PRP Pharmacology Research & Perspectives
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ORIGINAL ARTICLE

Long-lasting oral analgesic effects of *N*-protected aminophosphinic dual ENKephalinase inhibitors (DENKIs) in peripherally controlled pain

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Keywords
Enkephalinase inhibitors, enkephalin-gabapentin analgesic synergy, enkephalins, inflammation, neuropathic pain, peripherally controlled pain, pharmacological assay, physiological analgesia

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Funding Information
No funding information provided.

Received: 12 September 2014; Revised: 14 November 2014; Accepted: 20 November 2014

Pharma Res Per, 3(2), 2015, e00116, doi: 10.1002/prp2.116

doi: 10.1002/prp2.116

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Abstract

The peripheral endogenous opioid system is critically involved in neuropathic and inflammatory pain generation as suggested by the modulation of opioid receptors expression and enkephalins (ENKs) release observed in these painful conditions. Accordingly, an innovative approach in the treatment of these nocifensive events is to increase and maintain high local concentrations of extracellular pain-evoked ENKs, by preventing their physiological enzymatic inactivation by two Zn metallopeptidases, the neutral endopeptidase (NEP, neprilysin, EC 3.4.24.11) and the neutral aminopeptidase (APN, EC 3.4.11.2). With this aim, new orally active dual ENKephalinase inhibitors (DENKIs) were designed as soluble prodrugs by introducing a N-terminal cleavable carbamate in the previously described aminophosphinic inhibitors. This induces long-lasting antinociceptive responses after oral administration, in various rodent models of inflammatory and neuropathic pain. These responses are mediated through stimulation of peripheral opioid receptors by DENKIs-protected ENKs as demonstrated by naloxone methiodide reversion. In all tested models, the most efficient prodrug **2a** (PL265) was active, at least during 150–180 min, after single oral administration of 25–50 mg/kg in mice and of 100–200 mg/kg in rats. In models of neuropathic pain, both hyperalgesia and allodynia were markedly reduced. Interestingly, combination of inactive doses of **2a** (PL265) and of the anti-epileptic drug gabapentin had synergistic effect on neuropathic pain. Pharmacokinetic studies of **2a** (PL265) in rats show that the active drug is the only generated metabolite produced. These encouraging results have made **2a** (PL265) a suitable candidate for clinical development.

Abbreviations

APN, aminopeptidase N; BBB, blood brain barrier; CCI, chronic constrictive injury; CFA, complete Freund's adjuvant; DENKI, dual ENKephalinase inhibitor; DIEA, diisopropylethylamine; DOR, delta opioid receptor; DRG, dorsal root ganglion; ENKs, enkephalins; ESI, electro spray ionization; HPLC, high-performance liquid chromatography; HPT, hot plate test; i.v., intravenous; LC/MS, liquid chromatography/mass spectroscopy; Met-thiol, methionine thiol; MOR, mu opioid receptor; MPE, maximal possible effect; NEP, neprilysin; NHS, *N*-hydroxysuccinimide; Nlxe-Met, naloxone methiodide; Nlxe, naloxone; NMR, nuclear magnetic resonance; p.o., per os; PSNL, partial sciatic nerve ligation; PWL, paw withdrawal latencies; TLC, thin layer chromatography.

Introduction

Chronic pain, which includes neuropathic and inflammatory pain, can occur after various patho-physiological processes (of known or unknown origins) and is unsatisfactorily treated by the classical analgesic approaches. Thus, morphine and surrogates are partially effective (Rashid et al. 2004) and tricyclic antidepressants or anticonvulsants (such as gabapentin or pregabalin) have mediocre efficacy and tolerability (Offenbaecher and Ackenheil 2005).

On the other hand, many studies strongly support a critical role of the endogenous peripheral opioid system in the modulation of neuropathic and inflammatory pain (Przewlocki et al. 1992; Hassan et al. 1993; Maldonado et al. 1994).

These nocisponsive events, triggered by a noxious stimulation of nociceptors present on primary afferent nerve endings in skin, joints, muscles, and viscera, could be reduced at their origin by enhancing the extracellular concentrations of the endogenous opioids enkephalins (ENKs) (Maldonado et al. 1994; Stein et al. 2003; Tegeder et al. 2003). Nerve inflammation or injury increases local concentrations of ENKs via various mechanisms, such as migration of enkephalin-containing immune cells toward the injured site (Hassan et al. 1993) and enkephalin release from lymphocytes activated by inflammatory substances (chemokines, interleukins, leukotrienes such as LTB4, etc. (Machelska 2007), from inflamed keratinocytes (Gabrilovac et al. 2004), or from stimulated nerve fibers (Hassan et al. 1993; Rittner et al. 2001). Concomitantly, during inflammation and nerve injury, an upregulation of opioid receptors also occurs in the dorsal root ganglion (DRG) followed by their efficient transport to peripheral nerve endings (Hassan et al. 1993). In chronic constrictive injury (CCI) of the sciatic nerve, widely used as a rodent model of neuropathic pain, the amount of opioid receptors is also increased, on both sides of the nerve injury through neuromas which are expansions of nerve tissue (Truong et al. 2003), very painful when occurring in humans.

Accordingly, a promising approach in the treatment of chronic pain is to mobilize the endogenous opioid system by increasing local concentrations of extracellular ENKs at injury site. This was easily achieved by blocking two enzymatic activities accountable for physiological inactivation of ENKs, namely neprilysin (NEP, EC 3.4.24.11) (Roques et al. 1993) and aminopeptidase N (APN, EC 3.4.11.2) (Carenzi et al. 1983; Waksman et al. 1985; Roques et al. 1993). The dual inhibition of these two peptidases (Fournié-Zaluski et al. 1984), by dual ENKephalinase inhibitors (DENKIs), was shown to fully protect in vitro and in vivo the ENKs (Waksman et al. 1985; Bourgoin et al. 1986) and to induce in vivo antinociceptive responses associated with the activation of opioids receptors by endogenous ENKs selectively released in the painful area (Cesselin et al. 1982; Bourgoin et al. 1986; Dauge et al. 1996; Le Guen et al. 2003; Noble and Roques 2007; Roques et al. 2012). Another advantage of this approach is to avoid the severe or unpleasant side-effects of exogenous opiates. As previously demonstrated in mice chronically treated by another DENKI, RB101 (Fournié-Zaluski et al. 1992; Roques et al. 2012).

Two series of DENKIs endowed with antinociceptive properties in various animal models of pain after i.v. administration have been described: the first series comprised ester prodrugs of compounds combining through a disulfide bond a selective APN and a NEP inhibitor (Fournié-Zaluski et al. 1992; Noble et al. 1992, 1997; Poras et al. 2014), which are enzymatically released to interact with their own target according to their proper pharmacokinetics. A second series of DENKIs was made of esters prodrugs of truly dual aminophosphinic compounds inhibiting both APN and NEP with nanomolar affinities (Chen et al. 2000). In this case, only a single molecule was designed to interact with the two enzymatic targets, with a unique pharmacokinetics, suggesting a longer duration of action. The most potent inhibitor of this second series, RB3007, was esterified on both the carboxylate and the phosphinic groups (Chen et al. 2001). This series of DENKIs was poorly soluble in aqueous medium and was active only after intravenous administration, on centrally controlled nociceptive stimuli (Chen et al. 2001).

With the aim of increasing the oral bioavailability of these latter DENKIs, new prodrugs, with in vivo cleavable carbamate (Alexander et al. 1988) as N-terminal protection and containing or not a C-terminal ester were synthesized (Fig. 1) and tested for their ability to alleviate or reduce centrally or peripherally controlled nociceptive stimuli. The most efficient prodrug of this series, compound **2a**, PL265, was assessed in various animal models of inflammation and neuropathy.

