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Original Article

Analgesic effect of neohesperidin is mediated by TRPV1 antagonism

[El efecto analgésico de la neohesperidina está mediado por el antagonismo TRPV1]

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Abstract

Context: Transient receptor potential vanilloid type 1 (TRPV1) is a non-specific cation channel. It is one of the most important targets in pain research.

Aims: To evaluate new TRPV1 antagonists without altering body temperature.

Methods: Docking simulation was performed, and one of the candidate compounds, neohesperidin, was tested using thermal and chemical pain models in BALB/c mice. Rectal body temperature was measured using a temperature meter with a thermocouple probe detector, and the capsaicin-evoked calcium response was determined in dorsal root ganglia (DRG) neurons.

Results: Docking resulted in the identification of 30 compounds able to interact with the essential amino acids required for the antagonistic activity of TRPV1. Neohesperidin was chosen for further investigations because of its good binding energy (-6.63 kcal/mol) and because its TRPV1 antagonistic activity was not tested before. This study reported for the first time that neohesperidin exerted analgesic activity through TRPV1 antagonism without altering body temperature. Its activity was comparable to the known TRPV1 antagonist N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC). In the writhing test, acetic acid-induced abdominal cramps decreased by 66% using 30 mg/kg of neohesperidin. All tested doses of neohesperidin significantly decreased paw-licking time in the capsaicin-induced paw-licking test. A significant increase in the latency time in hot plate and tail flick tests was observed using 30 and 60 mg/kg of neohesperidin. In DRG neurons, neohesperidin reduced capsaicin-evoked calcium responses.

Conclusions: Neohesperidin exerts a significant analgesic activity without altering body temperature, which could be due, at least partially, to its antagonistic activity against TRPV1.

Keywords: analgesic; capsaicin; computer-aided design; molecular docking simulation; neohesperidin; TRPV1 receptor.

Resumen

Contexto: El receptor potencial transitorio vanilloide tipo 1 (TRPV1) es un canal de cationes inespecífico. Es una de las dianas más importantes en la investigación del dolor.

Objetivos: Evaluar nuevos antagonistas de TRPV1 sin alterar la temperatura corporal.

Métodos: Se realizó una simulación de acoplamiento y se probó uno de los compuestos candidatos, la neohesperidina, utilizando modelos de dolor térmico y químico en ratones BALB/c. Se midió la temperatura corporal rectal utilizando un medidor de temperatura con detector de sonda termopar y se determinó la respuesta de calcio evocada por la capsaicina en las neuronas de los ganglios de la raíz dorsal (GRD).

Resultados: Se identificaron 30 compuestos capaces de interactuar con los aminoácidos esenciales necesarios para la actividad antagonista de TRPV1. Se eligió la neohesperidina para futuras investigaciones por su buena energía de unión (-6,63 kcal/mol) y porque su actividad antagonista del TRPV1 no se había probado antes. Este estudio informó por primera vez de que la neohesperidina ejercía actividad analgésica a través del antagonismo TRPV1 sin alterar la temperatura corporal. Su actividad fue comparable a la del conocido antagonista del TRPV1 N-(4-terbutilfenil)-4-(3-cloropiridin-2-il)tetrahidropirazina-1(2H)-carbox-amida (BCTC). En la prueba de retorcimiento, los calambres abdominales inducidos por ácido acético disminuyeron en un 66% con 30 mg/kg de neohesperidina. Todas las dosis probadas de neohesperidina disminuyeron significativamente el tiempo de lamido de la pata en la prueba de lamido de la pata inducido por capsaicina. Con 30 y 60 mg/kg de neohesperidina se observó un aumento significativo del tiempo de latencia en las pruebas de la placa caliente y del movimiento de la cola. En las neuronas DRG, la neohesperidina redujo las respuestas de calcio provocadas por la capsaicina.

Conclusiones: La neohesperidina ejerce una actividad analgésica significativa sin alterar la temperatura corporal, lo que podría deberse, al menos parcialmente, a su actividad antagonista frente al TRPV1.

Palabras Clave: analgésico; capsaicina; diseño asistido por ordenador; simulación de acoplamiento molecular; neohesperidina; receptor TRPV1.

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INTRODUCTION

Pain is an unpleasant sensory and emotional experience that is associated with current or potential tissue damage. It is a response to the stimulation of the nervous system and gives a warning sign to the living organism of internal or external harm (Raja et al., 2020). Pain can be described as mild, moderate or severe depending on the nervous system response. It is also characterized as either acute or chronic, depending on how long it lasts (Basbaum et al., 2009). Patients are provided medications based on the sort of pain they are suffering from. Acute moderate to severe pain is treated with opioid analgesics, which operate primarily by activating opioid receptors (Trescot et al., 2008). Despite their efficient pain reduction, opioids may cause addiction, breathing problems, constipation, nausea, dizziness, and drowsiness (Virgen et al., 2022). Inflammatory pain is treated with a variety of non-steroidal anti-inflammatory drugs (NSAIDs). The side effects of NSAIDs vary with each medicine, but they commonly include an increased risk of gastrointestinal ulcers, bleedings, heart attacks, and kidney illness (Bally et al., 2017; Lanas and Chan, 2017). Accordingly, the pharmaceutical industry is putting a lot of effort into finding novel analgesics to overcome the currently available painkiller complications.

Transient receptor potential vanilloid type 1 (TRPV1), also called the capsaicin receptor, is a non-selective cation channel that can be activated by several endogenous and exogenous physical and chemical stimuli such as capsaicin, protons, temperature, and free reactive oxygen species released from inflammatory molecules after exposure to harmful stimuli (Eom et al., 2021; Everaerts et al., 2011; Laursen et al., 2016). It contributes to acute and chronic pain and can be modulated by inflammatory mediators (Abbas, 2020). For decades, capsaicin creams and patches were used clinically to treat acute and chronic painful conditions. They act by desensitizing TRPV1 channels. However, the wide spread of this treatment has been reduced due to its burning sensation and erythema (Fattori et al., 2016). Resiniferatoxin (RTX), the most potent TRPV1 agonist known, is about one thousand times more pungent than pure capsaicin. RTX is used to manage severe pain associated with cancer, but it can cause severe chemical burns and tissue damage (Brown, 2016).

