Pharmacology, Biochemistry and Behavior 97 (2011) 544–550

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Flupirtine antinociception in the rat orofacial formalin test: An analysis of combination therapies with morphine and tramadol

Alessandro Capuano [a,b], Alice De Corato [a], Mariangela Treglia [a], Giuseppe Tringali [a], Pierluigi Navarra [a,*]

[a] Institute of Pharmacology, Catholic University School of Medicine, Largo F. Vito 1, 00168 Rome, Italy
[b] Department of Neuroscience, Division of Neurology, Bambino Gesù Children's Hospital IRCCS, Piazza Sant'Onofrio 4, 00145 Rome, Italy

ARTICLE INFO

Article history:
Received 15 September 2010
Received in revised form 20 October 2010
Accepted 1 November 2010
Available online 10 November 2010

Keywords:
Flupirtine
Morphine
Tramadol
Isobolographic analysis
Orofacial formalin test
Rats

ABSTRACT

Combination therapy with two drugs is a straightforward strategy to improve the risk–benefit ratio of analgesic treatments. Flupirtine is a non-opioid analgesic drug acting via the enhancement of so-called M currents, associated to Kv7 potassium channels in the central nervous system. In this study we used the orofacial formalin test as a model of acute inflammatory pain in the rat; putative synergistic interactions between flupirtine and morphine or tramadol, given in various combinations, were investigated. We found that flupirtine exerts antinociception in the second phase of the test, whereas morphine and tramadol induced analgesia both in the first and in the second phase. An isobolographic analysis of data was carried out, showing a synergistic interaction between flupirtine and morphine, as well as between flupirtine and tramadol, in the second phase of the test. Conversely, in the first phase of the test only a single combination of morphine plus flupirtine, but not any of the combinations of tramadol and flupirtine, resulted in a synergistic interaction. Our data clearly indicate that flupirtine enhances in a synergistic manner the acute antinociceptive effects exerted by opioids in this paradigm.

1. Introduction

Opiates are broadly used in the treatment of malignant and non malignant pain (Flemming, 2010; Manchikanti et al., 2010). Important side effects are commonly associated to opioid treatments, including cognitive impairment, tolerance and dependence (Benyamin et al., 2008; Geppetti and Benemei, 2009). Such adverse effects are typically time- and dose-dependent; a possible strategy to improve the tolerability of treatments involves the use of lower doses of opioids, given in association with non-opiod analgesics in order to maintain an adequate level of analgesia (Smith, 2008). Indeed, a combination of drugs inducing analgesia through different mechanisms of action produces the classical "synergistic" effect; moreover, lowering the doses of each drug allows to reduce overall toxicity (Tallarida, 2001).

Both morphine and tramadol are widely used in clinical practice. A full μ-opioid agonist, morphine is considered — along with oxycodone — a gold standard among opiates. Morphine has been successfully investigated in combination with NSAIDs in various animal models of nociception (Déciga-Campos et al., 2003; Miranda et al., 2005; Zelcer et al., 2005). Tramadol is effective in the treatment of moderate to severe pain with a relatively low incidence of addiction; it was found to be effective in such different conditions as post-surgical pain, obstetric pain, terminal cancer pain and pain of coronary origin, and it has been used as adjuvant therapy in anesthesia (Scott and Perry, 2000). Tramadol activates opioid receptors and also appears to modify the transmission of pain impulses via the inhibition of monoamine reuptake (Dayer et al., 1997). The latter effect is observed in vitro at clinically relevant concentrations, and might at least in part account for the high antinociceptive efficacy of tramadol in spite of weak μ-opioid receptor affinity. Although tramadol presents a lower incidence and severity of opioid-like side effects compared to other opioid agents, the possible occurrence of adverse events related to interference with monoamine systems, such as a serotonergic syndrome, should be taken into account (Houlihan, 2004; Rojas-Corrales et al., 2005; Raffa, 2008).

In this study we investigated the antinociceptive activity of the combination of morphine plus flupirtine, and tramadol plus flupirtine, in the rat orofacial formalin test. Flupirtine is a centrally-acting non-opioid analgesic that has been used in Germany since 1984 for the treatment of several pain states (Klawe and Maschke, 2009). Despite a long history of clinical use, the mechanism of analgesic action as has not been characterized until recently. Indeed, early studies showed that flupirtine has no effect on serotonin, dopamine, nicotine or adrenoreceptors (Friedel and Fitton, 1993). Subsequently, the drug was found to decrease spinal polysynaptic reflexes mediated by NMDA receptors. However, flupirtine showed no binding affinity toward NMDA sites (Schwarz et al., 1994; Kornhuber et al., 1999). Such apparent discrepancy has been solved by the finding that flupirtine and the structurally related analog retigabine act as potassium-channel openers, binding neuronal potassium channels

* Corresponding author. Tel.: +39 630154367; fax: +39 6233235103.
E-mail address: pnavarra@rm.unicatt.it (P. Navarra).

0091-3057/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2010.11.002

(KCNQ 2–5 or Kv7.2–7.5) (Brown and Passmore, 2009). The opening of these channels is associated with an increase in so-called M current, which has been originally described as a hyperpolarizating current that operates around resting membrane potential (Wua and Dworetzky, 2005). The increase in M current is in turn associated with decreased neuronal excitability and reduced release of various neurotransmitters, including glutamate. Drugs acting on neuronal KCNQ might be useful in the treatment of a variety of clinical conditions including acute and neuropathic pain, and epilepsy (Gribkoff, 2003; Miceli et al., 2008). The new insights in the pharmacology of KCNQ channels and in the mechanism of action of flupirtine have renewed the interest in this compound, whose clinical use in the past was restricted to mild or moderate musculoskeletal pain syndromes because of dose-related side effects including somnolence, dizziness and rarely hallucinations (Klawe and Maschke, 2009). In particular, an interesting new perspective is the possible additive or synergistic interaction with other analgesics such as opioids (Goodchild et al., 2008a, b).

The rat orofacial formalin test proved to be a useful model to investigate the efficacy of analgesic compounds facial pain (Raboisson and Dallel, 2004). The test is based on a chemical stimulus (formalin) and induces a tissue damage that mimics acute post-injury pain in humans. During the test, two phases can be observed that are associated with two distinct mechanisms of nociception; the first phase is caused by the direct stimulation of C-nociceptors, whereas the second phase reflects integration between peripheral (nociceptors) and central (spinal/brainstem) signaling.

In this work, we used the rat orofacial formalin test as a model of post-acute inflammatory pain to investigate the efficacy of flupirtine given alone or in combination with morphine or tramadol. The experimental findings were subsequently analyzed by the isobolographic approach, a method widely used to analyze possible drug synergism in the formalin test.

