. Selected examples of TRPV1 (1 – 3), TRPV2 (4, 5), TRPV3 (6 – 8) and TRPV4 (9) modulators.

Furthermore, because of this complex regulation and pharmacological functional differences, the role of TRPV2 as a thermosensory channel in humans is still under investigation.

In the peripheral nervous system, TRPV2 is expressed in medium to large diameter (Aδ and Aβ fibers) DRG, trigeminal (TG) and nodose ganglia (NG) neurons and in the dorsal horn of the spinal cord [24]. TRPV2 mRNA or immunoreactivity has also been detected in the brain, autonomic ganglia, spinal cord, skeletal and vascular myocytes, blood cells and visceral organs including the intestine, pancreas, spleen, and bladder [13,25-30].

TRPV2 immunoreactivity in DRG neurons is augmented 2 days after complete Freund's adjuvant (CFA)-induced inflammation, suggesting a role in peripheral sensitization during inflammation [31]. Similarly, TRPV2 expression was increased in sciatic nerve ligation, constriction injury pain models [32], and in sympathetic neurons after peripheral axotomy, implying a potential role for TRPV2 in sympathetic-mediated neuropathic pain [33]. However, TRPV2 knock-out mice displayed normal behavioural responses to noxious heat over a broad range of temperatures and normal responses to punctate mechanical stimuli, both in the basal state and under hyperalgesic conditions, although showed reduced embryonic weight and perinatal viability [34]. Thus, TRPV2 appears important for perinatal viability but does not seem to be essential for heat or mechanical nociception or hypersensitivity in the adult mouse.

TRPV2 expression may also be involved in the migration and proliferation of cancer cells. Analysis of TRPV2 gene and protein expression in superficial and invasive tumours has shown that TRPV2 mRNA increased gradually as tumour grade and stage augmented. Given that both mRNA and protein levels were enhanced in urothelial carcinoma cell lines [35], modulators of TRPV2 channels may be attractive compounds for the treatment of malignant urothelial carcinomas. Similarly, high TRPV2 levels were observed in moderately and well-differentiated human hepatocarcinoma (HCC) compared with that of poorly differentiated tumours. In addition, TRPV2 mRNA was expressed in benign astrocyte tissues, and its expression progressively declined in high-grade gliomas as grade increased [36]. Furthermore, clinical assessment suggested a significant association between TRPV2 expression and portal vein invasion and histological differentiation [37]. Taken together, TRPV2 could represent a prospective prognostic marker and potential novel therapeutic target for cancer treatment. Due to the role of TRPV2 in the migration and proliferation of cancer cells, some patents have included this protein in methods that use gene expression markers for detection and prognosis of lung and pancreatic tumours [38,39].

Despite the potential therapeutic interest in TRPV2 modulation, the identification of specific channel antagonists is, surprisingly, non-existent. Up to this time, only general blockers such as ruthenium red, trivalent cations (La^{3+}), SKF96365 (4), citral (5) and potassium channel blockers have been shown to abolish 4-ABP evoked TRPV2 currents [40]. In addition, we could also found a Japanese patent application that claims TRPV2 inhibitors with therapeutic applications, but does not disclose the structures [41]. A plausible explanation for this lack of TRPV2 antagonists is probably due to the lack of an adequate HTS screening assay, and the pharmacological differences between TRPV2 orthologs from different species. Nonetheless, Janssen Pharmaceutica has recently patented a series of screening methods suitable for identifying TRPV2 modulators which include the expression of the protein in a recombinant host cell and the use of structural analogues of cannabinoids to activate the channel that is measured by automatic patch clamp methods [42,43]. It could be anticipated that TRPV2 modulators may be discovered in the near future.

4. TRPV3

TRPV3 is a polymodal receptor activated by warm, non-painful, temperatures with a threshold of 34°C to 38°C [44,45], and chemical stimuli such as the nonspecific TRP activator 2-APB, camphor and carvacrol or specific agonists such as thymol and eugenol [46]. TRPV3 is predominantly expressed in skin keratinocytes [47], although it has also been found in DRG and TG neurons, and in brain regions such as hippocampal, superior cervical neurons, ventral motor neurons and nigral dopaminergic neurons [44,45,48].

Mice lacking TRPV3 exhibit wavy hair coat and curly whiskers. It was recently shown that TRPV3 forms a signaling complex with TGF-α/EGFR, which regulates keratinocyte proliferation and differentiation [49]. TRPV3 is also required for the formation of the skin barrier by regulating the activity of transglutaminases, a family of Ca^{2+}-dependent cross-linking enzymes essential for keratinocyte cornification. Importantly, three missense mutations in TRPV3 that result in a gain-of-function channel phenotype have been detected in a syndrome characterized by skin keratinization and alopecia, known as Olmsted syndrome [50]. Altogether, TRPV3 seems to play a pivotal role controling hair morphogenesis and skin barrier formation.

Regarding TRPV3 contribution to somatosensation, TRPV3 deficient mice showed defects in thermal behaviour in response to innocuous temperature and displayed longer withdrawal latencies in response to high noxious temperatures thus suggesting an important role in pain transduction [51]. However, a recent report questioned the *in vivo* implication of TRPV3 in thermo-sensation under naïve conditions or following inflammation with CFA [52]. Nevertheless, augmented TRPV3 receptor levels have been found in the sciatic nerve constriction pain model [53]. In addition, increased TRPV3 immunoreactivity was found in DRG neurons after traumatic injury or in diabetic neuropathy [54], and in keratinocytes in breast pain patients [55]. Furthermore, stimulation of TRPV3 receptor in keratinocytes releases inflammatory mediators including ATP, prostaglandin PGE2 and IL-1β,

supporting a contribution to skin inflammatory signalling and pain transduction, and suggesting a therapeutic potential.

