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Antinociceptive and anti-allodynic effects of oral PL37, a complete inhibitor of enkephalin-catabolizing enzymes, in a rat model of peripheral neuropathic pain induced by vincristine

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ABSTRACT

Vincristine is a common anti-cancer therapy administered for the treatment of many types of tumors. Its dose-limiting side effect is the production of peripheral neuropathy, resulting in chronic neuropathic pain in many patients. An animal model of vincristine-induced sensory neuropathy was developed after repeated intraperitoneal injection in male rats and used in the present work to study the effects of PL37, an orally active complete dual inhibitor of enkephalin-catabolizing enzymes, on mechanical hypersensitivity and allodynia and on cold allodynia. We used the Electronic Von Frey filament (mechanical static allodynia), acetone test (cold allodynia), and a new behavioural test we first describe in this study, the "paint-brush test" which evaluates dynamic mechanical allodynia and dynamic mechanical hypersensitivity. We used a smooth paint brush leading to an innocuous stimulus, and a rough-one leading to an intense mechanical stimulus. Mechanical hypersensitivity and allodynia due to vincristine-induced neuropathy, but not cold allodynia, are strongly reduced by oral or i.p. injected PL37, the dose-dependent effects being reversed by naloxone-methiodide supporting the peripheral action of the dual inhibitor. These results show that enkephalins protected from degradation by PL37 could bind to peripheral opioid receptors expressed only on C- and Aδ-mechanonociceptors but not on cold thermonociceptors. The fact that PL37 is also active on small intensity mechanical stimulus could reveal an expression of opioid receptors on low threshold mechanoreceptors in the vincrisitine-evoked pathological conditions. Thus the increase in endogenous enkephalin levels induced by PL37 offers a new way to reduced neuropathic pain without the possible side effects of opiates.

1. Introduction

Vincristine is one of the most common anti-cancer therapy administered for the treatment of many types of cancers such as leukaemias, lymphomas and sarcomas. However, its dose-limiting side effect is the production of peripheral neuropathy, which in many patients is accompanied by a chronic neuropathic pain syndrome (reviewed in Dougherty et al., 2004) that requires discontinuation of treatment and thus greatly impacts on the survival of cancer patients (Casey et al., 1970). The incidence and severity of vincristine-induced neuropathic pain is positively correlated with the period and doses used. The anti-tumor action of vincristine is due to its binding to β-tubulin, which leads to its antimitotic property leading to aborted cell division and cell death (Wilson et al., 1975). Axonal microtubules also contain β-tubulin, and vincristine property to bind to β-tubulin is thought to induce peripheral neuropathies leading to chronic pain through its neurotoxic action on peripheral sensory fibres.

Using doses close to those used clinically, an animal model of vincristine-induced nociceptive sensory neuropathy after repeated intraperitoneal injection in male rats has been developed (Authier et al., 2003; Weng et al., 2003). The sensory behavioural assessment revealed mechanical hyperalgesia and allodynia associated with cold thermal hyperalgesia and allodynia (Authier et al., 2003). We developed this model of vincristine-induced painful neuropathy to test new analgesic drugs.

From a pharmacological standpoint, opioids in general and morphine in particular constitute the mainstay of treatment for pain resulting from excessive nociception, whether severe acute pain (such as post-operative pain), chronic cancer-related pain, or certain pain syndromes not related to cancer, e.g. pain resulting from chronic inflammation. Controversy over the use of morphine due to its limited efficacy for the treatment of neuropathic forms of pain has been building up for over a decade (Dellemijn, 1999). However studies using some experimental models of chronic neuropathic pain in animals based on the lesion of

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peripheral nerves (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992) have tended to demonstrate the efficacy of opiates both in elevating the thresholds or latencies of response of these "neuropathic" animals to mechanical or thermal nociceptive (hyperalgic) or non-nociceptive (allodynic) stimuli and in lowering the scores assessing the spontaneous behavioural reactions related to the pain experienced by these animals. These results are clearly in favour of the use of morphine or other selective opioid receptor agonists on the grounds of their efficacy in relieving chronic neuropathic pain (see, for example, Attal et al., 1991; Desmeules et al., 1993; and in particular, Martin et al., 1998).

Opioids, such as morphine, are widely used in the clinical management of pain, but unfortunately, limited by various side-effects (tolerance, dependence, respiratory depression, constipation …). To reduce such side-effects liability there is still considerable interest in the strategy based on the protection of extracellularly released endogenous opioid peptides (enkephalins) from their inactivation by two ectopeptidases : neprilysin and aminopeptidase N (review in Roques et al., 1993). Thus it has been shown that such protection by peptidase inhibitors provide analgesia with reduced side effects comparatively to exogenous opioids (reviews in Jutkiewicz, 2007 and in Noble and Roques, 2007). As previously studied, a dual inhibitor, RB101, induces in inflammatory pain potent antinociceptive responses in normal and mononeuropathic rats after systemic administration (Noble et al., 1992; Maldonado et al., 1994), by elevating the extracellular levels of enkephalins (Dauge et al., 1996; Nieto et al., 2001). Furthermore, dual inhibitors have been previously shown to produce antinociception in rats with a constriction of the sciatic nerve (Lee et al., 1994) and in diabetic neuropathic rats (Coudore-Civiale et al., 2001). However inhibitors of the two enkephalin inactivating enzymes have never been studied in vincristine-induced neuropathic pain. Accordingly, the present work was carried out to study the effects of PL37, an orally active dual inhibitor of neprilysin and aminopeptidase N on mechanical static allodynia and on cold thermal allodynia in neuropathic rats. Moreover, with the aim of studying mechanical dynamic allodynia and hypersensitivity we have developed a new original test: "the paint-brush test" which is described for the first time in this paper and used to investigate the effect of PL37. These parameters are highly relevant in patients suffering from neuropathic pain, and clinically different from mechanical static allodynia and hypersensitivity. Furthermore, the involvement of peripheral opioid receptors has been assessed using naloxone-methiodide, an opioid receptor antagonist which does not cross the blood-brain barrier (Milne et al., 1990).

