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Involvement of enkephalins in the inhibition of osteosarcoma-induced thermal hyperalgesia evoked by the blockade of peripheral $P2X_3$ receptors

Sara González-Rodríguez[a], Marta Pevida[b], Bernard P. Roques[c], Marie-Claude Fournié-Zaluski[c], Agustín Hidalgo[a], Luis Menéndez[a], Ana Baamonde[a,*]

[a] *Laboratorio de Farmacología, Facultad de Medicina, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, C/Julián Clavería 6, 33006 Oviedo, Asturias, Spain*
[b] *Centro Comunitario de Sangre y Tejidos del Principado de Asturias, Oviedo, Asturias, Spain*
[c] *Pharmaleads SAS, 11 rue Watt, 75013 Paris, France*

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ABSTRACT

Although previous studies describe the up-regulation of purinergic $P2X_3$ receptors expressed at peripheral nociceptive fibers in experimental painful neoplastic processes, the analgesic efficacy of $P2X_3$ receptor antagonists has not been tested in these settings. We study here the effect of the $P2X_3$ receptor antagonist, A-317491, on thermal hyperalgesia produced by the intratibial inoculation of NCTC 2472 fibrosarcoma cells to C3H/HeJ mice. The peritumoral administration of A-317491 (10–100 μg) dose-dependently attenuated osteosarcoma-induced thermal hyperalgesia without modifying thermal latencies measured in the contralateral paws. This antihyperalgesic effect was inhibited by the coadministration of naloxone-methiodide (0.1–1 μg) or the systemic injection of the selective μ-opioid receptor antagonist cyprodime (1 mg/kg), demonstrating the involvement of peripheral μ-opioid receptors. Furthermore, the antihyperalgesic effect induced by A-317491, was antagonised by the coadministration of an anti-enkephalin antibody supporting the participation of endogenous enkephalins. Consistent with this result, the antihyperalgesic effect induced by A-317491 was dramatically enhanced by the administration of an enkephalin-degrading inhibitor, Debio 0827, as demonstrated by isobolographic analysis. This synergism opens the theoretical possibility that the combination of both types of drugs could be useful to counteract some nociceptive symptoms derived from tumor development.

The nucleotide adenosine 5′-triphosphate (ATP) can modulate nociception by acting through particular purinergic receptors that, as $P2X_3$, are expressed at peripheral endings of nociceptors [3,4]. $P2X_3$ receptors are ligand-gated non-selective cation channels whose activation depolarises nociceptive fibers [6], producing an overt pain sensation [21]. The modulation by ATP of nociceptive responses is especially relevant in injured tissues, in which the elevated amounts of ATP present inside cells reach the extracellular fluid [4]. In fact, the stimulation of $P2X_3$ receptors plays a prominent role in the establishment of hyperalgesia from inflammatory or neuropathic origin [15,21]. In these pathological situations, $P2X_3$ receptors can be up-regulated [23,24] and the inhibition of the associated nociceptive symptoms by administering $P2X_3$ receptor antagonists has been reported [8,10,19,22]. Related to the putative role of $P2X_3$ receptors during painful neoplastic processes, an increased expression has been described at peripheral nerves. Thus, their up-regulation in CGRP-immunoreactive epidermal fibers has been shown in mice bearing a painful osteosarcoma evoked by intraosteal inoculation of NCTC 2472 cells [5] and an increase of $P2X_3$-positive cells in small trigeminal ganglion cells has also been detected in Fisher rats inoculated with squamous carcinoma cells [14]. Although these molecular data favour the involvement of $P2X_3$ receptors in cancer-induced pain, no behavioural study has tested so far the antihyperalgesic effects induced by blocking $P2X_3$ receptors in neoplastic models.

The present assays were designed to study whether the blockade of $P2X_3$ receptors could inhibit thermal hyperalgesia developed in a murine model of bone cancer pain. Since the up-regulation of $P2X_3$ receptors during tumoral processes has been described at peripheral level, we studied if the blockade of peripheral $P2X_3$ receptors with A-317491, a selective antagonist of $P2X_3$ homomeric and $P2X_{2/3}$ heteromeric receptors with a poor ability to cross the blood-brain barrier [18,22] could prevent tumoral hyperalgesia. Moreover, since a previous report described that some analgesic effects induced by this drug in inflammatory models are produced through the indirect activation of the opioid system [11], we explored the involvement of opioid peptides in the antihyperalgesic effects produced by A-317491 in tumor-bearing mice.

