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Research paper

Modulation of disulfide dual ENKephalinase inhibitors (DENKIs) activity by a transient N-protection for pain alleviation by oral route

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ABSTRACT

The endogenous opioid system, essentially constituted by two opioid receptors which are stimulated by the natural internal effectors enkephalins (Met-enkephalin and Leu-enkephalin), is present at the different sites (peripheral, spinal, central) of the control of pain. We have demonstrated that the protection of the enkephalin inactivation by the two metallopeptidases (neprilysin and neutral aminopeptidase) increases their local concentration selectively induced by pain stimuli triggering analgesic responses. With the aim of increasing the orally antinociceptive responses of the previously described disulfide DENKIs ($NH_3^+CH(R_1)CH_2-S-S-CH_2-C(R_2R_3)CONHCH(R_4)COOR_5$), we designed new pro-drugs, in the same chemical series, with a transient protection of the free amino group by an acyloxyalkyl carbamate, giving rise to ($((CH_3)_2CHCO_2CH(CH_3)OCONHCH(R_1)CH_2-S-S-CH_2-C(R_2R_3)CONHCH(R_4)COOR_5$) pro-drugs **2a**–**2g**. These compounds were easily prepared from their parent analogs, with a good yield. They were tested per os and shown to be highly efficient in peripherally-controlled inflammatory and neuropathic pain with long lasting effects but completely inactive in the acute centrally-controlled hot plate test, a model of pain by excess of nociception. This demonstrates that DENKIs are able to relieve pain at its source thanks to the increase of enkephalin levels.

1. Introduction

The physiological control of pain is essentially dependent on the three components of the enkephalinergic system: i) two opioid receptors designated μ and δ receptors (MOR and DOR respectively) [1–3] ii) their neuropeptide ligands -Met and Leu enkephalin (Tyr-Gly-Gly-Phe-Met/Leu) [4]- which are processed from a high molecular weight precursor -preproenkephalin- iii) an inactivating system, involving two zinc metallopeptidases: aminopeptidase N (APN, EC 3.4.11.2) and neprilysin (NEP, EC 3.4.24.11), which regulates the in vivo local concentration and half-life of opioid peptides by cleaving the Tyr–Gly and Gly–Phe bonds respectively to generate small inactive metabolites [5].

This physiological control of pain by the enkephalinergic system was demonstrated by the significant modulation in enkephalin concentration and opioid receptors located on nerve endings after nociceptive stimuli [6,7]. Therefore, the three components of the enkephalinergic system have been successively studied as targets for efficient painkillers development: the oldest approach has been to develop opiates (morphine and analogs) as non-peptide agonists of opioid receptors. The second approach was to design enzyme-resistant enkephalin analogs such as D-Ala2-N-Me-Phe4-Met(O) 5-OL)enkephalin (FK33-824) [8]. These strategies yielded potent antinociceptive compounds but their chronic use was associated with the well-known side-effects of opiates (constipation, sedation, respiratory depression, tolerance), therefore limiting their use. In

Abbreviations: ACE, angiotensin converting enzyme; APA, aminopeptidase A; APB, aminopeptidase B; APN, aminopeptidase N; DENKI, dual ENKephalinase inhibitor; DIEA, diisopropylethylamine; ECE, endothelin converting enzyme; ENK, enkephalin; EtOH, ethanol; HPLC, high performance liquid chromatography; iv, intravenous; i.pl., intraplantar; LC/MS, liquid chromatography/mass spectroscopy; met-thiol, methionine thiol; MPE, maximal possible effect; NEP, neprilysin; NHS, N-hydroxysuccinimide; Nlxe, naloxone; nlxe-met, methyl naloxonium; OR, opioid receptor; PSNL, partial sciatic nerve ligation; po, per os; TLC, thin layer chromatography.

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fact, these effects are mainly related to their exogenous administration that induces ubiquitous stimulation of all the opioid receptors present in the organism, even those not involved in pain control [7,9]. The latest approach in enkephalin-mediated pain alleviation was to develop efficient inhibitors of both NEP and APN. Inhibiting only one of these two peptidases is insufficient to induce significant antinociceptive responses as illustrated in pharmacological and clinical studies [10–12]. In contrast, the concomitant inhibition of both peptidases produces analgesic responses [11,13]. All together, this indicates that increasing enkephalin concentrations at the nociceptor level promotes "physiological analgesia" [6,12], as demonstrated by microdialysis experiments [12–14] and use of antibodies for measurement of enkephalins in injured tissues [15].

The intensity of these Dual ENKephalinase Inhibitors (DENKIs) [11,16–21] analgesic effects will be therefore dependent on various parameters, and more particularly how painful stimuli increase the secretion of endogenous enkephalins. The recruitment of peripheral opioid receptors was confirmed by previous use of methyl naloxonium antagonist [22,23].

The first DENKIs were ester pro-drugs of compounds combining, through a disulfide bond, nanomolar APN and NEP inhibitors [11,16–18]. After in vivo ester hydrolysis and disulfide bond cleavage, each inhibitor binds to its own enzyme. The presence of a free thiol group able to strongly interact with the zinc atom, present in the catalytic site of these zinc peptidases, is crucially required in both NEP and APN inhibition [24]. Moreover, we have shown that our designed inhibitors of NEP and APN have a high selectivity and do not bind to other physiological zinc metallopeptidases such as ACE (angiotensin converting enzyme), ECE (endothelin converting enzyme), APA (aminopeptidase A), APB (aminopeptidase B) … and have no affinity for the opioid receptors [24]. These DENKIs have shown antinociceptive properties in various animal models of pain but were only active after iv administration [11]. A dramatic improvement in their bioavailability was achieved by decreasing the lipophilicity of drug and ester pro-drug moieties, leading to the DENKI 1-(2-(1-Ethoxycarbonyloxy-ethoxycarbonylmethyl)-carbamoyl)-3-phenyl-propyldisulfanylmethyl)-3-methylsulfanylpropyl-ammonium fumarate **1a**, designated PL37 [22,25] (Scheme 1). This compound is highly active per os and iv on severe acute central pain as well as on inflammatory and neuropathic pain [22]. However, this compound harbors a relatively short duration of action (~1 h) in various animal tests of nociception. This inconvenience is very likely due to the presence of a free ammonium group. The aim of the present study was therefore to overcome this problem by introduction of a transient protection of this amino group.

2. Results and discussion

2.1. Synthesis

The N-(acyloxy)alkyl carbamates were described as bioreversible pro-drugs of amines by Alexander et al. [26]. This amine protection has a good chemical stability, is hydrolyzed in vivo by esterases and improves the permeation through biological membranes. More recently, the introduction of this labile protection in gabapentin significantly enhanced its oral activity [27], probably through improvement of the delivery of these pro-drugs by high capacity transporters located in the intestine [28,29].

