Characterization and evaluation of TRPV1 and TRPM8 antagonists as potential therapeutic tools for treating pain

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Doctoral thesis

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ELCHE 2016

Instituto de Biología Molecular y Celular

Universidad Miguel Hernandez

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DAN SU CONFORMIDAD a la lectura de la tesis doctoral titulada: **"**Characterization and evaluation of TRPV1 and TRPM8 antagonists as potential therapeutic tools for treating pain" presentada por D. Roberto de la Torre Martínez.

Elche, Junio 2016

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CERTIFICAN que el trabajo de investigación que lleva por título **"**Characterization and evaluation of TRPV1 and TRPM8 antagonists as potential therapeutic tools for treating pain", presentado por D. Roberto de la Torre Martínez para optar al grado de Doctor, ha sido realizado bajo su dirección en el Instituto de Biología Molecular y Celular de la Universidad Miguel Hernández de Elche. Considerando que la presente tesis se halla concluida, **AUTORIZAN** su presentación para que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste a los efectos oportunos, se expide el presente escrito.

Elche, Junio 2016

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El presente trabajo ha sido realizado en el Instituto de Biología Molecular y Celular (IBMC), de la Universidad Miguel Hernández de Elche.

Roberto de la Torre Martinez ha sido beneficiario de una beca predoctoral del programa Formación de Personal Investigador (FPI) con referencia BES-2010-037112 del Ministerio de Ciencia e Innovación.

Este trabajo se ha desarrollado gracias a la financiación procedente del Ministerio de Economía y Competitividad y de la Generalitat Valenciana.

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INDEX OF ABREVIATIONS

[Ca^{2+}]	Calcium concentration
AITC	Allyl isothiocyanate
ANOVA	Analysis Of Variance
ARD	Ankyrin Repeats Domain
ATP	Adenosine Triphosphate
BCTC	4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide
BDL	Bile Duct Ligation
c-AMP	Cyclic Adenosine Monophosphate
Caps	Capsaicin
CCI	Chronic Constriction Injury
CFA	Complete Freunds Adjuvant
CGRP	Calcitonin Gene Related Peptide
CHO	Chinese Hamster Ovary cell line
Co-app	Co-application of compound
cRNA	Complementary Ribonucleic Acid
C-ter	Carboxyl-terminal
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DRG	Dorsal Root Ganglion

EGTA	Ethylene Glycol Tetraacetic Acid
FCi	Fluorescence before the addition of menthol in the absence of the compound
FCo	Fluorescence after the addition of menthol in the absence of the compound
FCS	Fetal Calf Serum
Fi	Fluorescence before the addition of menthol in the presence of the compound
Fo	Fluorescence after the addition of menthol in the presence of the compound
GFRα3	Glial cell-line derived neurotrophic factor Family Receptor 3
HEK293	Human Embryonic Kidney cell line
HEK-CR1	HEK293 cell line expressing TRPM8 channel
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
Hz	Hertz
i.v.	Intravenous administration
I/V	Current-voltage relationship
IB4	Isolectin B4
IC_{50}	Half maximal inhibitory concentration
I_{com}	Current evoked with compound
I_{max}	Maximum evoked current
Ins	Inside application of compound
IP_3	Inositol 1,4,5-trisphosphate
iPLA2	Calcium-insensitive Phospholipase A2
Kv	Potassium channel family (Kv)

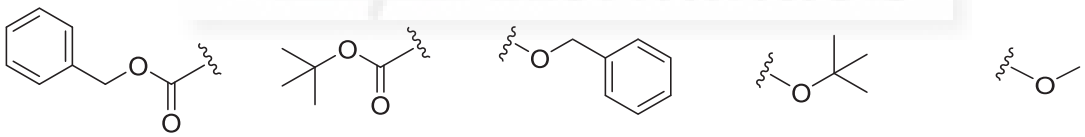
K(0 mV)	Constant in the modify Woodhull model
MAPKs	Mitogen-Activated Protein Kinases
MEA	Microelectrodes Arrays
$Mean_{max}$	Mean of the maximum fluorescence in the presence of agonist
$Mean_{min}$	Mean of the maximum fluorescence in the presence of agonist and antagonist
MEM	Earle's Minimum Essential Medium
MES	2-(N-Morpholino)ethanesulfonic acid hydrate
MS-222	Tricaine mesylate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
mV	Millivolts
MΩ	Megaohms
nA	Nanoampere
NaV	Sodium channel family (NaV)
NGF	Nerve Growth Factor
n_H	Hill coefficient
NMDA	N-methyl-D-aspartate
NSAIDs	NonSteroidal Anti-Inflammatory Drugs
N-ter	Amino terminal
Out	Outside application of compound
P1	First application of compound
P2	Second application of compound

PBMC	(S)-1-Phenylethyl(2-aminoethyl)(4-(benzyloxy)-3-methoxybenzyl)carbamate
PAR2	Proteinase-Activated Receptor-2
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PK-A	Protein Kinase A
PK-C	Protein Kinase C
PLC	Phospholipase C
PLIP	Protein-Ligand Interaction Profiler software
PPAHV	Phorbol-12-phenylacetate-13-acetate-20-homovanillate
Pre-app	Pre-application of compound
RTX	Resiniferatoxin
S1-S6	Transmembrane Segments
SAR	Structure-activity relationship
SD_{max}	Standard deviation of the maximum fluorescence in the presence of agonists
SD_{min}	Standard deviation of the maximum fluorescence in the presence of agonist and antagonist
SEM	*Standard Error* of the Mean
ShSy5y	Neuroblastoma cell line
TKs	Kinase Receptors
TRP	Transient Receptor Potential
TRPA	Transient Receptor Potential Ankyrin
TRPC	Transient Receptor Potential Canonical
TRPM	Transient Receptor Potential Melastatin

TRPML	Transient Receptor Potential Mucolipin
TRPP	Transient Receptor Potential Polycystic
TRPV	Transient Receptor Potential Vanilloid
U/ml	Concentration given as units of active enzyme/milliliter
v/v	Concentration given as volume/volume
Vh	Holding potential
w/v	Concentration given as weight/volume
w/w	Concentration given as weight/weight
ϛ	Valence of the molecule
δ	Electric distance for the binding site

Protective goups

N-Protecting groups C-Protecting groups



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SUMMARY

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SUMMARY

Although pain is a warning mechanism necessary for the defense against noxious stimuli, occasionally it loses its meaning and becomes pathological (inflammatory or neuropathic pain). Unfortunately, nowadays pathological pain is a global problem that affects a large number of people around the world and, although there are several drugs to treat it, they have undesirable side effects. Luckily, it has been discovered the implication of an ion channels family known as TRP (Transient Receptor Potential) that play an important role in pain transduction. Specifically, it has been shown that TRPV1 and TRPM8 channels are key proteins in pain transduction mechanisms, and through their modulation, analgesic effects might be achieved. Thus, in this thesis we addressed the modulation of these two ion channels looking for new potential modulators that could be develop as analgesics.

On one hand, in the first part of the thesis (chapter 1), we investigated the activity of compound *triazine 8aA*, as an antagonist of TRPV1 channel. We demonstrated its selectivity and specificity, the lack of toxicity in different cell lines and its analgesic and anti-pruritic properties in *in vivo* pain models. We also present evidence that the mechanism of action is that of an open-channel blocker, showing that it is possible to block TRPV1 with un-competitive modulators, opening new horizons for the next generation of analgesics and anti-pruritic therapies based on TRPV1.

On the other hand, in the second part (chapter 2), we addressed the search and characterization of TRPM8 channel antagonists. Using high-throughput screening techniques we found a potent blocker of TRPM8 (*compound 8-3*) and demonstrated its selectivity and specificity. The structure-activity relationship analysis suggests the minimum elements necessary for the β-lactam scaffold to block the TRMP8 channel activity. In addition, based on docking experiments, we postulated two potential binding sites for this potent, specific and selective TRPM8 antagonist that might help to complete the virtually inexistent literature regarding the mechanisms of action of TRPM8 antagonists.

RESUMEN

El dolor es un mecanismo de alerta y defensa necesario frente a estímulos nocivos. Sin embargo, es posible que en determinadas circunstancias pierda sus bondades y se convierta en una patología a tratar (dolor inflamatorio y dolor neuropático). Desafortunadamente, en la actualidad ese dolor patológico es un problema que afecta a un gran número de personas en todo el mundo y aunque existen fármacos para paliarlo, estos presentan importantes deficiencias. Por suerte, en los últimos años se ha descubierto la implicación de una familia de canales iónicos conocidos como TRP (de las siglas en ingles Transient Receptor Potential) que juegan un importante papel en la traducción del dolor. En concreto, se ha demostrado que los canales TRPV1 y TRPM8 son proteínas clave en el mecanismo de transducción del dolor y que a través de modulación se podría conseguir efectos analgésicos. Así, en esta tesis se aborda la búsqueda de nuevos moduladores de estos canales iónicos con potenciales propiedades analgésicas.

En la primera parte de la tesis (capítulo 1), se ha investigado la actividad antagonista del compuesto *triazine 8aA* sobre el canal TRPV1. Demostramos que el compuesto actúa de forma específica y selectiva sobre su diana sin presentar efectos tóxicos sobre diferentes tipos celulares. Además, mostramos que el compuesto tiene propiedades analgésicas en modelos de dolor in vivo. También presentamos evidencias de que el compuesto *triazine 8aA* es un bloqueador de canal abierto, probando que es posible bloquear TRPV1 con antagonistas acompetitivos y abriendo las puertas a una nueva generación de analgésicos y antipruriginosos basados en la modulación de la actividad de TRPV1.

Por otro lado, en el capítulo 2, se abordó la búsqueda y caracterización de antagonistas del canal iónico TRPM8. Mediante técnicas de cribado de alto rendimiento se encontró un potente bloqueador de TRPM8 (*compuesto 8-3*) y se demostró su selectividad frente a otros canales iónicos. El análisis de la relación estructura-actividad realizado en los derivados de β-lactama permitió identificar los elementos mínimos necesarios en dicho esqueleto químico para bloquear la actividad del canal iónico TRPM8. Además, utilizando modelos informáticos, se

postularon dos posibles sitios de unión para este potente y selectivo antagonista de TRPM8 que contribuyen a aumentar el conocimiento con respecto a los mecanismos de modulación de compuesto desarrollados para TRPM8.

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OVERVIEW

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OVERVIEW

The world surrounding us is full of different physical and chemical stimuli. All organisms, from bacteria to humans, have the ability to receive, transduce and transmit these signals to coordinate and modify their behavior. This is called **somatosensation,** and encompasses several modalities of detection that include light mechanical stimuli (touch), sense of the relative position of muscles and joints (proprioception) and detection of cool and warmth (thermosensation)[1]. Additionally, there is another modality of somatosensation that detect noxious mechanical, thermal, or chemical stimuli that produce pain sensation (**nociception**)[2, 3].

In complex animals, specifically in mammals, the process of detecting sensory stimuli relies on **primary sensory neurons**, specialized cells that innervate the whole body and collect sensory data (Figure 1)[4, 5]. Primary sensory neurons responsible for nociception are called **nociceptors**[3, 6], and when activated generate an unpleasant sensation called **pain**. The nociceptive process starts with the activation of pain receptors by noxious stimuli. This activation depolarizes the peripheral sensory neurons, generating an action potential that is propagated through primary sensory neurons to the spinal cord or dorsal horn. There they form synapsis with secondary sensory neurons, which bring the signal to the brain eliciting a perception of discomfort or pain. Then the signal is processed and the adequate decision is made to prevent damage[4, 6]. This pathway has been essential for living organisms not only to interact with the environment, but also to avoid potential harmful stimuli that could compromise their lives[7].

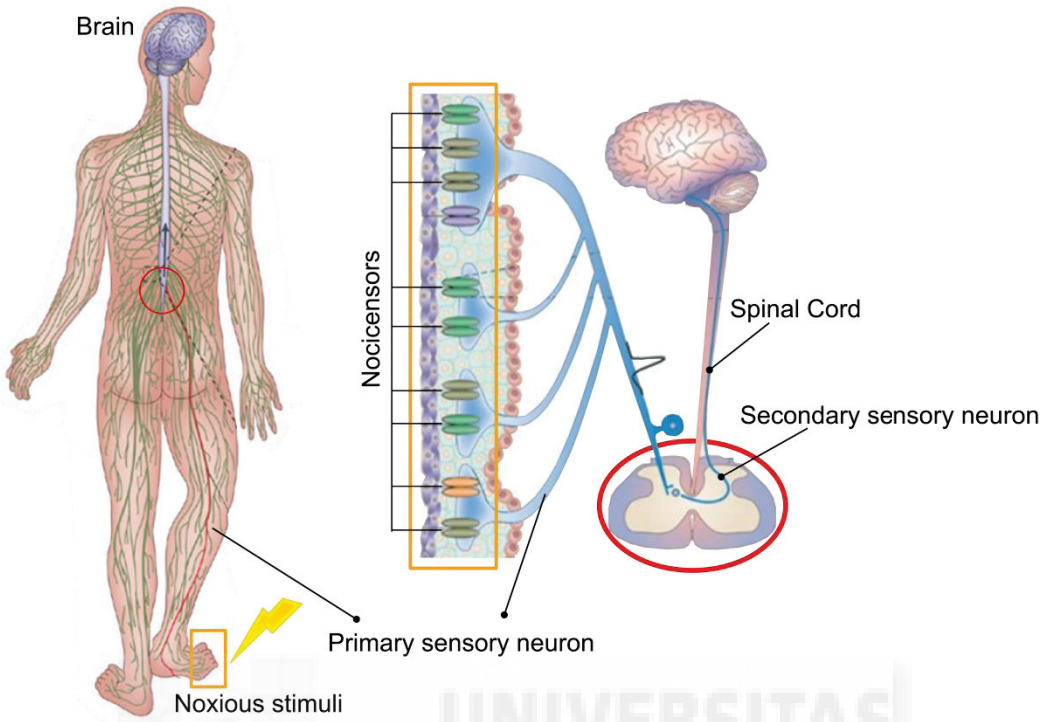

Figure 1 The nociceptive pathway adapted from Moran, M.M et al. 2011[5]

Under normal situations, **nociceptive** or physiological pain is an essential protective system that detects and minimizes contact with damaging or noxious stimuli[8]. However, occasionally pain can become pathological, losing its warning meaning. For example, if nociceptive pain is prolonged over time it can become chronic, producing tissue damage and sensitizing the affected region (**inflammatory pain**)[9]. The symptoms of this sensitized state are allodynia, a phenomenon where normally innocuous stimuli produce an unpleasant sensation, and hyperalgesia, an increment of pain suffered from a stimulus that normally provokes mild pain[10, 11]. Some examples of pain disorders where this sensitization has been described are conjunctivitis, psoriasis or rheumatoid arthritis. Another example of pathological pain is when it results from nerve damage (**neuropathic pain**)[12]. Contrary to inflammatory chronic pain, it is not a symptom of a disorder but rather a disease state. Herpes zoster, ischemia or neuropathies related to diabetes are some examples of this type of pain[13-15].

Unfortunately, and despite the fact that pathological pain is a global health problem that affects more than 20% of the adult population, little advances have been reached, especially for persistent pain syndromes[16]. Luckily, over the past two decades, the cloning and functional characterization of sensory receptors has provided a molecular framework for understanding peripheral mechanisms underlying stimulus detection and injury-evoked sensation[17]. These molecular entities are the **nocicensors**, and among them, the Transient Receptor Potential (TRP) ion channels have emerged as a family of ion channels that play crucial roles in the generation and development of pathological pain perception[18, 19]. This family has nowadays 28 members in mammals distributed in 6 subfamilies named as follow TRPC1-7 (Canonical), TRPM1-8 (Melastatin), TRPV1-6 (Vanilloid), TRPA1 (Ankyrin), TRPP1-3 (Polycistic), and TRPML1-3 (Mucolipin)[20]. They are widely expressed in several tissues, one of the reasons why they play an important role in sensory physiology, which in addition to nociception, include olfaction, hearing, vision, touch, and osmo- and thermosensation[21, 22]. Furthermore, several studies have highlighted the importance of TRP channels in a wide range of human disorders such as respiratory problems, skeletal dysplasia, neurodegenerative conditions or pain disorders[23, 24].

The discovery of the TRP channels represented a revolution for the pain research field since it revealed a new complex and dynamic regulatory system on the pain pathway[3, 18]. Traditionally, pain treatment has utilized two types of drugs: nonsteroidal anti-inflammatory drugs (NSAIDs) that include aspirin and paracetamol[25, 26], local anesthetics such as lidocaine or benzydamine[27, 28], and narcotics such as morphine or tramadol[29]. Although they are effective, undesired side effects including gastric and kidney problems in the case of NSAIDs or sedation, dizziness or loss of cognitive function to the anesthetic has been reported[9]. For its part, the continued use of narcotics can result in physical dependence and addiction producing restlessness, muscle and bone pain, insomnia, diarrhea, vomiting or cold flashes when the treatment is stopped[29]. The discovery of the TRP family led to the possibility of developing modulators of their activity that would have an analgesic effect. Acting directly on the initiators of the nociceptive process would allow for the

control of the generation of the pain signal at its very beginning. Thus, side effects associated to drugs acting more upstream on the nervous system would be avoided[5, 9, 16]. With this objective, a lot of effort is being made trying to develop modulators for TRP nociceptors[30].

In this direction, **TRPV1** has emerged as one of the most interesting members of TRP channels[17]. The *in vitro* studies of channel function as well as the deficiencies in nociceptive sensation and inflammatory processes showed by the TRPV1 knockout mice validated the vanilloid receptor as a therapeutic target[31]. Moreover, TRPV1 antagonists have shown analgesic effects with positive results in clinical pain trials[32]. However, on the negative side, TRPV1 antagonists are reported to diminish acute sensitivity to noxious heat and produce hyperthermia (increase in core body temperature), which compromise the integrity of the patients. As a result, up to date, no TRPV1 blocker has progressed further than phase II. It seems that the indiscriminate pharmacological blocking of the receptor with high affinity, and in a quasi-irreversible and competitive manner may be responsible for the observed side effects[33]. Thus, in this thesis a new approach was used to design novel antagonists with analgesic activity that primarily target pathological over-activated TRPV1 receptors (**Chapter 1**).

Similar relevance on the pain field has been suggested for **TRPM8** channel[34]. Genetic ablation of TRPM8 in rodents substantially attenuated the hypersensitivity produced by nerve injury. The implication of TRPM8 not only in this class of chronic pain syndrome, but also in some types of cancer and tearing regulation has motivated the development of different TRPM8 antagonists[35]. Unfortunately, a small number of compounds have entered the clinical trials, and the few of them that have done it reported hypothermia (decrease in core body temperature)[36]. As such, recent efforts have focused on discovering novel subtypes of pharmacophores. However, in contrast to TRPV1, little or nothing is known about the binding site of TRPM8 blockers.

The necessity of new TRPM8 antagonists and the lack of information regarding their mechanism of action have motivated the second part of this thesis. An extensive

study was done to identify TRPM8 antagonists which were later on characterized and computationally modeled to propose a binding sites (**Chapter 2**).

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CHAPTER 1

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INTRODUCTION

TRPV1 structure and modulation

TRPV1 is the founding member of a subfamily of thermoTRP channels that enable primary afferent nociceptors to detect harmful stimuli. It was cloned in 1997 from rat dorsal root ganglion (DRG) neurons and firstly described as the capsaicin receptor[17]. Further studies revealed TRPV1 as a molecular integrator for a broad range of physical and chemical stimuli. In addition to capsaicin, other vanilloid compounds such as resiniferatoxin (RTX) also activate TRPV1 channels [37]. Moreover, voltage, noxious temperatures > 42 ºC, and low pH (< 6) are TRPV1 channel activators as well [38, 39] (Figure 2).

TRPV1 is a tetrameric membrane protein with four identical subunits assembled around a central aqueous pore [40]. Each TRPV1 subunit protein shows a membrane domain composed of six transmembrane segments (S1-S6) [41], with an amphipathic region between the fifth and sixth segment that forms the channel conductive pore and intracellularly located amino and carboxyl-terminus (N-terminus and C-terminus respectively)[42] (Figure 2).

The C-terminus domain of TRPV1 is formed by 145 amino acids and contains a 25-residues sequence highly conserved referred to as TRP domain [43, 44](Figure 2A). This region has been widely described as a transduction domain important for channel gating[45]. Specifically, this domain includes a proline-rich region and a 6-mer conserved sequence termed TRP box that has been implicated in the allosteric coupling of stimuli sensing and pore opening[44, 46, 47]. The TRP domain has also been involved in channel tetramerization, however, this role still remains controversial, as other motifs in the C-terminus have also been identified to promote TRPV1 subunit association[48, 49].

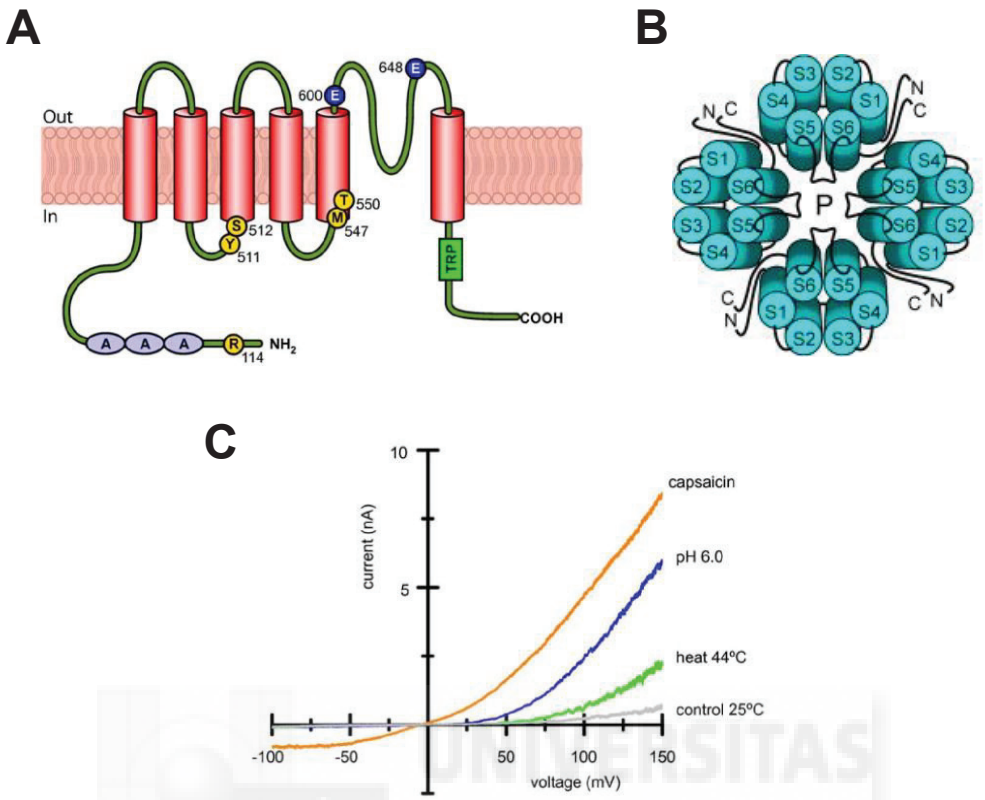

Figure 2. TRPV1 is a homotetramer activated by different stimuli. **A)** Schematic representation of the topology of a TRPV1 protein subunit. **B)** The functional channel is a tetramer formed by the ensemble of four such subunits. Residues involve in capsaicin binding are marked in orange. Marked in blue are two extracellular residues critical for activation by protons. **C)** Whole-cell I-V relationships of TRPV1 showing the activation of currents by low pH (6.0), heat (42°C) and capsaicin (100 nM). Modified from Belmonte, C. and Viana, F. 2008[1]

The 432-amino acid N-terminus contains potential protein-protein interacting domains such as a relatively proline-rich region and six ankyrin repeats domains (ARD) essential for channel function and whose structure has been determined with high resolution using X-ray crystallography[50,51]. Interestingly, the recent publication of a high-resolution cryo-electromicroscopy structure of an assembled TRPV1 channel has highlighted the interaction among the third and fourth ARD from one subunit and the pre-S1 and the C-terminus linker from an adjacent TRVP1 subunit[52]. The interaction of several proteins implicated in the trafficking to the membrane such as Snapin[53] , or in the stabilization of the channel in the membrane such as Whirlin[54] has also been reported in the C-terminus region.

The important role of TRPV1 as a molecular integrator for physical and chemical stimuli has motivated many structure-functional studies to identify the gating mechanism behind them[46, 55-59]. In relation to the vanilloids' binding site, studies using analogues of capsaicin and capsaicin-insensitive animal models demonstrated that these compounds bind to a putative vanilloid pocket through residues located in the cytoplasmic loop between the second, the third and the fourth transmembrane domains[60, 61]. Recent cryo-electromicroscopy studies identified distinct but overlapping binding sites for capsaicin and RTX, and suggested that the S4–S5 linker and the sixth transmembrane domain may also contribute to define the vanilloid binding site[62, 63] (Figure 3).

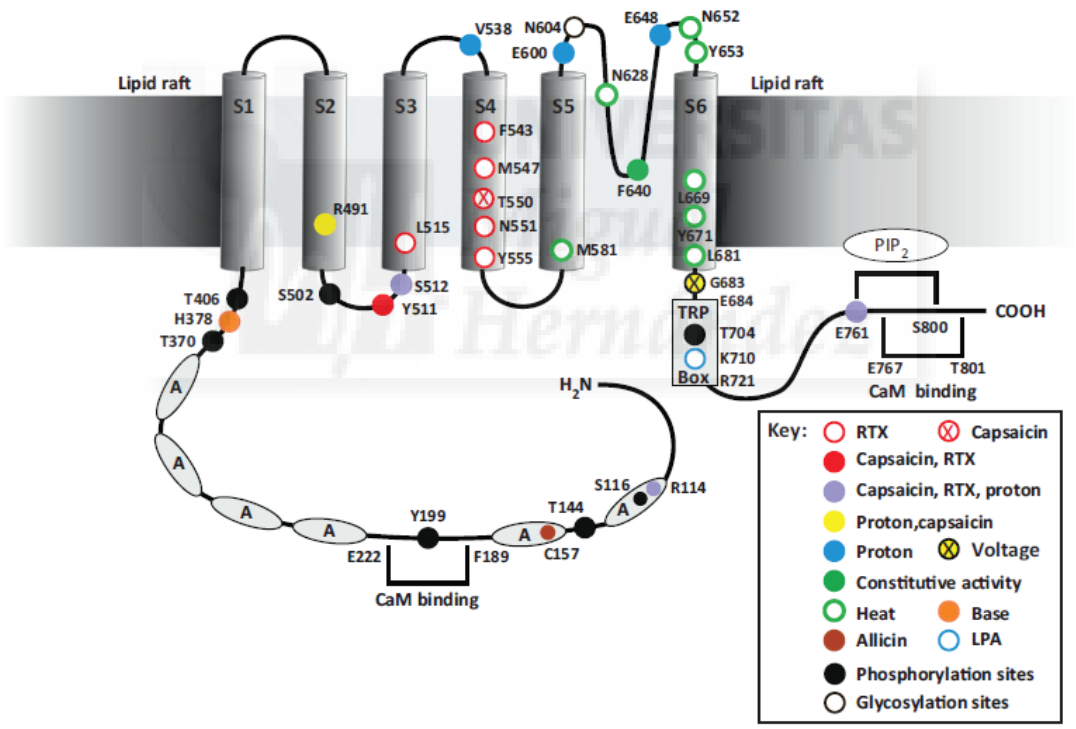

Figure 3. TRPV1 channel topology. The figure shows the key residues for channel gating by different stimuli as well as phosphorylation sites crucial for channel function modulation. Szolcsanyi, J. and Sandor, Z. 2012[59].

Regarding proton activation, two specific extracellular glutamate residues seem to be involved in pH sensitivity[64]. The first, E648, located at the loop between S5 and the S6, is crucial for direct activation of the channel by strong pH. The second, E600,

at the end of S5, is responsible for the potentiating effect of protons at milder acidic conditions[39, 64] (Figure 3). Interestingly, it has been shown that protons activate and potentiate TRPV1 by shifting the voltage dependence of the activation curves towards more physiological membrane potentials[38]. However, the structural basis for voltage sensing is not fully understood yet. A recent study has identified several basic and acid residues in S4 and the S4-S5 linker whose substitution altered the voltage gating, however, also significant effects on the capsaicin and temperature sensitivities were observed[65].

Similar to voltage sensor, and despite significant efforts, the temperature-sensitive gating of TRPV1 is still far from being fully understood, and widely diverging global views have been developed[55, 57, 66] (Figure 3). On one hand, some authors have implicated N- and C-termini domains and the outer pore region as the thermosensor[67]. On the other hand, other authors have suggested that temperature sensitivity is an integral property of the TRPV1 protein and no specific regions exist[55, 68].

An important characteristic of TRPV1 ion channels is its desensitization[17]. At the ion channel level, desensitization is defined as a decrease of channel activity due to a continuous stimulation. In vitro, is largely suppressed by buffering of intracellular [Ca^{2+}] or by absence of this ion in the extracellular solution[17]. This phenomenon can occur rapidly during single application of an agonist (desensitization) or slowly following repeated agonist applications (tachyphylaxis)[69]. Regarding the mechanism, it has been suggested that may be signaling via Ca^{2+}-calmodulin, since disruption of the proposed region for interacting with this protein in the C-terminal partially inhibits desensitization[70-72] (Figure 3).

Role of TRPV1 in nociception and pathological pain

Cumulative evidence shows that TRPV1 is a key player in the nociceptive sensation process[17, 39, 73-75]. First of all, TRPV1 has been mainly detected in small and medium diameter neurons in the primary sensory ganglia (trigeminal and DRG) from the

peripheral nervous system grouped in Aδ and C fibers[76]. Second, analysis of mice lacking TRPV1 channels not only revealed a complete loss of capsaicin sensitivity, but these animals also exhibited significant impairment in their ability to detect and respond to noxious heat[31, 73]. Moreover, TRPV1-knockout mice showed absence of thermal hyperalgesia development after peripheral administration of capsaicin or complete Freund's adjuvant (CFA)[31, 73]. Third, and as described in greater detail below, TRPV1-evoked responses are markedly enhanced by proalgesic or proinflammatory agents such as bradykinin or neurotrophins which produce hypersensitivity to heat in vivo[39, 77-83].

During any inflammatory process a wide variety of pro-inflammatory mediators are released sensitizing TRPV1 channel[84]. Cytokines[79], pruritogens[85, 86], ATP[87] and neuropeptides[76, 88] are some examples of these types of mediators. These mediators activate their respective receptors expressed on sensory neurons leading to a wide variety of intracellular signaling pathways that result in the activation of protein kinases such as protein kinase A (PKA) [89], protein kinase C (PKC)[77, 90], mitogen-activated protein kinases (MAPKs)[91, 92], and phospholipases such as phospholipase C (PLC) [93] . Protein kinases phosphorylate different residues of TRPV1 (Figure 4) and as a result TRPV1 channels are sensitized, increasing the probability of channel opening at normal membrane potentials or in response to other stimuli[94-96]. In addition, during an inflammatory process, increased expression of TRPV1 proteins and a subsequent increase in the number of TRPV1 in the membrane has been demonstrated[88, 96], contributing to an augmented TRPV1-mediated pain signalling.

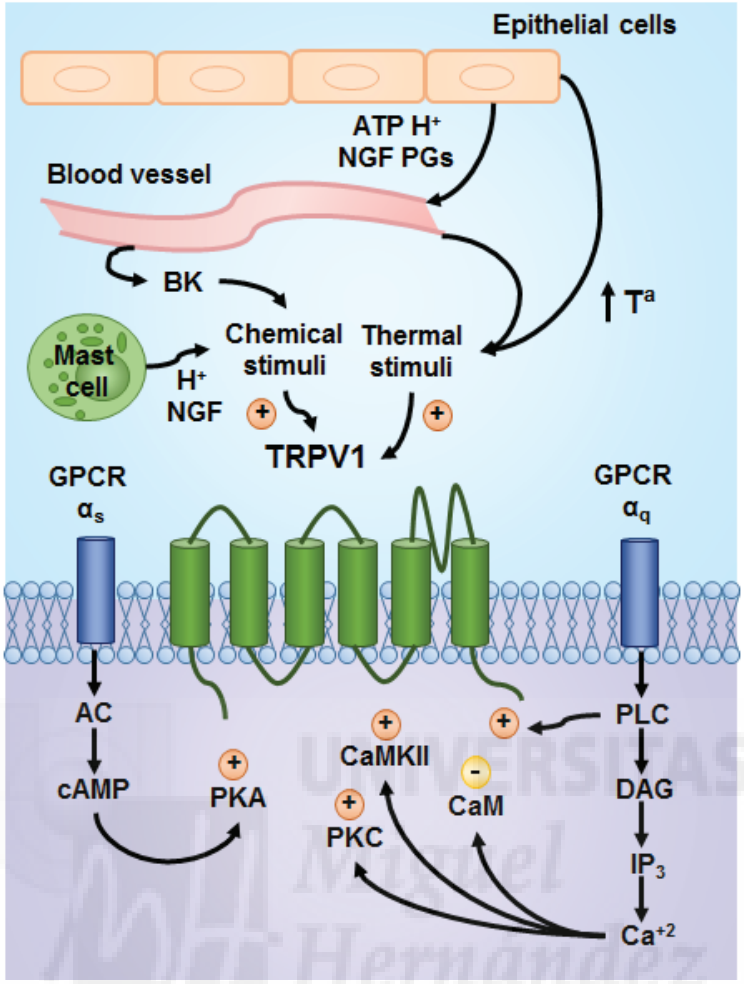

Figure 4. TRPV1 sensitization mechanism. Under pathological conditions, different pro-inflammatory mediators produces a wide variety of intracellular signaling pathways that result in potentiation of TRPV1 activity. PGs, prostaglandins; CaM, calmodulin; PLC, phospholipase C. DAG, diacylglycerol; IP3 inositol triphosphate; AC, adenylate cyclase; NGF, nerve growth factor. Adapted from Gold, M.S. and Gebhart, G.F. 2010[84]

TRPV1-based pain treatments

Due to the important role of TRPV1 in the pain pathway, several approaches have been taken to utilize it as a therapeutic target. On one hand, TRPV1 agonists have been used for many years for pain relief of peripheral origin. On the other hand, different TRPV1 antagonists are being investigated as new analgesics [30, 97].

Agonists

In addition to capsaicin, other vanilloids have been identified which also activate the TRPV1 channel. Resiniferatoxin (RTX), a diterpene related phorbol ester, is a potent analogue of capsaicin present in *Euphorbia resinifera* cactus and noted for having greater power-irritating[37]. Other chemical compounds that activate TRPV1 are 4-(thiophen-2-yl)butanoic acid[98] , camphor[99], 2-aminoethoxydiphenyl borate (2-APB)[100] and hydroxyl alpha sanshool[101], the active component of Sichuan pepper. TRPV1 is also activated by extracts of onion and garlic due to the molecule allicin, the main active component of these extracts[102]. Zingerone piperine and two compounds present in black pepper and ginger also activate TRPV1[103].

Despite the wide variety of TRPV1 agonists, nowadays the only one used in the pain relief is capsaicin, the canonical activator of TRPV1 channel[104]. Based on TRPV1-mediated defunctionalization of nociceptors, several creams and patches containing capsaicin have been used in the treatment of post-herpetic neuralgia, neuropathy, mastectomy, amputation and skin cancer among others, the best example being the 8 % capsaicin patch (Qutenza™)[105]. The prolonged activation of TRPV1 (> 60 min) produced a strong alteration of membrane potential that reduce neuronal excitability, inability to produce neurotrophic factors (Substance P) and retraction of epidermal and dermal nerve fibre terminals[106]. However, the initial pungency and irritation and modest or lack of efficacy observed in some clinical trials has created concern about their use[104]. For these reasons, pharmaceutical companies have tried to develop TRPV1 antagonists that could treat pathological pain avoiding the sides effects observed with TRPV1 agonists.

Antagonists

Few years after the TRPV1 cloning, several potential antagonists started to emerge based on the capsaicin structure[30, 107]. Those compounds shared a pharmacophore scaffold conformed by an A-region (aromatic ring similar to vanilloid) + Linker (ester, amida, urea, thiourea) + C-Region (aliphatic group)[30] (Figure 5).

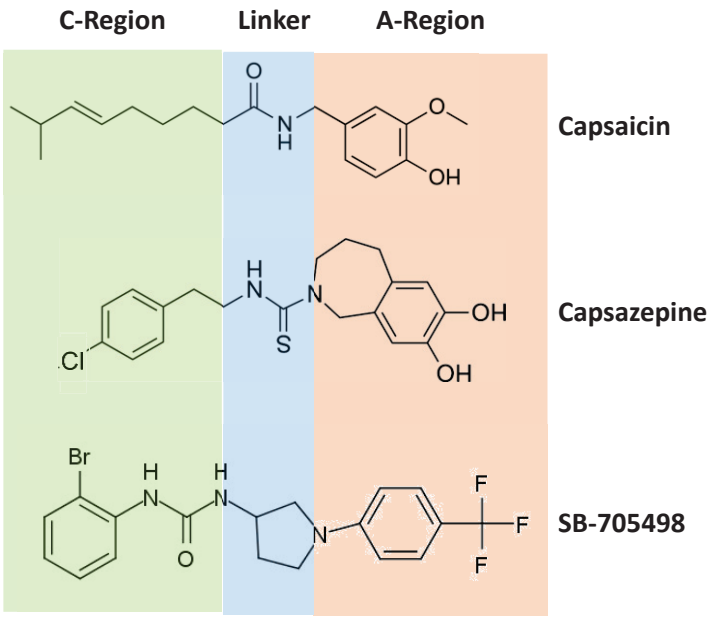

Figure 5. Pharmacophores of the representative TRPV1 ligands. For capsaicin, capsazepine and SB-705498, three important pharmacophore regions are marked. Modified from Lee,Y et al. 2015[30].

The first of these new antagonists was the capsazepine. This molecule was a competitive antagonist that blocked only the activation of TRPV1 channels by chemicals[107]. However, in addition to its effect on TRPV1 channels, it was also shown to inhibit the cold activated TRPM8 channel, voltage-activated calcium channels and nicotinic acetylcholine receptors[108-110]. After capsazepine, many other compounds appeared following this strategy[30] (Table 1).

Table 1. Status of clinical trials for TRPV1 antagonists

Name	Therapeutic	Development status	ClinicalTrials.gov ID
SB-705498 (GSK)	Atopic dermatitis	Phase II (Completed)	NCT01673529
AZD-1386 (AstraZeneca)	Pain	Phase II (Completed)	NCT00672646
AMG-517 (Amgen)	Pain	Phase I (Terminated)	1*
PHE-377 (PharmaEste)	Neuropathic pain	Phase I (Completed)	2*
GRC-6211 (Glenmark)	Neuropathic pain	Phase II (Suspended)	3*
MK-2295 (Merk)	Post-operative pain	Phase II (Completed)	NTC00387140
XEN-D0501 (Xention Ltd.)	Cough	Phase II (Completed)	NTC02233699
PAC-14028 (Amorepacific Corporation)	Pruritus	Phase II (Recruiting)	NCT02565134

1* http://www.ncbi.nlm.nih.gov/pubmed/18337008

2* http://www.mp-healthcare.com/pdf/20090714_Pharmeste.pdf

3* https://investor.lilly.com/releasedetail.cfm?ReleaseID=271993

Unfortunately, these competitive TRPV1 antagonists showed critical side effects such as hyperthermia and impaired noxious heat sensation in humans, leading to their withdrawal from clinical trials[32, 111]. It is not clear why some TRPV1 blockers elevate body temperature whereas others do not. It seems that indiscriminate pharmacological blocking of the receptor with high affinity, quasi-irreversible, competitive antagonists may be responsible for the observed side effect. Moreover, several studies suggested that compounds which prevented the activation of TRPV1 by capsaicin, but not by H^+ or temperature, had no effect on body temperature[33, 112]. However, the relation between these two conditions is not clear yet. Thus, the need for a different class of antagonists emerged: antagonists that would be activity-dependent primarily targeting over-activated receptors.

