

Transient Receptor Potential Melastatin 8 Channel (TRPM8) Modulation: Cool Entryway for Treating Pain and Cancer

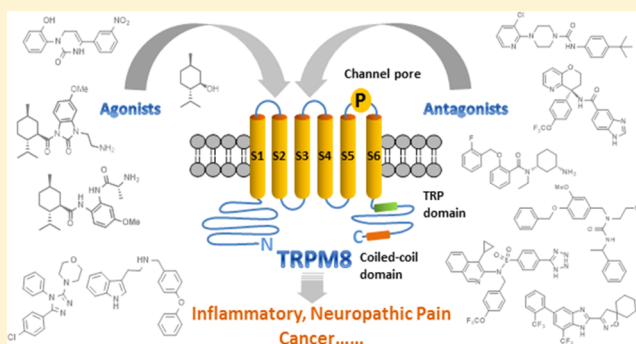
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ABSTRACT: TRPM8 ion channels, the primary cold sensors in humans, are activated by innocuous cooling (<28 °C) and cooling compounds (menthol, icilin) and are implicated in sensing unpleasant cold stimuli as well as in mammalian thermoregulation. Overexpression of these thermoregulators in prostate cancer and in other life-threatening tumors, along with their contribution to an increasing number of pathological conditions, opens a plethora of medicinal chemistry opportunities to develop receptor modulators. This Perspective seeks to describe current known modulators for this ion channel because both agonists and antagonists may be useful for the treatment of most TRPM8-mediated pathologies. We primarily focus on SAR data for the different families of compounds and the pharmacological properties of the most promising ligands. Furthermore, we also address the knowledge about the channel structure, although still in its infancy, and the role of the TRPM8 protein signalplex to channel function and dysfunction. We finally outline the potential future prospects of the challenging TRPM8 drug discovery field.



1. INTRODUCTION

The family of TRP channels comprises 28 members, clustered in eight subfamilies (TRPA, TRPC, TRPM, TRPML, TRPN, TRPP, TRPV, and TRPY) that function as molecular cellular signal integrators.^{1–4} Among them, the so-called thermoTRPs are activated by different temperatures, ranging from painful levels of heat (TRPV1 and TRPV2) to nonpainful warmth (TRPV3 and TRPV4), innocuous cool (TRPM8), and noxious cold (TRPA1). Despite their role as sensors for temperature, these channels also act in response to voltage, mechanical stimuli, and endogenous and exogenous molecules.^{5–9} It has been shown that different syndromes result from mutations or changes in the activities of TRP channels, including sensory deficits, abnormal sensitivity to pain, certain cancers, asthma, and even cardiovascular, gastrointestinal, and neurodegenerative diseases. This fact makes TRP channels exciting targets for drug development, although the efforts made in this field, especially in the search for TRPV1 antagonists, have not yet been fully successful, and the first compound in clinical use is still to come.^{9,10}

Along with TRPV1 and TRPA1, transient receptor potential melastatin 8 (TRPM8, also known as Trp-p8 or MCR1) is one of the most studied thermoTRP channels in relation to its physiological and pathological implications, and the search for new modulators occupies the efforts of different pharmaceutical companies and academic groups. Initially identified from the prostate,¹¹ TRPM8 channels are widely distributed in different

tissues (subsets of sensory neurons innervating skin and mucosae,^{12–14} the male urogenital tract,¹⁵ lung epithelium cells,¹⁶ and artery myocytes¹⁷), although their specific role in many cases still remains to be completely elucidated. This is a Ca²⁺-permeable, nonselective cation channel that exhibits polymodal gating mechanisms, being activated by innocuous cool to cold temperatures (10–28 °C),^{18,19} membrane depolarization,^{20,21} cooling agents like menthol and icilin,^{20,22} and different synthetic molecules.^{12,23–25} The enhanced sensitivity to innocuous cold (allodynia) and to mildly noxious stimuli (hyperalgesia), occurring after nerve injury or inflammation, have been correlated with an augmented expression of TRPM8 in sensory neurons.^{26,27} Other experimental evidence indicates that activation of TRPM8 channels resulted in attenuating pain in inflammatory and acute pain states,²⁸ as described in more detail in the following section. In recent years, as discussed in section 3, the up-regulation of TRPM8 expression has also been correlated with the evolution of androgen-sensitive prostate cells and other malignancies.^{11,29}

As for other ion channels, a point of debate arises from the possibility that both agonists and antagonists can be used for the same therapeutic indication. This comes from the fact that these channels do not have two discrete states only, open

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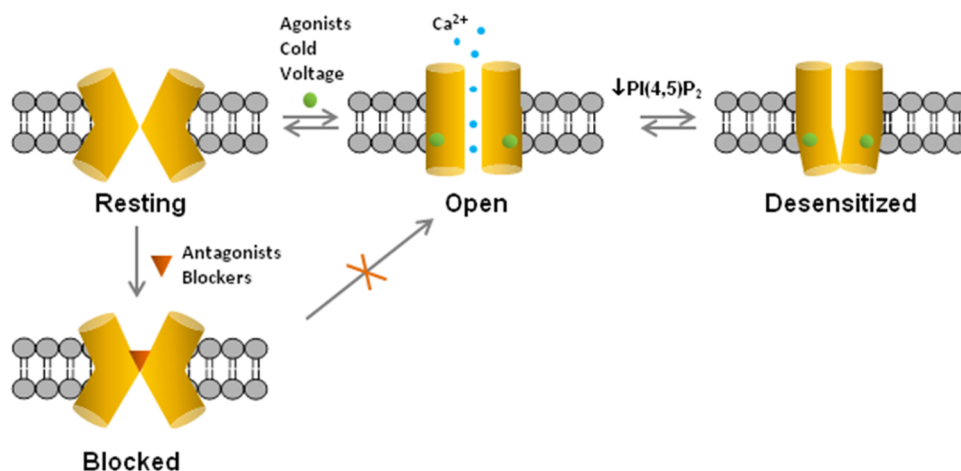


Figure 1. Simplified scheme of TRPM8 channels at different states (resting, open, desensitized, and blocked).

(conducting) and closed (nonconducting), but also an inactivated/desensitized state, which after long exposure to agonists or continuous stimulation are not able to conduct ions across because the channel gate closes (Figure 1).³⁰ For TRP channels, calcium entry into the cell seems to be the primary event leading to desensitization.³⁰ It has been demonstrated that TRPM8 channels are activated by PI(4,5)P₂ through putative interactions with the TRP domain and that cold or menthol increases the apparent affinity of TRPM8 channels for this endogenous ligand.³¹ More importantly, desensitization of the TRPM8 receptor, and probably of other TRP channels, is mediated by PI(4,5)P₂ depletion, triggered by a Ca²⁺-sensitive phospholipase C (most likely PLCδ1) stimulated after cell Ca²⁺ influx and that resulted in PI(4,5)P₂ hydrolysis.^{31,32}

The implications of TRPM8 channels in the above indicated physiopathological conditions underscore their potential as valuable pharmacological targets for therapeutic intervention, not only for the diagnosis/prognosis of prostate cancer and other tumors but also in the search for innovative analgesics and antitumor agents.^{10,33} Although comprehensive reviews on the pharmacology and therapeutic potential of TRPM8 modulators have already appeared,^{12,23–25,33} here we offer a general overview of the field, with focus on medicinal chemistry aspects extracted from primary research articles and patents. Sections 5 and 6 describe the most important agonist and antagonist chemotypes, with indication of structure–activity relationships, whenever possible, and the *in vitro* and *in vivo* pharmacological characterization of the most prominent derivatives. The progression of a few compounds into clinical development will be also commented upon. Some recent contributions related to the indirect assessment of the TRPM8 structure (section 7) and the advanced knowledge of the modulation of these channels by different endogenous molecules (section 8) are also included. This information could provide important clues for upcoming drug discovery programs based in this challenging but high potential therapeutic target.

2. TRPM8, INFLAMMATION AND PAIN

Pain is defined by the International Association for the Study of Pain (IASP) as an “unpleasant sensory and emotional experience associated with actual or potential tissues damage or described in terms of such damage”.³⁴ Pain results from activation of specialized peripheral nerve fibers, called

nociceptors, by a noxious stimulus.³⁵ The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) for the body and the trigeminal ganglion (TG) for the face and have both a peripheral and central axonal branch that innervates their target organ and the spinal cord, respectively. These receptors respond to various external stimuli: mechanical (i.e., pressure, tumor growth, incision) thermal (i.e., hot or cold), or chemical (i.e., ischemia or infection). Pain represents a key evolved survival mechanism to detect potentially damaging stimuli and thereby warn of the risk of injury. Unlike acute pain, pain arising after a lesion or disease of the peripheral or central nervous system (inflammatory and neuropathic pain) is often persistent, a chronic state. Hypersensitivity to mechanical, thermal, and/or chemical stimulation is one of the hallmarks of inflammatory and neuropathic pain, which can manifest in the form of allodynia and/or hyperalgesia.^{36–38} Hypersensitivity has been associated with increased excitability of nociceptor neurons as a result of changes in the expression of ion channels, receptors, and mediator substances involved in the transduction processes.^{36,39} TRP channels are involved in these processes and play an important role in the first step of primary afferent sensitization.⁴⁰ Therefore, their modulation has been considered, in the past decade, a prominent approach to develop new therapies for chronic pain syndromes.^{41,42} Identifying the mechanisms used by nociceptors to transduce noxious cold has lagged behind progress in understanding heat transduction mechanisms.^{43,44}

TRPM8 channels are expressed by ≈5% of all somatosensory neurons, including mostly small-diameter, unmyelinated C-fibers as well as a minor group of lightly myelinated Aδ fibers in dorsal root ganglia (DRG) and trigeminal ganglia (TG) and have been identified as candidates for cold sensing.^{45–48} A subset of the TRPM8-positive cells may coexpress TRPV1 and/or CGRP but are not labeled by other known somatosensory or nociceptive markers (e.g., purinergic receptors, IB4 lectin, or neurotrophin receptors), placing them in their own anatomically and functionally unique subpopulation of primary afferent neurons.^{49,50} *In vivo*, TRPM8 knockout mice are deficient in detecting innocuous cool temperatures and exhibit a partially defective phenotype in responding to both normal (acute) cold sensation and injury-evoked cold hypersensitivity. However, low temperature (>5 °C) induced cold pain was intact in these animals.^{51–54}

Activation of TRPM8 by the agonist menthol or icilin decrease nociceptive response in several animal pain models, including neuropathic pain caused by chronic constriction injury (CCI), inflammatory pain induced by injection of complete Freund's adjuvant (CFA), and a peripheral demyelination model of inflammatory and neuropathic pain.^{27,55} Also, TRPM8 activation with icilin leads to potent anti-inflammatory effects with decreased mucosal damage in two different models of chemically induced colitis (DSS, TNBS) that are not observed in TRPM8-null mice.⁵⁵ Paradoxically, TRPM8 antagonism may also provide relief from some forms of pain. For example, TRPM8 knockout mice failed to develop cold allodynia after chronic constriction injury or CFA injection.⁵⁶ Similarly, a TRPM8 antagonist reduced cold allodynia in a chronic constriction injury model of chronic neuropathic pain, thus recalling the phenotype of genetic deletion.⁵⁷ Lastly, it has been shown that the administration of TRPM8 agonist menthol induced behavioral visceral pain-like responses in a mouse colitis model, which decreased by pretreatment with a specific TRPM8 channel blocker.⁵⁸ Zou et al., observed also an exacerbated cold hypersensitivity in an orofacial neuropathic pain model rat after treatment with menthol, a cold allodynia/hyperalgesia that was alleviated after administration of a TRPM8 inhibitor.⁵⁹

Recently, different work showed that the ablation of TRPM8 afferents in the adult mouse leads to more profound deficits in cold behavior compared to TRPM8 knockout animals.⁶⁰ These results suggest the presence of TRPM8-independent transduction mechanisms in TRPM8 neurons, thereby providing cold sensitivity in the absence of TRPM8 channels. Other receptor molecules that have been linked to cold transduction include TRPA1,⁶¹ channels involved in signal propagation, such as Kv1 and two-pore (K2P) potassium channels,^{62,63} and the sodium channels Nav1.8 and Nav1.9.^{64,65} In addition to acute responses, cold pain associated with inflammation and nerve injury was significantly attenuated in ablated and knockout mice. Moreover, cooling-induced analgesia after nerve injury was abolished in both genotypes. These data confirm previous reports on the involvement of TRPM8 in cooling-induced pain relief²⁷ but show for the first time the absence of analgesia in TRPM8 knockout mice in a neuropathic pain model and demonstrate that this pain-relief mechanism requires signaling via TRPM8 afferent nerve fibers. Importantly, TRPM8-deficient mice retain normal sensitivity to noxious heat and mechanical stimuli, demonstrating specificity of the observed phenotypes for cold sensation as well as functional separation of these somatosensory/nociceptive modalities. This observation was confirmed in a recent study with spinal nerve-ligated (SNL) rats treated with a topical TRPM8 agonist, a 1,2,4-triazole derivative 1 (M8-Ag).⁶⁶ This agonist inhibited neuronal responses to innocuous and noxious cooling in SNL rats, with no effect in the control group, and did not alter neuronal responses to mechanical, heat, or brush stimulation.

