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Inhibitory effect of positively charged triazine antagonists of prokinecitin receptors on the transient receptor vanilloid type-1 (TRPV1) channel

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Graphical abstract

Abstract

Four positively charged compounds, previously shown to produce analgesic activity by interacting with prokinecitin receptor or T-type calcium channels, were tested for their ability to inhibit capsaicin-induced elevation of intracellular Ca^{2+} elevation in HEK-293 cells stably transfected with the human recombinant TRPV1, with the goal of identifying novel TRPV1 open-pore inhibitors. **KYS-05090** showed the highest potency as a TRPV1 antagonist, even higher than that of the open-pore triazine **8aA** inhibitor. The latter showed quite remarkable agonist/desensitizer activity at the rat recombinant TRPM8 channel. The activity of **KYS-05090** and the other compounds was selective because none of these compounds was able to modulate the rat TRPA1 channel. Open-pore inhibitors of TRPV1 may be a new class of multi-target analgesics with lesser side effects, such as loss of acute pain sensitivity and hyperthermia, than most TRPV1 antagonists developed so far.

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Keywords

TRPV1 receptor, TRPM8 receptor, open-pore inhibitors, prokinecitin receptors, Calcium channel assay.

1. Introduction

The transient receptor potential vanilloid 1, TRPV1 was the first identified member of a family of thermosensory receptors currently known as thermoTRPs, which encompass cold-, warm-, and heat-activated channels [1]. TRPV1 is a nonselective cation channel with high Ca^{2+} permeability, involved in somatosensation, chemical and thermal nociception and pain, and chronic neuroinflammatory conditions. It is also known as the 'capsaicin receptor', because capsaicin, the pungent principle of chili peppers, is a specific TRPV1 activator [2-4]. Endogenous mediators, like the endocannabinoid anandamide and 2-arachidonoyl glycerol [5-8], and some eicosanoids [9-10], also activate TRPV1 channels. The validation of TRPV1 as a therapeutic target for pain prompted the development of several TRPV1 antagonists that have entered clinical trials for the treatment of acute, chronic and neuropathic pain. [11-12]. However, complete pharmacological blocking of TRPV1 with high affinity irreversible, competitive vanilloid antagonists can result in hyperthermia and side effects [13].

Uncompetitive antagonists are activity-dependent inhibitors that specifically bind to the agonist-receptor complex or to the open state of the channel, blocking only highly activated receptors. Open-channel blockers can only access the channel when it is open, recognizing a binding site located deep within the pore. A class of compounds, which act as uncompetitive TRPV1 antagonists, are 1,3,5-triazine derivatives (Figure 1). Triazine **8aA** [(2,4-bis(2'-(3"-indolyl)ethylamino)-6-(3'-(*N*,*N*-dimethylamino)-propylamino)-1,3,5-triazine)] (Figure 1) has been recently reported as the most potent TRPV1 blocker [14]. **8aA** shares some structural features with a second family of 1,3,5-triazines derivatives (Figure 1), which have been shown to be antagonists at prokinecitin receptors (PKRs), as assessed by evaluating their inhibition of intracellular Ca^{2+} mobilization induced by the peptide Bv8 [15]. Bombina variegata 8 (Bv8) is a peptide (8 kDa) secreted by the frog skin, and acts as a selective agonist for two G-protein–coupled receptors, the prokineticin receptors of type 1 and 2, PKR1 and PKR2, expressed in dorsal root ganglia (DRG), in the outer layers of the dorsal horns of the spinal cord, and in peripheral terminals of nociceptor axons. In fact, studies in the mouse, rat, cattle, monkey, and man, identified orthologues of Bv8. Two human cDNAs have been cloned encoding two secreted proteins of 86 and 81 amino acids. [16]. These two mammalian proteins were named prokineticin 1 (PK1, or EG-VEGF) and prokineticin 2 (PK2 or mBv8), which is an orthologue of amphibian Bv8 (for reviews see [17-18]), and were later shown to

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act as ligands for PKR1 and PKR2 [19-21]. Affinity of Bv8 for the receptors is comparable to that of PK2 and is about 40 times higher than that of PK1 [18]. Bv8/PKs are peripheral and central pain modulators: activation of nociceptor PKRs by Bv8 in rats and mice produces sensitization to thermal and mechanical stimuli, and mice lacking the PKRs or PK2 are less sensitive to noxious stimuli than wild type mice [22-23]. Very recently our group described the synthesis of compounds capable to reduce *in vivo* the Bv8-induced thermal hyperalgesia when injected into the hind paw of mice 5 minutes before Bv8. In particular, some triazinediones (Figure 1) showed very high efficacy as prokinecitin receptor antagonists [24].

