Systemic synergism between codeine and morphine in three pain models in mice

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Abstract:

Background: The combination of two analgesic agents offers advantages in pain treatment. Codeine and morphine analgesia is due to activation of opioid receptor subtypes.

Methods: This study, performed in mice using isobolographic analysis, evaluated the type of interaction in intraperitoneal (*ip*) or intrathecal (*it*) coadministration of codeine and morphine, in three nociceptive behavioral models.

Results: Intrathecal morphine resulted to be 7.5 times more potent than *ip* morphine in the writhing test, 55.6 times in the tail flick test and 1.7 times in phase II of the orofacial formalin test; however, in phase I of the same test *ip* was 1.2 times more potent than *it* morphine. Intrathecal codeine resulted being 3.4 times more potent than *ip* codeine in the writhing test, 1.6 times in the tail flick test, 2.5 times in phase I and 6.7 times in phase II of the orofacial formalin test. Opioid coadministration had a synergistic effect in the acute tonic pain (acetic acid writhing test), acute phasic pain (tail flick test) and inflammatory pain (orofacial formalin test). The interaction index ranged between 0.284 (writhing *ip*) and 0.440 (orofacial formalin phase II *ip*).

Conclusion: This synergy may relate to the different pathways of pain transmission and to the different intracellular signal transduction. The present findings also raise the possibility of potential clinical advantages in combining opioids in pain management.

Key words:

opioids, algometer tests, isobolographic analysis, synergism

Abbreviations: DOR – delta opioid receptor, ED₅₀ – dosage that produced 50% of MPE, I.I. – interaction index, *ip* – intraperitoneal, *it* – intrathecal, KOR – κ opioid receptor, MPE – maximum possible effect, MOR – μ opioid receptor, NSAIDs – nonsteroidal anti-inflammatory drugs, NOP – nociceptin opioid receptor, SEM – standard mean error

Introduction

Opioids are frequently used in the treatment of moderate to severe pain. Opioids mimic the action of endogenous opioid peptides by interacting with opioid recep-

tor subtypes. Four opioid receptors have been cloned and are referred to as MOR, DOR, KOR, and NOR receptors, for mu (μ), delta (δ), kappa (κ), and nociceptin receptors, respectively [29]. Multimodal analgesia, which is the combination of analgesic agents, offers important benefits in the management of both acute and chronic pain. The mixture of different analgesic agents can achieve improved efficacy and/or tolerability and safety compared to equianalgesic doses of the individual drugs. Combining different agents also enhances efficacy in complex pain conditions involving multiple causes [22]. Interactions between opioids and nonsteroidal anti-inflammatory drugs (NSAIDs) have been previously reported [10, 11].

Furthermore, it has been communicated that coadministration of opioids induced a potent analgesic synergy in a mechanical nociceptive assay [9, 24]. Moreover, a marked antinociceptive synergy has been demonstrated by the coadministration of oxycodone and morphine [20]. When L-methadone was associated with several μ opioid ligands, a synergistic effect was observed. Out of the compounds examined, L-methadone selectively synergizes with morphine, morphine-6-glucuronide, codeine, and the active metabolite of heroin, 6-acetylmorphine [28]. On the other hand, since codeine is a morphine pro-drug and only 5% of the dose is O-demethylated to morphine, it is suggested that codeine analgesia does not depend on morphine formation [28, 29]. Pharmacokinetic features of codeine and the relative scarcity of behavioral model studies to evaluate the interaction between morphine and codeine justify this preclinical study. Furthermore, there have been clinical reports about differences in analgesic efficacy among MOR opioid agonists.

Considering the background mentioned above, the purpose of this study was to examine the analgesic interaction between morphine and codeine as not all opioid MOR agonists demonstrate synergism when ad-

ministered in combination [2]. In this study we used intraperitoneal (*ip*) or intrathecal (*it*) coadministration of codeine and morphine in three animal nociceptive behavioral models. The evaluation of interaction was performed through isobolographic analysis.

