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RESEARCH ARTICLE

Chalcone Derivatives Activate and Desensitize the Transient Receptor Potential Ankyrin 1 Cation Channel, Subfamily A, Member 1 TRPA1 Ion Channel: Structure-Activity Relationships *in vitro* and Anti-nociceptive and Anti-inflammatory Activity *in vivo*

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Abstract: Eleven compounds belonging to the chalcone family were tested for their ability to activate and subsequently desensitize the rat transient receptor potential ankyrin 1 cation channel, subfamily A, member 1 (TRPA1) in a heterologous expression system. Four of the tested compounds were more potent than the TRPA1 agonist mustard oil, and showed also a strong desensitizing effect. Some chalcone compounds were not pungent in the eye-wiping assay and quite remarkably inhibited in a long-lasting and dose-dependent manner the mustard oil-induced response in the formalin test. Chalcones can be considered as novel candidates for the development of anti-hyperalgesic preparations based on TRPA1 desensitization.

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1. INTRODUCTION

Chalcones are naturally-occurring compounds which display a wide range of biological properties. Chalcone (1,3-diphenyl-2E-propene-1-one) is an open chain intermediate precursor in the synthesis of flavones [1]. It contains two aromatic rings connected by a α-β-unsaturated ketone system [2]. The most important sources of chalcones are plant extracts, which are widely used in traditional Chinese Medicine, although the potential uses of this family of compounds have not been fully explored [3].

Synthetic chalcones and chalcone derivatives show a wide range of biological activities with therapeutic interest [for reviews see 3, 4]. One of the most studied applications of these compounds is as anticancer agents [5, 6], and a series of chalcone derivatives with excellent antitumor activity have been patented [3]. Very interestingly, compounds bearing electron-withdrawing groups (NO_2 or CN) on the ring near the α-β-unsaturation of chalcones were the most potent [7]. Chalcone analogs bearing an additional α,β-unsaturated arylketone were active against the resistant T-47D breast cancer cells [8]. The induction of apoptosis by intrinsic pathways, alterations in the cellular levels of Bcl-2

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family proteins, upregulation of p53 and PUMA, inhibition of nuclear factor-κB (NF-κB) and Akt, and blockage of oxidative stress might represent possible mechanisms [9-14]. The role of some chalcones as anti-inflammatory agents is summarized in a comprehensive review [15]. Several pure chalcones have been approved for clinical use and tested in humans, and are well-tollerated [16].

Despite the impressive pharmacological potential of chalcones, their mechanism of action has not been fully clarified. The presence of a double bond in conjugation with a carbonyl group is believed to be responsible for the biological activities of chalcones. The α,β-unsaturated carbonyl compounds, by being Michael acceptors and capable of trapping thiols, represent an important mechanism of bioactivity [17]. Nucleophilic cysteines and lysines located in the cytosolic N-terminus of the transient receptor potential ankyrin 1 cation channel, subfamily A, member 1 (TRPA1) undergo covalent attack by reactive electrophilic chemicals, leading to formation of Michael adducts and allosteric opening of the channel [18, 19]. TRPA1 agonists such as allyl isothiocyanate (mustard oil, MO) and several α,β-unsaturated aldehydes such as formaldehyde and its aqueous solution, formalin, directly activate TRPA1 and are widely used for testing analgesic compounds [20].

As the above data suggest that calchones might modulate TRPA1 activity we investigated 11 novel chalcone derivatives for TRPA1 activity as well as anti-nociceptive and anti-inflammatory activity *in vivo*.

2. MATERIAL AND METHODS

2.1. Chemistry

All reagents and solvents were of the highest commercial quality available and used as received. Chalcones were synthesized as follows: an aqueous solution of sodium hydroxide (30%, 25 mL) was slowly added to a methanol solution (30 mL) of the appropriate acetophenone (5.0 mmol). After the solution had been cooled to room temperature, the appropriate benzaldehyde (6.0 mmol) was added. The mixture was stirred at room temperature overnight and was then poured into water (100 mL). The obtained solid was filtered, washed with water until reaching neutral pH and recrystallized from ethanol. The chemical structure was confirmed base on reported data (references [21-27] in Table **1**).

