Synergistic interaction between rilmenidine and ibuprofen in the writhing test in mice

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Abstract

OBJECTIVES: The aim of the study was to ascertain whether rilmenidine, a second generation imidazoline-alpha-2-adrenoreceptor agonist, is able to increase analgesic effects of ibuprofen in the writhing test in mice. Experimental studies combining these agents have not yet been published.
METHODS: An acetic acid (0.7%) solution was injected into the peritoneal cavity and the number of writhes was counted. The influence on locomotor performance was tested using the rotarod test.
RESULTS: Rilmenidine, ibuprofen, and rilmenidine-ibuprofen fixed-ratio combinations produced dose-dependent antinociceptive effects. ED_{50} values were estimated for the individual drugs and an isobologram was constructed. The derived theoretical additive ED_{50} value for the rilmenidine-ibuprofen combination was 34.00 ± 9.39 mg/kg. This value was significantly greater than the observed ED_{50} value which was 18.07 ± 5.41 mg/kg, indicating a synergistic interaction. Rilmenidine did not impair motor coordination, as measured by the rotarod test, at antinociceptive and higher doses.
CONCLUSIONS: The present results suggest that rilmenidine enhances the analgesic activity of ibuprofen. If rilmenidine produces antinociception in humans, then the synergistic antinociception of rilmenidine with ibuprofen could offer therapeutic advantage for clinical treatment of pain.

1. INTRODUCTION

Rilmenidine is an imidazoline-α2-adrenoreceptor agonist, which, in some countries, is also used as a second-generation central antihypertensive drug. The first generation imidazoline-α2-adrenoreceptor agonist, clonidine, has been used not only as a central antihypertensive but also as an adjuvant analgesic in neuraxial analgesia (Eisenach, 1996; Millan, 2002; Schug *et al.* 2006). In addition, clonidine has been demonstrated to produce antinociception in synergy with various nonsteroidal anti-inflammatory drugs and paracetamol (Miranda & Pinardi, 2004), with benzodiazepines (Nishiyama & Hanaoka, 2001), N-methyl D-aspartate (NMDA) receptor antagonists (Nishiyama *et al.* 2001) and gabapentin (Cheng *et al.* 2000).

However, the therapeutic utility of clonidine, as an analgesic, is limited by its undesirable side effects including sedation, dry mouth, hypotension, and rebound hypertension (Dias *et al.* 1999;

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Abbreviations

ANOVA	– one-way analysis of variance
CL	– confidence limit
ED_{50}	– the fifty percent effective dose
ID_{50}	– the fifty percent inhibitory dose
i.p.	– intraperitoneally
MPE	– maximum possible effect
NMDA	– N-methyl-D-aspartate
NMRI	– Naval Medical Research Institute
NSAID(s)	– non-steroidal anti-inflammatory drug(s)
p.o.	– per oral
SEM	– standard error of the mean

Puskas *et al.* 2003). Rilmenidine exhibits fewer side-effects (including sedation) than clonidine, which is attributed to the more selective action of rilmenidine at cerebral imidazoline receptors (Gomez *et al.* 1991; Ernsberger *et al.* 1992; Harron *et al.* 1995; Yu & Frishman, 1996). If rilmenidine also has analgesic activity, the drug, with its good tolerability, might be of interest in the treatment of pain. However, surprisingly little has been reported on the analgesic activity of rilmenidine. It has only recently been shown that rilmenidine produced dose-dependent analgesia in the formalin test in mice (Sabetkasaie *et al.* 2007).

The aim of the present study was to determine whether rilmenidine in combination with ibuprofen has synergistic effects using the writhing test in mice and an isobolographic analysis.

2. MATERIAL AND METHODS

2.1. Animals

The experimental animals were male, Naval Medical Research Institute (NMRI), mice (VUFB Konarovice, Czech Republic) weighing 20–25 g, housed on a 12 h light-dark cycle at 22 ± 2 °C with access to food and water *ad libitum*. Food was withheld 12 h prior to the start of experimental procedures; access to water was not restricted. 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 Third Faculty of Medicine, Charles University. Animals were (i) acclimatized to the laboratory for at least 1 h before testing, (ii) were used only once during the protocol, and (iii) were sacrificed, by an anesthetic overdose, immediately after algesiometric testing. The duration of the experiments was as short as possible and the number of animals was the minimum compatible with consistent effects of drug treatments (6–9 mice per experimental group). Control animals (sterile water) were run interspersed concurrently with drug-treated animals.