Materials and Methods

Animals

Male CF-1 mice (30 g), housed on a 12 h light-dark cycle at $22 \pm 2^\circ\text{C}$ with *ad libitum* access to food and water were used. Experiments were performed according to 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 University of Chile Medical School. Animals that 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 test. The number of animals was kept at a minimum compatible with consistent effects of the drug treatments. All assays were conducted by an experimented observer who was unaware of the drug treatment of each individual mouse.

Dose-response curves for administration of codeine (1, 3, 10 and 30 mg/kg, *via ip* and *it*) and morphine (0.01, 0.03, 1, and 3 mg/kg *via ip* and the same doses *via it*) were obtained using at least six animals for each of at least four doses, 30 min after drug application. A least-square linear regression analysis of the log dose-response curve allowed the calculation of the doses that produced 50% (ED_{50}) antinociception when each drug was administered alone (Tab. 1).

Tab. 1. ED_{50} values $\pm$ SEM (mg/kg) for the antinociceptive effect of codeine and morphine administration *ip* and *it* in the writhing, tail flick and orofacial formalin tests in mice

Test	Codeine <i>ip</i>	Codeine <i>it</i>	Morphine <i>ip</i>	Morphine <i>it</i>
Writhing	6.17 ± 0.92	$1.84 \pm 0.21^*$	0.12 ± 0.01	$0.016 \pm 0.003^\diamond$
Tail flick	46.95 ± 4.42	$29.99 \pm 2.24^*$	5.01 ± 0.92	$0.09 \pm 0.003^\diamond$
Orofacial formalin Phase I	5.76 ± 0.61	$2.33 \pm 0.84^*$	0.17 ± 0.02	0.21 ± 0.02
Orofacial formalin Phase II	9.33 ± 0.67	$1.40 \pm 0.45^*$	0.34 ± 0.02	$0.19 \pm 0.06^\diamond$

* $p < 0.05$ compared with codeine *ip*; $^\diamond$ $p < 0.05$ compared with morphine *ip* (Student's *t*-test)

Tab. 2. Theoretical, experimental ED₅₀ ± SEM (mg/kg) and interaction indexes (I.I.) values for the antinociceptive activity of *ip* or *it* administration of codeine combined with morphine in the writhing, tail flick and orofacial formalin tests in mice

Test	ED ₅₀ ± SEM theoretical	ED ₅₀ ± SEM experimental	I. I.
Writhing <i>ip</i>	3.15 ± 0.46	0.89 ± 0.09	0.285
Writhing <i>it</i>	0.93 ± 0.10	0.40 ± 0.02	0.433
Tail flick <i>ip</i>	25.98 ± 2.25	10.68 ± 1.90	0.411
Tail flick <i>it</i>	15.05 ± 1.11	5.21 ± 0.97	0.346
Orofacial formalin Phase I <i>ip</i>	2.95 ± 0.30	1.27 ± 0.07	0.432
Orofacial formalin Phase I <i>it</i>	1.27 ± 0.42	0.49 ± 0.06	0.384
Orofacial formalin phase II <i>ip</i>	4.84 ± 0.33	2.13 ± 0.11	0.442
Orofacial formalin Phase II <i>it</i>	0.80 ± 0.12	0.28 ± 0.03	0.345

All the results are significant ($p < 0.05$) compared ED₅₀ theoretical with ED₅₀ experimental (Student's *t*-test)

ED₅₀ was used in tests as the equieffective dose for isobolographic analysis because higher doses did not show increased effects without motor impairment [14, 17]. Then, a similar dose-response curve was also obtained and analyzed after the co-administration of codeine and morphine, in fixed ratio (1:1) combinations based on the mixture of 1/2, 1/4, 1/8, 1/16 of their respective ED₅₀ values, 30 min after the co-administration of the mixture. The following doses expressed in mg/kg were used for the isobolographic study: in the writhing test: morphine 0.12 *via ip* and 0.016 *via it*, codeine 6.17 *via ip* and 1.84 *via it*; in the tail flick assay: morphine 5.01 *ip* and 0.09 *it*, codeine: 46.95 *ip* and 29.99 *it*; for the orofacial formalin assay, in Phase I, morphine 0.17 *ip* and 0.21 *it*, codeine 5.76 *ip* and 2.33 *it*; in Phase II, morphine 0.34 *ip* and 0.19 *it* and codeine 9.33 *via ip* and 1.40 *via it*, as can be seen in Table 2.