2.2. Cell Cultures

HEK-293 (human embryonic kidney) and HEK-293 heterologously transfected with recombinant rat TRPA1 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% fetal bovine serum and 2 mM glutamine, and maintained under 5% CO_2 at 37°C. HEK-293 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Berlin, Germany). Cell culture medium and supplements were from Invitrogen.

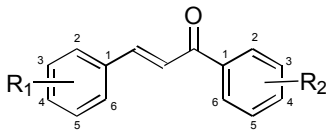
2.3. Assay of TRPA1-mediated Elevation of Intracellular [Ca^{2+}]

Compound effects on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were determined using the selective intracellular fluorescent probe Fluo-4. On the day of the experiment, cells were loaded for 1 h at room temperature with Fluo-4-AM methyl ester (4 μM in dimethyl sulfoxide containing 0.02% Pluronic F-127, Invitrogen) in Minimal Essential Medium without fetal bovine serum, then washed twice in a buffer containing 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 the same buffer, and transferred (about 100,000 cells) to the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS50B equipped with PTP-1 Fluorescence Peltier System; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring. The changes in $[Ca^{2+}]_i$ were determined before and after addition of various concentrations of test compounds by measuring cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 516 nm) at 25 °C. Curve fitting (sigmoidal dose-response variable slope) and parameter estimation were performed with GraphPad Prism® (GraphPad Software Inc., San Diego, CA). Potency was expressed as the concentration of test compound exerting a half-maximal agonist effect (i.e., half-maximal increases in $[Ca^{2+}]_i$) (EC_{50}). Agonist efficacy was expressed as a percentage of the effect on $[Ca^{2+}]_i$ observed with 100 μM mustard oil (MO). When significant, values of the effect on $[Ca^{2+}]_i$ in wild-type (i.e., not transfected with any construct) HEK293 cells were taken as baseline and subtracted from the values obtained with transfected cells. Antagonist/ desensitising behavior was evaluated against 100 μM MO by adding test compound to the quartz cuvette 5 min before agonist stimulation. Data are expressed as the concentration exerting half-maximal inhibition of agonist-induced $[Ca^{2+}]_i$ elevation (IC_{50}), which was calculated using GraphPad Prism® software. The effect on $[Ca^{2+}]_i$ exerted by MO 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 was performed by analysis of variance at each point using ANOVA followed by Bonferroni's test.

2.4. Animals

Male C57BL/6J mice, 8 weeks old (Harlan, Italy), were housed 5 per cage under controlled illumination (12:12 h light: dark cycle; light on 06.00 h) and standard environmental conditions (room temperature 22±1°C, humidity 60±10%) for at least one week before experimental use. Mouse chow and tap water were available ad libitum. Experimental procedures were in accordance with Italian and European regulations governing the care and treatment of laboratory animals (Permission no. 41/2007B). Animal care was in compliance with Ethical Guidelines of the IASP and European Community (E.C. L358/1 18/12/86) on the use and protection of animals in experimental research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Table 1. Activity of Chalcones at rat TRPA1. Efficacy and Inhibition were Measured Versus 100 µM Mustard Oil (MO).



