



DOCTORAL THESIS

Title	Chemical modulation of the nociceptive receptor TRPV1: Synthetic, biological and computational studies
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Summary

The ion channel Vanilloid receptor type 1 (TRPV1) is considered an important integrator of various pain stimuli. Among the products that interact with TRPV1, the ones blocking the “pain signal” in an uncompetitive manner have attracted the attention of researchers working in the field. Based on previously obtained results for several peptoid hits that showed activity as uncompetitive TRPV1 antagonists, and that allowed to propose a basic pharmacophoric hypothesis consisting on one cationic and two identical aromatic moieties, this thesis focused on the design and synthesis of new antagonists with a more rigid non-peptoid structure. Biological evaluation of a collection of new blockers and an in depth *in vitro* and *in vivo* study of the most active one provided knowledge about their mode of action. In addition, a 3D-quantitative structure-activity relationship study (3D-QSAR) allowed to generate models that explain the observed biological activity and can help to predict the activities of new compounds.

The 1,3,5-triazine skeleton was selected as rigid non-peptoid scaffold that can hold the three required pharmacophoric features. A total of 38 2,4,6-trisubstituted-1,3,5-triazines were synthesized using an optimized procedure. An in depth NMR study combined with theoretical calculations allowed to determine that most of these triazines present different conformers in equilibrium in solution, derived from the rotation of the bonds that joint the three side chains to the triazine ring. Ten of those triazines were particularly active blocking TRPV1 with submicromolar IC_{50} values. Triazine **46** exhibited a remarkably high activity ($IC_{50} = 50$ nM) being one of the most potent TRPV1 blockers described. It behaves as a polymodal antagonist against capsaicin and pH activation signals, exhibiting a low toxicity profile both *in vitro* and *in vivo*. Concerning its mechanism of action, **46** acts as an open channel blocker that locates relatively deep within the aqueous channel pore.

Statistically significant 3D-QSAR models were generated, using the CoMSIA methodology, for the anti-TRPV1 activity of the newly synthesized compounds. Those models reinforced the initial hypothesis on the structural requirements for uncompetitive TRPV1 antagonists and provided a quantitative tool that could help to predict the activity of new compounds. Using these models, an activity in the low micromolar range was estimated for a few members of a new family of pyrrolidine based TRPV1 antagonists. The synthesis and biological evaluation of some of them confirmed the activity prediction, further validating the 3D-QSAR models and establishing these pyrrolidine analogues as an interesting starting point for further development of new anti-TRPV1 compounds.

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Abbreviations

ACN	Acetonitrile
AcOH	Acetic acid
AcONa	Sodium acetate
AKAP150	"A" Kinase anchor protein 150
ANOVA	Analysis of Variance
API	Active Pharmaceutical Ingredient
ATP	Adenosine Triphosphate
BDL	Bile duct ligation
BK	Bradykinins
Boc	<i>Tert</i> -Butyloxycarbonyl protecting group
BHTHF	Borane-tetrahydrofuran complex
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase
cap	Capsaicin
CFA	Complete Freund's Adjuvant
CNS	Central Nervous System
CHO	Chinese Hamster Oocytes
CoMFA	Comparative Molecular Field Analysis
CoMSIA	Comparative Molecular Similarity Index Analysis
COSY	Homonuclear Correlation Spectroscopy
DCM	Dichloromethane
DFT	Density Functional Theory
DIC	Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethylsulfoxide
DRGs	Dorsal Root Ganglion
EC ₅₀	Half-maximal response
ESI	Electrospray Ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
EXSY	Exchange Spectroscopy
FDA	Food and Drug Administration
Fmoc	Fluorenylmethyloxycarbonyl
GI	Gastrointestinal

HPETE	Hydroperoxyeicosatetraenoic acid
HPLC	High Performance Liquid Chromatography
hERG	human Ether-à-go-go-Related Gene channel
HRMS	High Resolution Mass Spectroscopy
HSQC	Heteronuclear Single-Quantum Correlation Spectroscopy
HTS	High Throughput Screening
HIV	Human Immunodeficiency Virus
IASP	International Association for the Study of Pain
IC ₅₀	Half maximal inhibitory concentration
IPA	Isopropanol
i.p.	Intraperitoneal administration
i.v.	intravenous administration
LOO	Leave-one-out
LSO	Leave-several-out
MeOH	Methanol
MIF	Molecular Interaction Field
MLR	Multiple Linear Regression
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MS	Mass spectroscopy
NADA	<i>N</i> -arachidonoyl-dopamine
NMDA	N-methyl-D-aspartate receptor
NME	New Molecular Entity
NMR	Nuclear Magnetic Resonance
mw	Microwave
NOESY	Nuclear Overhauser Effect Spectroscopy
NSAIDs	Nonsteroidal anti-inflammatory drugs
OTC	Over The Counter
PBS	Phosphate-buffered saline
PC	Principal Component
PCA	Principal Component Analysis
PCR	Principal Component Regression
PDB	Protein Data Bank
PGs	Prostaglandins
PIP ₂	Phosphatidylinositol
PKA	Protein kinase A

PKC	Protein kinase C
PLS	Partial Least Square
PNS	Peripheral Nervous System
PP	Polypropilene
pt	Polarization Transfer
PRESS	Predicted Residual Sum of Squares
PWL	Paw Withdrawal Latency
R&D	Research and Development
RTX	Resiniferatoxin
r.t.	Room temperature
SAR	Structure-Activity Relationship
SAMPLS	Sample-Distance Partial Least Squares
SDEP	Standard Deviation Error in Prediction
S _N Ar	Nucleophilic Aromatic Substitution
QSAR	Quantitative Structure-Activity Relationship
TES	Test Set
TFA	Trifluoroacetic Acid
THF	Tetrahydrofurane
TLC	Thin Layer Chromatography
TNBS	2,4,6-trinitrobenzenesulfonic acid
TRP	Transient Receptor Potential channel
TRPM8	Transient Receptor Potential subfamily M8 channel
TRPV1	Transient Receptor Potential Vanilloid subunit I channel
TRS	Training Set
UPLC	Ultra-Performance Liquid Chromatography
VR1	Vanilloid type 1 receptor (synonym of TRPV1)
WHO	World Health Organization
WT	Wild Type
ZPE	Zero-Point Energies

Chapter 1 – Introduction.

1.1 The Foundation la Marató.

“La Marató de TV3” is a support project promoted by Televisió de Catalunya, a TV channel from Catalonia, focused in raising the public awareness and gathering resources for the scientific research of diseases without actual cure. The first telethon of “La Marató” was carried out in 1992. Nowadays, it is a well-established project. Usually the program is seen by more than 3 million people, lasts for 15 h and collects an average of around 7 million euros. Relying in experts advice, the foundation selects diseases or illnesses with great impact in society. Previous editions of this project were dedicated to Leukemia, Down’s syndrome, cancer, cardiovascular and neurological diseases, hereditary genetic disorders, diabetes, organ transplantation, mental diseases, AIDS, chronic inflammatory diseases, chronic respiratory diseases, chronic pain, cardiovascular diseases, rare diseases among others. In this list only chronic pain is a symptom, not an illness. In 2006 “la Marató” decided to change its scheme and selected pain as objective, relying on scientific societies and patient associations which support that pain is a central issue in almost every illness. In the European Union and the United States, pain and chronic low back pain are the first and second reason of consultation in primary healthcare, and migraine is the most common reason for consultation in neurology. The telethon took place on December 17th, 2006, and collected an amount of 6.993.480 €. Raised funds allowed the foundation to finance a total of 28 projects from 81 applications. The selected projects aimed to find out the basic mechanisms of pain, the reasons why it appears in the course of different diseases, and to discover new therapeutic tools to remove or mitigate pain.

The present doctoral thesis is enclosed in one of the multidisciplinary projects selected by the foundation. Molecular biology (University Miguel Hernandez), cellular physiology (Prince Felipe Research Center) and medicinal chemistry (Institute of Advanced Chemistry of Catalonia) were used to study pain modulation mechanisms. The global title of this project was “Pharmacological Intervention at TRPV1 for attenuation of chronic pain”.

1.2 Drug Discovery.

Research on active pharmaceutical ingredients (API) for the treatment of unmet pathological conditions is a social need and it is urgent for some diseases. There are many clinical conditions without effective cure or whose current treatments are under revision. Chronic pain, neurodegeneration and cancer associated diseases, autoimmune or inflammatory diseases are a few examples. Multiple medicinal chemistry programs are therefore focused on the design, synthesis and development of new molecules as therapeutic agents against these diseases.

Investment in drug research and development (R&D) has increased substantially in recent decades, but the annual number of new molecular entities (NME) approved by the US Food and Drug Administration (FDA) has not increased accordingly; on the contrary, it has diminished as attrition rates are very high.¹ A new molecular entity is defined as a drug that contains no active moiety that has been approved by the Food and Drug Administration (FDA) in any application submitted.