2. Material and methods

2.1. Drugs

Morphine sulfate was purchased from commercial source (Oramorph®) as oral solution (20 mg/5 mL). Dilutions to working concentrations were made in sterile saline. Flupirtine maleate was purchased from commercial source (Efiret®) as tablets containing 100 mg of flupirtine base. Flupirtine was dissolved in DMSO at concentration of 20 mg/mL. Subsequent dilutions were made in sterile saline. All drugs and vehicle were delivered by intraperitoneal injection 30 min before the formalin test was performed.

2.2. Animals, orofacial formalin test, and antinociceptive measurement

Male Wistar rats aged 7–8 weeks (weight range 165–180 g) were used in this study. Animals were obtained from the breeding facilities of Catholic University and were housed on a 12 h light–dark cycle at 22 ± 2 °C, with free access to food and drinking water. On the day of experiment, animals were acclimatized to the testing room for at least 2 h before testing. All animals were used only once and were sacrificed immediately after the formalin test. This study was conducted according to EC Directive 86/609/EEC for animal experiments and the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983). Additionally, the study protocol was approved by the Local Ethical Committee for Animal Care and Use of the Faculty of Medicine, Catholic University in Rome as well as by the Italian Ministry of Health.

The orofacial formalin test was performed as previously described (Capuano et al. 2009). Briefly, 50 μL of 1.5% diluted formalin solution were injected subcutaneously into the right side of upper lip, in proximity to the nostril, using a 100 μL Hamilton syringe. After the injection, the rat was put in an observation cage consisting in a glass chamber ($30 \times 30 \times 30$) with mirrored sides, and its behavior was video-recorded for 45 min. Videos were analyzed using JWatcher software (developed at Dan Blumstein's Lab University of California Los Angeles & The Animal Behavior Lab, Macquarie University, Sydney); recording time was divided into 15 blocks of 3 min and the nociceptive response was assessed as seconds spent by the animal in face rubbing during each 3-min block. Time-courses of nociceptive response for each drug and combination were constructed as mean number of seconds that rats spent rubbing, plotted for each 3-min block over the 45 min post-injection observation period. For the two phases of the formalin test, the areas under the curve (AUC) were calculated by the trapezoidal rule: for the first phase, the first 3 blocks of 3 min, for 9 min total, were considered. The following 2 blocks of 3 min were not considered for calculation (quiescent period) and the time between 15 and 30 min (five 3-min blocks) was taken for calculation of the second phase. Percentage of antinociception for each phase was calculated according to the following equation: Percentage of antinociception = (AUC vehicle − AUC drug treatment) / AUC vehicle] × 100 (Capuano et al., 2009).

We did not measure animal motor activity as a specific marker of CNS depression. Nevertheless, overall behavioral assessment was performed, and observation was carried out by a blinded researcher. Furthermore, doses of morphine, tramadol and flupirtine used for this study were tested previously without any experimental evidence of CNS depression (Solbrig et al., 2007; Munro, 2009).

2.3. Isobolographic analysis

Morphine, tramadol and flupirtine displayed different antinociceptive profiles. Indeed, morphine and tramadol, as expected, resulted antinociceptive both in the first and in the second phase, while flupirtine was effective in the second phase only. Thus, isobolographic analysis parameters were calculated on the basis of the second phase. Dose–response curves for second phases were constructed for flupirtine, morphine and tramadol using at least six animals for each level of dose. Log dose–response curves were fitted using a non linear regression analysis for each phase of orofacial formalin test. Moreover, morphine and tramadol were effective over an extended dose-range and achieved the maximal effect, while flupirtine produced a less-than-maximal effect and was effective over a narrower range of doses.

Considering only the second phase of the test, all test drugs were antinociceptive when given alone; therefore morphine/flupirtine and tramadol/flupirtine combinations met the criteria for a joint-action analysis for similar and independent action, according to Tallarida (2001). However, as stated above, the two compounds displayed a variable potency ratio, meeting the case of an interaction between a full (morphine and tramadol) and a partial (flupirtine) agonist. Thus, the isobole of additivity is represented by a curved line. According to Grabovsky and Tallarida (2004), the isobole of additivity for 50% effect is the set of combination (a,b) dose pairs calculated following the equation:

$$b = B50 - \frac{B50}{\left[\frac{100}{E_C}\left(1 + \frac{Ac^q}{a^q}\right) - 1\right]^{1/p}} \quad [1]$$

where B50 was the dose of morphine (or tramadol) (denoted as drug B) that gives the 50% of effect; Ac is the dose of flupirtine (denoted as drug A) that gives half of its maximum effect; p and q were Hill coefficients of B and A respectively; Ec was the maximum effect reached by drug A. Three combination dose pairs that theoretically give 50% of the effect ($ED50_{add}$) were chosen. Subsequently, an experimental dose–response curve was obtained treating the animals

with one of the following combination doses: $ED50_{add}$, $ED50_{add}/2$, $ED50_{add}/4$, for each fixed-ratio of morphine/flupirtine and tramadol/flupirtine, and an ED50 for each combination treatment ($ED50_{comb}$) could be calculated on the basis of the experimental dose–response curve. Theoretical and experimental ED50 values were then tested for statistical differences. Moreover, the interaction index (γ) was calculated as follows: $\gamma = ED50comb/ED50add$; an interaction index not significantly different from the unit corresponds to an additive interaction, whereas higher or lower values indicate sub-additivity or synergism, respectively (Tallarida, 2006).

To assess whether an interaction between the two drugs also occurred in the first phase, we considered this phase as the peculiar condition where a combination of an active (morphine or tramadol) and an inactive drug (flupirtine) takes place. In this paradigm, assuming that only the active drug contributes to the selected level of effect (e.g., 50% of antinociception), we calculated the $ED50_{add}$ of the first phase. Synergism requires that ED50comb<ED50add, and therefore the difference between these values was tested by the Fischer test for statistical difference (Tallarida, 2000).

2.4. Statistical analysis

Dose–response data were analyzed by one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test. Statistical significance between the theoretical additive ED50 and the experimentally derived ED50 was evaluated using Student's t test and/or Fisher test of significance. Statistical procedures were performed using Pharm Tools Pro (version 1.1.20, The McCary Group Inc.) and PrismTM (GraphPad, San Diego, CA, USA). The non linear isobole was obtained using an application developed in our lab for MATLAB software. P values lower than 0.05 ($P<0.05$) were considered significant.

