Long Lasting Antinociceptive Properties of Enkephalin Degrading Enzyme (NEP and APN) Inhibitor Prodrugs

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Prodrugs of phosphinic dual inhibitors of the enkephalin degrading enzymes, neutral endopeptidase (NEP) and aminopeptidase N (APN), corresponding to the formula H_3N^+CH-$(R_1)P(O)(OR)CH_2CH(CH_2Bip)CONHCH(CH_3)COOCH_2Ph$, with $R_1 = CH_3$ or Ph and R being a benzyl ester, a *S*-acyl-2-thioethyl derivative, or an acyloxyalkyl group, were synthesized to improve the poor central bioavailability of their precursors. As expected, these compounds (50 mg/kg, iv or ip) induced long lasting (∼2 h) antinociceptive responses in the hot plate test in mice with a ceiling effect varying between 25 and 42% of analgesia. A very rapid hydrolysis of the carboxylate ester contrasting with a slow deprotection of the phosphinate group ($t_{1/2} \sim 1$ h) was observed in serum while 80% of free drug was obtained after 1 h incubation with brain membranes. These results account for the long duration of action observed with these prodrugs.

Introduction

In the central nervous system (CNS), the modulation of nociceptive stimuli at both the spinal and central levels depends on the equilibrium between endogenous counteracting systems. The opioid system involves several peptides deriving from three independent precursors designated pro-enkephalin, pro-dynorphin, and proopiomelanocortin. These peptides induce analgesia through interaction with one or several of the three types of opioid receptors μ, δ, and κ, which seem to be differently involved in the treatment of nociceptive signals.[1]

Conversely, anti-opioid systems involve endogenous peptides able to potentiate pain sensation. This is the case of calcitonin gene releasing peptide (CGRP), substance P, and neurokinin A at the spinal cord level (review in 2) whereas the C-terminal fragment of cholecystokinin CCK_8,[3] the neuropeptide FF,[4] or the recently discovered peptide nociceptin[5] facilitate spinal and central nociceptive stimuli.

Pain control by these counteracting systems has generated numerous studies aimed at developing new analgesics with reduced side effects as compared to morphine and surrogates. However, synthetic analogues of opioid peptides were shown to produce the same type of drawbacks as morphine,[6] and antagonists of the anti-opioid systems were reported to have a low activity or to induce important undesirable side effects.[7]

Other approaches such as development of antagonists of bradykinin receptors and inhibitors of cyclo-oxygenases have been achieved to try to reduce nociceptive stimuli at the peripheral nociceptor level. Thus selective Cox-2 inhibitors behave as interesting new NSAIDs (nonsteroidal antiinflammatory drugs) since they are devoid of the gastrointestinal nuisance of most of these latter compounds.[8] However, none of these compounds resulted in analgesics able to fill the gap between NSAIDs and opioids and thus to alleviate the various types of nociceptive stimuli, in particular, severe osteoarticular, post-operative, or neuropathic pain.

Numerous experiments have shown that one of the most promising approaches appears to potentiate the antinociceptive effects induced by the endogenous opioid peptides Met- and Leu-enkephalin (review in 9). Indeed, intracerebroventricularly administered, these morphine-like peptides interact selectively with μ and δ opioid receptors to trigger a strong but brief analgesic response,[10] due to the rapid physiological inactivation of the peptides by two zinc metallopeptidases, the neutral endopeptidase (NEP, neprilysin, EC 3.4.24.11),[11] and the neutral aminopeptidase (APN, EC 3.4.11.2).[12] Joint inhibition of both enzymes allows the potent antinociceptive properties of enkephalins to be strongly potentiated.[13] Thus, icv or iv administration in mice and rats of a dual inhibitor such as kelatorphan, OHNH-CO-CH_2-$CH(CH_2Ph)$-CONH-$CH(CH_3)$-COOH (K_i values 1.7 nM and 7 μM on NEP and APN, respectively (Scheme 1)[14] or the prodrug RB 101, H_2N-$CH(CH_2CH_2SCH_3)$-CH_2-S-S-CH_2-$CH(CH_2Ph)$-CONH-$CH(CH_2Ph)$-CO_2CH_2Ph[15] (Scheme 1), produce antinociceptive responses, only slightly lower than those produced by morphine, in all animal models of pain used for screening new analgesics. In the case of RB 101, the biologically dependent reduction of the disulfide bond in brain tissue was shown to release, respectively, two selective thiol-containing inhibitors which display nanomolar affinities for NEP and APN, respectively.[16]

However, due to the short duration of action of these compounds, a new series of dual inhibitors has been designed. Among these molecules, α-aminophosphinic derivatives,[17,18] which behave as "transition state" analogues interacting with the S_1, S_1' and S_2' subsites of both NEP and APN and are endowed with K_i values in the nanomolar range for the two targeted enzymes, were shown to be the most interesting.

Thus, the two dual inhibitors **1** and **2** (Scheme 1) (compound **1**: K_i(NEP) 1.2 nM, K_i(APN) 2.9 nM; com-

