Antinociceptive activity of buprenorphine and lumiracoxib in the rat orofacial formalin test: A combination analysis study

Alessandro Capuano, Alice De Corato, Mariangela Treglia, Giuseppe Tringali, Cinzia Dello Russo, Pierluigi Navarra *

Institute of Pharmacology, Catholic University School of Medicine, largo F. Vito 1, 00168 Rome, Italy

ARTICLE INFO

Article history:
Received 22 September 2008
Received in revised form 5 December 2008
Accepted 18 December 2008
Available online 10 January 2009

Keywords:
Rat orofacial test
Formalin
Buprenorphine
Lumiracoxib
Isobolographic analysis

ABSTRACT

Combination of two or more analgesics is widely used for the treatment of moderate and severe pain syndromes, allowing usage of lower doses of each compound and thereby limiting side effects; there is currently a large interest in investigating the potential advantages of combinations between opioids and non-steroidal inflammatory drugs (NSAIDs), coxibs in particular. The rat orofacial formalin test is a useful pre-clinical model of inflammatory trigeminal pain for evaluating antinociceptive activity of analgesics and their combinations. Injection of formalin in the rat wiskerpad induces a stereotyped response (rubbing), consisting of two distinct phases: a first ‘phasic’ phase and a second ‘tonic’ phase. In this work we tested a partial agonist to μ-opioid receptors, buprenorphine, and a selective cyclo-oxygenase-2 inhibitor, lumiracoxib, each of which given i.p. either alone or in combination. Buprenorphine reduced nociception both in the first and in the second phase, whereas lumiracoxib induced antinociception in the second phase only. The interaction between the two drugs was assessed through isobolographic analysis after combined administration at a fixed dose ratio. Such combination produced a dose-dependent antinociceptive effect in both phases. We observed a statistical difference between the theoretical and the experimental ED50, which indicated synergistic interaction in the second phase. Concerning the first phase, we assumed that the antinociceptive effects were almost completely to be attributed to buprenorphine, since lumiracoxib was ineffective when administered alone. However, we found an unexpected difference between the theoretical and experimental ED50, suggesting synergism in the first phase as well.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The rat orofacial formalin test is a well-established pre-clinical model to investigate the efficacy of analgesic compounds in pain of the facial district (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 to two at least partially distinct mechanisms of nociception; the first phase is associated to direct stimulation of C-nociceptors, whereas the second phase reflects integration between peripheral (nociceptors) and central (spinal/brainstem) signaling (Dallel et al., 1995).

In this study, we used the orofacial formalin test to investigate the additive antinociceptive effect of the association buprenorphine-lumiracoxib. “Combination analgesia” based on drugs with different antinociceptive mechanisms affords “synergistic” interaction; using lower doses of each drug, it allows to reduce overall toxicity (Tallarida, 2001). Opiates and non steroidal inflammatory drugs (NSAIDs) act on different components of pain, and their useful combination has been extensively demonstrated in different pre-clinical and clinical studies (Fletcher et al., 1997; Elia et al., 2005; Marret et al., 2005; Dunbar et al., 2007). Buprenorphine is a synthetic opiate acting as a partial agonist to mu-opioid receptor. After its introduction, buprenorphine has been widely used in the treatment of different pain syndromes (Cowan, 2003). By the time its clinical use became less popular because of its ceiling effect, probably due to k-opioid receptor antagonism (Tejwani and Rattan, 2002) and, at least in part, to systemic activation of supra spinal nociceptin/orphanin peptide (NOP) receptors (Lutfy et al., 2003). However, buprenorphine has recently received renewed attention in the clinical practice, because of its effectiveness in the management of opiate addiction and its good profile of tolerability and safety, in particular as regards the development of tolerance (Heit and Gourlay, 2008). Furthermore, the drug showed anti-hyperalgesic effects in human models of pain (Koppert et al., 2005).