Materials and Methods

Synthesis

The procedure for the synthesis of *N*-isopropylcarbonyloxyethyloxycarbamate prodrugs **2a**–**2g** (Fig. 1, Table 1) is described in Data S1.

Prodrugs bioactivation in vitro and in vivo

In vitro bioactivation of prodrugs was monitored, in triplicate, by liquid chromatography/mass spectroscopy

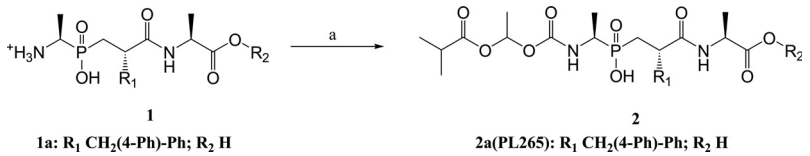
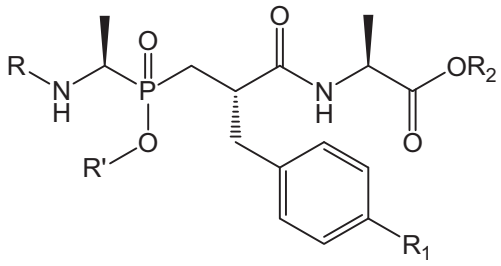
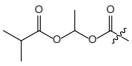
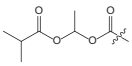
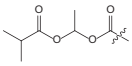
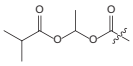
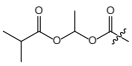
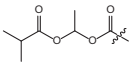
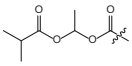
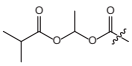
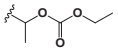


Figure 1. Synthesis of acyloxyalkylcarbamate amino-phosphinic prodrugs **2**. Reagents and conditions: (a) $(CH_3)_2CHCO_2CH(CH_3)OCO$-NHS, DIEA, CH_2Cl_2, 0°C then RT, 5 h.

Table 1. Antinociceptive effects of oral administration of DENKIs (50 mg/kg) measured during the phase 1 (0–5 min) of the formalin test in mice.



					% of control		
					25 mg/kg	50 mg/kg	
Compound	*R*	*R′*	R_1	R_2	90 min	90 min	150 min
RB3007					nd	nd	nd
1a	H	H	Ph	H	nd	92 ± 9 ^{ns}	nd
2a		H	Ph	H	63 ± 6**	70 ± 6**	69 ± 7**
2b		H	Br	H	nd	80 ± 6*	70 ± 7*
2c		H	Ph		nd	68 ± 6**	72 ± 6*
2d		H	Ph	CH_2Ph	60 ± 6**	67 ± 6***	73 ± 4*
2e		H	Ph	CH_2CH_3	nd	70 ± 4**	88 ± 8^{ns}
2f		H	Br	CH_2Ph	nd	69 ± 3***	71 ± 6***
2g			Ph	H	nd	95 ± 5 ^{ns}	60 ± 4***

DENKIs, dual ENKephalinase inhibitor; Nd, not determined; ns, not significant.
*P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle, two-way ANOVA followed by Bonferroni's test.

(LC/MS) in plasma. The results were expressed in percentage of the initial standard concentration (Fig. 2A and B).

In vivo bioactivation of **2a** in mice after oral administration was monitored by LC/MS/MS in plasma (Fig. 2C).

Pharmacological studies

Animals

Experiments were performed on adult male OF1 mice 22–30 g (Charles River Laboratories, L'Arbresle, France) and male Sprague–Dawley rats 220–300 g (Janvier, Le Genest,

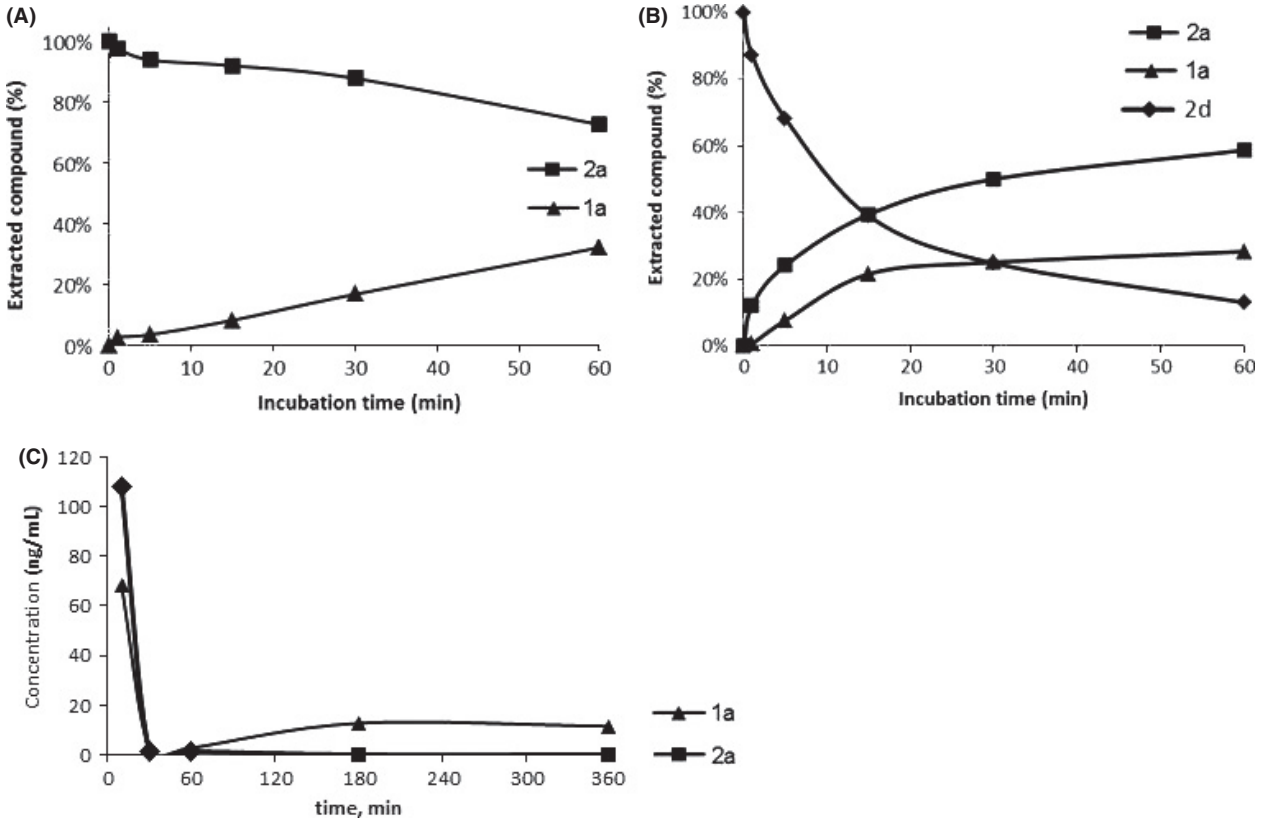


Figure 2. Biological transformation in 1a (▲) of 800 μmol/L of 2a (■) (A) and 2d (♦) (B) in male rat plasma (66 mg protein/mL) over 1 h, monitored in triplicate by LC-MS. (C) Pharmacokinetics hydrolysis in 1a (▲) in mice after oral administration of compound 2a (■) (20 mg/kg) in methyl cellulose (0.5%), monitored by LC-MS-MS. LC-MS, liquid chromatography-mass spectroscopy.

France). Animals were housed for at least 2 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 agreement with the European Communities Council Directive (89/609/CEE) and in accordance with the ethical guidelines of International Association of Pain.

