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

Elisabeth Bonnard1,a, Hervé Poras1,a, Xavier Nadal2, Rafael Maldonado2, Marie-Claude Fournié-Zaluski1 & Bernard P. Roques1,3

1Pharmaleads, 75013, Paris, France
2Laboratori de Neurofarmacologia, Universitat Pompeu Fabra, Parc de Recerca Biomedica de Barcelona (PRBB), Barcelona, Spain
3Université Paris-Descartes, 75006, Paris, France

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

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, di-isopropylethylamine; 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; NIH, 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.

© 2015 The Authors. Pharmacology Research & Perspectives published by John Wiley & Sons Ltd, British Pharmacological Society and American Society for Pharmacology and Experimental Therapeutics. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
Protected Endogenous Enkephalins in Chronic Pain

E. Bonnard et al.

Introduction

Chronic pain, which includes neuropathic and inflammatory pain, can occur after various pathophysiological 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-isopropylcarbonyloxyethylxycarbamate 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.
(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).

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.

| Compound | R | R' | R1 | R2 | % of control |
|----------|---|----|----|----|--------------|
|          |   |    |    |    | 25 mg/kg 90 min | 50 mg/kg 90 min | 150 min |
| RB3007   |   |    |    |    | 90 min | 150 min |
| 1a        | H | H  | Ph | H  | nd | nd | nd |
| 2a        | H | H  | Ph | H  | 63 ± 6** | 70 ± 6** | 69 ± 7** |
| 2b        |   | H  | Br | H  | nd | 80 ± 6* | 70 ± 7* |
| 2c        | H | H  | Ph | C6  | nd | 68 ± 6** | 72 ± 6* |
| 2d        | H | H  | CH3Ph | C6  | 60 ± 6** | 67 ± 6*** | 73 ± 4* |
| 2e        | H | H  | CH3CH3 | C6  | nd | 70 ± 4** | 88 ± 8** |
| 2f        | H | H  | Br | CH3Ph | nd | 69 ± 3*** | 71 ± 6*** |
| 2g        | H | H  | 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.

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,
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 κ-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 naïve 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-Quantin 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-yl oxy)carbonyloxy)ethyl isobutyrate (Cundy et al. 2004) in CH₂Cl₂ in presence of Et₃N (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-chloroethyl 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
reaction of the respective acids with one or two equivalents of NaHCO₃ in CH₃CN/H₂O 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 (Nlx), demonstrating the selective involvement of opioid receptors (Fig. 3C).
Further studies were performed in another vehicle, EtOH/Tween 80/H2O (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/H2O (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
salm (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 H2O (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 (Nlx-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 stimuli (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 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).
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 observed 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 pre-surgery 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 Nalx-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.
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.
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 alldynia 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/H2O (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 DENGIs 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 DENGIs 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 DENGK 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 DENGIs-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
simple in vivo metabolism leading only to the active
drugs have also several great advantages such as a very
receptors (Williams et al. 1987; Roques 2000).
ENKs local versus MO-ubiquitous recruitment of opioid
origin (Oshita et al. 1990; Schreiter et al. 2012; Stein
inflammatory or/and neuropathic pain, blocking it at the
peripheral concentrations of ENKs. These ENKs, selec-
tively released at the site of the noxious stimuli, will acti-
vely released at the site of the noxious stimuli, will acti-

In conclusion, the N-protected aminophosphinic DEN-
KIs prodrugs, orally administered, increase exclusively the
peripheral concentrations of ENKs. These ENKs, selec-
tively released at the site of the noxious stimuli, will acti-

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

Disclosure
None declared.

References
Alexander J, Cargill R, Michelson SR, Schwam H (1988).
(Acylxy)alkyl carbamates as novel bioreversible pro-drugs for
amines: increased permeation through biological membranes. J
Med Chem 31: 318–322.

Bennett GJ, Xie YK (1988). A peripheral mononeuropathy in
rat that produces disorders of pain sensation like those seen
in man. Pain 33: 87–107.

Bianchi G, Fiocchi R, Tavani A, Manara L (1982).
Quaternary narcotic antagonist relative ability to prevent
antinociception and gastrointestinal transit inhibition in
morphine-treated rats as an index of peripheral activity. Life
Sci 30: 1875–1883.

Bourgoign S, Le Bars D, Artaud F, Clot A-M, Bouboutou R,
Fournie-Zaluski M-C, et al. (1986). Effects of kelatorphan and
other peptidase inhibitors on the in vitro and in vivo release
of methionine-enkephalin-like material from the rat spinal
cord. J Pharm Exp Therap 238: 360–366.

Carenzi A, Frigeni V, Reggiani A, Della Bella D (1983). Effect
of inhibition of neuropeptidases on the pain threshold of mice
and rats. Neuropharmacology 22: 1315–1319.

Cesselin F, Oliveras JL, Bourgoign S, Sierralta F, Michelot R,
Besson JM, et al. (1982). Increased levels of
Met-enkephalin-like material in the CSF of anesthetized cats
after tooth pulp stimulation. Brain Res 237: 325–338.