2. Materials and methods

2.1. Experimental animals

Three experiments were performed on 87 adult male Sprague–Dawley rats (Charles River, France), weighing 175–200 g at their arrival. The guidelines proposed by the Ethical Committee of the IASP (Committee for Research and Ethical issues of the IASP, 1980) for investigations of experimental pain in conscious animals have been followed and all experiments were carried out in accordance with the Declaration of Helsinki and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Accordingly, the number of animals was kept to a minimum. Great care was taken, particularly with regard to housing conditions, to avoid or minimize discomfort of the animals: rats were housed four to a cage to minimize the possibility of painful interactions. The animals were kept on solid floor cages with a deep layer of sawdust to accommodate the excess of urination and cages were changed daily. They were kept at a constant temperature of 22 °C, with a 12 h alternative light/dark cycle. Food and water were available *ad libitum*. All animals were housed during two weeks before the beginning of the experiments, a period during which they were able to familiarize with the investigator and get used to the experimental room and to the various apparatus likely to be used for pain or allodynia tests. At the beginning of the experiment (D1) their weight was 312.3 ± 3.9 g.

2.2. Induction of peripheral neuropathic pain and drugs treatment

Rats were intraperitoneally injected with vincristine (or saline in control animals in the first experiment) according to the protocol proposed by Weng et al. (2003): they were treated with i.p. bolus injections of vincristine (Oncovin, EGLabo; 0.1 mg/kg/day diluted to 1 ml in saline, Lavoisier). The drug was again administered daily in two 5-day cycles (D1–D5 and D8–D12) with two days pause between cycles. Animals showed no acute reaction to the drug injection. Mortality level was 8% (7 rats upon 87), which occurred in the second cycle of vincristine injections (between D8 and D12). No noticeable motor disorder was observed during the all experimental period.

A preliminary experiment was first designed in order to evaluate neuropathic pain induced by the vincristine chemotherapeutic treatment with the various behavioural tests we used (vincristine-treated, $n=7$ and saline-treated, $n=6$).

Three experiments were then realized in order to evaluate the effects of PL37 1- 2-[(1-ethoxy-carbonyloxy-ethoxycarbonylmethyl)-carbamoyl]-3-phenyl-propyldisulfanylmethyl -3-methylsulfanyl-propyl-ammonium. On D15, PL37 was i.p. administrated to rats (70 mg/kg in saline solution) or *per os* (50 or 100 mg/kg in solvent: ethanol 10%, PEG 40% and water 50%). Naloxone-methiodide (2 mg/kg) or saline was i.p. administered immediately after PL37 administration. All drugs were freshly prepared daily.

2.3. Experimental design

Behavioural tests were performed on D15, and consisted of the "paint-brush" tests, the Electronic Von Frey test and finally the acetone test. All behavioural tests were realized at three time points: before the administration of PL37 or of its solvent, 20 min and 50 min after.

Three series of experiments were conducted according to the way of administration of PL37.

Drugs were injected as follows:

Experiment 1: we compared the effects of i.p. PL37 (70 mg/kg; $n=7$) and of saline (i.p.; $n=7$) in vincristine-pre treated animals.

Experiment 2: we tested the dose effects of PL37 administered *per os* in two doses (50 mg/kg, $n=6$, and 100 mg/kg, $n=7$) and of its solvent (ethanol 10%, PEG 400 40% and water 50%; $n=6$) in vincristine-pre treated animals.

Experiment 3: we tested the effects of naloxone-methiodide on PL37 administered *per os* (100 mg/kg, $n=7$) in vincristine-pre treated animals, comparing four groups of animals in which PL37 and its solvent were administered *per os* and naloxone-methiodide and saline administered i.p.: PL37 (100 mg/kg)/naloxone-methiodide (2 mg/kg) ($n=7$); PL37 (100 mg/kg)/saline ($n=7$); solvent/naloxone-methiodide (2 mg/kg) ($n=6$); solvent/saline ($n=6$).

All drugs were administered in a volume of 1 ml/kg.

All the experiments included for each treatment were realized at the same time in the afternoon (between 1 and 4 P.M.); they were performed blind by the same person using a randomized block to avoid any chronobiological effects, and to assess the effects of the different treatments under the same environmental conditions.