The transient receptor potential melastatin type-8 (TRPM8) ion channel was identified as the principal sensor for environmental cold in mammals [25-26]. No endogenous agonists for the TRPM8 channel have been so far identified, although there is recent evidence that testosterone exerts an ionotropic effect on TRPM8 at picomolar concentration [27], while the endocannabinoids anandamide and *N*-arachidonoyldopamine produce instead antagonism [28]. Apart from its role in thermosensation, acute activation of TRPM8 can have analgesic effects [29] suggesting that neuronal TRPM8 may play a neurogenic anti-inflammatory role. Very recently it has been shown that the synthetic TRPM8 agonist icilin (i) has a potent anti-inflammatory effect, and (ii) inhibits E2F1 transcription factor-mediated cell cycle regulatory programs in prostate cancer [30], thus suggesting the potential relevance of icilin and 'icilin-like' compounds as therapeutic tools for the treatment of a variety of pathological conditions [31].

Here we tested four positively charged compounds, previously shown to produce analgesic activity by interacting with PKRs or T-type calcium channels, for their ability to modulate TRPM8 and TRPV1 activity *in vitro* as potential open-pore inhibitors.

2. Material and methods

2.1. Materials

MEM medium, foetal bovine serum (FBS), Fluo-4 AM, Pluronic® F-127, Geneticin G-418 and ionomycin were obtained from Invitrogen (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), trypsin/EDTA, penicillin, streptomycin and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell Culture

Human embryonic kidney HEK-293 cells were purchased from ATCC (LGC Standards, Sesto San Giovanni, Milan, Italy).

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HEK-293 and HEK-293 stably over-expressing human TRPV1, or rat TRPA1 or rat TRPM8 and selected by G-418 (Geneticin; 600 μg/ml), were grown as monolayers on 100 mm diameter Petri dishes in minimum essential medium supplemented with non-essential amino acids, 10 % foetal bovine serum, and 2 mM glutamine, and maintained under 5% CO_2 at 37 °C.

2.3. TRP functional assays

Cell-based fluorescence assays of calcium influx upon TRPV1 activation were performed as described:

The effect of the substances on $[Ca^{2+}]_i$ was determined by using the selective intracellular fluorescent probe for Ca^{2+} Fluo-4. On the day of the experiment the cells were loaded for 1h at 25°C with 4 μM Fluo-4 methylester (Molecular Probes) in DMSO containing 0.03% Pluronic. After the loading, cells were washed with Tyrode pH=7.4 (145 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in Tyrode and transferred (~100,000 cells) to the cuvette of the fluorescence detector (Perkin-Elmer LS50B Waltham, MA, USA) under continuous stirring. Experiments were carried out by measuring cell fluorescence at 25 °C (λ_{EX}=488 nm, λ_{EM} = 516 nm) before and after the addition of the test compounds at various concentrations. Potency was expressed as the concentration of test compounds exerting a half-maximal agonist effect (i.e. half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}). The efficacy of TRPV1 and TRPM8 agonists was determined by normalizing their effect to the maximum Ca^{2+} influx effect on $[Ca^{2+}]_i$ observed with application of 4 μM ionomycin, while the effects of TRPA1 agonists are expressed as a percentage of the effect obtained with 100 μM allylisothiocyanate (AITC). When significant, the values of the effect on $[Ca^{2+}]_i$ in wild type HEK293 (i.e., not transfected with any TRP construct) were taken as baselines and subtracted from the values obtained from transfected cells.

Antagonist/desensitizing behaviour was evaluated against capsaicin (0.1 μM) for TRPV1 against AITC (100 μM) for TRPA1 and icilin (0.25 μM) for TRPM8, by adding the test compounds in the quartz cuvette 5 min before stimulation of cells with agonists. In another set of experiments the loading cells were washed with Tyrode pH=7.4 without calcium ions (145 mM NaCl, 2.5 mM KCl, 2.7 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4), resuspended in the same buffer and treated with 1 μM capsaicin for 1 hr (at the aim of dilating the pore and facilitate the entry of charged substances) in presence of different concentrations of the potential antagonist compound. After that the cells were extensively washed in order to remove capsaicin, balanced in Tyrode's buffer contained calcium and then treated with capsaicin 0.1 μM. Data are expressed as the concentration exerting a half-maximal inhibition of agonist-induced $[Ca^{2+}]_i$ elevation (IC_{50}), which

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was calculated using GraphPad Prism® software. The effect on $[Ca^{2+}]_i$ exerted by agonist alone was taken as 100%. Dose response curves were fitted by a sigmoidal regression with variable slope. All determinations were performed at least in triplicate. Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by the Bonferroni's test.

