Anxiolytic activity and the mechanism of action of deramciclane

Ph.D. Thesis

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INTRODUCTION

Fear and anxiety are parts of our life. Every time our existence or well-being is threatened by a putative or real danger, we experience an “alarm state”, which is called fear. We also feel threatened when we are faced with the demands of change. These presumed or true dangers are called stressors. We meet numerous stressors in everyday life; e.g. unexpected life events, persistent changes in our lives (e.g. marriage), permanent problems (e.g. poverty, chronic illness) or other traumas like catastrophes, aggression and verbal aggression.

Anxiety is an adaptive reaction in normal circumstances, but may become pathological and can impair our ability of coping with stress. Extreme anxiety may also cause somatic symptoms (e.g. gastric ulcer).

Anxiety disorders are characterized by similar symptoms, and are often combined with each other.

The role of serotonin in the pathomechanism of anxiety

The role of serotonin in the regulation of emotional states has been assumed for long time. The role of almost all of the 16 known mammalian receptors in anxiety or stress is supported by several research data (Lucki, 1996). However, despite the enormous amount of data available, the role of the 5-HT system in anxiety is rather contradictory. It is not evident if the activation of the 5-HT system increases or decreases anxiety, and vice versa. The reasons of this might be methodological, but might also be due to the fact that our knowledge of the extremely complicated functioning and interactions of the 5-HT system is still limited.

Buspirone, the firstly discovered 5-HT partial agonist, which was registered in 1986, is claimed to be anxiolytic (Weissmann et al., 1984) but there is also data about its anxiogenic activity (Collison et al., 1997). The reasons of this discrepancy are unknown, but the possible explanations may be different conditions of animal housing and the short term corticosterone-elevating effect of buspirone (Haller et al., 2000). Controversial results can be found in the case of other molecules acting on other serotonin receptors. The 5-$HT_{2C/2B}$ agonist m-chlorophenylpiperazine (mCPP) induces anxiety, and even evokes panic attacks accompanied by other neuropsychiatric symptoms in man (Charney et al., 1987). mCPP is anxiogenic in animal models (Kenneth et al., 1989). On the other hand, SSRIs that increase serotonin activity show anxiolytic effects in both man and animal models (Bagdy, 1998). At the same

time we can find data supporting the anxiogenic activity of SSRIs (Dekeyne et al., 2000; Overstreet et al. 2000).

Further evidence of the role of the 5-HT system in anxiety comes from the results of experiments with 5-HT knockout mice. There is comparative data on the anxious type knockout and the wild type mice with different genetic backgrounds published by 3 different research groups (Gingrich et al., 2001). 5-HT_{1A} knockout mice showed anxiety-like behaviour in behavioural tests, and deficiencies in experiments investigating the functioning of their autonomous nervous system (Pattij et al., 2002).

The most intensively investigated receptors in the regulation of behaviour are the 5-$HT_{2A/2C}$ receptors besides the 5-HT_{1A} receptors. 5-HT_{2C} receptor antagonists may provide an alternative therapy for anxiety in place of benzodiazepines. Acute administration of the 5-$HT_{2C/2B}$ agonist mCPP induces anxiety in both man and animals (Charney et al., 1987). Data from experiments with early, non-selective 5-HT_{2C} antagonists and with the selective 5-$HT_{2A/2C}$ antagonist ritanserin proves that the anxiogenic effect of mCPP is due to its 5-HT_{2C} agonist effect (Kalus et al., 1990; Piggot et al., 1991; Seibyl et al., 1991). Microinjection of the 5-HT_{2C} agonist MK-212 into the ventral area of the hippocampus caused anxiety-like behaviour in the elevated plus-maze test (Alves et al., 2004). Numerous clinical and experimental results prove that 5-HT_{2C} antagonists have anxiolytic effects as opposed to the anxiogenic property of 5-HT_{2C} agonists. Ritanserin was found to be anxiolytic in generalized anxiety disorder in Human Phase II studies, and it also had an anxiolytic-like effect in animal tests (Ceulemans et al., 1985; Meert et al., 1989). Kennett et al. demonstrated the anxiolytic effect of four non-selective 5-HT_{2C} antagonists (mianserine, 1-NP, ICI-169369, LY 53857) in the Geller-Seifter model of anxiety (Kennett et al., 1994). From the late 1990's an increasing number of 5-HT_{2C} antagonists with more selective profiles were developed, and they proved to be anxiolytic in animal models of anxiety. This type of molecules was developed mainly in GlaxoSmithKline Pharmaceuticals. The most widely known examples are SB-200646, SB-24084 and SB-243213 (Kennett et al., 1994; Martin et al., 2002; Wood et al., 2001).

5-HT_3 receptor antagonists were also supposed to possess an anxiolytic effect. Experiments showed that these molecules do not inhibit anxiety but are potent suppressors of the cytostatic-induced emesis. At present they are important tools in oncological therapy like e.g. ondansetron (Wolf, 2000).

The most recently identified 5-HT_6 and 5-HT_7 receptors are also supposed to play a role in anxiety. Yoshioka et al. were able to elicit freezing and 5-HT release in the rat frontal cortex by conditioned fear reactions. A 5-HT_6 antisense oligonucleotide can antagonize 5-HT release

but not the freezing reaction (Yoshioka et al., 1998). 5-HT_7 receptors are assumed to be involved in the pathomechanism of depression but they may have a role in anxiety, too. The selective 5-HT_7 antagonist SB-269970 showed anxiolytic activity in the lick-conflict, the elevated plus-maze and the four plate tests (Wesolowsaka et al., 2006).