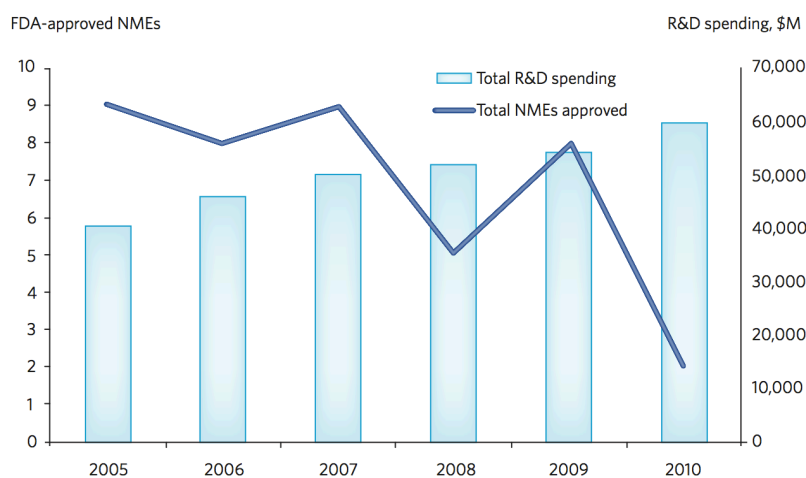


Figure 1. Major Pharma productivity from 2005–2010. Combined FDA-approved NMEs versus R&D spending for nine major pharmaceutical companies.¹

Historically, most drugs were discovered either identifying the active ingredient from traditional remedies or by serendipitous usage of plants, herbs or fungi in the chemical/pharmaceutical environment. Nowadays, although serendipity still exerts an important role in the global picture of NME/API discovery, the rational approach has proven to be a useful tool.^{2,3}

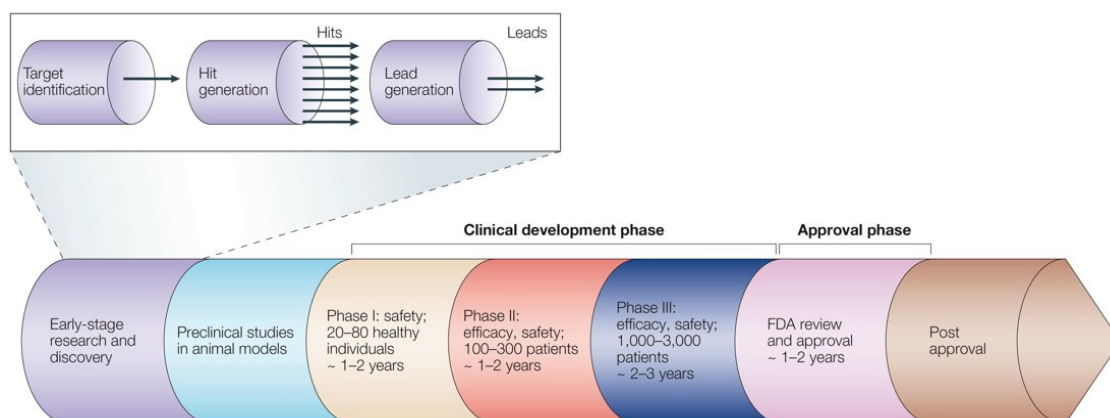


Figure 2. A timeline of traditional de novo drug discovery and development. De novo drug discovery and development is a 10 –15 year process from the idea to the marketed drug. The probability of success is lower than 10%.

Drug discovery can be separated basically in two main stages: discovery and clinical studies. The discovery of a NME/API usually begins with the isolation and/or characterization of a representative biological system with potential therapeutic value. After the target/system is defined, chemoinformatic tools can focus and reduce the costs and time by preselecting the candidates to assay. But in the end, the biological assay of chemical products is what will provide reliable information about the system. The candidates can proceed from different sources: synthetic or natural chemical commercial libraries, focused synthetic libraries, etc. In that context, combinatorial chemistry became relevant to generate large amount of chemically diverse subsets of products in short periods of time. Along with the development of chemical libraries, high throughput assays (HTS) allowed scientists to evaluate large numbers of products by automatizing, miniaturizing and robotizing the biological assay systems. When a product interacts positively with the system, it becomes a hit. It is very unlikely for a hit to proceed to further development without modifications on its chemical structure. For that reason, the hit structure is modified to improve its pharmacokinetic properties, trying to retain the pharmacodynamics profile in what is defined a “hit to lead” process. Then, the lead candidate, i.e. a product optimized to be active and non toxic in animal models, passes to clinical phases where its toxicity, dosage, efficacy and effectiveness will be studied in humans. It is at these stages where the “attrition curse” appears, making candidates to fall out from the pipeline at higher rate, especially in the phase 2 to phase 3 transitions.¹ On 2010 the total investment required to “launch” a new drug into the market was estimated to be around 1.800 M\$ and the process usually lasts for 10 - 15 years.⁴ Being a large and expensive process, rational selection of chemical

entities using available knowledge on target receptor can aid in the shortening of the process.

All of the above reflect the necessity of further research on new molecules that can treat or improve actual therapies. From the different unmet pathological conditions, chronic pain is one of most important as its incidence among population is large. As a reference, it was estimated that chronic pain affected around 100 millions of adults in the USA during 2008, having a cost of 600 M\$.⁵

1.3 Pain as a symptom.

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.⁶ It motivates the subject to evade and withdraw from potentially damaging situations, protect a damaged body part while it heals, and avoid harmful situations in the future. Usually pain resolves promptly once the painful stimulus is removed and the body has healed, but sometimes pain persists despite removal of the stimulus and apparent healing of the body, and sometimes pain arises in the absence of any detectable stimulus, damage or pathology.⁷

The classification system used by the International Association for the Study of Pain (IASP) describes pain according to five categories: duration and severity, anatomical location, body system involved, cause, and temporal characteristics (intermittent, constant, etc). In addition, some years ago the “neurochemical mechanism” category was proposed to the IASP in order to treat the symptom in a correct way, based on its molecular mechanism.⁸ In terms of duration, pain can be classified as acute or chronic. Acute pain occurs while the painful stimulus is present, and when it is removed it tends to disappear. When pain does not disappear within 6-12 months after the painful stimulus has been removed, it becomes chronic. Migraine and fibromyalgia are two illnesses where pain shows itself in a discontinuous way, making it difficult to classify it as acute or chronic.⁸ The IASP recognizes 11 levels of pain going from 0 (no pain) to 10, being 10 the worst possible pain that can be imagined.⁹

Pain that is generated by some damage done to the body is known as somatogenic pain, and pain that arises without any physical damage is known as psychogenic pain. Somatogenic pain appears when the body gets hurt (nociceptive pain) or when the neuronal network involved in sensing does not work properly

(neuropathic pain). Nociceptive pain is caused by nociceptor cells located in peripheral nerve fibers. Its main function is to act as a tool to protect and regenerate the damaged tissue. Nociceptor cells are a specific type of neurons with different molecular mechanisms that are able to transduce external stimuli into neuronal activity. Mechanical, thermal and chemical stimuli can be transduced using different receptors that can be stimulated in different ranges, showing a continuous response to those stimuli. Neuropathic pain, on the other hand, does not have any known function. The damaged neuronal cells trigger the pain stimuli without a noxious condition.⁸

According to the classical cartesian view, pain was considered to be a hard-wired system in which noxious input (either nociceptive or neuropathic) was passively transmitted along sensory channels to the brain. However, today it is generally accepted that this is far from truth and pain experience is not at all simply driven by noxious stimulus characteristics.¹⁰ Different types of pain arise from complex cellular and molecular mechanisms that can involve both the central nervous system (CNS) and/or the peripheral nervous system (PNS).¹¹ The advent of modern, noninvasive neuroimaging techniques in combination with many experimental paradigms has greatly expanded our knowledge concerning the CNS processes underlying pain perception and its modulation in humans.¹² But today we are still far from understanding the entire pain “picture”.

1.4 Pharmacological management of pain.

Although there are a large number of treatments for pain management, pharmacological pain therapies are carried out by the administration of two families of products: Opioids and nonsteroidal anti-inflammatory drugs (NSAIDs).

1.4.1 Opioids.

Opioids are natural or chemically synthesized products that interact with the opioid receptors located mainly in the brain, spinal cord and digestive tract. Natural occurring opioids, as codeine, morphine or thebaine, share a common partially hydrogenated phenanthrene scaffold, but other opioids show different structures, like fentanyl, pethidine or dextropropoxyphene (Figure 3).

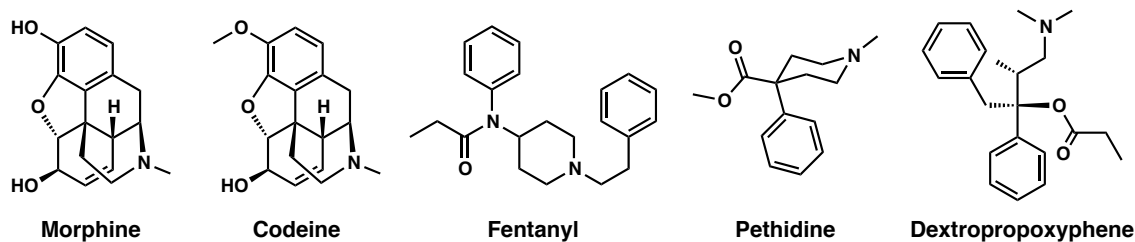


Figure 3. Molecular structure of some natural and synthetic opioids.