3. Results

3.1. Antinociceptive activity of flupirtine in the rat orofacial formalin test

Diluted formalin injected subcutaneously in the rat wisker pad exerted a stereotyped nocifensive behavior consisting of two distinct phase: first phase, a phasic period lasting 10 min after formalin injection and a second phase, a tonic long-lasting period attributed to sensitization mechanisms (Fig. 1, squared-vehicle). Intraperitoneal administration of flupirtine, given in the range 0.5–20 mg/kg, produced a dose-dependent reduction in face rubbing behavior (Figs. 1 and 2). In particular, flupirtine reduced face rubbing behavior only in the second phase (Figs. 1 and 2). Indeed, flupirtine increased antinociception in a dose-dependent manner in the second phase of formalin test, while in the first phase it did not exert any antinociceptive effect (Figs. 1 and 2).

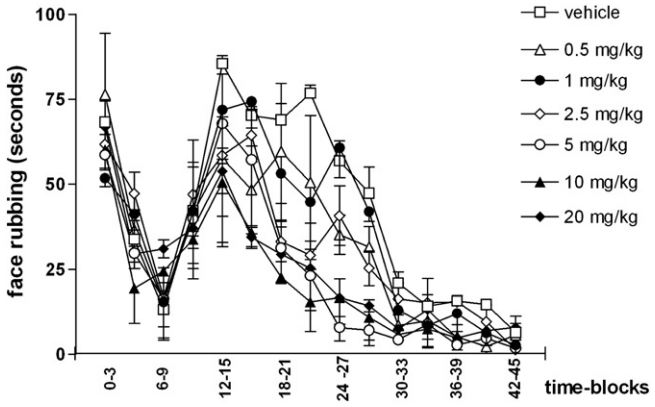

Fig. 1. Effects of flupirtine on the orofacial formalin test. Data are presented as mean (±S.E.M.) number of seconds that rats (n = 6 for each group) spent in face rubbing for each 3-min block over the 45 min post-injection observation period.

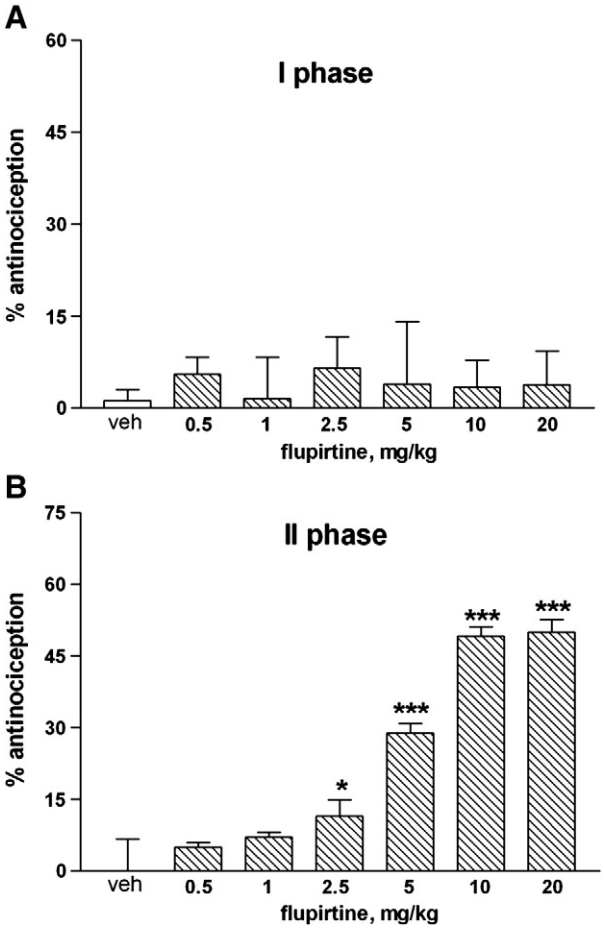

Fig. 2. Antinociceptive effects of flupirtine dose-range in the first (A) and in the second (B) phase of orofacial formalin test. Data are the means ± S.E.M. of n = 6 animals for each experimental group. * and***: P<0.05 and P<0.001 vs controls (vehicle), respectively.

3.2. Morphine and tramadol in the rat orofacial formalin test

As expected for opioid drugs, morphine and tramadol were both able to reduce face rubbing behavior (Fig. 3A and B). In particular, morphine, given in the range 0.5–4 mg/kg, increased antinociception in a dose-dependent manner both in the first (Fig. 4A) and in the second phase (Fig. 4B). Likewise, tramadol, given in the range 1–30 mg/kg, induced dose-dependent antinociception both in the first and in the second phase of rat formalin test (Fig. 5).

3.3. Isobolographic design

Since putative interactions between morphine or tramadol and flupirtine can be observed only when both test drugs are simultaneously effective, interaction parameters for isobolographic analysis were calculated on the basis of antinociceptive effects exerted in the second phase. Fig. 6 shows the (log)dose–response non linear regression curves for the three drugs. Morphine was more potent than tramadol and flupirtine, which displayed similar potency. However, morphine and tramadol reached different maxima (i.e. theoretically 100% of antinociception) compared with flupirtine, and isobolographic analysis fitted the case of interaction between drugs with different maxima (or the interaction between a full and a partial agonist), according to Tallarida (2006). Dose–response curve of morphine and tramadol (full agonist denoted as drug B) was fitted