Code	R_1	R_2	Ref.	TRPA1		
				Efficacy (% MO 100µM)	Potency µM (EC_{50})	Inhibition µM (IC_{50}) (vs MO 100µM)
C1	3-NO_2	4-OMe	[21]	107.7 ± 1.7	0.70 ± 0.05	0.49 ± 0.01
C2	4-Me	H	[22]	80.2 ± 3.3	1.4 ± 0.1	1.7 ± 0.1
C3	H	4-OMe	[23]	74.2 ± 2.9	2.3 ± 0.2	3.3 ± 0.2
C4	3-NO_2	H	[24]	111.8 ± 1.6	0.27 ± 0.02	0.21 ± 0.01
C5	H	4-OMe	[25]	88.2 ± 1.4	1.6 ± 0.1	2.2 ± 0.1
C6	4-OMe	H	[22]	69.3 ± 3.7	1.9 ± 0.2	2.3 ± 0.1
C7	4-OMe	3-NO_2	[26]	70.7 ± 1.5	0.94 ± 0.05	1.2 ± 0.1
C8	4-OMe	4-OMe	[23]	83.2 ± 5.7	10.6 ± 2.1	8.7 ± 1.1
C9	H	H	[23]	108.1 ± 3.3	2.6 ± 0.3	2.1 ± 0.1
C10	4-Cl	4-OMe	[27]	92.5 ± 3.0	1.0 ± 0.1	0.82 ± 0.05
C11	4-Cl	H	[22]	118.9 ± 4.2	1.4 ± 0.2	0.71 ± 0.04

2.5. Eye-Wiping Test

The pain-inducing potency of C1, C4, C10 and C11 was determined by the eye-wiping assay in mice, a sensitive animal model for acute trigeminal pain studies, using a protocol similar to that described in rats [28]. Male C57BL/6J mice were maintained in a controlled lighting environment and groups of 6-8 animals were used for each treatment. The animals received drug instillations (10 µl) in the left eye and were used for one treatment only. The number of eye-wiping movements following drug instillation into the eye was considered as an index of pungency. The eye-wiping reflexes in response to MO (10 µg/ml), or C1, C4, C10, C11 dropped in solution into the eye, was determined 10 min after the instillation.

In another set of experiments the effects of topical C4 and C1 on MO-induced eye-wiping were studied. Mice were treated as follows:

(A) MO (10 µg/ml)

(B) C1 (10 µg/ml, 30 or 120 min) + MO (10 µg/ml)

(C) C4 (10 µg/ml, 30 or 120 min) + MO (10 µg/ml)

2.6 Formalin Test

Formalin injection induces a biphasic stereotypical nocifensive behavior [29]. Nociceptive responses are divided into an early, short-lasting first phase (0-7 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period and then a second, prolonged phase (15-60 min) of tonic pain. Mice received formalin (1.25 % in saline, 30 µl) in the dorsal surface of one side of the hindpaw. Each mouse was randomly assigned to one of the experimental groups and placed in a plexiglas cage and allowed to move freely for 30 min. A mirror was placed at a 45-degree angle under the cage to allow full view of the hindpaws. Lifting, favoring, licking, shaking, and flinching of the injected paw were recorded as nociceptive responses. The duration of these noxious behaviors was monitored by an observer blind to the experimental treatment for periods of 0-10 min (early phase) and 20-60 min (late phase) after formalin administration. Groups of 6-8 animals per treatment were used with each animal being used for one treatment only. Mice received vehicle (5% dimethylsulfoxide in 0.9% NaCl) or different doses of C1, C4, C10 or C11 (0.75, 1.5 and 3 µg/paw, 30 µl) administered 10 min before formalin or saline.

3. RESULTS

3.1. Effect of Chalcones on MO-Induced rat TRPA1-Mediated Elevation of $[Ca^{2+}]_i$

We evaluated the possible inhibitory effect of chalcones on TRPA1-mediated $[Ca^{2+}]_i$ elevation induced by 100 µM MO. Compounds were added 5 min before cell exposure to MO (Fig. **1**). The observed inhibitory effect is likely due to desenitisation rather than antagonism, since: 1) chalcones *per se* added to cells produced a concentration-dependent increase in $[Ca^{2+}]_i$ at concentrations similar to those necessary to inhibit the MO effect (Fig. **2**), and 2) MO also inhibited its own effect when administered to cells in two consecutive times (IC_{50} 1.71 ± 0.06 µM) [30]. The values of efficacy (expressed as % of MO) and potency (EC_{50}) as well as the inhibition of increase in $[Ca^{2+}]_i$ elevation induced by MO (IC_{50}) are listed in Table **1**. The four compounds with

IC_{50} below 1 μM were selected for testing in the eye-wiping and formalin assays in mice.

