Enhancement of Lipolysis in 3T3-L1 Adipocytes by Nitroarene Capsaicinoid Analogs

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Abstract

Transient receptor potential vanilloid 1 (TRPV1) activation by capsaicin binding increased intracellular calcium influx and stimulated adipocyte-to-adipocyte communication, leading to lipolysis. Generally, enhancement of π-stacking capabilities improves certain binding interactions. Notably, nitroarenes exhibit strong binding interactions with aromatic amino acid side chains in proteins. New capsaicinoid analogs were designed by substitution of the OCH₃ group with a nitrogen dioxide (NO₂) group on the vanillyl ring to investigate how π-stacking interactions in capsaicinoid analogs contribute to lipolysis. Capsaicinoid analogs, nitro capsaicin (5), and nitro dihydrocapsaicin (6) were prepared in moderate yields via coupling of a nitroaromatic amine salt and fatty acids. Oil Red O staining and triglyceride assays with 10 µM loading of capsaicin (CAP), dihydrocapsaicin (DHC), 5, and 6 were performed to investigate their effect on lipolysis in 3T3-L1 adipocytes. Both assay results indicated that 5 and 6 decreased lipid accumulation by 13.6% and 14.7%, respectively, and significantly reduced triglyceride content by 26.9% and 28.4%, respectively, in comparison with the control experiment. Furthermore, the decrease in triglyceride content observed in response to nitroarene capsaicinoid analogs was approximately 2-folds higher than that of CAP and DHC. These results arose from the NO₂ group augmented π-π stacking with Tyr511 and the attractive charge interaction with Glu570 affecting binding interactions with TRPV1 receptors.

Keywords

nitroarene capsaicinoid analogs, alkaloids, 3T3-L1 adipocyte, Oil Red O staining, triglyceride, lipolysis

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The increasing number of overweight individuals has become a major global concern for maintaining healthy lifestyles. In 2016, in excess of 1.9 billion adults were overweight, with at least 650 million of them being obese, and the numbers continue to increase worldwide.1 Moreover, obesity is one of the chief factors leading to noncommunicable diseases,8 such as hyperglycemia,3 hypertension,4 coronary heart disease,5 ischemic stroke,6 respiratory complications,7 and atherosclerosis.8 Previous studies have revealed that excess weight and obesity are the result of energy disproportion between consumption and utilization of calories in the body, which leads to inordinate fat storage9-11 and increasing the numbers, size, and mass of adipocytes.12

Generally, adipocytes, the most prevalent cells among adipose tissues, control anabolism, by transforming energy intake into triglycerides, as well as catabolism, by converting triglycerides to free fatty acids.13-16 Furthermore, adipocytes regulate lipid mobilization and distribution in the body.17 Therefore, they play a key role in energy balance and body
weight management. Currently, functional food from natural sources is being utilized as an alternative approach for weight management. For example, coffee, tea, garlic, and soybeans have been demonstrated as being beneficial for ameliorating the energy balance in overweight people. Presently, several bioactive compounds are employed as ingredients in supplementary food for weight control, including resveratrol from grapes, quercetin from onions, genistein from soy, 6-shogaol from ginger, and piperine from pepper. Capsaicin (CAP) from chilies, which is generally used as a flavor ingredient in spicy foods, has also found use in the context of weight control. Furthermore, the beneficial role of CAP has been established for the treatment and prevention of several diseases, such as cardiovascular, gastrointestinal, and Alzheimer’s, as well as cancers. Moreover, CAP is well known as a treatment for inflammatory and pain symptoms, such as those associated with rheumatoid arthritis, posthepatic neuralgia, and osteoarthritis.

The mode of action of CAP involves the transient receptor potential vanilloid 1 (TRPV1) receptors. Based on the structure-activity relationships of CAP and TRPV1, CAP is generally divided into 3 sections (Figure 1): the head section (vanillyl residue), neck section (amide bond), and tail section (hydrocarbon chain). Previously, computational calculations of CAP-TRPV1 binding interactions had revealed that the vanillyl moiety and amide bond are involved through hydrogen bonding. Moreover, the unsaturated hydrocarbon chain displays nonspecific Van der Waals interactions with TRPV1 receptors. Recently, further evidence has suggested that TRPV1 activation from CAP increases intracellular calcium influx, which then stimulates adipocyte-to-adipocyte communication, leading to lipolysis. Enhancement of binding interactions between ligands and TRPV1 receptors has been achieved via various approaches, such as increasing the number of hydrogen bonding sites, alteration of the amide bond, and structural modification of the tail section.

Additionally, nondestructive and noncovalent π-stacking interactions are an important phenomenon that has been extensively investigated for application in chemistry and molecular biology, especially regarding binding interactions in enzymes and receptors. Computational calculations have established that the attachment of nitrogen dioxide (NO₂) groups on aromatic rings results in strong π-stacking interactions, particularly with tryptophan, tyrosine, phenylalanine, and histidine, which consequently significantly promotes binding interactions with proteins.

Structurally, the vanillyl residue represents a crucial pharmacophore that contributes to TRPV1 binding. Removal of the OH group, which acts as a hydrogen bond donor on the vanillyl residue, clearly diminishes binding interactions, thereby reducing TRPV1 activation. To avoid alteration of the OH group while promoting binding affinity with TRPV1 receptors, an appropriate approach for the modification of CAP in order to increase lipolysis involves the adjustment of π-stacking interactions of the vanillyl residue. The NO₂ group was therefore chosen to substitute the OCH₃ group on the vanillyl.

**Figure 1.** Segmentation of capsaicin (CAP) into 3 sections: (1) head section (vanillyl residue), (2) neck section (amide bond), and (3) tail section (hydrocarbon chain). Chemical structures of naturally occurring capsaicinoids: CAP, dihydrocapsaicin (DHC), and synthetic nitro capsainoid analogs: nitro capsaicin (5) and nitro dihydrocapsaicin (6).
residue, as its introduction onto the aromatic ring can be accomplished employing relatively straightforward chemistry. It should be noted that a CAP-like analog containing this modification of the aromatic region, as well as other structural modifications, has been shown previously to bind to the TRPV1. To explore this hypothesis, nitro capsaicin (5) and nitro dihydrocapsaicin (6) (Figure 1) were synthesized using standard peptide coupling between a nitroaromatic amine salt and the corresponding fatty acids. Next, lipolysis in 3T3-L1 adipocytes exposed to CAP, dihydrocapsaicin (DHC), 5, and 6 in 3T3-L1 adipocytes was investigated.