2.4. Pain behaviour testing

2.4.1. Assessment of mechanical static allodynia

Rats were individually placed on an elevated wire mesh floor in a clear plastic cylinder (22 cm diameter) and were adapted to the testing environment for 10 min. An Electronic Von Frey hair unit (EVF-3, Bioseb,

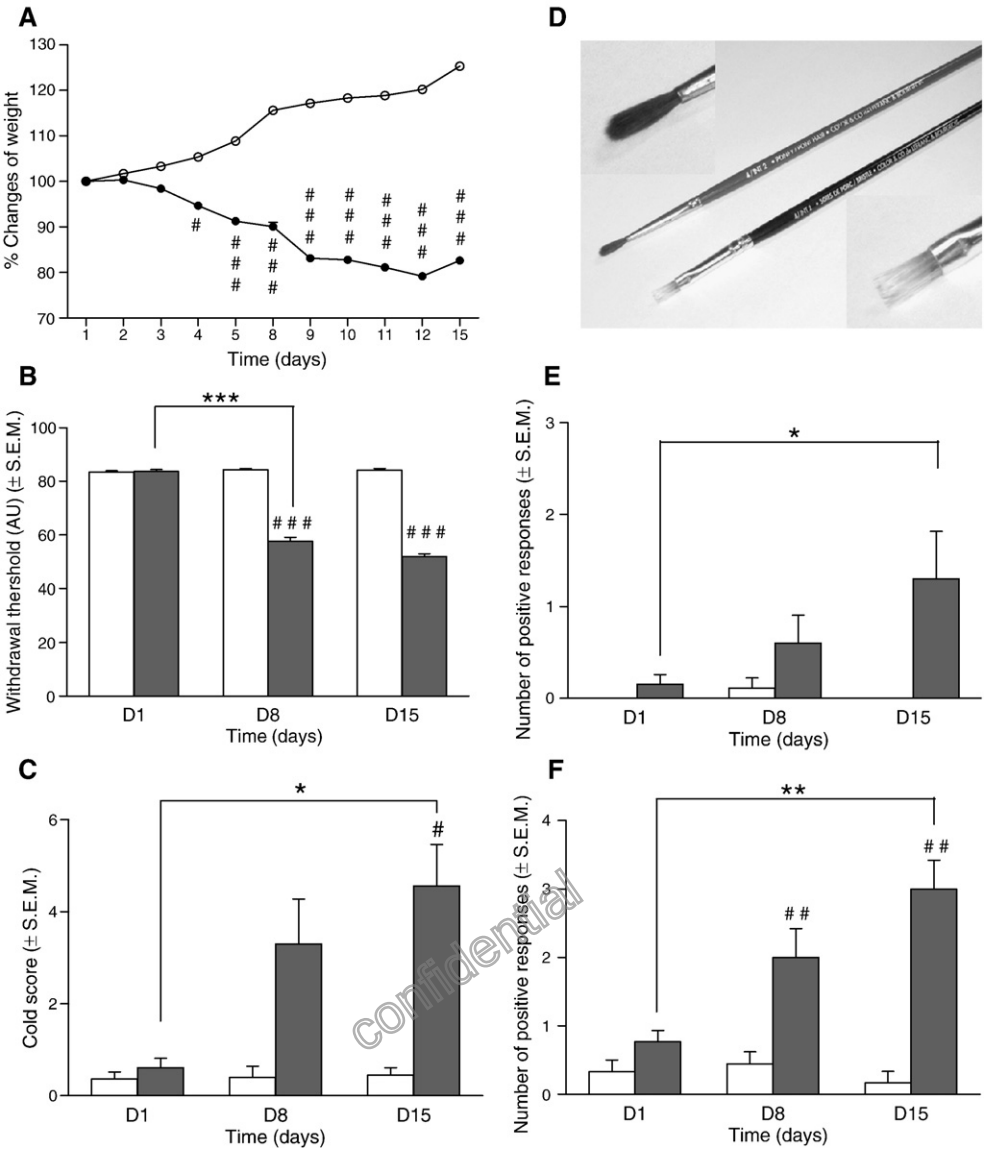

Fig. 1. Time-course of weight gain and pain-related behaviour, static and dynamic mechanical allodynia, dynamic mechanical hyperesthesia, and cold thermal allodynia, in control and vincristine-treated animals. A: Changes in the mean daily body weight in vincristine-treated (black dots) and saline-treated rats (empty dots). Vincristine-treated rats failed to gain weight as expected from the saline-treated group (#$P \leq 0.5$, ###$P \leq 0.001$ vincristine-treated rats *versus* controls rats). B: Mean withdrawal threshold to an increasing pressure on the hind-paw with Electronic Von Frey test. The withdrawal threshold was significantly different in vincristine-treated rats (dark grey bars) compared to control rats (empty bars) as soon as D 8 and maintained at D15 (***$P \leq 0.001$ vincristine-treated rats between D1 and D8, ###$P \leq 0.001$ vincristine-treated rats *versus* controls rats). C: Mean cold scores obtained with the acetone test were slightly significantly increased at day 8, but significantly higher at day 15 compared to control animals. Data are expressed in mean ± S.E.M. (*$P \leq 0.05$ vincristine-treated rats between D8 and D15, #$P \leq 0.05$ vincristine-treated rats *versus* controls rats). D: Photos of paint-brushes used to stimulate plantar area of the hind paw of the rats. In the upper left corner, magnification of the smooth paint-brush, and in the lower right corner, magnification of the rough paint-brush. E: Mean numbers of positive responses to the smooth paint-brush test. At D8 and D15, the mean number of positive response was significantly increased in vincristine-treated rats (dark grey bars) compared to control rats (#$P \leq 0.05$). F: Mean numbers of positive responses to the rough paint-brush test. The mean numbers of positive responses was significantly different in vincristine-treated rats (dark grey bars) compared to control rats (empty bars) as soon as D8 and was significantly increased in D15. Data are expressed in mean ± S.E.M. (**$P \leq 0.01$ vincristine-treated rats between D1 and D15; ##$P \leq 0.01$ vincristine-treated rats *versus* controls rats).

