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Antinociceptive effects of morphine, fentanyl, tramadol and their combination, in morphine-tolerant mice

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ABSTRACT

The development of morphine-tolerance after chronic administration, reduces analgesic efficacy and is a significant clinical problem in some patients; may be managed clinically by increasing the doses of morphine and/or the administration of a second mu-opioid agonist. In morphine-tolerant mice, we investigated the presence of an interaction when two opioids are administered simultaneously. We determined the antinociceptive effects of morphine (M), fentanyl (FEN), and tramadol (TRM) individually and combined in a 1:1 proportion, based on their potency. Nociceptive thresholds were evaluated in CD1 mice using the hot plate test. Morphine tolerance was induced by the subcutaneous implantation of a 75 mg morphine pellet, whereas control animals received a placebo pellet; the experiments were performed three days later. In both (placebo and morphine pellets), dose–response curves for M, FEN and TRM, individually and combined were obtained, and the doses that produced 50% inhibition (ED_{50}) were determined. Sustained exposure to morphine induced a significant decrease in antinociceptive potency to acute M or FEN administration (tolerance), which was of a lesser magnitude after acute TRM; in these experiments the analysis of the interaction between chronic morphine and each opioid, demonstrated functional antagonism. The simultaneous administration of two opioids in morphine-tolerant mice, demonstrated antagonism for the M:FEN combination, whereas the effects of TRM combined with M or FEN, remained additive. The results suggest that during morphine-tolerance, TRM could be a useful drug to induce effective analgesia when combined with FEN or M.

1. Introduction

Chronic exposure to morphine induces adaptive changes in cellular and molecular pathways, underlying the development of clinically tolerance and cross-tolerance to other mu-opioid agonists (Tumati et al., 2009; Zhang et al., 2009; Hernández et al., 2009). Clinically, the repeated administration of morphine to patients induces loss of analgesic efficacy requiring an increase in the doses to maintain the analgesic effect (Bailey and Connor, 2005; Mercadante and Bruera, 2006). Opioid switching (Slatkin, 2009; Vissers et al., 2010) and multimodal analgesia including the administration of a second opioid (Richebé and Beaulieu, 2009), are common strategies in the management of opioid-tolerance in clinical practice, but their efficacy remains unclear. Different opioids such as fentanyl or tramadol, alone or combined, are used to enhance analgesia in morphine tolerant patients (Portenoy et al., 2006; Simpson et al., 2007; Devulder et al., 2009); however, little is known about the possible pharmacological interactions of these drugs during morphine-tolerance.

In animal models of nociception, we have recently shown that the simultaneous administration (combination) of tramadol (TRM) plus fentanyl (FEN) or TRM plus morphine (M) are synergistic for antinociception in the writhing and formalin tests in mice, although they were only additive in the hot plate test; conversely, co-administration of FEN and M showed additive nociceptive effects regardless of the type of stimulus (Romero et al., 2010). However, these experiments were performed in opioid-naïve animal models. The aim of the present study was to assess in mice, whether the presence of morphine-tolerance would modify the type of interaction when two opioids were administered simultaneously. To this purpose, M, FEN and TRM were administered alone or combined in a 1:1 potency ratio on the basis of their antinociceptive potency.

2. Material and methods

2.1. Animals

Male CD1 mice, weighing 25–30 g (Charles River, France) were used. Procedures were performed in accordance with the Ethical

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Guidelines of the International Association for the Study of Pain and approved by the Ethical Committee for Animal Welfare of our Institution (CEEA-PRBB, *Comité Ético de Experimentación Animal*, IMIM-PRBB, Barcelona, Spain). Mice were housed in groups of 5 in individual ventilated cages (Technicplast), and had free access to food and water. Autoclaved poplar softwood bedding (Souralit S.L., Barcelona, Spain) was used. Additionally, all cages had autoclaved cellulose paper as nesting material. All these conditions provide maximum animal comfort. Animals were maintained in a room under a 12 h light/dark cycle (lights on at 08:00 a.m.), at controlled temperature (21 ± 1 °C), and relative humidity (55 ± 10%). Behavioral testing was performed between 9:00 a.m. and 5:00 p.m., in a quiet room, during the light cycle. Mice were used once and were sacrificed at the end of the experiment by cervical dislocation.

2.2. Drugs

Morphine hydrochloride and morphine base (for pellet preparation) were obtained from Alcaliber, S.A (Madrid, Spain); for the other opioids we used the same commercial drugs administered to humans in clinical practice: fentanyl was obtained from Kern Pharma (Barcelona, Spain) and tramadol from Grünenthal (Madrid, Spain). Individual drugs and their combinations were dissolved in saline solution (NaCl 0.9%) just before use. Drugs were administered subcutaneously (s.c.) at the nape of the neck in a final volume of 0.250 ml, 30 min before behavioral testing. Doses and time of administration were selected based on previous studies performed by our group (Dürsteler et al., 2006; Fernández-Dueñas et al., 2007).

