Title: Small molecule positive allosteric modulation of TRPV1 activation by vanilloids and acidic pH

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Non-standard Abbreviations:

TRPV1, transient receptor potential cation channel subfamily V member 1; DRG, dorsal root ganglion; PAM, positive allosteric modulator; RTX, resiniferatoxin; DHP, dihydropyridine; PMA, phorbol 12-myristate 13-acetate; RR, ruthenium red; HS, Hill Slope

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Abstract:

TRPV1 is a high-conductance, non-selective cation channel strongly expressed in nociceptive primary afferent neurons of the peripheral nervous system and functions as a multimodal nociceptor gated by temperatures above 43°C, protons, and small-molecule vanilloid ligands like capsaicin. The ability to respond to heat, low pH, vanilloids and endovanilloids, as well as altered sensitivity and expression in experimental inflammatory and neuropathic pain models made TRPV1 a major target for the development of novel, non-opioid analgesics, and resulted in the discovery of potent antagonists. In human clinical trials, observations of hyperthermia and the potential for thermal damage by suppressing the ability to sense noxious heat suggested that full-scale blockade of TRPV1 function can be counter-productive and subtler pharmacological approaches are necessary. Here we show that the dihydropyridine derivative MRS1477 behaves as a positive allosteric modulator of both proton and vanilloid activation of TRPV1. Under inflammatory-mimetic conditions of low pH (6.0) and PKC phosphorylation, addition of MRS1477 further increased sensitivity of already sensitized TRPV1 towards capsaicin. MRS1477 does not affect inhibition by capsazepine or ruthenium red and remains effective in potentiating activation by pH in the presence of an orthosteric vanilloid antagonist. These results indicate a distinct site on TRPV1 for positive allosteric modulation that may bind endogenous compounds or novel pharmacological agents. Positive modulation of TRPV1 sensitivity suggests that it may be possible to produce a selective analgesia through calcium overload restricted to highly active nociceptive nerve endings at sites of tissue damage and inflammation.
**Introduction:**

TRPV1 is a key ion channel for sensing noxious stimuli, is activated by capsaicin, protons or heat, and is highly expressed in nociceptive Aδ and C-fiber sensory afferent neurons of the peripheral nervous system (Caterina et al., 1997; Tominaga et al., 1998; Mitchell et al., 2010). These fibers are responsible for both the initiation and the transmission of nociceptive signals, and pathological pain states often correlate with their altered functioning (Gracely et al., 1992; Costigan et al., 2009; Bruehl, 2010). Increased amounts of TRPV1 in nerve endings at sites of neurogenic inflammation and in models of neuropathic pain, and sensitization of TRPV1 by NGF released from inflamed tissues, are consistent with the idea that TRPV1 plays a key role in chronic pain and hyperalgesia (Cortright and Szallasi, 2004). Observations that TRPV1 agonists such as RTX or capsaicin can either outright ablate (Karai et al., 2004; Brown et al., 2005) or render nociceptive afferent nerve endings unresponsive (Neubert et al., 2003) to a broad palette of noxious stimuli led to the development of therapeutic approaches by localized delivery. These include intrathecal administration of RTX to control pain in advanced cancer (Karai et al., 2004) and the topical use of high concentrations of capsaicin for post-herpetic neuralgia (Backonja et al., 2008), complex regional pain syndrome (Robbins et al., 1998), experimental inflammatory or neuropathic pain models (Nolano et al., 1999; Neubert et al., 2003; Bates et al., 2010).

Blockade of TRPV1 at the orthosteric capsaicin site also has therapeutic potential, and considerable effort has been directed at developing orally available TRPV1 antagonists as analgesics by the pharmaceutical industry and academia (Wong and Gavva, 2009). These efforts spanning the last decade, while producing extraordinarily potent and specific antagonists, fell
short of producing the expected analgesic drugs due to side-effects such as unpredictable levels of hyperthermia and whole-body suppression of thermosensation in the 48-50°C range, increasing the chance of burns associated with their use (Wong and Gavva, 2009; Rowbotham et al., 2011). Structural modifications to produce peripherally restricted antagonists, and thereby eliminate potential actions on CNS thermoregulatory mechanisms, did not solve the problem of hyperthermia (Tamayo et al., 2008). The aforementioned problems underscore the necessity to explore novel pharmacological approaches in the development of TPRV1-targeted analgesics.

Allosteric agonism is a well-known quality of TRPV1 since protons and small-molecule ligands with a vanilloid moiety activate the ion channel at spatially distinct and structurally divergent domains (Gavva et al., 2005; Ryu et al., 2007). Moderate proton concentrations of a slightly acidic extracellular milieu (~pH 6.0) will not gate the channel but will increase its sensitivity towards vanilloid ligands (Jordt et al., 2000), an example of positive allosteric modulation. Thus, a logical next step is to explore existing chemical libraries for compounds that are allosteric modulators of TRPV1, rather than direct inhibitors of the orthosteric capsaicin binding site. Here we characterize in detail the 1,4-dihydropyridine (DHP)-derived compound MRS1477 (Supplemental Figure 1), previously identified as an enhancer of capsaicin activation of TRPV1 (Roh et al., 2008). We show that it is a generalized potentiator of vanilloid and/or proton activation of TRPV1. Studies using different classes of TRPV1 inhibitors suggest that this compound (MRS1477) does not interact directly with ligands targeting the vanilloid binding site or with the channel pore. We propose that MRS1477 is a proof of concept compound showing the feasibility of small molecule positive allosteric modulation of TRPV1. The results also
suggest the presence of sites on TRPV1 that exhibit activity- or state-dependent properties and potentially novel analgesic pharmacology.
Methods:

Cell culture: The HEK-293 cell line expressing rat TRPV1 (HEK293-TRPV1) (Caterina et al., 1997) was maintained following ATCC guidelines for HEK-293 cells, supplemented with 200 µg/ml G418 sulfate.

