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Research Paper

Antinociceptive effect of natural and synthetic alkamides involves TRPV1 receptors

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Keywords
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Abstract

Objective To establish the role of TRPV1 receptor in the antinociceptive effect of natural alkamides (i.e. affinin, longipinamide A, longipenamide A and longipenamide B) isolated from *Heliopsis longipes* (A. Gray) S.F. Blake and some related synthetic alkamides (i.e. *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide).

Methods The orofacial formalin test was used to assess the antinociceptive activity of natural (1–30 μg, orofacial region) and synthetic alkamides (0.1–100 μg, orofacial region). The alkamide capsaicin was used as positive control, while capsazepine was used to evaluate the possible participation of TRPV1 receptor in alkamide-induced antinociception.

Key findings Natural (1–30 μg) and synthetic (0.1–100 μg) alkamides administered to the orofacial region produced antinociception in mice. The antinociceptive effect induced by affinin, *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide was antagonized by capsazepine but not by vehicle.

Conclusions These results suggest that alkamide affinin, longipinamide A, longipenamide A and longipenamide B isolated from *Heliopsis longipes* as well as the synthesized analogue compounds *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide produce their effects by activating TRPV1 receptor and they may have potential for the development of new analgesic drugs for the treatment of orofacial pain.

Introduction

Alkamides (*N*-alkylamides, alkenamides or alkenylamides) are bioactive compounds with potential pharmacological activity for the treatment of pain. More than 300 alkamides have been isolated as natural products. They represent a class of lipid compounds that are highly active in the central nervous system as they produce analgesic and immunomodulatory effects. These types of compounds are considered chemotaxonomic markers because special families of plants biosynthesize alkamides with unique structural characteristics.[1] Capsaicinoids are alkamides that are exclusively isolated from the fruits of the Capsicum genus (e.g. hot pepper, chile, chili). Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide, Figure 1), the most important capsaicinoid in nature, also participates in the nociceptive process. First, it activates the transient receptor potential cation channel vanilloid subfamily member 1 (TRPV1) receptor in sensory nerves, thereby inducing a sensation of hotness and burning due to depletion of substance P and other neurotransmitters such as somatostatin, neuronin A and kassinin, which leads to nociception.[2] A desensitization period then occurs in small sensory neurons due to the de-phosphorylation of TRPV1, which occurs in a Ca^{2+}-dependent manner via calmodulin–calcineurin.[3]

TRPV channels are a large family of nonselective cation channels that are involved in pain transmission. They function to detect external stimuli such as heat (<42 °C), protons, voltage and pressure.[4] TRPV1 is expressed in

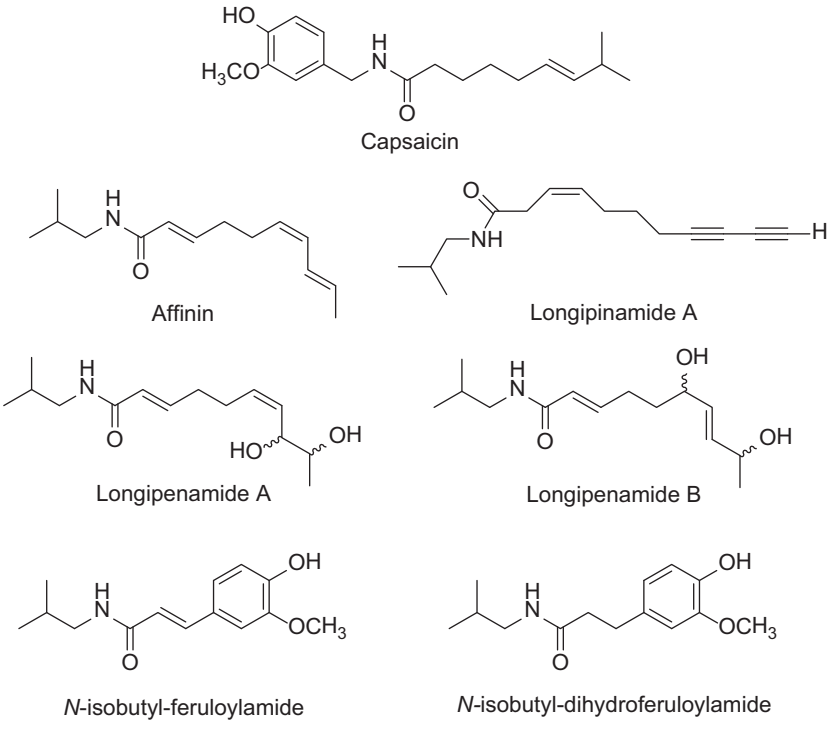

Figure 1 Chemical structures of natural capsaicin, affinin, longipinamide A, longipenamide A and longipenamide B alkamides and related synthetic, *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide alkamides.

the peripheral and central nervous system, especially in the trigeminal ganglion (TG) and dorsal root ganglion (DRG) neurons, hippocampus and dentate gyrus.[5] Activation of TRPV1 results in an increase of permeability towards Na^+ and Ca^{2+}, thereby increasing neuronal excitability.[6]

The roots of *Heliopsis longipes* (A. Gray) S.F. Blake are used in Mexico as a spice and for flavouring, just like the fruit of *Capsicum* species. These roots are also used as a buccal analgesic and anaesthetic in Mexican traditional medicine.[7] Affinin (i.e. spilanthol, *N*-isobutyl-2(E),6(Z),8(E)-decatrienamide, Figure 1) is responsible for these effects.[7] The antimutagenic, pharmacological and toxicological profile of affinin has been determined by our working group.[8,9] Its antinociceptive properties are related to the release of GABA[7] as well as to the activation of the opioid, serotoninergic and the nitric oxide pathways.[9,10] The intense, nonpungent numbness and tingling sensation that is caused by affinin when orally consumed suggests that this compound and the related alkamides could be useful for the treatment of pain. Capsaicin and affinin are related compounds that have structural similarities (Figure 1), such as the amide region and the fatty acid chain. These similarities may account for their shared analgesic and anaesthetic capacities.

The orofacial (i.e. the oral mucous, gums and face) perception of painful stimuli is performed by the primary afferent neurons of the TG system.[11] Their cell bodies are located in the TG in the face.[12] Orofacial pain is a condition that affects a large portion of the population, and it occurs as a result of health complications, such as neuralgia, headache and masticatory muscle disorders.[13] It is easily recognized when its origin is dental, such as with cavities and periodontal diseases. However, it may be produced by several causes (i.e. nerve injury).

Due to the high incidence of orofacial pain and the low efficacy of pharmacological treatments, we sought to obtain natural[14] and synthetic alkamides[15] and to evaluate their antinociceptive activity in mice. The purpose of the present work was to evaluate the antinociceptive effect of isolated natural alkamides of *Heliopsis longipes* and some related synthetic alkamides in the orofacial formalin test in mice and to establish the TRPV1 receptor participation on this activity.

