RESEARCH PAPER

Modulation of imidazoline I₂ binding sites by CR4056 relieves postoperative hyperalgesia in male and female rats

Marco Lanza, Flora Ferrari, Ilaria Menghetti, Dario Tremolada and Gianfranco Caselli

Department of Pharmacology & Toxicology, Rottapharm Biotech S.r.l., Monza, MB, Italy

BACKGROUND AND PURPOSE
CR4056 is a novel imidazoline-2 (I₂) ligand exhibiting potent analgesic activity in animal models of pain. In this study, we investigated the effects of CR4056 in a well-established model of postoperative pain where rats develop hyperalgesia in the injured hind paw.

EXPERIMENTAL APPROACH
By measuring paw withdrawal threshold to mechanical pressure, we studied the pharmacology of CR4056, potential sex differences in pain perception and response to treatment, and the pharmacodynamic interaction of CR4056 with morphine.

KEY RESULTS
Oral CR4056 and subcutaneous morphine dose-dependently reversed the hyperalgesic response. Analgesic effects of CR4056 were completely suppressed by the non-selective imidazoline I₂/α₂-adrenoceptor antagonist idazoxan, were partially reduced (∼30%; P < 0.05) by the selective α₂-adrenoceptor antagonist yohimbine, but were not influenced by the non-selective I₁/α₂-adrenoceptor antagonist efaroxan or by the μ opioid receptor antagonist naloxone. We found no differences in responses to CR4056 or morphine between male and female rats. However, females had a lower pain threshold than males, and needed lower doses of drugs to reach a significant analgesia. When CR4056 and morphine were combined, their median effective doses were lower than expected for additive effects, both in males and in females. Isobolographic analysis confirmed a synergism between CR4056 and morphine.

CONCLUSIONS AND IMPLICATIONS
CR4056 is a novel pharmacological agent under development for postoperative pain both as stand-alone treatment and in association with morphine. CR4056 has successfully completed Phase I studies for tolerability and pharmacokinetics in healthy volunteers, and is currently entering the first proof-of-concept study in patients.

Abbreviations
2-BFI, 2-(2-benzofuranyl)-2-imidazoline; 95% CI, 95% confidence interval; BU224, 2-(4, 5-dihydroimidazol-2-yl) quinolone; I₂, imidazoline-2; I.I., interaction index; RM, repeated measures
Introduction

Imidazoline-2 (I2) binding sites, also referred to as I2 receptors, are widely distributed in mammalian cells of the central and peripheral nervous system (Molderings, 1997; receptor nomenclature follows Alexander et al., 2013). At a cellular level, I2 receptor ligands have been mainly associated with an inhibitory modulation of MAO activity (Tesson et al., 1995; Ozaita et al., 1997). Remarkably, metabolism of catecholamines by sensory neurons contributes to peripheral neuropathies, suggesting that an MAO inhibitor, devoid of adverse effects in the CNS, could be useful for the treatment of chronic pain (Dina et al., 2008). Indeed, pharmacological manipulations that increase the synaptic levels of noradrenaline and 5-HT have gained prominence in the management of chronic pain, including neuropathic pain and fibromyalgia (Kuner, 2010). Another relevant activity of I2 receptors is the modulation of the opioid system, which may occur at different levels. First, recent evidence has shown how I2 ligands mimic the effect of opioid receptor agonists by increasing beta-endorphin secretion in the rat adrenal medulla (Hwang et al., 2005; Chang et al., 2010). Second, a role for I2 receptors in pain control stems from the interaction between imidazoline ligands and the opioid system in the locus ceruleus neurons (Ruiz-Durantez et al., 2003). This interaction is probably involved in the prevention of tolerance and addiction to opioids (Wu and Raja, 2011).

CR4056 is a novel I2 receptor ligand characterized by potent analgesic activity in different animal models of inflammatory and neuropathic pain (Ferrari et al., 2011; Thorn et al., 2012; Li et al., 2014). The efficacy of CR4056 was thoroughly investigated in a rat model of neuropathic pain that parallels the clinical condition of patients treated with the chemotherapeutic agent bortezomib (Meregalli et al., 2012).

In the present study, we investigated the effects of acute oral administration of CR4056 in a rat model of postoperative pain (Brennan et al., 1996). The pharmacology of the incisional pain in rats has been well characterized in a study by Whiteside et al. (2004). Their work shows how, from a pharmacological point of view, postoperative pain is clearly distinct from pure inflammatory or neuropathic pain. Indeed, when hyperalgesic or allodynic responses are measured 24 h after surgery, both inflammatory and neuropathic mechanisms contribute to the pain balance (Whiteside et al., 2004). This condition, from a translational point of view, is quite common in patients undergoing a major surgical event where, as expected, pain evoked by pressure is greater than pain experienced at rest.