* Corresponding author. Tel.: +34 985102755; fax: +34 985103551.
E-mail address: arbaiza@uniovi.es (A. Baamonde).

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Five- to six-week-old male C3H/HeJ mice (CRIFFA) maintained in the Animalario of the Universidad de Oviedo (Reg. 33044 13A) were used. The experimental procedures were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain).

A-317491 (Sigma), naloxone-methiodide (Sigma) and anti-met-enkephalin antibody (Chemicon) were dissolved in saline and subcutaneously (s.c.) administered over the tibial tumoral mass (peritumoral injection) or the corresponding region of control mice in a volume of 200 μl. Cyprodime (Sigma), naltrindole (Tocris) and nor-binaltorphimine (Tocris), dissolved in saline, were s.c. administered under the fur of the neck in a volume of 10 ml/kg. Debio 0827 (PL37), 1-2-[(1-ethoxy-carbonyloxy-ethoxycarbonylmethyl)-carbamoyl]-3-phenyl-propyldisulfanylmethyl-3-methylsulfanyl-propyl-ammonium (Debiopharm) was orally (p.o.) administered dissolved in a mixture of ethanol, polyethylene glycol 400 and distilled water (1/4/5). All drugs were administered 30 min before testing.

As previously described [2], NCTC 2472 cells (ATCC) were cultured and passaged weekly. Cells were detached by scraping, centrifuged and the remaining pellet suspended in PBS. For surgical procedures, anesthesia 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 after applying acrylic glue (Hystoacril®, Braun) on the tibial plateau incised area, surgery was finished with a stitch of the skin. Control mice were inoculated with 10^5 NCTC 2472 cells killed by quickly freezing and thawing. Mice were tested 4 weeks after cell inoculation.

Thermal withdrawal latencies were assessed by placing the plantar side of the tested paw on a hot plate surface (51 ± 0.5 °C) as previously described [12]. The mean of two measures was considered in each hind paw and a cut-off of 30 s was established.

The ED_{50} values, which represent the dose of drug that yields antihyperalgesic effects in the 50% of mice, were calculated. The antihyperalgesic effect was considered to be reached when the latency measured in the tumor-bearing paw surpasses the latency that represents the 50% of the maximal antihyperalgesic effect (L50). The latency obtained in the right paws of solvent-treated mice inoculated with killed cells was considered as the maximal antihyperalgesic effect (LK). Thus, L50 was calculated from the latency values obtained in the right paws of solvent-treated mice inoculated with live (LL) or killed (LK) cells by the following formula:

$$L50 = LL + \frac{LK - LL}{2}$$

Based on this fixed value, each tested mouse was classified as showing or not an antihyperalgesic effect and ED_{50} values were calculated by constructing quantal dose–effect curves with the computer program Pharm Tools Pro (version 1.27, The McCary Group Inc.).

Intergroup comparisons were made by an initial one-way analysis of variance followed by the Dunnett's *t*-test in the dose–effect curves or by the Newman–Keuls test for multiple groups. The Student's *t*-test was used for comparing two groups.

As described [13], for isobolographic analysis dose–response curves were obtained by concurrent delivery of A-317491 and Debio 0827 in fixed ratios of their ED_{50} doses (1/8, 1/7, 1/6 and 1/4). The experimental ED_{50} so obtained was compared with the theoretical additive ED_{50} value to determine significant differences and the interaction index was calculated as the experimental ED_{50} value/theoretical ED_{50} value, where values lower than 1 indicate a synergistic interaction [20].