Preclinically, TRPM8 has been functionally linked to pain in oxaliplatin-induced⁶⁷ and chronic nerve injury neuropathic pain models,^{28,68} where increased receptor expression and sensitivity to menthol or icilin were observed. In oxaliplatin-treated cancer patients, an increased sensitivity to menthol suggests TRPM8 overexpression or potentiation of its activity.⁶⁹ This preclinical evidence for TRPM8 ion channels as a novel analgesic target has been translated to clinical benefit in patients with cancer-associated neuropathic pain, one of the most feared symptoms in cancer, particularly in terminally ill patients. Recently, Fallon

et al.^{70–72} reported the analgesic benefit from the application of topical menthol to treat chemotherapy-induced peripheral neuropathy (CIPN) and post-mastectomy pain syndrome (PMPS). The first clinical results showed that the 82% of evaluable patients had an improvement in their total pain scores after 4–6 weeks of treatment. Fifty percent had a clinically relevant reduction in pain scores of at least 30%, allowing continuation of life-prolonging treatment.

Finally, patients with idiopathic detrusor overactivity have increased TRPM8 expression in bladder sensory neurons.⁷³ These patients also show involuntary sustained bladder contractions and reduced bladder threshold volumes in response to installation of ice-cold water into the bladder, an effect absent in healthy adults.⁷⁴ In addition to a potential role in cold detection and pain, TRPM8 has been demonstrated preclinically to have a fundamental homeostatic role in the control of core body temperature, with TRPM8 antagonists causing significant hypothermia.⁷⁵ However, Winchester et al.⁷⁶ have demonstrated that the use of selective TRPM8 antagonists induce analgesia in an experimental model of cold pain in humans, with efficacy equivalent to the therapeutically relevant semisynthetic opioid oxycodone and without a concomitant change in core body temperature.

The involvement of TRPM8 channels in migraine is one aspect of the pain field that still remains unclear. Migraine is a very common neurobiological headache disorder associated with nausea, vomiting, and photo- and phonophobia symptoms that is caused by increased excitability of the CNS. It ranks among the world's most disabling medical illnesses.⁷⁷ Indirect evidence, such as expression and function of TRP channels in the cortex and trigeminal afferent fibers, suggests a mechanism for the initiation of migraine pain by activation, among others, of TRP channels on meningeal nociceptors after cortical spreading depression (CSD) or local inflammation.^{78,79}

Identification of common genetic variants associated with migraine susceptibility has been a real breakthrough in the understanding of migraine molecular bases as well as in determining a potential TRPM8–migraine relationship.⁸⁰ The identification of a TRPM8 gene variant (2q37.1, rs10166942) is associated with increased susceptibility to common migraine. In 2015, a re-evaluation of key findings from candidate gene–migraine association studies provided clear evidence for the involvement of TRPM8 in migraine and showed no evidence for the participation of TRPV1 and TRPV3.^{81,82} The exact role of the TRPM8 variant is unknown, as is the association of TRPM8 and migraine. However, these data allow hypothesizing that, regardless of being or not being a migraine specific susceptibility gene, this TRPM8 variant may have a role in more general pain sensitivity mechanisms such as the cutaneous allodynia present in the majority of migraine patients.

3. TRPM8 IN CANCER

Cancer is a life threatening disease and, even though a plethora of cancer drugs exist for various ailment types, current treatment regimens have important associated problems, and for many cancer types there is still no known cure. In this respect, the number of TRP channels that have been linked to carcinogenesis and tumor progression has considerably increased in recent years.^{83–87} TRPM8 channels are overexpressed in a number of malignant tumors, especially solid tumors, being involved in different stages of cancer development, proliferation, migration, and invasion.^{88,89}

In 2001, L. Tsavaler and co-workers reported the identification of a new gene *trp-p8*, which was preferentially expressed in normal prostate epithelial tissue and up-regulated in prostate tumor cells.¹¹ The polypeptide (1104-mer) encoded by this gene showed a very high homology to a human-melastatin TRP channel identified a year later by G. Reid and M. L. Fonta,⁹⁰ who described a new ionic channel activated by cool and sensitized by menthol, the TRPM8 channel.¹¹ Tsavaler et al. observed that the overexpression of TRPM8 in prostate cells correlates with rate of prostate cancer progression. Their studies also revealed the overexpression of *trp-p8* gene in a number of neoplastic tissues other than prostatic, like breast, colon, lung, and skin.

The expression of TRPM8 in prostate cells is closely related to androgen receptor (AR) activity, being clearly down-regulated in cells in which this activity is cut out.^{91–93} In fact, Prevarskaya and co-workers demonstrated that TRPM8 expression is AR controlled in normal, hyperplastic, and cancerous human prostate apical cells, while the loss of TRPM8 expression can be connected to either a high grade of tumor development, with undifferentiated cells (metastasis), or to a AR-independent tumor, where androgen receptor is not expressed.⁹³ Moreover, TRPM8 expression has been observed in the androgen-dependent prostate cancer (PCa) cell line LNCaP but not in the androgen-independent PCa cell lines PC-3 and DU-145.^{11,91} However, expression of TRPM8 in the androgen-independent PC-3 cells was reported later by Zhang et al.⁹² Several experiments modulating TRPM8 expression in the androgen-sensitive cell line LNCaP by using menthol and cooling as activators of the channel, and capsazepine as channel antagonist, provided evidence of the role of TRPM8 as mediator of Ca²⁺ influx and release from intracellular stores, in both plasma membranes and in the endoplasmic reticulum.^{92,94} These results together with those of siRNA experiments confirmed that normal function of TRPM8 is required for tumor cell survival. Later research showed that TRPM8 is expressed both in the plasma membrane, TRPM8_{PM}, and in the endoplasmic reticulum, TRPM8_{ER}. TRPM8_{PM} channel is found only in highly differentiated prostate epithelial cells, while TRPM8_{ER} is not dependent on the differentiation status. Further experiments revealed that functional AR seems to be mandatory for TRPM8_{PM} activity, while TRPM8_{ER} activity is independent. They also observed that the transition to PCa upregulated TRPM8 expression is both in plasma membrane and reticulum. It was suggested that TRPM8_{PM} is important for the Ca²⁺ signaling involved in proliferation, while TRPM8_{ER} can be considered essential in controlling the growth of advanced PCa metastatic cells.⁹⁵ This hypothesis is consistent with the model proposed by Hendriksen et al., in which progression from high-grade PCa to metastasis is mediated by selective downregulation of the AR pathway genes.⁹⁶ It seems clear then, the benefit of androgen-ablation therapy in early stages of prostate cancer, but still there are doubts about the role of TRPM8 in the androgen-independent cell lines and in advanced grade tumors. Recent work of Zhu et al. proved that artificial overexpression of TRPM8 in the AR-independent cancer PC-3 cell line, in vivo, has a negative effect on the proliferation and angiogenesis progression. These results open new opportunities for the potentiation of TRPM8 channels as treatment for patients in advanced stages of cancer and metastasis.⁹⁷

Despite all these results that link TRPM8 to prostate carcinogenesis and tumor progression, there was still

controversy around whether the aberrant expression of TRPM8 in PCa is the origin or the result of tumor development. Pardo and co-workers designed several experiments that shed light on this question. They studied the consequences of TRPM8 silencing, both pharmacologically blocking and siRNA experiments, and showed that proliferation was reduced in all tumor prostate cells tested, but not in normal prostate cells. It seems that the proliferation of normal cells is not affected by TRPM8 downregulation. These interesting results support the idea that TRPM8 plays a tumor specific role in tumor proliferation instead of being a mere consequence of tumor development.⁹⁸

Concerning diagnoses, the biomarker currently used in clinic for prostate cancer, is the prostate-specific antigen (PSA). However, its usefulness is questionable because PSA can be found in serum samples of both PCa patients and patients with benign prostatic hyperplasia. Moreover, the PSA levels are quite similar in patients with localized/quiescent PCa and in patients with metastasis.⁹⁹ The higher presence of TRPM8 compared to PSA in malignant tissue samples in comparison to non-malignant ones suggests TRPM8 as a useful biomarker for PCa diagnosis.¹⁰⁰ Bai et al. found that TRPM8 levels in urine and blood of patients with metastasis were significantly higher than in patients with localized cancer that have undergone radical prostatectomy as well as than those of healthy men. These findings suggest that TRPM8 levels in body fluids can be a useful prognosis biomarker, which will allow clinically localized and metastatic prostate cancer to be distinguished. Their experiments also confirmed the role of TRPM8 in androgen regulated proliferation of prostate cancer cells, although they are in some ways contradictory with the results found for TRPM8 expression in organ tissues.¹⁰¹

The number of tumors in which upregulation of TRPM8 has been detected has increased in the past decade. Tsavaler et al. already reported it in lung, skin, breast, and colon cancer tissues.¹¹ Since then, it has also been identified in neuroblastoma, osteosarcoma, urothelial carcinoma, in which it was associated with poor survival, and pancreatic adenocarcinoma, where it was shown to correlate with tumor stages.⁸⁹ Chodon et al. found correlation between TRPM8 expression and the breast cancer grade in the MCF-7 cell line, and interestingly they also found correlation with estrogen receptor alpha (ER α) levels.¹⁰² This proves that the regulation of TRPM8 in breast cancer cells is hormonally dependent, which is in agreement with the androgen-dependent regulation of TRPM8 in prostate cancer cells.^{11,93} Additionally, experiments performed by Okamoto et al. suggest that TRPM8 channels are functional on the plasma membranes in human oral squamous carcinoma cell lines HSC3 and HSC4.¹⁰³ Experiments to assess the biological response to TRPM8 agonists and antagonists demonstrated that the stimulation of TRPM8 increases MMP-9 activity, while its blockade has the opposite effect, which is consistent with the increased metastatic potential induced by TRPM8.¹⁰³ The group of Yee has demonstrated that TRPM8 is consistently overexpressed in human pancreatic adenocarcinoma cell lines and tissues and that patients with this type of tumor aberrantly exhibit immunoreactivity against the TRPM8 channel.^{104,105} They also showed that RNAi-mediated silencing of the *TRPM8* gene stimulates replicative senescence, thus suggesting that abnormally expressed TRPM8 channels could contribute to pancreatic tumorigenesis.¹⁰⁵

4. TRPM8 IN OTHER PATHOLOGICAL CONDITIONS

Apart from inflammation and cancer, TRPM8 channels are related to other pathological conditions, although they have been less studied and their role is less established.⁸⁵

For instance, there is evidence that links TRPM8 channels to respiratory disorders. Thus, the mitigating effects of menthol in the irritant responses to certain tobacco smoke constituents had been reported.^{106,107} These effects were reversed by treatment with a TRPM8 antagonist. Stimulation of TRPM8 channels can be harmful in patients with asthma and also with chronic obstructive pulmonary disease (COPD). In fact, cold-induced airway hyperresponsiveness can either induce asthma or exacerbate COPD and asthma, increasing morbidity. Cold-air inhalation causes airway constriction and can induce asthma associated with, for instance, winter sports.^{107,108} These observations suggest the implication of TRPM8 channels both in physiological and pathological respiratory processes.

