Original experimental

Synergistic combinations of the dual enkephalinase inhibitor PL265 given orally with various analgesic compounds acting on different targets, in a murine model of cancer-induced bone pain

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HIGHLIGHTS

• Combination of DENKI with non-opioid analgesic drugs results in synergistic antihyperalgesic effects.
• Mechanism of action involves the direct activation of the enkephalinergic system.
• The multi-target based strategy allows the reduction of doses in the treatment of chronic pain.

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ABSTRACT

Background: The first line pharmacological treatment of cancer pain is morphine and surrogates but a significant pain relief and a reduction of the side-effects of these compounds makes it necessary to combine them with other drugs acting on different targets. The aim of this study was to measure the antinociceptive effect on cancer-induced bone pain resulting from the association of the endogenous opioids enkephalin and non-opioid analgesic drugs. For this purpose, PL265 a new orally active single dual inhibitor of the two degrading enkephalins enzymes, neprilysin (NEP) and aminopeptidase N (APN) was used. It strictly increased the levels of enkephalin at their sites of releases. The selected non-opioid compounds are: gabapentin, A-317491 (P2X3 receptor antagonist), ACEA (CB1 receptor antagonist), AM1241 (CB2 receptor antagonist), JWH-133 (CB2 receptor antagonist), URB937 (FAAH inhibitor), and NAV26 (Nav1.7 channel blocker).

Methods: Experiments. Experiments were performed in 5–6 weeks old (26–33 g weight) C57BL/6 mice.

Cell culture and cell inoculation. B16-F10 melanoma cells were cultured and when preconfluent, treated and detached. Finally related cells were resuspended to obtain a concentration of $2 \times 10^6$ cells/100 μL. Then $10^5$ cells were injected into the right tibial medullar cavity. Control mice were treated by killed cells by freezing.

Behavioural studies. Thermal withdrawal latencies were measured on a unilateral hot plate (UHP) maintained at 49 ± 0.2 °C. Mechanical threshold values were obtained by performing the von Frey test using the “up and down” method. To evaluate the nature (additive or synergistic) of the interactions between PL265 and different drugs, an isobolographic analysis following the method described by Tallarida was performed.

Results: The results demonstrate the ability of PL265, a DENKI that prevents the degradation of endogenous ENKs, to counteract cancer-induced bone thermal hyperalgesia in mice, by exclusively stimulating peripheral opioid receptors as demonstrated by used of an opioid antagonist unable to enter the brain. The development of such DENKIs, endowed with druggable pharmacokinetic characteristics, such as good

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Abbreviations: ACEA, arachidonyl-2′-chloroethylamide; AEA, N-arachidonoylethanolamide, anandamide; APN, aminopeptidase N; BNI, nor-binaltorphimine; CCI, chronic constrictive injury; CYP, cyprodime; DENKI, dual enkephalinase inhibitor; DOR, delta opioid receptor; ENK, enkephalin; FAAH, fatty acid amide hydrolase; i.p., intraperitoneal; KOR, kappa opioid receptor; MOR, mu opioid receptor; NEP, neprilysin; NTI, naltrindole; Nlx-Met, naloxone methiodide; UHP, unilateral hot plate test; p.o., per os; s.c., subcutaneous; S.E.M., standard error of mean.

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1. Introduction

Cancer pain associated to neoplastic processes [1–3] remains frequently difficult to treat [4], particularly in cancers affecting bones as primary or metastatic site [3,5]. Several factors, such as the release of mediators by tumoral cells, the inflammatory components, the presence of bone fractures or the accompanying neuropathy, may contribute to nociceptive symptoms [2,6–9]. In fact, painful symptoms can adopt different characteristics depending on the involvement of these factors [10,11]. Current analgesic therapies can satisfactorily achieve favourable pain control in 75% of cancer patients [3,12]; although some patients remain refractory to pharmacological treatment [4]. Furthermore, the better efficacy of antinociceptive treatments increases the duration of the neoplastic process, implying that analgesic drugs must be administered during long time periods, with adverse effects limiting their chronic use [2,4].

The first line pharmacological treatment of cancer pain is morphine and its synthetic derivatives. But the complexity of associated inflammatory and neuropathic components often makes it necessary, for a significant pain relief, to combine them with other drugs, acting on different targets [3,4,13], especially when synergistic interactions allow dose reductions of combined drugs [14–16]. Thus, we decided to assess the combination of a compound that increases endogenous opioids concentrations with a wide range of non-opioid analgesic drugs, using a rodent model of cancer-induced bone pain.

The endogenous peptides Met and Leu-enkephalin, tonically released at the injured site [17], bind with about the same high affinity to both mu (MOR) and delta receptors (DOR). However, Met- and Leu-enkephalin evoke only transient analgesic effects due to their rapid degradation by the concomitant action of two zinc metalloproteases: the neutral endopeptidase neprilysin (NEP, EC 3.4.24.11) and aminopeptidase N (APN, EC 3.4.11.2) [18,19]. Dual enkephalinase inhibitors (DENKIs) showed the interesting property that even when administered systemically and homogeneously distributed within the body, their antinociceptive effects are essentially a consequence of the stimulation of opioid receptors located near the injured site, where the local release of enkephalins (ENKs) occurs [17,20–22].

In previous preclinical studies, we have demonstrated antinociceptive effects induced by the oral administration of the DENKI PL37 [23–27]. In order to broaden our understanding of the antinociceptive effects elicited by the stimulation of peripheral opioid receptors where local enkephalins are protected from degradation, we studied the antinociceptive effects of a new DENKI, PL265 [19,28], a nanomolar single inhibitor of both NEP and APN, in a model of cancer-induced bone pain based on the intratibial inoculation of B16-F10 melanoma cells [29,30]. This model of cancer-induced bone pain shows a mixed osteoblastic–osteoclastic histopathological pattern [29] and tumours develop faster than in mice inoculated with NCTC2472 cells that produce osteolytic injury in bone [31].

