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Promising biomedical subcutaneous delivery system in opioid disaccustom process: In vitro/*in vivo* evaluation of naloxone microparticles on antagonist effect of morphine

M Cristina Benéitez García [a], Rocío Girón Moreno [b], Inés Colmena Crespo [c], Rosalía M Díez-Orejas [d], M Isabel Martín Fontelles [b], Carlos Goicoechea García [b,1], Eva M Sánchez-Robles [b,*], M Esther Gil-Alegre [e,f,1,*]

[a] *Dpt. Ciencias Básicas de la Salud, Área de Farmacia y Tecnología Farmacéutica. Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos, Alcorcón (Madrid) 28922, Spain*
[b] *Dpt. Ciencias Básicas de la Salud, Área de Farmacología, Nutrición y Bromatología Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos, High Performance Research Group in Experimental Pharmacology (PHARMAKOM), Unidad Asociada I+D+i al Instituto de Química Médica (CSIC), Alcorcón (Madrid) 28922, Spain*
[c] *Departamento de Farmacología, Facultad de Medicina, Universidad Autónoma de Madrid, 28029 Madrid, Spain*
[d] *Dpt. Microbiología y Parasitología, Facultad de Farmacia, Universidad Complutense de Madrid, 28040 Madrid, Spain*
[e] *Dpt. Farmacia Galénica y Tecnología Alimentaria. Universidad Complutense de Madrid, 28040 Madrid, Spain*
[f] *Instituto Universitario de Farmacia Industrial, Universidad Complutense de Madrid, 28040 Madrid, Spain*

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ABSTRACT

The addiction induced by the misuse of opioids, is not only a public health emergency but also a social and economic welfare. The main therapy is based on opioid antagonists. Oral and injectable naltrexone administration is the most widely used, presenting some inconveniences: poor patient adherence to the oral daily dosing schedule, cases of hepatitis and clinically significant liver dysfunction.

This study proposes the *in vitro e in vivo* evaluation of anti-opioid properties of naloxone loaded-poly(lactic-*co*-glycolic) acid microparticles (NX-MP). In previous studies, NX-MP showed *in vitro* sustained naloxone release for one week at least.

Our results demonstrate the *in vitro* efficacy of the NX-MP antagonizing for 7 days the morphine effect in SH-SY5Y cells and myenteric plexus-longitudinal muscle preparations isolated from guinea-pig ileum.

The *in vivo* evaluation of the NX-MP was carried out in mice testing the antagonism of the antinociceptive effect of morphine. Results showed that subcutaneous administration of NX-MP blocked the morphine effect.

The results of this work suggest that the subcutaneous administration of NX-MP enhances naloxone therapeutic efficacy as non-addictive medication and could be a promising alternative to naltrexone. Furthermore, the dose of NX-MP can be adapted to the patient necessities. It would be an interesting advantage to treat opioid-addiction.

1. Introduction

In October 2017, the U.S. Government declared the current opioid epidemic the worst drug crisis in American History (Averick, 2020). From 2013 to 2019, the synthetic opioid-involved death rate increased 1,040 %, from 1.0 to 11.4 per 100,000 age-adjusted (3,105 to 36,359). The psychostimulant-involved death rate increased 317 %, from 1.2 (3,627) in 2013 to 5.0 (16,167) in 2019 (Mattson et al.,2021). In recent years, this ratio is increasing (Kristin, 2021). Thus, the addiction induced by the misuse of opioids is a serious crisis that affects public health as well as social and economic welfare (National University of Distance Education, 2010).

* Corresponding authors at: Dpt. Farmacia Galénica y Tecnología Alimentaria. Universidad Complutense de Madrid, 28040 Madrid, Spain (M Esther Gil-Alegre) and Dpt. Ciencias Básicas de la salud, Área de Farmacología, Nutrición y Bromatología, Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos (Eva M Sánchez-Robles).
E-mail addresses: eva.sanchez@urjc.es (E.M. Sánchez-Robles), megil@ucm.es (M.E. Gil-Alegre).
[1] These authors contributed equally as senior authors to this work.