TRPM8 channels are also implicated, together with other TRPs (mainly TRPV1), in bladder sensation, showing high potential as therapeutic targets for lower urinary tract dysfunction (LUTd).¹⁰⁹ Several studies suggest the relevant role of TRPM8 in disorders like bladder pain syndrome and bladder hyperactivity.^{109,110} The observation of upregulation of TRPM8 in bladder tissues of patients with painful bladder syndrome or idiopathic detrusor overactivity (IDO), in comparison to healthy controls, points in this direction, but the correlation of TRPM8 expression and these abnormalities still remain unclear.¹¹¹

Concerning obesity, there are two types of adipose tissue in mammals, white (WAT) and brown (BAT). BAT is in charge of energy expenditure through the activation of the UCP1 protein, inducing thermogenesis, which can prevent development of obesity. Work reported by Ma et al. showed that menthol promotes the overexpression of UCP1 in cultured brown adipocytes from wild-type mice but not in TRPM8-deficient mice.¹¹² Recent work reported by Rossato et al.¹¹³ showed that menthol or icilin activation of TRPM8 in WAT enhanced UCP1 expression, having as a consequence an increase in glucose uptake and heat production. The authors interpret these results as induction of the brown phenotype in white adipocytes, which points to TRPM8 as a potential target for the treatment of obesity.¹¹³ Moreover, certain connections between TRPM8 gene polymorphisms and obesity-related metabolic syndrome have also been established.¹¹⁴

TRPM8 has also been detected in blood vessels, being the most abundant among the TRP channels in these tissues.^{115,116} TRPM8 expression has been observed in several rat arteries, and its activation by menthol or icilin promoted vasodilation. This vasodilation, induced by systemic administration of menthol, may be responsible for the fall in arterial blood pressure observed in an in vivo study in rabbits and cats. It has also been reported that the vasoconstriction or vasodilation depends on the previous vasomotor tone and that it is mainly mediated by TRPM8 activation on smooth muscle cells.¹⁷ Although results of other research in this area are in agreement with these observations,^{117–119} further research is still required to confirm the role of TRPM8 in vasodilation-related processes.

Experimental studies from the group of Belmonte and co-workers showed that reduction of temperature and subtle osmolality increases can stimulate DRG and TG neurons expressing cold-sensitive TRPM8 channels, which act as regulators of surface wetness of exposed body mucosae, like

the eye.^{120,121} Therefore, another function of the TRPM8-expressing neurons is to act as regulators of ocular wetness through the modulation of the blink rate, thus predicting new opportunities for the understanding and treatment of dry eye, a common ophthalmological disorder that increased with age.

5. TRPM8 AGONIST CHEMOTYPES

5.1. Menthol and Derivatives. Chemical modification of endogenous ligands or natural products, capable of interfering with a given receptor, is a widely used strategy in medicinal chemistry programs.^{122,123} Therefore, not surprisingly, the exploration of menthol-derived compounds emerged shortly after the discovery that menthol activates TRPM8 channels,⁴⁵ with ester derivatives like Frescolat ML,¹²⁴ carboxamide analogues such as **2** (WS-12, Figure 2),¹²⁵ described as the first high-affinity TRPM8 ligand,¹²⁵ and fluorinated analogues claimed as the basis for diagnostic agents.^{126,127} Sherkheli et al. also quantified the activation of TRPM8 channel by several

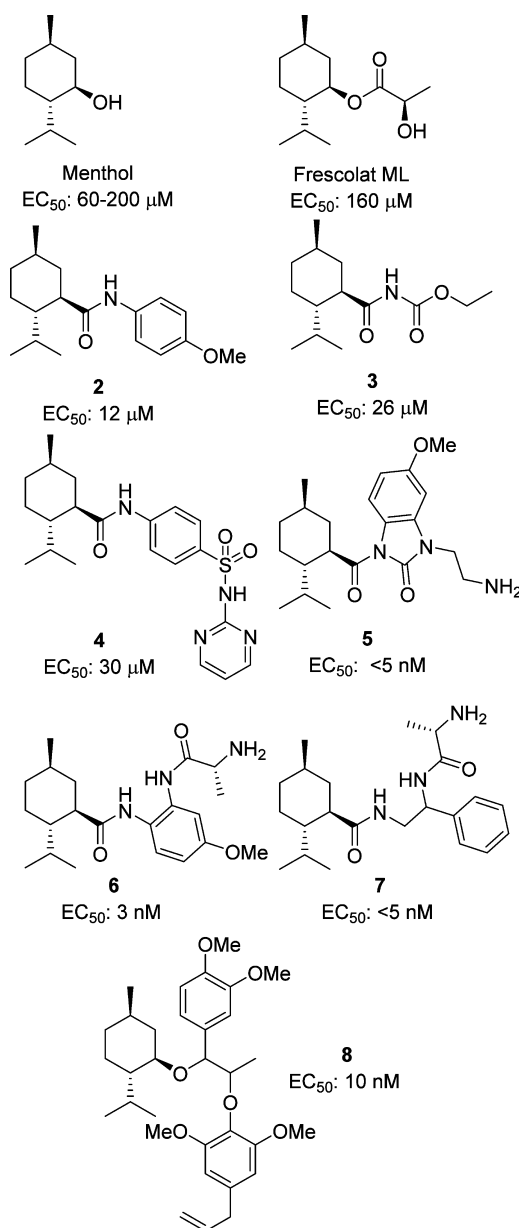


Figure 2. Menthol and derived TRPM8 agonists.

menthol carboxamide derivatives, some of which were initially prepared by Wei, E.T. et al., finding up to 10-fold lower EC_{50} values than of the parent menthol.¹²⁸ It seems that the cyclohexyl ring, characteristic of menthol, is behind the enhanced selectivity of the most representative derivatives in this series, 2, 3 (WS-5),¹²⁸ and 4 (CPS-125)¹²⁸ (Figure 2) when compared to lower homologues and linear analogues.

Dendreon Corporation described related derivatives in several patents. Their menthol-derived small-molecules, illustrated by compounds 5–7, are able to activate TRPM8 with nanomolar potency.^{129,130} In this family of compounds, the substituted cyclohexyl moiety and the free amino groups appear to be structural requirements for good agonist activity, while the carboxamide substituent can be replaced by bezimidazolone (D3263, 5), phenyl (6), and substituted ethyl (7) derivatives. In addition to increasing Ca^{2+} entry in CHO cells expressing TRPM8, selected compounds from this series prove to stimulate cancer cell apoptosis and substantially reduce xenograft tumors in animal models of human prostate cancer without showing apparent toxicity. Compound 5, which showed good oral bioavailability, entered phase I clinical trials in 2009 for the assessment of safety, determination of the maximum tolerated dose, and characterization of the pharmacokinetic profile after a single dose and repeated daily dosing (NCT00839631). In men with advanced prostate cancer, preliminary results indicated that this compound is able to stabilize the disease. Apart from elevations in Troponin-T, no signs of cardiac toxicity were observed (maximum tolerated dose: 100 mg/day).¹³¹ The last update of data at ClinicalTrials.gov was on April 30th, 2012. Some preclinical data also demonstrated the potential of 5 to treat benign prostatic hyperplasia by itself or in combination with the synthetic 5 α -reductase inhibitor finasteride (additive effect).

Some diastereomeric menthol ethers, like 8, have been described in a recent Japanese patent to display nanomolar activation of TRPM8 receptors.¹³² Menthol-related cyclohexane–hydantoin spirocyclic derivatives, as well as pyrazolyl-5-one and pyrimidinetrione analogues, have also been claimed as TRPM8 effectors (structures not disclosed).¹³³

In addition to numerous studies on its applications in cosmetics, which are not described here, there are several clinical trials evaluating the usefulness of menthol alone or in combination with other active principles as a therapeutic agent. A few of these studies are recorded here, including their ClinicalTrials.gov identifier number. Thus, menthol alone or Biofreeze, a topical lotion containing menthol and alcamphor, is being examined for treating pain symptoms in slaughterhouse workers with symptoms of carpal tunnel syndrome (NCT01716767), as a topical counter-irritant on cutaneous pain and hyperalgesia provoked by topical application of TRPA1-agonists (NCT02653703), to alleviate shoulder pain (NCT01827306), musculoskeletal disorders associated with neck pain (NCT01542827), and persistent neuropathic pain from chemotherapy among breast and colorectal cancer patients (NCT01855607).

An invention by researchers at the Edinburgh University also claims that activation of TRPM8 channels induces analgesia in chronic neuropathic pain states.¹³⁴ Therefore, activation agents including menthol and menthyl derivatives, among others, could be useful for the treatment of diseases and conditions which involve neuropathic pain, such as that induced by trauma, demyelination, cancer or inflammatory states, phantom-limb, back and bone pain, and chemotherapy neuropathy.

As a potential alternative for the prevention and treatment of hypertension, the Third Military Medical University is evaluating the effects of menthol on blood pressure in hypertensive patients (a double-blind phase II clinical study, NCT01408446).

Studies performed with two different human melanoma cell lines expressing TRPM8 reported the dose-dependent in vitro cytotoxic effects of menthol.^{29,135} Chanchao and co-workers had in fact stated an effectiveness comparable with that of 5-FU, with the advantage that menthol has no toxicity while the side effects of 5-FU are multiple and non-negligible.¹³⁵ Menthol also showed a significant decrease of viability of a bladder cell line T24, inducing cell death through TRPM8 activation.¹³⁶

Inventors from the Miguel Hernández University have patented that the TRPM8 receptor is linked to the control of lacrimation and that its activation with agonists, like menthol, increased tearing, thus leading to a promising remedial for the dry eye condition.¹³⁷ As agonists they studied menthol, frescolat, geraniol, eucaliptol, icilin, and other cooling agents. The invention also relates the use of TRPM8 antagonists as useful treatment for epiphora (excessive tear production).

The activity and interest of various natural and synthetic menthol derivatives, along with other cooling compounds that behave as activators of thermoTRPM8, have been compiled in a 2012 comprehensive review.¹³⁸

5.2. Pyrimidine Derivatives. Icilin is a synthetic compound that behaves as an agonist of TRPM8 (about 200-fold more potent than menthol)^{26,139} but also suppresses TRPM8 current activation by other agonists²² and modulates TRPV3¹⁴⁰ and TRPA1.²² Therefore, icilin has been and continues being used as a pharmacological tool for studying TRP channels.^{141–143} Icilin has anti-inflammatory activity,⁵⁵ has been claimed for the treatment of different neurological disorders, as menthol and linalool,¹⁴⁴ and reduces the activity and expression levels of E2F1,¹⁴⁵ a transcription factor controlling the cell cycle in prostate cancer cells. The group of De Petrocellis explored the preparation of new analogues by modification of the pyrimidin-2-one central scaffold of icilin in an attempt to improve the therapeutic potential.¹⁴⁶ Work at the three rings of the icilin molecule demonstrated very narrow limits for modification. SAR studies point to the importance of the presence and position of the *o*-OH and *m*-NO₂ substituents on the distal phenyl groups. The phenyl ring at the 1-position of the pyrimidine central scaffold tolerates some variations, like the addition of a methyl group in relative *para* position to the OH or the presence of *m*-halogens (I < Br > Cl) in non-OH analogues but always provided less potent compounds (Figure 3). Both a lower homologue of the central ring and conformationally restricted tricyclic derivatives were totally inactive.¹⁴⁶

Structurally unrelated pyrimidine derivatives, exemplified by compound 9, produce cooling effects on skin and mucous

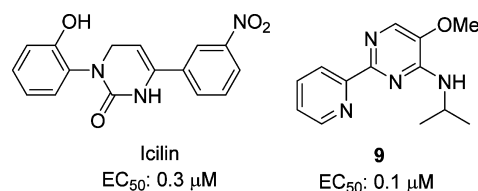


Figure 3. Pyrimidine-derived TRPM8 agonists.

membranes through the interaction with the TRPM8 receptor (submicromolar potencies).¹⁴⁷

5.3. Five-Membered Heterocycle Derivatives. In a recent paper by R. Patel and co-workers, compound **1**, with a 1,2,4-triazole central scaffold, was described to display more potent antihyperalgesic effects than menthol in a rat model of neuropathic pain.⁶⁶ This compound activates TRPM8 channels with a 45 nM EC₅₀ value and also TRPA1 thermoreceptors although with lower potency (4 μM). It attenuates neuronal responses after innocuous and noxious cooling and reverses hypersensitivity to acetone-induced cooling in spinal nerve-ligated (SNL) rats. Results from this study led the authors to suggest that TRPM8 agonists might be beneficial in neuropathy without affecting normal cold sensation.

The group of Ostacolo described the preparation of a family of isoxazole derivatives and their preliminary characterization as TRPM8 agonists.¹⁴⁸ Some of the isoxazoles were more potent than menthol (up to 200-fold), but most of them display lower efficacy (Figure 4). A few compounds, like **10**, elicit stronger antiallodynic responses than menthol in rats with chronic constriction injury of the sciatic nerve.

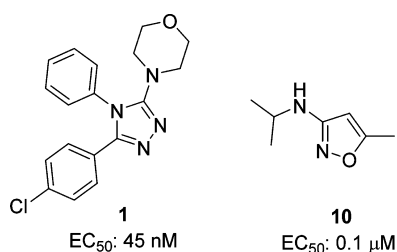


Figure 4. 1,2,4-Triazolyl- and isoxazolyl-derived TRPM8 agonists.