Furthermore, we tested whether synergistic interactions can occur through the combined administration of this drug with several other mechanistically unrelated painkillers acting peripherally. In the present study, we assess the possible interactions of PL265 combined with gabapentin, A-317491 (a P2X3 antagonist), ACEA (CB1 receptor agonist), AM1241 and JWH-133 (two structurally unrelated CB2 receptor agonists), URB937 (inhibitor of FAAH, that impedes endogenous cannabinoid degradation, AEA) or NAV26 (a NaV1.7 channel blocker).

2. Methods

2.1. Animals

Experiments were performed in 5–6 weeks old (26–33 g weight) C57BL/6 mice bred in the Animalario de la Universidad de Oviedo (Reg. 33044 13A), housed six per cage with a bedding of sawdust and maintained on a 12-h dark–light cycle with free access to food and water. All the experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain) and performed in accordance with the recommendations of the European Communities Council Directive of 24 November 1986 (86/609/EEC). Each animal was used only once and randomly allocated into a treatment group.

2.2. Cell culture and cell inoculation

B16-F10 melanoma cells (American Type Culture Collection) were cultured in DMEM (Gibco) enriched with 10% foetal calf serum (FCS, Gibco). When cells were preconfluent, they were treated with trypsin/EDTA (0.05%/0.02%) and detached. The trypsin/EDTA solution was recovered, neutralized with DMEM, supplemented with 10% FCS and centrifuged at 400 × g for 10 min. Finally, pellets were resuspended in PBS in order to obtain a concentration of 2 × 10^6 cells/100 μL [29].

For surgical procedures, anaesthesia was induced by spontaneous inhalation of 3% isofluorane (Isoflo® Esteve) and maintained by administering 1.5% isoflurane in oxygen through a breathing mask. A suspension of 10^5 cells in 5 μL of PBS was injected into the right tibial medullar cavity and next, acrylic glue (Hystoacril®, Braun) was applied on the tibial plateau incised area. Surgery was finished with a stitch of the skin. Control mice received the inoculation of 10^5 cells previously killed by quickly freezing them three
times without cryoprotection. Behavioural tests were performed one week after tumoral cell inoculation.

2.3. Cell viability

B16-F10 viability was assayed by using the alamarBlue® kit (Bio-Rad) according to the manufacturer’s protocol. In a 96-well microliter plate (Sarstedt), 90 μL of culture media only (blanks) or with 10^3 B16-F10 cells were kept in an incubator at 37 °C and 5% CO2 in sterile conditions. After 12 h, when cells reached approximately 50% of confluence, 10 μL of PL265, morphine or solvent were added to the well with 10 μL of the reagent and sealed with adhesive film. Changes in cell viability were measured 8 h after adding drugs by using fluorescence spectrophotometry read at 540/610 nm of excitation/emission wavelengths respectively, since it has been previously reported that at shorter incubation times growth alterations in 10^3 B16-F10 cells can be measured [32]. The recorded values in the presence of drug or solvent were subtracted from the mean of blank values obtained in media without cells and further considered for statistical analysis. The experiment was run 5 times independently and, in each experiment, blanks were performed in triplicate and wells bearing the cells in quintuplicate.

2.4. Behavioural studies

Thermal withdrawal latencies were measured by the unilateral hot plate (UHP) [33]. Briefly, mice were gently restrained and the plantar side of the tested paw placed on the hot plate surface (ITC Life Science) maintained at 49.1 ± 0.2 °C [34]. Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at 2 min intervals and the mean of two measures was considered. A cut-off of 20 s was established. Experiments were performed between 15:00 and 20:00 h in a thermostated (21 °C) and noise-isolated room.

Mechanical threshold values were obtained by performing the von Frey test as previously reported [35]. Mice were placed on a wire mesh platform and, after a 20 min habituation period, von Frey filaments (Stoelting) were applied to the plantar side of the paws Von Frey filaments 2.44, 2.83, 3.22, 3.61, 4.08 and 4.56 were used and, starting with the 3.61 filament, 6 measurements were taken in each animal randomly starting by the left or right paw. Based on the “up and down” method [36], the observation of a positive response (lifting, shaking or licking of the paw) was followed by the application of the immediate thinner filament or the immediate thicker one if the response was negative. The 50% response threshold was calculated using the following formula: 50% g threshold = (10^{Xf})/10,000; where \(Xf\) is the value of the last von Frey filament applied; \(x\) is a correction factor based on pattern of responses (from the Dixon’s calibration table); \(δ\) is the mean distance in log units between stimuli (here, 0.4).

2.5. Drugs

PL265, 2-(2-biphenyl-4-ylmethyl-3-[(hydroxyl-(1-(1-isobutyryloxy-ethoxycarbonylamino)-ethyl)-phosphinyl)-(propionylamino)])-propionic acid disodium salt, was dissolved in saline and administered by oral route (p.o.) in a final volume of 10 mL/kg. Its effects were measured at several times after administration for time course studies or 30 min after in the other experiments. The opioid receptors antagonists, naloxone-methiodide (Nlx-Met, Sigma), cyprodime hydrobromide (CYP, Sigma), naltrindole hydrochloride (NTI, Tocris) and nor-binaltorphimine dihydrochloride (BNI, Tocris) were dissolved in saline and administered subcutaneously (s.c.), 30 min before testing. Gabapentin (Sigma) dissolved in saline, was intraperitoneal (i.p.) administered 60 min before testing. A-317491 (Sigma), JWH-133 (Tocris) and ACEA (Tocris) were prepared in EtOH as stock solution and next, diluted in distilled water. AM1241 (Tocris) was prepared in EtOH as stock solution and further diluted to a final concentration of 1/18 of EtOH/cremophor EL/distilled water. URB937 (Sigma) and NAV26 (Tocris) were prepared in DMSO and diluted in distilled water. They were dissolved in 100 μL of saline and injected subcutaneously over the tibial tumoral mass (peritumoral administration), 30 min before the test. When studying control animals – inoculated with killed B16-F10 cells – the injections were performed in the same region of the limb which, in this case was tumour-free. For studies of cell viability, PL265 or morphine (morphine hydrochloride, Ministerio de Sanidad, Madrid, Spain) were dissolved in saline and directly added to wells.

