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Chalcone derivatives as non-canonical ligands of TRPV1

Bruna Benso[a,b,c], Daniel Bustos[d], Miguel O. Zarraga[e], Wendy Gonzalez[c,d], Julio Caballero[d], Sebastian Brauchi[b,c,*]

[a] School of Dentistry, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, RM, Chile
[b] Department of Physiology, Faculty of Medicine, Universidad Austral de Chile, Valdivia, Chile
[c] Millennium Nucleus of Ion Channels-Associated Diseases (MiNICAD), Valdivia, Chile
[d] Center for Bioinformatics and Molecular Simulation (CBSM), Universidad de Talca, Talca, Chile
[e] Department of Organic Chemistry, Faculty of Chemical Sciences, Universidad de Concepcion, Concepcion, Chile

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ABSTRACT

Transient receptor potential vanilloid 1 (TRPV1) is a polymodal cation channel activated by heat, voltage, and ligands. Also known as the capsaicin receptor, TRPV1 is expressed in numerous tissues by different cell types, including peripheral sensory fibers where acts as a thermal and chemical detector in nociceptive pathways. TRPV1 channels are able to bind a wide range of ligands, including a number of vanilloid derivatives all modulating channel's activity. When expressed by sensory neurons, activation of TRPV1 channels by heat ($> 40\ ^{\circ}C$), capsaicin (sub-micromolar), or acid environment ($pH < 6$), causes depolarization leading to burning pain sensation in mammals. Naturally occurring chalcones (1,3-diaryl-2-propen-1-ones) have been reported as effective inhibitors of TRPV1. Their relatively simple chemical structure and the possibility for handy chemical modification make them attractive ligands for the treatment of peripheral pain. By taking advantage of the structural information available, here we discuss pharmacological properties of chalcones and their putative mechanism of binding to TRPV1 channels.

1. TRP channels and the capsaicin receptor TRPV1

First described in *Drosophila* (Montell et al., 1985), *trp* genes encode a large family of cation permeable ion channels (Montell, 2005). TRP channel proteins are tetramers, composed by 6 transmembrane subunits and are considered distant relatives of voltage-gated potassium channels (Montell, 2005). Consistent to predictions, structural data revealed that the transmembrane region of each TRP channel subunit has six helical segments (TM1 through TM6) that can be divided into two major functional domains. TM1 through TM4 forms a domain that is structurally similar to the voltage sensor domain of voltage-gated ion channels. TM5 through TM6 assemble into the pore domain containing the gate and the selectivity filter (Fan et al., 2018; Gao et al., 2016; Paulsen et al., 2015; Yin et al., 2018). Sensitive to electric, chemical, mechanical, and thermal stimuli, many of them act as cellular receptors involved in environmental detection (Clapham, 2003; Julius, 2013).

TRPV1 not only belongs to the *Vanilloid* family of TRP channels, it is also the funding member of a group of temperature-activated TRP channels (*thermo*TRPs), composed by representatives from different TRP sub-families (i.e. **V**anilloid, **M**elastatin, **A**nkiryn, and **C**anonical) (Diaz-Franulic et al., 2016). ThermoTRPs are tightly associated with the detection and integration of peripheral sensory input (Caterina et al., 1997; Venkatachalam and Montell, 2007; Zheng, 2013). The close-open transition in thermoTRP channels can be explained by allosteric coupling of the multiple stimuli they detect (Brauchi et al., 2004; Matta and Ahern, 2007; Yao et al., 2010; Zheng, 2013; Diaz-Franulic et al., 2016). Thus, thermoTRP channels emerge as a model to study the polymodal activation of ion channel proteins, where interconnected molecular detectors transduce information to the channel's gate (Brauchi et al., 2004; Diaz-Franulic et al., 2016). The thermal detectors from the Vanilloid family (i.e members 1 through 4) are expressed in the sensory nerve and skin cells, transducing both proprioceptive and nociceptive information to the central nervous system (Julius, 2013).