The $P2X_3$ receptor antagonist, A-317491, was peritumorally administered (10–100 μg, corresponding to 0.3–3 mg/kg) 30 min before testing, a time at which analgesic effects have been previously measured [8,11]. After its administration, thermal hyperalgesia measured in the paw affected by the osteosarcoma was inhibited in a dose-dependent manner, whereas the withdrawal latencies measured in the left, non-injured, paw remained unaffected (Fig. 1A). The calculated ED_{50} value was 32.03 ± 3.9 μg (equivalent to 1.06 ± 0.13 mg/kg). When the dose of 100 μg of A-317491, that completely suppresses hyperalgesia when administered in tumor-bearing limbs, was administered to mice inoculated with killed NCTC 2472 cells, no change in withdrawal latencies was detected (Fig. 1B). In addition, the administration of 100 μg of A-317491 to the contralateral, non-inoculated, limbs of tumor-bearing mice did not modify tumoral hyperalgesia, although it induced an increase of latency values in the injected limbs (Fig. 1B).

The antihyperalgesic effect evoked by 100 μg of A-317491 administered in the inoculated limb was dose-dependently antagonised by the peritumoral coadministration of the quaternary opioid receptor antagonist naloxone-methiodide (0.1–1 μg, corresponding to 3–30 μg/kg) or the simultaneous systemic administration of the selective μ-opioid receptor antagonist cyprodime (1 mg/kg), but remained unaffected by the systemic administration of the δ- and κ-opioid receptor antagonists, naltrindole (0.1 mg/kg) or nor-binaltorphimine (10 mg/kg) (Fig. 1C).

In accordance with a previous report [13], the oral administration of 25 mg/kg of the enkephalinase inhibitor Debio 0827 30 min before testing completely inhibits osteosarcoma-induced thermal hyperalgesia (Fig. 2A). This effect was prevented by the peritumoral administration of an anti-met-enkephalin antibody (1:20,000 of the 1 mg/ml commercial solution) (Fig. 2A). When 100 μg of A-317491 were coadministered in the tumor-bearing limb together with the same concentration of this antibody, the antihyperalgesic effect induced by the $P2X_3$ receptor antagonist was also totally inhibited (Fig. 2B).

We next assessed whether the antihyperalgesic effect induced by A-317491 could be potentiated by the coadministration of Debio 0827. A dose–response curve of the antihyperalgesic effect induced by oral Debio 0827 (Fig. 3A) yield an ED_{50} value of 16.52 ± 0.51 mg/kg whereas, as described above, the ED_{50} of A-317491 was 1.06 ± 0.13 mg/kg. Dose–effect curves with fixed combinations of 1/8, 1/7, 1/6 and 1/4 of the ED_{50} of A-317491 and Debio 0827 were performed. As shown in Fig. 3B, a complete antihyperalgesic effect was already obtained when simultaneously administering 1/6 of the ED_{50} of each drug. The experimental ED_{50} value of the combination (2.51 ± 0.04 mg/kg) was significantly lower than the theoretical one (8.32 ± 0.44 mg/kg) (Fig. 3C), being the interaction index value of 0.284 ± 0.02.

The present results demonstrate that the blockade of peripheral $P2X_3$ receptors inhibits osteosarcoma-induced thermal hyperalgesia in mice. The dose-dependent antihyperalgesic effect induced by A-317491 when administered in tumor-bearing paws but not in the contralateral ones or in mice inoculated with killed cells, demonstrates the involvement of $P2X_3$ receptors expressed at the limb affected by the tumor. The slight local analgesic effect detected after the injection of the maximal antihyperalgesic dose of A-317491 in the contralateral paw of tumor-bearing mice could reflect the appearance of contralateral changes after unilateral tissue injury, as sometimes described in experimental pathological settings [9]. Previous reports have described antinociceptive effects in response to the blockade of peripheral $P2X_3$ receptors in inflammatory [10,16,22] or neuropathic [18,19] models. The present results obtained in a tumoral model could be related with the reported increased expression of $P2X_3$ receptors in nociceptive fibers of mice inoculated with NCTC 2472 cells [5], the same cell line