2.4. Electrophysiological studies

DRG neurons were isolated as previously described [32]. Neurons on a glass coverslip were transferred into an external bath solution of 150 NaCl, 5 KCl, 3.5 $MgCl_2$, 10 HEPES, and 10 glucose at pH 7.4. Borosilicate glass pipettes (Sutter Instrument Co., Novato, CA, USA) (3–5 MΩ) were filled with internal solution containing 140 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, 2 mM Na-ATP and 0.3 mM Na-GTP, pH 7.3. Whole-cell patch clamp recordings were performed by using an EPC 10 amplifier (HEKA Elektronik, Bellmore, NY, USA) linked to a personal computer equipped with Pulse (V8.65) software (HEKA Elektronik). After seal formation, the membrane beneath the pipette was ruptured and the pipette solution was allowed to dialyze into the cell for 3–5 min before recording. Currents were elicited by application of 1 μM capsaicin from a holding potential of -60 mV. Data were recorded at 10 kHz and filtered at 2.9 kHz. Data analysis was performed by using online analysis built in Pulse software, and graphs were prepared by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Error bars plotted represent the mean values ± standard error.

3. Results and discussion

3.1. TRPV1 antagonist activity

The compounds were evaluated for TRPV1 antagonist activity based on their ability to block capsaicin-induced elevation of intracellular Ca^{2+} in HEK-293 cells stably transfected with the human recombinant TRPV1 (hTRPV1-HEK-293 cells). The tests were carried out with a fluorescence-based intracellular calcium detection assay for capsaicin. We started our investigation with the compound **8aA** 2,4-Bis(4'-fluorophenethylamino)-6-(3'-(N,N-dimethylamino)-propylamino)-1,3,5-triazine, a triazine-based TRPV1 receptor open channel uncompetitive antagonist, among the strongest TRPV1 open channel blockers described to date [14]. The pKa value calculated with Epik (Epik, version 2.1, Schrödinger, L.L.C.: New York, NY, 2010.) [33-34] for this compound is 9.51, indicating that it is protonated at physiological pH (7.4, at which our test is performed). Because known charged blockers have previously displayed agonistic activity, we also sought to check whether **8aA** activates TRPV1. We observed that **8aA** shows no agonist activity up to 100 μM, confirming that this compound is a pure blocker. Indeed, we calculated an inhibitory activity of the capsaicin (0.1 μM) response of **8aA** with IC_{50} = 21.7 ± 0.7 μM from the

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corresponding dose-response curve in hTRPV1-HEK-293 cells. The IC_{50} value was therefore much higher than that measured by voltage-clamp against rat TRPV1 channels heterologously expressed in Xenopus oocytes (IC_{50} = 0.05 ± 0.007 μM), as reported by Vidal-Mosquera et al. [14].

The compound **8aA** is endowed with some features in common with some prokinecitin antagonists based on the triazinedione scaffold, among which compounds with EC_{50} analgesic activity values in picoM range, i.e. **PC-7** [1-(2-((5-(4-fluorobenzyl)-1-(4-methoxybenzyl)-4,6-dioxo-1,4,5,6-tetrahydro-1,3,5-triazin-2-yl)amino)ethyl)guanidine] (EC_{50} = 0.31 pM) and **PC-27** [1-(2-((1-(4-methylbenzyl)-5-(4-(trifluoromethyl)benzyl)-4,6-dioxo--1,4,5,6-tetrahydro-1,3,5-triazin-2-yl)amino)ethyl)guanidine] (EC_{50} = 0.033 pM), both containing an 4-halogen fluoride atom substituted benzyl group in position 5, which possibly explains why they are 10 and 100 times more potent, respectively than **PC-1** [1-(2-((5-(4-ethylbenzyl)-1-(4-methoxybenzyl)-4,6-dioxo-1,4,5,6-tetrahydro-1,3,5-triazin-2-yl)amino)ethyl)guanidine] (EC_{50} = 5.8 pM)**,** which bears a 4-ethylbenzyl group in position 5 [22]. These compounds mimic the structural features required for Bv8 binding to PKRs [17, 35]. They present a triazine-guanidine moiety that mimics the N-terminal AVITGA sequence, whereas the benzyl moiety is orientated like the conserved tryptophan residue in position 24. Importantly, **PC-1,** which preferentially binds PKR1, injected intraplantar at the dose of 30 ng, significantly reduced capsaicin-induced hyperalgesia [17], suggesting that it may also inhibit TRPV1. It is to be noted that peripheral nociceptors expressing the ion channels, TRPV1 and TRPA1, co-express PKR1, which most probably contributes to the development of inflammatory hyperalgesia [22, 36]. Indeed, some Bv8-responding DRG neurons also express neuromediators implicated in pain processing as calcitonin gene-related peptide (CGRP) and substance P and release these neuropeptides upon exposure to Bv8. The glial cell line-derived neurotrophic factor (GDNF) induced 'de novo' expression of functional PKRs suggesting a possible upregulation of PKRs after the tissue damage and inflammation that cause heat hyperalgesia by sensitizing TRPV1 through PKC activation [36]. PKR1 gene deletion or PKR1 blockade with **PC-1**, markedly reduced the inflammation-induced hypersensitivity and the up-regulation of Bv8/PK2 [15, 23, 37]. Furthermore, the prokineticin system plays a role in neuroinflammation and in the evolution of the neuropathic pain, and **PC-1** administration at peripheral or central levels alleviates an established neuropathic hyperalgesia and prevents the activation of glia and the increased production of inflammatory cytokines [38].