Originally cloned by using cDNA libraries, TRPV1 channels were identified as the molecular targets for capsaicin hence dubbed Vanilloid Receptor 1 (VR1; Caterina et al., 1997). Indeed, agonist-induced activation of TRPV1 channels has been critical not only to their identification but also for the functional description of their molecular inner workings. TRPV1 channels from human and rat are highly sensitive to capsaicin, widely expressed in peripheral fibers, and form part of pain pathways transducing nociceptive information to the central nervous system (Julius, 2013). In contrast, other vertebrates encode a less

* Corresponding author at: Department of Physiology, Faculty of Medicine, Universidad Austral de Chile, Valdivia, Chile.
E-mail address: sbrauchi@uach.cl (S. Brauchi).

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capsaicin-sensitive (e.g. rabbit) or a capsaicin-insensitive (e.g. chicken) orthologs of TRPV1, making primary sensory neurons from rabbit and chicken to be impervious to pungent natural vanilloids (Wood et al., 1988; Jordt and Julius, 2002; Gavva et al., 2004). Early comparative studies using chicken and rabbit TRPV1 orthologs underscore residues Tyr511, Ser512, and Thr550 as critical for capsaicin-induced activation of the channel (Jordt and Julius, 2002; Gavva et al., 2004). These naturally occurring mutations were mapped to the transmembrane region, within the TM1-TM4 domain, a portion of the protein that suffered high evolutionary pressure (Doñate-Macián and Perálvarez-Marín, 2014), and gives the impression to serve as a Ligand Binding Domain (LBD) in TRP channels. The lower part of such motif is the linker between TM4-TM5 that is in close proximity to the TRP domain helix (TDh) (Liao et al., 2013). The TDh is the extension of the TM6 that forms an amphipathic alpha-helix running parallel to the membrane (Liao et al., 2013). The integration of the different molecular detectors present in the channel (such as the LBD) with the TDh seems to provide a reasonable explanation for channel modulation and polimodal activation (Cao et al., 2013; Gregorio-Teruel et al., 2014; Steinberg et al., 2014; Yang et al., 2015; Elokely et al., 2016; Teng et al., 2016).

2. Ligand coordination in TRPV1 channels

The canonical TRPV1 agonist capsaicin binds to the channel with high affinity (Caterina et al., 1997; Yang et al., 2015). Multiple vanilloid-related compounds bind to the channel with different affinities, modulating the open-close equilibrium differently, sometimes causing dissimilar physiological outcomes. A good example can be drawn from capsaicin and capsiate, a non-pungent capsinoid that activates TRPV1 (Iida et al., 2003) (Fig. 1A). In this case the differences in affinity and hydrogen bonding are transduced into differences in potency, causing a different nociceptive response that is accompanied with the differential expression of thermogenic genes (Iida et al., 2003; Baskaran et al., 2018). Interestingly, a good deal of TRPV1 ligands are of natural origin and slight derivatives of the natural chemical structure often present differences in activity (Darré and Domene, 2015). Some examples of natural compounds that modulate TRPV1 activity include capsaicinoids (from pungent peppers), capsinoids (find in non-pungent peppers), piperines, eugenol, resiniferols, gingerols, phytocannabinoids (including tetrahydrocannabinol and cannabidiol), dialdeydes terpenes (e.g. polygodial and drimenol), and triprenyl phenols (e.g. grifolin and scutigeral) (Meotti et al., 2014). In traditional natural medicine these compounds are obtained from plants (i.e. seeds, bark, flowers, fruits, or rhizomes), fungus, or insects. These extracts are generally used in the form of crude preparations, essential oils, or infusions. Most of these naturally occurring compounds present high affinities for the capsaicin receptor (in the low μM to pM range), good water solubility, and in many cases adequate cellular bioavailability (Premkumar, 2014; Lu et al., 2017).