Materials and Methods

General

The progress of reactions and the purity of products were monitored using thin layer chromatography (TLC) and

visualized using UV light with subsequent application of $(NH_4)_4Ce(SO_4)_4$ in 2N H_2SO_4 and heat. All analytical samples were homogenized before application on TLC, which was performed in at least two different solvent systems. Products were purified by flash column chromatography (FCC, silica gel, 100–230 mesh), and melting points were determined using a Fisher-Johns apparatus. Infrared (IR) spectra were recorded in a NICOLET 6700 laser with a source of He/Ne and a spectral range of 525–4000 cm^{-1}. ^1H, ^{13}C and 2D NMR experiments were performed by dissolving the samples in 0.5 ml of $CDCl_3$ or acetone, as is indicated, and then measuring using an Inova Varian and Mercury Spectrometer, at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. Chemical shifts are in parts per million, and tetramethylsilane (TMS) was used as an internal standard. A JMS-700 high-resolution mass spectrometer was used to obtain mass spectrometry data by (+)-FABMS. Catalytic hydrogenation reactions were conducted in a Parr Instrument Hydro Ap auto 500 ml to measure temperature and solvents for each reaction, as indicated.

Chemicals: name (abbreviation, PubChem compound)

4-dimethylaminopyridine (DMAP, 14284), isobutylamine (6558), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDAC, 15908), *N*,*N*-diisopropylethylamine (DIPEA, 81531), palladium/carbon (Pd/C, 23938), *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid, 445858), capsazepine (2733484), pentylenetetrazole (PTZ, 5917) and Tween 80 were purchased from Sigma Aldrich (St. Louis, MO, USA). Formalin (712), which was purchased from J.T. Baker, and diazepam (Valium, 3016) were used in this study. Acetone, dichloromethane (CH_2Cl_2), hexane and methanol (MeOH) were distilled before use. All natural or synthetic alkamides and capsazepine were dissolved in 2% Tween 80 in 0.9% saline solution. Formalin was dissolved to 2% in 0.9% saline solution. In addition, all drugs were freshly prepared for each experiment and administered to the orofacial region (20 µl) or intraperitoneally (i.p., 0.1 ml/10 g). Control animals received the same volume of vehicle (2% Tween 80 in 0.9% saline solution).

Animals

Swiss Webster female mice (20–25 g) from Cinvestav, Sede Sur, Mexico City, were used in this study for behavioural experiments. All experiments followed the Guidelines on Ethical Standards for Investigations of Experimental Pain in Animals,[16] and their care was conducted in accordance with the Mexican Official Norm for Animal Care and Handing (NOM-062-ZOO-1999). The protocol was approved by the institutional ethics committee (Protocol SIP-20170861). All efforts were made to minimize animal suffering and to reduce the number of animals used. Hence, mice were used once only. Animals were given free access to standard chow and tap water while being housed in a climate-controlled room with a 12-h light/dark cycle. At the end of the experiment, animals were sacrificed in a CO_2 chamber.

Capsaicin extract preparation

Dried pericarp of *Capsicum chinense* (commonly named 'chile habanero', 5 kg) were extracted at room temperature with dichloromethane (3.5 l × 4). The extract was concentrated to dryness in a vacuum to produce 24.7 g of extract (oleoresin). Oleoresin fractionation was performed through an open FCC (silica gel, 100–230 mesh; 8 × 24 cm) with a step gradient of *n*-hexane-acetone 100 : 0 to 70 : 30 (yielding 140 fractions of 50 ml each). Capsaicin was isolated as an 8 : 2 unresolvable mixture of capsaicin : dihidrocapsaicin from fractions 105-13t. In our hands, the IR, ^1H, ^{13}C NMR and (+)-FABMS data for capsaicin are as follows: IR (film) ν_{max} 3306, 2954, 1650, 1625 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 6.85 (1H, d, J = 8.0 Hz, H-5′), 6.78 (1H, d, J = 1.8 Hz, H-2′), 6.74 (1H, dd, J = 8.0, 1.8 Hz, H-6′), 5.90 (1H, s, NH), 5.34 (1H, dd, J = 15.4, 5.6 Hz, H-7), 5.33 (1H, dt, J = 15.4, 5.8, H-6), 4.33 (2H, d, J = 4.0 Hz, H-7′), 3.85 (3H, s, -OCH_3), 2.20 (2H, t, J = 7.5 Hz, H-2′), 1.98 (2H, dt, J = 7.8, 2.0 Hz, H-5′), 1.64 (2H, q, J = 7.7 Hz, H-3′), 1.50 (1H, o, J = 6.7 Hz, H-8), 1.37 (2H, q, J = 7.8, 7.4 Hz, H-4), 0.94 (6H, d, J = 6.7 Hz, H-9,H-10); ^{13}C NMR ($CDCl_3$, 100 MHz): 173.07 (s, C-1), 146.94 (s, C-4′), 145.28 (s, C-3′), 138.19 (d, C-7), 130.45 (s, C-1′), 126.57 (d, C-6), 120.84 (d, C-6′), 114.58 (d, C-5′), 110.9 (d, C-2′), 56.12 (c, -OCH_3), 43.72 (t, C-7′), 36.82 (t, C-2), 32.33 (t, C-5), 31.06 (d, C-8), 29.38 (t, C-4), 25.42 (t, C-3), 22.80 (c, C-9, C-10); (+)-FABMS m/z 305 ($C_{18}H_{27}NO_3$). For IR and NMR spectra, see Appendix S1. These data agreed with previous reports.[14]

Natural alkamides isolated from *Heliopsis longipes*

Fresh roots from *Heliopsis longipes* (Compositae) were collected at 'Real de Xichu' in Guanajuato and were identified by M.Sc. Ramiro Rios-Gómez. A voucher specimen (number 5904) was deposited at the FEZA Herbarium at the Facultad de Estudios Superiores, Zaragoza (UNAM) in Mexico City. Affinin (90% purity), longipinamide A (95% purity), longipenamide A (95% purity) and longipenamide B, (98% purity) were purified from the acetone extract of the fresh roots from *Heliopsis longipes* (A. Gray) S.F. Blake following the methodology previously reported.[15] Approximately 30 mg of each natural alkamide was obtained. The ^1H and ^{13}C NMR profiles for each alkamide agreed with our previous reports.[4,15]

Affinin, IR (film): ν_{max} 3288, 2957, 1668, 1625 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 6.82 (1H, dt, *J* = 15.4, 6.4 Hz, H-3), 6.29 (1H, ddc, *J* = 15.2, 10.2, 1.0, H-8), 5.98 (1H, sa, NH), 5.97 (1H, dd, *J* = 8.0 Hz, H-7), 5.85 (1H, d, *J* = 15.4 Hz, H-2), 5.69 (1H, dc, *J* = 14.8, 6.7 Hz, H-9), 5.26 (1H, dt, *J* = 10.6, 7.4 Hz, H-6), 3.14 (2H,dd, *J* = 6.5 Hz, H-1′), 2.28 (4H, m, H-4, H-5), 1.78 (1H, n, *J* = 6.8 Hz, H-2′), 1.77 (3H, dd, *J* = 6.7, 1.2 Hz, H-10), 0.92 (6H, d, *J* = 6.7 Hz, H-3′, H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz): 166.15 (s, C-1), 143.43 (d, C-3), 129.94 (d, C-9), 129.49 (d, C-7), 127.77 (d, C-6), 126.77 (d, C-8), 124.34 (d, C-2), 47.02 (t, C-1′), 32.18 (t, C-4), 28.69 (d, C-2′), 26.46 (t, C-5), 20.29 (c, C-3′,C-5′), 18.38 (c, C-10); (+)-FABMS *m/z* 221 ($C_{14}H_{23}NO$) (See Appendix S1).

