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RESEARCH PAPER

Modulation of imidazoline I_2 binding sites by CR4056 relieves postoperative hyperalgesia in male and female rats

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BACKGROUND AND PURPOSE

CR4056 is a novel imidazoline-2 (I_2) 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 I_2/α_2-adrenoceptor antagonist idazoxan, were partially reduced (~30%; $P < 0.05$) by the selective α_2-adrenoceptor antagonist yohimbine, but were not influenced by the non-selective I_1/α_2-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; I_2, imidazoline-2; I.I., interaction index; RM, repeated measures

Introduction

Imidazoline-2 (I_2) binding sites, also referred to as I_2 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, I_2 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 I_2 receptors is the modulation of the opioid system, which may occur at different levels. First, recent evidence has shown how I_2 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 I_2 receptors in pain control stems from the interaction between imidazoline ligands and the opioid system in the locus ceruleus neurons (Ruiz-Durántez *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 I_2 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. Idazoxan, 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 ED_{50} 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:

$$ED_{50\,add} = ED_{50\,CR4056}/(P_1 + R * P_2),$$

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:

$$\mathrm{Var}\ ED_{50\,add} = \mathrm{Var}\ ED_{50\,CR4056} * (FR_{CR4056})^2 + \mathrm{Var}\ ED_{50\,morphine} * (FR_{morphine})^2$$

From this 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 $mL \cdot kg^{-1}$); naproxen was dissolved in distilled water and administered orally (5 $mL \cdot kg^{-1}$); 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 $mg \cdot kg^{-1}$) significantly [RM two-way ANOVA: $F(3, 20) = 13.99$; $P < 0.0001$] and dose-dependently reversed the established hyperalgesia (ED_{50} = 1.63 $mg \cdot kg^{-1}$; 95% CI = 1.07–2.47) (Figure 1A). Oral naproxen (30 $mg \cdot kg^{-1}$), 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 $mg \cdot kg^{-1}$) significantly [RM two-way ANOVA: $F(4, 25) = 14.40$; $P < 0.0001$] and dose-dependently reversed the established hyperalgesia (ED_{50} = 1.27 $mg \cdot kg^{-1}$; 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_2/α_2-adrenoceptor antagonist idazoxan (3 $mg \cdot kg^{-1}$, i.p.; Figure 2A). Yohimbine (2 $mg \cdot kg^{-1}$, i.p.; Figure 2C), a selective α_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 $mg \cdot kg^{-1}$, s.c.; data not shown), an α_2–adrenoceptor antagonist with negligible affinity for I_2 receptors (Diaz *et al.*, 1997; Pertovaara *et al.*, 2005). Conversely, the non-selective I_1/α_2-adrenoceptor antagonist efaroxan (1 $mg \cdot kg^{-1}$, i.p.; Figure 2B) and the μ opioid receptor antagonist naloxone (3 $mg \cdot kg^{-1}$, 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

