https://doi.org/10.31925/farmacia.2024.3.4 **ORIGINAL ARTICLE**

EFFECTS OF H4415 ON PAIN SENSITIVITY IN RAT

AURELIA MÂRZA [1], CĂTALIN CEZAR CĂRĂTAŞU [1], ANDREI CIOBICĂ [1]*, VERONICA BILD [1,2], ALEXANDRU VASINCU [1], RĂZVAN-NICOLAE RUSU [1], BOGDAN IONEL TAMBA [1,3], WALTHER BILD [1,2]

[1]*Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania*
[2]*Center of Biomedical Research, Romanian Academy, Iași branch, Romania*
[3]*Advanced Center for Research and Development in Experimental Medicine, Prof. Ostin C. Mungiu", CEMEX, "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania*

**corresponding author: andrei.ciobica@yahoo.com*

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Abstract

H4415, also known chemically as HC-030031, is a xanthine derivative that acts as an experimental antagonist of the TRPA1 (transient receptor potential ankyrin 1) channels, known to be involved in the transmission of pain signals, particularly in response to inflammatory stimuli. H4415 inhibits the entry of calcium ions into cells, inhibiting a crucial step in the pain signalling pathway. The compound has been studied for its potential in treating both inflammatory and neuropathic pain. This study aims to explore the efficacy of H4415 in reducing pain sensation by blocking TRPA1 channels. Various pain induction experiments such as tail flick, hot plate, cold plate, and pressure algesimetry tests were used, comparing the pain response at various doses of H4415 (0.4 mg/kg, 0.8 mg/kg, 1.2 mg/kg), dissolved in 5% glucose solution to controls. The results suggest that H4415 significantly alleviates pain across all tests, with notable decreases in pain response, mainly at lower doses. Statistical analysis of the results supports the compound's potential as a pain reliever, showing a marked increase in pain thresholds and a reduction in pain sensitivity, results that underscore the importance of TRPA1 channels in pain perception and position H4415 as a promising candidate for treating both inflammatory and neuropathic pain conditions.

Rezumat

H4415, cunoscut și sub denumirea chimică de HC-030031, este un derivat de xantină care acționează ca un antagonist experimental al canalelor TRPA1 (Transient Receptor Potential Ankyrin 1), cunoscute pentru implicarea lor în transmiterea semnalelor dureroase, în special ca răspuns la stimuli inflamatori. H4415 inhibă intrarea ionilor de calciu în celule, blocând astfel o etapa esențială în calea de semnalizare a durerii. Compusul a fost studiat pentru potențialul său în tratamentul durerii inflamatorii și neuropate. Acest studiu își propune să exploreze eficacitatea H4415 în reducerea senzației de durere prin blocarea canalelor TRPA1. Au fost utilizate diverse experimente de inducere a durerii, cum ar fi testul tail-flick, hot-plate, cold-plate și algezimetrie, comparând răspunsul la durere la diverse doze de H4415 (0,4 mg/kg, 0,8 mg/kg, 1,2 mg/kg), dizolvat în soluție de glucoză 5%, cu grupurile de control. Rezultatele sugerează că H4415 atenuează semnificativ durerea la toate testele, cu scăderi notabile ale răspunsului la durere, în special la dozele mai mici. Analiza statistică a rezultatelor susține potențialul compusului ca si analgezic, arătând o creștere semnificativă a pragurilor de durere și o reducere a sensibilității la durere, rezultate care subliniază importanța canalelor TRPA1 în percepția durerii și poziționează H4415 ca un candidat promițător pentru tratamentul condițiilor de durere inflamatorie și neuropată.

Keywords: pain, TRPV channel blockers, thermal stimulation

Introduction

Pain is a major symptom for which patients seek medical attention. It can be divided into three classes. The role of pain is to prevent tissue injury secondary to noxious stimuli. Inflammatory pain occurs due to tissue infiltration with cells belonging to the immune system, causing hypersensitivity until the affected tissue heals. The third class of pain is pathological, caused by the alteration of the nervous system called neuropathy, or dysfunctional secondary to the functional alteration of the nervous system [1].

