Naunyn-Schmied Arch Pharmacol (2009) 379:575–580
DOI 10.1007/s00210-009-0402-3

ORIGINAL ARTICLE

The synergistic interaction between rilmenidine and paracetamol in the writhing test in mice

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Received: 8 September 2008 / Accepted: 20 January 2009 / Published online: 11 February 2009

Abstract The aim of the study was to ascertain antinociceptive effects of rilmenidine, a second-generation imidazoline-alpha-2-adrenoreceptor agonist, and to see whether rilmenidine was able to increase the analgesic effects of paracetamol in the writhing test in mice. 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. Rilmenidine, paracetamol, and rilmenidine–paracetamol 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–paracetamol combination was 109.23± 35.05 mg/kg. This value was significantly greater than the observed ED_{50} value which was 56.35±20.86 mg/kg, indicating a synergistic interaction. Rilmenidine did not impair motor coordination, as measured by the rotarod test, at antinociceptive and higher doses.

Keywords Rilmenidine · Paracetamol · Antinociception · Pain · Imidazoline · Receptors · Alpha-2-adrenoceptors

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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 paracetamol and various nonsteroidal anti-inflammatory drugs (Miranda and Pinardi 2004), with benzodiazepines (Nishiyama and Hanaoka 2001), *N*-methyl-D-aspartate 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; 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 and 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 ascertain the antinociceptive effects of rilmenidine, using an acute visceral pain model (the writhing test), following systemic (oral) administration in mice. An additional aim was to determine whether rilmenidine in combination with paracetamol had synergistic effects, combinations were evaluated using the writhing test in mice and isobolographic analysis.

Materials and methods

Animals

The experimental animals were male Naval Medical Research Institute mice (VUFB Konárovice, 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 (1) acclimatized to the laboratory for at least 1 h before testing, (2) were used only once during the protocol, and (3) 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 (six to nine 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 the International Association for the Study of Pain for investigations of experimental pain in animals (Zimmermann 1983).

The writhing test

The writhing test was selected as a model of acute visceral pain because it is a 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 with 10 ml/kg of a 0.7% acetic acid solution, 30 min after oral (po) 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 three animals was observed by one observer who was blinded to the treatment.

Study design of analgesic activity measurement

Thirty minutes before the start of the writhing test, animals were orally administered with (1) the vehicle (sterile water), (2) increasing doses of rilmenidine (1.0–10.0 mg/kg), (3) increasing doses of paracetamol (10–300 mg/kg), or (4) rilmenidine–paracetamol combinations to asses the antinociceptive effect via isobolographic analysis (see "Data analysis" for dosing details).

Rotarod test

The animals (six 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 which 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 percent maximum possible effect (%MPE) which 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 50% inhibitory dose of diazepam, the dose causing failure in 50% of the animals, was calculated through linear regression analysis. A reference dose of 10 mg/kg po 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 (six animals per group). Comparisons of significance between the control and drug-treated groups were performed using one-way analysis of variance (ANOVA) on ranks, followed by Tukey's test; statistical significance was set at the 0.05 level.

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 the individual drugs. Nine animals were tested at each of four doses to determine a dose–response curve for the (rilmenidine + paracetamol) combinations. Antinociceptive activity (reduction in writhes) was expressed as the %MPE which 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 using least-squares linear regression and ED_{50}±standard error values were calculated according to Tallarida (2000). The interaction between rilmenidine and paracetamol 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 ED_{50} could be determined. Therefore, we estimated the ED_{50} of paracetamol and rilmenidine. Subsequently, a dose–response curve was obtained by concurrent delivery of both drugs (rilmenidine and paracetamol) 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: (1) (rilmenidine ED_{50} + paracetamol ED_{50}); (2) (rilmenidine ED_{50} + paracetamol ED_{50})/2; (3) (rilmenidine ED_{50} + paracetamol ED_{50})/4; or (4) (rilmenidine ED_{50} + paracetamol 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, i.e., 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, 2006, 2007). The theoretical and experimental ED_{50} values of the studied combination were also contrasted by calculating the interaction index (γ) as follows: $\gamma=ED_{50}$ 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).