$^{45}\text{Ca}^{2+}$ uptake assays: Cells were seeded a day before the experiment to 96-well plates at a density of $4 \times 10^4$ cells/well. Drugs were diluted on separate 96-well plates using $^{45}\text{Ca}^{2+}$ containing (0.5 µCi / well) buffer in a total volume of 100 µl/well. All experiments followed the same pattern, and were conducted using a Biomek FX liquid handling robot (Beckman): (1) removing cell culture medium, (2) washing the cells using Assay Buffer (3) simultaneous transfer of drugs and $^{45}\text{Ca}^{2+}$ to all 96 wells (4) 5–8 minutes of incubation at room temperature, depending on agonist (5) removing $^{45}\text{Ca}^{2+}$ and drugs (6) washing the cells (7) lysis in hypotonic lysis buffer [1% Triton X-100, 1% SDS] (8) transfer of 75 µl lysate to 96-well OptiPlate (white, PerkinElmer) already containing 125 µl Microscint-40 (PerkinElmer) scintillation cocktail. We used a TopCount NX (Packard) liquid scintillation counter to quantify the $^{45}\text{Ca}^{2+}$ signal. Treatment and control experiments were run on the same plate; we also included a full, six to seven point capsaicin dose-response curve for within-plate normalization and to check the plate-to-plate consistency of our data.

Intracellular $\text{Ca}^{2+}$ imaging: Cells were seeded on a coverslip the day before the experiment. A 4µM solution of the ratiometric $\text{Ca}^{2+}$ dye Fura4F-AM was used to load cells for 45 min at room temperature, followed by a 15 min period to permit for the complete cleavage of the AM groups from the dye. The imaging apparatus consisted of an Olympus BX60 microscope equipped with
a Hamamatsu ORCA 12-bit monochrome CCD camera. Illumination was provided by a Lambda LS xenon arc lamp light source, and switching between excitation and emission filters was performed with a Lambda 10-2 filter wheel controller, all from Sutter Instruments. Coverslips with cells were mounted on the microscope stage using the p21B perfusion chamber and P2 platform (both from Warner Instruments) and then imaged using the Olympus UApO/340 20× objective. Image acquisition and filter switching during the experiment were controlled through the Metafluor (Molecular Devices) software package. Perfusion speed was 0.732 ml/min using a Gilson Minipuls 3 peristaltic pump; drug injection was done by manual operation of a HPLC valve linked to a 0.5 ml sample loop.

**Electrophysiology**: HEK293-TRPV1 cells were plated on 35mm dishes at a density of $10^5$ cells per dish. Electrophysiological experiments were performed on cells at room temperature using the whole-cell patch-clamp recording technique. Currents were recorded using an Axopatch 200B patch clamp amplifier (Molecular Devices) and were filtered at 2 kHz using a low-pass Bessel filter. Patch electrodes, were fabricated from borosilicate glass (type 1B150F3; World Precision Instruments) on a Brown Flaming horizontal puller (P87; Sutter Instruments) and heat polished to a final tip resistance of 2–4 MΩ. All current records were captured and stored using the pClamp9 software packages in conjunction with a Digidata 1322A analog-to-digital converter (Molecular Devices). Patch electrodes were filled with a solution containing the following (in mM): 142 NaCl, 1 MgCl₂, 10 EGTA and 10 HEPES; pH was adjusted to 7.35 using 10 M NaOH. The osmolarity of the internal solutions was 306 mOsm. The bath solution contained the following (in mM): 148 NaCl, 3 KCl, 2 MgCl₂, 10 glucose and 10 HEPES, pH was adjusted to 7.35 using 10 M NaOH. No calcium was included in the extracellular solution in
order to prevent receptor desensitization. The osmolarity of this solution was 295–305 mOsm. Capsaicin, MRS1477 and acidic solutions were prepared daily in bath buffer and applied using a fast gravity-driven rapid solution changer (RSC-200, Biologic Instruments). The current responses were recorded from single cells clamped at -60 mV or in 3-second voltage-ramps ranging from -80 to +80 mV.

**Core body temperature measurements:** Procedures followed the NIH Guidelines for the care and use of laboratory animals, and were approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. Core body temperature was measured using intraperitoneal implanted telemetric temperature probes (Data Sciences International), telemeter signal was processed using a model RPC-1 receiver, data exchange matrix and a DATA Quest ART acquisition system (Data Sciences International). Telemetry devices were placed in the abdominal cavity of C57BL/6 mice (2–4 months old, 20–30g) as described (Mishra et al., 2011). Experiments were initiated at least 2 weeks after surgery. Drugs were administered by intraperitoneal injection in a solution of 10% ethanol, 0.5% Tween, in physiological saline. To facilitate dispersion, MRS1477 was sonicated prior injection.