Consequently, we have assumed that such type of pro-drug could enhance the oral bioavailability of dual NEP/APN inhibitors leading to a longer pharmacological effect. Therefore, the N-Isopropylcarbonyloxyethylcarbamate group was introduced in the DENKIs series **1a**–**1g** previously described [22] (Scheme 1), leading to compounds **2a**–**2g**. In addition, these new pro-drugs were esterified or not on the C-terminal position (see formula in Table 2). From a chemical point of view, this N-protecting group can be easily introduced with a good yield [28] (Scheme 1).

The pro-drugs **2a**–**2g** were obtained by reaction of the corresponding precursors **1a**–**1g** in CH_2Cl_2 in presence of Et_3N with 1-((2, 5-Dioxopyrrolidin-1-yloxy)carbonyloxy)ethyl isobutyrate, synthetized as described in the literature [27,30] (Scheme 1). This pro-drug moiety generates a supplementary asymmetric center, which can be possibly resolved [31]. Compound **2g**, containing a cyclopentyl moiety in P1' position of neprilysin active site [32], was prepared starting from 1-(Mercaptomethyl)cyclopentane carboxylic acid **5** (Scheme 2) [33]. The (S)-2-(1-(Mercaptomethyl) cyclopentanecarboxamido)succinic acid **9** (Table 1) was described as a good NEP inhibitor [22]. Its corresponding disulfide **6** was synthetized using protocol described in Poras et al. [22] (Scheme 2).

For pro-drugs **2d**–**2f**, oxidation of the methionine side-chain of **10** was performed with $NaIO_4$ in EtOH leading to **11** before the introduction of the carbamate (Scheme 3).

2.2. Inhibitory potency

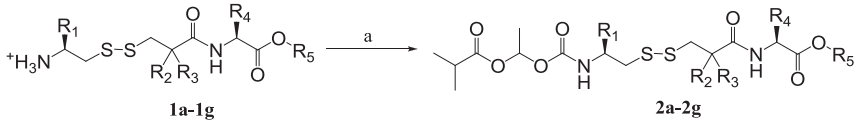
The inhibitory potencies of the NEP and APN inhibitors corresponding to the disulfide series **1** (Scheme 1) have been determined previously [22] and are reported (Table 1). All inhibitors are selective for their target enzyme.

2.3. Biotransformation

Compound **2a**, which harbors a double protection by an ester and an acyloxyalkyl carbamate moiety, was incubated at a final concentration of 800 μM in rat plasma (66 mg of protein/mL) at 37 °C (Fig. 1). Its biotransformation occurred by a two phase-process: a rapid hydrolysis of the ester, generating compound **2c**, followed by a slow hydrolysis of the carbamate, releasing the co-drug **1c** (Fig. 1). In these conditions, **1a** (PL37), which is the only compound able to cross the blood brain barrier and to induce antinociceptive responses in the hot plate test (after the biologically related disulfide cleavage) [22] was never detected.

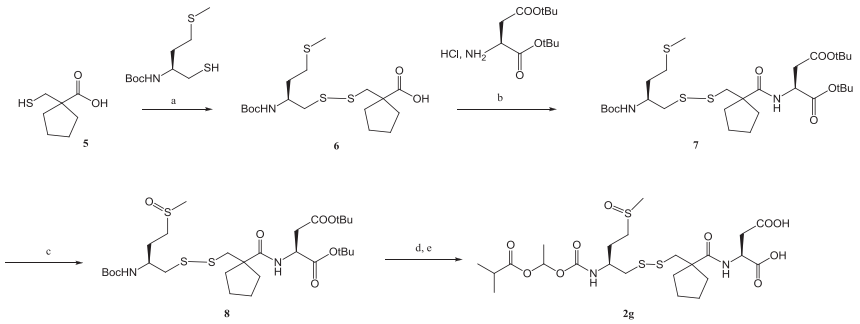
2.4. Behavioral study in mice

The new N-protected DENKIs have been tested on three animal models of pain to evaluate their efficacy in relieving acute and chronic pain, at the central or at the peripheral level.



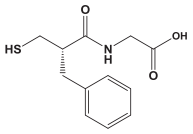
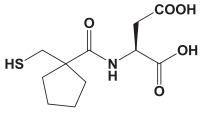
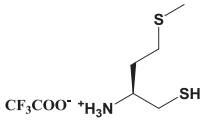
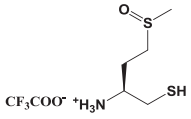
1a: PL37
R_1: $CH_2CH_2SCH_3$; R_2:H; R_3 (*S*)-CH_2Ph; R_4: H; R_5: $CH(CH_3)OCO_2Et$

Scheme 1. Synthesis of **2a**–**2g** starting from **1a**–**1g**. Reactions conditions: a) $(CH_3)_2CHCO_2CH(CH_3)OCO$–NHS, DIEA, CH_2Cl_2, 0 °C then RT, 5 h.



Scheme 2. Synthesis of (2S)-2-(1-((5S)-9, 12-Dimethyl-5-(2-(methylsulfinyl)ethyl)-7,11-dioxo-8,10-dioxa-2,3-dithia-6-azatridecyl)cyclopentanecarboxamido)succinic acid 2g. Reaction conditions: a) i) ClCOSCl, THF, MeOH ii) $CHCl_3$, Et_3N, RT; b) EDCI, HOBt, MeOH, DIEA, CH_2Cl_2; c) $NaIO_4$ (0.2 M water solution), EtOH, 4 °C; d) TFA, CH_2Cl_2, 0 °C; e) $(CH_3)_2CHCO_2CH(CH_3)OCO$–NHS, DIEA, CH_2Cl_2, 0 °C then RT, 1 h.

Table 1
Inhibitory potencies of inhibitors on NEP and/or APN.