TRPV1 channel is one of the most important targets in pain research (Jaffal et al., 2022). Unfortunately, the clinical use of TRPV1 antagonists was limited due to the alteration of body temperature. The majority of them cause hyperthermia. Although some cause hypothermia or being euthermic (Abbas, 2020). Therefore, the search for new euthermic TRPV1 modulators continues. Many natural and synthetic compounds were tested for their activity on TRPV1. Phytochemicals from different chemical groups like capsaicinoids, alkaloids, flavonoids, terpenoids, fatty acids, terpenyl phenols, and others modulate the activity of TRPV1 to varying degrees (Abbas, 2020). From ginger, *Zingiber officinale*, the pungent compounds gingerol, shogaol and zingerone were found to activate TRPV1 (Yin et al., 2019). Similarly, diallyl sulfides in garlic and piperine, the pungent component of black pepper, activate TRPV1 (Koizumi et al., 2009; McNamara et al., 2005). On the other hand, cinnamodial isolated from the bark of *Cinnamosma fragrans* acts as a partial TRPV1 agonist (Szallasi et al., 1998).

The discovery of novel therapeutic drugs is both a time-consuming and expensive process. It is estimated that a classical drug discovery cycle, from lead identification to clinical trials, can take 12-15 years with a cost of 1.2 billion USD (Kumar et al., 2019). Progresses in the fields of biochemistry, molecular biology, and cell biology are generating a large number of novel biological targets that may be subjected for therapeutic benefits (Lewis, 2010). Therefore, there was a need for computational techniques to facilitate the discovery of new active compounds. This study uses a structure-based drug design protocol to discover new TRPV1 antagonists. One of the hits with good binding energy was chosen to complete other experiments, namely neohesperidin (NHP). NHP is a dihydroxyflavone rich in the peels of oranges, lemons, and grapefruit and is widely used in traditional medicine (Wang et al., 2023). This citrus flavonoid was selected because its effect on TRPV1 activity was not tested before. Two approaches were utilized: (1) Study the effect of NHP on capsaicin-evoked calcium response in dorsal root ganglia (DRG) neurons. (2) Testing the analgesic activity of NHP *in vivo* using thermal (hot plate and tail immersion tests) and chemical (writhing and capsaicin tests) pain models in BALB/c mice. In addition, rectal body temperature was measured to check if NHP alters body temperature.

This study aims to evaluate new TRPV 1 antagonists utilizing Structure-Based Drug Design protocol. Molecular docking was carried out with AutoDock 4.2 Software of an in-house prepared small molecule database. The most promising candidates were further investigated *in vivo* for their TRPV1 antagonistic effect in mice using thermal (hot plate and tail immersion tests) and chemical (writhing and capsaicin) pain models.

MATERIAL AND METHODS

In silico study

The following software packages were utilized in this project: ACD/ChemSketch, (www.acdlabs.com), Autodock 4.2 (The Scripps Research Institute, San Diego, CA, USA), and Biovia Discovery Studio visualizer (http://accelrys.com). TRPV1's crystal structure (PDB entry: 5IS0) was obtained from the Protein Data Bank. Both proteins and chemical compounds received Kollman and Gasteiger charges (Gasteiger and Marsili, 1980). Chemical compounds used in this study were utilized from the in-house database (around 100 compounds) (Jaffal et al., 2021). These compounds were screened to determine the best docking candidate to bind TRPV1 as capsazepine within 5IS0. A set of grid maps were created using Auto Grid 4.2 within a grid box of 18.75, 18.75, and 18.75 Å (x, y, and z). Molecular docking was performed utilizing the Lamarckian Genetic Algorithm to find the most suitable docking poses. Intermolecular interactions between TRPV1 and each compound were analyzed according to the binding energy and amino acid interactions. Hits were further investigated *in vivo* for TRPV1 antagonistic activity. ChemSketch software was used to draw and save the chemical compounds as mol files, which were then minimized and converted to pdb files using Avogadro software (Hanwell et al., 2012).

For the preparation of the ligands, ChemSketch software (https://www.acdlabs.com/) was used to sketch and save the chemical compounds as mol files, which were then minimized and converted to 3D structure in pdb file format using Avogadro software (https://avogadro.cc/) (Hanwell et al., 2012).

Drugs and chemicals

NHP was supplied by Resonance Research Lab (CAS No: 13241-33-3). NHP was dissolved in a solution of Dimethyl sulfoxide (DMSO, Fisher BioReagents, USA). Further dilution of NHP was performed using normal saline so that the final concentration of DMSO in solutions injected to animals does not exceed 2%. N-(4-tertiarybutylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC), a high potency selective TRPV1 receptor antagonist, was supplied by Tocris Bioscience (CAS No: 393514-24-4, USA) and was dissolved in 2% DMSO in normal saline. Capsaicin was supplied by Tocris Bioscience (CAS No: 404-86-4, USA), and the stock solution was dissolved in absolute ethanol (GCC Diagnostics, CAS No: 05890, UK) and kept at -20°C until used. Upon use, capsaicin was then dissolved in 2% DMSO in normal saline.

In vivo study

All *in vivo* tests in this research complied with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee at Al-Ahliyya Amman University (ethical approval number: AUP: AAU/2/4/2021-2022). Male BALB/c mice (22 ± 2 g) were used. They were kept at standard laboratory conditions (23 ± 2°C) in 12 h dark/light cycle. Food and water were provided *ad libitum*.