**Solutions and Buffers:** The assay buffer used for $^{45}$Ca$^{2+}$ assays contained $1\times$ Ca$^{2+}$, Mg$^{2+}$ free HBSS (diluted from 10× HBSS, Gibco 14815) supplemented with 0.8 mM MgCl$_2$, 10 mM glucose with the pH set to 7.4 using 10 mM Hepes. The same buffer was used for diluting drugs and $^{45}$Ca$^{2+}$. When preparing pH gradients for proton activation experiments, an unbuffered physiological salt solution was made consisting of 138 mM NaCl, 5.33 mM KCl, 0.8 mM MgCl$_2$, 10 mM glucose and no buffering agent, pH was set by mixing the sodium salt of Hepes and MES hydrate to a total concentration of 10mM. Osmolality of the buffers was always set to
325mOsm using sucrose. All solutions containing capsaicin also contained 1mM ascorbic acid to protect it from oxidation. For intracellular Ca\(^{2+}\) imaging the assay buffer was supplemented with 2.5 mM CaCl\(_2\).

**Drugs and stock solutions:** Capsaicin (Caps, Sigma Cat#M2028) was prepared as 10mM stock solution in 100% ethanol; 1–2mM intermediate concentrations were prepared fresh on the day of the experiments in 75% ethanol, 2mM ascorbic acid. This solution was used to prepare working concentrations of capsaicin (1–2\(\mu\)M, ~0.08% ethanol), which were always discarded at the end of the day, the ascorbic acid was contributed by the assay buffer, see above). NADA (Tocris Cat#1568) was prepared as 12mM stock solution and resiniferatoxin (LC Laboratories, Cat#R-6712) was prepared as 3.18mM stock solution both in 100% ethanol. Ruthenium Red (RR, Tocris Cat#1439) was prepared as 50mM stock solution in Ultra Pure Water (KD Medical Cat#RGF-3410). MRS1477 (Roh et al., 2008), Capsazepine (CPZ, Tocris Cat#0464) and phorbol 12-myristate 13-acetate (PMA, Tocris Cat#1201) stock solutions were prepared at 20, 50 and 50mM concentrations, respectively, in 100% DMSO. Drugs used in \(^{45}\)Ca\(^{2+}\)-uptake, intracellular calcium imaging and electrophysiology experiments were diluted from these stock solutions at least 500-fold, with final DMSO and ethanol concentrations not exceeding 0.15% for DMSO and 0.08% for ethanol in buffers during experiments. The highest concentrations of MRS1477 in the above vehicle produced no effect on calcium uptake (see Supplementary Figure 1).

**Statistical Analyses:** Students’s \(t\)-test was used for pairwise comparisons, and one-way ANOVA test was used for multiple comparisons with Bonferoni post-hoc test except for the analysis of voltage-ramp results where Dunnett post-hoc test was used. Differences were considered
significant where $P<0.05$. Curve fit of dose-response experiments was done using Prism 5.0 (Graphpad) “least squares” fit.
Results:

**MRS1477 positively modulates both vanilloid and proton activation of TRPV1**

1,4-DHP compounds are known to interact with calcium channels (Triggle et al., 1989) and MRS1477 was identified as small-molecule potentiator of TRPV1 activation by capsaicin upon screening a library of 1,4-DHP compounds (Roh et al. 2008). In the present study our initial objectives were to probe the generality of the TRPV1 enhancing effect by (a) expanding the range of vanilloid ligands and (b) determining whether proton activation of TRPV1 could also be positively modulated. For this, $^{45}\text{Ca}^{2+}$-uptake assays were conducted using HEK293 cells expressing TRPV1 (HEK293-TRPV1, a kind gift from M. Caterina). We subsequently explored combinations of inflammatory-mimetic agents and the pharmacological actions of MRS1477 using electrophysiological, cell biological and in vivo approaches.

Capsaicin-induced calcium uptake through TRPV1 shows sensitivity enhancement with a decrease in the EC$_{50}$ of capsaicin from 77.7nM +/-3.72, HS 1.97+/0.19 to 30.2nM +/-1.46, HS 2.01+/0.19 in the presence of 20µM MRS1477 (Figure 1A). Potentiation of vanilloid agonist activity is a generalized feature of MRS1477 that is independent of agonist potency since both NADA, a proposed endogenous ligand with low potency (Figure 1B), and the ultra-potent agonist RTX (Figure 1C) show comparable potentiation in the presence of MRS1477, reducing their respective EC$_{50}$ values from 4.48µM +/-0.66, HS 1.34+/0.07 and 21.5nM +/-1.82, HS 1.33+/0.08 to 1.71µM +/-0.13, HS 1.72+/0.2 and 6.59nM +/-0.57, HS 1.09+/0.05, respectively. Beside sensitization, MRS1477 also produces a small but consistent increase in the
maximum response of TRPV1 at saturating doses of vanilloid agonists (~10% enhancement in the presence of 20 µM MRS1477, Figure 1A-C).