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Currently, the therapy for opioid disaccustoming can be achieved mainly by combining psychosocial management with pharmacological interventions, using opioid antagonists such as naltrexone or naloxone (Ndesendo et al., 2012) whose administration in patients warrant the need for their prolonged or sustained systemic availability.

Although the oral administration of naltrexone has proven effectiveness in the management of opioid disaccustoming, a poor patient adherence to the daily dosing schedule is detected (Ndesendo et al., 2012; Naltrexone Injection, 2010).

For this reason, VIVITROL®, non-addictive medication consisting of naltrexone, was approved in the United States to prevent relapse to opioid dependence after detox. It is given as gluteal intramuscular injection, once monthly. Before starting VIVITROL® treatment, the patient must be opioid-free for a minimum of 7–10 days to avoid sudden opioid withdrawal (U.S. Food& Drug Administration,2006). Up to date, this type of medication has not been authorized in European Union by the European Medicines Agency (EMA) (EMA. Medicines Search, 2022).

In addition, successful of naltrexone as pharmacological treatment is still limited by patient's liver damage. Patients should be warned of the risk of hepatic injury and advised to seek medical attention if they experience symptoms of acute hepatitis. The use of VIVITROL should be discontinued in cases of symptoms and/or signs of hepatic injury. Doctors will probably not recommend naltrexone injection if patients have liver disease (Skolnick, 2018).

This means that not all the patients may receive naltrexone-based treatment, so an alternative pharmacological treatment is needed.

Naloxone (NX) is an opioid receptor antagonist which acts on the μ, δ and κ receptors (Kjøniksen et al., 2014). Compared with naltrexone, NX has the advantage of lower dosage-dependent liver toxicity (Ferrari et al., 1998).

This work proposes the *in vitro* and *in vivo* evaluation of anti-opioid properties of naloxone loaded-poly(lactic-*co*-glycolic) acid (PLGA) microparticles (NX-MP). NX-MP are intended for a week sustained release of NX after their subcutaneous administration. The design of this injectable biomedical system, NX-MP, has been described in detail in our previous works (Benéitez and Gil-Alegre, 2017; Benéitez et al., 2014). These NX-MP are made of biocompatible and biodegradable polymers, approved both by the Food Drug Administration (FDA) and European Medicine Agency (EMA) for human use (Deshayes and Kasko, 2013). Naloxone release was sustained *in vitro* for one week at least, which could be of great interest in the clinic, as it could allow a prolonged therapeutic effect in patients after a single parenteral administration of the NX- MP suspended in PBS. Then, the evaluation of the pharmacological efficiency of the NX-MP is needed. For this, *in vitro* (cell cultures and organ bath) and *in vivo* assays have been performed in this study.

The ability of NX-MP to reverse the *in vitro* morphine effect at different concentrations in SH-SY5Y cells was investigated for the first time. Through a literature review, it was observed that SH-SY5Y human neuroblastoma cell line (Horner and Zadina, 2004; Fiore et al., 2013) expresses the μ opioid receptor, which is the main pharmacological target for morphine (Rang and Dale, 2012).

In addition, *in vitro* pharmacological activity of the amount of NX released from MP was studied in organ bath, and its ability to antagonize the morphine effect on the myenteric plexus-longitudinal muscle strips, isolated from guinea-pig ileum, was tested.

Once *in vitro* studies showed that NX-MP have opioid antagonist effect, it was carried out preliminary *in vivo* studies. CD-1 mice were used to determinate if the NX-MP can reverse the morphine effect.

2. Materials and methods

2.1. Animals and ethics statement

All experimental procedures were carried out according to the National (Law 32 / 2007, RD 53/2013 and order ECC /566/2015) and European (2010/63/EU) laws and governmental regulations. The procedures were approved by the Ethical Committee of the Universidad Rey Juan Carlos (Ref. 2806202216722). Efforts were made to reduce the number of animals used.

Animals were young adult (2.5 months old) female guinea-pigs (300–350 g) and male CD-1 mice (25–30 g) obtained from Envigo, S. A, Spain. All animals were housed in the Veterinary Unit of Universidad Rey Juan Carlos, in rooms with controlled conditions (22 ± 1 °C of temperature, 60 % of humidity and 12 h day/night cycle. Food and water were available *ad libitum*.