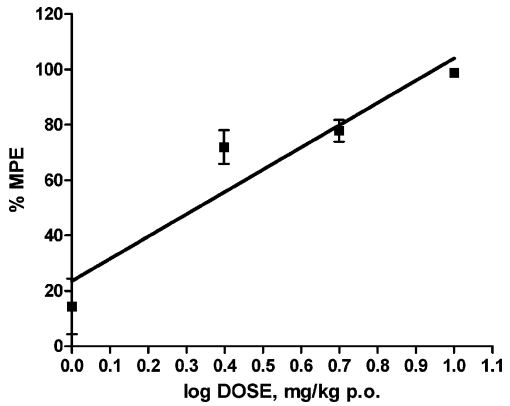

Fig. 1 Dose–response curve for the antinociceptive activity after oral administration of rilmenidine in mice. Antinociceptive activity (reduction in writhes) was expressed as percent maximum possible effect (%MPE) that was calculated by the following equation: %MPE=[100×(mean writhes in control group)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

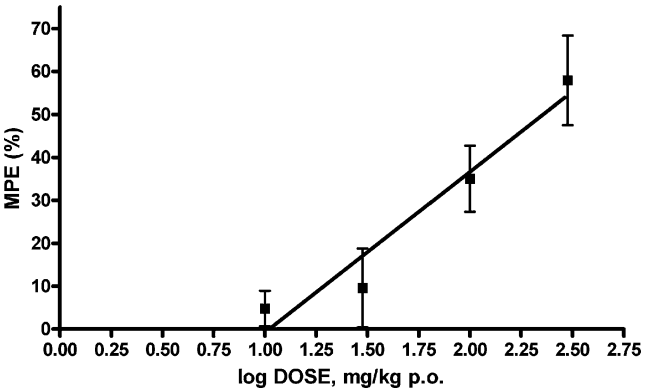

Fig. 2 Dose–response curve for the antinociceptive activity after oral administration of paracetamol in mice. Antinociceptive activity (reduction in writhes) was expressed as percent maximum possible effect (%MPE) that was calculated by the following equation: %MPE=[100×(mean writhes in control group)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

Statistical significance between the theoretical additive ED_{50} and the experimentally derived ED_{50} value was evaluated using 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 paracetamol. Statistical significance was considered to be achieved when $p<0.05$.

Drugs

Rilmenidine was provided by Sigma-Aldrich (USA); paracetamol 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 (one fourth 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.

Results

Antinociceptive effect of rilmenidine and paracetamol, dose–response relationship

Acetic acid administration produced a typical pattern of writhing behavior. Dose–response curves obtained for rilmenidine and paracetamol are depicted in Figs. 1 and 2, respectively. The ED_{50} value and 95% confidence limit

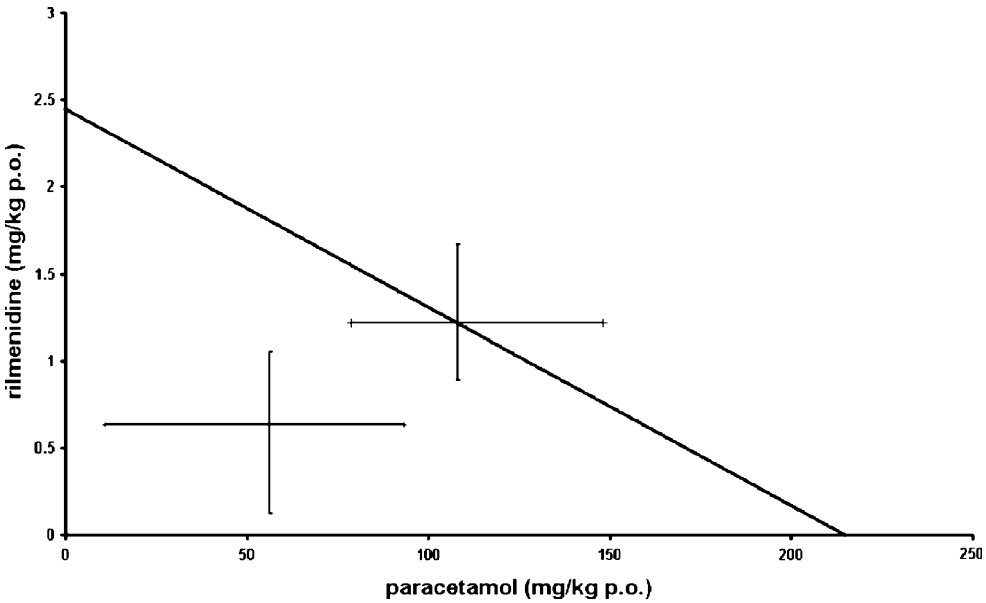

Fig. 3 Isobologram for the oral coadministration of rilmenidine and paracetamol. The point on the theoretical additive line corresponds to theoretical ED50± SEM; the point under the theoretical additive line corresponds to experimental ED50±SEM of the mixture. The experimental point was significantly different from the calculated additive point, indicating a synergistic interaction ($p<0.05$; Student's t test)

(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 paracetamol was 214.80 (127.33–445.40) mg/kg.