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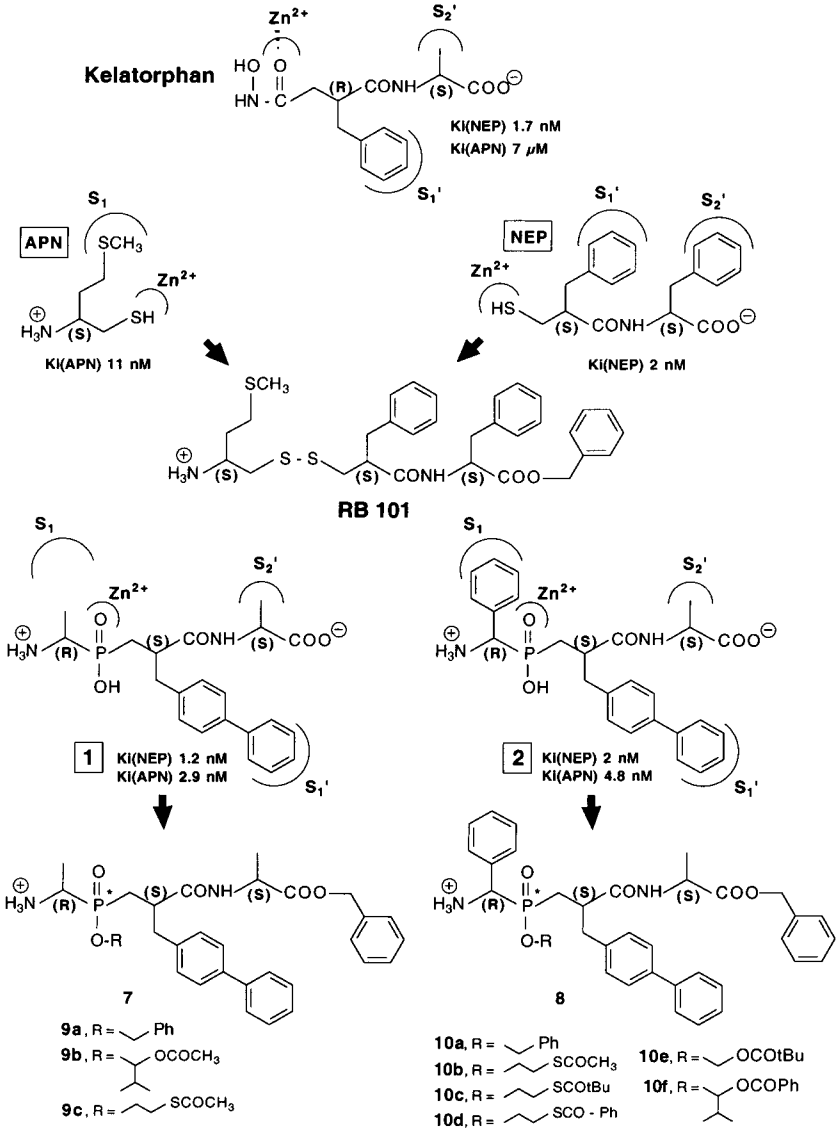
Scheme 1. Structure of the Two Selected Dual NEP/APN Inhibitors **1** and **2** and Their Respective Prodrugs **9a**−**c** and **10a**−**f**



pound **2**: K_i(NEP) 2.0 nM, K_i(APN) 4.8 nM) selected from structure−activity studies have been shown to induce strong dose-dependent analgesic responses following icv administration in mice in the hot plate and the writhing tests, but high doses were required to obtain these effects after iv administration. Thus at 100 mg/kg, compound **1** induced a maximal effect of 25% analgesia, 15 min after administration.[18] This was interpreted by a reduced passage into the brain of these compounds due to the presence of three highly polar functions. To increase their brain penetration, polar functions could be transiently protected by lipophilic groups, able to be cleaved in vivo by biologically dependent processes.

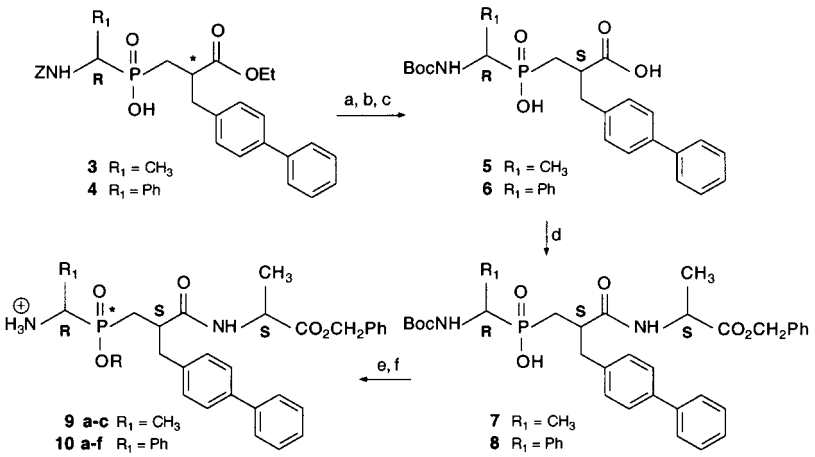
In this work, we describe the development of such prodrugs (Scheme 1) differing essentially by the nature of the phosphinic protecting groups. Their antinociceptive properties and duration of action were investigated by using the hot plate test in mice after iv or ip administration.

Several of these dual NEP/APN inhibitor prodrugs showed, for the first time, potent and long lasting (∼2 h) antinociceptive responses.

Results

I. Synthesis. The ethyl (2*R*,*S*)2-biphenylmethyl-3-[hydroxy[(1′*R*)1′-(*N*-benzyloxycarbonylamino)alkyl]phosphinyl]propanoate **3** and **4** were synthesized as previously described[18,19] by Michael condensation between the 2-biphenylmethylacrylate and a (1*R*)-1-(benzyloxycarbonylamino)alkylphosphinic acid[20] (Scheme 2). The Z-protection of the amino function in **3** and **4** was replaced by a Boc group, and saponification of the ethyl ester afforded **5** and **6** which are obtained as optically pure compounds by crystallization in a mixture of methanol and ethyl acetate, 90/10. These molecules were coupled with alanine benzyl ester using classical conditions (EDCI/HOBt or BOP) to give **7** and **8**. Esterification of the phosphinic function was achieved either by coupling an alcohol ROH using the BOP method or through alkylation by an alkyl halide in alkaline conditions as described in Petrillo, Jr., et al.[21] The final and selective deprotection of the amino group was performed with mild acid (HCOOH) to prevent competitive cleavage of the phosphinic ester. After purification by chromatography on silica gel column,

Scheme 2. Synthesis of Dual NEP/APN Inhibitor Prodrugs[a]



[a] Reagents: (a) 48% HBr; (b) Boc_2O; (c) 1 N LiOH, crystallization; (d) Ala-benzylester/BOP/DIEA (or EDC/HOBT); (e) BOP/DIEA/ROH or RBr/Et_3N; (f) HCO_2H.

prodrugs **9a**−**c** and **10a**−**f** were used as trifluoroacetate or methanesulfonate salts. These prodrugs were mixture of two diastereoisomers, due to the presence of the chiral phosphorus atom. This mixture is clearly evidenced by HPLC.

II. HPLC Study of the Bioactivation of Compound 10b in Rat Serum and Brain Homogenate. Prodrug **10b** was incubated at a final concentration of 25 μM with rat serum (3 mg protein/mL) at 37 °C in Tris HCl buffer pH 7.4. The analysis of the incubation mixture was performed by HPLC using a C_{18} kromasil column with a mixture of 50% CH_3CN/50% H_2O (0.05% TFA) as mobile phase.