Writhing test

The acetic acid-induced nociception was performed as described previously by Koster et al. (1959). Groups of mice (n = 6) were treated with vehicle (2% DMS in saline, 10 mL/kg, intraperitoneally (i.p.) or NHP (10, 30 and 60 mg/kg, i.p.) or BCTC (30 mg/kg, i.p.), a standard TRPV1 antagonist, 30 min before the administration of acetic acid (1%, 10 mL/kg, i.p.). The number of abdominal constrictions (writhes) was counted for each animal, starting 10 min after acetic acid injection over a period of 20 min using a mobile camera. Writhe is defined as a contraction of the abdominal muscles accompanied by the elongation of the body and extension of the forelimbs (Koster, 1959).

Capsaicin-induced paw-licking test

Mice were weighed and divided into four groups. Each group consisted of six animals. The tested mouse received either a vehicle solution (2% DMSO in normal saline) and serves as a control, 0.2 mL of BCTC 30 mg/kg (standard antagonist) or 0.2 mL of NHP (60, 30 or 10 mg/kg) i.p. Thirty minutes later, 6 µg capsaicin (20 µL/foot pad) was intra-plantarly injected to the right paw of the mouse. The total time spent in licking the injected paw, lifting the leg, or exhibiting a flinching behavior was recorded in the first five minutes after the administration of capsaicin (Sakurada et al., 1992).

Hot plate test

The acclimatization period of mice in a quiet room before the beginning of the tests was 60 min. Each animal was admitted to this procedure once. Thirty minutes before the experiment, mice were weighed and divided into four groups each containing eight animals. The animals received either vehicle (2% DMSO in normal saline, control), 0.2 mL of BCTC 30 mg/kg (standard antagonist) or 0.2 mL of NHP 10, 30 or 60 mg/kg i.p. The hot-plate test based on the method of Eddy and Leimbach (1953) was used with slight modifications. The time taken to jump up (reaction time) was recorded when the mouse was placed on the hot plate (Thermofisher, USA) maintained at

55 ± 1.0°C. A cut-off time of 30 s was used to avoid tissue damage (Duo et al., 2018).

Tail flicking test

The acclimatization period in a quiet room before the beginning of the test was 60 min. The tail-flick test based on the method of Janssen et al. (1963) was used with slight modifications to evaluate the antinociceptive effect of NHP. Mice were weighed and divided into four groups. Each group consisted of eight animals. The tested animal received either 0.2 mL vehicle solution of 2% DMSO in normal saline (control), 0.2 mL of BCTC 30 mg/kg (standard antagonist) or 0.2 mL of NHP 10, 30 or 60 mg/kg i.p. The time was taken to flick the tail (the reaction time) when it was immersed (3–4 cm from its tip) in a water bath at 55 ± 1.0°C was noted. A cut-off time of 10 s was used to avoid tissue damage (Hanlon and Vanderah, 2010).

Effects of NHP on body temperature

The body temperature of mice was measured for 1 min rectally using a TM-902C temperature meter with a thermocouple probe detector (Mikroelectron electronics online store).

Calcium imaging of dorsal root ganglion neurons

Dorsal root ganglia (DRGs) neurons were obtained after removing the spinal columns, and cell preparation and culture were performed as previously described (Jaffal et al., 2022). Cells were washed three times with calcium buffer (NaCl, 145 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgSO_4.7H_2O$, 1 mM; HEPES, 10 mM; glucose, 10 mM; pH, 7.4). Cells were then loaded with 5 µL Fura2-AM and incubated for 30 min in the dark with 895 µL of calcium buffer and 100 µL of fetal calf serum. Before the imaging, cells were washed with calcium buffer and left for at least 15 min. Peak fluorescence emission intensities (measured at 500 nm) at 340 nm and 380 nm excitation wavelengths were compared to determine changes in $[Ca^{2+}]_i$ using Nikon Eclipse Ti2 inverted microscope. DRG neurons were suprafused with calcium buffer at a rate of 2 mL/min. All treatments were applied by suprafusion in calcium buffer. The excitation signal was measured by subtracting the baseline from the reading of stimulation.

Effects of NHP on capsaicin-evoked calcium responses

DRG cells were exposed to 100 nM capsaicin for 1 min followed by a 5 min wash-out period with calcium buffer (Alsalem et al., 2016). The ratio of peak fluorescence emission intensities (measured at 500 nm) at excitation wavelengths of 340 nm and 380 nm (340/380) was used to evaluate changes in $[Ca^{2+}]_i$. Peak ratios were calculated by subtracting the baseline ratio from the ratio obtained during drug superfusion (Δ 340/380). To evaluate the effect of NHP on capsaicin-evoked calcium response, cells were pre-incubated with NHP (10 µM for 4 min) or capsazepine (10 µM for 4 min) or vehicle before capsaicin (100 nM). Results were expressed as the ratio of peak response difference and presented as mean ± SEM (standard error of the mean).

Statistical analysis

GraphPad Prism version 6 was used to perform statistical analysis. One-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test was used for all *in vivo* tests and calcium imaging studies. For body temperature measurements, a *t*-test was used. Values were represented as mean ± SEM. Results were considered statistically significant when $p<0.05$.

RESULTS

In silico study

The docking results showed that 30 compounds from the in-house database have both the top docking energies as well as docking the antagonist interactions in the TRPV1 binding site (Table 1). Among them, the top-ranked six compounds were selected for analysis to choose one compound as a candidate for further *in vivo* study (Fig. 1 and Table 2).