2.6. Isobolographic analysis

The use of two or more drugs in combination can result in an additive (a sum of the effect induced by each drug separately) or even superadditive (synergistic) effect, that is, their action given in combination is above what is expected from their individual potencies and efficacies [16,37]. In order to evaluate the possible interaction between PL265 and the different drugs studied in the UHP test, we performed an isobolographic analysis following the method described by Tallarida [37,38] by using the computer program Pharm Tools Pro (version 1.27, The McCay Group Inc.). With this aim, the dose-response curves of the thermal antihyperalgesic effects, induced by both drugs on their own were constructed and the ED50 ± standard error (S.E.M.) was calculated. In order to calculate the ED50 values, we considered that each mouse showed an antihyperalgesic effect in response to a drug when the increase in the latency value measured in the tumour-bearing paw surpassed the 50% of the maximal possible increase. This maximal antihyperalgesic effect (100%) was considered to be achieved when the latencies obtained in the injured paws reached the mean value obtained in the right paws of the control group (treated with killed tumoral cells). To perform an isobolographic analysis, dose–response curves were constructed after the administration of equipotent doses of both drugs, expressed as fractions of their respective ED50. Thus, to study the interaction index of PL265 with a given drug, a curve was obtained following the administration of specific ratio of the ED50 of both drugs. From the resulting dose–response curve of drugs administered in combination, the experimental ED50 was calculated. In addition, a theoretical additive ED50 must be estimated from the dose–response curves of each drug administered individually considering that the effect of drug combination results from the sum of the individual effects of each component. The theoretical and experimental ED50 values of the studied combinations were compared by calculating the interaction index (\(γ\)) as follows: \(γ =\) experimental ED50 value/theoretical ED50 value, where values lower than 1 indicate a synergistic interaction [38]. Finally, to determine if the interaction between two drugs given in combination is synergistic, the theoretical ED50 value is compared with the experimental ED50 to determine if there is statistically significant difference, as evaluated by using a Student’s t test [37].

2.7. Data analysis

The mean values and the corresponding standard errors were calculated for each assay. Data obtained in the UHP test were compared by the Student’s t test when only two groups were studied whereas the comparisons among several groups were done by using an initial one-way analysis of variance (ANOVA) followed by either the Dunnett’s t test when groups received different doses of a drug or by the Newman–Keuls test when groups received different
3. Results

3.1. Lack of effect of PL265 and morphine on B16-F10 cell viability in vitro

Opiates have been described to increase tumoral cell growth [39]. The viability of B16-F10 melanoma cells was therefore studied in the presence of PL265. The incubation of 10^3 B16-F10 cells with PL265 (10^{-9}–10^{-5} M), morphine (10^{-9}–10^{-5} M) or solvent yields very similar spectrophotometric readings 8 h after the presence of resazurin, the alamarBlue reagent® (Fig. 1). Thus, neither PL265 nor morphine alters B16-F10 cell viability in culture.

3.2. Antinociceptive effect induced by the systemic administration of PL265 on B16-F10-evoked thermal hyperalgesia and mechanical allodynia

Mice inoculated with B16-F10 melanoma cells into the tibia one week before the experiment showed decreased withdrawal latencies measured by the UHP test in the inoculated (right) paw compared with the non-inoculated (left) paw (Fig. 2A). Oral administration of PL265 (12.5–37.5 mg/kg, 30 min before testing) inhibited melanoma-induced thermal hyperalgesia in a dose-dependent manner (Fig. 2A). Withdrawal latency values were significantly increased following the administration of 25 mg/kg of PL265 and the maximal antihyperalgesic effects (100%) were observed at 37.5 mg/kg (the values obtained were indistinguishable from those of the contralateral paw). The administration of PL265 never modified withdrawal latency values of the non-inoculated paw. The ED_{50} value was 21.5 ± 1.2 mg/kg (Table 1). The thermal antihyperalgesic effect induced by 37.5 mg/kg of PL265 was inhibited by the systemic administration of the nonselective opioid antagonist Nlx-Met given at a dose unable to cross the blood brain barrier [40,41] (2 mg/kg s.c., 30 min before testing) (Fig. 2A). As control, neither the administration of Nlx-Met on its own in melanoma-bearing mice nor the administration of the highest dose of PL265 to mice inoculated with inactivated B16-F10 cells modified withdrawal latencies (data not shown).

Assessment of mechanical thresholds using von Frey filaments revealed the development of mechanical allodynia in mice inoculated one week before with B16-F10 melanoma cells (Fig. 2B). The administration of PL265 (25–100 mg/kg p.o., 30 min before testing), dose-dependently inhibited, melanoma-induced mechanical allodynia in this test (Fig. 2B). The antiallodynic effects induced by the highest dose of PL265 tested, 100 mg/kg, remained unaffected following the administration of Nlx-Met (2 mg/kg s.c., 30 min before testing) (Fig. 2B). Mechanical thresholds remained unaffected following the administration of the highest dose of PL265 to mice inoculated with inactivated B16-F10 cells (data not shown).

Fig. 1. Effect of PL265 or morphine on B16-F10 cell viability in vitro. Fluorescence intensity was measured 8 h after the addition of vehicle, PL265 (10^{-9}–10^{-5} M) or morphine (10^{-9}–10^{-5} M) to culture plates with 1000 B16-F10 cells. Each bar represents the mean and corresponding S.E.M. (n = 5).