Compound	Ki NEP (nM)	Ki APN (nM)
 Thiorphan	5.5 ± 0.2	>10,000
 **9**	3.4 ± 0.3	>10,000
 Methionine thiol	>	11 ± 2
 Methionine Thiol sulfoxide	>10,000	11 ± 2

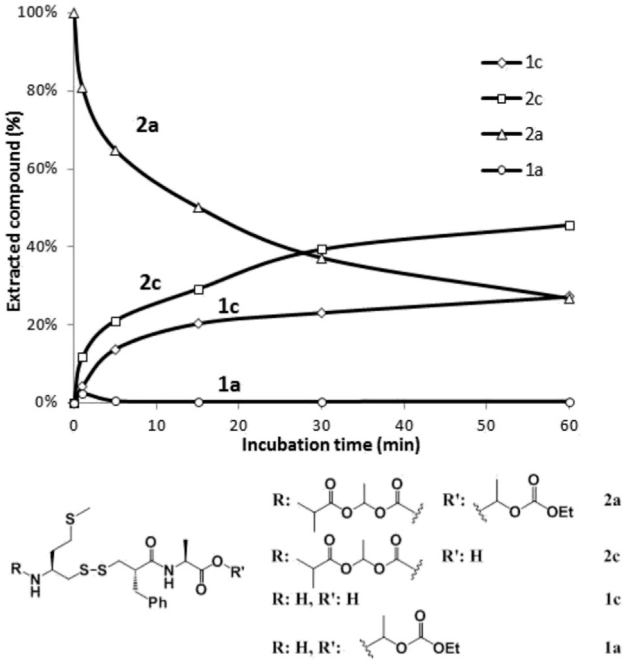
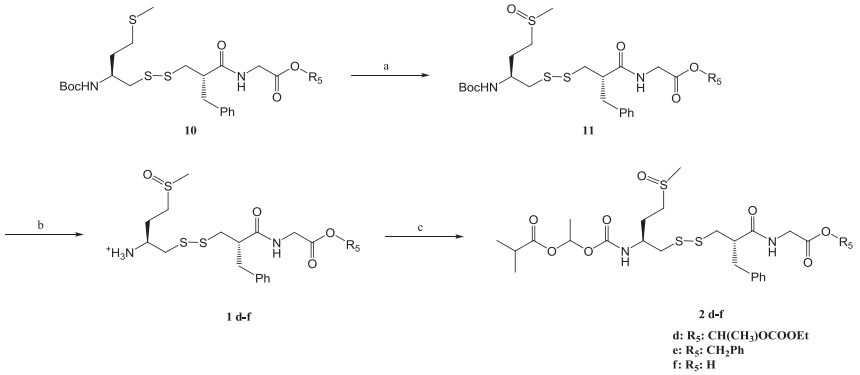


Fig. 1. Biotransformation of compound **2a** (800 μM) in male rat plasma (66 mg protein/mL) over 1 h.



Scheme 3. Synthesis of N-Isopropylcarbonyloxyethylcarbamate pro-drugs **2d**, **2e** and **2f**. Reaction conditions: a) $NaIO_4$, EtOH; b) HCOOH; c) $(CH_3)_2CHCO_2CH(CH_3)OCO$–NHS, DIEA, CH_2Cl_2, 0 °C then RT, 5 h.

2.4.1. Hot plate test

Firstly, antinociceptive responses of pro-drugs **2a**–**2g** were evaluated in the hot plate test in mice [34], as a model of centrally-controlled and integrated acute pain by excess of nociception [35]. All the N-protected pro-drugs **2a**–**2g** were not significantly active in this test by iv or oral route. In contrast, in the same conditions, all corresponding free amino C-protected analogs **1a**–**1g** were found effective [22]. This result indicates that compounds **2a**–**2g** do not cross the blood brain barrier unlike compounds **1a**–**1g** and/or are unable to generate the active free sulfhydryl NEP and APN inhibitors as illustrated by comparison of **1a** and **2a** (Fig. 2).

It can be deducted from these findings that the introduction of the N-terminal protection into the co-drug of this series prevents all central antinociceptive effects.

2.4.2. Formalin test

The analgesic effects of **2a**–**2g** were also evaluated in the formalin test in mice [36]. Compounds **1a** and its N-protected derivative **2a** were compared after oral administration, in the early phase (phase 1) of this test which involves an acute peripheral nociceptive stimulation [35]. Fifty mg/kg of both compounds, **1a** and **2a**, were administered at different time-points (20, 60 and 120 min), before intra-plantar (i.pl) formalin injection (Fig. 3). At 20 min, **1a** was significantly more effective than **2a** (62% versus 25%) in reducing lickings. At 60 min and 120 min, the relative efficacies of **1a** and **2a** were reversed: **2a** giving 56% and 31%, at 60 min and 120 min respectively, while **1a** had a decreased activity at 60 min (31%) and no significant activity at 120 min (Fig. 3). These results denotes a delayed and long lasting effect of **2a**, which may be due to a reduced transport and/or a slower release of the free NEP/APN inhibitors, consistent with the results reported (Fig. 1).

All pro-drugs **2a**–**2g** were then compared at the dose of 50 mg/kg in the early phase of the formalin test, at two time-points, 90 and 150 min before formalin administration (Table 2).

These compounds induced antinociceptive responses at 90 min (between 30 and 50%) and these effects were present 150 min after administration. When these compounds were administered 210 min before formalin, the responses were not different from the control (data not shown). It should be emphasized that with these N-protected compounds, the various C-terminal esterification did not modify the antinociceptive properties, as shown by the similarity the effects of **2a**, **2b** and **2c**. Methionine sulfoxide derivatives (compounds **2d**–**2f**) gave similar results (Table 2) with analgesic effects of around 40% at 90 min and 150 min. Compound **2g**, which contains a cyclopentyl moiety, is the most active of this series, with a constant analgesic effect (46% and 47%) at 90 min and 150 min respectively. This long duration of action was actually observed with all pro-drugs **2a**–**2g** (Table 2) indicating that the N-protection prolongs the antinociceptive effect of DENKIs in acute peripheral pain. Moreover, a C-protection does not improve their efficiencies, consistently with the very rapid plasmatic hydrolysis of the C-terminal ester (Fig. 1). It can be underlined that the presence of a free carboxylate group in these molecules (**2c**, **2f**, **2g**) is a favorable element for the development of various salts (sodium or lysine for example), increasing their aqueous solubility.

2.4.3. Partial sciatic nerve ligation

Partial sciatic nerve ligation (PSNL) [37], here adapted to mice [38], is a widely-used predictive model of chronic neuropathic pain. It produces a profound and lasting decrease in mechanical threshold related to chronic nerve injury-evoked pain. This neuropathic pain induces both intense and prolonged mechanical allodynia and thermal hyperalgesia, characterized by lowered paw withdrawal thresholds to pressure (von Frey test) [39] and to thermal stimuli (plantar test) [40] respectively. The potencies of **2c** and its oxidized form **2f** were compared at the same doses, using von Frey filaments. As shown (Fig. 4A), oral administration of pro-drug **2c** (50 mg/kg) induced strong, long lasting, antiallodynic effects at 45 min (0.97 ± 0.15 g) and 90 min (0.88 ± 0.17 g), as illustrated by an increase in mechanical threshold by 64% and 56%, respectively. At 150 min, the antiallodynic effect was markedly reduced (0.55 ± 0.10 g; 18%). Similar results were obtained after oral administration of compound **2f** in a dose-dependent manner (Fig. 4B): **2f** (25 mg/kg po) induced a strong antiallodynic effect (0.69 ± 0.08 g at 30 min, 0.82 ± 0.11 g at 60 min, 0.94 ± 0.08 g at 120 min, corresponding to 40, 52 and 63% analgesia respectively, whereas vehicle-treated mice displayed a constant mechanical