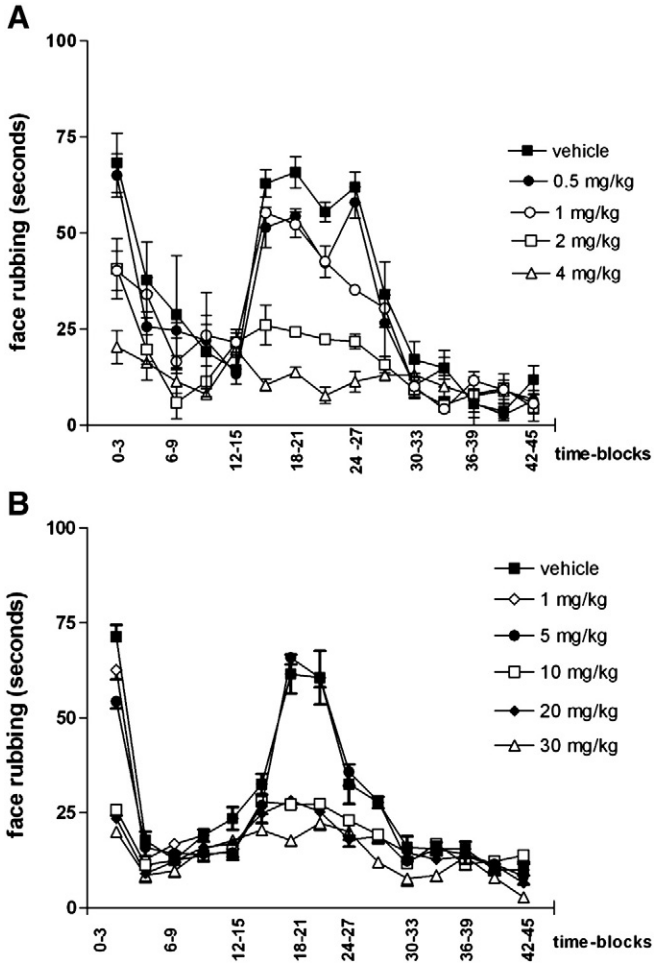

Fig. 3. Effects of morphine (A) and tramadol (B) on the orofacial formalin test. Data are presented as mean (±S.E.M.) number of seconds that rats (n = 6 for each group) spent in face rubbing for each 3-min block over the 45 min post-injection observation period.

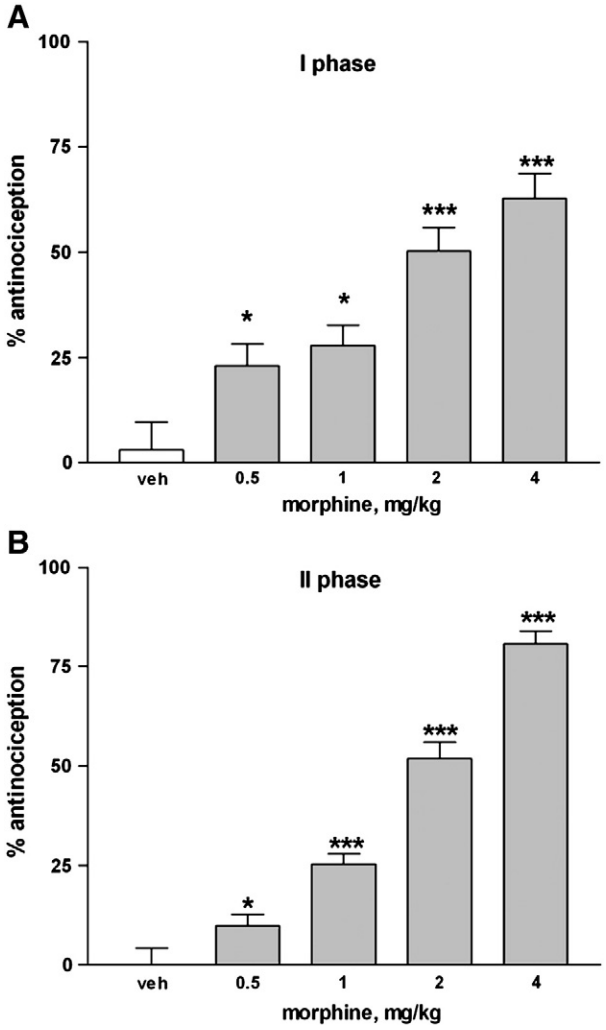

Fig. 4. Antinociceptive effects of morphine dose-range in the first (A) and in the second (B) phase of orofacial formalin test. Data are the means ± S.E.M. of n = 6 animals for each experimental group. * and ***: P<0.05 and P<0.001 vs controls (vehicle), respectively.

by: $E = EbB^p/(B^p + B50^p)$, whereas flupirtine (partial agonist denoted as drug A was fitted by $E = EbA^q/(A^q + Ac^q)$, where Ac denoted the dose that gives half of its maximum (Ec). Due to variable potency ratio, the isobole of additivity was constructed by using Eq. [1] (see Material and methods) and resulted in a non linear isobole. For each dose (b) of morphine or tramadol in the combination dose pairs, the dose of flupirtine (a) was calculated as an equivalent dose b′ of drug B (morphine). Fig. 7 shows 50% antinociception curved isobole (additivity line) for morphine (A) and for tramadol (B). Three additive dose pairs for 50% of the antinociceptive effect were chosen to be experimentally tested. The additive ED50 ($ED50_{add}$) are denoted as the points A, B and C in Fig. 7A and B for morphine and tramadol, respectively. For morphine/flupirtine combination $ED50_{add}$ values were 4.16, 6.35, and 9.008 mg/kg containing different proportion of morphine: 50%, 16.1% and 3.23%, respectively. For tramadol, $ED50_{add}$ values were 16.56, 13.46, and 11.13 mg/kg containing different proportion of tramadol: 83.5%, 61.35% and 18.16% respectively.

3.4. Isobolographic analysis for morphine and tramadol combinations

Dose–response curves were constructed for each combination testing the doses $ED50_{add}$, $ED50_{add}/2$, and $ED50_{add}/4$. Linear regression was performed to obtain experimental ED50 ($ED50_{comb}$). We found that combination doses were able to induce antinociception both in the first (Table 1) and in the second phase of formalin test (Fig. 8). In particular, as regard morphine combinations $ED50_{comb}$ (±S.E.M.) values were 1.854 ± 0.26, 4.076 ± 0.62, 7.85 ± 3.71 mg/kg, for combination A, B and C respectively (denoted as A′, B′ and C′ in Fig. 7A). Interaction analysis demonstrated that synergism occurred only for combination A (ED50 A′ vs ED50 A P<0.001) and B (ED50 B′ vs ED50 B P<0.001), whereas combination C resulted in a simple additive interaction. Moreover, the interaction index (see Material and methods) confirmed synergistic interaction for A and B (γ = 0.46 and γ = 0.64 for A and B, respectively). As far as the tramadol/flupirtine association is concerned, experimental doses were able to induce antinociception both in the first (Table 1) and in the second phase of formalin test (Fig. 8B). In particular, $ED50_{comb}$ (±SEM) values were 17.43 ± 1.130, 10.15 ± 1.05 and 9.29 ± 0.74 mg/kg, for combination A, B and C respectively (denoted as A′, B′ and C′ in Fig. 7B). Interaction analysis demonstrated that synergistic interaction occurred only for combination B (ED50 A′ vs ED50 A, P<0.05), while A and C resulted in a simple additive interaction. The interaction index confirmed synergistic interaction for B (γ = 0.75).