Concerning the putative role of TRPV3 in the central nervous system, incensole acetate a potent TRPV3 agonist has been shown to produce anxiolytic and antidepressive effects in wildtype but not in TRPV3 knockout mice, suggesting its participation in emotional control [56]. Moreover, it appears that TRPV3 may be also implicated in the pathophysiology of migraine [57].

The therapeutic potential of this thermoTRP has inspired drug discovery programs aimed at finding selective antagonists. Hydra Biosciences was the first company to disclose the efficacy of their TRPV3 antagonists *in vivo* [58]. Compound **6** at 50 mg/Kg intraperitoneal (ip) or 200 mg/Kg oral (po) was capable of reversing formalin-induced flinches, thermal injury pain, and inflammatory pain in the CFA model. In 2007 Hydra Biosciences signed a collaboration agreement with Pfizer Global Research & Development aimed at developing TRPV3 antagonists for treatment of pain, although no further developments have been disclosed thus far.

Glenmark Pharmaceuticals has reported a series of potent and selective TRPV3 antagonists such as compound **7**. They include series of chromane-, fused pyrimidine-, fused pyrimidinones-, chromanone- and fused imidazole-derivatives that have been tested by [^{45}Ca] uptake assays [59-65]. The antihyperalgesic activity of two different TRPV3 blockers, GRC15133 and GRC17173 (structures not yet disclosed), has been demonstrated. They attenuate the mechanical hyperalgesia in chronic constriction-induced injury with an ED_{50} of 4.8 mg/Kg, the CFA-induced mechanical hyperalgesia with an ED_{50} of 8 mg/Kg (ip), and the thermal hyperalgesia at 30 mg/Kg. Notably, GRC15300, a potent, selective and orally bioavailable TRPV3 antagonist, has been the first TRPV3 blocker that successfully finished Phase-I clinical trials. The drug seems to be well tolerated, non-habit forming and has a good pharmacokinetic profile. Glenmark Pharmaceuticals out-licensed GRC15300 to Sanofi, who plans to start clinical proof of concept studies (Phase-II) in neuropathic and inflammatory pain models in the near future.

In 2012, Abbott patented a series of TRPV3 modulators (represented by compound **8**) [66]. Some of these antagonists showed IC_{50} < 1 μM. The analgesic effect is claimed being tested in an osteoarthritis model of pain using 300 μmoles/Kg although the specific compounds tested are not detailed. Collectively, it appears that therapeutic modulation of TRPV3 is progressing and it might provide us with the first thermoTRP therapeutics.

5. TRPV4

TRPV4 is activated by warm temperatures (27°C to 35°C) [67,68], physical cell stress [69], phospholipase A2 [70], exogenous natural plant extracts (i.e., bisandrographolide A) and synthetic ligands [71]. TRPV4 functions include temperature monitoring in skin keratinocytes, osmolarity sensing in kidneys, sheer stress detection in blood vessels, and osteoclast differentiation control in bones. TRPV4 is expressed in DRG, TG and NG neurons [72], and in the lung [73], where it has been shown to mediate Ca^{2+} entry in isolated endothelial cells [74]. Under inflammatory pain conditions, TRPV4 activity is enhanced upon hypotonic stimulus of C-fibres in wild type, but not in TRPV4 knockout mice [75]. The physiological roles of TRPV4 expand the interest in targeting TRPV4 modulation for therapeutic purposes, which is now focused on several areas [76,77].

Clinical benefit of inhibiting TRPV4 function has been proposed in the treatment of heart failure associated with lung congestion, and in other pathological conditions with symptoms of lung edema/congestion, including a number of respiratory disorders [76,77]. Notably, activation of TRPV4 channels results in cardiovascular collapse due, at least partially, to enhanced filtration at the septal barrier, resulting in lung edema and hemorrage [78].

TRPV4 channels have been also implicated in urinary bladder function [71,79], and are expected to provide therapeutic benefit for conditions of bladder overactivity. TRPV4 channels expressed within bladder smooth muscle cells probably act as sensors of bladder pressure/stretch/filling, contributing to bladder contraction and hyperactivity [71]. TRPV4 is also likely to be expressed directly on afferent nerves providing a direct neuronal stimulation of the bladder [54]. In addition, TRPV4 antagonists could provide therapeutic benefit to other pathologies such as pain [80-82], cardiovascular disease [83], osteoarthritis [84] and ventilator-induced lung injury [85].

GlaxoSmithKline developed a series of diazabicyclo[2.2.1]hept-2-yl derivatives [86-94] to act as TRPV4 antagonists (illustrated by compound **9**, **Figure 2**). These compounds are claimed to be useful in the treatment or prevention of atherosclerosis, disorders related to intestinal edema, postsurgical abdominal edema, local and systemic edema, fluid retention, hypertension, inflammation, bone loss associated with immobilization, congestive heart failure, pulmonary disorders, sinusitis/rhinitis, asthma, overactive bladder, pain, cardiovascular disease, renal dysfunction, and osteoarthritis. A hypotonicity assay was used to measure changes in intracellular Ca^{2+} using the fluorescent dye Fluo4. All examples described possessed TRPV4 biological activity with IC_{50} ranges from 1 nM - 10 μM.

Additional inventions expanded the substituent of the diazabicyclo[2.2.1]hept-2-yl scaffold, or used other chemical skeletons such as naphthyridine, and benzothiophene, and their corresponding derivatives as TRPV4 antagonists [86-94]. These new compounds were additionally indicated for the treatment of chronic obstructive pulmonary disorder, ventilator induced lung injury, high altitude provocked pulmonary edema, acute respiratory distress syndrome, pulmonary fibrosis, motor neuron disorders, and genetic gain of function disorders. Clearly, TRPV4 is also emerging as an interesting therapeutic target for the treatment of several human diseases.