Chaville, France) was used: the sensitivity threshold is measured in one test, measurement ranging from 0.1 to 100 g with a 0.2 g accuracy. Punctuate stimulus is delivered to the mid-plantar area of each hind paw from below the mesh floor through a plastic spring tip and sensibility threshold result is displayed on a screen. Paw sensitivity threshold was defined as the minimum pressure required eliciting a robust and immediate withdrawal reflex of the paw. Voluntary movement associated with locomotion was not taken as a withdrawal response. The stimulus was applied on each hind paw five times with a five seconds interval and the value adopted as a threshold for a rat was the average of the ten values measured. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to EVF-3 application. For pharmacological studies, the results were expressed for each group as follow: Δ-paw withdrawal threshold (mean ± S.E.M.) in arbitrary unit (AU), calculated from individual paw withdrawal thresholds at each time, as followed:

Δ- paw withdrawal = t20 (or t50) paw withdrawal value - baseline value

Analgesia effect on mechanical allodynia was defined as a significant increase in Δ-paw withdrawal threshold to EVF-3 application.

2.4.2. Assessment of mechanical dynamic allodynia and mechanical dynamic hypersensitivity: the "paint-brush test"

This behavioural test, the "paint-brush" test allowed to explore dynamic responses to a mechanical stimulus. Animals were placed in

the plastic cylinder on the wire mesh floor and a paint-brush was used to rub the plantar area of each hind paw from the heel to the toes as a stimulus. Two paint-brushes were used, one very smooth made of marten hairs and the other rough made of bristles pig (Fig. 1D). The smooth paint-brush was firstly used then followed by the rough paint-brush. The stimulus was applied on each hind paw five times with a five seconds interval and the value adopted using each paint-brush for a rat was the average score of withdrawals (for each paw between 0 and 5) got from each hind paw. Vincristine-induced responses to smooth paint-brush are best described as allodynia because normal rats never withdraw from this stimulus and responses to rough paint-brush are described as mechanical hypersensitivity because normal rats withdraw from this stimulus 5–10% of the time.

2.4.3. Assessment of cold thermal allodynia

Animals were placed in the plastic cylinder on the wire mesh floor and a drop (0.05 ml) of acetone was placed against the centre of the ventral side of the hind paw. In the following 40 s after acetone application, the rat's response was monitored. Responses of the rat to acetone were graded to a 4-point scale as defined by Flatters and Bennett (2004): 0, no response; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking; 3, repeated flicking of the paw with licking directed at the ventral side of the paw. Acetone was applied alternately three times to each paw and the responses scored categorically. Cumulative scores were then generated by adding the six scores for each rat together, the minimum score being 0 and the maximum possible score being 18.

2.5. Statistical analysis

The behavioural data are presented as mean±S.E.M. at different time point after the beginning of the vincristine treatment or drug administrations. Statistical analysis of changes in mechanical and cold thresholds were carried out using a two-way ANOVA (electrical von Frey) or a Kruskal–Wallis test (paint-brush test, acetone test) following by Student Newman–Keuls test (electrical von Frey test) or Wilcoxon matches pairs test and Mann–Whitney *U* test (paint-brush test, acetone test), to compared specific data points. $P<0.05$ was considered statistically significant in all tests.

3. Results

3.1. Vincristine chemotherapeutic treatment induced neuropathic pain

The vincristine-injected rats (V-rats) lost a mean of 17.36% of their body weight during treatment (82.64±2.18%) whereas, in the first experiment, saline-injected control animals (C-rats) increased of their body weight 25% (125±1.2%) during the same period (Fig. 1A).

As measured with the EVF-3, the mean withdrawal reflex of the paw was significantly reduced in vincristine-treated rats (57.74±0.83 in arbitrary units (AU) at D8 *versus* 83.28±0.63 at D1) ($P\leq0.001$) (dark grey bar) when compared with control rats (empty bar), a decrease which persisted at D15 (51.93±0.99) ($P\leq0.001$) (Fig. 1B). This result is interpreted as a mechanical static allodynia.

Control rats only rarely responded to acetone, their mean "cold score" was between 0.36±0.15 and 0.45±0.16. At day 15, vincristine-treated rats responded to acetone, with a "cold score" of 4.55±0.98, which was statistically different from the score of control rats ($P\leq0.05$) (Fig. 1C). This result is interpreted as a cold thermal allodynia.