Recent progress on TRPV1 pharmacology includes three approaches aimed at developing inhibitors offering an alternative to classical competitive antagonists. The first approach aims to target the TRP domain as a region that modulates channel gating with compounds that would act as allosteric modulators named as TRPducins[113]. These short peptides mimic the sequence of the N-end region of the TRP domain and selectively block the channel by interacting with cytosolic binding sites. This line of research has rendered one compound called (TRPducin TRP-p5) that targets TRPV1 and displayed *in vivo* anti-nociceptive effect[97, 113].

The second approach for the development of new analgesics consists of targeting the overexpression of TRPV1 during inflammation[114]. One example is a peptide that mimics the SNAP25 protein (DD04107)[114] and modulate the TRPV1 recruitment in inflammatory conditions by blocking its exocytotic incorporation to the plasma membrane. This peptide has shown an important and long-lasting anti-nociceptive activity in models of chronic neuropathic and inflammatory pain and is currently in Phase II of clinical trials[114, 115].

The third strategy is to develop TRPV1 non-commpetitive antagonists that exert an activity-dependent inhibitory effect specifically binding to the agonist-receptor complex or to the open state of the channel[116]. Their interaction with active receptors

enables them to preferentially block highly activated receptors while only interacting minimally with physiologically working or silent channels. This characteristic makes these compounds to attract sizable interest as potent and safe drugs. An example showing that this strategy can be successful is memantine[116], an open-channel blocker of the NMDA receptor that has been approved for the treatment of Alzheimer's disease[117].

Open-channel blockers are compounds that enter the mouth of the channel when is open and do not allow the pass of positively charged ions through the pore[118]. They usually interact at the location of the pore where several negatively charged amino acid residues control ion permeability, and those are only exposed on the open state. To interact with these residues, open-channel blockers normally have positive charges and show a strong voltage-dependence inhibiting at negative potentials. Furthermore, the time that the binding site is accessible is directly proportional to the time the channel remains in its open conformation[119]. This is a clear advantage for TRPV1-mediated inflammatory pain since the drug-binding site would be more exposed in over activated channels.

Following the approach of designing open-channel blockers, previous studies in our lab identified two compounds, DD161515 and DD191515 (Figure 6, left)[120]. These peptoids conformed by two aryl moieties and one cationic group, were selective TRPV1 antagonists with micromolar efficacy. Moreover, administration of DD161515 and DD191515 into mice significantly attenuated the irritant activity of capsaicin and reversed the thermal hyperalgesia induced by tissue irritation[120]. Thus, they came out as compounds with high therapeutic potential at the same time that supported the relevance of TRPV1 in the pain perception. However, their moderated antagonist activity together with an unexpected toxicity interrupted their development into useful analgesics even though they were active *in vivo* in animal models of pain. Nevertheless, these peptoids established the bases of a new generation of non-competitive TRPV1 blockers. In parallel, other laboratories were also developing non-competitive capsaicin antagonist such as methoctramine[121]. However, a moderate potency (IC_{50} of 2 μM), together with the lack of receptor selectivity has restrained the use of these compounds *in vivo*.

The promising results observed with DD161515 and DD191515 led, few years later, to the design of the second-generation compound DD01050 (Figure 6, right)[122]. The relationship observed between the positive charges of the parenteral compounds and TRPV1 antagonism, motivated the incorporation of a strong positive charged amino acid (arginine) on the DD01050 structure. Similar to DD161515 and DD191515, compound DD01050 preferentially blocked TRPV1 over other neuronal receptors albeit its potency was 10-foldhigher than the original peptoids. As expected by an open-channel blocker, DD01050 exerted its activity in a voltage-dependent manner. Interestingly, as its antecessors, DD01050 prevented the irritant activity of capsaicin and reduced the thermal hyperalgesia[122]. However, despite the chemical modifications done in DD01050 respect to DD161515 and DD191515, the toxicity remained, preventing its further development as analgesic. Moreover, it was found that DD01050 not only blocked TRPV1 activity, but also was a potent antagonist for cold-evoked responses in mouse and human TRPM8 channel[123].

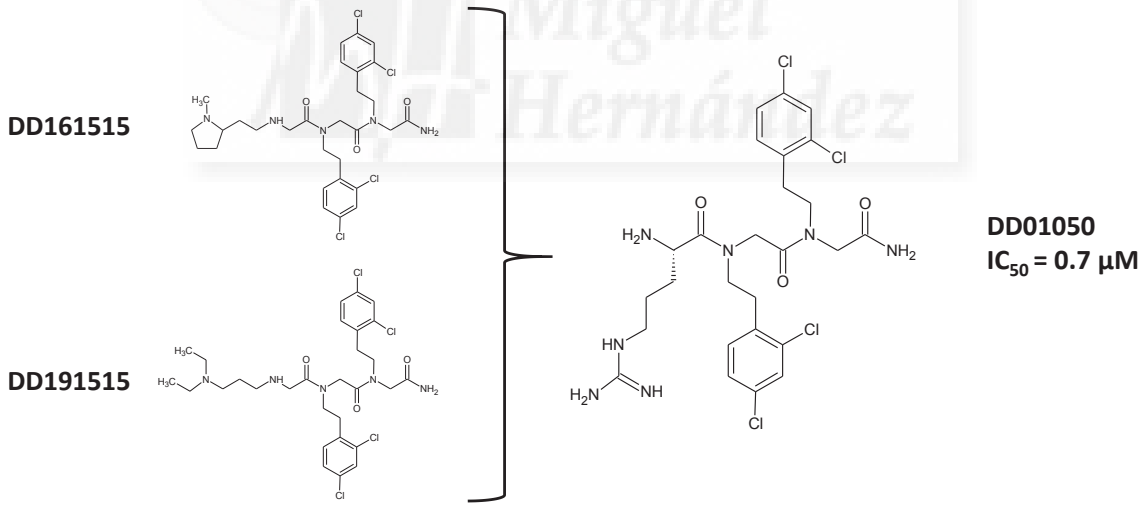

Figure 6. N-alkylglycine trimers **DD161515** and **DD191515** (left) to a new TRPV1 antagonist, **DD01050** (right) by addition of an arginine amino acid. Adapted from Garcia-Martinez, C. et al. 2002 and Garcia-Martinez, C. et al. 2006[120,122].

Despite the fact that the challenges faced with compounds DD161515, DD191515 and DD01050 have limited their therapeutic potential, the analgesia displayed in preclinical models of pain validated this class of TRPV1 antagonist as promising

molecules for analgesic drug development. Based on this evidence, the flexible scaffold present in the peptoids (Figure 7, left) was substituted by a more rigid and symmetric moiety (2,4,6-trisubstituted-1,3,5-triazine, Figure 7, right) since more rigid structures usually increase the potency and selectivity of the candidates[124].

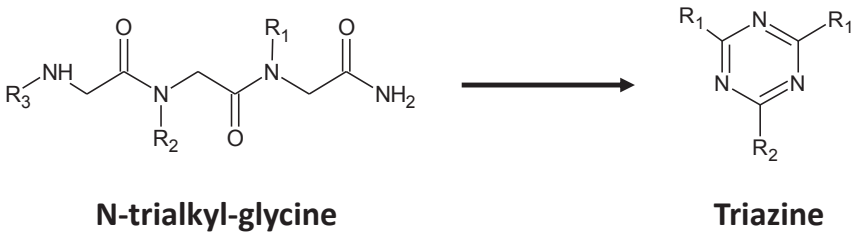

Figure 7. General chemical structure from previous antagonists (**N-trialkyl-glycine**) and the new scaffold used on the synthesis (**2,4,6-trisubstituted-1,3,5-triazine**).

Then, this improved scaffold was utilized to synthetize a third generation of open-channel blockers with 35 new compounds, and their activity was tested using voltage-clamp experiments in amphibian oocytes from *Xenopus laevis* heterologously expressing rat TRPV1 (rTRPV1) channels. Among all the compounds, 15 presented blockade activity higher than 75%. Modifications around the common triazine scaffold, and the biological activity assays of these derivatives, allowed us to establish the Structure-Activity Relationship (SAR) of this chemical library[124]. These studies showed that aromatic rings together with a positively charged group were required to exert the antagonistic activity. Moreover, an increase in the electronegativity of the phenethylamino region increased the potency. Taking in to account these results, the study of three derivatives, 8aA, 8bA, and 8cA was continued (Figure 8).

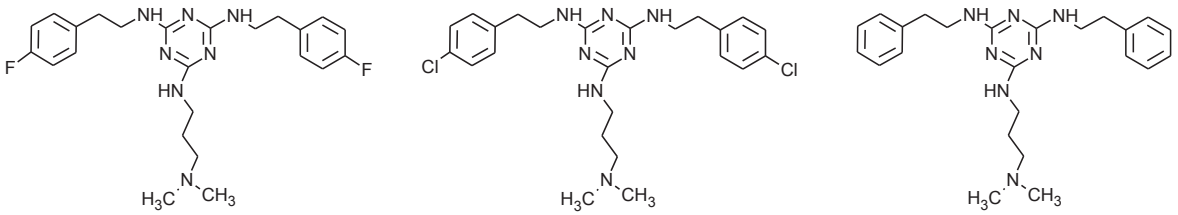

Figure 8. Chemical structure from the triazine derivatives 8aA, 8bA and 8cA from Vidal-Mosquera, M. et al.[124].

The blockade showed by these three triazine-derivatives was dose-dependent and reversible. In the bibliography it has been reported that some charged antagonists also presented agonist effects[125]. As the structure of the triazines allows their protonation at physiological pH, the possibility of them having a similar behavior was explored. Unfortunately, derivatives 8cA and 8bA presented significant agonist effect even at the lowest concentration. In contrast, triazine 8aA did not activate the channel at any of the tested concentrations[124].

According to the results previously described, the main objective of this project was to continue the characterization of the trizaine-derivative 8aA. The aim was to develop a novel TRPV1 open-channel blocker with high pharmacological potential that could avoid the toxicity displayed by other TRPV1 antagonists.

OBJECTIVES

Main objective

The main objective of this study was to characterize a new TRPV1 open channel blocker with analgesic properties. With this aim we evaluate the therapeutic potential of triazine 8aA, previously identified as a TRPV1 antagonist.

Specific objectives

- Characterize the pharmacological properties (efficacy, potency, selectivity, neuronal excitability) and blockade mechanism of triazine 8aA.

- Study the anti-nociceptive activity of the candidate in different in vivo models of acute and chronic pain.

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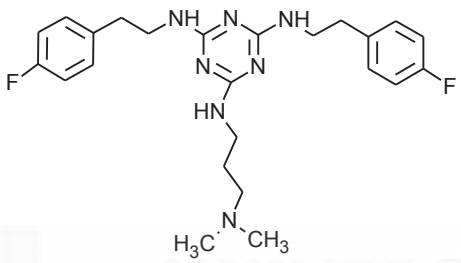
RESULTS

Triazine 8aA preferentially blocked capsaicin-evoked rTRPV1 activity

As it has been described in the introduction, previous studies in our group revealed the triazine-based TRPV1 antagonist compound 8aA (triazine 8aA from now on) as a promising TRPV1 open-channel blocker[124] (Figure 9).



8aA

Figure 9. Chemical structure from the triazine derivatives 8aA.

To further investigate the antagonistic properties of this compound, we firstly addressed its potency as blocker of capsaicin-induced TRPV1 activity. In order to prevent the desensitization process, we utilized a Ringer's solution without Ca^{2+}. We obtained a dose-response curve from the ionic currents acquired at -60 mV after the application of different concentrations (from 0.001 µM to 10 µM) of triazine 8aA in presence of 10 µM capsaicin (solid red line in Figure 10). The IC_{50} obtained was 50 nM with a 95% confidence interval (95% CI) between 46 nM and 71 nM. The Hill coefficient was close to 1, n_H=0.61 (95% CI=0.59 – 0.69), suggesting a single binding site for triazine 8aA in TRPV1.

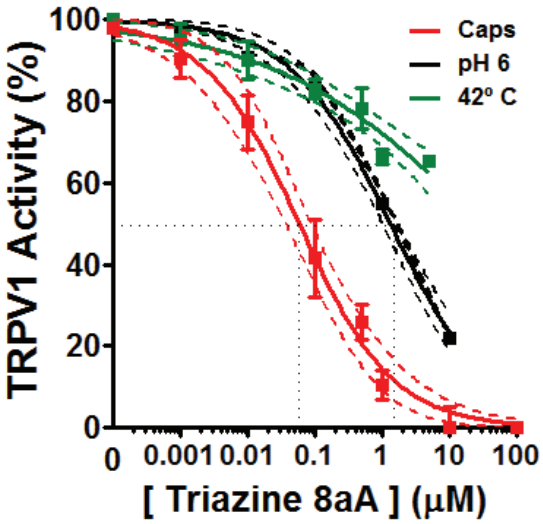


Figure 10. Triazine 8aA blockade of capsaicin, acidic pH and heat-evoked rTRPV1 activity. Red line) Dose-response curve for triazine 8aA blockade activity of 10 µM capsaicin-activated TRPV1 activity. **Black line)** Dose-response curve for triazine 8aA on TRPV1 channel activated with external solution at pH 6. **Green line)** Dose-response curve for triazine 8aA on TRPV1 channel activated with external solution at 42 ºC. In capsaicin and acidic experiments, rTRPV1 channels were expressed in amphibian oocytes and currents were measured using voltage clamp in absence of Ca^{2+} (Vh= - 60 mV). In heat activation, rTRPV1 channels were expressed in Sh-Sy5y cells and the changes in the Ca^{2+}-dependent fluorescence was measured. Responses were normalized with respect to that in the absence of antagonist. Each point represents the mean ± SEM, with n≥4. Dotted line represents the 95% CI.

As mention previously, it has been reported that compounds that fully blockade of TRPV1 activation by capsaicin, but only partially blockade of TRPV1 activation by other stimuli, avoid certain side effects such as hyperthermia in most of the cases[33]. Thus, we explored whether triazine 8aA would be active in presence of external solution at pH 6.

In Ringer's solution without Ca^{2+} and the voltage held at -60 mV, we first applied buffer at pH 6 as a control to corroborate that low pH activated the channel. We next tested triazine 8aA at different increasing concentrations from 0.001 µM to 10 µM in presence of acidic solution. Similar to capsaicin modality of activation, we obtained a dose-response curve (solid black line in Figure 10). The IC_{50} was 1.31 µM (95% CI = 1.13 – 1.52 µM) and the Hill coefficient was n_H = 0.58 (95% CI = 0.53 to 0.63). The difference of orders of magnitude between the concentration necessary to block

capsaicin activation (50 nM) and to block acidic pH activation (1.31 μM) shows a clear modality preference.

We also evaluated its possible antagonist effect on heat-induced TRPV1 activity. We obtained a dose response curve after the application of different concentrations of triazine 8aA in presence of the thermal stimulus (42ºC) (solid green line in Figure 10). None of the concentrations tested blocked more than 50% of the heat evoked TRPV1 activity, suggesting an IC_{50} higher than 10 μM.

These results supported triazine 8aA as a potent TRPV1 antagonist with preference to block capsaicin modality of activation.

Triazine 8aA blocked TRPV1 activity in a selective manner

For the development of new drugs, not only high potency is desirable, but also specificity and selectivity against the target. To analyze these parameters for triazine 8aA we assayed its inhibitory activity on TRPM8 and TRPA1 channels, members from the same family as TRPV1, and N-methyl-D-aspartate receptor (NMDA), a non-related channel in absence of Ca^{2+}. The results from these experiments are collected in Table 2.

Table 2. Effect of triazine 8aA in TRPV1, TRPM8, TRPA1 and NMDA receptors.

Ion channel (Oocytes)	Triazine 8aA IC_{50} (μM) at -60mV
TRPV1 (vs 10 μM capsaicin)	0.05 ± 0.01
TRPM8 (vs 300 μM menthol)	7.50 ± 1.50
NMDA (vs 100 μM L-glutamate /10 μM glycine)	9.20 ± 2.10

Ion channel (HEK cells)	Triazine 8aA IC_{50} (μM) at -60mV
TRPA1 (vs 100 μM AITC)	> 10.00

Values derived from corresponding dose response curves. TRPV1, TRPM8 and NMDA were heterologously expressed in *Xenopus* oocytes and tested at different increasing concentrations from 0.001 μM to 10 μM of triazine 8aA. Responses were recorded at -60 mV in Ringer's solution without Ca^{2+} and normalized respect to that elicited by 10 μM Caps, 300 μM menthol and 100 μM L-glutamate/10 μM glycine respectively. TRPA1 channel was expressed in HEK cells and recorded at -60mV in Standard solution without Ca^{2+}. Responses were normalized respect to that elicited by 100 μM AITC. Data are shown as the mean ± SEM, n≥4.

The dose-response of triazine 8aA in presence of 10 μM capsaicin reported an IC_{50} in the nanomolar range (see Figure 10 and Table 2). Next, we tested the same range of concentrations of triazine 8aA in menthol-activated TRPM8 channel. As shown in Table 2, the IC_{50} was 7.50 ± 1.50 μM, two order of magnitude higher than the one observed in TRPV1 suggesting that triazine 8aA could be a specific TRPV1 modulator. Interestingly, when we tested up to 10 μM triazine 8aA on TRPA1 channel expressed in human embryonic kidney (HEK293) cell line activated by 100 μM AITC, the blockade was almost inexistent (IC_{50} > 10 μM, Table 2). Although the expression system was different and may slightly differ in oocytes, the wide-ranging differences observed between the IC_{50} in TRPV1 and TRPA1 support the possible specificity of triazine 8aA. In order to also test the selectivity, we studied the effect the previous mentioned range of concentrations of triazine 8aA in the NMDA receptor activated by 100 μM L-glutamate/10 μM glycine. Comparable to the results observed with TRPM8 and TRPA1, triazine 8aA blocked only marginally the activity of this channel and the IC_{50} was 9.20 ± 2.10 μM (Table 2). Thus, triazine 8aA, showed a preferential TRPV1 blockade.

Triazine 8aA blocked capsaicin- and acidic pH-evoked hTRPV1 activity

Compounds that block the channel in one species could be inactive or even act as an agonist on its orthologue in other species[126-129]. This information brings up to question whether triazine 8aA, active on rat TRPV1, could block human TRPV1 (hTRPV1). For this task, hTRPV1 was expressed in HEK293 cell line. We observed that the blockade activity showed by triazine 8aA over capsaicin-evoked currents was reproduced in hTRPV1 channels, where almost all the current was abolished at 10 µM (Figure 11A).

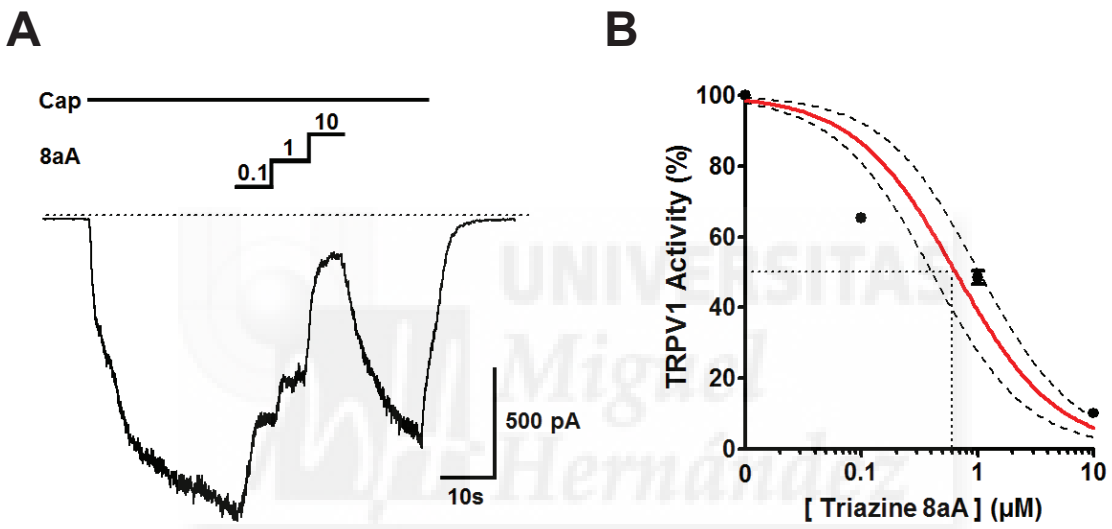


Figure 11. Triazine 8aA blockade of capsaicin-evoked hTRPV1 activity. A) Representative capsaicin-evoked ionic currents from hTRPV1 in presence of 1 µM capsaicin and 0.1 µM, 1 µM and 10 µM triazine 8aA measured by patch-clamp in whole cell configuration held at -60mV. Ca^{2+} was removed from the buffer to prevent the desensitization process. The horizontal bars indicate the experimental paradigm used for agonist stimulation and channel blocking. **B)** Dose-response curve of the inhibitory activity showed by triazine 8aA at -60mV. Data are given as means ± SEM; n≥4 cells. Dotted line represents the 95% CI.

Triazine 8aA also showed a dose-dependent of hTRPV1. The dose-response curve (solid red line in Figure 11B) reported an IC_{50} for triazine 8aA of 506 nM (95%CI=345-741 nM). We also were able to detect a recovery on the current once the inhibitor was removed from the medium, an indication of a reversible mechanism of blockade (Figure 11A).

The effect previously observed with triazine 8aA over pH activation in rTRPV1 made us question whether the results would be reproducible in hTRPV1. Hence, we tested the activity of the antagonist in the presence of a moderate acidic pH. The application of a first pulse of buffer at pH 6 produced inward currents that were reproduced when we applied a second pulse of acidic pH in absence of triazine 8aA (Figure 12A). On the contrary, when we applied 10 μM triazine 8aA, the pH-evoked current was blocked with a similar potency than the observed in oocytes (73.1 ± 4.2% in hTRPV1 vs 78.0 ± 0.5% in rTRPV1) (Figure 12B). Thus, these results confirmed a similar antagonistic effect on both TRPV1 channels.

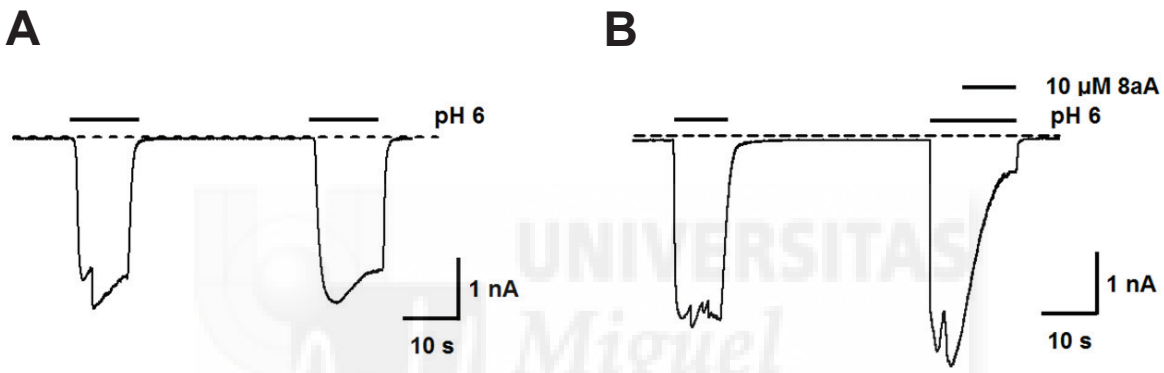


Figure 12. Triazine 8aA blockade activity on acidic pH-evoked hTRPV1 activity. Currents were obtained by patch clamp in a whole-cell configuration at -60mV in Ca^{2+} free buffer. The horizontal bars indicate the experimental paradigm used for agonist stimulation and channel blocking. **A)** Representative currents activated by two pulses of pH 6. **B)** Representative recording in absence (first pulse) or presence of 10 μM triazine 8aA (second pulse).

Triazine 8aA blocked capsaicin-evoked TRPV1 activity in voltage-dependent manner

Triazine 8aA at pH 7.4 is positively charged molecule that could be sensing the pore electrostatic field. In order to explorer this possibility, we studied the voltage dependence of exerted by the compound. As it shown In Figure 13A, current-to-voltage relationship revealed that triazine 8aA inhibited capsaicin-evoked TRPV1 responses potently at negative membrane potential, yet it was nearly inactive at depolarized voltages. These results indicate that TRPV1 receptor blockade by triazine 8aA is voltage dependent, and suggest that the triazine 8aA binding site senses the pore electrostatic field.

Seeking to further corroborate this observation, we obtained the fraction of unblocked response (**$I_{blocker}/I_{control}$**) as a function of the membrane potential (Figure 13B, dots). The fraction of unblocked response-voltage relationship is related with the location of the blocker binding site within the membrane electrostatic field [130,131]. Experimental data exhibited a dependence on the applied membrane voltage in the range of -60 to -15 mV. It is common to use the Woodhull model to describe the voltage-dependent blockade of compounds. In this model it is assumed that the charged blocking particle enters the channel pore to a certain distance, and sense part of the transmembrane electric field. According to the Woodhull model, the IC_{50} of a molecule with valence **ʒ**, binding to a site within the membrane electric field is described by the relations stated in Equation 1:

$$IC_{50}(V_m) = IC_{50}(0\ mV)exp^{\frac{-\zeta\delta V_m F}{RT}}$$

Equation 1. Woodhull model

where the **IC_{50} (Vm)** and **IC_{50} (0 mV)** are the unblocked response fraction at transmembrane voltage Vm and at 0 mV. In this equation, **δ** is expressed as “part per unit” of the way across the membrane’s potential from extracellular side to the

cytoplasmic side. It represents the location of the energy barrier for block (i.e. the blocker binding site) expressed as a fraction of the electrostatic field gradient sensed by the blocking site. For its part, **RT/F** is a constant of value 25.3 mV at 20ºC.

In our study, we utilized an approximation to the Woodhull model previously described by Planells-Cases et al [132] (Equation 2). In this model **($I_{blocker}/I_{control}$)** was utilized as an approximation of the **IC_{50} (Vm)** and **IC_{50} (0 mV)** was substituted by the constant **K (0 mV).**

$$\frac{I_{(c)}}{I_{(O)}}(V_m) \approx K(0\ mV)exp^{\frac{-z\delta V_m F}{RT}}$$

Equation 2. Fraction of unblocked response of triazine 8aA (modified from Woodhull model)

The inferred electrical distance of the triazine 8aA binding site from the mouth of the channel, δ, was ~0.36 (Figure 13B, solid line). Together, these results imply that the drug binding site is located within the aqueous pore, and hint that triazine 8aA acts as a TRPV1 open channel blocker with moderate affinity.

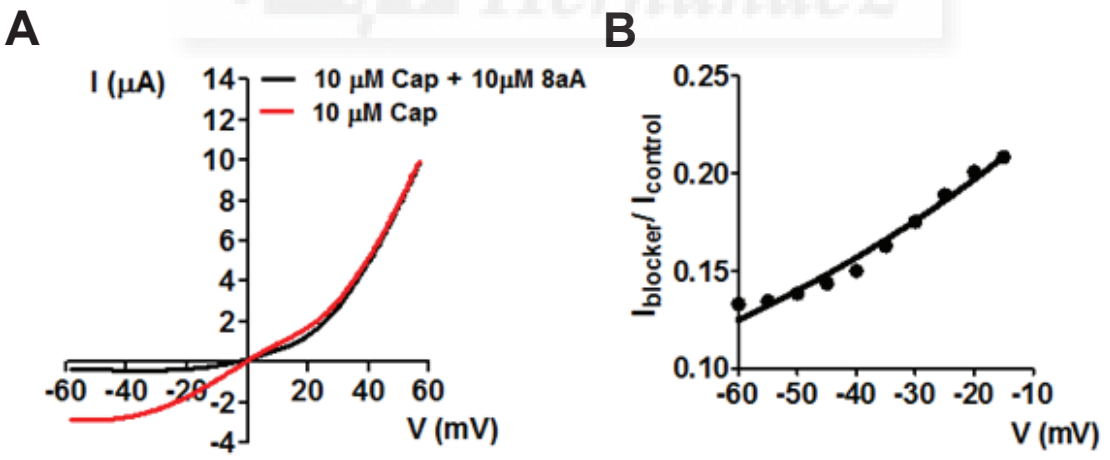


Figure 13. Voltage dependency of triazine 8aA blockade. **A**) Representative ionic currents evoked by 10 µM capsaicin using a linear ramp from -60 to +60 mV in the absence (red line) or presence (black line) of triazine 8aA at 10 µM. **B**) Fractional blocking of TRPV1 by triazine 8aA as a function of the voltage. The solid line depicts the fitting to the Woodhull model, which for the triazine **8aA** binding site, yielded an electric distance (δ) value of 0.36 within the membrane electric field.

Triazine 8aA docking suggested interactions in the pore region of TRPV1 channel

In collaboration with Jordi Bujons from the Institute of Advanced Chemistry of Catalonia (IQAC) in Barcelona we performed molecular docking to locate the regions involved in the binding of triazine 8aA. We utilized a simplify model of the transmembrane domains S5 and S6 and their extracellular loops of TRPV1 on the open state based on the Kv1.2 structure.

In Figure 14 can be observed that triazine 8aA is localized into the pore region of TRPV1 channel. The amino groups of the molecule formed hydrogen bonds with G645 from a subunit and the G645 from the opposite one on the S5-S6 loop. Moreover, the amino groups of the alkyl chain also established hydrogen bonds with M644 and I642 stabilizing the complex triazine 8aA-TRPV1 channel. Although we performed the experiments on a structure based on Kv1.2, we expect similar results on the recent and almost complete published 3,4 Å-resolution structure of TRPV1[52] since no differences has been proposed for this region.

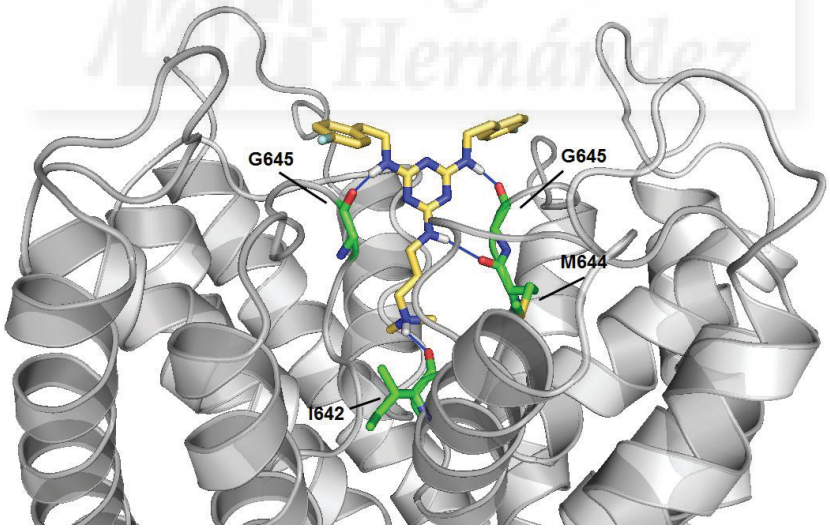


Figure 14. Molecular modelling of triazine 8aA where a binding site is proposed into the pore region of TRPV1 receptor. Parts of the S1 and S2 domains of TRPM8 are depicted as well as the TRP domain. Boc group of the compound 8-3 makes a hydrogen bond with the residue W693 (blue line). The hydrophobic interactions between compound and residues are represented as grey doted lines.

Triazine 8aA did not affect the cellular viability

Once triazine 8aA was characterized, the next step was to evaluate its cytotoxicity on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay[133]. We performed the experiments on two different systems: HEK293 cells, as generic model to evaluate general toxicity as it does not express TRPV1 channels; and ShSy5y-TRPV1, a neuroblastoma cell line used as a model of neuronal function that heterologously expressed rTRPV1. We utilized a range of concentrations from 1 nM to 100 µM triazine 8aA, which includes the IC_{50} determined on previous experiments. We measured the cell viability by spectrophotometry after 24h of incubation with triazine 8aA.

We observed that triazine 8aA showed complete absence of toxicity from 1 nM to 10 µM on HEK cells and only at 100 µM can be detected a significant reduction of the cell viability (27%) (Figure 15A). Similar to HEK results, triazine 8aA was no toxic up to 1 µM in Sh-Sy5y-TRPV1.

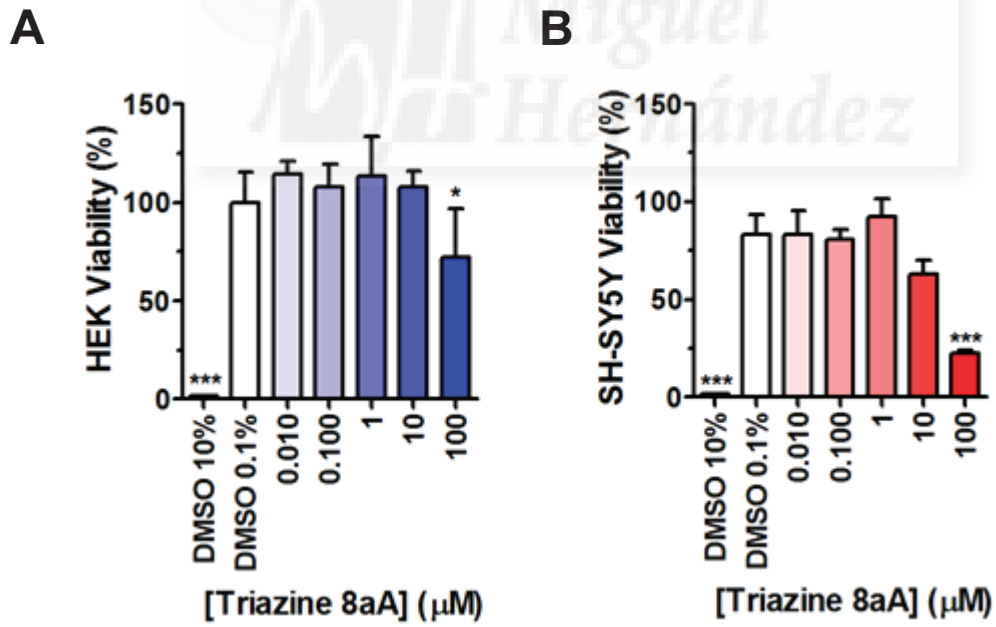


Figure 15. Effect of triazine 8aA on MTT-based assay of cytotoxicity in HEK293 cells and Sh-Sy5y-TRPV1. A) HEK293 and **B)** SH-SY5Y-TRPV1 cell were cultured in 96-well plates and incubated with and without the indicated concentrations of triazine 8aA for 24 h. Each value is expressed as mean ± SEM. n=4. ANOVA with Bonferroni post hoc test (*** $p < 0.001$).

Contrary, when we tested the compound at 10 μM and 100 μM we obtained a moderate (20%) and strong (61%) reduction on the cell viability. However, the statistical analysis did not detect significantly differences at 10 μM with control conditions (DMSO 0.1%) (Figure 15B). Thus, from these experiments we concluded that triazine 8aA presented a moderate cytotoxicity.

Triazine 8aA inhibited neuronal TRPV1 channel activity

We demonstrated the antagonistic activity of triazine 8aA in heterologous systems, however, a question that emerges is whether the compound will block TRPV1 channel in its neuronal environment, i.e., sensory neurons. Thus, we investigated the efficacy of triazine 8aA modulating TRPV1 function in primary cultures of rat DRG sensory neurons. The neuronal cultures were tested in absence (vehicle) or in presence of 10 μM triazine 8aA, and TRPV1 channel activity was measured by Microelectrodes Arrays (MEA) chambers.

As shown in Figure 16A, in absence of triazine 8aA, DRG neurons responded to capsaicin instillation producing action potentials with a mean spike frequency of 2.06 Hz. Interestingly, when we applied capsaicin in presence of triazine 8aA, the mean spike frequency was attenuated (1.03 Hz). A quantitative assessment of the inhibitory activity is displayed in Figure 16B, demonstrating the significant reduction on the neuronal TRPV1 activity (50%) and corroborating the results observed in heterologous systems.

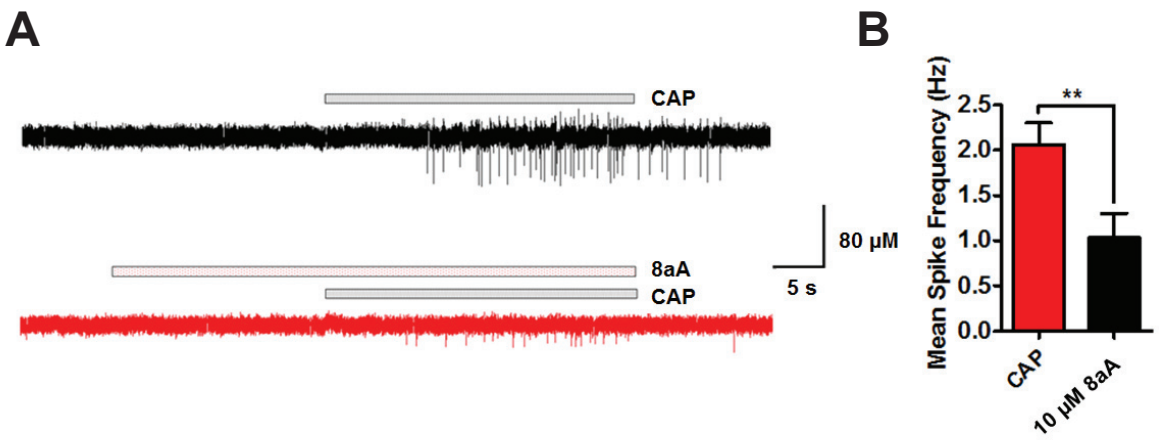


Figure 16. Effect of triazine 8aA in neurons activated by capsaicin. A) Representative recordings of evoked action potentials in rat DRG neurons. We applied a pulse of 30 seconds of 1 µM capsaicin to evoke action potentials in absence (top, black) or in presence of 10 µM triazine 8aA. **B)** Mean spikes frequency measured from the pulse of capsaicin in absence and presence of 10 µM triazine 8aA. Data are given as means ± SEM; n ≥ 15 cells. Statistical analysis was made by t-test, ns no significance **P < 0.01.

Triazine 8aA did not affect normal sensory transduction

Triazine 8aA has demonstrated to block preferentially TRPV1 when it was compared with other channels. However, its potent inhibitory effect may create concerns of whether it is also acting on channels present in neurons responsible for action potential generation and propagation (e.g. voltage-gated Na^+ and K^+ channels). Thus, we decided to test it on neurons isolated from neonatal rat DRG cultured on MEA chambers.