The construction of 5 and 6 was inspired by the biosynthesis of CAP, which involves fatty acid derivatives, vanillyl amine, and the action of the key enzyme, CAP synthase, which connects the residues via amide bond formation. Likewise, the preparation of capsiate, an unstable capsinoid, has been achieved via an analogous approach by coupling a fatty acid and vanillyl alcohol with lipase. In this investigation, we performed the synthesis in 2 parts: (1) the preparation of nitrobenzylamine salt 2 and (2) the coupling reaction between the nitrobenzylamine salt 2 and an appropriate fatty acid 3 or 4 using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl) as the coupling agent (Scheme 1).

First, commercially available 4-hydroxybenzaldehyde (1) underwent nitration with nitric acid and sulfuric acid under reflux for 30 minutes to deliver the NO2 group at the C-3 position on the ring. Without purification, the reaction mixtures were then reduced with ammonium formate at 180 °C for 4 hours. Under these conditions, the aldehyde underwent selective reductive amination to give the requisite amine in moderate yield, while the NO2 group remained intact. Subsequently, the reaction mixture was acidified with HCl to give nitrobenzylamine salt 2 with an overall yield of 68% over the 3 steps, after recrystallization from absolute ethanol. The infrared (IR) signals for 2 appearing at 1534 and 1332 cm−1 indicated N-O stretching, thus confirming the presence of the NO2 group on the aromatic ring.

**Figure 2.** (A) Effect of CAP at 5 different concentrations (5, 10, 25, 50, and 100 µM) on cell viability. (B) Effect of CAP, DHC, 5, and 6 at 10 µM on cell viability. Differentiated 3T3-L1 adipocytes were treated for 24 hours. The cell viability was quantified and compared with control by measurement using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at an absorbance of 595 nm. Data are expressed as mean ± SE from 3 independent experiments. *P < 0.05 versus the vehicle control. CAP, capsaicin; DHC, dihydrocapsaicin.
Next, 5 and 6 were prepared via standard amide coupling between the nitrobenzylamine salt 2 and either (E)-8-methyl-6-noneoic acid (3) or 8-methylnonanoic acid (4), respectively. Both 3 and 4 were readily synthesized according to a literature procedure and obtained after high-performance liquid chromatography (HPLC) purification as light yellow oils. The corresponding fatty acids 3 or 4 were activated with EDC.HCl and 1-hydroxy benzotriazole (HOBt), while the nitrobenzylamine salt 2 was treated with triethylamine. The 2 solutions were then mixed under a nitrogen atmosphere and reacted for 6 hours. The purification was achieved by column chromatography using ethyl acetate (EtOAc)/hexane 1:1 as eluent, followed by further purification by HPLC with acetonitrile:water:acetic acid (1:1:0.1) as the mobile phase to afford the target compounds 5 and 6 in high purity. Under these unoptimized conditions, the capsaicinoid analogs 5 and 6 were obtained in 47% and 50% yields, respectively.

A previous report had established that CAP, the most pungent of the capsaicinoid analogs, induced apoptosis of 3T3-L1 adipocytes in the concentration range from 50 to 250 µM after 24 hour exposure. Unlike CAP, the nitroarene capsaicinoid analogs are less pungent, which was evident during the synthesis. Therefore, CAP was selected for the initial screen to establish a suitable concentration for use in the biological studies. With the mentioned criteria, we explored the appropriate concentration of CAP using its cytotoxicity with differentiated 3T3-L1 adipocytes, as determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differentiated 3T3-L1 adipocytes were tested with various concentrations of CAP (5, 10, 25, 50, and 100 µM) for 24 hours. The results shown in Figure 2(A) demonstrate that concentrations of CAP under 10 µM exhibited no significant cytotoxicity to differentiated 3T3-L1 adipocytes in comparison with the vehicle control (no CAP treatment). In order to observe a distinct change in biological activities, 10 µM CAP, DHC, 5, and 6 was deemed an optimal concentration for cell

![Figure 3](image)

**Figure 3.** Effect of CAP, DHC, 5, and 6 on the lipid content of differentiated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were incubated with CAP, DHC, 5, and 6 at 10 µM for 24 hours. The cells were stained with Oil Red O and quantified by measuring absorbances at 500 nm. Data are expressed as mean ± SE from 3 independent experiments. *P < 0.05 versus the respective parent compounds. CAP, capsaicin; DHC, dihydrocapsaicin.

| Abbreviations: CAP, capsaicin; DHC, dihydrocapsaicin. All measurements were obtained from at least 3 independent experiments, each of which was performed in triplicate (n = 3). The data are presented as mean ± SE. *P < 0.05 versus the control. **P <0.05 versus CAP. ***P < 0.05 versus DHC. |
viability, lipid accumulation, and triglyceride studies. A 10 μM concentration of CAP provides maximum activation of TRPV1 receptors after 24 hours of exposure, as previously reported. As can be seen in Figure 2(B), the cell viabilities of differentiated 3T3-L1 adipocytes treated with CAP, DHC, 5, and 6 exceeded 96% in comparison with cell viability in the control group, and there were no significant differences in cell viability between cells treated with CAP, DHC, 5, and 6.

Oil Red O staining of differentiated 3T3-L1 adipocytes treated with 10 μM CAP, DHC, 5, and 6 was performed to observe lipid accumulation. After 24 hours, the results revealed that after treating the cells with 5 and 6 for 24 hours, lipid accumulation had significantly decreased in comparison to the dimethylsulfoxide (DMSO)-treated control cells. The observed decreases in lipid accumulation were 13.6 and 14.7 % for compounds 5 and 6, respectively, as shown in Figure 3. On the other hand, CAP and DHC reduced lipid accumulation by only 5.6 and 8.1 %, respectively, compared with the DMSO-treated control cells. Structurally, CAP and 5, as well as DHC and 6, are isosteres in the tail region and it appears that the NO2 group on the aromatic ring leads to significantly reduced lipid accumulation elicited by 5, in comparison with the corresponding parent structure (CAP). The analogs result was obtained for 6 and its parent structure (DHC).