Rats treated with vincristine, but not those treated with saline, showed significant increased responses to smooth and rough paint-brush stimulation at D8 and D15 of vincristine treatment respectively. This increased response persisted throughout the treatment. Vincristine-treated rats exhibited a number of positive responses of 1.3±0.5 at D15, significantly different to the beginning of the treatment (0.15±0.1) at D1, to smooth paint-brush ($P\leq0.05$) (Fig. 1E). This result is interpreted as a mechanical dynamic allodynia. In the same way, vincristine-treated rats presented a significant increased number of positive responses to rough paint-brush stimulation at D8 and D15 of the treatment significantly different from the saline injected group ($P\leq0.01$). In fact, vincristine-treated rats exhibited a number of positive response of 2±0.4 at D8 and 3±0.16 at D15, significantly different to the beginning of the treatment at D1, to rough paint-brush stimulation ($P\leq0.01$) (Fig. 1F). This result is interpreted as a dynamic mechanical hypersensitivity.

3.2. Experiment 1: Effects of PL37 (70 mg/kg i.p.) or saline alone in vincristine-treated rats

As evaluated with the EVF-3, before drug injection (*T*0), the mean withdrawal reflex threshold of the paw was not significantly different in both V-rats groups (PL37 and saline). PL37 i.p. injected significantly increased this threshold in V-rats 20 min after, whereas saline was without effect ($P\leq0.001$). In fact, in PL37-treated V-rats, we observed an increased of Δ-paw withdrawal threshold of 8.14±2.07 at 20 min when compared with *T*0 value; the Δ-paw withdrawal change in saline V-rats between *T*0 and *T*20 min was of −0.31±0.55. Δ-paw withdrawal value between *T*0 and *T*50 min was not significantly different in both V-groups (PL37 and saline) (1.08±0.92 *versus* −2.26±1.38 respectively) (Fig. 2).

PL37 was without effect on the cold scores in both groups of rats (data not presented).

3.3. Experiment 2: Effects of two doses of PL37 administered per os in V-rats

Two doses of PL37 were tested in this experiment (PL37 50 mg/kg and 100 mg/kg *per os*/solvent) in V-rats. The baseline values were determined before oral administration of compounds and no statistical differences were observed between the three tested groups.

In the electrical von Frey test, both PL37 orally-administered doses (50 mg/kg and 100 mg/kg) induced an antiallodynic dose-dependant effect at *T*20 min. We observed an increased of Δ-paw withdrawal thresholds at *T*20 min in V-rats who received oral administration of PL37 at 50 mg/kg and 100 mg/kg (3.4±2.99 and 14.36±1.02 respectively *versus* −2.77±1.17 for saline group) ($P\leq0.05$; $P\leq0.001$). Δ-paw withdrawal thresholds at *T*50 min was not significantly different in both PL37 groups when compared with saline group (−6.43±2.9 and −1.9±0.65 respectively for the two doses *versus* −0.43±1.17) (Fig. 3A).

With the smooth paint-brush test, before drug injection the average score was not significantly different between the three groups of rats (Fig. 3B). PL37 100 mg/kg significantly decreased the score

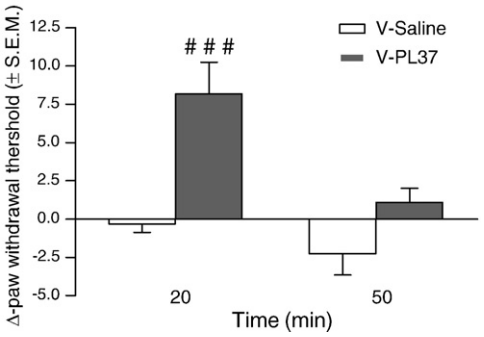

Fig. 2. Effects of PL37 (70 mg/kg i.p.) in vincristine-treated *versus* control rats: mean withdrawal threshold to an increasing pressure on the hind-paw with Electronic Von Frey procedure. Twenty minutes after i.p. injection of PL37, the Δ-paw withdrawal threshold was significantly increased in vincristine-pretreated rats and this effect was abolished 50 min after injection (dark grey bars). The Δ-paw withdrawal was not significantly different in saline-injected vincristine-treated rats (empty bars). Data are expressed in mean±S.E.M. (###$P\leq0.001$).