In these patients, opioid analgesics still remain the treatment of choice during the intraoperative and postoperative period. Morphine is by far the most commonly used opioid, although its long-term use in chronic pain conditions is limited by a relevant spectrum of adverse effects, including constipation, dependence and tolerance (Anderson and Palmer, 2006; Oderda et al., 2007; Sadhasivam and Chidambaran, 2012). Because multimodal analgesia has been shown to be an effective strategy to improve postoperative pain management (Buvanendran and Kroin, 2009), we included in this study a protocol of co-administration of morphine and CR4056. If different drugs contribute to pain control through different mechanisms, the optimal analgesia could be reached at lower doses of each drug, thus decreasing the chance for adverse effects to occur. Further work in our study was devoted to analysing potential sex differences in the perception of pain and response to treatment in this animal model. This is actually a matter of debate because relevant discrepancies do exist among animal as well as human studies (Craft, 2003; Kroin et al., 2003; Aubrun et al., 2005; Dahan et al., 2008; Fillingim et al., 2009).

Methods

Animal subjects

All animal care and experimental procedures described were in compliance with international laws and policies (Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996), and were approved by the Rottapharm Review Board. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny et al., 2010; McGrath et al., 2010). A total of N = 336 animals were used in the experiments described here. Male and female Sprague-Dawley rats (Charles River, Calco, Italy) weighing 250–300 g were housed with ad libitum access to food and water, in a temperature-controlled room with a 12 h light/dark cycle, at least 1 week before the surgical procedure.

Brennan’s model of postoperative pain

Rats were anaesthetized with 2% isoflurane in pure oxygen inside an induction chamber. Once unconscious, rats were removed and placed on a non-rebreathing anaesthetic circuit with mask delivery of isoflurane in pure oxygen throughout the procedure. Paw incision was performed as described by Brennan et al. (1996), with minor modifications. A 1 cm longitudinal incision was made through skin and fascia of the plantar face of the right hind paw, starting at 0.5 cm from the proximal edge of the heel. The plantar muscle was then elevated with forceps and incised longitudinally with the blade, keeping attention to leave muscle origins intact. A group of un-operated sham rats was always present in each experiment. Paw withdrawal threshold (i.e. the pain threshold) to mechanical pressure was determined by the Randall-Selitto method (Analgesy-Meter, Ugo Basile, Comerio, VA, Italy) 24 h after surgery. According to the weight of the rats enrolled in the study, we set the Randall-Selitto device on level 2 (range: 0–750 g). CR4056 was suspended in 0.5% methyl cellulose (MC) and administered orally. Mechanical hyperalgesia was measured 30, 90, 180 and 360 min after CR4056 administration. Lidoxan, efaroxan, yohimbine, atipamezole and naloxone were administered i.p. or s.c. 15 min before CR4056 administration. Morphine was administered s.c.

The interaction of CR4056 with morphine was evaluated by administering fixed proportions of CR4056 and morphine (1:1 for male rats, 3:1 for female rats) selected on the basis of their respective ED50 when administered alone. When CR4056 and morphine were co-administered, since these
drugs induce their analgesic effect with a different temporal response, we synchronized the peak effect of each drug by administering CR4056 1 h before morphine. Thus, in these types of experiments, mechanical hyperalgesia was measured 90, 180 and 360 min after CR4056 administration corresponding to 30, 120 and 300 min after morphine administration.

**Data analysis**

Throughout the manuscript, data are given either as mean and SEM or as \( ED_{50} \) and 95% confidence interval (95% CI). To evaluate the statistical significance of the anti-hyperalgesic effects of drugs (alone or in combination), data analysis was performed on the crude mechanical threshold values expressed in grams. Dose-response curves and experiments with receptor antagonists were analysed by two-way analysis of variance: repeated measures (RM) two-way ANOVA with treatment as the inter-subject variable and time as the intra-subject variable. \( F \) and \( P \) values for the main effect of treatment are given in the text. *Post hoc* comparisons were made using a multiple comparison within each experimental time point (Tukey’s multiple comparisons test), with \( P < 0.05 \) considered statistically significant (GraphPad Prism software, version 6.0; GraphPad Software Inc., San Diego, CA, USA). A Student’s \( t \)-test was run when mean withdrawal threshold was to be compared between two experimental groups (i.e. sham un-operated rats vs. control operated rats; male rats vs. female rats), with \( P < 0.05 \) considered statistically significant.