In addition to lipid accumulation, intracellular lipolysis metabolism of differentiated 3T3-L1 adipocytes was examined by measuring triglyceride levels, using the Cayman triglyceride assay kit. Differentiated 3T3-L1 adipocytes were treated with CAP, DHC, 5, and 6 for 48 hours to evaluate and compare their lipolysis properties. The results indicated that differentiated 3T3-L1 adipocytes treated with 10 μM CAP, DHC, 5, and 6 exhibited significant decreases in triglyceride content. The observed decreases in triglyceride content were 12.7%, 11.5%, 26.9%, and 28.4% for CAP, DHC, 5, and 6, respectively, as compared with the control (Table 1). Analogous to the results of lipid accumulation, both 5 and 6 revealed an approximately 2-fold higher reduction in triglyceride content when compared with that of their corresponding parent structures. The results of both lipid accumulation and triglyceride content experiments emphasize the effect of the NO2 group and its significant contribution to lipolysis in differentiated 3T3-L1 adipocytes.

π-Stacking interactions can be manipulated by attenuating hydrophobicity, surface area of substituents, electronegativity, and dipole moment of the participating residues. The inclusion of a NO2 group presumably increases the dipole moment on 5 and 6, which contributes to enhanced π-stacking interactions, compared with those in their original parent structure. To observe and compare electron distribution in the aromatic region of the studied compounds, density functional theory was applied. In order to focus on the aromatic region, the calculations were performed with model compounds in which the long-chain hydrocarbon unit was simplified by a CH3 group. The geometry optimizations, Hessian calculations, and molecular property calculations were conducted at the B3LYP/6-31+G(d) level of theory to locate the equilibrium geometries located on the local minimum with no imaginary frequency. It was found that the calculated dipole moment of the model compound representing the parent structures (CAP and DHC) was equal to 2.09 Debye with a volume of 149.44 cm3/mol and was directed from the aromatic ring to the amide bond. In the
case of the model compound representing the novel nitro compounds 5 and 6, the calculated dipole moment was 6.16 Debye with volume 150.51 cm$^3$/mol and the direction of the dipole moment was pointed from the aromatic ring to the methylene group located between the aromatic residue and the amide bond. The significant increase in the calculated dipole moment in the model compounds bearing a nitro group resulted from the appreciable negative charge of the NO$_2$ group. The HOMO orbital plot illustrates that the electrons are predominantly localized on the carbon atom of the aromatic ring for both model compounds. However, only the model structure for the nitro-containing compounds 5 and 6 exhibited electrons localized in the methylene carbon and amide bond, as shown in Figure 4. Thus, the model structure for the nitro-containing products displays a more conjugated double bond character, distributing electrons from the aromatic ring to the outer regions. This likely creates a lower π-electron density at the aromatic ring, which is analogous to a previous report.\textsuperscript{57} Not only were the calculated dipole moment values and predicted electron distribution on the model structure for the nitro-containing compounds 5 and 6 different from the model compound representing the parent structures (CAP and DHC) but also previous work by Walpole and co-workers\textsuperscript{33} demonstrated that capsaicinoid analogs containing the aromatic region substituted with a nitro group exhibit binding interactions with TRPV1. Therefore, the binding interactions were then analyzed to confirm how the nitro group affected the π-stacking interactions. Additionally, the predicted binding affinity values were also calculated while binding to the TRPV1 receptor. Due to the widespread use of CAP for several applications as a supplement and functional compound, 5 and CAP were selected first as the representative compounds to observe the difference between them in terms of their binding interactions. To obtain this information, molecular dynamics simulations (for the flexible ligands) were performed using a semi-rigid docking method (using the CHARMM program) to explore the possible binding conformations of the molecules. Then, the energy minimized structures were refined using a grid-based simulated annealing algorithm.

The total number of docking poses for 5 and CAP in the defined vanilloid binding pocket of the TRPV1 receptor were 14 and 19, respectively. The successful binding conformations of 5 and CAP, obtained by molecular docking experiments, in the vanilloid binding site of the TRPV1 receptor, were sorted according to their CDOCKER Interaction Energy values (from higher to lower energy values). The docking poses of 5 and CAP with the highest CDOCKER Interaction Energy

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{molecular_docking_diagram.png}
\caption{Molecular docking diagram of (A) CAP and (B) 5 with TRPV1 receptor. The figure depicts the best binding conformations and possible binding modes at the vanilloid binding site of the TRPV1 receptor. The graphical representations of binding interactions are presented as a 3-dimensional structure and 2-dimensional interaction diagrams. CAP, capsaicin; TRPV1, transient receptor potential vanilloid 1.}
\end{figure}
scores of $-42.96$ and $-39.94$ kcal/mol, respectively, were selected for further energy minimization calculations. Following this, the binding interactions between the receptor and the ligand molecules were analyzed. The superposition of the energy-minimized structures of 5 and CAP and the corresponding key amino acid residues of the TRVP1 receptor that interact with these ligands are shown in Figure 5.

The overall binding free energies of 5 and CAP were $-83.47$ and $-79.60$ kcal/mol, respectively, indicating that the predicted binding affinity value of 5 was higher than that of CAP (in the vanilloid binding pocket of the TRVP1 receptor). Moreover, the nonbonded interaction energy values (the van der Waals and electrostatic energies) calculated for the interaction between the amino acid residues (within 4 Å of 5 and CAP) and the ligands in the binding pocket are $-43.33$ kcal/mol for CAP and $-53.93$ kcal/mol for 5. The difference in the nonbonded interaction energies of the ligands arises from the electrostatic energy, potentially originating from the interaction of charges between the nitro group-containing molecule and the nearby amino acid residues. The interactions between the 5 and CAP molecules and the corresponding key amino acid residues of the TRVP1 receptor are shown as 3-dimensional structures and 2-dimensional interaction diagrams in Figure 6.

