Intestinal Inflammation and Morphine Tolerance Alter the Interaction between Morphine and Clonidine on Gastrointestinal Transit in Mice

Margarita M. Puig, M.D., Ph.D.*, William Warner, Ph.D.,† Olga Pol, Ph.D.‡

Background: Morphine and clonidine show synergy or antagonism inhibiting gastrointestinal transit depending on their proportion and level of effect. Their interaction during morphine tolerance and intestinal inflammation were assessed.

Methods: Gastrointestinal transit in mice was evaluated with charcoal and antitransit effects expressed as percent mean values ± SEM. Tolerance was induced with a morphine pellet (75 mg) implanted for 72 h, and inflammation with intragastric croton oil. Dose–response curves for morphine and clonidine alone and combined at a 1:1 potency ratio were obtained, and doses producing a 50% and 60% inhibition were calculated (ED50, ED60). Interaction was established by isobolograms, interaction indexes, and analysis of variance.

Results: In naive and tolerant mice, the combination induced linear dose–response curves up to the ED50, and then reached a plateau. In naive mice, ED50 values were as follows: morphine 1.52 ± 0.15 mg/kg, clonidine 0.09 ± 0.008 mg/kg, and combined 0.506 ± 0.084 mg/kg (0.478 ± 0.08 mg/kg morphine plus 0.028 ± 0.004 mg/kg clonidine). During tolerance, ED50 values were as follows: morphine 0.75 ± 0.8 mg/kg, clonidine 0.09 ± 0.007 mg/kg, combination 0.131 ± 0.09 mg/kg (morphine 0.13 ± 0.09 mg/kg plus clonidine 0.0013 ± 0.0005 mg/kg). In both groups, the interaction was synergistic up to the ED50 and antagonistic thereafter; synergy was enhanced during tolerance.

During inflammation, ED50 values were as follows: morphine 0.17 ± 0.04 mg/kg, clonidine 0.015 ± 0.006 mg/kg, combined 0.62 ± 0.04 mg/kg (morphine 0.568 ± 0.04 mg/kg plus clonidine 0.052 ± 0.004 mg/kg); thus, potencies of morphine and clonidine increased 9.3 and 7.1 times, while the combination remained unaltered. Moreover, inflammation transformed synergy into antagonism.

Conclusions: The interaction between morphine and clonidine was significantly altered during tolerance and inflammation. During tolerance, synergy was present up to 60% effect and then became antagonistic. Inflammation converted synergy to antagonism. A common pathway in signal transduction could partially explain the results. (Key words: α2-Agonists; antitransit effects; drug combinations; drug interactions; gut; opioids.)

THE antinociceptive effects of the combination of morphine and clonidine have been reported to be synergistic in different animal models.1,2 In humans, opioids and α2-adrenergic agonists are both used as analgesics, and recent reports suggest that when combined they produce a more potent and longer-lasting analgesia3,4; however, the demonstration of synergy in humans has been elusive.5 When morphine or clonidine are used as analgesics in humans, ileus and constipation are common side effects.6–12 Thus, the evaluation of the antitransit effects of these drugs, individually or combined, could help to anticipate the degree and characteristics of gastrointestinal effects in humans.

In different animal species, morphine and clonidine each produce inhibition of gastrointestinal transit (GIT)13,14 and permeability.15 In our laboratory, we have evaluated the interaction between morphine and clonidine on GIT, demonstrating that the type of interaction depends on the ratio of the doses of the drugs used and the level of response.16 It has been postulated that different experimental and pathologic conditions might alter the type of interaction between two or more agents.17–19 In the present study, we evaluated morphine–clonidine interactions on the inhibition of GIT in morphine-tolerant mice and in the presence of intestinal inflammation. Tolerance to the analgesic effect of mor-
Tolerance occurs after chronic morphine administration, and clonidine has been used to delay or lessen its development; however, tolerance to the antitransit effects of morphine, clonidine, or their combinations is not well defined. In addition, inflammation of the gut enhances the antitransit effects of opioids and α2-adrenergic agonists, but the effect of the combination in animals with inflammatory diarrhea has not been evaluated.

The aim of the present investigation was to characterize the morphine-clonidine interaction on GIT in two nonphysiologic conditions: tolerance to morphine and the presence of intestinal inflammation.

Materials and Methods

The protocol was approved by the Institutional Committee of Animal Use and Care. Male Swiss CD-1 mice (20-25 g), housed under controlled standard conditions (12 h light/dark, 22°C temperature, and 66% humidity) were used in the study. Animals had free access to food and water and were acclimated to the housing conditions for at least 1 week before use. All experiments were conducted between 9 AM and 3 PM.

