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Fentanyl–trazodone–paracetamol triple drug combination: Multimodal analgesia in a mouse model of visceral pain

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ABSTRACT

Multimodal or balanced analgesia is commonly used in the management of acute and chronic pain in humans, in order to achieve the best analgesic/safety profile. Here, by using a model of visceral acute tonic pain, the acetic acid-induced writhing test of mice, we show a synergistic interaction between fentanyl, trazodone and paracetamol on the inhibition of nociception. First of all, once assessed that all drugs induced dose-related antinociceptive effects, they were mixed in fixed ratio (1:1) combinations and a synergistic drug–drug interaction was obtained in all circumstances. Thereafter, we assayed the effects of the triple combination of fentanyl–trazodone–paracetamol and it was demonstrated that they displayed a potent synergistic interaction on the inhibition of acetic acid-mediated nociception. Interestingly, drug dosage reduction permitted to reduce the incidence of possible adverse effects, namely exploratory activity and motor coordination, thus it was demonstrated that it improved the benefit/risk profile of such treatment. Afterwards, we attempted to elucidate the mechanism of action of such interaction, by means of the non-selective opioid receptor antagonist naloxone. Interestingly, naloxone completely antagonized the antinociceptive effects of fentanyl, and it also partially reversed paracetamol and trazodone mediated analgesia. Furthermore, when naloxone was co-administered with the triple-drug treatment it blocked the previously observed enhanced antinociceptive effects of the combination. Thus, these results indicated that the endogenous opioid system played a main role in the present drug–drug interaction. Overall, the triple combination of fentanyl–trazodone–paracetamol induced a potent synergistic antinociceptive effect, which could be of interest for optimal multimodal clinical analgesia.

1. Introduction

An effective control of pain is difficult to achieve when using one drug individually. Most drugs (i.e. opioids) cannot be prescribed at high doses, not only because they have a ceiling on its efficacy and security but they also may induce tolerance. Drug combinations are a powerful alternative. In fact, multiple analgesic-drug combinations are commonly used in the management of acute and chronic pain in humans during multimodal or balanced analgesia, in order to improve beneficial effects and/or reduce adverse ones (Elia et al., 2005; Skinner and Shintani, 2004; Troster et al., 2006). However, an empirical and not precise approximation is usually followed in clinical practice, and the pre-clinical characterization of the associations with the best analgesic/safety profile would be desirable. Indeed, while interactions between two drugs have been quite studied and are considered to be beneficial for the patient, combinations of three drugs have been not, scarcely in pre-clinical experiments, thus the characterization of such approaches would be of interest for the clinical management of pain.

Fentanyl (FEN) is a synthetic short-acting μ-opioid receptor agonist, which has a 50–100 times more potent analgesic activity than morphine (Clotz and Nahata, 1991). This opioid derivative modifies both central and peripheral nociception but when therapeutically used it also has undesirable side effects (drowsiness, nausea, vomiting, etc.) that may preclude its continuous usage. New therapeutic approaches have been developed, such as transdermal patches or intrathecal administration, and the concept of opioid multimodal analgesia in which the inclusion of analgesic adjuvants might provide a superior dynamic pain relief with reduced opioid-related side effects also has become a widespread strategy (Goodwin et al., 2009).

Antidepressant drugs are widely used in the treatment of chronic pain states as an adjuvant or alone (Fishbain et al., 2000). Trazodone (TRZ) is an atypical antidepressant, which has been shown to block the postsynaptic serotonin (5-HT) receptors, 5-HT2A and 5-HT2C, and weakly inhibit presynaptic 5-HT transporters (Marcoli et al., 1998). As

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a result, it has been suggested that this triazolopyridine derivate could exert its antinociceptive action either increasing the activity of descending monoamine inhibitory fibers or alternatively attenuating the increase in descending facilitatory inputs involved in maintaining pain after peripheral nerve injury, in both of which 5-HT receptors play a key role (Bomholt et al., 2005; Burgess et al., 2002; Robinson et al., 2004). Moreover, TRZ has been shown to bind to opioid receptors, but only at much higher concentrations than those needed for the interaction with the 5-HT receptors. In such way, it was reported that TRZ antinociceptive activity in rodent models appeared to require intact μ-opioid receptors (Schreiber et al., 1999). Thus, it would seem likely that the analgesic effect of TRZ would be driven, together with the stimulation of descending monoamine transmission, by an interaction with the endogenous opioid system.