5.4. Other TRPM8 Agonists. Different plant-derived natural products have been identified as TRPM8 agonists, including eugenol,¹² eucalyptol,¹²⁴ geraniol,¹²⁴ and neferine,¹⁴⁹ all of them displaying moderate to low potency ranging from μM to mM EC₅₀ values (Figure 5). The activation/sensitization to cold of mammalian TRPM8 seems to be responsible for the enhancement of innocuous cold and noxious cold sensations induced by camphor, which also potentiates heat sensations through activation of TRPV1 and TRPV3 channels.¹⁵⁰

5-Methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (**11**), isolated from roasted malt,¹⁵¹ and some hybrid derivatives¹⁵² were identified as intense cooling compounds. Although authors mentioned TRPM8 channels as cold-sensitive receptors, no data is provided regarding the direct action of these compounds on the mentioned thermoreceptor.

In patch-clamp experiments, eucalyptol, from eucalyptus oil, evokes inward currents in TRPM8-expressing cells and also behaves as a TRPA1 antagonist, while its regioisomer 1,4-cineole activates both receptor types.¹⁵³ Studying different small-molecules BASF SE identified new chemotypes able to activate TRPM8 channels.^{147,154} Thus, a series of tetrahydrofuro[3',4':5,6]pyrido[2,1-*a*]isoquinoline-dione derivatives, exemplified by compound **12**, and different secondary caffeic-derived amides, like derivative **13**, show submicromolar potencies (Figure 5).

We described in a recent patent a series of new di-isopropyl-phosphinoyl alkene (DIPA) compounds for the topical treatment of sensory discomfort (itch, dermatitis, psoriasis, and ocular discomfort, among others).¹⁵⁵ In this series, the *i*Pr group is preferred over *sec*- or *iso*-butyl homologues and

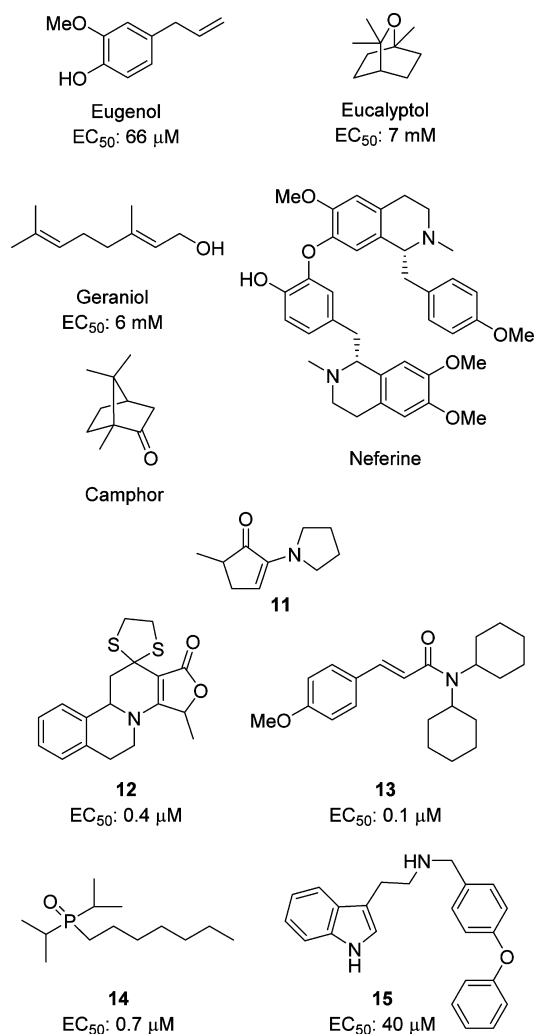


Figure 5. Natural products and other TRPM8 agonists.

optimal length for the linear aliphatic alkene is 6–9 carbon atoms. These compounds elicit submicromolar EC₅₀ values and >5-fold increased potency compared to menthol (i.e., **14**, Figure 5). A related phosphine oxide, containing *sec*-butyl substituents instead of *i*Pr, was previously studied, showing a dose-dependent and reversible activation of TRPM8 with an EC₅₀ value of 4.1 μM.¹⁵⁶

While studying a series of *N*-aryl and *N*-benzyl triptamine derivatives to explore the structural requirements determining TRPM8 modulation, Bertamino and co-workers described a new compound able to activate the channels under investigation (**15**, Figure 5).¹⁵⁷ Thus, tryptamine derivative **15** displays significantly higher potency (EC₅₀ = 40 μM) than menthol and a similar efficacy under patch-clamp experiments.

6. TRPM8 ANTAGONIST CHEMOTYPES

During the past decade, several pharmaceutical companies and a few academic groups have made enormous efforts in the search for new antagonists of the TRPM8 channel. The different families of compounds described to date are recorded in this section, with an intended rational classification, based first on the central structural scaffold and then, if necessary, subcategorizing them by other secondary motifs.

6.1. *N*-Heteroaryl-*N*-benzyl Sulfonamides. **6.1.1. Benzothioephene Derivatives.** Several patents from Janssen claimed a

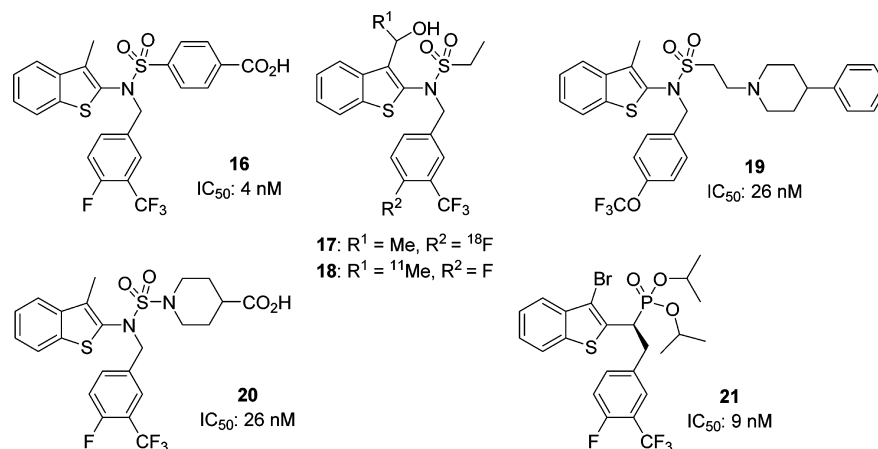


Figure 6. TRPM8 antagonists: Compilation of representative *N*-benzothiophene sulfonamides and related compounds.

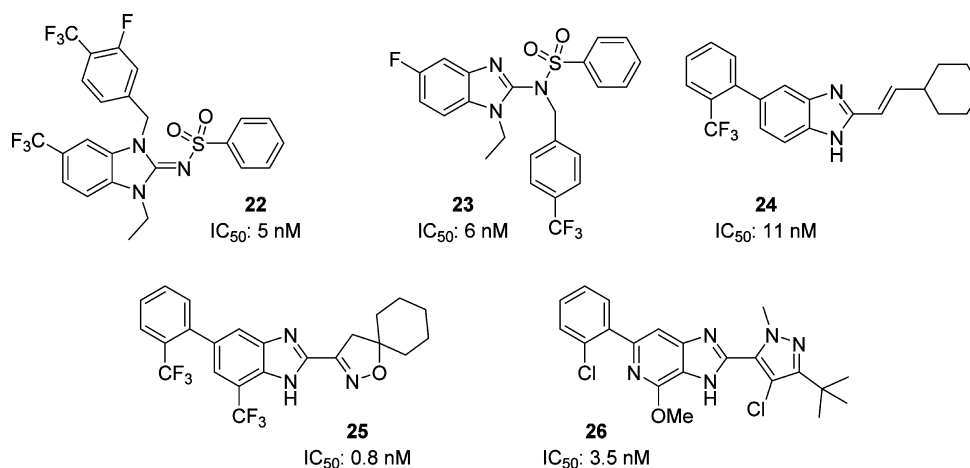


Figure 7. TRPM8 antagonists: representative *N*-benzimidazole sulfonamides and related compounds.

big family of benzothiophene-bearing *N*-benzyl sulfonamides (examples 16–20) and related *N,N'*-disubstituted analogues (21) as potent TRPM8 antagonists (Figure 6). A first patent describes 850 benzo[*b*]thiophene derivatives and some azanalogues through the combination of different substituents at position 3 of the bicyclic skeleton at the *N*-benzyl substituent and at the sulfonyl moiety.¹⁵⁸ Preliminary HTS evaluation was performed in an *in vitro* canine TRPM8 functional assay, measuring the changes produced by compounds in the intracellular Ca^{2+} concentration induced by the agonist (menthol or icilin). From the results of this assay, it can be said that chlorine, bromine, or small alkyl groups are preferred at position 3, while at the benzyl moiety a *m*- or *p*-fluorine or trifluoromethyl group, or a combination of them, favored TRPM8 antagonist properties. Either alkyl or aryl groups at the sulfonyl moiety are well tolerated. The antagonist properties for the most promising compounds were confirmed through patch clamp experiments. Thus, representative compound 16 exhibits high potency either in the hTRPM8 HTS assay (IC_{50} : 4 nM) or in patch clamp experiments (IC_{50} : 1.1 and 2.3 nM, after menthol and cold activation, respectively). The potent TRPM8 antagonist properties were confirmed in different animal models by reversing carrageenan-induced heat hypersensitivity (64% at 30 mg/kg, po) and CFA-induced thermal and mechanical hyperalgesia (91% at 10 mg/kg, po). This compound was also able to reduce icilin-stimulated “wet-dog” shakes (WDS) in rats with an ED_{50} of 2.5 mg/kg, cold-evoked

cardiovascular pressor responses (75% of inhibition), and acetone-induced hypersensitivity, an *in vivo* model of neuropathic pain (69% pain relief, 30 mg/kg, po). A ${}^{18}F$ labeled analogue of 16, its related derivative 17, and the ${}^{11}C$ labeled compound 18 were also protected as useful tools for the labeling and imaging of TRPM8 channels.¹⁵⁹ One year after the initial patent in this series, Janssen published an extension that covered potent aminoethylsulfonyl derivatives, like 19, and some less active naphthalene analogues.¹⁶⁰ Closely related sulfamides, exemplified by compound 20 in Figure 6, also afforded TRPM8 antagonists with nanomolar potencies in the HTS assay, although their efficacy in animal models was not revealed.¹⁶¹

At the beginning of 2012, Janssen published the US patent 20120053347 describing a new series of benzothiophene derivatives, resembling to some extent previous series in this section but bearing a phosphonate moiety instead of the initial sulfonamide.¹⁶² The 3-bromothiophene 21, with a 9 nM IC_{50} value in the HTS functional assay and good antagonist *in vivo* activities, is one of the most active compounds in this series. Thus, this compound reverses up to 80% the icilin-induced behaviors in rats (30 mg/kg, po), and several compounds within this family are also active in the CFA-induced model of hyperalgesia (ED_{50} up to 5.2 mg/kg). As it could be expected, the configuration of the asymmetric center of 21 is important for the antagonistic activity, with its *R*-enantiomer showing one order of magnitude lower potency. An initial SAR study,

including a part of the compounds covered by the patent, has been published and highlights the preference for small alkyl groups at position 3 of the benzothiazophene ring and either ethyl or isopropyl esters alongside the phosphonate.¹⁶³

6.1.2. Benzimidazole Derivatives. A second family of TRPM8 antagonists with a central *N*-sulfonamide motif includes compounds substituted with a benzimidazole moiety (examples 22, 23, Figure 7). Two patent applications protect the TRPM8 antagonist activity of the mixtures of 1,3- and 1,2-*N*-disubstituted derivatives, initially obtained after benzylation of the corresponding 1-monosubstituted analogues.^{164,165} In these series, lower alkyl groups at the N1 atom are well tolerated (ethyl preferred), and one or two fluorine atoms or a CF₃, or a combination of both, at the phenyl ring of the benzyl moiety increase potencies with respect to unsubstituted analogues, as it was also found for the incorporation of F or CF₃ at position 5 of the benzimidazole nucleus.