Effective and safe treatment of inflammatory and neuropathic pain remains a medical necessity [2]. Ion channels that have on their surface vanilloid receptors for ankyrin 1 (TRPA, transient receptor potential action ankyrin 1), present at the level of sensitive nerve endings, contribute to the amplification of transmission of pain signals due to inflammation [3]. Membrane receptor sensitivity depends on specialised receptor subtypes, which play a role in various noxious, mechanical, and/or chemical signal transduction. Among the membrane receptors involved in pain processing are the vanilloid receptors belonging to the voltage-gated ion channels (TRP, transient receptor potential). A member of this family is the receptor for ankyrin1, TRPA1 (transient action potential receptor ankyrin 1), which is activated by the action of heat and acidic pH

and is involved in the detection of hypersensitivity to cold and capsaicin [4, 5]. The activation of this channel causes an influx of sodium and calcium, generating an action potential that will propagate further towards the central nervous system. At the level of the dorsal horn, TRPA1 amplifies the release of glutamate [6]. Non-neuronal cells that express this receptor include keratinocytes, vascular endothelium [7] and astrocytes [8]. The TRPA1 channel is a homo-tetramer composed of six transmembrane helical sub-units (TM1-TM6), ankyrin repeat regions, and intra-cellular amino and carboxyl termini [9]. The amino portion (NH_2) represents more than half of the composition of the receptor protein. It is the one that holds an elongated repetitive domain of ankyrin, having the role of controlling protein interactions and the membrane insertion of the ion channel [10]. The endogenous activators of TRPA1 are represented by the oxidative stress given by different compounds, such as nitric oxide [11], zinc [12] and hydrogen peroxide [13]. The best-known agonists of TRPA1 are oleocanthal, acrolein, allyl isocyanate, nicotine, thymol, 4-hydroxy-noneal, prostaglandin A2, crotyl aldehyde, methyl isocyanate, dibutyl phthalate, methyl-glyoxal, cinnamaldehyde, acetaldehyde, flufenamic acid, isoflurane, hydrogen peroxide, formalin, zinc, eugenol, cinnamon, methyl salicylate, icilin, allicin, lidocaine, propofol, nitric oxide, thymol, clotrimazole, nifedipine and diclofenac [14].

H4415 (HC 030031) is a xanthine derivative [15] experimental antagonist of TRPA1 channels that blocks the intracellular penetration of calcium determined by the action of formalin [16]. This compound could have a role in neuropathic and inflammatory pain. Research results suggest its potential for pain treatment in current medical practice. In 2008, Eid et al. orally administered H4415 to mice and provided information on relieving mechanical and inflammatory pain secondary to Freund's adjuvant and spinal nerve ligation [2].

Materials and Methods

The algesiology experimental models were carried out in the Advanced Centre for Research and Development in Experimental Medicine, Prof. Ostin C. Mungiu", CEMEX, from the "Grigore T. Popa" University of Medicine and Pharmacy, Iași, Romania.

Substances administered

The compound H4415 (HC-030031), chemical formula $C_{18}H_{21}N_5O_3$ (Sigma Aldrich®), with a molecular weight of 355.39 g/mol, was dispersed in a 5% glucose solution. H4415 is a substance that has not been used extensively in research, and its mention in the literature is quite rare. According to previous studies, we have selected a series of more closely probable doses to generate effects in an experimental setting [17-20]. An average dosage has been chosen: 0.4 mg/kg bw, 0.8 mg/kg bw, 1.2 mg/kg bw. All references mention low water solubility, needing the administration of DMSO. However, not wanting to introduce a well-known experimental bias due to DMSO, which has effects on neurotransmission and ion channel activity [21, 22]. We used 5% glucose saline, in which the mentioned doses of H4415 dissolved after 10 minutes of gentle stirring and heating at 37 C.

Animals

Experimental animals were purchased from the "Cantacuzino" Institute, Bucharest, Romania. Adult male Wistar rats weighing 200.00 - 400.00 g and at least 14 weeks of age were grouped into five animals for each of the three doses mentioned, plus a control group treated with 5% glucose solution. Before beginning the algesiology experiments, the animals were housed in a quarantine room, which ensures a constant ambient temperature of 21.00 ± 2°C, with caloric and water intake *ad libitum*, placed two animals in a cage, with bedding changed every two days. At the end of the experiments, the animals were euthanised with an overdose of anaesthetic. The presence of neurological deficits and motor or tail lesions were exclusion criteria from the experiments. The working protocols of this study are carried out in accordance with the International Association for the Study of Pain (IASP) guidelines for the care of laboratory animals and European Union legislation (directive 2010/63/EU, the experiments were performed according to the approval of the Commission for the Ethics of Research of the Grigore T. Popa University of Medicine and Pharmacy.