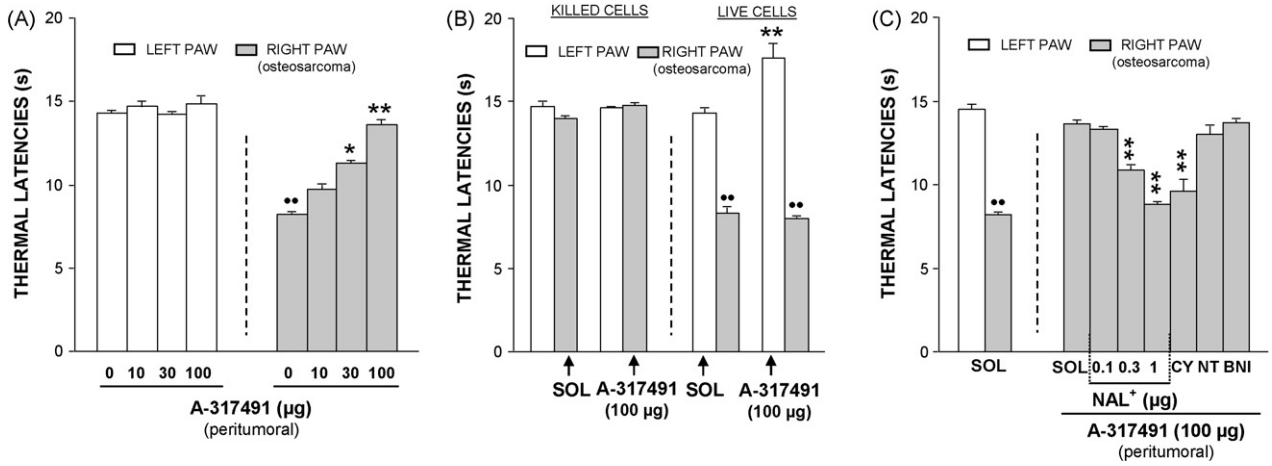

Fig. 1. Peripheral opioid-mediated inhibition of osteosarcoma-induced thermal hyperalgesia by peritumoral A-317491 measured by the unilateral hot plate test. (A) Antihyperalgesic effect induced by A-317491 (10–100 μg, 30 min before testing) in mice inoculated with NCTC 2472 cells in their right paws. $^{*}P<0.05$; $^{**}P<0.01$, compared with solvent-treated right paws, Dunnett's *t*-test. $^{\bullet\bullet}P<0.01$, compared with the corresponding left paw of solvent-treated mice, Student's *t*-test. (B) On the left hand side of the graph, lack of effect of the s.c. administration at the tibial region 30 min before testing of A-317491 (100 μg) or solvent (SOL) in mice inoculated with killed NCTC 2472 cells in their right paws. On the right hand side of the graph, the effect of the s.c. administration at the tibial region of A-317491 (100 μg) in the left paw of mice inoculated with live NCTC 2472 cells in their right paws is shown. $^{**}P<0.01$, compared with the corresponding solvent-treated group; $^{\bullet\bullet}P<0.01$, compared with the value obtained in the corresponding left paw, Student's *t*-test. (C) Inhibition of the antihyperalgesic effect induced by A-317491 (100 μg, 30 min before testing) after the coadministration of naloxone-methiodide (NAL^{+}; 0.1–1 μg, 30 min before testing) or the s.c. injection of cyprodime (CY; 1 mg/kg, 30 min before testing) but not naltrindole (NT; 0.1 mg/kg, 30 min before testing) or nor-binaltorphimine (BNI; 10 mg/kg, 30 min before testing). $^{**}P<0.01$, compared with the values obtained in right paws treated exclusively with A-317491, Dunnett's *t*-test. $^{\bullet\bullet}P<0.01$, compared with the value obtained in the corresponding left paws of solvent-treated mice, Student's *t*-test. In all cases, means and their corresponding S.E.M. are represented ($n=5$–6 per group).

used in the present experiments, or rats inoculated with squamous carcinoma cells [14].