In the view of the above reported *in vivo* activity of the PKR1 antagonist **PC-1** against capsaicin-induced hyperalgesia, and of its chemical similarity with **8aA**, we extended the *in vitro* studies on

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TRPV1 to all three PKR antagonists, **PC-1**, **PC-7** and **PC-27**. The pKa values calculated with Epik (Epik, version 2.1, Schrödinger, L.L.C.: New York, NY, 2010.) [33-34] for these compounds is 12.44, indicating that they can be protonated at physiological pH more than **8aA**. The compounds showed no or very low (**PC-27** EC_{50} > 50 μM with an efficacy at 100 μM ~ 20 %) agonist activity (Table 1). However, **PC-27** and **PC-1** inhibited the capsaicin (0.1 μM)-induced response in hTRPV1-HEK-293 cells, although this effect was weaker than that observed for **8aA,** whereas **PC-7** was not active as blocker (Figure 2, Table 1).

Most of the exploration so far has been focused on the 5-position benzyl group: consistent with calcium influx data from recombinant TRPV1 (Table 1), the substitution of the ethyl group with a fluorine atom in position 4 on the 5-benzyl group was not tolerated and the resulting compound did not show *in vitro* potency at blocking capsaicin activation of TRPV1. The introduction of a CF_3 in position 4 on the 5-benzyl group together with the simultaneous replacement of the methoxy group with a less polar methyl in position 4 on the 1-benzyl group resulted in the regaining *in vitro* activity, with slightly higher potency at blocking capsaicin activation of TRPV1 (IC_{50} = 27.2 ± 1.9 μM).

3.2. TRPA1 activity

Responses to Bv8 were colocalized with responses to mustard oil, an activator of the channel TRPA1, which like TRPV1 is involved in nociception [39-41]. Furthermore, inflammatory heat hyperalgesia caused by mustard oil was also significantly lower in $PKR1^{-/-}$ mice [22], suggesting that these nonspecific proinflammatory stimuli act in part via release of PK and activation of PKR1 receptors. It seemed therefore interesting to study whether the test compounds interacted with TRPA1. However, none of the compounds blocked mustard oil isothiocyanate-induced and TRPA1-mediated elevation of intracellular Ca^{2+} in HEK-293 cells stably transfected with the rat recombinant TRPA1 channel, as is shown in Table 1.

3.3. KYS05090 activity

The T-type calcium channel blocker **KYS-05090** [2-(3-(1,1'-biphenyl-4-yl)-2-((5-(*N*,*N*-dimethylamino)pentyl)-*N*'-(methyl)amino)-3,4-dihydroquinazolin-4-yl)-*N*''-benzylacetamide] 2-hydrochloride was included in this study. Very recently the ability of **KYS-05090** to block the Ca^{2+} channel, and the dependence of **KYS-05090**-induced cell death on the $[Ca^{2+}]_i$ in a concentration- and time-dependent manner have been shown [42]. **KYS-05090** is not a trisubstituted triazine but a quite rigid dihydroquinazoline containing a cationic tertiary *N*,*N*-dimethylaminopentyl-*N*'-methyl-

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amine group (p*K*a value calculated with Epik for this compound is 9.6, suggesting its protonation at physiological pH). This compound exhibited significant albeit very low agonist activity at both TRPV1 and TRPA1. However, **KYS-05090** showed the highest potency as an antagonist against TRPV1, even higher than that of **8aA**, with an IC_{50} value of 11.6 μM. Electrophysiological experiments performed on acutely dissociated dorsal root ganglion (DRG) neurons from adult mice confirmed the results of the calcium experiments. Figure 3 shows that DRG neurons positive for TRPV1 exhibit significantly reduced capsaicin-evoked currents after a 3-min incubation with KYS-05090 (10 μM). For comparison, the IC_{50} found for **KYS-05090** when screened against TRPA1 was > 50 μM.

TRPV1 is permeable by different ions with a preference for divalent species (Ca^{2+} and Mg^{2+}) over monovalent ions (Na^{+}, K^{+} and Cs^{+}) [1]. Studies with ATP-gated ion channels of the P2X family suggested that prolonged agonist exposure increases the pore size, leading to enhanced permeability of the channel to cations [43-44]. In the case of TRPV1 the permeability to different cationic species varies in a time-dependent and an agonist concentration-dependent manner, allowing permeation of large organic cations [45]. During prolonged activation with vanilloids, permeability to large cations increases, although the channel continues to exclude anions. One worth noting characteristic of TRPV channels that has been proposed to explain Ca^{2+} selectivity in TRPV1 is the presence of negatively charged residues in the outer region of the selectivity filter, due to the presence of acidic residue E648 and D646, which exert an electrostatic attraction on cations [46]. It has been shown that also TRPV3 [47], and TRPA1 activation resulted in dynamic changes in permeability to cations [48-50]. Alkaline pH has been found to cause helix dilation of the pore for TRPV5 [51], whereas TRPM8 activation does not seem produce significant changes in ion selectivity [48]. The three-dimensional structure of the TRPV1 channel was recently determined by single particle electron cryo-microscopy, allowing us to explore ionic conduction in TRP channels at atomic detail [52]. Pore dilation may facilitate the entry of otherwise impermeant drugs into cells [53], although the quaternary derivative of lidocaine QX-314 seems to enter through the standard pore and does not require pore dilation [54].