As illustrated in Figure 17A, top and bottom, the first 40 mM KCl application (P1) evoked action potentials on the neurons due to membrane depolarization that we could also measure when we applied the second KCl pulse (P2). Due to desensitizing processes, we observed a significant reduction on the spikes frequency when we compared P1 and P2 under control conditions (0.67 ± 0.19 and 0.35 ± 0.11 respectively, Figure 17B). Similarly, in presence of 10 µM triazine 8aA the mean spike frequency obtained at the P1 (0.79 ±0.17) was higher than the one in P2 (0.52 ± 0.12) (Figure 17B). Then we analyzed the ratio established between

P2 and P1 in the presence and absence of triazine 8aA (Figure 17C). Depolarizing effects produced by the compound would be represented as a decrease on the ratio P2/P1 compare to control conditions, meanwhile a hyperpolarization would produce an increase on the ratio. A statistical t-test revealed no significant differences between neurons treated with vehicle or in presence of the antagonist (Figure 17C) indicating that triazine 8aA did not modify the neuronal excitability by itself.

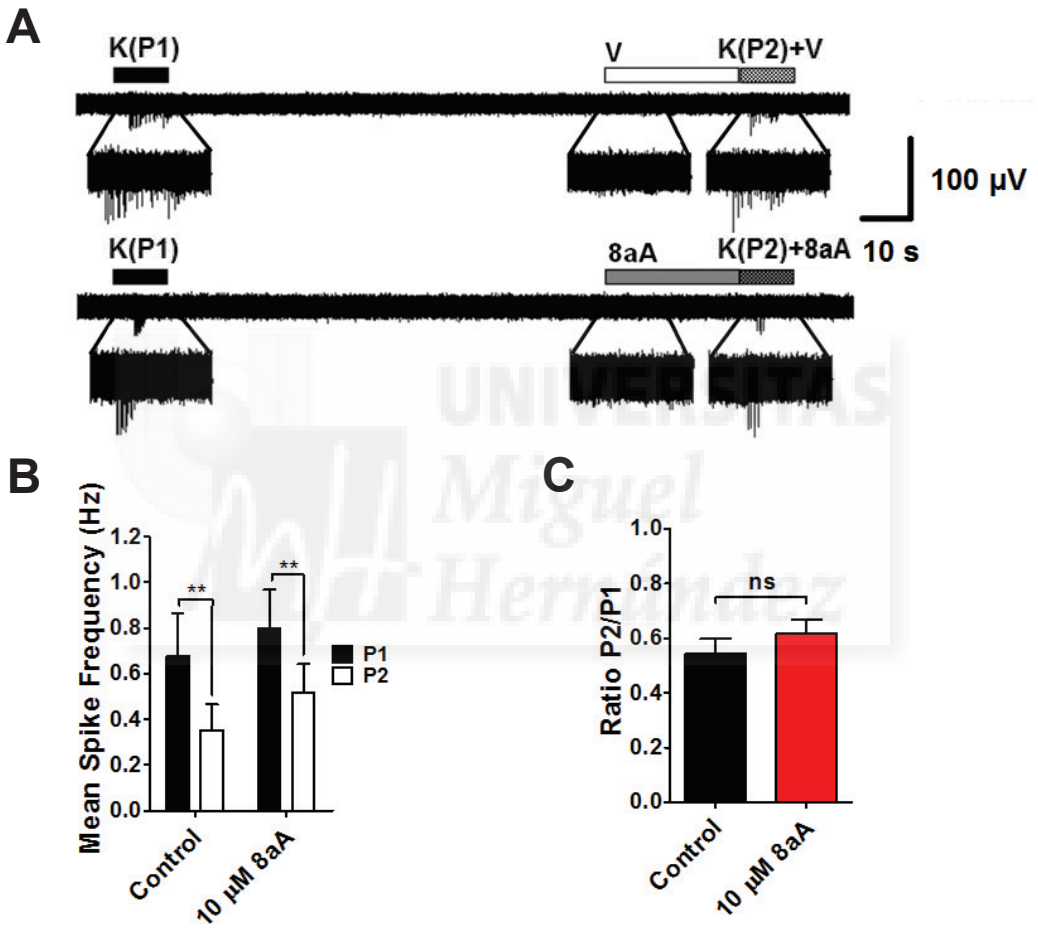


Figure 17. Effect of triazine 8aA in neuronal action potentials. **A)** Representative recordings of evoked action potentials in rat DRG neurons. We applied a first 20-second pulse (P1) of 40 mM KCl (K) to evoke action potentials. After a recovery period of 3 minutes, we applied a second KCl pulse (P2) in absence (vehicle, V) or presence of 10 µM triazine 8aA (8aA). **B)** Mean spikes frequency measured from the first pulse (P1) and second pulse (P2) of 40 mM KCl in absence and presence of 10 µM triazine 8aA. **C)** Ratio established between P2 and P1 in absence (black bar) and presence of 10 µM triazine 8aA (red bar). Data are given as means ± SEM; n ≥ 15 electrodes. Statistical analysis was made by t-test, ns no significance **P < 0.01.

Triazine 8aA attenuated capsaicin effect in nociceptive fibers

The promising outcome showed by triazine 8aA when we characterized it by electrophysiological techniques, encouraged us to consider testing it in a more complex biological environment. With this aim, we used nociceptive rat knee joint nerve fibers to evaluate its inhibitory effect on TRPV1 function[134, 135]. These experiments where performed in collaboration with Ana Gomis from the Institute of Neuroscience in San Juan.

We measured electrical responses from multiunit filaments that discharged in reaction to two different stimuli: chemical, produced by the intra-arterial injection of capsaicin; and physical, produced by noxious rotation of the knee joint. On the performed protocol, we applied four doses of 100 µl of 10 µM capsaicin, with 15-minute intervals between injections to reduce the well-known effect of desensitization (Figure 18A-D). In absence of triazine 8aA, capsaicin induced fiber response which, after the third and fourth application presented a small reduction on the firing frequency due to the desensitization process (Figure 18A-D and 19A, black bars). Importantly, when we administered 100 µl of 10 µM triazine 8aA, followed by washing with saline to avoid cumulative effect, the capsaicin-evoked impulse discharge was strongly reduced (up to 83%) (Figure 19A, red bars).

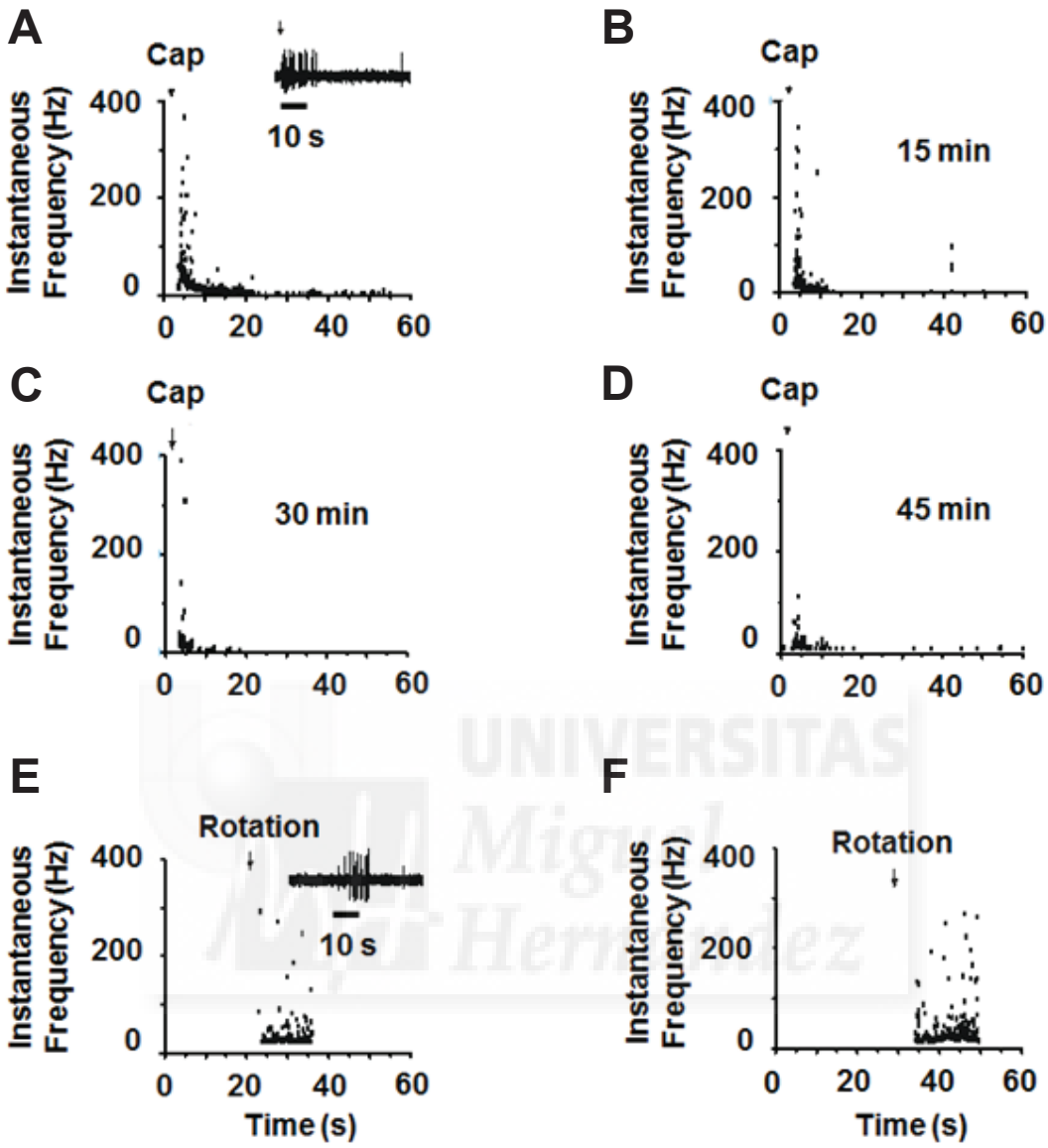


Figure 18. Triazine 8aA blockade of capsaicin-evoked neural activity in knee joint nociceptor fibers. A–D) Instantaneous frequency on the nerve impulse discharge evoked by intraarterial injections of 100 µl of 10 µM capsaicin (arrows) before (**A**) and 15 min (**B**), 30 min (**C**), and 45 min (**D**) after administration of 100 µl of 10 µM triazine 8aA. **E, F**) Impulse discharge elicited by a 10-s knee joint rotation (starting at the arrow) applied before injection of capsaicin and triazine 8aA (**E**) and 15 min after the last injection of capsaicin (**F**). Insets: sample records of multiunit impulse activity evoked by capsaicin (**A**) and by mechanical stimulation (**E**).

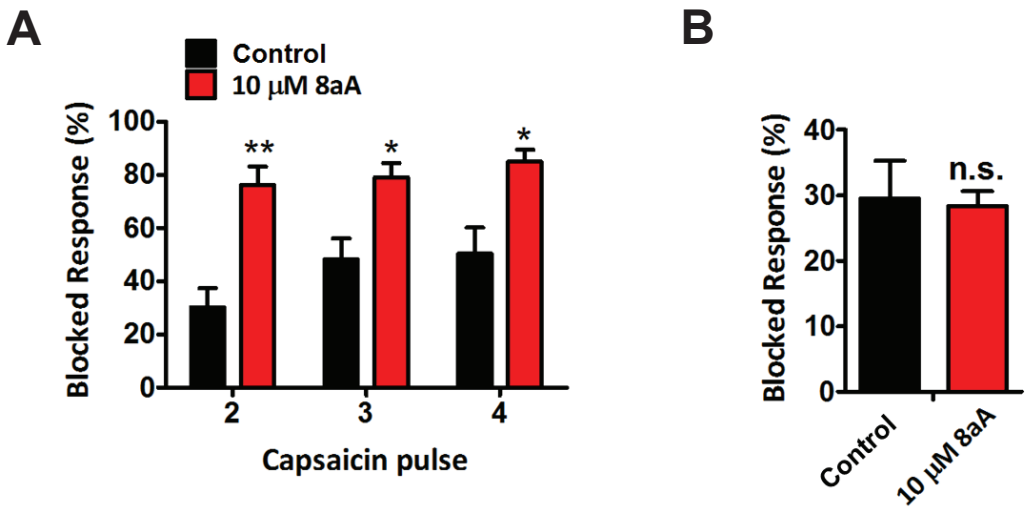


Figure 19. Triazine 8aA blocked capsaicin-evoked neural activity in knee joint nociceptor fibers without alteration on mechanical sensitivity. A) Quantitative assessment of 10 μM triazine 8aA blocked response to capsaicin and **B)** mechanically evoked responses on nociceptive fibers. Data are given as means ± SEM; n=4 animals. Two-way ANOVA with Bonferroni post hoc test; * $P \leq 0.05$, ** $P \leq 0.01$.

When we quantitatively evaluated the capsaicin-activated responses in absence and presence of the 10 μM triazine 8aA, we observed a significant blockade activity. For instance, in the second administration of capsaicin, triazine 8aA blocked more than 75% of the capsaicin response. We observed a similar blockade effect after the third (80%), and the fourth (83%) application of capsaicin (Figure 19A, red bars). However, this blocked response was not completely due to the compound activity. In absence of compound, desensitization produced by the second, third and fourth capsaicin administration blocked 27%, 48% and 51% of the response respectively (Figure 19A, black bars).

In contrast, the impulse discharge evoked by mechanical stimulation was practically unaffected by administration of triazine 8aA (Figure 18E, F and 19B). Collectively, these findings proved the anti-nociceptive activity of triazine 8aA modulating the activity of TRPV1 channels without affecting mechanical sensation.

Triazine 8aA exhibited analgesic effect in an acute pain model

Collectively, the results obtained from the *in vitro* experiments and nerve fibers, suggested a possible analgesic activity of trizine 8aA *in vivo*. In order to investigate attenuation of pain-related behavior by triazine 8aA, we evaluated its analgesic effect in different pain models.

Under blind conditions, we utilized an acute pain model where intraplantar injection of 10 µL of 2% solution of capsaicin into the rat hindpaw evoked pain in the animals. This pain was demonstrated by licking and shaking of the paw (flinches) in the animal and it disappears 5 minutes post injection. Figure 20 summarizes the obtained results as number of flinches/5 min. The control group, treated with vehicle, showed a high number of flinches (107 ± 16 flinches/5min) as a response of the pain experienced. In contrast, intravenous administration of 10 mg/Kg triazine 8aA 30 min prior capsaicin injection reduced the total number of flinches (66 ± 4 flinches/5min). These results indicate that triazine 8aA has analgesic activity in acute pain models.

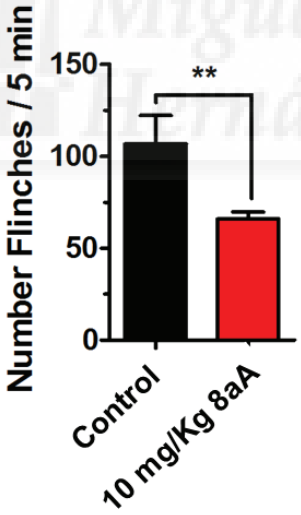


Figure 20. Triazine 8aA exhibits analgesic activity in vivo in capsaicin induced model of pain. Total number of flinches in the first 5 minutes after capsaicin injection was counted and plotted for each condition (n=6; unpaired t test, ** $p < 0.01$). All data are given as mean ± SEM.

After capsaicin administration, the region affected by the vanilloid, increases its sensitivity to thermal stimuli[136]. This process is called thermal hyperalgesia. Thus, we questioned whether triazine 8aA could reduce the pain produced by heat in animals sensitized by capsaicin administration. In this model, we applied a radiant noxious heat stimulus to the hind paw of the animal and measured the time necessary for eliciting a withdrawal response (thermal latency). Under basal conditions, prior to capsaicin and treatment injection, all the groups showed similar thermal latencies with values between 12 ± 1 s (Figure 21, Basal). Then, we administered intravenously 10 mg/kg triazine 8aA or vehicle in absence of capsaicin sensitization. After 30 min, we observed that the thermal latency was similar to the basal conditions, with values between 12 ± 1 s (Figure 21, 0 min). This result suggested no anesthetic or pungent effect of triazine 8aA by itself.

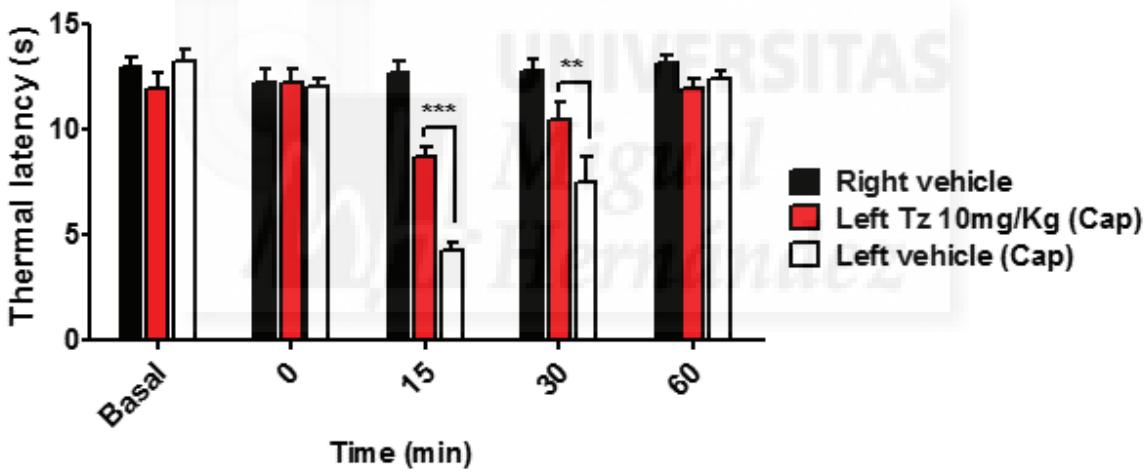


Figure 21. Triazine 8aA exhibits analgesic *in vivo* activity in thermal noxious stimuli. Latency for paw withdrawal from controlled radian heat stimulus. Treatment was administered intravenously 30 minutes prior time 0 min (Basal). Capsaicin was injected on the left hindpaw of the animals in absence (vehicle, white) or presence of 10 mg/Kg triazine 8aA (red) inmediately after time 0 min. Right hindpaw was used to evaluated the activity in a non sensitized context (black). Data are given as means + SEM; n=6. ** $p < 0.01$; *** $p < 0.001$. Two-way ANOVA with Bonferroni post hoc test.

Then, we sensitized the hind paw of the animals with an injection of capsaicin and measured the effect of triazine 8aA 15 min, 30 min and 60 min after capsaicin administration. As we expected, after 15 min, capsaicin sensitization produced, in the animal treated with vehicle, a decrease on the thermal latency response (4 ± 1 s; Figure 21, 15 min, white). This reduction was significantly prevented in the animals treated with triazine 8aA (9 ± 1 s; Figure 21, 15 min, red). After 30 min, the thermal latency in the animals treated with vehicle was significantly lower (7 ± 1 s; Figure 21, 30 min, white) than the group treated with triazine 8aA (10 ± 1 s; Figure 21, 30 min, red). Sixty minutes post capsaicin injection, the sensitization effect disappeared and the responses were again similar in all the groups (Figure 21, 60 min). These results showed a clear analgesic effect of triazine 8aA suggesting a possible therapeutic activity in a more complex inflammatory pain context.

Triazine 8aA reduced chronic pruritus in rat model with bile duct ligation

The analgesia exhibited by triazine 8aA in different acute pain models, prompted us to explore its possible effect in a chronic and more complex model. In recent years, chronic Bile Duct Ligation (BDL) model has been established as a new animal model for pruritus associated with hepatic diseases[137]. In pruritus, TRPV1 channel activity is potentiated presumably by inflammatory release of proteases from cutaneous mast cells found in close proximity to nerve terminals. This process sensitizes the nociceptors by augmenting the expression and activity of neuronal TRPV1 channels. As a result, animals exhibited augmented scratching accompanied by thermal hyperalgesia[137].

In order to investigate whether triazine 8aA may reduce the itch sensation, we utilized the BDL model in Wistar rats. These experiments were conducted in collaboration with the group of Rosa Planells at the Príncipe Felipe Institute in Valencia. Three weeks after the ligation surgery, we recorded the cumulative spontaneous scratching bouts during 1 hour in sham-operated group (control) and BDL animals. As Figure 22A shows, in the absence of triazine 8aA (vehicle), BDL

rats exhibited a significant increase in the number of scratches (96 ± 8 scratches/h) compared to sham animals (46 ± 5 scratches/h). Then, we administered intravenously either 10mg/kg triazine 8aA to BDL animals. One hour post administration, BDL group treated with the antagonist showed a decrease in the total scratches ($8aA_{1h}$ = 32 ± 4 scratches/h) (figure 22A, $8aA_{1h}$). This value was comparable to the one observed in the control group (Sham, Veh) suggesting a potent anti-itching effect. The effect of triazine 8aA was reversible, and 120 hours post treatment the reduction on scratching bouts disappeared showing no significant differences with the BDL group with vehicle (Figure 22A).

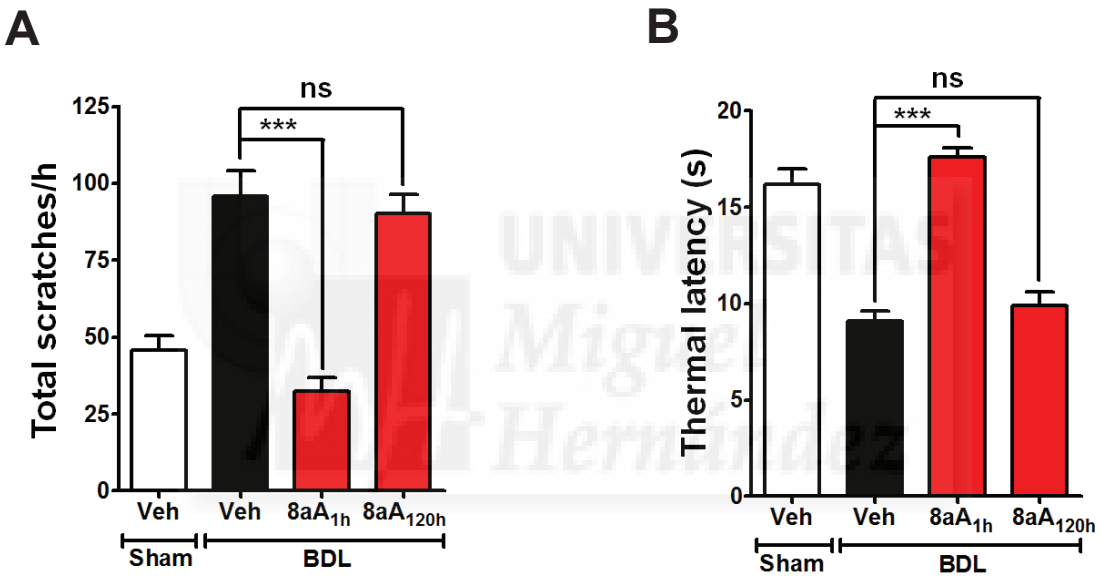


Figure 22. Triazine effect in BDL rats. A and **B**, effect on spontaneous scratching bouts/hour (**A**) and on paw withdrawal latency from a heat source (**B**) of the triazine 8aA at 1 hour ($8aA_{1h}$) and at 120 hours ($8aA_{120h}$) and vehicle (Veh). n ≥ 4. Each data point represents mean ± S.E.M. ANOVA with Bonferroni post hoc test (*** $p < 0.001$).

As we mentioned before, chronic BDL rats display thermal hyperalgesia due to, among others factors, TRPV1 potentiation. Thus, we investigated whether triazine 8aA could produce analgesia against thermal sensitivity produced by the BDL model in Wistar rats. To answer this question, we applied noxious heat stimulus to the hind paw and measured the thermal latency. We observed that, in the absence of triazine 8aA (Veh), BDL animals removed the hind paw faster than the control group (9 ± 1

s vs 16 ± 1 s respectively, Figure 22B). Then, we administered intravenously 10mg/kg triazine 8aA or vehicle to each group. One hour post administration, BDL animals treated with vehicle still showed thermal sensitization. On the contrary, the BDL group treated with triazine 8aA exhibited a potent increase in the thermal latency. This value was comparable to the one observed in the control group pre-treatment (18 ± 1 s vs 16 ± 1 s respectively, Figure 22B). The action of triazine 8aA was reversible and 120 hours post treatment, the analgesic effect disappeared showing no significant differences with the BDL group treated with vehicle (Figure 22B). These results demonstrated the *in vivo* application of triazine 8aA as anti-pruritus agent and to treat thermal hyperalgesia,

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DISCUSSION

The essential role of TRPV1 on pain transduction turned this channel into a crucial pharmaceutical target[5, 138]. A great effort is being focused on identifying high-affinity, competitive vanilloid antagonists that exhibit oral bioavailability[18, 30]. Many important companies produced compounds that in preclinical studies blocked the activity evoked by the vanilloid receptor in the presence of noxious stimulus[32, 112, 139]. However, although, they were able to reduce pain in different models, they also showed some side reactions like increase in noxious heat perception threshold or hyperthermia in human volunteers[30, 111, 140]. This is partially due to an equal inhibition of physiological and pathological working receptors. This deficiency might be defeated by non-competitive antagonists such as open-channel blockers. In this case, the binding site is accessible only in the open state conformation, acting preferentially on over-activated receptors[141].

Our group has previously reported arginine-rich peptides and peptoid molecules as non-competitive TRPV1 channel blockers with moderate blockade potency[120, 122]. Excitingly, the peptoids showed analgesic and anti-inflammatory activity in preclinical models of pain, thus validating this class of antagonists as potential leads for analgesic and anti-inflammatory drug development[120, 122]. However, their high $IC_{50,}$ in the micromolar, range is a serious challenge that limits their therapeutic potential.

Based on previous studies in our lab with a small library of 2,4,6-trisubstitued-1,3,5-triazines, compound 8aA was identified as a potent TRPV1 channel antagonist. Here, we have further characterized triazine 8aA and found that this compound blocked capsaicin-induced TRPV1 activity with high potency in absence of Ca^{2+}, being one of the most potent open-channel blocker describe to date [122]. Additionally, triazine 8aA also abrogated heat and pH-activated responses although the potency showed was 100-fold lower than in the capsaicin activation. Albeit this low activity against pH modality of activation could seem problematic, it has been described that it could contribute to prevent hyperthermia[33, 112, 142]. These results were obtained

without affecting channels from the same family, as shown by the low activity in TRPM8 and TRPA1; or against members from a different one such as NMDA receptor. Moreover, recent studies from other groups showed absence of activity of triazine 8aA against nicotinic receptors α4β2 and α3β4 (IC_{50} > 5 μM) supporting the possible selectivity of the compound[143].

Several evidences for species selectivity in the activity of some compounds in TRPV1 orthologues have been previously described[126, 144]. For example, phorbol-12-phenylacetate-13-acetate-20-homovanillate (PPAHV) shows agonist effect in rTRPV1, but is virtually inactive at human TRPV1[126]. In order to confirm the effect of triazine 8aA observed in rTRPV1, we also tested it in its human orthologue. Although the IC_{50} was slightly higher in hTRPV1 than in rTRPV1 (506 nM and 50 nM, respectively), triazine 8aA showed potent activity at the nanomolar range on both channels. It is possible that the observed 10-fold lower potency blocking hTRPV1 than rTRPV1 way arise, at least in part, from the different expression systems used (HEK cells and oocytes respectively). Considering that difference, this result is of great relevance because the absence of results' translation from rTRPV1 to hTRPV1 could lead to failure good preclinical candidates when they enter clinical trials.

Regarding the mechanism of action, the experimental data together with the molecular docking in TRPV1, suggested that triazine 8aA could be an open-channel blocker. Voltage dependency of the inhibitory activity is an essential characteristic of open-channel blockers that sense the membrane electric field and exert their activity within a defined range of voltages[122]. In the case of triazine 8aA, this voltage dependency was shown by clearly stronger efficacy at negative membrane potentials than at positive ones. Indeed, the Woodhull model yields an electric distance δ ≈ 0.36 for the binding site of triazine 8aA within the membrane electric field. This parameter indicates that the compound is penetrating a third of the electric field from the extracellular side to the cytoplasmic side. In addition, molecular docking of triazine 8aA on TRPV1 channel also supported the open-channel blocker mechanism for the compound. The binding model of triazine 8aA shows that the dimethylaminopropyl moiety of substituent R2, fit into the channel pore, establishing polar interactions with the amino acids G645 from opposite subunits. Moreover, the

alkyl chain of triazine 8aA interact with M644 and I642 from the pore region stabilizing the complex formed between triazine 8aA and TRPV1 preventing the ion flux inside the cell.

All the previously discussed results support triazine 8aA as a promising TRPV1 antagonist. However, from a pharmacological point of view, the toxicity represents a threat to the safety profile of any compound[123]. For example, clotrimazole that blocks TRPM8 activity and activates TRPV1 channel[145], produce toxicity in eukaryotic cells[123, 146]. We performed preliminary studies on the toxicity of triazine 8aA that did not show significant effect in cell viability in two different cellular models, even at 1 μM, a concentration 100-fold higher the IC_{50} in absence of Ca^{2+}. Contrary, at 10 μM and more potently at 100 μM we observed a reduction in the Sh-Sy5y-TRPV1 cells viability suggesting a moderate cytotoxicity.

The previous pharmacological characterization performed with triazine 8aA was done in oocytes and HEK-TRPV1 cells in absence of Ca^{2+}. Thus, we decided to evaluate the activity of triazine 8aA in a native neuronal system (DRG neurons) with standard external solution. Unexpectedly, the potency of the compound in this system was notably lower compare to the one observed in the heterologous systems. In fact, when we tested 50 nM triazine 8aA, we could not observed any antagonist activity against capsaicin activation. It is possible that the decrease in the potency could be in part attributable to differences in the expression system (heterologous vs native). However, we hypothesized that in some way, triazine 8aA and Ca^{2+} should be competing reducing the activity of the compound when calcium is present. Thus, we decided to increase the concentration of triazine 8aA up to 10 μM. At this concentration, triazine 8aA reduced 50% of the neuronal activity evoked by capsaicin suggesting a moderate TRPV1 antagonist effect. Furthermore, the application of the compound on DRG neurons did not affect spontaneously firing of action potentials by itself. This discarded possible alterations on the membrane potential and indicate that triazine 8aA does not modify the neuronal excitability.

Moving forward in the development of triazine 8aA, we tested whether it exhibited analgesic activity *in vivo* in preclinical models of pain. Akin other TRPV1

antagonists[113, 122], triazine 8aA displayed *in vivo* activity by attenuating the capsaicin-evoked responses in polymodal endings of nociceptor nerve fibers, mediating pain at the knee joint, without altering mechanically triggered neuronal firing[113, 134]. This analgesic effect was corroborated with the acute capsaicin pain model[147]. Our experiments show that intravenous pre-administration of triazine 8aA reduce the number of flinches provoked by capsaicin and prevent significantly the burning pain sensation. Moreover, the administration of capsaicin into the dorsal surface of the hind paw generates hyperalgesia in response to thermal stimuli[148, 149]. The application of triazine 8aA also produced an increase in the thermal latency, indicating a reduction in the thermal hyperalgesia. These results demonstrated that triazine 8aA has an analgesic effect comparable to the competitive TRPV1 antagonists[150, 151].

Interestingly, TRPV1 has also been involved in the pruritus associated with the neuroinflammation produced during the end-stage of hepatic diseases or during cholestasis of pregnancy[137, 152]. These conditions often present chronic itch due, directly or indirectly, to a pathological activation of unmyelinated C-fibers that innervate the skin[153, 154]. To evaluate the potential anti-itching activity of triazine 8aA, we utilized the BDL chronic model of pruritus. In this model, based on bile duct ligation in Wistar rats, chronic itch is mediated by the increase and potentiation of TRPV1 activity on the nerve terminals, presumably by inflammatory release of proteases mediated by PAR2[155-157]. As a result, the animals exhibit augmented scratching accompanied by peripheral sensitization of primary afferents as revealed by thermal hyperalgesia[137]. When we administered triazine 8aA to the BDL animals a potent anti-itching effect was exhibit after 1 hour. Similar to the acute pain model, the administration of triazine 8aA also mitigated the thermal hyperalgesia produced in the BDL animals, restoring the basal level of heat sensation.

All the evidence shown in this study indicates that triazine 8aA is a potent and selective TRPV1 antagonist with *in vivo* activity in preclinical models of acute pain and chronic pruritus. Up to date, most of the TRPV1 antagonists that showed analgesic activity in pre-clinical models of pain where competitive blockers that in posterior clinical trials produced side effects[158, 159]. For example, AMG-517 was a

potent TRPV1 competitive antagonist that blocked potently all TRPV1 modalities of activation in both, closed and open state[111]. However, the administration of the compound in patients reported an increase in the body temperature that interrupted the clinical trials in phase I. More recent compounds such as JTS-653[160], that elicited hyperthermia in animal models, were disclosed in 2011 from phase II for unknown reasons[161]. In the case of pruritus, anti-itching drugs traditionally formed part of the antihistamines family that, in several situations, has proved to be insufficient to relieve the itching sensation[162]. Corticosteroids, are also used in the treatment of pruritus, however, their numerous side effects, some of them sever, advice against their application[163-165]. Moreover, using classical TRPV1 antagonists will render the same side effect observed for pain treatment.

What distinguishes triazine 8aA is its mechanism of action. Oppositely to the classical TRPV1 competitive antagonists, results shown in this thesis support an open-channel blockade mechanism. Our data indicates that triazine 8aA interacts with a site located within the pore and thus, it mainly interacts with the open channel state. In this way, it would preferentially block pathologically sensitized TRPV1 channels, potentially avoiding undesired side effects. The *in vivo* analgesic and anti-pruritus activity that we showed here is an encouraging demonstration that it is possible to block TRPV1 with other type of modulators, opening new horizons for the next generation of analgesics and anti-pruritic therapies based on TRPV1.

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CHAPTER 2

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INTRODUCTION

TRPM8 structure and modulation

The discovery of TRPV1 as heat-activated ion channel triggered the discovery of many more nocisensors[3, 34]. At that time, it was well known that neurons in DRG and TG exhibited cold- and menthol-activated currents, and it was postulated that both cold and menthol presumably activate the same receptors[166, 167]. The search for these cold-sensing receptors on sensory neurons led to the identification of TRPM8[34, 168]. TRPM8 is activated by cold temperatures (< 26ºC in heterologous systems[34, 168] and < 30ºC in sensory neurons[166, 169, 170]), the agonists menthol and icilin, and voltage[168,171, 172] (Figure 23). Surprisingly, TRPM8 previously designated as Trp-p8, had already been described to be overexpressed in prostate tumors[173]. However, TRPM8 is predominantly expressed in peripheral nervous system neurons, mostly in C and Aδ fibers in DRGs[174-176]. Other than sensory neurons and prostate tissue, TRPM8 is expressed in bladder, lung and urogenital tract[177, 178].

In the absence of a crystal structure, TRPM8 has been predicted to be a tetrameric membrane protein with four identical subunits assembled around a central aqueous pore[179-181]. Similar to TRPV1, each TRPM8 subunit shows a membrane domain composed of six transmembrane segments (S1-S6)[168, 179], with an amphipathic region between the fifth and sixth segment that forms the channel conductive pore. It presents intracellular amino and carboxyl-termini (N-terminus and C-terminus respectively)[182, 183] (Figure 23).

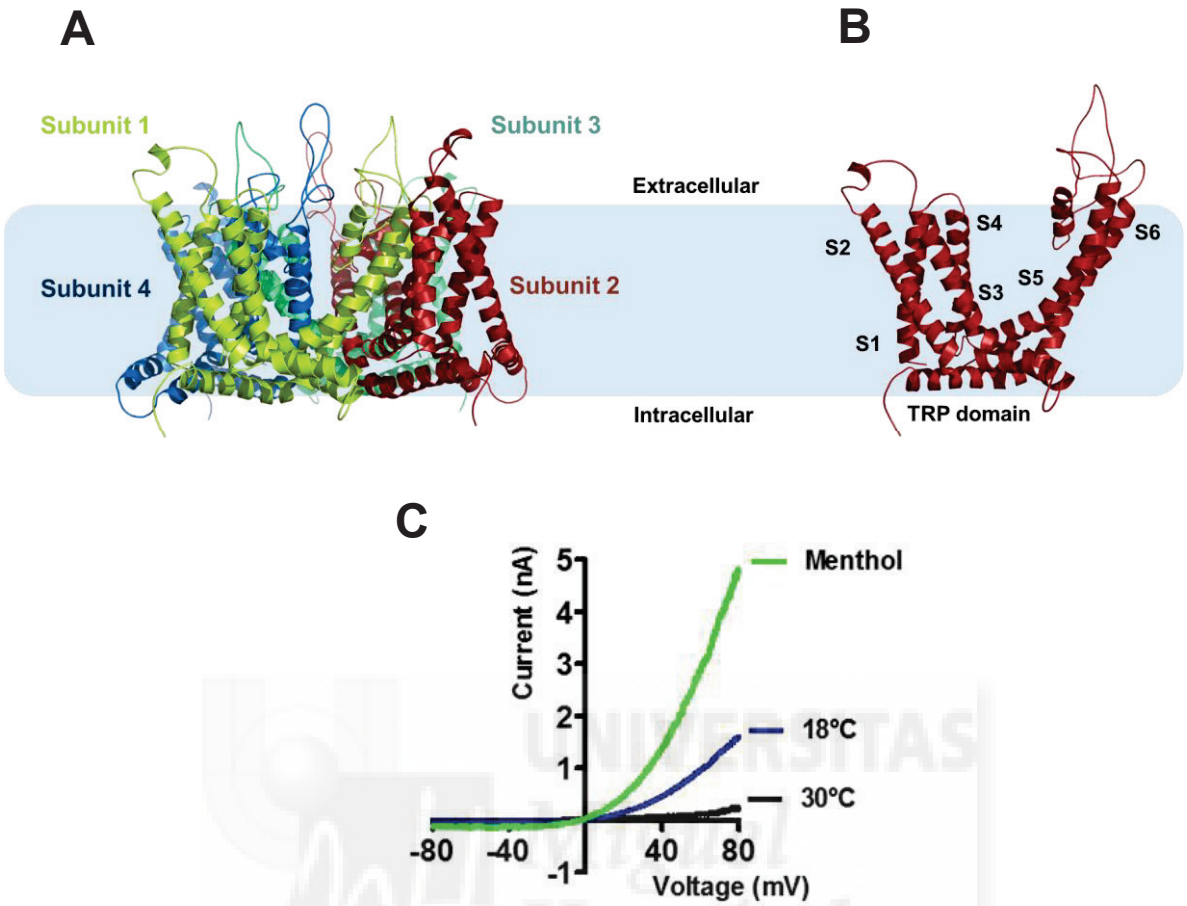

Figure 23. TRPM8 is a homotetramer activated by different stimuli. A) The functional channel is a tetramer formed by the ensemble of four such subunits. **B)**. Schematic representation of the topology of a TRPM8 protein subunit. **C)** Whole-cell I-V relationships of TRPM8 showing the activation of currents by cold temperature and menthol. Adapted from Taberner, FJ. et al. 2014[182].

As characteristic in the TRP ion channel family, the C-terminus of TRPM8 presents a TRP domain adjacent to the S6 transmembrane segment that structures the channel inner gate. Recent studies suggest that S6-TRP box linker region is important for the allosteric coupling of stimuli sensing to channel activation[182]. Moreover, the C-terminus present several regions involve in the PIP_2 binding[184, 185].

The N-terminus domain, the largest intracellular part of the protein with 693 amino acids, seems to be involved in the stabilization of the tetramer[186]. Moreover, recent studies revealed that punctual mutation of this region yield channels with augmented responses to stimuli[183]. Additionally, mutations within the positions 40-60 produced

nonfunctional channels that are retained in the endoplasmic reticulum suggesting that this region could be necessary for the proper trafficking of TRPM8 to the plasma membrane[186]. Nevertheless, the role of the large N-terminus domain is still poorly understood.