Similar to the mechanism of action proposed for TRZ, the analgesic activity of paracetamol (PRC) has been related to many neurotransmitter systems. Thus, in addition to the inhibition of prostaglandin biosynthesis, in which the isoenzyme COX-3 would play a major role, the antinociceptive action of PRC has been postulated to occur by means of the modulation of the serotonergic and opioidergic systems (Botting and Ayoub, 2005; Pini et al., 1996, 1997). Hence, since the antinociceptive effects of the three drugs described may be explained by the fact that all can affect either the serotonergic or the opioidergic systems at various degrees, it can be hypothesized that they could interact in a synergic way if administered concomitantly.

The aim of the present work was to characterize through isobolographic analysis the antinociceptive interaction of the three-drug combination (FEN, TRZ, and PRC), using a model of visceral acute tonic pain, the acetic acid-induced writhing test of mice. Different proportions of the mixture were used trying to attempt the best analgesic/safety profile, since we evaluated psychomotor effects through the rota-rod and open-field tests. Furthermore, in order to elucidate the mechanism of the possible drug–drug interaction, the role of the endogenous opioid system was evaluated by the use of the non-selective opioid receptor antagonist naloxone.

2. Materials and methods

2.1. Animals

The Institutional Committee on Animal Use and Care, in accordance with the International Association for the Study of Pain guidelines on ethical standards for investigation in animals, approved the protocol. Male Swiss CD1 mice (20–25 g) housed under controlled standard conditions (12 h dark/light cycle, 22 °C temperature and 66% humidity) were used. Animals were acclimated (free access to food and water) for at least 1 week before use. All experiments were conducted between 9 a.m. and 3 p.m. During the study, animal welfare (feeding, posture, grooming, and motor activity) was verified daily. Mice were used only once, and were sacrificed immediately after the experiments by cervical dislocation.

2.2. Drugs

Fentanyl (KernPharma, Barcelona, Spain), trazodone (Angelini Farmacéutica, Barcelona, Spain), paracetamol (Bristol-Myers Squibb, Madrid, Spain) and naloxone (Sigma-Aldrich, St. Louis, MO, USA) were used. These drugs and their combinations were dissolved in 0.9% NaCl and injected subcutaneously. Acetic acid was purchased from Panreac Química (Castellar del Vallès, Barcelona, Spain), dissolved in 0.9% NaCl and injected intraperitoneally.

2.3. Writhing test

Visceral chemical nociception was evaluated using the writhing test (Collier et al., 1968). Briefly, an acetic acid solution (0.6% v/v, 10 ml/kg) was injected intraperitoneally and the mouse was placed in a clear plexiglass cylinder to observe the writhes produced by the irritant solution (a single writhe is defined as a wave of contraction passing caudally along the abdominal wall, accompanied by a twisting of the trunk and followed by extension of the hind limbs). The number of writhes in a 5 min period was counted, starting 5 min after the acetic acid administration. Drug-mediated antinociception was expressed as percent inhibition of the number of writhes observed in control animals (saline-injected), according to the following expression: % Inhibition = [(writhes in control mice − writhes post-drug mice)/writhes in control mice] × 100. Saline, single drugs or their combinations were injected subcutaneously 30 min before the administration of acetic acid.

2.4. Rota-rod test

Motor coordination was assessed by means of the rota-rod test (Dunham and Miya, 1957). Briefly, animals were placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor, which was turning at 16 rpm. For each animal, the number of falls for 1 min was registered. Drugs were injected 30 min before the test. But importantly, approximately 4 h before the experiment, mice were trained on the rota-rod apparatus, and those animals scoring more than three falls after two consecutive 1 min periods were rejected.