Longipinamide A, IR ($CHCl_3$): ν_{max} = 3433, 3305, 2959, 2932, 2871, 2224, 1646, 1552, 1266, 971, 739 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 5.93 (1H, bs, NH), 5.60 (1H, d, *J* = 5.6 Hz, H-3), 5.59 (1H, ddd, *J* = 5.6, 2.0, 0.8 Hz, H-4), 3.07 (2H, dd, *J* = 6.8, 6.0 Hz, H-1′), 2.96 (1H, dd, *J* = 4.8, 2.0 Hz, H-2a), 2.95 (1H, dd, *J* = 4.8, 0.8 Hz, H-2b), 2.28 (2H, td, *J* = 6.8, 1.2 Hz, H-7), 2.18 (2H, m, H-5), 2.01 (1H, t, *J* = 1.2 Hz, H-11), 1.77 (1H, h, 6.8 Hz, H-2′), 1.64 (2H, q, *J* = 6.8 Hz, H-6), 0.90 (6H, d, *J* = 6.8 Hz, H-3′, H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz): 171.1 (s, C-1), 134.3 (d, C-4), 124.3 (d, C-3), 77.9 (s, C-8), 68.5 (s, C-9), 65.3 (s, C-10), 65.0 (d, C-11), 47.0 (t, C-1′), 40.7 (t, C-2), 31.6 (t, C-5), 28.6 (d, C-2′), 27.5 (t, C-7), 20.2 (q, C-3′,C-4′), 18.6 (t, C-6); EIMS *m/z* 231 ($C_{15}H_{21}NO$).

Longipenamide A, IR ($CHCl_3$): ν_{max} = 3315, 2961, 2926, 2871, 1669, 1627, 1514, 1370, 1255, 979 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 6.79 (1H, ddd, *J* = 15.6, 6.4, 6.4 Hz, H-3), 6.04 (1H, bs, NH), 5.85 (1H, d, *J* = 15.6 Hz, H-2), 5.55 (1H, dd, *J* = 11.2, 5.2 Hz, H-6), 5.54 (1H, dd, *J* = 11.2, 8.4 Hz, H-7), 4.34 (1H, dd, *J* = 8.4, 4.0 Hz, H-8), 3.82 (1H, dq, *J* = 6.4, 4.0 Hz, H-9), 3.13 (2H, dd, *J* = 6.4, 6.4 Hz, H-1′), 2.25 (2H, m, H-4), 1.79 (1H, h, 6.8 Hz, H-2′), 1.30 (3H, d, *J* = 6.4 Hz, H-10), 0.92 (6H, d, *J* = 6.8 Hz, H-3′,H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz): 166.5 (s, C-1), 143.8 (d, C-3), 132.2 (d, C-7), 129.4 (d, C-6), 124.4 (d, C-2), 71.3 (d, C-8), 70.5 (d, C-9), 47.2 (t, C-1′), 31.7 (t, C-4), 28.8 (d, C-2′), 26.9 (t, C-5), 20.4 (q, C-3′, C-4′), 17.7 (q, C-10). EIMS *m/z* 255 ($C_{14}H_{25}NO_3$).

Longipenamide B, IR ($CHCl_3$): ν_{max} = 3309, 2960, 2925, 2869, 1669, 1628, 1555, 1370, 1255, 979 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 6.94 (1H, bs, NH), 6.77 (1H, ddd, *J* = 15.6, 6.8, 6.8 Hz, H-3), 5.90 (1H, d, *J* = 15.6 Hz, H-2), 5.68 (1H, ddd, *J* = 15.6, 12.4, 5.6 Hz, H-8), 5.61 (1H, ddd, *J* = 15.6, 12.4, 5.6 Hz, H-8), 4.26 (1H, dq, *J* = 12.4, 6.4 Hz, H-9), 4.06 (1H, ddd, *J* = 13.2, 6.8, 5.6 Hz, H-6), 3.08 (2H, dd, *J* = 6.4, 6.4 Hz, H-1′), 2.22 (2H, m, H-4), 1.78 (1H, h, 6.8 Hz, H-2′), 1.64 (2H, m, H-5), 1.23 (3H, d, *J* = 6.4 Hz, H-10), 0.90 (6H, d, *J* = 6.8 Hz, H-3′,H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz): 166.8 (s, C-1), 144.0 (d, C-3), 135.0 (d, C-8), 131.8 (d, C-7), 124.1 (d, C-2), 71.1 (d, C-6), 67.7 (d, C-9), 47.1 (t, C-1′), 35.6 (t, C-5), 28.7 (d, C-2′), 28.1 (t, C-4), 23.3 (q, C-10), 20.4 (q, C-3′,C-4′); (+)-FABMS *m/z* 255 ($C_{14}H_{25}NO_3$).

HPLC analysis

The acetone extract of H. longipes was injected in a Dionex LC System Model Ultimate 3000, a WPS-3000SL Ultimate 3000 analytical Split loop autosampler, an LPG3400SD pump system, a DAD 3000 UV/VIS detector, a computerized data station equipped with Chromeleon 6.80 SR10 version software serial number 17056 (US patent 7747402) Munich, Germering. Separation was achieved on an Eclipse Plus C18 column (150 × 2.1 mm I.D.; 3.5 mm particle size) operating at 30°C. The mobile phase consisted of acetonitrile (55%) and water (45%). It was applied in an isocratic form. The flow rate was adjusted to 0.6 mL/min, during 10 min, monitoring at 213 nm.

Synthetic alkamides

N-isobutyl-feruloylamide [(*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-isobutylacrylamide]: To a well-stirred solution of ferulic acid (1498 mg, 7.74 mmol) in CH_2Cl_2 (45.0 ml), DIPEA (2.0 ml, 11.61 mmol) was added dropwise, followed by EDAC (1480 mg, 7.74 mmol) and DMAP (1418 mg, 11.61 mmol). The reaction was stirred at room temperature for 5 min. Then, isobuthylamine (0.9 ml, 7.74 mmol) was added. The reaction mixture was stirred at room temperature for another 12 h. Brine solution (120.0 ml) was added to the reaction mixture, and the organic phase was separated. Next, the aqueous phase was extracted using CH_2Cl_2 (90 ml × 3). The organic solvent was concentrated in vacuum. The residue was purified by FCC (silica gel, 100–230 mesh; 24 × 3 cm id), using *n*-hexane-acetone 90 : 10, to collect 32 fractions of 20 ml each, to produce 1.68 g of C7 (82.5% yield, 94% purity).