Hot plate test in mice

The test was based on that described in literature (Eddy and Leimbach 1953). Animals were placed into a glass cylinder on a heated surface maintained at 52°C ± 0.1°C and jump latency in seconds was recorded. Cut-off time was set to 240 sec. Each mouse was tested once. Data are expressed as the maximal possible effect (% MPE) calculated as: % MPE = 100 × [(drug latency − vehicle latency)/(cutoff time − vehicle latency)].

Formalin test in mice

The formalin test, adapted from literature (Hunskaar et al. 1985), was carried out in a clear plastic box with a mirror placed behind it to allow unobstructed observations of animals. Mice were injected with formalin (5% in 20 μL saline, s.c.) into the plantar right hindpaw and placed immediately into the observation chamber for nocifensive responses observation (time spent licking and biting) during the early phase (0–5 min) and the late phase (10–35 min) of the test. DENKIs or vehicle were orally given 60, 90, or 150 min before formalin injection.

Carrageenan-induced inflammatory pain

Animals were habituated for 20 min per day to the testing environment of the thermal hyperalgesia paradigm during 1 week. At the end of the habituation period, baseline responses were established for two consecutive days. For carrageenan-induced inflammatory pain, acute inflammation was induced by an injection of a λ-carrageenan solution (1% in 150 μL saline, s.c.) into the plantar surface of the right hindpaw (day 1) under isoflurane anesthesia. Compound **2a** or vehicle was given by oral route, 3 h after carrageenan injection. The thermal nociceptive threshold was measured before (t0) and 40 min after oral administration.

CFA-induced inflammatory pain

For complete Freund's adjuvant-induced hyperalgesia in rat, inflammation was produced by a s.c. injection, on day 1 of 150 μL of a 1 mg/mL dose of heat-killed and dried *Mycobacterium butyricum* (CFA; Calbiochem, Saint-Quentin Fallavier, France) into the plantar surface of the right hindpaw (ipsilateral side), under isoflurane anesthesia. The thermal nociceptive threshold was measured on day 5 (96 h post-CFA injection) before (t0) and 45, 90, 120, and 180 min after oral administration of compound **2a** or vehicle.

Kaolin-induced arthritis in rats

Gait score was determined in naive rats before arthritis induction (Hertz et al. 1980). Animals were placed into the observation chamber during 30 sec and discomfort was evaluated for each animal according to a score (0–3) attributed to the posture of the painful leg as follow: *score 0*: normal gait, *score 1*: mid disability, *score 2*: intermittent rising of the paw, *score 3*: elevated paw. Then, arthritis was induced by an intra-articular injection (100 μL) of 10% kaolin (Sigma, Saint-Quentin Fallavier, France) suspension (w/v, saline) into the knee joint of the right hindpaw under isoflurane anesthesia. Compound **2a** or vehicle was injected by intravenous route, 3 h after kaolin injection. The gait score was measured before (t0) and 30, 60, 120, and 240 min after compound **2a** or vehicle injection.

Partial Sciatic Nerve Injury in mice and rats

Animals were habituated to the testing environment during two consecutive days for 2 h. After the habituation period, baseline responses were measured during two consecutive days. One day after baseline measurements, sciatic nerve surgery was carried out (day 0). For experiments conducted in mice, tactile and thermal nociceptive thresholds were evaluated by von Frey and plantar tests on days 6 and 8 to observe modification in tactile and heat sensitivity on the ipsilateral paw.

Then, compound **2a** or vehicle was given orally by gavage and paw withdrawal threshold was measured before (t0) and 45, 90, and 150 min after oral administration according to a Latin square design. Experiments were conducted in test sessions performed between day 10 and day 18 after surgery. Mechanical thresholds were evaluated in mice in each test session before drug administration to ensure that sensitive thresholds were not influenced by previous treatments. In rats' studies, the effects of acute administration of compound **2a** or vehicle were evaluated on the expression of neuropathic pain on days 8–18 after surgery. Postsurgery responses of rats were obtained after vehicle administration on day 8 after surgery. The effects of a single oral dose of compound **2a** (100 mg/kg) were measured 30, 60, 90, 120, and 150 min after administration on days 12–15 postsurgery, according to a Latin square design. Finally, rats were treated by vehicle on day 18 postsurgery and the responses were evaluated (posttreatment values) as an internal control to ensure that sensitive thresholds were not influenced by previous treatments. Nociceptive thresholds were evaluated on ipsilateral and contralateral sides.

CCI model in rats

Animals were habituated to the testing environment during three consecutive days for 2 h. Then rats were evaluated for mechanical sensitivity to von Frey filaments application (presurgery thresholds). One day after baseline measurements, sciatic nerve surgery was carried out (day 0). Postsurgery responses of rats in the von Frey test were obtained on day 12 after surgery. Then, mechanical allodynia was assessed before (t0), 30 and 60 min after i.v. injection of compound **2a** or vehicle, on days 14–16 postsurgery according to a Latin square design. Mechanical thresholds were evaluated in rats in each test session before drug administration to ensure that sensitive thresholds were not influenced by previous treatments. Nociceptive thresholds were evaluated on ipsilateral and contralateral sides.

Statistical analyses

Student's *t*-test or ANOVA were used for comparison of multiple groups with Bonferroni's post hoc analysis to determine statistical significant difference between groups. The corresponding nonparametric analyses were used when data were not normally distributed, Kruskal–Wallis test with Mann–Whitney's or Wilcoxon's post hoc analyses. The level of significance was set at $P < 0.05$.

Results

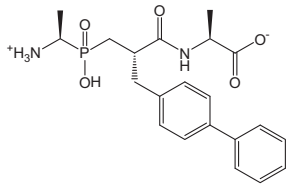
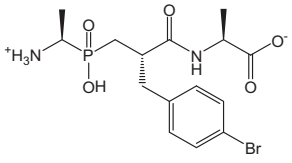
Synthesis of carbamate prodrugs

The prodrugs **2a**–**2f** (Table 1), protected on the N-terminal position, were obtained by reaction of **1a**–**1f** with 1-((2, 5-dioxopyrrolidin-1-yloxy) carbonyloxy)ethyl isobutyrate (Cundy et al. 2004) in CH_2Cl_2 in presence of Et_3N (Fig. 1).

The prodrug **2g**, protected on the N-terminal and P positions, was obtained by protection of the phosphinic acid function of **2d** with Isobutyric 1-chloroethylic ester, followed by the benzyl ester deprotection.

Due to the presence of two free acid functions in compounds **2a** and **2b**, monosodium (carboxylate) or disodium (carboxylate and phosphonate) salts were prepared by

Table 2. Inhibitory potencies of inhibitors on NEP, APN, and LTA4H.

Compound	Structure	Ki NEP (nmol/L)	Ki APN (nmol/L)	Ki LTA4H (nmol/L)
1a		1.4 ± 0.2	2.9 ± 0.3	5.4 ± 0.3
1b		5.0 ± 0.5	1.9 ± 0.2	8.6 ± 0.2

NEP, neprilysin; APN, aminopeptidase N.

reaction of the respective acids with one or two equivalents of $NaHCO_3$ in CH_3CN/H_2O followed by freeze-drying process. The sodium salts were highly soluble in aqueous medium: the solubility of **2a** in water is of around 5 mg/mL, but rises to around 100 mg/mL for the monosodium salt and above 500 mg/mL for the disodium salt.

Inhibitory potencies

The inhibitory activities of drugs **1a** and **b** on both NEP and APN have been previously published (Chen et al. 2000) and are reported in Table 2. Moreover, the inhibitory potency of drug **1a** which binds to LTA4 hydrolase (Tholander et al. 2008) and inhibits the formation of LTB4 was performed as described (Poras et al. 2013) with their Ki reported in Table 2.

