

Isobolographic analysis of multimodal analgesia in an animal model of visceral acute pain

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Abstract

Multiple analgesic–drug combinations are commonly used in the management of acute and chronic pain in humans during multimodal or balanced analgesia. At present, these combinations are used empirically in clinical practice and are considered to be beneficial for the patient. Interactions between two antinociceptive drugs have been thoroughly examined, but the nature of interactions between three analgesics has not been studied. The antinociceptive interaction of ketoprofen (K) with a mixture of morphine (M) and paracetamol (P) was evaluated using a model of visceral acute tonic pain, the acetic-acid writhing test in mice. The i.p. administration of the combination M/P+K resulted in a significant potentiation of the antinociception induced either by K or by the synergic two-drug mixtures M/K, P/K and M/P. The effect of opioid, cholinergic, adrenergic and serotonergic antagonists on the analgesic interaction was assessed. The pretreatment of mice with atropine (1 mg/kg) did not produce any change in the synergistic interaction of the triple combination. The pretreatment with naltrexone (1 mg/kg) or tropisetron (1 mg/kg) reduced the intensity of M/P+K synergic interaction, while prazosin (0.1 mg/kg) significantly potentiated the synergy. The findings of this work suggest that the two major pathways of descending inhibitory systems, noradrenergic and serotonergic are significantly involved in the mechanism of the antinociceptive synergy induced by the triple combination. On the other hand, opioid pathways also seem to participate, since pretreatment with naltrexone reduced the synergy. In conclusion, the triple combination M/P+K induced a strong synergistic antinociceptive effect, which could be of interest for optimal multimodal clinical analgesia.

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1. Introduction

Pain is the net effect of multidimensional mechanisms that involve most parts of the central nervous system (Le Bars et al., 2001) and its treatment is probably one of the major challenges in clinical medicine. According to this view, a variety of drugs, alone or in combination, have been tested in different preclinical models of pain with variable results, depending on the models and tests used. For instance, opioids show good antinociceptive effects in models of thermal stimulation, where non-steroidal anti-inflammatory drugs (NSAIDs) produce inconsistent results. Several drug combinations which have synergistic analgesic interactions have been tested preclinically and clinically (Elia

et al., 2005). For example, it has been reported that gabapentin, the (*S*)-ketoprofen isomer, and the cannabinoid agonist CP 55,940, enhance the analgesic effect of morphine (Ossipov et al., 2000; Pakulska and Czarnecha., 2004; Tham et al., 2005). Combinations of NSAIDs and adrenergic drugs are also synergic (Miranda et al., 2001; Pinardi et al., 2001). Moreover, it has been recently reported that NSAIDs and morphine show a synergistic interaction (Miranda et al., 2004, 2005; Pinardi et al., 2005). At present, interactions between two analgesic drugs have been thoroughly examined, but to date, the nature of interactions between three analgesic drugs has not been studied extensively. However, multiple analgesic–drug combinations (mainly an NSAID+paracetamol+an opioid) are commonly used in clinical practice to manage acute and chronic pain syndromes (Elia et al., 2005; Skinner and Shintani, 2004). The aim of the present work was to experimentally evaluate the antinociceptive

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interaction of ketoprofen with the clinically frequently used combination of morphine and paracetamol (M/P), using a model of visceral acute tonic pain, the acetic acid-induced writhing test of mice. In this test, the combination M/P has shown a strong supra-additive interaction (Miranda et al., 2004, 2005). Furthermore, attempting to clarify the mechanisms of the interaction, the effect of opioid, cholinergic, adrenergic and serotonergic system pathways, usually involved in analgesia, was assessed by the use of specific antagonists. This investigation attempts to use isobolographic analysis to examine a triple combination of analgesic drugs.

2. Materials and methods

2.1. Animals

Male CF-1 mice (30 g), housed on a 12 h light–dark cycle at 22 ± 2 °C and with access to food and water *ad libitum* were used. Experiments were performed in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigation of experimental pain approved by the Animal Care and Use Committee of the Faculty of Medicine, University of Chile. Animals were acclimatized to the laboratory for at least 2 h before testing, were used only once during the protocol and were sacrificed immediately after the algesiometric test. The number of animals was kept at a minimum compatible with consistent effects of the drug treatments (6–8 mice per experimental group).