The amide group of the CAP is in close proximity to the Leu669 and Thr550 amino acid residues of the receptor molecule. However, no interaction between the ligand and the receptor amino acid units was predicted. Moreover, the terminal methylene carbon of the CAP tail interacted with the Phe543 and Met547 receptor residues in the extracellular region of the TRVP1 receptor. At the vanilloid binding pocket of the intracellular region of the TRVP1 receptor, a T-shaped π-π stacking interaction was observed between the Tyr511 residue and the vanilloid ring of the CAP molecule (Figure 6(A)). The calculated interaction values agreed well with the experimentally obtained ones. The results were also consistent with those obtained from previous molecular modeling studies (binding studies between the CAP molecule and the TRVP1 receptor).31,60-62

The binding conformation of 5 was similar to that exhibited by the CAP molecule (Figure 6(B)). All residues interact with 5 via the same type of binding interactions (even though no interaction was theoretically predicted). In addition, a well-defined T-shaped π-π stacking interaction was observed between 5 and the aromatic ring of the Tyr551 residue. A higher nonbonding interaction (compared with CAP) was observed between the Tyr511 residue and 5 (Table 2). The result supports the hypothesis that stronger π-π stacking interaction is present inside the binding pocket of the TRVP1 receptor when the binding ligand is 5. This might be explained by the lower π-electron density of the aromatic ring of the

Table 2. Interaction Energies Per-Residue Within 4 Å of CAP and 5 in the Capsaicin-Binding Pocket of TRVP1 Ion Channel.

| Residue | CAP interaction energy (kcal/mol) | 5 interaction energy (kcal/mol) |
|---------|----------------------------------|--------------------------------|
|         | Total interaction | VDW interaction | Electrostatic interaction | Total interaction | VDW interaction | Electrostatic interaction |
| TYR511  | $-5.82$ | $-3.44$ | $-2.38$ | $-6.55$ | $-3.28$ | $-3.27$ |
| SER512  | $1.43$ | $-2.23$ | $3.66$ | $1.64$ | $-0.69$ | $2.33$ |
| LEU515  | $-4.58$ | $-3.27$ | $-1.30$ | $-3.49$ | $-2.90$ | $-0.59$ |
| PHE516  | $1.44$ | $-0.59$ | $2.03$ | - | - | - |
| VAL518  | $-1.83$ | $-0.37$ | $-1.46$ | - | - | - |
| PHE522  | $-0.63$ | $-1.12$ | $0.49$ | - | - | - |
| PHE543  | $-4.44$ | $-2.39$ | $-2.05$ | $-6.21$ | $-2.87$ | $-3.34$ |
| ALA546  | - | - | - | $-1.86$ | $-1.31$ | $-0.55$ |
| MET547  | $-3.72$ | $-4.06$ | $0.34$ | $-3.11$ | $-3.79$ | $0.68$ |
| THR550  | $-3.20$ | $-2.80$ | $-0.40$ | $-4.75$ | $-3.24$ | $-1.51$ |
| ASN551  | $-7.07$ | $-1.79$ | $-5.28$ | $-3.84$ | $-2.25$ | $-1.59$ |
| LEU553  | $-1.41$ | $-1.68$ | $0.27$ | $-1.13$ | $-1.65$ | $0.52$ |
| TYR554  | $-1.43$ | $-1.34$ | $-0.09$ | $-1.15$ | $-1.53$ | $0.38$ |
| ARG557  | $3.88$ | $-0.38$ | $4.26$ | $0.03$ | $-0.58$ | $0.61$ |
| ALA566  | $0.77$ | $-0.38$ | $-0.39$ | $-0.96$ | $-0.45$ | $-0.51$ |
| ILE569  | $-1.22$ | $-1.38$ | $0.16$ | $-2.81$ | $-2.69$ | $-0.12$ |
| GLU570  | $-8.02$ | $-1.29$ | $-6.73$ | $-8.67$ | $-1.95$ | $-6.72$ |
| ILE573  | $-2.07$ | $-2.22$ | $0.15$ | $-3.18$ | $-2.05$ | $-1.13$ |
| PHE587  | $-1.19$ | $-0.76$ | $-0.43$ | $-1.76$ | $-1.47$ | $-0.29$ |
| PHE591  | $-1.09$ | $-0.39$ | $-0.70$ | $-2.29$ | $-1.12$ | $-1.17$ |
| LEU662  | - | - | - | $-0.42$ | $-0.37$ | $-0.05$ |
| ALA665  | - | - | - | $-1.91$ | $-0.40$ | $-1.50$ |
| LEU669  | $-1.60$ | $-1.46$ | $-0.14$ | $-1.52$ | $-1.82$ | $0.30$ |
| Total   | $-43.33$ | $-33.34$ | $-9.99$ | $-53.93$ | $-36.41$ | $-17.52$ |

Abbreviations: CAP, capsaicin; TRVP1, transient receptor potential vanilloid 1; VDW, Van der Waals.
nitro-containing molecule. Additionally, an attractive charge interaction between the nitro group and the Glu570 residue is also observed and contributes to the strong binding interaction of 5 when compared with that of CAP.

Conclusions

Herein we demonstrate a facile synthetic route for synthesizing the respective CAP and DHC analogs 5 and 6. The analogs were synthesized via the amide coupling reaction between fatty acids and an aromatic amine using EDC.HCl and HOBt as the coupling reagents. The results from the lipolysis study demonstrated that compared with the cells treated with the parent compounds, those treated with 5 and 6 showed a lesser degree of lipid accumulation and triglyceride contents (in differentiated 3T3-L1 adipocytes).

The introduction of the nitro group in the CAP skeleton had a significant effect on the lipolysis process. The nitro-substituted vanilloid ring could potentially improve the lipolytic activity due to the optimal π-π stacking and attractive charge interactions present between the substituted vanilloid ring and the Tyr511 and Glu570 residues, respectively. This specific interaction could increase the binding affinity and stability.

These preliminary findings suggest that the nitroarene capsaicinoid analogs can be used to reduce the triglyceride levels in cells. They may also help in overcoming obesity. Presently, a comprehensive study on the pungency of the nitroarene capsaicinoid analogs is being carried out. Their potential role as skin irritants is also being explored. These factors must be accounted for before the compounds can be developed as supplements for topical applications.

Experimental

General

CAP, DHC, 4-hydroxybenzaldehyde, MTT, Oil Red O solution, DMSO, and human recombinant insulin were obtained from Sigma-Aldrich (St. Louis, MO, USA); high glucose Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), bovine calf serum (BCS), phosphate-buffered saline (PBS), antibiotics (100 unit/mL penicillin and 100 µg/mL streptomycin) from Gibco (Grand Island, NY, USA); dexamethasone (DEX) and 3-isobutyl-1-methyl xanthine (IBMX) from Merck (Kenilworth, NJ, USA); HOBt from Acros Organic (Geel, Belgium); EDC.HCl from GL Biochem (Shanghai, China); Triton X-100 from VWR international (Radnor, PA, USA); 3T3-L1 preadipocytes (ATCC CL-173) from the American Type Culture Collection (Manassas, VA, USA); and the triglyceride colorimetric assay kit from Cayman Chemical (Ann Arbor, MI, USA). All chemical reagents were used as received from vendors unless otherwise noted.