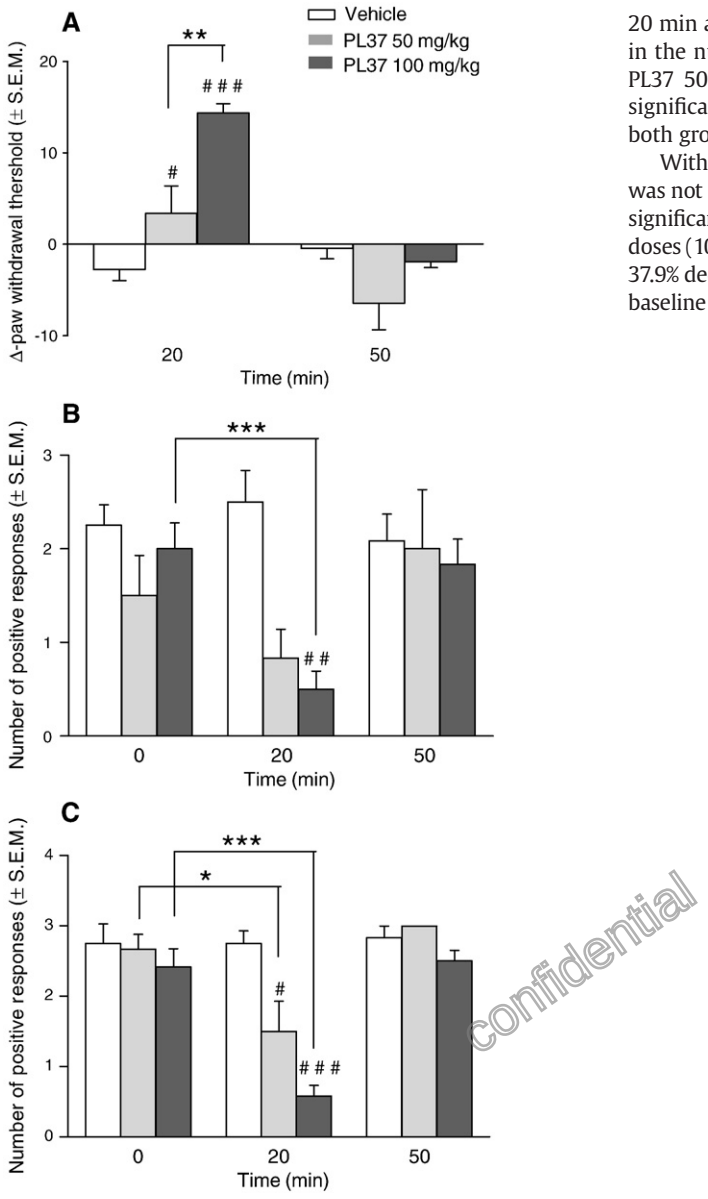

Fig. 3. Dose dependent effects of PL37 administered *per os* in vincristine-treated rats. A: Mean withdrawal threshold to an increasing pressure on the hind-paw with Electronic Von Frey procedure. Twenty minutes after injection of PL37 the Δ-paw withdrawal was significantly increased in vincristine-treated rats receiving the two doses of PL37 (50 and 100 m/kg) (#$P \leq 0.05$; ###$P \leq 0.001$); this effect was dose-dependent (**$P \leq 0.01$ vincristine-treated rats between 50 mg/kg and 100 mg/kg) and abolished 50 min after injection (light grey bars and dark grey bars). The Δ-paw withdrawal did not significantly differ at *T*20 and *T*50 in vincristine-treated rats who received saline injection (empty bars). Fifty minutes after administration of PL37, thresholds values return to their baseline values. B: Positive mean number responses to the smooth paint-brush. Before administration of PL37 (*T*0) this mean number was not significantly different in the three groups of rats. Twenty minutes after administration of PL37, the mean number of positive responses was significantly decreased in PL37 100 mg/kg (##$P \leq 0.01$) treated (V-rats dark grey bars) compared with control V-rats (empty bars). There is a clear tendency to a reduction in the mean number of positive responses in PL37-injected 50 mg/kg vincristine-treated rats (light grey bars) but the decrease was not statistically significant. Fifty minutes after administration of PL37, thresholds values return to their baseline. C: Positive mean number responses to the rough paint-brush tests. Before administration of PL37 (*T*0) this mean number was not significantly different in the three groups of rats. Twenty minutes after administration of PL37, the mean number of positive responses was dose-dependently significantly decreased in V-rats receiving one of the two doses of PL37 (50 and 100 m/kg) (light grey bars and dark grey bars) (*$P \leq 0.05$; ***$P \leq 0.001$) and significantly different in comparison with control V-rats (#$P \leq 0.05$; ###$P \leq 0.001$). Fifty minutes after administration of PL37, thresholds values return to their baseline. Data was expressed in mean ± S.E.M.

20 min after injection (75% decrease; $P \leq 0.001$) and a clear reduction in the number of positive responses was also observed 20 min after PL37 50 mg/kg injection (Fig. 3B) but the result was not statically significant. Fifty min later, threshold values return to their baseline in both groups of rats.

With the rough paint-brush, before drug injection the average score was not significantly different in the three groups of rats (Fig. 3C). PL37 significantly decreased dose-dependently the score 20 min after at both doses (100 mg/kg *per os*, 75.86% decrease, $P \leq 0.001$, and 50 mg/kg *per os*, 37.9% decrease, $P \leq 0.05$). Fifty min later, threshold values return to their baseline in both groups of rats.