2.3. Induction of tolerance to morphine

Under sevoflurane anesthesia, mice were randomly implanted subcutaneously with a 75 mg morphine base or placebo pellet, at the nape of the neck. In all instances, experiments were performed 72 h after pellet implantation. We have previously reported that the s.c. implantation of a 75 mg morphine-pellet in mice, induces high plasma concentrations of morphine that peak and remained unaltered from days 2 to 3, after implantation (7.3 ± 1.4 μg/ml and 8.2 ± 1.3 μg/ml, respectively); under these conditions, we could demonstrate intense tolerance to the antinociceptive, anti-extravasation (Fernández-Dueñas et al., 2007) and anti-transit effects of morphine (Pol and Puig, 1997).

2.4. Antinociceptive test

Drug-induced antinociception was assessed in the hot plate test, using a commercial analgesiometer (Columbus Instruments, Columbus, OH, USA), heated to 52 ± 1 °C (Castañé et al., 2006; Bredeloux et al., 2006). A 240 s cut-off time was selected on the basis of the work of other investigators (Castañé et al., 2006; Bredeloux et al., 2006; Contet et al., 2006). Moreover, it has also been suggested that the cut-off should be set at 3 times the reaction time of the control (Le Bars et al., 2001), and our mean baseline latency value in naïve mice (PL) was 82.41 ± 4.05 s ($n = 29$).

In the initial pilot experiments we were unable to obtain reliable dose–response relationships when assessing hindpaw licking, and thus we utilized as endpoint the jumping behavior, that is considered less variable (Carter, 1991; Wilson and Mogil, 2001). Robust curves could be generated when evaluating jumping (see Fig. 1). However, we are aware that evaluating the antinociceptive behavior by means of licking or jumping may produce different results (Carter, 1991). None of the animals tested using our experimental protocol presented noticeable lesions or redness of the plantar tissue. Percent antinociception was calculated as:

$$\%MPE = [(\text{latency postdrug} - \text{control latency}) / (240 - \text{control latency})] \times 100$$