1H NMR and 13C NMR spectra were recorded on a 400 MHz NMR spectrophotometer (AVANCE, Bruker, Switzerland) in appropriate deuterated solvents. Chemical shifts (δ) are reported in parts per million (ppm) relative to either tetramethylsilane or the residual protonated solvent signal as a reference. Mass spectra were measured in positive ion mode with a high-resolution mass spectrometer (micrO-TOF, Bruker, Switzerland) with electrospray ionization using sodium formate for calibration. IR spectra were obtained on a Fourier-transform infrared (FT-IR) spectrophotometer (Model 1600, Perkin Elmer, USA) in the wavenumber range of 4000–400 cm⁻¹. The absorbance was measured using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader, BioTek, USA).

Synthesis of 4-Hydroxy-3-Nitrobenzylamine Hydrochloride (2)

4-Hydroxybenzaldehyde (1) (1.02 g, 8.19 mmol) was dissolved in 98% sulfuric acid (2 mL) and cooled in an ice bath. The resultant mixture was then added dropwise into a cold nitric acid solution (0.41 mL, 9.01 mmol of 98% sulfuric acid and 0.42 mL, 9.02 mmol of 65% nitric acid). The reaction mixture was stirred at room temperature for 30 minutes, poured onto ice, and stirred until a yellow precipitate formed. This was filtered, washed with cold ethanol, and dried in vacuo.

The dried yellow precipitate was reacted with ammonium formate (0.38 g, 5.98 mmol) in a round-bottom flask connected to a sealed reflux apparatus. The reaction mixture was then refluxed at 180 °C for 4 hours, after which time it was cooled to room temperature and conc. HCl (2 mL) was added. The reaction mixture was then refluxed at 100 °C for 1 hour. The mixture was cooled to room temperature and the formed precipitate was collected by filtration, washed with ice-cold absolute ethanol (3 × 5 mL), and recrystallized from absolute ethanol to give compound 2 (0.86 g, 51% yield).

Compound 2, light-yellow crystals, Rf = 0.10 (10% MeOH in CH2Cl2);1H NMR (400 MHz, D2O) δ (ppm): 8.25 (1H, d, J = 4.0 Hz), 7.74 (1H, dd, J = 8.0, 4.0 Hz), 7.26 (1H, d, J = 8.0 Hz), 4.23 (2H, s); 13C NMR (100 MHz, D2O) δ (ppm): 153.8, 138.0, 127.4, 126.3, 125.1, 120.7, 42.0; FT-IR (ATR) (cm⁻¹): 2955, 1631, 1534, 1332, 1240.

Synthesis of (E)-8-Methyl-6-Nonenoic Acid (3)

Unsaturated fatty acid 3 was successfully synthesized via a Wittig reaction between an appropriate phosphonium salt and isobutyraldehyde, followed by Z-E isomerization with nitrous acid, according to a literature procedure.53

Compound 3, clear oil, 24% yield, Rf = 0.64 (10% EtOAc in n-hexane):1H NMR (400 MHz, deuterated chloroform [CDCl3] δ (ppm): 5.42-5.30 (2H, m), 2.35 (2H, t, J = 7.6 Hz), 2.25-2.20 (1H, m), 2.02-1.97 (2H, m), 1.67-1.60 (2H, m), 1.44-1.37 (2H, m), 0.95 (6H, d, J = 6.8 Hz), 13C NMR (100 MHz, CDCl3) δ (ppm): 180.6, 138.3, 126.5, 34.1, 32.2, 31.1, 29.1, 24.3, 22.8; FT-IR (ATR) (cm⁻¹): 2955, 1705, 968.
Synthesis of 8-Methylnonanoic Acid (4)

Saturated fatty acid 4 was readily prepared via the copper-catalyzed cross-coupling alkylation with a Grignard reagent and ethyl ω-haloalkyloxycarboxylate, followed by acid hydrolysis, as previously described.\textsuperscript{63}

Compound 4, a clear oil, 19% yield, \( R_e \) = 0.64 (10% EtOAc in \( n \)-hexane): \( ^{1} \)H NMR (400 MHz, CDCl\(_3 \)) \( \delta \) (ppm): 2.35 (2H, \( t, J = 7.5 \) Hz), 1.65-1.59 (2H, m), 1.53-1.48 (1H, m), 1.35-1.26 (6H, m), 1.18-1.14 (2H, m), 0.86 (6H, \( d, J = 6.8 \) Hz); \( ^{13} \)C NMR (100 MHz, CDCl\(_3 \)) \( \delta \) (ppm): 180.7, 39.1, 34.3, 29.6, 29.2, 28.1, 27.3, 24.8, 22.8; FT-IR (ATR) (cm\(^{-1}\)): 2924, 1705, 1542, 1316; HRMS (ESI-TOF) \( m/z \) 323.1978 [M + H]\(^+\) (calcd for C\(_{17}\)H\(_{27}\)N\(_2\)O\(_4\), 323.1965).

Cell Culture and Differentiation

3T3-L1 Preadipocytes (ATCC CL-173) were cultured in DMEM supplemented with 3.7 g/L sodium bicarbonate, 10% BCS, and antibiotics at 37 °C in a humidified atmosphere of 5% \( \text{CO}_2 \). The medium was changed every 3 days. The cells were subcultured after reaching 80% confluence, seeded in a 96-well plate at a density of \( 2 \times 10^4 \) cells/well, and cultured in maintenance medium (DMEM containing 10% BCS and 1% antibiotics) until 90%-95% confluence. After reaching the desired confluence (defined as day 0), the cells were further induced with initiation medium (1 \( \mu \)M DEX, 0.5 mM IBMX, 10 \( \mu \)g/mL insulin in 10% FBS-DMEM) for 3 days. At the end of the third day, the initiation medium was replaced with the progression medium (10 \( \mu \)g/mL insulin in 10% FBS-DMEM). The media was changed every 2 days until day 9, and then the cells were differentiated into a rounded shape with lipid droplets. The morphology of mature adipocytes was examined using a microscope.