Prodrugs 2a and 2d bioactivation in plasma

Both compounds were incubated at a final concentration of 800 μmol/L in rat plasma (66 mg of protein/mL) at 37°C (Fig. 2).

Compound **2a** undergoes a slow hydrolysis (30% in 1 h) of the carbamate moiety generating the NEP/APN inhibitor **1a** (Fig. 2A).

Compound **2d**, which possesses a benzyl ester and an acyloxyalkyl carbamate moiety, showed a rapid hydrolysis of the benzyl ester, leading to **2a** with 10% remaining compound **2d** after 1 h (Fig. 2B). Concomitantly, the *N*-protection of the intermediate **2a** was hydrolyzed, generating compound **1a**, with a similar activation profile as observed in Figure 2A.

After 1 h, in both cases, the proportion of the active dual NEP/APN inhibitor **1a** was around 30%.

Pharmacokinetics of 2a after oral administration in mice

The experiments were carried out by sampling blood from 10 male OF1 mice after a single oral administration of compound **2a** (disodium salt), 20 mg/kg, in 0.5% methyl cellulose in water. Two mice were sampled at each time point: 10, 30, 60, 180, and 360 min. Measurements of compound **2a** plasma levels showed a rapid hydrolysis of the carbamate moiety leading only to the drug **1a** (Fig. 2C).

Antinociceptive responses in the hot plate test in mice

A preliminary assay was performed to compare the drug **1a** and its corresponding prodrug **2a** after i.v. administration. The time-course of their responses was measured at the same concentration (28.4 μmol/kg) (**1a** 15 mg/kg; **2a** 17.5 mg/kg) after solubilization in 0.9% NaCl (Fig. 3A). The two curves were almost superimposable until 60 min with around 50% analgesia. Then at 90 min, the response to **1a** decreased significantly, while that of **2a** remained unchanged.

In the same solvent, a dose–response curve (4, 8 and 16 mg/kg) of **2a** was determined and the % of analgesia calculated 20 min after i.v. injection (Fig. 3B). The same antinociceptive effects (~50% analgesia) were obtained at 8 and 16 mg/kg and similar responses were observed when a higher dose (50 mg/kg) was injected, showing a ceiling effect. This response was completely reversed by administration of naloxone (Nlxe), demonstrating the selective involvement of opioid receptors (Fig. 3C).

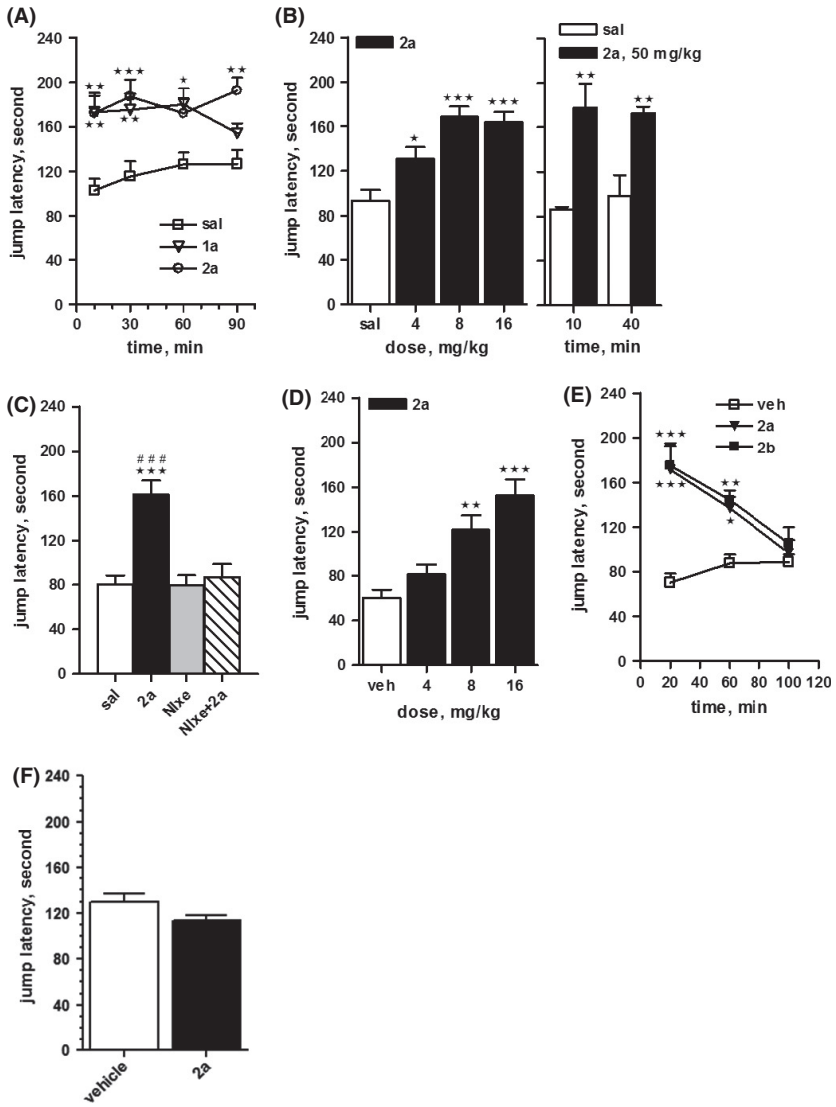


Figure 3. Antinociceptive effects of DENKIs were evaluated after intravenous injection in the hot plate test in mice. (A) Antinociceptive effects of the prodrug 2a (○) and its corresponding drug 1a were evaluated (jump latency) after i.v. injection of an equimolar dose (28 μmol/kg, which corresponds to 15 mg/kg of compound 2a) for 90 min (n = 6–9 per group). (B) Compound 2a was injected at different doses in saline vehicle 20 min before jump latency measurement (n = 8–10 per group). (C) Compound 2a injected by i.v. route at the dose of 16 mg/kg induced significant antinociceptive responses that were fully reversed by coinjection of naloxone (0.5 mg/kg i.p. in saline, n = 8 per group). (D) Jump latency was measured in mice 20 min after i.v. injection of different doses of compound 2a solubilized in EtOH/Tween80/H_2O (1/1/8) vehicle (n = 7–9 per group). (E) Time-course of compounds 2a (▼) and 2b (■), 15 mg/kg, solubilized in EtOH/Tween80/H_2O 1/1/8 (n = 7–8 per group). Values are mean ± SEM. ★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.001$ versus vehicle; ###$P < 0.001$ versus treated group, one-way ANOVA (two-way ANOVA in B and E) followed by Bonferroni's test, n = 6–10 per group. (F) Antinociceptive effect of the prodrug 2a was evaluated 90 min after oral administration (50 mg/kg of compound 2a) by jump latency measurement (n = 7 per group). DENKIs, dual ENKephalinase inhibitor; i.v., intravenous.

Further studies were performed in another vehicle, EtOH/Tween 80/H_2O (10/10/80), which is described to enhance BBB crossing of small molecules (Pardridge 2005). The dose–effect curve obtained with **2a** (Fig. 3D) did not show any improvement of antinociceptive responses as compared to the assay in saline (Fig. 3B).

To complete this study, the time-courses of two prodrugs **2a** and **2b** were compared (20, 60, and 100 min) at the same dose (15 mg/kg) in EtOH/Tween 80/H_2O (10/10/80). Similar profiles (Fig. 3E) were obtained for both compounds with activity until 60 min disappearing at 100 min, showing a shorter time-course as compared to

saline (Fig. 3A) where compound **2a**-induced analgesia after 60 min (~50%).