In general, the best compounds within these collections were less potent than 2-alkene substituted benzimidazole related derivatives, which displayed nanomolar potency in the canine hTRPM8 Ca²⁺ flux assay.¹⁶⁶ A representative compound in this new series, 24, is able to reverse cold hypersensitivity in a model of neuropathic pain (oral 10 mg/kg, 91%) and icilin-induced WDS (oral 30 mg/kg, 98%), even if it does not show optimal PK properties regarding metabolism.¹⁶⁷

Analogues of 24 bearing spiroheterocyclic systems in position 2 of the central skeleton provided highly potent TRPM8 antagonists, with IC₅₀ values in the subnanomolar range.¹⁶⁸ In this series, the dihydroisoxazole ring is preferred over the oxazole and imidazole at the spiro moiety. Compound 25 inhibits cold-stimulated currents in HEK cells expressing hTRPM8, reverses icilin-induced behaviors in rats (98%, 10 mg/kg, po), and reduces neuropathic pain (acetone-mediated hypersensitivity, 83%). This compound exhibits a good pharmacokinetic profile, with excellent oral bioavailability and relatively high metabolic stability.⁵⁷

Related azabenzimidazole derivatives, substituted with different five-membered ring heterocycles at position 2, here represented by compound 26, are also claimed in a patent application.¹⁶⁹ Chlorine, alkyloxy, and substituted amines are the groups of choice at the 7-position, while pyrazole ring provided the most potent compounds among all the 2-pentaheterocycles incorporated in this series.

6.1.3. Pyridine Derivatives. Two patents on *N*-benzyl-*N*-pyridylsulfonamide structures, similar to those described in the previous sections, have been disclosed by RaQualia Pharma.^{170,171} In a first series of 199 compounds, exemplified by the *N*-pyridine derivative 27 (RQ00203078, Figure 8),¹⁷⁰ the observed activities indicated the need for a 3-Cl atom on the pyridine ring, combined with CF₃, CH₃, or Cl groups at position 5. In addition, the incorporation of different substituents at the phenyl ring of the benzyl moiety either maintain or improve the activity, as it does replacement of the carboxylic acid by a hydroxymethyl group or differently substituted carboxamides. In the second set of compounds, the phenyl ring of the benzyl substituent has successfully been replaced by diverse heterobicycles, like benzofuran, indole, indazole, and benzotriazole derivatives. In these patents, the inventors claimed good in vitro antagonist properties in HEK293 and human malignant melanoma cell lines and also indicated potent activities in different in vivo models, although there is no disclosed data. They also claim beneficial effects of their compounds for neuropathic pain of cold- and static-

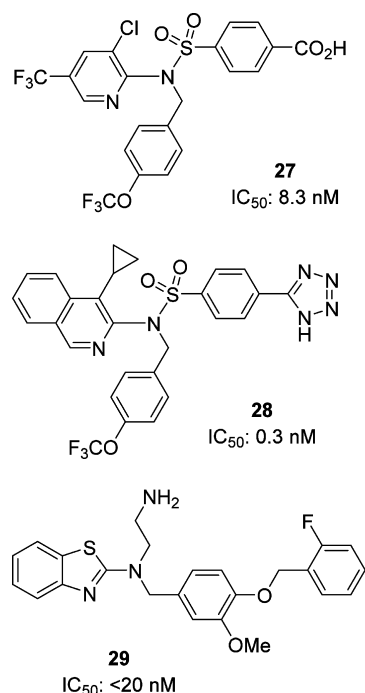


Figure 8. TRPM8 antagonists: representative pyridine-derived *N*-heteroaryl sulfonamides and related compounds.

allodynia, icilin-caused WDS, oxaliplatin-induced pain, and overactive bladder in rats suffering from cystitis. A recent paper by this group reports that compound 27 attenuates the shaking behavior with an ED₅₀ of 0.65 mg/kg (po), indicating that this sulfonamide derivative is a potent and orally active TRPM8 antagonist. This compound, which is commercially available as a pharmacological tool, is also selective over other TRP channels (A1, V1, V4) and has been selected as an advanced compound for further investigation.¹⁷²

Related analogues having a condensed pyridine moiety (isoquinoline) have been patented by Mitsubishi Tanabe Pharma Corporation (see compound 28 in Figure 8).¹⁷³ Among these very potent compounds, the presence of the cyclopropyl group is an important structural requirement, while the tetrazole group (a carboxylic acid isostere) can be substituted by other nitrogen-containing heterocycles, like tetrazolone, 1,2,4-triazole, and 1,2,4-oxadiazole-5-one, and even by CO₂H, CO₂Me, and CN groups without significant loss of antagonist activity. Similarly, different groups are allowed at the ortho position of the tetrazole moiety, with Me, OMe, Cl, and NMe₂ within the preferred ones.

In 2010, Glenmark patented a new family of compounds, which keeps an *N*-heteroaryl (benzothiazole or benzoxazole) and an *N*-benzyl moiety on a nitrogen atom but differs from previous series in the third *N*-substituent.¹⁷⁴ Instead of the sulfonyl appendage, these tertiary amines bear an alkylamino moiety, as illustrated by compound 29. In this series, unsubstituted benzo[*d*]thiazol-2-yl and benzo[*d*]oxazol-2-yl led to potent derivatives and some aza analogues are acceptable. The presence of F atoms (2- or 2,4-) or a 2-methyl group in the benzyloxy moiety is coupled to high activity, as the *m*-OMe group on the *N*-benzyl substituent.

6.2. Urea Derivatives. The (3-chloro-pyridin-2-yl)-piperazine derived urea (BCTC, 38) has been one of the first TRPM8 antagonists used for studying the pharmacology of this channel (IC₅₀: 0.8 μM, Figure 9).¹²⁴ This compound, which is

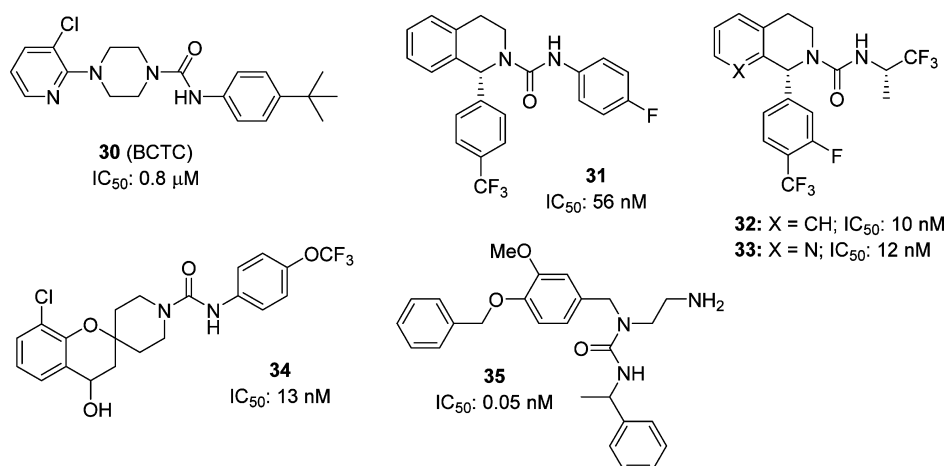


Figure 9. TRPM8 antagonists: representative urea derivatives.

commercially available, was initially prepared as a TRPV1 modulator (IC₅₀: 35 nM) and therefore is a nonselective TRPM8 antagonist. Probably, in the search for more potent and selective TRPM8 antagonists, this compound inspired the design and synthesis of other urea derivatives as those described in the two following subsections.

6.2.1. Tetrahydroisoquinolines and Aza-analogues. In 2009, Amgen Inc. published two patents describing tetrahydroisoquinolines and aza-analogues as TRPM8 antagonists and claimed their potential value for the treatment of diverse pathologic conditions.^{175,176} In their efforts to validate TRPM8 channel as a novel target for pain, they discovered a tetrahydrothienopyridine derivative after a high-throughput screening campaign. The initial hit was then modified to lead to tetrahydroisoquinoline-urea analogues with higher potency and increased metabolic stability.¹⁷⁷ In this series, modification at the *N*-phenyl ring is restricted to the incorporation of F atoms or its replacement by unsubstituted pyridine rings. Although with suboptimal pharmacokinetic properties, compound **31** (Figure 9) reduces icilin-induced WDS in a dose-dependent manner (80%, 30 mg/kg, po). Further optimization of potency and pharmacokinetic properties afforded analogues **32** and **33**, with hTRPM8 IC₅₀ values of 10 and 12 nM, respectively, and balanced properties that made them ideal for in vivo evaluation.¹⁷⁸ In the icilin induced rat “wet dog” shakes (WDS) model, compound **32** achieves maximal inhibition at 10 mg/kg (po), while **33** results in 68% inhibition of WDS at 1 mg/kg and the icilin effect was completely blocked at 3 mg/kg. Compared to the initial hit **31**, the higher in vivo activity of **33** was attributed to the increased drug plasma levels, which results from the improved pharmacokinetic properties of the latter molecule. This compound was selected for further progression in preclinical models of pain.

6.2.2. Spiro[chromene-2,4'-piperidine] Derivatives. Following an analogue-based rational design, synthesis, and screening, Glenmark Pharmaceuticals discovered a set of spiro[chromene-2,4'-piperidine]phenylureas that behave as TRPM8 antagonists (see derivative **34** in Figure 9).^{179,180} Selected compound **34** shows nanomolar in vitro TRPM8 potency, moderate to high metabolic stability in different species, and quantifiable levels in plasma up to 8 h postdose following oral administration. The *R*-stereoisomer is more potent and more selective over other TRP channels than its optical *S*-antipode. In in vivo models of neuropathic pain, **34** is able to inhibit the acetone-induced

allodynia in rats (74%, 10 mg/kg, po), significantly reduced the icilin-induced WDS in mice (72% efficacy, 30 mg/kg, po), and produced a dose-dependent inhibition of paw licking after oxaliplatin injury (100% effect, 30 mg/kg, po).¹⁸⁰

6.2.3. Other Urea Derivatives. Very potent TRPM8 antagonists, containing a central urea motif have been patented by Bayer.¹⁸¹ Compound **35** is one of the most potent TRPM8 antagonist described to date, displaying subnanomolar potency in the menthol-induced Ca²⁺ influx in TRPM8-expressing HEK293 cell assay (Figure 9).

6.3. Phenylglycine Derivatives. Another collection of TRPM8 antagonists is that of *N*-arylphenylglycine amides, described by Janssen Pharmaceutica (i.e., **36**, Figure 10).^{182,183}

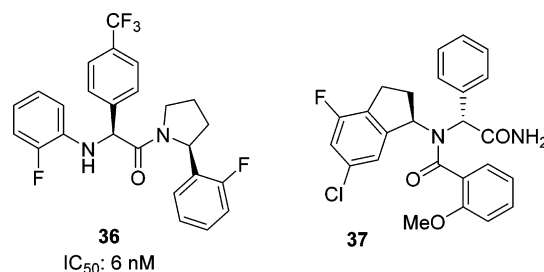


Figure 10. TRPM8 antagonists: illustrative phenylglycine derivatives.

This family comprises two libraries of 375 and 55 compounds in which 2-arylpyrrolidine and isoquinoline substituents gave better activities than other mono- and bis-heterocyclic systems. Substituted phenyl and benzothiophene are preferred aryl moieties at the α position of the amino acid. As expected, the influence of the configuration was crucial for the activity of these Phg derivatives. Thus, compound **36** (*S,S*) shows 1 order of magnitude higher potency than its (*R,R*) and (*R,S*) isomers, while the (*S,R*) diastereoisomer undergoes more than 100-fold drop in the antagonist activity, indicating a larger influence on the activity of the pyrrolidine stereogenic center than that of the Phg residue. Compound **36** demonstrated good in vivo efficacy, reducing WDS in rats produced after administration of icilin.¹⁸³

Related phenylglycine amides, combining either a differently substituted *N*-(*R*)-inden-1-yl ring or a 1-phenylethyl group with an *N*-acyl moiety, bearing aryl- or heteroaryl groups, have been recorded in two recent patents by Kissei Pharmaceutical.^{184,185} Preferred compounds from these series are able to reduce the

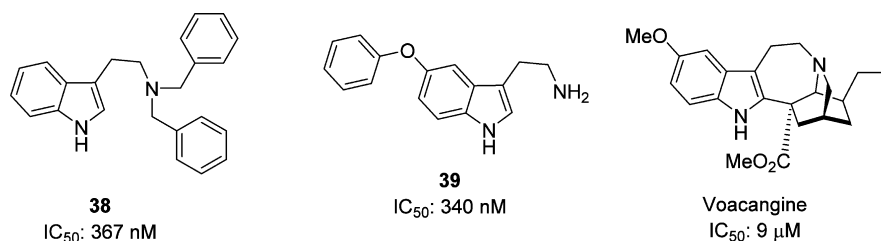


Figure 11. TRPM8 antagonists: selected tryptamine derivatives.