Estimating Gastrointestinal Transit

Gastrointestinal transit was measured according to procedures used in our laboratory. Briefly, food was removed 18 h before the experiment, but animals had free access to water. After 18 h of fasting, a charcoal meal (0.25 ml of a suspension of 10% vegetable charcoal in 5% gum acacia) was administered using a rigid canula introduced through the mouth into the esophagus (orally) in awake animals. In control animals, GIT was evaluated 20 min after charcoal administration, because at this time the marker has traveled approximately 50% of the length of the small intestine, thus permitting the evaluation of the inhibition or increase in GIT (GIT time in the small intestine for control animals was 42-45 min). Animals were killed by cervical dislocation, and the small intestine was separated from the omentum, avoiding stretching. The length of intestine from pyloric sphincter to the ileocecal junction and the distance traveled by the charcoal meal were measured. For each animal, GIT was calculated as the percent of the distance traveled by the charcoal, relative to the total length of the intestine (percent of GIT). The inhibitory effects of drugs on GIT are expressed as a percentage of inhibition of the GIT in a drug-treated animal (test GIT) when compared with the mean GIT measured in a group of vehicle-treated mice (n = 10):

\[
\% \text{ inhibition} = \left\{ \frac{\text{vehicle GIT} - \text{test GIT}}{\text{vehicle GIT}} \right\} \times 100.
\]

Induction of Tolerance to Morphine

Under light ether anesthesia, mice were randomly implanted with 75 mg morphine base or placebo pellets at the nape of the neck. GIT was determined 72 h after pellet implantation. Figure 1 shows the experimental design used for the study.

Intestinal Inflammation

In this study we used a model of intestinal inflammation produced after the intragastric administration of an irritant and cathartic agent (croton oil). Intestinal inflammation was induced by the oral administration of two doses (0.05 ml) of croton oil, 24 h apart, according to the method; control animals received the same volume of saline. In both instances, animals were fasted for 18 h before croton oil or GIT testing (charcoal), except for free access to water. GIT was measured 96 h after the first dose of croton oil. Morphologic changes induced by croton oil have been previously reported by our group and were established by optical microscopy. They included a disruption of the mucosa and the infiltration of lymphocytes in the submucosa. In addition, animals treated with croton oil (but not those receiving saline)
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Table 1. Groups of Experiments (Dose–Response Curves)

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Morphine</th>
<th>Clonidine</th>
<th>Morphine + Clonidine (1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive (placebo pellet)</td>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 3 (one ED₉₀ from group 1 plus one ED₉₀ from group 2)</td>
</tr>
<tr>
<td>Tolerant (morphine pellet)</td>
<td>Group 4</td>
<td>Group 5</td>
<td>Group 6 (one ED₉₀ from group 4 plus one ED₉₀ from group 5)</td>
</tr>
<tr>
<td>Tolerant fixed clonidine (0.02 mg/kg)</td>
<td>Group 7</td>
<td>—</td>
<td>Group 10 (one ED₉₀ from group 8 plus one ED₉₀ from group 9)</td>
</tr>
<tr>
<td>No inflammation (saline by mouth)</td>
<td>Group 8</td>
<td>Group 9</td>
<td>Group 13 (one ED₉₀ from group 11 plus one ED₉₀ from group 12)</td>
</tr>
<tr>
<td>Inflammation (croton oil by mouth)</td>
<td>Group 11</td>
<td>Group 12</td>
<td>—</td>
</tr>
</tbody>
</table>

Dose-response curves to morphine or clonidine alone were obtained in the different experimental conditions (naive, tolerant, noninflamed, inflamed), and the ED₉₀s were calculated. For the morphine–clonidine combinations, one ED₉₀ of morphine plus one ED₉₀ of clonidine were combined (from their corresponding dose–response curves), and the dose–response curve of the combinations was obtained (1:1 potency ratio). The different experimental conditions (tolerance and inflammation) changed the potency of morphine and clonidine (ED₉₀s) and, therefore, the actual doses combined (mg/kg) were different (all producing a 50% inhibition of gastrointestinal transit). In experiments in group 7, a dose-response curve to morphine was obtained in the presence of 0.02 mg/kg clonidine (see Methods).

Presented with weight loss (20.8 ± 0.8%) and increased GIT (25.2 ± 2.0%).

Experiments Performed

Dose-response relationships were first obtained for morphine and clonidine individually, and their median effective doses (ED₉₀s) were calculated. In all experiments, six to eight mice were tested per each dose point. Initially, six mice were used for each point; however, when the observed effects in one of the animals deviated from the other five, two additional mice were tested, and the mean value of the eight mice was used for evaluation of the results.