Chen H, Noble F, Mothé A, Meudal H, Coric P,
Danascimento S, et al. (2000). Phosphinic derivatives as new
dual enkephalin-degrading enzyme inhibitors: synthesis,
biological properties, and antinociceptive activities. J Med
Chem 43: 1398–1408.

Chen H, Noble F, Roques BP, Fournie-Zaluski MC (2001).
Long lasting antinociceptive properties of enkephalin
degrading enzyme (NEP and APN) inhibitor prodrugs. J Med
Chem 44: 3523–3530.
Cundy KC, Branch R, Chernov-Rogan T, Dias T, Estrada T, Hold K, et al. (2004). XP13512[(±)-1[(z-Isobutanoxyfuroxythoxy) carbonyl][aminomethyl]-1-cyclohexane acetic acid], a novel gabapentin pro-drug: I. Design, synthesis, enzymatic conversion to gabapentin, and transport by intestinal solute transporters. J Pharm Exp Therap 311: 315–323.

Dauge V, Mauborgne A, Cesselin F, Fournie-Zaluski MC, Roques BP (1996). The dual peptidase inhibitor RB101 induces a long-lasting increase in the extracellular level of Met-enkephalin-like material in the nucleus accumbens of freely moving rats. J Neurochem 67: 1301–1308.

Dhawan BN, Cesselin F, Raghubir R, Reisine T, Bradley PB, Portoghese PS, et al. (1996). International union of Pharmacology. XII. Classification of opioid receptors. Pharmacol Rev 48: 567–592.

Eddy NB, Leimbach D (1953). Synthetic analgesics: diethynylbutenyl and diethynylbutylamines. J Pharm Exp Therap 107: 385–389.

Fournié-Zaluski MC, Chaillet P, Bouboutou R, Coulaud A, Cherot P, Waksman G, et al. (1984). Analogic effects of kelatorphan, a new highly potent inhibitor of multiple enkephalin degrading enzymes. Eur J Pharmacol 102: 525–528.

Fournié-Zaluski MC, Coric P, Turcaud S, Lucas E, Noble F, Maldonado R, et al. (1992). “Mixed inhibitor-pro-drug” as a new approach toward systemically active inhibitors of enkephalin-degrading enzyme. J Med Chem 35: 2473–2481.

Gabrilovic J, Cupic B, Brejda J, Zekus C, Boranic M (2004). Expression of CD13/aminopeptidase N and CD10/neutral endopeptidase on cultured human keratinocytes. Immunol Lett 91: 39–47.

Gavéraux-Ruff C, Karchewski LA, Hever X, Matífas A, Kieffer BL (2008). Inflammation pain is enhanced in delta opioid receptor-knockout mice. Eur J Neurosci 27: 2558–2567.

Gonzalez-Rodriguez S, Pevida M, Roques BP, Fournié-Zaluski MC, Hidalgo A, Menendez L, et al. (2009). Involvement of enkephalins in the inhibition of osteosarcoma-induced thermal hyperalgesia evoked by the blockade of peripheral P2X3 receptors. Neurosci Lett 465: 285–289.

Gregory NS, Harris AL, Robinson CR, Dougherty FM, Fuchs PN, Sluka KA (2013). An overview of animal models of pain: disease models and outcome measures. J Pain 14: 1255–1269.

Hassan AH, Ableitner A, Stein C, Herz A (1993). Inflammation of the rat enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. Neuroscience 55: 185–195.

Hertz F, Ranson M, Lwoff JM (1980). Pharmacological properties of a new anti-inflammatory agent: 2-(2-isopropyl-5-indanyl) propionic acid (UP517-03). Arzneimittelforschung 30: 1549–1557.

Hunskaar S, Fasmer OB, Hole K (1985). Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Methods 14: 69–76.

Le Bars D, Gozariu M, Cadden S (2001). Animal models of nociception. Pharmacol Rev 53: 597–652.

Le Guen S, Mas Nieto M, Canestrelli C, Chen H, Fournie-Zaluski MC, Cupo A, et al. (2003). Pain management by a new series of dual inhibitors of enkephalin degrading enzymes: long lasting antinociceptive properties and potentiation by CCK2 antagonist and methadone. Pain 104: 139–148.

Machelska H (2007). Targeting of opioid-producing leukocytes for pain control. Neuropeptides 41: 355–363.

Maldonado R, Valverde O, Turcaud S, Fournie-Zaluski MC, Roques BP (1994). Antinociceptive response induced by mixed inhibitors of enkephalin catabolism in peripheral inflammation. Pain 58: 77–83.

Malmborg AB, Basbaum AI (1998). Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. Pain 76: 215–222.

Martin M, Matífas A, Maldonado R, Kieffer BL (2003). Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct mu, delta and kappa tones. Eur J Neurosci 17: 701–708.

Matthes HW, Maldonado R, Simon F, Valverde O, Slowé S, Kitchen I, et al. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature 383: 819–823.

Menendez L, Hidalgo A, Meana A, Poras H, Fournie-Zaluski MC, Roques BP, et al. (2008). Inhibition of osteosarcoma-induced thermal hyperalgesia in mice by orally active dual enkephalinase inhibitor PL37. Potentiation by gabapentin. Eur J Pharmacol 596: 50–55.