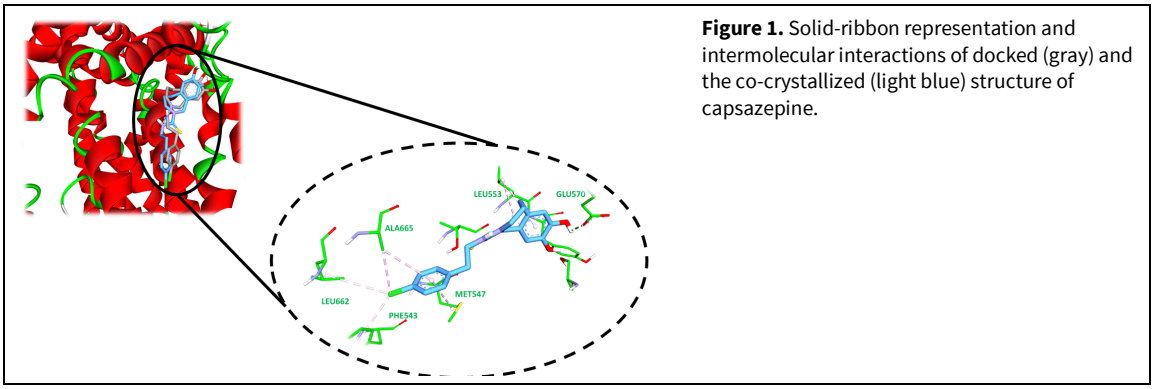


Figure 1. Solid-ribbon representation and intermolecular interactions of docked (gray) and the co-crystallized (light blue) structure of capsazepine.

Table 1. List of the analyzed compounds.

No	Ligand	Molecular formula	Molecular weight (g/mol)	PubChem ID
1	Pregnenolone sulfate	$C_{21}H_{32}O_5S$	396.5	105074
2	BCTC	$C_{20}H_{25}ClN_4O$	372.9	9929425
3	Rhamnetin	$C_{16}H_{12}O_7$	316.3	5281691
4	Hesperidin	$C_{28}H_{34}O_{15}$	610.6	10621
5	Isorhamnetin	$C_{16}H_{12}O_7$	316.3	5281654
6	Neohesperidin	$C_{28}H_{34}O_{15}$	610.6	442439
7	NSC39911	$C_{29}H_{14}ClNO_5$	491.9	236883
8	NSC618165	$C_{35}H_{32}N_4O_4$	572.7	358241
9	NSC110323	$C_{34}H_{24}N_2O_4$	524.6	269361
10	NSC354684	$C_{36}H_{44}N_2O_2$	536.7	337301
11	NSC380324	$C_{31}H_{24}N_4O_4$	516.5	54600782
12	NSC100877	$C_{32}H_{34}N_8O_4$	594.7	418440
13	NSC38283	$C_{30}H_{28}N_6O_5S_2$	616.7	54609483
14	NSC67441	$C_{38}H_{48}N_6O_2$	620.8	54609838
15	NSC68125	$C_{38}H_{36}N_6O_2$	608.7	54601266
16	Glimepiride	$C_{24}H_{34}N_4O_5S$	490.6	3476
17	Nortriptyline	$C_{19}H_{21}N$	263.4	4543
18	NSC68126	$C_{40}H_{40}N_6O_2$	636.8	54603628
19	NSC337392	$C_{23}H_{28}N_6O_5S_4$	596.8	5384478
20	NSC119937	$C_{38}H_{52}BrN_3O_5$	710.7	24191124
21	Terfenadine	$C_{32}H_{41}NO_2$	471.7	5405
22	Fexofenadine	$C_{32}H_{39}NO_4$	501.7	3348
23	Maslinic acid	$C_{30}H_{48}O_4$	472.7	73659
24	NSC26837	$C_{32}H_{42}N_4O_8$	610.7	24183371
25	NSC127487	$C_{28}H_{30}O_{14}$	590.5	278171
26	NSC203914	$C_{28}H_{36}N_2O_6$	496.6	306237
27	Doxorubicin	$C_{27}H_{29}NO_{11}$	543.5	31703
28	NSC2382	$C_{31}H_{36}N_2O_{11}$	612.6	54695425
29	NSC642056	$C_{28}H_{20}N_4O_8$	540.5	369860
30	NSC646802	$C_{30}H_{24}N_6O_9$	612.5	9571346

Table 2. The lowest binding energies to TRPV1 and the interacting amino acids using AutoDock 4.2.

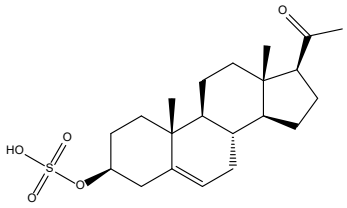
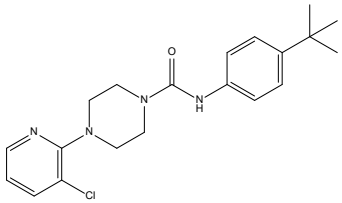
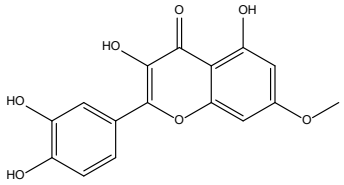
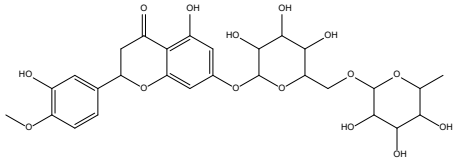
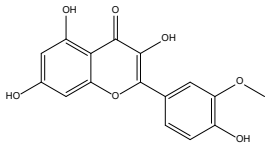
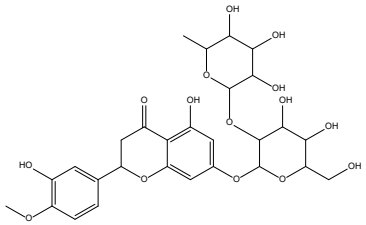
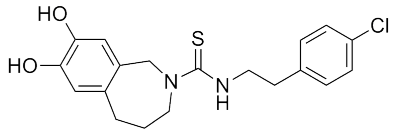
Compound name	Chemical structure	Docking energy (Kcal/mol)	Amino acid interactions
Pregnenolone sulfate		-12.31	TYR511, LEU553, THR556, ARG557
BCTC		-9.53	LEU515, SER521, MET547, THR550, LEU553, ALA566, GLU570, PHE591
Rhamnetin		-8.03	SER512, ASN551, LEU553, THR556, ARG557, ILE573, LEU669
Hesperidin		-7.96	TYR511, SER512 THR550, LEU553, ARG557, ALA566, GLU570
Isorhamnetin		-7.06	THR550, LEU553, ARG557, ALA566, VAL567, GLU570
Neohesperidin		-6.63	TYR511, SER512, LEU553, ARG557, ALA665, GLU570
Capsazipine (reference ligand)		-8.68	GLU570, HE543, MET547, LEU553, LEU662, ALA665