In the present study, ED₉₀ (a measure of potency) is defined as the dose of a drug or a combination of drugs that produces a 50% inhibition of GIT. Similarly, the ED₂₀, ED₆₀, and ED₉₀ correspond to the doses that produce 20%, 60%, and 80% inhibition, respectively. The term Eₘₐₓ is used to designate the maximal inhibitory effect observed after the administration of a single drug or their combination; actually, the Eₘₐₓ indicates the maximal effect that can be obtained when increasing the doses of the drugs or their combination. The value is calculated on the basis of the experimental points that form the linear segments of the curves.

Dose-response curves were also obtained with combinations of both drugs. The doses used in fixed potency ratio experiments were multiples of their respective ED₉₀s, e.g., in saline-treated animals, multiples of 1.6 and 0.107 mg/kg of morphine and clonidine, respectively. These mixtures were termed 1:1 combinations (one ED₉₀ of morphine combined with one ED₉₀ of clonidine, indicating a 1:1 potency ratio). From the dose–response curves to the combination, the ED₉₀ and ED₀ were calculated by linear regression analysis, using the points that formed the linear segments of the curves. Because the dose–response curves of the combination were obtained with a mixture of the two drugs, the results are expressed as total milligrams (resulting from adding up the milligrams of morphine plus the milligrams of clonidine). Similar experiments were also performed in animals that were tolerant to morphine or that had croton oil–induced intestinal inflammation. In each of these cases, different doses were used, but they were always combined at a 1:1 potency ratio (using the ED₉₀s obtained under each of the two experimental conditions).

A total of 13 groups of experiments were conducted (table 1). We evaluated dose–response relations for morphine, clonidine, and their 1:1 combination (one ED₉₀ of morphine plus one ED₉₀ of clonidine) in the following experimental protocols: (1) naive and (2) morphine-tolerant mice, and (3) saline- and croton oil–treated mice. An additional group of experiments was conducted in tolerant animals in which a complete dose–response curve to morphine was obtained in the presence of a fixed dose of clonidine.

To maintain standardized conditions and minimize errors, control and treated animals were evaluated in parallel on the same day. Animals were randomly assigned to the control or treatment groups, and the technician performing the test (GIT) was blinded as to the treatment administered.

Data Analysis

The results are expressed as percent inhibition of GIT. Statistical calculations were performed as described by Tallarida and Murray. Because all dose–response curves had a linear segment, effective doses (EDs) were determined by linear regression analysis of this segment, based on at least five different doses (data points) and six to eight mice per dose. Individual slopes of the dose–response curves were compared by Student t test, according to the test for parallelism described in Tal-
larida and Murray. To evaluate the interaction of morphine and clonidine, we used two methods of analysis: isobolographic analysis and fixed-dose analysis.

**Construction of Isoboles.** Isoboles are graphic representations of the doses of two (or more) drugs used individually or in combination that are needed to produce a specified level of response (e.g., ED₅₀). The doses were obtained from their respective dose-response curves. In constructing an isobole, the dose of each drug that individually produces a given level of response is plotted on the axes of the graph. A diagonal line is then drawn to join the isoeffective doses. The doses of each drug used in combinations producing the same effect are then plotted. Points falling on the diagonal line represent zero interaction (additivity), whereas those located above and below are antagonistic and synergistic, respectively. Mean and SEM values were calculated for all the doses plotted, and the doses expected in the absence of interaction (those on the isobole line) were compared with those obtained experimentally (observed) using a Student t test. The diagonal noninteraction line of the isobole is described by the equation \( \frac{da}{Da} + \frac{db}{Db} = 1 \). In this equation, \( Da \) and \( Db \) are the doses of the two drugs that individually produce the specified level of response; \( da \) and \( db \) are the combined doses that also produce that response. The sum of these quotients is the interaction index. An interaction index of 1 indicates additivity (no interaction). If the index differs from 1 an interaction exists: either synergy (index < 1) or antagonism (index > 1). Using this type of analysis, an interaction can be established only for a given drug ratio and level of effect, and the results cannot be extrapolated to other drug combinations.

**Fixed-dose Analysis.** In the fixed-dose experiments, we compared a dose-response curve of morphine alone with a dose-response curve to morphine in the presence of a small, fixed dose of clonidine (0.02 mg/kg). This dose was selected because it produces a 10% inhibitory effect that, although small, can be measured and evaluated for statistical purposes. In these experiments, the data was plotted in a log dose-response manner, and the \( E_{\text{max}} \) was estimated. In this type of analysis, when the dose-response curve of the combination is shifted to the right, an antagonistic interaction is presumed, whereas a displacement to the left suggests synergy. The drug interaction is statistically corroborated when the sum of the effects produced by each agent alone (the expected effect) is significantly different from the observed effect of the combination. Two-factor analysis of variance was used to demonstrate the presence of statistical differences between two treatment groups. We compared the responses obtained with morphine alone with those of morphine in the presence of 0.02 mg/kg of clonidine, and from these data the effects of the drugs, dose, and their interaction were determined. In this calculation, a significant effect of the drugs indicates statistical differences between the two treatment groups; a significant effect of the dose indicates that the observed responses vary significantly between different doses tested, and a significant interaction shows that the differences between the treatment groups depend on the dose administered. If all factors (drug, dose, and interaction) are significantly different, the analysis indicates that the effects of the combination are different from additivity, whereas if the interaction is not statistically significant, the effects are considered to be additive. To identify the type of interaction, the dose-response curves obtained with each treatment are also shown. This method is not applicable when dose-response curves are parallel, because it cannot differentiate between parallel-additive and parallel-synergistic or -antagonistic combinations.

