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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

Sara González-Rodríguez[a,1], Hervé Poras[b,1], Luis Menéndez[a], Ana Lastra[a], Tanja Ouimet[b], Marie-Claude Fournié-Zaluski[b], Bernard P. Roques[b,c,*], Ana Baamonde[a]

[a] Laboratorio de Farmacología, Facultad de Medicina, Instituto Universitario de Oncologia del Principado de Asturias (IUOPA), Universidad de Oviedo, C/ Julián Clavería, 6, 33006 Oviedo, Asturias, Spain
[b] Pharmaleads, 11 rue Watt, 75013 Paris, France
[c] Université Paris Descartes, 4 avenue de l'Observatoire, 75006 Paris, France

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 ($P2X_3$ 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 2×10^6 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 unilatered 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 absorption by oral route, can be considered as an important step in the development of much needed novel

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.

* Corresponding author at: Pharmaleads, 11 rue Watt, 75013 Paris, France.
E-mail address: bernard.roques@pharmaleads.com (B.P. Roques).
[1] Joint first authors.

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antihyperalgesic drugs. Furthermore, all the tested combinations resulted in synergistic antihyperalgesic effects. As shown here, the greatest synergistic antinociceptive effect (doses could be lowered by 70%) was produced by the combination of PL265 with the $P2X_3$ receptor antagonist (A-317491), cannabinoid CB1 receptor agonist (exogenous, ACEA and endogenous URB937-protected-AEA) and $Na_v1.7$ blocker (NAV26) whose mechanism of action involves the direct activation of the enkephalinergic system.

Conclusions: These multi-target-based antinociceptive strategies using combinations of non-opioid drugs 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.

Implications: This article presents synergistic antinociceptive effect produced by the combination of PL265 with non-opioid analgesic drugs acting via unrelated mechanisms. These multi-target-based antinociceptive strategies may bring therapeutic advantages by allowing the reduction of doses, which is of great interest in the chronic treatment of cancer pain.

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 antitumour 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 $P2X_3$ 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 $Na_v1.7$ 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 $400 \times g$ 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% isoflurane (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