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Analysis of the opioid–opioid combinations according to the nociceptive stimulus in mice

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ABSTRACT

The purpose of the present study was to characterize the antinociceptive effects of tramadol, fentanyl and morphine, when two of them were systemically combined in a 1:1 potency ratio, in the hot plate, the acetic acid writhing, and the formalin tests in mice. Interaction indexes and isobolographic analysis were used to assess the type of interaction. Fentanyl was the most potent drug, followed by morphine and tramadol, with the exception in the phase I of formalin test. Synergistic interactions were obtained when tramadol was combined with fentanyl or with morphine in the writhing and formalin tests. But, in the hot plate only additive interactions were obtained. Changes were induced on the type of interaction depending on the level of effect of opioid–opioid combinations. Moreover, co-administration of fentanyl with morphine showed additivity, regardless of the type of stimulus. Standard rotarod test analysis confirmed intact motor coordination. The present findings suggest that the type of interaction between opioids is not only related to the nature of nociceptive stimulus but also to non-opioid analgesic pathways.

1. Introduction

In pain management, not enough analgesia is achieved using monotherapy. The administration of two or more drugs (multimodal analgesia) is a widely used strategy to improve the analgesic efficacy and to reduce adverse side effects of drugs. Although the usefulness of the co-administration of drugs of the same pharmacological group is controversial, it is effective in some pathologies such as depression [1] and epilepsy [2]. In humans, opioids are frequently used for the relief of moderate to severe pain of different etiology, and empirically combined, without knowing if they interact. Some clinical evidences in cancer patients suggest that the combination of two opioids (morphine plus oxycodone, morphine plus fentanyl or methadone) can be a useful alternative to opioid monotherapy [3,4]. In addition, clinical data in the management of moderate to severe pain in postoperative patients, show that morphine combined with tramadol improves analgesia and decreases morphine requirements after abdominal surgery compared with morphine alone [5]. Similarly, the addition of intrathecal morphine to spinal fentanyl plus bupivacaine, significantly reduced persistent pain and prolonged the time to analgesic request [6]. However, Friedman et al. [7] did not find benefits when combining morphine and fentanyl administered by intravenous PCA after bowel surgery and Marcou et al. [8] described the presence of antagonism between morphine and tramadol.

Thus, the benefits of two or more drugs simultaneously administered, should be evaluated before the combination can be considered useful. The pharmacological effects (beneficial and/or adverse) attained using combination treatment can be studied and additive, or non-additive effects (synergy or antagonism interaction) between drugs can occur [9].

Studies in rodents using different nociceptive assays have reported synergistic pain relief (supraadditive antinociceptive effects) following simultaneous administration of opioid agonists with different selectivity for μ-, δ and κ-OR at spinal site [10], or combining supraspinal and spinal sites [11,12], spinal and systemic routes [13–15]. Hence, synergy is usual in opioid pharmacology, but not all μ opioid agonists in combination interact [16]. Studies in animal models have reported that drug–drug interactions can be altered by different factors such as the ratio of the combinations [17], the presence of inflammation or morphine tolerance [18]. Moreover, Loomis et al. [19] described that the nature of the nociceptive stimulus evaluated could change the type of interaction between opioids co-administered in rats. All these results indicate a complex analgesic interaction between opioids.

The present study reports the analysis of the interaction when two opioids such as morphine (standard reference drug), fentanyl or tramadol are combined in mice. These drug combinations are often used in clinical practise in different situations. For example,

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cancer pain patients often receive chronic slow release morphine and oral transmucosal fentanyl for breakthrough pain [20,21]. In the management of acute postoperative pain tramadol is frequently used as analgesic, and morphine or fentanyl administered as rescue medication [22–24]. Thus, the drugs used in our study were selected on the basis of their clinical use. However, these and other opioid–opioid combinations are used empirically in humans, without knowing if the drugs interact with each other.

It has been found that synergy (when it happens) is a function of the proportions in the combination [9], and the fixed-ratio design was used in this study. In this protocol, the drugs are administered in amounts (doses) that keep the proportions of each constant. Although the drugs studied induce their antinociceptive effects by opioidergic mechanisms mainly, other possible pathways could be implicated. Then, the analgesic effects could change not only with the drug pair and the ratio of the two components, but also according to the level of effect, a fact that may be relevant when attempting to introduce drug combinations in clinical practise. Moreover, we also studied if the nature of noxious stimulus could modify the type of interaction between those opioids combined.

2. Material and methods

2.1. Animals

Male CD1 mice, weighting 25–30 g (Charles River, France) were used in this study. The experiments were performed according to the Ethical Guidelines of the International Association for the Study of Pain and the Ethical Committee for Animal Welfare of the Institution approved the protocols. Mice were housed in plastic cages (five mice per cage) with soft bedding and free access to food and water. They were maintained in a controlled temperature ($22 \pm 1\,^{\circ}C$ and 60% relative humidity) and light (12:12 dark:light cycle with light on at 8:00) environment. Behavioural testing was performed between 09:00 a.m. and 17:00 p.m., in a quiet room. Mice were used only once and were killed at the end of the experiment by cervical dislocation.