The overall action of opioids is a sum of activation and depression of different body functions depending on the somesthetic state of the subject. A dose of 10 mg of morphine (prototypical opioid) in subjects coursing pain can erase this situation, leading to a sedated and euphoric state, reducing disgusting sensations. If the dose is increased, the subject enters in a state of torpor and sleepiness. On the other hand, if the same dose is administered to a healthy subject, it causes dysphoria, nausea, vomiting and weakness.¹⁴ The analgesic effect of opioids is dose dependent and can erase pain in all its extension (except some cases of neuralgias and phantom limb pain, both related with deafferentation pain). The effect of opioids over pain is quite selective and does not alter sensorial perception. The analgesia elicited by opioids is subsequent to its action over spinal and supraspinal structures of the CNS.¹⁵

Opioids are the most potent analgesics known today but, due to different issues with undesired effects, their usage is restricted. Aside from dependency, the most known side effect, common adverse reactions can include nausea and vomit, drowsiness, itching, dry mouth, miosis and constipation. It can also compromise the function of the immune system. Among male subjects, long-term users of opioids have lower levels of testosterone, leading to bone diseases like osteoporosis or decreased muscle strength.^{8,13,14}

1.4.2 Non-steroidal anti-inflammatory drug (NSAIDs).

Composed by a large number of families, with a non-common chemical scaffold, these products are also known as minor analgesics or aspirin-like analgesics. Along with aspirin, ibuprofen, acetaminophen (paracetamol) and naproxen are the most used over the counter analgesics (Figure 4). These products act as analgesics, antipyretics, antithermics and some of them as antirheumatics.^{8,14} The main differences between NSAIDs and opioids are:

1.- Their action is fundamentally peripheral (except for the antithermic component).

2.- Their analgesic action has mild potency, being specially indicated for pain related with the integumentary system, somatic system and non related visceral pain.

3.- NSAIDs do not cause addiction nor physical or psychical dependence.

4.- Undesired reactions are similar to all of them, being the most extended related with gastrointestinal diseases and the hematopoietic system.

5.- The general mechanism of action of NSAIDs implies the inhibition of prostaglandins, leukotrienes and autacoids.

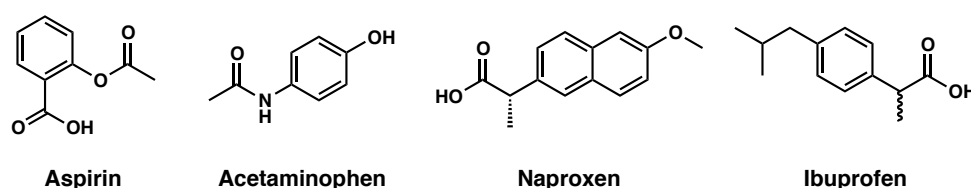


Figure 4. Most sold over the counter (OTC) analgesics.

NSAIDs act in a non-selective way on different cyclooxygenase isozymes. These enzymes catalyse the formation of prostanoids (prostaglandins and thromboxanes) from arachidonic acid. Prostaglandins (PGs) are mediators and have a variety of strong physiological effects, such as regulating the contraction and relaxation of smooth muscle tissue, aggregation or disaggregation of platelets or sensitization of spinal neurons to pain.¹⁵

The usual undesired effects are related to direct and indirect irritation of the gastrointestinal (GI) tract due to the acid character of most NSAIDs. In addition, due to the inhibition of protective prostaglandins, the acid secretion is increased along with a decrease of mucus, bicarbonate secretion, and a decrease of trophic effects on epithelial mucosa. This leads to ulceration of GI tract, nausea and vomiting, dyspepsia gastric ulceration (with corresponding bleeding) and diarrhea.

Although most types of acute pain can be treated using different types of analgesics like opioids and NSAIDs, neuropathic pain, bone pain¹⁶, some cases of myalgia and cancer pain remain as a widely recognized unmet medical need. Neuropathic pain for example is relatively resistant to opioids and NSAIDs but respond to some extent to ion channel-blocking drugs such as gabapentin, mexiletine and amitriptyline.¹⁷ The search for new analgesic agents is being intensively investigated by the pharmaceutical industry, and there is particular interest in identifying novel

mechanisms of action that play a role in pain sensation/transmission. One relatively new molecular target that has attracted significant attention in this matter in the past decade is TRPV1 (transient receptor potential vanilloid type 1).

1.5 Transient Receptor Potential Vanilloid – I (TRPV1).

Transient Receptor Potential ion channels are tetrameric integral membrane proteins grouped in 8 subfamilies, namely TRPC, TRPV, TRPM, TRPN, TRPA, TRPML, TRPP, and the recently identified TRPY in yeast.¹⁸ Members of the TRPV (V1-V4), TRPM (M2, M3, M5 and M8) and TRPA (A1) subfamilies are gated by temperature changes, extending its function from noxious cold (< 15 °C) to injurious heat (> 42 °C), and are therefore known as thermoTRPs. Furthermore, these channels may respond to chemical compounds, voltage, and some of them are activated by mechanical stimuli. ThermoTRPs are cation selective ion channels that permeate Na⁺, K⁺, and some of them display a notable Ca²⁺ permeability. These channels act as integrators of several environmental cues and signaling pathways, including those mediated by cell surface expressed receptors.

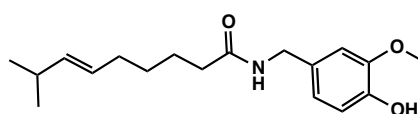
At the molecular level, these ion channels are generally homotetrameric proteins, although the formation of heterotetramers has also been proposed.¹⁹ The basic channel subunit displays a modular organization having N- and C-terminal cytosolic domains and a six-transmembrane region that contains a large extracellular domain between the fifth and sixth transmembrane segments. The cytosolic N-terminus of the thermoTRPs may contain ankyrin repeats or TRPM homology regions, and phosphorylation consensus sites for serine/threonine and tyrosine kinases.¹⁸ Ankyrin repeats are known for their role in mediating protein–protein interactions. The C-terminus of thermoTRPs is also a modular domain that contains a multimerization motif (known as the TRP domain), calmodulin and tubulin binding regions, and consensus sequences for protein kinases.²⁰ Furthermore, it may have molecular determinants of the channel temperature sensor. The TRP domain appears to be critical for functional coupling between stimuli sensing and pore opening. ThermoTRP dysfunction has been implicated in several pathologies, including diverse kinds of pain, inflammation, pulmonary disorders, gastrointestinal hypersensitivity, bladder dysfunction, cardiac hypertrophy, skin sensitivity, ischaemic cell-death, and neurodegeneration. These pathological processes either display an increase of channel activity, an augmented channel expression or both.²⁰ Furthermore, gene association studies have reported

that some of the thermoTRP members display gain-of-function mutations in human inherited diseases.²¹

The cloning of the Transient Receptor Potential Vanilloid Subunit 1 channel (TRPV1) has notably contributed to our current knowledge on the molecular and cellular mechanisms underlying chemical and thermal nociception, as well as pain transduction.^{22,23} TRPV1 is the first identified member of a family of thermosensory receptors that is mainly expressed in both the peripheral nervous system and the CNS of superior animals.¹⁸

Genetic and pharmacological suppression of TRPV1 activity notably reduces the thermal hyperalgesia characteristic of inflammatory pain. Noteworthy, an enhanced expression of this thermoTRP channel has been observed in human chronic pathologies such as arthritis and cancer pain. TRPV1 plays a pivotal role in the peripheral sensitization of nociceptors upon tissue injury and/or inflammation produced by trauma, infection, surgery, burns or diseases with an inflammatory component.^{24,25} Therefore, the vanilloid receptor is gaining a great interest in pain pharmacology for the treatment of neuropathic, postoperative and chronic pain, as well as target for bone cancer pain.

TRPV1 is a ligand-gated nonselective cation channel with high Ca^{2+} permeability, which is activated by noxious heat ($> 42\text{ }^{\circ}\text{C}$), low pH and different endogenous compounds, as well as exogenous ones, like capsaicin (Figure 5). Capsaicin is a vanilloid found mainly in the fruits of the *Capsicum* genus, like jalapeño and chili pepper among others.



Capsaicin

Figure 5. Chemical structure of Capsaicin.

TRPV1 is a homotetrameric membrane protein. Each monomer contains 839 aa, six predicted α -transmembrane domains and three ankyrine repeat domains.²⁶ These features can also be observed in other TRPV family channels (Figure 6) with some differences in distribution along their primary sequence.