During late years, several research groups have obtained TRP channel structures either by single particle cryoEM or X-ray crystallography. These include the six members of the TRPV family (Deng et al., 2018; Gao et al., 2016; Hughes et al., 2018; Huynh et al., 2016; Saotome et al., 2016; Singh et al., 2018). Special importance has been given to the cryo-EM data available for TRPV1, obtained in lipid nanodisks and in the presence or absence of different ligands (Gao et al., 2016). All this structural information available benefits the formulation of more elaborate questions such as how agonists and antagonists bind to a similar region, but might transduce opposite information (Darré and Domene, 2015) and to put forward reasonable models for channel activation by ligands (Yang et al., 2015; Elokely et al., 2016).

Archetypal TRPV1 ligands (such as capsaicin, capsazepine, and resiniferotoxin) can be structurally divided in three regions defined by a vanilloid or catechol group (head), a linker (neck), and a hydrophobic group (tail) (Fig. 1A). The binding mode of capsaicin likely adopts a configuration of tail-up in the direction of the TM4 helix and head-

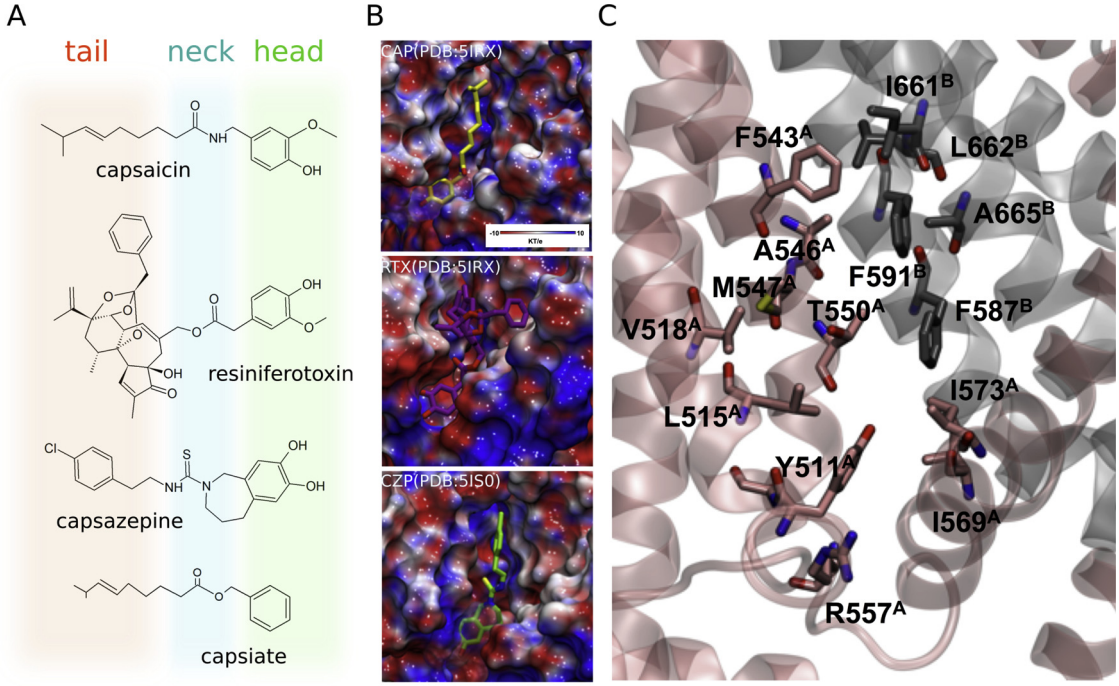

Fig. 1. Canonical modulators of TRPV1. A. Chemical structures of canonical TRPV1 ligands. These compounds are composed of three pharmacophoric regions: 4-hydroxyphenyl (*head*), a connecting amide, ester or thiourea groups (*neck*), lipophilic side chains (*tail*). The *tail*, *neck*, and *head* groups are shaded in orange, blue, and green respectively. **B.** Eletrostatic potencial maps for the binding cavity occupied by capsaicin (top), resiniferotoxin (middle), or capsazepine (bottom). The structures were prepared with the Protein Preparation Wizard in the Schrödinger Suite. The electrostatic potential surfaces were computed on the TRPV1 structures bound to resiniferotoxin (RTX; PDB: 5IRX), CZP (PDB: 5IS0), and a docked capsaicin (CAP) molecule on ligand stripped 5IRX structure, using the APBS software. **C.** The vanilloid-binding site highlighting important residues. Suffixes “a” and “b” denote different side chains of the tetramer. Images were prepared on VMD.