2.2. Drugs

Naloxone hydrochloride and morphine sulphate were obtained from Sigma-Aldrich, Spain and Alcaliber, Spain, respectively. The purity of both drugs is 98.0 per cent to 102.0 per cent (anhydrous substance).

2.3. Preparation of naloxone microparticles

Spherical and nonporous NX-MP (SEM microscopy, EOS 6335F, JEOL, Japan) were prepared by doble emulsion–solvent evaporation method, described in detail in previous works (Benéitez and Gil-Alegre, 2017; Benéitez et al., 2014). The MP were freeze-dried for 12 h at − 60 °C, 200 mT (Flexi-Dry MP, FTS® Systems, NY, USA) to prevent polymer hydrolysis during storage.

The diameter of the MP was found to be 45 μm. Laser Diffraction Particle Size analysis indicated a monomodal distribution of particle size, with a size distribution of 8.80 μm.The drug loading was determined by spectrophotometry method (Benéitez et al., 2014), dissolving accurately weighed amounts of MP in THF: water (7:1) and subsequent UV drug detection at λ 288 nm (Spectrophotometer Beckmann DU7). All measurements were carried out in triplicate. The drug loading of the MP was 7 ± 0.81 mg of NX in 100 mg of MP.

Encapsulation efficiency was calculated from equation: $EE = (M_{in}/M_{tot}) \times 100$, where EE was the encapsulation efficiency, M_{in} the mass of NX in MP, and M_{tot} the mass of NX used in the.

formulation. The experiments were accomplished in triplicate. The EE % was 58.7 ± 2,3.

NX-loaded MP appears to generate tri-phasic release profile with a rapid release phase I (12 h). Drug release was 70 % over 7 days and the *in vitro* release studies showed concentrations about 200 μM of NX within the first 24 h. The lowest concentration was obtained in the 7th day (103 μM of NX). The dose or amount of NX-MP used in the *in vitro/in vivo* evaluations was calculated from the amount of NX able to revert the effect of morphine according to the results of naloxone control in the different studies.

2.4. In vitro assays: Cell culture

The activity of the NX-MP incubated in the physiological cell culture conditions was determined at different morphine concentrations: from therapeutic doses to over-doses.

SH-SY5Y human neuroblastome cell line was purchased from Novartis (Basel, Switzerland). Cells were grown at 37 °C in medium containing a 1:1 ratio of Eagle's minimum essential medium and Ham's/F^2 nutrient mixture, and 10 % fetal bovine serum in a humidified atmosphere containing 5 % of carbon dioxide. Cells were harvested, after three rinses with Dulbecco's phosphate buffered saline.

Cell viability was determinate using the MTT assay. Briefly, the day of the analysis, MTT (5 mg/mL) was added to each well and the cells were incubated at 37 °C for 3 h. Then, blue formazan crystals were dissolved by adding lyses buffer pH 4.7 consisting of 20 % w/v sodium dodecyl sulfate in 1:1 dimethylformamide: water. After 6 h incubation period at 37 °C, the optical densities were measured with absorbance, luminescence and fluorescence (FLUOstar OPTIMA, BMG LABTECH) at a wavelength of 562 nm. Data were expressed as the percentage of cell viability in relation to vehicle or placebo MP-treated wells. The cell

viability was calculated by the following formula (Hernán Pérez de la Ossa et al, 2012):

$$Cellviability(\%) = \frac{OpticalDensityofthetreatedcells}{OpticalDensityofthevehicle - treatedcells}$$

All the experiments were performed a minimum of eight times.

2.5. In vitro assays: Organ bath

Experiments were designed to evaluate the effect of NX (control) and NX delivered from MP on the effect of morphine on the myenteric plexus–longitudinal muscle strips (MP–LM) isolated from guinea pig ileum.