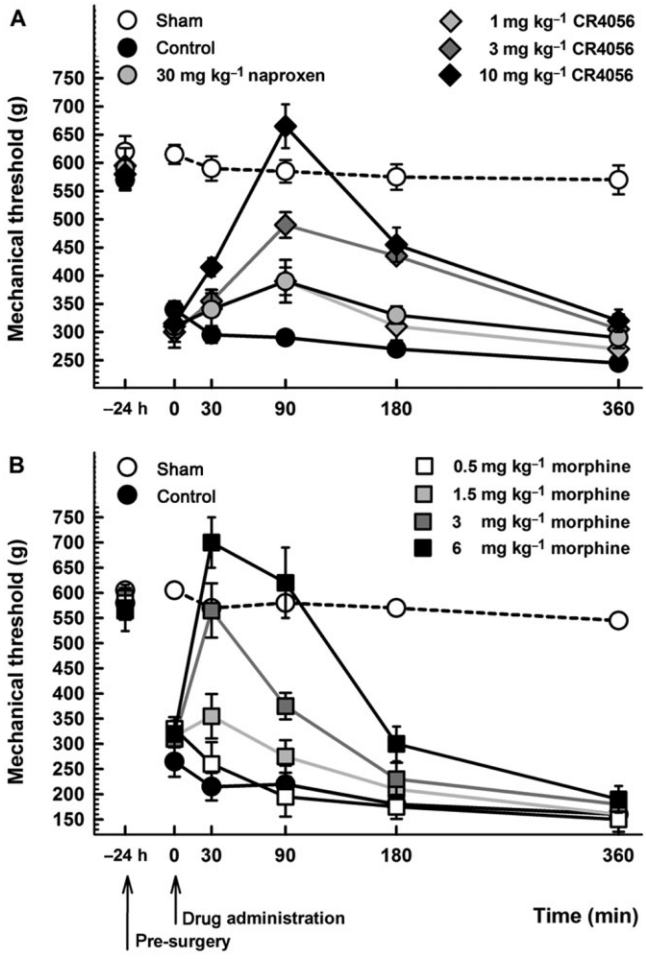

Figure 1

(A) Anti-hyperalgesic effect of CR4056 on postoperative pain-induced mechanical hyperalgesia in male rats (Randall-Selitto test). CR4056 was orally administered 24 h after surgery. Naproxen (30 mg·kg^{-1}; oral) was used as comparison. Data represent the mean withdrawal threshold expressed in grams ± SEM (n = 6 per group). (B) Anti-hyperalgesic effects of morphine on postoperative pain-induced mechanical hyperalgesia in male 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).

versus male rats (P < 0.01; Figure 3). Twenty-four hours after surgery, female rats still showed lower threshold levels than those recorded in male rats, so that the effect of surgery on mechanical threshold was similar in female and male rats (Figure 3). And even in females, oral CR4056 significantly [RM two-way ANOVA: $F(3, 20) = 41.91$; $P < 0.0001$] and dose-dependently reversed the established hyperalgesia (ED_{50} = 0.93 mg·kg^{-1}; 95% CI = 0.75–1.14) (Figure 4A). According to the protocol adopted for male rats, we administered to female rats a subcutaneous treatment with morphine (range 0.125–1 mg·kg^{-1}). Morphine significantly [RM two-way ANOVA: F(4, 25 = 19.09, P < 0.0001] and dose-dependently reversed the established hyperalgesia (ED_{50} = 0.25 mg·kg^{-1}; 95% CI = 0.18–0.35) (Figure 4B).