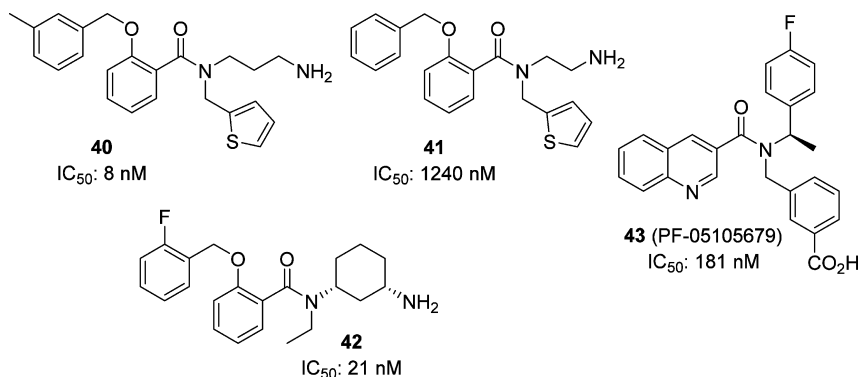


Figure 12. TRPM8 antagonists: archetypal tertiary amide derivatives.

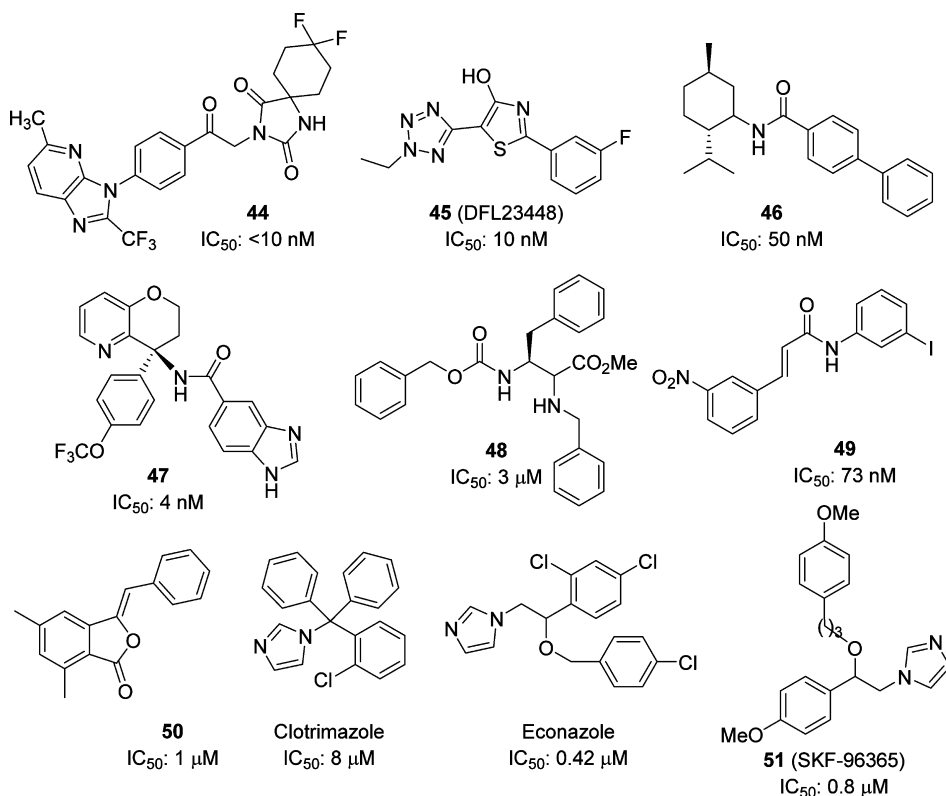


Figure 13. Structure of other structurally diverse TRPM8 antagonists.

icilin-evoked wet dog shakes in rats (i.e., derivative 37, 100% inhibition at 3 mg/kg). These compounds also elongate significantly the micturition interval in guinea pigs (up to 221% at 0.5 mg/kg/mL).

6.4. Tryptamine Derivatives. The structural requirements behind TRPM8 modulation were also explored by means of a series of N-substituted tryptamines. Apart from the already

mentioned agonist 15, most derivatives within this series behave as TRPM8 antagonists, with the (N,N-dibenzyl-2-(1H-indol-3-yl)ethanamine (38) as the most potent inhibitor of calcium influx in HEK293 cells.¹⁵⁷ Perfusion of tryptamine derivative 38 (3 μ M) produced a complete inhibition of menthol-induced TRPM8 currents measured at +80 mV, with

kinetics slower than that observed for **30** (BCTC) but increased potency (**38**, $IC_{50} = 367$ nM).

5-Substituted tryptamine derivatives were also identified as antagonists of TRPM8 after screening of the Renovis corporate collection and further evaluation of commercial analogues.¹⁸⁶ Compound **39**, identified as the most potent tryptamine derivative within this family, lacks selectivity because it is a known ligand of 5-HT receptors (Figure 11).¹⁸⁷

Voacangine, a conformationally restricted tryptamine-derived natural product, competitively displaces menthol, but not icilin, from TRPM8 receptors ($IC_{50} = 9$ μ M).¹⁸⁸ This compound is a stimulus-selective antagonist because it did not abrogate cold-induced channel opening but inhibited chemical agonist-provoked TRPM8 activation. In addition, voacangine competitively inhibits capsaicin binding on TRPV1 and blocks capsaicin- and heat-induced activation of this thermoreceptor, and shows some TRPA1 agonist activity.

6.5. Tertiary Amide Derivatives. A collection of 177 2-benzyloxy-benzoic acid amide derivatives were covered in a patent application by Bayer Healthcare AG.¹⁸⁹ The prototype *N*-(2-thienylmethyl) derivative (AMTB, **40**, $IC_{50} = 8$ nM, Figure 12)¹⁸⁹ in this series was used as a pharmacological tool to show that, in rats, this TRPM8 blocker attenuated bladder micturition reflex without affecting the contraction amplitude and reduced nociceptive reflex responses to noxious bladder distension.¹⁹⁰

More recently, Pfizer and collaborators described new salicyl amide derivatives with improved properties.¹⁹¹ Thus, following a lead-hopping strategy from the closely related **40** (AMTB) analogue **41**, they prepared three libraries with decreasing degree of similarity. Compound **42** from one of these libraries displays improved potency ($IC_{50} = 21$ nM) and better lipophilic efficiency compared to **41**.

The HTS screening of the Pfizer in-house collection allowed the identification of another tertiary amide derivative with weak inhibitory properties of TRPM8 receptors, which upon optimization resulted in the quinolin-3-carboxamide derivative PF-05105679 (**43**).¹⁹² Modifications at the acidic group resulted in reduced potency, while at the other benzylic residue only the incorporation of a *m*-chlorine atom is well tolerated. Its potency against TRPM8 was confirmed by patch-clamp electrophysiology studies, and selectivity was assessed through evaluation in a set of different receptors, enzymes, and ion channels, including related thermoreceptors (TRPV1 and TRPA1) and hERG. In preclinical studies, compound **43** exhibited good oral absorption, moderate blood clearance, and low toxicity. In humans, a single 900 mg dose was effective in a cold pressor test (phase I, NCT01393652) without significant changes in core body temperature, but intolerable hot sensation side effects precluded further progression into the clinic.

6.6. Other Recent TRPM8 Antagonists with Variable Central Scaffolds. **6.6.1. Cyclohexane-hydantoin Spirocyclic Derivatives.** Patent WO2015136947 from Raqualia Pharma Inc. communicates the preparation of a large collection of 1,3-diazaspiro[4.5]decane-2,4-dione derivatives and their 1-oxa-3-azaspiro analogues (~600 examples), among which an array of 42 compounds potently inhibited the Ca^{2+} influx through HEK293 cell expressing hTRPM8 ($IC_{50} < 10$ nM, i.e., **44**, Figure 13).¹⁹³ Although a central phenyl ring seems to be preferred, some heterocyclic moieties, like pyridine, imidazole, and pyrrole, are allowed at this position. The phenyl ring could bear a diverse set of nitrogen heterocycles, including indole, 1-benzimidazole, isoquinoline, and different aza analogues, with

the 7-aza-1-benzimidazole as the most repetitive motif in highly active compounds. Although without displaying specific data, the inventors claimed *in vitro* inhibition of the Ca^{2+} influx through a melanoma cell line (G-31) and potent *in vivo* activities in different neuropathic pain models and on micturition frequency.

6.6.2. Thiazole Derivatives. A patent application from Dompé Farmaceutici SpA revealed a series of compounds with a thiazole or isoxazole central scaffold with interesting activities as TRPM8 antagonists *in vitro* and *in vivo*.¹⁹⁴ The phenyl thiazole derivative (DFL23448, **45**) was then fully characterized in collaboration with different academic groups.¹⁹⁵ It shows an IC_{50} value of 10 nM in cold-activated hTRPM8 HEK-293 cells, being selective against other TRP channels (TRPV1, TRPA1, TRPV4) and different G-protein-coupled receptors. In *in vivo* experiments in rats, **45** is able to reduce icilin-induced wet dog shakes (10 mg/kg, *iv*), prolongs the storage phase of micturition, and inhibits bladder overactivity.

6.6.3. Menthol Derivatives. A series of menthylamine derivatives, embodied by compound **46**, elicited potent TRPM8 antagonist properties, being between 4- and 150-fold selective against TRPV1 and TRPA1 channels.¹⁹⁶ When tested on a human androgen-responsive prostate carcinoma cell line, which overexpresses TRPM8, compound **46** induces apoptosis, measured by the release of caspase 3/7. However, in the androgen-unresponsive DU-145 tumor cell line, this compound is inactive, indicating a TRPM8-dependent antitumor activity. This compound was also progressed to Ph I clinical trials.

6.6.4. Chroman Derivatives. A large series of azachroman derivatives was protected under patent application WO 2014025651 by Amgen Inc.¹⁹⁷ Exemplified here by compound **47**, a number of these derivatives displayed nanomolar activity in the luminescence assay of intracellular calcium (CHO expressing hTRPM8). The most potent antagonists were able to inhibit >90% the wet dog shakes in rats at 1 mg/kg dose. These compounds combine in the molecule key structural elements of other families of TRPM8 antagonists, akin to the chroman heterocyclic ring present in **34** (Figure 9) and a heterocyclic moiety, like the benzimidazole profusely used within the sulfonamide derivatives.

Clinical trials (phase I, NCT01953341, NCT02132429) have been started with AMG333 (structure not disclosed) to define safety and tolerability and to characterize the pharmacokinetic (PK) profile as well as its effect on the cold pressor test (CPT)-induced increase in blood pressure after single oral doses in healthy individuals and subjects with migraine.¹⁹⁷

6.6.5. β,γ -Diaminoesters. Selective antagonists for TRPV1, TRPM8 (**48**), and TRPA1 channels, as well as dual TRPV1/TRPM8 and TRPM8/TRPA1 ligands, were obtained after modification of a linear β,γ -diaminoester scaffold.¹⁹⁸ In this family, no significant differences in activity were found among diastereoisomers of the same compound, suggesting that they could accommodate within a big pocket within these TRP channels. Lessons extracted from the preferred substituents for the selective blockade of these three TRP channels could serve to design new potent and selective TRP blockers.