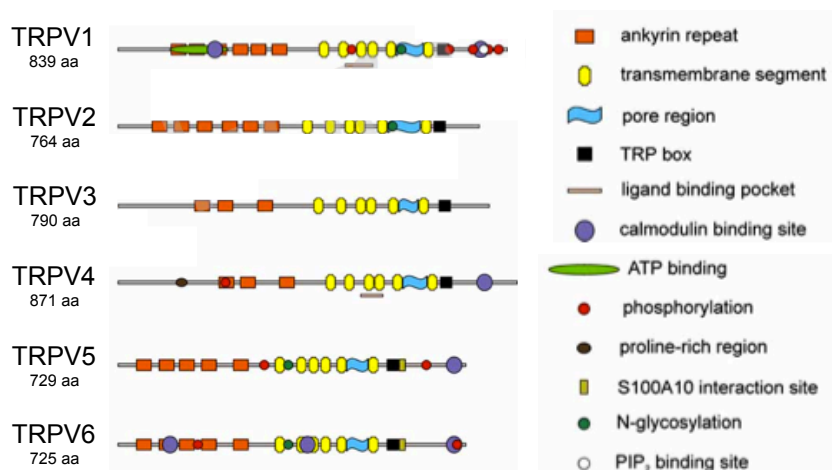


Figure 6. Prediction of the structural topology of TRPVs as described by Rudi et al.²⁶

The high-resolution three-dimensional structure of full TRPV1 is not available, only the structure of the ankyrin repeat domain has been solved.²⁷ Moiseenkova-Bell et al. reported a low resolution structure of rat TRPV1 obtained by electron cryomicroscopy,²⁸ and very recently much better resolution ($< 4 \text{ \AA}$) structures of TRPV1, alone and in complex with different modulators, have been obtained by single particle electron cryomicroscopy.²⁹

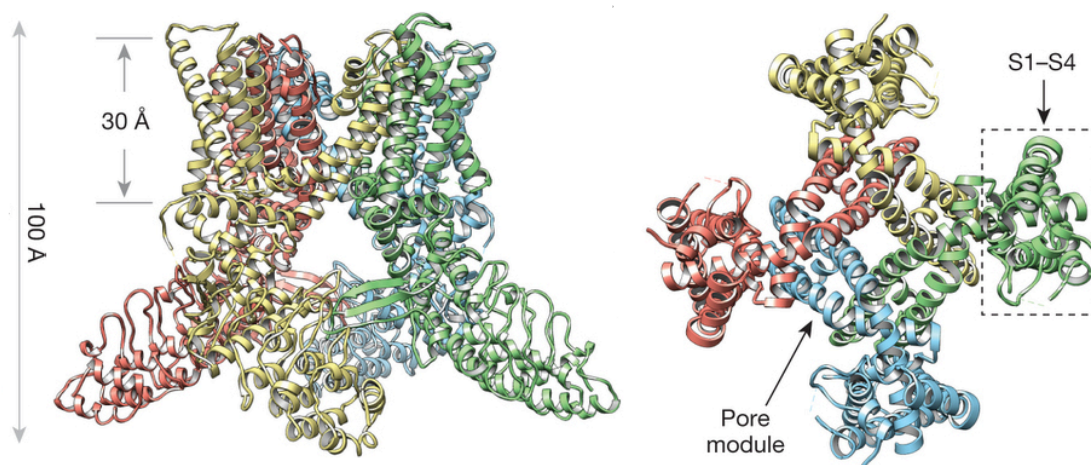


Figure 7. Ribbon diagram of TRPV1 atomic model with each of the four identical subunits color-coded, showing views from side (left) and bottom (right).²⁹

Homology modelling has also been used to obtain atomic-resolution models of TRPV1. In this concept, the three-dimensional structures of K⁺ channels Kv1.2 and KcsA in the open and closed states have been used for that purpose.^{30,31} Kv1.2 is an eukaryotic and KcsA is a prokaryotic channel which both share several features with TRPV1. Both of them are known to be voltage gated positive ion channels, formed by

four identical monomers which present a selectivity pore between the fifth and the sixth transmembrane segment. Their primary structures show a moderate sequence homology with TRPV1 for the transmembrane domains and the selectivity filter: around 12% sequence identity and 34% sequence similarity.³² Using those channels as templates different homology models have been generated.³²⁻³⁵

On the other hand, site directed mutagenesis performed on TRPV1 allowed to identify the role of a diverse set of amino acids implied in pH sensing, the capsaicin binding site and the heat sensing domains. Protons activate and potentiate TRPV1 by shifting the voltage dependence of the activation curves towards more physiological membrane potentials. Mutants V538L, E600Q, T633A, E648Q, F660S, F660A modulate the response of the channel from activation and potentiation to inactivation (Figure 8).

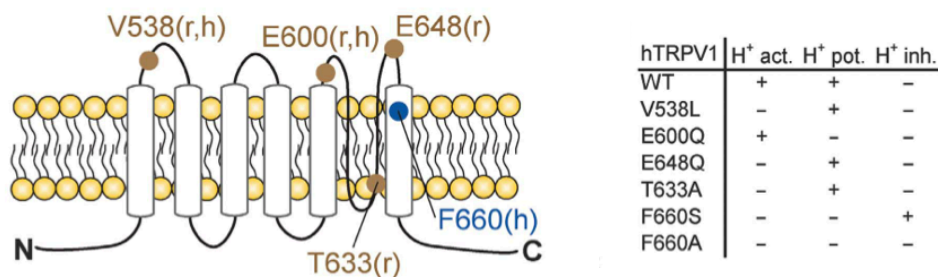


Figure 8. Scheme of TRPV1 topology showing the location of residues involved in proton activation or potentiation (brown) or activation and potentiation (blue). Letter(s) in parentheses indicate whether the respective position is in human (h) and/or rat (r) TRPV1. The table shows the effect of TRPV1 point mutations on proton activation and/or potentiation of the channel.³⁶

Temperature sensing domains are located on the cytoplasmic C-terminal region of the ion channel. Work performed by Brauchi et al. where the C-terminal domain of TRPV1 was exchanged by the homologous sequence of TRPM8 and vice versa, led to chimeras showing exchanged temperature behavior: a cold activated TRPV1 and a hot activated TRPM8. This led to the conclusion that the C-terminal domain is able to define the phenotype of heat activation of the TRPV1 channel.³⁷

1.5.1 Endogenous regulation of TRPV1 activity.

TRPV1 exhibits a dynamic threshold of activation that could be significantly lowered under inflammatory conditions. TRPV1 is thought to mediate the phenomenon of peripheral sensitization that involves a reduction in the threshold of activation and an increase in the responsiveness of the peripheral termini of nociceptors. Indeed, agents in the 'inflammatory soup' e.g. (bradykinin (BK), substance P, lactic acid, leukotrienes) act together to lower the activation threshold of TRPV1. Other agents that can activate and/or sensitize TRPV1 include: nerve growth factor 21 (NGF 21), anandamide, arachidonic acid metabolites such as *N*-arachidonoyl-dopamine (NADA) and *N*-oleoyldopamine, lipoxygenase products (12 and 15-hydroperoxyeicosatetra-enoic acid (12-HPETE and 15-HPETE), leukotriene B₄, PGs, adenosine and ATP, prokineticins, and some polyamines (such as spermine, spermidine and putrescine) (Figure 9).²⁴

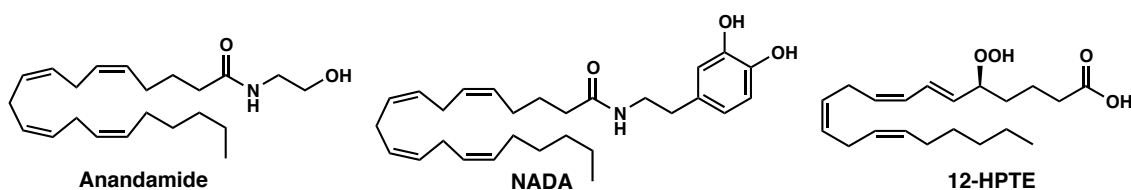


Figure 9. Different structures of endogen and exogenous agonists of TRPV1.

Phosphorylation of TRPV1 is a major mechanism that accounts for TRPV1 sensitization and various second messenger pathways have been associated with TRPV1 phosphorylation (see Figure 10).³⁸⁻⁴⁰ Protein kinase C (PKC) phosphorylates TRPV1 at S502 and S800 whereas protein kinase A (PKA) activation results in the phosphorylation of S502, S116, T144 and T370 residues.^{38,41} Ca²⁺/calmodulin-dependent protein kinase (CaMKII) activation also leads to phosphorylation of TRPV1 at S502, T370 and T704.³⁹ Activation of NMDA receptors sensitizes TRPV1 by serine phosphorylation via pathways involving PKC and CaMKII.⁴² The modulation of TRPV1 by intracellular enzymes such as PKA, PKC, and calcineurin depend on the formation of signalling complex between TRPV1 and the scaffolding protein AKAP150. AKAP150 binds to TRPV1 C-terminal in nociceptors enhancing its function.⁴³⁻⁴⁵

In summary, nociceptive pain is produced under physiological conditions only by noxious stimuli acting on high-threshold nociceptors. But when inflammation occurs proinflammatory compounds and activated kinases lower the threshold of activation of transducer receptors such as TRPV1, and the excitability of the peripheral terminal

membrane increases producing a state of heightened sensitivity termed 'peripheral sensitization' (Figure 11).⁴⁶

These properties conform TRPV1 as a principal integrator of nociceptive stimuli. First TRPV1 detects the chemical and thermic nociceptive stimuli and, second, it contributes to establish a persistent peripheral inflammation and central sensitization that aids to protect the affected zone against further damage.