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-

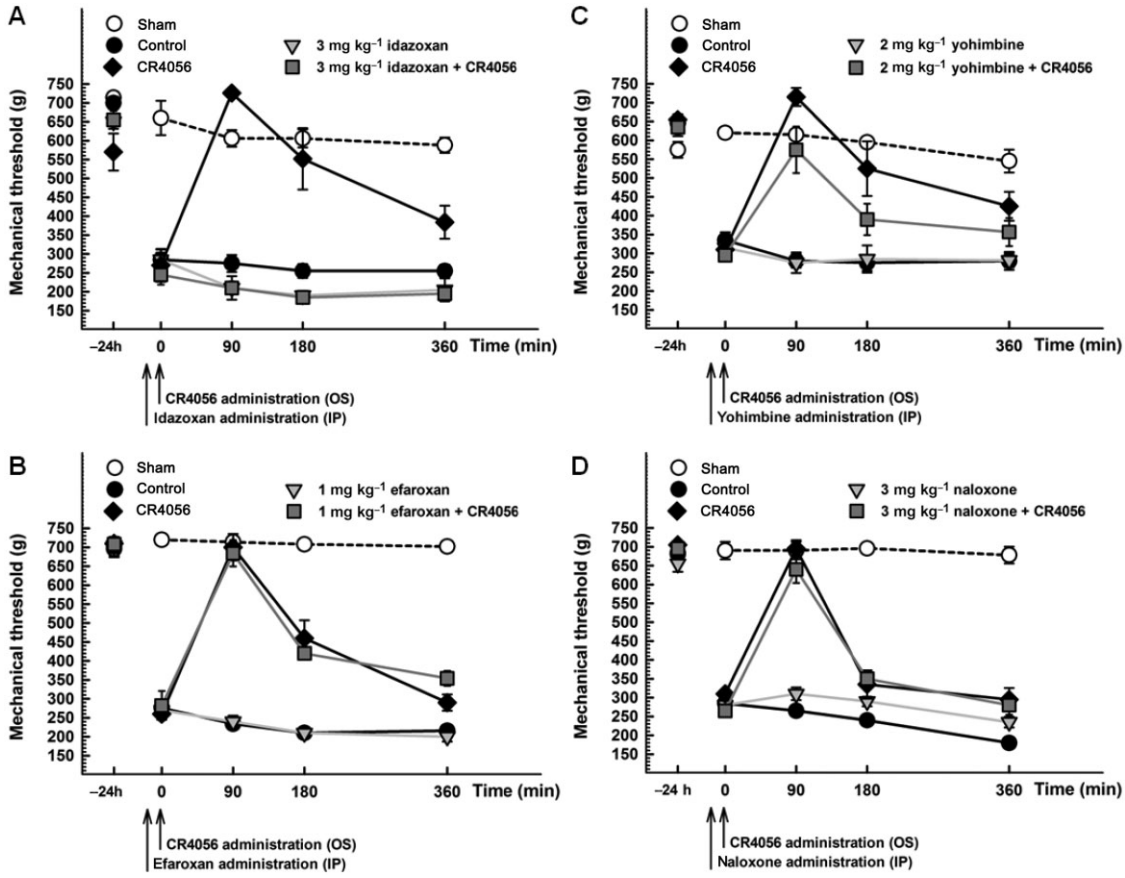

Figure 2

Effects of idazoxan [A: RM two-way ANOVA: $F(3, 20) = 41.77$; $P < 0.0001$], efaroxan [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).

nize the analgesic effect of CR4056 in the capsaicin model (Ferrari *et al.*, 2011), we decided to test in the postoperative model an additional α_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 neurones 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, α_2-adrenoceptor antagonists, such as yohimbine and atipamezole, antagonized the *in vivo* effects of drugs modulating noradrenergic pathways either directly by interacting with α_2-adrenoceptors or indirectly by inhibiting the noradrenergic catabolism (Schreiber *et al.*, 1998; Onttonen and Pertovaara, 2000; Li *et al.*, 2007). Like other imidazoline receptor modulators, CR4056 is a functional and reversible inhibitor of MAO-A, because it modulates enzyme activity through allosteric I_2 sites present in a discrete population of MAO proteins (Carpéné *et al.*, 1995; Tesson *et al.*, 1995; Ferrari *et al.*, 2011). Accordingly, it is possible that the increased levels of endogenous noradrenaline found in different areas of the central nervous system after oral administration of CR4056 in rats (Ferrari *et al.*, 2011) activate presynaptic α_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

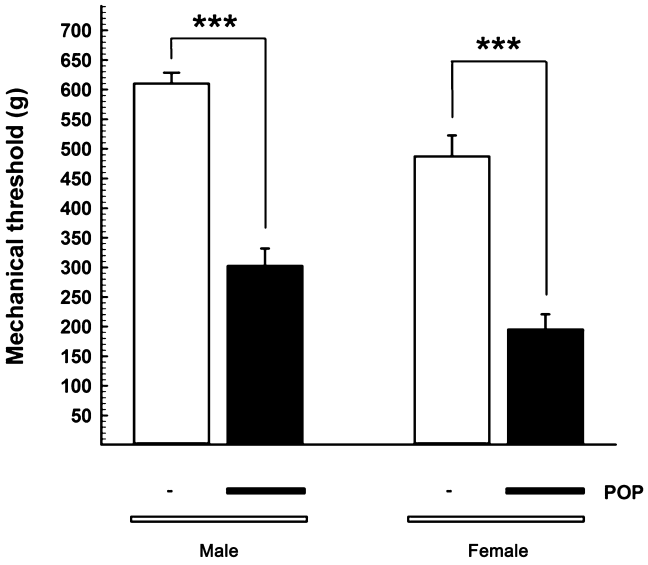

Figure 3

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.