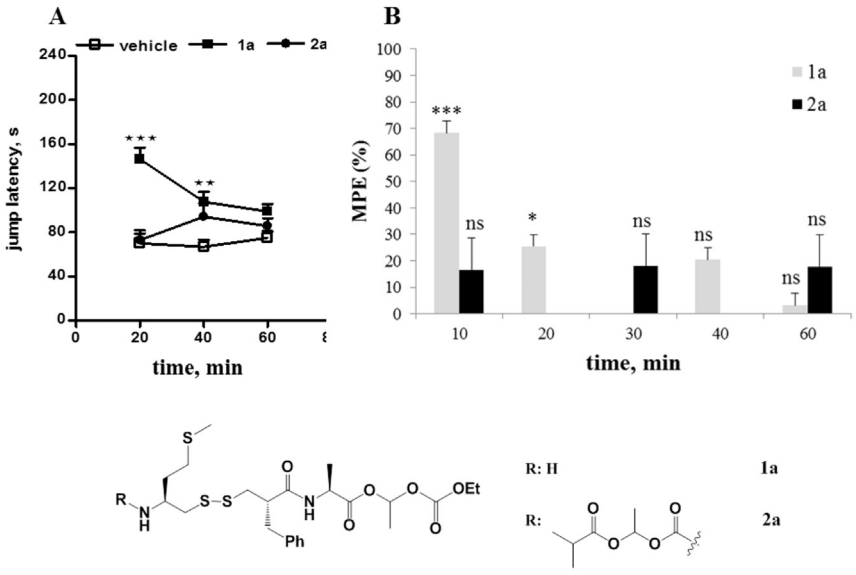


Fig. 2. Antinociceptive effects of compounds **1a** and **2a** in the hot plate test (jump latency) in mice. (A) Time-course of **1a** (■) or **2a** (●) administered per os at 150 mg/kg in EtOH/PEG400/H_2O (1/4/5), n = 7–8 per group. Results are expressed as mean jump latency (s) ± sem. (B) Time-course of **1a** (grey bar, EtOH/Tween 80/H_2O (1/1/8)) or **2a** (black bar, EtOH/Cremophor/H_2O (1/1/8)) injected by iv route at 10 mg/kg, n = 6 per group. Results are expressed as MPE (%) ± sem. ★p < 0.05, ★★p < 0.01, ★★★p < 0.001, ns: non-significant versus vehicle, ANOVA followed by Bonferroni test.

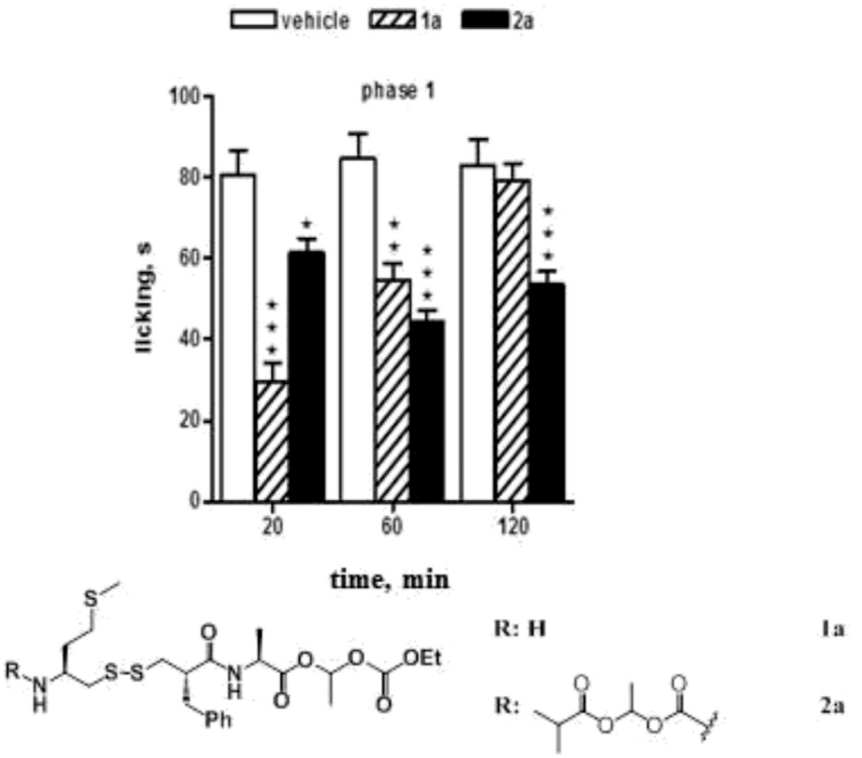
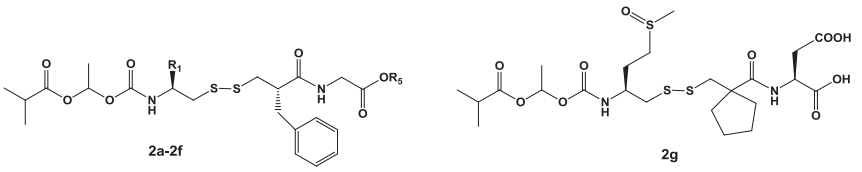
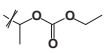
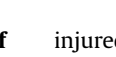


Fig. 3. Comparison of antinociceptive effects of compounds **1a** and **2a** in the formalin test in mice. Compounds **1a** (50 mg/kg), **2a** (50 mg/kg) or vehicle (ethanol/methylcellulose 0.5% in distilled water, 1.5/98.5) were orally given 20 min, 60 min or 90 min before i.pl formalin injection (5%, 20 μL) and nociceptive behavior observation (paw licking) was conducted during Phase 1 of the test (0–5 min after formalin injection). ★p < 0.05, ★★p < 0.01, ★★★p < 0.001 versus vehicle, 2-way ANOVA followed by Bonferroni's test, n = 6–8 per group.

Table 2
Comparison of antinociceptive effects of compounds **2a**–**2g** in the formalin test in mice.