Compound *N*-isobutyl-feruloylamide was obtained as a waxy yellow powder from fractions 13–18: Rf 0.47 (*n*-hexane-acetone 1 : 1); IR (film) ν_{max} 3275, 3100, 2958, 1652, 1625 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): 7.52 (1H, d, *J* = 15.4 Hz, H-3), 7.02 (1H, dd, *J* = 8.2, 1.9 Hz, H-6″), 6.96 (1H, d, *J* = 1.9 Hz, H-2″), 6.88 (1H, d, *J* = 8.2 Hz, H-5″), 6.32 (1H, d, *J* = 15.4 Hz, H-2), 6.03 (1H, s, NH), 3.85 (3H, s, -OCH3), 3.21 (2H, t, *J* = 6.8 Hz, H-1′), 1.84 (1H, n, *J* = 6.8 Hz, H-2′), 0.94 (6H, d, *J* = 6.8 Hz, H-3′, H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz): 166.48 (s, C-1), 147.45 (s, C-4″), 146.83 (s, C-3″), 140.88 (s, C-1″), 127.30 (d, C-6″), 121.91 (d, C-5″), 118.35 (d, C-2″), 114.83 (d, -OCH3), 109.81 (t, C-1′), 55.84 (d, C-2), 47.10 (d, C-3′), 28.61 (d, C-3), 20.13 (q, C-4′ and C-5′); (+)-FABMS *m/z*: 249 ($C_{14}H_{19}NO_3$) (See Appendix S1).

N-isobutyl-dihydroferuloylamide [(*E*)-3-(4-hydroxy-3-methoxyphenyl)-N-isobutylpropanamide]: To a solution of *N*-isobutyl-feruloylamide (535 mg, 2.15 mmol) in CH_2Cl_2 (10.0 ml), 5 mg of 5% Pd/C was added. A flux of hydrogen was bubbled through the solution at 50 °C and 55 KPa for 8 h. The mixture reaction was filtered through celite, thereby adding CH_2Cl_2 (15.0 ml × 2). The organic solvent was concentrated in vacuum. The residue was purified by FCC (silica gel, 100–230 mesh; 24 × 3 cm id), using *n*-hexane-acetone 70 : 30, to collect 21 fractions of 20 ml each, to produce 480 mg of C8 (89.0% yield, 99% purity). Compound *N*-isobutyl-dihydroferuloylamide was obtained as a white powder from fractions 15–21: Rf 0.53 (*n*-hexane-acetone 1 : 1); m.p. 101–103 °C; IR (film) ν_{max} 3515, 3300, 2957, 1639 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) 7.40 (1H, s, NH), 6.81 (1H, d, J = 1.9 Hz, H-2″), 6.71 (1H, d, J = 8.0 Hz, H-5″), 6.64 (1H, dd, J = 8.0, 2.0 Hz, H-6″), 3.80 (3H, s, -OCH_3), 2.98 (2H, t, J = 6.0 Hz, H-1′), 2.81 (2H, t, J = 8.0 Hz, H-3), 2.41 (2H, t, J = 8.0 Hz, H-2), 1.70 (1H, n, J = 6.7 Hz, H-2′), 0.83 (6H, d, J = 6.7 Hz, H-3′,H-4′); ^{13}C NMR ($CDCl_3$, 100 MHz) 171.52 (s, C-1), 147.22 (s, C-4″), 144.78 (s, C-3″), 132.91 (s, C-1″), 120.56 (d, C-6″), 114.72 (d, C-5″), 111.90 (d, C-2″), 55.19 (d, -OCH3), 46.40 (t, C-1′), 38.13 (t, C-2), 31.25 (d, C-2′), 28.42 (t, C-3), 19.53 (q, C-3′,C-4′); (+)-FABMS *m/z*: 251 ($C_{14}H_{21}NO_3$) (See Appendix S1).

Pharmacological assay

Antinociceptive evaluation

The orofacial formalin test procedure was performed as described by Raboisson,[12] with slight modifications. Mice were placed in an open plexiglass observation chamber for 30 min to become familiarized with the testing equipment. Then, 20 µl of 2% formalin solution was subcutaneously injected into the upper lip lateral to the nose surface (orofacial region) using a 30-gauge needle. The animals were then returned to the chamber for observation, and nociceptive behaviour was observed immediately after injection. A mirror was placed behind the chamber to enable unhindered observation. Nociceptive behaviour was quantified as the time that mice spent rubbing the orofacial region during a 30-min period with 1-min measurements every 3 min after the irritant injection. Rubbing was readily identified and was characterized as face-rubbing episodes with vigorous face-wash strokes directed to the orofacial area. The time (in seconds) that animals spent rubbing the injected orofacial area is defined as the nociceptive response. Formalin-induced rubbing behaviour is biphasic: an initial, acute phase (0–5 min), followed by a relatively short quiescent period, before the terminal prolonged tonic response phase (9–30 min).

Participation of TRPV1 on alkamide-induced antinociception

Mice received increasing doses of natural (1–30 µg) and synthetic (0.1–100 µg) alkamides or vehicle (20 µl) 20 min before formalin injection into the orofacial region. Then, rubbing behaviour was assessed for 30 min. The concentration at which each alkamide showed its maximal effect in the orofacial region was selected to assess the mechanism of action. Capsaicin was tested at 3 µg, affinin at 30 µg and *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide at 10 µg. Two hours before the administration of the different alkamides (−2 h), animals were treated with capsazepine (a TRPV1 receptor antagonist). For the capsaicin group, 10 µg of capsazepine was administered, and for affinin and *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide, 100 µg of capsazepine was administered. The time and concentration schedule for capsazepine injection was determined in a pilot experiment in our laboratory (data not shown). Two hours following capsazepine injection, capsaicin, affinin and *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide or the vehicle were administered. After 15 min, each animal received an orofacial injection of 20 µl of 2% formalin to induce nociceptive behaviour, as previously performed in the experiment.

Molecular docking study

To investigate the binding interaction between alkamides and pain receptor TRPV1, we performed a molecular docking study with SPARTAN 08 program (Wavefunction, Inc., Irvin, California USA) to determine the minimum energy conformers through semiempirical calculations using the parametric method number 3 (PM3). TRPV1 channel model was taken from PDB entry 5IRX. Only the transmembrane domains (residues: 394–719) were used in the computational model as binding of capsaicin occurs in a pocket located between the voltage-sensor-like domain and the pore, above the S4-S5 linker.

The molecular docking calculation was focused on the binding pocket where the capsaicin molecule was previously bonded[17] using AutoDock4.0 software (The Scripps Research Institute. Molecular Graphics Loboratory. La Jolla, California USA). To obtain initial models of the structure of alkamides in the binding pocket, a grid maps representing the protein during the docking process were calculated with AutoGrid 4.0, with grid dimensions of 20 × 20 × 40 grid points, a spacing of 0.375Å, and the centre of the box placed at the reported essential residues for capsaicin (T550 and E570). A Lamarckian genetic algorithm search and an empirical free energy function were used. Docking parameters included an initial population of 200 randomly placed individuals, a maximum number of 3

million energy evaluations, a maximum of 27 000 generations, mutation and crossover rates of 0.02 and 0.80, respectively, and an elitism value of 1. Thirteen independent runs were carried out, and the docking solutions were clustered by root-mean-square deviation, using a threshold of 2Å. The resulting clusters were ranked according to the values of the free energy of binding. The best docked conformations were those found to have the lowest binding energy and the largest number of members in the cluster, indicating good convergence.