down towards the TM4-TM5 linker (Yang et al., 2015) (Fig. 1B). When bound, the polar the methoxy group of the vanillyl head is hydrogen bonded to Tyr511. The hydroxyl group and amide nitrogen are hydrogen bonded to Glu570, Tyr511, and Thr550 (Fig. 1C). The rest of the molecule is stabilized by hydrophobic interactions (Fig. 1C). The larger binding affinity of RTX compared to capsaicin is likely supported by strong hydrogen bonds between the vanilloid head group with Glu570, Arg557, and Ser512 together with interactions of the ester group (i.e. the *neck* of the ligand) with Tyr511 and Thr550 (Elokely et al., 2016). Functional studies on capsazepine (a competitive vanilloid antagonist) suggest a similar occupancy within the hydrophobic pocket with the exception of interactions with Arg557 and Glu570 that are seemingly essential to facilitate the movement of the intracellular linker TM4-TM5. Thus, important interactions between ligands and the receptor include hydrogen bonds between the polar *head* group and the residues Tyr511, Ser512, and Arg557 (located deep down into the binding cavity); hydrogen bonds between the functional group at the *neck* segment with residues Thr550 and Tyr511. On the other hand, hydrophobic interactions are formed between the *tail* of the ligand and the hydrophobic residues Leu515, Val518, Phe543, Ala546, Met547, Ile569, and Ile573. Interestingly, the group at the tail can also interact with hydrophobic residues of the neighboring subunit, including Phe587, Phe591, Ile661, Leu662, Ala665, and Leu669 (Fig. 1B and C). The presence of multiple sites of intra- and inter-subunit contact helps to understand whether slight changes in the structure of the ligand may result in different conformations of the protein.

3. Structure-directed design of novel modulators of TRPV1

The diversity of the functional groups present in the *head*, *neck*, and *tail* regions modulates potency and pharmacokinetic properties of TRPV1 ligands (Lee et al., 2011). In this context, the properties of the neck region in both agonists and antagonists play an important role in modulating potency and affinity. The neck region usually contains functional groups such as *amine* and *oxo* having hydrogen bond donor/acceptor properties (Appendino et al., 2007; Cheung et al., 2008; Norman et al., 2007; Xi et al., 2005). The substitution of the linker by non-isosteric groups or its deletion decreases or even removes both agonist and antagonist activity (Walpole et al., 1993; Suh et al., 2003). The synthesis of *N*-acylcinammide derivatives showed that *s-cis* double bond confers rigidity to the ligand molecule and contribute to the antagonist activity (Norman et al., 2007). Thereby, the incorporation of functional groups introducing conformational restrictions between the *neck* and *tail* regions (e.g. pirazynes or pirimidines) correlates well with obtaining antagonistic drugs with improved potency and selectivity (Hodgetts et al., 2010; Yang et al., 2015).