Fig. 1 shows the stick representation of capsazepine (light blue) bound within TRPV1 crystal structure in the active site (green). Capsazepine was found to perform one hydrogen bond interaction with GLU570 and hydrophobic interactions with five amino acids: PHE543, MET547, LEU553, LEU662, ALA665. Additionally, the solid-ribbon representation of docked (gray) and the co-crystallized (light blue) structure of capsazepine showed an RMSD of 1.4 Å.

Pregnenolone sulfate, rhamnetin, hesperidin, and isorhamnetin were among the six compounds that revealed good binding energy, with -12.31, -8.03, -7.96, -7.06 kcal/mol, respectively. Pregnenolone sulfate interacted with four amino acids via conventional hydrogen bonding with LEU553, THR556, and ARG557 and hydrophobic interactions with LEU553 and TYR556, as shown in Fig. 2A, whereas rhamnetin interacted with seven amino acids via conventional hydrogen bonding with SER512, ASN551, LEU553,

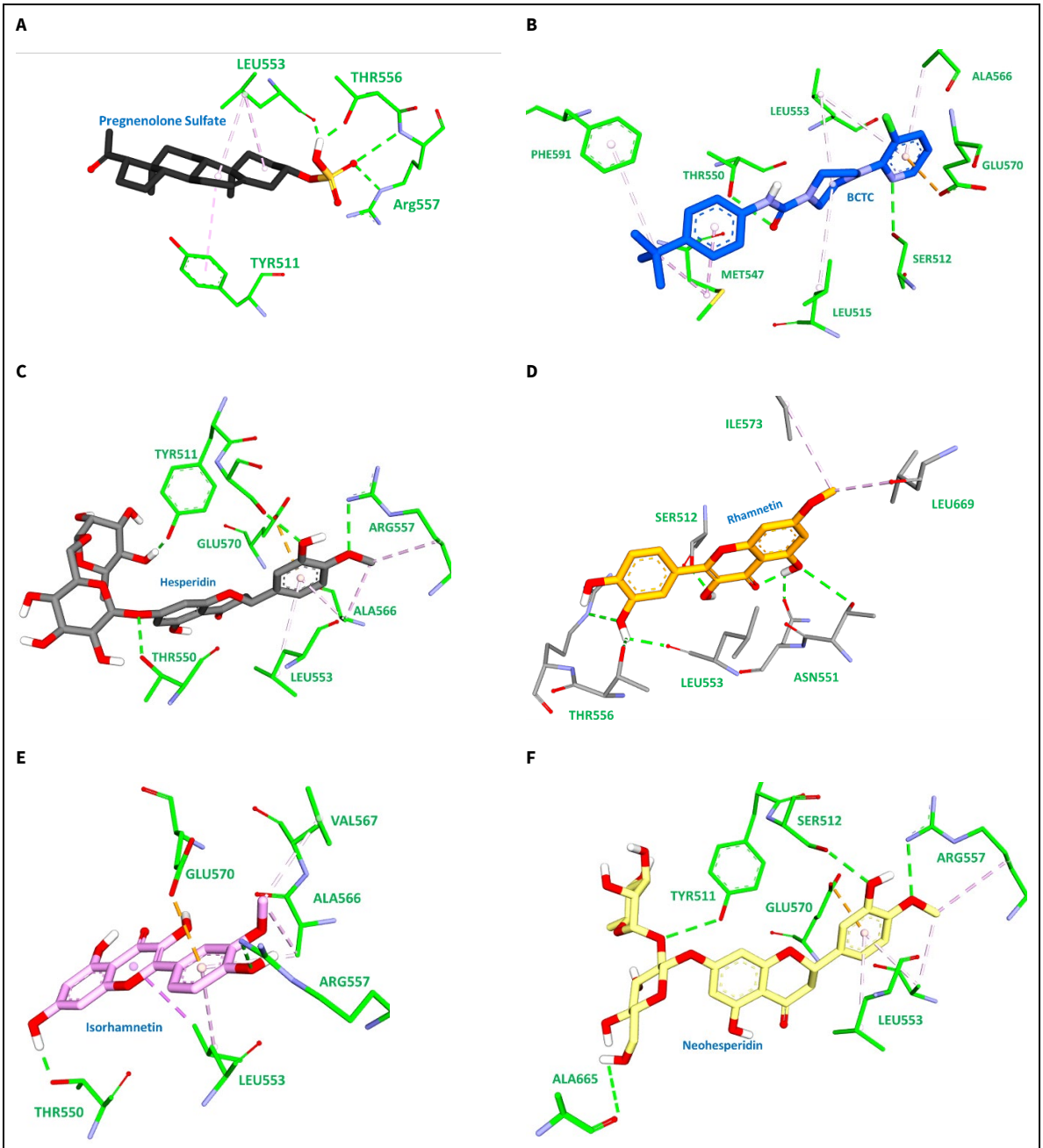


Figure 2. Stick representation of docked poses for **(A)** pregnenolone sulfate (black); **(B)** BCTC (blue); **(C)** hesperidin (gray); **(D)** rhamnetin (orange); **(E)** isorhamnetin (pink); and **(F)** neohesperidin (yellow).