Fig. 2. (A) Antihyperalgesic effect induced by the oral administration of PL265 (12.5–37.5 mg/kg, 30 min before testing) in mice intratibially inoculated with B16-F10 cells one week before measured by unilateral hot plate (UHP) test. The antihyperalgesic effect is inhibited by naloxone-methiodide (Nlx-Met 2 mg/kg s.c., 30 min before testing). Each bar represents the mean withdrawal latency value with its corresponding standard error (n = 5–6). **p < 0.01 versus corresponding left paw, Student’s t test; *p < 0.01 versus vehicle-treated right paw, Dunnett’s t test. (B) Antiallodynic effect induced by PL265 (25–100 mg/kg p.o., 30 min before testing) in mice intratibially inoculated with B16-F10 cells one week before measured by the von Frey test. The administration of naloxone-methiodide (Nlx-Met 2 mg/kg s.c., 30 min before testing) does not modify the antiallodynic effect. Each bar represents the 50% mechanical threshold value with its corresponding standard error (n = 7–9). *p < 0.05, **p < 0.01 versus vehicle-treated right paw, Dunn’s test.
Effects of the administration of PL265 alone and combined drugs in mice intratibially inoculated with B16-F10 measured by the unilateral hot plate test.

<table>
<thead>
<tr>
<th>Drug (ED50, mg/kg)</th>
<th>PL265 (21.5 ± 1.2 mg/kg)</th>
<th>Experimental ED50 (mg/kg)</th>
<th>Interaction index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gabapentin (10.8 ± 0.8)</td>
<td>16.1 ± 0.7</td>
<td>10.8 ± 0.41</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>A-317491 (0.22 ± 0.02)</td>
<td>10.9 ± 0.6</td>
<td>3.7 ± 0.11</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>AM1241 (0.34 ± 0.04)</td>
<td>10.9 ± 0.6</td>
<td>7.5 ± 0.3</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>JWH 133 (0.5 ± 0.2)</td>
<td>10.9 ± 0.6</td>
<td>9.2 ± 1.9</td>
<td>0.85 ± 0.14</td>
</tr>
<tr>
<td>ACEA (0.005 ± 0.0005)</td>
<td>11.1 ± 0.6</td>
<td>3.7 ± 0.8</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>UR8937 (0.10 ± 0.01)</td>
<td>10.5 ± 0.6</td>
<td>3.6 ± 0.5</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>NAV26 (2.0 ± 0.4)</td>
<td>11.4 ± 0.6</td>
<td>3.5 ± 0.9</td>
<td>0.31 ± 0.03</td>
</tr>
</tbody>
</table>

ED50: Effective dose resulting in a 50% of the antihyperalgesic effect. Data are the mean ± S.E. of the estimate.

3.3. Effects of selective opioid receptor antagonists CYP, NTI and BNI on the peripheral antihyperalgesic effect produced by oral PL265 on B16-F10-evoked thermal hyperalgesia

The antihyperalgesic effect produced by 37.5 mg/kg of oral PL265 measured by UHP was inhibited by the s.c. administration, 30 min before testing, of the MOR antagonist CYP (1 mg/kg) but not by the administration of a DOR antagonist NTI (0.1 mg/kg) or a kappa opioid receptor (KOR) antagonist BNI (10 mg/kg) (Fig. 3). These doses are currently used to check such antagonistic effects [23,24]. The s.c. administration of the same doses of opioid antagonists on their own did not modify thermal latencies (data not shown).

3.4. Synergistic interactions with PL265

3.4.1. Antihyperalgesic effect induced by the systemic administration of gabapentin on B16-F10-evoked thermal hyperalgesia

The anticonvulsant gabapentin [26,42] administered i.p. 60 min before testing increased thermal latencies in a dose-dependent manner (6.25–25 mg/kg) in melanoma bearing mice (Fig. 4A) and the maximal antihyperalgesic effect was achieved at 25 mg/kg. None of the tested doses modified withdrawal latencies observed in contralateral paws. The calculated ED50 value of gabapentin was 10.8 ± 0.8 mg/kg. The antihyperalgesic effect induced by 25 mg/kg of gabapentin remained unmodified when 2 mg/kg of Nlx-Met were administered s.c. 30 min before testing (Fig. 4B).

As shown in Fig. 4C, the combined administration of PL265 and gabapentin at fixed fractions (1/2, 1/3, 1/4) of their corresponding ED50 dose-dependently inhibited thermal hyperalgesia in mice intratibially inoculated with live B16-F10 cells, without altering the latency values of the contralateral paws (not shown). The calculated experimental ED50 value from this combination of PL265 and gabapentin was 10.8 ± 0.4 mg/kg, that was significantly lower than the theoretical one (16.1 ± 0.7 mg/kg) (Fig. 4D), leading to the interaction index value of 0.67 ± 0.03 (Table 1).

3.4.2. Antihyperalgesic effect induced by the peritumoral administration of the P2X3 purinergic receptor antagonist A-317491 on B16-F10-evoked thermal hyperalgesia

The purinergic receptor antagonist A-317491 [43] was peritumorally administered (1.5–15 μg) in melanoma bearing mice 30 min before testing. The maximal antihyperalgesic effect was achieved after the administration of 15 μg of A-317491 (Fig. 5A) and the experimental ED50 value obtained was 6.5 ± 0.7 μg (corresponding to 0.22 ± 0.02 mg/kg) (Table 1). The antihyperalgesic effect produced by 15 μg of A-317491 was inhibited when Nlx-Met (2 mg/kg) or CYP (1 mg/kg) were administered s.c. 30 min before testing. In contrast, this antihyperalgesic effect remained unaltered after s.c. administration of NTI (0.1 mg/kg) or BNI (10 mg/kg) (Fig. 5B), demonstrating the exclusive involvement of MOR in this response.

Following the administration of a combination of both drugs at fixed fractions (1/4, 1/6, 1/8) of their respective ED50 values, the calculated experimental ED50 value (3.7 ± 0.1 mg/kg) was significantly lower than the theoretical ED50 (10.9 ± 0.6 mg/kg) (Fig. 5D), the value of the interaction index being 0.34 ± 0.02 (Table 1).