Animals were sacrificed and the MP–LM were isolated from the ileum as described by Ambache (Ambache et al., 1969). Tissues were suspended in a 10 mL organ bath that contained 5 mL of Krebs solution (NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11 mM) at 37 °C and continuously gassed with carbogen (95 % O_2 and 5 % CO_2). Tissues were kept under 1 g of resting tension and were electrically stimulated through two platinum ring electrodes (rectangular pulses of 70 V, 0.1 ms duration and 0.3 Hz frequency). The isometric force was monitored through a computer using a MacLab data recording and analysis system (Cibertec, S.A., Spain).

The NX-MP were incubated in a pH-regulating phosphate saline solution at a value of 7.4 for 7 days and the supernatant, containing the released NX, was collected at different times (1, 2, 4, 5, 6 and 7 days) for evaluate its pharmacological effect.

The evaluation of the opioid-antagonistic activity of the NX or NX-MP supernatants on the inhibitory effect of morphine on the tissue contractions was carried out as follows: after a 15 min stabilization period of the MP–LM strips in the organ bath, electrical stimulation was applied, and 5 min after, a dose of morphine (2×10^{-6} M) was added to the organ bath and its effect on the electrically induced contractions was evaluated, once the response reached a plateau. Next, a cumulative concentration–response curve of the supernatants with seven concentrations in the range of $10^{-8} - 6.4 \times 10^{-7}$ M was constructed in a step by step manner. These concentrations were chosen according to previous studies of our research group (Goicoechea et al., 2008). The effect of NX and NX-MP was expressed as the percentage (%) of contraction recovery, taking the mean amplitude of the last five contractions after the addition of morphine as 100 %.

Each tissue was employed only once, and all the experiments were performed a minimum of six times.

2.6. In vivo assays: hot-plate test

The hot-plate test was performed according to a modification of the method described by Eddy and Leimbach (Eddy and Leimbach, 1953) using a commercial device (Ugo Basile, Italy). Each mouse was placed on the heated surface at 55 °C, in a Plexiglas cylinder, and the nociceptive response latency (s) to the thermal stimulus was measured, being the licking of forepaws the nociceptive threshold. To avoid paw damage, a cut-off time of 30 s was considered. Response latencies were determined twice, before and after treatment administration. Data were converted to percentage of the maximum possible effect (% M.P.E.) that was calculated as: 100 × [post-drug latency] − [baseline latency] / [cut-off time − baseline latency].

Different animal groups were used to evaluate the antinociceptive effect of morphine and its antagonism by NX-MP. Doses were chosen according to previous studies of our research group (Goicoechea et al., 2008; Giron et al., 2002; Mercado et al., 2022). All treatments were administered to the mice, dissolved in 0.9 % saline solution. Morphine 5 mg/kg was administered by intraperitoneal (i.p.) via and NX-MP subcutaneously (s.c.), in a volume of 0.5 mL. The amount of NX-MP was 0.85 mg per animal (0.06 mg of NX).

Naloxone microparticles was administered once per group in previous days (1, 2, 4, 5, 6 and 7) to the evaluation day and morphine administration. The antinociceptive effect of morphine was evaluated 30 min after its injection. (See protocol in Fig. 1).

The experimental groups (N ≥ 10 mice/group) were:

- Control Group: Morphine 5 mg/kg
- Group 1: Naloxone microparticles (NX-MP) administered 1 day prior to morphine.
- Group 2: NX-MP administered 2 days prior to morphine.
- Group 3: NX-MP administered 4 days prior to morphine.
- Group 4: NX-MP administered 5 days prior to morphine.
- Group 5: NX-MP administered 6 days prior to morphine.
- Group 6: NX-MP administered 7 days prior to morphine.

2.7. Statistical analysis

In vitro cell studies: Statistical analysis was performed using Statgraphics® software. The results were expressed as mean ± S.D. Statistically significant differences were calculated by one-way analysis of variance (ANOVA) following the posthoc test least significant difference (LSD).

In vitro organ bath and *in vivo* studies: The sample size was calculated by G-power 3.1 software; considering an alpha error of 0.05, a power of 0.8 and an effect size of 0.25, the minimum number of animals in each experimental group was 10 to obtain reliable results.