Writhing test

The procedure used in this test has been previously described [13]. Mice were injected *ip* with 10 ml/kg of 0.6% acetic acid solution, 30 min after the *ip* or *it* administration of the drugs, time at which preliminary experiments showed occurrence of the maximum effect. A writhes is characterized by a contraction wave 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 a percentage of the maximum possible effect (% MPE) and converted as a percent inhibition of the number of writhes observed in control animals (20.15 ± 0.28 , $n = 8$, *ip* and 19.30 ± 0.35 , $n = 10$, *it*).

Tail flick test

The algosimetric test was similar to that described previously [17]. A radiant heat, automatic tail flick algosimeter (U. Basile, Comerio, Italy) was used to measure response latencies. The light beam was focused on the animal's tail about 4 cm from the tip and the intensity was adjusted so that baseline readings were between 2 and 3 s. An 8 s cut-off time was imposed to avoid damage to the tail. Control reaction time or latency of the response was recorded twice, with an interval of 15 min between readings, the second reading being similar to the first. Only animals with baseline reaction times between 2 and 3 s were used in the experiments. The values of control latencies were: 2.54 ± 0.07 , $n = 12$ *ip* and 2.45 ± 0.09 , $n = 12$ *it*. Tail flick latencies were converted to a % maximum possible effect (MPE) as follows:

$$\%MPE = \frac{(\text{post drug latency} - \text{pre drug latency})}{(\text{cut - off time} - \text{pre drug latency})}$$

Each animal was used as its own control. Drugs were administered *ip* or *it* 30 min before the experimental

protocol, time at which preliminary experiments showed occurrence of the maximum effect. The dose that produced 50% of MPE (ED_{50}) was calculated from the linear regression analysis of the curve obtained by plotting log dose vs. %MPE.

Orofacial formalin test

The method described by Miranda et al. [14] was used. Orofacial formalin-induced responses showed two distinct phases that were separated by a period of relative inactivity with an early short-lasting response (0–5 min, phase I) and a continuous prolonged response (20–30 min, phase II). To perform the test, mice were randomly assigned to different groups (6–8 per group) and 20 μ l of 2% formalin solution was injected into the upper lip, right next to the nose with a 27-gauge needle attached to a 50 μ l Hamilton syringe. The chemical stimulus (formalin) applied can be considered noxious since it produces tissue injury, activates A δ and C nociceptors as well as trigeminal and spinal nociceptive neurons and produces a painful sensation in humans [6]. Each mouse was immediately returned to the observation chamber. The test shows two clear cut phases: Phase I corresponds to the 5-min period starting immediately after the formalin injection and represents a tonic acute pain due to peripheral nociceptor sensitization. Phase II was recorded as the 10 min period starting 20 min after the formalin injection and represents inflammatory pain. The nociceptive score was determined for each phase by measuring the total number of seconds that the animals spent grooming the injected area with the ipsilateral fore or hind paw [7]. Drug or saline ($n = 8$) was administered *ip* or *it* to animals 30 min before formalin injection. Total grooming time in each period was converted to a percentage of maximum possible effect (MPE) as follows:

$$\%MPE = 100 - (\text{post drug grooming time/control grooming time saline}) \times 100$$

The dose that produced 50% of MPE (ED_{50}) was calculated from linear regression analysis of a dose-response curve obtained by plotting log doses vs. %MPE.