2.2. Drugs

We used the same commercial drugs administered to humans in the clinical practise. Drugs were obtained from the following sources: tramadol (TRM) (Grünenthal, Madrid, Spain); fentanyl (FEN) (Kern Pharma, Barcelona, Spain) and morphine hydrochloride (M) (Alcaliber, Madrid, Spain). Individual drugs and their combinations were dissolved in saline solution (0.9%) just before use, and the drugs were administered subcutaneously (s.c.) at the nape of the neck in a volume of 0.250 ml, 30 min before behavioural testing, on the basis of previous reports [25,26].

2.3. Nociceptive tests

Each drug was administered at the following doses: TRM (1, 3, 5, 10, 30 and 50 mg/kg in the hot plate; 3,7, 8, 10 and 30 mg/kg in the writhing test and 1, 3,10, 30 and 100 mg/kg in both phases of the formalin test), FEN (0.01, 0.02, 0.03, 0.04, 0.05 and 0.07 mg/kg in each of the tests) and M (0.5, 1, 2, 3, 4 and 7 mg/kg in the hot plate; 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/kg in the writhing and 0.05, 0.1, 0.3, 1 and 3 mg/kg in the phase I and 0.3, 1, 3, 7 and 10 mg/kg in the phase II of the formalin test).

2.3.1. Formalin test

The method described by Rosland et al. [27] was used. To carry out the test 20 μl of a 5% formalin solution, was injected into the dorsal surface of the mice right hind paw, with a 27-gauge needle attached to a 50 μl Hamilton syringe. Each mouse was immediately returned to a plexiglass observation cylinder especially designed. The degree of pain intensity was recorded as the total time spent by the animal licking the injected paw, measured by visual observation and a digital time-out stopwatch. The test shows two clear cut phases: *Phase I* corresponds to the 5 min period starting immediately after the formalin injection. This initial phase represents a tonic acute pain due to peripheral nociceptor sensitization. *Phase II* was recorded as the 10 min period starting 20 min after the formalin administration and corresponds to inflammatory pain. The time of both phases was not registered due to that corresponds to a period of stillness or of not-activity. Control animals ($n = 34$) were injected with saline. For each drug, analgesic effects were characterized after the administration of a minimum of five doses. The licking times observed were converted to % maximum possible effect (MPE) as follows:

$$\%\text{MPE} = \left[\frac{\text{control licking time} - \text{postdrug licking time}}{\text{control licking time}}\right] \times 100$$

2.3.2. Writhing test

The procedure used has been described previously [28]. Briefly, mice were injected intraperitoneally (i.p.) with 10 ml/kg of 0.6% acetic acid solution, 30 min after the subcutaneous (s.c.) administration of the drugs, time at which preliminary experiments showed occurrence of the maximum effect of all drugs used. Each mouse was then placed in an individual clear plexiglass observation cylinder. A writhe is characterized by a wave of contraction of the abdominal musculature, accompanied by the elongation of the body and extension of one or both hind limbs. The number of writhes in a 5 min period was counted, starting 5 min after the acetic acid administration. Antinociception was expressed as percent inhibition of the number of writhes observed in control animals ($n = 23$). The results are expressed as maximum possible effect (%MPE) according to the following expression:

$$\%\text{MPE} = \left[\frac{\text{writhes in control mice} - \text{writhes postdrug mice}}{\text{writhes in control mice}}\right] \times 100$$

2.3.3. Hot plate test

The hot plate test was performed using an electronically controlled hot plate analgesia meter (Columbus Instruments, Columbus, OH, USA) heated to $52 \pm 1\,^{\circ}C$, described by Castañé et al. [29]. The nociceptive threshold evaluated was the time of the jumping response as the latency period. In absence of jumps, a 240 s cut-off was used to prevent tissue damage. Control animals ($n = 37$) were injected with saline. Percent analgesia was calculated as

$$\%\text{MPE} = \left[\frac{\text{latency postdrug} - \text{control latency}}{240 - \text{control latency}}\right] \times 100$$

2.4. Determination of motor functions: rotarod test

The rotarod test [30] was used to evaluate the effects of each opioid, individually (ED_{80}'s) and combined in 1:1 proportion, on motor coordination behaviour (LSI-Letica Scientific Instruments, Barcelona, Spain). We tested the highest values of the $ED_{80's}$ obtained for each individual opioid (in the different nociceptive tests. The doses used for drug combinations were those that induced synergistic antinociceptive effect at 80% of effect, to discriminate a possible synergism in impairment to motor function. The drugs were administered s.c. (at the nape of the neck), in a volume of 0.250 ml, 30 min before behavioural testing. Initially, all animals were trained to run on the rotarod apparatus on day 1, at a constant 10 rpm. Those 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 on day 2. The time each animal remained on the rotarod was recorded (s).

2.5. Protocol

In each test, dose–response curves were generated for tramadol (TRM), fentanyl (FEN) and morphine (M) alone. To obtain the dose–response curves, a range between five and six different doses were used, and at least 8–10 animals per dose. A least-square linear regression analysis of the log dose–response curves allowed the calculation of the dose that produced 20, 50 and 80% of effect ($ED_{20,50,80}$) of antinociception for each drug alone, according to the method of Tallarida [31]. 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. Afterwards, dose–response curves were obtained in the different nociceptive tests with the following drugs (combined in a 1:1 proportion): (i) TRM:FEN; (ii) TRM:M, and (iii) FEN:M. We used the following fractions $1ED_{50} + 1ED_{50}$, $(1/2)\,ED_{50} + (1/2)\,ED_{50}$, $(1/4)\,ED_{50} + (1/4)\,ED_{50}$, $(1/8)\,ED_{50} + 1/8\,ED_{50}$, and $(1/16)\,ED_{50} + 1/16ED_{50}$, depending on the results obtained.