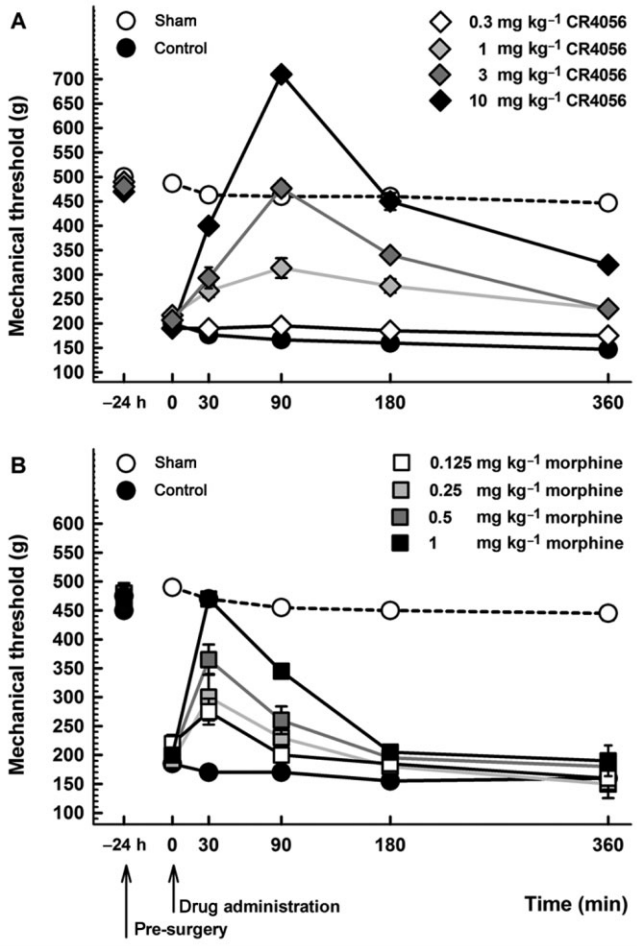

Figure 4

(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).

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 to mechanical stimuli than male rats, before and after 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 ED_{50} 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