3.4. Experiments on hTRPV1-HEK-293 cells pre-treated with capsaicin

When the experiments were performed on cells pre-treated with 1 μM capsaicin for 1 hr at the aim of dilating the pore and facilitate the entry of charged substances, all the tested compounds inhibited the capsaicin (0.1 μM)-induced response in hTRPV1-HEK-293 cells with about the same IC_{50} (Table 1).

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The pore dilation previously measured in capsaicin-bound TRPV1 is still not sufficient to account for TRPV1 permeability to the large cations used in the present study. One possible explanation for our findings is that the capsaicin-bound channel represents an intermediate state, and that conformations of a flexible TRPV1 selectivity filter exhibiting even greater pore expansion are transient and possibly inducible by large cations themselves as they pass through the activated channel [55].

3.5 *TRPM8 activity*

To assess channel selectivity, the five compounds tested here were screened also against TRPM8 (Table 2) by measuring their effect on TRPM8-mediated elevation of intracellular Ca^{2+} in HEK-293 cells stably transfected with the rat recombinant TRPM8 channel. The triazine **8aA** activated and subsequently desensitized TRPM8 and the IC_{50} value observed desensitization was quite remarkable (0.95 ± 0.03 μ M) (Figure 4 and Table 2). This effect was surprising since it has been shown that pore dilation does not occur in TRPM8 channels [48]. The interaction of **8aA** with TRPM8 might be explained by evidence showing that this channel can be activated at the outer pore (EC_{50} 1.1 ± 0.1 μM). Menthol interacts with a hydrophobic pocket within the S1-S4 transmembrane bundle, and causes conformational rearrangements that lead to gate opening [56]. Furthermore, TRPV1 can be activated at the outer pore by a bivalent tarantula toxin involving the extracellular domains of S5 and S6 [57]. Vanillotoxins activate TRPV1 channel in the outside-out, but not inside-out configuration, consistent with an extracellular site of action, and ruling against a role for the S3-S4 domain in TRPV1-vanillotoxin interaction. Recent reports have shown a hydrophobic binding pocket for capsaicin and anandamide that is accessible from the extracellular side [52, 58], thus indicating that these compounds access TRPV1 also from the outside [59]. Our results suggest that some TRPM8 negative charged aminoacids at extracellular domains could interact with the protonated **8aA** at physiological pH. Similarly, but to a lesser extent, also **PC-27** and **KYS-05090**, but not **PC-1** and **PC-7**, activate and consequently desensitize TRPM8 (Table 2).

4. Conclusions

Based on past evidence obtained with **8aA**, we have conducted studies on previously reported analgesic and positively charged compounds, such as three triazine inhibitors of PKRs, and a dihydroquinazoline T-type calcium selective channel blocker, with the goal of identifying novel TRPV1 open-pore inhibitors with acceptable *in vitro* potency and possible use as analgesics with lesser side effects than most TRPV1 antagonists developed so far. The latter compounds antagonize TRPV1 regardless of its endogenous activators, whereas TRPV1 open-pore inhibitors only act when

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the channel is prolongedly activated by endogenous noxious ligands, thus being potentially less prone to produce side effects such as loss of acute pain sensitivity and hyperthermia. Further chemical modification of **KYS-05090,** on the one hand, and **PC-27** and **PC-1**, other hand, with the aim of enhancing their potencies as potential open-pore inhibitors of TRPV1 without diminishing their capability of antagonizing T-type channels or PKRs, respectively, might lead to efficacious multi-target analgesics. None of the compounds tested showed significant activity at rat TRPA1 channels. However, we revealed that the previously developed open-pore TRPV1 antagonist, **8aA**, behaves as a relatively potent agonist/desensitizer of the rat recombinant TRPM8 channel.