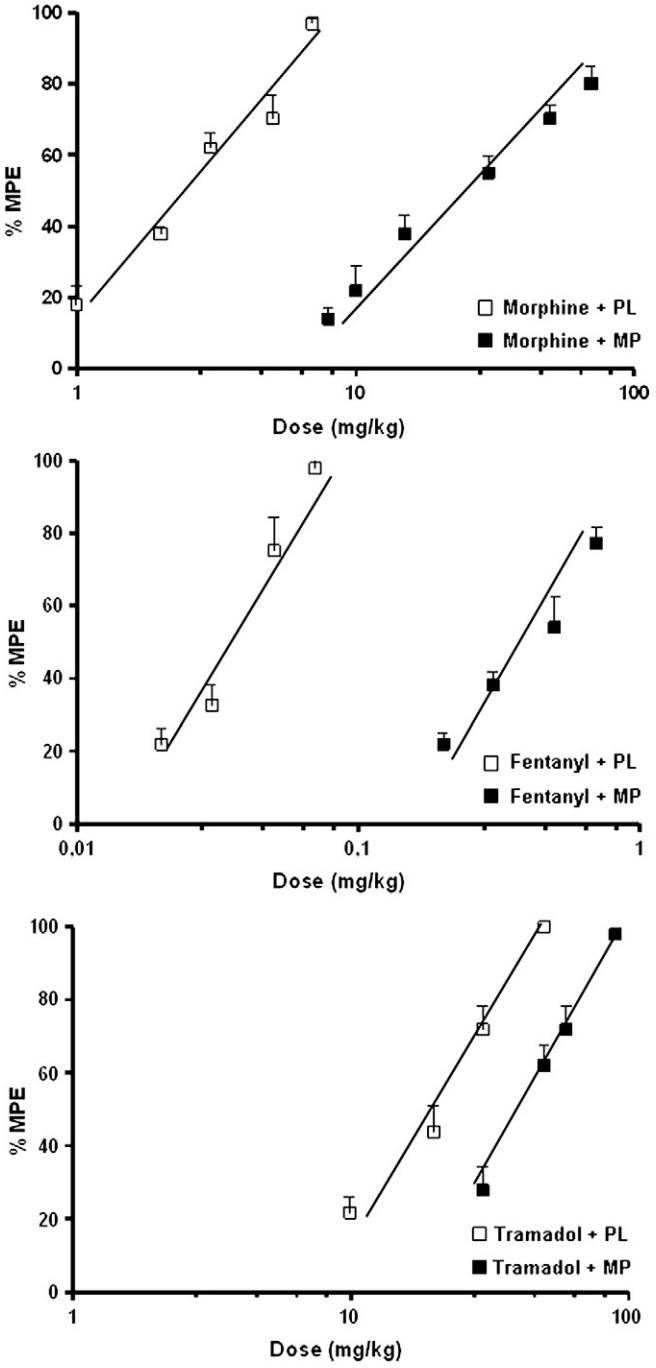

Fig. 1. Dose–response curves for the antinociceptive effect of subcutaneous morphine, fentanyl or tramadol, in placebo (PL,□) and morphine-pellet (MP,■) implanted mice. Each point represents the mean value of 10–12 animals and the vertical bars indicate the S.E.M.% MPE, percent of maximum possible effect.

2.5. Determination of motor coordination: Rotarod test

The rotarod test (Cristiano et al., 2008) was used to evaluate the effects of the opioids, individually and combined in a 1:1 proportion, on motor coordination behavior (LSI — Letica Scientific Instruments, Barcelona, Spain). Based in the antinociceptive responses obtained in tolerant mice, the ED_{80} values obtained from tolerant mice were tested. Drugs were subcutaneously administered in a volume of 0.250 ml, 30 min before behavioral testing. Prior to rotarod testing, all animals (placebo and morphine pellet) were trained to run on the

rotarod equipment at a constant velocity of 10 rpm. Mice that were unable to remain on the rod for two consecutive periods of 240 s (cut-off) were discarded. After a baseline trial of 240 s, the effects of the different drugs were tested, and the time that the animal remained on the rotarod was recorded.

2.6. Experimental protocol and groups of experiments

Initially, we tested the antinociceptive effects induced by the s.c. administration of M (dose range 1–7 mg/kg and 7–70 mg/kg in mice implanted with a placebo or morphine-pellet respectively), FEN (0.02–0.07 mg/kg in placebo-pellet, or 0.2–0.7 mg/kg in morphine-pellet) and TRM (range 10–50 mg/kg in placebo-pellet and 20–100 mg/kg in morphine-pellet mice). Dose–response relationships were obtained for each drug individually in placebo and morphine-pellet implanted mice. A least-square linear regression analysis of the log dose–response curves allowed the calculation of the effective doses (ED) as a measure of potency, according to the method of Tallarida (2000). In the present study, the ED_{50} is defined as the dose of a drug or a combination of drugs that produces a 50% of the maximum effect. Similarly, the ED_{20} and ED_{80} correspond to the doses that produce a 20% and 80% of effect, respectively. However, ED_{20} and ED_{80} data are not shown. The term E_{max} is used to designate the maximum inhibitory effect observed after the administration of a single drug or their combination; the value is calculated on the basis of the experimental points that form the linear segments of the curves.

Dose–response curves were also obtained for the combinations of morphine with fentanyl (M:FEN), morphine with tramadol (M:TRM) and fentanyl with tramadol (FEN:TRM), in placebo and morphine pellet implanted mice. To obtain the 1:1 mixtures we combined one ED_{50} of one of the opioids (i.e. morphine) with one ED_{50} of another opioid (i.e. fentanyl or tramadol) thus obtaining a 1:1 potency ratio. The actual doses used in the fixed potency ratio were multiples or fractions of their respective ED_{50} values. Depending on the opioids combined and the results obtained, the following fractions were used: $1ED_{50}+1ED_{50}$, 1/2 $ED_{50}+1/2$ ED_{50}, 1/4 $ED_{50}+1/4$ ED_{50}, 1/8 $ED_{50}+1/8$ ED_{50}, and 1/16 $ED_{50}+1/16$ ED_{50}, To maintain standardized conditions and minimize errors, placebo and morphine pellet implanted animals were evaluated in parallel on the same day.

To generate the different dose–response relationships, we tested 4–6 doses of each drug or combination, using 10–12 mice per dose.

2.7. Statistical data analysis

The results are expressed as mean ED_{50} values ± S.E.M., or 95% confidence limits (95% CL). Effective doses were determined by linear regression analysis of dose–response curves. Individual slopes of the dose–response curves were compared by Student *t* test, according to the test of parallelism, and isobolographic calculations were performed using the PharmTools Pro (version 1.27, McCary Group Inc) as described by Tallarida (2000). The data from the rotarod test were compared using one-way analysis of variance (ANOVA) followed by a post-hoc Student–Newman–Keuls test (SPSS version 12.0 for windows; SPSS Inc, Chicago, IL). *P* values below 0.05 ($P<0.05$) were considered significant.

To assess the type of interaction between drugs two different methods of evaluation were used: a *fixed-dose* analysis or the *isobolographic* analysis when testing the effects of the opioid–opioid combinations.