2.5. Open field test

Exploratory activity was evaluated using the open field test (Archer, 1973). This apparatus consists of an area of black floor delimited with acrylic transparent walls (30 × 30 × 15 cm) and divided into nine squares of equal area. Drugs were injected 30 min before the test, and thereafter during a 5 min period the number of squares crossed (with the four paws) was observed.

2.6. Experiments performed

2.6.1. Antinociception

i) In the writhing test dose–response curves were generated for FEN (0.01–0.1 mg/kg), TRZ (0.5–5 mg/kg) and PRC (20–200 mg/kg) alone, in order to perform a linear regression analysis and calculate the dose that produced a 20, 50 and 80% of the maximal antinociceptive effect ($ED_{20,50,80}$). For all dose–response curves, at least eight mice were tested per dose, and at least five points were used to define each curve. Dose–response curves were then obtained combining two drugs in fixed ratio (1:1) combinations based on ED_{50} fractions (1/2, 1/4, 1/8, and 1/16) of each drug in the mixture (i.e. for the FEN + PRC combination: $ED_{50\ FEN} + ED_{50\ PRC}$; 1/2 $ED_{50\ FEN}$ + 1/2 $ED_{50\ PRC}$; 1/4 $ED_{50\ FEN}$ + 1/4 $ED_{50\ PRC}$; etc.). Afterwards, the two-drug combinations were treated as a single drug, and the calculated ED_{50} of these mixtures were combined in the same fractions as above with the ED_{50} of the remaining drug (i.e. for the FEN-PRC + TRZ combination: $ED_{50\ FEN\text{-}PRC} + ED_{50\ TRZ}$; 1/2 $ED_{50\ FEN\text{-}PRC}$ + 1/2 $ED_{50\ TRZ}$; 1/4 $ED_{50\ FEN\text{-}PRC}$ + 1/4 $ED_{50\ TRZ}$; etc.). Thus, the ED_{50} of the different three-drug combinations (FEN:PRC + TRZ; FEN:TRZ + PRC; TRZ:PRC + FEN) were obtained. *ii*) The non-selective opioid receptor antagonist naloxone was used in order to study the mechanism of action of the interaction between the different drugs. In the writhing test, a single fixed dose of naloxone (1 mg/kg) was administered alone and together with the determined effective dose (ED_{80}) of the individual drugs or of the combined treatments.

2.6.2. Motor coordination and exploratory activity

In order to discriminate possible synergism impairment to motor function, since a deficit in motor coordination and general activity would hamper the usefulness of the present drug combinations, the determined effective doses (ED_{80}) of the individual and combined

treatments were challenged in the behavioral tests (rota-rod and open field), and the results compared to those obtained in control animals.

2.7. Isobolographic analysis

Isobolographic analysis was used as described previously (Poveda et al., 2003) according to the method described by Tallarida (2001). Briefly, isobolograms were constructed by plotting 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 point represented in the isobole line is the theoretical additive point of the combined treatment. Then, the experimental point of the combination is plotted in the graph and, if it falls below or above the isobole synergy or antagonism, respectively, occur between the combined drugs. The presence of an interaction was also evaluated calculating the interaction index. The diagonal non-interaction (additivity) line of the isobole is described by the equation: $da/dA + db/dB = 1$, where dA and dB are the doses (mg) of each drug individually that induce a 50% inhibition of nociception, and da and db are the doses (mg) of each drug in the combination that produce the same level of inhibition. The sum of these quotients is the interaction index. When the interaction index value is close to 1, there is no interaction and the final effect is additive. Values lower than 1 are an indication of the magnitude of supra-additive or synergistic interactions, and values higher than 1 correspond to sub-additive or antagonistic interactions (Tallarida, 2001).

2.8. Statistical analysis

The interaction between the different drugs on the inhibition of nociception was analyzed using the procedure previously described (Fernandez-Duenas et al., 2008) based on the method developed by Tallarida (1992, 2001). Briefly, we compared the dose–response curve of the combination experimentally determined, with that of the theoretical curve of additivity in the same proportional mixture. An interaction occurred when the experimental dose–response curves showed a significant leftward or rightward shift compared to the theoretical additive dose–response curves ($P<0.05$, Student's t test). Similarly, in the isobolographic analysis, the statistical differences between theoretical and experimental values were assessed by the Student's t-test ($P<0.05$). The data from the rota-rod and open field tests were compared using one-way analysis of variance (ANOVA), followed by a post hoc Student–Newman–Keuls test ($P<0.05$).