Author contributions

Luciano De Petrocellis, Aniello Schiano Moriello, Joon Seok Byun, Joo Mi Sohn, Jae Yeol Lee, Ana Vázquez-Romero, Maria Garrido, Angel Messeguer, Alessandro Deplano, Cenzo Congiu, Valentina Onnis, Gianfranco Balboni and Vincenzo Di Marzo designed the study; Gerald W Zamponi and Fang-Xiong Zhang designed and performed the electrophysiological studies; Luciano De Petrocellis and Aniello Schiano Moriello performed the experiments and analyzed the data; Luciano De Petrocellis, Gianfranco Balboni and Vincenzo Di Marzo wrote the paper. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Fig. 1

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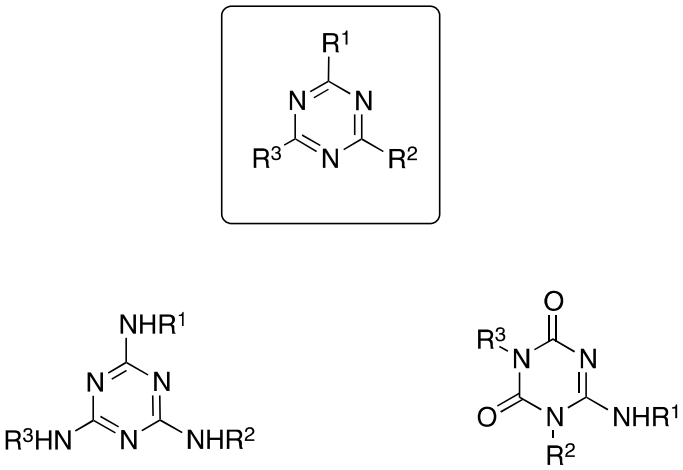

Figure 1. General structure of triazines. On the left: triazine derivative type **8aA**. On the right: triazinedione type derivative.

Fig. 2

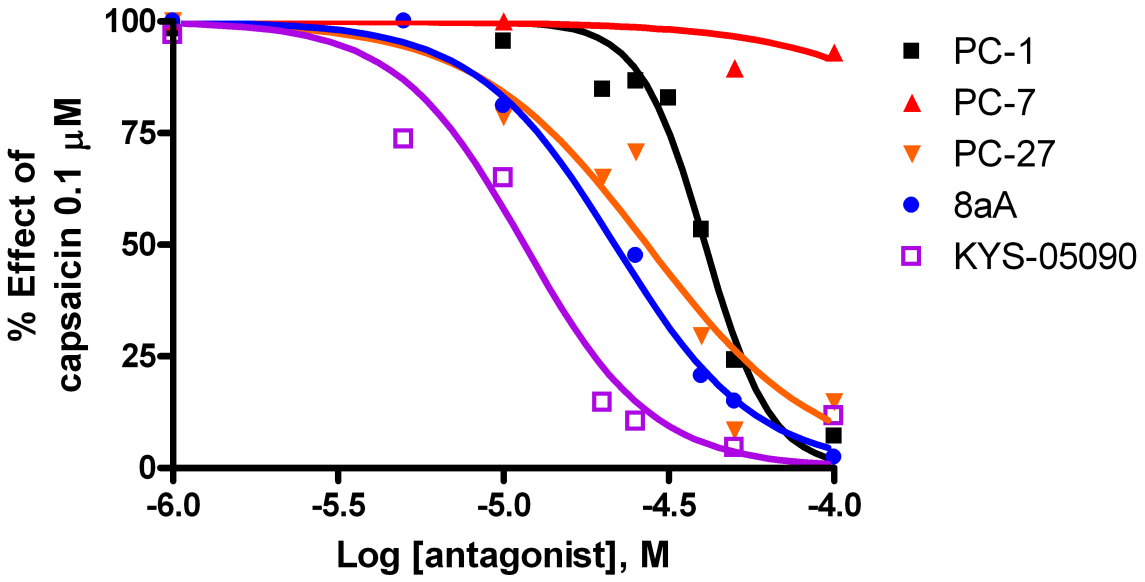

Fig 2: Effect of a 5 min pre-incubation with the tested compound on capsaicin (100 nM)-induced Ca^{2+} elevation in HEK-293 cells over-expressing the human TRPV1. The effect on $[Ca^{2+}]_i$ exerted by capsaicin 100 nM alone was considered as 100%.

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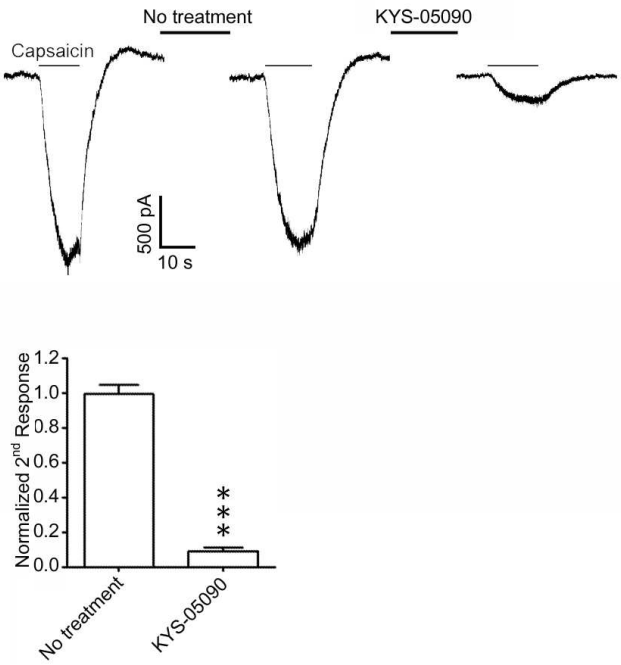

Fig 3: KYS-05090 modulates capsaicin-evoked currents.