Isobolographic analysis

Isobolographic analysis was used to characterize drug interactions. The method of isobolographic analysis

has been described previously in detail [11, 12]. The isobologram was built by connecting the ED_{50} of the codeine plotted on the abscissa with the ED_{50} of the morphine plotted on the ordinate to obtain the additivity line. For each drug mixture, the ED_{50} and its associated 95% confidence intervals were determined by a linear regression analysis of the log dose-response curve (six or eight animals at each dose of at least 4 doses) and compared by a *t*-test to a theoretical additive ED_{50} obtained from the calculation:

$$ED_{50} \text{ add} = ED_{50} \text{ codeine} / (P1 + RP2),$$

where R is the potency ratio of the codeine alone to morphine alone, P1 is the proportion of codeine and P2 is the proportion of morphine in the total mixture. In this study, fixed-ratio proportions were selected by first combining the ED_{50} of each compound and then constructing a dose-response curve in which ED_{50} fractions (1/2, 1/4, 1/8 and 1/16) of codeine and morphine combinations were administered, in the equation above, ED_{50} add is the total dose and the variance of ED_{50} add was calculated from the fraction of the ED_{50} 's (i.e., 0.5) in the combination as:

$$\text{Var } ED_{50} \text{ add} = (0.5)^2 \text{Var } ED_{50} \text{ codeine} + (0.5)^2 \text{Var } ED_{50} \text{ morphine}$$

Confidence limits are calculated from these variances and resolved according to the ratio of the individual drugs in the combination. The ED_{50} for the drug combinations was obtained by linear regression analysis of the dose-response curves. A supra-additive or synergistic effect is defined as the effect of a drug combination that is higher and statistically different (ED_{50} significantly lower) than the theoretically ED_{50} calculated of a drug combination in the same proportion. 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 effect.

Furthermore, the interaction index (I.I.) or ratio of combination potency to additive potency, indicates the magnitude and nature of the interaction. The interaction index (I.I.) was calculated as:

$$I.I. = \text{experimental } ED_{50} / \text{theoretical } ED_{50}$$

If the value is close to 1, the interaction is additive. Values below 1 are an indication of the magnitude of supra-additive or synergistic interactions and values above 1 correspond to sub-additive or antagonistic interactions [25].

Drugs

All drugs were freshly dissolved in saline solution in a constant volume of 10 ml/kg when administered *ip*. When the administration was *it*, a constant volume of 5 μ l of a slightly hyper osmotic solution of glucose (6%, to limit diffusion) was used [9]. Codeine phosphate and morphine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO, USA. Doses were expressed based on the salts.

Statistical analysis

Results are presented as ED₅₀ values $\pm$ 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. PA, USA) based on Tallarida [26]. Results were analyzed by Student's *t*-test for independent means; *p* values less than 0.05 (*p* < 0.05) were considered statistically significant.

Results

The different doses of opioids used in this work did not produce significant behavioral or motor dysfunctions in the animals tested.

Antinociception induced by opioids

Both *ip* and *it* administration of codeine or morphine produced a dose-related antinociceptive activity with different potencies on the writhing, tail flick tests and in phases I and II of the orofacial formalin assays.

When administered *it*, codeine resulted to be 3.4 times more potent than when administered *ip* in the writhing test, 1.6 times more potent in the tail flick, 2.5 times in phase I and 6.7 times in phase II of the orofacial formalin test (see Figs. 1 and 2).

Relative potency of morphine administered *it* resulted to be 7.5 times higher than when administering *ip* on the writhing test, 55.6 times higher in the tail flick and 1.7 times higher in phase II of the orofacial formalin, however, in phase I of the same test, *ip* morphine was 1.2 times more potent than when was administered *it*, as it can be seen in Figures 1 and 2. Table 1 shows the corresponding ED₅₀ values of codeine and morphine for each algesiometric test.