All data, from *in vitro* and *in vivo* assays were analyzed and plotted using GraphPad Prism 7.0 (GraphPad Software, San Diego, USA). They were checked for normality by DAgostino-Pearson test and expressed as mean ± Standard Error of the Mean (S.E.M.). Two-tailed Unpaired *t*-test (for following normality data) or Mann Whitney test (for non-following normality data) were used to compare results obtained in the hot-plate test.

Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. In vitro assays: Evaluation of NX-MP on the morphine effect in cell cultures

3.1.1. Effect of morphine on SH-SY5Y live cells

Treatment of SH-SY5Y cells with varying concentrations of morphine was studied from morphine clinical analgesic doses to abuse values. Thus, morphine is used clinically in dosages of 10–2400 mg/day resulting in serum concentration of 2 nM − 3.5 μM (Fiore et al., 2013). Then, the tested concentrations were: 1 nM, 10 nM, 100 nM, 1 μM, 10 μM, 100 μM and 1 mM.

It was observed that all the morphine concentrations studied except two, 10 nM and 1 μM, affected significantly to the cell viability in comparison with control cells (untreated cells) ($p < 0.05$) (Fig. 2).

The morphine concentrations with the greatest influence in the results were 100 nM (115.6 ± 3.7 %) and 10 μM (83,5 ± 2.4 %) that increased and decreased cell viability, respectively. Therefore, concentrations of morphine of 100 nM and 10 μM, were selected for the *in vitro* evaluation of the efficacy of NX-MP.

3.1.2. Effect of NX on SH-SY5Y live cells

Cell exposure to NX had no effect on cell proliferation for all the concentrations studied compared to control cells (untreated cells) ($p < 0.05$), as shown in Fig. 3.

Treatments with 100 nM, 1 μM and 10 μM NX resulted in values of 98.06 ± 3.1 %, 99.14 ± 2.7 % and 101.0 ± 2.1 %, respectively. No significant differences to the control value (100 %) were detected. All the NX concentrations studied were selected.

3.1.3. Effect of NX on SH-SY5Y live cells in presence of morphine

Cells were co-incubated with morphine at the selected two

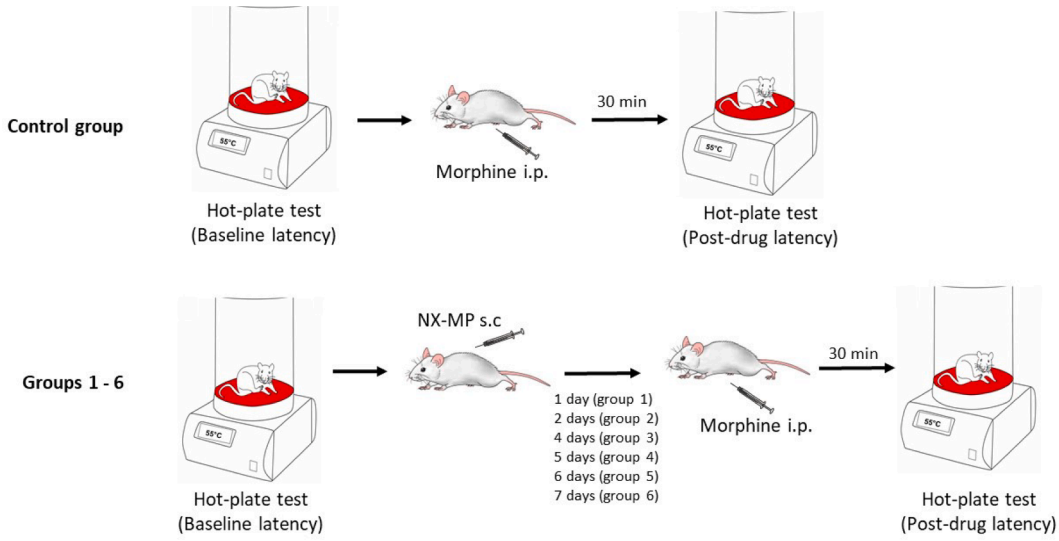

Fig. 1. Scheme illustrating the *in vivo* evaluation protocol in different animal experimental groups. In the control group the antinociceptive effect of morphine 5 mg/kg was tested. In groups 1–6, naloxone-microparticles (NX-MP) were administered, prior to morphine to study their antagonist effect, at different time (1, 2, 4, 5, 6 and 7 days, post administration). (The hot-plate drawing is modified from that of Deuis et al., 2017).