The dose that produced 50% of the anti-hyperalgesic effect \( (ED_{50}) \) was calculated at the time corresponding to the peak effect (90 min for CR4056, 30 min for morphine, either alone or in combination) using a standard linear regression analysis of the log dose-response curve, constrained between 100% (i.e. the mean withdrawal threshold in sham un-operated rats) and 0% (i.e. the mean withdrawal threshold in control operated rats). The regression analyses were performed on the single data points (six animals at each of at least three doses) and not on the group means.

The interaction of CR4056 with morphine was evaluated by isobolographic analysis, which was carried out as described by Tallarida et al. (1989). The isobologram was constructed by connecting the \( ED_{50} \) of CR4056 plotted on the abscissa with the \( ED_{50} \) of morphine plotted on the ordinate to obtain the additivity line. On this line, a theoretical additive \( ED_{50} \) \( (ED_{50,add}) \) was calculated (Pinardi et al., 2001; Miranda et al., 2002) using the following formula:

\[
ED_{50,add} = ED_{50,CR4056} \left( P_1 + R \cdot P_2 \right),
\]

where \( R \) is the potency ratio of CR4056 alone to morphine alone, \( P_1 \) is the proportion of CR4056 and \( P_2 \) is the proportion of morphine in the total mixture.

The variance of \( ED_{50,add} \) was calculated from the fraction (FR) of the \( ED_{50} \) in the combination as:

\[
Var ED_{50,add} = Var ED_{50,CR4056} \cdot \left( FR_{CR4056} \right)^2 + Var ED_{50,morphine} \cdot \left( FR_{morphine} \right)^2
\]

From these variances, 95% confidence limits of the theoretical additive \( ED_{50} \) were calculated. To evaluate the statistical significance of the synergistic effect, the theoretical values calculated as described above were compared by a Student’s \( t \)-test with the \( ED_{50} \) values experimentally obtained for the drug mixture. The interaction index (I.I.) was calculated as the ratio experimental \( ED_{50}/theoretical \ ED_{50} \) (Miranda et al., 2008). Values lower than 1 indicate synergistic interactions.

Isobolograms and dose-response curves were plotted using SigmaPlot version 12.0 (Systat Software Inc., San Jose, CA, USA).

**Materials**

The following chemicals were used: morphine (S.A.L.A.R.S., Como, Italy), naloxone, efaroxan, yohimbine, idazoxan, naproxen (SigmaAldrich, Milan, Italy) and atipamezole (Abcam PLC, Cambridge, UK). CR4056 was synthesized by the Medicinal Chemistry Department of Rottapharm. CR4056 was suspended in 0.5% methyl cellulose (MC) and administered orally (5 mL·kg\(^{-1}\)); naproxen was dissolved in distilled water and administered orally (5 mL·kg\(^{-1}\)); all the other drugs were dissolved in saline for i.p. or s.c. administration.

**Results**

**CR4056 dose-response efficacy in male rats: comparison with morphine**

Twenty-four hours after surgery, male rats showed hyperalgesia to mechanical stimuli. The mean withdrawal threshold in the injured paw was halved compared with that measured in the hind paw of sham rats (302.5 ± 29.7 g vs. 610.0 ± 18.5 g; Student’s \( t \)-test: \( P < 0.01 \)). Under these experimental conditions, oral CR4056 (range 1–10 mg·kg\(^{-1}\)) significantly [RM two-way ANOVA: \( F(3, 20) = 13.99; P < 0.0001 \)] and dose-dependently reversed the established hyperalgesia (ED\(_{50} = 1.63\) mg·kg\(^{-1}\); 95% CI = 1.07–2.47) (Figure 1A). Oral naproxen (30 mg·kg\(^{-1}\)), previously reported to be poorly active in reducing postoperative pain (Whiteside et al., 2004), did not show significant effects at any time point analysed. Conversely, subcutaneous morphine (range 0.5–6 mg·kg\(^{-1}\)) significantly [RM two-way ANOVA: \( F(4, 25) = 14.40; P < 0.0001 \)] and dose-dependently reversed the established hyperalgesia (ED\(_{50} = 1.27\) mg·kg\(^{-1}\); 95% CI = 0.93–1.73) (Figure 1B).