Proton activation of TRPV1 is also enhanced by MRS1477: calcium uptake is progressively potentiated as pH decreases, producing between 90% to 80% enhancement of Ca\(^{2+}\) uptake at pH values of 5.5 and 4.8, respectively (Figure 1D). The EC\(_{50}\) value for proton activation also shows a small but significant leftward shift from pH 5.65 +/-0.03 under control conditions to pH 5.87 +/-0.03 in the presence of 20 µM MRS1477; however, the effect resembles an increase in efficacy rather than a change in sensitivity as seen with vanilloid agonists.

Dose-response studies performed with MRS1477 also reveal modality-dependent differences in potentiation. Low micromolar concentrations of MRS1477 produced potentiation of a fixed concentration (100 nM) of capsaicin. The potentiation exhibited a steady increase, with no sign of saturation, up to the maximum MRS1477 concentration of 40 µM (Figure 1E “100nM Caps”, EC\(_{50}\) 22.4µM +/-14.1). The potentiation observed upon activation by protons (pH 5.5) requires slightly higher concentrations of MRS1477 and the potentiation shows saturation for doses above 20 µM (Figure 1E “pH5.5”, EC\(_{50}\) 14.2µM +/-2.82). In the absence of TRPV1 agonists, MRS1477 did not elicit \(^{45}\)Ca\(^{2+}\) uptake at any concentration (Supplementary Information Figure 2).

**MRS1477 potentiation is not influenced by calcium efflux mechanisms**

The generalized positive modulation of TRPV1 activation, regardless of the vanilloid compound or modality of activation used, raised the question of whether the action was directly on TRPV1 or indirectly on molecules such as calcium pumps. The 1,4-DHP structure is a chemical scaffold
for L-type calcium channel antagonists (Triggle et al., 1989) and we conducted several experiments to determine whether or not the enhanced $^{45}$Ca$^{2+}$ uptake observed was due to off-target inhibition of the plasma membrane Ca$^{2+}$ ATPase (PMCA) and sodium-calcium exchanger (NCX) that could result in increased retention of $^{45}$Ca$^{2+}$ following TRPV1 activation. We performed $^{45}$Ca$^{2+}$ uptake assays under “triple-block” conditions, simultaneously inhibiting the majority of calcium-efflux mechanisms of HEK293-TRPV1 cells. Our “triple block” buffer was a modified Assay Buffer where NaCl was replaced with LiCl (to block NCX), pH was set to 9.0 (to reduce PMCA efficiency) and 1 µM Gd$^{3+}$ was added to inhibit capacitative calcium entry (Duman et al., 2008). Although we observe an expected leftward shift in the capsaicin dose-response curve when inhibiting calcium efflux using “triple-block” conditions (Figure 1F, control vs. triple block, EC$_{50}$: 132.7nM +/-14.9, HS 1.11 +/-0.13 vs. 59.6nM +/-7.13, HS 1.29 +/-0.19), the degree of potentiation by MRS1477 is unaffected and remains comparable between normal and blocked conditions (Figure 1F).

**Sensitization by serine phosphorylation and extracellular protons is further enhanced by MRS1477**

Serine phosphorylation by protein kinase C (PKC) on residues S502 and S800 sensitizes TRPV1 (Mandadi et al., 2006) and produces a leftward shift of the capsaicin dose-response curve similar to MRS1477 (Bhave et al., 2003). Similarly, a low concentration of extracellular protons corresponding to pH~6.0 will not activate the receptor but will sensitize it towards vanilloid ligands (Jordt et al., 2000). To determine if positive modulation by protons, serine-phosphorylation and MRS1477 produce additive effects or share mutually exclusive
mechanisms, we compared the ability of MRS1477 to sensitize TRPV1 in the presence or absence of PMA, both at neutral and at slightly acidic pH (pH6.0). We found that MRS1477 is equally effective at potentiating capsaicin-stimulated $^{45}\text{Ca}^{2+}$-uptake at either pH 7.4 (Figure 1G, compare pH 7.4 vs. pH 7.4 +MRS1477) or pH 6.0 (Figure 1G, compare pH 6.0 vs. pH 6.0 + MRS1477). Potentiation of vanilloid activation by serine phosphorylation appeared independent of and additive with pH 6.0 sensitization in the $^{45}\text{Ca}^{2+}$-uptake assay, resulting in a reduction of capsaicin EC$_{50}$ values from 133 nM at pH 7.4 through 31.4 nM at pH 6.0, to 21 nM at pH 6.0 with 10 µM PMA present. This could be further enhanced by the addition of MRS1477, reducing the EC$_{50}$ of capsaicin to 6.52 nM (Figure 1G, pH6.0+PMA+MRS1477). Figure 1H is a graphic depiction of the EC$_{50}$ shifts in the combination conditions tested in panel 1G (detailed curve-fit data are presented in Supplementary Table I), illustrating enhanced TRPV1 sensitivity to the same concentration of vanilloid agonist with the progressive combination of low pH, serine phosphorylation and MRS1477. These data suggest the possibility that the addition of a positive allosteric modulating agent may “overdrive” TRPV1 under inflammatory conditions. This overdrive could produce local nerve terminal inactivation and analgesia through calcium overload, similar to what has been observed with subcutaneous injections of direct acting TRPV1 agonists such as capsaicin or resiniferatoxin (Neubert et al., 2003; Mitchell et al., 2010).