THR556, and ARG557 and hydrophobic interactions with ILE573 and LEU669, as shown in Fig. 2D. Furthermore, hesperidin showed to interact with seven amino acids via conventional hydrogen bonding with TYR511, SER512, THR550, and ARG557 and hydrophobic bonding with LEU553, ARG557, ALA566, and GLU570, as shown in Fig. 2C. Isorhamnetin interacts with six amino acids via conventional hydrogen bonding with THR550 and ARG557 and hydrophobic interactions with GLU570, VAL567, ALA566, and LEU553, as shown in Fig. 2E. The other two compounds were BCTC and NHP with binding energies of -9.53 and -6.63 kcal/mol, respectively. BCTC interacts with eight amino acids via hydrogen bonds with SER521 and THR550 and hydrophobic interactions with GLU570, ALA566, LEU515, ET547, LEU553, and PHE591, as shown in Fig. 2B. NHP was found to interact with six amino acids by conventional hydrogen bond interactions with TYR511, SER512, ARG557 and ALA665 and hydrophobic bonding with LEU553, ARG557 and GLU570, as shown in Fig. 2F.

TRPV1 crystallographic structure (PDB code 5IS0) was used in this study, which co-crystallized with capsazepine. In order to validate the docking procedure, re-docking of the co-crystallized ligand, capsaz-

epine, was performed and resulted in RMSD of 1.4 Å. Generally, molecular docking simulations that produce an RMSD less than 2.0 Å are considered acceptable (Hevener et al., 2009) as shown in Fig. 2.

In vivo evaluation of NHP analgesic activity

All the tested doses of NHP decreased the mean number of writhes, when compared to vehicle-treated control group (Fig. 3A). Abdominal cramps produced by acetic acid decreased by 32%, 66% and 51% using 10 mg/kg, 30 mg/kg and 60 mg/kg of NHP, respectively compared to 58% decrease produced by BCTC (30 mg/kg). No significant difference was found between 30 and 60 mg/kg doses.

In the capsaicin-induced paw-licking test, all the tested doses of NHP decreased significantly paw licking time (Fig. 3B). The percentage of decrease was 19%, 53%, and 55% for 10 mg/kg, 30 mg/kg and 60 mg/kg NHP, respectively. BCTC (30 mg/kg) produces 61% inhibition. No significant difference was found between 30 and 60 mg/kg doses.

A significant increase in the latency time required for jumping in hot plate test was observed after using 30 mg/kg, 60 mg/kg of NHP, or 30 mg/kg of BCTC compared to control. However, there was no significant difference in latency time between them (Fig. 4A). In tail-flick test, NHP (60 mg/kg), NHP (30 mg/kg) or BCTC (30 mg/kg) increased latency significantly compared with the control, while no significant difference was found when compared to each other (Fig. 4B). On the other hand, NHP (10 mg/kg) showed no significant effect compared to control.

Effects of NHP on body temperature

The average body temperature of mice treated with 30 mg/kg NHP was 36.88 ± 0.25°C compared to 36.91 ± 0.19°C in the control group. No significant difference between the 2 groups was found.

Effects of NHP on capsaicin-evoked calcium responses

Capsaicin (100 nM) produced a robust increase in $[Ca^{2+}]_i$ compared to basaline (0.35 ± 0.05 of Δ 340/380) (Fig. 5A). Pre-incubation of DRG neurons with NHP (10 µM), or capsazepine (10 µM) for 4 min before stimulation with capsaicin, significantly reduced capsaicin-evoked calcium responses (0.06 ± 0.01 and 0.01 ± 0.01 of Δ 340/380, respectively) (Fig. 5B).

DISCUSSION

The results obtained from AutoDock 4 revealed that only 19 out of 70 of the chemical compounds had low binding energy values and similar intermolecular interactions with TRPV1 antagonists. Notably, previously proven potent TRPV1 antagonists are more likely to form hydrogen bonds with ARG557 and LEU553 amino acids (Carnevale and Rohacs, 2016). Pregnenolone sulfate and rhamnetin showed the lowest binding energy values of -12.31 and -8.03 kcal/mol, respectively. Both interact with one amino acid similar to capsazepine, LEU553, as shown in Fig 4A-D.

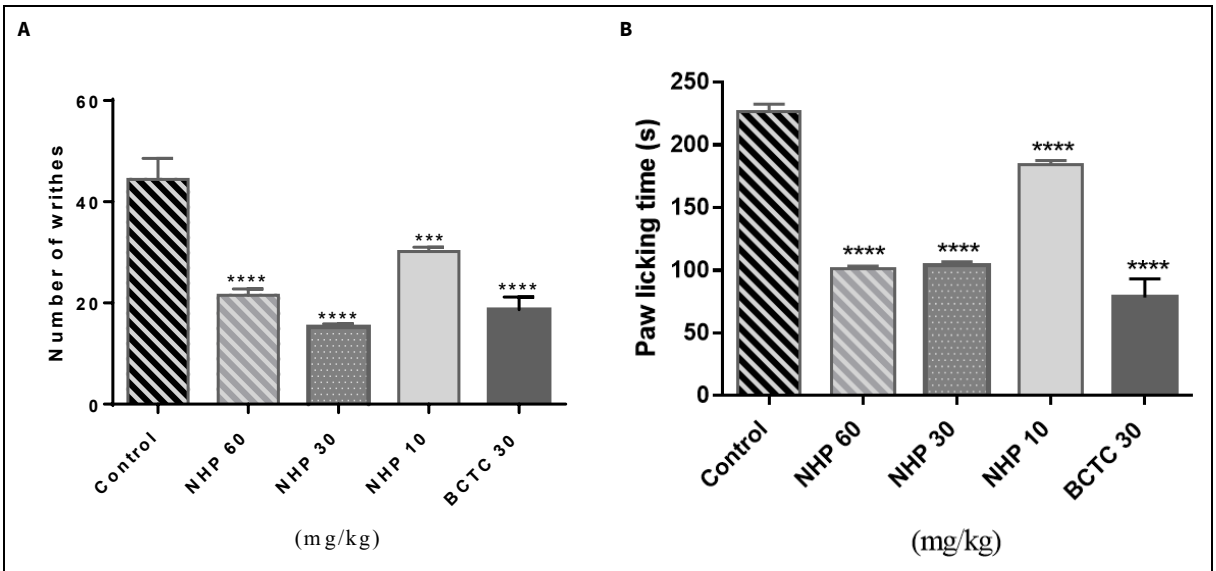


Figure 3. (A) Effect of a single dose of NHP (10–60 mg/kg, 10 mL/kg, i.p), BCTC 30 mg/kg or vehicle (2% DMSO) on the number of writhes. (**B**) Capsaicin induced-paw licking test.