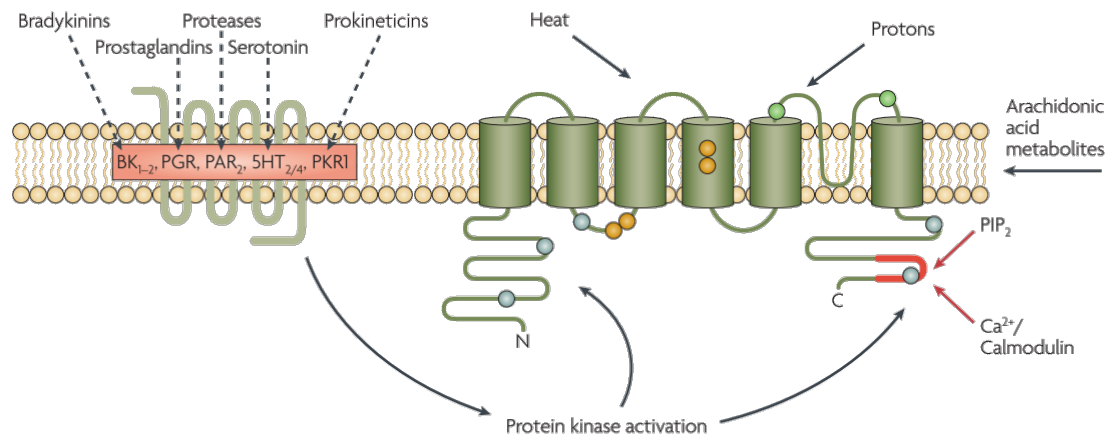


Figure 10. Schematic summary of TRPV1 signal integration in the peripheral nociceptor terminal. Solid arrows indicate TRPV1-sensitizing stimuli. The red arrows indicate negative regulation. Receptors and corresponding ligands known to mediate the sensitization of TRPV1 are shown on the left. Colored circles represent amino-acid residues that have been identified to be important in particular functions: orange, vanilloid binding (Y511, S512, L547, T550); blue, protein kinase phosphorylation sites (S116, T370, S502, T704, S800); and green, low-pH activation (E600, E646). The red line indicates the carboxy-terminal domain of TRPV1, which has been shown to interact with both PIP₂ and calmodulin.²⁴

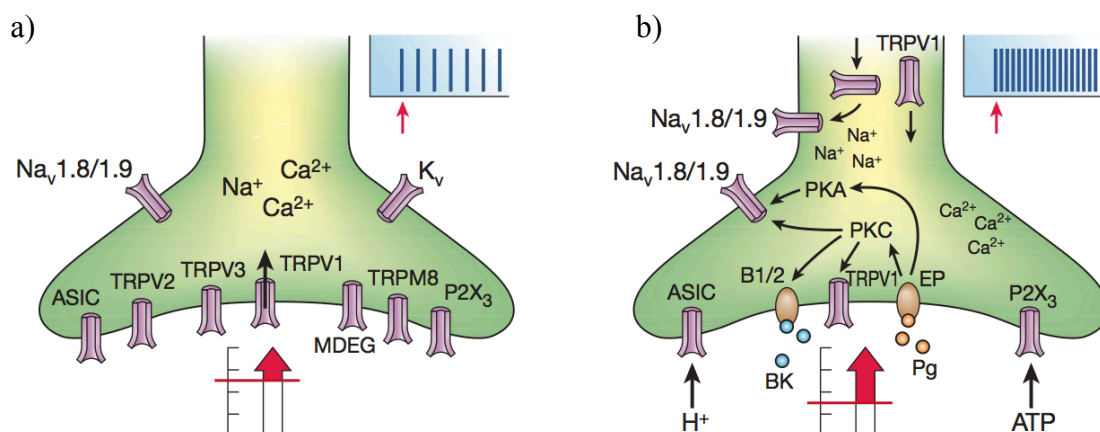


Figure 11. a) Physiological conditions with a normal threshold for TRPV1. b) In peripheral inflammation conditions, compounds like BK or PGs activate kinases A and C. As a result, the threshold of activation of transducer receptors such as TRPV1 is reduced leading to a peripheral sensitization. This is represented in the upper right side as blue lines.

1.5.2 Pharmacological modulation of TRPV1 activity.

Capsaicin, the active component of chili peppers, is an irritant for mammals, including humans. It produces a burning sensation on contact with any tissue. Upon prolonged exposure to capsaicin, TRPV1 activity decreases, a phenomenon called desensitization. Influx of calcium and the consequential increase of its intracellular concentration mediate this effect. This desensitization is thought to underlie the paradoxical analgesic effect of capsaicin.²⁴

According to their mode of action there exist three major types of compounds that can modulate the activity of TRPV1: agonists, competitive antagonists and uncompetitive antagonists. Agonists can interact at the binding site of capsaicin producing a desensitization of the receptor after a fast onset. Competitive antagonists bind to the capsaicin binding site, blocking the currents evoked by capsaicin, heat or pH. Uncompetitive inhibitors can block the evoked currents (by capsaicin, heat or pH) independently of capsaicin concentration.

1.5.2.1 Agonists.

Capsaicin is the representative example of this family. This molecule bears the 4-hydroxy-3-methoxybenzyl moiety characteristic of homovanillin derivatives. Agonists directly gate the channel by a mechanism that involves the reduction of the heat threshold of activation.⁴⁷ Prolonged exposure of the receptor to the agonist in the presence of Ca^{2+} induces channel closure by desensitisation mediated by phosphorylation of key residues at the C-terminus of the protein (Ser774 and Ser820).^{48,49} In addition, overload of intracellular calcium resolves in a deletion of TRPV1 expression giving a second path to the desensitization mechanism.⁵⁰

The structures of capsaicin and most of its analogues are formed by a polar, a linker and a hydrophobic moieties (Figure 12). Structure-activity relationships (SAR) studies have shown that the polar head, which in capsaicine is the 4-hydroxy-3-methoxybenzyl moiety characteristic of homovanillin derivatives, is essential for exciting sensory neurons.⁴⁷ On the other hand, the hydrophobic region interacts with a hydrophobic region in the receptor. Diverse site directed mutagenesis and docking studies using TRPV1 homology models suggest that the hydrophobic region where vanilloids bind spans the third transmembrane domain of the receptor.⁵¹⁻⁵³ Residues

Tyr511, Ser512, Thr550 and Tyr667 were identified as key residues for the interaction (Figure 13).

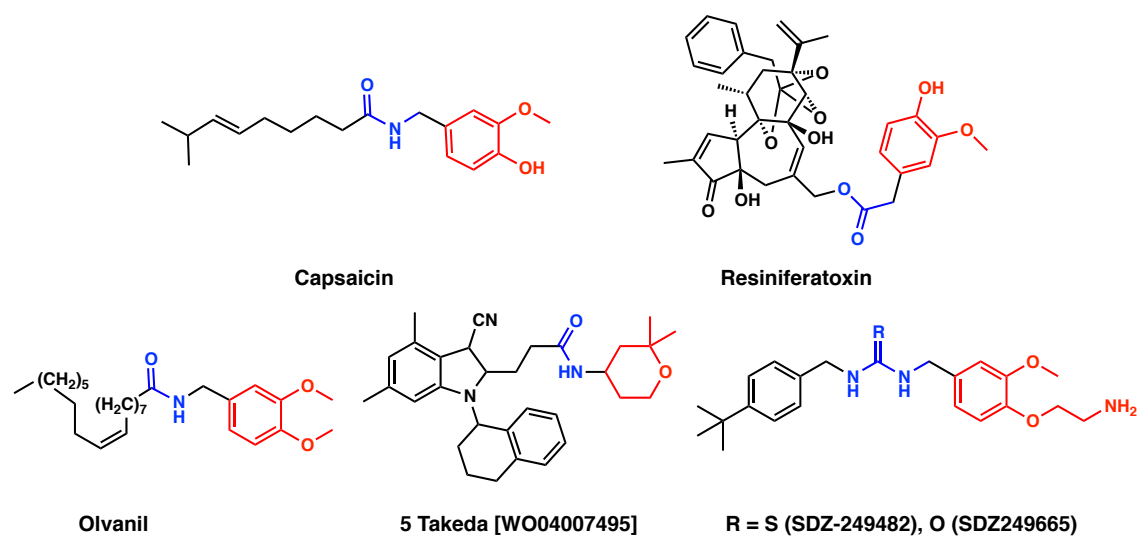


Figure 12. Chemical structures of TRPV1 agonists showing relevant regions: polar (red), linker (blue) and hydrophobic (black) moieties.