Small molecules from secondary metabolism of land plants appear to provide a simple and modular scaffold useful for the design of novel TRPV1 ligands. These natural compounds are widely distributed in plants and classified in six mayor groups: chalcones, flavones, flavonols, flavandiols, anthocyanins, and proanthocyanidins (Nowakowska, 2007). Chalcones (1,3-diaryl-2-propen-1-ones) are open chain flavonoids in which two aromatic rings are joined by a short three-atom carbon chain (Batovska and Todorova, 2010) (Fig. 2A). They exhibit anti-inflammatory, antimicrobial, and antifungal properties and have been suggested as pharmacological targets of ion channels (e.g voltage-gated sodium and potassium channels), cellular receptors (e.g. Toll-like receptor 4), and enzymes (e.g. COX-2/5-LOX) (Nowakowska, 2007; Mahapatra et al., 2015; Seleem et al., 2016). Structural analogues such as cinnamides or pentadiamines have been explored in more detail, suggesting an inhibitory activity against TRPV1 channels (Saku et al., 2012). Dragon's blood (from *Dracaena cochinchinensis*) is an ancient mixture of compounds, traditionally obtained directly by grinding parts of the plant. The extract has been reported to have analgesic properties among other therapeutic uses (Gupta et al., 2008). Loureirin B, cochinchinenin A, and cochinchinenin B are naturally occurring chalcones, isolated from Dragon's blood extracts (Fig. 2A), all having an inhibitory effect against TRPV1 (Wei et al., 2013).

In looking for simple molecular scaffolds to design inhibitory molecules, our rational design aimed first to restrict conformations by introducing a double bond around of the neck (Fig. 2A, pink *box*), and second, to promote minimum but localized hydrogen bonding to the protein receptor by eliminating the majority of functional groups along the molecule (Fig. 2A). After such stripping off of properties, the tested molecules, (*E*)-1-(4-bromophenyl)-3-phenylprop-2-en-1-one (Chalcone 1, CH1) and (*E*)-1-(4-bromophenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (Chalcone 2, CH2) still preserve the hydrophobic spacer and the size of previously reported chalcones of natural origin (Fig. 2A). With the addition of a single polar functional group for the case of CH2 we aimed to promote localized dipole interactions with a restricted set of side chains.

Molecular docking simulations (using Glide; Friesner et al., 2004) yielded two preferential poses for CH1 and CH2 with scoring energies of -32.4 and -44.6 kcal/mol respectively (Fig. 2B). In all cases keeping the head-down tail-up orientation, suggesting a conserved mechanism for binding when compared to canonical ligands (Fig. 2B, *top left*). When compared to loureirin B (scoring energy of -44.8 kcal/mol), subtle but important differences can be appreciated. In contrast to CH1 and CH2, loureirin B establish hydrogen bonds with Ser512 and Thr550 (Fig. 2B, *top right*), absent in the new chalcones (Fig. 2B, *bottom panels*). Interestingly, the 4-bromophenyl groups of both CH1 and CH2 are found close to Thr550 suggesting hydrophobic interactions with this essential residue (Fig. 2B, *bottom panels*). The polar head of CH2 is hydrogen bonded to residues Ser512 and Arg557, somewhat resembling interactions observed between vanilloid-related agonists and the receptor (Fig. 2B, *bottom right*). For the case of CH2, the obtained poses are different regarding the position of the 4-bromophenyl group, which is located near the residue Thr550 in the first pose, and near the residues of the neighboring subunit in the second pose (Fig. 2B, *bottom right*). To investigate the effect of these ligands we performed a preliminary evaluation by calcium imaging experiments. Fluo4AM-loaded HEK293 T cells expressing TRPV1 channels were stimulated with capsaicin (1 μM) and the $[Ca^{2+}]_i$ response was recorded (Fig. 2C). Incubations with both chalcone ligands (sub-micromolar) were effective to prevent the sustained capsaicin-induced increase in $[Ca^{2+}]_i$ (Fig. 2D). Interestingly, cells treated with supersaturating concentrations (10μM) of CH1 showed a brief initial elevation of $[Ca^{2+}]_i$, followed by a rapid and strong decrease of the signal (Fig. 2C). This might suggest that CH1 is able to transit between coordination states and that the initial orientation of CH1 within the binding pocket is simply not able to stabilize the open state of the channel, preventing a sustained calcium entry. Such chalcone-induced activation was not observed for CH2 at the same concentration (Fig. 2C). Still, when the cells are incubated with the compounds at sub-micromolar concentrations, both CH1 and CH2 function as potent antagonists, probably by just occupying the binding site, preventing later capsaicin-induced channel activation. It has been suggested that vanilloids and chalcone compounds may exhibit a cytotoxic effect against tumor and cancer cells (Prevarskaya et al., 2007; Mahapatra et al., 2015). Therefore, we performed a cell viability assay, normalized to vehicle treated control group. Transfected and naïve HEK293 T cells were incubated for 24 h with either CH1 or CH2 compounds. MTT assay confirmed that the concentrations used in our experiments were safe below 300 nM (> 95% viability at 100 nM). For all the conditions IC50 was in the low-micromolar range (2–5 μM). Moreover, at the saturating concentration of 30 μM viability drops down up to 40% and 25% for CH1 and CH2 respectively. This suggests a more general and non-specific cytotoxic effect of the compounds in the micromolar concentration range. More thorough experimental evidence will be needed to address the modes of action of these and other chalcone derivatives.