6.6.6. Cinnamamide Derivatives. Some open-chain cinnamamide derivatives, as **49**, primarily prepared as intermediates in the synthesis of icilin derivatives, displayed pronounced TRPM8 antagonist activity and acceptable selectivity over TRPV1.¹⁴⁶ Halogens or aliphatic groups at *meta*-position of the

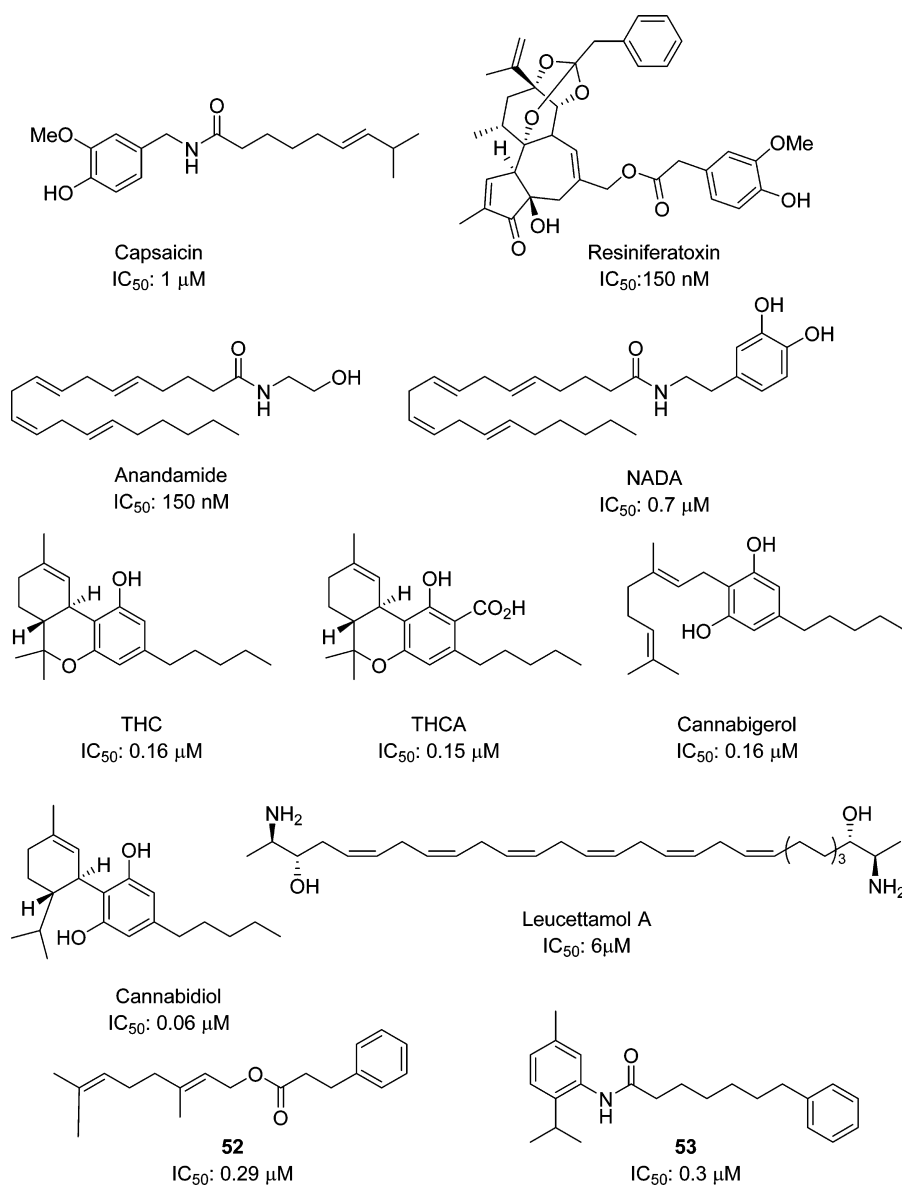


Figure 14. Structure of phytochemicals and derivatives acting as TRPM8 antagonists.

aniline-derived ring provide the most potent antagonists, with the *m*-iodo derivative **49** showing a 73 nM IC_{50} value.

6.6.7. 3-Ylidenepthalides. A new template that resulted in novel TRP channel modulators was the 3-ylidenepthalides. Thus, Ortar and co-workers described that these compounds act as TRPM8 antagonists (**50**, Figure 13) but also as strong modulators of TRPA1 (activators and desensitizers).¹⁹⁹

6.6.8. Others. Some approved antifungal drugs as clotrimazole and econazole are also good blockers of TRPM8 channels.^{200,201} The phenylethyl imidazole (SKF-96365, **51**),²⁰² an inhibitor of receptor-mediated Ca^{2+} entry with blocking actions on several TRPC channels, also targets and blocks TRPM8 receptors (IC_{50} = 0.8 μ M).⁵¹

6.7. Natural Products and Derivatives as TRPM8 Antagonists. The well-known TRPV1 activators capsaicin and resiniferatoxin inhibit the effect on Ca^{2+} induced by icilin, but not by menthol, on TRPM8-HEK-293 cells (micromolar and submicromolar potencies, respectively).²⁰³ Anandamide and *N*-arachidonoyl-dopamine (NADA, Figure 14),²⁰⁴ endogenous ligands of TRPV1 and CB1 receptors, behaved as

TRPM8 antagonists by inhibiting the Ca^{2+} entry stimulated both by icilin and menthol.²⁰³ Similarly, other cannabinoid structures of natural origin, like THC, THCA, cannabigerol, and cannabidiol, are able to block the TRPM8 channel activation but in general are nonselective against other TRP channels.²⁰⁵ Cannabigerol, a known nonpsychotropic cannabinoid, is able to reduce cell viability in the colorectal cancer cell line CACO-2 by promoting apoptosis, which was confirmed by the increased activity of caspase 3/7.²⁰⁶ This effect on cell viability was mimicked by two other phytocannabinoids, which also block TRPM8, and what is more important, TRPM8 silencing resulted in a lower cytotoxic effect. In vivo experiments performed both in xenograft and AOM (azoxymethane) models of colon cancer showed that cannabigerol reduces and even prevents the tumor growth. The antitumor activity of cannabidiol on prostate cancer cell lines has also been reported.²⁰⁷ These results suggest cannabigerol, cannabidiol, and related nonhabit analogues could be promising agents in cancer treatment.

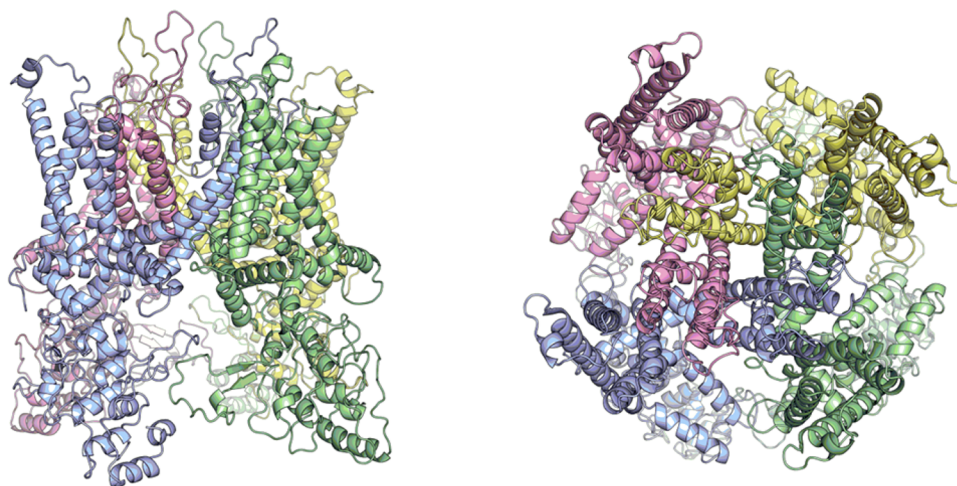


Figure 15. Homology model of the tetrameric assembly of TRPM8 channel viewed from the lateral (left) and the intracellular (right) side. Each monomer is differently colored.

Sphingoid-like compounds, like leucettamols A and B obtained from a marine sponge, inhibit icilin-induced activation of TRPM8 channels.²⁰⁸ These compounds also target the cold-sensitive TRPA1 receptors but are inactive on TRPV1 and the cannabinoid receptors.

Some geraniol, nerol, and citronellol derivatives behave as TRPM8 antagonists on TRPM8-HEK293 cells, the nerol chain being particularly effective in this respect. Compound **52**, with an IC_{50} value of $0.29 \mu\text{M}$ in the TRPM8 assay, was selective against TRPV1 but acts as a strong modulator of TRPA1 channels (EC_{50} and/or IC_{50} values $<1 \mu\text{M}$).²⁰⁹ Similarly, thymol derivatives, here exemplified by compound **53**, are true TRPM8 antagonists, but as for most phytochemicals with TRPM8 activity, also display strong TRPA1 modulation.²¹⁰

7. INSIGHTS INTO THE TRPM8 STRUCTURE

Due to the lack of a crystal structure, little is known about the structure–function of TRPM8, and the scarce structural information available comes from mutagenesis experiments and molecular modeling studies.

Similarly to voltage-gated potassium channels (Kv) and like other TRPs, TRPM8 channels have six α -helices forming the transmembrane architecture (S1–S6). Among them, S1–S4 segments constitute the sensing domain (SD) and S5 and S6 constitute the pore (PD). The intracellular N-domain, with an important role in targeting TRPM8 to the plasma membrane and also the inner C-terminal domain, containing the TRP and coiled-coil subdomains required for gating and channel assembly, complete the protein.^{211,212} The S4 transmembrane and the S4–S5 linker are responsible for voltage sensing, S6 is involved in the selectivity for cations and the S2–S3 linker seems to be implicated in the interaction with icilin, the same region that has been recognized in the activation of other TRPs by chemical agonists.²¹³ It has been demonstrated that the initial N-terminal region of TRPM8 is important for correct biogenesis and function, because truncation of the 1–40 segment augment TRPM8 responses to menthol and cold and elimination of the 40–60 region afforded nonfunctional channels that are retained in the ER.²¹⁴ The group of Edwardson, by means of atomic force microscopy (AFM) imaging, has showed that the TRPM8 channel assembles as a tetramer, with a centrally located pore.²¹⁵ These authors suggest that this assembly pattern is common to other TRP

members, as they had previously shown for TRPC1,²¹⁶ and recently has been elegantly confirmed by the group of Julius, which has solved the structure of TRPV1 through cryoelectron microscopy.²¹⁷

High-throughput random mutagenesis studies on the TRPM8 thermosensor proved that residues in S2 and S4 transmembrane segments are important for menthol-induced channel opening and voltage and temperature activation.²¹⁸ Namely, Y745 in S2 and R842 in S4 were designated as chief residues for channel opening and menthol dependent currents through TRPM8. Y745 is also critical for the inhibition of cold- and voltage-activated TRPM8 currents mediated by **51** but not by other antagonists like **30**, clotrimazole, and econazole, suggesting multiple binding sites on these channels.²⁰¹ Very recent NMR experiments on the interaction between menthol and the hTRPM8-SD corroborate that R842 and Y745 are essential residues for the interaction and indicated that more likely ligand binding produces a conformational change in the sensor domain that results in channel gating.²¹⁹

Chimeric TRPM8 channels containing segments of the S6-TRP box linker of TRPV1 permitted the identification of residue Tyr981 as a key molecular determinant of channel function, while mutations in the 986–990 region influenced channel gating by voltage and menthol. On the basis of these results and on the cryoelectron microscopy model of TRPV1, the group of Ferrer–Montiel generated a molecular model of TRPM8 and hypothesized that residue Tyr981 and region 986–990 may be involved in intersubunit interactions between the TRP domain and the S4–S5 linker (Figure 15). If the TRP domain participates in channel opening, the modulation of protein–protein interactions within this domain may constitute a new strategy for channel modulation and/or drug intervention.²²⁰

On the basis of the above model, Bertamino et al. proposed two pharmacophore prototypes for their series of tryptamine-based TRPM8 modulators, identifying a unique binding site for both agonist and antagonist derivatives (**15** and **38**, Figures 5 and 11, respectively) but differing in the interaction network.¹⁵⁷ TRPM8 complexes with these compounds are stabilized by similar hydrophobic interactions with Leu843, Ile701, Leu697, a π – π stacking with Tyr754, and electrostatic pairs with the Glu1004 side chain. The indole NH proton of agonist **23** makes hydrogen bonds with Tyr754 phenoxy group and with

the backbone of Arg1008, increasing the Arg1008–Glu1004 distance when compared to that measured for the TRPM8–38 complex and promoting an evident shift of the TRP domain helix. Further mutagenesis studies are needed to corroborate the structural hypothesis depicted from these pharmacophore models.

Pedretti and co-workers generated a full-length computer model for the hTRPM8 channel, following a fragmental strategy, exploiting the homology with other channel to construct the monomer, namely the voltage-dependent Shaker family of K^+ channel for the transmembrane region and the structure of HCN2 channel for the C-terminal domain.²²¹ Protein–protein docking was then used to assemble monomers into the corresponding homotetramer. Docking with menthol, icilin, and other ligands resulted in good agreement with mutagenesis studies, confirming that S4 and S4–S5 loop are crucial for channel activation.

The TRPM8 transmembrane and pore helix region has been studied by Bidaux et al. using a combination of molecular modeling and experimental techniques.²²² As for other members of the TRP family, they identified that the DDDD ring was essential for the pore of TRPM8, with the length of side chains and the negative charges of these residues being crucial in TRPM8. However, unlike for other TRP channels, the negative charge of TRPM8 D920 residue is not enough to select divalent cations but participates in the facilitation of cation conductance. This study also demonstrated that residue Y908 in the P-helix is implicated in menthol and cold-mediated TRPM8 activity but not in icilin recognition. Whether menthol binds directly to the P-helix or to the binding site on the S2 pocket, inducing a conformational change on the pore domain remains to be elucidated.