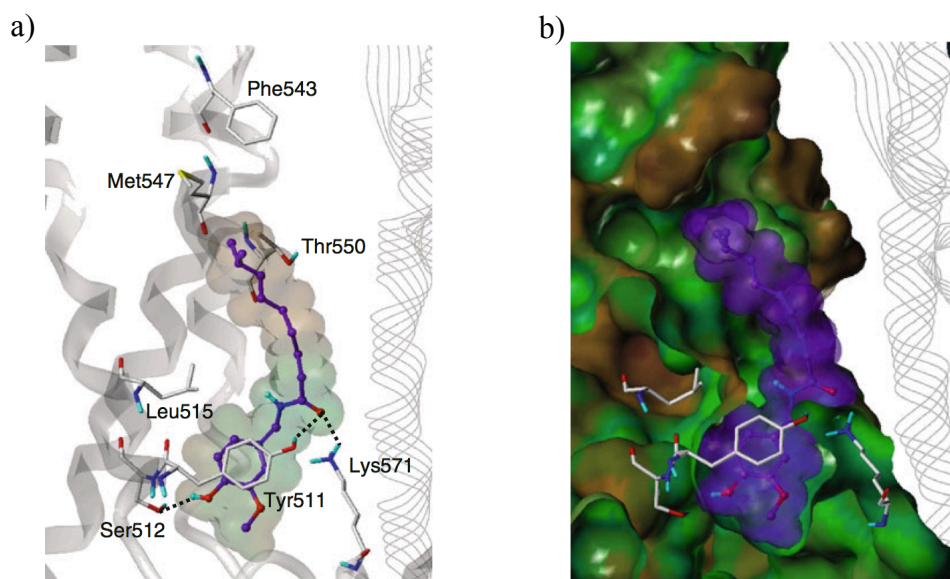


Figure 13. a) Proposed binding mode of capsaicin. The key interacting residues (capped-sticks) of rat TRPV1 are labelled. B) The same structure represented with surfaces: purple for capsaicin and green-orange (green = lipophilic, orange = lipophobic) for the protein.³²

Aside from capsaicin, there are other natural products, like resiniferatoxin (RTX), that possess TRPV1 agonist activity. RTX, isolated from *Euphorbia Resinifera*, exhibits a more potent response and has a more complex structure than capsaicin, as shown in Figure 12.⁵⁴ RTX has been useful for the treatment of urinary urge

incontinence and pain associated with diabetic neuropathy, two human pathologies mediated by TRPV1 dysfunction.⁵⁵

Nonetheless, natural and synthetic availability of RTX is reduced, difficulting its application in therapy. In addition TRPV1 agonists generate an initial burning-itching sensation due to the activation of the ion channel, which also discourages from using them. Despite this fact, the analgesic effect of capsaicin is currently used in topical ointments, as well as a high-dose dermal patch, to relieve the pain of peripheral neuropathy such as post-herpetic neuralgia caused by shingles.⁵⁶ In addition, the potency and activity of both capsaicin and RTX has launched several pharmaceutical discovery programs in order to find synthetic vanilloid derivatives with small or low sensitization of TRPV1 and a good activity as a desensitizer. Derived from these programs, products like olvanil, SDZ-249482, SDZ-249665 or the capsaicin analogue **5** were discovered (Figure 12).^{47,57} Although they did not fully circumvent the discomfort of the side effects derived from irritation, they shed some light in the pharmacodynamics behavior of TRPV1 agonists.

1.5.2.2 Competitive antagonists.

Competitive antagonists bind to the agonist binding site and lock the channel in the closed, nonconductive state, having the capability to reduce pain and preventing inflammation state without the discomforting sensation of TRPV1 agonists. They have generated a great interest on pharmaceutical industry and nowadays the antagonistic mode of interaction with the receptor is the most studied area of TRPV1 to block pain signaling.

The first capsaicin competitive antagonist, capsazepine, was described in 1994.⁵⁸ It can be considered as a conformationally restricted thiourea analogue of capsaicin (Figure 14). Although it exhibits a good activity as TRPV1 competitive blocker, its fast metabolization prevented further clinical development.⁵⁹ Modification of RTX by halogenation yielded different products among which 5-iodoresiniferatoxin (5-iodo-RTX) has a potent activity as TRPV1 capsaicin antagonist.⁶⁰ But as for RTX, problems regarding the availability of source material dismissed the use of this product. For some years both capsazepine and 5-iodo-RTX were the only high profile antagonists known for TRPV1.⁶¹

Due to the interest in this pharmacological profile, different discovery programs were set up in order to find new TRPV1 antagonist. However, although very potent compounds were developed (Figure 14), none of the clinical trial candidates has

arrived to the market due to different undesired effects, like hyperthermia (e.g. AMG-517),⁶² increased levels of liver enzymes,⁶³ or tumorigenic effects (e.g. AMG-9810).⁶⁴

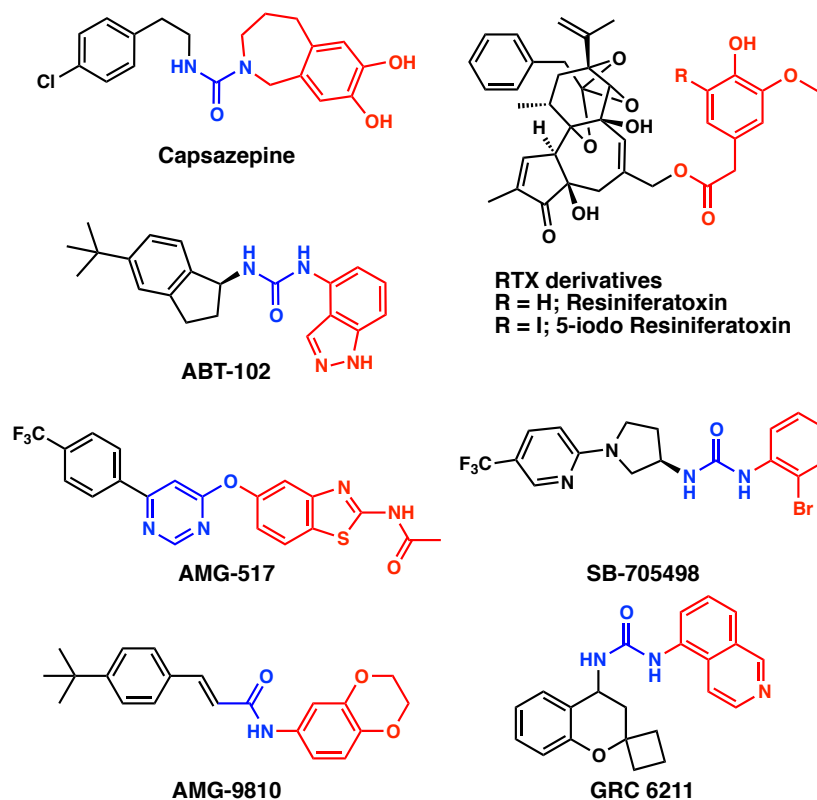


Figure 14. Chemical structures of TRPV1 competitive antagonists showing relevant regions related to capsaicin-receptor interaction: polar (red), linker (blue) and hydrophobic (black) moieties.

As blocking of TRPV1 activity results in a dysregulation of many signaling pathways, temperature neutral TRPV1 antagonists and selective blockers are being developed trying to minimize the undesired effects (Figure 15). AS1928370, developed by Astellas, displays a selective blocking of TRPV1 relative to other TRP channels and little impact on core temperature when given to rats.^{65,66} Products reported by Pharmeste (P14 and P15) claim to have little (< 0,5 Δt) or no impact in body core temperature in rodent assays, with potencies under nanomolar level.^{67,68} These products open new hopes on antagonists as TRPV1 blockers for pain treatment.

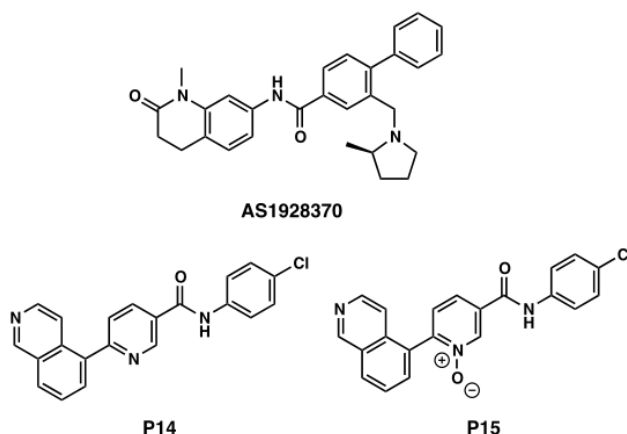


Figure 15. Some examples of TRPV1 selective blockers. AS1928370 developed by Astellas and P14, P15 developed by Pharmeste.

1.5.2.3 Uncompetitive antagonists.

Uncompetitive antagonists interact with additional binding sites on the receptor structure, thereby preventing opening of the receptor by an agonist or blocking its aqueous pore.^{69,70} Among the noncompetitive blockers, open channel blockers are a class of antagonists that recognize a site within the channel aqueous pore, thus being only accessible when the channel is in the open, conductive state. Because of their interaction with active/overactivated receptors, these compounds have attracted a notable interest as potential drugs with better therapeutic index. Ruthenium red, polyarginine peptides and methoctramine are some examples of known uncompetitive antagonists with high to mid potency (100 nM ruthenium red and 2 μ M methoctramine, Figure 16). These products show a low drug profile due to the high flexibility of their structure and exhibit low specificity towards the receptor. In addition some of these products, like the arginine hexapeptides, have agonistic activity at low doses.⁴⁷

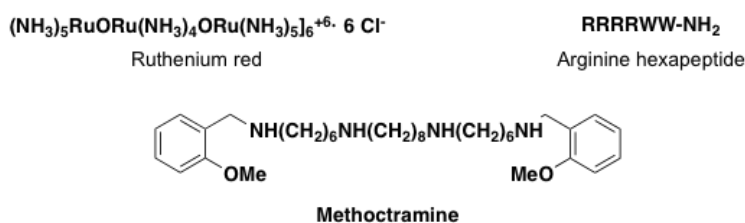


Figure 16. Chemical structures some uncompetitive antagonists of TRPV1.