3. Results

3.1. Antinociception induced by fentanyl (FEN), trazodone (TRZ), paracetamol (PRC) and the mixtures FEN:PRC, FEN:TRZ and TRZ:PRC

In the writhing test, we analyzed the antinociceptive effect of the subcutaneous administration of FEN (0.01–0.1 mg/kg), TRZ (0.5–5 mg/kg) and PRC (20–200 mg/kg). All drugs induced a dose-dependent inhibition of writhes with similar efficacy (100%) and the following $ED_{50's}$: FEN = 0.021 ± 0.003, TRZ = 1.16 ± 0.02 and PRC = 109.3 ± 2.5 mg/kg (Fig. 1). Interestingly, dose–response curves were not significantly different ($P>0.05$), indicating that the relative drug-potency was equal at all levels of effect in the same order of magnitude. Control animals were injected with saline and the number of writhes was 35.5 ± 4.6.

On the basis of the obtained $ED_{50's}$, drugs were combined in fixed ratio (1:1) combinations (FEN:PRC, FEN:TRZ and TRZ:PRC). In all cases, the mixtures induced dose-dependent antinociceptive effects, with similar efficacy (100%), and dose–response curves were shifted to the left with respect to the theoretical additive dose–response curves that would be obtained if no synergism or antagonism would occur. When comparing the different combinations, synergism was

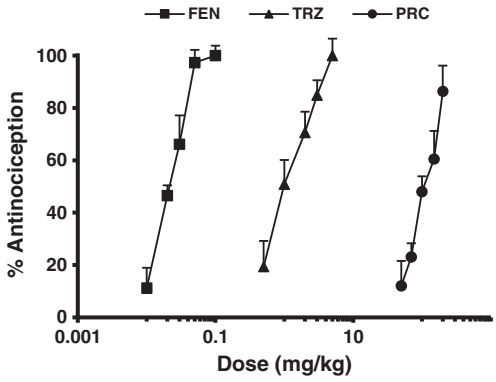

Fig. 1. Dose–response regression lines for the administration of fentanyl (FEN, squares), trazodone (TRZ, triangles) and paracetamol (PRC, circles) on the inhibition of acetic acid-mediated nociception (% inhibition). Each point represents mean values of at least eight mice, and vertical bars indicate S.E.M.

achieved at different degrees, since the interaction indexes for FEN:TRZ and TRZ:PRC mixtures were 0.21 ± 0.06 and 0.20 ± 0.07, respectively, while the interaction index for FEN:PRC was 0.61 ± 0.09. Nevertheless, in the isobolographic analysis (Fig. 2), there were significant differences ($P<0.05$; Student t test) between the experimental and the additive values in all cases.

3.2. Triple drug combination: synergistic interaction between fentanyl, trazodone and paracetamol

In order to further reduce drug dosage, aiming to obtain similar antinociceptive efficacy but with a reduction of possible undesirable effects, triple drug combinations were performed. Thus, the double combinations were treated as a single drug, and mixed with the remaining drug in a fixed ratio (1:1) combinations based on ED_{50} fractions as follows: FEN:TRZ + PRC; FEN:PRC + TRZ; PRC:TRZ + FEN. In all cases, dose–response curves were significantly shifted to the left regarding the curves calculated from the sum of the individual effects. Synergism was substantiated by means of the isobolographic analysis and interaction index calculation. In Fig. 3 the isobolographic analysis when adding PRC to the mixture FEN:TRZ is shown. Interestingly, this was the combination with the lowest interaction index (0.22 ± 0.05), although when adding TRZ to FEN:PRC, or when adding FEN to TRZ:PRC there also were values significantly lower than 1 (0.49 ± 0.08 and 0.46 ± 0.06 respectively; $P<0.05$ Student t test). Thus, these results clearly demonstrated that FEN:TRZ:PRC, when used in combination, had a potent synergistic effect on the inhibition of acetic acid-mediated nociception.