Interaction between codeine and morphine

The interactions between the combination of codeine and morphine, with either *ip* or *it* administration, on the basis of the fixed ratio (1:1) of their ED₅₀ values alone, were calculated by isobolographic analysis. Fixed ratios (1:3 and 3:1) of their ED₅₀ were also analyzed, but the data are not shown, since this mixture was also synergic. The theoretical additive ED₅₀ values and the experimental ED₅₀ values for the fixed ratio (1:1) combination are shown in Table 2.

Statistical analysis using the data from the isobolographic analysis indicated that synergistic interactions occurred between codeine and morphine *ip* or *it* administered in the writhing, tail flick and orofacial formalin tests. These results are shown in Figures 3 and 4.

Furthermore, the I.I. indicating the magnitude and nature of the interaction when two drugs are combined, demonstrated the following rank of potencies for the combination of codeine and morphine in the different algesiometric tests: 0.284 (writhing *ip*), 0.346 (orofacial formalin phase II *it*), 0.350 (tail flick *it*), 0.385 (orofacial formalin phase I *it*), 0.411 (tail flick *ip*), 0.430 (orofacial formalin phase I *ip*), 0.430 (writhing *it*) and 0.440 (orofacial formalin phase II *ip*). All these results are shown in Table 2.

Discussion

The addition of a second agent to an opioid, which may or not also be an analgesic, is a combination that may prolong analgesic duration, enhance analgesic efficacy, diminish or minimize adverse effects and could reduce opioid tolerance or dependence [21]. Thus, the combinations that enhance opioid analgesic efficacy (e.g., synergism) include association with norepinephrine transport modulators, anti-inflammatory drugs, calcium channels α_2 - δ ligands, local anesthetics, α_2 -adrenergic agonists, calcium channel blockers, cannabinoids, GABA_B agonists, glial inhibitors, histamine and venlafaxine [4, 21, 27].

The findings of this study demonstrated that codeine and morphine, typical MOR agonists, possesses a marked antinociceptive dose-dependent activity, independently of the animal models of nociception or the nociceptive stimulus, i.e., acute tonic pain (acetic acid writhing test), acute phasic pain (tail flick test)

Fig. 1. Dose-response curves for the antinociceptive activity in mice induced by codeine and morphine administered *ip* (●) or *it* (○) in the writhing test (**A** and **B**) and in the tail flick assay (**C** and **D**). Each point is the mean $\pm$ SEM of 6–8 animals. %MPE = antinociception represented as a percentage of maximum possible effect, see Materials and Methods

Fig. 2. Dose-response curves for the antinociceptive activity in mice induced by codeine and morphine administered *ip* (●) or *it* (○) in the orofacial formalin phase I (**A** and **B**) and in the orofacial formalin phase II (**C** and **D**). Each point is the mean $\pm$ SEM of 6–8 animals. %MPE = antinociception represented as percentage of maximum possible effect, see Materials and Methods

Fig. 3. Isobolograms for the administration of the combination of codeine and morphine in the acetic acid writhing test in mice (**A**, *ip*, **B**, *it*); in the tail flick test in mice (**C**, *ip*, **D**, *it*). Filled circles (●) correspond to the theoretical ED_{50} with 95% CL and open circles (○) correspond to the experimental ED_{50} with 95% CL.

Fig. 4. Isobolograms for the administration of the combination of codeine and morphine in phase I (**A**, *ip*, **B**, *it*) and phase II (**C**, *ip*, **D**, *it*) of the orofacial formalin test in mice. Each point represents the mean $\pm$ SEM of 6–8 animals. %MPE = antinociception represented as percentage of the maximum

and inflammatory pain (orofacial formalin test) [6]. In addition, this work confirmed the different relative potency of codeine or morphine in several types of tests in mice [10, 19]. This antinociceptive action of codeine and morphine is reflected in parallel dose response curves for the different assays with the exception of the orofacial formalin test in phase II. This could be explained by the inflammatory nature of this phase of the assay.