Each column represents the reactivity time of 6 animals per group as mean ± SEM. For statistical analysis: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

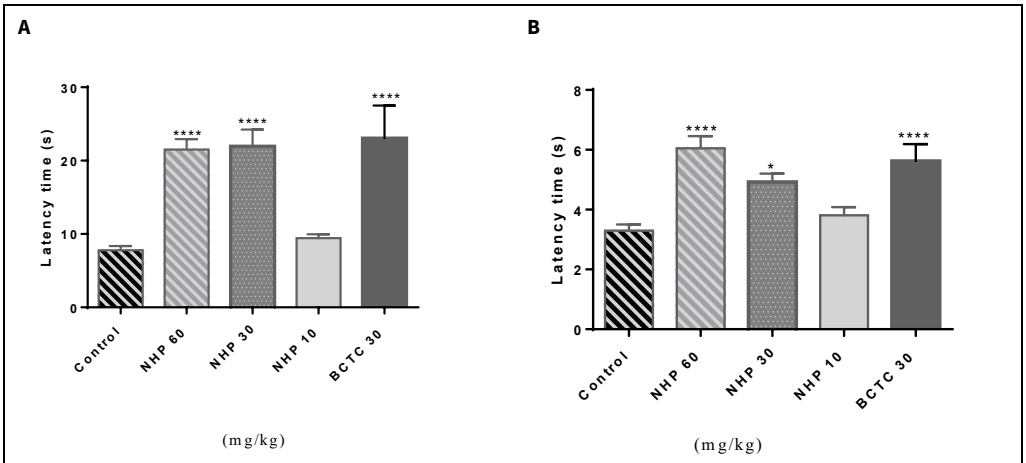


Figure 4. (A) Effect of a single dose of NHP (10–60 mg/kg, 10 mL/kg, i.p), BCTC 30 mg/kg or vehicle (2% DMSO) on latency time in hot plate test. **(B)** Tail flick test.

Each column represents the reactivity time of 8 animals per group as mean ± SEM. For statistical analysis: *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

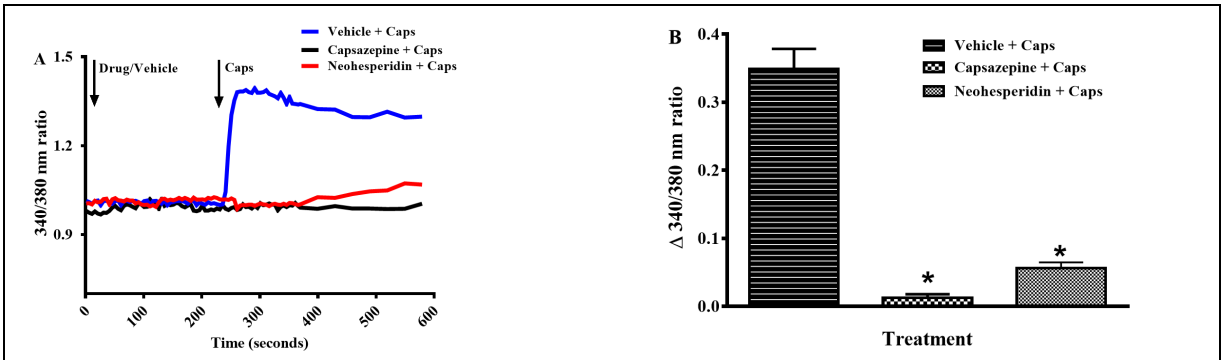


Figure 5. (**A)** Representative traces illustrating changes in intracellular calcium concentrations (340/380 nm ratios) in three different DRG cells responding to 100 nM capsaicin (Caps). The cells were exposed for 1 min to 100 nM capsaicin, then 5 min of wash with calcium buffer. The blue trace represents capsaicin-evoked calcium responses in a cell exposed to the vehicle for 4 min before capsaicin application. The black trace represents capsaicin-evoked calcium responses in cells exposed to 10 µM capsazepine for 4 min before capsaicin application, and the red trace represents capsaicin-evoked calcium responses in cells exposed to 10 µM NHP for 4 min before capsaicin application. (**B)** The amplitude of Ca^{2+} peak after stimulation with 100 nM capsaicin in DRG cells pre-treated with vehicle (n = 88 cells) or 10 µM capsazepine (n = 79 cells) or 10 µM NHP (n = 99 cells) for 4 min before capsaicin application. Data are expressed as mean ± SEM of (Δ 340/380) ratio (*p<0.05).

On the other hand, hesperidin and isorhamnetin showed binding energy values of -7.96 and -7.06 kcal/mol, and they were interacting with two amino acids that are similar to capsazepine (LEU553, GLU570), as shown in Fig. 4C-E. It was decided not to proceed with the mentioned compounds through *in vivo* studies since they have been previously reported for their antinociceptive activity (Kilts et al., 2010; Vabeiryureilai et al., 2015).