3.4.3. Antihyperalgesic effect induced by the peritumoral administration of the CB1 agonist ACEA on B16-F10-evoked thermal hyperalgesia

The peritumoral administration of the CB1 agonist, ACEA [1], (0.03–0.3 μg) in melanoma-bearing mice yielded antihyperalgesic effects with the maximal response detected after the administration of 0.3 μg of ACEA (Fig. 6A). The calculated ED50 value was 0.16 ± 0.02 μg (corresponding to 5 ± 0.5 μg/kg) (Table 1). The s.c. administration of 2 mg/kg of Nlx-Met, 1 mg/kg of CYP or 0.1 mg/kg of NTI inhibited the antihyperalgesic effect induced by 0.3 μg of ACEA.
Fig. 4. Potentiation of the antihyperalgesic effect induced by gabapentin by the simultaneous oral administration of PL265 measured by the unilateral hot plate (UHP) test in mice intratibially inoculated with B16-F10 cells into the right paw one week before. (A) Antihyperalgesic effect induced by gabapentin (6.25–25 mg/kg i.p., 60 min before testing). Bars represent means and S.E.M. (n=5); **p<0.01 versus corresponding left paw of vehicle-treated group, Student’s t test; *p<0.05, **p<0.01, compared with vehicle-treated right paws, Dunnett’s t test. (B) Lack of inhibition of the antihyperalgesic effect induced by 25 mg/kg of gabapentin by s.c. administration 30 min before testing of naloxone-methiodide (Nlx-Met, 2 mg/kg). Bars represent means and S.E.M. (n=5). **p<0.01 versus vehicle, Newman–Keuls test. (C) Antihyperalgesic effect induced by the combined administration of gabapentin and PL265 at fixed doses that represent 1/4, 1/3 and 1/2 of their ED50 values. Each point represents the mean with the corresponding S.E.M. (n=4–5). *p<0.05, **p<0.01, compared with vehicle-treated mice, Dunnett’s t test. (D) Isobologram showing the interaction between gabapentin and PL265 administered 60 and 30 min, respectively, before testing. The oblique line between the x and y axes is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the individual drug ED50 values. The point below the line is the experimental ED50 value obtained with the combination. Horizontal and vertical bars indicate S.E.M.

ACEA peritumorally administered (Fig. 6B), showing the involvement of both peripheral MOR and DORs in this response.

The experimental ED50 value calculated from antihyperalgesic effect following the combined administration of ACEA and PL265 (Fig. 6C) was 3.7 ± 0.8 mg/kg. This value is significantly lower than the theoretical ED50 one, 11.1 ± 0.6 mg/kg (Fig. 6D) and, as shown in Table 1, the value of the corresponding interaction index is 0.34 ± 0.02.

3.4.4. Antihyperalgesic effect induced by the peritumoral administration of the CB2 agonist AM1241 on B16-F10-evoked thermal hyperalgesia

The peritumoral administration of the CB2 receptor agonist, AM1241 [44], evoked a dose-dependent (3–30 μg) antihyperalgesic effect (Fig. 7A) and the calculated experimental ED50 value was 10.0 ± 1.2 μg (corresponding to 0.34 ± 0.04 mg/kg) (Table 1). The antihyperalgesic effect induced by 30 μg of AM1241 was completely blocked by the s.c. administration of Nlx-Met (2 mg/kg), CYP (1 mg/kg) or NTI (0.1 mg/kg) but it remained unaffected when BNI (10 mg/kg) was administered (Fig. 7B), demonstrating the involvement of both peripheral MORs and DORs in this response.

The combined administration of AM1241 and PL265 at fixed fractions of their corresponding ED50 induced a dose-dependent antihyperalgesic effect (Fig. 7C) that yielded an experimental ED50 value of 7.5 ± 0.3 mg/kg. A significant difference was obtained when comparing the experimental ED50 value with the theoretical one (10.9 ± 0.6 mg/kg) (Fig. 7D). As shown in Table 1, the interaction index obtained was 0.68 ± 0.05.

3.4.5. Antihyperalgesic effect induced by the peritumoral administration of the CB2 agonist JWH-133 on B16-F10-evoked thermal hyperalgesia

Another, more selective CB2 receptor agonist, JWH-133 [45], was administered (10–100 μg) in melanoma-bearing mice (Fig. 8A). Significant increases of right paw withdrawal latencies were obtained following the peritumoral administration of 10 μg of JWH-133, and the maximal antihyperalgesic effect was achieved following the administration of 100 μg (Fig. 8A). The calculated ED50 value of this thermal antihyperalgesic effect was 14.4 ± 4.9 μg
Fig. 5. Potentiation of the antihyperalgesic effect induced by A-317491 by the simultaneous oral administration of PL265 measured by the unilateral hot plate (UHP) test in mice intratibially inoculated with B16-F10 cells into the right paw one week before. (A) Antihyperalgesic effect induced by the peritumoral administration of A-317491 (1.5–15 μg, 30 min before testing). Bars represent means and S.E.M. (n = 5). **p < 0.01 versus corresponding left paw of vehicle-treated group, Student’s t test. *p < 0.01, compared with solvent-treated right paws, Dunnett’s t test. (B) Inhibition of the antihyperalgesic effect induced by 15 μg of A-317491 by s.c. administration 30 min before testing of naloxone-methiodide (Nlx-Met, 2 mg/kg) and cyprodime (CYP, 1 mg/kg) but not naltrindole (NTI, 0.1 mg/kg) or nor-binaltorphimine (BNI, 10 mg/kg). Bars represent means and S.E.M. (n = 5–6). **p < 0.01 versus gabapentin-treated mice, Newman–Keuls test. (C) Antihyperalgesic effect induced by the combined administration of A-317491 and PL265 at fixed doses that represent 1/8, 1/6 and 1/4 of their ED50 values. Each point represents the mean with the corresponding S.E.M. (n = 6 per group). **p < 0.01, compared with vehicle-treated mice, Dunnett’s t test. (D) Isobologram showing the interaction between A-317491 and PL265 administered 30 min before testing. The oblique line between the x and y axes is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the individual drug ED50 values. The point below the line is the experimental ED50 value obtained with the combination. Horizontal and vertical bars indicate S.E.M.