Prodrug **10b** disappeared rapidly in less than 10 min leading, at nearly 95%, to a compound derived from benzyl ester hydrolysis (Figure 1). Then, the amount of this intermediate decreased slowly with concomitant formation of the completely deprotected and more polar dual inhibitor. After 1 h, the incubation mixture contains about 40% of the partially protected form and 55% of the final inhibitor (Figure 1).

Incubation of **10b** with a homogenate of brain membranes in Tris HCl buffer pH 7.4 showed a HPLC profile different from that observed in the previous experiment (Figure 2). The prodrug disappears slowly and requires about 1 h for a complete conversion. The two metabolites appear simultaneously, and the amount of the partially protected inhibitor culminates after 20 min of incubation at around 40%. Then, this intermediate decreases slowly down to 20% after 1 h, leading to 80% of completely deprotected drug (Figure 2).

III. Antinociceptive Properties of the Various Prodrugs after iv Administration. Antinociceptive properties of the various prodrugs were studied after mice iv administration by the hot plate test. Two parameters were measured, the jump and the paw lick latencies. All compounds were tested at the unique dose of 50 mg/kg ($\sim 6 \times 10^{-5}$ mol/kg). In these conditions, the three prodrugs **9a**−**c** present a significant antinociceptive activity (Figure 3). The response was relatively low for **9a**, affording less than 20% analgesia 30 min after administration, but this effect was maintained for 30 min. With prodrug **9b**, a significant response appeared 40 min after injection with a maximum of 25% analgesia at 50−60 min, the effect remaining significant for 40 min. The antinociceptive profile of **9c** was similar to that of **9a** for the duration of action, but a slightly improved percentage of analgesia (30% at 30 min) was obtained each time during the study. Moreover, this latter prodrug induced a significant response (20% analgesia) on the paw lick latency 30 and 40 min after administration (data not shown), whereas the two other prodrugs were inactive in the same conditions.

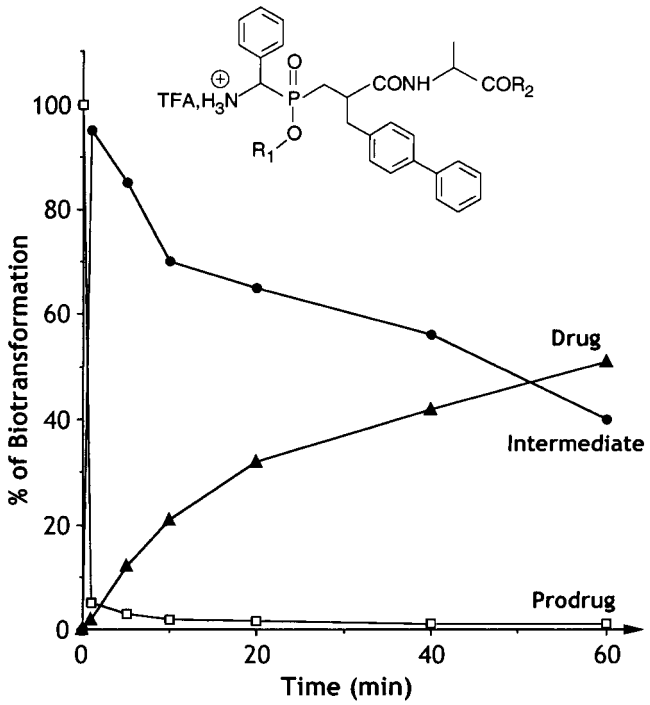


Figure 1. Time dependent biotransformation of prodrug **10b** incubated with rat serum (pH 7.4). Values are the mean of three independent experiments with variations remaining in the range of ± 5% for each point. □ R_1 = $CH_2CH_2SCOCH_3$, R_2 = OCH_2−Ph; ● R_1 = $CH_2CH_2SCOCH_3$, R_2 = OH; ▲ R_1 = H, R_2 = OH.

For the second series of prodrugs **10a**−**d**, the phosphinic acid was protected by a benzyl group (**10a**) or various SATE (*S*-acyl-2-thioethyl derivatives (**10b**−**d**). A very long duration of action was observed with the four derivatives with significant responses obtained from 20−30 min to 90−100 min after injection (Figure 4A,B). Compound **10b** emerges from this study with

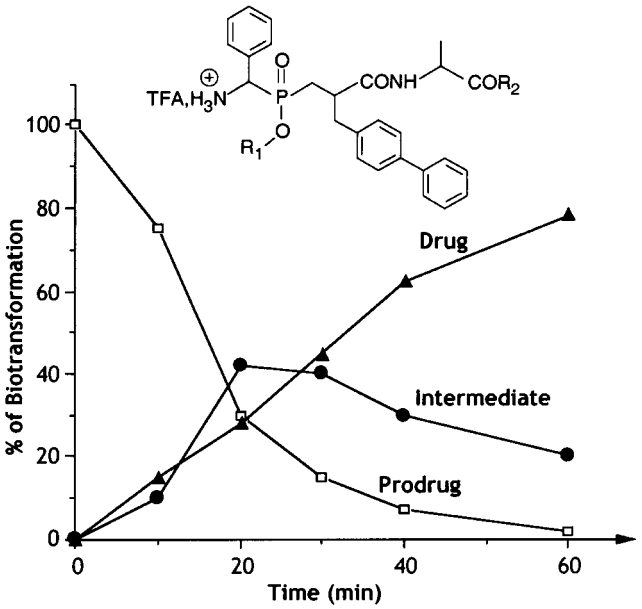


Figure 2. Time dependent biotransformation of prodrug **10b** incubated with rat brain membranes (pH 7.4). Values are the mean of three independent experiments with variations remaining in the range of ± 5% for each point. □ $R_1 = CH_2CH_2SCOCH_3$, $R_2 = OCH_2-Ph$; ● $R_1 = CH_2CH_2SCOCH_3$, $R_2 = OH$; ▲ $R_1 = H$, $R_2 = OH$.