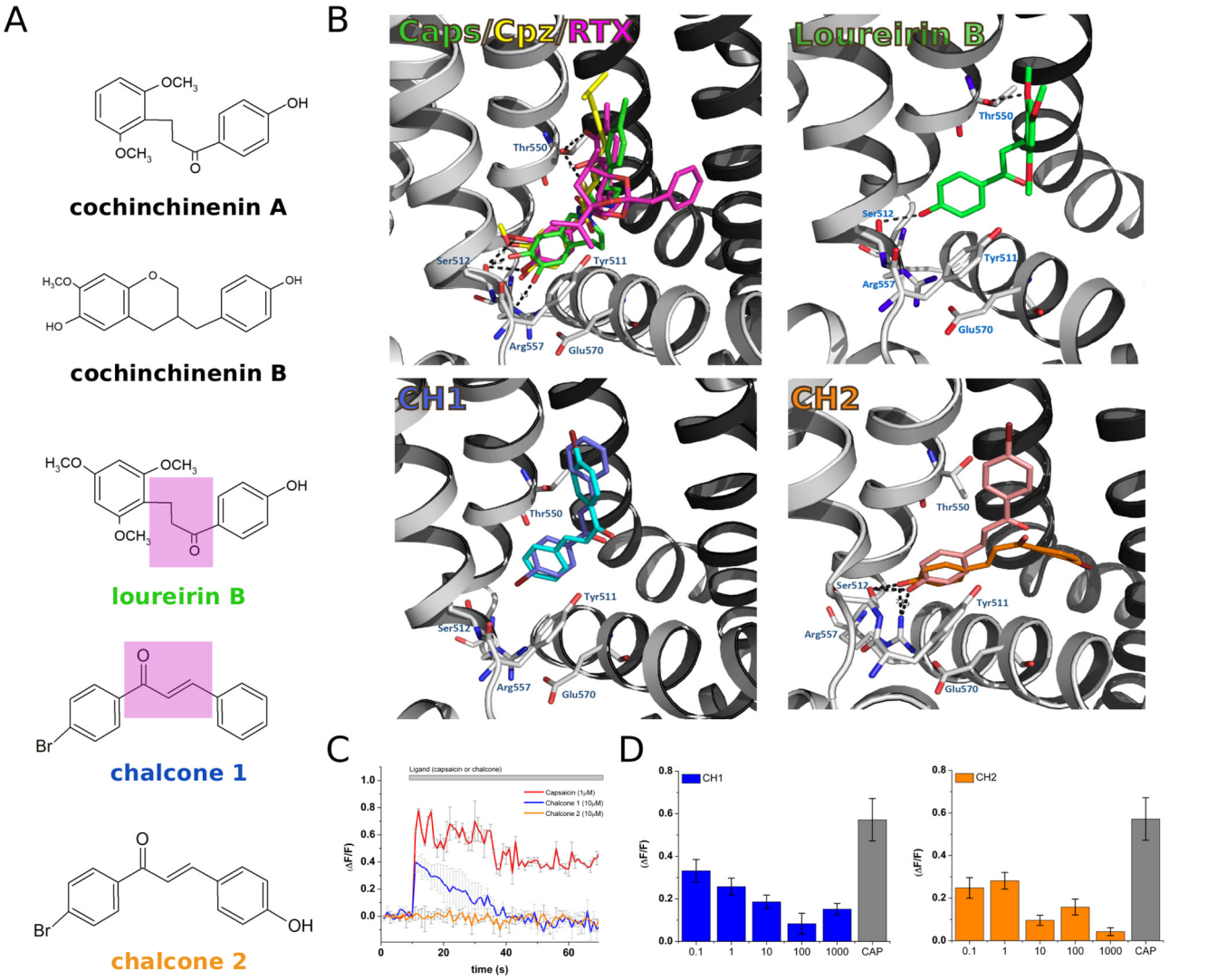

Fig. 2. Chalcone interaction with TRPV1. **A.** Chemical structures of chalcone ligands. Pink shades highlight the difference in the *neck* group between loureirin B and the designed novel chalcones. **B.** *Top left*. Docking simulations showing the orientations of RTX (purple), capsaicin (yellow), and capsazepin (green) inside the TRPV1 binding site (PDB:5IRX). *Top right*. Docking simulation for loureirin B (green). *Bottom panels.* The most favorable docking orientations for CH1 (*left,* blue shades) and CH2 (*right,* orange shades) inside TRPV1's binding site. *Docking simulation procedure*. The prepared molecules were docked in TRPV1 using the Standard Precision (SP) mode of Glide and the best two poses (as ranked by the docking scoring energies) are reported in the figure. The crystallized ligand was removed and a grid box ($22 \times 22 \times 22$ Å^3) was set in the binding pocket near the residues Tyr511, Ser512, Thr550, Arg557 and Glu570, covering the reported binding site for capsaicinoid-type molecules. CAP, CH1, CH2, and loureirin B were sketched and prepared with LigPrep using the force field OPLS v2005. **C.** Acute ligand-induced response. Time-lapse of fluorescence in cells loaded with Fluo4AM and exposed to capsaicin (1 μM), chalcone 1 (10 μM), or chalcone 2 (10 μM). The gray bar on top indicates when the ligand was added to the bath solution. All traces correspond to the average of three independent experiments; bars correspond to SD. **D.