3.4.6. Antihyperalgesic effect induced by the peritumoral administration of the FAAH inhibitor URB937 on B16-F10-evoked thermal hyperalgesia

The peripherally acting FAAH inhibitor URB937 [46], was peritumorally administered (1–10 μg) to melanoma-bearing mice. The maximal antihyperalgesic effect was detected after the administration of 10 μg of this drug (Fig. 9A) and the calculated ED50 value was 3.2 ± 0.4 μg (corresponding to 0.10 ± 0.01 mg/kg) (Table 1). The antihyperalgesic effect induced by 10 μg of peritumorally administered URB937 was completely blocked when Nlx-Met (2 mg/kg) or CYP (1 mg/kg) were administered s.c. (Fig. 9B), demonstrating the involvement of peripheral MOR in the antihyperalgesic effects of URB937.

The experimental ED50 value (3.6 ± 0.5 mg/kg) calculated from the dose–effect curve from the combined administration of URB937 with PL265 (Fig. 9C), was significantly lower than the theoretical ED50 one, 10.5 ± 0.6 mg/kg (Fig. 9D) and the interaction index obtained was 0.35 ± 0.02 (Table 1).

3.4.7. Antihyperalgesic effect induced by the peritumoral administration of the Na+1.7 channel inhibitor NAV26 on B16-F10-evoked thermal hyperalgesia

The selective Na+1.7 channel blocker, NAV26 [47], was peritumorally administered (0.01–1 μmol) in mice bearing B16-F10 cells. Its maximal antihyperalgesic effect was detected after the administration of 1 μmol (Fig. 10A) and the ED50 obtained value was 0.14 ± 0.03 μmol (corresponding to 2.0 ± 0.4 mg/kg) (Table 1). The...
antihyperalgesic effect induced by 3 μmol of NAV26 peritumorally administered was completely inhibited by the s.c. administration of Nlx-Met (2 mg/kg), CYP (1 mg/kg) or NTI (0.1 mg/kg) (Fig. 10B), demonstrating the involvement of both peripheral MOPRs and DORs in this antinociceptive response.

The combined administration of NAV 26 and PL265 at fixed fractions (1/8, 1/4 and 1/2) of the corresponding ED50 values yielded a dose-dependent antihyperalgesic effect (Fig. 10C) whose calculated ED50 value, 3.5 ± 0.9 mg/kg, was significantly lower than the theoretical ED50, 11.4 ± 0.6 mg/kg (Fig. 10D) and the derived interaction index was 0.31 ± 0.03 (Table 1).

4. Discussion

In the present study, we show the antinociceptive effects of PL265, in a model of cancer-induced bone pain based on the intratibial inoculation of B16-F10 melanoma cells [7]. This study reveals that the stimulation of peripheral opioid receptors is an effective strategy to counteract the hypernociceptive responses in these tumour-bearing mice. In addition, this study describes synergistic interactions that occur when PL265 is administered in combination with several drugs which alleviate pain via different mechanisms. Protection of endogenous enkephalins from their physiological degradation, by PL265 effectively counteracts thermal hyperalgesia in mice bearing B16-F10 melanoma cells with 100% alleviation at 37.5 mg/kg. This antihyperalgesic effect induced by PL265-protected ENKs depends on the specific stimulation of peripheral MOPRs (Fig. 3) as demonstrated by its inhibition by Nlx-Met (Fig. 2B). Furthermore, PL265 also induced anti-mechanical hypersensitivity effects, but at higher doses (>25 mg/kg). These anti-mechanical hypersensitivity effects observed in tumour-bearing mice seem to involve central opioid receptors, since they are not affected by Nlx-Met (Fig. 2B). This contrasts with previous results observed in a partial sciatic nerve ligation model (CCI), when PL265 evoked peripherally mediated anti-mechanical hypersensitivity responses [28]. To avoid these potential central effects, the current study of the hypernociceptive effects of PL265, focused on its peripheral thermal hyperalgesic effects, by using doses lower than 25 mg/kg. This peripheral opioid-related effect is probably due to the stimulation of mu receptors since only cyprodime, but not naltrindole or nor-binaltorphimine, inhibited it (Fig. 3).
Fig. 7. Potentiation of the antihyperalgesic effect induced by the peritumoral administration of AM1241 by the simultaneous oral administration of PL265 measured by the unilateral hot plate (UHP) test in mice one week after the intratibially inoculation of B16-F10 cells in their right hind paws. (A) Antihyperalgesic effect induced by AM1241 (3–30 µg, 30 min before testing). Bars represent means and S.E.M. (n = 5). **p < 0.01 versus corresponding left paw, Student’s t test of vehicle-treated group; *p < 0.01 compared with vehicle-treated right paws, Dunnett’s t test. (B) Inhibition of the antihyperalgesic effect induced by 30 µg of AM1241 by s.c. administration 30 min before testing of naloxone-methiodide (Nlx-Met, 2 mg/kg), cyprodime (CYP, 1 mg/kg) and naltrindole (NTI, 0.1 mg/kg) but not nor-binaltorphimine (BNI, 10 mg/kg). Bars represent means and S.E.M. (n = 5). **p < 0.01 versus AM1241-treated mice, Newman–Keuls test. (C) Antihyperalgesic effect induced by the combined administration of AM1241 and PL265 at fixed doses that represent the 1/4, 1/3 and 1/2 of their ED50. Each point represents the mean with the corresponding S.E.M. (n = 4–5). *p < 0.05, **p < 0.01, compared with vehicle-treated mice, Dunnett’s t test. (D) Isobologram showing the interaction between AM1241 and PL265 administered 30 min before testing. The oblique line between the x and y axes is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the individual drug ED50 values. The point below the line is the experimental ED50 value obtained with the combination. Horizontal and vertical bars indicate S.E.M.

Another aim of this study was to determine whether combining PL265 with compounds acting via different mechanisms of action could decrease the effective dose of both drugs. The targets of these substances are calcium channel sub-unit α2δ, ATP sensitive nerve fibres, cannabinoid receptors and voltage-gated sodium channel Nav1.7.