**Drugs**

Drugs used in the study were obtained from several sources: morphine sulphate from Alcaiber S.A., Madrid, Spain, and clonidine hydrochloride from Research Biochemicals, Wayuland, MA. All drugs and their combinations were prepared in pyrogen-free water just before use and administered in a final volume of 10 ml/kg. Drugs were injected subcutaneously at the nape of the neck 30 min before the administration of charcoal, and GIT was assessed 20 min later. The time of injection was selected to coincide with the peak effect for gastrointestinal inhibition after subcutaneous administration of the drugs, occurring approximately 45 min after injection.

**Results**

**Naïve Mice**

Dose-response relationships to morphine, clonidine, and their 1:1 combination were obtained in naïve mice (implanted with a placebo pellet), and the results are shown in figure 2 (top). In these animals, calculated ED₅₀ values for morphine and clonidine were 1.52 ± 0.15 and 0.09 ± 0.008 mg/kg, respectively (table 2); thus, clonidine was approximately 16.8 times more potent than morphine as an inhibitor of GIT. Unlike the
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Table 2. ED$_{50}$ Values (mg/kg) of Morphine (MS), Clonidine (CL), and Their 1:1 Combination (MS + CL) in Naive (Placebo Pellet) and Morphine-tolerant (Morphine Pellet) Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MS</th>
<th>CL</th>
<th>MS + CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo pellet</td>
<td>1.52 ± 0.15</td>
<td>0.09 ± 0.008</td>
<td>0.506 ± 0.084</td>
</tr>
<tr>
<td>(PL, naive)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphine pellet</td>
<td>9.73 ± 0.80</td>
<td>0.0942 ± 0.0068</td>
<td>0.131 ± 0.09</td>
</tr>
<tr>
<td>(TOL, tolerant)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio (TOL/PL)</td>
<td>6.4</td>
<td>1.05</td>
<td>0.259</td>
</tr>
</tbody>
</table>

ED$_{50}$ values were obtained from the log dose–response curves shown in figure 2. For the MS + CL combination, the results are expressed as total (MS + CL) mg/kg.

Doses of up to 2.41 mg/kg of the 1:1 combination failed to produce a greater effect. These results coincide with those previously reported by our group in nontreated animals. In the present experiments, the dose–response curve for the combination morphine–clonidine in naive animals had an ED$_{50}$ value of 0.506 ± 0.084 mg/kg (total milligrams of the combination), of which 0.478 ± 0.08 mg/kg was morphine and 0.028 ± 0.004 mg/kg was clonidine. The linear segments of all of the three curves were parallel, thus permitting the comparison of their potencies.

**Fig. 2.** Log dose–response curves to morphine (circles) and clonidine (squares) individually or combined (triangles) in a 1:1 proportion. In ordinates we represent the percent inhibition of gastrointestinal transit and in abscissa, the doses of morphine or clonidine alone or their combination (mg/kg). Each point is the mean ± SEM of six to eight mice. Naive (top) and tolerant mice (bottom).

**Morphine-tolerant Mice**

**Dose–Response Curves to Morphine, Clonidine, and Their 1:1 Combination.** Dose–response relationships for morphine, clonidine, and their 1:1 combination were also obtained in morphine-tolerant mice (implanted with a morphine pellet), and the results obtained in these animals are shown in the lower panel of figure 2. In tolerant mice, the dose–response curve to morphine was shifted to the right, and the potency of morphine (ED$_{50}$ 9.73 ± 0.80 mg/kg) decreased approximately 6.4 times when compared with placebo, thus demonstrating the presence of tolerance to morphine. No significant changes in the dose–response curve to clonidine were observed in morphine-tolerant animals (ED$_{50}$ 0.09 ± 0.006 mg/kg; table 2). The results show the absence of cross-tolerance between clonidine and morphine. When morphine–clonidine combinations (1:1 potency ratio) were administered to tolerant animals, the resulting dose–response curve had an $E_{max}$ of 67.6%, after which the curve showed a plateau; the response remained unaltered even after doses of 14.6 mg/kg of the combination were administered. The ED$_{50}$ of the 1:1 combination in tolerant animals was 0.131 ± 0.09 mg/kg, of which 0.13 ± 0.09 was morphine and 0.0013 ± 0.0005 mg/kg was clonidine. Thus, in tolerant animals, 50% inhibition of GIT was achieved by significantly lower doses of the drug mixture.
Table 3. Type of Interaction between Morphine and Clonidine (1:1 Potency Ratio) in Placebo-implanted (Naive) and Morphine-tolerant Mice