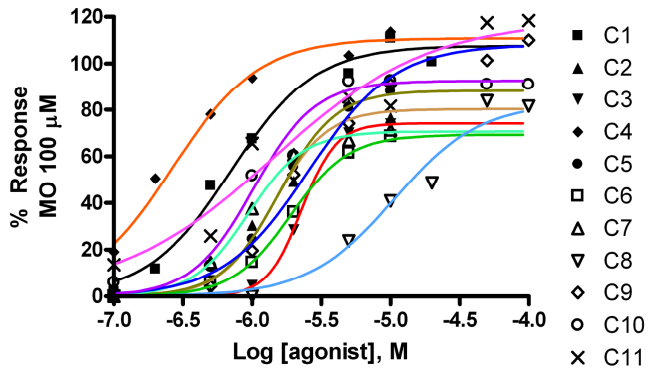


Fig. (1). Dose-response effects of chalcones on the effect on intracellular Ca^{2+} in HEK-293-TRPA1 cells by 100 μM mustard oil (MO). Data are means ± SEM of n = 4 different determinations.

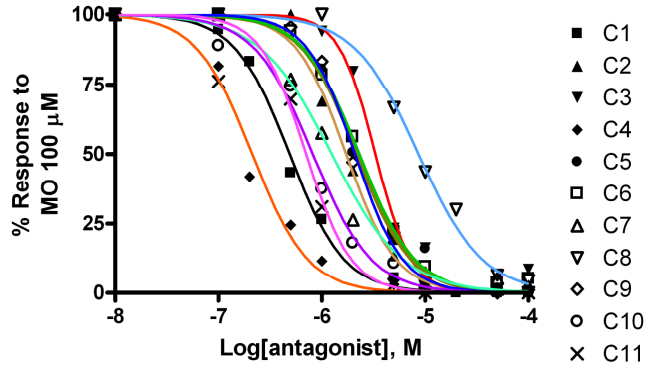


Fig. (2). Dose-dependent effects of chalcones on elevation of intracellular calcium in HEK-293 cells overexpressing rat TRPA1. Data are means ± SEM of n = 4 different determinations. The compounds were tested also on HEK-293 cells not transfected with TRPA1: none produced a significant elevation of intracellular Ca^{2+}.

3.2. Effect of Topical MO and C1, C4, C10 and C11 on Eye-Wiping in Mice

The eye-wiping test was employed as an *in vivo* pungency test to assess the pain-producing effects of topical drugs. The instillation of 10 μl of allyl isothiocyanate solution (MO, 10 μg/ml), used as comparator drug, evoked 28.9 ± 2.3 wiping movements monitored within 10 min. Moreover, we observed different pungency profiles for the various compounds tested. In particular, C10 proved to be the most pungent. No significant changes were seen following C10 topical application (10 and 30 μg/ml) as compared with MO. However, compounds C1, C4 and C11 showed significantly lower pungent properties. In particular, the wiping movements were 8.7 ± 2.2 and 6.8 ± 2.9 for C1 (10 and 30 μg/ml, respectively); 8.7 ± 2.3 and 5.8 ± 2.0 for C4 (10 and 30 μg/ml, respectively); and 25.2 ± 5.6 and 6 ± 2.7 for C11 (10 and 30 μg/ml, respectively) (Fig. **3**).

3.3. Effect of Topical C4 and C1 on MO-Induced Eye-Wiping in Mice

The two compounds with lowest eye-wiping activity, C1 and C4, were also tested on MO (10 μg/ml) (Fig. **4**) induction of such activity, given their TRPA1 desensitising activity in transfected HEK-293 cells. C4 pre-treatment (30 and 120 min) significantly reduced (12.5 ± 3.2 and 11.6 ± 4.0, respectively) the number of eye-wiping movements induced by application of MO (41.5 ± 2.8). Interestingly C1, applied 30 min before MO in the same eye, increased pain behavior (68.6 ± 11.0), although this was significantly decreased (10 ± 0.89) at 120 min in pre-treated mice, as compared to MO alone (33.2 ± 2.0).