Cell Viability Assay

Cell viability was determined by the MTT assay to define a suitable concentration for all testing. Differentiated 3T3-L1 adipocytes were treated with 5, 10, 25, 50, and 100 \( \mu \)M CAP dissolved in DMSO for 24 hours in a humidified 5% carbon dioxide (CO\(_2\)) atmosphere at 37 °C. The final concentration of DMSO in the culture medium was 0.2% v/v. The vehicle control contained the same concentration of DMSO, but no CAP. After incubation, MTT reagent in PBS solution was added to each well plate, and the plate was incubated for another 3 hours. Subsequently, the culture medium containing MTT reagent was removed. The formazan produced in the viable cells was dissolved by the addition of DMSO. The absorbance at 595 nm was measured using a microplate reader. The values were presented as a percentage of those for control cells without capsaicinoid and analogs. The effects of CAP, DHC, \( 5 \), and \( 6 \) on the viability of differentiated 3T3-L1 adipocytes were evaluated by treatment of the cells with 10 \( \mu \)M of each compound according to the above-mentioned procedure. All measurements were performed in at least 3 independent experiments, each of which was performed in triplicate (\( n = 3 \)).

Determination of Lipid Accumulation in 3T3-L1 (Oil Red O Staining)

To examine the effects of CAP, DHC, \( 5 \), and \( 6 \) on lipid accumulation, serum-free DMEM was added to the microplate wells of the mature adipocytes, after which the cells were treated with 10 \( \mu \)M of CAP, DHC, \( 5 \), and \( 6 \) for 24 hours. The lipid droplets were then stained with Oil Red O.
Differentiated 3T3-L1 adipocytes were treated with 10 μM of CAP, DHC, 5, and 6 in serum-free DMEM for 48 hours. The cells were collected by the addition of Triton X-100 (0.1% in 0.1 M sodium hydroxide). The cellular triglyceride content was determined using a triglyceride colorimetric assay kit (Cayman Chemical Co., USA), following the manufacturer’s protocols. Triglyceride levels were analyzed in at least 3 independent experiments, each of which was performed in triplicate (N = 3). The values were expressed as a percentage of triglycerides in the control without capsaicinoid and analogs.

**Triglyceride Assay**

Differentiated 3T3-L1 adipocytes were treated with 10 μM of CAP, DHC, 5, and 6 in a 96-well plate with CAP, DHC, 5, and 6. Following the treatment, the medium was removed from the plates, and the cells were rinsed twice with PBS to remove any remaining medium and fixed with 10% formaldehyde for 45 minutes at room temperature. Formaldehyde was then removed, and the cells were refixed with 10% formaldehyde for 1 hour at room temperature, and then washed with 60% isopropanol. A fresh Oil Red O solution (0.5% Oil Red O prepared in isopropanol: water, 3:2 v/v) was filtered through a 0.45-µm nylon filter, and 50 µL of the filtrate was added to each well of the well plate and left to stain the cells for 1 hour at room temperature. After staining, the cells were thoroughly rinsed with distilled water. The morphology of the stained cells was then examined using a microscope. The stained lipid droplets were then denatured and dissolved using isopropanol and mixed well until the dye was homogeneously distributed in the isopropanol. The absorbance was measured at 500 nm using a microplate reader. Lipid accumulation measurements were performed in at least 3 independent experiments, each of which was performed in triplicate (N = 3). The values were expressed as a percentage of the result for control cells lacking capsaicinoid and analogs.

**Structure Preparations and Molecular Docking**

In this study, the vanilloid binding site in the structure of the open TRPV1 channel in complex with RTX and DkTx (PDB ID: 3J5Q) was used as the target for docking of nitro capsaicin and CAP. The 6-dimensional structure of this TRPV1 was retrieved from the Research Collaboratory for Structural Bioinformatics protein data bank. Before docking, the TRPV1 structure (chains B, D, E, and G) was cleaned up by removing double-knot toxin molecules (chains A, C, F, and H). The missing loop regions of the TRPV1 chains (503-LKSLF-507) were inserted based on the SEQRES data. The amino acid residues in TRPV1 were protonated at pH 7.4 according to the predicted pKₐ values. The structure of TRPV1 was submitted for energy minimization with the conjugate gradient algorithms applying CHARMM forcefield with protein backbone constraints to reduce the atomic clash of amino acid side chains. The structures of 5 and CAP as ligands for docking were retrieved from quantum mechanics optimization at the B3LYP/6-31+G(d) level of theory. The 20 Å binding site sphere in the TRPV1 channel was generated by using the center coordinate obtaining from the arithmetic mean coordinates of the residues Y511, M547, T550, E570, and L669 of chain D to cover the whole vanilloid binding pocket. The binding modes of 5 and CAP were predicted using the CDOCKER module in Discovery Studio 2.5 software. In CDOCKER, 100 random ligand conformations were generated in the vanilloid binding site of the TRPV1 structure. The temperature was set to 700 K for 2000 steps and cooled to 300 K for 5000 steps. The grid extension was set to 8 Å, and 10 ligand-binding poses were ranked according to their CDOCKER Energies scores (kcal/mol). The CDOCKER Interaction Energy scores (kcal/mol) of docking poses were recorded. After docking, the successful conformations were sorted from high to low according to their CDOCKER Interaction Energy score towards the TRPV1 target. The docking pose with the highest CDOCKER Interaction Energy score was selected as an active conformation for further analyses. The structures of complexes were submitted for energy minimization with CHARMM forcefield using the Steepest Descent (100 000 steps) followed by the Adopted Basis Newton Raphson (ABNR) algorithms (50 000 steps) to obtain the stable structure with a convergence criterion of 0.001 kcal/(molÅ) energy RMS Gradient. Harmonic restraints were applied on the protein backbone during the minimization steps with 40 kcal/mol/Å². The overall binding free energy of the obtained complex was estimated between ligands and target using CHARMM-based energies methods. The nonbonded interactions (ie, van der Waals and electrostatic terms) between the ligand (5 and CAP) and each amino acid residue within 4 Å around the ligand were calculated using the CHARMM-based Interaction Energy protocol. All molecular modeling techniques and calculations were performed using the program Discovery Studio 2.5. The graphics visualization tool for viewing, analyzing protein, and modeling data were carried out using the free DS Visualizer 2020.