**Electrophysiology and calcium imaging disclose a rapid action for MRS1477**

Intracellular Ca$^{2+}$ imaging experiments revealed that co-application of MRS1477 significantly increases the peak intracellular calcium concentration ([Ca$^{2+}]_i$) following capsaicin activation. The kinetics of the rise and the decay of the transient do not show substantial alterations apart
from the increase in peak height (Figure 2A). As with $^{45}\text{Ca}^{2+}$ uptake, the potentiation by MRS1477 occurred in a dose-dependent manner (Figure 2B). Application of MRS1477, regardless of the presence of capsaicin, does not elicit a detectable change in the [Ca$^{2+}$]$_i$ of untransfected HEK293 cells (Figure 2A, “293 – 100nM Caps + 20µM MRS1477”), confirming the results of $^{45}\text{Ca}^{2+}$ uptake experiments under the “triple-block” condition, showing that MRS1477 does not interfere with intracellular calcium homeostasis.

The experimental methods used so far provided us either with end-point data of calcium entry through activated TRPV1 present in the plasma membrane ($^{45}\text{Ca}^{2+}$-uptake assays) or with real-time low-frequency readout of cytoplasmic calcium concentrations, regardless if it is the result of calcium release from the endoplasmic reticulum or entry through plasma membrane calcium channels (intracellular calcium imaging) (Kárai et al., 2004). To obtain better temporal resolution we studied the interaction between MRS1477 and TRPV1 using the whole-cell patch clamp method.

As we observed in calcium imaging studies, MRS1477 significantly increased peak currents activated by the administration of 200nM capsaicin. Application of the agonist alone over a five second period resulted in a strong inward current (Figure 3A, 200nM Caps), the peak amplitude of this current increased ~two-fold in the presence of 20 µM MRS1477 (Figure 3A, 200nM Caps +20µM MRS1477). This increase was highly significant with a peak current of ~1nA on average when applying capsaicin only, compared to >2nA when co-applying 20µM MRS1477 (Figure 3B, $P<0.01$). Pre-incubation with MRS1477 was not necessary to observe potentiation and the
positive modulation was reversible by a three-minute washing step, resulting in currents comparable to controls when applying capsaicin only (Figure 3A).

Applying a voltage ramp to the capsaicin-activated receptor revealed no change in the reversal potential of the activated current when co-applying MRS1477, indicating that MRS1477 does not change the relative permeability of TRPV1 to cations. Current flow through the ion channel shows, however, a generalized increase both below and above the reversal potential suggesting that the action of MRS1477 is of allosteric nature (Figure 3C). Although the potentiation was observed at all membrane potentials, the magnitude of this effect was significantly higher at negative potentials (Fig. 3D), suggesting that the allosteric site for MRS1477 might be influenced by the channel’s electric field.

**MRS1477 enhances capsaicin-induced hypothermia in vivo**

The ability of MRS1477 to enhance the actions of capsaicin in vivo was tested using capsaicin-evoked hypothermia. Intraperitoneal administration of 5µg capsaicin provoked a drop in core body temperature (CBT) and statistically significant hypothermia (compared to vehicle) at time points 15 and 20 min after drug injection (Figure 4, Capsaicin). Co-injection of 200µg MRS1477 extended the duration of hypothermia by a factor of two with CBT significantly below control values at time points 20 – 35min (Figure 4, Capsaicin +MRS1477). The presence of MRS1477 had no effect on the amplitude of the peak decrease in CBT. Minimum CBTs were 35.31°C and 35.38°C for capsaicin and capsaicin with MRS1477, respectively. These data show that MRS1477 extended the duration but not the amplitude of capsaicin-evoked hypothermia.

**Positive modulation is independent of the vanilloid-binding site**
We tested if MRS1477 interacts with the channel pore or with the vanilloid binding site and determined dose-inhibition profiles of capsazepine (CPZ) and ruthenium red (RR) in the presence of increasing concentrations of MRS1477. The results of the dose-response studies are presented in two formats. First, we show dose-inhibition data normalized to their respective minimums and maximums to better visualize any change in the IC₅₀ values of inhibitors (Figure 5, detailed curve-fit data presented in Supplementary Table 2). We also included the same set of results normalized to the minimum and maximum values of their respective controls to show the potentiation by increasing doses of MRS1477 (Supplementary Figure 3, detailed curve-fit data presented in Supplementary Table 3).

CPZ is a competitive (orthosteric) vanilloid antagonist, capable of inhibiting capsaicin activation with an IC₅₀ in the 10⁻⁷M range. However, it is an inefficient antagonist of low pH activation at similar concentrations (Gavva et al., 2005). Although TRPV1 response to 50nM capsaicin shows gradual potentiation in the presence of increasing concentrations of MRS1477 (>three-fold with 30µM MRS1477, Supplementary Figure 3), normalized results reveal no change in CPZ IC₅₀ values (Figure 5A). Inhibition of proton activation by CPZ is only partial and we could not obtain a full dose-inhibition curve (Supplementary Figure 3B) but normalization of calcium uptake at each CPZ concentration to CPZ only (control) values revealed that the extent of potentiation by 30µM MRS1477 is not altered by different concentrations of this antagonist, with 50% enhancement observed at any concentration of CPZ (Figure 5B).