Seizures as adverse effect of alkamides

High doses of affinin (90 mg/kg, i.p.), capsaicin (90 mg/kg, i.p.), *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide (90 and 180 mg/kg, i.p.) were intraperitoneally administered in mice, and latency of mortality by seizures was quantified. Evaluation of pentylenetetrazole (PTZ)-induced seizures was used as a positive control to demonstrate the seizure activity in the mice used in this study. Diazepam (90 mg/kg, −10 min, i.p.) was used to reduce seizures induced by PTZ. If no convulsions or mortality occurred during the 30 min time period, animals were considered to be protected from seizures in the diazepam group. In the case of alkamides, we used the same time period. Natural alkamides were not evaluated, as the quantity isolated was low. The alkamides were administered without PTZ.

Data and statistical analysis

All experimental results are given as the mean ± SEM of the data obtained in six animals per group. Curves were constructed by plotting the time that mice spent rubbing the orofacial area (rubbing time). The area under the rubbing time against time curve (AUC), an expression of the duration and intensity of the effect, was calculated by the trapezoidal rule. One-way analysis of variance (ANOVA) followed by Dunnett test was used to compare differences between treatments. Differences were considered to reach statistical significance when $P < 0.05$.

Results and Discussion

Formalin injection produced immediate withdrawal of their head, which was often accompanied with vocalization. When returned to the test box and after a short delay of 15–30 s, the animals displayed sustained face-rubbing episodes with vigorous face-wash strokes that were directed to the orofacial area (i.e. whisker pad, upper lip and nostril) with their forepaw and hindpaw (Figure 2). The first phase commenced immediately following formalin injection and declined gradually over approximately 5 min. The second phase initiated 8 min after formalin injection, became maximal at approximately 18 min, and gradually declined over approximately 30 min (Figure 2a). Capsaicin (3 μg) injection, a positive control for alkamide compounds, significantly reduced formalin-induced nociception (Figure 2a). In addition, capsaicin (0.1–3 μg) produced a concentration-dependent antinociceptive effect during the second phase of the formalin test (Figure 2b). At the tested doses, capsaicin (0.1–3 μg) did not show nocifensive responses before formalin injection.

Local peripheral injection of affinin (1–30 μg), longipinamide A, longipenamide A and longipenamide B (1–30 μg) produced a concentration-dependent antinociceptive effect (Figure 3). The maximum antinociceptive effect for natural alkamides at the 30 μg concentration was

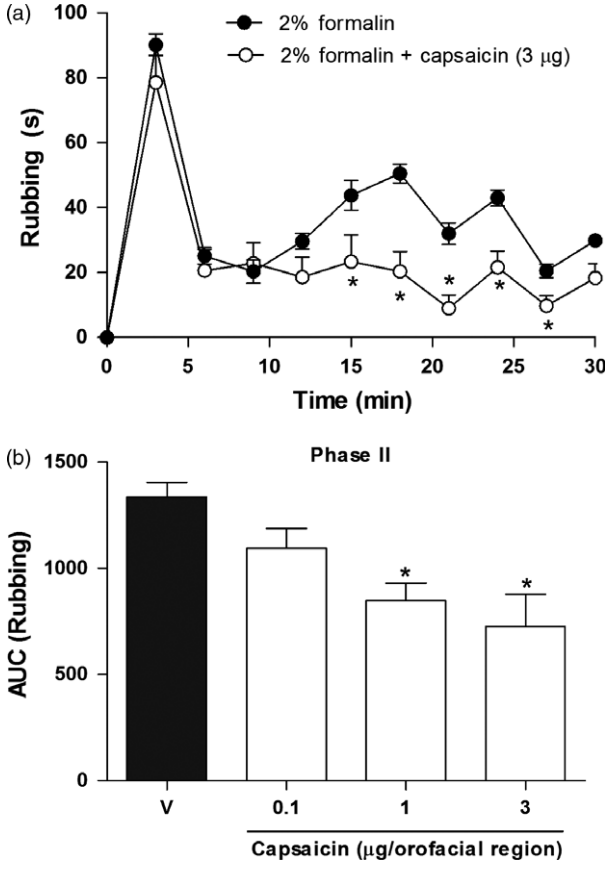

Figure 2 Time course of the rubbing behaviour observed after local orofacial administration of vehicle and capsaicin (3 μg/20 μl) in mice submitted to the 2% formalin test (a). The antinociceptive effect of local orofacial administration of capsaicin (0.1–3 μg/20 μl) during the second phase of the formalin test (b). Each bar indicates the area under the rubbing time against time curve (AUC) in mice submitted to the formalin test. Each value represents the mean of at least six animals ± SEM. *Significantly different with respect to the vehicle ($P < 0.05$), determined by the Student's *t*-test (a) and one-way ANOVA, followed by the Dunnett's test (b).

similar: affinin (65.8%), longipinamide A (56.3%), longipenamide A (60.5%) and longipenamide B (52.8%).

Local peripheral injection of synthetic compounds *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide (0.1–100 μg) in the orofacial region significantly ($P < 0.05$) reduced in a concentration-dependent fashion the time spent in rubbing during the first and second phase of the formalin test (Figure 4).

Local peripheral administration of capsazepine (10 μg) in the capsaicin-treated group or capsazepine (100 μg) in the affinin-, *N*-isobutyl-feruloylamide- and *N*-isobutyl-dihydroferuloylamide-treated groups prevented alkamide-induced antinociception during both phases of the formalin test (Figure 5).

Structurally, the TRPV1 channel is a homotetramer, symmetrically organized around a solvent exposed central pore, each subunit is formed by six transmembrane helices (S1-S6).[18] The hybrid approach that combines computational docking and functional studies has been employed to identify the capsaicin-binding pocket.[19] However, although the study revealed a 'tail-up, head-down' configuration according to previously reported,[20] and explained that the lack of clear electron density showed in cryo-electron microscopy (cryo-EM) technic was due to a very flexible tail that adopted more than one fixed conformations. Nevertheless, the atomic interaction was related on one side to the van der Waal's interactions and two hydrogen bonds between its Neck and Head with THR550 and GLU 570, respectively, and in another study argues that the two hydrogen bonds interactions were mediated by water molecules.[17] Although the specific interactions were not detailed, this supports the position of capsaicin in a 'tail-up, head-down' configuration and the assistance of THR550 and GLU570 residues as confidence position in TRPV1 activation.