As mentioned previously, it has been found that the thermal threshold of TRPM8 channel expressed in heterologous system is lower than in native neurons (21ºC-26ºC and 27ºC-31ºC, respectively)[34, 166, 168-170] This discrepancy suggests an endogenous modulation of TRPM8 activity in vivo, and numerous studies have described different modes of modulation of its activity (Figure 24)[187-190]. For example, it has been demonstrated that rising intracellular Ca^{2+}-levels modulate the activity of TRPM8 via the activation of Ca^{2+} dependent phospholipase C (PLC)[189]. TRPM8 channels contain several PIP_2 binding sites (Figure 24)[188, 191, 192], and this molecule is required for channel activation. In fact, depleting intracellular PIP_2 results in channel desensitization by shifting the voltage dependence of TRPM8 towards more positive potentials[185, 188]. The phospholipase PLC hydrolyzes PIP_2 to form diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), thus mimicking PIP_2 depletion and desensitizing TRPM8[193].

Another way in which intracellular Ca^{2+} levels modulate TRPM8 is by calcium-dependent phosphokinase C (PKC)[194-196]. An increase in intracellular Ca^{2+} levels produces the dephosphorylation and desensitization of TRPM8 through the activation of PKC[195, 196]. Furthermore, increases in cyclic adenosine monophosphate (c-AMP) through activation of G-protein coupled receptors leads to the activation of PKA that desensitizes the response of TRPM8 to menthol and icilin (Figure 24)[197]. Moreover, it has been described that the alpha subunit of the G-protein binds directly to TRPM8, preventing its activation[198].

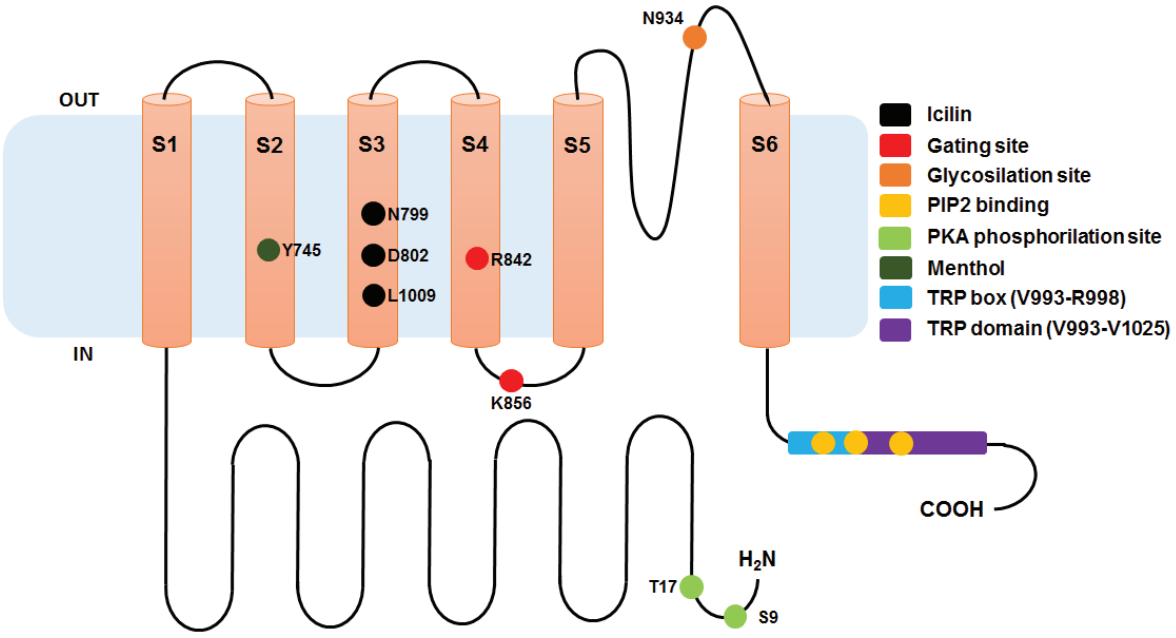

Figure 24. Structure of TRPM8. Schematic representation of human TRPM8 channel subunit topology, showing relevant residues for channel gating and modulation. Individual residues involved in particular aspects of TRPM8 function are highlighted in a color code. Modified from Malkia, A. et al. 2007[188].

However, not only mechanisms that reduce TRPM8 activity have been described. For instance, the activation of calcium-insensitive phospholipase A2 (iPLA2) results into TRPM8 activation[199-201]. Furthermore, an increase in intracellular pH enhances icilin and cold activation, whilst lowering the pH decreases channel activation[172]. Additionally, glycosylation of the channel in specific residues (Figure 24) results in a significant potentiation of menthol- and cold-mediated responses in heterologous systems and sensory neurons[202, 203]. This potentiation is based on a shift in the voltage dependent activation of TRPM8 towards more negative potentials[202].

Physiological and pathophysiological roles of TRMP8

As mentioned previously, TRPM8 was originally identified in a screening for up regulated genes in prostate cancer tissue, and it was proposed to be an ion channel

with oncogene or tumor promoter potential[173]. Recent studies showed that menthol-evoked activation of TRPM8 channel inhibits the proliferation and motility of prostate cancer cells[204]. Surprisingly, other studies that used two TRPM8 blockers (AMTB and JNJ41876666) demonstrated that TRPM8-antagonists were able to reduce proliferation in prostate tumor cells tested, but not in non-tumor cells[205, 206]. Thus, the above research indicated that TRPM8 played critical role in mediating biological behavior of prostate tumors.

In addition to its role in prostate cancer, TRPM8 channels located in the ocular cold fibers appear to be critical in tearing production[207,208]. Genetic ablation of this channel in mice renders cold thermoreceptors' endings of the cornea silent and irresponsive to cooling[207]. In pathologies such as dry eye, where basal tearing is reduced partially due to a decrease of the reflex input from cold receptors[209], the application of TRPM8 agonists would increase tear volume[210]. On the contrary, in pathological processes where tearing is augmented, such as corneal irritation, allergies or bacterial conjunctivitis[211], the application of TRPM8 antagonists could be a valuable therapeutic tool[212].

Due to its sensitivity to cold, TRPM8 is also expected to play an important role in cold sensation and thermoregulation[178]. Supporting this, TRPM8 knockout mice showed a reduction on cold sensation[174]. In addition, the pro-algesic glial cell-line derived neurotrophic factor family receptor 3 (GFRα3) is preferentially localized to a subset of putative nociceptive TRPM8-expressing neurons[213]. Injecting artemin, the specific natural ligand of GFRα3, increases cold-sensitivity in wild type but not TRPM8-knockout mice[213]. Interestingly, artemin expression is increased in inflamed skin, supporting the notion that TRPM8 is involved in cold hypersensitivity associated to inflammatory conditions[213, 214]. This modulation of TRPM8 demonstrates that this channel do not only regulate physiological cold sensation, but also has an important role in pathological processes.

Moreover, TRPM8 has been implicated in cold-hypersensitivity in the context of chronic pain caused by nerve injury (neuropathic pain)[4, 215]. For example, some platinum-based chemotherapeutic agents utilized in the treatment of some types of

cancer, such as Oxaliplatin, cause cold hypersensitivity, which severely restricts its dosage and duration of treatment[216-218]. Cold hypersensitivity has also been reported in mice models of chronic constriction injury (CCI) of the sciatic nerve[219]. In this particular case, TRPM8-knockout mice do not develop cold hypersensitivity in contrast to wild type mice[219]. Similar results in CCI-induced cold hypersensitivity were obtained for mice in which TRPM8-expressing neurons were conditionally ablated[220].

TRPM8 antagonists

The involvement of TRPM8 in diverse physiological and pathophysiological processes reveals this channel as a promising therapeutic target. In particular, as inferred from the previous section, the development of TRPM8 antagonists is of great interest[36, 178, 221-229]. In this regard, some compounds have been identified. For example, capsazepine, a potent TRPV1 antagonist and a non-specific TRPM8 blocker, significantly attenuates CCI induced cold allodynia[230]. Similarly, (S)-1-phenylethyl (2-aminoethyl)(4-(benzyloxy)-3-methoxybenzyl)carbamate (PBMC), a novel TRPM8 antagonist also significantly attenuates CCI induced cold allodynia[36]. However, as in the case of TRPV1, critical side effects have been reported[36, 222, 226]. The TRPM8 antagonist PBMC produced a reduction in core body temperature of more than two degrees when it was tested in mice[36]. Moreover, many of the current TRPM8 antagonists also act on other ion channels. Capsazepine, although a TRPM8 antagonist with demonstrated efficacy on cold allodynia treatment, also has non-specific activity on voltage-gated calcium channels, nicotinic acetylcholine receptors, and TRPV1[107, 108, 110]. Similarly, while 4-(3-chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide (BCTC) inhibits TRPM8-mediated Ca^{2+} influx, this compound also functions as a TRPA1 agonist[169]. Likewise, the anti-fungal medication clotrimazole has strong TRPM8 antagonistic activity, but also robustly activates TRPV1 and TRPA1, actions consistent with the commonly reported side effects of irritation and burning[123, 145]. SKF96365, a non-specific blocker of several types of calcium channels, receptor-operated channels, and inwardly rectifying potassium channels also inhibits TRPM8 in vitro[231, 232]. The poor

selectivity and side effects of the TRPM8 antagonists described until now hinders not only their therapeutic utility, but also their use in the investigation of the role of TRPM8 as a therapeutic target.

Moreover, a key challenge in the development of new TRPM8 antagonists is the deficiency in information that exists related to their mechanism of action. Up to date, only few papers have emerged and two main binding regions for antagonists have been suggested[233, 234]. Competitive TRPM8 antagonists such as SKF96365, have been found to bind the Y745 located between S2 and S3 domains of one subunit, preventing conformational changes necessary for channel activation (Figure 25A)[233]. An alternative region formed by S1, S2, S3, S4 and the residues 990 to 1010 in the TRP domain has also been described to modulate TRPM8 activity (Figure 25B)[234]. Probably, compounds interacting in this region would exert a negative allosteric modulation of the channel. Further investigations in this field might contribute considerably to the rational design of antagonists to avoid the typical side effects

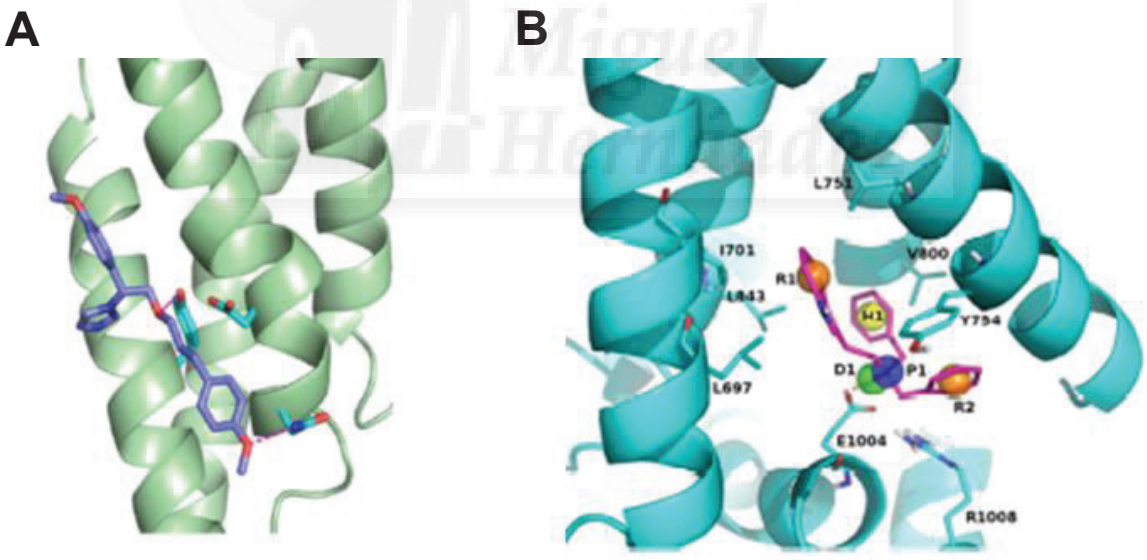

Figure 25. Molecular modeling of TRPM8 antagonists. A) A competitive TRPM8 antagonist (SKF96365) interacts with Y745 and N799 at S2 and S3 domains **B)** Tryptamine-based TRPM8 antagonist binds a region between S1, S2, S3, S4 and the residues 990 to 1010, a different location proposed for the menthol binding site. Adapted from Malkia, A. et al. 2009 and Bertamino, A. et al. 2016[233, 234].

Despite the discouraging results obtained up to date in the search of TRPM8 antagonists, the value of this ion channel as a therapeutic target is unquestionable. Therefore, the need of more selective antagonists based on different strategies of modulation of TRPM8 is the main focus of the second part of this project. With the objective of finding new TRPM8 antagonists we conducted a high-throughput screening assay of a set of chemical libraries followed by the study of the structure-activity relationship of the most promising candidates. Then, we applied the obtained knowledge to chemically improve the design of the TRPM8 antagonist candidates. In addition, aiming at filling the lack of information regarding the mechanisms of action of TRPM8 antagonists, an exploration by *in vitro* experiments and computational approaches was done to identify the interactions established between the best antagonist and the receptor.

OBJECTIVES

Main objective

The main objective of this study was to develop and characterize new antagonists for TRPM8 channel and to study their mechanism of action. With this aim, several experiments, from *in silico* to *in vitro*, were performed to identify and evaluate the candidates.

Specific objectives

- To identify new inhibitors for TRPM8 by high-throughput screening techniques.

- To establish the minimal requirements of β-lactam scaffold to block TRPM8 channel through structure-activity relationships.

- To evaluate the pharmacological properties of the best candidate by electrophysiological techniques (patch clamp and microelectrodes arrays) and colorimetric assays (MTT).

- To study the mechanism of action of the best candidate by computational approaches.

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RESULTS

β–lactam ring was revealed as a central scaffold for TRPM8 antagonists

In order to identify new molecules with an acceptable potency against TRPM8 channel we implemented a high-throughput screening using a fluorescent Ca^{2+} indicator that allowed the measurement of the compounds' effect on TRPM8 activity. This indicator (Fluo4-NW) emitted fluorescence upon binding Ca^{2+} when the channel was open, in our case, when TRPM8 channel was activated by 300 μM menthol (Figure 26A). Contrary, in presence of an antagonist, the channel remained close and the fluorescence did not show increments even in presence of an agonist stimulus (Figure 26B).

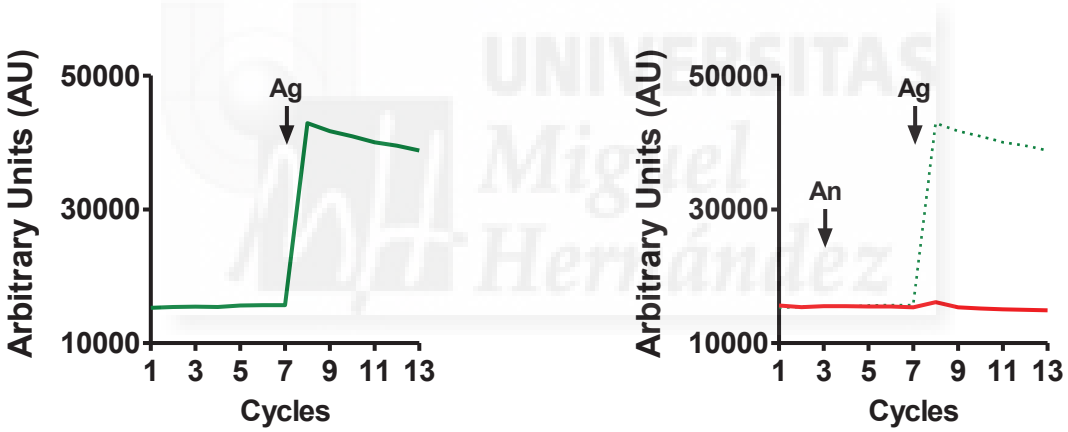

Figure 26. Representative fluorescence assay of the TRPM8 channels activity in stable cell line. TPR-mediated Ca+2 influx observed upon addition of agonist (Ag, green) in the seventh cycle, while the addition of the antagonist (An, red) at the third cycle blocked the response.

We evaluated the antagonistic effect of 33 chemical libraries (326 compounds) with different chemical scaffolds against 300 μM menthol-evoked activity in TRPM8 channel. We tested two different concentrations (50 and 5 μM), calculated the blocking percentage (see Material and Methods for more details) and selected as the best candidates those that showed more than 50% blockade at 5 μM. Table 3 summarizes the number of compounds that showed the highest antagonistic potential for TRPM8 in each library.

Table 3. Chemical libraries and the number of found TRPM8 antagonists

Chemical library	Sub-libraries	Compounds	Antagonists at 50 µM	Antagonists at 5 µM
PG	2	21	0	0
RH	20	102	11	0
↳	RH-18	16	6	0
RGM	11	203	20	7
↳	RGM-8	8	5	3

Arrows indicate the best sub-library of each group

As it is shown in Table 3, the most promising library was RGM, with seven potential candidates with a blockade higher than 50% at 5 µM. Interestingly, inside this chemical library we identified RGM-8 as the best sub-library with three of those seven candidates (8-3, 8-4 and 8-6).

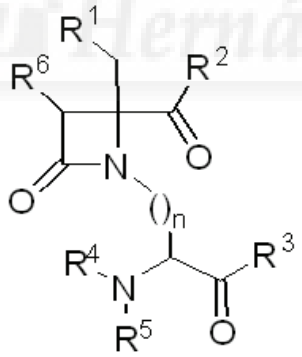

Figure 27. β–lactam ring as a central scaffold for TRPM8 antagonists. R^n represent each substituent used in the design of compounds. ( )n represents the length of the alkyl chain.

To further understand the effect of the structure of the RGM-8 family in the antagonistic activity, in collaboration with the group of Rosario Gonzalez Muñiz from Institute of Medicinal Chemistry in Madrid we expanded the library with more compounds and we performed a SAR analysis. Compounds in the RGM-8 library

belong to the family of β-lactams (Figure 27). The β-lactam ring is formed by a cyclic amide, with the nitrogen atom attached to the β-carbon relative to the carbonyl.

The results of the SAR evaluation are displayed in Table 4. Compounds 8-1, 8-2 and 8-3 are similar, sharing a phenyl group in R1, hydrogens in positions R4 and R6 and the protective group tert-butoxycarbonyl (Boc) as substituent R5. Their differences are small modifications on the ester moieties R2 and R3. According to this evaluation, the presence of benzyl esters in R2 and R3 (8-3) confers the molecule a high antagonist effect against TRPM8 (95.4% blockade at 5 μM). This activity decreases considerably in presence of a methyl group on R2 (8-2), or drastically when the same modification was done on both positions R2 and R3 (8-1). The importance of the two hydrophobic benzyl esters is reinforced with the results from derivative 8-6. Compared to 8-2, it incorporates conservative substitutions on R3, with a terc-butyl (tBu) group, and a benzyl-zyloxycarbonyl (Z) on R5. The activity of 8-6 was slightly higher compared to 8-2, 56.9% and 27.0% of blockade respectively, but far from the potent 8-3.

Comparison of compounds 8-3 and 8-29, with the same substituents but different length of the N-alkyl chain (2 and 3 carbons, respectively) indicates that a small increment on the chain length reduces the blockade activity up to 50%. As expected, derivative 8-20, which maintains the 3 carbon chain but replaces the benzyl ester on R3 by a methyl group, while keeping conservative modification of R2 (tBu instead of Bn) and R5 (Z instead of Boc), reported a decrease in the antagonist activity compared to 8-29. Derivative 8-9, which incorporates a small modification on R4 (a Me group), slightly increases the blockade activity. The antagonist activity of analogue 8-10 (R5 = H), resulting from the removal of the Z group of 8-9, was almost inexistent. Thus, the results showed by 8-29, 8-20, 8-9 an 8-10 support that main requirements for TRPM8 blockade are a short N-alkyl chain and, additionally, emphasize the relevance of hydrophobic groups at positions R2, R3, and R5.

Table 4. Activity of RGM8 compounds in TRM8 channel.

Com	R^1	R^2	R^3	R^4	R^5	R^6	n	% Block TRPM8 5 µM	% Block TRPV1 5 µM
8-1	Ph	Me	Me	H	Boc	H	2	15.9±15.2	3.9±2.1
8-2	Ph	Me	Bn	H	Boc	H	2	27.0±12.7	18.0±2.2
8-3	Ph	Bn	Bn	H	Boc	H	2	95.4±9.9	28.7±7.3
8-6	Ph	Me	tBu	H	Z	H	2	56.9±10.8	26.5±21.2
8-29	Ph	Bn	Bn	H	Boc	H	3	50.4±17.8	21.7±12.2
8-20	Ph	tBu	Me	H	Z	H	3	24.9±17.8	28.4±2.0
8-9	Ph	tBu	Me	Me	Z	H	3	53.4±17.0	1.3±7.2
8-10	Ph	tBu	Me	Me	H	H	3	13.0±7.0	4.5±10.7
8-65	H	Me	Bn	H	Boc	H	2	42.7±12.6	--
8-5	H	Me	tBu	H	Z	H	2	18.2±13.9	16.7±10.5
8-23	H	tBu	Me	H	Z	H	3	4.4±23.8	17.3±4.7
8-14	Ph	Bn	Bn	H	Boc	Me	2	91.1±17.0	15.0±8.1
8-41	Ph	$NHCH_2$(4-Py)	$NHCH_2$(4-Py)	H	Boc	H	2	39.3±14.7	15.9±22.3
8-42	Ph	NH(3-Py)	NH(3-Py)	H	Boc	H	2	64.6±13.2	43.6±10.4
8-43	Ph	NH(4-Py)	NH(4-Py)	H	Boc	H(S)	2	52.3±7.6	43.0±14.3
8-44	Ph	NH(4-Py)	NH(4-Py)	H	Boc	H(R)	2	43.5±9.8	38.5±12.4
8-18	Ph	Bn	Ph	H	Z	Me	1	97.2±1.9	--

Remarked in grey color the compounds selected for the characterization

To further understand the structure-activity relationship in this series, we tested compounds 8-65, 8-5 and 8-23, Ala analogues of 8-2, 8-6 and 8-20, respectively. Substituting the phenyl group by hydrogen on R1 on these three derivatives causes a dramatically decrease in activity, suggesting that an aromatic ring on this position is essential for the antagonist effect. Regarding the R6 substituent, the replacement of the hydrogen in 8-3 by a methyl group gives derivative 8-14. This substitution

showed little influence on the activity, since both compounds displayed similar potency. These results support the premise that high TRPM8-blocking activity requires hydrophobic moieties on R1, R2, R3 and R5 and a short N-alkyl chain. To corroborate the relevance of the hydrophobic groups, we designed and evaluated pyridine derivatives 8-41, 8-42, 8-43 and 8-44, compounds obtained from the precursor 8-3 that present hydrophilic groups. Thus, the substitution of the benzyl ester of R2 and R3 of 8-3 by a 4-aminopyridine gave to compound 8-41 that revealed a strong reduction in the activity compared to 8-3 (39% and 96% of TRPM8 blockade respectively, at 5 μM). Interestingly, the total substitution of the benzyl group by either a 3-aminopyridine in 8-42 or a 4-aminopyridine in 8-43 and its stereoisomer 8-44 recovered slightly the blockade activity (64%, 52% and 43% at 5 μM respectively) compared to 8-3. We also explore the contribution of the N-alkyl chain. When the β–lactam 8-18 with the shorter alkyl chain was evaluated at 5 μM its TRPM8 antagonist potency was comparable to that observed for 8-3. These results seem to suggest that all these compounds interact with the receptor in a large binding pocket, able to accommodate different structures, and that probably the main forces maintaining the interaction are hydrophobic.

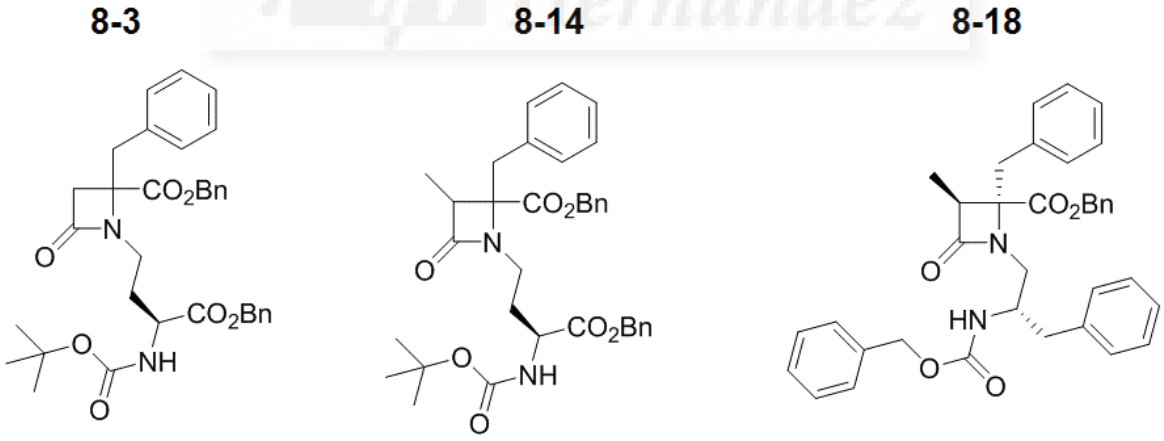

Figure 28. Representation of the most potent derivatives of the RGM8 chemical library

In conclusion, the study of the structure-activity relationship on this series contributed to the establishment of the minimal requirements for a potent TRPM8 blocker. Based

on the β-lactam scaffold (Figure 27), the preferred structural elements are two: 1) the presence of hydrophobic groups (benzyl ester or tBu) on the substituents R1, R2, R3 and R5; and, 2) a short N-alkyl chain (≤ 2 carbons). The best compounds in this library (8-3, 8-14 and 8-18) meet the criteria (Figure 28).

Compounds 8-3, 8-14, and 8-18 were potent TRPM8 antagonists

After the initial screening and the SAR analysis to identify potential candidates, we investigated the antagonist efficacy of the compounds 8-3, 8-14, and 8-18 on TRPM8 activity by patch clamp in whole cell configuration.

As depicted in Figure 29, the blockade activity showed by the compounds was dose-dependent with totally absence of current at 1 μM in all of them. We fit the dose-response curve to obtain the IC_{50} and the Hill coefficient

Against TRPM8 activity evoked by 300 μM menthol at -60 mV, derivative 8-3 blocked potently the receptor with an IC_{50} of 46.0 nM (95% CI = 38.9 - 55.3) and n_H=1.3 (95% CI =1.6 - 0.9) (Figure 29A). In derivative 8-14, the hydrogen of R6 substituent present in derivative 8-3 was replaced by a methyl group seeking an increase on the potency. However, the dose-response of the compound 8-14 reported an IC_{50} of 82.9 nM (95% CI = 53.8 - 127.5) with n_H= 1.1 (95% CI = 1.4 - 0.6) what supposed a slightly decrease in the potency compare to derivative 8-3 (Figure 29B). Similarly, derivative 8-18, a compound with a shorter N-alkyl chain than derivative 8-3, reported an IC_{50} 104.9 nM (95% CI = 68.5 - 160.6) with n_H= 1.1 (95% CI = 1.4 - 0.6) (Figure 29C) that confirmed that these derivatives (8-3, 8-14 and 8-18) were potent TRPM8 antagonists with activity on the nanomolar range situated then within the most potent blockers of TRPM8 channel described to date.

We decided to further explore compound 8-3 because it was the most potent among the β–lactam group.

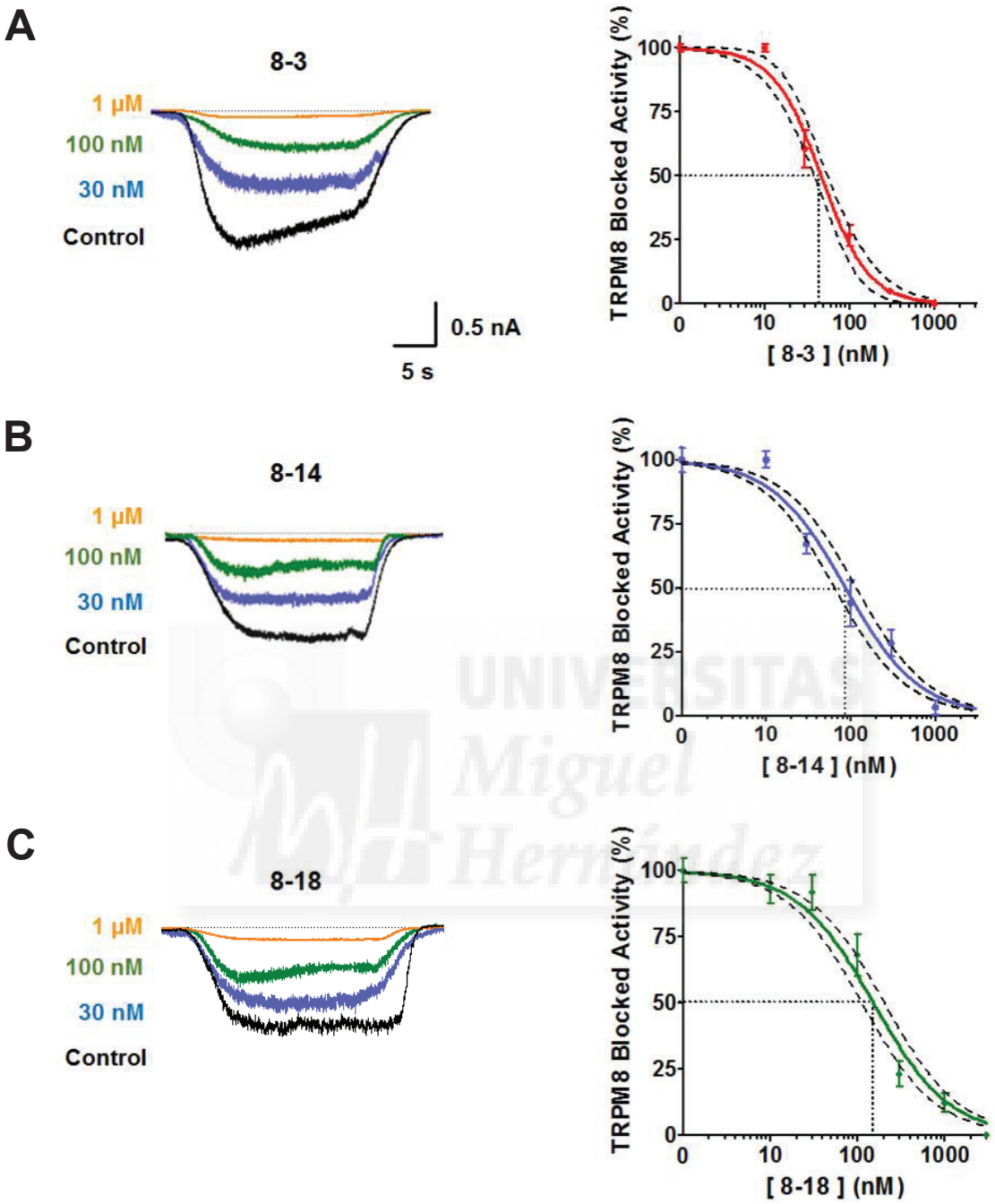

Figure 29. Selected compounds blocked TRPM8 at nanomolar range. **Left:** Representative whole-cell patch clamp recordings from TRPM8-expressing HEK-CR1 cells and compound 8-3 (**A**), 8-14 (**B**) or 8-18 (**C**). Voltage was held at -60 mV. Pre-application of compounds (20 s) were followed by co-application with 300 µM menthol (Control) for 20s. Current traces of different colors denote the different concentrations of compounds tested. **Right:** Dose response of compound 8-3 (**A**, red), 8-14 (**B**, blue) or 8-18 (**C**, green) TRPM8 blocked activity. Solid line depicts the fitting to a Hill equation. Data are given as mean ± sem, with n>4 cells per data point.

Compound 8-3 showed promising pharmacological properties

In the development of new drugs, it is key to ensure that potential candidates do not show high cellular toxicity. Thus, we evaluated the cell viability of HEK-CR1 in presence of the compounds 8-3 with the MTT assay using a range of concentrations (0.1; 1 and 10 μM). As seen in Figure 30, any of the four selected compounds showed a significant effect on the cell viability, even at the highest concentration (10 μM). The lack of toxicity for these concentrations, several orders of magnitude higher than the IC_{50}, suggested a non-toxic profile interesting on the development of the compounds.

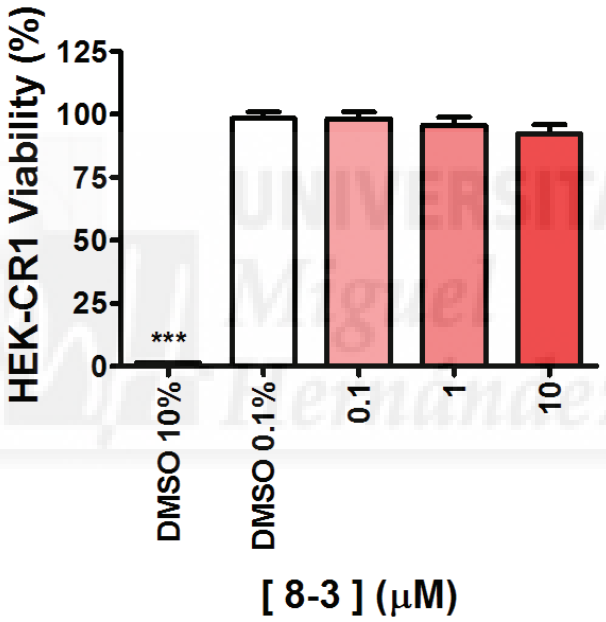

Figure 30. Effect of compound 8-3 **on MTT-based assay of cytotoxicity in HEK-CR1 cells.** Cell survival after 24 hours incubation at three concentrations of 8-3. Each value is expressed as mean ± SEM. n ≥ 15, ANOVA with Bonferroni post hoc test *** $p < 0.001$.

In order to probe the specificity and selectivity of compound 8-3, we measured its activity on different heterologous systems: hTRPV1 and hTRPA1, two ion channels from the same family as TRPM8; and Kv1.1 (potassium ion channel) and NaV1.6 (sodium ion channel), two ion channels from different families. The results are represented in Figure 31. The application of 1 μM 8-3 to channels from the TRP family produced a minimal blockade of capsaicin-evoked activity in TRPV1 and

AITC-evoked activity in TRPA1. Moreover, the experiments on Kv1.1-expressing cells revealed a slightly higher blockade respect to TRP channels V1 and A1, although it was non-significant. When we evaluated the effect on NaV1.6, compound 8-3 did not report blockade activity. Together, these data indicate that compound 8-3 was a specific and selective TRPM8 antagonist.

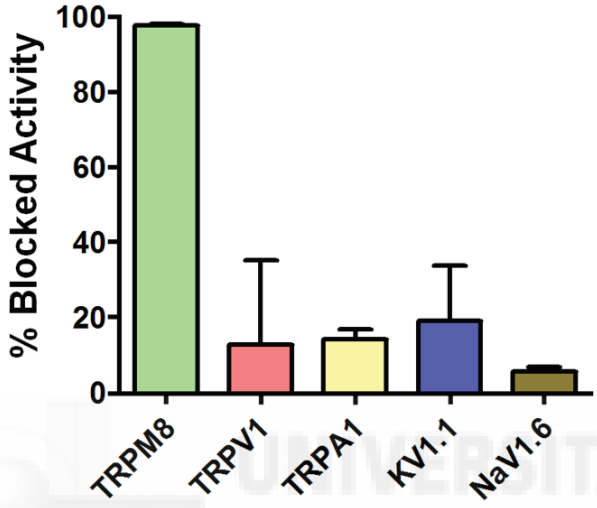

Figure 31. Compound 8-3 blocked selectively TRPM8 channel activity. Blocked activity of 10 µM 8-3 after 300 µM menthol (TRPM8), 1 µM capsaicin (TRPV1), 100 µM AITC (TRPA1) and voltage (Kv1.1 and NaV1.6). Responses were normalized with respect to that obtained in the absence of compound. Each point represents the mean ± SEM, n≥4.

To further prove the selectivity of compound 8-3 we measured electrical activity of DRG neurons on MEA chambers in response to depolarizing stimuli (Figure 32) to study the effect on neuronal excitability. Figure 32A shows a representative recording with MEA on DRG neurons. We applied a first depolarizing pulse (P1) of 20 seconds of 40 mM KCl (K in the Figure 32A) followed by recovery period and a second pulse (P2) equivalent to the first one, in the absence (vehicle, V, upper part of Figure 32A) or presence of the 10 µM 8-3 (lower part of Figure 32A).

Figure 32B show the ratio established between the P2 and P1 for compounds 8-3. The presence of 10 µM 8-3 (0.6 ± 0.1) did not show any difference when compared to the vehicle (0.5 ± 0.1) (Figure 32B). These results indicate that this compound do

not have a significant effect on nociceptors' excitability, data supported by the specific and selective inhibitory activity reported on the previous experiments.

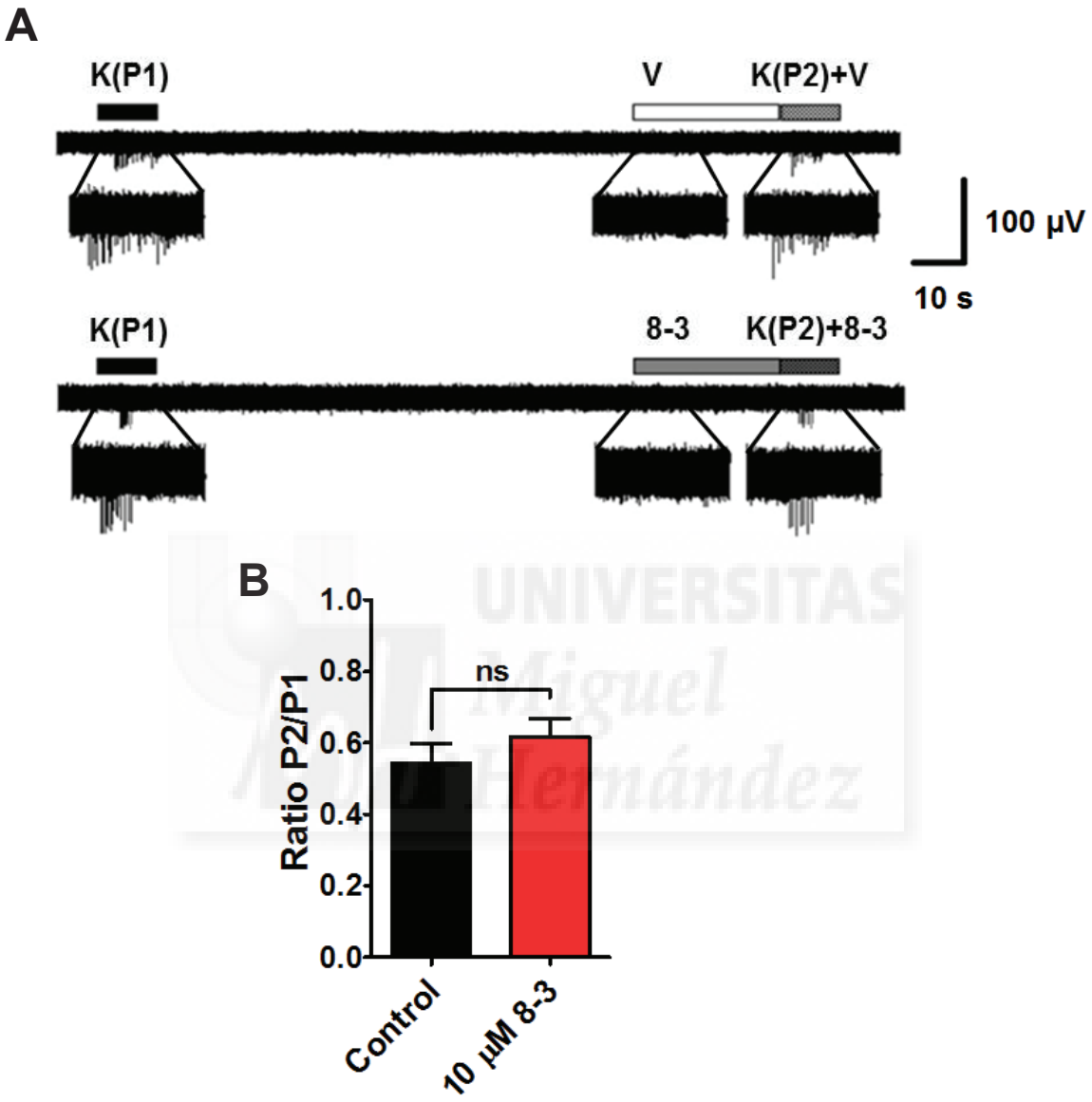

Figure 32. Compound 8-3 **did not affect neuronal action potentials. A)** Representative recordings of evoked action potentials in rat DRG neurons with 40 mM KCl (**K**) in the absence (**V**) and presence of compound. **B)** Ratio established between **P2** and **P1** in the absence and presence of 10 µM 8-3. Data are given as means ± SEM; n ≥ 15 elecrodes. Statistical analysis was made by t-test, ns depicts no significance.