3.5. Isobolographic analysis in the first phase of formalin test

Concerning the first phase (where flupirtine was not effective), we postulated that the observed effects were almost completely to be attributed to morphine or tramadol. For each combination tested, we calculated theoretical ED20 (a level of antinociception to be compared without extrapolation for all tested doses) that was compared with actual ED20 values. In particular, with the morphine/flupirtine

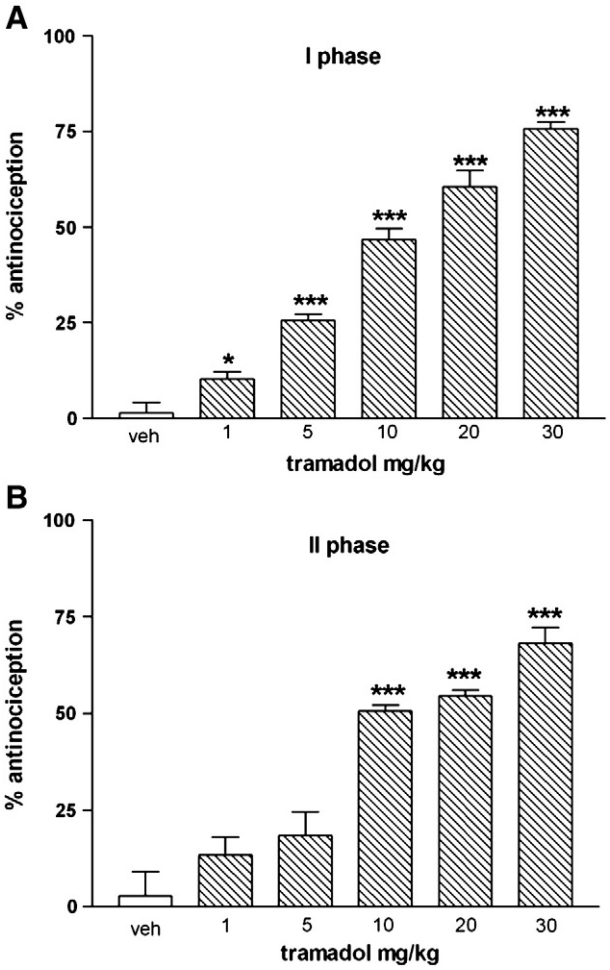

Fig. 5. Antinociceptive effects of tramadol dose-range in the first (A) and in the second (B) phase of orofacial formalin test. Data are the means ± S.E.M. of n = 6 animals for each experimental group. * and***: P<0.05 and P<0.001 vs controls (vehicle), respectively.

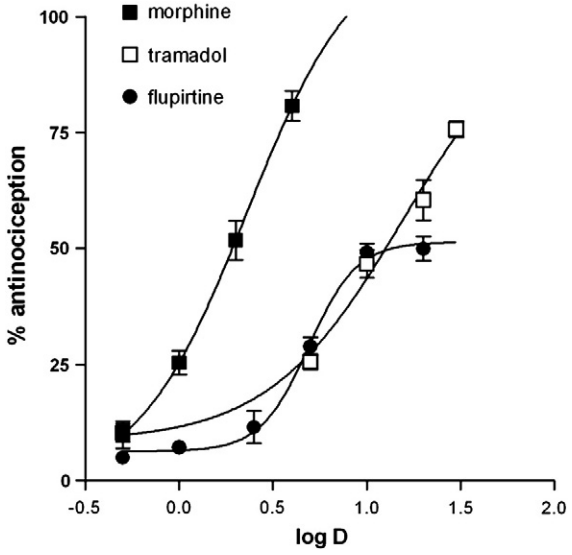

Fig. 6. (Log)dose–response of morphine, tramadol and flupirtine in the second phase of rat orofacial formalin test. The dose–response curves were fitted by $E = EbB^p/(B^p + B50^p)$ and $E = EbA^q/(A^q + Ac^q)$ for morphine and tramadol (full agonist) and flupirtine (partial agonist), respectively. Thus, morphine and tramadol displayed different maxima and had a variable potency ratio, compared with flupirtine. Parameters obtained from dose–response curves were used to construct the isobole for 50% of the antinociceptive effect (see Material and methods and Results).

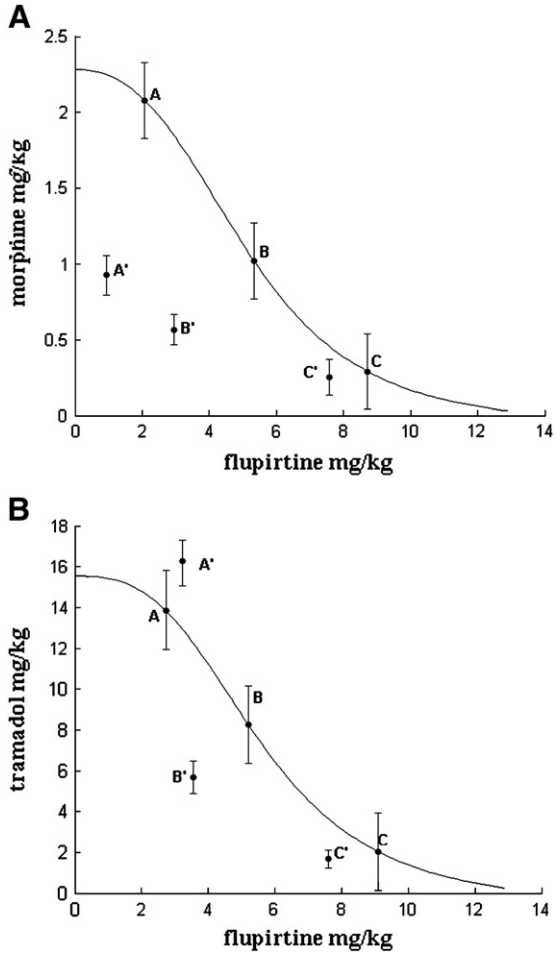

Fig. 7. Isobologram for the 50% of the effect for combination doses of morphine/flupirtine (A) and tramadol/flupirtine (B). X- and Y-intercept are the doses that displayed 50% of the antinociceptive effect for each drug in the combination, when administered alone. The smooth curve is the line of additivity where all combination dose pairs lie. ED50add (additive) values are denoted as A, B and C, for combination containing different percentage of morphine and flupirtine, or tramadol and flupirtine, respectively. ED50comb (experimentally obtained) are plotted as A′, B′ and C′. For morphine combinations, A′ and B′ resulted significantly below the isobole, thus indicating synergism of these combinations. As regard tramadol combination only B′ significantly showed synergism. Data are expressed as the means ± S.E.M.

interaction we found that $ED20_{comb}$ was statistically different from $ED20_{add}$ for combination A, suggesting a synergistic interaction in the first phase as well for this combination. On the contrary, $ED20_{comb}$ did not significantly differ from $ED20_{add}$ for combinations B and C, thus suggesting that the observed effect was due solely to morphine (no synergism was detected). Likewise, tested doses of tramadol/flupirtine combinations did not show a synergistic interaction. Detailed results of isobolographic analysis in the first phase are summarized in Table 1.