6. TRPA1

TRPA1 is expressed in the inner ear, in sensory neurons of TG and DRG and co-localizes with TRPV1 channels in a subset of sensory neurons [95,96]. TRPA1 is the receptor for multiple noxious stimuli like environmental pollutants and oxidants [97-101]. Experimental findings also indicate that TRPA1 contributes to cold sensation, being activated at cold temperatures close to the threshold of noxious cold for humans [102,103], while its role as detector of mechanical stimuli is still a matter of controversy [104]. Cumulative evidence indicates that TRPA1 is the effector of sensory neuron activation and most respiratory reflexes triggered by lung irritants [105], alone or interplaying with nicotine acetylcholine receptors [106]. Furthermore, it has been suggested that the activation of TRPA1 may contribute to chronic obstructive pulmonary disease [107].

Glenmark Pharmaceuticals is the most active company in patent applications from 2009. They focused in fused pyrimidindione derivatives of general formula **I** (**Figure 3**) [108-115]. These heterocyclic molecules were screened for human and rat TRPA1 antagonism, measuring the inhibition of the allyl isothiocyanate (AITC)-induced cellular uptake of radioactive [$^{45}Ca^{2+}$]. Several tetrahydroquinazolidinones were assayed for the *in vivo* efficacy in various models of pain/hyperalgesia [108-115]. Compound **10** potently blocked the AITC- and formalin-induced nocifensive behavior in rats, and displayed good efficacy against CFA-induced mechanical hyperalgesia. In the chronic constriction injury of sciatic nerve in male rats, acute (10 mg/Kg) and chronic (3.5 mg/Kg) administration of quinazolinedione derivative **10** showed a maximum efficacy of 75% reversing the mechanical hyperalgesia. Notably, compound GRC-17536 (structure not yet disclosed), has successfully completed Phase-I clinical trials and is now registered for Phase-II studies in patients with painful diabetic neuropathy (**Table 1**). This highly selective compound, administered via the inhalation route, is also planned to enter Phase-I trials for different respiratory indications, starting by mild asthma.

Hydra Biosciences has protected new families of xanthine-7-ylacetamide TRPA1 antagonists **11 – 13**, and related analogues (**Figure 3**) [116-123]. Compound **11** inhibited the rise in intracellular Ca^{2+} induced by AITC and displayed high selectivity for TRPA1. Compound **12** showed an IC_{50} of 4 nM for hTRPA1 antagonism [116-123], and was able to reduce by 30% the AITC- and formalin-induced pain behavior in rats (50 mg/Kg). Attempts to improve the pharmacokinetic properties of HC-030031 resulted in the heptafluoro analogue **13** that exhibited higher potency, and better solubility and bioavailability than the parental compound [116-123]. A small library of pyrrolo[3,2-d]pyrimidinone derivatives, related to **11**, was recently described by an academic group at Ferrara University [124]. The best compounds displayed antagonist potencies in the low micromolar concentration. Furthermore, Hydra Biosciences, in collaboration with Cubist Pharmaceuticals, has identified a small-molecule, CB-625 (structure not disclosed yet), that blocks peripheral TRPA1 channels and attenuates inflammatory mediated pain in animal models. This molecule has been selected as a clinical candidate.

Janssen Pharmaceutica has patented two families of heterocyclic derivatives illustrated by compounds **14** and **15** (**Figure 4**) [125-127]. The first series of 2-thioxotetrahydroindeno[1,2-*d*]pyrimidin-5-one derivatives showed TRPA1 antagonistic properties. Structure-activity relationships indicated the importance of the thiocarbonyl group (**14**) and the absolute need for a 4*R* configuration (IC_{50} = 4 nM) as compared with the 4S enantiomer (IC_{50} > 10 μM). However, this family of compounds displayed a poor drug-like profile [128]. A library of 417 proline derivatives, bearing Nα-phenylsulfonyl or thiophenesulfonyl groups and disubstituted pyridine or pyrimidine moieties at the N-amide group, was assayed *in vitro* as modulators of hTRPA1 [125-127], showing potent antagonist properties (**15**, IC_{50} < 10 nM)

Abbott Laboratories has patent protected oxime derivatives as TRPA1 antagonists [129,130]. The most potent molecule (**16**, **Figure 4**), inhibited Ca^{2+} influx with an IC_{50} of 74 nM, displayed good oral biovailability (38%), and excellent plasma and brain exposure. In addition, oxime **16** was fully efficacious in a rat model of knee joint osteoarthritis [129,130]. A new series of oxime analogues, similar to compound **16**, showing either modest TRPA1 agonist or antagonist activity was described by Renovis, Inc. [131]. Octahydronaphthalene derivatives (**17**) acting as TRPA1 antagonists (**Figure 4**) have been patented by Merck [132]. Two molecules from the Korea University, the dual TRPV3/TRPA1 inhibitor isopentenyl pyrophosphate (**18**) and the 7S,8R,17S-trihydroxy-4Z,9E,13Z,15E,19Z-docosahexaenoic acid (**19**) were reported to ameliorate TRPA1-mediated pain signaling in animal models [133-135]. A similar activity was shown for a recent piperidineurea derivative (**20**) from AstraZeneca [136]. This plethora of TRPA1 antagonists awaits clinical validation, with many expectations on GRC-17536 (**Table 1**).