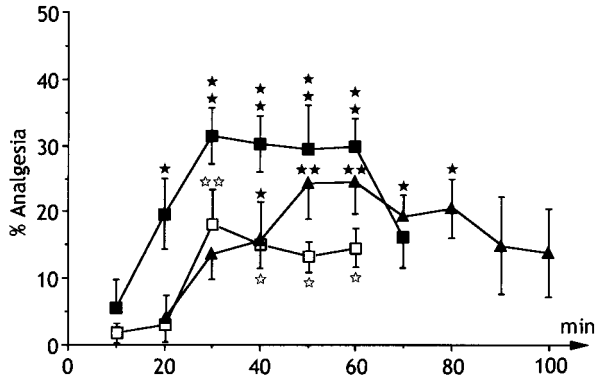


Figure 3. Time course of the antinociceptive responses induced after iv administration of 50 mg/kg (6×10^{-5} mol/kg) of prodrugs **9 a−c** in the hot plate test (52 °C) in mice (n = 10). Prodrugs **9a** (□), **9b** (▲), **9c** (■). Results are expressed as percentage of analgesia ± SEM. *P < 0.05, **P < 0.01 as compared to control (Dunnett's t test).

more than 40% analgesia at 60 min, and a long-lasting effect exceeding 100 min.

IV. Antinociceptive Properties of the Prodrugs after ip Administration. The antinociceptive properties of two of the most efficient prodrugs **10c** and **10d** were studied after i.p. administration under methanesulfonate salts at the unique dose of 50 mg/kg (~6×10^{-5} mol/kg). As shown in Figure 5, they induced antinociceptive responses with a ceiling effect at 30−35% analgesia, but with a long duration of action since both prodrugs were still active 100 min after administration. Maximum activity was observed in the 70−80 min range. Moreover, a significant response was observed on the paw lick latency (28% analgesia at 60 min) with compound **10c** (data not shown).

Two other prodrugs **10e** and **10f** containing an acyloxyalkyl ester as the phosphinic protective group were also tested. Compound **10e** gave a maximum activity

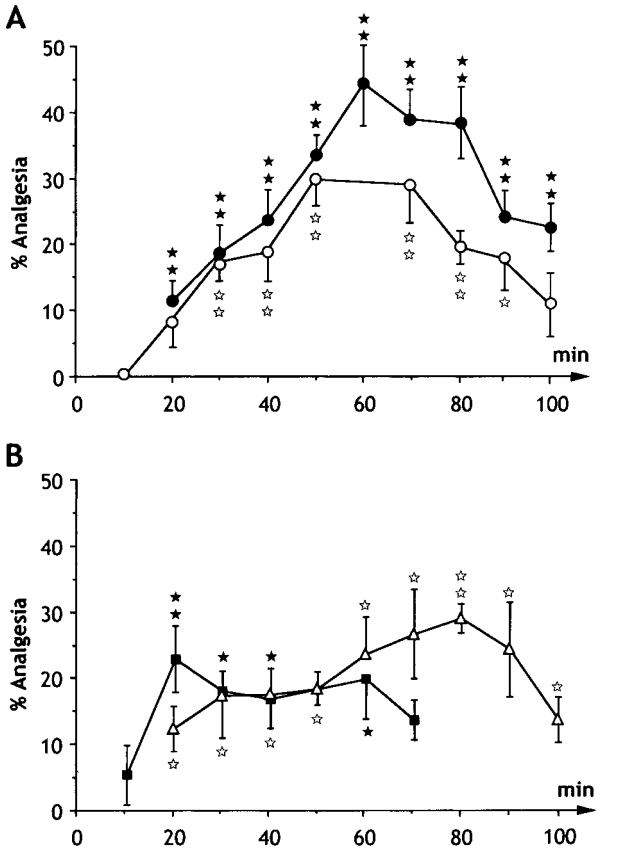


Figure 4. Time course of antinociceptive responses induced after iv administration of 50 mg/kg (6×10^{-5} mol/kg) of prodrugs **10 a−d** in the hot plate test (52 °C) in mice (n = 10). (A) Prodrugs **10a** (○) and **10b** (●). (B) Prodrugs **10c** (■) and **10d** (Δ). Results are expressed as percentage of analgesia ± SEM. *P < 0.05, **P < 0.01 as compared to control (Dunnett's t test).

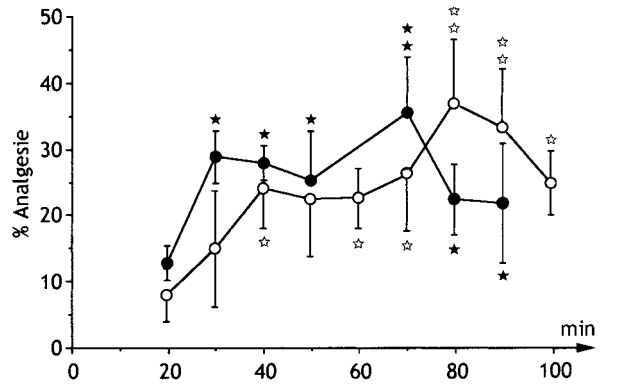


Figure 5. Time course of antinociceptive responses after i.p. administration (50 mg/kg) (6×10^{-5} mol/kg) of prodrugs **10 c−d** in the hot plate test (52 °C) in mice (n = 10). Prodrug **10c** (●) and **10d** (○). Results are expressed as percentage of analgesia ± SEM. *P < 0.05, **P < 0.01 as compared to control (Dunnett's t test).

at 50 min with about 35% analgesia, while **10f** exhibited a very delayed response leading to a significant activity starting 80 min after administration (data not shown).

Discussion

The aim of this study was to design prodrugs of the recently reported[17,18] dual phosphinic inhibitors of the two physiological enkephalin degrading enzymes, NEP and APN, with the purpose of improving the duration

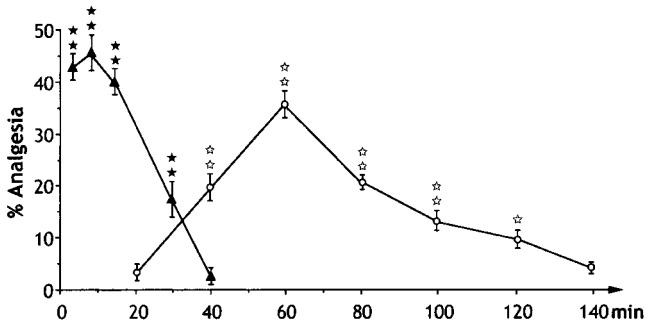


Figure 6. Comparison of the antinociceptive responses induced by iv administration of 10 mg/kg (15 μmol/kg) of RB 101 (▲) and 25 mg/kg (30 μmol/kg) of compound **10b** (○) in the hot plate test (52 °C) in mice (n = 10). RB 101, AUC = 970 (0−30 min); compound **10b**, AUC = 1760 (20−120 min). Results are expressed as percentage of analgesia ± SEM. *P < 0.05, **P < 0.01 as compared to control (Dunnett's t test).