Next, in order to study the mechanism of action of such interaction we tested the effect of the non-selective opioid receptor antagonist naloxone on FEN:TRZ:PRC mediated antinociception. Interestingly, naloxone not only completely reversed the analgesic effects of FEN but it also partially diminished the effects of PRC and TRZ when singly administered (Fig. 4). Afterwards, the opioid receptor antagonist was challenged in the presence of the $ED_{80's}$ of the triple combinations. In Fig. 4 the effect of naloxone on antinociception produced by the addition of PRC to the mixture FEN:TRZ is shown. Interestingly, naloxone significantly reduced the synergic effect of the combined treatment, since antinociception decreased in a higher extent compared with the sum of the reversal effects obtained when administrating the individual drugs (Fig. 4).

3.3. Open field and rota-rod tests

Exploratory activity (open field test) and motor coordination (rota-rod test) were evaluated in order to characterize the benefit/risk profile of the present drug combinations. The effects of individual

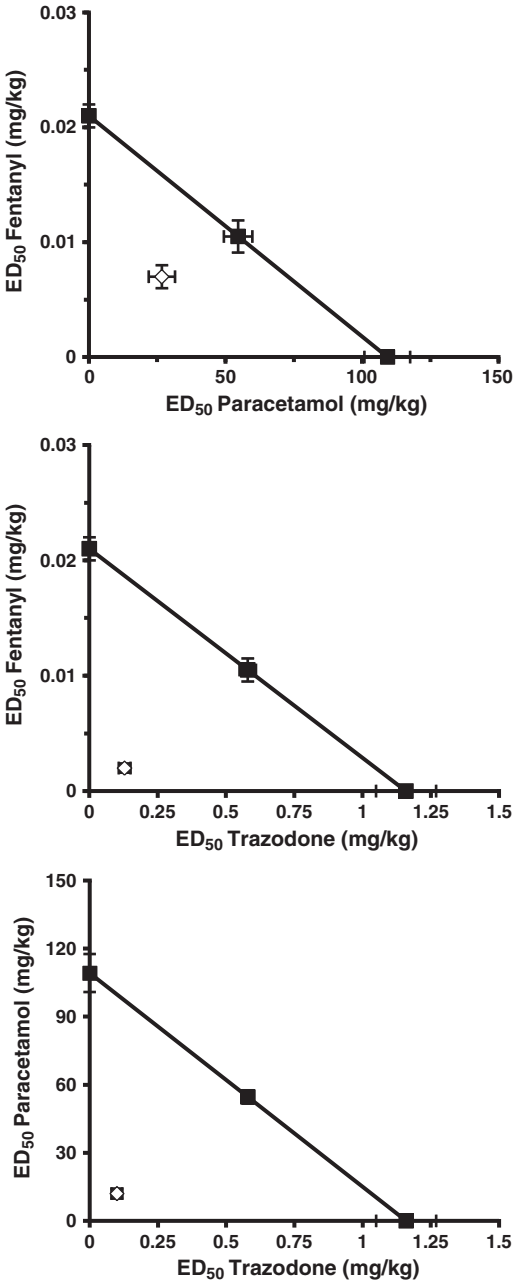

Fig. 2. Isobolographic representation of the antinociceptive effects of the combination of fentanyl/paracetamol, fentanyl/trazodone and trazodone/paracetamol, at the ED_{50} level of effect, in the writhing test. Filled circles represented in the line joining the x- and y-axes indicate the theoretical ED_{50} with 95% confidence limits (CL); open circles below the isobole correspond to the experimental ED_{50} with 95% CL.

drugs and double and triple combinations are shown in Table 1. Thus, the determined ED_{80} of the individual drugs and of the combined treatments ($ED_{80\ FEN}$, $ED_{80\ TRZ}$, $ED_{80\ PRC}$, $ED_{80\ FEN\text{-}PRC}$, $ED_{80\ FEN\text{-}TRZ}$, etc.) were assayed in the open field and rota-rod tests. Compared with control animals (saline-injected), FEN and PRC treatments did not significantly alter locomotor activity and motor coordination, while TRZ decreased the numbers of squares crossed in the open field test and it augmented the number of falls from the rota-rod apparatus. We also performed behavioral testing three days after drug administration, and there were no significant differences between drug-treated and saline-injected animals (data not shown). Regarding the double combinations, squares crossed and the number of falls were altered when TRZ was present in the mixture. Interestingly, despite dose reduction in the TRZ:PRC mixture, motor coordination was affected at