Compounds 8-3 was a polymodal antagonist

Similar to other members of the TRP family, TRPM8 is a polymodal channel gated by chemical and physical stimuli. We already knew that 8-3 was able to block menthol-mediated TRPM8 activity; thus, we investigated whether it could inhibit voltage and cold TRPM8 activation in HEK-CR1 cells using patch clamp in whole cell configuration.

As illustrated in Figure 33, the application of 1 μM 8-3 notably reduced voltage evoked TRPM8 response at depolarizing potentials (90 ± 2% of TRPM8 blockade), both in pulse (Figure 33A) and ramp protocol (Figure 33B).

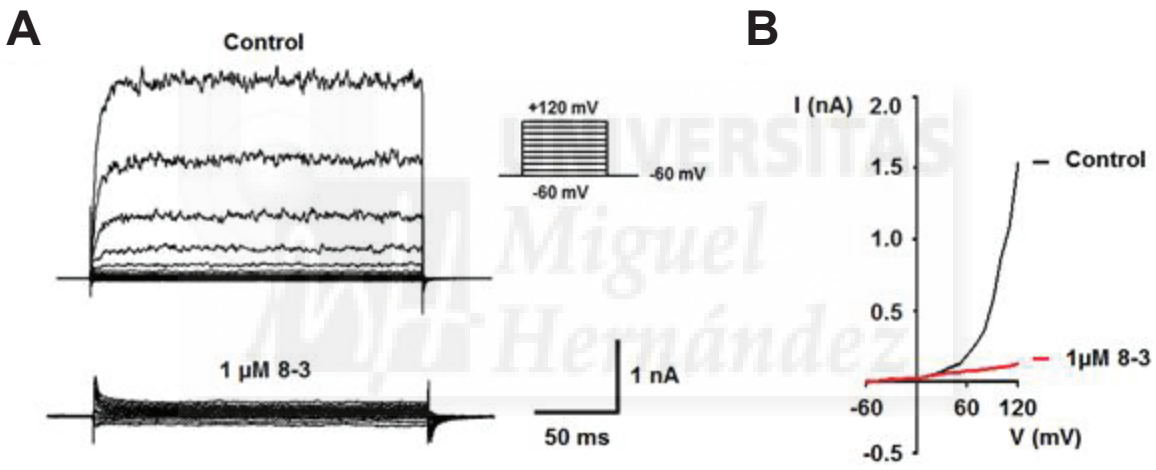

Figure 33. Compound 8-3 **abolished the voltage-evoked response on TRPM8.** **A**) Family of voltage-gated ionic currents evoked from TRPM8 channels in the absence (top) and presence of 1 μM 8-3 (bottom). Currents were activated with 200-ms pulses from -60 to +120 mV (Vh=-60 mV). **B**) Representative I-V relationship of TRPM8 channel activity in the absence (black curve) and presence 1 μM 8-3. Currents were obtained with 350-ms ramp from -60 mV to +120 mV.

We next addressed the question of whether 8-3 might affect the thermal modality of TRPM8 activation by reducing its activation when mild cold is applied (Figure 34). We measured the ionic currents evoked when we applied buffer at 16 ºC. We applied a second pulse of cold buffer to ensure that the current was similar and no desensitization occurred. Under control conditions, both pulses produced a similar

current increase (Figure 34A). In contrast, the application of 1 μM 8-3 (Figure 34B) at the second pulse together with the thermal stimulus revealed a significant decrease on the current intensity (75 ± 12%). The fact that compound 8-3 is a polymodal antagonist suggests that this compound could be interfering with channel gating.

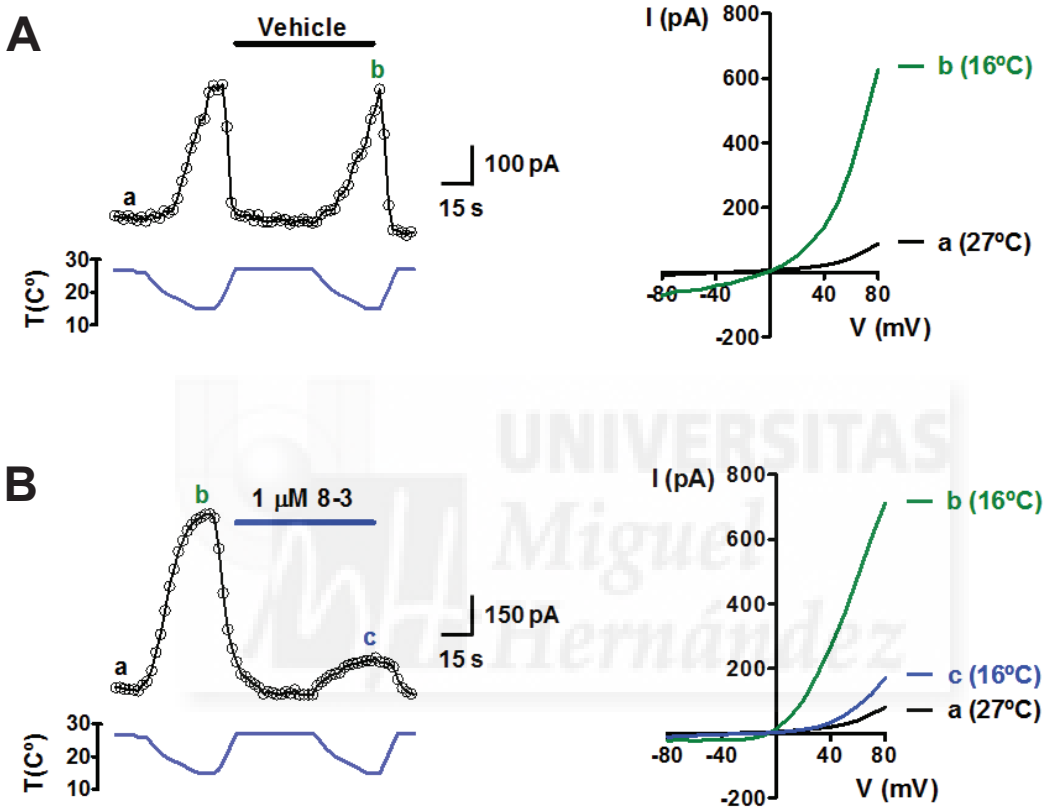

Figure 34. Derivative 8-3 **abolished the cold thermal activity response on TRPM8. Left:** Representative families of TRPM8 ionic currents at +80mV in absence (**A**) or presence of 1μM 8-3 (**B**) activated by cold temperature (16ºC) obtained with a 300-ms ramp from -80 to +80 mV in intervals of 2 s during 3 minutes. Holding potential -60 mV. **Right:** I-V relationships of TRPM8 channel in basal conditions (a, black line), activated by cold in the absence (b, green line) and presence of 1 μM 8-3 (c, blue line). n≥4.

Compound 8-3 was more potent when applied from the outside

In order to elucidate the mechanism of action of 8-3 we questioned whether the binding site of the selected compounds would be located on the extracellular or into the cytosolic region. With this aim we introduced the compound inside the cell through the patch pipette to study the activity in the intracellular region (Figure 35). To guarantee the complete distribution of the compound inside the cell, we started the recordings 3 minutes after entering whole cell. In this condition we observed that compound 8-3 blocked 65 ± 6% of the TRPM8 activity evoked by menthol (Figure 35; red bar). Contrary, the extracellular application of 1 μM 8-3 (Figure 35; blue bar) revealed a strong decrease on the activity, blocking more than 95% of the activity evoked by menthol on TRPM8.

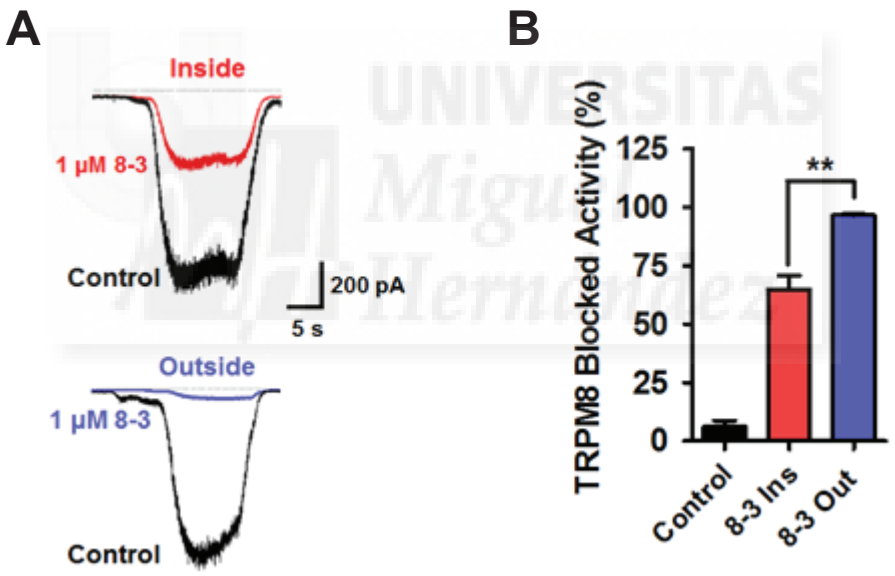

Figure 35. Compound 8-3 **showed different blockade when it is applied inside or outside the cell.** A) Representative whole-cell currents from TRPM8 channel activated with 300 μM in absence (control, black line) and presence of 1 μM 8-3 applied inside the pipette (top, red line) or applied externally (bottom, outside). B) TRPM8 blocked activity in absence (black), internal application of 1 μM 8-3 (red) and external application of 1 μM 8-3 (blue). Voltage held at -60mV. Data are given as mean ± SEM, n≥4. ANOVA with Bonferroni post hoc test ** $p < 0.05$.

Compound 8-3 blocked potently TRPM8 activity after pre-incubation

In all previous experiments, the compound was always pre-incubated prior to menthol application. Then, we investigated whether it might require a period of incubation to develop its activity or, contrary to this, it could have a direct effect when it is co-applied with menthol. In Figure 36A, we can see representative recordings under co-application (top, red) and pre-application (bottom, blue) of compound 8-3. As it has been shown before, the pre-application of 1 μM 8-3 produced an almost total blockade of menthol-evoked TRPM8 activity (Figure 36B; blue bar). By contrast, when we co-applied 300 μM menthol and 1 μM 8-3 for 20 seconds, the blockade was 24 ± 4 % (Figure 36B; red bar), a significant reduction of the inhibitory effect.

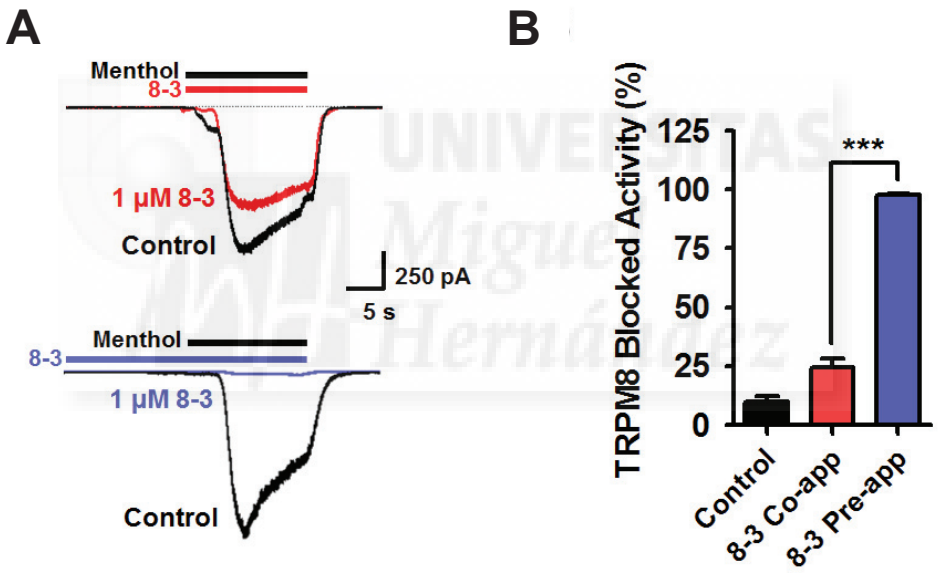

Figure 36. Compound 8-3 **requires from a pre-application to exert its blockade activity on TRPM8. A, top)** Representative whole-cell voltage clamp recordings from TRPM8-expressing HEK-CR1 cells activated by 300 μM menthol in absence (black) or co-applied with compound 8-3 at 1μM for 20 s (red). **A, bottom)** Pre-application of compound 8-3 at 1μM (20 s) followed by co-application with 300 μM menthol (Control) for 20s. Voltage was held at -60 mV. **B)** TRPM8 blocked activity in absence (black), co-application (red) or pre-application (blue) of compound 8-3 at 1 μM. Data are given as mean ± SEM, with n ≥ 5 cells per data point. ANOVA with Bonferroni post hoc test *** p<0,001.

Docking studies on TRPM8 channel and compound 8-3

In order to locate the regions involved in the binding of the compound 8-3, we first built a homology model of TRPM8. Since the high-resolution structure of TRPM8 has not been solved yet, the channel was modeled by Gregorio Fernández Ballester (UMH, IBMC) based on the recently published 3,4 Å-resolution structure of TRPV1. Figure 37A shows a side view of the TRPM8 structural model in the closed state where the four subunits are differently colored. The intracellular domains corresponding to the cytoplasmic N- and C-termini fragments were removed for simplicity. In Figure 37B we show a detailed view of a single subunit, indicating the location of the six transmembrane segments, as well as the TRP domain. In Figure 37C we show top (extracellular) and bottom (cytoplasmic) views of the TRPM8 model.

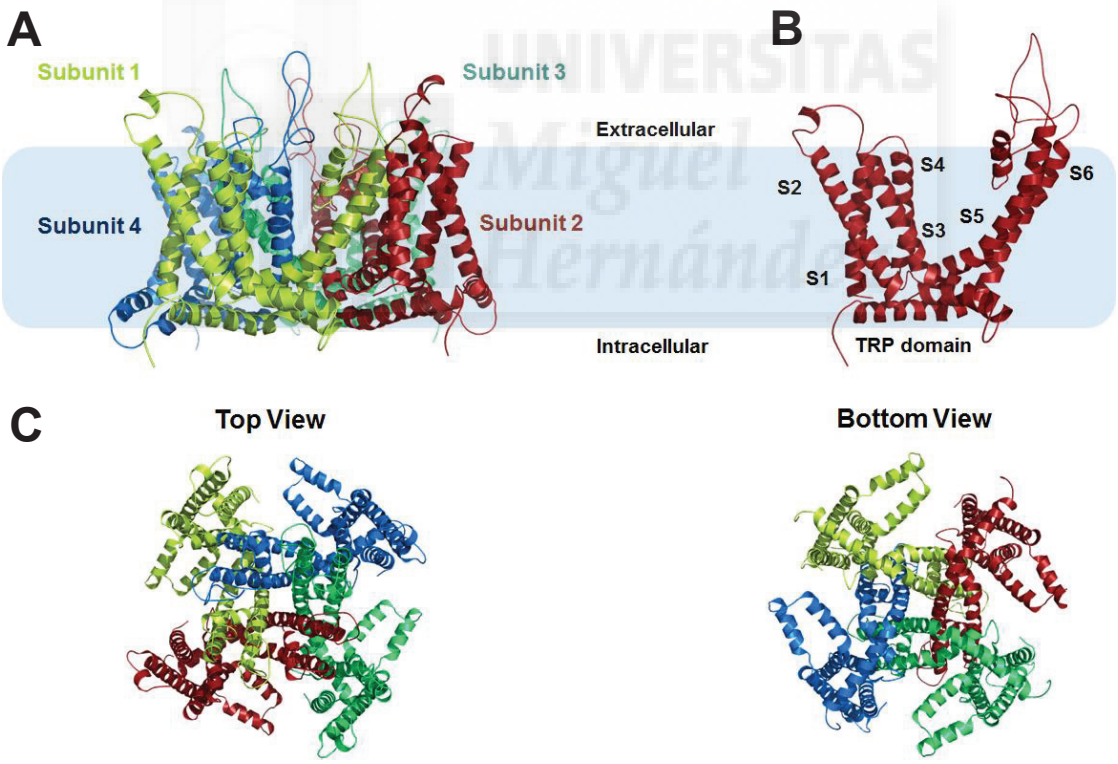

Figure 37. Homology model for TRPM8 channel. A) Side view of the structural model of TRPM8 based on the TRPV1 structure. The four different subunits are depicted in yellow, red, green and blue. **B)** Detail of a subunit where the different transmembrane segments are indicated as well as the TRP domain in parallel to the membrane plane. **C)** Top and bottom view in the modelled closed state of TRPM8.

Similarly, we modeled the structures of the compound 8-3 according to their chemical scaffold. Then, we ran a series of docking simulations to investigate possible binding sites for the newly-synthesized small molecules in the TRPM8 tetramer and to test the influence of this binding on the conformation. We performed the docking simulations with the software Autodock[235] implemented in YASARA[236, 237] (http://www.yasara.org/index.html). After 500 trials of simulations, the docking predicted four major solutions named A, B, C and D (Figure 38). We continue studying solution A and D. Solution B, located in the extracellular region of the channels was discarded due to the hydrophobic properties of compound 8-3 and its low binding energy (6.7 Kcal/mol). Similarly we also discarded solution C based on the electrophysiological recordings that showed a lower TRPM8 blockade activity when compound 8-3 was applied internally compare to the external application.

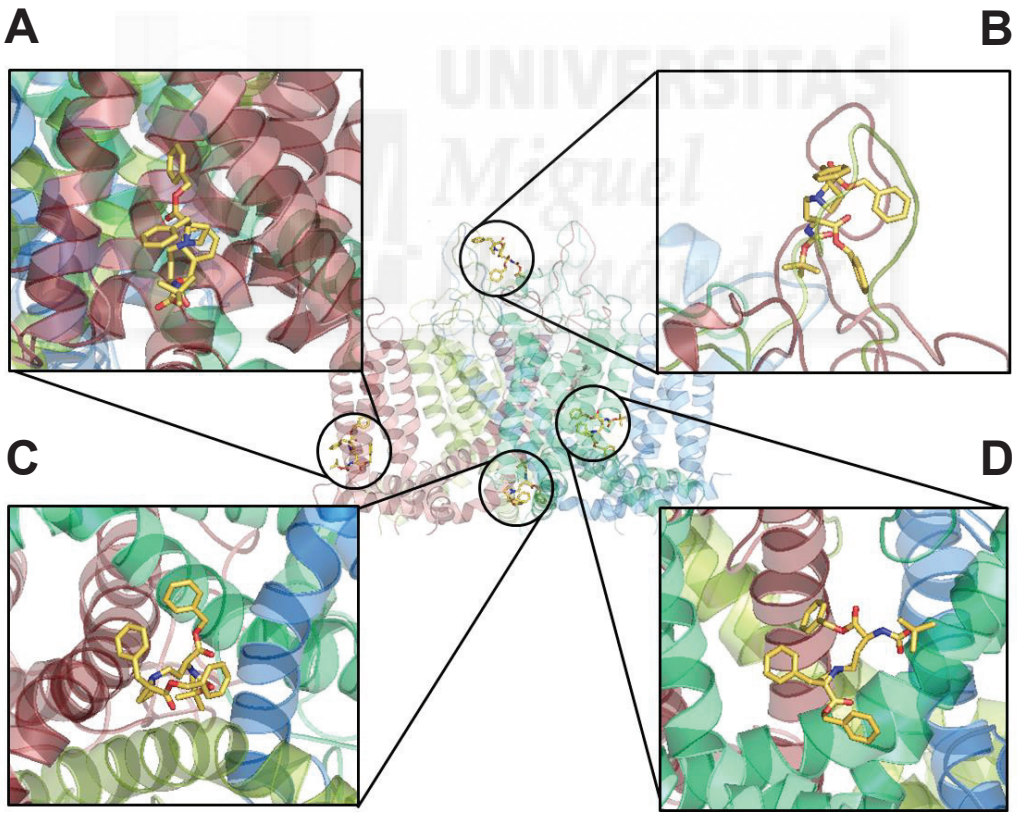

Figure 38. Docking predicted four possible binding sites for compound 8-3 **in the TRPM8 channel. A**) Site A, compound 8-3 is localized close to the S1, S2 and TRP domain. **B)** Site B, compound 8-3 binds extracellular loops of TRPM8 channel. **C)** Site C, compound 8-3 interact intracellular region close to the pore. **D)** Site D, compound 8-3 binds in a hydrophobic cavity located among S3, S4, S5 from a subunit and S6 from an adjacent one.

On solution A, from now site 1, compound 8-3 is localized in a hydrophobic pocket formed by the transmembrane segments S1, S2 and the TRP domain (Figure 39). Inside this region, compound 8-3 is stabilized by noncovalent interactions. Thus, simulations using Protein-Ligand Interaction Profiler (PLIP) software[238] identified hydrophobic contacts with S1 (W693, F700, I701, L704), S2 (A747, L750, L751, Y754, T803), and TRP domain (L1009) (Figure 39 as a grey dots).We also identified a hydrogen bond between compound 8-3 and S1 (W693) (Figure 39 as blue line). Results of all the interactions, site 1 showed a binding energy of 8.16 Kcal/mol.

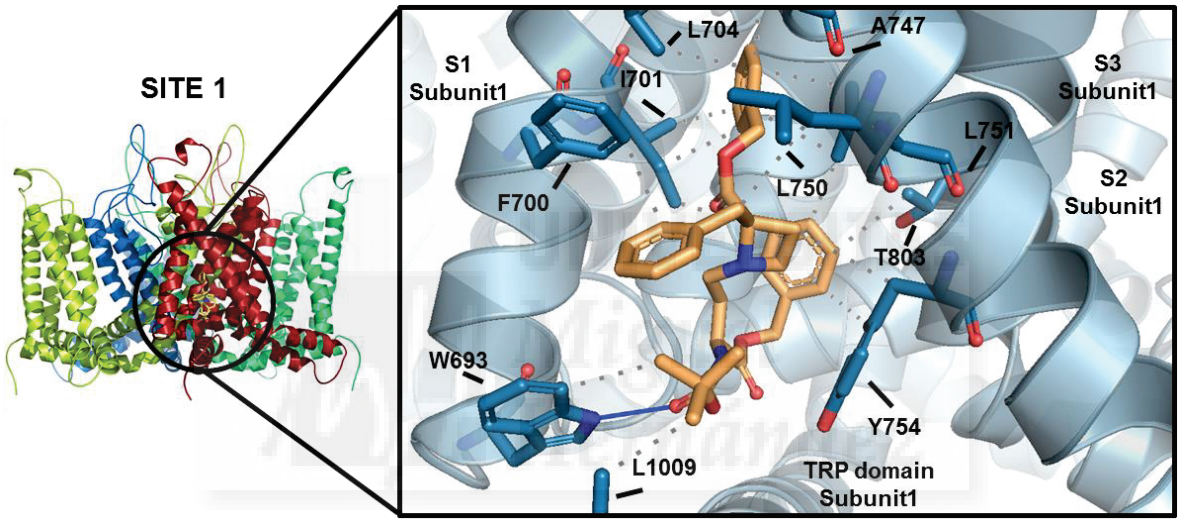

Figure 39. Molecular modelling of site 1 where a binding site is proposed to compound 8-3 **into the TRPM8 receptor**. Parts of the S1 and S2 domains of TRPM8 are depicted as well as the TRP domain. Boc group of the compound 8-3 makes a hydrogen bond with the residue W693 (blue line). The hydrophobic interactions between compound and residues are represented as grey doted lines.

On site 2, previously named solution D, compound 8-3 is localized in a wide region with accessibility for compounds with hydrophobic groups. Similar to site 1, site 2 is a hydrophobic pocket formed by the transmembrane segments S3, S4, and S5 from a subunit and interestingly, also by the S6 from the contiguous subunit (Figure 40). The docked compound is stabilized by hydrophobic interactions with S3 (F794, W798) S4 (F839) and S5 (L864, I865, F868, F869) from a subunit and S6 (L965) from the contiguous subunit. (Figure 40 as a grey dots). Contrary to site 1, we could not find any hydrogen bond in site 2, although compound 8-3 binds to the F869 through π-stacking between the aromatic rings (Figure 40, green line). All these interactions yield a binding energy of 8.09 Kcal/mol.

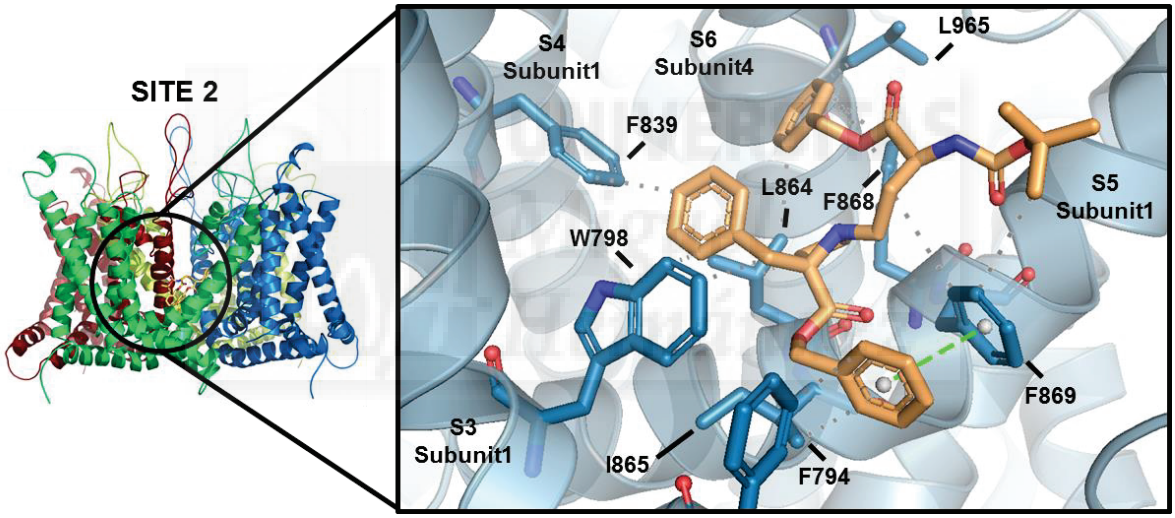

Figure 40. Molecular modelling of the alternative solution 2 for compound 8-3 **and TRPM8 interaction**. Parts of the S3, S4 and S5 domains from subunit 1 of TRPM8 and S6 from subunit 4 are depicted. The phenyl ester of 8-3 makes a π-staking with the aromatic ring of the residue F869 (green line). The hydrophobic interactions between compound and residues are represented as grey dots.

DISCUSSION

TRPM8 channels have been implicated in the aethiology of painful cold hypersensitivity produced under inflammatory and neuropathic conditions, as well as platinum-based chemotherapy drugs[142, 222, 223, 239]. Knockout mice studies and the use of TRPM8 antagonists demonstrated to be valuable to attenuate the symptoms observed after peripheral nerve injuries[36, 219]. Moreover, the implication of TRPM8 on prostate cancer, melanoma proliferation and tear regulation positioned this channel as an emergent and essential pharmaceutical target[35]. For these reasons several companies and academic groups have produced TRPM8 modulators. Unfortunately, many of them have been proved to provoke hypothermia when they were administered in *in vivo* models acting on other somatosensory ion channels, such as TRPV1 and TRPA1. PBMC, BCTC or the anti-fungal clotrimazole are some examples of potent TRPM8 antagonists that robustly affect TRPV1 or TRPA1[36, 145]. This overlap is, in some way, due to the lack of information related to the mechanism of action of the antagonists that difficult the design of specific molecules to inhibit TRPM8 activity. Thus, new compounds that block TRPM8 channel function are needed both as therapeutic molecules as well as pharmacological tools for further study the modulation of the channel activity.

To identify new candidates with high TRPM8 inhibitory activity we performed a high throughput screening where more than 350 compounds were evaluated. The most interesting compounds belonged to RGM-8 family, a chemical library based on β-lactams. The study of the structure-activity relationship on this series contributed to the establishment of the minimal requirements for a potent TRPM8 blocker (Table 3). Based on the β-lactam scaffold, the preferred structural elements are hydrophobic groups (benzyl ester or tBu) in R1, R2, R3 and R5 and a short N-alkyl chain (≤ 2 carbons). The best compounds in this library (**8-13**, 8-14 and 8-18) met the criteria. Interestingly, β-lactams have been traditionally employed as antibiotics (penicillin)[240]. Thus, these discoveries suppose an advance on that field, but also lead the progress of new drugs based on β-lactams with novel properties.

The activity of compounds 8-3, 8-14, and 8-18 was substained by patch clamp showing that the potency against menthol activation of all of them was on the nanomolar range. Notably, the IC_{50} of compound 8-3 (46 nM) underlined it as one of the most potent antagonists of TRPM8 channel described to date[35]. Furthermore, concentrations 1,000-fold higher than the IC_{50} of compound 8-3 maintained similar percentages of viability compared to the control conditions for HEK-CR1 cells. Among the set of ion channels evaluated to determine the selectivity of the compounds, members from the potassium family as well as other TRPs channels were tested. Importantly, and unlike other TRPM8 antagonists, we did not observe any cross reactivity with other ion channels for any of the selected compounds[145]. A demonstration of the specificity and selectivity of compound 8-3 was the absence of effect on voltage-gated Na+ and K+ channels on DRG neurons of newborn rats.

Regarding the modality of blockade, we found that compound 8-3 was able to reduce the activation of TRPM8 channel by menthol, voltage and cold in a strong manner. Akin to other TRPM8 blockers such as BCTC[233], the polymodal activity showed by compound 8-3 suggests that this molecule could be acting as a negative allosteric modulator affecting the channel gating instead of individual sensors. To explore this option, we ran molecular modeling analysis to identify possible binding sites for compound 8-3 in the TRPM8 structure. Interestingly, although pre-incubation and co-application experiments with compound 8-3 suggested a competitive mechanism of action, none of the four predicted solutions showed a competitive binding site. Similar results have been described with the antagonist BCTC[233]. Competition experiments with this TRPM8 blocker suggested that this molecule should be competing with menthol for the binding site[188]. However, BCTC blocked cold- and voltage-evoked TRPM8 activity when it was tested in the Y745H mutant, a mutant that prevents binding of menthol, indicating a different binding site for allosteric modulation that the authors could not identify[233].

Out of the four solutions found by the molecular modeling analysis, two of them (B and C) were localized at the extracellular and intracellular regions respectively which are widely expose to an aqueous environment. Contrary, solution A and D are situated in the transmembrane region characterized by its high hydrophobic

environment. Taking into account that compound 8-3 is composed mainly by hydrophobic moieties, it is tentative to discard options B and C. Moreover, the low energy obtained with the docking and the decrease observed in the activity of more hydrophilic compounds supports this decision.

The most energetically favorable solution was site 1 localized in a hydrophobic pocket formed by the transmembrane segments S1, S2 and the TRP domain. There, compound 8-3 could establish a wide network of hydrophobic interactions. In addition, compound 8-3 also formed a hydrogen bond with TRPM8 channel. All these interactions reported a binding energy of 8.16 Kcal/mol, and potentially stabilized the molecule-channel complex in the close state preventing channel activation.

Interestingly, the molecular modeling analysis also showed another option, with a similar energy (8.09 Kcal/mol), the site 2. In this solution, the compound 8-3 established hydrophobic interactions with residues localized in a cavity formed by the transmembrane segments S3-S4-S5 from one of the subunits and the segment S6 from the contiguous subunit. Moreover, it formed a π-stacking between its aromatic ring of and the one present in the S5. This interaction, similar to a weak hydrogen bond, could be responsible of the selectivity to the compound 8-3.

The relevance of these interactions was observed when we performed the docking experiment of compound 8-1 on sites 1 and 2 (Figure 41). This compound, which barely blocked TRPM8 activity, differs from compound 8-3 on the positions R2 and R3, where it has two methyl moieties instead of two aromatic rings (Table 4). As we expected, the absence of these two benzyl ester groups reduced considerably the hydrophobic interactions between compound 8-1 and TRPM8 channel in site 1 (10 hydrophobic interactions with compound 8-3 and 8 with compound 8-1). In site 2, results were similar, a decrease from 8 to 5 interactions with compound 8-3 and 8-1 respectively. If we take into account that hydrophobic bonds usually increase the affinity of the compounds[241], a lower number of them could explain the decrease on the TRPM8 blockade observed with compound 8-1. Thus, although compound 8-1 presented two hydrogen bonds on site 1 (S1(W693) and S2(Y754)), they could not compensate the decrease on hydrophobic interactions and reported a final binding

energy of 6.71 Kcal/mol. A similar result was obtained on site 2. Compound 8-1 was able to form a hydrogen bond with S3 (D802) and another one with S4 (Y836) and also presented a π-stacking with S4 (F839). However, the significant decrease on hydrophobic interactions could not be compensated by the described interactions and the complex compound-TRPM8 reported a binding energy of 6.03 Kcal/mol.

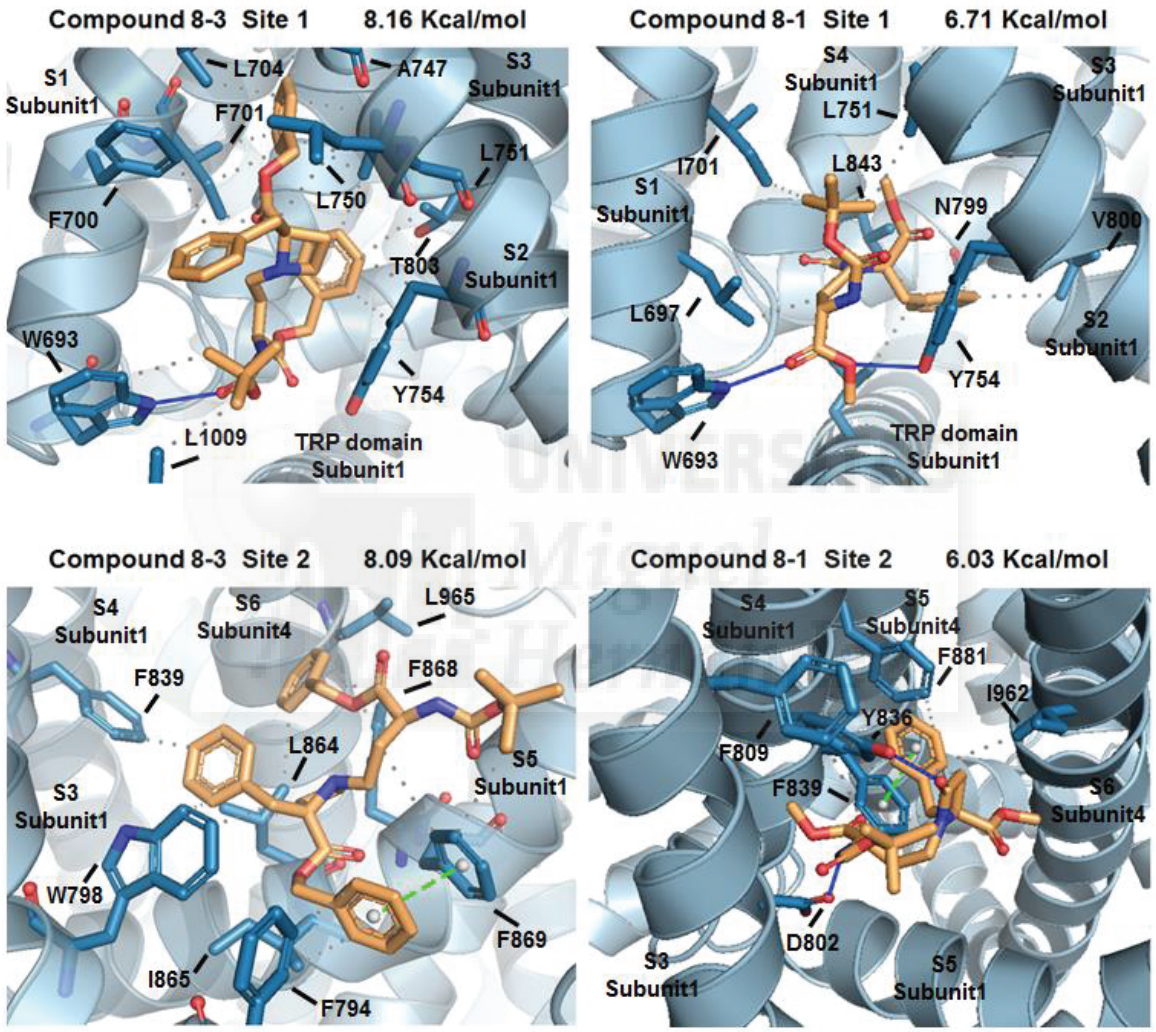

Figure 41. Molecular modelling of interaction sites 1 and 2 in TRPM8 for compounds 8-3 **(left) 8-1 (right).** Parts of the S3, S4 and S5 domains from subunit 1 of TRPM8 and S6 from subunit 4 are depicted. Pi-stacking are denoted as green lines and hydrophobic interactions between compound and residues are represented as grey doted lines. Hydrogen bonds are represented by a solid blue line.

Hence, these computational results explain the potency observed with compound 8-3 in patch clamp experiments and demonstrate the relevance of the aromatic rings for the TRPM8 blockade activity.

We also performed computational studies with compound 8-3 on the TRPV1 structure to explorer the selectivity of the molecule (Figure 42). In concordance with micro-fluorography and patch clamp experiments, the complex compound-TRPV1 channel on site 1 presented a binding energy significantly lower than the one observed with compound 8-3 and TRPM8 channel (4.83 Kcal/mol vs 8.16 Kcal/mol respectively). That is, in part, due to the diminution on hydrophobic interactions formed between the compound and TRPV1 channel (10 and 5 hydrophobic interactions in TRPM8 and TRPV1 channel respectively). Interestingly, compound 8-3 presented a hydrogen bond with S1 (W693) of TRPM8 channel that disappeared when it was docked in the TRPV1 structure. It has been reported that hydrogen bonds confer selectivity to the compounds [241]. Therefore, the lack of this hydrogen bond together with the reduction on the hydrophobic interactions could explain the absence of blockade activity observed with compound 8-3 in TRPV1 channel.