(Upper panel) Representative current responses to consecutive applications of 1 μM capsaicin without or with an intervening 3-min incubation of KYS-05090 (10 μM) in acutely dissociated dorsal root ganglion neurons from adult mice.

(Lower panel) Ratio of the second to the initial peak capsaicin response (mean ± SEM) in the absence or presence of an intervening KYS-05090 (10 μM) application. Capsaicin-evoked currents were significantly reduced after 3-min KYS-05090 pre-treatment ($p < 0.0001$, compared with no KYS-05090 treatment, n=4).

Fig. 4

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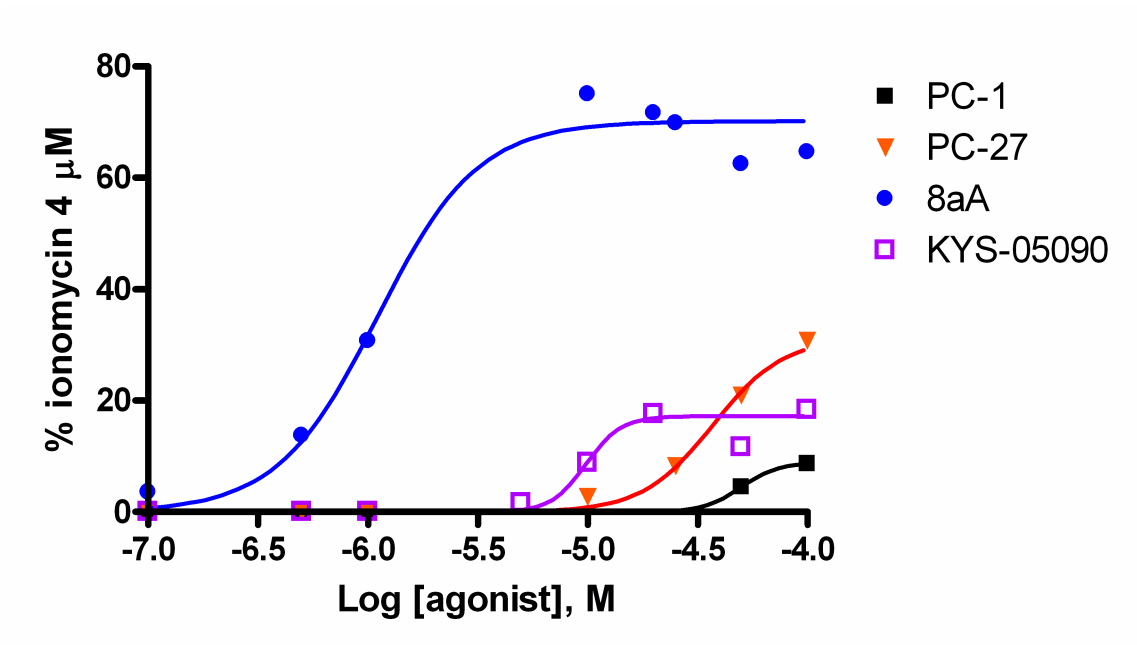
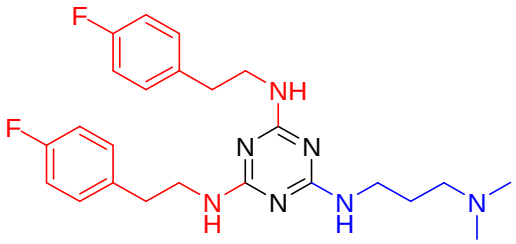


Fig 4: Effect on intracellular Ca^{2+} elevation in HEK-293 cells over-expressing rat TRPM8. Data are expressed as % of the effect observed with 4 µM ionomycin

Table 1. Structures and in vitro activity of studied compounds.

Code	Structure	TRPV1 IC_{50}, µM vs. capsaicin 0.1 µM (*cells pre-treated with capsaicin)*	TRPV1 EC_{50}, µM (efficacy at 100 µM)	TRPA1 IC_{50}, µM vs. AITC 100 µM	TRPA1 EC_{50}, µM (efficacy at 100 µM vs AITC 100 µM)
8aA		21.7 ± 0.7 (20.5 ± 1.9)	NA (< 10)	> 100	> 50 (52.7 ± 2.4)