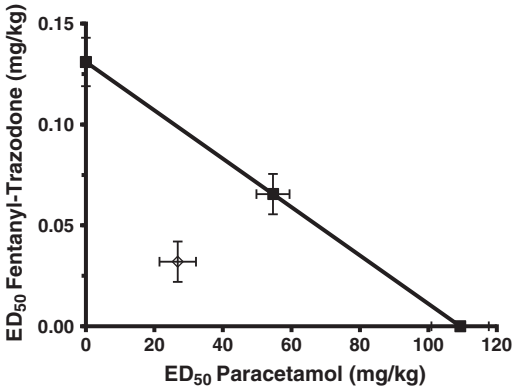

Fig. 3. Isobolographic representation of the antinociceptive effects of the combination of a mixture of fentanyl–trazodone plus paracetamol, at the ED_{50} level of effect, in the writhing test. The filled circle represented in the line joining the x- and y-axes corresponds to the theoretical ED_{50} with 95% CL; the open circle below the isobole corresponds to the experimental ED_{50} with 95% CL.

the same level that with TRZ alone. However, when the triple drug combination TRZ:PRC + FEN was performed the reduction of the amount of TRZ permitted to significantly reduce these adverse effects. Similarly, in the triple combination in which TRZ predominated (FEN:PRC + TRZ) motor coordination and exploratory activity were also altered compared with saline-injected animals, although in a lesser extent than with TRZ alone. Finally, the FEN:TRZ + PRC mixture presented the best benefit/risk profile, since behavioral parameters did not differ from that obtained in control animals.

4. Discussion

The aim of the present work was to fully characterize the beneficial/risk profile of a triple drug combination (FEN:TRZ:PRC) as a balanced approach in the management of pain. Our results show that a synergistic interaction occurs between the three drugs in the relieving of acetic acid-mediated nociception, and that the endogenous opioid system may be involved in the enhanced antinociceptive effects of such combined treatment. Furthermore, as expected, multimodal analgesia permitted decreasing drug dosage and consequently diminishing the appearance of adverse effects, namely alterations of motor coordination and exploratory activity.

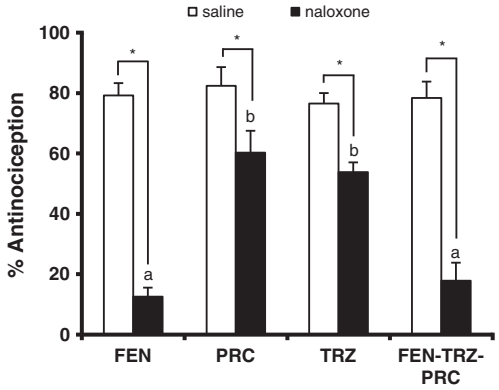

Fig. 4. Effects of the non-selective opioid receptor antagonist naloxone (1 mg/kg), on the inhibition of acetic acid-mediated nociception (% inhibition), by the determined effective dose (ED_{80}) of the individual drugs (fentanyl, 0.037 ± 0.008; trazodone, 2.05 ± 0.04; paracetamol, 193.1 ± 3.2 mg/kg) and of a mixture containing the three drugs. In control animals saline was injected instead of the antagonist. Each column represents mean values of at least eight mice, and vertical bars indicate S.E.M. (*) indicates statistically significant differences ($P<0.05$; Student t test) between saline and naloxone treatments for each treatment. (a and b) indicate statistically significant differences ($P<0.05$; one way ANOVA followed by Student–Newman–Keuls test) between the effects of naloxone in the different experimental conditions.