2.6. Drug interaction

The presence of an interaction between the two drugs (TRM:FEN, TRM:M, FEN:M) was evaluated using: (i) interaction indexes (I.I.) and (ii) isobolographic analysis:

(i) The magnitude of the interaction was calculated as: $da/dA + db/dB = 1$ where A and B are the doses (mg) of each drug individually that induce a 20, 50 or 80% inhibition of nociception, and a and b are the doses (mg) of each drug in the combination that produce the same level of inhibition. For the interaction index (I.I.) a value close to 1 indicates no interaction (additive effects), whereas values below and above 1 suggest synergy and antagonism, respectively.

(ii) Isobolographic analysis was used according to the method described by Tallarida [31]. In order to reduce the extension of the manuscript we represented the isobolograms corresponding only to the ED_{50}'s. In brief, we plotted on the x- and y-axes the ED_{50}'s values of each drug alone. Then, the line joining the x- and y-axes corresponds to the theoretical additive line (isobole). The doses of the combination were also plotted. If the experimental point falls below or above the isobole, synergy or antagonism is present, respectively. The point represented in the line is the theoretical additive point and the point from the combination is the experimental point.

2.7. Statistical analysis

Results are presented as ED_{50} values ± S.E.M., or 95% confidence limits (95% CL). Statistical significance between theoretical additive ED's and the experimentally derived ED's values was assessed by Student's t-test for independent means, and P values less than 0.05 ($P < 0.05$) were considered statistically significant. Slopes were obtained from dose–responses curves by lineal regression. The ratio between the ED_{50} of tramadol and the ED_{50}s of other drugs, and the ratio between the ED_{50}s of morphine and fentanyl, was used to establish the relative potencies. Statistical analysis of parallelism of dose–response curves and isobolographic calculations were performed with the PharmTools Pro (version 1.27, McCary Group Inc), based on Tallarida [31]. 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) and a $P < 0.05$ was considered statistically significant.

3. Results

3.1. Antinociception induced by tramadol (TRM), fentanyl (FEN), and morphine (M)

We tested the antinociceptive effects induced by the s.c. administration of TRM (dose range 1–100 mg/kg), FEN (0.01–0.07 mg/kg) and M (0.05–10 mg/kg), in the hot plate, the writhing and the formalin tests. TRM, FEN and M, each one individually, induced dose–response curves.

Control animals were injected with saline and the baseline values obtained for each antinociceptive tests were: 80.70 ± 8.20 s and 58.30 ± 9.80 s in phase I and phase II (licking time) of formalin, respectively; 38.60 ± 7.56 (number of writhes) in the writhing test, and 81.56 ± 5.23 s (latency period) in the hot plate.

The effective doses (ED, see Section 2) of each drug at different levels of effect (20, 50 and 80%) were obtained from their individual log dose–response curves. The ED_{50}'s values, E_{max} and slope are shown in Table 1. When the slopes of curves were compared, significant differences ($P < 0.05$, Student's t-test), were found between TRM vs. FEN or M obtained in the hot plate test, in the writhing and in the phase I of formalin tests, demonstrating lack of parallelism. Consequently, the relative potency of these drugs varied at each level of effect. The order of potency was FEN > M > TRM in all behavioural assays, except in the phase II of formalin test, where M showed a decrease on the potency, values included in Table 1.

3.2. Dose–response curves of combined TRM, FEN, and M at equieffective doses, in 1:1 proportion

In the hot plate, the writhing and the formalin tests, combinations of TRM:FEN, TRM:M and FEN:M, on the basis of their potency, in a 1:1 proportion were tested (see Section 2). In all tests, the systemic opioids co-administrated induced dose-dependent response curves, with similar efficacy (100% E_{max}) and the combined curve was shifted to the right from the more potent drug. For the different nociceptive tests, the ED_{50}'s values of the combinations (TRM:FEN, TRM:M and FEN:M) are included in Table 2.

In the hot plate, all combinations demonstrated additivity at all levels of effect.

However, in the writhing test synergism at all level of effect was obtained when TRM:M and TRM:FEN were combined, with the exception of the combination TRM:FEN in which no interaction (additivity) was observed at the higher level of effect (ED_{80}). The FEN:M mixture was additive at all levels of effect.

In the phase I of the formalin test the co-administration of TRM with FEN and FEN with M induced antagonism, additivity and synergism at each level of effect ($ED_{20,50,80}$), respectively. Nevertheless, TRM:M produced synergism, except at lower doses of the combination (ED_{20}), that induced additivity.

On the other hand, in the phase II of the formalin test, the mixture of TRM:FEN, induced synergism at all level of effect ($ED_{20,50,80}$). The combination of TRM with M, produced similar synergistic interactions at ED_{50} and ED_{80}, while no interaction was obtained at lower doses of the combination (ED_{20}). As in the phase I, the co-administration of FEN:M induced antagonism, additivity and synergism at the level of $ED_{20,50,80}$, respectively.