The study compound was administered intraperitoneally, using single-use syringes with a volume of 1 mL with a 25 - 27 G needle, the administration being carried out in a volume of 0.2 mL/100g animal. This route of administration is preferred for isotonic solutions and provides an absorption rate of ½- ¼ compared to intravenous absorption. Without sedation, one rat at a time was immobilised in a transparent polyethylene device with spaces for injection manoeuvres. Later, it was positioned in a supinen position, with the trunk lower than the cephalic extremity, thus pushing the internal organs caudally to avoid the risk of accidental puncture. The administration was made in a single dose, followed by the testing.

Algesiology Tests

The tail flick test

The tail flick test is part of the tests that provide information on the transmission of pain in the spinal pathways [23]. The Tail Flick 37360© Ugo Basile device was used for testing. The test refers to the placement of a radiant heat source with the intensity set at 90 at the level of the distal third of the tail of the experimental animal [24], which will cause it to move its tail in a flipping motion when it feels pain. The intensity value of the radiant heat source of the beam was adjusted according to the baseline with values

between 3 and 6 seconds [25]. The duration of time from applying the painful stimulus to the movement of the tail is noted in seconds, and the timer automatically stops [26]. The maximum (cut-off) time allowed for a determination was 12 seconds [26]. Recordings were performed at time zero (T0), 15 minutes (T1), 30 minutes (T2), 45 minutes (T3) and 60 minutes (T4).

The hot plate test

This test evaluates the transmission of pain through a central mechanism from the spinal and supraspinal pathways [23]. The Hot Plate 7280 (Ugo Basile) apparatus was used for testing. The testing involved placing the rat on a plate heated to 54 ± 2°C and observing the behaviour denoting the presence of pain, such as shaking the hind paws, jumping from the heat source, and vocalisation [27]. The cut-off time was set to 30 seconds. Recordings were made at time zero (T0), 15 minutes (T1), 30 minutes (T2), 45 minutes (T3) and 60 minutes (T4).

Cold plate test

The cold plate test used the Hot/Cold Plate 35100 apparatus (Ugo Basile). This test was carried out by passing an experimental animal on a plate cooled to 5°C for 3 minutes (180 seconds). The number of lifts of the right posterior limb was quantified [28, 29]. Recordings were performed at time zero (T0), 10 minutes (T1), 20 minutes.

Algesimetric test with pressure stimulus

The analgesimeter 35100 apparatus (Ugo Basile) was employed for this test. A mechanical force measured in gram force was placed at the level of the right hind limb [30]. Rats with baseline measurements above 80 gF were excluded from the determinations [31]. Recordings were made at time zero (T_0), 15 minutes (T_1), 30 minutes (T_2), 45 minutes (T_3) and 60 minutes (T_4) [32, 33].

Statistical analysis

To determine the maximum percentage inhibition, the calculation formula $(Lx-L_B)/(CO-L_B) \times 100$ was used, with L_X representing the latency at a time "x", L_B the baseline latency, CO and the time of cut-off. By normalising the recorded values, T_0 became 0%, plotting the means of percentage inhibitions (MIP) ± standard error of the mean (SD). All data were compared with control groups and with values obtained at T_0. The potential antinociceptive effect was considered when there was a statistically significant increase in response latency after applying the painful stimulus. The statistical analysis was done using GraphPad Prism software© 1992 - 2021 Software, LLC, Version 9.30 (463) Windows 64-bit. The tail flick, hot plate, cold plate, and algesimetry tests performed following the administration of the three doses of the H4415 compound have results graphs that include the mean percentage inhibitions (MIP) ± the standard error (SEM) of the mean from each determination (T_0- T_4) of each test batch of animals.

The Gaussian distribution of the data was checked using the Kolmogorov-Smirnov normality test, and then the analysis of multiple variants ANOVA was performed, in which the differences in time and the efficiency of pain inhibition were compared. Furthermore, differences between doses and between determination times (T_0 - T_4) were subjected to Dunnett's post hoc multiple comparisons test. The threshold of statistical significance was considered for a $p < 0.05$.

For data without a Gaussian distribution, the Kruskall-Wallis test was performed, followed by Dunn's post hoc test for multiple comparisons.