All procedures involving animals strictly adhered to the guidelines proposed by the Committee on Research and Ethical Issues of IASP for investigations in experimental pain in animals (Zimmermann, 1983).

2.2. The writhing test

The writhing test was selected as a model of acute visceral pain, because it is feasibly reproducible, widely accepted and well established pain test used in laboratories around the world. The procedure has been previously described (Millan, 1994; Miranda *et al.* 2001). Briefly, mice were injected intraperitoneally (i.p.) with 10 ml/kg of a 0.7% acetic acid solution, 30 min after oral (p.o.) administration of the test drug. The 30 min interval was established during preliminary experiments as the optimal interval for achieving the maximal effect of rilmenidine.

Mice were injected with acetic acid in groups of three, which were then placed in a clear Plexiglas cage (20 × 30 × 20 cm) for observation. A writhe was defined as a wave of contraction of abdominal muscles followed by dorsiflexion and extension of the hind limbs. The number of writhes in a 20 min period was counted, starting immediately after administration of the acetic acid. Antinociception was expressed as percent inhibition in the number of writhes observed in sterile water control animals during the 20 min period. Each group of 3 animals was observed by one observer, who was blinded to the treatment.

2.3. Study design of analgesic activity measurement

Thirty minutes before the start of the writhing test, animals were orally administered with (i) the vehicle (sterile water), (ii) increasing doses of rilmenidine (1.0–10.0 mg/kg), (iii) increasing doses of ibuprofen (3–100 mg/kg), or (iv) rilmenidine-ibuprofen combinations to asses the antinociceptive effect via isobolographic analysis (see 2.5. for dosing details).

2.4. Rotarod test

The animals (6 per group) were trained, 1 day before the experiment, to stay on the rotarod apparatus for 120 s (25 mm diameter rod rotating at 6 rpm) (Ugo-Basile, Varese, Italy; model 7650). Two or three trials were usually sufficient for the animals to learn the task. Drugs were tested only on those mice that were able to reproduce this performance the following morning. The vehicle (sterile water), doses of rilmenidine (2.63 and 5.20 mg/kg) and increasing doses of diazepam (5–20 mg/kg) were administered orally 30 min before testing.

The ability of the test animal to remain on the rotarod for 120 s was evaluated at 30, 60, 90 and 120 min after drug administration. A reduction in time spent on the rotarod (presumably reflecting sedation and/or reduced motor coordination) was expressed as a percent of the maximum possible effect (%MPE) and was calculated using the following equation, where time represents time spent on the rotarod: %MPE = [100 × (mean time in control group – mean time in drug treated group)] / mean of time in control group. The fifty percent inhibitory dose (ID_{50}) of diazepam, the dose causing failure in 50% of the animals, was calculated by using linear regression analysis. A reference dose of 10 mg/kg p.o.

diazepam was established for comparison with rilmenidine. Results describing the effect of rilmenidine in comparison with diazepam (10 mg/kg) are presented as mean ± SEM (standard error of the mean) for each group (6 animals per group). Comparison of significance between the control and drug-treated groups was performed using one-way analysis of variance (ANOVA) on ranks, followed by Tukey's test; statistical significance was set at the 0.05 level.

2.5. Data analysis

Results are presented as mean ± SEM or the dose resulting in 50% of the effect (ED_{50}) values with 95% confidence intervals. Six animals were tested at each of, at least, four doses to determine a dose-response curve for individual drugs. Nine animals were tested at each of four doses to determine a dose-response curve for the (rilmenidine + ibuprofen) combinations. Antinociceptive activity (reduction in writhes) was expressed as a percent of the maximum possible effect (%MPE) and was calculated using the following equation: %MPE = [100 × (mean writhes in control group – mean writhes in drug(s) treated group)] / mean of writhes in control group.