3.4. C1, C4, C10 and C11 Inhibit Formalin-Induced Nocifensive Behavior in Mice

The activity of compounds C1, C4, C10 and C11 was evaluated in the formalin test of acute peripheral and inflammatory pain in mice. Formalin-treated mice showed the typical nociceptive behavior characterized by an early, short-lasting first phase (0-7 min), followed by a quiescent period, and then a second, prolonged phase (15-60 min) of tonic pain [29]. Ten minutes before injection of formalin (1.25%, 30 μl), mice received intrapaw administration of vehicle or one of the four compounds (0.75, 1.5 and 3 μg/30 μl). In spite of their diverse efficacies, all drugs exhibited antinociceptive activity in formalin-treated animals. In fact, we observed a significant dose-dependent reduction of both the first and the second phase of nocifensive response, as compared with vehicle-treated mice. Interestingly, C4-mediated analgesic effects exhibited an inverted dose-activity relationship, being most active at the lowest dose tested (0.75 μg/paw), but ineffective at the highest dose (Fig. **5**).

4. DISCUSSION

TRPA1 [31] is a polymodal nociceptor that detects noxious chemical agents, whether exogenous or produced endogenously during tissue injury, inflammation and oxidative stress [32, 33]. The structure of TRPA1 suggests that it functions as a sensitive, low-threshold electrophile receptor [34]. The contribution of TRPA1 to the initial phase of the inflammatory process and its participation in chronic inflammatory pain has been explored [35, 36]. Antagonists of this ion channel have the potential for treating neurogenic inflammatory conditions. TRPA1 is up-regulated following inflammatory injury [37], whereas in nerve endings TRPA1 is activated by inflammatory mediators contributing to hyperalgesia [38]. Further, TRPA1 is a key molecular target in both neuropathic pain [39] and diabetic neuropathy [40, 41]. In the current study, we demonstrate a potential novel mechanism of action for 11 compounds belonging to the chalcone family, i.e. their ability to mediate elevation of $[Ca^{2+}]_i$ in HEK293-TRPA1 cells and subsequently desensitise TRPA1 in a heterologous expression system.

Four of the tested chalcone derivatives, C4, C1, C7 and C10 were more potent than mustard oil. At least four of the

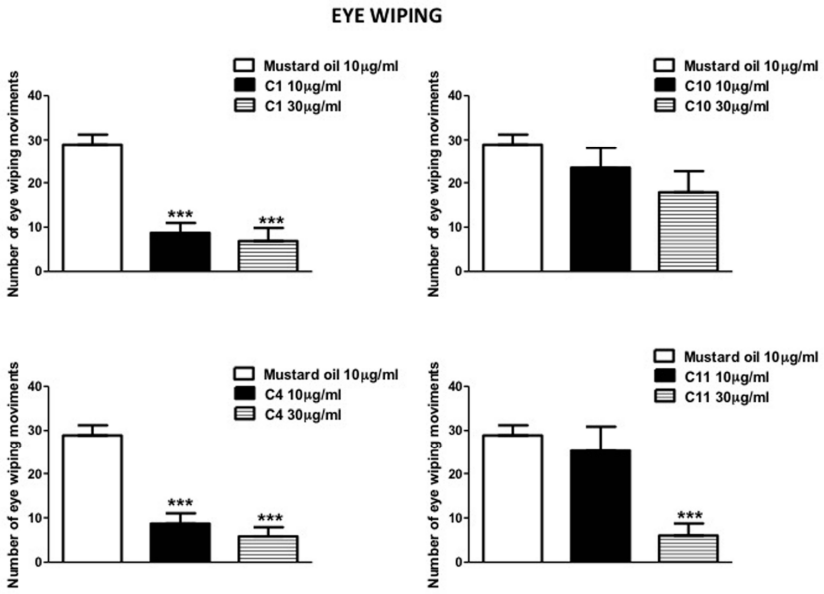


Fig. (3). Effect of C1, C4, C10 and C11 (10 and 30 µg/ml) topical administration (10 µl) on the number of wiping movements into the left eye. Data are means ± SEM of n = 6-8 mice per group. Statistical significance was determined by one-way ANOVA, followed by Tukey's post-hoc test. ***P<0.001 vs mustard oil-treated animals.