Interestingly, when administered by oral route, these prodrugs were completely inactive in the hot plate test in mice (Fig. 3F).

Antinociceptive responses in the formalin test

Antinociceptive responses of compound **2a** (50 mg/kg, p.o.), in EtOH/0.5% methylcellulose in H_2O (1.5/98.5) as vehicle, were assessed 60 min after administration, in the two phases of the formalin test in mice (Fig. 4): 30% inhibition of the nocifensive response in the early phase (Phase 1, Fig. 4A) and 50% inhibition in the late phase (Phase 2, Fig. 4B) were observed. Moreover, the specific involvement of peripheral opioid receptors in this test was demonstrated by the reversion of the antinociceptive response with naloxone methiodide (Nlxe-Met) (2 mg/kg, s.c.), an opioid receptor antagonist that does not cross the blood brain barrier at this dose (Bianchi et al. 1982) (Fig. 4C and D).

Then, prodrugs **2a** to **2f** were compared in the early phase of the formalin test at the same dose (50 mg/kg, p.o.) at 90 and 150 min after administration (Table 1). No significant differences were observed within *N*-protected aminophosphinic prodrugs, which induced moderate (around 30%), but long-lasting antinociceptive responses. Moreover, the inhibition of licking observed 90 min after administration of 25 mg/kg of compounds **2a** and **2d** was similar, showing here again, a ceiling effect for this series of inhibitors (Table 1).

Prodrug **2g**, with an *N*- and a phosphinic acid group protection, showed a delayed analgesic response until 90 min and 40% analgesia at 150 min (Table 1).

Carrageenan- and CFA-induced thermal hyperalgesia

Carrageenan model is useful in investigating acute inflammatory pain, whereas CFA is used to mimic chronic inflammatory pain conditions (Gregory et al. 2013).

In rats receiving local carrageenan, paw withdrawal latencies (PWL) to thermal noxious stimulus (plantar test) were strongly decreased to a mean PWL threshold of 1.8 ± 0.2 and 2.9 ± 0.4 sec on ipsilateral side in vehicle and treated groups (t0, Fig. 5A). Contralateral side displayed a heat-sensitive threshold (8.5 ± 0.2 sec) similar to that observed before carrageenan injection (8.5 ± 0.2 sec) and remained constant along the experiment. Compound **2a** (100 mg/kg, p.o.) elicited a significant increase in the PWL threshold on the ipsilateral side (5.3 ± 0.8 sec), 40 min after administration, without any

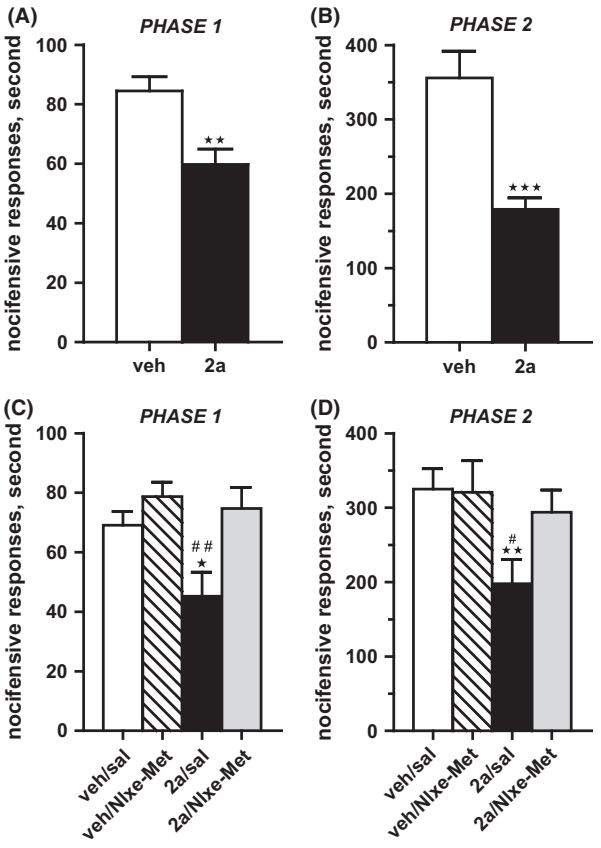


Figure 4. Oral administration of compound 2a inhibited formalin-induced nocifensive responses in mice. (A, B) Nocifencive responses (licking and biting) induced by subcutaneous injection of formalin (5%, in 20 μL saline) in the plantar hindpaw were reduced in mice pretreated with compound 2a (50 mg/kg p.o.) in a vehicle EtOH/0.5% methylcellulose in H_2O (1.5/98.5), 60 min before formalin (n = 7–8 per group). Antinociceptive responses were observed on formalin-induced phase 1 (0–5 min) (A) and phase 2 (10–35 min) (B). (C, D) Inhibition of nocifensive responses by compound 2a was fully reversed in mice pretreated with naloxone methiodide (Nlxe-Met) (2 mg/kg, s.c.). Compound 2a (50 mg/kg) and vehicle were administrated by gavage 60 min before formalin. Nlxe-Met was injected 30 or 5 min before formalin, according to phase 1 (C) or phase 2 (D) observations, respectively (n = 8–10 per group). Values are mean ± SEM. ★★$P < 0.01$, ★★★$P < 0.001$ versus vehicle, unpaired t-test, ★$P < 0.05$, ★★$P < 0.01$ versus vehicle, #$P < 0.05$, ##$P < 0.01$ versus treated group, one-way ANOVA followed by Bonferroni's test.

modification of the contralateral side threshold (Fig. 5A), suggesting that antihyperalgesic effects of compound **2a** observed at this dose were mainly produced by recruitment of peripheral opioid receptors.

In the CFA-induced inflammatory pain assay, thermal hyperalgesia was established 96 h after CFA injection and rats displayed a highly decreased PWL threshold on the ipsilateral side (4.7 ± 0.2 sec) significantly different from PWL threshold on the contralateral side (9.5 ± 0.8 sec)

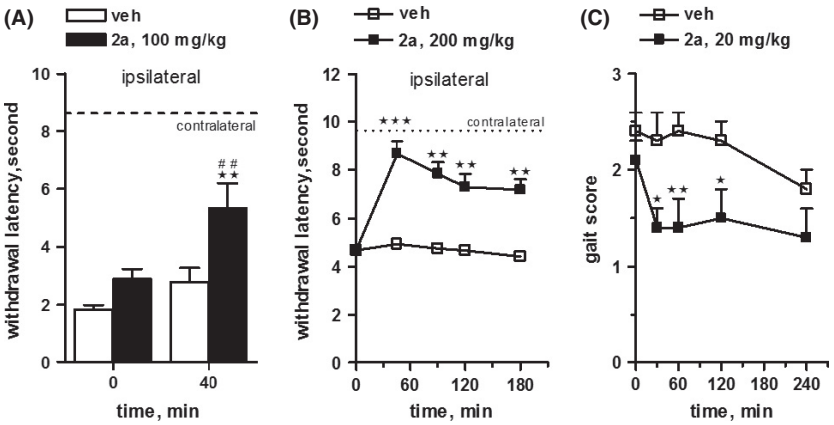


Figure 5. Effect of compound **2a** in inflammatory- and knee joint arthritis-induced pain models in rats. Thermal hyperalgesia was assessed 2 h after s.c. injection of λ-carrageenan (A), or 96 h after s.c. injection of CFA (B) in the plantar hindpaw by determining paw withdrawal latency thresholds using the plantar test (t0). Animals then received a gavage of compound **2a** (■) or vehicle (□) (distilled water) and paw withdrawal latency thresholds were measured 40 min after treatment (**A**) or 30, 90, 120, and 180 min after administration (**B**), n = 7–10 per group. **C**. Effect of compound **2a** on the gait score evaluated in knee joint arthritis model in rats. Gait score was observed 3 h after kaolin intra-articular injection (t0). Compound **2a** (20 mg/kg) (■) or vehicle (saline) (□) were then injected by intravenous route and gait score was observed 30, 60, 120, and 240 min after injection (n = 10 per group). Values are mean ± SEM. ★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.001$ versus vehicle, ##$P < 0.01$ versus treated group at t0, one-way ANOVA (A) or two-way repeated measures ANOVA (B, C) followed by Bonferroni's test. CFA, complete Freund's adjuvant.

or before CFA injection (9.4 ± 0.3 sec) (Fig. 5B). Compound **2a** administered at 200 mg/kg per os strongly increased PWL threshold 45 min after administration to value (8.7 ± 0.5 sec) (83% MPE) closed to that measured on the contralateral side. The time-course of **2a** action was followed during 180 min showing a significant thermal antihyperalgesic effect all over this period.