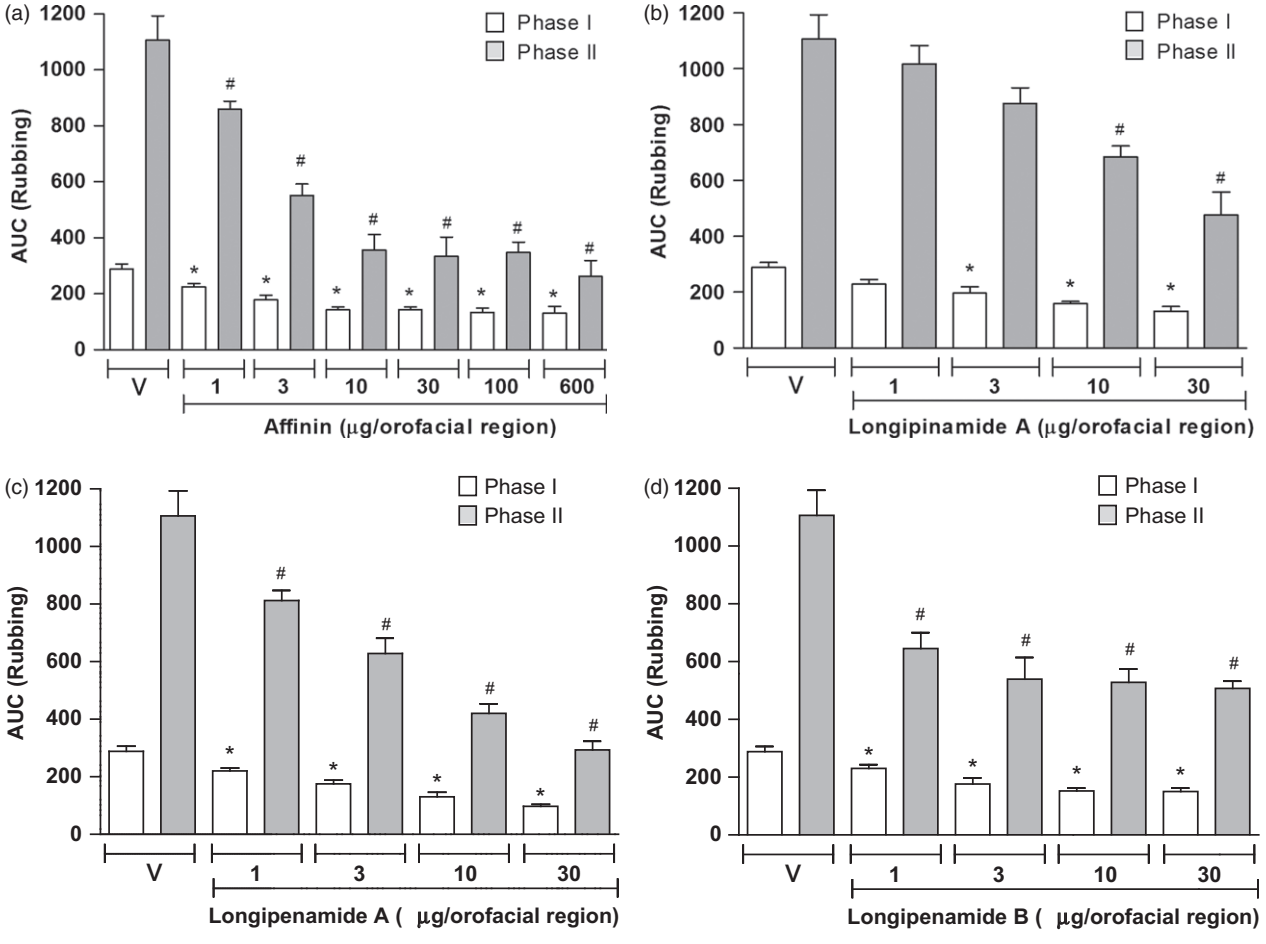

Figure 3 Antinociceptive effect of orofacial administration of natural alkamides isolated from *Heliopsis longipes*: affinin, longipinamide A, longipenamide A and longipenamide B during the first and second phase of the 2% formalin test in mice. Data are expressed as the area under the rubbing time against time curve (AUC). Bars represent the means ± SEM for at least six animals. *Significantly different ($P < 0.05$) from the vehicle group (V) in phase I, and #significantly different ($P < 0.05$) from the vehicle group in phase II, as determined by one-way ANOVA followed by the Dunnett's test.

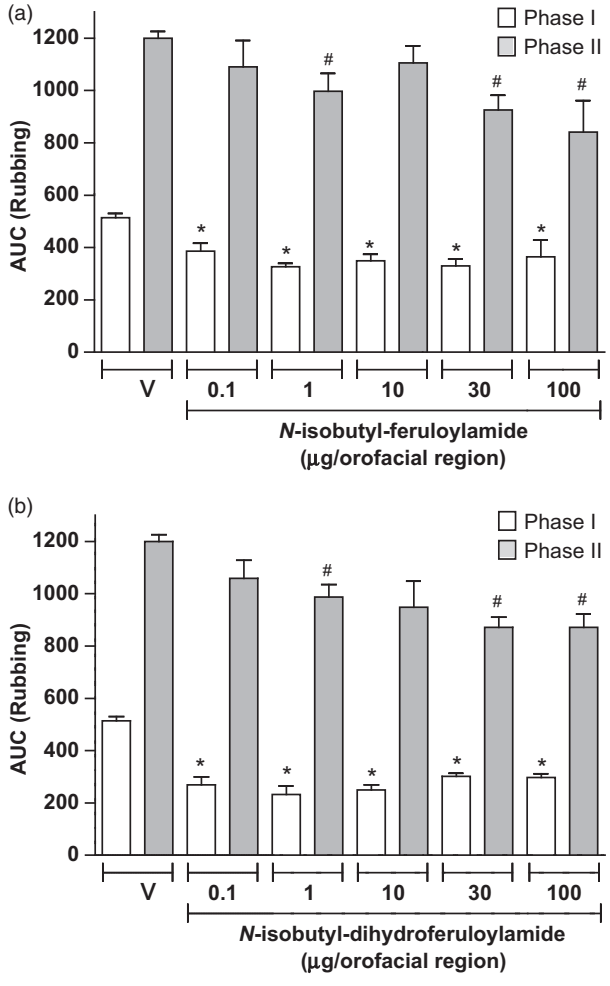

Figure 4 Antinociceptive effect of orofacial administration of synthetic alkamides: *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide during the first and second phase of the 2% formalin test in mice. Data are expressed as the area under the rubbing latency against time curve (AUC). Bars represent the means ± SEM for at least six animals. *Significantly different ($P < 0.05$) from the vehicle group (V) in phase I, and #significantly different ($P < 0.05$) from the vehicle group in phase II, as determined by one-way ANOVA, followed by the Dunnett's test.