2.7.1. Fixed-dose analysis

We compared the effects of each opioid (M, FEN or TRM) administered alone (placebo) with the responses of the same individual drug in the presence of constant plasma concentration of morphine (morphine pellet). From these data, the effects of the treatment (placebo or morphine pellet), the dose, and their combination were determined (Caudle and Williams, 1993). In this evaluation, a significant effect of the treatment indicates a statistical difference between the two groups of study (placebo vs. morphine pellet), a significant effect of the dose indicates that the observed responses vary significantly in a dose-dependent manner, and a significant interaction shows that the differences between the treatment groups depend on the dose administered. If all factors (treatment, dose and interaction) are significantly different, the analysis indicates that the effects of the combination are different from additivity, whereas if the interaction is not statistically significant, the effects are considered additive. Moreover, when a dose–response curve is shifted to the right, antagonism is indicated while a displacement to the left suggests synergy (Berenbaum, 1989; Tallarida, 2001).

2.7.2. Isobolographic analysis

Isoboles are graphic representations of the doses of two (or more) drugs used individually or in combination that are required to produce a specified level of response (*e.g.*, ED_{50}). In brief, we plotted on the *x*- and *y*-axes the ED_{50} values of each drug alone, obtained from their respective dose–response curves. The line joining the *x*- and *y*-axes corresponds to the theoretical additive line (isobole). Then the doses of the combination are also plotted. If the experimental point falls below or above the isobole, synergy or antagonism, respectively, may be present. The point represented in the isobole line is the theoretical additive point, and the point obtained after the administration of the combination is the experimental point. Mean and S.E.M values were calculated for the theoretical and experimental points and compared using a Student *t* test.

The diagonal non-interaction (additivity) line of the isobole is described by the equation $da/dA+db/dB=1$ that reflects the presence and/or the magnitude of the interaction. In this equation, dA and dB are the doses (mg) of each drug individually that induce the specific level of effect (i.e. ED_{50}), and da and db are the doses (mg) of each drug in the combination that also produce that response (ED_{50}). The sum of these quotients is the interaction index (I.I.). An interaction index value not statistically different from 1 demonstrates no interaction (additive effects), whereas values below and above 1 show synergy and antagonism, respectively. Using this type of analysis, an interaction can be established only for a given drug ratio and level of effect, and the results cannot be extrapolated to other drug combinations.

3. Results

3.1. Antinociceptive effects of acute morphine (M), fentanyl (FEN), or tramadol (TRM), administered in animals implanted with a placebo pellet

On day 3 after pellet implantation, the mean latency value (baseline behavioral nociceptive response) was 82.41 ± 4.05 s ($n=29$). The effective ED_{50} doses of each drug shown in Table 1 were obtained from the individual log dose–response curves (Fig. 1). From the data, we calculated the relative potency of the opioids showing that fentanyl was approximately 80 and 500 times more potent that morphine and tramadol, respectively, while morphine was approximately 5 times more potent than tramadol (Table 1). Thus, the order of potency at all levels of effect was established as follows: FEN>M>TRM.

3.2. Antinociceptive effects of acute morphine (M), fentanyl (FEN) or tramadol (TRM), administered in animals implanted with a morphine pellet

On day 1 after morphine pellet-implantation, nociceptive thresholds significantly increased (180.71 ± 17 s) when compared to placebo ($P<0.0001$). However on day 3, these values returned to baseline

Table 1
Antinociceptive potencies (ED_{50} values, in mg/kg ± S.E.M) of morphine, fentanyl and tramadol, administered individually, in placebo or morphine pellet-implanted mice, in the hot plate test.

Drugs	Placebo (PL)	Morphine pellet (MP)	MP/PL ratio
Morphine (M)			
ED_{50}	2.67 ± 0.11	33.86 ± 3.80*	12.68
Fentanyl (FEN)			
ED_{50}	0.03 ± 0.002	0.38 ± 0.03*	12.67
Tramadol (TRM)			
ED_{50}	14.73 ± 1.08	37.42 ± 3.11*	2.54
Relative potency			
M/FEN			
ED_{50}	89	89	
TRM/M			
ED_{50}	5	1.1	
TRM/FEN			
ED_{50}	491	98	

Data are shown as means ± standard error of mean (S.E.M.). Lower values of ED_{50}s indicate higher potency of drugs. For each drug, the (*) indicates a $P<0.001$ (Student's t test) comparing MP vs. PL. The ratios between ED_{50}s indicate the level of cross-tolerance to morphine (ratio >1).

Table 2
Antinociceptive potencies (ED_{50} values in mg/kg ± S.E.M) and interaction indexes (I.I.) of the morphine:fentanyl (M:FEN), morphine:tramadol (M:TRM) and fentanyl:tramadol (FEN:TRM) combinations.