8. ENDOGENOUS REGULATION OF TRPM8

Traditionally, a large effort has been devoted to modulating ion channels, including TRPM8, considering that they are isolated proteins inserted in a cell membrane. However, it is becoming evident that ion channels are members of protein networks that notably influence their activity. Furthermore, channel activity is highly regulated in a context-dependent manner, with the interplay of diverse cellular components, including lipids, proteins, and metabolites. In this regard, the activity of TRPM8 is prone to endogenous regulation for fine-tuning its activity to a proper cellular response. Accordingly, TRPM8 dysfunction appears also affected by regulatory pathways, as their malfunction may impact TRPM8 function.²²³ Undoubtedly, the most studied endogenous TRPM8 regulatory mechanism is its dependence on $PI(4,5)P_2$.³¹ It is well documented that $PI(4,5)P_2$ modulates both activation and desensitization of the channel.³¹ Hydrolysis of $PI(4,5)P_2$ by phospholipases type C ($PLC\gamma$, $PLC\beta$, and $PLC\delta$) abrogates channel function, while resynthesis of the original PIP_2 restores its channel activation.²²³ Regulation of TRPM8 by phosphoinositides can also be mediated by the phosphoinositide interacting regulator of TRP (Pirt), a protein that increases the sensitivity of TRPM8 to menthol and cool temperatures.²²⁴ This protein acts synergistically with $PI(4,5)P_2$ to augment TRPM8 channel activity.²²⁵

$PI(4,5)P_2$ levels are largely modulated by the activation of PLC through G-protein coupled receptors (GPCR) and receptor tyrosine kinases (RTK). Activated PLC enzymes hydrolyze $PI(4,5)P_2$ to phosphoinositol trisphosphate (PIP_3) and diacylglycerol (DAG). These two components act

synergistically to activate PKC, which in turn may phosphorylate TRPM8. Furthermore, PIP_3 -induced release of Ca^{2+} from the endoplasmic reticulum activates calmodulin kinase that can also phosphorylate TRPM8.²²⁶ A consequence of TRPM8 phosphorylation appears to be reduction of channel activity that further augments the inhibition produced by the hydrolysis of $PI(4,5)P_2$. Complementarily, an increase in PKA activity also appears to inhibit TRPM8 channel activity. Recently, a novel mechanism of TRPM8 inhibition by GPCRs that implies the direct inhibition of the channel by $G\alpha_q$ has been described.²²⁷ It is notable that most RTK and GPCRs sensitize TRPV1 channels, and concomitantly desensitize TRPM8 channels, which may underlie the heat hyperalgesia and abnormal cold sensations caused by inflammation.²²⁷

The activity of TRPM8 is additionally modulated through two additional membrane-related mechanisms. First, its regulation by phospholipase A_2 (PLA_2) has been documented, an enzyme that generates lysophospholipids and arachidonic acid.²²³ Intriguingly, these lipids modulate TRPM8 activity in some cellular systems but not in planar lipid bilayers. A plausible explanation is that PLA_2 generates two antagonistic compounds, as lysophospholipids tend to enhance TRPM8 activity and arachidonic acid inhibits the channel function.²²⁸ Because PLA_2 normally enhances menthol activity, the result is a prevalence of the lysophospholipid stimulating effect over the polyunsaturated fatty acids inhibitory action. Second, TRPM8 activity is also modulated by its location in membrane microdomains. For instance, it was reported that TRPM8 activity is downregulated by its partition in lipid rafts.²²⁹ However, a recent study reported just an opposite result as TRPM8 activity was reduced after disrupting the lipid rafts in trigeminal neurons.²³⁰ These results indicate a role of membrane microdomains modulating channel function although further studies are needed to clarify whether the discrepancies reported are due to different experimental conditions or determined by the cellular context. Finally, the oxidative metabolite of linoleic acid 9(S)-HODE acts a nonselective TRPM8 antagonist, which also modulates TRPV1 and TRPA1 channels.²³¹

Apart from the lipid-mediated endogenous regulation, there is evidence that TRPM8 function can be additionally modulated by short isoforms that emerge from post-transcriptional regulation of the main protein transcript.²³² These shorter channel isoforms inhibit TRPM8 activity by mimicking an increase in temperature as compared with the inhibitory activity exerted by chemical antagonists.²³² Interestingly, a novel TRPM8 isoform has been identified in keratinocytes that is expressed in the endoplasmic reticulum of these cells and endogenously contributes to the cold sensitivity of epidermal homeostasis by controlling the balance between keratinocyte proliferation and differentiation in a cold-dependent manner.²³³ Recently, it has been reported that the vesicle-associated membrane protein 7 (VAMP7) is implicated in the functional plasma membrane incorporation of TRPM8 and therefore in regulating thermosensitivity.²³⁴

Another important endogenous regulatory mechanism of TRPM8 arises from its sensitivity to sex hormones. As already mentioned, this channel was first identified as a biomarker of hormone-dependent prostate cancer cells, as its levels were high in these tumor cells.¹¹ Asuthkar et al. report the interesting observation that the TRPM8 channel is a receptor for testosterone, suggesting a direct role of the hormone regulating the receptor activity.^{235,236} Related to this observation, Gkika et

al. discovered that the prostate cancer marker, prostate-specific antigen (PSA), is also a modulator of TRPM8 activity.²³⁷ Indeed, they reported that PSA acts as a natural agonist of TRPM8 in the prostate and the channel activation by PSA results in a reduction of cancer cell motility. Overall, these findings suggest that modulation of TRPM8 may be an important strategy for anticancer drug intervention.

In summary, endogenous regulation of TRPM8 channels provides new opportunities to modulate the receptor activity by acting on signaling pathways that modulate the channel.

9. SUMMARY AND FUTURE PERSPECTIVES

Over the past few years, there has been significant development in the field of TRPM8 channels, regarding expression, polymodal activation, implication in diverse pathological conditions, and discovery of diverse modulators, both agonists and antagonists.^{25,33} Cumulative evidence indicates the essential role of TRPM8 in virtually all aspects of cold thermal transduction, including innocuous and noxious cold, hypersensitivity to cold caused by inflammation, nerve injury, or chemotherapeutic neuropathy, and regulation of core body temperature.⁴⁰ Therefore, modulation of TRPM8 channel function to tune the excitability of cold-sensing neurons could have a broad clinical benefit in the management of chronic and/or neuropathic pain.⁴⁴ Thus, the identification of new compounds targeting different binding sites of its signalplex may be anticipated as a realistic strategy for effectively and safely treating acute and chronic pain states, which is still a major unmet challenge in biomedical research. Regarding cancer, it is documented that in prostate tissues, TRPM8 channels play a different role depending of the cell type and also on the tumor grade and progression, showing even different and opposite effects.⁸⁹ TRPM8 expression in prostate cancer cells is androgen-regulated, but its overexpression appears to be hormone-independent. TRPM8 expression is substantially decreased in androgen-independent and metastatic prostate cancer. A number of other life-threatening tumors, like melanoma, neuroblastoma, and pancreatic adenocarcinoma, also overexpress TRPM8 channels, although their particular role is mostly unknown.⁸⁸ Therefore, there is still further research needed to completely elucidate the mechanisms by which TRPM8 exerts its biological activity and to understand its implications in tumorigenesis and cancer progression. Provided that activation or blockade of the TRPM8 channels may have opposite effects, depending on the type and stage of the tumor, their clinical significance remains to be proved. The proven involvement of TRPM8 channels in pain, cancer, and cancer-induced pain and its validation as a molecular target, open the door to new global strategies for effective management of these pathologies. Concerning diagnosis, TRPM8 levels could be exploited as a clinical biomarker for the diagnosis of different cancer types. In fact, the TRPM8 detection in patient fluids samples (urine and blood) proved to be useful to discriminate between clinically localized prostate cancer and metastatic tumors.^{86,101} Furthermore, molecular imaging agents based on TRPM8 selective modulators could also have a major effect on the detection of cancer, as already proposed.¹²⁶

Increasing experimental evidence has also revealed that stimulation of TRPM8 channels is involved in a number of pathological conditions, including thermogenesis of brown adipose tissue,¹¹³ asthma and COPD,²³⁸ urinary tract dysfunction,²³⁹ and eye wetness.¹²¹ Thus, TRPM8 provides

promising new approaches to treat obesity, respiratory processes, bladder dysfunction, and certain ophthalmologic problems.

Although a number of diverse TRPM8 modulators have been described and resulted in an improved understanding of the structure–activity relationships within different families, their selectivity over other TRP channels and in general over panels of diverse biological targets, is mostly unknown or not yet disclosed. Furthermore, taking into account that a unique chemotype could result in promiscuous ligands for different TRP channels,¹⁹⁸ the existing pharmacological knowledge about TRPM8 modulators should be taken with caution. Another factor of paramount importance for the advancement in TRPM8 drug discovery will be to elucidate the receptor binding sites for the different families of modulators. At present, this is partly precluded by the scarce structural knowledge available, although progress in homology modeling may contribute in the near future.¹⁵⁷ It is expected that further advances in structural biology could help in characterizing accurately TRPM8 ion channel–ligand interactions, thus increasing our understanding of ligand binding. In addition, it will contribute to apply rational design methodologies to hit discovery and to hit-to-lead optimization toward safer and more selective modulators. Nonetheless, until this information becomes available, existing screening methodologies provide a useful approach to discover novel TRPM8 modulators and to develop analogues with improved therapeutic properties.

As reviewed in this perspective, there are few TRPM8 modulators that have reached clinical studies and most of these have not progressed beyond phase I trials. Although the expectations are high, prediction of a clinical success based only on animal data appears premature at present. We have to wait for the results of proof-of-concept clinical studies to learn on the relevance of therapies based on TRPM8 modulators.

Finally, the information accrued thus far also suggests that the operational function of TRPM8 depends on the cellular context and that different strategies, from membrane localization and dynamics to translation of short isoforms, are involved in defining the pathophysiological function of the receptor. This information should be taken into account in the design of the next generation of TRPM8 therapeutic drug candidates. Structure–function studies have unveiled that the TRP domain,³¹ a protein region adjacent to the channel gate, contributes to the allosteric coupling of stimuli sensing to gate opening, and it is the binding site of PI (4,5)P₂.^{220,240} Notably, peptides patterned after this domain in TRPV1 channels inhibit receptor function,²²⁸ indicating that this domain may also be a novel site for drug intervention in TRPM8 channels. To date, the destabilization of protein–protein interactions (PPI) within ion channels has barely been studied,²⁴¹ but most probably the application of strategies followed for other PPIs could also be relevant for TRPM8 drug discovery.

Another important point to be explored in the near future would be to analyze the clinical benefit of combined therapies or multifunctional drugs acting simultaneously on several TRPs because sometimes more than one TRP channel is involved in the same pathological disorder (i.e., TRPM8 and TRPA1 in cold-induced pain and asthma).^{107,108,242}

Although ion channels remain underexploited in drug development, continued investigation into the discovery of new, selective TRPM8 agonists and antagonists will undoubtedly contribute to future progress in the field. The ability to combine advances in functional screening, biophysical charac-

terization, and structure-based design techniques, along with the generation of new chemical space to target ion channels, will constitute a substantial challenge for the drug discovery community in the following decade. We should eagerly await the clinical success of currently tested TRPM8 modulators for the treatment of human diseases from pain to cancer.

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Notes

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ABBREVIATIONS USED

AFM, atomic force microscopy; AMTB, *N*-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-*N*-(2-thienylmethyl)-benzamide HCl; AOM, azoxymethane; AR, androgen receptor; BAT, brown adipose tissue; BCTC, 3-chloro-pyridin-2-yl)-piperazine derived urea; CBI, cannabinoid receptor 1; CCI, chronic constrictive injury; CFA, complete Freund's adjuvant; CGRP, calcitonin gene-related peptide; COPD, chronic obstructive pulmonary disease; CPT, cold pressor test; CSD, cortical spreading depression; DAG, diacylglycerol; DRG, dorsal root ganglia; ER, estrogen receptor; 5-FU, 5-fluorouracil; hERG, human ether-à-go-go related gene; HTS, high throughput screening; IB4, isolectin B4; IDO, idiopathic detrusor over-activity; LUTd, lower urinary tract dysfunction; MMP-9, matrix metalloproteinase 9; PCa, prostate cancer; PD, pore domain; Phg, phenylglycine; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; Pirt, phosphoinositide interacting regulator of TRP; PLCδ1, phospholipase C δ1; PKA, protein kinase A; PKC, protein kinase C; PLA₂, phospholipase A₂; PSA, prostate-specific antigen; RTK, receptor tyrosine kinase; SD, sensing domain; SNL, spinal nerve-ligated; TG, trigeminal ganglion; THC, tetrahydrocannabinol; THCA, tetrahydrocannabinolic acid; TNBS, trinitrobenzenesulfonic acid; TRP, transient receptor potential; TRPM8, Trp-p8 = MCR1 = transient receptor potential melastatin 8; UCPI1, uncoupling protein 1; VAMP7, vesicle-associated membrane protein 7; WAT, white adipose tissue; WDS, "wet-dog" shakes

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