** Incubation chalcones inhibits capsaicin-induced calcium elevation. Averaged cell response to 1 μM capsaicin after 5 min incubation with CH1 (*top*) or CH2 (*bottom*). Incubation was performed at different concentrations (ranging from 0.1–1000 nM, as indicated). The recording media was a physiological extracellular buffer containing 2.5 mM $CaCl_2$. Changes in fluorescence intensity correspond to the difference between the equilibrium level before and after stimulation and normalized by the initial average value (ΔF/F).

4. Concluding remarks

TRP channels have been directly related to a wide spectrum of physiological traits and pathological conditions (Rosenbaum and Simon, 2007; Nilius and Owsianik, 2010). Current research links TRPs to hereditary neuropathies, neuronal disorders, and other channelopathies (Kiselyov et al., 2007; Gees et al., 2012; Julius, 2013). Thus, the understanding of the molecular mechanisms associated to TRPV1-ligand interaction may provide important information leading to the development of novel tools, useful for clinical therapeutics (Cui et al., 2016). Our preliminary findings put forward chalcones as novel chemical structure scaffolds. Presenting a variety of possibilities for chemical modification, this family of ligands allows simple rational design. Exploring their modes of action promoting either activation or inhibition of TRPV1 activity makes them attractive for biophysical research and pharmacological exploration. Research in chalcone derivatives may well serve to design novel and more selective ligands, not only for TRPV1 receptors, but also for other channels of the TRPV family.

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