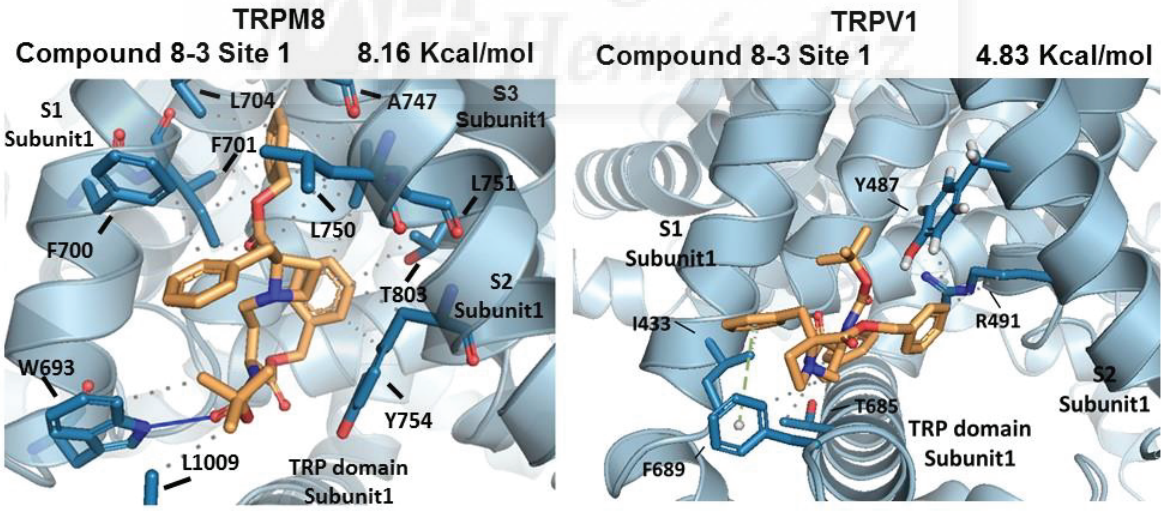

Figure 42. Molecular modelling of interaction sites 1 in TRPM8 and TRPV1 channels for compounds 8-3**. Left:** Compound 8-3 in the TRPM8 structure on site 1. **Right**: Compound 8-3 in the TRPV1 structure on site 1. Pi-stacking are denoted as green lines and hydrophobic interactions between compound and residues are represented as grey doted lines. Hydrogen bonds are represented by a solid blue line.

For site 2 in TRPV1 the results obtained were similar. The wide network of hydrophobic interactions observed with compound 8-3 on the TRPM8 structure was significantly diminished when this compound was docked in the TRPV1 channel (Figure 43). Moreover, the π-stacking formed between compound 8-3 and TRPM8 channel was not established on the compound-TRPV1 complex. As a result, the binding energy decreased dramatically on the TRPV1 structure (from 8.09 Kcal/mol for TRPM8 to 4.83 Kcal/mol), explaining the absence of activity of the compound 8-3 on the vanilloid receptor. Furthermore, these results corroborated and emphasized the relevance of hydrophobic and π-stacking interactions in the affinity and selectivity of compounds respectively.

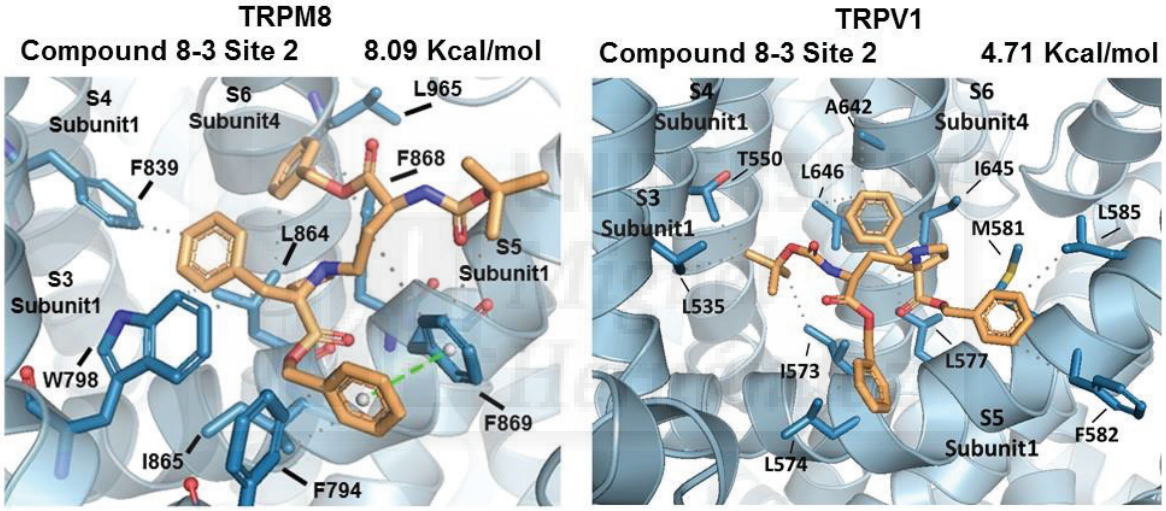

Figure 43. Molecular modelling of interaction sites 2 in TRPM8 and TRPV1 channels for compounds 8-3**. Left:** Compound 8-3 in the TRPM8 structure on site 2. **Right**: Compound 8-3 in the TRPV1 structure on site 2.Pi-stacking are denoted as green lines and hydrophobic interactions between compound and residues are represented as grey doted lines. Hydrogen bonds are represented by a solid blue line.

The results from the docking experiments suggest a possible negative allosteric mechanism of action of compound 8-3. On site 1, the physical proximity of this hypothetical binding site to the TRP box (980-992) region, together with the interactions with the S1-S2-TRP domain, suggest a stabilization of the closed state by avoiding the transition to the open state. First of all, it has been reported that structural changes in the S2 can severely affect the activity of the channel[242].

Therefore, the interactions with the S1-S2 region could prevent channel opening. Regarding the importance of the TRP domain, recent papers remarked the central role of this region in the gating mechanism of TRPM8 channel[182, 234]. Activating stimuli seems to disrupt the interactions in the 980-992 region of the TRP domain favoring channel opening[182]. Moreover, other TRP channel such as TRPV1 or TRPV4 showed a similar mechanism of gating suggesting a conserved function for this region[46, 47, 243]. Thus, the interaction of compound 8-3 with the TRP domain could prevent channel opening. Furthermore, the SAR results revealed that substitutions on the R1, R2 and R3 (8-41, 8-42, 8-43 and 8-44) that abolish the hydrophobic interactions, suppress the activity of the compound. These results support the hypothesis of the stabilization of the closed state on TRPM8 as a likely mechanism of action.

In the predicted binding site 2, as it has been mentioned, the compound 8-3 was localized in a hydrophobic pocket formed by the transmembrane segments S3-S4-S5 from one of the subunits and the segment S6 from the contiguous subunit of TRPM8 channel. Several evidences suggest that in the closed state, the S4-S5 linker is interacting with the S6 from an adjacent subunit[55, 182]. Upon addition of an activating stimulus, this interaction disappears producing the gating of the channel[55 244]. Based on these facts, we proposed that compound 8-3 blockade activity on site 2 is exerted via the π-stacking and hydrophobic interactions which maintained the S6 locked, avoiding the gating of the channel. Interestingly, this region has also been proposed as an allosteric regulatory site in TRPV1 channels (positively and negatively)[56, 63]. For example, it seems that upon binding a region between S3 and S4, capsaicin induces structural rearrangements to stabilize the open state. Remarkably, it has been reported that small modifications of the A-region of capsaicin turns these TRPV1 agonist into a competitive antagonist such as capsazepine[245, 246]. This effect occurs because changes in the A-region of capsaicin compromise its interaction with the S4 segment and allow the C-region and the S4-S5 linker to establish new interactions that hold the channel in the close state. That remarks the relevance of determined interactions on this site as we observed with

the β-lactam derivatives where modifying only one position can dramatically reduce the activity of the compound (8-1 and 8-2 vs 8-3, Table 4). Moreover, based on the TRPV1 evidences should be expectable that future modifications could deliver activators of TRPM8 channels.

As shown before, these two binding sites are located deep in the plasmatic membrane in a region relatively close to the cytoplasm. In contrast, electrophysiological experiments reported that extracellular application of compound 8-3 blocked more potently TRPM8 activity than the intracellular application, suggesting a binding site located more externally. One possibility to explain this controversy could be related to the faster diffusion through the membrane when compound 8-3 is applied externally. Although site 1 and site 2 are located far from the external region, the access of the compound 8-3 to these binding sites would be facilitated by the absence of structural impediments when it is applied externally. In this sense, binding site 2 would be the most suitable candidate. In Figure 44 it is shown that site 2 is localized in a deep hydrophobic cavity of the TRPM8 channel, being less accessible from the intracellular side than site 1. The top view of the channel suggests that site 2 is easily accessible from the extracellular part, where almost nothing disrupts the entrance of the compound 8-3 to the cavity. Similarly, site 1 is also easily accessible extracellularly.

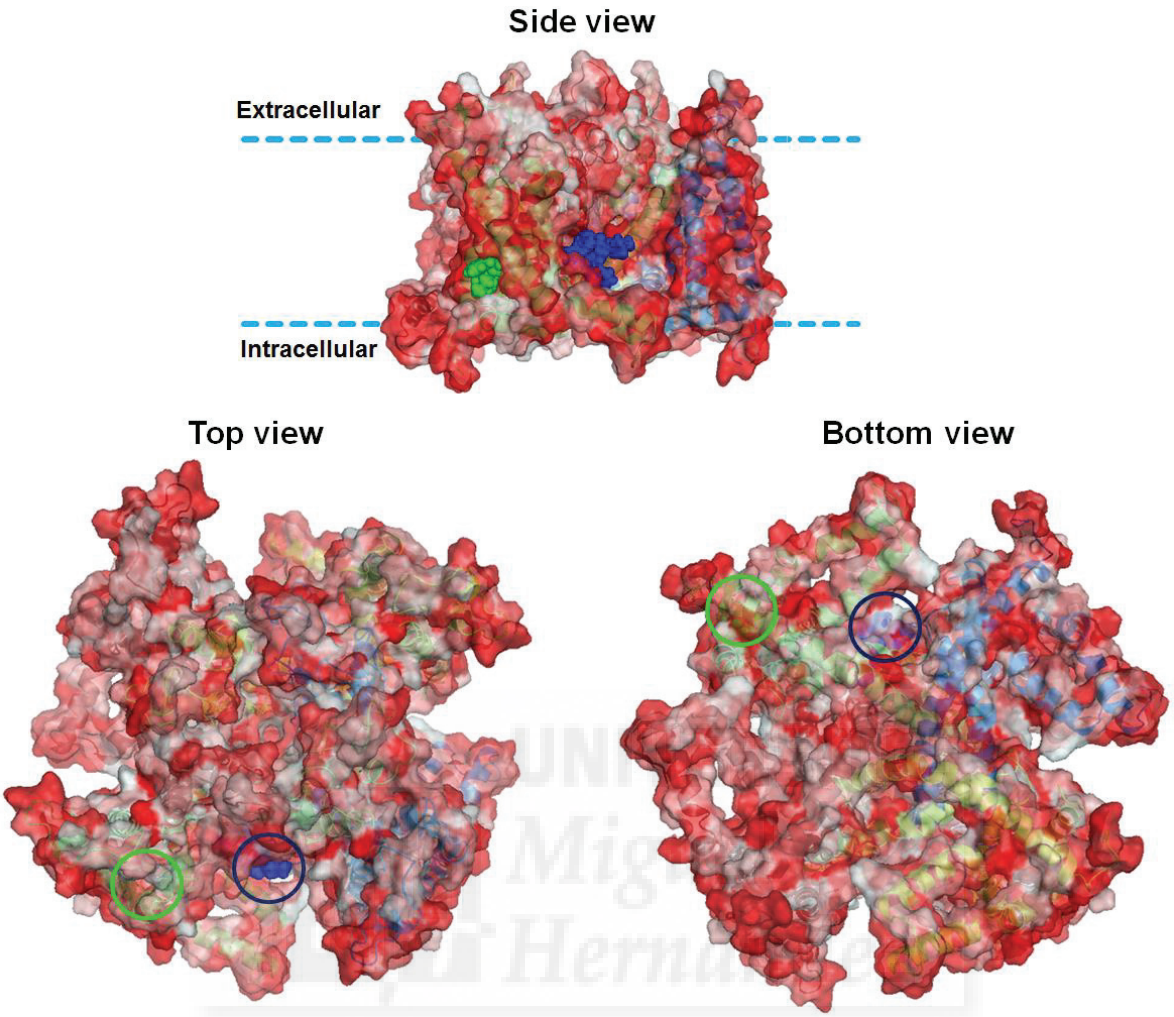

Figure 44. Representation of Van der Waals radii and hydrophobicity of TRPM8 channel and compound 8-3 **on sites 1 and 2.** Side view of TRPM8 channel and compound 8-3 in site 1 (green spheres) and site 2 (blue spheres). Hydrophobic regions are depicted in red color. Top view of TRPM8-compound 8-3 complex. Bottom view of TRPM8 channel and compound 8-3. Site 1 and site 2 are depicted with green and blue spheres respectively. In order to facilitate the identification of site1 and site 2 for compound 8-3 when is not visible, they have been circled in green and blue colors respectively.

On the contrary, the bottom view shows how the S2-S3 linker might obstruct the pass of the molecule from the inner part of the cell to the site 2. This does not seem to be the case with site 1, or at least not as dramatically as for site 2, since it is located more external than site 2. In agreement with this, recent studies have demonstrated that large structures could impede the binding of some compounds. For example, the N terminus of calcium-sensing receptor prevents the modulator

cinacalcet from directly activating the receptor[247]. Hence, it is possible that longer internal application of compound 8-3 (>3 minutes) would allow a better distribution, resulting in large TRPM8 blockade activity.

Another hypothesis to explain the differences observed when compound 8-3 is applied internally and externally is the possible interference of the intracellular organelles with the activity of the compound. Since compound 8-3 present a hydrophobic component, it may be retain on the membrane of different organelles (e.g. endoplasmic reticulum, Golgi apparatus and mitochondria). This effect will reduce the active concentration of compound 8-3 explaining its loss of blockade activity. Probably, experiments where is possible the direct application of compound on the cytosolic surface of the membrane (inside-out), will help to corroborate this hypothesis.

Regarding the two binding sites, since the similar binding energy predicted by the docking models suggests an almost identical affinity, we cannot differentiate between them based on in and therefore, both should be considered as valid solutions. Nevertheless, if the hypothesis of the structural impediments is correct, binding site 2 would be the most suitable candidate since compound 8-3 could access easily to the cavity where exert its blockade activity. However, further investigations would be required to confirm the binding site for compound 8-3. It is possible that mutagenesis work of the TRPM8 channel in site 1 and site 2 will help in the identification and the development of novel TRPM8 modulators.

CONCLUDING REMARKS

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CONCLUDING REMARKS

Current analgesics are effective pain suppressors, but they are plagued by serious side effects, in part because their targets are also implicated in many physiological functions. Alternatively, acting directly on the noxious stimuli transducers at the peripheral terminals of nociceptors would allow for the control of the generation of the pain signal at its very beginning. Thus, side effects associated to traditional analgesics would be avoided. In this direction, a lot of effort is being made trying to develop modulators for TRP nocicensors. Furthermore, knockout mice lacking TRPV1 and TRPM8 have shown absence of responses against different pain stimuli. The expectation is that TRPV1 blockers should be effective for inflammatory pain syndromes whereas TRPM8 antagonists should be efficacious in treating conditions of cold hypersensitivity brought on by nerve injury or treatment with chemotherapeutic agents. For these reason, many pharmaceutical companies have develop antagonist for these channels. Unfortunately, some TRPV1 antagonists produced adverse effects such as hyperthermia meanwhile TRPM8 blockers produced hypothermia. It seems that indiscriminate pharmacological blocking of the receptors with high affinity and competitive antagonists may be responsible for the observed side effects. Hence, high affinity antagonists that bind to the receptor in an activity-independent manner should show limited therapeutic indices, since these compounds would interact with both resting and active channels. Moreover, in the case of TRPM8 almost nothing is known about the antagonist mechanism of the current molecules. Our attempt in the first part of this work was to design and study new open channels blockers of TRPV1 channels with analgesic activity that primarily target over-activated TRPV1 receptors. In the second part, we identify and characterized a new class of negative allosteric modulators of TRPM8 channel. These approaches might avoid the side effects observed with the competitive antagonists. Thus, triazine 8aA, the TRPV1 blocker and compound 8-3, the TRPM8 blocker represent a novel class of antagonist that could be used as a pillar to evolve a new generation of antagonists of these TRP channels with higher therapeutic index that, in due turn, could be developed into potent analgesic drugs.

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CONCLUSIONS

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CONCLUSIONS

CHAPTER 1

- Triazine 8aA, is selective TRPV1 antagonist that preferably blocked capsaicin activity over pH either on rat or human orthologue.

- Triazine 8aA is an open channel blocker of TRPV1 that binds in the pore region.

- Triazine 8aA blocks TRPV1 in the peripheral nerves and reduced the firings evoked by capsaicin in the knee joint of the rat showing effect in vivo models of pain.

- Triazine 8aA has anti-nociceptive effect in vivo attenuating the nociception evoked by the intraplantar injection of capsaicin in rats.

- Triazine 8aA reduce considerably the scratching in the rat model of pruritus.

CHAPTER 2

- The high throughput screening identify the β-Lactam scaffold as a good candidate to develop TRPM8 antagonists.

- The Structure-Activity Relationship allowed to establish the minimal requirements on the β-lactam ring to design TRPM8 antagonists.

- Compounds 8-3, 8-14 and 8-18 are potent, selective and non-toxic TRPM8 antagonists that exert their activity in the nanomolar range.

- Molecular docking suggest two different places for 8-3 to modulate TRPM8:

    - A hydrophobic pocket formed by the transmembrane segments S1, S2 and the TRP domain

    - A hydrophobic pocket formed by the transmembrane segments S3, S4, and S5 from a subunit and interestingly, also by the S6 from an adjacent subunit.

- The bindings sites proposed in the molecular docking suggest that compound 8-3 could be a negative allosteric modulator of TRPM8

CONCLUSIONES

CAPITULO 1

- La triazina 8aA es un antagonista selectivo de TRPV1 que bloquea preferentemente la activación del canal por capsaicina tanto en rata como en el ortólogo humano.

- La triazina 8aA es un bloqueador de canal abierto que se une a TRPV1 en la región del poro.

- La triazina 8aA bloquea la actividad de TRPV1 en fibras nerviosas del sistema periférico y presenta actividad analgésica *in vivo* al reducir la generación de potenciales de acción evocados por capsaicina en la rodilla de la rata.

- La triazina 8aA tiene efecto analgésico *in vivo* reduciendo la nocicepción evocada por la inyección intraplantar de capsaicina en ratas.

- La Triazine 8aA reduce considerablemente el picor en el modelo de prurito desarrollado en ratas.

CAPITULO 2

- Mediante cribados de alto rendimiento identificamos una familia de β-lactamas como un esqueleto químico interesante para desarrollar antagonistas de TRPM8.

- La relación estructura-actividad de los compuestos derivados de la β-lactamas permitieron establecer los requisitos mínimos para diseñar antagonistas de TRPM8 basados en este esqueleto químico.

- Los compuestos 8-3, 8-14 y 8-18 son potentes y selectivos antagonistas de TRPM8 no tóxicos que ejercen su actividad a concentraciones en el rango nanomolar.

- Los estudios computacionales de unión del compuesto 8-3 a TRPM8 sugirieron dos posibles sitios de unión:
    - Uno localizado en un bolsillo hidrofóbico situado en la región transmembrana y formado por los segmentos S1, S2 y el TRP domain.
    - Otro localizado en un bolsillo hidrofóbico situado en la región transmembrana y formado por los segmentos S3, S4 y S5 de una subunidad y el S6 de la subunidad adyacente.

- Los sitios de unión propuestos en los estudios computacionales sugieren que el compuesto 8-3 podría ser un modulador alostérico negativo de TRPM8.

MATERIAL AND METHODS

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MATERIAL AND METHODS

Oocyte preparation and microinjection

Adult female *Xenopus laevis* (purchased from Harlan Interfauna Ibérica S.L., Barcelona, Spain) were immersed in cold 0.17% MS-222 for 20 min and a piece of ovary was drawn out aseptically. Animal handling was carried out in accordance with the guidelines for the care and use of experimental animals adopted by the E.U. and the animal protocol was approved by the ethic committee of Universidad Miguel Hernandez de Elche. Stage V and VI oocytes were isolated and their surrounding layers removed manually. Cells were kept at 15–16°C in a modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.40 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 mM HEPES (pH 7.4), 100 U ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin] until used. Oocytes were microinjected with 5 ng of wild-type rat TRPV1 cRNA kindly gifted from David Julius

Recombinant rat TRPV1 channels expression in *Xenopus* oocytes and channel blocking

Whole-cell currents from rat TRPV1-injected oocytes were recorded in standard Ringer's solution (in mM: 10 HEPES pH 7.4, 115 NaCl, 2.8 KCl, 2.8 $BaCl_2$) with a 2-microelectrode voltage-clamp amplifier at 20°C. TRPV1 channels were activated by application of 10 µM capsaicin in absence or presence of individual compounds at a holding potential (Vh) of -60 mV. The application of capsaicin produced the gating of rTRPV1 and the influx of ions inside the oocyte. We considered that current as the maximum capsaicin-evoked current ($\mathbf{I_{max}}$) and normalized against this value. To determine the blockade activity of the candidates, we applied them at 10 µM combined with 1 µM capsaicin ($\mathbf{I_{com}}$).

Then, we plotted the relationship establish on the Equation 3:

$$\%\ \boldsymbol{Blocked\ Response} = \left[1 - \left(\frac{Icom}{Imax}\right)\right] * 100$$

Equation 3. Percentage of blocked response normalized to the maximum current (Imax)

Dose-response curve for individual compounds were fitted to the Hill equation:

$$\frac{\boldsymbol{I}}{\boldsymbol{I_{max}}} = \frac{1}{1 + \left(\frac{[blocker]}{IC_{50}}\right)^{n_H}}$$

Equation 4. Normalized dose-response curve fitted with Hill equation

Where, the $\mathbf{IC_{50}}$ is the half maximal inhibitory concentration and $\mathbf{n_H}$ is the Hill coefficient describes the cooperativity of ligand binding.

Receptor selectivity of triazine 8aA was evaluated on heterologously expressed N-methyl-D-aspartate (NMDA) receptors (rat NR1:NR2A) and human TRPM8 ion channels. All these ion channels were heterologously expressed in *Xenopus* oocytes, and the extent of channel blockade by triazine 8aA was investigated 3 to 5 days after injection of cRNAs. Holding potential was kept at -80 mV. NMDA receptors (NR1:NR2A, 1:3 w/w) and TRPM8 were assayed in standard Ringer's solution. Recombinant NMDA receptor responses were activated with 100 µM-glutamate plus 20 µM glycine and TRPM8 with 300 µM menthol in the absence and presence of the compound at the indicated concentrations. Responses were normalized with respect to that evoked in the absence of triazine 8aA and fitted to Hill equation (Equation 2).

Cell culture

SH-SY5Y cells stably expressing rat TRPV1 channel (SH-SY5Y TRPV1) were grown in Earle's minimum essential medium (MEM) containing 10% (v/v) of Fetal Calf Serum (FCS), 1% nonessential amino acids, 2 mM l-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin (P/S), and 0.4 µg/ml puromycin in a humidity controlled incubator with 5% CO_2 and at 37°C.

Human embryonic kidney HEK293-TRPM8 (stably expressing human TRPM8 receptor[57]); HEK293-TRPV1 (stably expressing human TRPV1 receptor[248]), HBK1 (stably expressing human KV1.1), HEK-NaV1.6 (stably expressing rat NaV1.1) and Chinese hamster ovary (CHO)-TRPA1 (stably expressing the mouse TRPA1 receptor[249]) cells were cultured in DMEM supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin, and 100 U/ml penicillin, and maintained in a humidity controlled incubator (5% CO_2).

Temperature response assay

Media was removed from the attached rTRPV1 Sh-Sy5Y expressing cells and a 1-µM Fluo-4 AM dye solution in PBS was applied for 30 min at 37°C. Cells were then detached from the tissue culture dish using EDTA, centrifuged, resuspended in PBS, counted and plated at 100,000 cells/well in a 96-well conical bottom PCR plate (Model no. EK-19280, Greiner, Monroe, NC, USA). Temperature responses were assayed one column at a time as follows: 30ºC for 2 min followed by a temperature increase to 50ºC, in 2ºC increments for 5 min [250]. Antagonist was added to cells following the dye loading and cell plating steps. Temperature control over the cellular environment was accomplished with an ABI 7700 instrument (Applied Biosystems, Foster City, CA, USA). Data were analyzed with Equation 3 and 4.

Cell viability assay (MTT)

Cell viability was assessed by the detection of mitochondrial activity in living cells using a modified colorimetric analysis of Blue Tetrazolium Bromide Thiazolyl (MTT). Briefly, HEK293 cells (2 × 10^4 cells/well) were subcultured in 96-well plates, grown until 80–90% confluence, and incubated with increasing concentrations of testing compounds for 24 h. Following treatment, 10 µL of MTT solution (5 mg/mL in phosphate buffered saline) was added to each well and further incubated for 4 h at 37 °C. Next, 100 µL of DMSO was added to each well to dissolve any deposited formazan resulting from cleavage and reduction of MTT by active mitochondrial dehydrogenases. The optical density of each well was measured at 540 nm with a microplate reader (Polastar BMG LABTECH, Offenberg, Germany).

Electrophysiology (Patch clamp)

Electrophysiological recording was carried out 1–3 d after cells seeded. Membrane currents and voltages were recorded by patch clamp using the whole-cell configuration. For whole-cell recordings of HEK-hTRPV1 and HEK-TRPM8 cells, pipette solution contained (in mM) 140 CsCl, 5 EGTA, and 10 HEPES, adjusted to pH 7.2 with CsOH, and bath solution contained (in mM) 140 NaCl, 5 KCl, 2 MgCl2,5 EGTA ,10 d-glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. In acidic pH solution, HEPES was replaced by MES and pH was set at 6. In calcium-free bath solution, $CaCl_2$ was replaced with 5mM EGTA. Patch pipettes were prepared from thin-walled borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA), pulled with a horizontal puller (P-97, Sutter Instruments, Novato, CA, USA) to have a tip resistance of 2–4 MΩ when filled with internal solutions. Data were sampled at 10 kHz (EPC10 amplifier with PatchMaster 2.53 software; HEKA Electronics, Lambrecht, Germany) and low-pass filtered at 3 kHz for analysis (PatchMaster 2.53 and GraphPad Prism 5, Graphpad Software, USA). The series resistance was <10 MΩ and to minimize voltage errors was compensated to 60–80%. All measurements were performed at 24–25°C.

Primary culture of sensory neurons

Neonatal Wistar rats were purchased from in house bred stock (originally from Harlan Laboratories). DRG from neonatal Wistar rats (3-5 days old) were digested with 0.25% (w/v) collagenase (type IA) in DMEM-glutamax (Invitrogen) with 1% penicillin-streptomycin (5000 U/mL, Invitrogen) for 1 h (37 ºC, 5% CO2). After digestion, DRG were mechanically dissociated using a glass Pasteur pipette. Single cell suspension was passed through a 100 µm cell strainer, and washed with DMEM glutamax plus 10% fetal bovine serum (FBS)(Invitrogen) and 1% P/S. Cells were seeded at the required density for each experiment on microelectrode array chambers previously coated with poly-L-lysine (8.33 µg/ml) and laminin (5 µg/ml). After 2 h, medium was replaced with DMEM glutamax, 10% FBS and 1% P/S, supplemented with mouse 2.5s Nerve Growth Factor (NGF) 50 ng/mL (Promega), and 1.25 µg/mL cytosine arabinoside when required (37 ºC, 5% CO2).All experiments were made 48 h after cell seeding.

Microelectrode array (MEA)

Extracellular recordings were made using multiple electrode planar arrays of 60-electrode thin MEA chips, with 30 µm diameter electrodes and, 200 µm inter-electrode spacing with an integrated reference electrode (Multichannel Systems GmbH). The electrical activity of primary sensory neuron was recorded by the MEA1060 System (Multi Channel Systems GmbH, http://www.multichannelsystems.com), and MC_Rack software version 4.3.0 at a sampling rate of 25 kHz. TRPV1-mediated neuronal firing activity was evoked by 15s-applications of 100 mM KCl, using continuous perfusion system (2 mL/min flux). 10 µM Triazine 8aA in external solution was perfused in the presence or absence of KCl. Data were analyzed using MC_RACK spike sorter and Neuroexplorer Software (Nex Technologies). An evoked spike was defined when the amplitude of the neuronal electrical activity overcame a threshold set at -25 µV. The recorded signals were then processed to extract mean spike frequency.

Animals

All procedures were approved by the Institutional Animal and Ethical Committee of the University Miguel Hernández de Elche, in accordance with the guidelines of the Economic European Community, the National Institutes of Health, and the Committee for Research and Ethical Issues of the International Association for the Study of Pain. Animals were kept in a controlled environment (21-23 ºC, 12h light/dark cycle), and had food and water available ad libitum.

Rat knee joint nociceptor fiber preparation and *in vivo* recording

Adult male Wistar rats (Harlan, Holland) weighing 250–300 g were anesthetized, and a catheter was inserted into the right saphenous artery for local intraarterial injection of substances into the joint area[135, 251]. The saphenous nerve was dissected, and fine filaments were subdissected from the peripheral end. Nerve fibers innervating the knee joint were identified by the location of their receptive field, which was determined by the firing response to probing the structures in and around the knee joint with a handheld glass. The mechanical stimuli consisted of normal and noxious outward and inward rotation of the knee joint lasting 10 s. Successful experiments included complete recordings in 20 multiunit filaments containing 2–5 identifiable units.

Capsaicin-induced flinch model

Male Wistar rats (Janvier, France) weighing 250 to 300 g were allowed at least 3 days of acclimation in Laboratory Animal before start of the experiment. Experimental procedures were approved by the Ethics Committee and met European Union guidelines for care and management of experimental animals.

Animals (six per group) were pretreated with vehicle (10% ethanol, 10% Tween 80, and 80% saline) or triazine 8aA (i.v. 10 mg/kg), 30 min before intraplantar injection of 10 µL of capsaicin (at 0.06% in 10% ethanol, 10% Tween 80, and 80% saline). Immediately after the injection of capsaicin, the latency and duration times of licking and shaking the paw in response to the injection was recorded. Data were statistically analyzed using the unpaired *t* test or one-way ANOVA test. Data are presented as mean ± S.E.M with a minimum of six animals/group. n≥6

BDL inflammatory model

Male Wistar rats were operated as described in order to obtain BDL (bile duct ligation) rats. Briefly, a midline incision was made in rats under general anesthesia with diazepam (3 mg/kg) and ketamine (100 mg/kg) administered intraperitoneally. The common bile duct was localized, doubly ligate, and cut between these two ligatures. In sham animals, a midline incision was performed, but without BDL. Behavioral studies started 48 h after surgery and only when no signs of pain or distress were apparent. The animals were treated with triazine 8aA (10 mg/kg) 3 weeks after surgery.

Rats were acclimatized in a measuring cage for 30 min, followed by videotaping of scratching behavior for 30 min or 1 h. Spontaneous scratching was quantified by counting the number of scratches of any region of the body performed by forepaws or hindpaws. For Hargreaves' Plantar Test a standard apparatus (Ugo Basile, Italy) was used that automatically measured the thermal latency to a thermal radiant stimulus[252]. To avoid tissue injury in refractory animals, stimulation was automatically terminated after 32 s. Thermal latency was determined before and after triazine 8aA or vehicle treatment in BDL and sham control rats. Data were statistically analyzed using the unpaired *t* test or one-way ANOVA test. Data are presented as mean ± S.E.M with a minimum of six animals/group. n≥6

High-throughput screening with calcium microfluorography

For fluorescence assays, cells expressing TRP channels (rTRPV1-SH-SY5Y, hTRPV1-HEK and hTRPM8-HEK) were seeded in 96-well plates (Corning Incorporated, Corning, NY) at a cell density of 40,000 cells 2 days before treatment. The day of treatment the medium was replaced with 100 µL of the dye loading solution Fluo-4 NW supplemented with probenecid 2.5 mM. Then the compounds dissolved in DMSO were added at the desired concentrations and the plate(s) were incubated at 37˚C in a humidified atmosphere of 5% CO2 for 60 minutes.

The fluorescence was measured using instrument settings appropriate for excitation at 485 nm and emission at 535 nm (POLARstar Omega BMG LABtech). A baseline recording of 4 cycles was recorded prior to stimulation with the agonist (10 µM capsaicin for TRPV1 and 100 µM menthol for TRPM8). The corresponding antagonist (10 µM Ruthenium Red forTRPV1 and 100 µM AMTB for TRPM8) was added for the blockade. The changes in fluorescence intensity were recorded during 15 cycles more. DMSO, at the higher concentration used in the experiment, was added to the control wells.

The blocking percentage was calculated with the Equation 3

$$\%\ \boldsymbol{TRPM8\ Blocked\ Response} = \left(1 - \frac{Fo - Fi}{FCo - FCi}\right) * 100$$

Equation 5. Percentage of TRPM8 blocked response normalized to the maximal fluorescence.

Where **Fo** is the fluorescence after the addition of menthol in the presence of the compound, **Fi** is the fluorescence before the addition of menthol in the presence of the compound, **FCo** is the fluorescence after the addition of menthol in the absence of the compound, **FCi** is the fluorescence before the addition of menthol in the absence of the compound.

The statistical Z-factor to determine the quality of the high-throughput screening experiment was calculated using the following equation:

$$\boldsymbol{Z-factor} = 1 - \frac{3 * (SD_{max} + SD_{\min})}{Mean_{max} - Mean_{min}}$$

Equation 6. Z-factor used to determine the quality of the HTS experiments.

Where: $Mean_{max}$ is the mean of the maximum fluorescence in the presence of agonist, SD_{max} is the standard deviation of the maximum fluorescence in the presence of agonists, $Mean_{min}$ is the mean of the maximum fluorescence in the presence of agonist and antagonist and SD_{min} is the standard deviation of the maximum fluorescence in the presence of agonist and antagonist.

To guarantee that our results were validated, we only used assays with a Z-factor higher than 0.5.

Molecular modeling

TRPV1 Molecular Model Building – The automatic multiple sequence alignment of the TRPV1and Kv1.2 transmembrane regions was performed with CLUSTALW at the European Bioinformatics Institute site (http://www.ebi.ac.uk) using Gonnet matrices. Manual alignment of the transmembrane region was accomplished by the alignment editor BioEdit v7.0.9 using PAM250matrices. After visual inspection, the transmembrane alignments were adjusted manually. The visualization and editing of the molecules were done with Yasara (http://www.yasara.org). The homology modeling was performed with the standard homology modeling protocol implemented in Yasara (version 13.9.8). After side chain construction, optimization, and fine-tuning, all new modeled parts were subjected to a combination of steepest

descent and simulated annealing minimization, keeping the backbone atoms fixed to avoid molecule damage. Finally, a full-unrestrained simulated annealing minimization was run for the entire model, obtaining a satisfactory -1.597 quality Z-score for dihedrals, and -2.992 for the overall model.

TRPM8 Molecular Model Building—The molecular model forTRPM8 was modeled using the structures of the TRPV1 ion channel in the closed state (Protein Data Bank code 3J5P) determined by electron microscopy at 3.4-Å resolution. Sequence alignment between rat TRPV1 and TRPM8 was performed with ClustalO from the European Bioinformatic Institute (EBI, http://www.ebi.ac.uk). After side chain construction, optimization, and fine-tuning, all new modeled parts were subjected to a combination of steepest descent and simulated annealing minimization, keeping the backbone atoms fixed to avoid molecule damage. The homology modeling was performed with the standard homology modeling protocol implemented in Yasara (version 13.9.8).

The protein-ligand docking and the analysis of interactions was accomplished with Autodock implemented in the general purpose molecular modelling software Yasara, and optimized with AMBER 99 force field. Docking trials were optimized and clustered to remove redundancy and sorted by binding energy.

The non-covalent interactions in protein-ligand complexes from the options obtained with the docking were studied with the web service Protein-Ligand Interaction Profiler (PLIP, https://projects.biotec.tu-dresden.de/plip-web/plip/index). After analyzing the complex, the results were represented Pymol (http://www.pymol.org).

BIBLIOGRAPHY

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1. Belmonte, C.; Viana, F., Molecular and cellular limits to somatosensory specificity. *Molecular pain* **2008,** *4*, 14.
2. Julius, D.; Basbaum, A. I., Molecular mechanisms of nociception. *Nature* **2001,** *413* (6852), 203-10.
3. Julius, D., TRP channels and pain. *Annual review of cell and developmental biology* **2013,** *29*, 355-84.
4. Basbaum, A. I.; Bautista, D. M.; Scherrer, G.; Julius, D., Cellular and Molecular Mechanisms of Pain. *Cell* **2009,** *139* (2), 267-284.
5. Moran, M. M.; McAlexander, M. A.; Biro, T.; Szallasi, A., Transient receptor potential channels as therapeutic targets. *Nature reviews. Drug discovery* **2011,** *10* (8), 601-20.
6. Dubin, A. E.; Patapoutian, A., Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation* **2010,** *120* (11), 3760-72.
7. Crook, R. J.; Dickson, K.; Hanlon, R. T.; Walters, E. T., Nociceptive sensitization reduces predation risk. *Current biology : CB* **2014,** *24* (10), 1121-5.
8. Nicholson, B., Differential diagnosis: nociceptive and neuropathic pain. *The American journal of managed care* **2006,** *12* (9 Suppl), S256-62.
9. Patapoutian, A.; Tate, S.; Woolf, C. J., Transient receptor potential channels: targeting pain at the source. *Nature reviews. Drug discovery* **2009,** *8* (1), 55-68.
10. Coutaux, A.; Adam, F.; Willer, J. C.; Le Bars, D., Hyperalgesia and allodynia: peripheral mechanisms. *Joint, bone, spine : revue du rhumatisme* **2005,** *72* (5), 359-71.
11. Woolf, C. J., Pain: moving from symptom control toward mechanism-specific pharmacologic management. *Annals of internal medicine* **2004,** *140* (6), 441-51.
12. Baron, R., Peripheral neuropathic pain: from mechanisms to symptoms. *The Clinical journal of pain* **2000,** *16* (2 Suppl), S12-20.
13. Nukada, H., Ischemia and diabetic neuropathy. *Handbook of clinical neurology* **2014,** *126*, 469-87.