2.2. Writhing test

The procedure used has been described previously (Miranda et al., 2001). Briefly, mice were injected intraperitoneally (i.p.) with 10 mL/kg of 0.6% acetic acid solution, 30 min after the i.p. administration of the drugs, a time at which preliminary experiments showed to be the correct time interval for the maximal effect of all drugs used. Each mouse was then placed in an individual clear plexiglass observation cylinder (20 × 20 cm). A writhing was defined as a wave of contraction of the abdominal musculature followed by the extension of the hind limbs. The number of writhes in a 5 min period was counted, starting 5 min after the acetic acid administration. Antinociception was expressed as percent inhibition of the number of writhes observed in saline control animals (19.7 ± 0.27 , $n = 22$).

2.3. Protocol

Individual dose–response curves for paracetamol (P), morphine (M) and ketoprofen (K) were obtained using at least six animals per dose and at least four doses. A least-squares linear regression analysis of the log dose–response curve allowed the calculation of the dose that produced 50% of control writhes (ED_{50}) when each drug was administered alone. Dose–response curves were also obtained and analyzed for the mixtures of morphine plus ketoprofen (M/K), paracetamol plus ketoprofen (P/K) and morphine plus paracetamol (M/P), administered in fixed ratio combinations based on fractions

(1/2, 1/4, 1/8, 1/16) of the ED_{50} of each drug in the combination (Pinardi et al., 2003). Afterwards, the calculated ED_{50} of the dose–response curve originated from the M/P mixture, was combined with the ED_{50} of K (Table 1) and co-administered to mice in the same fractions as above. The new dose–response curve (three-drug combination) was then analyzed to obtain the ED_{50} of the triple combination. With the ED_{50} 's of K and M/P, an isobolographic analysis was performed to characterize the interaction, treating M/P as a single drug and comparing the isobologram to the one calculated for the M/P combination.

Dose–response curves for the three-drug combination were also obtained after the animals were pretreated with 1 mg/kg of i.p. atropine, tropisetron, naltrexone or 0.1 mg/kg of i.p. prazosin. The antagonists doses were selected from literature references (Pinardi et al., 1998; Miranda et al., 2004; Giordano and Gerstmann, 2004; McQueen et al., 2007). A similar isobolographic analysis was used to characterize the drug interactions after these pretreatments.

A detailed description of the isobolographic analysis has been previously published (Miranda et al., 2002, 2004; Pinardi et al., 2005). Supra-additivity or synergy is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretically calculated equieffect of drugs combined in the same proportions. If the ED_{50} 's are not statistically different, the effect of the combination is additive, meaning that each constituent contributes with its own potency to the total analgesic effect (Tallarida, 2001). The interaction index was calculated as the ratio between the experimental ED_{50} /the theoretical ED_{50} . When this value is close to 1, there is no interaction and the final effect is additive. Values lower than 1 are an indication of the magnitude of supra-additive or synergistic interactions, and values higher than 1 correspond to sub-additive or antagonistic interactions (Tallarida, 2001).

2.4. Drugs

All drugs were freshly dissolved in saline. Ketoprofen was provided by Rhone-Poulenc Rohrer, Chile; paracetamol by Bristol–Myers–Squibb, France; tropisetron hydrochloride by Novartis Chile S.A.; atropine sulfate, morphine sulfate,

Table 1

ED_{50} values \pm SEM for the antinociceptive effect of i. p. morphine, paracetamol, ketoprofen and the combinations M/K, P/K, M/P and M/P+K in the writhing test of mice

	$ED_{50} \pm$ SEM (mg/kg)	Combinations drug ratio
Morphine	0.12 \pm 0.011	
Paracetamol	49.46 \pm 3.32	
Ketoprofen	30.30 \pm 3.85	
M/K	3.98 \pm 0.22	1: 0.004
P/K	20.37 \pm 1.12	1: 1.63
M/P	10.11 \pm 0.9	1: 0.003
M/P+K	3.73 \pm 0.21	1: 0.33

M/K: combination of the ED_{50} of morphine + the ED_{50} of ketoprofen.

P/K: combination of the ED_{50} of paracetamol + the ED_{50} of ketoprofen.

M/P: combination of the ED_{50} of morphine + the ED_{50} of paracetamol.

M/P+K: combination of the ED_{50} of M/P + the ED_{50} of ketoprofen.