The combination of PL265 with gabapentin yields synergistic antihyperalgesic effects (Fig. 4). As previously shown [24,48–50], gabapentin alone was effective in counteracting excessive nociception in mice inoculated with B16-F10 cells (Fig. 4A). It has been shown that at doses lower than 30 mg/kg, gabapentin acts only at the peripheral level [51]. Its antinociceptive effects [52] are supposed to be related to the peripheral release of nitric oxide (NO), a mediator shown to induce analgesia at this level [53,54]. Consequently, one possible mechanism to explain the observed synergy could be related to the peripheral MORs stimulation by PL265-protected-ENKs potentiated by the activation of NO/cGMP/K+ATP cascade [29,54,55].

In a second experiment, PL265 was combined with peritumoral A-317491, a P2X3 receptor antagonist [56]. Initial reports have shown the antinociceptive effects derived from the acute blockade of this receptor in inflammatory and neuropathic settings [57,58], as well as in other bone-induced cancer models in rats [59–61] and mice [23,62].

A-317491 dose-dependently inhibits thermal hyperalgesia (Fig. 5A) apparently through the specific stimulation of peripheral MORs, as the effect is blocked by Nlx-Met and CYP (Fig. 5B). Considering that A-317491, peritumorally administered, poorly crosses the blood–brain barrier [61,63] and has a low affinity for MORs [56], the most likely explanation for its antihyperalgesic synergistic effects with PL265-protected ENKs is a large increase in peripheral endogenous opioids release that stimulate local MORs, as demonstrated with an anti-ENK antibody [23]. Therefore, the doses of drugs necessary to achieve antihyperalgesic effects when administered in combination are reduced by about 70% (Fig. 5D).

Many studies have demonstrated that the combination of cannabinoids and opioids produced synergistic antinociceptive effects [64–66], irrespective of the route of administration (i.t., i.c.v., s.c., p.o.), in various animal models of pain. This synergistic effect can be explained by the initial release by cannabinoid [67] of dynorphin A and its subsequent breakdown to Leu-enkephalin as well as by the direct interactions between cannabinoid and opioid receptors, including allosteric interactions [68] or co-localization on the same neurons [69] or by the fact that CB1 receptor stimulation is
related to the potentiation of DORs coupling to [Ca^{2+}], [70]. Three approaches were chosen in the present study: a combination of PL265-protected-ENKs with a selective CB1 or a CB2 agonist or use of the AEA endogenous cannabinoid ligand, protected by a selective inhibitor from physiological inactivation by its fatty acid amide hydrolase (FAAH) [71].

In mice inoculated with B16-F10 cells, the peritumoral administration of the selective CB1 receptor agonist ACEA [1] inhibited thermal hyperalgesia at very low doses (0.3 μg per mouse). It has been previously reported that the stimulation of peripheral CB1 receptors counteract hypernociceptive responses in mice inoculated with NCTC2472 sarcoma-derived cells [34,72–76]. We demonstrated here the participation of peripheral MORs and DORs in the antihyperalgesic effect induced by ACEA since it was antagonized by Nlx-Met, CYP or NTI and not by BNI. The involvement of opioid mechanisms in the effects of ACEA probably explains that the combination of ACEA and PL265 shows sizeable synergistic effects (interaction index: 0.34).

The local peritumoral administration of two CB2 receptor agonists, AM1241 and JWH-133 counteracted thermal hyperalgesia (Figs. 7A and 8A respectively) in mice inoculated with B16-F10 melanoma cells, demonstrating, as previously shown in mice inoculated with sarcoma cells [30,75,76], the stimulation of peripheral CB2 receptors. Interestingly, synergistic antihyperalgesic effects were only obtained when PL265 was combined with AM1241 (30% lower; Fig. 7D) but not with JWH-133, whose effects are only additive (Fig. 8D). The effects of peritumoral administration of the former and not of the latter were inhibited by Nlx-Met (Fig. 7B), confirming the involvement of peripheral opioid receptors in the response. At the peripheral level, the stimulation by the agonist AM1241 of CB2 receptors located in keratinocytes leads to the release of beta-endorphin [77], and thus probably explains the inhibition of the antihyperalgesic effects by CYP and NTI, since beta-endorphin possesses affinity for both MORs and DORs. On the contrary, the absence of inhibiting effects by naloxone of the antihyperalgesic effects of JWH-133 excludes an opioid component, which also explains why this CB2 agonist shows no synergy with PL265.

URB937 is a potent FAAH inhibitor that does not cross the blood–brain barrier and therefore prevents the deactivation of the endogenous cannabinoid anandamide, AEA [19,71], only in peripheral tissues [46]. It has been previously demonstrated that this drug alleviated pain in mice via the specific activation of peripheral CB1 receptors [46,78]. The antihyperalgesic effects of URB937-protected AEA are dose-dependent (Fig. 9A) and are mediated by the stimulation of peripheral MORs as they are blocked by...
Fig. 9. Potentiation of the antihyperalgesic effect induced by the peritumoral administration of URB937 by the simultaneous oral administration of PL265 measured by the unilateral hot plate (UHP) test in mice one week after the intratibially inoculation of B16-F10 cells in their right hind paws. (A) Antihyperalgesic effect induced by URB937 (1–10 μg, 30 min before testing). Bars represent means and S.E.M. (n = 5). **p < 0.01 versus corresponding left paw of vehicle-treated group, Student’s t test; ***p < 0.01 versus vehicle-treated right paws, Dunnett’s t test. (B) Inhibition of the antihyperalgesic effect induced by 10 μg of URB937 by s.c. administration 30 min before testing of naloxone-methiodide (Nlx-Met, 2 mg/kg), cyprodime (CYP, 1 mg/kg) but not naltrindole (NTI, 0.1 mg/kg) and nor-binaltorphimine (BNI, 10 mg/kg). Bars represent means and S.E.M. (n = 4–5). **p < 0.01 versus URB937-treated mice, Newman–Keuls test. (C) Antihyperalgesic effect induced by the combined administration of URB937 and PL265 at fixed doses that represent 1/8, 1/4 and 1/3 of their ED50. Each point represents the mean with the corresponding S.E.M. (n = 6–7). **p < 0.01, compared with vehicle-treated mice, Dunnett’s t test. (D) Isobologram showing the interaction between URB937 and PL265 administered 30 min, before testing. The oblique line between the x and y axes is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the individual drug ED50 values. The point below the line is the experimental ED50 value obtained with the combination. Horizontal and vertical bars indicate S.E.M.