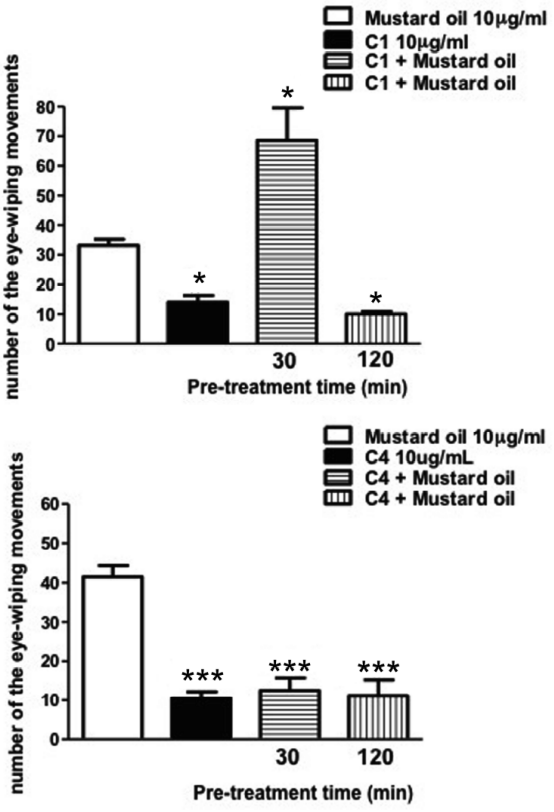


Fig. (4). Effect of C1 or C4 (10 µg/ml) topical administration (10 µl) on the number of wiping movements induced by Mustard oil (MO, 10 µg/ml) into the left eye. C1 or C4 were applied 30 or 120 min before mustard oil. Data are means ± SEM of n = 6-8 mice per group. Statistical significance was determined by One-way ANOVA followed by Tukey's post-hoc test. *P<0.05 and ***P<0.001 vs MO-treated animals.

tested chalcones were significantly more potent at desensitising mustard oil than mustard oil itself. Unlike mustard oil, some chalcones were not pungent in the eye-wiping assay, possibly due to their lipophilic nature. Indeed, several TRPV1 agonists which exhibit a slower onset of activation and high lipophilicity are also non-pungent [49-52]. Although non-pungent per se, some chalcones inhibit in a long-lasting and dose-dependent manner the mustard oil-induced response in the formalin test, likely due to TRPA1 desensitisation to the action of mustard oil. The formalin test shows a biphasic pain-related behavior, with the first phase been attributed to TRPA1-mediated excitation of nociceptors, and the second phase to their inflammatory and/or spinal sensitisation. [53]. Either pharmacological blockade or genetic deletion of TRPA1 markedly reduces both the first and second phases of the nociceptive response induced by formalin in the rat and mouse paw [20]. The TRPA1 antagonist HC-030031 inhibits the first phase of the formalin response, confirming a role for TRPA1 in this response [20].

At first glance these findings may appear difficult to rationalise given that chalcones activate TRPA1, a target of numerous hazardous/reactive chemicals such the pro-nociceptive and pro-inflammatory agent mustard oil. The structurally similar compound, curcumin, a bis-α,β-unsaturated β-diketone of two ferulic acid units connected through a methylene group, is a typical Michael acceptor [42]. Curcumin selectively activates and subsequently desensitises human [43] and rat TRPA1 in heterologous expression systems [44]. Curcumin has antioxidant, anti-inflammatory, anticancer, antiviral, antibacterial, anti-diabetic, anti-proliferative and pro-apoptotic properties, and clinical trials are underway to identify its potential beneficial effects [45]. Most of the these activities have been linked to curcumin's α,β-unsaturated carbonyl moiety [46]. Curcumin inhibits the proliferation and survival of almost all types of