All the results explained above and the corresponding interaction indexes of all the combination are shown in Table 3.

The isobolograms obtained with the mixtures at the ED_{50} level in the hot plate test are represented in Fig. 1, showing TRM with FEN (Fig. 1A), TRM:M (Fig. 1B), and FEN with M (Fig. 1C).

Table 1
ED_{50} values (in mg/kg ± SEM), E_{max}, slope and relative potency of tramadol, fentanyl and morphine, administered individually in the hot plate, writhing and formalin (phase I and II) tests in mice.

Drugs	Hot plate	Writhing	Formalin (phase I)	Formalin (phase II)
Potency				
Tramadol				
ED_{50}	15.09 ± 1.58	7.6 ± 0.55	2.79 ± 0.31	1.41 ± 0.41
E_{max} (%)	100	100	98	100
Slope	88*	115*	32*	45
Fentanyl				
ED_{50}	0.031 ± 0.003	0.024 ± 0.001	0.055 ± 0.008	0.030 ± 0.002
E_{max} (%)	100	99	97	99
Slope	111	95	65	52
Morphine				
ED_{50}	2.55 ± 0.11	0.25 ± 0.01	0.12 ± 0.005	3.01 ± 0.53
E_{max} (%)	88	96	90	85
Slope	100	92	52	42
Relative potency				
TRM/FEN	487	317	51	47
TRM/M	6	30	23	0.47
M/FEN	82	10	2	100

Lower values indicate higher potency of the drugs.
* $P < 0.05$ (Student's t-test) comparing tramadol vs. fentanyl or morphine.

Table 2
ED_{50} values (in mg/kg ± SEM), for the antinociceptive effects of combined tramadol:fentanyl (TRM:FEN), tramadol:morphine (TRM:M) and fentanyl:morphine (FEN:M), administered s.c., in the hot plate, writhing and formalin (phase I and II) tests in mice.

Drugs ED_{50} (total mg/kg)	Hot plate	Writhing	Formalin (phase I)	Formalin (phase II)
TRM:FEN	9.3 ± 0.43	1.61 ± 0.13	1.51 ± 0.08	0.329 ± 0.06
TRM:M	10.62 ± 0.67	1.63 ± 0.095	0.41 ± 0.014	0.74 ± 0.03
FEN:M	1.66 ± 0.26	0.13 ± 0.007	0.078 ± 0.001	1.02 ± 0.023

Lower values indicate higher potency of the drugs.

In Fig. 2, are illustrated the isobolograms obtained with the mixtures at the ED_{50} level in the acetic acid writhing test, thus Fig. 2A corresponding to the combination of TRM with FEN, Fig. 2B to TRM with M and Fig. 2C to FEN with M.

The isobolograms of the co-administration at the ED_{50} level in phase I and phase II of formalin test are represented in Figs. 3 and 4, respectively showing TRM with FEN (Figs. 3A and 4A), TRM with M (Figs. 3B and 4B), and FEN with M (Figs. 3C and 4C).

3.3. Rotarod test

None of the doses tested (each drug individually (ED_{80}'s), and the combined in 1:1 proportion) altered the locomotor activity (performance time) of the animals on the apparatus [$F_{6,63} = 1.345$ $P = 0.251$]. The results are showed in Table 4. Moreover, no changes in exploratory behaviour or muscle tone were observed in any of the mice after the administration of the opioids individually.

Table 3
Isobolographic parameters for the antinociceptive activity of equieffective doses combined of tramadol:fentanyl (TRM:FEN), tramadol:morphine (TRM:M) and fentanyl:morphine (FEN:M), in a 1:1 proportion, in the hot plate, the writhing and the formalin tests in mice.

Drug combination Level of effect	Interaction indexes (I.I.) type of interaction			
	Hot plate test	Writhing test	Formalin test (phase I)	Formalin test (phase II)
TRM:FEN				
ED_{20}	1.48 ± 0.14	0.53 ± 0.03	4.3 ± 0.59	0.34 ± 0.11
	Additive	*Synergy*	*Antagonism*	*Synergy*
ED_{50}	1.21 ± 0.08	0.42 ± 0.04	1.02 ± 0.09	0.46 ± 0.09
	Additive	*Synergy*	*Additive*	*Synergy*
ED_{80}	0.95 ± 0.08	1.36 ± 0.09	0.23 ± 0.03	0.24 ± 0.06
	Additive	*Additive*	*Synergy*	*Synergy*
TRM:M				
ED_{20}	1.46 ± 0.15	0.26 ± 0.02	1.32 ± 0.3	1.41 ± 0.5
	Additive	*Synergy*	*Additive*	*Additive*
ED_{50}	1.31 ± 0.09	0.40 ± 0.02	0.30 ± 0.04	0.30 ± 0.07
	Additive	*Synergy*	*Synergy*	*Synergy*
ED_{80}	1.16 ± 0.11	0.62 ± 0.04	0.5 ± 0.07	0.4 ± 0.05
	Additive	*Synergy*	*Synergy*	*Synergy*
FEN:M				
ED_{20}	1.24 ± 0.11	1.09 ± 0.05	2.45 ± 0.4	2.6 ± 0.56
	Additive	*Additive*	*Antagonism*	*Antagonism*
ED_{50}	1.04 ± 0.06	0.97 ± 0.03	0.89 ± 0.06	0.67 ± 0.08
	Additive	*Additive*	*Additive*	*Additive*
ED_{80}	0.86 ± 0.07	0.84 ± 0.03	0.33 ± 0.03	0.44 ± 0.12
	Additive	*Additive*	*Synergy*	*Synergy*