4. Discussion

The rat orofacial formalin test is a suitable animal model to investigate acute inflammatory nociception in the trigeminal region (Raboisson and Dallel, 2004). Diluted formalin solutions, injected subcutaneously into the rat upper lip, elicit a stereotyped nocifensive behavior (face rubbing) consisting of two distinct phases: a short-lasting response referred to as first phase and a tonic, longer-lasting phase, named second phase. These two phases are related to different underlying pathophysiological mechanisms. The first phase is thought to be related to a direct stimulation of nociceptors, whereas the second phase is believed to be associated to a combination of ongoing

Table 1
Results of isobolographic analysis for the first phase of rat orofacial formalin test.

First phase			
Combination[a]	$ED20_{comb}$[b] (mean ± S.E.M.)	$ED20_{add}$[c] (mean ± S.E.M.)	$F_{0.5}$-statistics
Morphine			
w/o flupirtine	0.528 ± 0.115	–	–
A: 50%	0.552 ± 0.176*	1.10 ± 0.23	F(calc) : 12.676 F(tab) : 3.44 (P<0.05)
B:16.1%	2.805 ± 0.374	3.240 ± 0.70	F(calc) : 2.25 F(tab) : 3.44 (P>0.05)
C:3.23%	17.49 ± 6.13	16.50 ± 3.60	F(calc) : 3.798 F(tab) : 3.44 (P>0.05)
Tramadol			
w/o flupirtine	2.19 ± 0.49	–	–
A: 83.5%	2.47 ± 1.11	2.63 ± 0.58	F(calc) : 2.522 F(tab) : 3.340 (P>0.05)
B: 61.35%	3.171 ± 0.46	3.573 ± 0.80	F(calc) : 0.167 F(tab) : 3.34 (P>0.05)
C: 18.16%	32.40 ± 5.70[c]	27.40 ± 7.70[c]	F(calc) : 3.56 F(tab) : 3.34 (P>0.05)

[a] It is indicated the proportion (ρ) expressed as percentage of morphine or tramadol in the combination.
[b] ED20 value obtained experimentally.
[c] ED20 value theoretically calculated on the basis of proportion of morphine in the combination as follow: $ED50_{morphine}/\rho_{morphine}$.

inflammatory inputs from peripheral sites and central sensitization at brainstem level (Dallel et al., 1995). In this experimental paradigm, we tested flupirtine, morphine, and tramadol, administered alone or in combination to assess antinociceptive activity and putative synergistic interaction. The major findings of our study were: 1) flupirtine, morphine and tramadol, when administered alone, exerted antinociception in the rat orofacial formalin test; morphine and tramadol were effective in the first as well as in the second phase of test, while flupirtine was effective in the second phase only; 2) considering the second phase of the test, morphine was more potent than flupirtine, and both morphine and tramadol exerted antinociception over an extended dose-range; 3) due to variable potency ratio displayed by the two opioid drugs compared with flupirtine, isobolographic analysis was based on a non linear interaction, leading to plot a curved line of additivity (isobole); 4) drugs given in combination exerted antinociception both in the first and in the second phase of the test. However, only two combination doses of morphine (containing 50% and 16.1% respectively) and one combination dose of tramadol (containing 61.35%) showed a synergistic interaction in the second phase; 5) although flupirtine was ineffective in the first phase, the combination dose containing 50% of morphine showed a synergistic interaction also in the first phase, while no synergistic interaction occurred in the first phase for all of the tramadol/flupirtine combination doses.

Flupirtine is a non-opioid analgesic acting at the level of KCNQ channels in the CNS. The hyperpolarizating M current which follows KCNQ channel opening by flupirtine inhibits the release of neurotransmitters such as glutamate from synaptic terminals, thereby reducing neuronal firing (Klawe and Maschke, 2009). Drugs modulating KCNQ channels may be useful in the treatment of several clinical conditions including pain syndromes. Flupirtine was released decades ago. However, its use so far has been limited to mild or moderate musculoskeletal pain, and no evidence is available of its efficacy in combination therapies. Indeed, clinical trials with flupirtine were designed to test comparison with other analgesic in mono-therapies (Moore et al., 1983; Heusinger, 1987; Mastronardi et al., 1988; Hummel et al., 1991; Pothmann and Lobisch, 2000; Li et al., 2008). In such studies, flupirtine is reported to induce equivalent or slightly superior analgesia when compared with paracetamol, diclofenac or opioid such as dihydrocodeine, pentazocine or tramadol. However, the safety profile of flupirtine, including dizziness, somnolence and rare cognitive impairment, limits its use in pain states (Herrmann et al., 1993). On the other hand, the novel evidence concerning its action on M current led to reconsider flupirtine as an useful drug in pain therapy. Recently, flupirtine was shown to display a synergistic interaction with morphine in a model of diabetic neuropathic pain and in a model of inflammatory hyperalgesia induced by carrageenan injection (Goodchild et al., 2008a, b). Furthermore, Goodchild et al. (2008b) reported the efficacy of flupirtine given in combination with opioids in a case series of cancer patients with neuropathic pain.

To the best of our knowledge, this is the first report of antinociception induced by flupirtine in an acute model of inflammatory pain. Flupirtine showed some differences compared with other analgesics, including opioids and non-opioid drugs. Indeed, the degree of antinociception was week if compared with that induced by morphine or tramadol. Moreover, flupirtine was effective only in the second phase of formalin test. The latter is thought to be related, at least in part, to central sensitization phenomena, and glutamatergic transmission plays an important role in such mechanisms (Latremoliere and Woolf, 2009). Flupirtine, by enhancing M current, might counteract the glutamatergic overflow during the second phase of the test.