<table>
<thead>
<tr>
<th></th>
<th>MS + CL</th>
<th>ED(_{50})</th>
<th>ED(_{90})</th>
<th>60% Effect (plateau)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>Synergy</td>
<td>(0.60*')</td>
<td>(0.58*')</td>
<td>Synergy Antagonism</td>
</tr>
<tr>
<td>Tolerant</td>
<td>Synergy</td>
<td>(0.027**')</td>
<td>(0.052**')</td>
<td>Synergy Antagonism</td>
</tr>
</tbody>
</table>

\(ED_{50}\) and \(ED_{90}\) values were obtained from the linear segments of dose-response curves in naive and tolerant mice; the curves showed a plateau after 60% effect (fig. 2). The type of interaction at 60% inhibition was calculated from the linear dose-response curve (\(ED_{50}\)) and the plateau (see text). Synergy and antagonism represent statistically significant deviations from additivity. In parentheses, values of the interaction indexes.

\* \(P < 0.05\) (Student t test).
\** \(P < 0.01\) (Student t test).

MS = morphine; CL = clonidine.

When the \(ED_{50}\)s in naive and tolerant mice were compared, the data showed: (1) a sixfold decrease in the potency of morphine in tolerant mice (a demonstration of tolerance); (2) the absence of cross-tolerance between morphine and clonidine; (3) that the combination regimen was 3.18 times more potent than morphine alone in placebo-treated animals; and (4) that the combination regimen was 74.8 times more potent than morphine alone in tolerant animals.

The isobolographic and interaction index analysis of the morphine:clonidine combination showed that for the linear portion of the curve (i.e., levels of effect up to the \(E_{\text{max}}\)), the interaction was synergistic, both in naive and tolerant mice. However, the interaction became antagonistic when the doses of the combination were further increased (on the plateau). Table 3 shows the type of interaction obtained at different levels of effect and that the magnitude of synergy seems to be enhanced in morphine-tolerant animals.

Because the \(E_{\text{max}}\) value of the combination was approximately 60% in both naive and tolerant animals, the interaction at the 60% level of response was evaluated using both interaction indexes and isobolographic analysis. Two sets of values were used: the estimated \(ED_{50}\) from the linear segments of the dose-response curves (fig. 2), and the doses (obtained experimentally) that produced a 60% effect on the plateau, where the dose-response curves of the combination cross the curve of morphine (fig. 2). The experimentally obtained values used in this calculation were 2.41 mg/kg in naive animals, which produced a 60.3 ± 1.9% inhibition, and 14.6 mg/kg in tolerant animals, which produced 59.7 ± 1.7% inhibition. Our results show that at the 60% level of response, the interaction was synergistic with respect to the linear segment of the curve, but it became antagonistic on the plateau. Figure 3 graphically represents the isobolographic analysis of the interaction at the 60% effect level in naive and tolerant animals. The isoboles graphically illustrate that the degree of synergy between morphine and clonidine is significantly increased in morphine-tolerant mice (Student \(t\) test, \(P < 0.05\)), and that at the 60% level of response the interaction can be synergistic or antagonistic, depending on the doses evaluated.
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Fig. 4. Log dose–response curves to morphine alone (circles) and in the presence of a fixed dose (0.02 mg/kg) of clonidine (triangles) in animals tolerant to morphine. In the ordinates we have represented percent inhibition of gastrointestinal transit, and in the abscissa, doses in milligrams per kilogram. Each point is the mean ± SEM of six to eight mice.

Fixed-dose Analysis of the Morphine–Clonidine Interaction. To further characterize the morphine–clonidine interaction in tolerant mice, we obtained a dose-response curve to morphine in the presence of a fixed dose of clonidine (0.02 mg/kg) that produced approximately a 10% inhibition of GIT (fig. 4). In these experiments, clonidine induced a shift to the left of the dose-response curve of the opioid at morphine doses of 0.6–3 mg/kg. When larger doses of morphine were used (5–60 mg/kg) the dose-response curve reached a plateau. In constructing the curves, the responses were separated into two segments, the first one comprising the linear segment of the dose–response, and a plateau segment starting at 3 mg/kg (the inflection point of the dose–response curve) and including all of the higher doses tested (up to 60 mg/kg). The dose-response curves obtained in these experiments (fig. 4) were not parallel, and consequently we were able to use a two-factor analysis of variance to establish the presence of an interaction. We compared the dose–response curve of morphine alone to the ascending linear segment of the dose-response curve of the drug combination and to the horizontal line that formed the plateau. Each segment was analyzed separately. Analysis of the ascending segment showed significant effects attributable to the drugs, the dose, and their interaction (P < 0.0001). However, at the plateau, no significant effect attributable to the drugs was observed (P < 0.147), whereas the dose (P < 0.0001) and the interaction (P < 0.0001) remained highly significant. The results demonstrate the presence of an interaction between morphine and clonidine at both segments of the response.