RR is a non-specific calcium channel inhibitor binding directly to the extracellular side of the pore, inhibiting all modalities of TRPV1 (Caterina et al., 1997; García-Martínez et al., 2000).
Similar to results obtained with CPZ, ascending concentrations of MRS1477 produced robust potentiation of capsaicin and proton-induced calcium uptake (Supplementary Figure 3C-D). No substantial differences are seen in the RR inhibition profiles for either capsaicin or pH activation (Figure 5C-D), although there is a small decrease in RR potency inhibiting capsaicin. These data are consistent with the idea that MSR1477 does not interact directly with either the CPZ or RR binding sites.
Discussion:

The present studies describe positive allosteric modulator effects of a 1,4-DHP on the vanilloid receptor TRPV1. We show ligand-specific positive modulation using three independent methodologies: \(^{45}\text{Ca}^{2+}\)-uptake assays, ratiometric calcium imaging, and patch clamp recording. Positive modulation of a capsaicin-induced physiological response was also confirmed using an \textit{in vivo} assay. Dose-response results from \(^{45}\text{Ca}^{2+}\)-uptake experiments suggest that vanilloid agonists undergo sensitivity modulation regardless of their potencies in addition the maximum response from proton-induced activation is increased. Such generalized positive modulation of TRPV1 has been observed in slightly acidic extracellular environments (Jordt et al., 2000), in response to elevated concentration of extracellular divalent cations (Ahern et al., 2005), or as the result of direct phosphorylation from constitutive PKC activation (Bhave et al., 2003). All of these conditions are present during inflammation or tissue damage. Although there are known orthosteric vanilloid \textit{antagonists} that potentiate receptor activation by protons (Wong and Gavva, 2009), MRS1477 is the first known small molecule positive modulator of TRPV1 without detectable agonist or antagonist activity.

Multiple factors can affect TRPV1 sensitivity especially in inflammatory conditions. The combined effects of multiple sensitizing agents are expected to be additive if the underlying mechanisms are independent. We observed decreases of capsaicin EC50 values upon treatment with either PMA, low pH, low pH with PMA, or MRS1477 alone. However, under inflammatory-mimetic conditions (low pH + PMA), MRS1477 caused a further 3.1-fold reduction of the capsaicin EC50 value reaching a cumulative EC50 decrease 20-fold below
control (Figure 1H). This shows that MRS1477 can act independently of, but also in concert with, either proton- or PKC-mediated sensitization. This is consistent with observations that the potency of TRPV1 ligands and endovanilloids can vary greatly depending on the physiological or pathophysiological context (i.e., at sites of inflammation and tissue damage) (Olah et al., 2001). Importantly, their actions can be further enhanced by small-molecule compounds.

Interference with mechanisms of active calcium extrusion (PMCA and NCX) or the stimulation of capacitative calcium entry (CCE) by MRS1477 could result in an apparent increase of $^{45}\text{Ca}^{2+}$-uptake in our assay. However, inhibition of these major calcium transport mechanisms did not affect the capacity of MRS1477 to potentiate TRPV1 activation. Intracellular calcium imaging experiments show that increasing doses of MRS1477 correlate with increasing peak $[\text{Ca}^{2+}]_{i}$ after TRPV1 activation but have no effect on other kinetic parameters of the calcium transient such as peak rise and decay times. Calcium imaging also showed that MRS1477 alone does not induce detectable changes in baseline $[\text{Ca}^{2+}]_{i}$. Whole-cell patch clamp experiments using HEK293-TRPV1 cells show increased amplitude of capsaicin-activated current in the presence of MRS1477 with no change in other kinetic parameters. MRS1477 alone causes no detectable transmembrane current and no effects were seen on the parental cell line. These three methods encompass a broad range of temporal resolution and consistently demonstrate positive modulation of TRPV1 by MRS1477 with no detectable effect on calcium transport mechanisms involved in Ca$^{2+}$ homeostasis.

In our initial evaluations of the in vivo activity of MRS1477 using acute nociceptive tests, we found that nociceptive behavioral responses evoked by acute capsaicin application, such as the
eye wipe test or intraplantar injection, lack assay sensitivity for detecting enhancement by MRS1477. In place of an acute pain assay, we used capsaicin-evoked hypothermia as a test model, since we were able to accurately measure small changes in core body temperature (CBT) during this more gradually evolving physiological response. Reduction in CBT following systemic administration of capsaicin occurs over several minutes and this extended onset provided a less volatile baseline. Co-administration of MRS1477 with capsaicin significantly extends the duration of hypothermia. Although recovery time was prolonged with MRS1477, we did not observe a significant change in the amplitude of CBT. Since administration of 20µg capsaicin can provoke a further drop of 1°C in CBT, the results with 5µg of capsaicin are within the dynamic range of the assay. Although it is difficult to extrapolate from our in vitro observations which parameters – duration, intensity, or both – should have been affected, nevertheless, the nature of the effect, an enhancement of capsaicin-evoked hypothermia by MRS1477, is consistent with our in vitro findings. It is also interesting to note that a previous study showed a similar prolongation of hypothermia, without a change in amplitude, when comparing the TRPV1 agonists RTX and capsaicin (de Vries and Blumberg, 1989).