Dose-response curves were constructed by least-squares linear regression and ED_{50} ± standard error (SE) values were calculated according to Tallarida (2000). The interaction between rilmenidine and ibuprofen was characterized by isobolographic analysis assuming that the combinations were constituted by equally-effective doses of the individual drugs. Thus, from the dose-response curves of each individual agent, the dose resulting in 50% of the effect (ED_{50}) could be determined. Therefore, we estimated the ED_{50} of ibuprofen and rilmenidine. Subsequently, a dose-response curve was obtained by concurrent delivery of both drugs (rilmenidine and ibuprofen) in fixed-ratios, based on the ED_{50} values of each individual agent. To construct this curve, groups of animals received one dose of one of the following combinations: (i) (rilmenidine ED_{50} + ibuprofen ED_{50}); (ii) (rilmenidine ED_{50} + ibuprofen ED_{50})/2; (iii) (rilmenidine ED_{50} + ibuprofen ED_{50})/4; or (iv) (rilmenidine ED_{50} + ibuprofen ED_{50})/8. The experimental ED_{50} value for the combination was calculated from this curve.

The theoretical additive ED_{50} was estimated from the dose-response curve of each drug administered individually, which presupposes that the observed effect of the combination is the sum of the effects of each individual drug. This theoretical ED_{50} value is then compared with the experimentally derived ED_{50} value to determine if there is a statistically significant difference (Tallarida, 2001; Tallarida, 2006; Tallarida, 2007). The theoretical and experimental ED_{50} values of the studied combination were also contrasted by calculating the interaction index (γ) as follows: γ = ED_{50} of combination (experimental) / ED_{50} of combination (theoretical). An interaction index not significantly different from unity corresponds to an additive interaction whereas values higher and lower than unity imply antagonistic and synergistic interactions, respectively (Tallarida, 2002).

Statistical significance between the theoretical additive ED_{50} and the experimentally derived ED_{50} value was evaluated using the Student's t-test. An experimental ED_{50} significantly lower than the theoretical additive ED_{50} was considered to indicate a synergistic interaction between rilmenidine and ibuprofen. Statistical significance was considered to be achieved when $p < 0.05$.

2.6. Drugs

Rilmenidine was provided by Sigma-Aldrich (USA); ibuprofen by Léčiva a.s. (Czech Republic) and diazepam by Kulich a.s. (Czech Republic). All drugs were freshly suspended in sterile water. Suspensions were made using an appropriate amount of arabic gum (1/4 the weight of the amount of substance to be suspended). Drugs were prepared and administered in a volume of 10 ml/kg. Suspensions were thoroughly vortexed before administration. Control groups received an equal amount of sterile water.

3. RESULTS

3.1. Antinociceptive effect of rilmenidine and ibuprofen, dose-response relationship

Acetic acid administration produced a typical pattern of writhing behavior. Dose-response curves obtained for rilmenidine and ibuprofen are shown in the Fig. 1 and 2, respectively. The ED_{50} value and 95% confidence limit (CL) for the writhing test for oral rilmenidine was 2.45 (2.34–2.55) mg/kg. The ED_{50} value and 95% CL for oral ibuprofen was 61.13 (35.85–95.37) mg/kg.

3.2. Interaction of rilmenidine and ibuprofen

The antinociceptive activity of p.o. co-administration of fixed ratio combinations of ED_{50} fractions of rilmenidine with ibuprofen was assessed by calculating the ED_{50} value of the mixture from the corresponding dose-response curves (see section 3.1.). Fixed-dose ratio combinations were prepared, as described in the material and methods section (2.5.). The experimental ED_{50} value was calculated as 18.07 ± 5.41 mg/kg. This value was significantly lower ($p < 0.05$) than the theoretical ED_{50} value expected for a purely additive interaction, which was 34.00 ± 9.39 mg/kg. As can be seen in Fig. 3, the experimental ED_{50} value is located below the additive dose line.