It should be noted that these findings, which agree with previously reported compounds, were found among the top-ranked hits, thus improving the confidence in the docking results and analysis. Furthermore, BCTC, a well-known TRPV1 antagonist, was used as a control in the docking study and showed a binding energy of -9.53 kcal/mol. The docking pose was found to interact with three amino acids similar to capsazepine: MET547, LEU553, and GLU570, as shown in Fig. 4B. Moreover, NHP showed binding energy of -6.63 kcal/mol and found to interact with three amino acids similar as capsazepine; LEU553, GLU570, and ALA665 (see Fig. 4F). Interestingly, up to our best knowledge, this is the first study of such activity of NHP, and it has not previously been investigated as a promising therapeutic hit for the development of pain reducers as a TRPV1 antagonist. NHP was selected for evaluation for its analgesic action potential according to its binding energy and intermolecular interactions, as shown with ligand-protein docking approaches. In general, the results of this study support many success stories that can be found in the literature related to CADD.

NHP is a flavanone glycoside found in citrus fruits, which has a strong bitter flavor (Zhang et al., 2012). Previous studies showed that NHP has a wide range of pharmacological effects, including neuropro-

tection, anti-inflammatory, antidiabetic, antimicrobial, and anticancer activities (Akhter et al., 2022). To our knowledge, this study represents the first record of the analgesic activity of NHP involving TRPV1 antagonism. A recent study reported that NHP decreased neuropathic pain in rats via downregulating the P2X4 receptor (Wang et al., 2023). Most importantly, NHP produced no hyperthermia in mice. This finding is very important since few TRPV1 antagonists lack this side effect (Abbas, 2020).

According to the results obtained from molecular docking study against TRPV1 protein, NHP is a good candidate for further investigations. Calcium imaging studies, as well as *in vivo* studies, supported that the mechanism of the analgesic activity of NHP is through TRPV1 antagonism. NHP activity was comparable to the standard TRPV1 antagonist (BCTC) in pain relief in mouse models. Similar to NHP, several other flavonoids exerted antagonistic activity against TRPV1. Eriodictyol, a flavonoid present in citrus fruits, vegetables, and other plants, acted as an antagonist of the TRPV1. It induced analgesic effects without causing hyperthermia (Rossato et al., 2011). Hesperidin, the flavanone glycoside found in citrus fruits, induced analgesic activities partially via interaction with TRPV1 (Martínez et al., 2011). The citrus flavonoid naringin inhibited TRPV1 selectively (Eom et al., 2021). Moreover, hesperidin methyl chalcone inhibited capsaicin-induced paw flinching and licking, suggesting that its mechanism of action through blocking TRPV1 channel (Pinho-Ribeiro et al., 2015).

The abdominal writhing test utilized in this study to evaluate NHP analgesic activity is used as a screening tool to evaluate the antinociceptive activity of potential analgesics acting peripherally (Eddy and Leimbach, 1953). The writhing response is induced by the activation of acid-sensitive ion channels and/or TRPV1 localized in afferent primary fibers (Ping et al., 2018). Similar to NHP, other flavonoids acting via TRPV1 channels such as vitexin and hesperidin methyl chalcone inhibited acetic acid-induced writhing (Borghi et al., 2013; Pinho-Ribeiro et al., 2015).

Upon injection in the foot pad of the animal, capsaicin, the pungent ingredient of hot chili peppers, directly stimulates TRPV1 (Ping et al., 2018). Capsaicin-induced nociceptive behavior can be blocked by TRPV1 antagonists at the peripheral level and thus the licking response after capsaicin injection into the paw (Sakurada et al., 2003). NHP reduced paw licking in the present study. This agrees with previous studies reporting that the citrus flavonoid naringenin reduced flinching and the time spent licking the paw induced by capsaicin (Pinho-Ribeiro et al., 2016). Also, hesperidin methyl chalcone inhibited capsaicin-induced paw flinching and licking (Pinho-Ribeiro et al., 2015).

Similar to chemical pain models, NHP was an effective analgesic agent in alleviating pain in thermal models. A hot plate test was used since it is a useful technique for the evaluation of analgesics with central action (Eddy and Leimbach, 1953). TRPV1 knockout mice exhibited prolonged withdrawal latencies in response to acute noxious heat temperatures in thermosensory tests such as the hot plate and the tail immersion (Marics et al., 2014). In our study, a significant increase in the latency time required for jumping in the hot plate test was observed using NHP and BCTC. Similar results were obtained by hesperidin methyl chalcone that inhibited thermal hyperalgesia (Pinho-Ribeiro et al., 2015).

Calcium imaging studies of NHP suggest that it functions as an antagonist on TRPV1 channels as it blocks calcium-induced currents evoked by capsaicin. Similar effects were produced by the well-known antagonist capsazepine (Alsalem et al., 2016). Similar to NHP, naringenin reduced TRPV1 activation. However, it produces its effect only at high concentrations (Straub et al., 2013). Conversely, hesperidin partially reduced the capsaicin-induced nociceptive response (Martínez et al., 2011).

CONCLUSION

The results of this study suggest that NHP could be an interesting candidate based on its *in silico* interaction with the receptor. Also, NHP showed TRPV1 antagonistic functionality *in vivo* with its corresponding analgesic effect in pain models. This was further supported by the reduction of calcium currents induced by the activation of TRPV1 signaling in sensory neurons. This significantly affects nociceptive signaling when TRPV1 is activated under pathological conditions due to the release of inflammatory compounds.

NHP produced an analgesic effect without causing hyperthermia, the side effect that limits the use of TRPV1 antagonists as analgesics. NHP can be considered as a prototype for the development of more potent agents for the treatment of pain. However, much remains to be done to make this assertion. Toxicological studies need to be performed as well.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Abdulwahed O	Al-Najjar B	Alsalem M	Abbas M
Concepts or ideas		x		x
Design		x		x
Definition of intellectual content		x		
Literature search	x			
Experimental studies	x		x	x
Data acquisition	x		x	x
Data analysis		x	x	x
Statistical analysis			x	x
Manuscript preparation	x			x
Manuscript editing				x
Manuscript review	x	x	x	x

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