of their antinociceptive properties. For this purpose, the carboxylate and phosphinate functions of the dual inhibitors were substituted with hydrophobic groups. We have previously shown that introduction of a benzyl ester on the C-terminal carboxyl group of inhibitors increase the bioavailability but delays the antinociceptive effects of the phosphinic inhibitors.[18]

Three different protecting groups have been introduced on the phosphinic function: a benzyl group as described for NEP/ECE phosphonate inhibitors,[22] *S*-acyl-2-thioethyl (SATE) derivatives developed for the protection of the phosphate group of nucleotides,[23] and acyloxyalkyl esters previously used in the ACE inhibitor fosinoprilat for the protection of the phosphinic moiety[24] (Scheme 1).

Prodrugs **9a**−**c** and **10a**−**d** iv administration induced intermediate antinociceptive responses, in the hot plate test, a typical animal model of severe pain, with a relatively low intensity (20 to 40% analgesia), but with a long duration of action since some of these compounds were still active 2 h after administration.

In the two series, the most efficient prodrug contains an acetylthioethyl group (**9c** and **10b**), the latter being more active. This is the reverse in the case of the precursor **1** of prodrugs **9a**−**c** which were found more efficient after icv administration[19] than the precursor **2** of prodrugs **10a**−**d,** in agreement with the better inhibitory potencies of the latter. This illustrates the importance of the whole lipophilic content of prodrugs on their pharmacokinetics properties (Scheme 1).

Due to the very different time course of nociceptive responses observed after iv administration of prodrugs issued from dual phosphinic inhibitors and from those obtained with RB 101,[16] a comparison of their analgesic profile was performed. This was achieved by measuring the area under the curve (AUC) (Figure 6), which reflects the relationship between the time course and the percentage of analgesia induced by one dose of RB 101 (10 mg/kg = 15 μmol/kg) and a 2-fold higher dose of **10b** (25 mg/kg = 30 μmol/kg). The AUC for **10b** is twice as high as that obtained with RB 101 (Figure 6). This result indicates that both compounds have a quite similar in vivo potency, which could be related to a complete protection of endogenous enkephalins.[25] However, the different time courses of RB 101 and **10b** analgesic effects suggest the occurrence of differences during the elimination from the brain.

To investigate the fate of **10b**, we have studied its bioactivation in serum and in homogenates of brain membranes. A very rapid cleavage of the benzyl ester occurs in plasma, but hydrolysis of the phosphinic ester is very slow since after 1 h incubation only 50% of the final unprotected inhibitor **2** was obtained (Figure 1). Incubation with a brain membrane homogenate showed a longer lifetime of the benzyl ester and a more efficient phosphinic deprotection (Figure 2). Taken together, these results suggest than an important proportion of the prodrug is probably rapidly eliminated following removal of the benzyl ester group, whereas the remaining unchanged prodrug could cross the blood brain barrier before its slow conversion in brain structures.

Due to the better activity of iv administered prodrugs issued from inhibitor B, only compounds **10d** and **10e** were tested after ip administration, at the same dose of 50 mg/kg in order to achieve a direct comparison of their relative efficiencies. Interestingly, both compounds showed antinociceptive responses not very different from those obtained after iv administration as illustrated in Figures 4 and 5. This suggests that the two phosphinic prodrugs are only slowly deprotected before their entrance in circulation.

In conclusion, iv and ip administration of prodrugs derived from phosphinic dual inhibitors of NEP and APN show interesting pharmacological properties. Indeed, even if their antinociceptive activity is lower than that observed with RB 101, the best compounds induced at least 40% analgesia in the hot plate test, considered as a highly predictive model of severe pain alleviation in humans. Nevertheless, the most interesting result is the time course of the pharmacological response which is much more favorable than that of RB 101 as illustrated by the ceiling antinociceptive effect and the slow disappearance of this response.

The results obtained in this study confirm, once more, that complete protection of endogenous enkephalins could induce antinociceptive responses needed for the treatment of all types of pain which cannot be treated with NSAIDs. In addition, as widely demonstrated with RB 101, dual inhibitors are devoid of the major drawbacks of opiates including constipation and respiratory depression.[26] Accordingly, it could be now interesting to investigate the preclinical properties of these compounds and to search for an appropriate form of administration in humans.

Experimental Section

I. Chemistry. Natural amino acid derivatives were purchased from Bachem (Bubbendorf, Switzerland). Reagents were from Aldrich-chimie (Strasbourg, France). Solvents were from SDS (Peypin, France). TLC were revealed with UV, iodine vapor, or ninhydrin. The purity of the final compounds was checked by HPLC on a reverse phase kromasil C_8 (5 μm, 100 Å) column (4.6 × 250 mm) with 0.05% TFA in H_2O (solvent A)/CH_3CN (solvent B), as the mobile phase, in isocratic conditions at a flow rate of 1 mL/min on a Shimadzu apparatus (detector SPD 6AV, pumps LC9A, recorder CR6A). Eluted peaks were monitored at 210 nm.

The structure of all compounds was confirmed by ^{1}H NMR spectroscopy (Brüker AC 270 MHz) in DMSO-d_6 using HMDS as an internal reference (values in δ, ppm). Melting points of the compounds were determined on an Electrothermal ap-

paratus (Bûchi Melting Point B-450) and are reported uncorrected. Mass spectra were performed at the Pierre & Marie Curie University (CNRS EP 103, Paris, France), on a Esquier-Brüker spectrometer using the electrospray ionization (ESI) technique. Satisfactory analysis were obtained (C, H, N) for all final compounds.

Abbreviations: AcOH, acetic acid; Bip, biphenyl; BOP, [1]H-benzotriazol-1-yloxytri(dimethylamino)phosphonium hexafluorophosphate; BSA, *N,O*-bistrimethylsilylacetamide; Chex, cyclohexane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide; EtOAc, ethyl acetate; Et_2O, ethyl ether; Hex, hexane; HMSD, hexamethyldisiloxane; HOBT, 1-hydroxybenzotriazole; MeOH, methanol; SATE, *S*-acyl-2-thioethyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Et_3N, triethylamine.