Compound	R_1	R_5	Analgesia (%)[a] 90 min	150 min
2a	$CH_2CH_2SCH_3$		32 ± 3**	30 ± 8**
2b	$CH_2CH_2SCH_3$		34 ± 9**	26 ± 4*
2c	$CH_2CH_2SCH_3$	H	34 ± 9**	31 ± 7*
2d	$CH_2CH_2SOCH_3$		39 ± 4**	38 ± 8**
2e	$CH_2CH_2SOCH_3$	CH_2Ph	39 ± 8**	38 ± 9**
2f	$CH_2CH_2SOCH_3$	H	39 ± 10**	38 ± 8**
2g			46 ± 6***	47 ± 4***

a) ANOVA + Newman Keuls: ***p < 0.001; **p < 0.01; *p < 0.05.

hypersensitivity. At the highest dose tested (50 mg/kg po), **2f** induced also a large increase in mechanical threshold (Fig. 4B) which was not significantly different from that obtained at 25 mg/kg (compared to contralateral paw). Compounds **2c** and **2f** elicited a significant increase of the mechanical threshold on the injured side, 45 or 60 min after administration respectively, without any modification of the mechanical threshold on the contralateral non injured side, suggesting that mechanical hypersensitivity effects observed were mainly produced locally from painful-induced release of ENKs by stimulation of opioid receptors occurring selectively at the injured site. This is consistent with previous results where the same unilateral effect was observed [23]. Indeed, the involvement of ENKs acting on peripheral receptors has been demonstrated by reversion of the anti-neuropathic effects after

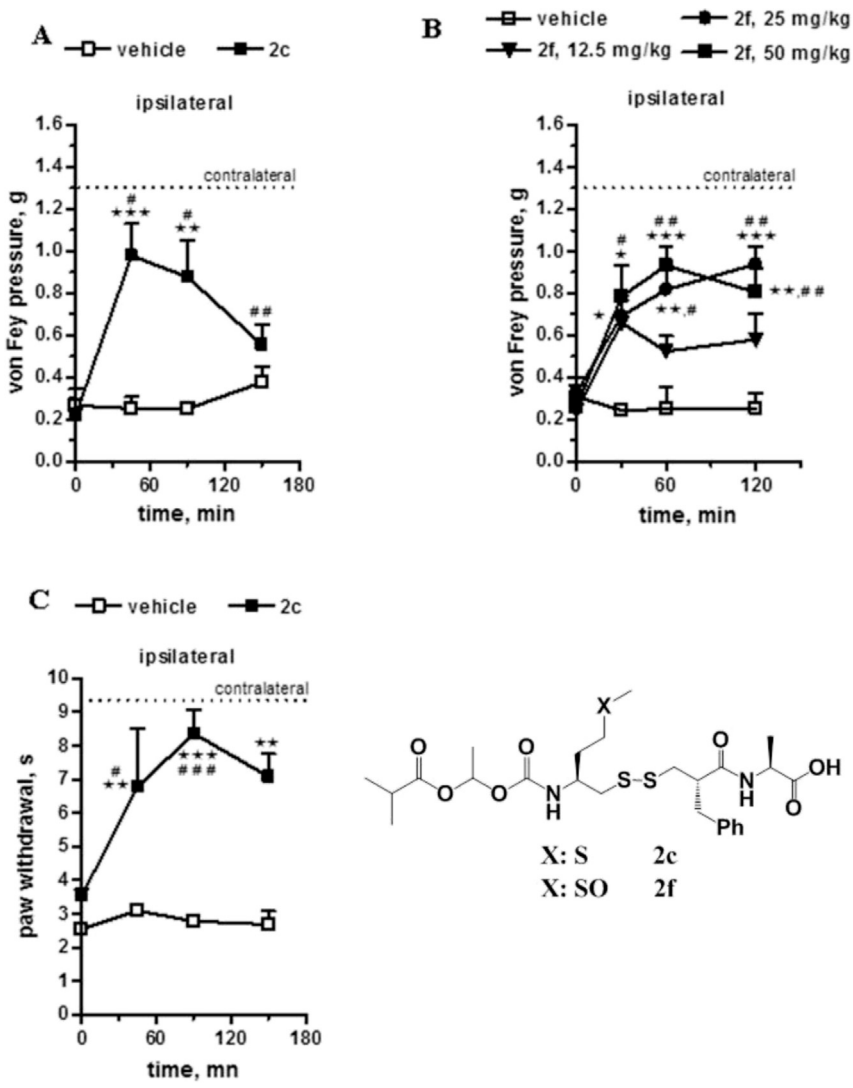


Fig. 4. Antiallodynic and antihyperalgesic effects of compounds **2c** and **2f** after PSNL. (A) Compound **2c**, 50 mg/kg or vehicle (EtOH/methylcellulose 0.5% in distilled water, 1.5/98.5) were given orally and paw withdrawal threshold values were measured before administration (t0) and 45 min, 90 min, 150 min after oral gavage using von Frey filaments (n = 5–7 per group). (B) Compound **2f** (12.5, 25 or 50 mg/kg) or vehicle (EtOH/methylcellulose 0.5% in distilled water, 1.5/98.5) were administrated by oral route and paw withdrawal threshold values were measured before (t0) and 30 min, 60 min, 120 min after oral gavage on both ipsilateral and contralateral side. (C) Plantar test of compound **2c** (50 mg/kg), solubilized in EtOH/methylcellulose 0.5% in distilled water, 1.5/98.5. ★p < 0.05, ★★p < 0.01, ★★★p < 0.001 versus vehicle, #p < 0.05, # #p < 0.01 versus t0, Kruskal–Wallis followed by Dunn's Multiple Comparison test.

prior administration of methyl-naloxonium, an opioid antagonist unable to produce a central effect at the dose used [23].

Compound **2c** was tested using the plantar test, at 50 mg/kg. An anti-hyperalgesic response was measured, with a maximal effect at 90 min (100% as compared to contralateral paw) remaining highly significant up to 150 min after pro-drug administration (Fig. 4C).

It was interesting to compare the activities of the carbamate protected DENKIs **2c** and **2f**, without C-terminal protection, with **1a** (PL37) [22], which has only a C-terminal protection. The intensity of **1a** antiallodynic response in the von Frey test was 51% at 25 mg/kg per os at 20 min [22]. A similar response (64% antiallodynia) was observed at 45 min for **2c** (Fig. 4A) but the effect of **1a** disappeared after 30 min [22] whereas as expected the reversion of allodynia by **2c** is still significant at 150 min (Fig. 4A). Moreover, **2c** shows 64% reversion of allodynia and 100% of hyperalgesia at 50 mg/kg per os (Fig. 4A and C). The dose–response curve obtained with **2f** in von Frey pressure (Fig. 4B) shows a ceiling effect from 25 mg/kg. The apparent plateau effect upon the tests is consistent with the lack of further augmentation of protected ENKs, an effect which is very likely due to a maximum inhibition of the two enkephalin inactivating enzymes from 25 mg/kg [5,41,42].