Nlx-Met, as well as by the selective MOR antagonist CYP (Fig. 9B). The combined administration of URB937 with PL265 yielded such high synergistic effects that the active doses were reduced by about 65% (Fig. 9D), similar to what was observed when PL265 and the CB1 agonist, ACEA were combined (Fig. 6D). Considering that URB937 does not cross the blood–brain barrier [19], the most likely explanation for its synergistic antihyperalgesic effects with PL265-protected-ENKs is that opioid mechanisms are involved in the effects of URB937-protected-AEAs in this setting.

The voltage-gated sodium channel Nav1.7 represents an interesting drug target for the treatment of pain. Clinical genetic studies by several groups have established strong genetic links between mutations in the gene SCN9A, coding for Nav1.7, and pain related observations [79,80]. It was shown that the iv administration of naloxone, to an individual insensitive to pain bearing loss-of-function Nav1.7 mutations resulted in a dramatic sensitization of pain thresholds [81], supporting the fact that endogenous enkephalins substantially contribute to the pain-free state found in human Nav1.7-null mutants. Moreover, it was shown that continuous DORs activation by ENKs reduces neuronal Nav1.7 levels in painful diabetic neuropathy [82]. It seemed therefore relevant to study the combination of PL265 with the Nav1.7 blocker, NAV26. In contrast with the previously reported lack of participation of Na,1.7 sodium channel positive receptors in mice with cancer-induced bone pain due to the intrafemoral injection of LL2 lung carcinoma cells [83], the administration of NAV26 induced a dose-dependent antihyperalgesic effect in mice inoculated with B16-F10 cells (Fig. 10A), thus reflecting the previously noted heterogeneous affectation of the nociceptive system when different types tumoral cells are inoculated in bones [84,85]. This antihyperalgesic effect exerted by the administration of NAV26 is related to the stimulation of peripheral MORs and DORs, since it is blocked by naloxone-methiodide, as well as by the selective MOR antagonist NTI and DOR antagonist NTI (Fig. 10B). In the present study, the doses of PL265 and NAV26 necessary to achieve antihyperalgesic effects when administered in combination were reduced by about 70% (Fig. 10D). It was recently demonstrated that preproenkephalin (Penk1) mRNA expression in murine sensory neurons is massively increased in mice lacking the sodium channel Nav1.7 [81]. Therefore, the synergistic antihyperalgesic effect of the combination of PL265 and NAV26 observed in this study (Fig. 10D) are possibly related to the hyperexpression of the ENK-precursor secondary to Nav1.7 blockade, combined with PL265 protecting the tonic release of ENKs.
Fig. 10. Potentiation of the antihyperalgesic effect induced by the peritumoral administration of NAV26 by the simultaneous oral administration of PL265 measured by the unilateral hot plate (UHP) test in mice one week after the intratibially inoculation of B16-F10 cells in their right hind paws. (A) Antihyperalgesic effect induced by NAV26 (0.01–1 μmol, 30 min before testing). Bars represent means and S.E.M. (n = 5–6). **p < 0.01 versus corresponding left paw of vehicle-treated group, Student’s t test; *p < 0.01 versus vehicle-treated right paws, Dunnett’s t test. (B) Inhibition of the antihyperalgesic effect induced by 3 μmol of NAV26 by s.c. administration 30 min before testing of naloxone-methiodide (Nlx-Met, 2 mg/kg), cyprodime (CYP, 1 mg/kg) and naltrindole (NTI, 0.1 mg/kg) but not nor-binaltorphimine (BNI, 10 mg/kg). Bars represent means and S.E.M. (n = 6–7). **p < 0.01 versus NAV26-treated mice, Newman–Keuls test. (C) Antihyperalgesic effect induced by the combined administration of NAV26 and PL265 at fixed doses that represent 1/8, 1/4 and 1/2 of their ED50. Each point represents the mean with the corresponding S.E.M. (n = 5). *p < 0.05, **p < 0.01, compared with vehicle-treated mice, Dunnett’s t test. (D) Isobologram showing the interaction between NAV26 and PL265 administered 30 min, before testing. The oblique line between the x and y axes is the theoretical additive line. The point in the middle of this line is the theoretical additive point calculated from the individual drug ED50 values. The point below the line is the experimental ED50 value obtained with the combination. Horizontal and vertical bars indicate S.E.M.

5. Conclusions

Our results demonstrate the ability of PL265, to counteract bone cancer-induced thermal hyperalgesia in mice, by exclusively stimulating peripheral opioid receptors. The development of such DENKIs, endowed with druggable pharmacokinetic characteristics can be considered as an important step in the development of much needed novel antihyperalgesic drugs. Furthermore, all the tested combinations, except with the CB2 receptor agonist JWH-133, resulted in synergistic antihyperalgesic effects. Although the study of drugs combinations by isobolographic analysis shows limitations since it does not provide an explicit model of a combination’s effect, and thus cannot be used to estimate the effect of a given dose or set of doses [86], as shown here, the greatest synergistic antinociceptive effect (doses could be lowered around 70%) was produced by the combination of PL265 with drugs whose mechanism of action involves the direct activation of the enkephalinergic system. These multi-target-based antinociceptive strategies using combinations with dual inhibitors of enkephalin degrading enzymes may bring therapeutic advantages in terms of efficacy and safety by allowing the reduction of doses of one of the compounds or of both, which is of the utmost interest in the chronic treatment of cancer pain.

Ethical issues

See section 2.1.

Conflict of interest statement

None of the authors have any conflict of interest to declare.

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