General Procedure for the Synthesis of (2*S*)-2-Biphenylmethyl-3-{hydroxy[(1′*R*)1′-(*N*-*tert*-butoxycarbonylamino)-alkyl]phosphinyl}propanoic Acid 5 and 6. A solution of ethyl (2*R,S*)2-biphenylmethyl-3-{hydroxy[1′-(*N*-benzyloxycarbonylamino)alkyl]phosphinyl}propanoate **3** or **4** (1 mM) in HBr (33%) was stirred for 30 min at room temperature. After evaporation of the solvent, the bromhydrate of ethyl (2*R,S*)2-biphenylmethyl-3-{hydroxy[1′-aminoalkyl]phosphinyl}propanoate was obtained without purification.

To a solution of ethyl (2*R,S*)2-biphenylmethyl-3-{hydroxy-[1′-aminoalkyl]phosphinyl}propanoate (1 equiv) in DMF were added $(Boc)_2O$ (1 equiv) and Et_3N (4 equiv). The mixture was stirred for 6−8 h at room temperature. The mixture was taken off with water and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH/AcOH (9:1:0.2) as eluents.

To a solution of ethyl (2*R,S*)2-biphenylmethyl-3-[hydroxy-[1′-(*N*-*tert*-butoxycarbonylamino)alkyl]phosphinyl]propanoate (1 equiv) in ethanol was added 5 equiv of 1 N NaOH. The mixture was stirred for 6−8 h at room temperature. After acidification with 2 N HCl, the ethanolic layers were evaporated, diluted in water, and extracted with EtOAc. The organic layers were washed with water and brine, dried over Na_2SO_4, and evaporated in vacuo.

The crude compounds were dissolved in a mixture of methanol and ethyl acetate 90/10, and the pure (2*S*)2-biphenylmethyl-3-hydroxy[(1′*R*)1′-*N*-*tert*-butoxycarbonylamino)alkyl]-phosphinyl propanoic acids **5** and **6** crystallized and were isolated by filtration.

Compound 5: white solid; mp 182−184 °C (73%); HPLC (60% B) 5.1 min; [1]H NMR (DMSO-d_6 and TFA): 1.1 (m, 3H, $CH_3\beta$); 1.25 (s, 9H, tBu); 1.6−2.1 (m, 2H, P-CH_2); 2.9 (m, 3H, CH_2Ar, $CHCO_2$); 3.68 (m, 1H, CHα); 7.1−7.6 (m, 10H, Ar, NH).

Compound 6: white solid; mp 201−202 °C (70%); HPLC (60% B) 6.7 min; [1]H NMR (DMSO-d_6 and TFA): 1.35 (s, 9H, tBu); 1.5−2.1 (m, 2H, P-CH_2); 2.85 (m, 3H, CH_2Ar, $CHCO_2$); 4.85 (m, 1H, CHα); 7.1−7.7 (m, 15H, Ar, NH).

General Procedure for the Synthesis of (2*S*)2-Biphenylmethyl-3-{hydroxy[(1′*R*)1′-(*N*-*tert*-butoxycarbonylamino)alkyl]phosphinyl}propanoyl Alanine Benzyl Ester 7 and 8. To a solution of (2*S*)2-biphenylmethyl-3-{hydroxy[(1′*R*)-1′-(*N*-*tert*-butoxycarbonylamino)alkyl] phosphinyl}propanoic acid (1 equiv) and DIEA (1 equiv) in THF was added a solution of the chlorhydrate of alanine benzyl ester (1 equiv) and DIEA (1 equiv) in THF, a solution of HOBT (1 equiv) in CH_2Cl_2, EDCI (4 equiv), and DIEA (1 equiv). After being stirred for 30 min at room temperature and evaporation of the solvent, the mixture was taken off with water and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH (9:1) as eluents.

Compound 7: white solid; mp 138−139 °C (47%); HPLC (60% B) 11.2 min; [1]H NMR (DMSO-d_6 and TFA): 1.1 (m, 3H, $CH_3\beta$); 1.25 (d, 3H, CH_3); 1.3 (s, 9H, tBu); 2.65−3.05 (m, 2H, P-CH_2); 2.7 (m, 1H, $CHCO_2$); 2.95 (m, 2H, CH_2Ar); 3.65 (m, 1H, CHα); 4.25 (m, 1H, CH); 5.05 (s, 2H, CH_2Ph); 7.0−7.7 (m, 15H, Ar, NH); 8.4 (d, 1H, NH).

Compound 8: white solid; mp 174−176 °C (40%); HPLC (60% B) 19.9 min; [1]H NMR (DMSO-d_6 and TFA): 1.2 (d, 3H, CH_3); 1.25 (s, 9H, tBu); 1.4−2.1 (m, 2H, P-CH_2); 2.65 (m, 1H, $CHCO_2$); 2.9 (m, 2H, CH_2Ar); 4.3 (m, 1H, CH); 4.8 (m, 1H, CHα); 5.05 (s, 2H, CH_2Ph); 7.1−7.55 (m, 20H, Ar, NH); 8.4 (d, 1H, NH).

General Procedure for the Synthesis of (2*S*)2-Biphenylmethyl-3-{alkyloxy[(1′*R*)1′-aminoalkyl)]phosphinyl}-propanoyl Alanine Benzyl Ester 9 and 10. Method A: To a solution of (2*S*)2-biphenylmethyl-3-{hydroxy[(1′*R*)1′-(*N*-*tert*-butoxycarbonylamino)alkyl] phosphinyl} propanoyl alanine benzyl ester **7** or **8** (1 equiv) and ROH (3−5 equiv) in DMF were added DIEA (3−4 equiv) and BOP (3−4 equiv). Different alcohols ROH were used, benzyl alcohol or three *S*-acyl-2-thioethanols described in Lefebvre et al.[22]; *S*-(2-hydroxyethyl)-thioacetate, *S*-(2-hydroxyethyl)thiopivaloate, and *S*-(2-hydroxyethyl)thiobenzoate. The mixture was stirred for 2−3 h at room temperature, taken off with water, and extracted with EtOAc. The organic layer was washed with water and brine and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography on silica gel using Chex/EtOAc (1.5:1) as eluents.