3. Conclusion

The aim of this study was essentially to increase the duration of action of the disulfide NEP/APN inhibitors previously described [22]. All these new DENKIs were found very active after oral administration in animal models of inflammatory and neuropathic pain. N-protected DENKIs, presented in this paper, do not cross the blood brain barrier and act only on peripheral nociceptors. They are devoid of central side-effects constantly observed with opiates, enabling their administration to patients with persistent pain. The peak effect of these DENKIs is lower than the one of disulfide NEP/APN inhibitors described previously [22], but, as expected, their antinociceptive responses last longer. Other experiments are in progress to determine the most appropriate clinical indications for these compounds.

4. Materials and methods

4.1. General

All reagents were from Sigma–Aldrich and the solvents from Carlo Erba-SDS (France). TLC plates were Merck TLC aluminum sheets coated with silica gel 60F254. Compounds were purified by flash chromatography (Silica gel Si 60, 40–63 μm) or by semi-preparative HPLC purification. ^{1}H NMR spectra were recorded on 200 MHz Bruker instrument. Chemical shift were reported in ppm with the solvent as internal standard ($CDCl_3$: 7.26 ppm; DMSO d6: 2.54 ppm). Data are reported as followed: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet). Electron spray mass spectroscopy (ESI) was performed with an LCMS Quad Electrospray Agilent 6120. RP-HPLC separations were respectively performed on Shimadzu Prominence HPLC at a flow rate of 1 mL/min for analytic column (Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm) and on Waters prep 600 HPLC at a flow rate of 10 mL/min on the corresponding semi-preparative column (Kromasil C18, 100 Å, 5 μm, 250 × 21.2 mm). Purity (%) was determined from a surface integral of detected peaks by reversed-phase HPLC using UV detection (210 nm) and all compounds showed purities greater than 95%. All commercial reagents and solvents were used without further purification.

Compounds **1a**, **1b** and **1c** were synthesized as described [22].

4.2. Synthesis of pro-drugs (**2a**–**2g**)

4.2.1. General procedure for the synthesis of N-Isopropylcarbonyloxyethyloxycarbamate pro-drug

Compound **1** (2.82 mmol) was solubilized in CH_2Cl_2 (20 mL) and then N-isopropylcarbonyloxyethoxy carbonyl derivative (1.5eq) was added, followed by DIEA (3eq, 1.4 mL). The reaction mixture was stirred for 1 h at room temperature and the solvent was removed under reduced pressure. The crude mixture was portioned in AcOEt-10% citric acid solution (50 mL/50 mL). The organic layer was washed by water (50 mL), brine (50 mL) and dried over Na_2SO_4. The crude compound **2** was purified by semi-preparative HPLC on C18 column, 5 μm, 100 Å, 250 × 21.2 mm using CH_3CN/H_2O (0.1% TFA) as elution system.

*4.2.1.1. 1-(1-{2-[(1-Ethoxycarbonyloxy-ethoxycarbonylmethyl)-carbamoyl]-3-phenyl-propyldisulfanylmethyl}-3-methylsulfanyl-propylcarbamoyloxy)-ethyl isobutyrate (**2a**) (R_1 = $CH_2CH_2SCH_3$; R_2 = (S)–CH_2Ph; R_3 = H; R_4 = H; R_5 = $CH(CH_3)OCO_2CH_2CH_3$).* Solid (yield: 50.4%); ^{1}H NMR ($CDCl_3$) δ 1.10 (2 × 3H, d), 1.25 (3H, t), 1.50 (2 × 3H, d), 1.65–1.85 (2H, m), 2.40 (4H, m), 2.50 (1H, m), 2.50–3.00 (5H, m), 3.90–4.00 (3H, m), 4.15 (2H, q), 4.90 (1H, d), 6.40 (1H, t), 6.75 (2 × 1H, q), 7,20 (10H, m); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 70-30: 10.68 & 11.52 min; ESI(+): $[M + H]^+ = 677$.

*4.2.1.2. 1-{1-[2-(Benzyloxycarbonylmethyl-carbamoyl)-3-phenyl-propyldisulfanylmethyl]-3-methylsulfanyl-propylcarbamoyloxy}-ethyl isobutyrate (**2b**) (R_1 = $CH_2CH_2SCH_3$; R_2 = (S)–CH_2Ph; R_3 = H; R_4 = H; R_5 = CH_2Ph).* Solid (yield: 48.5%); ^{1}H NMR ($CDCl_3$) 1.10 (2 × 3H, d), 1.50 (3H, d), 1.70–1.90 (2H, m), 2.00 (3H, s), 2.50 (3H, m), 2.70–3.00 (7H, m), 3.70–4.20 (3H, m), 4.95 (1H, d), 5.15 (2H, s), 6.40 (1H, t), 6.70 (1H, q), 7.20 (10H, m); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 70-30: 12.39 & 12.80 min; ESI(+): $[M + H]^+ = 651$.

*4.2.1.3. 1-{1-[2-(Carboxymethyl-carbamoyl)-3-phenyl-propyldisulfanylmethyl]-3-methylsulfanyl-propylcarbamoyloxy}-ethyl isobutyrate (**2c**) (R_1 = $CH_2CH_2SCH_3$; R_2 = (S)–CH_2Ph; R_3 = H; R_4 = H; R_5 = H).* Solid (yield: 42.7%); ^{1}H NMR ($CDCl_3$) δ 1.10 (2 × 3H, d), 1.40 (3H, d), 1.60–1.75 (2H, m), 2.00 (3H, s), 2.50 (3H, m), 2.80–3.10 (7H, m), 3.80–4.10 (3H, m), 5.00 (1H, d), 6.70 (1H, q), 7.20 (5H, m); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 60-40: 7.81 & 8.23 min; ESI(+): $[M + H]^+ = 561$.

*4.2.2. Synthesis of N-Isopropylcarbonyloxyethyloxycarbamate pro-drugs(**2d**), (**2e**) and (**2f**)*

Compounds **1d**, **1e** and **1f** were synthesized as described [16] and last step leading to compounds **2d**, **2e** and **2f** as previously described.

*4.2.2.1. 1-(1-{2-[(1-Ethoxycarbonyloxy-ethoxycarbonylméthyl)-carbamoyl]-3-phenyl-propyldisulfanylmethyl}-3-methanesulfinyl-propylcarbamoloxy)-ethyl isobutyrate (**2d**) (R_4 = H; R_5 = $CH(CH_3)OCO_2CH_2CH_3$).* Solid (yield: 54.3%); RMN (DMSOd6) δ: 1.00 (2 × 3H, d), 1.15 (3H, t), 1.40 (2 × 3H, d), 1.65–1.90 (2H, m), 2.40–3.00 (13H, m), 3.75 (1H, m), 3.90 (2H, d), 4.00 (2H, q), 5.10 (2H, s), 6.65 (2H, m), 7.10–7.30 (5H, m), 7.50 (1H, d), 8.50 (1H, t); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 50–50:14.55 min; ESI(+): $[M + H]^+ = 692$.