7. TRPM8

TRPM8 was first identified as a marker of prostate cancer cells, and thereafter as a sensory ion channel gated by menthol and icilin and innocuous cool temperatures (15 – 28°C) [137-139]. Cumulative evidence substantiate that an increased TRPM8 expression in sensory neurons after nerve injury or inflammation, underlies the enhanced sensitivity to cold allodynia and hyperalgesia [140,141]. Similarly, TRPM8 expression is up-regulated in androgen-sensitive prostate and skin melanoma cancer cells [142,143]. Activation of TRPM8 channel activity appears to attenuate pain transduction in certain inflammatory conditions [144]. Therefore, TRPM8 is considered as an attractive target for therapeutic intervention, mainly for developing new analgesics and antitumor agents [145-147].

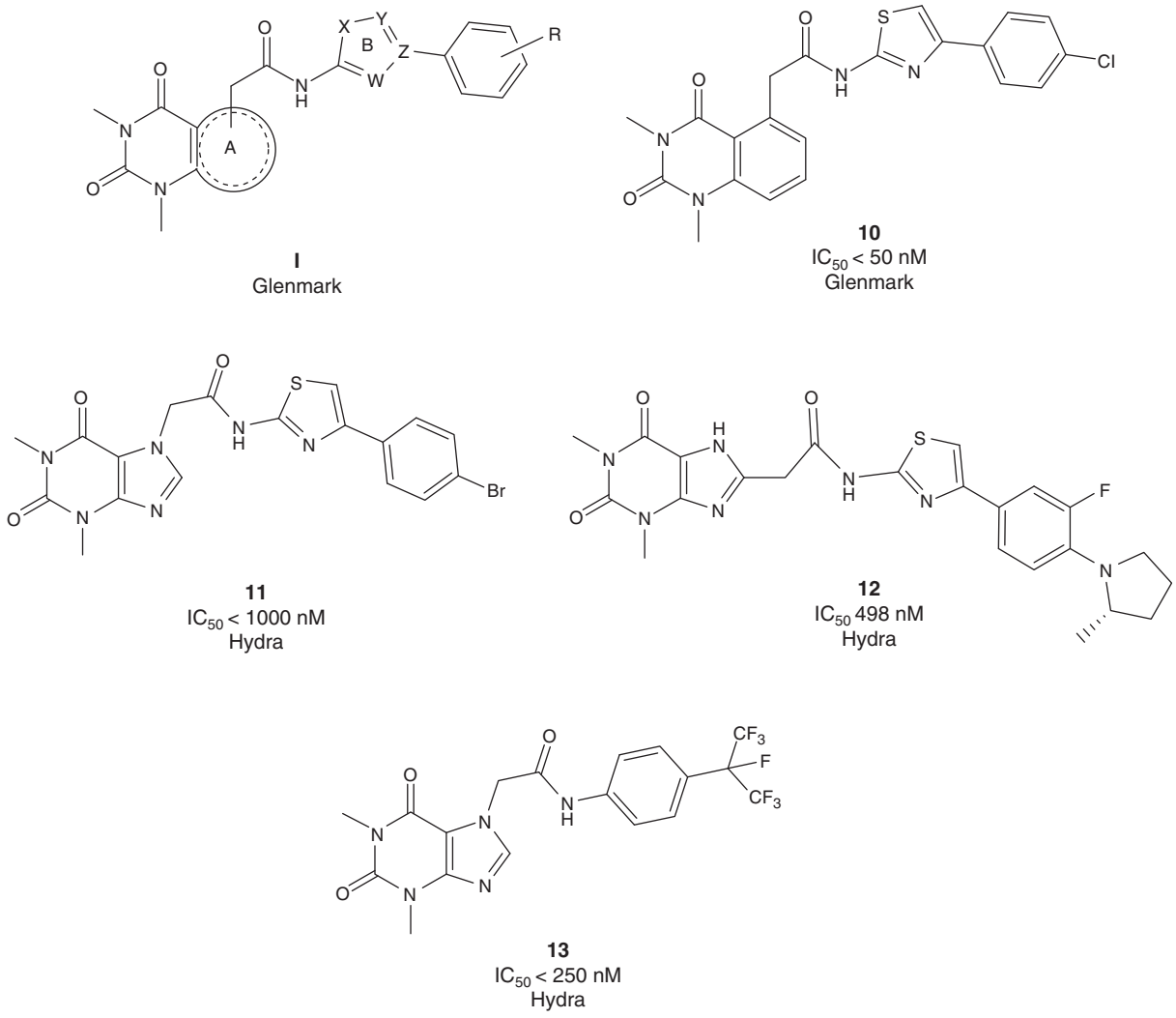


Figure 3. Representative examples of TRPA1 antagonists recently protected by Glenmark Pharmaceuticals S.A. (I, 10) and by Hydra Biosciences, Inc. (11 – 13).

Janssen Pharmaceutica has made a tremendous effort in the search for TRPM8 antagonists (**Figure 5**). The first large family comprises several patents on benzothiophene derivatives, bearing N-benzyl sulfonamides (**21**), N,N'-disubstituted sulfamides (**22**), and phosphono(benzyl)methyl appendages (**23**) at position 2 [148-154]. Biological evaluation of compound **21** in hTRPM8 yielded an IC_{50} of 1.1 nM and 2.3 nM for menthol and cold activation, respectively. This compound also displayed *in vivo* activity in different animal models, being able to reduce icilin-induced "wet-dog" shakes (WDS) in rats (ED_{50} = 2.5 mg/Kg), and to reverse carrageenan-induced heat hypersensitivity (64% at 30 mg/Kg, po), as well as CFA-induced thermal and mechanical hyperalgesia (91% at 10 mg/Kg, po). Bezothiophene **21** (30 mg/Kg, po), was also effective in an *in vivo* model of neuropathic pain (acetone-induced hypersensitivity, 69% of pain relief), and in the model of cold-evoked cardiovascular pressor responses (75% attenuation). One of the most active compound was 3-bromothiophene (**23**), with IC_{50}= 9 nM, and good *in vivo* activity. In the CFA-induced model, this compound showed an ED_{50} of 21 mg/Kg reversing cold hyperalgesia. Similarly, an 80% reduction of the icilin-induced cold sensations in rats was observed at 30 mg/Kg (po).