Furthermore, the interaction index (γ) for the rilmenidine-ibuprofen combination was 0.53 ± 0.06, which is statistically different from unity. These data indicate that the interaction between the antinociceptive actions of rilmenidine and ibuprofen is synergistic, with the resulting effect being approximately twice that expected from the sum of the effects of the individual components.

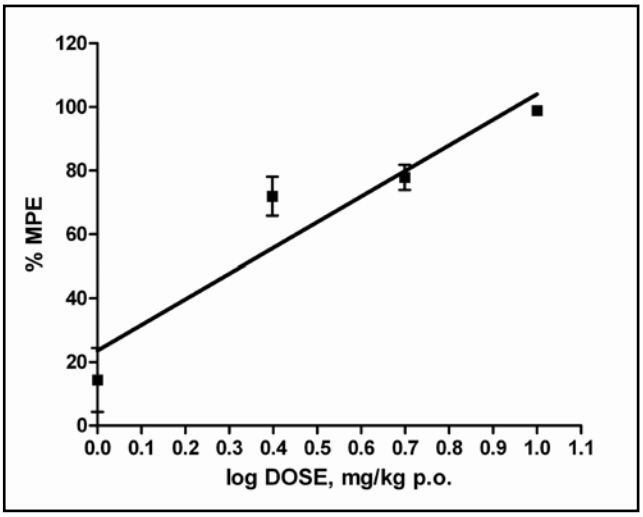

Fig. 1. Dose-response curve for antinociceptive activity following oral administration of rilmenidine in mice. Antinociceptive activity (reduction in writhes) was expressed as a percent of the maximum possible effect (%MPE) and was calculated using the following equation: %MPE = [100 x (mean writhes in control group – mean writhes in drug treated group)]/mean of writhes in control group. Each point represents data from six animals per group ± SEM.

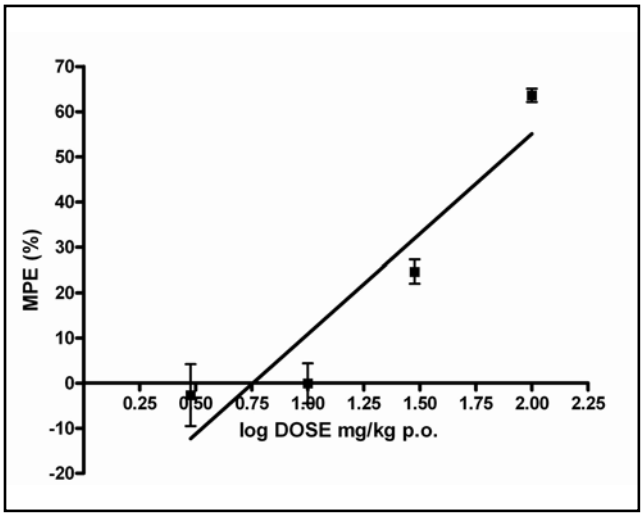

Fig. 2. Dose-response curve for antinociceptive activity following oral administration of ibuprofen in mice. Antinociceptive activity (reduction in writhes) was expressed as a percent of the maximum possible effect (%MPE) and was calculated using the following equation: %MPE = [100 x (mean writhes in control group – mean writhes in drug treated group)]/mean of writhes in control group. Each point represents data from six animals per group ± SEM.

3.3. Rotarod test

The mean (± SEM) time spent on the revolving rotarod by vehicle-treated animals was 120 ± 0 s (Fig. 4). Oral administration of rilmenidine (2.63 mg/kg and 5.20 mg/kg) did not decrease time spent on the revolving rotarod at 30 min (Fig. 4), or at 60, 90, 120 min (data not shown). On the other hand diazepam (10 mg/kg p.o.), used as a positive control, significantly reduced time spent on the rotarod (Fig. 4). This dose of diazepam was selected based on preliminary experimentation, where diazepam dose-dependently reduced time spent on the rotarod (ED_{50} = 11.2 mg/kg).

4. DISCUSSION

In the present study, rilmenidine and ibuprofen administered alone showed dose-dependent antinociceptive effects in the writhing test in mice. Moreover, oral co-administration of rilmenidine with ibuprofen produced synergistic antinociceptive effects in this model of visceral pain.