*4.2.2.2. 1-{1-[2-Benzyloxycarbonylmethyl-carbamoyl)-3-phenyl-propyldisulfanylmethyl]-3-methanesulfinyl-propylcarbamoyloxy}-ethyl isobutyrate (**2e**) (R_4 = H; R_5 = CH_2Ph).* Solid (yield: 43.%); RMN (DMSOd6) δ: 1.00 (2 × 3H, d), 1.40 (3H, d), 1.65–1.90 (2H, m), 2.40–3.00 (13H, m), 3.75 (1H, m), 3.90 (2H, d), 5.10 (2H, s), 6.65 (1H, m), 7.10–7.30 (10H, m), 7.50 (1H, d), 8.50 (1H, t); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 60-40: 8.21 min; ESI(+): $[M + H]^+ = 666$.

*4.2.2.3. 1-{1-2[2-(Carboxymethyl-carbamoyl)-3-phenyl-propyldisulfanylmethyl]-3-methanesulfinyl-propylcarbamoyloxy}-ethyl isobutyrate (**2f**) (R_4 = H; R_5 = H).* Solid (yield: 28.1%); RMN (DMSOd6) δ: 1.00 (2 × 3H, d), 1.40 (3H, d), 1.70–1.90 (2H, m), 2.50–3.00 (13H, m), 3.70 (2H, m), 6.65 (1H, m), 7.20 (5H, m), 7.50 (1H, d), 8.40 (1H, t); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 40–60:11.99 min; ESI(+): $[M + H]^+ = 576$.

4.2.3. Synthesis of 2-({1-[2-(1-Isobutyryloxy-ethoxycarbonylamino)-4-methanesulfinyl-butyldisulfanyl methyl]-cyclopentanecarbonyl}-amino)-succinic acid (**2g**)

*4.2.3.1. Synthesis of (S)-1-(((2-(tert-Butoxycarbonylamino)-4-(methylthio)butyl)disulfanyl) methyl) cyclopentanecarboxylic acid (**6**).* 1-(Mercaptomethyl)cyclopentanecarboxylic acid **5** was synthesized as described [33].

A mixture of MeOH (19 mL) and THF (19 mL) was cooled at 4 °C, under inert atmosphere. Then chlorosulfonylchloride (1.11 mL, 13.25 mmol, 1.06 eq) was added dropwise. The reaction mixture was stirred for 15 min at 4 °C to give the methoxycarbonylsulfenyl chloride. Compound **4** (13.25 mmol, 1.06 eq) in 12 mL THF/MeOH was added once. The reaction mixture was warmed to room temperature and was stirred for 30 min. The previous solution was added dropwise to a solution of compound **14** (14.02 mmol, 1eq) in 100 mL of degassed CH_2Cl_2 in presence of Et_3N (1eq). The reaction mixture was stirred for 1 h at room temperature. The solvent was removed under reduced pressure and the product was portioned in CH_2Cl_2 and 10% citric acid solution. The organic layer was washed with brine and dried over Na_2SO_4 to give a crude product, which is purified on silica gel with CHex/AcOEt 8/2 then 6/4 as eluent.

White solid (3.8 g; yield: 75%); RMN ($CDCl_3$) δ: 1.40 (9H, s), 1.40–1.80 (10H, m), 1.90 (3H, s), 2.38 (2H, m), 2.75 (2H, d), 3.15 (2H, d), 4.30 (1H, m), 4.70 (1H, m); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 50-50: 31.6 min; ESI(+): $[M + Na]^+ = 432$.

*4.2.3.2. Synthesis of (S)-di-tert-butyl 2-(1-((((S)-2-(tert-butoxycarbonylamino)-4-(methylthio)butyl)disulfanyl)methyl)cyclopentanecarboxamido)succinate (**7**).* The aspartic acid di-tert-butyl ester was coupled using TBTU (1.2 eq) and DIEA (3eq) as reagent in DMF as solvent.

Solid (yield: 77.5%); RMN (DMSO d6) δ: 1.40–1.50 (27H, m), 1.40–1.80 (10H, m), 1.90 (3H, s), 2.40 (2H, m), 2.80 (2H, m), 3.10 (2H, d), 3.75 (1H, m), 4.45 (1H, m), 6.80 (1H, d); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 80-20: 15.6 min; ESI(+): $[M + H]^+ = 637$.

*4.2.3.3. Oxidation of the methionine, synthesis of (2S)-di-tert-butyl 2-(1-((((2S)-2-(tert-butoxycarbonylamino)-4-(methylsulfinyl)butyl) disulfanyl)methyl)cyclopentanecarboxamido) succinate (**8**).* Compound **7** was solubilized in EtOH (41 mL) and cooled to 0 °C. $NaIO_4$ (0.2 M in water) (33 mL) was added. The reaction became trouble with pale yellow color. The reaction mixture was concentrated under reduced pressure and portioned in $AcOEt/H_2O$ (50 mL/50 mL). The organic layer was washed with brine (50 mL), dried over Na_2SO_4 and concentrated under reduced pressure to give a crude product which was purified with semi-preparative HPLC yielding a white solid (94.9%).

HPLC Kromasil C18, 5 μm, 100 Å, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 80-20: 5.68 min.

$$ESI(+): [M + H]^+ = 653$$

*4.2.3.4. Synthesis of 2-({1-[2-(1-Isobutyryloxy-ethoxycarbonylamino)-4-methanesulfinyl-butyldisulfanyl methyl]-cyclopentanecarbonyl}-amino)-succinic acid (**2g**).* Compound **8** (3.12 mmol) was solubilized in CH_2Cl_2 (20 mL) and TFA (20 mL) was added at 0 °C. The reaction mixture was stirred for 3 h at 0 °C and concentrated under reduced pressure.

The previous compound (3.12 mmol) was solubilized in CH_2Cl_2 (30 mL) and then N-isopropylcarbonyloxyethoxy carbonyl derivative (1.5eq) was added, followed by DIEA (3eq, 3.14 mL). The reaction mixture was stirred 1 h at room temperature and the solvent was removed under reduced pressure. The crude mixture was portioned in AcOEt-10% citric acid solution (50 mL/50 mL). The organic layer was washed by water (50 mL), brine (50 mL) and dried over Na_2SO_4. The crude compound **2**g was purified by semi-preparative HPLC on Kromasil C18 column, 5 μm, 100 Å, 250 × 21.2 mm using CH_3CN/H_2O (0.1% TFA) 30–70 as elution system.