Drugs	Placebo (PL)	Morphine pellet (MP)	MP/PL ratio
M:FEN			
ED_{50}	1.48 ± 0.26	30.05 ± 1.22*	18.10
I.I.	1.51 ± 0.08	1.78 ± 0.18#	
M:TRM			
ED_{50}	9.59 ± 0.35	32.68 ± 2.60*	3.41
I.I.	1.18 ± 0.07	0.93 ± 0.09	
FEN:TRM			
ED_{50}	8.38 ± 0.44	13.94 ± 0.94*	1.66
I.I.	1.13 ± 0.10	0.73 ± 0.04	

Drugs were administered s.c., in placebo (PL) or morphine (MP) pellet-implanted mice. The (*) indicates a $P<0.001$ (Student's t test) comparing MP vs. PL conditions for each drug-mixture. The ratio between the ED values in MP and PL indicates the presence of cross-tolerance to morphine (ratio >1). The (#) indicates the presence of statistically significant antagonism.

(80.46 ± 9.7 s, $P=0.83$ s when compared placebo mice). To characterize the effects of M, FEN or TRM in morphine-pellet implanted mice (MP), dose–response curves were generated to each opioid on day 3 after pellet implantation. For the same opioid, the resulting curves were parallel and shifted to the right when compared to those obtained in PL mice (Fig. 1), thus indicating the presence of tolerance; these results suggest the same mechanism of action in both experimental conditions. E_{max} values in all curves were between 80 and 85%. The ED_{50} values obtained for M, FEN and TRM in tolerant mice are shown in Table 1. During tolerance, FEN was 89 and 98 times more potent than morphine or tramadol, respectively, whereas M and TRM had a similar relative potency. During morphine-tolerance, the order of potency at all levels of effect was: FEN>M=TRM.

For the same drug, the ratio between the ED values in tolerant (MP) and naïve (PL) mice indicate the magnitude of tolerance and cross-tolerance to morphine (ratio >1). The results show that exposure to chronic morphine induces a lesser degree of cross-tolerance to TRM than to FEN (Table 1). Moreover, acute morphine administration to MP implanted mice demonstrated robust tolerance.

To characterize the type of interaction in these experiments, we compared the effects of each opioid in naïve (PL) and morphine-tolerant mice (MP) with a two way ANOVA (dose–response curves shown in Fig. 1). For each one of the drugs used in the study (M, FEN and TRM), the statistical analysis demonstrated significant effects of the treatment (placebo or morphine pellet) ($P<0.0001$), the dose ($P<0.0001$), and their interaction ($P<0.05$). The level of significance of the statistical evaluation (ANOVA) was similar for the three drugs. These results together with the shift to the right of the dose–response curves in MP mice, indicate the presence of *functional* antagonism.

3.3. Dose–responses curves to M, FEN and TRM, combined at equieffective doses (1:1 proportion) in animals implanted with a placebo or a morphine pellet

Using the ED_{50} values obtained in placebo and morphine pellet implanted mice (Table 1), we performed dose–response curves to M:FEN, M:TRM and FEN:TRM, combined in a 1:1 proportion. All dose–response curves had E_{max} values between 85 and 100%; from the linear portion of the curves, the total doses (in milligrams) of each combination that produced a 50% response were calculated (Table 2). The results show that the antinociceptive potency of all combinations tested decreased in morphine-tolerant animals. Potency ratios between MP/PL were greater in the M:FEN combination, suggesting a higher degree of cross-tolerance. Similar results were obtained at 20% (ED_{20}) and 80% (ED_{80}) levels of effect (data not shown).

For all drugs and their combination/s similar results were obtained when plotting the values obtained using the equation where the cut-off time is taken into account (%MPE, see Materials and methods), and the raw data values expressed in seconds.

3.4. Analysis of the interactions between M and FEN, M and TRM or M and FEN in animals implanted with a placebo or a morphine pellet

For the analysis of the interaction between the different opioids when combined in 1:1 proportion, we determined the interaction indexes (I.I.) as described in the Materials and methods section. The results show that in animals implanted with a PL pellet (naïve), the mixtures of M:FEN, M:TRM or FEN:TRM were not significantly different from 1, demonstrating additivity ($P>0.05$) (Table 2). However, when analyzing the same drug interactions in tolerant animals (MP), we observed that in the M:FEN group the I.I. were significantly greater than 1, demonstrating antagonism ($P<0.05$). On the other hand, the combinations of M:TRM and FEN:TRM were always additive (Table 2). From the data obtained in tolerant animals (MP), we constructed isobolograms at the 50% level of effect for all drug combinations, and the results are shown in Fig. 2. To obtain the isobolograms, first we plotted the equieffective ED_{50} doses of each drug individually in the X and Y axes, and the theoretical line of additivity (isobole) is represented. The ED values obtained from the dose–response curves of the combination/s were also plotted in the same axes and represented in the graph; dose-points that are significantly different (Student's t test) from the theoretical additive line and fall above or below of the isobole, demonstrate antagonism and synergy respectively (see Materials and methods). In Fig. 2 we can observe that the M:FEN combination (ED_{50}) induced antagonism, which was also similar when ED_{20} and ED_{80} were tested (data not shown). However, for the M:TRM and FEN:TRM combinations all experimental points were not significantly different from the line of additivity, demonstrating summation of their antinociceptive effects. Thus the present results show that in naïve mice (PL) the antinociceptive effects of the M:FEN combination are additive, since in these experimental conditions the drugs do not interact (Table 2). However, when animals have been previously exposed to morphine (MP), the same M:FEN combination shows antagonism, suggesting that the concurrent administration of these two opioids would increase the doses required to obtain effective analgesia.