Interaction of rilmenidine and paracetamol

The antinociceptive activity of po coadministration of fixed-ratio combinations of ED_{50} fractions of rilmenidine with paracetamol was assessed by calculating the ED_{50} value of the combination from the corresponding dose–response curves (see "Antinociceptive effect of rilmenidine and paracetamol, dose–response relationship"). Fixed-dose ratio combinations were prepared, as described in the "Materials and methods" section ("Data analysis"). The experimental ED_{50} value was calculated as 56.35± 20.86 mg/kg. This value was significantly lower ($p<0.05$) than the theoretical ED_{50} value expected for a purely additive interaction, which was 109.23±35.05 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–paracetamol combination was 0.52±0.07, which is statistically different from unity. These data indicate that the interaction between the antinociceptive actions of rilmenidine and paracetamol is synergistic, with the resulting effect being approximately twice that expected from the sum of the effects of the individual components.

Rotarod

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 and 5.20 mg/kg) did not decrease time spent on the revolving rotarod at 30 min (Fig. 4) or at 60, 90, and 120 min (data not shown). On the other hand, diazepam (10 mg/kg po), 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).

Discussion

In the present study, rilmenidine alone showed dose-dependent antinociceptive effects in the writhing test in

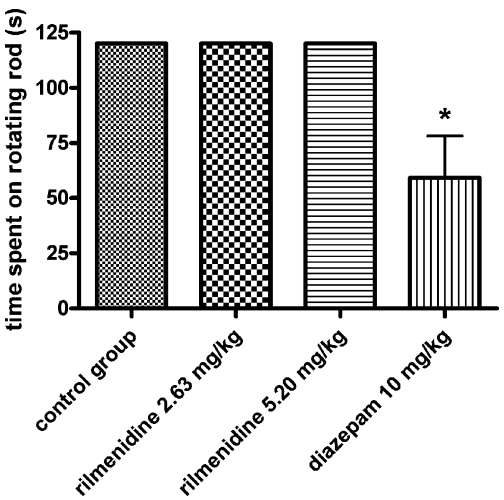

Fig. 4 Effect of rilmenidine (2.63 and 5.20 mg/kg) and diazepam (10 mg/kg) in the rotarod test in mice. Test compounds were administered po 30 min before performing the test. Results are expressed as mean (six animals per group) time spent on the rotating rod (*vertical line* visible for diazepam represents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

mice. Moreover, oral coadministration of rilmenidine with paracetamol 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. 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 and Pinardi 2004; Sabetkasaie et al. 2004), the tail-flick test (Dogrul and Uzbay 2004; Nishiyama et al. 2001; Ozdogan et al. 2004), the formalin test (Nishiyama and Hanaoka 2001; Yoon et al. 2004; Zarrindast and Sahebgharani 2002), and the substance P nociceptive test (Fairbanks and Wilcox 1999). Clonidine has also been shown to have analgesic effects in humans, particularly after epidural administration (Bernard and 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 and 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 and Regunathan 2000), produced dose-dependent inhibition of acetic-acid-induced visceral pain in mice (Santos et al. 2005).

As shown by isobolographic analysis, coadministration of rilmenidine with paracetamol, a well-established analgesic with central antinociceptive effect but with still unclear and disputed mechanism of action, produced synergistic or supra-additive antinociception, the experimental ED_{50} being significantly less than the theoretically calculated ED_{50}. Our results are consistent with a previous study which demonstrated that the simultaneous administration of paracetamol with clonidine resulted in synergistic interactions in the writhing test in mice (Miranda and Pinardi 2004).

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. Rilmenidine did not cause sedation at doses up to 10 mg/kg in mice and rats; additionally, it did not prolong barbiturate-induced sleeping time or modify spontaneous locomotor activity in rats at doses up to 2.5 mg/kg (Montastruc et al. 1989). In rats, rilmenidine did not decrease motor activity at doses up to 50 times the antihypertensive dose (Koenigberard et al. 1988). In contrast to clonidine, saccadic eye movements and other measures of sedation were not significantly impaired after hypotensive doses of rilmenidine in healthy male volunteers (Harron et al. 1995; Mahieux 1989).

Rilmenidine has been reported to produce antihypertensive effects in hypertensive rats but such an effect has not been observed in normotensive rats (Briaud et al. 2005; Cechetto and Kline 1997, 1998; Mao et al. 2003; Monassier et al. 2004; Wang et al. 2005). Thus, it does not seem that rilmenidine produced 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 effect profile. Moreover, the synergistic antinociception of rilmenidine with paracetamol 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.

Acknowledgments This project was supported by research grants MSM0021620816 and IGA NR/9072-3. We thank Dr. Zdenek Roth for his expert statistical analysis assistance.

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