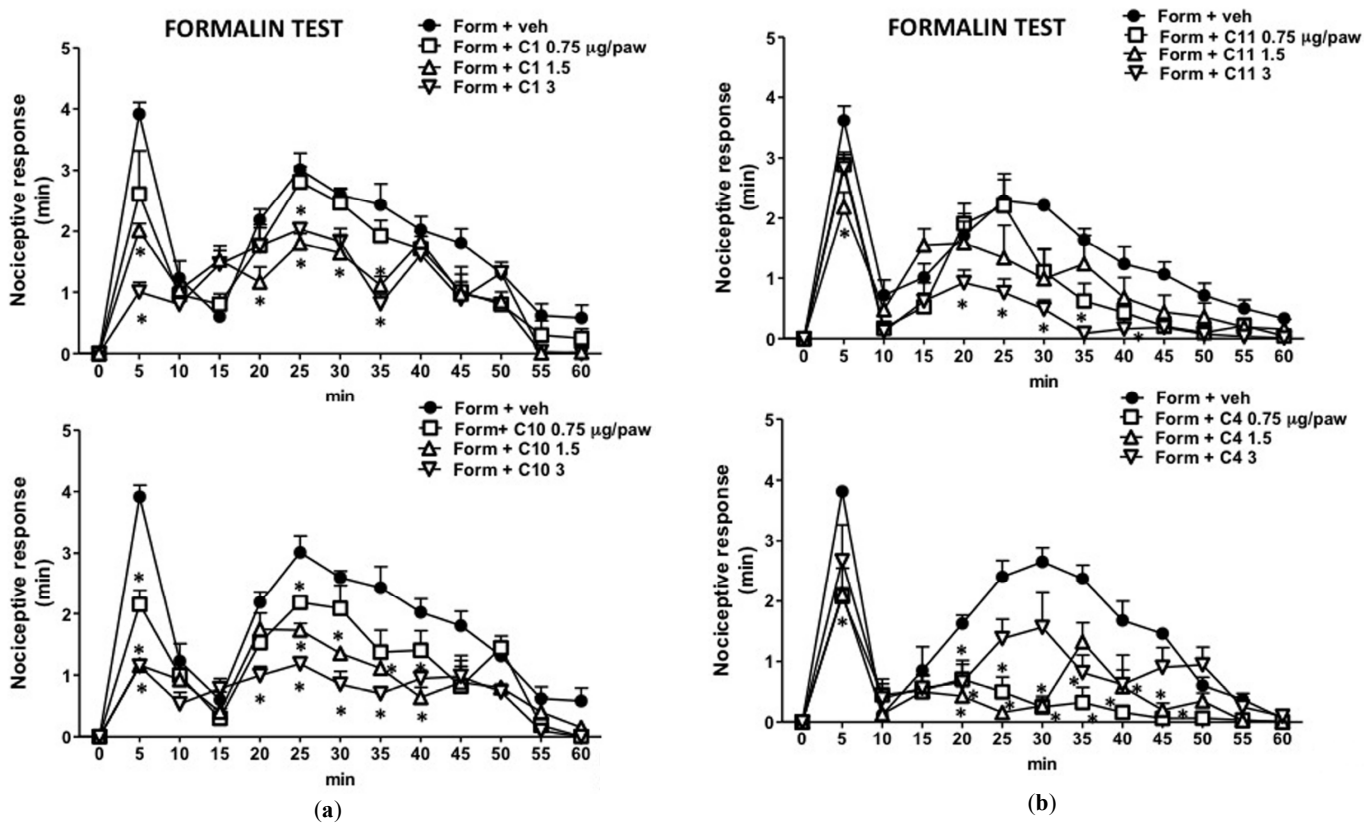


Fig. (5). Effect of C1, C4, C10 and C11 (0.75, 1.5 and 3 μg/paw) in the formalin test in mice. The total time of the nociceptive response was measured every 5 min and expressed in minutes. Statistical significance was determined by two-way analysis of variance followed by Turkey-Kramer post-hoc test. *P<0.05 vs formalin+ vehicle-treated animals.

tumour cells so far examined [47]. Its anti-inflammatory effects are mediated by suppression of the transcription factor NF-kB, which is activated in response to various carcinogens, growth factors and inflammatory cytokines [48]. It is worth noting that curcumin caused a strong desensitization and tachyphylaxis in a recombinant TRPA1 system and may activate the channel efficaciously without producing noxious sensations.