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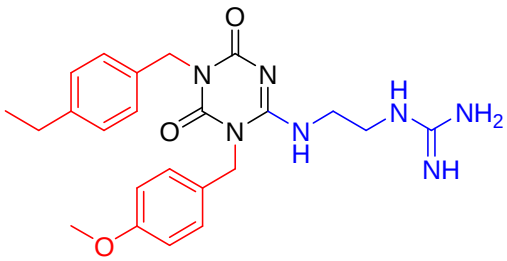
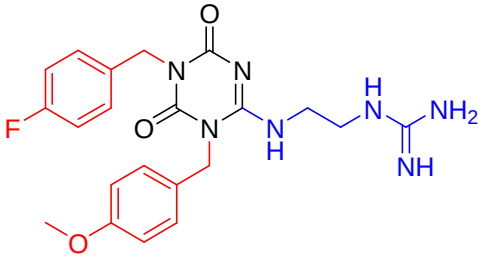
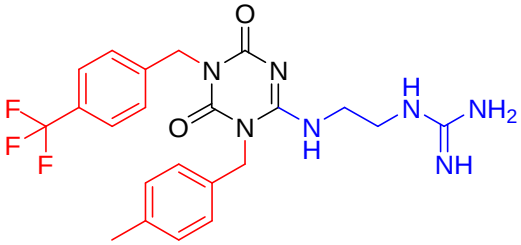
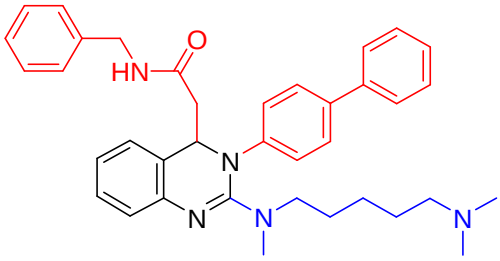
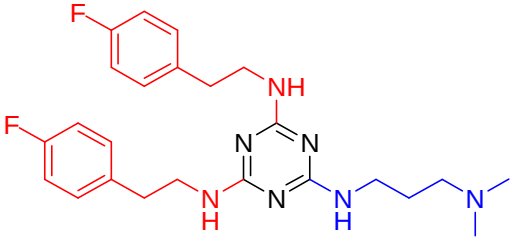
PC-1		40.8 ± 0.9 (40.5 ± 1.1)	NA (< 10)	> 100	NA (18.2 ± 0.1)
PC-7		>100 (> 100)	NA (< 10)	> 100	NA (0)
PC-27		27.2 ± 1.9 (31.0 ± 1.0)	> 50 (20.8 ± 0.1)	> 50	> 50 (42.6 ± 2.6)
KYS-05090		11.6 ± 0.7 (10.1 ± 0.5)	7.1 ± 1.9 (15.4 ± 2.0)	> 50	13.3 ± 1.1 (27.6 ± 0.7)

NA = not active

Table 2. Structures and in vitro TRPM8 activity of studied compounds.

Code	Structure	TRPM8 IC_{50}, µM vs icilin 0.25 µM	TRPM8 EC_{50}, µM (efficacy at 100 µM)
8aA		0.95 ± 0.03	1.1 ± 0.1 (70.2 ± 1.1)

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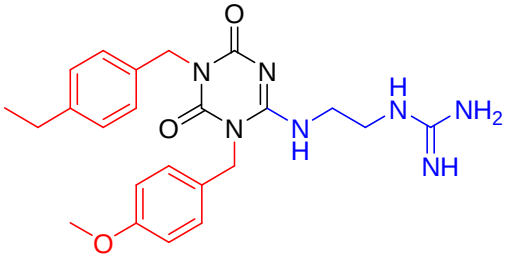
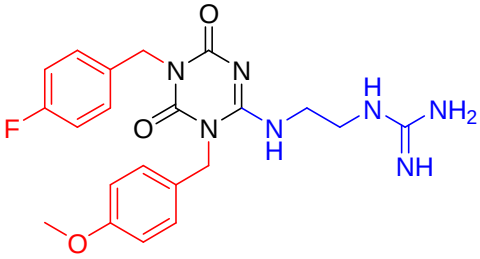
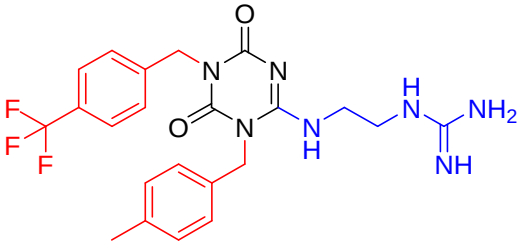
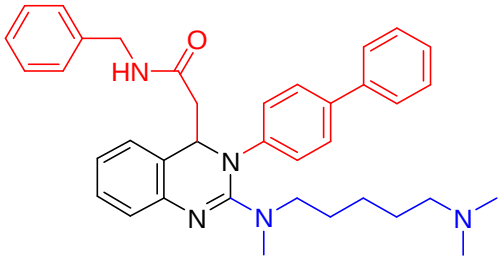
PC-1		57.5 ± 0.8	NA (< 10)
PC-7		> 100	NA (< 10)
PC-27		34.2 ± 1.5	37.0 ± 4.7 (31.1 ± 1.7)
KYS-05090		11.0 ± 0.8	9.8 ± 1.2 (17.2 ± 1.3)