Table 2

Theoretical additive ED₅₀, experimental ED₅₀, confidence limits (CL) and interaction index (I.I.) for the i. p. combinations of morphine (M), paracetamol (P) and ketoprofen (K) and the effect of pretreatment with antagonists in the writhing test of mice

ED ₅₀ (mg/kg)	Theoretical (95% CL)	Experimental (95% CL)	I. I.
P/K	35.80 (28.6–44.7)	20.37 * (15.2–25.6)	0.569
M/P	20.71 (15.7–27.2)	10.11 * (7.1–16.6)	0.407
M/K	15.21 (10.9–21.0)	3.98 (2.9–5.0)	0.258
M/P+K	26.32 (20.7–33.4)	3.73 (2.8–4.6)	0.185
M/P+K		4.86	0.241
+ Atropine (1 mg/kg)		(2.6–7.1)	
M/P+K		7.53 *	0.373
+ Naltrexone (1 mg/kg)		(6.9–8.2)	
M/P+K		6.06 *	0.300
+ Tropisetron (1 mg/kg)		(4.9–7.3)	
M/P+K		0.752 *	0.037
+ Prazosin (0.1 mg/kg)		(0.026–1.8)	

* $P < 0.05$ versus M/P+K.

naltrexone hydrochloride and prazosin hydrochloride were purchased from Sigma Chemical Co, St. Louis, MO, USA.

2.5. Statistical analysis

Results are presented as ED₅₀ values ± SEM or with 95% confidence limits (95% CL). The program used to perform statistical procedures was Pharm Tools Pro (version 1.27, The

McCary Group Inc.). Results were analyzed by one-way ANOVA followed by Student–Newman–Keuls test or by Student's "t" test when appropriate. P values less than 0.05 ($P < 0.05$) were considered statistically significant.

3. Results

3.1. Antinociception induced by morphine, paracetamol, ketoprofen and the mixtures M/K, P/K and M/P

The i.p. administration of M, P, K, and the mixtures M/K, P/K and M/P (ED₅₀'s fixed ratios, Table 1) in the writhing test resulted in dose-dependent antinociception. On the basis of the ED₅₀'s, the potency of the two-drug combinations was $P/K < M/P < M/K$ (Table 1). The addition of K to the mixture M/P significantly decreased the ED₅₀ of M/P, so the triple combination was almost 3 times more potent than M/P (Table 1). There were no obvious gross observable motor disturbances or abnormal behaviours in the drug-treated mice in this study.

3.2. Interaction between M/P and ketoprofen

The interaction between the combinations of M/K, P/K, M/P and M/P+K at fixed ratio (on the basis of their ED₅₀'s), was assessed by isobolographic analysis of the dose–response curves obtained after i.p. administration, as indicated in the Materials and methods section. As previously reported (Miranda et al., 2004, 2005, 2006), the combinations M/K, P/K and M/P were synergic, with interaction indexes of 0.258, 0.569 and 0.407, respectively. When K was co-administered with the mixture M/P, the isobologram at the 50% maximum effect, demonstrated a more intense synergism, shown by an interaction index of 0.185 (Table 2, Fig. 1).

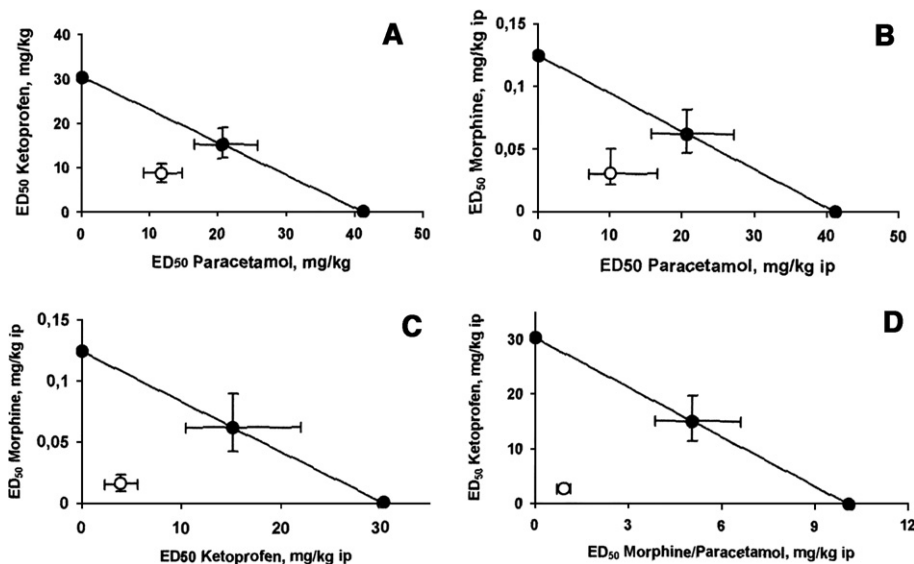


Fig. 1. Isobolograms of the antinociception induced by the combinations of ketoprofen/paracetamol (A, interaction index=0.569), morphine/paracetamol (B, interaction index=0.407), morphine/ketoprofen (C, interaction index=0.268) and morphine/paracetamol+ketoprofen (D, interaction index=0.185). Filled circles indicate the theoretical ED₅₀ with 95% confidence limits; open circles correspond to the experimental ED₅₀ with 95% confidence limits.