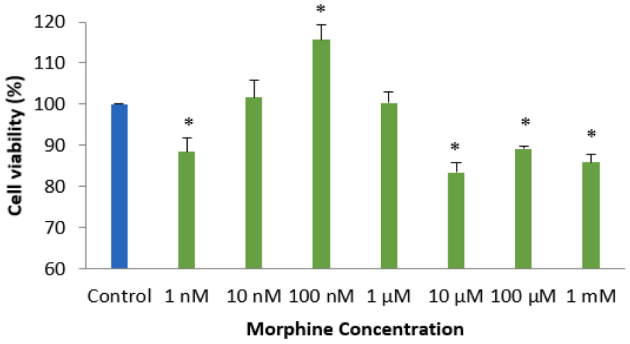

Fig. 2. Effect of different concentrations of morphine on SH-SY5Y cells. Data are presented as the percentage of the basal cell viability (mean ± SD) (N = 8). *$p < 0.05$ (1-way ANOVA *vs* control, posthoc test LSD).

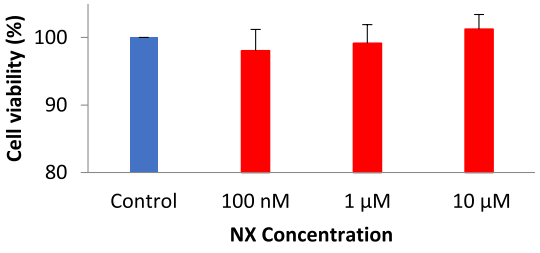

Fig. 3. Effect of different concentrations of naloxone (NX) on SH-SY5Y cells. Data are presented as the percentage of the basal cell viability (mean ± SD) (N = 8).

concentrations (100 nM and 10 µM) and with NX at the three selected concentrations (100 nM, 1 µM and 10 µM) and results are shown in Fig. 4a and 4b. These results showed that only NX at 10 µM was able to revert morphine effects not only at 100 nM (Fig. 4a) but also at 10 µM (Fig. 4b) ($p < 0.05$, one-way ANOVA, post-hoc test LSD).

3.1.4. Effect of NX-MP on SH-SY5Y live cells in presence of morphine

According to previous results, it was calculated the amount of MP for a week cell-incubation reaching a daily NX concentration at least of 10 µM. Then, 6 mg of NX-MP were co-incubated with morphine for the *in vitro* study in cell culture.

The results obtained when morphine, at both concentrations 100 nM and 10 µM, was incubated simultaneously with the NX-MP (7th day) were presented in Fig. 4a and 4b. As can be observed, the effect of NX-MP after 7 days of co-incubation with morphine was not significant different from the effect of free NX and control.

Then NX-MP was able to antagonize the morphine effect on cells, at morphine concentrations of 100 nM and 10 µM, during 7 days of co-incubation ($p < 0.05$, one-way ANOVA).

3.2. In vitro assays: Evaluation of NX-MP on the morphine effect on MP-LM in the organ bath

Morphine $2x10^{-6}$ M decreased the contractile activity of MP-LM (decrease of 68.8 ± 3.5 %, data not shown). Both the control NX and all supernatants with NX released from the MP, at different times, were able to antagonize the morphine effect, on a concentration dependent manner (Fig. 5). This effect was very similar amongst all samples and no significant statistically differences were obtained with respect to the NX used as control. Therefore, these results confirm the validity of the MP as a modified NX release system and their efficacy antagonizing the opioid agonist effect on the *in vitro* assays of organ bath.

3.3. In vivo assays: Evaluation of NX-MP on the morphine effect in the hot-plate test in mice

Morphine 5 mg/kg produced an antinociceptive effect of 76.5 ± 5.8 % on the hot-plate test. When the effect of NX-MP was evaluated at different days of release, it was observed that they antagonized the antinociceptive effect of morphine at days 1, 2, 4, 5 and 6 ($p < 0.01$, $p < 0.05$, $p < 0.01p < 0.01p < 0.001$, respectively *vs* morphine, unpaired-t or Mann Whitney tests), but not at day 7 ($p > 0.05$) (Fig. 6). These results demonstrate the efficacy of the NX-MP in antagonizing the opioid effect and that this pharmacological effect lasts for at least 6 days.