The graphs indicate the moment when significant statistical differences between the determination times (T_0 - T_4) appeared with an asterisk (*).

Results and Discussion

Testing for antinociceptive potential produced the following data (Figure 1).

In the *hot plate test*, H4415 causes analgesia at all three doses (0.4 mg/kg bw, 0.8 mg/kg bw and 1.2 mg/kg bw). The minimum dose, 0.4 mg/kg bw, at T_2, has the most significant analgesic effect, compared to 0.8 mg/kg bw and 1.2 mg/kg bw compared to T_0 (38.57 ± 7.98% at T_2).

In *algesimetry*, 0.4 mg/kg bw H4415 is obvious from T1 with a strong analgesic effect, which amplifies at T_3 and T_4 (96.67 ± 3.33% at T_3 and 93.33 ± 6.67% at T_4). The differences in T_3 and T_4 of 0.4 mg/kg bw and 0.8 mg/kg bw are relatively small, but the nociceptive effect registers a slight decrease at T_4 for 0.8 mg/kg bw.

In the *cold plate* test, H4415 had a global anti-nociceptive effect, but the most important was seen at 0.4 mg/kg bw at T_3 and T_4 compared to T_0 (90.32 ± 1.51% at T3 and 98.4 ± 1.6% at T_4).

In the *tail-flick test*, the used doses of the compound H4415 determined an antinociceptive effect but did not exceed the statistical significance threshold below 5%.

The experimental evaluation of pain in the present study uses behavioural algesiology models such as tail flick, hot plate, cold plate and algesimetry to test the antinociceptive properties of H4415. Their use is widespread because they provide information regarding the animal's behaviour when experiencing acute pain.

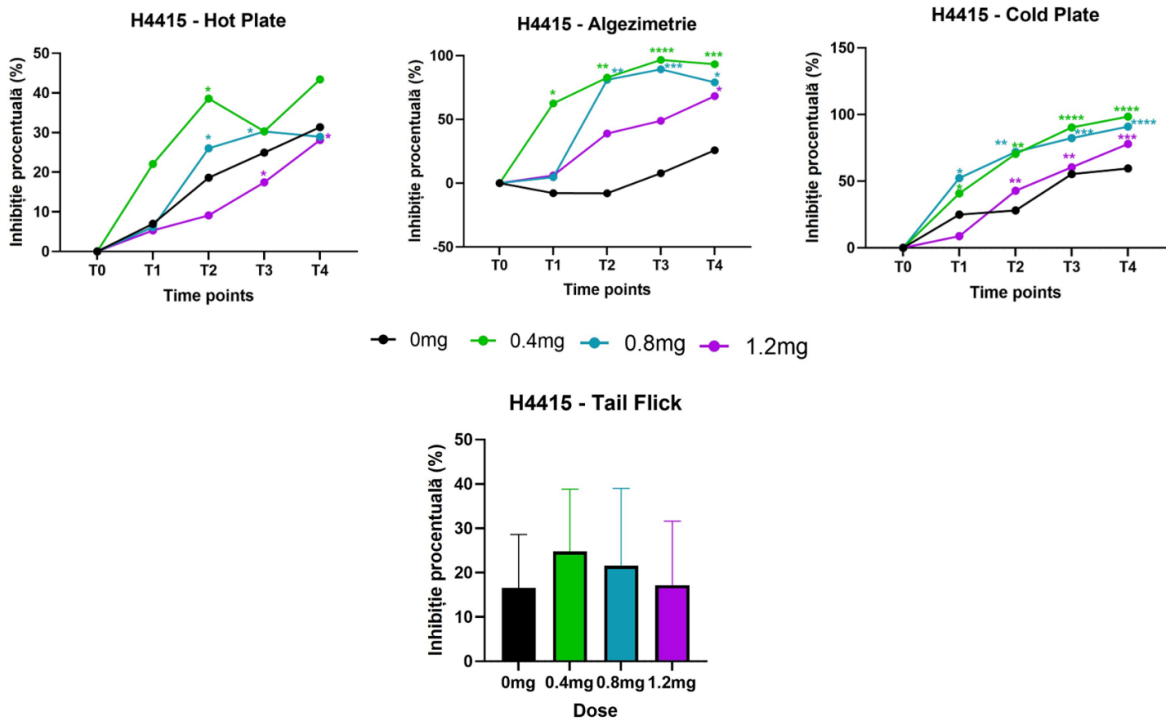

Figure 1.