Table 1
Effects of the determined effective doses (ED_{80}) of the individual and combined treatments of fentanyl, trazodone and paracetamol ($ED_{80\ FEN}$, $ED_{80\ TRZ}$, $ED_{80\ PRC}$, $ED_{80\ FEN\text{-}PRC}$, $ED_{80\ FEN\text{-}TRZ}$, etc.), on motor performance (open-field and rota-rod tests). The number of squares for the open-field test and the number of falls for the rota-rod test are expressed as mean values ± S.E.M. of at least eight mice. In each test, (a, b, and c) indicate statistically significant differences ($P<0.05$; one way ANOVA followed by Student–Newman–Keuls test) between the different treatments.

Drugs (treatments)	Number of squares (open field)	Number of falls (rota-rod)
Saline	82.4 ± 7.9^{a}	0.3 ± 0.2^{a}
FEN	66.4 ± 9.0^{a}	0.3 ± 0.1^{a}
PRC	76.7 ± 7.2^{a}	0.5 ± 0.2^{a}
TRZ	31.8 ± 4.8^{b}	3.8 ± 0.7^{b}
FEN:PRC	74.2 ± 8.0^{a}	0.6 ± 0.3^{a}
FEN:TRZ	44.2 ± 7.5^{c}	1.9 ± 0.6^{c}
TRZ:PRC	45.3 ± 4.9^{c}	4.2 ± 0.7^{b}
FEN:PRC + TRZ	51.8 ± 5.4^{c}	2.2 ± 0.3^{c}
FEN:TRZ + PRC	72.4 ± 7.3^{a}	0.7 ± 0.3^{a}
TRZ:PRC + FEN	68.7 ± 7.0^{a}	1.7 ± 0.4^{c}

First of all, we tested the individual antinociceptive effects of FEN, TRZ and PRC. In the writhing test, FEN and PRC produced dose-dependent antinociceptive effects, as it has been previously reported (Ayoub et al., 2006; Giron et al., 2002). Interestingly, TRZ was also able to generate a dose–response curve inhibiting acetic acid induced-nociception. These results are consistent with those reported by other authors, in which TRZ was found effective as analgesic (Abdel-Salam et al., 2003; Okuda et al., 2003; Schreiber et al., 2000). In fact, the clinical effectiveness of antidepressants as analgesics is widely accepted, and, indeed, it has been reported that the acetic acid-induced writhing test is more sensitive to antidepressants than other tests using thermal, mechanical or electrical stimuli (Aoki et al., 2006; Rojas-Corrales et al., 2003). Subsequently, drugs were mixed in fixed ratio (1:1) combinations, and in all cases, dose-dependent antinociceptive effects were obtained. Noteworthy, all dose–response curves were shifted to the left with respect to the expected additive dose–response curves, and synergism could be demonstrated for all the combinations. It is interesting to mention that the isobolographic analysis from the mixture containing FEN and PRC revealed synergy in a lesser extent than the FEN:TRZ and TRZ:PRC combinations, despite the fact that the combination of PRC with opioids has been further explored (Miranda et al., 2008). Nevertheless, these results are in accordance with those reported previously, where the two-drug combination PRC plus morphine was shown to be synergic and presented a similar interaction index (Miranda et al., 2008). Next, our aim consisted of performing a multimodal analgesic paradigm, in which the three drugs were combined. The data obtained demonstrated that the triple combination of FEN:TRZ:PRC displayed a potent synergistic interaction on the inhibition of acetic acid-mediated nociception. Hence, the three-drug protocol allowed antinociception reducing individual drug doses, thus increasing, as discussed below, its analgesic/safety profile. On the other hand, this study permitted to obtain data graphically and mathematically validated from the administration of three drugs concomitantly, a fact that has became to be explored recently (Miranda et al., 2008), even though it is quite frequent and well-established in clinical practice managing acute and chronic pain by means of multiple analgesic drug combinations (Raffa, 2006; White, 2008).