The analgesic activity of rilmenidine, a preferential imidazoline receptor and a weak alpha-2 adrenergic receptor agonist, has, to date, received little attention. Only recently it has been reported that rilmenidine produced dose-dependent analgesia in the formalin test in mice (Sabetkasaie *et al.* 2007). In general there is a lack of clinical evidence regarding the analgesic activity of rilmenidine.

On the other hand, the antinociceptive activity of clonidine, the reference drug for alpha-2 adrenergic and imidazoline receptor agonists, has been extensively studied both preclinically and clinically. Clonidine has been shown to induce antinociception in the writhing test (Jain *et al.* 2002; Miranda & Pinardi, 2004; Sabetkasaie *et al.* 2004), the tail-flick test (Dogrul & Uzbay, 2004; Nishiyama *et al.* 2001; Ozdogan *et al.* 2004), the formalin test (Nishiyama & Hanaoka, 2001; Yoon *et al.* 2004; Zarrindast & Sahebgharani, 2002) and the substance P nociceptive test (Fairbanks & Wilcox, 1999). Clonidine has also been shown to have analgesic effects in humans, particularly after epidural administration (Bernard & Macaire, 1997; DeKock *et al.* 1997; Hood *et al.* 1996).

Several other α_2-adrenergic and imidazoline receptor agonists such as tizanidine, fadolmidine, medetomidine, and dexmedetomidine have shown antinociceptive effects in both animals and humans (Jain *et al.* 2002; Pertovaara & Kalmari, 2003; Kauppila *et al.* 1991; Hall *et al.* 2000; Angst *et al.* 2004; Schug *et al.* 2006). A recent study demonstrated that agmatine, a presumed endogenous ligand at imidazole receptors which also binds to alpha-2 adrenoceptors (Reis & Regunathan, 2000), produced dose-dependent inhibition of acetic acid-induced visceral pain in mice (Santos *et al.* 2005).

As shown by the isobolographic analysis, co-administration of rilmenidine with ibuprofen, a well-established non-steroidal anti-inflammatory drug (NSAID) which has been shown to act as an inhibitor of the enzyme cyclooxygenase (Blain *et al.* 2002), produced synergistic or supra-additive antinociception. (experimental ED_{50} were significantly less than the theoretically calculated ED_{50}). Our results are consistent with a previous study which demonstrated that the simultaneous administration of various NSAIDs (naproxen, piroxicam, nimesulide) with clonidine resulted in synergistic interactions in the writhing test in mice (Miranda & Pinardi, 2004). Furthermore, the systemic

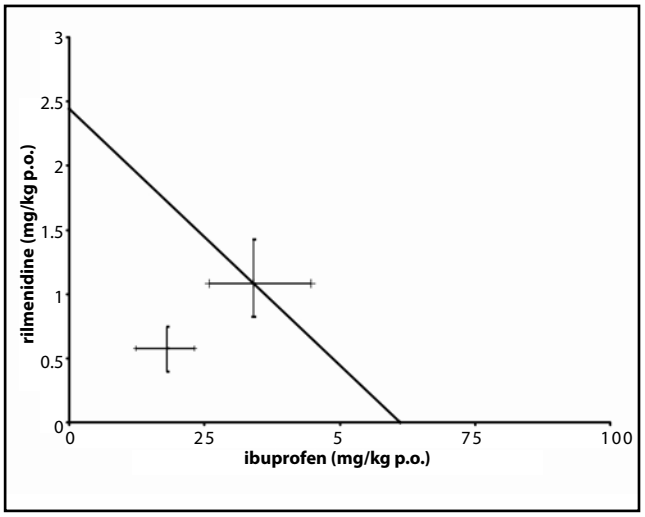

Fig. 3. Isobologram for oral co-administration of rilmenidine and ibuprofen. The point on the theoretical additive line corresponds to the theoretical ED_{50} ± SEM, the point under the theoretical additive line corresponds to experimental ED_{50} ± SEM of the mixture. The experimental point was significantly different from the calculated additive point, indicating a synergistic interaction ($p < 0.05$; Student's t-test).