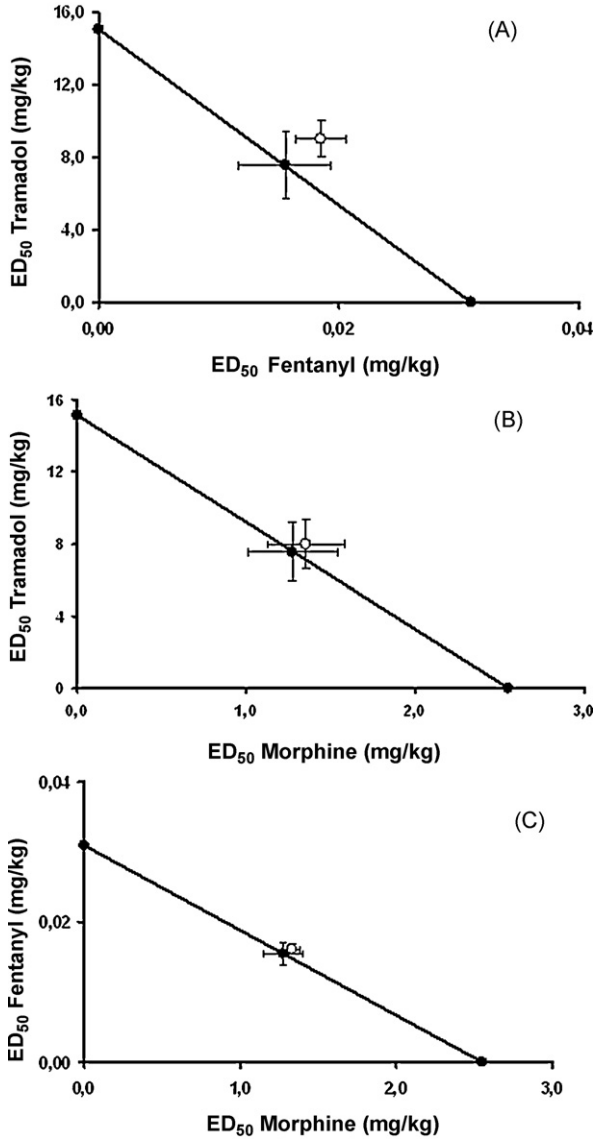

Fig. 1. Isobolograms for the subcutaneous administration of tramadol:fentanyl (A), tramadol:morphine (B) and fentanyl:morphine (C), in the hot plate test in mice. Filled circles represent the theoretical ED_{50} with 95% confidence limits (CL). Open circles the experimental ED_{50} with 95% confidence limits (CL).

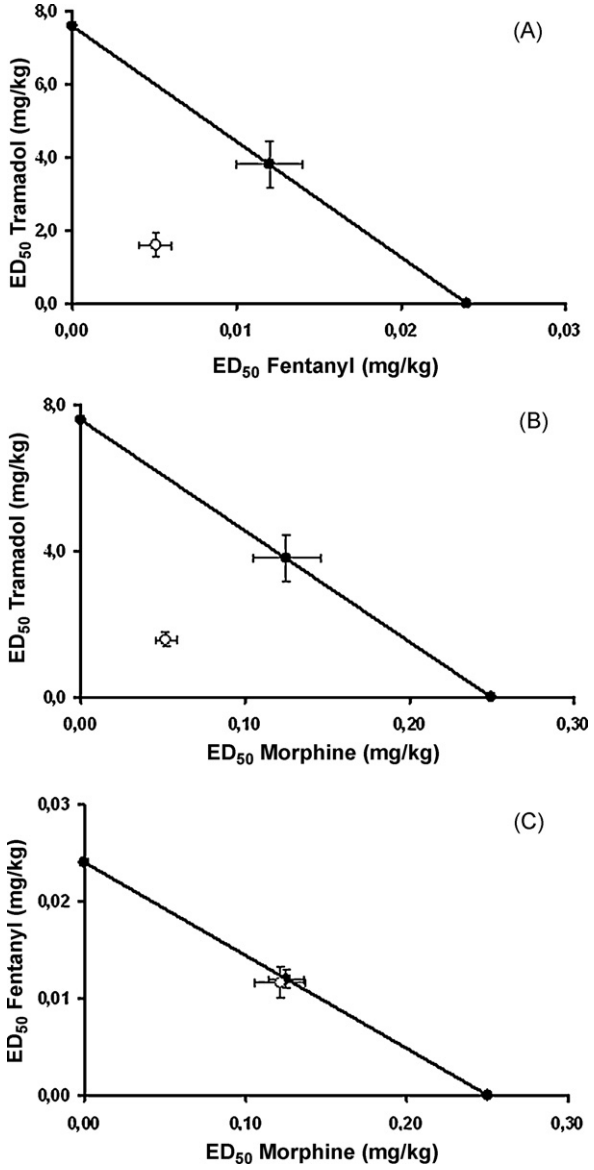

Fig. 2. Isobolograms for the subcutaneous administration of tramadol:fentanyl (A), tramadol:morphine (B) and fentanyl:morphine (C), in the writhing test in mice. Filled circles represent the theoretical ED_{50} with 95% confidence limits (CLs). Open circles the experimental ED_{50} with 95% confidence limits (CLs).