4. Discussion and conclusion

The design of an injectable biomedical system as MP with NX described in detail in previous works (Benéitez and Gil-Alegre, 2017; Benéitez et al., 2014) demonstrated a control sustained release of NX for 7 days. According to this promising result, the preclinical evaluation of the NX-MP efficacy was performed for 7 days using *in vitro* and *in vivo*

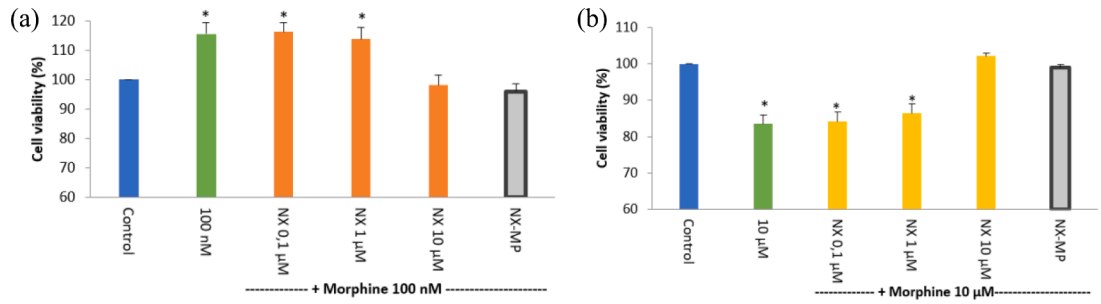

Fig. 4. Effect of NX (0.1 µM, 1 µM and 10 µM) co-incubated with morphine on SH-SY5Y cell viability, and effect of NX-MP co-incubated during 7 seven days with morphine on SH-SY5Y cell viability. Data are presented as the percentage of the basal cell viability (mean ± SD) (N = 8). Control = untreated cells. *$p < 0.05$ (1-way ANOVA *vs* control following the post-hoc test LSD). Fig. 4a co-incubated with morphine 100 nM and Fig. 4b co-incubated with morphine 10 µM.

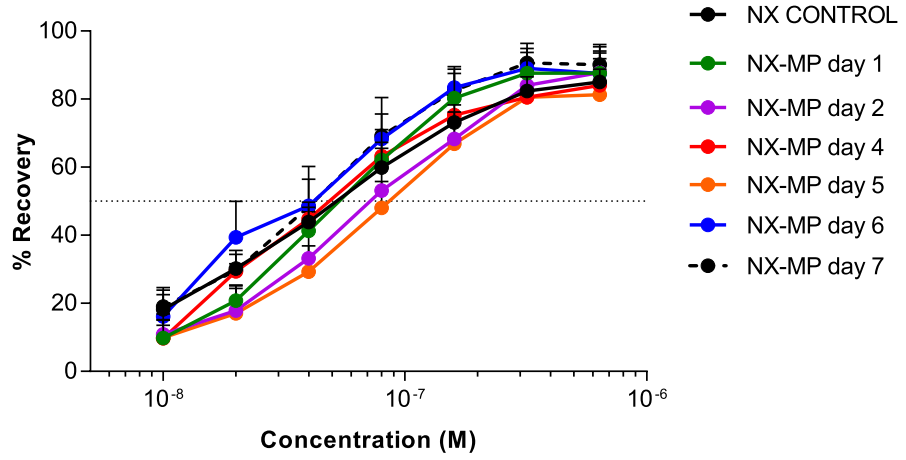

Fig. 5. The graph shows the blockade of the inhibitory effect of morphine induced by the addition to the organ bath of control naloxone (NX) or the supernatants containing NX released from the naloxone microparticles (NX-MP) at 1, 2, 4, 5, 6 and 7 days of the *in vitro* release assays. Each line shows the concentration-dependent recovery (%) of the basal contractile activity of the strips expressed as the mean ± S.E.M. of 6–8 experiments. 1-way ANOVA *vs* NX control.