The inhibition of the antihyperalgesic effect induced by A-317491 when coadministered with naloxone-methiodide, a quaternary opioid antagonist which does not cross the blood-brain barrier, further reinforces the idea of a peripheral effect and demonstrates that the inhibition of tumoral hyperalgesia by A-317491 occurs through the stimulation of peripheral opioid receptors. Opioid mechanisms have been involved in the central antihyperalgesic effects produced by this $P2X_3$ receptor antagonist in inflammatory models [11]. Our results indicate that this opioid mechanism triggered secondary to the blockade of $P2X_3$ receptors can also be activated at peripheral level in a bone cancer situation. The reversion of the antihyperalgesic effects induced by A-317491 by the coadministration of a low dose of the selective μ-opioid receptor antagonist cyprodime and the lack of effect of naltrindole and nor-binaltorphimine at doses previously reported to block the analgesic effects produced through the stimulation of δ- and κ-opioid receptors in this same model [1], indicate that the antihyperalgesic effect evoked by A-317491 exclusively occurs through the activation of μ-opioid receptors. Considering the low affinity of A-317491 for μ-opioid receptors [8], the most likely explanation seems to be the involvement of endogenous opioids.

Several opioid peptides, such as endomorphins, β-endorphin or enkephalins, bind to μ-opioid receptors and, thus, could participate in this antihyperalgesic effect. Although enkephalins can

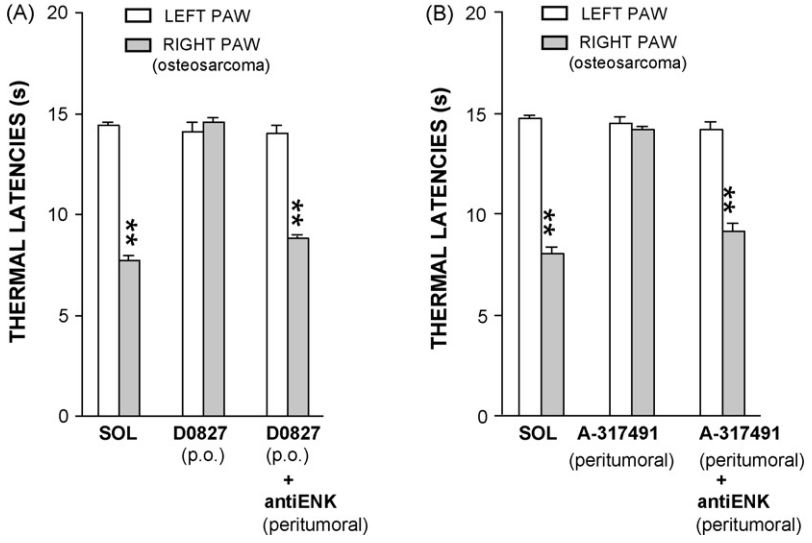

Fig. 2. The peritumoral administration of an anti-met-enkephalin antibody inhibits the antihyperalgesic effect evoked by oral Debio 0827 or peritumoral A-31749 measured by the unilateral hot plate test.
Inhibition evoked by an anti-met-enkephalin antibody (anti-ENK; 1:20,000, 200 μl, 30 min before testing) on the antihyperalgesic effect induced by Debio 0827 (D0827, 25 mg/kg, 30 min before testing) (A) or A-317491 (100 μg, 30 min before testing) (B) in mice inoculated with NCTC 2472 cells in their right paws. In both cases, means and S.E.M. are represented ($n=6$–7 per group). $^{**}P<0.01$, compared with the corresponding left paws, Student's *t*-test.