4. Discussion

The results of the present study demonstrated that fentanyl, a typical MOR agonist, possesses a higher antinociceptive potency than morphine or tramadol, independently if the nature of stimulus induces phasic or tonic pain [32]. The order of potency obtained with each drug used in this work, may be related to the affinity of each drug with their receptor, considering that the analgesia is principally due to MOR activation.

The type of interaction between drugs according to the theory of drug interaction can be expressed as: antagonism, additivity or synergy, when the combined effect of two drugs is lower, equal or greater than the sum of the effect of each agent given alone, respectively [33]. Synergism requires that drugs have different mechanism of action [34].

In the present study, we have obtained the three types of interaction, and changes from antagonism to synergy have been also described, depending on the level of effect evaluated of the same combination.

In the writhing test, the mixture of tramadol either with fentanyl or with morphine induced synergistic effect at all levels of effect, except when tramadol and fentanyl were combined at the level of ED_{80} in which additivity was observed. It is recognized that tramadol is a centrally acting analgesic, with very low affinity by MOR. By this reason, the antinociception of tramadol must be due to another cooperative mechanism than opioidergic pathway, in which it has been included, besides the inhibition of reuptake of monoamines, the role on muscarinic and nicotinic acetylcholine receptors or serotonin receptors, e.g. 5-HT_2, 5-HT_3 [35]. Moreover, the activity of tramadol on GABA receptors is controversial [33,36]. Ide et al. [37] reported, using the hot plate test, that the analgesic activity of tramadol is mediated by MOR and α_2-adrenoceptor. Both receptors belong to the rhodopsin-like family of heptahelical cell membrane receptors that are coupled to similar $G_{i/o}$-type G-proteins and mediate antinociceptive effects via similar signal transduction pathways. Antinociceptive synergy between opioid and α_2-adrenergic mechanisms has been described [38,39]. Then,

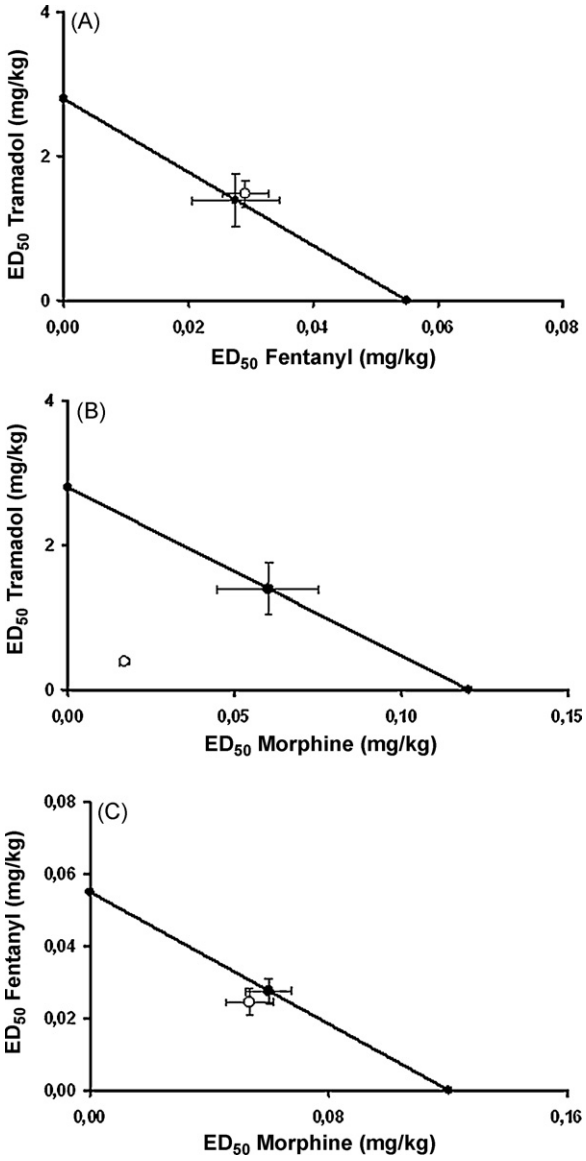

Fig. 3. Isobolograms for the subcutaneous administration of tramadol:fentanyl (A), tramadol:morphine (B) and fentanyl:morphine (C), in the phase I of formalin test in mice. Filled circles represent the theoretical ED_{50} with 95% confidence limits (CL). Open circles the experimental ED_{50} with 95% confidence limits (CLs).