**Statistical Analysis**

The values are expressed as mean ± SE. Statistical analyses were performed using SPSS software version 17.0 (SPSS Inc, Chicago, IL, USA). Significant differences between groups were analyzed using a one-way analysis of variance followed by Tukey’s multiple comparison tests. A value of P < 0.05 indicated a significant difference.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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References

1. World Health Organization. Obesity and overweight. Accessed March 31, 2019. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
2. World Health Organization. Noncommunicable diseases. Accessed May 01, 2019. https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases
3. Martyn JAJ, Kaneki M, Yasuhara S. Obesity-Induced insulin resistance and hyperglycemia: etiologic factors and molecular mechanisms. Anesthesiology. 2008;109(1):137-148. doi: 10.1097/ALN.0b013e3181799d45
4. Re RN. Obesity-related hypertension. Ochsner J. 2009;9(3):133-136.
5. Cho E, Manson JE, Stampfer MJ, et al. A prospective study of obesity and risk of coronary heart disease among diabetic women. Diabetes Care. 2002;25(7):1142-1148. doi: 10.2337/diabetes.52.5.757-759
6. Kurth T, Gaziano JM, Berger K, et al. Body mass index and the risk of stroke in men. Arch Intern Med. 2002;162(22):2557-2562. doi:10.1001/archinte.162.22.2557
7. Rabec C, de Lucas Ramos P, Veale D. Respiratory complications of obesity. Arch Bronconeumol. 2011;47(5):252-261. doi: 10.1016/j.arbes.2011.01.012
8. Angelo Carpi FG. Obesity and increased risk for atherosclerosis and cancer. Internal Medicine: Open Access. 2014;3(3):1-8.
9. Flatt J-P. Differences in basal energy expenditure and obesity. Obesity. 2007;15(11):2546-2548. doi:10.1038/oby.2007.304
10. Redinger RN. Fat storage and the biology of energy expenditure. Transl Res. 2009;154(2):52-60. doi: 10.1016/j.trsl.2009.05.003
11. Choe SS, Huh JY, Hvang IJ, Kim JI, Kim JB. Adipose tissue remodeling: its role in energy metabolism and metabolic disorders. Front Endocrinol. 2016;7(Suppl 2):1-16. doi:10.3389/fendo.2016.00030
12. Jo J, Shreif Z, Pertwal V. Quantitative dynamics of adipose cells. Adipocytes. 2012;1(2):80-88. doi:10.4161/adip.19705
13. Luo L, Liu M. Adipose tissue in control of metabolism. J Endocrinol. 2016;231(3):R77-R99. doi:10.1530/JOE-16-0211
14. Zechner R, Zimmermann R, Eichmann TO, et al. FAT SIG-NALS—lipases and lipolysis in lipid metabolism and signaling. Cell Metab. 2012;15(3):279-291. doi: 10.1016/j.cmet.2012.02.018
15. Lass A, Zimmermann R, Oberer M, Zechner R. Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. Prog Lipid Res. 2011;50(1):14-27. doi:10.1016/j.plipres.2010.10.004
16. Camp HS, Ren D, Leff T. Adipogenesis and fat-cell function in obesity and diabetes. Trends Mol Med. 2002;8(9):442-447. doi:10.1016/S1471-4914(02)02396-1
17. Ntambi JM, Young-Choel K. Adipocyte differentiation and gene expression. J Nutr. 2000;130(12):3122S-3126. doi:10.1093/jn/130.12.3122S
18. Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. J Clin Invest. 2011;121(6):2094-2101. doi:10.1172/JCI45887
19. Mohamed GA, Ibrahim SRM, Elkhayat ES, El Dine RS. Natural anti-obesity agents. Bulletin of Faculty of Pharmacy, Cairo University. 2014;52(2):269-284. doi:10.1016/j.butfopcu.2014.05.001
20. Aguirre L, Fernández-Quintela A, Arias N, Portillo MP. Resveratrol: anti-obesity mechanisms of action. Molecules. 2014;19(11):18632-18655. doi:10.3390/molecules19118632
21. Bae C-R, Park Y-K, Cha Y-S. Quercetin-rich onion peel extract suppresses adipogenesis by down-regulating adipogenic transcription factors and gene expression in 3T3-L1 adipocytes. J Sci Food Agric. 2014;94(13):2655-2660. doi:10.1002/jsfa.6604
22. Behloul N, Wu G. Genistein: a promising therapeutic agent for obesity and diabetes treatment. Eur J Pharmacol. 2013;698(1-3):31-38. doi:10.1016/j.ejphar.2012.11.013
23. Suk S, Seo SG, Yu JG, et al. A bioactive constituent of ginger, 6-shogaol, prevents adipogenesis and stimulates lipolysis in 3T3-L1 adipocytes. J Food Biochem. 2016;40(1):84-90. doi:10.1111/jfbc.12191
24. Park U-H, Jeong H-S, Jo E-Y, et al. Piperine, a component of black pepper, inhibits adipogenesis by antagonizing PPARγ activity in 3T3-L1 cells. J Agric Food Chem. 2012;60(15):3853-3860. doi:10.1021/jf204514a
25. Varghese S, Kubatka P, Rodrigo L, et al. Chili pepper as a body weight-loss food. Int J Food Sci Nutr. 2017;68(4):392-401. doi:10.1080/09637486.2016.1258044
26. Sun F, Xiong S, Zhu Z. Dietary capsaicin protects cardiometabolic organs from dysfunction. Nutrients. 2016;8(5):174-13. doi:10.3390/nu8050174
27. Aniwan S, Gonlachanvit S. Effects of chili treatment on gastrointestinal and rectal sensation in diarrhea-predominant irritable bowel syndrome: a randomized, double-blinded, crossover study. J Neurogastroenterol Motil. 2016;36(3):837-843. doi:10.5009/jnm14022
28. Chen J, Li L, Li Y, et al. Activation of TRPV1 channel by dietary capsaicin against inflammatory bowel disease. J Neurogastroenterol Motil. 2016;23(1):R77-R99. doi:10.1530/JOE-16-0211
29. Clark R, Lee S-H. Anticancer properties of capsaicin against melanoma and obesity. J Agric Food Chem. 2009;57(5):133-136. doi:10.1021/jf802183b
30. Engler A, Aeschlimann A, Simmen BR, et al. Expression of transcription factors and gene expression in 3T3-L1 adipocytes. J Cell Biochem. 2014;130.12:3122S-3126. doi:10.1002/jcb.258174
31. Yang F, Xiao X, Cheng W, et al. Activation of TRPV1 channel by dietary capsaicin improves visceral fat remodeling through connexin43-mediated Ca2+ influx. Cardiovasc Diabetol. 2015;14(1):22-14. doi:10.1186/s12933-015-0183-6
33. Walpole CS, Wrigglesworth R, Bevan S, et al. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 1. The aromatic “A-region”. J Med Chem. 1993;36(16):2362-2372. doi:10.1021/jmc00068a014
34. Thomas KC, Ethisraj M, Shahrokhi K, et al. Structure-activity relationship of capsaicin analogs and transient receptor potential vanilloid 1-mediated human epidermal cell toxicity. J Pharmacol Exp Ther. 2011;337(2):400-410. doi:10.1124/jpet.110.178491
35. Li J, Nie C, Qiao Y, et al. Design, synthesis and biological evaluation of novel 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole triazole derivatives as potent TRPV1 antagonists. Eur J Med Chem. 2019;178:443-445. doi:10.1016/j.ejmech.2019.06.007
36. Fattori V, Hohmann MSN, Rossanesi AC, Pinho-Ribeiro FA, Verri WA. Capsaicin: current understandings of its mechanisms and therapy of pain and other pre-clinical and clinical uses. Molecules. 2016;21(7):844-33. doi:10.3390/molecules21078844
37. McDonald HA, Neelands TR, Kort M, et al. Characterization of A-425619 at native TRPV1 receptors: a comparison between dorsal root ganglia and trigeminal ganglia. Eur J Pharmacol. 2008;596(1-3):62-69. doi:10.1016/j.ejphar.2008.07.063
38. Pomonis JD, Harrison JE, Mark L, Bristol DR, Valenzano KJ, Walker K. N-(4-Tertiarybutylphenyl)-4-(3-cholorphyridine-2-yl) tetrahydroprazin-1(2H)-carboxamide (BCTC), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: II. in vivo characterization in rat models of inflammatory and neuropathic pain. J Pharmacol Exp Ther. 2003;306(1):387-393. doi:10.1124/jpet.102.046268
39. Surowy CS, Neelands TR, Bianchi BR, et al. (R)-(5-tert-buty1-2,3-dihydro-1H-inden-1-yl)-3-(1H-indazol-4-yl)-urea (ABT-102) blocks polymodal activation of transient receptor potential vanilloid 1 receptors in vitro and heat-evoked firing of spinal dorsal horn neurons in vivo. J Pharmacol Exp Ther. 2008;326(3):879-888. doi:10.1124/jpet.108.138511
40. Walpole CSJ, Wrigglesworth R, Bevan S, et al. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 2. The amide bond “B-region”. J Med Chem. 1993;36(16):2373-2380. doi:10.1021/jmc00068a015
41. Walpole CS, Bevan S, Bloomfield G, et al. Similarities and differences in the structure-activity relationships of capsaicin and resiniferatoxane analogues. J Med Chem. 1996;39(15):2939-2952. doi:10.1021/jm960139d
42. Lee J, Kang S-U, Choi H-K, et al. Analysis of structure-activity relationships for the ‘B-region’ of N-(3-acyloxy-2-benzylpropyl)-N’-[4-(methylsulfonylaminol)benzyl]thiourea analogues as vanilloid receptor antagonists: discovery of an N-hydroxythiourea analogue with potent analgesic activity. Biorg Med Chem Lett. 2004;14(9):2291-2297. doi:10.1016/j.bmcl.2004.02.002
43. Sun W, Liu K, Ryu H, et al. 2-(4-Methylsulfonylaminophenyl) propanamide TRPV1 antagonists: Structure-activity relationships in the B and C-regions. Biorg Med Chem. 2012;20(3):1310-1318. doi:10.1016/j.bmc.2011.12.014
44. Walpole CS, Wrigglesworth R, Bevan S, et al. Analogues of capsaicin with agonist activity as novel analgesic agents; structure-activity studies. 3. The hydrophobic side-chain “C-region”. J Med Chem. 1993;36(16):2381-2389. doi:10.1021/jm00068a016
45. Barbero GF, Molinillo JMG, Varela RM, Palma M, Macías FA, Barroso CG. Application of Hansch’s model to capsaicinoids and capsinoids: a study using the quantitative structure-activity relationship. A novel method for the synthesis of capsinoids. J Agric Food Chem. 2010;58(6):3342-3349. doi:10.1021/jf9035029
46. Appendino G, De Petrocellis L, Trevisani M, et al. Development of the first ultra-potent “capsaicinoid” agonist at transient receptor potential vanilloid type 1 (TRPV1) channels and its therapeutic potential. J Pharmacol Exp Ther. 2005;312(2):561-570. doi:10.1124/jpet.104.074864
60. Darré L, Domene C. Binding of capsaicin to the TRPV1 ion channel. *Mol Pharm*. 2015;12(12):4454-4465. doi:10.1021/acs.molpharmaceut.5b00641