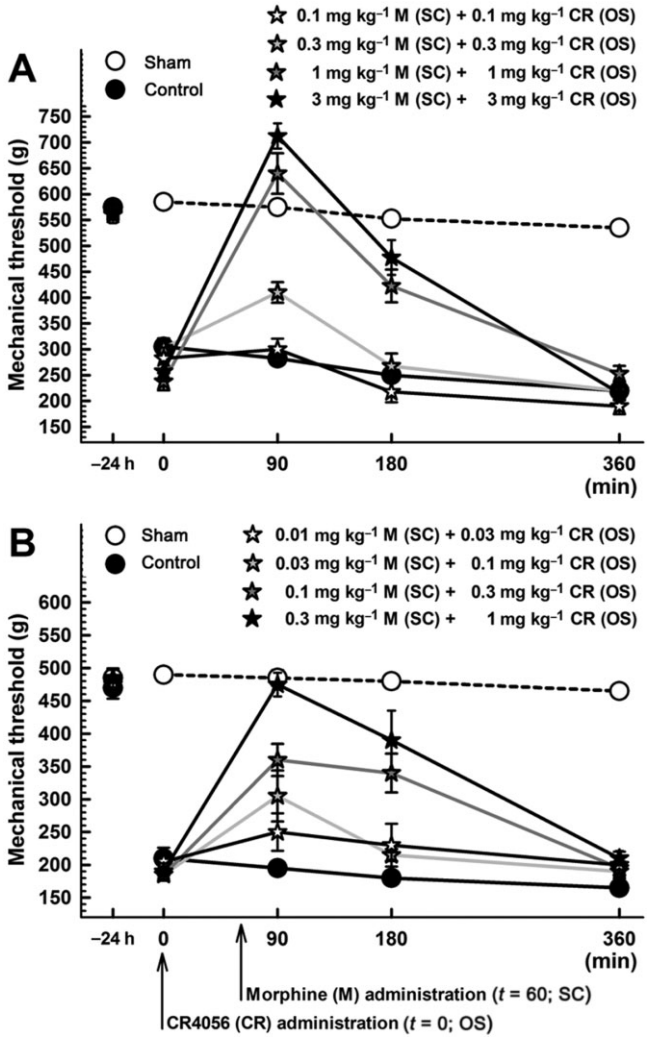

Figure 5

Anti-hyperalgesic effect of the combination oral CR4056/subcutaneous morphine on postoperative pain-induced mechanical hyperalgesia (Randall-Selitto test) in male (A) and female (B) rats. Since CR4056 and morphine showed a different temporal response when administered alone, we synchronized the peak effect of each drug by administering CR4056 1 h before morphine. Thus, mechanical hyperalgesia was measured 90, 180 and 360 min after CR4056 administration corresponding to 30, 120 and 300 min after morphine administration. Data represent the mean withdrawal threshold expressed in grams ± SEM ($n = 6$ per group). OS, oral administration; SC, subcutaneous administration.

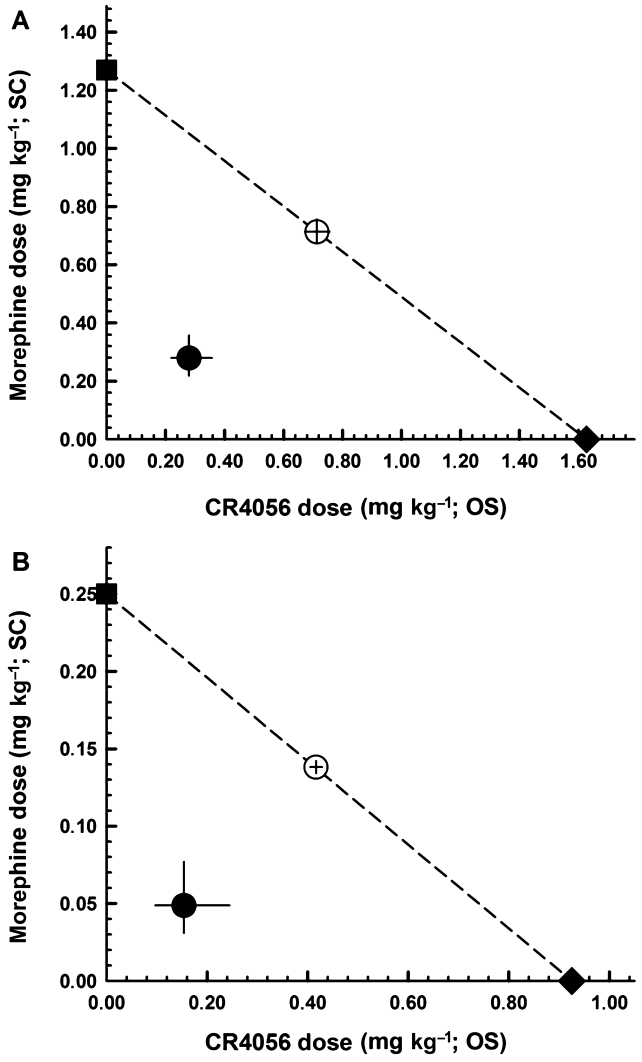

Figure 6

Isobolograms for the combination of oral CR4056 with subcutaneous morphine in male (A) and female (B) rats. Open circles correspond to the theoretical ED_{50} with 95% CI; filled circles corresponds to the experimental co-treatment ED_{50} with 95% CI; filled square corresponds to the experimental ED_{50} for morphine alone; and filled diamond corresponds to the experimental ED_{50} for CR4056 alone. I.I. was 0.39 for both CR4056 and morphine in male rats. In female rats, the calculated I.I. was 0.37 and 0.35 for CR4056 and morphine respectively. OS, oral administration; SC, subcutaneous administration.

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.

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