Regarding the possible drug-induced behavioral undesirable effects, they were measured using the open field and rota-rod tests. Neither PRC nor FEN did modify exploratory activity and motor coordination, as previously shown (Romero et al., 2010). It is important to mention here that opioid effects (i.e. FEN) on locomotion have been described to occur at doses higher than the obtained antinociceptive ED_{50} (Meert and Vermeirsch, 2005), and moreover, FEN doses were even lower when drugs were combined, thus still reducing more the probability of the appearance of undesirable effects. Consequently, from the individual drugs, at the range of doses used, only TRZ impaired exploratory activity and motor coordination. These undesirable motor effects of TRZ have been shown previously (Brocco et al., 2002), and in fact TRZ antidepressant therapy has usually been associated with sedation, particularly at high doses (Mayers and Baldwin, 2005). Conversely, other authors did not find a sedative effect of TRZ when used in the treatment of neuropathic pain in rats (Okuda et al., 2003); however, in this study the motor effects of the drug were evaluated during the seven day-period after the drug treatment and not just after the post-injection episode. In fact, we also examined in preliminary studies exploratory activity and motor coordination three days after drug administration and we did not find drug-induced changes (data not shown), thus indicating that a single dose of TRZ did not permanently alter psychomotor behavior. Interestingly, when double and triple combinations were assayed exploratory activity and motor coordination impairment was significantly reduced at the same time as TRZ doses diminished. In fact, the FEN:TRZ + PRC mixture, in which the doses of TRZ and FEN were the lowest, presented behavioral parameters similar to that obtained in control animals. Furthermore, this mixture in which PRC predominated had, as described above, the lowest interaction index, thus it had the best benefit/risk profile, and at last demonstrated the benefit of performing such triple drug combination paradigm.

Once completed the study of the synergistic antinociceptive effects of the combination we attempted to elucidate the mechanism of action of such interaction by means of the non-selective opioid receptor antagonist naloxone. Firstly, we assayed the effects of naloxone on the antinociceptive effects of the individual drugs. Interestingly, naloxone not only was able to completely antagonize the antinociceptive effects of FEN but it also partially reversed PRC and TRZ mediated analgesia. Up to that time, the capacity of naloxone to block the antinociceptive action of systemically administered PRC or antidepressants has been described (Ardid and Guilbaud, 1992; Pini et al., 1997). Accordingly, it has been hypothesized that among the possible mechanisms explaining the antinociceptive action of PRC and antidepressants, namely TRZ, the endogenous opioid system, either via an increase in the release of endogenous opioid peptides or via a direct activation of opioid receptors is activated (Gray et al., 1998; Schreiber et al., 1999; Sprott et al., 2005). The involvement of opioid receptors in the filtering and modulation of nociceptive transmission in the dorsal horn has been fully characterized; furthermore, the existence, in descending inhibitory pathways, of several neurotransmitters, which receptors are in certain cases co-localized (i.e. opioid and serotonin receptors), thus permitting the possibility of their interaction in order to effectively achieve the control of pain is well-known (Millan, 2002; Ren and Dubner, 2002). Hence, since PRC and TRZ have been shown to induce their antinociceptive effects, along with other mechanisms, via the inhibitory descending pathways, it would be likely that they could interact in a synergistic manner with opioid agonists (i.e. FEN). Accordingly, when we examined the effects of naloxone on the antinociceptive action of the triple-drug combination FEN:TRZ:PRC, the non-selective opioid receptor antagonist was able to completely reverse the synergistic effect of the combined treatment. Thus, from these results, one mechanism that could explain such drug–drug interaction would consist of a regulatory role of the opioidergic system, which would integrate antinociceptive drug effects after the stimulation of several receptors (i.e. opioid and serotonin receptors). In such way, the activation of opioid receptors located in the distinct inhibitory and facilitatory fibers would potentiate the effects of the different drugs, for instance TRZ or PRC, thus exerting a powerful modulatory influence upon the onward transfer of nociceptive information from the periphery to the central nervous system.

In conclusion, our study shows that the combination of FEN, TRZ and PRC leads to a synergistic drug–drug interaction that might be useful for

the treatment of pain. Furthermore, we have substantiated the involvement of the endogenous opioid system potentiating the antinociceptive effects of the FEN:TRZ:PRC combination. Thus, this approach might represent a novel approach in the management of pain in which opioid drugs are used, since it permits achieving enhanced antinociceptive effects concomitantly with a reduction of possible undesired secondary effects associated with high drug dosages.

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