Based on the above, the binding pocket was determined considering the 'tail-up, head-down' configuration and the THR550 and GLU570 residues to validate the docking studios with capsaicin and hence studies on derivatives after. According to docking results, (1) the Figure 6a showed the docking with capsaicin well-situated in binding-site, in a 'tail-up, head-down' configuration and near to THR550 and GLU570 residues.[17] This related with de antinociceptive activity results (36% at 3 μg) where capsaicin showed outstanding activity. (2) For natural and synthetic alkamide docking analysis, (Figure 6b–6g) displayed a 'tail-up, head-down' configuration similar to capsaicin at the same binding-site and neither of them showed GLU570 residue interactions. The antinociceptive activity below to capsaicin (60% at 30 μg for natural alkamides and 35% at 10 μg, for synthetic alkamides) could be explained by the unique THR550 residue interaction; however, the pungency absence shown in the biological assay could be explained by the lack of interaction at GLU570 residue too. This would place to GLU570 as key residue in the process pungency. (3) The synthetic alkamide docking analysis (Figure 6f and 6g) displayed the amide group is plainly close to THR550 residue and the p-OH interacts with LEU553 residue for both, even N-isobutyl-feruloylamide showed a hydrogen bond interaction. The antinociceptive activity results showed a ceiling effect, which is characterized by partial agonists. The synthetic alkamides occupied receptors but only partially activating it, while at the same time displacing or blocking full agonist's binding-site from receptors. The LEU553 residue interaction could be responsibly of this effect. Finally, (4) In the natural alkamide docking analysis, the ligand flexibility let to adopted a conformation that let them interacting with TYR511 residue considered by some reports as important residue for capsaicin activation,[19] and thus, the nociceptive activity is comparable between them.

Intraperitoneal administration of PTZ (90 mg/kg, i.p.) produced seizures and 100% mortality in mice (Table 1), which were prevented by diazepam. The administration of capsaicin and affinin (90 mg/kg, i.p.) produced a mortality rate of 100 and 83%, respectively. In both cases, the animals died at a faster rate (latency, Table 1) when compared to PTZ administration. None of the synthetic compounds (i.e. *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide) generated seizures and mortality at 90 mg/kg, i.p. However, when the dose was increased to 180 mg/kg, i.p., *N*-isobutyl-dihydroferuloylamide produced a mortality rate of 66% in mice (Table 1).

Results indicate that affinin, the principal alkamide from *Heliopsis longipes*, showed the best antinociceptive activity, followed by longipenamide B, with 65.8 and 60.5%, respectively. These results suggest that the *N*-isobutyl-feruloylamide double bond contributed to the antinociceptive activity. Our data extend previous observations showing that the systemic administration of affinin inhibits acetic acid- and capsaicin-induced nociception in mice.[10] In addition, this antinociceptive effect was demonstrated in the rat formalin test. Thus, data suggest that affinin inhibits nociception in inflammatory pain models following its local and systemic administration in rats and mice.[9]

The natural alkamide longipinamide A and longipenamide B showed an % of antinociception of 56.3 and 52.8, respectively. Their range of activity is close to that of affinin and longipenamide A, thereby confirming the hypothesis that a long chain (8–11 carbons) is ideal for exerting the

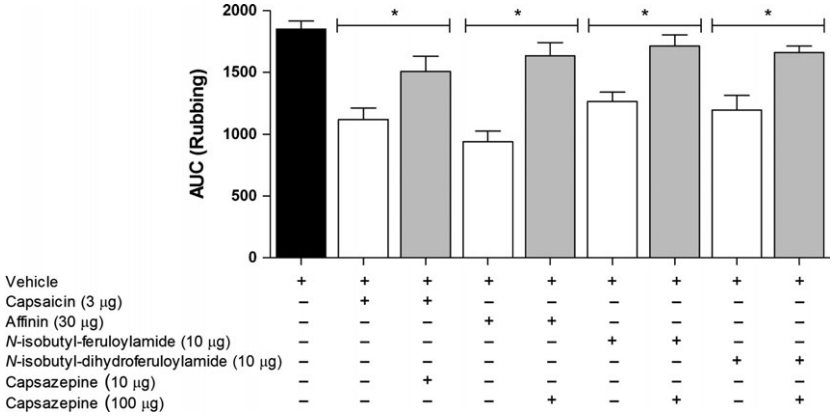

Figure 5 Effect of capsazepine (10 μg/20 μl) and (100 μg/20 μl) on the antinociceptive effect of capsaicin (3 μg/20 μl), affinin (30 μg/20 μl), *N*-isobutyl-feruloylamide (10 μg/20 μl) and *N*-isobutyl-dihydroferuloylamide (10 μg/20 μl) in the orofacial formalin test. Data are expressed as the area under the rubbing time against time curve (AUC). Bars represent the means ± SEM for at least six animals. *Significantly different ($P < 0.05$) from the individual administration, respectively, on each group, determined by one-way ANOVA and Tukey's test.

most effective antinociceptive effect.[21] Despite the effective antinociceptive properties of alkamide longipinamide A, longipenamide A and longipenamide B, their low quantities in nature could be a limitation for their use as alternative analgesic compounds. Obtaining these compounds with synthetic or bioengineering methods could be a solution to this problem. The finding that longipenamide B has antinociceptive activity further demonstrates that *Heliopsis longipes* (A. Gray) extracts present anti-inflammatory and antinociceptive properties that are even greater than those of affinin.[7,10] In contrast, related alkamides *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide displayed a % of antinociception between 25 and 35%, which is smaller than that of the natural alkamides. Furthermore, its evaluation demonstrated that the removal of the double bonds in the fatty acid chain allowed the creation of a stable derivative. This was most likely due to the presence of reducing epoxidation sites during metabolism, hence its toxicity.

While the mechanism of TRPV receptor activity remains to be elucidated, there is evidence suggesting that the satellite cell activation that follows dental pulp inflammation may be involved in the enhancement of the activity of TG neurons. This activation causes the innervation of the adjacent noninflamed teeth, the enhancement of TRPV1 expression and neural excitation, which manifests as ectopic persistent tooth-pulp pain.[22] In addition, the up-regulation or sensitization of TRPV1 channel receptors may also have an important role in mechanical allodynia and heat hyperalgesia on TG C-fibre neurons.[23]

It has been previously reported that the antinociceptive effect of capsaicin is related to its interaction with the TRPV1 receptor.[24] Confirming this finding, our experiments have shown that the capsaicin-induced antinociception was blocked by capsazepine, a TRPV1 receptor antagonist. Capsazepine also blocked the antinociceptive effect of affinin and that of the related synthetic alkamides in the formalin test, thereby implicating an activation of the vanilloid system. This blockade can be attributed to the structural similarity between these alkamides (Figure 1): (1) all include an amide group, (2) all include a short lateral chain as the fatty acid residue, and (3) alkamide compounds include an isobutylamine as the amine residue. This mechanism is also partly responsible for the antinociceptive activity that is observed in the alkamides *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide. Thus, the structural similarities between affinin and capsaicin and more generally between the natural and related alkamides that were assayed in this study might account for the significant interaction with the vanilloid system, particularly with the TRPV1 receptor. In the present study, we provide the first evidence that shows the antinociceptive properties of both natural and synthetic alkamides, with the participation of the TRPV1 channel. Previous studies have shown that affinin interacts with GABA, opioid and serotoninergic receptors[7,10] and it activates the cGMP-K^+ channel pathway to modulate the nociceptive process.[10] These observations suggest a need for future studies to determine the interaction of longipinamide A, longipenamide A, longipenamide, *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide with these signalling pathways. Among alkamides, capsaicin is the only pungent compound. Its initial burning pain is considered a potential limitation for use and development as active principle in formulations for the treatment of pain and itch.[25] This pungent property can therefore be attributed to the vanillylamine residue. Alkamide compounds lack this residue as well as the pungent property. Therefore,