The type of interaction can also be determined by comparing the inhibitory effects of morphine to the effects of morphine plus a fixed dose of clonidine. For each dose, synergy can be established when the sum of the observed effects of each agent individually (at each point tested) is smaller than the effect of the combination. In our experiments, a dose of 0.02 mg/kg clonidine produced a 10.8 ± 1.9% inhibition of GIT. When the effect produced by clonidine alone was added to the effect produced by each dose of morphine alone (from 1 to 3 mg/kg), the observed effects of the combination were always greater than expected (Student t test, P < 0.01). Antagonism was shown in these experiments by the existence of a plateau in the combination dose–response curve; increasing doses of morphine (from 5 to 60 mg/kg) in the presence of clonidine produced smaller effects than morphine alone (fig. 4). These experiments confirm the results obtained with the isobolographic analysis of the interaction.

Intestinal Inflammation

Dose–response curves to morphine, clonidine, and morphine:clonidine (1:1 potency ratio) were obtained in mice with intestinal inflammation. Animals receiving oral saline served as control. No significant differences were observed between the curves obtained in placebo-implemented mice (fig. 2) and oral saline controls; thus, the dose–response curve to the combination was linear up to an E_max of 60% and then reached a plateau. Figure 5 shows the dose–response curves obtained in controls and during inflammation. In the latter experiments, the dose–response curves to morphine and clonidine individually were each shifted to the left, demonstrating that inflammation increased the potency of the agonists. However, the dose–response curve of the morphine-clonidine combination was shifted to the right and had a similar E_max (80%) as those of the individual drugs. Thus, the presence of inflammation significantly altered the characteristics of the dose–response curve to the mor-
phine–clonidine combination. Table 4 shows the potency (ED$_{50}$ values) of morphine, clonidine, and their combination in control animals and during inflammation. In controls, the calculated ED$_{50}$ values for morphine and clonidine were 1.62 ± 0.16 and 0.107 ± 0.01 mg/kg, respectively, whereas the ED$_{50}$ of the morphine–clonidine combination was 0.555 ± 0.075 mg/kg. The presence of inflammation increased the potency of morphine approximately 9.3 times, and that of clonidine was increased 7.1 times, whereas the potency of the combination remained unaltered.

The morphine–clonidine interaction was evaluated in the presence of intestinal inflammation and saline controls, using both isobolograms and interaction indexes. In control animals, the interaction was synergistic up to the ED$_{50}$ value and became antagonistic when the dose–response curve reached a plateau (fig. 5, top). During inflammation, the analysis of the morphine:clonidine interaction demonstrated antagonism at all levels of effect, and the values of the interaction indexes at the ED$_{50}$ are shown in table 4. Figure 6 shows the isoboles obtained for the morphine:clonidine interaction in controls and during inflammation, both at the 50% level of response. The results demonstrate that the presence of intestinal inflammation changes the type of interaction between morphine–clonidine when they are administered at a 1:1 potency ratio.

Discussion

Morphine and clonidine are clinically used in humans, alone or in combination, in the treatment of acute and chronic pain. Although the drugs have independent mechanisms of action, they share several properties such as analgesia, sedation, ileus, constipation, and the development of tolerance after repeated administration. Beneficial (e.g., analgesia) and side effects occur simultaneously, and to decrease the latter, morphine and clonidine have been successfully used in combination. When two or more drugs are administered simultaneously, the type of interaction (synergy, antagonism, no interaction) for all pharmacologic effects should be evaluated before the combination can be considered useful.

Intestinal disorders are very frequent in patients treated with opioids. Although the precise incidence and extent of the GIT effects in humans is unknown, studies performed in experimental animal models generate information that can serve as guidelines for human studies. Sensitivity of mice to morphine is quantitatively different than in humans (less than one tenth as potent), but their effects on pain perception and inhibition of GIT are qualitatively similar. The gastrointestinal effects of opioids in small mammals (guinea pig, rat, mice) and humans share a common characteristic: they all slow the propulsion of intestinal contents and cause constipation.
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Several animal models have been successfully used to screen the effects of opioids for their antidiarrheal activity, and extrapolations to humans using the transit of a charcoal meal in rodents have been instrumental in the development of gut selective, clinically effective, antidiarrheal opioids. Moreover, the same types of opioid receptors, mainly μ and δ, located both at central and peripheral (intestinal) sites mediate the GIT effects of opioids in rodents and humans. However, the receptors differ considerably in their relative density and distribution in the gut. Thus, the results obtained in animals can be used only as a guideline or to complement clinical studies.