1.5.3 Recent advances on TRPV1 uncompetitive inhibitors.

Peptoids, or *N*-alkylglycine polymers, are a class of peptidomimetic derivatives whose side chains are appended to the nitrogen atom of the peptide backbone, rather than to the α -carbons.

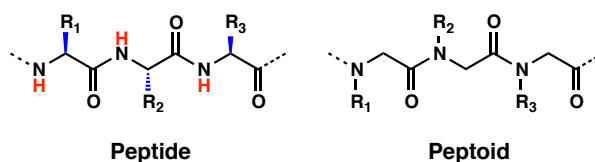


Figure 17. Comparison of the chemical structures of a peptide and a peptoid.

Because of this particular structural feature, peptoids have some physicochemical properties that make them very attractive from a pharmacological point of view. Like β -peptides, peptoids, are resistant to proteolysis in vivo and in vitro and they are also less polar than the respective peptides. This gives peptoids a more interesting bioavailability profile due to lower degradation in stomach and a better gastrointestinal absorption. Exchange of the hydrogen atom by a side chain on the nitrogen blocks the capability to establish hydrogen bonds. Peptide secondary structure is build up thanks to this intramolecular hydrogen bond formation. For that reason peptoids have a larger conformational freedom than peptides. Furthermore, the peptoid scaffold is achiral, avoiding the racemization problems of peptides and tough resolutions of individual products.

Peptoids were first used by Simon *et al.*⁷¹ to mimic protein/peptide interactions to aid in the discovery of protease-stable small molecule drugs. The classic synthetic protocol was performed as a conventional peptide synthesis, on solid phase using Fmoc *N*-substituted amino acids (Figure 18 route a).

Zuckermann *et al.*⁷² redesigned this synthetic method in what is known today as the submonomer protocol (Figure 18, route b), which was based on the use of amines, for the nucleophilic substitution of previously introduced bromoacetic subunits. This protocol had the advantage of avoiding the requirements of the soluble phase preparation of the Fmoc *N*-substituted amino acids, allowing the use of commercial amines which permit to introduce a larger chemical diversity in the final peptoid.

Thus, peptoids are very interesting as pharmacological tools since they can be synthesized as combinatorial collections with great chemical diversity and with a wide capacity of optimization. On the other hand, having a large conformational freedom,

peptoids may interact with different targets, which converts them in promiscuous ligands with frequent selectivity problems, and lack of the structural originality required to be protected by patents.

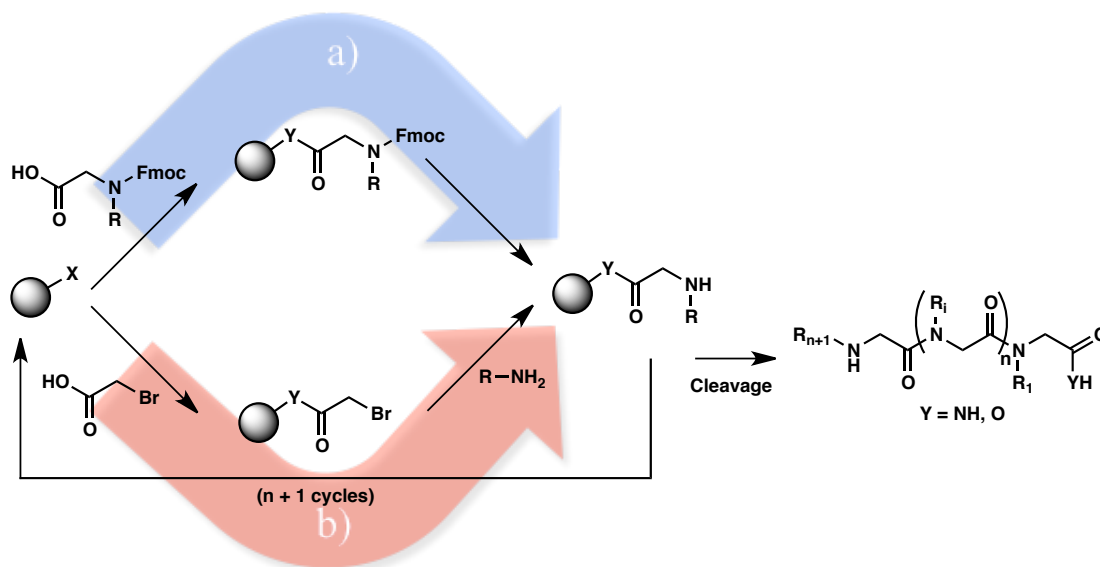


Figure 18. Different strategies for the solid phase synthesis of peptoids, through a) cycles of acylation with Fmoc N-substituted glycines followed by Fmoc group removal, or b) through cycles of acylation with bromoacetic acid and nucleophilic substitution with amines.

The first demonstration of the use of peptoids was in the screening of a combinatorial library of structurally diverse peptoids which yielded novel high-affinity ligands for 7-transmembrane G-protein-coupled receptors.⁷³ After that, the use of peptoids was extended to other pharmacological targets: as antimicrobial agents,⁷⁴ as synthetic lung surfactants,⁷⁵ as ligands for various proteins like the Src Homology 3⁷⁶ or the Vascular Endothelial Growth Factor (VEGF) receptor 2,⁷⁷ and as antibody Immunoglobulin G biomarkers for the identification of Alzheimer's disease.⁷⁸

In our group, two chemical libraries, the first one containing 10648 and the second with 5120 peptoid trimers, were synthesized. These libraries allowed to identify new compounds with a variety of biological activities, ranging from antimicrobial,⁷⁴ antiapoptotic,⁷⁹ anti-TAT and anti-HIV,⁸⁰ and antiSema3A activities,⁸¹ among others.

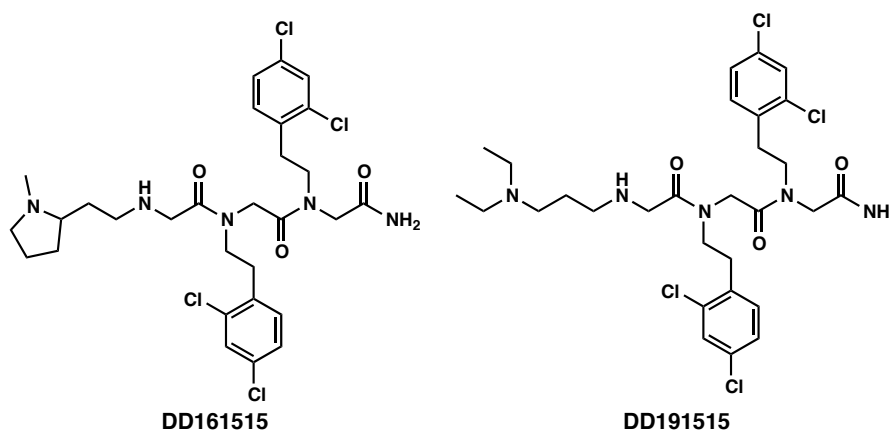


Figure 19. Structure of peptides DD161515 and DD191515.

In addition, two molecules referred as DD161515 and DD191515 (Figure 19) were also identified from those libraries which selectively block the TRPV1 channel with micromolar efficacy ($IC_{50} = 0.7$ and $2.6 \mu\text{M}$, respectively), rivaling the potency of vanilloid-related inhibitors (Figure 20).⁶⁹

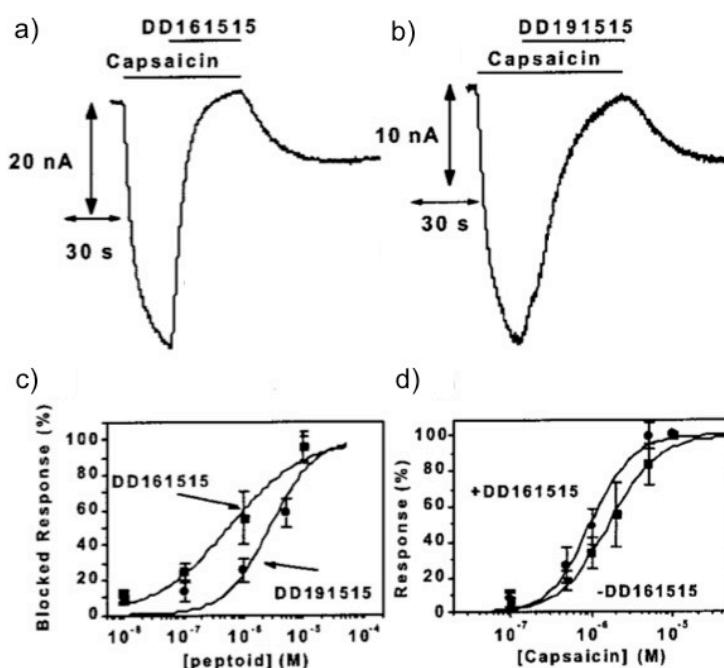


Figure 20. Blockade activity of peptides (a) DD161515 and (b) DD191515 at $10 \mu\text{M}$. (c) Dose/response curves of DD161515 and DD191515. (d) Dose - response curves of VR1 activation by capsaicin in the absence (-DD161515) and presence (+DD161515) of $1 \mu\text{M}$ peptide.⁶⁹

These peptides appeared to be noncompetitive capsaicin antagonists, as suggested by the similar capsaicin concentration required to activate half-maximal response (EC_{50}) in the absence or presence of peptide, which bind from the

extracellular side of the channel at a site close to the aqueous vestibule of the ionic pore (Figure 21).