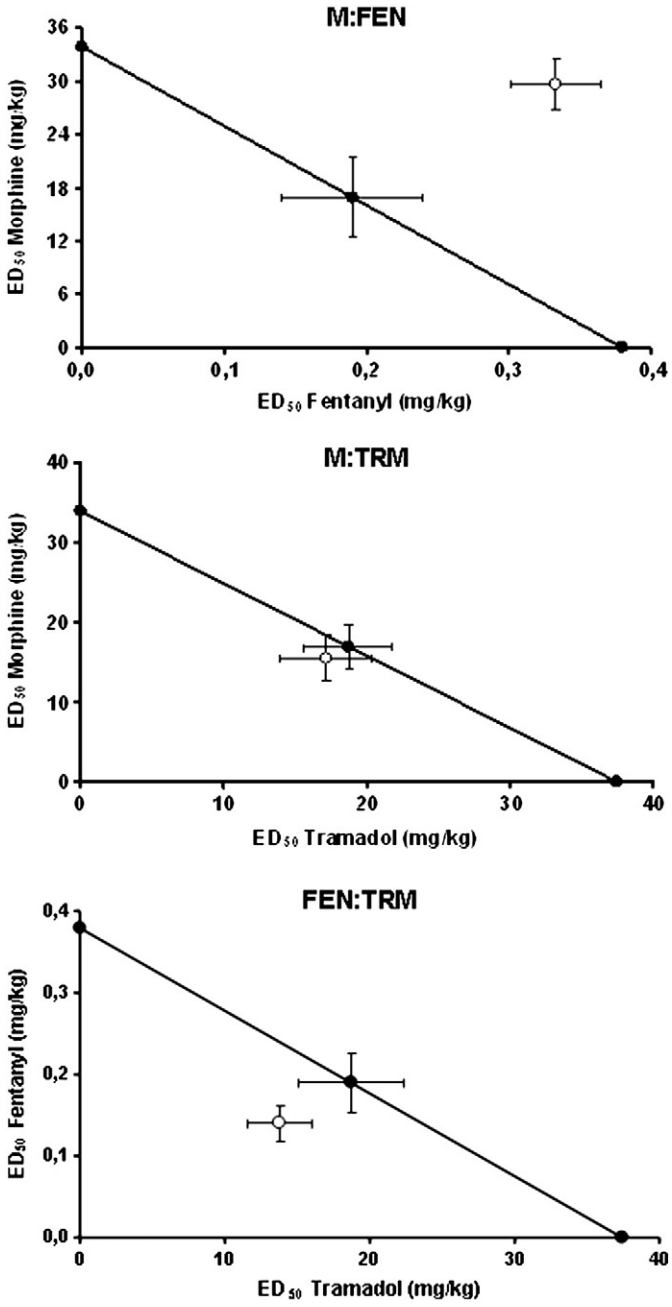

Fig. 2. Isobolographic representation of the antinociceptive effects of the combination of morphine:fentanyl (M:FEN), morphine:tramadol (M:TRM) and fentanyl:tramadol (FEN:TRM), at the ED_{50} level of effect in the hot plate test, in tolerant mice. Filled and open circles represent the theoretical and experimental ED values respectively, with 95% confidence limits (CL).

3.5. Effects of M, FEN and TRM, individually (ED_{80} values) and the M:FEN combination on motor performance (rotarod test), in naïve and morphine-tolerant mice

In these experiments we tested the ED_{80} values obtained in the hot plate test in tolerant mice for M (65 mg/kg), FEN (0.84 mg/kg), and TRM (71.92 mg/kg) individually; in addition we tested the ED_{80} for the M:FEN combination (71.32 mg/kg of M plus 0.86 mg/kg of FEN) also obtained in tolerant animals. The rationale for selecting these doses was to test the highest amount of opioids to assess a possible effect on motor coordination that could interfere with nociceptive evaluation. None of the doses tested (each drug individually or the M:FEN combination) altered performance time in the motor coordination test [$F_{(4,34)}=1.271$ $P=0.300$], and the results are shown in Table 3. Moreover, no noticeable changes in exploratory behaviour were observed in any of the mice.