A solution of the previous compound (2−3 mM) in HCO_2H was stirred for 2−4 h at room temperature. After evaporation of the solvent, the residue was dissolved in ethyl acetate, washed with 10% $NaHCO_3$ until pH = 7, washed with water and brine, and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography on silica gel using CH_2Cl_2/MeOH (9:1) as eluents.

Then, the pure compounds were dissolved in Et_2O, and 1 equiv of CF_3COOH or CH_3SO_3H was added. After the mixture was stirred for 15 min, the precipitate formed was filtered, washed with Et_2O and a 50/50 mixture of Et_2O/Hex, and dried under vacuo.

Method B: This procedure is described in Petrillo, Jr., et al.[21] To a solution of 2-biphenylmethyl-3-{hydroxy[(1′*R*)1′-(*N*-*tert*-butoxycarbonylamino)alkyl]phosphinyl}propanoyl alanine benzyl ester (1 equiv) and various alkyl halides RX (3−5 equiv) in $CHCl_3$ were added NaI (1 equiv), $(n\text{-}Bu)_4NHSO_4$ (0.5 equiv), and Et_3N (3 equiv). The mixture was maintained at reflux for 7−8 h, cooled, washed with water, and dried over Na_2SO_4. After filtration and evaporation of the solvent, the residue was purified by chromatography on silica gel using Chex/EtOAc (1.5:1) as eluents.

Three alkyl halides RX were used: benzyl bromide and *tert*-butylchloroacetate, which were commercially available (Aldrich, France), and isobutylchlorobenzoate which was prepared from 2-methylpropionaldehyde and benzoyl chloride (Aldrich, France) as described in ref 27.

The compounds obtained were then deprotected as described in method A.

Compound 9a (method A or B): white solid; mp 107−109 °C (56%); HPLC (50% B) 7.9 and 8.7 min; [1]H NMR (DMSO-d_6 and TFA): 1.2 (m, 6H, $CH_3\beta$, CH_3); 1.6−2.4 (m, 2H, P-CH_2); 2.6 (m, 1H, CHCO); 2.95 (m, 2H, CH_2Ar); 3.5 (m, 1H, CHα); 4.15−4.3 (m, 1H, $CHCO_2$); 4.95 (d, 2H, $POCH_2$); 5.05 (d, 2H, CH_2Ph); 7.15−7.6 (m, 19H, Ar); 8. (s, br, 3H, NH_3); 8. (dd, 1H, NH), MS (ESI) $(M + 1)^+$ m/z = 599.1. Anal. ($C_{35}H_{39}N_2O_5P$) C, H, N.

Compound 9b (method B): white solid; mp 104−106 °C (47%); HPLC (50% B) 7.1 and 8.3 min; [1]H NMR (DMSO-d_6 and TFA): 0.8 (m, 6H, 2xCH_3); 1.1−1.35 (m, 7H, $CH_3\beta$, CH_3, CH); 1.5−2.0 (m, 2H, P-CH_2); 2.55 (m, 1H, CHCO); 2.95 (m, 2H, CH_2Ar); 3.45 (m, 1H, CHα); 4.35 (m, 1H, $CHCO_2$); 5.05 (s, 2H, CH_2Ph); 6.0−6.2 (m, 1H, OCHO); 7.2−7.6 (m, 14H, Ar); 8.15 (s, br, 3H, NH_3); 8.7 (m, 1H, NH), MS (ESI) $(M + 1)^+$ m/z = 623.1. Anal. ($C_{34}H_{43}N_2O_7P$) C, H, N.

Compound 9c (method A): white solid; mp 113−114 °C (66.4%); HPLC (50% B) 6.4 and 6.9 min; [1]H NMR (DMSO-d_6 and TFA): 1.0−1.25 (m, 6H, $CH_3\beta$); 1.3 (m, 3H, CH_3); 1.65−2.4 (m, 2H, P-CH_2); 2.2 (d, 3H, $COCH_3$); 2.6 (m, 1H, CHCO); 2.95 (m, 4H, CH_2S, CH_2Ar); 3.45 (m, 1H, CHα); 3.95 (m, 2H,

$POCH_2$); 4.3 (m, 1H, $CHCO_2$); 5.05 (s, 2H, CH_2Ph); 7.15−7.6 (m, 14H, Ar); 8.12 (s, br, 3H, NH_3); 8.4 (dd, 1H, NH), MS (ESI) $(M + 1)^+$ m/z = 611.4. Anal. ($C_{32}H_{39}N_2O_6SP$) C, H, N.

Compound 10a (method A or B): white solid; mp 102−104 °C (67%); HPLC (50% B) 12.4 and 13.9 min; ^{1}H NMR (DMSO-d_6 and TFA): 1.15 (m, 3H, CH_3); 1.4−2.4 (m, 2H, P-CH_2); 2.5−3.05 (m, 3H, CHCO, CH_2Ar); 4.15 (m, 1H, $CHCO_2$); 4.3−5.0 (m, 2H, $POCH_2$); 4.8 (m, 1H, CHα); 5.05 (d, 2H, CH_2Ph); 7.0−7.6 (m, 24H, Ar); 8.5 (d, 1H, NH); 8.8 (s, br, 3H, NH_3), MS (ESI) $(M + 1)^+$ m/z = 661.4. Anal. ($C_{40}H_{41}N_2O_5P$) C, H, N.

Compound 10b (method A): white solid; mp 106−107 °C (72%); HPLC (50% B) 10.1 and 11.2 min; ^{1}H NMR (DMSO-d_6 and TFA): 1.28 (m, 3H, CH_3); 1.35−2.15 (m, 2H, P-CH_2); 2.25 (d, 3H, $COCH_3$); 2.6−3.1 (m, 5H, CH_2S, CH_2Ar, CHCO); 3.3−4.0 (m, 2H, $POCH_2$); 4.35 (m, 1H, $CHCO_2$); 4.78 (m, 1H, CHα); 5.05 (s, 2H, CH_2Ph); 7.05−7.6 (m, 19H, Ar); 8.55 (dd, 1H, NH); 8.85 (s, br, 3H, NH_3), MS (ESI) $(M + 1)^+$ m/z = 673.4. Anal. ($C_{37}H_{41}N_2O_6SP$) C, H, N.