3.3. Effect of different antagonists on the synergism of M/P+K

In our model, the doses of the antagonists acting on different receptors administered i.p. did not induce significant antinociception *per se*. Naltrexone (1 mg/kg, $n=6$), prazosin (0.1 mg/kg, $n=6$), atropine (1 mg/kg, $n=6$) and tropisetron (1 mg/kg, $n=6$) induced 18.7 ± 0.85 , 19.1 ± 1.23 , 19.3 ± 0.45 and 18.6 ± 1.14 writhes, respectively, values not statistically different from saline control (19.7 ± 0.27).

Pretreatment with atropine did not change the synergistic antinociceptive activity of the mixture M/P+K, while tropisetron and naltrexone significantly reduced the synergic effect, without antagonizing it completely (Table 2 and Fig. 2). The pretreatment with prazosin, on the contrary, significantly enhanced the synergic effect of the triple combination. Table 2 shows the theoretically calculated additive and the experimentally obtained ED₅₀ values with 95% CL for the combinations, with their corresponding interaction indexes.

4. Discussion

Multiple analgesic–drug combinations are commonly used in the management of acute and chronic pain in humans during multimodal or balanced analgesia. At present, these combinations are used empirically in clinical practice and are considered to be beneficial for the patient (Elia et al., 2005). The aims of analgesic–drug combinations are to improve analgesia, and at the same time decrease the incidence and severity of adverse effects. It is generally accepted that the more efficient combinations are those in which analgesics with different mechanisms of action are used. Thus, in the clinical management of acute postoperative pain an opioid (usually morphine), is often combined with an NSAID and with paracetamol in empirical proportions. In the present investigation we have evaluated in a model of acute abdominal pain in mice, if the addition of a third drug, an NSAID, to the combination of two analgesics (morphine+paracetamol) would

enhance analgesia, allowing to decrease the doses necessary to induce similar antinociceptive effects.

The individual i.p. administration of M, P or K produced dose-dependent antinociceptive effects in the acetic acid writhing test in mice, confirming previous findings (Miranda et al., 2001, 2002, 2004, 2006). It is accepted that these drugs have different mechanisms of action, a theoretical necessity for synergistic interactions (Tallarida, 2001). The two-drug combinations M/K, P/K and M/P have been previously shown to be synergic (Miranda et al., 2004, 2005, 2006), a fact confirmed in the present work. When K was added to M/P and the three analgesics (M/P+K) were administered simultaneously, the interaction was more synergic than the M/P combination alone. The approach to the three-drug analysis, by dissecting the three-drug combinations into two-drug combinations, has been suggested and analyzed by Chou (2006). In the present investigation, the combination M/P was used as a starting point for the analysis, because it is one of the most frequently used empirically in clinical situations (Elia et al., 2005; Skinner and Shintani, 2004). The combination M/P used was treated as a single drug and it is assumed that the relative potency of the individual components is maintained in the mixture. Pretreatment with atropine did not change the synergistic nature of the interaction of the triple combination M/P+K, while the pretreatment with tropisetron and naltrexone significantly reduced the intensity of the interaction. Prazosin, on the other hand, significantly incremented the synergy. The control of the expression of molecular receptors for chemical messengers and the modulation of the activity of these receptors has been involved in the etiology of pain (Perl, 1999). In the white and gray matter of the spinal cord (superficial dorsal horn and dorsal root ganglion neurons) the presence of α_1 -adrenoceptors has been documented (Nicholson et al., 2005; Venugopalan et al., 2006). The involvement of the α_1 -adrenoceptor subtypes has been described in the formalin test (Nalepa et al., 2005), in the tail-flick and hot-plate (Tasker et al., 1992; Zarrindast and