61. Poblete H, Oyarzún I, Olivero P, et al. Molecular determinants of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) binding to transient receptor potential V1 (TRPV1) channels. *J Biol Chem*. 2015;290(4):2086-2098. doi:10.1074/jbc.M114.613620

62. Duarte Y, Cáceres J, Sepúlveda RV, et al. Novel TRPV1 channel agonists with faster and more potent analgesic properties than capsaicin. *Front Pharmacol*. 2020;11(1040):1-14. doi:10.3389/fphar.2020.01040

63. Kurosawa W, Nakano T, Amino Y. Practical large-scale production of dihydrocapsiate, a nonpungent capsaicinoid-like substance. *Biosci Biotechnol Biochem*. 2017;81(2):211-221. doi:10.1080/09168451.2016.1254533

64. Liao M, Cao E, Julius D, Cheng Y. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature*. 2013;504(7478):107-112. doi:10.1038/nature12822

65. Wu G, Robertson DH, Brooks CL, Vieth M. Detailed analysis of GRID-based molecular docking: a case study of CDOCKER-A CHARMm-based MD docking algorithm. *J Comput Chem*. 2003;24(13):1549-1562. doi:10.1002/jcc.10306

66. Discovery Studio 2.5, Accelrys, Inc., San Diego, CA, USA. 2010

67. Tirado-Rives J, Jorgensen WL, Julian Tirado-Rives WLJ. Contribution of conformer focusing to the uncertainty in predicting free energies for protein-ligand binding. *J Med Chem*. 2006;49(20):5880-5884. doi:10.1021/jm060763i

68. BIOVIA. *Discovery Visualizer* (v.20.1.0,19295). San Diego; 2020.