**Pharmacology of CR4056-induced analgesia**

The analgesic effect induced by CR4056 was completely suppressed by the non-selective imidazoline I\(_{1}/\alpha_2\)-adrenoceptor antagonist idazoxan (3 mg·kg\(^{-1}\), i.p.; Figure 2A). Yohimbine (2 mg·kg\(^{-1}\), i.p.; Figure 2C), a selective \( \alpha_2 \)-adrenoceptor antagonist, partly but significantly reduced (by about 30%); Tukey’s multiple comparisons test: \( P < 0.05 \) the effect of CR4056. Similar results were obtained with atipamezole (1 mg·kg\(^{-1}\), s.c.; data not shown), an \( \alpha_2 \)-adrenoceptor antagonist with negligible affinity for I\(_3\) receptors (Diaz et al., 1997; Pertovaara et al., 2005). Conversely, the non-selective I\(_1/\alpha_2\)-adrenoceptor antagonist efaroxan (1 mg·kg\(^{-1}\), i.p.; Figure 2B) and the \( \mu \)-opioid receptor antagonist naloxone (3 mg·kg\(^{-1}\), i.p.; Figure 2D) were unable to alter the analgesic response induced by CR4056.

**CR4056 dose-response efficacy in female rats: comparison with morphine**

At baseline, female Sprague-Dawley rats showed a significantly lower paw withdrawal threshold to mechanical stimuli

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Morphine/CR4056 co-treatment: dose-response efficacy and isobolographic analysis

We examined the effects of co-administered morphine and CR4056, and determined the type of interaction between the two treatments. Similarly to morphine or CR4056 alone, their combination produced a significant, dose-dependent analgesic effect 24 h after surgery both in male rats [Figure 5A; RM two-way ANOVA: $F(4, 25) = 27.61; P < 0.0001$] and in female rats [Figure 5B; RM two-way ANOVA: $F(4, 25) = 9.89; P < 0.0001$]. Isobolographic analysis revealed a significant synergistic interaction that is independent of sex. In fact, when CR4056 and morphine were combined, their ED$_{50}$ values were about fivefold lower than those measured after administration of each drug alone, both in male rats (0.28 mg·kg$^{-1}$; 95% CI = 0.22–0.36 vs. 1.63 mg·kg$^{-1}$; 95% CI = 1.07–2.47 for CR4056; 0.28 mg·kg$^{-1}$; 95% CI = 0.22–0.36 vs. 1.27 mg·kg$^{-1}$; 95% CI = 0.93–1.73 for morphine) and in female rats (0.15 mg·kg$^{-1}$; 95% CI = 0.10–0.24 vs. 0.93 mg·kg$^{-1}$; 95% CI = 0.75–1.14 for CR4056; 0.05 mg·kg$^{-1}$; 95% CI = 0.03–0.08 vs. 0.25 mg·kg$^{-1}$; 95% CI = 0.18–0.35 for morphine). And they were significantly lower than the ED$_{50}$ values predicted assuming an additive effect, both in male rats (Figure 6A; Student’s $t$-test: $P < 0.01$) and in female rats (Figure 6B; Student’s $t$-test: $P < 0.01$). The I.I. was <0.4 for both drugs.

Discussion

Imidazoline I$_{2}$ receptors have been studied for more than two decades (Mallard et al., 1992; Miralles et al., 1993; Carpéné et al., 1995; Tesson et al., 1995), but only in recent years they have truly emerged as a promising target for the treatment of different conditions, including depression and pain (Ferrari et al., 2011; Li and Zhang, 2011; Li et al., 2011; 2014; Han et al., 2012; Meregalli et al., 2012; Tonello et al., 2012; Garau et al., 2013; Min et al., 2013). The main finding of this study is that a single oral treatment with the I$_{2}$ receptor ligand CR4056 significantly reduced mechanical hyperalgesia caused by surgical lesion of the hind paw in rats, using Brennan’s model of postoperative pain. Since CR4056 was previously reported to be effective in both inflammatory and neuropathic pain models (Ferrari et al., 2011; Meregalli et al., 2012; Li et al., 2014), this study identified CR4056 as a broad-spectrum analgesic drug.