14. Vinik, A.; Casellini, C.; Nevoret, M. L., Diabetic Neuropathies. In *Endotext*, De Groot, L. J.; Beck-Peccoz, P.; Chrousos, G.; Dungan, K.; Grossman, A.; Hershman, J. M.; Koch, C.; McLachlan, R.; New, M.; Rebar, R.; Singer, F.; Vinik, A.; Weickert, M. O., Eds. MDText.com, Inc.: South Dartmouth (MA), 2000.
15. Gilden, D.; Nagel, M.; Cohrs, R.; Mahalingam, R.; Baird, N., Varicella Zoster Virus in the Nervous System. *F1000Research* **2015,** *4*.
16. Schumacher, M. A., Transient Receptor Potential Channels in Pain and Inflammation: Therapeutic Opportunities. *Pain Practice* **2010,** *10* (3), 185-200.
17. Caterina, M. J.; Schumacher, M. A.; Tominaga, M.; Rosen, T. A.; Levine, J. D.; Julius, D., The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **1997,** *389* (6653), 816-24.
18. Ferrer-Montiel, A.; Fernandez-Carvajal, A.; Planells-Cases, R.; Fernandez-Ballester, G.; Gonzalez-Ros, J. M.; Messeguer, A.; Gonzalez-Muniz, R., Advances in modulating thermosensory TRP channels. *Expert opinion on therapeutic patents* **2012,** *22* (9), 999-1017.
19. Cortright, D. N.; Szallasi, A., TRP channels and pain. *Current pharmaceutical design* **2009,** *15* (15), 1736-49.
20. Venkatachalam, K.; Montell, C., TRP channels. *Annual review of biochemistry* **2007,** *76*, 387-417.
21. Talavera, K.; Yasumatsu, K.; Yoshida, R.; Margolskee, R. F.; Voets, T.; Ninomiya, Y.; Nilius, B., The taste transduction channel TRPM5 is a locus for bitter-sweet taste interactions. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2008,** *22* (5), 1343-55.
22. Pedersen, S. F.; Nilius, B., Transient receptor potential channels in mechanosensing and cell volume regulation. *Methods in enzymology* **2007,** *428*, 183-207.
23. Nilius, B.; Owsianik, G.; Voets, T.; Peters, J. A., Transient receptor potential cation channels in disease. *Physiological reviews* **2007,** *87* (1), 165-217.

24. Nilius, B., TRP channels in disease. *Biochimica et biophysica acta* **2007,** *1772* (8), 805-12.
25. Hinz, B.; Cheremina, O.; Brune, K., Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2008,** *22* (2), 383-90.
26. Green, G. A., Understanding NSAIDs: From aspirin to COX-2. *Clinical Cornerstone* **2001,** *3* (5), 50-59.
27. Brinkrolf, P.; Hahnenkamp, K., Systemic lidocaine in surgical procedures: effects beyond sodium channel blockade. *Current opinion in anaesthesiology* **2014,** *27* (4), 420-5.
28. Quane, P. A.; Graham, G. G.; Ziegler, J. B., Pharmacology of benzydamine. *Inflammopharmacology* **1998,** *6* (2), 95-107.
29. Trescot, A. M.; Datta, S.; Lee, M.; Hansen, H., Opioid pharmacology. *Pain physician* **2008,** *11* (2 Suppl), S133-53.
30. Lee, Y.; Hong, S.; Cui, M.; Sharma, P. K.; Lee, J.; Choi, S., Transient receptor potential vanilloid type 1 antagonists: a patent review (2011 - 2014). *Expert opinion on therapeutic patents* **2015,** *25* (3), 291-318.
31. Caterina, M. J.; Leffler, A.; Malmberg, A. B.; Martin, W. J.; Trafton, J.; Petersen-Zeitz, K. R.; Koltzenburg, M.; Basbaum, A. I.; Julius, D., Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **2000,** *288* (5464), 306-13.
32. Gavva, N. R.; Tamir, R.; Qu, Y.; Klionsky, L.; Zhang, T. J.; Immke, D.; Wang, J.; Zhu, D.; Vanderah, T. W.; Porreca, F.; Doherty, E. M.; Norman, M. H.; Wild, K. D.; Bannon, A. W.; Louis, J. C.; Treanor, J. J., AMG 9810 [(E)-3-(4-t-butylphenyl)-N-(2,3-dihydrobenzo[b][1,4] dioxin-6-yl)acrylamide], a novel vanilloid receptor 1 (TRPV1) antagonist with antihyperalgesic properties. *The Journal of pharmacology and experimental therapeutics* **2005,** *313* (1), 474-84.
33. Reilly, R. M.; McDonald, H. A.; Puttfarcken, P. S.; Joshi, S. K.; Lewis, L.; Pai, M.; Franklin, P. H.; Segreti, J. A.; Neelands, T. R.; Han, P.; Chen, J.; Mantyh,

P. W.; Ghilardi, J. R.; Turner, T. M.; Voight, E. A.; Daanen, J. F.; Schmidt, R. G.; Gomtsyan, A.; Kort, M. E.; Faltynek, C. R.; Kym, P. R., Pharmacology of modality-specific transient receptor potential vanilloid-1 antagonists that do not alter body temperature. *The Journal of pharmacology and experimental therapeutics* **2012,** *342* (2), 416-28.

34. Peier, A. M.; Moqrich, A.; Hergarden, A. C.; Reeve, A. J.; Andersson, D. A.; Story, G. M.; Earley, T. J.; Dragoni, I.; McIntyre, P.; Bevan, S.; Patapoutian, A., A TRP channel that senses cold stimuli and menthol. *Cell* **2002,** *108* (5), 705-15.
35. Almaraz, L.; Manenschijn, J. A.; de la Pena, E.; Viana, F., TRPM8. *Handbook of experimental pharmacology* **2014,** *222*, 547-79.
36. Knowlton, W. M.; Daniels, R. L.; Palkar, R.; McCoy, D. D.; McKemy, D. D., Pharmacological blockade of TRPM8 ion channels alters cold and cold pain responses in mice. *PloS one* **2011,** *6* (9), e25894.
37. Szallasi, A.; Goso, C.; Manzini, S., Resiniferatoxin binding to vanilloid receptors in guinea pig and human airways. *American journal of respiratory and critical care medicine* **1995,** *152* (1), 59-63.
38. Aneiros, E.; Cao, L.; Papakosta, M.; Stevens, E. B.; Phillips, S.; Grimm, C., The biophysical and molecular basis of TRPV1 proton gating. *The EMBO journal* **2011,** *30* (6), 994-1002.
39. Tominaga, M.; Caterina, M. J.; Malmberg, A. B.; Rosen, T. A.; Gilbert, H.; Skinner, K.; Raumann, B. E.; Basbaum, A. I.; Julius, D., The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* **1998,** *21* (3), 531-43.
40. Ferrer-Montiel, A.; Garcia-Martinez, C.; Morenilla-Palao, C.; Garcia-Sanz, N.; Fernandez-Carvajal, A.; Fernandez-Ballester, G.; Planells-Cases, R., Molecular architecture of the vanilloid receptor. Insights for drug design. *European journal of biochemistry / FEBS* **2004,** *271* (10), 1820-6.
41. Messeguer, A.; Planells-Cases, R.; Ferrer-Montiel, A., Physiology and pharmacology of the vanilloid receptor. *Current neuropharmacology* **2006,** *4* (1), 1-15.

42. Clapham, D. E., TRP channels as cellular sensors. *Nature* **2003,** *426* (6966), 517-24.
43. Garcia-Sanz, N.; Valente, P.; Gomis, A.; Fernandez-Carvajal, A.; Fernandez-Ballester, G.; Viana, F.; Belmonte, C.; Ferrer-Montiel, A., A role of the transient receptor potential domain of vanilloid receptor I in channel gating. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2007,** *27* (43), 11641-50.
44. Garcia-Sanz, N.; Fernandez-Carvajal, A.; Morenilla-Palao, C.; Planells-Cases, R.; Fajardo-Sanchez, E.; Fernandez-Ballester, G.; Ferrer-Montiel, A., Identification of a tetramerization domain in the C terminus of the vanilloid receptor. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2004,** *24* (23), 5307-14.
45. Gregorio-Teruel, L.; Valente, P.; Liu, B.; Fernandez-Ballester, G.; Qin, F.; Ferrer-Montiel, A., The Integrity of the TRP Domain Is Pivotal for Correct TRPV1 Channel Gating. *Biophys J* **2015,** *109* (3), 529-41.
46. Valente, P.; Garcia-Sanz, N.; Gomis, A.; Fernandez-Carvajal, A.; Fernandez-Ballester, G.; Viana, F.; Belmonte, C.; Ferrer-Montiel, A., Identification of molecular determinants of channel gating in the transient receptor potential box of vanilloid receptor I. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2008,** *22* (9), 3298-309.
47. Gregorio-Teruel, L.; Valente, P.; Gonzalez-Ros, J. M.; Fernandez-Ballester, G.; Ferrer-Montiel, A., Mutation of I696 and W697 in the TRP box of vanilloid receptor subtype I modulates allosteric channel activation. *The Journal of general physiology* **2014,** *143* (3), 361-75.
48. Zhang, F.; Liu, S.; Yang, F.; Zheng, J.; Wang, K., Identification of a tetrameric assembly domain in the C terminus of heat-activated TRPV1 channels. *The Journal of biological chemistry* **2011,** *286* (17), 15308-16.
49. Flynn, R.; Chapman, K.; Iftinca, M.; Aboushousha, R.; Varela, D.; Altier, C., Targeting the transient receptor potential vanilloid type 1 (TRPV1) assembly

domain attenuates inflammation-induced hypersensitivity. *The Journal of biological chemistry* **2014,** *289* (24), 16675-87.

50. Lishko, P. V.; Procko, E.; Jin, X.; Phelps, C. B.; Gaudet, R., The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron* **2007,** *54* (6), 905-18.
51. Mosavi, L. K.; Cammett, T. J.; Desrosiers, D. C.; Peng, Z. Y., The ankyrin repeat as molecular architecture for protein recognition. *Protein science : a publication of the Protein Society* **2004,** *13* (6), 1435-48.
52. Liao, M.; Cao, E.; Julius, D.; Cheng, Y., Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature* **2013,** *504* (7478), 107-12.
53. Morenilla-Palao, C.; Planells-Cases, R.; Garcia-Sanz, N.; Ferrer-Montiel, A., Regulated exocytosis contributes to protein kinase C potentiation of vanilloid receptor activity. *The Journal of biological chemistry* **2004,** *279* (24), 25665-72.
54. Ciardo, M. G.; Andres-Borderia, A.; Cuesta, N.; Valente, P.; Camprubi-Robles, M.; Yang, J.; Planells-Cases, R.; Ferrer-Montiel, A., Whirlin increases TRPV1 channel expression and cellular stability. *Biochimica et biophysica acta* **2015,** *1863* (1), 115-127.
55. Voets, T.; Droogmans, G.; Wissenbach, U.; Janssens, A.; Flockerzi, V.; Nilius, B., The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature* **2004,** *430* (7001), 748-54.
56. Yang, F.; Xiao, X.; Cheng, W.; Yang, W.; Yu, P.; Song, Z.; Yarov-Yarovoy, V.; Zheng, J., Structural mechanism underlying capsaicin binding and activation of the TRPV1 ion channel. *Nature chemical biology* **2015,** *11* (7), 518-24.
57. Brauchi, S.; Orta, G.; Salazar, M.; Rosenmann, E.; Latorre, R., A hot-sensing cold receptor: C-terminal domain determines thermosensation in transient receptor potential channels. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2006,** *26* (18), 4835-40.
58. Welch, J. M.; Simon, S. A.; Reinhart, P. H., The activation mechanism of rat vanilloid receptor 1 by capsaicin involves the pore domain and differs from

the activation by either acid or heat. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** *97* (25), 13889-94.

59. Szolcsanyi, J.; Sandor, Z., Multisteric TRPV1 nocisensor: a target for analgesics. *Trends in pharmacological sciences* **2012,** *33* (12), 646-55.
60. Jordt, S. E.; Julius, D., Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell* **2002,** *108* (3), 421-30.
61. Gavva, N. R.; Klionsky, L.; Qu, Y.; Shi, L.; Tamir, R.; Edenson, S.; Zhang, T. J.; Viswanadhan, V. N.; Toth, A.; Pearce, L. V.; Vanderah, T. W.; Porreca, F.; Blumberg, P. M.; Lile, J.; Sun, Y.; Wild, K.; Louis, J. C.; Treanor, J. J., Molecular determinants of vanilloid sensitivity in TRPV1. *The Journal of biological chemistry* **2004,** *279* (19), 20283-95.
62. Cao, E.; Liao, M.; Cheng, Y.; Julius, D., TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature* **2013,** *504* (7478), 113-8.
63. Darre, L.; Domene, C., Binding of Capsaicin to the TRPV1 Ion Channel. *Molecular pharmaceutics* **2015,** *12* (12), 4454-65.
64. Jordt, S. E.; Tominaga, M.; Julius, D., Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** *97* (14), 8134-9.
65. Boukalova, S.; Marsakova, L.; Teisinger, J.; Vlachova, V., Conserved residues within the putative S4-S5 region serve distinct functions among thermosensitive vanilloid transient receptor potential (TRPV) channels. *The Journal of biological chemistry* **2010,** *285* (53), 41455-62.
66. Grandl, J.; Kim, S. E.; Uzzell, V.; Bursulaya, B.; Petrus, M.; Bandell, M.; Patapoutian, A., Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain. *Nature neuroscience* **2010,** *13* (6), 708-714.
67. Vriens, J.; Nilius, B.; Voets, T., Peripheral thermosensation in mammals. *Nature reviews. Neuroscience* **2014,** *15* (9), 573-89.
68. Clapham, D. E.; Miller, C., A thermodynamic framework for understanding temperature sensing by transient receptor potential (TRP) channels.

Proceedings of the National Academy of Sciences of the United States of America **2011,** *108* (49), 19492-7.

69. Cortright, D. N.; Szallasi, A., Biochemical pharmacology of the vanilloid receptor TRPV1. An update. *European journal of biochemistry / FEBS* **2004,** *271* (10), 1814-9.
70. Numazaki, M.; Tominaga, T.; Takeuchi, K.; Murayama, N.; Toyooka, H.; Tominaga, M., Structural determinant of TRPV1 desensitization interacts with calmodulin. *Proceedings of the National Academy of Sciences of the United States of America* **2003,** *100* (13), 8002-6.
71. Rosenbaum, T.; Gordon-Shaag, A.; Munari, M.; Gordon, S. E., Ca2+/calmodulin modulates TRPV1 activation by capsaicin. *The Journal of general physiology* **2004,** *123* (1), 53-62.
72. Klein, R. M.; Ufret-Vincenty, C. A.; Hua, L.; Gordon, S. E., Determinants of molecular specificity in phosphoinositide regulation. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P2) is the endogenous lipid regulating TRPV1. *The Journal of biological chemistry* **2008,** *283* (38), 26208-16.
73. Davis, J. B.; Gray, J.; Gunthorpe, M. J.; Hatcher, J. P.; Davey, P. T.; Overend, P.; Harries, M. H.; Latcham, J.; Clapham, C.; Atkinson, K.; Hughes, S. A.; Rance, K.; Grau, E.; Harper, A. J.; Pugh, P. L.; Rogers, D. C.; Bingham, S.; Randall, A.; Sheardown, S. A., Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **2000,** *405* (6783), 183-7.
74. Caterina, M. J.; Julius, D., The vanilloid receptor: a molecular gateway to the pain pathway. *Annual review of neuroscience* **2001,** *24*, 487-517.
75. Cesare, P.; McNaughton, P., Peripheral pain mechanisms. *Current opinion in neurobiology* **1997,** *7* (4), 493-9.
76. Price, T. J.; Flores, C. M., Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *The journal of pain : official journal of the American Pain Society* **2007,** *8* (3), 263-72.

77. Cesare, P.; Dekker, L. V.; Sardini, A.; Parker, P. J.; McNaughton, P. A., Specific involvement of PKC-epsilon in sensitization of the neuronal response to painful heat. *Neuron* **1999,** *23* (3), 617-24.
78. Zhang, X.; Huang, J.; McNaughton, P. A., NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *The EMBO journal* **2005,** *24* (24), 4211-23.
79. Zhang, N.; Inan, S.; Cowan, A.; Sun, R.; Wang, J. M.; Rogers, T. J.; Caterina, M.; Oppenheim, J. J., A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1. *Proceedings of the National Academy of Sciences of the United States of America* **2005,** *102* (12), 4536-41.
80. Mohapatra, D. P.; Nau, C., Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *The Journal of biological chemistry* **2003,** *278* (50), 50080-90.
81. Schaible, H. G.; von Banchet, G. S.; Boettger, M. K.; Brauer, R.; Gajda, M.; Richter, F.; Hensellek, S.; Brenn, D.; Natura, G., The role of proinflammatory cytokines in the generation and maintenance of joint pain. *Annals of the New York Academy of Sciences* **2010,** *1193*, 60-9.
82. Zhang, X.; Li, L.; McNaughton, P. A., Proinflammatory mediators modulate the heat-activated ion channel TRPV1 via the scaffolding protein AKAP79/150. *Neuron* **2008,** *59* (3), 450-61.
83. Cheng, J. K.; Ji, R. R., Intracellular signaling in primary sensory neurons and persistent pain. *Neurochemical research* **2008,** *33* (10), 1970-8.
84. Gold, M. S.; Gebhart, G. F., Nociceptor sensitization in pain pathogenesis. *Nature medicine* **2010,** *16* (11), 1248-57.
85. Than, J. Y.; Li, L.; Hasan, R.; Zhang, X., Excitation and modulation of TRPA1, TRPV1, and TRPM8 channel-expressing sensory neurons by the pruritogen chloroquine. *The Journal of biological chemistry* **2013,** *288* (18), 12818-27.
86. Kim, S. J.; Park, G. H.; Kim, D.; Lee, J.; Min, H.; Wall, E.; Lee, C. J.; Simon, M. I.; Lee, S. J.; Han, S. K., Analysis of cellular and behavioral responses to

imiquimod reveals a unique itch pathway in transient receptor potential vanilloid 1 (TRPV1)-expressing neurons. *Proceedings of the National Academy of Sciences of the United States of America* **2011,** *108* (8), 3371-6.

87. Grycova, L.; Lansky, Z.; Friedlova, E.; Vlachova, V.; Kubala, M.; Obsilova, V.; Obsil, T.; Teisinger, J., ATP binding site on the C-terminus of the vanilloid receptor. *Archives of biochemistry and biophysics* **2007,** *465* (2), 389-98.
88. Devesa, I.; Ferrandiz-Huertas, C.; Mathivanan, S.; Wolf, C.; Lujan, R.; Changeux, J. P.; Ferrer-Montiel, A., alphaCGRP is essential for algesic exocytotic mobilization of TRPV1 channels in peptidergic nociceptors. *Proceedings of the National Academy of Sciences of the United States of America* **2014,** *111* (51), 18345-50.
89. Lee, S. Y.; Lee, J. H.; Kang, K. K.; Hwang, S. Y.; Choi, K. D.; Oh, U., Sensitization of vanilloid receptor involves an increase in the phosphorylated form of the channel. *Archives of pharmacal research* **2005,** *28* (4), 405-12.
90. Numazaki, M.; Tominaga, T.; Toyooka, H.; Tominaga, M., Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *The Journal of biological chemistry* **2002,** *277* (16), 13375-8.
91. Mizushima, T.; Obata, K.; Yamanaka, H.; Dai, Y.; Fukuoka, T.; Tokunaga, A.; Mashimo, T.; Noguchi, K., Activation of p38 MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. *Pain* **2005,** *113* (1-2), 51-60.
92. Obata, K.; Yamanaka, H.; Kobayashi, K.; Dai, Y.; Mizushima, T.; Katsura, H.; Fukuoka, T.; Tokunaga, A.; Noguchi, K., Role of mitogen-activated protein kinase activation in injured and intact primary afferent neurons for mechanical and heat hypersensitivity after spinal nerve ligation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2004,** *24* (45), 10211-22.
93. Rohacs, T.; Thyagarajan, B.; Lukacs, V., Phospholipase C mediated modulation of TRPV1 channels. *Molecular neurobiology* **2008,** *37* (2-3), 153-63.

94. Mandadi, S.; Numazaki, M.; Tominaga, M.; Bhat, M. B.; Armati, P. J.; Roufogalis, B. D., Activation of protein kinase C reverses capsaicin-induced calcium-dependent desensitization of TRPV1 ion channels. *Cell calcium* **2004,** *35* (5), 471-8.

95. Bhave, G.; Hu, H. J.; Glauner, K. S.; Zhu, W.; Wang, H.; Brasier, D. J.; Oxford, G. S.; Gereau, R. W. t., Protein kinase C phosphorylation sensitizes but does not activate the capsaicin receptor transient receptor potential vanilloid 1 (TRPV1). *Proceedings of the National Academy of Sciences of the United States of America* **2003,** *100* (21), 12480-5.

96. Liu, J.; Du, J.; Yang, Y.; Wang, Y., Phosphorylation of TRPV1 by cyclin-dependent kinase 5 promotes TRPV1 surface localization, leading to inflammatory thermal hyperalgesia. *Experimental neurology* **2015,** *273*, 253-62.

97. Fernandez-Carvajal, A.; Fernandez-Ballester, G.; Devesa, I.; Gonzalez-Ros, J. M.; Ferrer-Montiel, A., New strategies to develop novel pain therapies: addressing thermoreceptors from different points of view. *Pharmaceuticals (Basel, Switzerland)* **2011,** *5* (1), 16-48.

98. Aiello, F.; Badolato, M.; Pessina, F.; Sticozzi, C.; Maestrini, V.; Aldinucci, C.; Luongo, L.; Guida, F.; Ligresti, A.; Artese, A.; Allara, M.; Costa, G.; Frosini, M.; Schiano Moriello, A.; De Petrocellis, L.; Valacchi, G.; Alcaro, S.; Maione, S.; Di Marzo, V.; Corelli, F.; Brizzi, A., Design and Synthesis of New Transient Receptor Potential Vanilloid Type-1 (TRPV1) Channel Modulators: Identification, Molecular Modeling Analysis, and Pharmacological Characterization of the N-(4-Hydroxy-3-methoxybenzyl)-4-(thiophen-2-yl)butanamide, a Small Molecule Endowed with Agonist TRPV1 Activity and Protective Effects against Oxidative Stress. *ACS chemical neuroscience* **2016**.

99. Xu, H.; Blair, N. T.; Clapham, D. E., Camphor activates and strongly desensitizes the transient receptor potential vanilloid subtype 1 channel in a vanilloid-independent mechanism. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2005,** *25* (39), 8924-37.

100. Hu, H. Z.; Gu, Q.; Wang, C.; Colton, C. K.; Tang, J.; Kinoshita-Kawada, M.; Lee, L. Y.; Wood, J. D.; Zhu, M. X., 2-aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. *The Journal of biological chemistry* **2004,** *279* (34), 35741-8.
101. Sugai, E.; Morimitsu, Y.; Iwasaki, Y.; Morita, A.; Watanabe, T.; Kubota, K., Pungent qualities of sanshool-related compounds evaluated by a sensory test and activation of rat TRPV1. *Bioscience, biotechnology, and biochemistry* **2005,** *69* (10), 1951-7.
102. Salazar, H.; Llorente, I.; Jara-Oseguera, A.; Garcia-Villegas, R.; Munari, M.; Gordon, S. E.; Islas, L. D.; Rosenbaum, T., A single N-terminal cysteine in TRPV1 determines activation by pungent compounds from onion and garlic. *Nature neuroscience* **2008,** *11* (3), 255-61.
103. McNamara, F. N.; Randall, A.; Gunthorpe, M. J., Effects of piperine, the pungent component of black pepper, at the human vanilloid receptor (TRPV1). *British journal of pharmacology* **2005,** *144* (6), 781-90.
104. Burness, C. B.; McCormack, P. L., Capsaicin 8 % Patch: A Review in Peripheral Neuropathic Pain. *Drugs* **2016,** *76* (1), 123-34.
105. Anand, P.; Bley, K., Topical capsaicin for pain management: therapeutic potential and mechanisms of action of the new high-concentration capsaicin 8% patch. *British journal of anaesthesia* **2011,** *107* (4), 490-502.
106. Polydefkis, M.; Hauer, P.; Sheth, S.; Sirdofsky, M.; Griffin, J. W.; McArthur, J. C., The time course of epidermal nerve fibre regeneration: studies in normal controls and in people with diabetes, with and without neuropathy. *Brain : a journal of neurology* **2004,** *127* (Pt 7), 1606-15.
107. Walpole, C. S.; Bevan, S.; Bovermann, G.; Boelsterli, J. J.; Breckenridge, R.; Davies, J. W.; Hughes, G. A.; James, I.; Oberer, L.; Winter, J.; et al., The discovery of capsazepine, the first competitive antagonist of the sensory neuron excitants capsaicin and resiniferatoxin. *J Med Chem* **1994,** *37* (13), 1942-54.

108. Docherty, R. J.; Yeats, J. C.; Piper, A. S., Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture. *British journal of pharmacology* **1997,** *121* (7), 1461-7.

109. Weil, A.; Moore, S. E.; Waite, N. J.; Randall, A.; Gunthorpe, M. J., Conservation of functional and pharmacological properties in the distantly related temperature sensors TRVP1 and TRPM8. *Molecular pharmacology* **2005,** *68* (2), 518-27.

110. Liu, L.; Simon, S. A., Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. *Neuroscience letters* **1997,** *228* (1), 29-32.

111. Gavva, N. R.; Treanor, J. J.; Garami, A.; Fang, L.; Surapaneni, S.; Akrami, A.; Alvarez, F.; Bak, A.; Darling, M.; Gore, A.; Jang, G. R.; Kesslak, J. P.; Ni, L.; Norman, M. H.; Palluconi, G.; Rose, M. J.; Salfi, M.; Tan, E.; Romanovsky, A. A.; Banfield, C.; Davar, G., Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans. *Pain* **2008,** *136* (1-2), 202-10.

112. Lehto, S. G.; Tamir, R.; Deng, H.; Klionsky, L.; Kuang, R.; Le, A.; Lee, D.; Louis, J. C.; Magal, E.; Manning, B. H.; Rubino, J.; Surapaneni, S.; Tamayo, N.; Wang, T.; Wang, J.; Wang, J.; Wang, W.; Youngblood, B.; Zhang, M.; Zhu, D.; Norman, M. H.; Gavva, N. R., Antihyperalgesic effects of (R,E)-N-(2-hydroxy-2,3-dihydro-1H-inden-4-yl)-3-(2-(piperidin-1-yl)-4-(trifluorom ethyl)phenyl)-acrylamide (AMG8562), a novel transient receptor potential vanilloid type 1 modulator that does not cause hyperthermia in rats. *The Journal of pharmacology and experimental therapeutics* **2008,** *326* (1), 218-29.

113. Valente, P.; Fernandez-Carvajal, A.; Camprubi-Robles, M.; Gomis, A.; Quirce, S.; Viana, F.; Fernandez-Ballester, G.; Gonzalez-Ros, J. M.; Belmonte, C.; Planells-Cases, R.; Ferrer-Montiel, A., Membrane-tethered peptides patterned after the TRP domain (TRPducins) selectively inhibit TRPV1 channel activity. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2011,** *25* (5), 1628-40.

114. Camprubi-Robles, M.; Planells-Cases, R.; Ferrer-Montiel, A., Differential contribution of SNARE-dependent exocytosis to inflammatory potentiation of TRPV1 in nociceptors. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **2009,** *23* (11), 3722-33.
115. Ponsati, B.; Carreno, C.; Curto-Reyes, V.; Valenzuela, B.; Duart, M. J.; Van den Nest, W.; Cauli, O.; Beltran, B.; Fernandez, J.; Borsini, F.; Caprioli, A.; Di Serio, S.; Veretchy, M.; Baamonde, A.; Menendez, L.; Barros, F.; de la Pena, P.; Borges, R.; Felipo, V.; Planells-Cases, R.; Ferrer-Montiel, A., An inhibitor of neuronal exocytosis (DD04107) displays long-lasting in vivo activity against chronic inflammatory and neuropathic pain. *The Journal of pharmacology and experimental therapeutics* **2012,** *341* (3), 634-45.
116. Johnson, J. W.; Kotermanski, S. E., Mechanism of action of memantine. *Current opinion in pharmacology* **2006,** *6* (1), 61-7.
117. Greig, S. L., Memantine ER/Donepezil: A Review in Alzheimer's Disease. *CNS drugs* **2015,** *29* (11), 963-70.
118. Jackson, M. B., Open channel block and beyond. *The Journal of physiology* **2010,** *588* (Pt 4), 553-4.
119. Neher, E.; Steinbach, J. H., Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *The Journal of physiology* **1978,** *277*, 153-76.
120. Garcia-Martinez, C.; Humet, M.; Planells-Cases, R.; Gomis, A.; Caprini, M.; Viana, F.; De La Pena, E.; Sanchez-Baeza, F.; Carbonell, T.; De Felipe, C.; Perez-Paya, E.; Belmonte, C.; Messeguer, A.; Ferrer-Montiel, A., Attenuation of thermal nociception and hyperalgesia by VR1 blockers. *Proceedings of the National Academy of Sciences of the United States of America* **2002,** *99* (4), 2374-9.
121. Mellor, I. R.; Ogilvie, J.; Pluteanu, F.; Clothier, R. H.; Parker, T. L.; Rosini, M.; Minarini, A.; Tumiatti, V.; Melchiorre, C., Methoctramine analogues inhibit responses to capsaicin and protons in rat dorsal root ganglion neurons. *European journal of pharmacology* **2004,** *505* (1-3), 37-50.

122. Garcia-Martinez, C.; Fernandez-Carvajal, A.; Valenzuela, B.; Gomis, A.; Van Den Nest, W.; Ferroni, S.; Carreno, C.; Belmonte, C.; Ferrer-Montiel, A., Design and characterization of a noncompetitive antagonist of the transient receptor potential vanilloid subunit 1 channel with in vivo analgesic and anti-inflammatory activity. *The journal of pain : official journal of the American Pain Society* **2006,** *7* (10), 735-46.

123. Valero, M.; Morenilla-Palao, C.; Belmonte, C.; Viana, F., Pharmacological and functional properties of TRPM8 channels in prostate tumor cells. *Pflugers Archiv : European journal of physiology* **2011,** *461* (1), 99-114.

124. Vidal-Mosquera, M.; Fernandez-Carvajal, A.; Moure, A.; Valente, P.; Planells-Cases, R.; Gonzalez-Ros, J. M.; Bujons, J.; Ferrer-Montiel, A.; Messeguer, A., Triazine-based vanilloid 1 receptor open channel blockers: design, synthesis, evaluation, and SAR analysis. *J Med Chem* **2011,** *54* (21), 7441-52.

125. Rivera-Acevedo, R. E.; Pless, S. A.; Ahern, C. A.; Schwarz, S. K., The quaternary lidocaine derivative, QX-314, exerts biphasic effects on transient receptor potential vanilloid subtype 1 channels in vitro. *Anesthesiology* **2011,** *114* (6), 1425-34.

126. McIntyre, P.; McLatchie, L. M.; Chambers, A.; Phillips, E.; Clarke, M.; Savidge, J.; Toms, C.; Peacock, M.; Shah, K.; Winter, J.; Weerasakera, N.; Webb, M.; Rang, H. P.; Bevan, S.; James, I. F., Pharmacological differences between the human and rat vanilloid receptor 1 (VR1). *British journal of pharmacology* **2001,** *132* (5), 1084-94.

127. Savidge, J.; Davis, C.; Shah, K.; Colley, S.; Phillips, E.; Ranasinghe, S.; Winter, J.; Kotsonis, P.; Rang, H.; McIntyre, P., Cloning and functional characterization of the guinea pig vanilloid receptor 1. *Neuropharmacology* **2002,** *43* (3), 450-6.

128. Seabrook, G. R.; Sutton, K. G.; Jarolimek, W.; Hollingworth, G. J.; Teague, S.; Webb, J.; Clark, N.; Boyce, S.; Kerby, J.; Ali, Z.; Chou, M.; Middleton, R.; Kaczorowski, G.; Jones, A. B., Functional properties of the high-affinity TRPV1 (VR1) vanilloid receptor antagonist (4-hydroxy-5-iodo-3-

methoxyphenylacetate ester) iodo-resiniferatoxin. *The Journal of pharmacology and experimental therapeutics* **2002,** *303* (3), 1052-60.

129. Miller, S.; Rao, S.; Wang, W.; Liu, H.; Wang, J.; Gavva, N. R., Antibodies to the extracellular pore loop of TRPM8 act as antagonists of channel activation. *PloS one* **2014,** *9* (9), e107151.
130. Woodhull, A. M., Ionic blockage of sodium channels in nerve. *The Journal of general physiology* **1973,** *61* (6), 687-708.
131. Ferrer-Montiel, A. V.; Merino, J. M.; Planells-Cases, R.; Sun, W.; Montal, M., Structural determinants of the blocker binding site in glutamate and NMDA receptor channels. *Neuropharmacology* **1998,** *37* (2), 139-147.
132. Planells-Cases, R.; Montoliu, C.; Humet, M.; Fernandez, A. M.; Garcia-Martinez, C.; Valera, E.; Merino, J. M.; Perez-Paya, E.; Messeguer, A.; Felipo, V.; Ferrer-Montiel, A., A novel N-methyl-D-aspartate receptor open channel blocker with in vivo neuroprotectant activity. *The Journal of pharmacology and experimental therapeutics* **2002,** *302* (1), 163-73.
133. Balsera, B.; Mulet, J.; Fernandez-Carvajal, A.; de la Torre-Martinez, R.; Ferrer-Montiel, A.; Hernandez-Jimenez, J. G.; Estevez-Herrera, J.; Borges, R.; Freitas, A. E.; Lopez, M. G.; Garcia-Lopez, M. T.; Gonzalez-Muniz, R.; Perez de Vega, M. J.; Valor, L. M.; Svobodova, L.; Sala, S.; Sala, F.; Criado, M., Chalcones as positive allosteric modulators of alpha7 nicotinic acetylcholine receptors: a new target for a privileged structure. *European journal of medicinal chemistry* **2014,** *86*, 724-39.
134. Caires, R.; Luis, E.; Taberner, F. J.; Fernandez-Ballester, G.; Ferrer-Montiel, A.; Balazs, E. A.; Gomis, A.; Belmonte, C.; de la Pena, E., Hyaluronan modulates TRPV1 channel opening, reducing peripheral nociceptor activity and pain. *Nature communications* **2015,** *6*, 8095.
135. Gomis, A.; Miralles, A.; Schmidt, R. F.; Belmonte, C., Nociceptive nerve activity in an experimental model of knee joint osteoarthritis of the guinea pig: effect of intra-articular hyaluronan application. *Pain* **2007,** *130* (1-2), 126-36.

136. Binshtok, A. M.; Bean, B. P.; Woolf, C. J., Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* **2007,** *449* (7162), 607-10.

137. Belghiti, M.; Estevez-Herrera, J.; Gimenez-Garzo, C.; Gonzalez-Usano, A.; Montoliu, C.; Ferrer-Montiel, A.; Felipo, V.; Planells-Cases, R., Potentiation of the transient receptor potential vanilloid 1 channel contributes to pruritogenesis in a rat model of liver disease. *The Journal of biological chemistry* **2013,** *288* (14), 9675-85.

138. Bevan, S.; Quallo, T.; Andersson, D. A., TRPV1. *Handbook of experimental pharmacology* **2014,** *222*, 207-45.

139. Ognyanov, V. I.; Balan, C.; Bannon, A. W.; Bo, Y.; Dominguez, C.; Fotsch, C.; Gore, V. K.; Klionsky, L.; Ma, V. V.; Qian, Y. X.; Tamir, R.; Wang, X.; Xi, N.; Xu, S.; Zhu, D.; Gavva, N. R.; Treanor, J. J.; Norman, M. H., Design of potent, orally available antagonists of the transient receptor potential vanilloid 1. Structure-activity relationships of 2-piperazin-1-yl-1H-benzimidazoles. *J Med Chem* **2006,** *49* (12), 3719-42.

140. Eid, S. R., Therapeutic targeting of TRP channels--the TR(i)P to pain relief. *Current topics in medicinal chemistry* **2011,** *11* (17), 2118-30.

141. Chen, H. S.; Pellegrini, J. W.; Aggarwal, S. K.; Lei, S. Z.; Warach, S.; Jensen, F. E.; Lipton, S. A., Open-channel block of N-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **1992,** *12* (11), 4427-36.

142. Brederson, J. D.; Kym, P. R.; Szallasi, A., Targeting TRP channels for pain relief. *European journal of pharmacology* **2013,** *716* (1-3), 61-76.

143. Vazquez-Romero, A.; Criado, M.; Messeguer, A.; Vidal-Mosquera, M.; Mulet, J.; Sala, F.; Sala, S., Effect of triazine derivatives on neuronal nicotinic receptors. *ACS chemical neuroscience* **2014,** *5* (8), 683-9.

144. Geisthovel, E.; Ludwig, O.; Simon, E., Capsaicin fails to produce disturbances of autonomic heat and cold defence in an avian species (Anas platyrhynchos). *Pflugers Archiv : European journal of physiology* **1986,** *406* (4), 343-50.

145. Meseguer, V.; Karashima, Y.; Talavera, K.; D'Hoedt, D.; Donovan-Rodriguez, T.; Viana, F.; Nilius, B.; Voets, T., Transient receptor potential channels in sensory neurons are targets of the antimycotic agent clotrimazole. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2008,** *28* (3), 576-86.

146. Yong, C. S.; Li, D. X.; Prabagar, B.; Park, B. C.; Yi, S. J.; Yoo, B. K.; Lyoo, W. S.; Woo, J. S.; Rhee, J. D.; Kim, J. A.; Choi, H. G., The effect of beta-cyclodextrin complexation on the bioavailability and hepatotoxicity of clotrimazole. *Die Pharmazie* **2007,** *62* (10), 756-9.

147. Santos, A. R.; Calixto, J. B., Ruthenium red and capsazepine antinociceptive effect in formalin and capsaicin models of pain in mice. *Neuroscience letters* **1997,** *235* (1-2), 73-6.

148. Callsen, M. G.; Moller, A. T.; Sorensen, K.; Jensen, T. S.; Finnerup, N. B., Cold hyposensitivity after topical application of capsaicin in humans. *Experimental brain research* **2008,** *191* (4), 447-52.

149. O'Neill, J.; Brock, C.; Olesen, A. E.; Andresen, T.; Nilsson, M.; Dickenson, A. H., Unravelling the mystery of capsaicin: a tool to understand and treat pain. *Pharmacological reviews* **2012,** *64* (4), 939-71.

150. Gavva, N. R.; Bannon, A. W.; Hovland, D. N., Jr.; Lehto, S. G.; Klionsky, L.; Surapaneni, S.; Immke, D. C.; Henley, C.; Arik, L.; Bak, A.; Davis, J.; Ernst, N.; Hever, G.; Kuang, R.; Shi, L.; Tamir, R.; Wang, J.; Wang, W.; Zajic, G.; Zhu, D.; Norman, M. H.; Louis, J. C.; Magal, E.; Treanor, J. J., Repeated administration of vanilloid receptor TRPV1 antagonists attenuates hyperthermia elicited by TRPV1 blockade. *The Journal of pharmacology and experimental therapeutics* **2007,** *323* (1), 128-37.

151. Gunthorpe, M. J.; Rami, H. K.; Jerman, J. C.; Smart, D.; Gill, C. H.; Soffin, E. M.; Luis Hannan, S.; Lappin, S. C.; Egerton, J.; Smith, G. D.; Worby, A.; Howett, L.; Owen, D.; Nasir, S.; Davies, C. H.; Thompson, M.; Wyman, P. A.; Randall, A. D.; Davis, J. B., Identification and characterisation of SB-366791, a potent and selective vanilloid receptor (VR1/TRPV1) antagonist. *Neuropharmacology* **2004,** *46* (1), 133-49.