Lumiracoxib is a selective COX-2 inhibitor that was originally developed for the treatment of chronic inflammatory pain. Lumiracoxib displays a distinct pharmacological profile compared to other COX-2 inhibitors. In fact, due to its carboxylic group, lumiracoxib possesses weak acidic properties that favor concentration of the drug...
2.2. Animals

intraperitoneally.

sterile saline,

cial source. Each tablet, containing 200 mg of drug, was dissolved in

Lumiracoxib was provided by Novartis (Italy) as tablets of commer-

as hydrochloride salt, was freshly dissolved in sterile saline.

2.1. Drugs

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

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 the Guidelines on Ethical Standards for

Investigation of Experimental Pain in Animals (Zimmermann, 1983).

Additionally, the study protocol was approved by 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 (authorization to P. Navarra).

2.2. Animals

Male Wistar rats aged 6–7 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 the Guidelines on Ethical Standards for

Investigation of Experimental Pain in Animals (Zimmermann, 1983).

Additionally, the study protocol was approved by 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 (authorization to P. Navarra).

2.3. Orofacial formalin test

Orofacial formalin test was performed according to the methods

described by Raboisson and Dallel (2004). Fifty μL of 1.5% diluted

formalin solution were injected into the right side of upper lip

subcutaneously just lateral to the nose using a 100 μL Hamilton

syringe. After injection, the rat was put in an observation cage at

consistent in a glass chamber (30×30×30) with mirrored sides and

its behavior was video-recorded for 45 min. Videos were analyzed

using JWatcher software (developed at Animal Behavior Laboratory,

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 for each 3-min block. Formalin

injection induced a stereotyped response characterized by two well

distinct phases; phase I started almost immediately and was short-

lasting (0–9 min) followed, after a quiescent period, by phase II

lasting 20–40 min. All test drugs, or normal saline for the control

group, were administered intraperitoneally (i.p.) 30 min before

formalin injection.

Each experimental session included a group of control animals,

and the results with study drugs were expressed in relationship to the

respective controls for each experimental session, in order to reduce

to a minimum confounding bias due to environmental variability or

animal handling.

2.4. Antinociception measurement and data analysis

Time-courses of nociceptive response for each drug and combina-
tion were constructed as mean number of seconds that rats spent

rubbing, plotted for each 3-min block over 45 min post-injection

observation period. For the two phases of formalin test, the areas

under the curve (AUC) were calculated by trapezoidal rule: for the

first phase, the first 3 blocks of 3 min, for 9 min total, were considered.

The following 2 block of 3 min were not considered for calculation

(quiessent 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 (Jiménez-Andrade et al., 2003):

Percentage of antinociception = \left(\frac{\text{AUC saline} - \text{AUC drug treatment}}{\text{AUC saline}}\right) × 100.

Dose–response curves for each phase were constructed for

buprenorphine and lumiracoxib using at least six animals for each
dose. Four doses of each drug were tested. A linear regression analysis

of the log dose–response curve allowed calculating the doses that

produced 50% of antinociception (ED50) when each drug was

administered alone.
An isobolographic analysis was performed to characterize drug interaction according to the method originally described by Tallarida (2001). As expected, lumiracoxib did not reach a valuable percentage of antinociception in the first phase, thus it was considered as an “inactive” drug for this phase. In the second phase, both drugs achieved comparable levels of antinociception so that ED50 values were used to obtain a theoretical dose–response curve of a fixed-ratio combination of buprenorphine and lumiracoxib (Tallarida, 2001). From the theoretical dose–response curve we calculated a theoretical ED50, otherwise called “additive” (add). Subsequently, an experimental dose response curve was obtained treating animals with one of the following combination doses: ED50add, ED50add/2, ED50add/4, ED50/8 in a fixed-ratio of buprenorphine and lumiracoxib. Ratio (0.4/0.6 buprenorphine/lumiracoxib) was calculated on the basis of variances of individual ED50 values according to the following equation (Tallarida, 2000a):

\[ f = \frac{V_B}{VA + VB} \]