In agreement with what was reported for the capsaicin-induced hyperalgesia model in the rat (Ferrari et al., 2011), the effect of CR4056 in Brennan’s model of postoperative pain seems to be mechanism-related. In fact, idazoxan, a mixed I$_{2}$/α$_{2}$-adrenoceptor antagonist, completely suppressed the analgesic activity of CR4056, suggesting that binding to the I$_{2}$ receptor is a primary requirement for its efficacy. Conversely, when we tried to block the analgesic effect of CR4056 with substances that do not interact with imidazoline I$_{2}$ receptors, we failed to show a significant antagonism. A relevant exception was the moderate (about 30%) but significant effect of yohimbine, a classical α$_{2}$-adrenoceptor antagonist, administered at the maximum possible dose not inducing obvious behavioural changes (Arrant et al., 2013; Zheng and Rinaman, 2013). As yohimbine did not antago-
nize the analgesic effect of CR4056 in the capsaicin model (Ferrari et al., 2011), we decided to test in the postoperative model an additional $\alpha_2$-adrenoceptor antagonist, atipamezole, which has negligible affinity for $I_2$ receptors (Pertovaara et al., 2005), still obtaining a partial but significant block of the effects of CR4056. A similar pattern of $I_2$ receptor signal modulation was previously shown by Diaz et al. (1997) in a different pain model. They reported that BU224, a potent $I_2$ receptor ligand, inhibited the response of dorsal horn neurons in a dose-dependent manner, and that this effect was completely reversed by idazoxan but only partly reversed by yohimbine and atipamezole. In their discussion, these authors suggested that the actions of BU224 were mostly mediated through spinal imidazoline receptors selectively blocked by a putative antagonist to these receptors, such as idazoxan. Conversely, $\alpha_2$-adrenoceptor antagonists, such as yohimbine and atipamezole, antagonized the pre-synaptic $\alpha_2$ autoreceptors, which in turn are blocked by exogenous yohimbine and/or atipamezole. But the different levels of inhibition observed in our experiments with yohimbine and idazoxan (i.e. partial vs. complete) supports the hypothesis that CR4056 exerts its analgesic effects mainly through a mechanism strictly related to $I_2$ receptors.

Remarkably, the recent interest in the $I_2$ receptor as a promising target in drug discovery prompted Min et al. (2013) to assess for the first time the safety profile of two widely used $I_2$ receptor ligands, namely 2-(2-benzofuranyl)-2-imidazoline (2-BFI) and 2-(4, 5-dihydroimidazol-2-yl)quinolone (BU224). They found that these compounds produce epileptic seizures in two strains of mice. Because these effects were not antagonized by the prototypical $I_2$ receptor antagonist idazoxan, the authors suggested that the epileptogenic potential of 2-BFI and BU224 is not related to $I_2$ receptors but rather to a different shared mechanism. No

Figure 2
Effects of idazoxan [A: RM two-way ANOVA: $F(3, 20) = 41.77; P < 0.0001$], efaxan [B: RM two-way ANOVA: $F(3, 20) = 53.71; P < 0.0001$], yohimbine [C: RM two-way ANOVA: $F(3, 20) = 25.17; P < 0.0001$] and naloxone [D: RM two-way ANOVA: $F(3, 20) = 26.25; P < 0.0001$] on the analgesic activity induced by 10 mg·kg$^{-1}$ oral CR4056. All the antagonists were administered i.p. 15 min before CR4056. Data represent the mean withdrawal threshold expressed in grams ± SEM ($n = 6$ per group).
neurobehavioral changes were instead associated with CR4056 during preclinical safety pharmacology studies, and specifically in the behavioural Irwin test in rodents, where the compound was administered orally up to a dose 100 times greater than the dose producing analgesic activity (Meregalli et al., 2012). Despite the different safety profile of CR4056 compared with 2-BFI and BU224, which makes the first one a candidate drug, all of them markedly attenuated the place escape/avoidance behaviour at a dose that significantly attenuated the hyperalgesic response in rat models of inflammatory and neuropathic pain (Li et al., 2014). This result is particularly relevant since it represents the first evidence that the efficacy of this novel class of putative analgesics may not be limited to the evoked component of pain but may also include the spontaneous/affective component, that is the one commonly measured in clinical trials of postoperative pain.

According to a recent review, postoperative pain in humans is clinically treated with a mixture of drugs, including non-steroidal anti-inflammatory drugs, opioids and peripheral anaesthetics (Wu and Raja, 2011). Interestingly, epidemiological and clinical studies show that women are at greater risk for many pain conditions, and there is some suggestion that postoperative pain may be more severe in women than in men (Fillingim and Maixner, 1995; Riley et al., 1998; Fillingim et al., 2009). In addition, sex differences in the response to stimuli or in the response to analgesics have been claimed in a number of human and animal studies (Fillingim and Maixner, 1995; Aubrun et al., 2005; Dahan et al., 2008; Campesi et al., 2012; Patil et al., 2013). So we did an additional series of experiments in female rats and compared the results with those obtained in male rats.