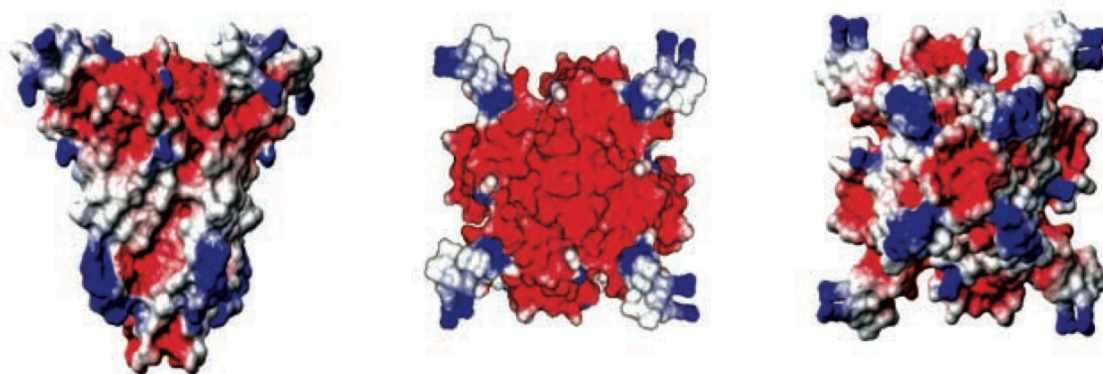


Figure 21. Electrostatic potential mapped on the surface of an homology model of the TRPV1 pore domain, showing three different views of the channel: side (left), extracellular (center) and intracellular (right) perspectives. Color scale goes from red (negative) to blue (positive). The structure of the *Streptomyces lividans* K⁺ channel was used as scaffold to build the homology model.⁸²

Although both peptoids were active *in vivo* in animal models of pain, their development was precluded because of unanticipated side effects arising from the release of calcitonin gene-related peptide from nociceptive neurons when used at submicromolar concentration. It could be speculated that their binding site location was also accessible in the closed state of the channel and that this could account for the undesired effects observed.

Peptoids DD161515 and DD191515 opened a new line for analgesia research. Those were the first small organic, drug-like uncompetitive antagonists of TRPV1 identified. Some years after the work of Garcia-Martinez *et al.* new peptoid derivatives were published with higher potencies. H-Arg-15-15-C⁸³ and an indole containing analog⁷⁰ (Figure 22) are examples, being the last one the most potent uncompetitive peptoid antagonist discovered up to that point, exhibiting an IC₅₀ of 180 nM, about four-fold more potent than peptoid DD161515. Figure 23 summarizes the structures of other peptoid and peptoid-like derivatives designed to block TRPV1.

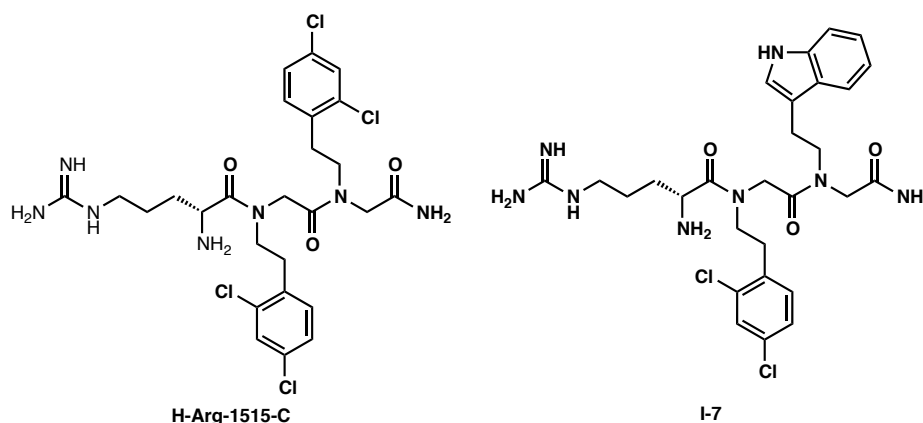


Figure 22. Peptoids derived from the original DD191515 and DD161515.^{70,83}

All of these compounds shared as common structural feature the presence of a positively charged moiety (basic amino or guanidinium group), which might enhance the interaction with the outer pore region of the channel, where acidic residues are known to be located. Furthermore, they also included two aromatic moieties that could interact with hydrophobic residues present at the vestibule of the channel. Consistent with this notion, acidification of the extracellular medium or mutation of D646 to asparagine, a residue in the pore forming region of VR1, reduced the peptoid blockade activity.⁶⁹

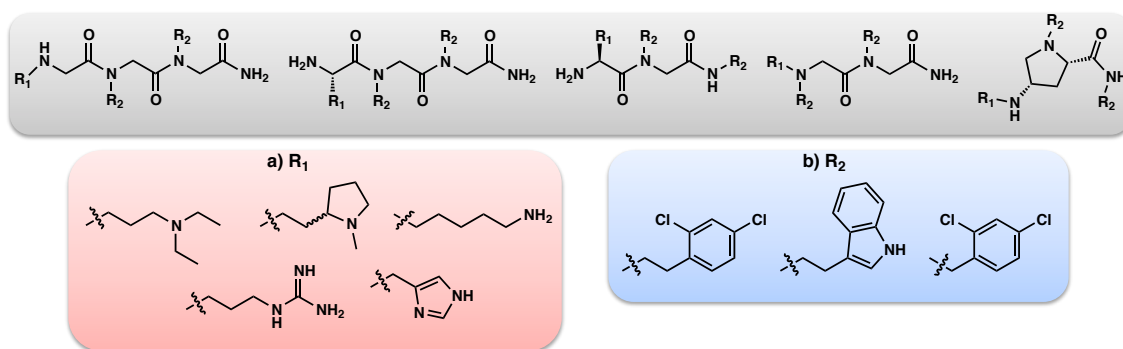


Figure 23. Summary of relevant scaffolds and side chains reported in the literature as TRPV1 uncompetitive antagonists.^{69,70,83}

Hence, a basic 3 point pharmacophore, formed by two aromatic moieties and one cationic moiety separated from the peptoid scaffold by 1-3 carbon atoms, constituted a starting point for the design of new molecules with improved TRPV1 antagonistic properties. Since, as already noted, the high conformational flexibility of peptoids can reduce their biomolecular selectivity as a result of undesired off-target interactions, it was envisaged that it would be advantageous to incorporate conformational restrictions in the design of new molecules, in order to increase the

affinity and pharmacological selectivity, as well as to improve the pharmacokinetic profile of the molecules. An initial result in this sense, was the preparation of dipeptide analogs containing an azetidine-derived arginine residue, which showed high potency and selectivity for TRPV1 relative to N-methyl-D-aspartate receptor (NMDA), although they lacked of analgesic activity *in vivo*, mostly due to their high toxicity.⁸⁴ More recently, during the course of this thesis, additional molecules, i.e. linear and hydantoin-containing dipeptide derivatives, have been designed and evaluated as TRPV1 antagonists based on the above pharmacophore.⁸⁵⁻⁸⁷

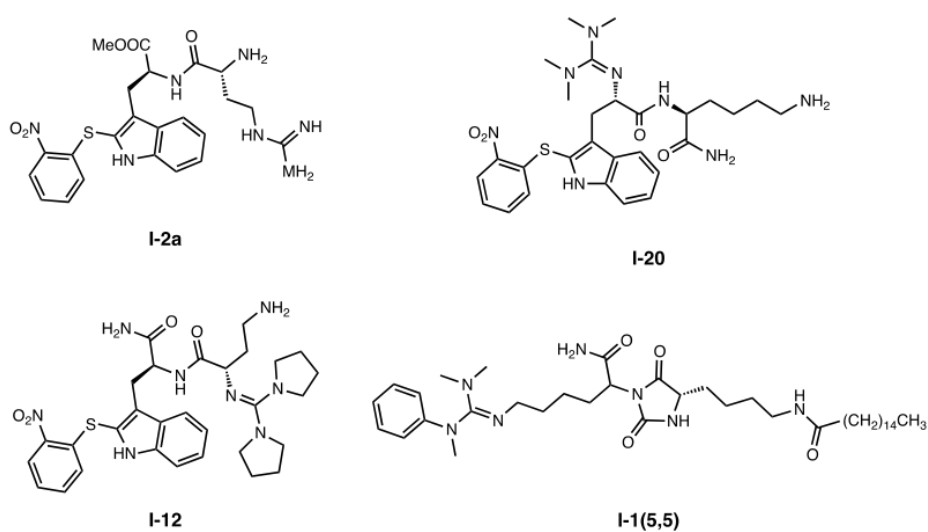


Figure 24. Examples of more rigid peptoid analogues tested against TRPV1. Blocking activities (IC_{50}) of all four compounds are in the low μM range, blocking more than 90 % of the current elicited by capsaicine at 10 μM concentration of antagonist.⁸⁵⁻⁸⁷