The effects of the combination of morphine and clonidine on GIT in situations such as morphine tolerance or intestinal inflammation have not been investigated. Because these circumstances occur in clinical practice, a preliminary assessment of the effects of the combination using a well-validated animal model could be useful to complement (but not replace) subsequent studies in humans. Similarly, tolerance to the constipating effects of morphine in humans has not been carefully evaluated or quantified. Chronic treatment with morphine produces tolerance to the analgesic effects of the drug. Consequently, increasing doses of opioids are required to obtain adequate analgesia. These patients present with constipation that is probably related to the higher doses of opioids administered. Thus, it is possible that tolerance develops to the constipating effects of morphine at a different rate, and possibly to a lesser degree, than to analgesia. For example, in mice, the development of tolerance to the gastrointestinal effects of opioids has been shown to follow a different pattern than tolerance to other pharmacologic effects such as antinociception. In our model, tolerance was manifested by a sixfold decrease in the potency of morphine, thus supporting the results obtained by other investigators in small rodents. Until the pattern and extent of tolerance to the GIT effects of morphine in humans is fully investigated, we could assume that the human gut behaves in a qualitatively similar manner to other species.

The gastrointestinal effects of α2-adrenergic agonists, such as clonidine, and the presence of α2-adrenergic receptors in the intestine of small rodents have been clearly demonstrated. However, in humans, the intestinal effects of systemic clonidine are poorly characterized, although several studies demonstrate the constipating/antidiarrheal effects of the drug in humans. The results of the present study show that the antitransit effects of the combination of morphine and clonidine can be synergistic or antagonistic according to the level of effect of the drugs combined. After defining the interaction between morphine and clonidine on GIT, we wanted to determine if the combination would behave in a similar manner in two distinct conditions: previous exposure to opioids (tolerance) and intestinal inflammation. Chronic administration of morphine induces tolerance to the antinociceptive and antitransit effects of morphine. Clonidine has been reported to delay and reduce the development of tolerance to the analgesic effects of morphine, although the mechanisms and clinical relevance of these findings are not well established. In the present study we investigated the effects of a 1:1 (potency ratio) morphine-clonidine combination in morphine-tolerant animals to establish the possible benefits of drug combinations on the inhibition of GIT.

The drug ratio used in this study (1:1) was selected because preliminary studies revealed a decrease on the inhibition of GIT with increasing doses of the combination. Such antagonism was considered particularly relevant because it could reflect a dissociation of the beneficial and adverse effects of the drugs involved. In control conditions, our results showed that clonidine was approximately 17 times more potent than morphine as an inhibitor of transit. The dose–response curve to the combination was linear but had an E_{max} of 60%. The decrease in the E_{max} of the combination suggests (1) a

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Table 4. ED_{50} Values (mg/kg) of Morphine (MS), Clonidine (CL), and Their 1:1 Combination (MS + CL) in Controls (SS) and Mice with Intestinal Inflammation (INF): Type of Interaction of the Morphine–Clonidine Combination at the ED_{50}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MS</th>
<th>CL</th>
<th>MS + CL</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (SS)</td>
<td>1.627 ± 0.16</td>
<td>0.107 ± 0.01</td>
<td>0.55 ± 0.075</td>
<td>Synergy (0.65*)</td>
</tr>
<tr>
<td>Inflamed (INF)</td>
<td>0.175 ± 0.04</td>
<td>0.015 ± 0.006</td>
<td>0.62 ± 0.044</td>
<td>Antagonism (6.7**)</td>
</tr>
</tbody>
</table>

ED_{50} values were obtained from the linear segments of the log dose–response curves shown in figure 5. Synergy and antagonism represent statistically significant deviations from additivity. The numbers in parentheses are the values of the interaction indexes.

*P < 0.05 (Student t test).