Results were obtained using compound H4415 in the hot plate (top left - a), algesimetry (top middle - b), cold plate (top right - c), and tail flick (bottom middle - d) assays. The obtained graphs represent the analgesic effect obtained for the doses used: 0.4 mg/kg (green), 0.8 mg/kg (blue), and 1.2 mg/kg (pink), compared to the control group (black, glucose solution). T_0 - determinations performed before H4415 administration. T_1 - determinations at 15 minutes, T_2 - determinations at 30 minutes, T_3 - determinations at 45 minutes, T_4 - determinations at 60 minutes for tail flick, hot plate and algesimetry. In the cold plate test, T_1 - at 10 minutes, T_2 - at 20 minutes.

When it was first discovered in 2003, the TRPA1 receptor was incriminated to be related to detecting temperatures at a threshold of 17.00°C [34]. Later, in the literature, TRPA1 was described as a sensor for cold [25, 36], a statement supported by other studies [37-40]. Despite these publications, it has not been established with certainty that TRPA1 senses cold temperature. What can be said is that there are other receptors involved, *e.g.* TRPM8.

In this study, we demonstrated pain inhibition by antagonising TRPA1 receptors. To our knowledge, this study is the first to evaluate the antinociceptive effect of H4415 in the rat using the tail flick, hot plate, cold plate and pressure algesimetric tests.

The TRPA1 channel is involved in sensing various external and internal stimuli, such as different chemical agonists, possibly temperature and calcium ions. In 2007, the hypothesis emerged that TRPA1 receptors are activated when intracellular action potentials reach a high positive level [39, 41, 42]. Voltage-gated ion channels, such as K^+ and Na^+, are activated by the movement of positively charged ions. Compared to other channels in the TRP family, the electrical activation of TRPA1 is weak, approximately 0.4 (1). The -COOH terminus seems more plausible to act as a voltage sensor via two positively charged residues, R975 and K989 [43]. Other acidic residues influencing electrical charge are K969, T1078, K1071, K1009, R1009 and E1077 [43, 44]. Possessing a small number of electrical charges, the TRPA1 receptor-bound ion channel opens at physiological action potentials only in the presence of an agonist [42]. Separate from the activation at high action potentials, the literature mentions that inactivation is also given by high values of the intracellular action potential [42, 45]. Considering this information, it can be hypothesised that these channels are both voltage-dependent and independent. Studies have shown that pharmacological inhibitors of TRPA1 are effective in attenuating mechanical hyperalgesia associated with neuropathic pain conditions. Additionally, TRPA1 is required for normal mechanosensation and is modulated by algesic stimuli. Pharmacological inhibitors of TRPA1 are effective in attenuating mechanical hyperalgesia associated with neuropathic pain conditions. Additionally, TRPA1-deficient mice develop normal mechanical hyperalgesia, indicating that TRPA1 is not essential for developing mechanical hyperalgesia but is required for sensitising nociceptors in response to injury and inflammation [46]. The channel is also known to be activated by direct calcium ions and is involved in the sensation of inflammatory pain. Pharmacological inhibitors of TRPA1 are effective in attenuating mechanical hyperalgesia associated with neuropathic

pain conditions. Additionally, TRPA1-deficient mice develop normal mechanical hyperalgesia, indicating that TRPA1 is not essential for developing mechanical hyperalgesia, but is required for sensitising nociceptors in response to injury and inflammation [47].

Clinical interest in TRPA1 receptors has increased recently due to their expression in nociceptive neurons. Their sensitivity to reactive oxygen and carbonyl species incriminates them as having a role in diabetic neuropathy [48] or that secondary to chemotherapy [49]. Prolonged exposure to low doses of atmospheric or food pollutants causes agonist-induced sensitisation to painful stimuli [50, 51]. This mechanism could be useful in the management of chronic pain conditions. Heat-induced pain was suppressed at all three doses (0.4 mg/kg bw, 0.8 mg/kg bw and 1.2 mg/kg bw). The antinociceptive effect obtained in the hot plate test is an inverse-response type. The same effect was also observed in *algesimetry*, where a mechanical stimulus was tolerated longer after the intraperitoneal administration of 0.4 mg/kg H4415. It is possible that the coupling of H4415 on the TRPV would not act as a perfect antagonist. Still, its binding might reduce the affinity further binding, thus inhibiting its biological response at higher doses. Such an effect might shift the dose-response curve to the left, indicating a decrease in the potency of the substances. This type of response is particularly relevant when the receptor is constitutively active, thus having a basal activity that does not need to be inhibited or stimulated. This can be particularly important in cases where the channel is spontaneously open, and the blockage will counteract its activity. Although the hypothesis that thermal and chemical stimuli activate TRPA1 receptors is unanimously accepted, mechano-nociception is rarely described in the literature with the TRP vanilloid receptor family, of which the TRPA1 receptor is also a part [51].