Besides, a straightforward rationale for association of flupirtine and opioids does exist; the antinociceptive mechanisms of opioids involve several cellular and sub cellular targets in the sensory neuron. In particular, many studies have suggested that K^+ channels play an important role in antinociception induced by morphine at supraspinal, spinal and peripheral (primary afferent nerve ending) level (Ocaña et al.,

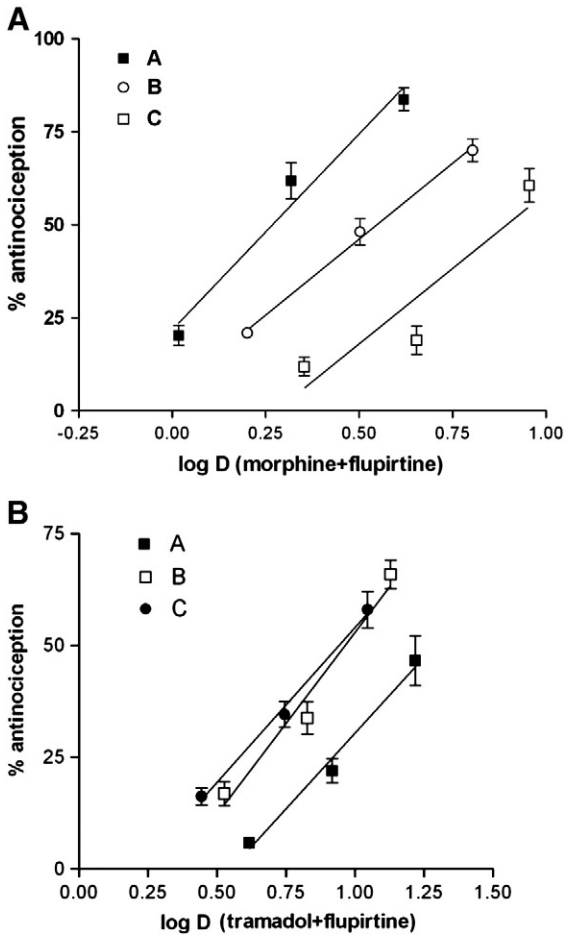

Fig. 8. (Log)dose–response curve of combination dose pairs of morphine+flupirtine (A) and tramadol + flupirtine (B) in the second phase of orofacial formalin test. Data were analyzed by linear regression and experimental ED50 values were obtained. Data are expressed as the means ± S.E.M. of n = 4–6 animals for each experimental group.

2004). The regulation of potassium current channels by morphine could be due to its ability to activate Gi/o proteins, as it has been clearly demonstrated for K_{ATP}, K_{ir} (Sanchez et al., 1998; Wada et al., 2000; Marker et al., 2004) and also, in few studies, for K_v channels (Vaughan and Christie, 1997; Vaughan et al., 1997). Thus, enhancing K^+ channels currents via flupirtine given in association with an opioid drug could result in a synergistic interaction of the overall antinociceptive effect.

In this study we tested tramadol in association with flupirtine. Tramadol acts both as a μ-opioid receptor agonist and a monoaminergic reuptake modulator; it is successfully used in the treatment of acute pain (Dayer et al., 1997). Although tramadol displays a more favorable safety profile compared to morphine, important toxic effects are reported, especially in the elderly. Formulations containing a fixed combination of tramadol and a non steroidal anti-inflammatory drug are commercially available (Dhillon, 2010)

Due to the different profiles of efficacy and potency, isobolographic analysis was adopted to study whether flupirtine–morphine association fit the case of an interaction between a full (morphine) and partial (flupirtine) agonist; in this case the isobole of additivity is not linear (Grabovsky and Tallarida, 2004). Moreover in the first phase, where flupirtine was ineffective, the interaction between the two drugs was considered to fit the case of an association between an 'active' (morphine or tramadol) and an 'inactive' (flupirtine) drug.

We found a synergistic interaction between flupirtine on the one hand and morphine or tramadol on the other hand. This supra-additive effect depended on morphine or tramadol. Indeed, only two morphine/flupirtine combination ratios were synergistic and the measure of this synergism was greater for the combination containing a higher proportion of morphine (γ for combination A$<\gamma$ for combination B). On the other hand, the tramadol/flupirtine combination containing the highest proportion of tramadol resulted in an additive-sub additive effect, while the combination containing 61.35% of tramadol resulted in a synergistic interaction, thus indicating that the mechanisms underlying the interaction between tramadol and flupirtine are different from those underlying the interaction between tramadol and morphine. Consistent with this notion, one morphine/flupirtine combination resulted in a synergistic interaction also in the first phase of the test, whereas no such interaction was observed at any of the flupirtine–tramadol combinations tested.

In conclusion, here we showed that the rat orofacial formalin test is a suitable model to investigate interactions between analgesic drugs belonging to different pharmacological classes; our data demonstrate that the combinations of flupirtine and morphine, and flupirtine and tramadol, result in a synergistic antinociceptive activity in vivo in the rat. On the basis in this clear pre-clinical evidence, it might be worth testing the effectiveness of flupirtine-containing combination therapies in the clinical setting.

References

Benyamin R, Trescot AM, Datta S, Buenaventura R, Adlaka R, Sehgal N, et al. Opioid complications and side effects. Pain Physician 2008;11:S105–20.

Brown DA, Passmore GM. Neural KCNQ (Kv7) channels. Br J Pharmacol 2009;156:1185–95.

Capuano A, De Corato A, Treglia M, Tringali G, Dello Russo C, Navarra P. Antinociceptive activity of buprenorphine and lumiracoxib in the rat orofacial formalin test: a combination analysis study. Eur J Pharmacol 2009;605:57–62.

Dallel R, Raboisson P, Clavelou P, Saade M, Woda A. Evidence for a peripheral origin of the tonic nociceptive response to subcutaneous formalin. Pain 1995;61:11–6.

Dayer P, Desmeules J, Collart L. Pharmacology of tramadol. Drugs 1997;53:18–24.

Déciga-Campos M, López UG, Reval MI, López-Muñoz FJ. Enhancement of antinociception by co-administration of an opioid drug (morphine) and a preferential cyclooxygenase-2 inhibitor (rofecoxib) in rats. Eur J Pharmacol 2003;460:99-107.

Dhillon S. Tramadol/paracetamol fixed-dose combination: a review of its use in the management of moderate to severe pain. Clin Drug Investig 2010;30:711–38.

Flemming K. The use of morphine to treat cancer-related pain: a synthesis of quantitative and qualitative research. J Pain Symptom Manage 2010;39:139–54.

Friedel HA, Fitton A. Flupirtine. A review of its pharmacological properties, and therapeutic efficacy in pain states. Drugs 1993;45:548–69.

Geppetti P, Benemei S. Pain treatment with opioids: achieving the minimal effective and the minimal interacting dose. Clin Drug Investig 2009;29:3-16.

Goodchild CS, Kolosov A, Tucker AP, Cooke I. Combination therapy with flupirtine and opioid: studies in rat pain models. Pain Med 2008a;9:928–38.

Goodchild CS, Nelson J, Cooke I, Ashby M, Jackson K. Combination therapy with flupirtine and opioid: open-label case series in the treatment of neuropathic pain associated with cancer. Pain Med 2008b;9:939–49.