4. Discussion

The present report shows that chronic exposure to morphine induced severe and similar tolerance and cross-tolerance to the antinociceptive effects of acute morphine or fentanyl administration, and that cross-tolerance was of a lesser magnitude for tramadol. Thus, the degree of cross-tolerance (ratio ED_{50}MP/ED_{50}PL) observed in morphine-tolerant mice was higher after the acute administration of fentanyl (a strong mu-opioid agonist) than that of tramadol (a weak mu-opioid agonist).

Indeed, in morphine-tolerant mice a 13 times increase in the dose of morphine or fentanyl was required to induce the same level of analgesia, while only a 2.5 fold increase was required for tramadol. However, although chronic morphine exposure induced a lower reduction in the potency of TRM, the order of potency of the opioids (fentanyl>morphine>tramadol) remained unaltered in naïve and morphine-tolerant mice, suggesting that antinociception is mainly mediated by the activation of mu-opioid receptors (MOR), besides other likely changes in the opioid system induced during tolerance.

The differences in cross-tolerance to morphine observed after the acute administration of TRM could be related to the dual mechanism of action of the drug, that in addition of binding to mu opioid receptors (MOR) induces antinociception by activating endogenous monoaminergic systems (Minami et al., 2007; Leppert, 2009). Tramadol, is a mixture of two enantiomers; (+)-tramadol and the metabolite (+)-o-desmethyl-tramadol (M1) which are MOR agonists; moreover, (+)-tramadol and (−)-tramadol also inhibit serotonin and noradrenaline reuptake respectively, most likely enhancing the inhibition of nociceptive transmission in the spinal cord. Our results suggest that chronic exposure to morphine (MP) induces tolerance to the opioid but not the monoaminergic component of TRM. It could be hypothesized that while MOR are adapted to a new *state* induced by sustained morphine exposure, the non-opioid component is preserved or even *sensitized;* thus during morphine-tolerance the analgesic potency of tramadol is decreased to a lesser degree than the pure MOR agonists such as FEN or M (Table 1). Accordingly, TRM would seem a better alternative to obtain analgesia in the presence of morphine tolerance.

The development of opioid tolerance in animal models by morphine pellet implantation has been well documented by our as well as other groups of investigators (Pol and Puig, 1997; Fernández-Dueñas et al., 2007). Dighe et al. (2009) showed that the magnitude of tolerance after morphine-pellet implantation in mice, increased for the first 3 days and then remained stable, suggesting that despite

Table 3
Effects of morphine (M), fentanyl (FEN), and tramadol (TRM) individually and of the combination of M:FEN in 1:1 proportion, in placebo (PL) and morphine (MP) pellet-implanted mice on motor performance.

Drugs	ED_{80} (total mg/kg)	Time on rotarod (s)			
		Baseline		Test	
		PL	MP	PL	MP
CTL	–	240 ± 0.0	240 ± 0.0	233 ± 5.7	216 ± 13.9
M	65.20 ± 27.20	240 ± 0.0	240 ± 0.0	240 ± 0.0	240 ± 0.0
FEN	0.84 ± 0.10	240 ± 0.0	240 ± 0.0	240 ± 0.0	218 ± 21.8
TRM	71.92 ± 9.93	240 ± 0.0	240 ± 0.0	240 ± 0.0	200 ± 23.0
M: FEN	72.18 ± 4.58	240 ± 0.0	240 ± 0.0	240 ± 0.0	240 ± 0.0

In the control group (CTL), animals implanted with a PL or MP pellet received s.c. saline. The ED_{80} values (mg/kg) and the time on rotarod (s) are expressed as mean ± S.E.M of 8–10 animals.
ED_{80}s were obtained in MP implanted animals. No significant differences between treatment groups were observed on the rotarod test (One-way ANOVA, $P=0.300$).

continued exposure to morphine, the mechanisms that mediate antinociceptive tolerance reached equilibrium at this time point. In the present work, nociceptive thresholds returned to baseline 3 days after morphine pellet implantation, confirming the presence of tolerance when experiments were performed.

The underlying mechanisms of opioid tolerance are poorly understood. Adaptive changes related to receptor downregulation (associated with increased receptor degradation), desensitization (uncoupling or disruption of the capacity of the receptors to interact with second messenger systems), internalization/endocytosis (decreased functional sites available for binding of agonists), or changes in gene expression have been suggested, among others, as possible mechanisms involved in the development of opioid tolerance (Martini and Whistler, 2007).