Compound 10c (method A): white solid; mp 117−118 °C (63%); HPLC (60% B) 8.0 and 8.9 min; ^{1}H NMR (DMSO-d_6 and TFA): 1.05 (d, 9H, tBu); 1.25 (m, 3H, CH_3); 1.4−2.3 (m, 2H, P-CH_2); 2.6−2.95 (m, 3H, CH_2Ar, CHCO); 3.0−4.0 (m, 4H, $POCH_2CH_2$); 4.3 (m, 1H, $CHCO_2$); 4.75 (m, 1H, CHα); 5.05 (s, 2H, CH_2Ph); 7.05−7.6 (m, 19H, Ar); 8.55 (dd, 1H, NH); 8.8 (s, br, 3H, NH_3), MS (ESI) $(M + 1)^+$ m/z = 715.4. Anal. ($C_{40}H_{47}N_2O_6SP$) C, H, N.

Compound 10d (method A): white solid; mp 113−114 °C (77%); HPLC (55% B) 10.9 and 11.8 min; ^{1}H NMR (DMSO-d_6 and TFA): 1.25 (m, 3H, CH_3); 1.4−2.4 (m, 2H, P-CH_2); 2.5−3.1 (m, 3H, CH_2Ar, CHCO); 3.0−4.15 (m, 4H, $POCH_2CH_2$); 4.35 (m, 1H, $CHCO_2$); 4.8 (m, 1H, CHα); 5.05 (s, 2H, CH_2Ph); 7.05−7.9 (m, 24H, Ar); 8.57 (dd, 1H, NH); 8.85 (s, br, 3H, NH_3), MS (ESI) $(M + 1)^+$ m/z = 735.4. Anal. ($C_{42}H_{43}N_2O_6SP$) C, H, N.

Compound 10e (method B): white solid; mp 97−99 °C (31%); HPLC (60% B) 9.7 and 10.3 min; ^{1}H NMR (DMSO-d_6 and TFA): 1.0 (m, 3H, tBu); 1.3 (m, 3H, CH_3); 1.4−2.4 (m, 2H, P-CH_2); 2.6−3.1 (m, 3H, CH_2Ar, CHCO); 4.35 (m, 1H, $CHCO_2$); 4.75 (m, 1H, CHα); 5.05 (s, 2H, CH_2Ph); 5.1−5.6 (m, 2H, OCH_2O); 7.0−7.6 (m, 19H, Ar); 8.6 (dd, 1H, NH); 8.9 (s, br, 3H, NH_3), MS (ESI) $(M + 1)^+$ m/z = 685.8. Anal. ($C_{39}H_{45}N_2O_7P$) C, H, N.

Compound 10f (method B): white solid; mp 100−101 °C (49%); HPLC (60% B) 17.4, 21.7, and 22.5 min; ^{1}H NMR (DMSO-d_6 and TFA): 0.85 (m, 6H, 2 × CH_3); 1.3 (m, 3H, CH_3); 1.4−2.4 (m, 3H, P-CH_2, CH); 2.5−3.1 (m, 3H, CH_2Ar, CHCO); 4.4 (m, 1H, $CHCO_2$); 4.8 (m, 1H, CHα); 5.05 (d, 2H, CH_2Ph); 5.9−6.5 (m, 1H, OCHO); 7.0−7.95 (m, 24H, Ar); 8.7 (dd, 1H, NH); 8.85 (s, br, 3H, NH_3), MS (ESI) $(M + 1)^+$ m/z = 747.4. Anal. ($C_{44}H_{47}N_2O_7P$) C, H, N.

II. HPLC Studies of Compound 10b Bioactivation. The in vitro bioactivation of prodrug **10b** was monitored by HPLC. This compound (25 μM final concentration) was incubated at 37 °C in the presence of rat serum (3.2 mg protein/mL) or rat brain membranes (5 mg protein/mL) in 450 μL of 50 mM Tris-HCl buffer pH 7.4. The metabolic process was stopped by addition of 50 μL of 4 M $HClO_4$. The preparation was cooled at 0 °C for 10 min, and 200 μL of CH_3CN was added. After being stirred vigorously, the mixture was centrifuged for 5 min at 100000*g*. Controls were performed in the same conditions with proteins inactivated by prior addition of 4 M $HClO_4$. The supernatants were analyzed by HPLC on a kromasil C_{18} column (5 μm, 100 Å) with a mixture of 0.05% TFA in H_2O and acetonitrile as mobile phase. Elution of the prodrug **10b** was obtained with 50% acetonitrile. Two peaks were observed due to the presence of a chiral phosphorus atom (t_R = 19.4 and 22.8 min). The metabolites formed during the reaction were analyzed by comparison with synthetic markers. The intermediate metabolite was identified as H_2N-CH(Ph)-P(O)-($OCH_2CH_2SCOCH_3$)-CH_2CH(CH_2-Bip)-CONH-CH(CH_3)-CO-OH by coelution with the synthetic analogue obtained by selective clivage, with TFA, of both the Boc and *tert*-butyl groups of Boc-NH-CH(Ph)-P(O)-($OCH_2CH_2SCOCH_3$)-CH_2-CH-(CH_2-Bip)-CONH-CH(CH_3)-COOtBu. This intermediate has a chiral phosphorus atom and gives two peaks in HPLC (t_R = 4.8 and 5.5 min in 50% CH_3CN); t_R = 8.3 min (in 35% CH_3-CN) for the deprotected drug **2**.[18]

III. Pharmacological Assays. Animals. Male Swiss albino mice (20−22 g) (Charles River, France) were used. Animals were housed in groups of 50 mice for at least 2 days before the experiments, and food and water were available ad libitum. Each animal was used only once. All antinociceptive measurements were recorded between 9:00 a.m. and 7:00 p.m.

Hot Plate Test. The test was based on that described by Eddy and Leimbach.[28] A glass cylinder (16 cm high, 16 cm diameter) was used to keep the mouse on the heated surface of the plate which was kept at a temperature of 52 ± 0.5 °C using a thermoregulated water circulating pump. The latency period until the mouse licked its paws or jumped was registered by a stop watch (cut-off time, 240 s). The data are expressed in percent of analgesia using the following equation: percent analgesia (test latency − control latency)/(cut-off time − control latency) × 100. Statistical analysis was carried out by ANOVA followed by the Dunnett's *t* test.

Systemic Injections. Prodrugs or vehicle were slowly (15 s) iv or ip injected in mice in a volume of 0.1 mL/10 g in mice as previously described.[15,18] Prodrugs were solubilized in a mixture of cremophore/ethanol/water = 10/10/80.

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