152. Pongcharoen, P.; Fleischer, A. B., Jr., An evidence-based review of systemic treatments for itch. *European journal of pain (London, England)* **2016,** *20* (1), 24-31.
153. Steinhoff, M.; Neisius, U.; Ikoma, A.; Fartasch, M.; Heyer, G.; Skov, P. S.; Luger, T. A.; Schmelz, M., Proteinase-activated receptor-2 mediates itch: a novel pathway for pruritus in human skin. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2003,** *23* (15), 6176-80.
154. Imamachi, N.; Park, G. H.; Lee, H.; Anderson, D. J.; Simon, M. I.; Basbaum, A. I.; Han, S. K., TRPV1-expressing primary afferents generate behavioral responses to pruritogens via multiple mechanisms. *Proceedings of the National Academy of Sciences of the United States of America* **2009,** *106* (27), 11330-5.
155. Mollanazar, N. K.; Smith, P. K.; Yosipovitch, G., Mediators of Chronic Pruritus in Atopic Dermatitis: Getting the Itch Out? *Clinical reviews in allergy & immunology* **2015**.
156. Amadesi, S.; Cottrell, G. S.; Divino, L.; Chapman, K.; Grady, E. F.; Bautista, F.; Karanjia, R.; Barajas-Lopez, C.; Vanner, S.; Vergnolle, N.; Bunnett, N. W., Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. *The Journal of physiology* **2006,** *575* (Pt 2), 555-71.
157. Amadesi, S.; Nie, J.; Vergnolle, N.; Cottrell, G. S.; Grady, E. F.; Trevisani, M.; Manni, C.; Geppetti, P.; McRoberts, J. A.; Ennes, H.; Davis, J. B.; Mayer, E. A.; Bunnett, N. W., Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2004,** *24* (18), 4300-12.
158. Quiding, H.; Jonzon, B.; Svensson, O.; Webster, L.; Reimfelt, A.; Karin, A.; Karlsten, R.; Segerdahl, M., TRPV1 antagonistic analgesic effect: a randomized study of AZD1386 in pain after third molar extraction. *Pain* **2013,** *154* (6), 808-12.

159. Krarup, A. L.; Ny, L.; Gunnarsson, J.; Hvid-Jensen, F.; Zetterstrand, S.; Simren, M.; Funch-Jensen, P.; Hansen, M. B.; Drewes, A. M., Randomized clinical trial: inhibition of the TRPV1 system in patients with nonerosive gastroesophageal reflux disease and a partial response to PPI treatment is not associated with analgesia to esophageal experimental pain. *Scandinavian journal of gastroenterology* **2013,** *48* (3), 274-84.

160. Kitagawa, Y.; Miyai, A.; Usui, K.; Hamada, Y.; Deai, K.; Wada, M.; Koga, Y.; Sakata, M.; Hayashi, M.; Tominaga, M.; Matsushita, M., Pharmacological characterization of (3S)-3-(hydroxymethyl)-4-(5-methylpyridin-2-yl)-N-[6-(2,2,2-trifluoroethoxy)pyrid in-3-yl]-3,4-dihydro-2H-benzo[b][1,4]oxazine-8-carboxamide (JTS-653), a novel transient receptor potential vanilloid 1 antagonist. *The Journal of pharmacology and experimental therapeutics* **2012,** *342* (2), 520-8.

161. Kitagawa, Y.; Tamai, I.; Hamada, Y.; Usui, K.; Wada, M.; Sakata, M.; Matsushita, M., Orally administered selective TRPV1 antagonist, JTS-653, attenuates chronic pain refractory to non-steroidal anti-inflammatory drugs in rats and mice including post-herpetic pain. *Journal of pharmacological sciences* **2013,** *122* (2), 128-37.

162. Wang, H.; Yosipovitch, G., New insights into the pathophysiology and treatment of chronic itch in patients with end-stage renal disease, chronic liver disease, and lymphoma. *International journal of dermatology* **2010,** *49* (1), 1-11.

163. Korte, S. M., Corticosteroids in relation to fear, anxiety and psychopathology. *Neuroscience and biobehavioral reviews* **2001,** *25* (2), 117-42.

164. Fukushima, C.; Matsuse, H.; Tomari, S.; Obase, Y.; Miyazaki, Y.; Shimoda, T.; Kohno, S., Oral candidiasis associated with inhaled corticosteroid use: comparison of fluticasone and beclomethasone. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **2003,** *90* (6), 646-51.

165. Donihi, A. C.; Raval, D.; Saul, M.; Korytkowski, M. T.; DeVita, M. A., Prevalence and predictors of corticosteroid-related hyperglycemia in

hospitalized patients. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists* **2006,** *12* (4), 358-62.

166. Reid, G.; Babes, A.; Pluteanu, F., A cold- and menthol-activated current in rat dorsal root ganglion neurones: properties and role in cold transduction. *The Journal of physiology* **2002,** *545* (Pt 2), 595-614.
167. Reid, G.; Flonta, M. L., Physiology. Cold current in thermoreceptive neurons. *Nature* **2001,** *413* (6855), 480.
168. McKemy, D. D.; Neuhausser, W. M.; Julius, D., Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature* **2002,** *416* (6876), 52-8.
169. Madrid, R.; Donovan-Rodriguez, T.; Meseguer, V.; Acosta, M. C.; Belmonte, C.; Viana, F., Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2006,** *26* (48), 12512-25.
170. Viana, F.; de la Pena, E.; Belmonte, C., Specificity of cold thermotransduction is determined by differential ionic channel expression. *Nature neuroscience* **2002,** *5* (3), 254-60.
171. Chuang, H. H.; Neuhausser, W. M.; Julius, D., The super-cooling agent icilin reveals a mechanism of coincidence detection by a temperature-sensitive TRP channel. *Neuron* **2004,** *43* (6), 859-69.
172. Andersson, D. A.; Chase, H. W.; Bevan, S., TRPM8 activation by menthol, icilin, and cold is differentially modulated by intracellular pH. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2004,** *24* (23), 5364-9.
173. Tsavaler, L.; Shapero, M. H.; Morkowski, S.; Laus, R., Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer research* **2001,** *61* (9), 3760-9.

174. Bautista, D. M.; Siemens, J.; Glazer, J. M.; Tsuruda, P. R.; Basbaum, A. I.; Stucky, C. L.; Jordt, S. E.; Julius, D., The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature* **2007,** *448* (7150), 204-8.
175. Dhaka, A.; Earley, T. J.; Watson, J.; Patapoutian, A., Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2008,** *28* (3), 566-75.
176. Takashima, Y.; Ma, L.; McKemy, D. D., The development of peripheral cold neural circuits based on TRPM8 expression. *Neuroscience* **2010,** *169* (2), 828-42.
177. Yudin, Y.; Rohacs, T., Regulation of TRPM8 channel activity. *Molecular and cellular endocrinology* **2012,** *353* (1-2), 68-74.
178. Knowlton, W. M.; McKemy, D. D., TRPM8: from cold to cancer, peppermint to pain. *Current pharmaceutical biotechnology* **2011,** *12* (1), 68-77.
179. Stewart, A. P.; Egressy, K.; Lim, A.; Edwardson, J. M., AFM imaging reveals the tetrameric structure of the TRPM8 channel. *Biochemical and biophysical research communications* **2010,** *394* (2), 383-6.
180. Pedretti, A.; Marconi, C.; Bettinelli, I.; Vistoli, G., Comparative modeling of the quaternary structure for the human TRPM8 channel and analysis of its binding features. *Biochimica et biophysica acta* **2009,** *1788* (5), 973-82.
181. Latorre, R.; Brauchi, S.; Orta, G.; Zaelzer, C.; Vargas, G., ThermoTRP channels as modular proteins with allosteric gating. *Cell calcium* **2007,** *42* (4-5), 427-38.
182. Taberner, F. J.; Lopez-Cordoba, A.; Fernandez-Ballester, G.; Korchev, Y.; Ferrer-Montiel, A., The region adjacent to the C-end of the inner gate in transient receptor potential melastatin 8 (TRPM8) channels plays a central role in allosteric channel activation. *The Journal of biological chemistry* **2014,** *289* (41), 28579-94.
183. Pertusa, M.; Gonzalez, A.; Hardy, P.; Madrid, R.; Viana, F., Bidirectional modulation of thermal and chemical sensitivity of TRPM8 channels by the

initial region of the N-terminal domain. *The Journal of biological chemistry* **2014,** *289* (32), 21828-43.

184. Rohacs, T.; Lopes, C. M.; Michailidis, I.; Logothetis, D. E., PI(4,5)P2 regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nature neuroscience* **2005,** *8* (5), 626-34.

185. Liu, B.; Qin, F., Functional control of cold- and menthol-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2005,** *25* (7), 1674-81.

186. Phelps, C. B.; Gaudet, R., The role of the N terminus and transmembrane domain of TRPM8 in channel localization and tetramerization. *The Journal of biological chemistry* **2007,** *282* (50), 36474-80.

187. de la Pena, E.; Malkia, A.; Cabedo, H.; Belmonte, C.; Viana, F., The contribution of TRPM8 channels to cold sensing in mammalian neurones. *The Journal of physiology* **2005,** *567* (Pt 2), 415-26.

188. Malkia, A.; Madrid, R.; Meseguer, V.; de la Pena, E.; Valero, M.; Belmonte, C.; Viana, F., Bidirectional shifts of TRPM8 channel gating by temperature and chemical agents modulate the cold sensitivity of mammalian thermoreceptors. *The Journal of physiology* **2007,** *581* (Pt 1), 155-74.

189. Daniels, R. L.; Takashima, Y.; McKemy, D. D., Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. *The Journal of biological chemistry* **2009,** *284* (3), 1570-82.

190. Tang, M.; Wu, G. Y.; Dong, X. Z.; Tang, Z. X., Phosphoinositide interacting regulator of TRP (Pirt) enhances TRPM8 channel activity in vitro via increasing channel conductance. *Acta pharmacologica Sinica* **2016,** *37* (1), 98-104.

191. Rohacs, T.; Nilius, B., Regulation of transient receptor potential (TRP) channels by phosphoinositides. *Pflugers Archiv : European journal of physiology* **2007,** *455* (1), 157-68.

192. Brauchi, S.; Orta, G.; Mascayano, C.; Salazar, M.; Raddatz, N.; Urbina, H.; Rosenmann, E.; Gonzalez-Nilo, F.; Latorre, R., Dissection of the components for PIP2 activation and thermosensation in TRP channels. *Proceedings of the National Academy of Sciences of the United States of America* **2007,** *104* (24), 10246-51.

193. Yudin, Y.; Lukacs, V.; Cao, C.; Rohacs, T., Decrease in phosphatidylinositol 4,5-bisphosphate levels mediates desensitization of the cold sensor TRPM8 channels. *The Journal of physiology* **2011,** *589* (Pt 24), 6007-27.

194. Mandadi, S.; Armati, P. J.; Roufogalis, B. D., Protein kinase C modulation of thermo-sensitive transient receptor potential channels: Implications for pain signaling. *Journal of natural science, biology, and medicine* **2011,** *2* (1), 13-25.

195. Abe, J.; Hosokawa, H.; Sawada, Y.; Matsumura, K.; Kobayashi, S., Ca2+-dependent PKC activation mediates menthol-induced desensitization of transient receptor potential M8. *Neuroscience letters* **2006,** *397* (1-2), 140-4.

196. Premkumar, L. S.; Raisinghani, M.; Pingle, S. C.; Long, C.; Pimentel, F., Downregulation of transient receptor potential melastatin 8 by protein kinase C-mediated dephosphorylation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2005,** *25* (49), 11322-9.

197. De Petrocellis, L.; Starowicz, K.; Moriello, A. S.; Vivese, M.; Orlando, P.; Di Marzo, V., Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): effect of cAMP, cannabinoid CB(1) receptors and endovanilloids. *Experimental cell research* **2007,** *313* (9), 1911-20.

198. Zhang, X.; Mak, S.; Li, L.; Parra, A.; Denlinger, B.; Belmonte, C.; McNaughton, P. A., Direct inhibition of the cold-activated TRPM8 ion channel by Galphaq. *Nature cell biology* **2012,** *14* (8), 851-8.

199. Andersson, D. A.; Nash, M.; Bevan, S., Modulation of the cold-activated channel TRPM8 by lysophospholipids and polyunsaturated fatty acids. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2007,** *27* (12), 3347-55.

200. Gentry, C.; Stoakley, N.; Andersson, D. A.; Bevan, S., The roles of iPLA2, TRPM8 and TRPA1 in chemically induced cold hypersensitivity. *Molecular pain* **2010,** *6*, 4.

201. Vanden Abeele, F.; Zholos, A.; Bidaux, G.; Shuba, Y.; Thebault, S.; Beck, B.; Flourakis, M.; Panchin, Y.; Skryma, R.; Prevarskaya, N., Ca2+-independent phospholipase A2-dependent gating of TRPM8 by lysophospholipids. *The Journal of biological chemistry* **2006,** *281* (52), 40174-82.

202. Pertusa, M.; Madrid, R.; Morenilla-Palao, C.; Belmonte, C.; Viana, F., N-glycosylation of TRPM8 ion channels modulates temperature sensitivity of cold thermoreceptor neurons. *The Journal of biological chemistry* **2012,** *287* (22), 18218-29.

203. Morenilla-Palao, C.; Pertusa, M.; Meseguer, V.; Cabedo, H.; Viana, F., Lipid raft segregation modulates TRPM8 channel activity. *The Journal of biological chemistry* **2009,** *284* (14), 9215-24.

204. Wang, Y.; Wang, X.; Yang, Z.; Zhu, G.; Chen, D.; Meng, Z., Menthol inhibits the proliferation and motility of prostate cancer DU145 cells. *Pathology oncology research : POR* **2012,** *18* (4), 903-10.

205. Valero, M. L.; Mello de Queiroz, F.; Stuhmer, W.; Viana, F.; Pardo, L. A., TRPM8 ion channels differentially modulate proliferation and cell cycle distribution of normal and cancer prostate cells. *PloS one* **2012,** *7* (12), e51825.

206. Okamoto, Y.; Ohkubo, T.; Ikebe, T.; Yamazaki, J., Blockade of TRPM8 activity reduces the invasion potential of oral squamous carcinoma cell lines. *International journal of oncology* **2012,** *40* (5), 1431-40.

207. Parra, A.; Madrid, R.; Echevarria, D.; del Olmo, S.; Morenilla-Palao, C.; Acosta, M. C.; Gallar, J.; Dhaka, A.; Viana, F.; Belmonte, C., Ocular surface wetness is regulated by TRPM8-dependent cold thermoreceptors of the cornea. *Nature medicine* **2010,** *16* (12), 1396-9.

208. Quallo, T.; Vastani, N.; Horridge, E.; Gentry, C.; Parra, A.; Moss, S.; Viana, F.; Belmonte, C.; Andersson, D. A., TRPM8 is a neuronal osmosensor that regulates eye blinking in mice. **2015,** *6*, 7150.

209. Kurose, M.; Meng, I. D., Dry eye modifies the thermal and menthol responses in rat corneal primary afferent cool cells. *Journal of neurophysiology* **2013,** *110* (2), 495-504.
210. Robbins, A.; Kurose, M.; Winterson, B. J.; Meng, I. D., Menthol activation of corneal cool cells induces TRPM8-mediated lacrimation but not nociceptive responses in rodents. *Investigative ophthalmology & visual science* **2012,** *53* (11), 7034-42.
211. Belmonte, C.; Acosta, M. C.; Merayo-Lloves, J.; Gallar, J., What Causes Eye Pain? *Current ophthalmology reports* **2015,** *3* (2), 111-121.
212. Belmonte, C.; Gallar, J.; Ferrer Montiel, A. V.; Fernandez Carvajal, A.; Viana, F. Pharmaceutical composition for the treatment of dry eye. 2012.
213. Lippoldt, E. K.; Elmes, R. R.; McCoy, D. D.; Knowlton, W. M.; McKemy, D. D., Artemin, a glial cell line-derived neurotrophic factor family member, induces TRPM8-dependent cold pain. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2013,** *33* (30), 12543-52.
214. Elitt, C. M.; McIlwrath, S. L.; Lawson, J. J.; Malin, S. A.; Molliver, D. C.; Cornuet, P. K.; Koerber, H. R.; Davis, B. M.; Albers, K. M., Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2006,** *26* (33), 8578-87.
215. Su, L.; Wang, C.; Yu, Y. H.; Ren, Y. Y.; Xie, K. L.; Wang, G. L., Role of TRPM8 in dorsal root ganglion in nerve injury-induced chronic pain. *BMC neuroscience* **2011,** *12*, 120.
216. Gauchan, P.; Andoh, T.; Kato, A.; Kuraishi, Y., Involvement of increased expression of transient receptor potential melastatin 8 in oxaliplatin-induced cold allodynia in mice. *Neuroscience letters* **2009,** *458* (2), 93-5.
217. Sakurai, M.; Egashira, N.; Kawashiri, T.; Yano, T.; Ikesue, H.; Oishi, R., Oxaliplatin-induced neuropathy in the rat: involvement of oxalate in cold hyperalgesia but not mechanical allodynia. *Pain* **2009,** *147* (1-3), 165-74.

218. Lehky, T. J.; Leonard, G. D.; Wilson, R. H.; Grem, J. L.; Floeter, M. K., Oxaliplatin-induced neurotoxicity: acute hyperexcitability and chronic neuropathy. *Muscle & nerve* **2004,** *29* (3), 387-92.

219. Colburn, R. W.; Lubin, M. L.; Stone, D. J., Jr.; Wang, Y.; Lawrence, D.; D'Andrea, M. R.; Brandt, M. R.; Liu, Y.; Flores, C. M.; Qin, N., Attenuated cold sensitivity in TRPM8 null mice. *Neuron* **2007,** *54* (3), 379-86.

220. Knowlton, W. M.; Palkar, R.; Lippoldt, E. K.; McCoy, D. D.; Baluch, F.; Chen, J.; McKemy, D. D., A sensory-labeled line for cold: TRPM8-expressing sensory neurons define the cellular basis for cold, cold pain, and cooling-mediated analgesia. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2013,** *33* (7), 2837-48.

221. Salat, K.; Filipek, B., Antinociceptive activity of transient receptor potential channel TRPV1, TRPA1, and TRPM8 antagonists in neurogenic and neuropathic pain models in mice. *Journal of Zhejiang University. Science. B* **2015,** *16* (3), 167-78.

222. Winchester, W. J.; Gore, K.; Glatt, S.; Petit, W.; Gardiner, J. C.; Conlon, K.; Postlethwaite, M.; Saintot, P. P.; Roberts, S.; Gosset, J. R.; Matsuura, T.; Andrews, M. D.; Glossop, P. A.; Palmer, M. J.; Clear, N.; Collins, S.; Beaumont, K.; Reynolds, D. S., Inhibition of TRPM8 channels reduces pain in the cold pressor test in humans. *The Journal of pharmacology and experimental therapeutics* **2014,** *351* (2), 259-69.

223. Patel, R.; Goncalves, L.; Newman, R.; Jiang, F. L.; Goldby, A.; Reeve, J.; Hendrick, A.; Teall, M.; Hannah, D.; Almond, S.; Brice, N.; Dickenson, A. H., Novel TRPM8 antagonist attenuates cold hypersensitivity after peripheral nerve injury in rats. *The Journal of pharmacology and experimental therapeutics* **2014,** *349* (1), 47-55.

224. Borrelli, F.; Pagano, E.; Romano, B.; Panzera, S.; Maiello, F.; Coppola, D.; De Petrocellis, L.; Buono, L.; Orlando, P.; Izzo, A. A., Colon carcinogenesis is inhibited by the TRPM8 antagonist cannabigerol, a Cannabis-derived non-psychotropic cannabinoid. *Carcinogenesis* **2014,** *35* (12), 2787-97.

225. Liu, T.; Fang, Z.; Wang, G.; Shi, M.; Wang, X.; Jiang, K.; Yang, Z.; Cao, R.; Tao, H.; Wang, X.; Zhou, J., Anti-tumor activity of the TRPM8 inhibitor BCTC in prostate cancer DU145 cells. *Oncology letters* **2016,** *11* (1), 182-188.
226. Andrews, M. D.; Af Forselles, K.; Beaumont, K.; Galan, S. R.; Glossop, P. A.; Grenie, M.; Jessiman, A.; Kenyon, A. S.; Lunn, G.; Maw, G.; Owen, R. M.; Pryde, D. C.; Roberts, D.; Tran, T. D., Discovery of a Selective TRPM8 Antagonist with Clinical Efficacy in Cold-Related Pain. *ACS medicinal chemistry letters* **2015,** *6* (4), 419-24.
227. Calvo, R. R.; Meegalla, S. K.; Parks, D. J.; Parsons, W. H.; Ballentine, S. K.; Lubin, M. L.; Schneider, C.; Colburn, R. W.; Flores, C. M.; Player, M. R., Discovery of vinylcycloalkyl-substituted benzimidazole TRPM8 antagonists effective in the treatment of cold allodynia. *Bioorganic & medicinal chemistry letters* **2012,** *22* (5), 1903-7.
228. Lashinger, E. S.; Steiginga, M. S.; Hieble, J. P.; Leon, L. A.; Gardner, S. D.; Nagilla, R.; Davenport, E. A.; Hoffman, B. E.; Laping, N. J.; Su, X., AMTB, a TRPM8 channel blocker: evidence in rats for activity in overactive bladder and painful bladder syndrome. *American journal of physiology. Renal physiology* **2008,** *295* (3), F803-10.
229. Bodding, M.; Wissenbach, U.; Flockerzi, V., Characterisation of TRPM8 as a pharmacophore receptor. *Cell calcium* **2007,** *42* (6), 618-28.
230. Xing, H.; Chen, M.; Ling, J.; Tan, W.; Gu, J. G., TRPM8 mechanism of cold allodynia after chronic nerve injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **2007,** *27* (50), 13680-90.
231. Merritt, J. E.; Armstrong, W. P.; Benham, C. D.; Hallam, T. J.; Jacob, R.; Jaxa-Chamiec, A.; Leigh, B. K.; McCarthy, S. A.; Moores, K. E.; Rink, T. J., SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *The Biochemical journal* **1990,** *271* (2), 515-22.
232. Singh, A.; Hildebrand, M. E.; Garcia, E.; Snutch, T. P., The transient receptor potential channel antagonist SKF96365 is a potent blocker of low-voltage-activated T-type calcium channels. *British journal of pharmacology* **2010,** *160* (6), 1464-75.

233. Malkia, A.; Pertusa, M.; Fernandez-Ballester, G.; Ferrer-Montiel, A.; Viana, F., Differential role of the menthol-binding residue Y745 in the antagonism of thermally gated TRPM8 channels. *Molecular pain* **2009,** *5*, 62.

234. Bertamino, A.; Ostacolo, C.; Ambrosino, P.; Musella, S.; Di Sarno, V.; Ciaglia, T.; Soldovieri, M. V.; Iraci, N.; Fernandez Carvajal, A.; de la Torre-Martinez, R.; Ferrer-Montiel, A.; Gonzalez Muniz, R.; Novellino, E.; Taglialatela, M.; Campiglia, P.; Gomez-Monterrey, I., Tryptamine-Based Derivatives as Transient Receptor Potential Melastatin Type 8 (TRPM8) Channel Modulators. *J Med Chem* **2016**.

235. Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J., Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *Journal of Computational Chemistry* **1998,** *19* (14), 1639-1662.

236. Krieger, E.; Koraimann, G.; Vriend, G., Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. *Proteins* **2002,** *47* (3), 393-402.

237. Krieger, E.; Darden, T.; Nabuurs, S. B.; Finkelstein, A.; Vriend, G., Making optimal use of empirical energy functions: force-field parameterization in crystal space. *Proteins* **2004,** *57* (4), 678-83.

238. Salentin, S.; Schreiber, S.; Haupt, V. J.; Adasme, M. F.; Schroeder, M., PLIP: fully automated protein-ligand interaction profiler. *Nucleic Acids Research* **2015,** *43* (W1), W443-7.

239. Wilson, S. R.; Bautista, D. M., Frontiers in Neuroscience

Role of Transient Receptor Potential Channels in Acute and Chronic Itch. In *Itch: Mechanisms and Treatment*, Carstens, E.; Akiyama, T., Eds. CRC Press/Taylor & Francis

(c) 2014 by Taylor & Francis Group, LLC.: Boca Raton (FL), 2014.

240. Bush, K., beta-Lactamases of increasing clinical importance. *Current pharmaceutical design* **1999,** *5* (11), 839-45.

241. Agnieszka, K. B., *Thermodynamics of Ligand-Protein Interactions: Implications for Molecular Design*. 2011.

242. Bandell, M.; Dubin, A. E.; Petrus, M. J.; Orth, A.; Mathur, J.; Hwang, S. W.; Patapoutian, A., High-throughput random mutagenesis screen reveals TRPM8 residues specifically required for activation by menthol. *Nature neuroscience* **2006,** *9* (4), 493-500.

243. Teng, J.; Loukin, S. H.; Anishkin, A.; Kung, C., L596-W733 bond between the start of the S4-S5 linker and the TRP box stabilizes the closed state of TRPV4 channel. *Proceedings of the National Academy of Sciences of the United States of America* **2015,** *112* (11), 3386-91.

244. Raddatz, N.; Castillo, J. P.; Gonzalez, C.; Alvarez, O.; Latorre, R., Temperature and voltage coupling to channel opening in transient receptor potential melastatin 8 (TRPM8). *The Journal of biological chemistry* **2014,** *289* (51), 35438-54.

245. Appendino, G.; Daddario, N.; Minassi, A.; Moriello, A. S.; De Petrocellis, L.; Di Marzo, V., The taming of capsaicin. Reversal of the vanilloid activity of N-acylvanillamines by aromatic iodination. *J Med Chem* **2005,** *48* (14), 4663-9.

246. Appendino, G.; Harrison, S.; De Petrocellis, L.; Daddario, N.; Bianchi, F.; Schiano Moriello, A.; Trevisani, M.; Benvenuti, F.; Geppetti, P.; Di Marzo, V., Halogenation of a capsaicin analogue leads to novel vanilloid TRPV1 receptor antagonists. *British journal of pharmacology* **2003,** *139* (8), 1417-24.

247. Leach, K.; Gregory, K. J.; Kufareva, I.; Khajehali, E.; Cook, A. E.; Abagyan, R.; Conigrave, A. D.; Sexton, P. M.; Christopoulos, A., Towards a structural understanding of allosteric drugs at the human calcium-sensing receptor. *Cell research* **2016**.

248. Bianchi, B. R.; Lee, C. H.; Jarvis, M. F.; El Kouhen, R.; Moreland, R. B.; Faltynek, C. R.; Puttfarcken, P. S., Modulation of human TRPV1 receptor activity by extracellular protons and host cell expression system. *European journal of pharmacology* **2006,** *537* (1-3), 20-30.

249. Bandell, M.; Story, G. M.; Hwang, S. W.; Viswanath, V.; Eid, S. R.; Petrus, M. J.; Earley, T. J.; Patapoutian, A., Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron* **2004,** *41* (6), 849-57.
250. Reubish, D.; Emerling, D.; Defalco, J.; Steiger, D.; Victoria, C.; Vincent, F., Functional assessment of temperature-gated ion-channel activity using a real-time PCR machine. *BioTechniques* **2009,** *47* (3), iii-ix.
251. Gomis, A.; Pawlak, M.; Balazs, E. A.; Schmidt, R. F.; Belmonte, C., Effects of different molecular weight elastoviscous hyaluronan solutions on articular nociceptive afferents. *Arthritis and rheumatism* **2004,** *50* (1), 314-26.
252. Hargreaves, K.; Dubner, R.; Brown, F.; Flores, C.; Joris, J., A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **1988,** *32* (1), 77-88.

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PUBLICATIONS

1. **de la Torre-Martinez R***, Bonache MA*, LLabres PJ, Balsera B, Fernandez-Carvajal A, Fernandez-Ballester G, Ferrer-Montiel A, Perez de Vega MJ, Gonzalez-Muñiz R. *Exploring the β–lactam ring as a central scaffold for TRPM8 antagonists. Highly functionalized derivatives starting from amino acids. (*MANUSCRIPT UNDER PREPARATION*)*

2. **de la Torre-Martinez R**, Vazquez-Romero A, Belghiti M, de la Figuera N, Valente P, Planells-Cases R, Gomis A, Vidal-Mosquera A, Messeguer A, Ferrer-Montiel A, Fernandez-Carvajal A. *Characterization of a triazine-based open channel blocker of the TRPV1 receptor with in vivo analgesic and anti-pruritus activity.* (MANUSCRIPT UNDER PREPARATION)

3. Criado M, Balsera B, Mulet J, Sala S, Sala S, **de la Torre-Martínez R**, Fernández-Carvajal A, Ferrer-Montiel A, Moreno-Fernández S, Miguel M, Jesús Pérez de Vega MJ, González-Muñiz R. 1,3-*Diphenylpropan-1-one Derivatives as Allosteric Modulators of α-7 nACh Receptors with Analgesic and Antioxidant Properties*. Future Med. Chem. 2016, 731-49

4. Bertamino A, Ostacolo C, Ambrosino P, Musella S, Di Sarno V, Ciaglia T, Soldovieri MV, Iraci N, Fernandez-Carvajal A, **de la Torre-Martinez R**, Ferrer-Montiel A, Gonzalez Muñiz R, Novellino E, Taglialatela M, Campiglia P, Gomez-Monterrey I. *Tryptamine-based derivatives as Transient Receptor Potential Melastatin type-8 (TRPM8) channel modulators*. J. Med. Chem. 2016, 2179–2191

5. Perez-Faginas P, Aranda MT, **de la Torre-Martínez R**, Quirce S, Fernandez-Carvajal A, Ferrer-Montiel A, Gonzalez-Muñiz R. *New transient receptor potential TRPV1, TRPM8 and TRPA1 channels antagonists from a single linear b,g-diamino ester scaffold*. RSC Adv. 2016, 6868-6877

6. Fresno N, Pérez-Fernández R, Goicoechea C, Alkorta I, Fernández-Carvajal A, **de la Torre-Martínez R**, Quirce S, Ferrer-Montiel A, Martín MI, Goya P, Elguero J. *Adamantyl analogues of paracetamol as potent analgesic drugs via inhibition of TRPA1*. PLoS One 2014, e113841

7. Balsera B, Mulet J, Fernández-Carvajal A, **de la Torre-Martínez R**, Ferrer-Montiel A, Hernández-Jiménez JG, Estévez-Herrera J, Borges R, Freitas AE, López MG, García-López MT, González-Muñiz R, Pérez de Vega MJ, Valor LM, Svobodová L, Sala S, Sala F, Criado M. *Chalcones as positive allosteric modulators of α-7 nicotinic acetylcholine receptors: a new target for a privileged structure*. Eur. J. Med. Chem. 2014, 724-739

8. Bonache MÁ, Balsera B, López-Méndez B, Millet O, Brancaccio D, Gómez-Monterrey I, Carotenuto A, Pavone LM, Reille-Seroussi M, Gagey-Eilstein N, Vidal M, **de la Torre-Martínez R**, Fernández-Carvajal A, Ferrer-Montiel A, García-López MT, Martín-Martínez M, de Vega MJ, González-Muñiz R. *De novo designed library of lineal helical peptides: an exploratoty tool in the discovery of protein-protein interaction modulators*. ACS Comb. Sci. 2014, 250-258

PATENTS

1 Ferrer-Montiel A, Fernández-Carvajal A, **de la Torre-Martínez R**, González-Muñiz R, Pérez-de Vega MJ, Martín-Martínez M, Bonache de Marcos MA, Balsera-Paredes B inventors; 2015 March 31. *Péptidos bloqueantes de termorreceptores y sus usos*. Spain ES2532749

ACKNOWLEDGEMENTS

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AGRADECIMIENTOS

Hace ya algunos años que empecé un camino. En el he aprendido infinidad de cosas, desde lo puramente científico hasta cosas tan triviales y en principio básicas como son el relacionarse con diferentes tipos de personas. Sin embargo, este periplo, conocido como tesis, no lo he realizado solo y por ello me gustaría dar gracias a todos los que han contribuido de una manera u otra a que este viaje haya sido un éxito.

Gracias a Antonio por haberme dado la oportunidad de hacer la tesis doctoral en tu grupo. Durante estos años he aprendido muchísima ciencia y también me has mostrado diferentes posibilidades que antes no contemplaba. Por todo ello, muchísimas gracias.

También quiero agradecer a Asia por haber estado ahí en todo momento, en los buenos y en los malos. Contigo también he aprendido muchísimas cosas tanto en el ámbito científico como en lo personal. Agradecer la paciencia que has tenido conmigo así como el esfuerzo depositado en la co-dirección de esta tesis.

Gracias a Gregorio no solo por toda la ayuda con el modelado molecular sino también por darme la oportunidad de dar clases y por tu buen humor! Te debo unos cruasanes bien rellenos de chocolate.

Gracias a Charo y su grupo por la síntesis de los compuestos del Capítulo 2, por la buena acogida que tuve en Madrid cuando estuve en su lab y por responder siempre a todas mis preguntas. Ah! Y gracias por recomendarme Platea.

Gracias a Ángel y su grupo tanto por la síntesis de las triazinas como por el modelado molecular en TRPV1 del Capítulo 1.

Gracias a Ana y su grupo por los experimentos realizados en fibra nerviosa del Capítulo 1.

Gracias también a Rosa y los miembros de su laboratorio por los experimentos de BDL del Capítulo 1.

Gracias a los miembros de secretaria del IBMC! Raquel, May, Javier, Carmen. Sois increíbles! Nunca he visto ni creo que vaya a ver a gente tan maja y tan buena en su trabajo como vosotros!!

Gracias a todos Loramiguicos, a los antiguos y a los nuevos: Ainara, Sakthi, Maria Grazia, Rober, Isa, Aarón, Angeles (Wen!!), Cloti, Efrén, Gema, Lucia, Maite, Nuria, Moni, Christoph, Vero, Paco, Jan Albert...se me olvida alguien y no sé quién es… ah si! Pier ;). Lo dicho, muchísimas gracias amiguicos! Sois los mejores del mundo mundial y por ello quería dedicaros una palabrillas a cada uno.

A Ainara darle millonitas de gracias por todo (verde)! Si tú necesitabas escribir otra tesis para dejar constancia de tu agradecimiento, yo necesito (turquesa) diez! Gracias por todos tus consejos, por todo tu apoyo (verde), por esos días intensivos de correcciones que sin ti hubiesen sido imposibles (turquesa), por los días en Copenhague, por estar siempre a mi lado. Es realmente difícil parar de agradecerte cosas, pero en algún sitio tengo que hacerlo…así que por último, gracias por ser como eres! Pingus 4ever.

Thanks to Sakthi! You have been a great colleague and better friend. We have learnt a lot together and I'm not only speaking about science, but also funny things! For example, thanks to you I check the water every time I go to Arenales :P I will miss you a lot man! I hope to see you in the future to tell you: Ola k ase! Comemos o k ase?

A Mery! Has sido una gran amiga, a pesar de que intentases matarme con tus pasteles quemados o tu petropizza. Y aunque a ti no te hiciese mucha gracia, agradezco que esos pocos meses que ibas a estar se convirtiesen en años. La verdad es que al final hasta te cogí cariño, jaja. Muchas gracias por todo Mery! Un abrazaco.

A Robertaco! Tú siempre has sido un ejemplo para mí, negativo, pero un ejemplo, jaja. Es broma hombre, sabes que te aprecio un montonazo y que tu vuelta de Bilbao fue uno de los mejores regalos del mundo! Muchas gracias por estos años!

A la Wen! Por esos conversaciones locas y divertidas que empezaban siendo actas para todos los públicos y tras pocos minutos eran dignas de cuarto milenio en el mejor de los casos.

A Maite! Porque es una repostera espectacular (además de ser una gran científica). Muchas gracias por esos pasteles y por esas chuches a mitad de tarde que te alegran el día.

A Gemi! Porque molas un montón! Gracias por toda la ayuda en cultivo y sobre todo, gracias por tus "ánimos",jaja, nunca los olvidare.

A Luci! Porque serás la chunga de Sanvi, crearas grupos de whatsapp para tocarme las narices, me chincharas, pero aun así… te haces de querer y te aprecio muchísimo!!

A Pierluigi! Aunque no hayamos coincidido prácticamente nada en el laboratorio siempre has estado al otro lado para ayudarme, para aconsejarme y de vez en cuando para recordarme no sé qué de los camareros, jaja. Muchas gracias por todo Pier.

A Cloti! Gracias por tus palabras de ánimo cuando a lo mejor he estado un poco de bajona. Y gracias por esas fiestas en el campo! Son geniales!!

A Isa! Porque junto a Roberto has sido un ejemplo para mi tanto en el laboratorio como fuera de él. Si al principio de tesis me hubiesen preguntado como quería ser en unos años, hubiese dicho como tú.

A Vero! Porque nada une más que estar peleándose contra el mismo cacharro, jaja. Muchas gracias por tus abrazicos pajarito!

A Jan Albert "Brillo de luna"! Porque en la vida es importante reírse y contigo cerca, eso está asegurado.

A mi “alumno” Paco! El aprendiz de electrofisiología que ahora es todo un maestro del patch.

A Nuri! Por esas conversaciones mañaneras desde el marco de la puerta que tanto me gustan.

A mi vecino Aarón, que suena como a mi vecino Totoro y sois igual de entrañables!

A Christoph, el master de los juegos de mesa!

A Efrén y a Moni, porque cada uno de vosotros cuando ha estado en el laboratorio me ha mimado un montón (incluido pedido de células vía telefónica).

A las nuevas generaciones, Laura, Magdalena, Jose y en especial a Eva, con la que he aprendido un montón! En tus manos dejo el set-up, cuídalo!!

Muchas gracias Loramiguicos!!

También quiero darles las gracias a Ángela, Pablo y Mónica.

A Ángela, porque hemos sido amigos desde que empezamos el instituto allá por el año 2001, incluso a pesar de la distancia (primero Granada, luego San Diego…). Muchas gracias Angelina!

A Pablo, porque gran culpa de que hoy este en el mundo de la investigación es suya. Gracias a su ayuda y a su apoyo, no solo termine la carrera sino que año tras año fui superándome. Desde entonces, hemos sido grandes amigos, a pesar de la distancia. Muchas gracias Pablo!

Y especialmente a Mónica, porque siempre has estado a mi lado, siempre me has apoyado, siempre has creído en mí (incluso más que yo mismo) y has sufrido no solo tu tesis sino también la mía. Sin ti, esta tesis nunca hubiese sido posible. Por todo eso y por millones de cosas más, infinitas gracias Mónica!

Gracias también a un pequeño monito llamado Wyslla. Este monito es de lo más bonito del mundo y aunque esta como una cabra, la quiero un montón. Un abrazo muy fuerte monito!

Y por supuesto gracias a mi familia. Gracias a mis hermanos Carlos y Javi por estar siempre a mi lado, preocupándose, animándome, sacándome a dar una vuelta cuando más lo necesitaba o preparándome una tarta de queso con base de Chips-Ahoy cuando menos falta me hacía. Gracias mamá y papá por preocuparos tantísimo por mí, por darme lo mejor de vosotros, por padecer con esta tesis como si fuese vuestra, por estar siempre a mi lado… gracias por todo mamá, gracias por todo papá! Os quiero!

Y por supuesto… gracias a mi mismo por haber llegado hasta el final de este camino.

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