Conclusions

In conclusion, we can state that inhibition of TRPA1 receptors improves tolerance to painful mechanical and thermal stimuli, including high and low temperatures. The increase in pain threshold secondary to systemic administration of H4415 indicates that TRPA1 inhibition represents a potential alternative for pain treatment. New studies are needed to fully characterise the molecular mechanisms underlying the analgesic effects of H4415.

Conflict of interest

The authors declare no conflict of interest.

References

1. Woolf CJ, What is this thing called pain? *J Clin Invest.*, 2010; 120(11): 3742-3744.
2. Eid SR, Crown ED, Moore EL, Liang HA, Choong KC, Dima S, Henze DA, Kane SA, Urban MO, HC-030031, a TRPA1 selective antagonist, attenuates inflammatory- and neuropathy-induced mechanical hypersensitivity. *Mol Pain*, 2008; 4: 48.
3. Koivisto A, Pertovaara A, Transient receptor potential ankyrin 1 (TRPA1) ion channel in the pathophysiology of peripheral diabetic neuropathy. *Scand J Pain*, 2013; 4(3): 129-136.
4. Patapoutian A, Tate S, Woolf CJ, Transient receptor potential channels: targeting pain at the source. *Nat Rev Drug Discov.*, 2009; 8(1): 55-68.
5. del Camino D, Murphy S, Heiry M, Barrett LB, Earley TJ, Cook CA, Petrus MJ, Zhao M, D'Amours M, Deering N, Brenner GJ, Costigan M, Hayward NJ, Chong JA, Fanger CM, Woolf CJ, Patapoutian A, TRPA1 contributes to cold hypersensitivity. *J Neurosci.*, 2010; 30(45): 15165-15174.
6. Kosugi M, Nakatsuka T, Fujita T, Kuroda Y, Kumamoto E, Activation of TRPA1 channel facilitates excitatory synaptic transmission in substantia gelatinosa neurons of the adult rat spinal cord. *J Neurosci.*, 2007; 27(16): 4443-4451.
7. Fernandes ES, Fernandes MA, Keeble JE, The functions of TRPA1 and TRPV1: moving away from sensory nerves. *Br J Pharmacol.*, 2012; 166(2): 510-521.
8. Shigetomi E, Tong X, Kwan KY, Corey DP, Khakh BS, TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. *Nat Neurosci.*, 2011; 15(1): 70-80.
9. Paulsen CE, Armache JP, Gao Y, Cheng Y, Julius D, Structure of the TRPA1 ion channel suggests regulatory mechanisms. *Nature*, 2015; 520(7548): 511-517.
10. Gaudet R, TRP channels entering the structural era. *J Physiol.*, 2008; 586(15): 3565-3575
11. Miyamoto T, Dubin AE, Petrus MJ, Patapoutian A, TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. *PLoS One*, 2009; 4(10): e7596.
12. Andersson DA, Gentry C, Moss S, Bevan S, Clioquinol and pyrithione activate TRPA1 by increasing intracellular Zn2+. *Proc Natl Acad Sci USA*, 2009; 106(20): 8374-8379.
13. Andersson DA, Gentry C, Moss S, Bevan S, Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci.*, 2008; 28(10): 2485-2494.
14. Souza Monteiro de Araujo D, Nassini R, Geppetti P, De Logu F, TRPA1 as a therapeutic target for nociceptive pain. *Expert Opin Ther Targets.*, 2020; 24(10): 997-1008.
15. Basbaum AI, Bautista DM, Scherrer G, Julius D, Cellular and molecular mechanisms of pain. *Cell*, 2009; 139(2): 267-284.
16. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM, Fanger CM, TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci USA*, 2007; 104(33): 13525-13530.
17. Xu Y, Huang C, Deng H, Jia J, Wu Y, Yang J, Tu W, TRPA1 and substance P mediate stress induced duodenal lesions in water immersion restraint stress