The second family of TRPM8 antagonists reported by Janssen Pharmaceutica encompasses compounds with a benzimidazole moiety as the central scaffold (**24** – **27**) and its aza-analogues [155]. Model compound **24** inhibited icilin-induced WDS (98%) in a dose-dependent manner (30 mg/Kg, po), and reversed cold hypersensitivity (91%) in the chronic condition injury model of neuropathic pain (10 mg/Kg, po) [156]. Two patents protect the TRPM8 antagonist activity of 1,3- and N,N-disubstituted derivatives, (**26** and **27**), respectively [157-160]. Compound **27**, inhibited the cold-stimulated currents in HEK cells expessing hTRPM8, and suppressed

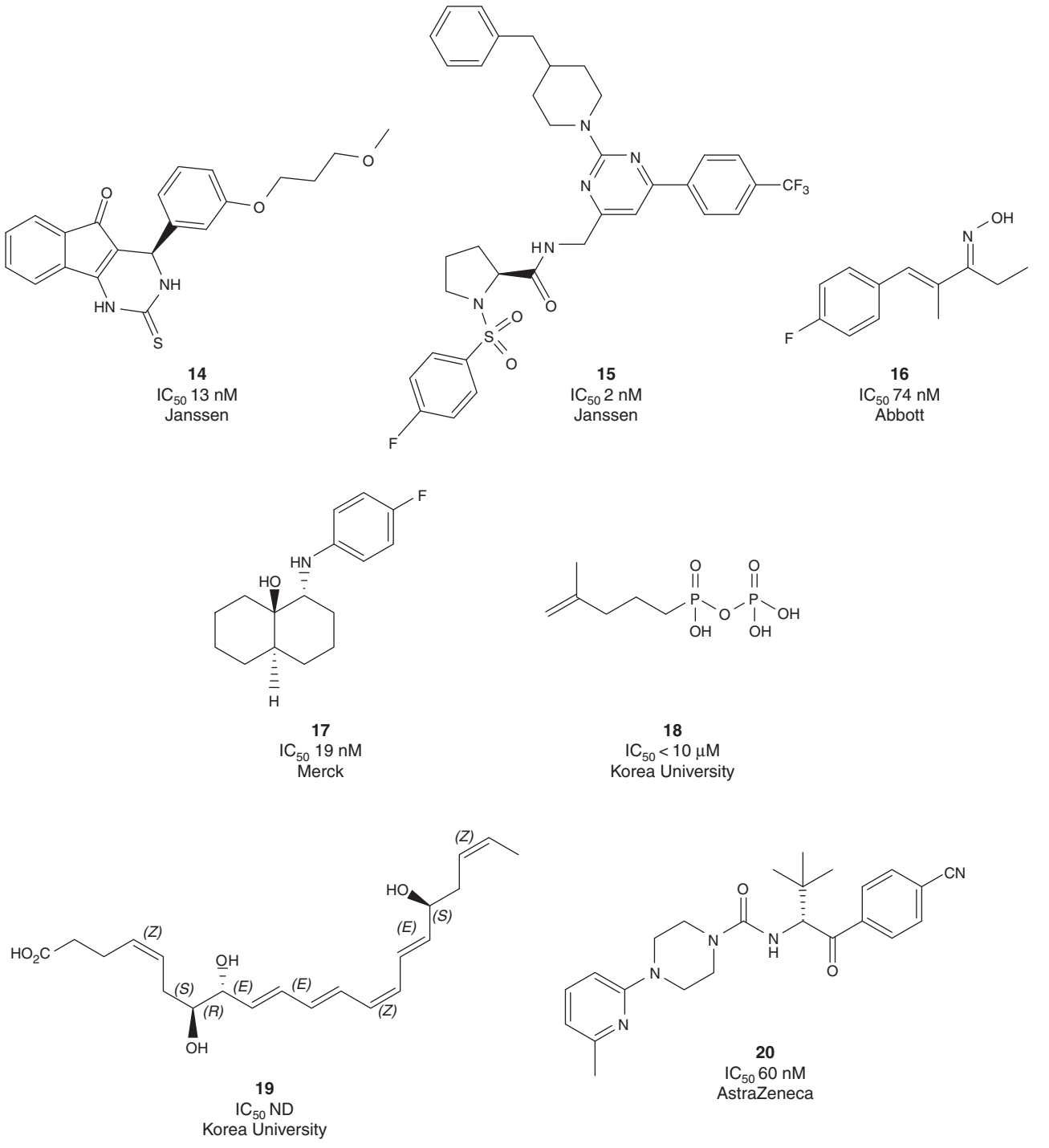


Figure 4. Additional TRPA1 antagonists recently protected.

icilin-induced behaviors in rats at 10 mg/Kg (po), as well as the acetone-mediated hypersensitivity in a model of neuropathic pain [157-160]. The third family of TRPM8 antagonists refers to N-arylphenylglycine amides (i.e., **28**) [157-160].