**P < 0.01 (Student t test).
Fig. 6. Isobolographic representation of the interaction of morphine (MS) and clonidine (CL) (1:1 ratio) at the ED$_{50}$ value. The straight line indicates the zero interaction isobole, and the points drawn on the left and right of the additive line show synergy and antagonism, respectively. Points on the axes represent isoeffective doses of morphine and clonidine used individually, whereas points on the right and left of the isobole illustrate isoeffective combinations derived from the dose-response curves. (Top) Saline control animals; (bottom) animals with intestinal inflammation.

decrease in receptor affinity or density, $^{42}$ (2) a partial antagonism revealed when the drugs are used in combination, and/or (3) a common pathway in the intracellular signal transduction mechanisms that mediate the antitransit response. $^{43}$

The results in tolerant animals showed that no cross-tolerance existed between morphine and clonidine. In these animals, the dose-response curve of the combination was shifted to the left and also had an $E_{\text{max}}$ of 60%. When the doses of the combination were increased, the dose-response curve developed a plateau. The $E_{\text{max}}$ and the plateau were similar in naive and tolerant animals, showing that morphine tolerance did not alter the response when high doses of the combination were used (fig. 2). The analysis of the interaction showed that synergy was significantly enhanced in tolerant animals.

Changes in the type of interaction (from synergistic to additive) between morphine and clonidine administered intrathecally have been reported in tolerant mice for antinociception. $^{18}$ In this report, linear dose-response curves for the morphine–clonidine combination (1:1) with $E_{\text{max}}$ values of 80% or higher were obtained. These results, together with our findings, suggest that morphine and clonidine (1:1 potency ratio) interact in a different manner when producing spinal antinociception and inhibition of GIT (a combination of central and peripheral-intestinal effects). Tolerance converts synergy to additivity for the antinociceptive effects, while the synergistic interaction for the antitransit action (< a 60% response) is significantly enhanced. The different interaction between morphine and clonidine for the antinociceptive and antitransit effects in tolerant mice could be related to the different routes of administration and/or to other methodologic variables. However, our results support those of other investigators when evaluating the antinociceptive effects of morphine and clonidine combined in morphine-tolerant mice, after spinal administration. $^{44}$

The effects of the combination of morphine and clonidine were also evaluated in a model of intestinal inflammation. In this model, peripheral opioid and $\alpha_2$-adrenergic receptors have been reported to be "sensitized" or "up-regulated" according to the type and duration of the inflammatory process. $^{21,22}$ Our results show that during inflammation, the potencies of morphine and clonidine increased nine and seven times, respectively. However, the potency of the combination remained unaltered, and the dose–response curve of the combination was linear up to an $E_{\text{max}}$ value $> 80%$. This observation would be consistent with an increase in the number of "functional" receptors. Further analysis of the data showed that during inflammation, the interaction between morphine and clonidine was transformed from synergistic to antagonistic at all levels of response. These data suggest that even in the presence of a higher "density" of receptors, both agonists could interact and compete with limited levels ("pools") of common membrane/intracellular substrates required for signal transduction.

In conclusion, our results show that interaction between morphine and clonidine on GIT is altered during morphine tolerance and in the presence of intestinal inflammation. The effect of the combination is increased during morphine tolerance, where it becomes highly
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synergistic; however, in the presence of inflammation, the synergistic interaction is converted to antagonistic. The antagonism between morphine and clonidine in both experimental conditions could be explained by a common pathway in the intracellular signal transduction mechanisms that mediate the antitransit effects of both drugs.

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References

32. Shook J, Lemcke PK, Gehrig CA, Hruby VJ, Burks TF: Antidiarrheal properties of supraspinal mu and delta and peripheral mu, delta and kappa opioid receptors: Inhibition of diarrhoea without constipation. J Pharmacol Exp Ther 1989; 249:83-90
38. Ling GS, Paul D, Simantov R, Pasternak GW: Differential development of acute tolerance to analgesia, respiratory depression, gastro-

Anesthesia, V 93, No 1, Jul 2000
intestinal transit and hormone release in a morphine infusion model. 
Life Sci 1989; 45:1627-36
39. Wong CL, Roberts MB, Wai MK: Effect of morphine and nalox-
one on intestinal transit in mice. Eur J Pharmacol 1980; 64:289-95
40. Brown NJ, Coupar IM, Rumsey DE: The effect of acute and 
chronic administration of morphine and morphine withdrawal on 
41. Ruffolo RR, Nichols AJ, Stadel JM, Hieble JP: Pharmacologic and 
therapeutic applications of \( \alpha_2 \)-adrenoceptor subtypes. Ann Rev Phar-
macol Toxicol 1993; 32:243-79
42. Pazos A: Acciones de los fármacos I: Interacción fármaco y 
receptor. Farmacología Humana. Edited by Flórez J, Armigo JA, Media-
villa A. Barcelona, Ediciones Masson-Salvat, 1992, pp 2-17
43. Wei ZY, Roerig SC: Spinal morphine/clonidine antinociceptive 
synergism is regulated by protein kinase C, but not protein kinase A 
44. Fairbanks CA, Wilcox GL: Spinal antinociceptive synergism be-
tween morphine and clonidine persists in mice made acutely and 
chronically tolerant to morphine. J Pharmacol Exp Ther 1999; 288: 
1107-16