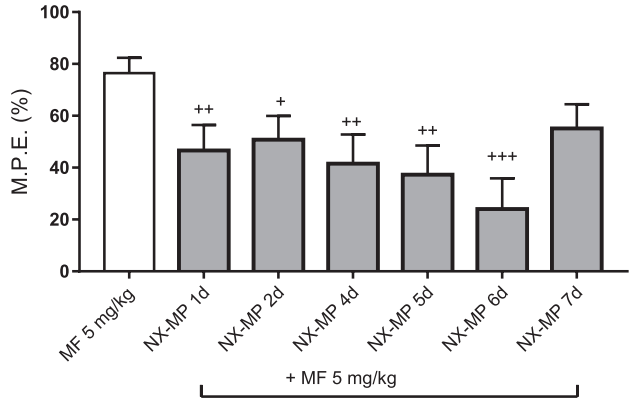

Fig. 6. Antinociceptive effect of morphine (MF) 5 mg/kg alone and with naloxone microparticles (NX-MP), at different days post administration, in mice. Each bar shows the mean ± S.E.M of maximum possible effect (M.P.E.) induced by morphine on the hot-plate test. + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ *vs* MF, Unpaired *t*-test/ Mann Whitney test, N ≥ 10.

experiments.

The *in-vitro* efficacy evaluation was carried out using SH-SY5Y cells that express the µ opioid receptor, being the first time that a cell culture is used to show the *in vitro* evaluation of naloxone in sustained release systems to antagonize the morphine effect. The results proved the ability of NX-MP to effectively reverse the morphine effect for 7 days. The evaluation period of 7 days could not be extended because cells in the control wells reached 100 % confluence and started to die immediately.

Moreover, the *in vitro* assays were carried out using the myenteric plexus–longitudinal muscle strips (MP–LM) isolated from guinea pig ileum in an organ bath. The results showed that the control NX and all supernatants with NX released from the MP, at the different times studied (1–7 days), were able to antagonize the morphine inhibitory effect on the contractile activity of the strips.

The blockade induced by NX-MP was comparable to that observed with the daily administration of equivalent amounts of NX in both *in vitro* assays, cell cultures and organ bath.

In vivo assays were performed on mice using the hot-plate test to evaluate the efficacy of NX-MP on antagonizing the morphine effect and, results show that NX-MP reduced the antinociception induced by morphine up to day 6. Although other studies have been already performed in mice to evaluate the effect of naloxone in sustained delivery systems, this is the first time that the subcutaneous administration of naloxone microparticles has been studied for a long period of 7 days (Madison et al., 2020; Akeemat et al., 2022; Andrew et al., 2019).

Thus, the results of the present work show the efficacy of NX-MP in antagonizing the opioid effect of morphine. This pharmacological effect lasts for at least 6 days, which demonstrated that NX activity was preserved after microencapsulation. The ineffectiveness on day 7 may be due to the polymer degradation, acidifying the environment and slowing down the release of naloxone from de MP (Benéitez and Gil-Alegre, 2017; Benéitez et al., 2014).

According to the data presented in this work, for the first time, PLGA MP allowed the development of a subcutaneous formulation of naloxone-microparticles suspended in an aqueous vehicle that manage to release an amount of drug with promising antagonist morphine effect

for 7 days, not only at clinically analgesic doses but also at abuse doses.

In conclusion, NX-MP could be a promising alternative to naltrexone, useful, specially, for patients with hepatic problems. The amount of NX-MP can be adapted to the patient necessities to avoid withdrawal behaviors and to answer the health problem of opioid over consumption.

CRediT authorship contribution statement

M Cristina Benéitez García: Investigation. **Rocío Girón Moreno:** Investigation. **Inés Colmena Crespo:** Investigation. **Rosalía M Díez-Orejas:** Methodology, Supervision. **M Isabel Martín Fontelles:** Methodology, Supervision. **Carlos Goicoechea García:** Methodology, Supervision. **Eva M Sánchez-Robles:** Investigation. **M Esther Gil-Alegre:** Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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