Noble F, Roques BP (2007). Protection of endogenous enkephalin catabolism as natural approach to novel analgesic and antidepressant drug. Expert Opin Ther Targets 11: 145–159.

Noble F, Soleilhac JM, Soroca-Lucas E, Turcaud S, Fournié-Zaluski MC, Roques BP (1992). Inhibition of the enkephalin metabolizing enzymes by the first systemically active mixed inhibitor pro-drug RB101 induces potent analgesic responses in mice and rats. J Pharm Exp Therap 261: 181–190.

Noble F, Smadja C, Valverde O, Maldonado R, Coric P, Turcaud S, et al. (1997). Pain-suppressive effects on various nociceptive stimuli (thermal, chemical, electrical and inflammatory) of the first orally active enkephalin-metabolizing enzyme inhibitor RB120. Pain 73: 383–391.

Offenbaecher M, Ackenheil M (2005). Current trends in neuropathic pain treatments with special reference to fibromyalgia. CNS Spectr 10: 285–297.
Oshita S, Yaksh TL, Chipkin R (1990). The antinociceptive effects of intrathecally administered SCH32615, an enkephalinase inhibitor in the rat. Brain Res 515: 143–148.

Pardridge WM (2005). The blood-brain barrier: bottleneck in brain drug development. NeuroRx 2: 3–14.

Poras H, Duquesnoy S, Fournie-Zaluski MC, Ratinaud-Giraud C, Roques BP, Ouimet T (2013). A sensitive fluorogenic substrate for selective in vitro and in vivo assay of leukotriene A4 hydrolase. Anal Biochem 441: 152–161.

Poras H, Bonnard E, Dange E, Fournie-Zaluski MC, Roques BP (2014). New orally active dual enkephalinase inhibitors (DENKIs) for central and peripheral pain treatment. J Med Chem 57: 5748–5783.

Porreca F, Ossipov MH, Gebhart GF (2002). Chronic pain and medullary descending facilitation. Trends Neurosci 25: 319–325.

Przewlocki R, Hassan AH, Lason W, Epplen C, Herz A, Stein C (1992). Gene expression and localization of opioid peptides in immune cells of inflamed tissue: functional role in antinociception. Neuroscience 48: 491–500.

Rashid MH, Inoue M, Toda K, Ueda H (2004). Loss of peripheral morphine analgesia contributes to the reduced effectiveness of systemic morphine in neuropathic pain. J Pharmacol Exp Ther 309: 380–387.

Rittner HL, Brack A, Machelska A, Moussa M, Schäfer M, et al. (2001). Identification, recruitment, and simultaneously increasing inhibition of inflammatory pain. Anesthesiology 95: 500–508.

Roques BP (2000). Novel approaches to targeting neuropeptide systems. Trends Pharmacol Sci 21: 475–483.

Roques BP, Noble F, Daugé V, Fournie-Zaluski MC, Beaumont A (1993). Neutral endopeptidase 24.11: structure, inhibition, and experimental and clinical pharmacology. Pharmacol Rev 45: 87–146.

Roques BP, Fournie-Zaluski M-C, Wurm M (2012). Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. Nat Rev Drug Discov 11: 293–310.

Schreiter A, Gore C, Labuz D, Fournie-Zaluski M-C, Roques BP, Stein C, et al. (2012). Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue. FASEB J 26: 5161–5171.

Stein C (2013). Targeting pain and inflammation by peripherally acting opioids. Front Pharmacol 123: 1–3.

Stein C, Schäfer M, Machelska H (2003). Attacking pain at its source: new perspectives on opioids. Nat Med 9: 1003–1008.

Tegeder I, Meier S, Burian M, Schmidt H, Geisslinger G, Lötsch J (2003). Peripheral opioid analgesia in experimental human pain models. Brain 126: 1092–1102.

Tholander F, Muroya A, Roques BP, Fournie-Zaluski MC, Thunissen MGM, Haeggström JZ (2008). Structure-based dissection of the active site chemistry of leukotriene A4 hydrolase: implications for M1 aminopeptidases and inhibitor design. Chem Biol 15: 920–929.

Truong W, Cheng C, Xu QG, Li XQ, Zochodne DW (2003). Mu opioid receptors and analgesia at the site of a peripheral nerve injury. Ann Neurol 53: 366–375.

Waksman G, Bouboutou R, Devin J, Bourgois S, Cesselin F, Hamon M, et al. (1985). In vitro and in vivo effects of ketorolac on enkephalin metabolism in rodent brain. Eur J Pharmacol 117: 233–243.

Williams JT, Christie MJ, North RA, Roques BP (1987). Potentiation of enkephalin action by peptidase inhibitors in rat locus ceruleus in vitro. J Pharmacol Exp Ther 243: 397–401.

Yaksh TL, Elde RP (1981). Factors governing release of methionine enkephalin-like immunoreactivity from mesencephalon and spinal cord of the cat in vivo. J Neurophysiol 46: 1056–1075.

Supporting Information

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

Data S1. Material and methods and references.