These findings strongly suggest that chalcones can be considered as novel candidates for the development of anti-hyperalgesic preparations based on TRPA1 desensitization. Indeed, the combination of significantly higher potency as a TRPA1-desensitizing agent and lower pungency should make these compounds useful tools for the treatment of neuropathic pain. In will be important in future studies to establish if chalcones are capable of producing anti-hyperalgesic actions in animal models of chronic inflammatory and neuropathic pain. The present study already shows that intraplantar injection of some chalcones in mice not only fails to cause nociception but also inhibits the second phase of the nocifensive response to formalin, a widely used model of acute inflammatory pain.

Interestingly, the four most potent compounds at desensitising TRPA1 are chalcones with a substituent electron-acceptor in the B ring which is closer to the α,β-unsaturated carbon, and whose nitro group is more efficient than a chlorine. The presence of a methoxy group in the other ring renders the compounds less active. This is in agreement with the hypothesis that the α-β-unsaturated ketone system in the structure of chalcones activates and desensitize TRPA1 via the formation of a covalent Michael adduct with cysteine residues within the channel [18, 19]. The IC_{50} rank is:

C4 < C1 < C11 < C10

Although the presence of an electron-acceptor nitro group on the other ring does not appear to markedly impact TRPA1 agonist activity (EC_{50} **C1** = 0.70±0,05 EC_{50} **C7** = 0.94±0.05), it approximately doubles the IC_{50} (IC_{50} **C1** = 0.49±0.01 IC_{50} **C7** = 1.2±0.1). It is intriguing to note that a series of chalcone derivatives tested for their anti-proliferative activities against a panel of cancer cells showed, in general, that compounds containing nitro groups on the ring near the α-β-unsaturation were more potent and selective [54].

The importance of a nitro substituent in the ring for chalcone biological activity has been shown *in vivo* and *in vitro*. Chalcone derivatives with an electron-acceptor nitro group substituent in the A and B rings have anti-hyperglycemic activity, increasing insulin secretion together with serum glucose-lowering in the presence of functional β cells. Only chalcones with a nitro group at position 3 on ring B, the same position as our C4, showed significant and acute anti-hyperglycemic activity in short-term treatment, in contrast to when the electron-acceptor nitro group is at position 2' in ring A [55]. Indeed, TRPA1 is abundantly

expressed in a rat pancreatic β cell line, and its activation by endogenous and exogenous ligands stimulates insulin release [56]. Chalcones are being considered for the management not only of diabetes but also treatment of several pathologies [3, 57]. A series of chalcone derivatives (curcumin analogs) showed anti-inflammatory activity in mouse RAW 264.7 macrophages, inhibiting the lipopolysaccharide-induced expression of tumour necrosis-α and interleukin-6 [58]. Structure-activity studies show that the asymmetric compounds possessed higher anti-inflammatory activity, while electro-negativity was an important factor for inhibiting lipopolysaccharide-induced interleukin-6 expression. Some nitro-substituted chalcones have also been proposed for the treatment of neurodegenerative diseases such as parkinsonian syndromes [59].

5. CONCLUSION

Eleven compounds, belonging to the chalcone family, were able to desensitise TRPA1. Several were not pungent in the eye-wiping assay and quite remarkably inhibited, in a long-lasting and dose-dependent manner the mustard oil-induced response in the formalin test. Chalcones can be considered as novel candidates for the development of anti-hyperalgesic preparations based on TRPA1 desensitization.

LIST OF ABBREVIATIONS

MO	=	Allyl isothiocyanate (mustard oil,);
NF-κB	=	Nuclear factor-κB;
TRPA1	=	Transient receptor potential ankyrin 1 cation channel, subfamily A, member 1.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

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