Glenmark Pharmaceuticals patented two new families of compounds [161,162]. The first series comprises tertiary amines substituted with a heterocycle (benzothiazole or bezooxazole), a benzylic group and an alkylamino moiety, as illustrated by compound **29** (**Figure 5**). The second set includes phenylureas of spiro[chromene-2,4′-piperidine] (**30**). Similarly, Amgen, Inc. protected tetrahydroquinoline and several aza-analogues as TRPM8 anatagonists [163-165]. Compound **31** from this

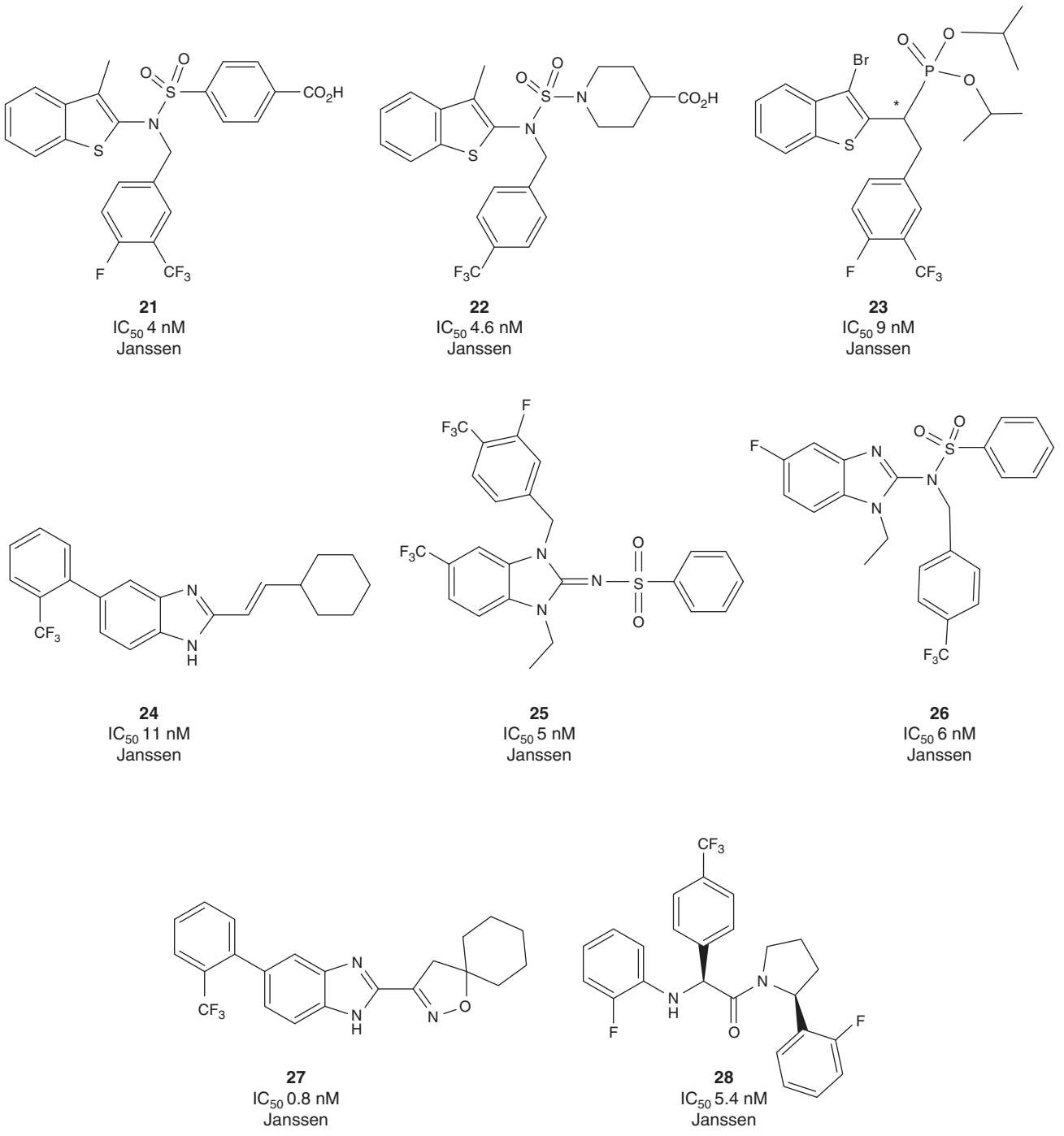
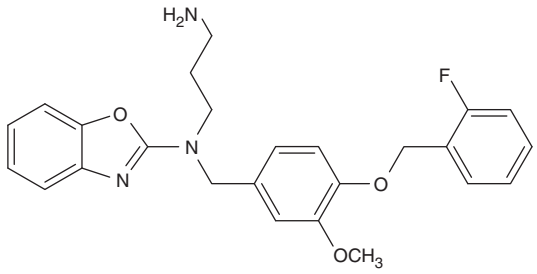


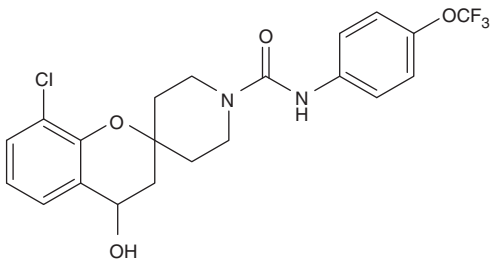
Figure 5. Compilation of representative TRPM8 antagonists described by Janssen Pharmaceutica (21 – 28), Glenmark Pharmaceuticals (29,30), Amgen (31) and Raqualia (32).

series abolished icilin-induced WDS (98%) in a dose dependent manner. However, further development of **31** is precluded due to its poor pharmacokinetic properties.

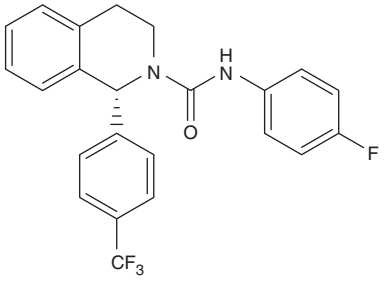
Raqualia Pharma disclosed two patents on N,N-arylsulfonamides structures (**32**) [166,167]. Although functional data have not been reported, inventors claimed good *in vitro* antagonist IC_{50} values in TRPM8-HEK293 and human malignant melanoma cell lines, as well as potent activities in a number of *in vivo* models, including chronic constriction injury-induced neuropathic pain (cold- and static-allodynia), oxaliplatin-induced pain, icilin-induced WDS, and significant effects on overactive bladder in anesthetized cystitis rats.