Grabovsky Y, Tallarida RJ. Isobolographic analysis for combinations of a full and partial agonist: curved isoboles. J Pharmacol Exp Ther 2004;310:981–6.

Gribkoff VK. The therapeutic potential of neuronal KCNQ channel modulators. Expert Opin Ther Targets 2003;7:737–48.

Herrmann WM, Hiersemenzel R, Aigner M, Lobisch M, Riethmüller-Winzen H, Michel I. Long-term tolerance of flupirtine. Open multicenter study over one year. Fortschr Med 1993;111:266–70.

Heusinger JH. Efficacy and tolerance of flupirtine and pentazocine in two multicentre trials. Postgrad Med J 1987;633:71–9.

Houlihan DJ. Serotonin syndrome resulting from coadministration of tramadol, venlafaxine, and mirtazapine. Ann Pharmacother 2004;38:411–3.

Hummel T, Friedmann T, Pauli E, Niebch G, Borbe HO, Kobal G. Dose-related analgesic effects of flupirtine. Br J Clin Pharmacol. 1991;32:69–76.

Klawe C, Maschke M. Flupirtine: Pharmacology and clinical applications of a nonopioid analgesic and potentially neuroprotective compound. Expert Opin Pharmacother 2009;10:1495–500.

Kornhuber J, Bleich S, Wiltfang J, Maler M, Parsons CG. Flupirtine shows functional NMDA receptor antagonism by enhancing Mg2+ block via activation of voltage independent potassium channels. Rapid communication. J Neural Transm 1999;106:857–67.

Latremoliere A, Woolf CJ. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. J Pain 2009;10:895–926.

Li C, Ni J, Wang Z, Li M, Gasparic M, Terhaag B, et al. Analgesic efficacy and tolerability of flupirtine vs. tramadol in patients with subacute low back pain: a double-blind multicentre trial. Curr Med Res Opin 2008;24:3523–30.

Manchikanti L, Benyamin R, Datta S, Vallejo R, Smith H. Opioids in chronic noncancer pain. Expert Rev Neurother 2010;10:775–89.

Marker CL, Stoffel M, Wickman K. Spinal G-protein-gated K+ channels formed by GIRK1 and GIRK2 subunits modulate thermal nociception and contribute to morphine analgesia. J Neurosci 2004;24:2806–12.

Mastronardi P, D'Onofrio M, Scanni E, Pinto M, Frontespezi S, Ceccarelli MG, et al. Analgesic activity of flupirtine maleate: a controlled double-blind study with diclofenac sodium in orthopaedics. J Int Med Res 1988;16:338–48.

Miceli F, Soldovieri MV, Martire M, Taglialatela M. Molecular pharmacology and therapeutic potential of neuronal Kv7-modulating drugs. Curr Opin Pharmacol 2008;8:65–74.

Miranda HF, Prieto JC, Pinardi G. Spinal synergy between nonselective cyclooxygenase inhibitors and morphine antinociception in mice. Brain Res 2005;1049:165–70.

Moore RA, Bullingham RE, Simpson S, O'Sullivan G, Evans PJ, McQuay HJ, et al. Comparison of flupirtine maleate and dihydrocodeine in patients following surgery. Br J Anaesth 1983;55:429–32.

Munro G. Pharmacological assessment of the rat formalin test utilizing the clinically used analgesic drugs gabapentin, lamotrigine, morphine, duloxetine, tramadol and ibuprofen: Influence of low and high formalin concentrations. Eur J Pharmacol 2009;605:95-102.

Ocaña M, Cendán CM, Cobos EJ, Entrena JM, Baeyens JM. Potassium channels and pain: present realities and future opportunities. Eur J Pharmacol 2004;500:203–19.

Pothmann R, Lobisch M. Acute treatment of episodic childhood tension-type headache with flupirtine and paracetamol — a double-blind crossover-study. Schmerz 2000;14:1–4.

Raboisson P, Dallel R. The orofacial formalin test. Neurosci Biobehav Rev 2004;28:219–26.

Raffa RB. Basic pharmacology relevant to drug abuse assessment: tramadol as example. J Clin Pharm Ther 2008;33:101–8.

Rojas-Corrales MO, Berrocoso E, Mico JA. Role of 5-HT1A and 5-HT1B receptors in the antinociceptive effect of tramadol. Eur J Pharmacol 2005;511:21–6.

Sanchez JA, Gonoi T, Inagaki N, Katada T, Seino S. Modulation of reconstituted ATP-sensitive K+ channels by GTP-binding proteins in a mammalian cell line. J Physiol 1998;507:315–24.

Schwarz M, Block F, Pergande G. N-methyl-D-aspartate (NMDA)-mediated muscle relaxant action of flupirtine in rats. NeuroReport 1994;5(15):1981–4 Oct 3.

Scott LJ, Perry CM. Tramadol: a review of its use in perioperative pain. Drugs 2000;60:139–76.

Smith HS. Combination opioid analgesics. Pain Physician 2008;11:201–14.

Solbrig MV, Adrian R, Wechsler SL, Koob GF. Activators of potassium M currents have anticonvulsant actions in two rat models of encephalitis. Eur J Pharmacol 2007;555:23–9.

Tallarida RJ. Different experimental design. In: Tallarida RJ, editor. Drug synergism and dose–effect data analysis. Chapman&Hall/CRC; 2000. p. 143–4.

Tallarida RJ. Drug synergism: its detection and applications. J Pharmacol Exp Ther 2001;298: 865–72.

Tallarida RJ. An overview of drug combination analysis with isobolograms. J Pharmacol Exp Ther 2006;319:1–7.

Vaughan CW, Christie MJ. Presynaptic inhibitory action of opioids on synaptic transmission in the rat periaqueductal grey in vitro. J Physiol 1997;498:463–72.

Vaughan CW, Ingram SL, Connor MA, Christie MJ. How opioids inhibit GABA-mediated neurotransmission. Nature 1997;390:611–4.

Wada Y, Yamashita T, Imai K, Miura R, Takao K, Nishi M, et al. A region of the sulfonylurea receptor critical for a modulation of ATP-sensitive K+ channels by G-protein hg-subunits. EMBO J 2000;19:4915–25.

Wua YJ, Dworetzky SI. Recent developments on KCNQ potassium channel openers. Curr Med Chem 2005;12:453–60.

Zelcer S, Kolesnikov Y, Kovalyshyn I, Pasternak DA, Pasternak GW. Selective potentiation of opioid analgesia by nonsteroidal anti-inflammatory drugs. Brain Res 2005;1040:151–6.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.