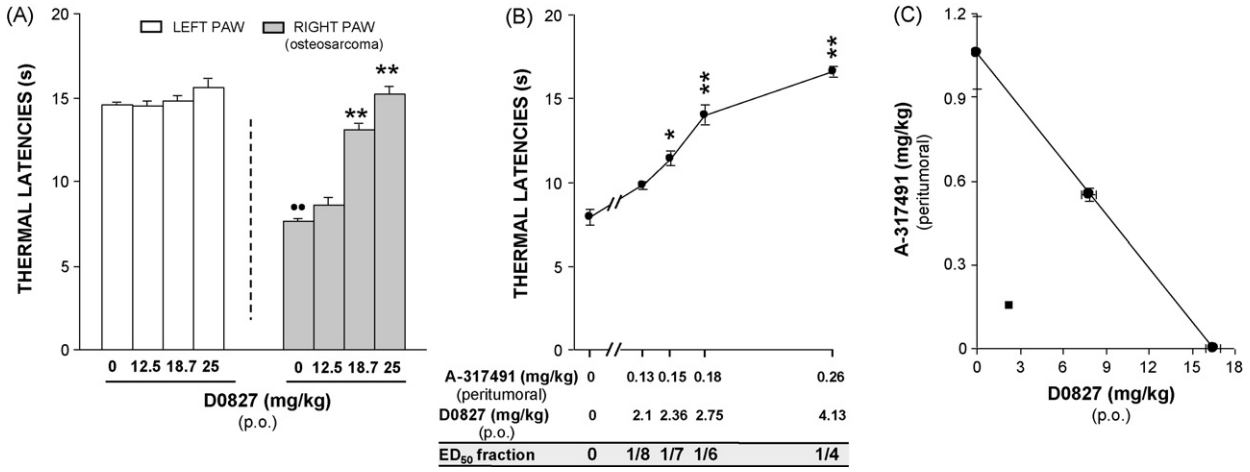

Fig. 3. Potentiation of the antihyperalgesic effect induced by peritumoral A-317491 by the simultaneous oral administration of Debio 0827 measured by the unilateral hot plate test. (A) Antihyperalgesic effect induced by Debio 0827 (D0827, 12.5–25 mg/kg, 30 min before testing) in mice inoculated with NCTC 2472 cells in their right paws. Vertical bars represent means and S.E.M. ($n = 5$–7 per group). $^{**}P < 0.01$, compared with solvent-treated right paws, Dunnett's t-test. $^{\bullet\bullet}P < 0.01$, compared with the corresponding left paws of solvent-treated mice, Student's t-test. (B) Antihyperalgesic effect induced by the combined administration of A-317491 and Debio 0827 (D0827) at fixed doses that represent the 1/8, 1/7, 1/6 and 1/4 of their ED_{50} values in mice inoculated with NCTC 2472 cells. Each point represents the mean with the corresponding S.E.M. ($n = 7$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with solvent-treated mice, Dunnett's t-test. (C) Isobologram showing the interaction between A-317491 and Debio 0827 (D0827) 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 ED_{50} values. The point below the line is the experimental ED_{50} value obtained with the combination. Horizontal and vertical bars indicate S.E.M.

bind both to μ- and δ-opioid receptors, since the inhibition of enkephalins degradation induces μ-opioid receptor-mediated antihyperalgesic effects in this model of bone cancer-induced pain [13], we designed experiments with an anti-enkephalin antibody and an inhibitor of enkephalin catabolism. The suppression of the effect induced by A-317491 by the coadministration of a dose of an anti-enkephalin antibody able to prevent the antinociception induced by an enkephalin-degrading inhibitor demonstrates the involvement of locally released enkephalins in the antihyperalgesic effect of A-317491. Furthermore, as revealed by isobolographic analysis, the effect induced by A-317491 was greatly potentiated when administering by the enkephalin-degrading inhibitor, Debio 0827. Since endomorphins or β-endorphin are not metabolized by the enzymes protected by enkephalin-degrading inhibitors [7], this result further supports that enkephalins are the peptides involved in the antihyperalgesic effect induced by A-317491. Our results raise the intriguing question of the mechanism by which the antagonism of $P2X_3$ receptors could lead to the release of enkephalins. Although the blockade of $P2X_3$ receptors modulates transductional mechanisms that, such as the extracellular signal-regulated kinase (ERK), are involved in the control of protein synthesis in peripheral sensory neurons [17], new experiments seem necessary to clarify this topic.

Overall, our results demonstrate that osteosarcoma-induced thermal hyperalgesia can be inhibited in response to the blockade of peripheral $P2X_3$ receptors through the release of endogenous enkephalins and that the combination of low doses of a $P2X_3$ antagonist together with a enkephalin-degrading inhibitor can be envisaged as a useful strategy to counteract some hyperalgesic symptoms associated to bone cancer.

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