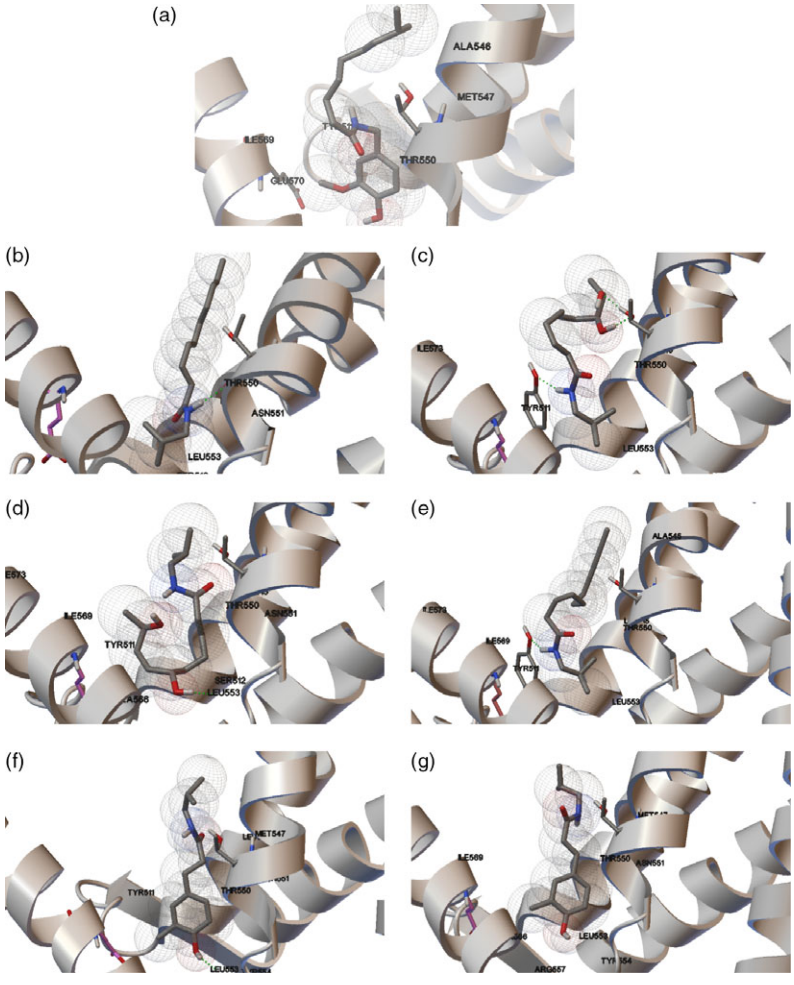

Figure 6 Interaction between alkamides and the binding-pocket recognition site TRPV1 considering the 'tail-up, head-down' configuration (THR550 and GLU570 residues). (a) Capsaicin; (b) affinin; (c) longipinamide A; (d) longipenamide A; (e) longipenamide A; (f) *N*-isobutyl-feruloylamide and (g) *N*-isobutyl-dihydroferuloylamide alkamides.

Table 1 Effect of alkamides on seizure activity in mice

Group	Doses mg/kg (i.p.)	Mortality	
		Frequency	Latency (s)
Vehicle + PTZ[a]		6/6	266 ± 46.5
Diazepam + PTZ[a]	1	0/6	0
Capsaicin	90	6/6	132 ± 19.8
Affinin	90	5/6	88 ± 25
N-isobutyl-feruloylamide	90	0/6	0
	180	0/6	0
N-isobutyl-dihydroferuloylamide	90	0/6	0
	180	4/6	224 ± 21.3

[a]PTZ, Pentylenetetrazole (80 mg/kg, i.p.).

these alkamides are excellent candidates for the development of compounds which could be useful for the treatment of pain.

Seizures are produced by neuronal activity that is unbalanced between inhibitory (GABA-mediated) and excitatory (glutamate-mediated) neurotransmission.[26] Evidence indicates that glutamate has a crucial role in seizures initiation and propagation. An abnormal glutamate release causes synchronous firing of large populations of neurons, leading to seizures.[27] Importantly, glutamate and NMDA receptors are involved in seizures aetiology and dopamine (D_2) and serotonin (5-HT_{1A}) monoamines are involved in the anticonvulsant effects.[28] On the other hand, the TRPV1 channels are expressed in the DRG and hippocampus, the main epileptogenic region in the brain. Ca^{2+} accumulation in the cytosol of the hippocampus and DRG is important in the aetiology of epilepsy. Furthermore, antagonist capsazepine suppresses epileptiform activity by decreasing the amplitude of nonsynaptic antidromic potentials, thereby interacting with residues from all four

monomers of the tetrameric channel.[29] Capsazepine interferes with the propagation of seizure activity along the hippocampal pathway. Blockade of TRPV1 channels by capsazepine administration remarkably suppressed in vivo ongoing ictal activity.[30] Recently, Nazıroğlu presents a revision that support a role for Ca^{2+} accumulation through TRPV1 channels in the aetiology of epileptic seizures, indicating that inhibition of TRPV1 in the hippocampus may possibly be a novel target for prevention of epileptic seizures.[31]

TRPV1 channels could be involved in the aetiology of seizures that are generated by capsaicin and affinin (90 mg/kg) as well as by *N*-isobutyl-dihydroferuloylamide at high doses (180 mg/kg). Affinin does not produce seizures at doses of 80 mg/kg, and alkamides *N*-isobutyl-feruloylamide and *N*-isobutyl-dihydroferuloylamide do not produce seizures at doses of 90 mg/kg. Furthermore, their antinociceptive effect was decreased by capsazepine treatment. Thus, affinin and *N*-isobutyl-feruloylamide (at doses lower than 90 mg/kg) and *N*-isobutyl-dihydroferuloylamide (at doses lower than 180 mg/kg) have antinociceptive activity, which acts on TRPV1 without seizure activity, perhaps due to different intrinsic activity and affinity towards the TRPV1 receptor or by activation of other mechanisms. More studies are necessary to determine the molecular mechanism mediating this behaviour.

Conclusions

Our results indicate that all natural and related alkamides produce peripheral antinociception in the orofacial formalin test. Docking analysis suggest that amide group play an important role on antinociceptive activity, due to a relevant interaction with THR550; additionally, the GLU570 residue could be involved in the pungent property. Data show that these compounds produce their antinociceptive effect also by activation of the TRPV1 receptor. At high doses (180 mg/kg), these alkamides induce seizures and death in mice, perhaps also due to their action on the TRPV1 receptor. Thus, blockade of TRPV1 activation in the DRG may be a target for the prevention of epileptic seizures and peripheral pain. It is important to consider that high doses of the natural extract of *Heliopsis longipes* could lead to intoxication. Notwithstanding, alkamides could be good candidates for future pharmaceutical development.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Alkamides chemical synthesis schemes and spectra data.