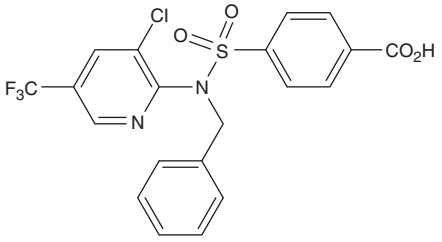
29
IC_{50} < 20 nM
Glenmark



30
IC_{50} < 20 nM
Glenmark



31
IC_{50} < 56 nM
Amgen



32
IC_{50} < 1 μM
Raqualia

Figure 5. (continued). Compilation of representative TRPM8 antagonists described by Janssen Pharmaceutica (21 – 28), Glenmark Pharmaceuticals (29,30), Amgen (31) and Raqualia (32).

Finally, blockade of the TRPM8 channel activity by cannabinoid structures, like THC and THCA, among others, has been protected by GW Pharma [168], but their antagonism was not selective with respect to the inhibition of other TRP channels.

TRPM8 agonists (**Figure 6**) are also important therapeutic compounds for attenuating pain and inducing apoptosis of cancer cells expressing TRPM8. In several patent applications, Dendreon Coorporation disclosed the agonist activity of small-molecule derivatives structurally related to menthol [155,169-171]. Compounds **33**, **34** and its analogues increased the Ca^{2+} influx in TRPM8-CHO cells with nanomolar potency, stimulated apoptosis of cancer cells, and showed significant activity in animal models for cancer (i.e., xenograft of human prostate cancer). A new orally bioavailable chemical entity, D3263 from Dendreon (structure not disclosed yet), showed good potency inhibiting the growth of tumor cells expressing TRPM8 in preclinical studies. This compound has already completed Phase-I clinical trials in healthy volunteers, and the company announced recently the initiation of clinical studies on patients with advanced solid tumors (**Table 1**).

A new activity for neferine **35**, a natural bis-benzylisoquinoline alkaloid, as agonist of TRPM8 and TRPV1, and its use for the treatment of diseases related to these channels has been protected by the Institute of Chinese Materia Medica [172]. The application of different TRPM8 agonists (i.e., **36**, **37**) for achieving cooling effects on skin and mucous membranes, and for reducing perspiration and unpleasant odors has also been described [173-175]. In a double blind, randomized, and parallel-group study, the Third Military Medical University is evaluating the effects of menthol on blood pressure in prehypertensive (Phase-II clinical studies) and hypertensive (Phase-III trials) patients (**Table 1**), as prospective alternative for the prevention and treatment of hypertension. Biofreeze®, a topical lotion containing menthol and alcamphor, and having analgesic effects through the activation of TRPM8, is being investigated to alleviate symptoms associated with knee osteoarthritis (**Table 1**).

8. Expert opinion

There is no doubt that thermoTRP channels are pivotal therapeutic targets involved in the etiology of several human