Additional evidence for an allosteric mechanism derives from our studies with TRPV1 inhibitors. The decrease in the EC$_{50}$ values we observe with vanilloid ligands suggests that MRS1477 may act by altering the affinity of the vanilloid binding site. Increasing concentrations of the positive modulator, however, did not affect CPZ inhibition of capsaicin activation in $^{45}$Ca$^{2+}$-uptake experiments. This apparent contradiction can be explained by the fact that the $^{45}$Ca$^{2+}$-uptake assay is a functional assay and measures TRPV1 channel opening over time as opposed to directly quantifying receptor-ligand interactions as with radioligand-binding...
assays. The lack of effect on capsaicin – capsazepine interaction is consistent with the idea that MRS1477 does not substantially alter the vanilloid binding site. This being the case, then positive modulation by MRS1477 likely is due to modulation of channel gating. It’s important to note that the present studies were conducted with CPZ concentrations between $10^{-8}$M and $10^{-5}$M. Some studies have shown that high micromolar concentrations of CPZ can inhibit voltage gated calcium channels in cultured rat DRGs (Docherty et al., 1997) and nicotinic acetylcholine receptors in rat trigeminal ganglia (Liu and Simon, 1997). However, based on our dose-inhibition curves, this non-specific antagonism is not sufficient to explain our results obtained using HEK cells expressing high levels of TRPV1.

Although the crystal structure of TRPV1 is not known, homology models based either on the structure of the Kv1.2 Shaker $K^+$ channel (Salazar et al., 2009) or the Streptomyces lividans $K^+$ channel (KcsA)(Ryu et al., 2007) are regularly used to model the pore region of TRPV1. These models use sequences encompassing transmembrane helices (TM) 5 and 6 with the pore-loop sequence in between. The structure of KcsA also served as the prototype structure for the homology model of the pore region of 1,4-DHP sensitive L-type calcium channels (Huber et al., 2000). 1,4-DHP binding was localized to a pocket formed by sequences that correspond to TM 5 and 6 in TRPV1 (Ryu et al., 2007). In light of the above findings, combined with our results showing no interaction between MRS1477 and CPZ or RR, we hypothesize that MRS1477 interacts with the pore-forming TM 5 and 6, directly influencing channel gating regardless of the potency of agonists. Further experiments examining the dynamics of open and closed states of TRPV1 at the single-channel level will be used to further explore the mechanism of action of this compound.
The potentiation of vanilloid and endovanilloid agonists and low pH suggests a new potential mechanism for amplification of nociceptive stimuli in primary afferent endings at sites of peripheral inflammation and tissue damage. This possibility may be operative if the MRS1477 allosteric site is a target for endogenous compounds generated during tissue damage or inflammation. Positive allosteric modulation of TRPV1 may provide an additional mechanism for peripheral sensitization processes, to sustain TRPV1 transduction capabilities in chronic pain states, or to override a desensitized TRPV1 to allow the neuron to respond to new changes in status at sites of peripheral inflammation or pathology. However, lessons from capsaicin and RTX demonstrate that a balance needs to occur between active and “over-active” TRPV1 in order to avoid complete incapacitation of the nerve ending due to calcium overload. Peripheral application of vanilloid agonists has been abundantly demonstrated to produce a selective, TRPV1-mediated axonopathy and prolonged analgesia (Robbins et al., 1998; Nolano et al., 1999; Neubert et al., 2003; Karai et al., 2004; Backonja et al., 2008; Mitchell et al., 2010). Therapeutically, a TRPV1 PAM could produce just such a calcium overload in highly active endings causing a pharmacological transition to an inactive nerve terminal. Studies of capsaicin or RTX action suggest that this acute effect would last for several days or longer until the ending is functionally reconstituted (Bates et al., 2010), thereby providing a defined, long duration analgesic action after a single administration. The TRPV1 PAM approach is predicated on the participation of a sufficiently potent endogenous agonist, combined with a potent PAM, to drive the afferent ending into an inactive state, rather than prolonging or exacerbating nociceptive processes. However, the very same nociceptive processes seen in inflammation or tissue damage also bias the system to potential MRS1477 actions on TRPV1.
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Authorship Contributions:

Participated in research design: KK, JMK, CC, SKM, MAH, SS, MJI

Conducted experiments: KK, CC, SKM, MAH

Contributed new reagents or analytic tools: KAJ

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Footnotes:

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FIGURE LEGENDS:

Figure 1: MRS1477 positively modulates vanilloid and proton activation of TRPV1 when studied using $^{45}$Ca$^{2+}$ uptake of HEK293-TRPV1 cells. (A) Capsaicin activation undergoes sensitization with a slight increase in $^{45}$Ca$^{2+}$ uptake at saturating concentrations of agonists in the presence of 20µM MRS1477. (B) Activation of TRPV1 by NADA shows a similar enhancement in the presence of MRS1477. (C) Positive modulation of TRPV1 sensitivity towards vanilloid ligands is also observed when using the ultra-potent agonist RTX. (D) The potentiation of proton activation rather resembles efficacy modulation — the EC$_{50}$ value for proton activation changes only slightly while maximum $^{45}$Ca$^{2+}$ uptake almost doubles, compared to controls. (E) Modulation by MRS1477 reaches saturation at ~20µM concentration when enhancing proton activation, while enhancement of capsaicin activation is limited only by the solubility of the test compound. Potentiation by MRS1477 is independent of major calcium efflux mechanisms (F) Inhibition of major plasma membrane Ca$^{2+}$ pumps and channels resulted in a left-shift of the capsaicin dose-response curve (control vs. triple-block control). This apparent sensitization did not interfere with modulation by MRS1477 that remained comparable both under control (control vs. +20µM MRS1477) and triple-block conditions (triple-block control vs. triple-block +20µM MRS1477). Positive modulation by MRS1477 is independent of sensitization by protons or serine-phosphorylation (G) TRPV1 is sensitized towards vanilloid ligands by slightly acidic pH (pH 7.4 vs. pH 6.0). This sensitization is further enhanced both by the PKC activator PMA (pH 6.0 + PMA) and by MRS1477 (pH 6.0 + MRS1477). The positive
modulation by these three agents (pH 6.0 + PMA + MRS1477) is cumulative. (H) EC$_{50}$ values for capsaicin in the presence of various positive modulators and their combinations, as shown in graph G.