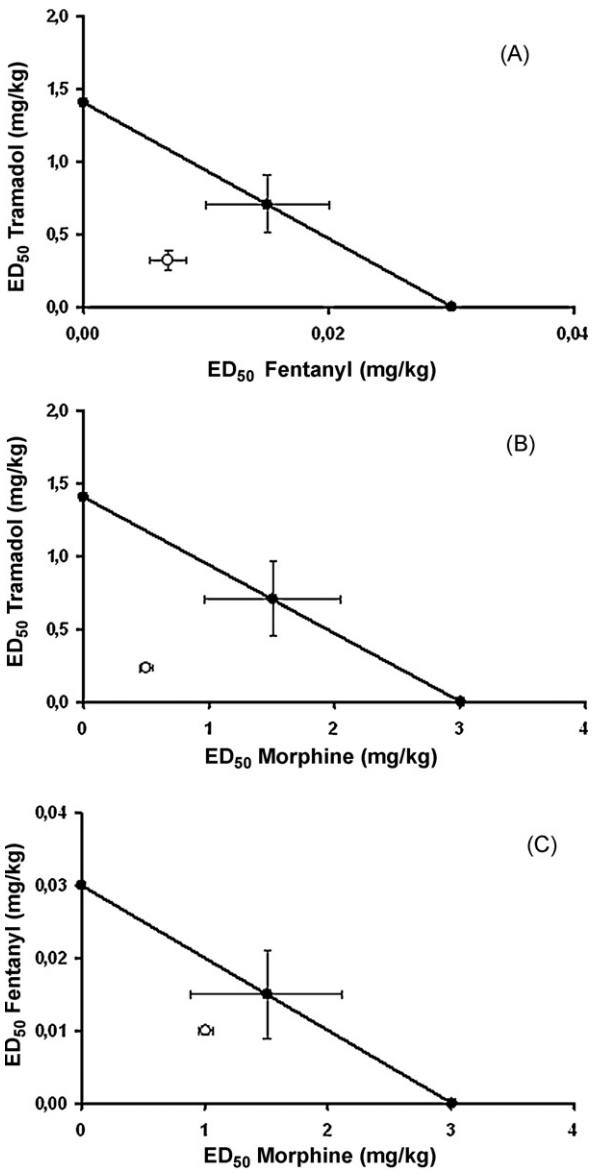

Fig. 4. Isobolograms for the subcutaneous administration of tramadol:fentanyl (A), tramadol:morphine (B) and fentanyl:morphine (C), in the phase II of formalin test in mice. Filled circles represent the theoretical ED_{50} with 95% confidence limits (CL). Open circles the experimental ED_{50} with 95% confidence limits (CLs).

the synergy obtained when tramadol is combined with fentanyl or morphine at high levels of effect, could be related with the activation of similar pathways that they could amplify the antinociceptive effect at these doses (synergy). The no interaction between tramadol and fentanyl at ED_{80} could be related to the different relative potency at this level of effect comparing to lower EDs. It is known that synergy is not only a property of the drug pair but also depends on the relative amounts in the combination tested [9]. Furthermore, the combination of fentanyl with morphine induced only additive effects, in the writhing test at all levels of effect. This finding could be due to the similar activation of MOR by both drugs.

The lack of synergism (no interaction) obtained in the hot plate test, after the co-administration of tramadol with fentanyl or morphine, or fentanyl with morphine it is hard to be explained. Possible justification to these observations may be that they share similar mechanism of action through the activation of MOR after thermal noxious stimuli. Additionally, the explanation could be ascribed to the different subtypes of opioid receptors that could be activated by morphine or fentanyl. Clinical and animal models observations [40,41] sustain that individual opioids may interact, at least in part, with different OR subpopulations (splice variants) or modulate OR signalling in subtly different ways [42,43]. The differences obtained with the combinations administered in this study, could be also related with the endogenous physiological response evoked by thermal or chemical stimuli. These antinociceptive effects were simply additive at all levels of effect, providing less potential clinical utility of these combinations after thermal noxious stimuli.

Then, the variations in the type of interaction with the same combination evaluated by different nociceptive tests, may be more selective for the clinical utility of each combination. In the phase I of the formalin test, where the nociceptive component is more relevant, the presence of fentanyl in the combination is determinant to modify the type of interaction. Changes from antagonism to synergism have been obtained, depending on the level of ED combined. The analysis of data showed that the mixtures of tramadol with fentanyl and fentanyl with morphine induced interactions that changed from antagonism, to additive and finally synergy was

Table 4
Effects of tramadol (TRM), fentanyl (FEN), and morphine (M) individually (ED_{80}'s) and combined in 1:1 proportion, on motor performance on the rotarod in mice.

Drugs (treatments)	ED_{80} (total mg/kg)	Time on rotarod (sec)	
		Baseline	Test
CTL	–	240 ± 0.0	232.82 ± 5.68
A			
TRM	33.04 ± 4.50	240 ± 0.0	217.80 ± 11.91
FEN	0.13 ± 0.04	240 ± 0.0	233.30 ± 4.90
M	15.36 ± 0.61	240 ± 0.0	240.00 ± 0.0
B			
TRM:FEN	5.95 ± 0.68	240 ± 0.0	233.33 ± 6.67
TRM:M	14.65 ± 1.23	240 ± 0.0	237.90 ± 1.45
FEN:M	1.63 ± 0.32	240 ± 0.0	227.30 ± 6.32