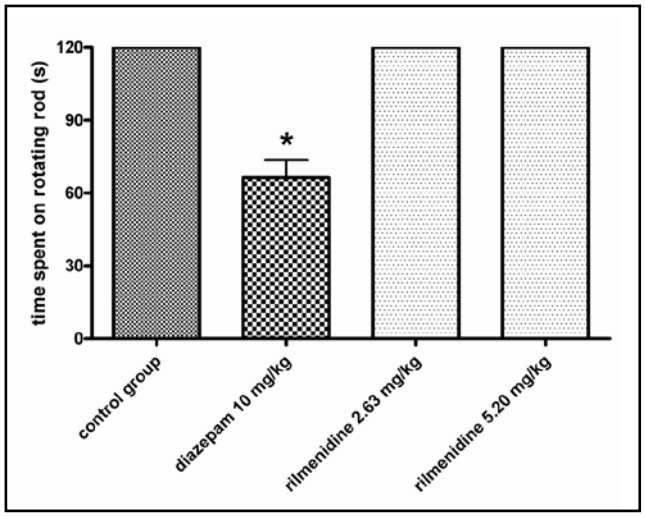

Fig. 4. Effect of rilmenidine (2.63 mg/kg and 5.20 mg/kg) and diazepam (10 mg/kg) in the rotarod test in mice. Test compounds were administered orally 30 min before performing the test. Results are expressed as mean (6 animals per group) time spent on the rotating rod (vertical line visible for diazepam represents SEM). * $p < 0.05$, significantly different from vehicle treated control group of animals (ANOVA on ranks followed by Tukey's test).

co-administration of diclofenac with clonidine showed supraadditivity in the same test (Miranda *et al.* 2001). Recently, we have also found a synergistic interaction between rilmenidine and paracetamol in the writhing test in mice (Soukupová, to be published).

Rilmenidine did not influence performance of mice on the rotarod at twice the antinociceptive ED_{50} dose of the drug, while diazepam, as expected, reduced rotarod times. Thus, doses of rilmenidine that were antinociceptive did not impair motor coordination as measured using the rotarod test. Most of the published data indicates that rilmenidine does cause sedation in animal models, at doses up to 10.0 mg/kg in mice and rats, and it did not prolong barbiturate-induced sleeping time. Additionally, it did not modify spontaneous locomotor activity in rats at doses up to 2.5 mg/kg (Montastruc *et al.* 1989). Substance S 3341 (matching rilmenidine) did not prolong the hexobarbitone-induced loss of righting reflex in mice (van Zweiten *et al.* 1986). In rats, S 3341 decreased the rate of discharge of noradrenergic cells located in the locus coeruleus, which is believed to be involved in wake/sleep mechanisms, however, depression was 63 times less than that of the reference drug, clonidine. At effective hypotensive doses, rilmenidine produced no sedation (loss of righting reflex) in 2 day old chicks (Laubie *et al.* 1985). These findings are consistent with the lack of effect of rilmenidine on the rotarod test in the present study.

Rilmenidine has been reported to produce antihypertensive effects in hypertensive rats but the effect has not been observed in normotensive rats (Briaud *et al.* 2005; Cechetto and Kline 1997; Cechetto and Kline 1998; Mao *et al.* 2003; Monassier *et al.* 2004; Wang *et al.* 2005). Thus, it is assumed that rilmenidine did not produce hypotension in the present study. It is well established that rilmenidine is an effective antihypertensive agent in hypertensive human subjects, while data on its effects on blood-pressure in healthy humans are limited (Dollery *et al.* 1988; Teixeira de Astro *et al.* 2006).

In conclusion, if rilmenidine produces antinociception in humans, then it could represent a good alternative to clonidine in the treatment of pain, especially considering its superior side-effects profile. Moreover, the synergistic antinociception of rilmenidine with ibuprofen could offer another therapeutic advantage for clinical treatment of pain. Therefore further studies assessing the analgesic potential of rilmenidine alone or in combination with analgesics are warranted and eagerly awaited.

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