Kaolin-induced knee joint arthritis

Acute joint inflammation was produced by intra-articular injection of 10% kaolin in rat knee joint. Before arthritis induction, all rats exhibited expected normal gait to which a score of 0 was assigned and animals observed 3 h after intra knee injection (t0) exhibited a painful leg and a limited mobility of the inflamed knee, as illustrated by a marked increase in the gait score (2.4 ± 0.2 and 2.1 ± 0.2 for vehicle and treated groups) (Fig. 5C). Intravenous injection of compound **2a**, 20 mg/kg, significantly reduced gait score by 40% for 120 min, with a maximum effect obtained at 30 and 60 min (1.4 ± 0.2) (Fig. 5C).

PSNL-induced neuropathic pain in mice

As shown in Figure 6A, mechanical allodynia was evidenced by a dramatic decrease in the paw withdrawal threshold to von Frey filament application in all nerve-injured mice (t0, 0.32 ± 0.02 g) compared to baseline presurgery threshold (1.42 ± 0.04 g) or contralateral side (1.24 ± 0.06 g). Oral administration of compound **2a** (12.5–50 mg/kg) increased dose dependently mechanical threshold measured in the von Frey test. At the higher dose tested (50 mg/kg), compound **2a** almost completely reversed PWT for 90 min (1.0 ± 0.12 g), producing 74% inhibition of mechanical allodynia. This effect was still significant at 150 min (31% inhibition).

In addition to mechanical allodynia, hypersensitivity to heat stimulation was measured in PSNL mice using the plantar test. As shown in Figure 6B, vehicle-treated mice displayed largely decreased PWL (3.18 ± 0.17 sec vs. contralateral 8.6 ± 0.3 sec). Compound **2a**, at 50 mg/kg p.o., reversed thermal hyperalgesia 45 min after administration, increasing heat-sensitive threshold values (8.6 ± 0.3 sec), not significantly different from the contralateral side (8.5 ± 0.6 sec), thus bringing heat sensitivity back to normal. These antihyperalgesic effects persisted after 150 min (6.0 ± 0.5 sec). These effects **2a** were dose dependent, since lower doses, 12.5 and 25 mg/kg, induced moderate but significant thermal antihyperalgesia at each time (Fig. 6B). Mechanical and heat sensitivity evaluated on the contralateral side were not modified by **2a**.

Oral administration of the most effective dose of compound **2a** (50 mg/kg) induced a marked antiallodynic effect that was fully reversed by coinjection of Nlxe-Met (5 mg/kg, i.p.), indicating peripheral opioid receptors recruitment in alleviation of nerve-injured-evoked pain (Fig. 6C).

The potentially effective **2a**/gabapentin combination (Menendez et al. 2008; Gonzalez-Rodriguez et al. 2009) was thus assessed on mechanical allodynia in PSNL mice.

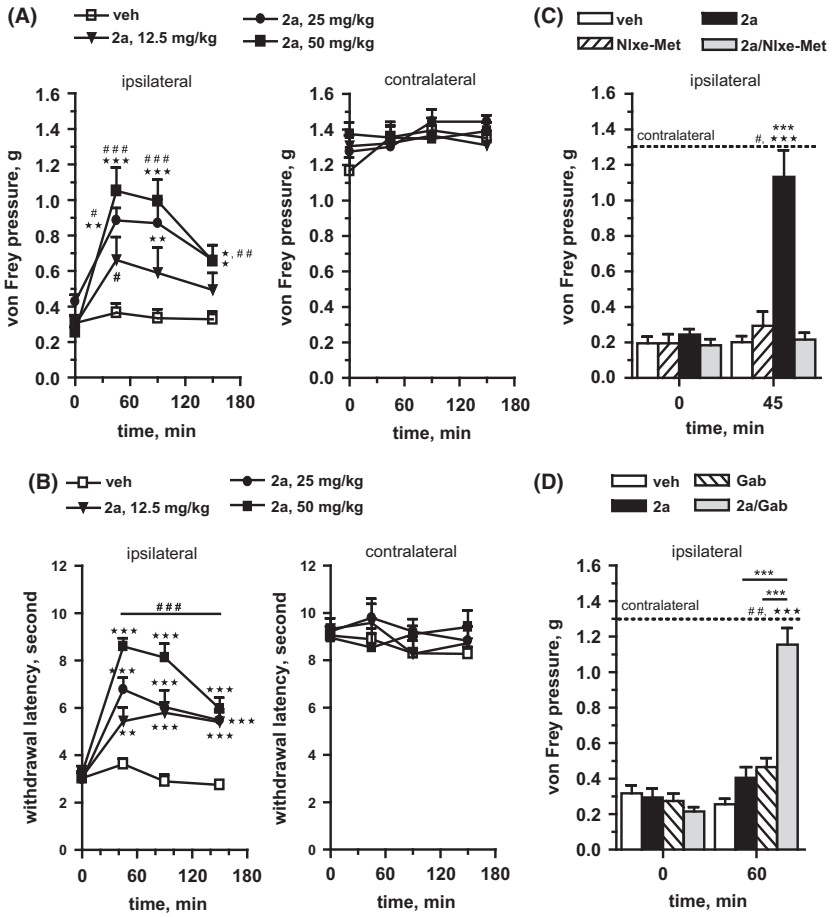


Figure 6. Peripheral opioid-mediated reduction in PSNL-induced neuropathic pain in mice by the orally active compound **2a**. (A) Dose–response effect of compound **2a** (12.5 mg/kg ▼, 25 mg/kg ● and 50 mg/kg ■) on tactile allodynia and (B) thermal hyperalgesia in nerve-injured mice. Mechanical and thermal hypersensitivity were assessed before (t0) and 45, 60, 90, and 150 min after oral administration of compound **2a** (12.5 mg/kg ▼, 25 mg/kg ● and 50 mg/kg ■) or vehicle (EtOH/0.5% methylcellulose in H_2O, 1.5/98.5) (□), in a Latin square design. Paw withdrawal responses were measured on both ipsilateral and contralateral sides. A total number of 15 mice exposed to nerve injury were used. (C) Reversion of antiallodynic effects evoked by compound **2a** (50 mg/kg p.o.) measured in the von Frey test by injection of naloxone methiodide (Nlxe-Met) (5 mg/kg i.p. in saline) in nerve-injured mice. Nlxe-Met was injected 25 min after gavage of compound **2a** or vehicle. Pressure thresholds were measured before (t0) and 45 min after oral administration in a Latin square design. A total number of 14 mice exposed to nerve injury were used. (D) Assessment of the antiallodynic effect of oral administration of a combination compound **2a**/gabapentin in nerve-injured mice. Pressure threshold to von Frey filaments application on ipsilateral hindpaw was measured before (t0) and 60 min after the oral administration of vehicle (EtOH/0.5% methylcellulose in H_2O, 1.5/98.5) or compound **2a** (10 mg/kg) or gabapentin (30 mg/kg) or the combination compound **2a**/gabapentin (10/30 mg/kg) in a Latin square design. A total number of 16 mice exposed to nerve injury were used. Values are mean ± SEM. ★$P < 0.05$, ★★$P < 0.01$, ★★★$P < 0.001$ versus vehicle, ***$P < 0.001$ versus **2a**/Nlxe-Met-treated group and Nlxe-Met-treated group at 45 min Kruskal–Wallis followed by Mann–Whitney's test. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus t0, Kruskal–Wallis followed by Wilcoxon's test.