rat model. *Turk J Gastroenterol.*, 2018; 29(6): 692-700.
18. Zhu H, Wang Y, He Y, Yu W, Inflammation-mediated macrophage polarization induces TRPV1/ TRPA1 heteromers in endometriosis. *Am J Transl Res.*, 2022; 14(5): 3066-3078.
19. Legrand C, Merlini JM, de Senarclens-Bezençon C, Michlig S, New natural agonists of the transient receptor potential Ankyrin 1 (TRPA`) channel. *Sci Rep.*, 2020; 10(1): 11238.
20. Maqboul A, Elsadek B, A Novel Model of Cancer-Induced Peripheral Neuropathy and the Role of TRPA1 in Pain Transduction. *Pain Res Manag.*, 2017; 2017: 3157207.
21. Jacob S, de la Torre J, Pharmacology of dimethyl sulfoxide in cardiac and CNS damage. *Pharmacol Rep.*, 2009; 61(2): 225-235.
22. Attarian S, Janakiram M, Ezzati A, Gucalp RA, Dimethyl sulfoxide (DMSO) cryopreserved stem cell infusion-related seizures: A single institution experience. *J Clin Oncol.*, 2017; 35(15_suppl): e19002-e19002.
23. Alexa T, Marza A, Voloseniuc T, Tamba B, Enhanced analgesic effects of tramadol and common trace element coadministration in mice. *J Neurosci Res.*, 2015; 93(10): 1534-1541.
24. Tamba BI, Leon MM, Petreus T, Common trace elements alleviate pain in an experimental mouse model. *J Neurosci Res.*, 2013; 91(4): 554-561.
25. Singh P, Kongara K, Harding D, Ward N, Dukkipati VSR, Johnson C, Chambers P, Comparison of electroencephalographic changes in response to acute electrical and thermal stimuli with the tail flick and hot plate test in rats administered with opiorphin. *BMC Neurol.*, 2018; 18(1): 43.
26. Bannon AW, Malmberg AB, Models of nociception: hot-plate, tail-flick, and formalin tests in rodents. *Curr Protoc Neurosci.*, 2007; 41: 8.9.1-8.9.16.
27. Vasincu A, Miron A, Bild V, Preliminary research concerning antinociceptive and antiinflammatory effects of two extracts from *Glinus oppositifolius* (L.) Aug. DC. *Rev Med Chir Soc Med Nat Iasi*, 2014; 118(3): 866-872
28. Leon-Constantin MM, Alexa-Stratulat T, Luca A, Tamba BI, Trandafir LM, Harabagiu V, Cojocaru E, The morphofunctional impact of topical Lidocaine formulation in inflammatory pain - experimental study. *Rom J Morphol Embryol.*, 2019; 60(3): 869-874.
29. Micheli L, Rajagopalan R, Lucarini E, Toti A, Parisio C, Carrino D, Pacini A, Ghelardini C, Rajagopalan P, Di Cesare Mannelli L, Pain Relieving and Neuro-protective Effects of Non-opioid Compound, DDD-028, in the Rat Model of Paclitaxel-Induced Neuropathy. *Neurotherapeutics*, 2021; 18(3): 2008-2020.
30. Miyamura S, Iwahashi T, Sayanagi J, Hirai Y, Okada K, Oka K, Niiyama E, Uto K, Ebara M, Yoshikawa H, Murase T, Tanaka H, A Nanofiber Sheet Incorporating Vitamin B12 Promotes Nerve Regeneration in a Rat Neurorrhaphy Model. *Plast Reconstr Surg Glob Open*, 2019; 7(12): e2538.
31. Chen J, Luo C, Li H, Chen H, Primary hyperalgesia to mechanical and heat stimuli following subcutaneous bee venom injection into the plantar surface of hindpaw in the conscious rat: a comparative study with the formalin test. *Pain*, 1999; 83(1): 67-76.
32. Qnais EY, Alqudah A, Wedyan M, Athamneh RY, Abudalo R, Oqal M, Gammoh O, The analgesic properties of the flavonoid galangin in experimental animal models of nociception. *Farmacia*, 2023; 71(5): 1054-1063.
33. Spataru MC, Coman C, Bild V, Stanciu G-D, Velescu BS, Beșchea Chiriac SI, Spataru C, The Romanian student perception about experimentation on animals. *Farmacia*, 2022; 70(6): 1194-1203.
34. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A, ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, 2003; 112(6): 819-829.
35. Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Högestätt ED, Meng ID, Julius D, Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature*, 2004; 427(6971): 260-265.
36. Nagata K, Duggan A, Kumar G, García-Añoveros J, Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. *J Neurosci.*, 2005; 25(16): 4052-4061.
37. Bandell M, Story GM, Hwang SW, Viswanath V, Eid SR, Petrus MJ, Earley TJ, Patapoutian A, Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron*, 2004; 41(6): 849-857.
38. Fajardo O, Meseguer V, Belmonte C, Viana F, TRPA1 channels mediate cold temperature sensing in mammalian vagal sensory neurons: pharmacological and genetic evidence. *J Neurosci.*, 2008; 28(31): 7863-7875.
39. Karashima Y, Damann N, Prenen J, Talavera K, Segal A, Voets T, Nilius B, Bimodal action of menthol on the transient receptor potential channel TRPA1. *J Neurosci.*, 2007; 27(37): 9874-9884.
40. Moparthi L, Survery S, Kreir M, Simonsen C, Kjellbom P, Högestätt ED, Johanson U, Zygmunt PM, Human TRPA1 is intrinsically cold- and chemosensitive with and without its N-terminal ankyrin repeat domain. *Proc Natl Acad Sci USA*, 2014; 111(47): 16901-16906.
41. Zurborg S, Yurgionas B, Jira JA, Caspani O, Heppenstall PA, Direct activation of the ion channel TRPA1 by Ca2+. *Nat Neurosci.*, 2007; 10(3): 277-279.
42. Meents JE, Fischer MJ, McNaughton PA, Agonist-induced sensitisation of the irritant receptor ion channel TRPA1. *J Physiol.*, 2016; 594(22): 6643-6660.
43. Samad A, Sura L, Benedikt J, Ettrich R, Minofar B, Teisinger J, Vlachova V, The C-terminal basic residues contribute to the chemical- and voltage-dependent activation of TRPA1. *Biochem J.*, 2011; 433(1): 197-204.
44. Sura L, Zíma V, Marsakova L, Hynkova A, Barvík I, Vlachova V, C-terminal acidic cluster is involved in $Ca2^{+}$-induced regulation of human transient receptor potential ankyrin 1 channel. *J Biol Chem.*, 2012; 287(22): 18067-18077.