CTL: control group (saline). The values of ED_{80}'s (mg/kg) and time on rotarod (s) are expressed as mean ± S.E.M of 9–11 animals.
We tested the highest values of the ED_{80}'s obtained for each individual drug, in the different nociceptive tests (Panel A): TRM (hot plate test), FEN (formalin test, phase I), M (formalin test, phase II). In panel B, synergistic doses for each opioid–opioid combination evaluated are showed: TRM:FEN (formalin test, phase I), TRM:M (formalin test, phase I) and FEN:M (formalin test, phase II). For the TRM:FEN combination, 5.92 ± 0.67 mg/kg corresponded to tramadol and 0.03 ± 0.003 mg/kg corresponded to fentanyl; for the TRM:M combination, 14.59 ± 1.22 mg/kg corresponded to tramadol and 0.06 ± 0.005 mg/kg corresponded to morphine; and for the FEN:M combination, 0.02 ± 0.005 mg/kg corresponded to fentanyl and 1.61 ± 0.31 mg/kg corresponded to morphine.
One-way ANOVA did no show significant differences among treatments ($P = 0.251$).

obtained when doses in both combinations were increased. The reason why it happens is not clear. But could be explain due to the fact that fentanyl is only a MOR agonist. In addition, it has been recognized that differences in activity and efficacy appears to be related to the type or nature of the stimulus and the relative activation of the opioid receptors, e.g. MOR, KOR or DOR, as well as genetic differences in opioid receptor sensitivity [36]. Explanations of the synergy, additive or antagonism interactions observed would be highly approximate. Since fentanyl and tramadol activate the same opioid receptors (MOR), the antagonism is induced when the combination is administered at low doses (ED_{20}). In this case, fentanyl would act as a full agonist antagonizing the MOR activity of tramadol and morphine. While tramadol is only a partial MOR agonist, it might be interacting with other non-opioid receptors with negative effects. As in the case of tramadol, the activation of several receptors MOR, DOR and KOR by morphine combined with fentanyl, cannot be excluded [36,44–46]. This antagonism is changed to additive or synergism when increasing the doses of the fentanyl and tramadol or morphine in the respective combinations. These changes can happen by the lack of parallelism by variable potency ratios of the two agonists at each level of effect.

In the phase II of the same test, in which the inflammatory events are important, synergism was obtained after combining tramadol with fentanyl at all levels of effect. The synergy obtained is concordant with the general theory of drug interaction [34]. The combination of fentanyl with morphine, induced the same results obtained in phase I. This mixture induced antagonism at lower doses (ED_{20}) that it changed after increasing these doses from additivity (ED_{50}) to synergism (ED_{80}). Then, different types of interactions were observed in the same combination at different levels of effect. The interaction can occur at one or at more levels of cell function, and these events are dependent on the local concentration of the drugs and on the nature of the nociceptive stimulus and its transduction mechanisms [34]. Moreover, the description of the association among MOR, DOR, or KOR by dimerization mechanisms could cause conformational changes of the receptors that possibly could affect the binding site, reason that might explaining these results [43,44].

In both phases of the formalin test, tramadol combined with morphine induced the same results. No interaction (additive) was obtained at lower doses (ED_{20}) of the combination, when the doses of combination were increased, and consequently the effect (ED_{50} and ED_{80}), synergy was induced. These results are in agreement with the previously reported mechanisms of action of the each drug.

Besides, pharmacodynamics of each drug could explain the differences obtained with the combinations administered in this study. These changes could be also related with the endogenous physiological response evoked by thermal or chemical stimuli. Acute pain tests, such as the hot plate the writhing and the formalin tests, define several substrates that are activated by acute and high intensity stimulus.

In our study, motor coordination was assessed using the rotarod and no significant impairment of motor function occurred after each drug, individually (ED_{80}'s) and combined in 1:1 proportion (Table 4). Then, the observed effects were due to the antinociceptive opioid actions in presence of a non-sedative condition. However, other pre-clinical studies, described a lack on motor performance at higher doses for fentanyl [47], but at similar dose range for morphine or tramadol [48,49]. These discrepancies could be explained by the variability among species or strains used in those studies [50].

In addition, we have been unable to find in the literature any studies reporting changes in motor coordination after the administration of opioid–opioid combinations. In the present study, the doses of each opioid administered in the combination (in 1:1 proportion) were always lower than those reported to induce changes in motor coordination when drugs were given individually. Then, since we observed synergy between the different opioids in some nociceptive tests (Table 3), these interactions had no effect on motor coordination in mice.

In the management of pain in humans, doses that decrease pain by 50–80% are utilized in order to obtain clinically relevant analgesia. Our results show that, at these levels of effect, the antinociceptive action of opioids is either additive or synergistic when assessed in the different nociceptive tests. Interactions at low levels of effect (i.e. ED_{20}) showed antagonism in some nociceptive tests, but they are not clinically important since they would not provide significant analgesia. Synergy is obtained following lower doses of either drug, thereby reducing side effects. We can conclude that there is no interaction among the possible side effects, although in the present study only signs of sedation or central nervous system depression were evaluated using the rotarod. The potential utility of the results would be that when using opioid–opioid combinations (in a 1:1 proportion based on their antinociceptive potency), they should be combined at doses

that induce over a 50% response, since no antagonism of analgesia was observed over the ED_{50}'s.

In conclusion, the present results suggest that the interaction between opioids depends not only on the type of noxious stimulus but also of the non-opioid antinociceptive pathways. For a given combination (at a fixed ratio), the type of interaction may change according to the level of effect, a fact that may be relevant when attempting to introduce drug combinations in clinical practise.

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