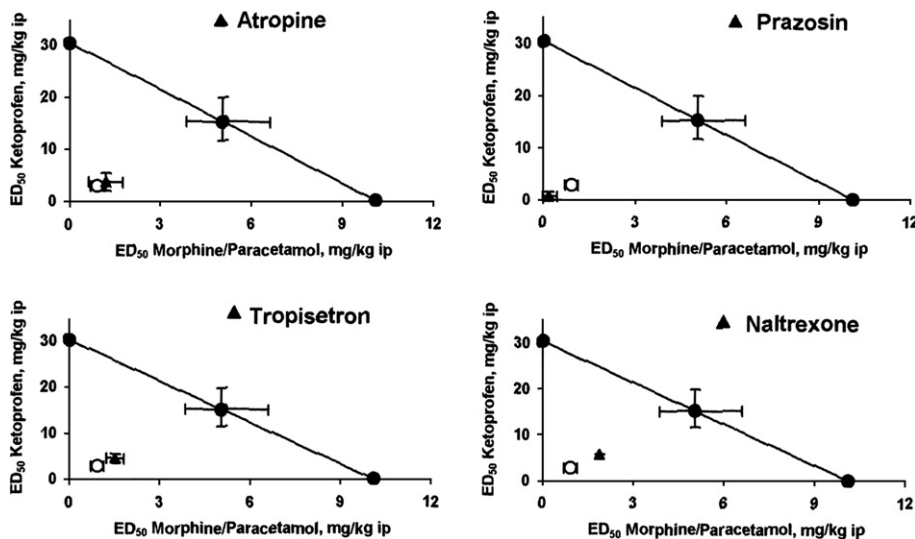


Fig. 2. Isobolographic representation of the effects of receptor antagonists on the antinociception induced by the combination of morphine/paracetamol+ketoprofen. Filled circles indicate the theoretical ED₅₀ with 95% confidence limits; open circles correspond to the experimental ED₅₀ with 95% confidence limits; filled triangles correspond to the experimental point after administration of the antagonist. Values are listed in Table 2.

Sahebgharani, 2002) and in the writhing test (Miranda et al., 2001; Pinardi et al., 1998). The results of the present study indicate that blockade of α_1 -adrenoceptors modulates the synergy of the triple combination, increasing the strength of the interaction. This is likely to be due to an interference with the activation of the noradrenergic descending inhibitory system at spinal level.

Several lines of research indicate that serotonin (5-HT) exerts its effects via several subtypes of receptors, and among them, the 5-HT₃ subtype. Its localization in axon terminals puts this receptor subtype in a prime location for the modulation of pain transmission and processing (Farber et al., 2004; Tham et al., 2005; Wolf, 2000). In addition, 5-HT produced a dose-dependent pro-nociceptive writhing response that was attenuated by the administration of tropisetron (Giordano and Gerstmann, 2004). The strength of the synergic interaction of M/P+K was also significantly attenuated by pretreatment with tropisetron. The findings of the present work with prazosin and tropisetron, suggest that the two major pathways of descending inhibitory control of pain at spinal level, noradrenergic and serotonergic, are significantly involved in the mechanism of the antinociceptive synergy induced by the co-administration of the triple mixture M/P+K.

The role of the cholinergic system in nociception has been recognized (Abdel-Salam, 2006; Ghelardini et al., 2002; Miranda et al., 2002; Pinardi et al., 2003). However, a dose of the non-selective antagonist atropine (1 mg/kg), which completely blocks cholinergic receptors (McQueen et al., 2007), did not modify the synergism of the triple combination, indicating that these receptors do not participate in the process and cholinergic activation is not a requisite for this interaction.

Finally, the involvement of opioidergic mechanisms modulating pain transmission is supported by multiple studies (Bodnar and Klein, 2005; Chevlen, 2003; Miranda et al., 2004, 2005). Moreover, the presence of three distinct opioid receptors (MOP-R, DOP-R and KOP-R) with different pharmacological effects was identified on the basis of the effect of selective agonists and antagonists. In the present work, the non-selective opioid antagonist naltrexone significantly attenuated the strength of the synergistic interaction of the triple combination. Since it has been shown that naltrexone does not alter the antinociception induced by NSAIDs in the writhing test (Miranda et al., 2004), the present results suggest that the effect could be related to the antagonism of morphine antinociception.

It should be noted that the addition of K to the M/P mixture significantly improves the synergy of M/P, but the observed interaction index value is very similar to that of the M/K combination, so a conclusion might be that the triple combination tested in this work is not much better than the mixture of M/K. This would indicate that the choice of drugs may be a critical fact in clinical multimodal analgesia.

In conclusion, the findings of the present work suggest that central and peripheral opioid receptor activation, together with the two major pathways of pain descending inhibitory systems in the CNS, noradrenergic and serotonergic, are significantly involved in the antinociceptive synergy induced by the co-administration of the mixture M/P+K. The potent synergy displayed by triple

combinations has potential clinical importance for the treatment of pain syndromes with multimodal analgesia.

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