where \( f \) is the fraction, \( V \) is the variance, \( A \) and \( B \) the less and more potent drug respectively. This procedure allowed us to obtain a variance of ED50add as small as possible, facilitating statistical analysis. Thus, an ED50 for combination treatment (ED50comb) was obtained from the experimental dose–response curve. Theoretical and experimental ED50 values were then tested for statistical differences (Tallarida, 2001). Moreover, the interaction index (\( \gamma \)) was calculated as follows: \( \gamma = \frac{ED_{50\text{comb}}}{ED_{50\text{add}}} \); an interaction index not significantly different from the unit corresponds to an additive interaction, whereas higher or lower values indicates sub-additivity or synergism, respectively.

To assess whether an interaction between the two drugs occurred also in the first phase, we considered this phase as the peculiar condition where a combination of an active (buprenorphine) and an inactive drug (lumiracoxib) takes place (Tallarida, 2000b). In this paradigm, assuming that only the active drug contributes to the selected level of effect (e.g., 50% of antinociception), we calculated the ED50add of the first phase. Synergy requires that ED50comb < ED50add, and therefore the difference between these values was tested by the Student’s t-test for statistical difference.

2.5. Statistical analysis

Dose response data were analyzed by one-way analysis of variance (ANOVA) with Newman–Keuls post hoc test. Statistical significance between the theoretical additive ED50 and the experimentally derived one was evaluated using Student’s t test. Statistical procedures were performed using Pharm Tools Pro (version 1.1.20, The McCary Group Inc.) and Prism™ (GraphPad, San Diego, CA, USA). \( P \) values lower than 0.05 (\( P < 0.05 \)) were considered significant.
3. Results

3.1. Nociceptive behavior

Subcutaneous injection of diluted formalin into the rat wiskerpad produced a typical pattern of face rubbing behavior. Nociceptive response is characterized by a biphasic time course (Fig. 1A and B, white squares): phase 1 began immediately after formalin administration and declined gradually in approximately 10 min, while phase 2 began about 15 min after formalin administration and lasted about 30 min. In preliminary experiments to assess orofacial formalin test in our laboratory we found that saline injection in the rat wiskerpad did not elicit any nociceptive response (data not shown).

3.2. Effects of buprenorphine and lumiracoxib on orofacial formalin test

Intraperitoneal administration of buprenorphine produced a dose-dependent reduction in face rubbing behavior (Fig. 1A). Fig. 2 shows that all tested doses of buprenorphine increased antinociception both in the first and second phase in a statistically significant manner compared to controls. Lumiracoxib was able to reduce nociceptive behavior only in the second phase; indeed, lumiracoxib reduced in a dose-dependent manner the time spent from rats in face rubbing (Fig. 1B) and increased the percentage of antinociception in the second phase of formalin test (Fig. 3B), whereas in the first phase it was ineffective (Fig. 3A).

![Antinociceptive effects of fixed ratio buprenorphine/lumiracoxib combination doses 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, respectively.](Image)

**Fig. 4.** Antinociceptive effects of fixed ratio buprenorphine/lumiracoxib combination doses 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, respectively.

3.3. Interaction analysis

Due to the different profile of action of buprenorphine and lumiracoxib, interaction parameters were calculated on the basis of the antinociceptive effects exerted by the two drugs in the second phase. Log–dose response curves of both compounds were linear, and the test for parallelism showed that indeed the two regression lines were significantly parallel (t-test: calculated T 2.0259 vs tabular T 2.0360).

Thus, a composite additive curve was constructed. Additive regression allowed us to calculate theoretical ED$_{50}$ for a fixed-ratio combination of buprenorphine and lumiracoxib (ED$_{50}$add=0.353±0.024).