Single oral administration of inactive doses of compound **2a** (10 mg/kg) and gabapentin (30 mg/kg) 60 min before testing, did not produce any antiallodynic effect in nerve-injured mice when administered alone (Fig. 6D). In contrast, mice receiving a single oral administration of the **2a**/gabapentin combination (10 mg/kg and 30 mg/kg, respectively) displayed a largely increased mechanical sensitive threshold, 60 min after gavage (Fig. 6D) (1.15 ± 0.09 g vs. 0.26 ± 0.03 g, for combined **2a**/gabapentin vs. vehicle, respectively), suggesting a synergistic action of both compounds on mechanical hypersensitivity in nerve-injured mice.

CCI- and PSNL-induced neuropathic pain in rat

As shown in Figure 7A, CCI induced a dramatic decrease in the mechanical threshold measured on ipsilateral side

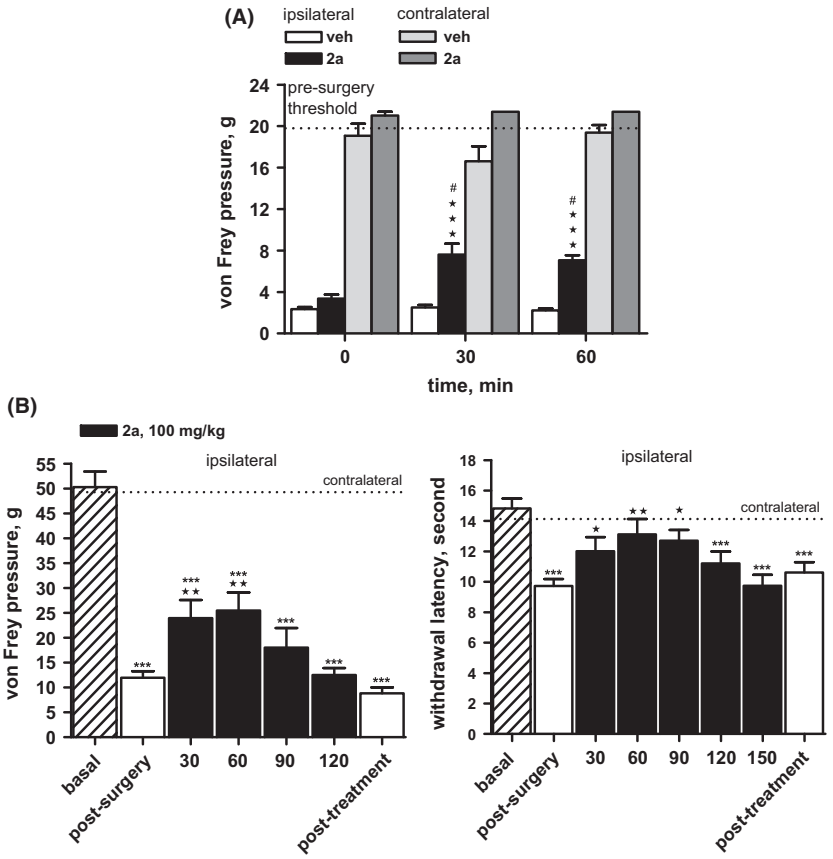


Figure 7. Effects of intravenous and oral administration of compound **2a** on tactile allodynia and thermal hyperalgesia induced by CCI models and partial sciatic nerve ligation in rats. (A) Mechanical hypersensitivity was assessed by the von Frey test on nerve-injured rats (CCI model) before (t0), 30 and 60 min after i.v. injection of compound **2a** (10 mg/kg) or vehicle (saline). Both ipsilateral and contralateral sides were evaluated (n = 7 per group). (B**)** Time-course of antiallodynic (left) and antihyperalgesic effects (right) of compound **2a**, given orally (100 mg/kg) in PSNL models in rats. Paw withdrawal thresholds to paw pressure by von Frey filaments application (left) or heat sensitivity thresholds to thermal stimuli (right) were measured on both ipsilateral and contralateral sides before surgery (basal), on day 8 after surgery following vehicle (saline) treatment (postsurgery), on days 9–15 postsurgery in a Latin square design 30, 60, 90, and 120 min after the gavage of compound **2a** and finally, on day 18 postsurgery after vehicle administration (posttreatment). A total number of 14 rats exposed to nerve injury were used. Values are mean ± SEM. $^{\star}P < 0.05$, $^{\star\star}P < 0.01$, $^{\star\star\star}P < 0.001$ versus vehicle, ###$P < 0.001$ versus treated group at t0, ***$P < 0.001$ versus basal, Kruskal–Wallis followed by Mann–Whitney's test. i.v., intravenous; CCI, chronic constrictive injury.

on day 12 after surgery in all groups of rats (t0; 2.8 ± 0.4 g vs. 19.4 ± 0.4 g, postsurgery vs. presurgery values, respectively) compared to contralateral side (20.4 ± 0.6 g). Compound **2a** (10 mg/kg, i.v.) significantly increased mechanical threshold (30%) on ipsilateral side after a single injection at 30 and 60 min, compared to the vehicle-treated group.

In rats exposed to PSNL-evoked peripheral neuropathy, mechanical and thermal nociceptive effects were evaluated by von Frey and plantar tests respectively on days 8–18 after surgery. The expression of both mechanical allodynia and thermal hyperalgesia was similar during the whole experimental session, since no relevant differences were observed when comparing responses in vehicle-treated group on days 8 and 18 after surgery (Fig. 7B). Acute oral administration of compound **2a** (100 mg/kg) induced a significant increase in the paw withdrawal threshold to von Frey stimulation 30 min (23.9 ± 3.7 g) and 60 min (25.5 ± 3.6 g) after gavage compared to vehicle-treated group (11.9 ± 1.3 g). The inhibition of the tactile allodynia induced by compound **2a** was ~30% and was observed for 60 min and remains slightly active at 120 min (Fig. 7B). Likewise, compound **2a**, given p.o. at the same dose, partially reversed sciatic nerve injury lowering PWL thresholds at 30 min (12 ± 0.9 sec), 60 min (13.1 ± 1.0 sec), and 90 min (12.7 ± 0.7 sec) compared to vehicle-treated group (9.7 ± 0.4 sec). The thermal antihyperalgesic responses (Fig. 7B, right) measured at 60 min after oral gavage of compound **2a** were not significantly different to those obtained under basal conditions,

indicating that compound **2a** fully reversed thermal hyperalgesia at this dose.

Discussion

The *N*-(acyloxy)alkyl carbamates have been investigated as bio-reversible prodrugs of amines by Alexander and coworkers (Alexander et al. 1988). This amine protection is characterized by a good chemical stability, is hydrolyzed in vivo by esterases and increases the biological membrane permeation. More recently, the introduction of this labile protection in gabapentin induced a significant enhancement in its oral activity (Cundy et al. 2004). The authors explain this property by the ability of these prodrugs to be transferred by high-capacity transporters located in intestine (Cundy et al. 2004).