It is well accepted that not all agonist ligands at the MOR promote the same degree of receptor desensitization and internalization/endocytosis. Some in vitro experiments (Finn and Whistler, 2001; He et al., 2002; Koch et al., 2005) suggest that morphine induces weak or partial desensitization and endocytosis, as well as lesser phosphorylation of the MOR (Schulz et al., 2004) than other agonists. As a consequence, signal transduction prompted by morphine is less dynamic than that induced by other MOR agonists such as fentanyl or ethorphine (Zuo, 2005). The cellular and/or molecular adaptive changes induced by sustained exposure to morphine diminish the capability of other opioids to interact with the MOR or to induce the necessary activation/inhibition of downstream pathways. Consequently, increased doses of other MOR agonists are needed to maintain the same degree of antinociception. In the present work, fentanyl (a strong opioid) significantly decreased its antinociceptive potency during morphine tolerance, and higher doses were needed to maintain the same levels of effect. Regarding morphine-induced MOR phosphorylation, different in vitro studies reported complete reversal between 30 min and 6 h after removal of morphine (Yu et al., 1997; Schulz et al., 2004). Since in our experimental model, morphine was not removed during nociceptive evaluation, MOR desensitization was probably long-lasting, partially explaining the decreased antinociceptive potency of the opioids.

Our results also show for the first time that in morphine-tolerant mice (MP), the co-administration of morphine and fentanyl shows antagonism, while no interaction exists when tramadol is combined either with morphine or fentanyl (additive effects); there is also a lesser degree of cross-tolerance to chronic morphine when tramadol is combined with a strong agonist. The results seem to indicate that in morphine-tolerant subjects, combining tramadol with a strong opioid (morphine or fentanyl) may be a better alternative to the concomitant use of two strong agonists. At present, this hypothesis is being tested by our group.

Besides opioid switching, opioid–opioid combinations are used in clinical practice for maintaining or restoring the analgesic response in morphine-tolerant patients. Pre-clinical studies in naïve rats have demonstrated antinociceptive synergy after the co-administration of low doses of different opioids (Sutters et al., 1990; Ross et al., 2000; Nemirovsky et al., 2001). In naïve mice, we have also recently described that the type of opioid–opioid interaction changes depending on the nature of the noxious stimulus and the percentage (or level) of antinociceptive effect evaluated (Romero et al., 2010). In the present work we show that the interaction between morphine and fentanyl changes form additive to antagonistic during morphine-tolerance. In tolerant mice, the isobolographic analysis (Fig. 2) clearly shows antagonism between morphine and fentanyl corroborating that tolerance is another factor that can modify the type of interaction when two opioids are administrated simultaneously. Although the antinociceptive effects of tramadol combined with morphine or fentanyl are additive, the presence of this weak opioid in the mixtures reduced the magnitude of cross-tolerance of both combinations in morphine tolerant mice (Table 2). We did not attempt to antagonize the aminergic effects of tramadol by α_2-noradrenergic or serotoninergic antagonists, and thus we do not have at present an explanation for this finding, other than a possible enhancement or *sensitization* of the aminergic component of tramadol analgesia.

As previously discussed, both tramadol and its metabolite M1, bind to MOR with different affinities and induce opioid analgesia. Moreover, tramadol activates pain descending serotoninergic and noradrenergic inhibitory systems, modulating nociceptive transmission in the spinal cord (Raffa and Friederichs, 1996). Noradrenergic and opioid receptors are coupled to analogous signal transduction pathways and synergy has been demonstrated between the endogenous α_2-noradrenergic and opioid systems in the spinal cord in mice (Ossipov et al., 1990; Fairbanks and Wilcox, 1999). Furthermore, the activation of α_2-adrenergic receptors by clonidine decreased fentanyl requirements in humans during surgical anaesthesia (Murga et al., 1994). Interestingly, Jordan et al. (2003) reported a hetero-oligomerization between α_{2A}-adrenoceptors and MOR, increasing physical interactions that seem to modulate receptor function. We hypothesize that during morphine tolerance a decreased function/activity of the MOR and the endogenous opioid system occurs, that is partially compensated by increased activity or *sensitization* of the α_2-adrenergic antinociceptive system.

In conclusion, the present report reveals for the first time, that in morphine-tolerant mice, the co-administration of morphine and fentanyl is antagonistic, while no interaction exists when tramadol is combined either with morphine or fentanyl (additive effects); interestingly, the type of interaction between morphine and fentanyl changes from additive to antagonistic in naïve and tolerant mice, respectively. Our work also shows a lesser degree of cross-tolerance to chronic morphine when tramadol is used individually or combined with a strong agonist. The results indicate that in morphine-tolerant subjects, the administration of tramadol may induce effective analgesia and that the combination of tramadol with a strong opioid (morphine or fentanyl) could be a better alternative to the concomitant use of two strong agonists.

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