Experimental data on the effect of combination doses are showed in Fig. 4. We observed a dose-dependent increase in antinociceptive activity both in the first (panel A) and in the second phase (panel B) of orofacial formalin test. Isobolographic analysis of buprenorphine and lumiracoxib combination effect in the second phase of nociceptive test showed that the interaction between the two drugs was synergistic (Fig. 5). In fact, the ED$_{50}$comb was lower than the ED$_{50}$add (0.146±0.009 vs 0.353±0.024, P<0.001). The interaction index (γ) calculated as described above (see Methods) was 0.41±0.012.

Concerning the first phase (where lumiracoxib resulted ineffective when administered alone), we postulated that the observed effects were almost completely to be attributed to buprenorphine. The resulting theoretical ED$_{50}$ value was 0.358±0.049, whereas experimental ED$_{50}$ was 0.131±0.007. The difference between theoretical and experimental ED$_{50}$ was statistically significant (t-test, P<0.001), suggesting synergistic interaction in the first phase as well (γ=0.36±0.021).

![Isobologram showing the interaction between buprenorphine and lumiracoxib. The slope from x- to y-axis represents the theoretical additive line. The point on the line indicated as ED$_{50}$add is the theoretical additive ED$_{50}$ calculated from individual drug ED$_{50}$ values. ED$_{50}$comb is the observed experimental value of the combination. Positioning under the theoretical additive line indicates synergistic interaction. Horizontal and vertical bars represent the relevant S.E.M.](Image)

**Fig. 5.** A: Isobologram showing the interaction between buprenorphine and lumiracoxib. The slope from x- to y-axis represents the theoretical additive line. The point on the line indicated as ED$_{50}$add is the theoretical additive ED$_{50}$ calculated from individual drug ED$_{50}$ values. ED$_{50}$comb is the observed experimental value of the combination. Positioning under the theoretical additive line indicates synergistic interaction. Horizontal and vertical bars represent the relevant S.E.M.

4. Discussion

The orofacial formalin test represents a useful animal model of acute inflammatory nociception in the trigeminal region. Diluted formaldehyde, injected subcutaneously into the rat upper lip, produces a nocifensive behavior (face rubbing) consisting in a biphasic response: a short-lasting response referred to as phase I, and a longer-lasting phase, caused by inflammatory processes, called phase II (Raboisson and Dallel, 2004). The biphasic component of formalin-induced nociception reflects different underlying mechanisms; the first phase appears to be related to the direct chemical stimulation of nociceptive nerve endings (Dallel et al., 1995), while the second phase depends on a combination of ongoing inputs from nociceptive afferents and, at least in part, of central sensitization. In this work, we used the orofacial formalin test to study putative additive interaction between buprenorphine and lumiracoxib in generating antinociception. The major findings of our study are: 1) buprenorphine and lumiracoxib were able to reduce nociception when
administered alone by i.p. injection; 2) the two drugs, given in a fixed ratio combination, show a synergistic interaction in the second phase of the test; 3) although lumiracoxib given alone was ineffective in the first phase, it was found to potentiate in a synergistic manner the effects of buprenorphine when the two drug were given in a fixed ratio combination ($\gamma = 0.36$).

In order to achieve a better level of analgesia, while on the same time reducing limiting side effects, a well established therapeutic strategy is represented by the combination of an opioid with another agent (that may be or not an analgesic compound) (Curatolo and Sveticic, 2002). Most of these combinations consist of NSAIDs (Marret et al., 2005); more recently, selective COX-2 inhibitors (coxibs) raised a large interest in this field (Reuben, 2007).

Opioids are widely used as analgesics in acute and chronic treatment of pain syndromes, and buprenorphine is widely used in clinical practice (Heit and Gourlay, 2008). As expected for opioids (Duale et al., 1996; Raboisson and Dallel, 2004), buprenorphine was able to induce antinociception in both phases of the formalin test. In vitro and in vivo evidence shows that buprenorphine possesses a complex pharmacological profile. It is a partial agonist to mu-opioid receptors and a full agonist to NOP receptors. Such dual component could explain some features of clinically observed effects. In particular, buprenorphine shows the so-called ceiling effect (Walsh et al., 1994), which is dependent on supra spinal activation of NOP receptor and do limit its own analgesic effects (Yamamoto et al., 2006). Combination of buprenorphine with other analgesics might be an useful approach to overcome such limitation.