The synergism obtained in this study conflicts with previous reports in which the interactions between agonists acting on the same opioid receptor subtype have shown only additive effects [2].

The synergism found in this study, could possibly relate to the activation of different groups of opioid receptors and their subtypes which could be mediated by modulation of membrane potential, deactivation of adenylate cyclase, a receptor trafficking or descending inhibition effect. The mechanism of synergism is complex and these findings may be influenced by other antinociceptive systems such as GABAergic and glycinergic neurons which are important factors related to opioid's antinociceptive potency.

Furthermore, if each opioid ligand has its own intrinsic efficacy or maximal effect [5], these properties may be used by codeine and morphine to interact with different opioid receptor subpopulation or modulating MOR receptor signalling in slightly different ways [3]. Up to date, the molecular biological approach has identified 15 splice variants of MOR receptors [22, 29]. On the other hand, synergism has been demonstrated between agonists that activate receptors in different G protein-coupled receptor families [2, 29].

Additionally, the differences in the pharmacological profiles of morphine and codeine could help to explain the synergism obtained in this study. Thus, the inhibition of central GABA synaptic transmission by morphine produces activation of central pain inhibiting neurons, which is one of the major actions responsible for opioid-induced analgesia [8]. The combination of morphine and codeine, can induce lesser inhibition of central pain inhibiting neurons that produces a supra additive effect in their antinociceptive activity, as it has been reported with the coadministration of other opioids agonists [15, 30]. On the other hand, the supra additive action obtained with the co-administration of codeine and morphine may be correlated to the reported synergism between agonists that activate receptors in different G protein-coupled receptor families [2, 5] by decreasing intracellular cAMP that

modulates the release of nociceptive neurotransmitters, i.e., substance P [3].

Besides, the opioid receptors are coupled to $G_{i/s}$ proteins and their actions are mainly inhibitory. Furthermore, these actions could be exerted by codeine and morphine with different efficacy and consequently it is possible to obtain a synergistic antinociceptive activity. In addition, the results of this study, in concordance with previous studies using the orofacial formalin test, demonstrated that activation of peripheral MOR provides greater anti-nociception in inflamed tissue, and that the enhanced MOR effect can be partly explained by significant up-regulation of MOR expression in the tissue [16]. Moreover, it has been reported that opioid agonists can decrease swelling due to increased excitability of primary afferent neurons and the release of proinflammatory neuropeptides as substance P [3]. In injured tissue, as well as in orofacial formalin and acetic acid writhing tests, these event leads to antinociceptive and anti-inflammatory effects [23]. Furthermore, peripheral inflammation can induce differential up-regulation of opioid receptor mRNA and protein in dorsal root ganglia. Thus, the expression of opioid receptors depends on receptor type and the duration of inflammation [2, 19].

Although the mechanisms underlying synergistic interactions are not well understood, synergy could be the result of the simultaneous action of the two agents at two distinct sites, such as a common receptor located at different anatomical sites or distinct receptors corresponding to a common anatomical location. Additionally, another explanation for the synergism obtained in this study, could be consistent with the proposed multiple subpopulation of MOR receptors, activated by codeine and morphine, which are colocalized in a single subcellular compartment (i.e., primary afferent terminals). This event enables the formation of heterodimeric complexes accounting for the change in G protein coupling. An interesting aspect of this heterodimeric association is the possibility of novel pharmacological properties distinct from either component receptor alone [23].

In conclusion, our findings suggest the presence of functional interactions among MOR opioid analgesics. These observations seem consistent with the involvement of multiple subpopulations of MOR opioid receptors. These findings also raise the possibility of potential clinical advantages in combining opioids in pain management.

Acknowledgments:

This work was partially supported by project DI-02-11/CB from Universidad Andrés Bello. The expert technical assistance of José López and Alejandro Correa is gratefully acknowledged.

Competing interests:

The authors have no conflicts of interests.

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Received: November 9, 2011; **in the revised form:** June 26, 2012; **accepted:** September 4, 2012.