In 2003, Kroin and colleagues had already analysed potential differences between male and female Sprague-Dawley rats with regard to postoperative pain and analgesic response. That study had shown no differences in postoperative pain perception or in the response to various analgesic drugs, such as morphine, gabapentin and clonidine (Kroin et al., 2003). In the present study, female Sprague-Dawley rats had a significantly lower paw withdrawal threshold before surgery. As a consequence, there were no sex differences in the net hyperalgesic response. Conversely, female rats were significantly more sensitive to treatments than males, as the ED50 of subcutaneous morphine and oral CR4056 was five- and twofold lower, respectively, in females versus males. In this regard, the differences between our results and those obtained by Kroin et al. (2003) may be

![Figure 3](image_url)

**Figure 3**

Sex differences in postoperative pain-induced mechanical hyperalgesia in Sprague-Dawley rats. The mean withdrawal threshold (expressed in grams ± SEM) 24 h after surgery, shown by the black bar below the columns (POP, postoperative pain), was compared with that in sham un-operated rats (no black bar). Thresholds were lower in female rats both in sham and POP groups. ***P < 0.001. Student’s t-test; n = 12 per group.

![Figure 4](image_url)

**Figure 4**

(A) Anti-hyperalgesic effect of CR4056 on postoperative pain-induced mechanical hyperalgesia in female rats (Randall-Selitto test). CR4056 was orally administered 24 h after surgery. Data represent the mean withdrawal threshold expressed in grams ± SEM (n = 6 per group). (B) Anti-hyperalgesic effect of morphine on postoperative pain-induced mechanical hyperalgesia in female rats (Randall-Selitto test). Morphine was subcutaneously administered 24 h after surgery. Data represent the mean withdrawal threshold expressed in grams ± SEM (n = 6 per group).
justified on the basis of the following considerations. First, we examined mechanical hyperalgesic responses registered by a Randall-Selitto apparatus while they reported on mechanical allodynic responses registered manually by von Frey filaments. Compared with monofilaments, the Randall-Selitto apparatus has a bigger contact surface that can recruit sensory nerve endings in a different manner (Khalsa, 2004). Second, we performed dose-response experiments (four increasing doses of morphine) with a time-dependent (30, 90, 180 and 360 min) evaluation of mechanical threshold, while Kroin et al. (2003) evaluated the effect of two doses of morphine at a single time point (30 min), and the route of morphine administration was also different (s.c. vs. i.p.). However, it is relevant to emphasize that the sex differences we found were limited to drug potency, as there were no differences in the efficacy of treatments.

Another relevant finding of our study was the synergistic interaction between morphine and CR4056. In a previous study (Ferrari et al., 2011), we already analysed the nature of the morphine–CR4056 interaction in the capsaicin model of pain in male Wistar rats. In that study, the combination of morphine with CR4056 was clearly synergistic, being more...
effective than predicted on the basis of a simple additive effect. The present results confirmed the synergistic nature of morphine–CR4056 interaction also in the postoperative model of pain in both male and female Sprague-Dawley rats. When these agents were co-administered, the doses required to achieve the ED_{50} were about five times lower than those required when each drug was administered alone. This finding is important from a translational point of view because combination therapy may decrease the required dose of individual drugs, thus limiting the occurrence of adverse effects.

In conclusion, this study has demonstrated a significant analgesic effect of CR4056 in a surgical model of pain in rats, and a synergistic interaction between CR4056 and morphine that was independent of sex.

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Contributions

M. L., F. F., D. T. and I. M. contributed in the process of data acquisition and in drafting the article. M. L., F. F. and G. C. contributed in the conception and design of the study, in the analysis and interpretation of data, and in the final approval of the version to be submitted. G. C. contributed also in obtaining the funding. M. L. (marco.lanza@rottapharm.com) and G. C. (gianfranco.caselli@rottapharm.com) declare to take responsibility for the integrity of the work as a whole, from inception to finished article.

Conflict of interest

IM is a graduate visiting student with no competing interests. All the other authors are scientists from the research unit of the Rottapharm group.

This study was funded by the Rottapharm group. However, the Rottapharm group as a corporate entity had no role in the conduct of the study; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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