Several studies on in vivo animal models, as well as important clinical trials, have confirmed the anti-inflammatory and anti-hyperalgesic activity of coxibs (Reuben, 2007). Their proven efficacy in acute pain conditions is currently limited by important side effects of long-term therapy. Lumiracoxib is a selective inhibitor of COX-2 with potent anti-inflammatory and anti-hyperalgesic activities (Mysler, 2004). It differs from other coxibs not only for its favorable pharmacokinetic profile (Bannwarth and Berenbaum, 2005), but also for certain pharmacodynamic features (Lozano-Cuenca et al., 2005).

In this study, lumiracoxib given alone was able to increase antinociception only in the second phase of the formalin test. Although our findings agree with current knowledge about opioids and NSAIDs in animal models of inflammatory nociception (Fletcher et al., 1997; Déciga-Campos et al., 2003; Zelcer et al., 2005), some issues need to be addressed here. In our study, apart from the efficacy of buprenorphine and lumiracoxib in reducing nociception when given alone, the isobolographic analysis of a fixed ratio combination demonstrates the occurrence of synergistic interaction. To our knowledge, this is the first report of a super-additive analgesic effect of buprenorphine and lumiracoxib after systemic delivery. Such synergism suggests that it should be possible to reduce the individual doses, and the interaction index ($\gamma$) is a measure of the potency of this combination (Tallarida, 2001). In our model, the $\gamma$ value is in the same order of magnitude that found in a previous study with another atypical opioid, nalbuphine, in combination with lumiracoxib (Ortiz and Castañeda-Hernández, 2008).

The role of COX-2 in mediating nociception in the formalin test remain controversial. Apart from the inhibition of prostaglandin synthesis, additional mechanisms of action have been suggested for these agents. Indeed, it has reported that celecoxib or rofecoxib reduce the second phase of formalin test but the onset of antinociception observed in these studies is faster than the time required for up-regulation of COX-2 mRNA expression (Torres-Lopez et al., 2002; Miranda et al., 2006). Additional (or alternative) mechanism of antinociceptive action have been postulated for classical NSAIDs as well. In fact, there is evidence that NSAIDs are able to activate the nitric oxide (NO)-cyclicGMP pathway, the serotoninergic system and the opioid system (Ortiz et al., 2005; Miranda et al., 2003). Moreover, the efficacy of these drugs appears to be strictly dependent on the route of administration, since it is less evident after systemic administration compared to local infiltration (Yamamoto and Nozaki-Taguchi, 2002).

The above limitation does not seem to apply to lumiracoxib, which was found to be effective after systemic administration by us and by other groups (Lozano-Cuenca et al., 2005). Recent findings on the efficacy of lumiracoxib in the hind paw formalin test (Lozano-Cuenca et al., 2005; Ortiz and Castañeda-Hernández, 2008) pointed out that antinociception associated to both peripheral and systemic (i.t.) lumiracoxib administration may be mediated by mechanisms different from those involving COX-2 pathways. Lozano-Cuenca et al. (2005) demonstrated that lumiracoxib is able to inhibit COX-2 at spinal level and to activate the nitric (NO)-cyclicGMP-K+ pathway, as well as to enhance the serotonergic drive. However, the relative contribution of the above mentioned components in mediating nociception in the trigeminal region might be different compared to that observed in the hind paw model (Chichorro et al., 2004).

Finally, one can speculate that mechanism(s) of action alternative to COX-2 inhibition may underlie the observed potentiation of buprenorphine effects brought about by lumiracoxib in the first phase of the test. 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 clearly show that the combination of buprenorphine and lumiracoxib results in synergistic antinociceptive activity.

Acknowledgments

We wish to thank Mr. R. Cricchi for his technical assistance. This work was supported in part by Grünenthal-Formenti.

References