Consequently, it was interesting to verify that such type of prodrug could significantly increase the oral bioavailability of the present dual NEP/APN inhibitors.

The *N*-protection largely enhances the solubility in aqueous medium, as compared to the previously described prodrugs (Chen et al. 2001), in which, transient protections were introduced on both carboxylic and phosphinic acid functions, leading to very hydrophobic compounds which requested solubilization in EtOH/Cremophor/H_2O (1/1/8) and were found active only by i.v. route (Chen et al. 2001). By contrast, the new *N*-protected prodrugs, (compounds **2a**–**2g**), can be dissolved in various vehicles suitable for human administration such as water.

These new generated DENKIs have been tested on several animal models of pain which assessed their ability to act either at the central or peripheral level on acute, chronic, and neuropathic pains. The critical requirement to observe behavioral antinociceptive responses to enkephalinase inhibitors is that ENKs are released by the given test-evoked stimulus. The strength of the antinociceptive effect will be proportional to the concentration of the endogenous opioid peptides and their subsequent stimulation of the ORs (Roques et al. 1993; Yaksh and Elde 1981). This was clearly demonstrated by the ED50 values at least 10 times higher after specific NEP (Noble et al. 1992; Oshita et al. 1990) or APN inhibition (Noble et al. 1992) to that observed with DENKIs as in this paper. This is observed whatever the type of painful stimulus used.

The hot plate test is a model of centrally integrated acute pain (Le Bars et al. 2001), which implies that the tested compounds are able to cross the blood brain barrier. In this test, the prodrug **2a** (Fig. 3A)-induced antinociceptive responses after i.v. administration, similar to those observed with the corresponding drug **1a**, showing that the prodrug form has no significant effect on central bioavailability. This is consistent with the very rapid plasmatic transformation of **2a** in the active DENKI **1a** (Fig. 2C), accounting for the time-course of antinociceptive effects of **2a** in the HPT (Fig. 3A). On the contrary, **2a** tested after oral administration, was found inactive (Fig. 3F). This indicates that the amount of prodrug (or of drug) which successfully crosses the intestinal barrier and then the blood brain barrier is too low to elicit a protected level of ENKs high enough to yield a positive response in this centrally controlled test.

Intraplantar injection of formalin in mice induces a biphasic behavioral reaction: a painful early phase resulting from the direct stimulation of nociceptors present on C and Aδ fibers and a late phase involving a period of sensitization during which inflammatory process and chronic nociception occur (Le Bars et al. 2001).

In this test, compounds **2a**–**2f** were active on the early phase (Fig. 4A, Table 1). The inhibition of the nociceptive stimulus was partial (~30% analgesia) but the effect was long lasting (until 150 min). The pretreatment with Nlxe-Met blocked the analgesic response (Fig. 4C and 4D), demonstrating the involvement of the peripheral endogenous opioidergic system with stimulation of the opioid receptors by DENKIs-protected ENKs. The analgesic response in the early phase of the assay is independent of the presence or not of an ester group in the prodrugs **2a**–**2f** (Table 1), as expected from a rapid hydrolysis of the ester group (Chen et al. 2001). Interestingly, compounds **2a** and **2d** gave the same antinociceptive response when tested at 25 and 50 mg/kg p.o. (Table 1) suggesting a ceiling effect probably associated with the saturation of an active delivery system (Cundy et al. 2004) and/or the complete inhibition of the NEP/APN system inducing similar local concentrations of ENKs at the two doses resulting in almost identical antinociceptive effects (Yaksh and Elde 1981; Roques et al. 1993). Moreover, in prodrugs, such as **2g**, containing both a *N*- and a *P*- protection by an (acyloxy)alkyl anhydride group, the analgesic response is delayed (Table 1). This is consistent with the slow enzymatic deprotection of the phosphinic group in plasma, preliminary observed in the RB3007 series (Chen et al. 2001). On the late phase of the test, which represents the inflammatory response to the s.c. injection of formalin (Fig. 4B), **2a** was more active (50% analgesia) than on the early phase. This is in consistent with the genetic evidence for the involvement of only mu opioid receptor (MOR) in the early phase and both MOR and delta opioid receptor (DOR) in the late phase (Matthes et al. 1996; Martin et al. 2003; Gavériaux-Ruff et al. 2008) and the higher affinity of ENKs for DOR than for MOR (Dhawan et al. 1996).

In the very often used model of neuropathic pain (PSNL), a dose-dependent and long-lasting reversion of allodynia and hyperalgesia was obtained in mice with an

almost complete antinociceptive effect at 50 mg/kg per os (Fig. 6A and B). In this test, the lack of changes in the contralateral side indicated the absence of central stimulation of opioid receptors by protected released ENKs- after oral administration of **2a**. Moreover, the involvement of the peripheral opioidergic system (Przewlocki et al. 1992; Hassan et al. 1993; Maldonado et al. 1994) was confirmed by the absence of analgesic responses of the DENKI-protected ENKs after pretreatment with Nlxe methiodide (Fig. 6C).

In the inflammatory-induced pain models in rats using λ-carragenan (Fig. 5A), CFA model (Fig. 5B) and Kaolin irritant inducing-knee joint arthritis (Fig. 5C), *N*-(Acyloxy)alkyl carbamates prodrugs, such as **2a**, induced good analgesic response with a long duration of action (over 120 min) after either oral or i.v. administration. In these assays, the i.v. administration of the prodrugs requires a 10-fold lower dose for a similar efficacy.

Same results were observed in rats CCI neuropathic pain model (Bennett and Xie 1988) after intravenous administration (10 mg/kg) (Fig. 7A) or in the PSNL model (Malmberg and Basbaum 1998) after oral administration (100 mg/kg) of **2a** (Fig. 7B).

In a neuropathic pain model of tibial osteosarcoma in mice, the combined administration of ineffective doses of oral disulfide DENKIs and s.c. gabapentin induced a synergistic complete alleviation of thermal hyperalgesia (Menendez et al. 2008). This result was extended to this new series of DENKI prodrugs using the PSNL model in mice. Thus, oral co-administration of **2a** (10 mg/kg) and gabapentin (30 mg/kg) produced an almost complete antiallodynic response (Fig. 6D), which was reached with 50 mg/kg of **2a** alone, suggesting synergistic antineuropathic effects of the combination. This synergistic effect is probably related to the action of DENKI-protected ENKs at the pain source (periphery) and to the gabapentin reduction in the reinforcing noxious messages occurring at the spino-bulbo spinal descending pathway (Porreca et al. 2002).

In conclusion, the *N*-protected aminophosphinic DENKIs prodrugs, orally administered, increase exclusively the peripheral concentrations of ENKs. These ENKs, selectively released at the site of the noxious stimuli, will activate the opioid receptors reducing or alleviating inflammatory or/and neuropathic pain, blocking it at the origin (Oshita et al. 1990; Schreiter et al. 2012; Stein 2013). This approach has been shown to give new analgesics and active at single dose and devoid of side effects as previously discussed (Roques et al. 2012). This is due to ENKs local versus MO-ubiquitous recruitment of opioid receptors (Williams et al. 1987; Roques 2000).

As compared to previous DENKIs, these new prodrugs have also several great advantages such as a very simple in vivo metabolism leading only to the active inhibitor and a very large increase in duration of action. Based on these results, compound **2a**, the new highly efficient DENKI prodrug, PL265, was selected for clinical development, the results of which will be published elsewhere.

Acknowledgements

The authors thank Sophie Duquesnoy, Annette Fayet, and Céline Ratinaud-Giraud for their technical assistance and Michel Wurm, M.D., for English revision of the manuscript.

Disclosure

None declared.

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Supporting Information

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

Data S1. Material and methods and references.