**Figure 2: Intracellular Calcium Imaging** (A) We recorded intracellular calcium levels for individual cells over the course of experiments, and plotted the average [Ca$^{2+}$]$_i$ of the population at each time point to obtain a kinetic trace. Non-transfected HEK293 cells did not show any change in their [Ca$^{2+}$]$_i$ when exposed to 100nM capsaicin and 20µM MRS1477 (dotted line). The increase of [Ca$^{2+}$]$_i$ in HEK293-TRPV1 cells was more important in the presence of 100nM capsaicin and 20µM MRS1477 (dashed line) than in the presence of 100 nM capsaicin only (continuous line) (B) Plotting the maximum increase in [Ca$^{2+}$]$_i$ of individual cells following the administration of 100nM capsaicin reveals a dose-dependent increase in average peak height when increasing concentrations of MRS1477 are present. Significant differences are present at 10µM MRS1477 and above ($P<$0.001, number of cells per treatment, from left to right: 59, 90, 55, 74, 44, 85 and 139, respectively).

**Figure 3. Electrophysiology experiments demonstrate increased current through TRPV1** (A) Whole-cell patch clamp experiments using HEK293-TRPV1 cells show that co-application of 10 µM MRS1477 results in a two-fold increase of the peak current activated by 200 nM capsaicin. The action of MRS1477 is rapid and reversible - it does not require pre-incubation and can be washed out. (B) Statistical analysis of peak currents show that this increase is highly
significant (p<0.01) (C) Voltage-ramp experiments show the generalized enhancement of capsaicin-activated currents with no apparent change in the reversal potential of the current. In the absence of both capsaicin and MRS1477 (control) there was no measurable current. (D) The enhancement is more important at negative holding potentials; the difference is significant when comparing the extreme values of the holding potentials examined.

Figure 4. Capsaicin-induced hypothermia is enhanced by the co-administration of MRS1477. Intraperitoneal injection of 5µg capsaicin provoked a significant drop in core body temperature (CBT). Hypothermia started to develop 10 min after the injection, peaked within the following 10 min then returned to control values during the subsequent 15 min period (Capsaicin). Co-administration of MRS1477 significantly extends the duration of the hypothermia with no change in amplitude of the drop in CBT (Capsaicin + MRS1477). Mice receiving vehicle only did not show significant changes in their CBT (Vehicle). CBT values were recorded every 5 min, starting at the time of injection, for 120 min total.

Figure 5: MRS1477 does not affect TRPV1 inhibition by capsazepine or ruthenium red (A) Increasing concentrations of MRS1477 potentiate activation of TRPV1 by 50nM capsaicin up to three-fold (Supplementary Figure 3A). CPZ interaction with TRPV1 remains unaffected, as the normalized dose-inhibition (D-I) curves show no change in CPZ IC_{50} values. (B) CPZ is a partial inhibitor of TRPV1 activation by protons, yielding incomplete D-I curves that show a linear increase in inhibition with increasing doses of CPZ (Supplementary Figure 3B). Normalizing
calcium uptake to each CPZ concentration without MRS1477 produces a horizontal line at 100% for control. Potentiation is apparent at all CPZ concentrations, ~50% above control in the presence of 30 µM MRS1477 (dashed lines represent upper and lower 95% CI). (C) RR inhibition of capsaicin-activated TRPV1 in the presence of increasing amounts of MRS1477 shows a progressive potentiation of TRPV1 response (Supplementary Figure 3C). Normalized curves show a small but non-significant reduction in RR IC<sub>50</sub> values with high concentrations of MRS1477. (D) RR is also an inhibitor of proton activation of TRPV1. It does not interfere with potentiation by MRS1477 (Supplementary Figure 3D), while normalized D-I curves of RR show no change in RR potency in the presence of MRS1477.
Figure 1
**Figure 2**

(A) Intracellular calcium ([Ca²⁺]) response over time for HEK293 cells transiently expressing TRPV1 receptors and HEK293 cells without TRPV1 expression. Treatment with 100 nM capsaicin alone or with 20 μM MRS1477 is shown.

(B) Distribution of peak [Ca²⁺] changes (Δ[Ca²⁺]) for HEK293 and HEK293-TRPV1 cells treated with 100 nM capsaicin and various concentrations of MRS1477 (1 μM, 5 μM, 10 μM, 20 μM). Statistical significance indicated by asterisks (***).
Figure 3
Figure 5