45. Fajardo O, Meseguer V, Belmonte C, Viana F, TRPA1 channels: novel targets of 1,4-dihydropyridines. *Channels (Austin)*, 2008; 2(6): 429-438.
46. Petrus M, Peier AM, Bandell M, Hwang SW, Huynh T, Olney N, Jegla T, Patapoutian A, A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain.*, 2007; 3: 40.
47. Mickle AD, Shepherd AJ, Mohapatra DP, Nociceptive TRP Channels: Sensory detectors and Transducers in Multiple Pain Pathologies. *Pharmaceuticals*, 2016; 9(4): 72.
48. Andersson DA, Gentry C, Light E, Vastani N, Vallortigara J, Bierhaus A, Fleming T, Bevan S. Methylglyoxal evokes pain by stimulating TRPA1. *PLoS One*, 2013; 8(10): e77986.
49. Nassini R, Gees M, Harrison S, De Siena G, Materazzi S, Moretto N, Failli P, Preti D, Marchetti N, Cavazzini A, Mancini F, Pedretti P, Nilius B, Patacchini R, Geppetti P, Oxaliplatin elicits mechanical and cold allodynia in rodents *via* TRPA1 receptor stimulation. *Pain*, 2011; 152(7): 1621-1631.
50. Kunkler PE, Zhang L, Pellman JJ, Oxford GS, Hurley JH, Sensitization of the trigeminovascular system following environmental irritant exposure. *Cephalalgia*, 2015; 35(13): 1192-201.
51. Meents JE, Ciotu CI, Fischer MJM, TRPA1: a molecular view. *J Neurophysiol.*, 2019; 121(2): 427-443.
52. Moore C, Gupta R, Jordt SE, Chen Y, Liedtke WB, Regulation of Pain and Itch by TRP Channels. *Neurosci Bull.*, 2018; 34(1): 120-142.