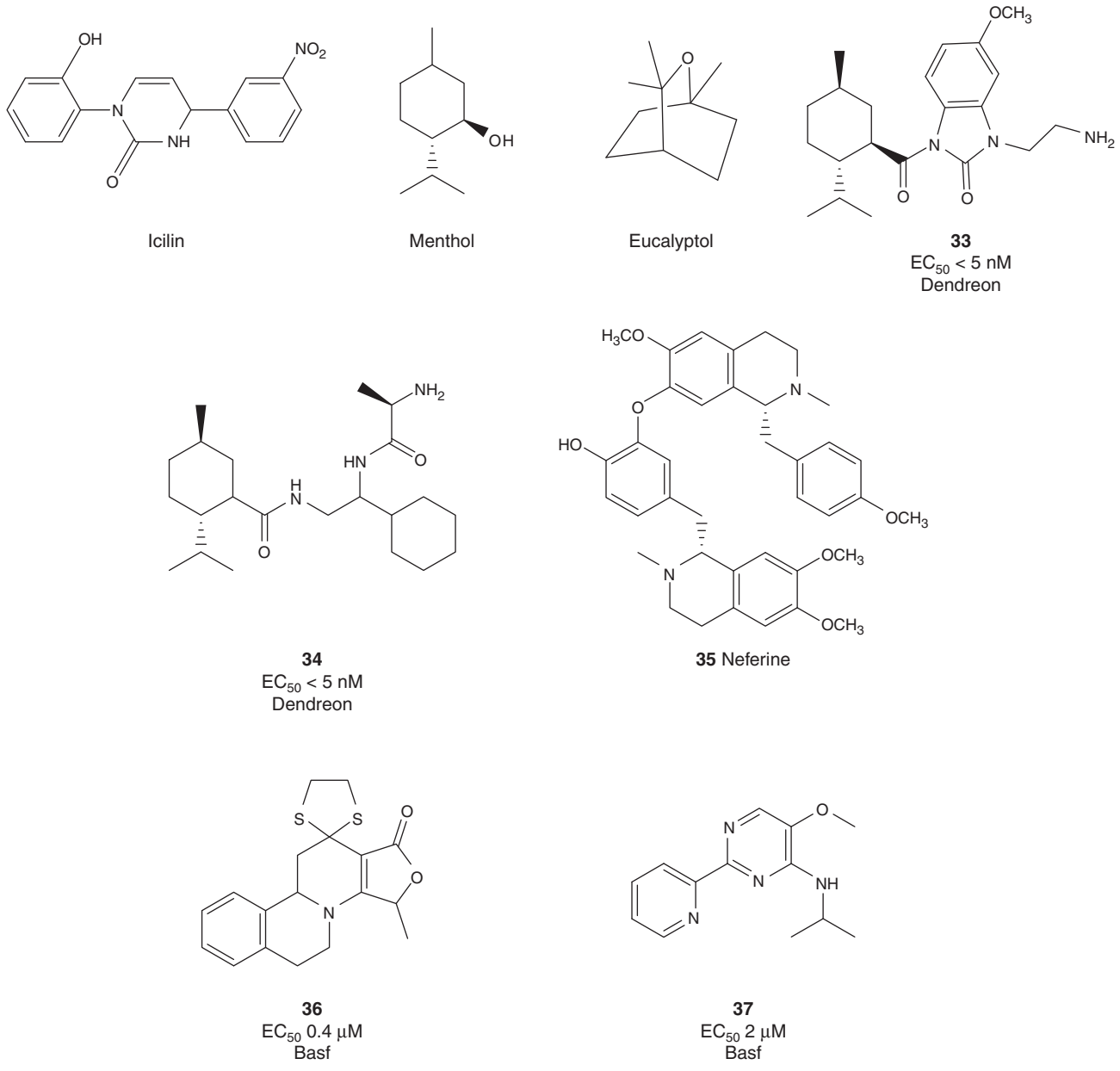


Figure 6. Selected known and recently reported TRPM8 agonists.

diseases. Although the first validated channel was TRPV1, and a large constellation of compounds has been developed that atttenuate its channel activity, we still are awating a clinical success (**Table 1**). Most compounds that progressed into clinical trials failed due to unanticipated side effects, primarily hyperthermia [176]. Since then, a significant effort has been thrust to understand the molecular basis of this side effect and to develop compounds that are devoid of it. We have learned that antagonists that do not affect TRPV1 activation by acidic pH do not increase the body temperature. However, the acidic pH present in the ischaemia that accompanies inflammation raises concerns on the *in vivo* activity of these compounds in some inflammatory disorders [6]. Clearly, additional work is necessary to understand the molecular and cellular basis of TRPV1 inhibition that leads to hyperthermia. Nonetheless, the identification of TRPV1 antagonists that block all modes of channel activation and do not produce significant hyperthermia [9] paves the way to the development of antagonists with enchanced therapeutic index.

Thus far, most of the drug discovery programs have focused on developing small molecules that act as competitive or non-competitive antagonists of these channels. These sort of compounds display high binding affinities, as well as

eficacies and potencies. A potential weakness of these molecules is that they bind to both the closed and open states of the channels. While interacting with the open state will target the active channels, binding to the closed state will act on all receptors, which may contribute to the manifestation of unwanted side effects. Accordingly, it seems reasonable that drug discovery programs focus on developing activity-dependent antagonists such as open channel blockers to preferentially block pathologically activated receptors. However, this strategy does not appear so straightforward because of the pore dilatation that some thermoTRPs display in the open state. This pore dynamics complicates the stable interaction of small molecules with binding sites inside the permeation pathway. Nevertheless, the recent identification of triazine-based compounds that act as open channel blockers provides pharmacological scaffolds to develop therapeutically useful uncompetitive antagonists.

The discovery and validation of new drug binding sites in thermoTRPs is another promising approach that may yield therapeuticatlly relevant compounds. Akin to G protein coupled receptors [177], allosteric modulators that interfere with the channel activation process may emerge as a valuable strategy. Hence, the recent identification of the TRPducins provides a powerful strategy to identify TRP allosteric modulators by exploiting the protein–protein interactions occurring during channel gating. This concept, illustrated for TRPV1, is being extended to TRPM8 and TRPA1, and may be further applied to other thermoTRPs as well as to the entire TRP family. Whether TRPducins will produce hyperthermia is yet unknow, although ongoing pre-clinical assays will provide a blueprint for the potential side effects of this novel pharmacological approach.

Last, but not least, the activity of a thermoTRP depends on both the amount of receptor residing at the plasma membrane and its degree of activation. Thus, modulation of thermoTRP recruitment to the cell surface under pathological conditions is a complementary approach to attenuate channel dysfunction. In this regard, the anti-nociceptive activity of botulinum neurotoxin and compound DD04107 in chronic inflammatory and neuropathic pain substantiates this strategy, and lend support to Ca^{2+}-dependent-exocytosis of thermoTRPs as an important cellular mechanism involved in pain transduction [12]. Similarly, inhibition of p38 (**Table 1**), a protein that modulates NGF-induced translation of TRPV1 in chronic inflammatory pain [178], further supports that regulation of receptor levels is a valuable pharmacological strategy that combined with modulation of channel gating may lead to better therapies.

Despite the initial clinical setbacks and dissapointment of the first generation of TRPV1 antagonists, the future of thermoTRP molecular pharmacology looks quite bright. The lessons learnt are helping in the design of therapeutically improved compounds that tackle down both the channel activity and its delivery to the plasma membrane. We will witness in the near future the clinical success of some of these compounds for the treatment of human diseases from pain to cancer. Because more than one thermoTRP may be involved in a given pathological disorder, it would be also important to test the clinical benefit of combined therapies that act simultaneously on several dysfunctional TRP channels. Furthermore, it may appear that small molecules that interact with more than one thermoTRP (for instance on TRPV1 and TRPA1) could exhibit higher therapeutic potential. In this regard, inhibition of both the inflammatory recruitment of TRPV1 and the release of proinflammatory peptides by compound DD04107 [12] contributes to its anti-nociceptive activity against several chronic pain states. We trust that the thermoTRP pharmacological armamentarium will stand up to our therapeutic expectations.

Acknowledgements

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Declaration of interest

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