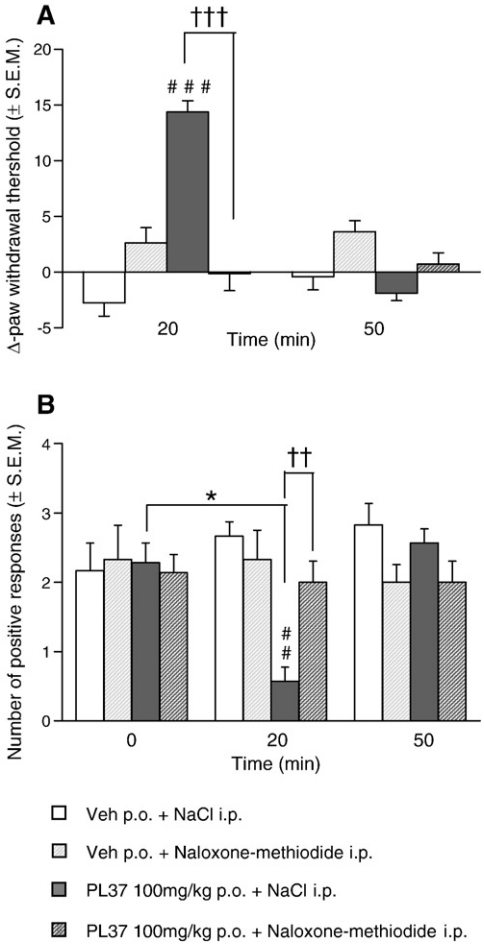

Fig. 4. Antagonizing effects of naloxone-methiodide i.p. injected on the antinociceptive effect of PL37 administered *per os* in vincristine-treated rats. A: Mean withdrawal threshold to an increasing pressure on the hind-paw as evaluated with Electronic Von Frey test. Twenty minutes after PL37 administration (*T*20), the Δ-paw withdrawal significantly increased in PL37 (100 m/kg)-saline vincristine-treated rats (dark grey bars) (###$P \leq 0.001$), when compared with the three other groups [solvent-saline (empty bars); solvent-naloxone-methiodide (hatched light grey bars); PL37-naloxone-methiodide treated rats (hatched black bars)]. The Δ-paw withdrawal significantly differ in PL37 (100 m/kg) vincristine-treated rats and PL37-naloxone-methiodide treated rats (†††$P \leq 0.001$). Fifty minutes after PL37 administration (*T*50), thresholds values return to their baseline values. Data are expressed in mean ± S.E.M. B: Mean positive number responses to the rough paint-brush. Before administration of PL37 (*T*0), the withdrawal threshold was not significantly different in the four groups of rats. Twenty minutes after PL37 administration (*T*20), the mean number of positive responses significantly decreased in PL37 (100 mg/kg)-saline vincristine-treated rats (dark grey bars) (##$P \leq 0.001$) and was significantly different when compared with the three other groups [solvent-saline (empty bars); solvent-naloxone-methiodide (hatched light grey bars); PL37-naloxone-methiodide treated rats (hatched black bars)]. The mean number of positive responses significantly differ in PL37 (100 m/kg) vincristine-treated rats and PL37-naloxone-methiodide treated rats (††$P \leq 0.01$). Fifty minutes after PL37 administration (*T*50), thresholds values return to their baseline values. Data are expressed in mean ± S.E.M.

PL37 was without effect on the cold scores in both groups of rats (data not presented).

3.4. Experiment 3: Effects of naloxone-methiodide i.p. injected on the antinociceptive effect of PL37 administered per os in V-rats

For testing the possible peripheral effects of PL37, naloxone-methiodide (2 mg/kg) was administered *per os* (100 mg/kg, $n=7$), and investigated with two behavioural tests in vincristine-pretreated animals: the Electronic Von Frey test and the rough paint-brush test.

As measured with the EVF-3, before drug injection (*T*0), the mean withdrawal reflex threshold of the paw was not significantly different in the four groups of rats. PL37 (100 mg/kg *per os*) significantly increased this threshold 20 min after ($P \leq 0.001$). Fifty min later, threshold values return to their baseline. Naloxone-methiodide i.p. injected antagonized the PL37 antiallodynic effects, measured at 100 mg/kg but *per se* had no effect on the threshold (Fig. 4A).

With the rough paint-brush, before drug injection (*T*0) the average score was not significantly different in the four groups of rats (Fig. 4B). As previously observed, PL37 significantly decreased the score 20 min later (*T*20) ($P \leq 0.01$), whereas these threshold values return to their baseline fifty min later. Naloxone-methiodide injected i.p. antagonized PL37 effects, but *per se* had no effect on the threshold (Fig. 4B).

As PL37 was without effect on the cold scores in experiments 1 and 2, we did not perform this test in experiment 3.

4. Discussion

In the present study, we investigated the anti-allodynic effects of a new mixed inhibitor of enkephalin degrading enzymes, PL37, systemically administered to neuropathic rats (intraperitoneal and oral route). With this aim, we used a classical test, the Electronic Von Frey filament (static allodynia) and a new behavioural test we first describe in this study, the "paint-brush test". This test evaluates dynamic mechanical allodynia and dynamic mechanical hypersensitivity by two different paint brushes, one smooth leading to an innocuous stimulus, the other rough triggering an intense mechanical stimulus. Furthermore, PL37 effect is dose-dependent and naloxone-methiodide reversible. Contrarily, PL37 was without any effect on cold allodynia. These results are in good agreement with those obtained with various dual inhibitors previously studied in other chronic neuropathic pain models such as in mononeuropathic (Attal et al., 1991; Lee et al., 1994) and neuropathic diabetic (Coudore-Civiale et al., 2001) models.

The analgesic potency of the peptidase inhibitors may directly depend on the importance of the extracellular release of endogenous opioid peptides caused by nociceptive stimuli (Bourgoin et al., 1986). The effects of endogenous enkephalins protected from degradation by PL37 on mechanical stimuli could result from their direct action on opioid receptors overexpressed at terminals of peripheral fibres. This is consistent with the complete reversion of responses induced by mechanical stimuli by naloxone-methiodide, a quaternary analogue of naloxone when it is used at a dose (2 mg/kg) unable to cross the blood brain barrier (Milne et al., 1990). Nevertheless one cannot completely exclude a weak participation of pre- or post-synaptic opioid receptors located at the lumbar spinal cord (Duggan and North, 1983; Dickenson et al., 1986; Lombard and Besson, 1989). Thus, these results show that by blocking enkephalin catabolism, mechanical hypersensitivity and allodynia due to vincristine-induced neuropathy are strongly reduced suggesting an increased tonic release of enkephalins in conditions of vincristine-induced neuropathy, the synaptic levels of which is still enhanced by inhibition of their catabolism.