Solid (yield: 22.3%); RMN (DMSOd6) δ: 1.00 (2 × 3H, d), 1.40 (3H, d), 1.70–1.90 (8H, m), 2.10 (2H, m), 2.50–3.00 (12H, m), 3.70 (1H, m), 4.60 (1H, m), 6.65 (1H, m), 7.30 (5H, m), 7.50 (1H, d), 8.40 (1H, m); HPLC Kromasil C18, 100 Å, 5 μm, 250 × 4.6 mm, CH_3CN/H_2O (0.1% TFA) 10–90 in 30 min: 19.35 min; ESI(+): $[M + H]^+ = 598.9$.

4.3. Biochemistry

Ki values on neprilysin (NEP) and aminopeptidase N (APN) were determined as described by Poras et al. [22] (Table 1).

4.4. Biotransformation of pro-drugs in plasma

In vitro biotransformation of pro-drugs were monitored by LC/MS, in triplicate. All compounds (800 μM final concentration) were incubated for 1, 5, 15, 30 and 60 min at 37 °C in the presence of commercial Sprague Dawley male rat plasma (heparin lithium) PR45.03.11 [66 mg protein/mL plasma] from Charles River. The reaction was stopped by addition of absolute EtOH (80% of final volume). The mixture was vigorously stirred and then kept at 0 °C for 1 h to extract products adsorbed on proteins. The suspension was centrifuged and then supernatant was analyzed by LC/MS and compared to a blank assay and to standard concentration.

4.5. Animals

Male OF1 mice (Charles River Laboratories, France), weighing approximately 20–25 g (or 16–18 g for PSNI studies) were housed for at least two days before experiments in a room with controlled temperature (21 ± 2 °C) under a 12 h light/dark cycle. Food and water were provided ad libitum. Animal experiments were carried out in accordance with the European Communities Council Directive (89/609/CEE) and in accordance with the ethical guidelines of International Association of Pain.

4.6. Pharmacological assays

4.6.1. Hot plate test

A glass cylinder (16 × 16 cm) was used to keep the mouse on the heated surface of the plate, which was kept at a temperature of 52 ± 1 °C using a thermo-regulated water-circulating pump. Jump latency was registered by a stop-watch and cut-off time was set at 240 s. Results are expressed as jump latency in seconds.

4.6.2. Formalin test

OF1 mice (Charles River Laboratories, France), 25–32 g were used in this study. Animals were housed in a temperature (19–24 °C) controlled room with a relative humidity (25–50%), a 12 h light/dark cycle, with ad libitum access to standard pelleted laboratory food and water throughout the study. Animals were housed 8 per cage and acclimatization period were observed (2 days at least). Each mouse was identified by body markings. The test adapted from Hunskaar et al. [36] was carried out in a glass cylinder chamber (16 cm diameter). The mice were placed in the test chamber for 30 min. After this period of adaptation, 20 μL of 5% formalin was injected *s.c.* into the dorsal surface of the right hindpaw of the mouse, using a 26-gauge needle connected to a Hamilton micro-syringe. Each mouse was immediately returned to the observation chamber after injection and its nociceptive response was recorded. After formalin injection, mice were then observed for licking behavior of their injected hindpaw in translucent plastic observation chambers. The time spend in licking the injected paw was counted continuously every 5 min, starting immediately after the formalin injection for 35 min. Recording of the early response (early phase) started immediately and lasted 5 min (0–5 min) and recording of the late response (late phase) started 15 min after formalin injection and lasted for 15 min (15–30 min). In both phases only licking or biting of the injected hindpaw was defined as a nociceptive response and the total duration of the response was defined by means of a stop-watch. Results, expressed as licking time (seconds), were analysed with ANOVA, followed by the Dunnett's t-test.

4.6.3. Partial sciatic nerve injury

A partial ligation of the sciatic nerve at mid-thigh level was used to induce neuropathic pain. Briefly, mice were anaesthetized with isoflurane (induction 4%, surgery, 1.5%) and the common sciatic nerve was exposed at the level of the mid-thigh of the right hindpaw. At 1 cm proximally to the nerve trifurcation, a tight ligation was created around 33–50% of the sciatic nerve using 8-0 18 inch non-absorbable virgin silk suture (Alcon® France), leaving the rest of the nerve “uninjured”. The muscle was then stitched and the incision was closed with wound clips. Control animals (sham-operated mice) underwent the same surgical procedure except that

the sciatic nerve was not ligated.

Mice were habituated to the experimental environment during two consecutive days. After habituation period, responses to von Frey filament stimulation or plantar test were measured on two consecutive days to obtain baseline values. One day after baseline measurement, surgery was carried out to generate nerve injury (day 0). Tactile and thermal thresholds were measured twice in the week following surgery to observe modification in tactile and heat sensitivity on the ipsilateral paw. Mice were treated in successive experimental sessions performed between day 10 and day 18 after surgery. Neuropathic pain evaluation was done on consecutive test sessions following a Latin square design. In the morning of each test session, before test, mechanical or thermal threshold levels were measured in order to ensure that previous injection did not influence the daily test session.

4.6.4. General mechanical allodynia: von Frey test

Mice were placed individually into a cylinder disposed on a framed metal mesh floor. Mice were allowed to acclimate for 2 h before testing. Mechanical allodynia was quantified by measuring the hindpaw withdrawal response to von Frey filament stimulation (Bioseb, France). The filament strength of 0.4 g was first used. Then, the strength of the next filament was decreased when the animal withdrew its paw or increased when the animal did not respond. This up-down procedure was stopped four measures after the first change in animal responding. Mechanical threshold was calculated by using the up-down Excel program generously provided by the Basbaum's laboratory (UCSF, San Francisco, USA). Only clear paw withdrawal, shaking or licking were considered as nociceptive-like response. Both ipsilateral and contralateral hindpaws were tested. As example, the application of von Frey filament on hindpaw, 13 days after surgery on ipsilateral side, evidenced a dramatic decrease of paw withdrawal threshold values (0.24 ± 0.04 g), compared to contralateral side values (1.38 ± 0.05 g) or values before ligature (1.42 ± 0.04 g).

4.6.5. Hyperalgesia to noxious thermal stimulus: plantar test

Mice were placed individually into a cylinder disposed on the plantar test apparatus (Bioseb, France) and paw withdrawal latency in response to a radiant heat stimulus was measured. The intensity of the noxious stimulus was calibrated to obtain paw withdrawal latencies in a range of 8–10 s in mice. A cut-off time of 15 s was used to prevent tissue damage. The mean paw withdrawal latencies for the ipsilateral and contralateral hindpaws were determined from the average of three separate trials, taken at 5 min intervals, to prevent thermal sensitization and behavioral disturbances.

Author contributions

HP was in charge of compounds synthesis. EB was in charge of pharmacological assays. The manuscript was written through contributions of all authors. All authors have given approval of the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.07.027.

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