In our study PL37 has a short-duration of action since at all the doses studied and with the two modes of administration used (i.p and *per os*), the effect was maximum at 20 min post-injection with a return to baseline levels at 50 min. This duration of action, can be compared with the one of RB101, since a dose of 10 mg/kg (i.v.) lasted less than 30 min (Le Guen et al., 2003).

PL37 was also highly effective against static and dynamic mechanical allodynia, but failed to alleviate cold allodynia. This difference of activity may be due to the characteristics of the applied stimulus, the fibres involved in nociception or allodynia being not the same according to the intensity (innocuous with the smooth paint brush, or intense with the rough paint-brush) and to the nature (mechanical or thermal) of the stimulus. It is generally admitted that small diameter C- and Aδ-fibres are mainly involved in the response to cold stimuli, and in the response to intense mechanical stimuli whereas large Aβ-fibres (low threshold fibres) respond to tactile stimuli. Vincristine treatment-induced neuropathy involved degeneration of myelinated fibres (Authier et al., 2003); but unmyelinated fibres also degenerate since occasionally swollen non-myelinated fibres (Authier et al., 2003), and a significant decrease in microtubule density suggesting swelling unmyelinated axons (Tanner et al., 1998) have been described. The present results showed that enkephalins protected from degradation by PL37 could bind to opioid receptors expressed only on C- and Aδ-mechanonociceptors but not on cold thermonociceptors. The fact that PL37 is also active on small intensity mechanical stimulus (smooth paint brush) could reveal an expression of opioid receptors on large Aβ-fibres (low threshold mechanoreceptors) in the pathological conditions resulting from vincristine treatment. In fact it has been shown that opioid analgesics can act outside the central nervous system, targeting opioid receptors expressed at sites of peripheral damaged tissue (Stein et al., 2003). Thus the preproenkephalin gene was shown to be expressed in dorsal root ganglia and then enkephalins transported to the nociceptor terminals (Antunes-Bras et al., 2001). Besides enkephalins were reported to be released from leukocytes recruited in injured tissue (Rittner et al., 2006) and able to express neprilysin and aminopeptidase N at their membrane level. In neuroinflammatory and neuropathic conditions the phasic increase of locally secreted enkephalins could interact with opioid receptors located on sensory endings (Machelska et al., 2003). As in the central nervous system, the dual inhibitors could enhance the amounts of enkephalins at the peripheral receptor level thus potentiating the antinociceptive response.

From a clinical point of view, the management of chronic neuropathic pain is one of the major concerns of clinicians. Nevertheless, observations in the clinical context tend to indicate that neuropathic pain is not a homogeneous concept: the numerous studies performed in recent years have demonstrated that the chronic pain associated with neuropathic syndromes results from a wide variety of underlying pathophysiological mechanisms, the large number of mechanisms involved constituting a major therapeutic issue. From a clinical standpoint, this finding makes it even more vital to thoroughly explore the origin of the neuropathic symptoms experienced by individual patients in order to assign them to homogeneous groups sharing the same aetiology and the same pathophysiological mechanisms. And from an experimental standpoint, this is why we developed and describe in this paper the "paint-brush" test, due to the reported multiplicity of clinical factors involved in human neuropathic pain syndromes, such as differences between the syndromes involved, more precisely differences between static and dynamic hyperalgesia and allodynia.

In this context, even within the pharmacological family of opioids, the results obtained are highly contradictory. A review by Dellemijn (1999) resumed the current status of this controversy, emphasizing that the disparities in the results described are largely due to the multiplicity of factors involved: definitions of neuropathic pain, differences between the mechanisms involved in human neuropathic syndromes and in experimental animal models, differences in response according to the nature of the pain studied: spontaneous or evoked; inter-individual variability in analgesic response according to the nature of the opiate used and the doses administered, etc. The disparity of the results presented in our study could emphasize the

importance of the origin of the peripheral events related with the pathological conditions resulting from vincristine treatment.

To conclude, our results offer new insights into the controversies surrounding the utility of opioid analgesics in the treatment of neuropathic pain syndromes and suggest new pharmacological tools for neuropathic pain therapy. The present results confirm that the antinociceptive and antiallodynic responses induced by an inhibitor of enkephalin metabolism are important in chronic painful conditions, probably as a consequence of the different adaptative changes occurring during chronic pain, since previous studies have shown that their effects are different in acute and chronic painful situations (see ref in Noble and Roques, 2007). It can be underlined that inhibition of the enzymes implicated in the inactivation of the endogenous enkephalins is a useful tool to assess the physiological role of the endogenous opioid system in neuropathic conditions. In addition, the oral use of PL37 is expected to allow a critical evaluation in clinic of the analgesic effects caused by potentiation of the endogenous opioids without the side effects of morphine.

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