Based on the above evidence, we can state that neither the therapy of anxiety nor the role of the 5-HT system has been fully worked out with respect to anxiety. Therefore, each new pharmacon that exerts its effect through the 5-HT system, and is curative in anxiety disorders can bring us closer to understanding the pathomechanism of anxiety and the role of the 5HT-system.

AIMS OF THE STUDY

Deramciclane is an original molecule synthesized and developed at EGIS Pharmaceuticals as part of a serotonin project. To understand the therapeutic effects of deramciclane and its mechanism of action the following experiments were necessary:

1. Investigation of the receptor profile of deramciclane, with special regards to the 5-HT receptors.

2. Further demonstration of its effects on the 5-HT system *in vivo* and *in vitro*.

3. Comparison of the serotonergic effects elicited by deramciclane on the central and the peripheral nervous systems.

4. *In vivo* and *in vitro* investigation of the agonist or antagonist actions of deramciclane on the 5-HT system.

5. Investigation of the anxiolytic activity of deramciclane in 3 experimental models of anxiety.

6. Investigation of the effects of deramciclane on the spontaneous motor activity of mice in order to reveal its possible sedative potential.

MATERIALS AND METHODS

Animal experiments were performed according to the Law of Animal Care and Use (1998. XXVIII.) and international guidelines.

Determination of receptor binding profile

These experiments were performed in isolated rat, guinea pig or porcine brains. Membrane preparations from whole brain or specific brain regions were used (rat cortex for α_1, α_2, β receptors; whole rat brain for $GABA_A$, benzodiazepine receptors; rat striatum for D_2, 5-HT_{1B} receptors; rat frontal cortex for 5-HT_{1A}, 5-HT_{2A} receptors; guinea pig cerebellum for H_1 receptors; guinea pig cortex for CCK_B receptors and porcine choroid plexus for 5-HT_{2C} receptors). Protein content was measured by the method of Bradford (Bradford, 1976). Human cloned receptors we expressed in CHO cells (5-HT_7 receptors), HEK cells (5-HT_6 receptors) and NIH-3T3 (CCK_A receptors).

Effects on the 5-HT system

Effects on the peripheral 5-HT system

Investigation of the 5-HT_{2A} receptor-antagonist effect in the rabbit aortic strip

Male, white New Zealand rabbits were used. 30 mm long helical strips were cut out of the aorta and mounted into an organ bath containing Krebs buffer solution. The strips were equilibrated under 1.0 g resting force, and incubated at 37 degree C for 90 min prior to the experiment. The isolated organ preparation was gassed with a 95% O_2/5% CO_2 mixture.

Concentrations of deramciclane tested were: $5x10^{-6}$, 10^{-6}, $5x10^{-7}$, 10^{-7}, $5x10^{-8}$, 10^{-8}, 10^{-9} M. Concentrations of the reference compounds were as follows: ritanserin: 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M; ketanserin: 10^{-7}, 10^{-8}, $5x10^{-9}$, 10^{-9} M; mianserine: 10^{-6}, 10^{-7}, $5x10^{-8}$, $3x10^{-8}$, 10^{-8}, 10^{-9} M.

IC_{50} values of the inhibition of the 5-HT response were calculated from the effects of incremental concentrations of the compound by nonlinear regression analysis (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA).

5-HT-induced paw oedema

Wistar rats were used in this experiment. Six animals per group received 30 ml/kg water through a gastric probe. Vehicle and drugs tested were administered in a volume of 10 ml/kg per os 60 min later. A further 60 min later 0.1 ml 5-HT dissolved in saline in the volume of 30 μg/ml was injected into the plantar surface of the right hind paw. The volume of the right hind paw was measured by a plethysmometer 30 min before and 30 min after the injection of the inflammatory drug (5-HT). The volume of the swelling, drug-treated paw was compared to that of the vehicle-treated control. Statistical analysis was done by ANOVA followed by Dunnett-test (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA). ID_{50} values were calculated by linear regression analysis.

Effects on the central nervous system

Inhibition of the DOI-induced head-twitch in the rat

Wistar rats (10 in a treatment group) were used in this study. Deramciclane (1.0, 3.0, 10.0 mg/kg), ritanserin (1.0, 3.0, 10.0 mg/kg) and vehicle (0.4% methyl-cellulose suspension) were administered per os 60 min prior to DOI treatment. The number of head-twitches was counted for 30 min. Counting began 5 min after DOI administration.

Statistical analysis was done by one-way ANOVA. For comparison of groups Dunnett-test was used (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA).

Anxiolytic activity

Vogel lick-conflict test

Long-Evans rats were deprived of water for 48 and of food for 24 h prior to the test session. Each treatment group consisted of a minimum of 8 animals. Vehicle and drugs tested were given per os 60 min before testing.

Anxiolytic activity was measured in a 8-chamber apparatus (LIKOSYS, Experimetria, Budapest) according to a slightly modified method of Vogel (Vogel et al., 1971). An electric shock of 2 mA was applied through the drinking tube for 1 sec. The number of accepted shocks during the 5-min test period was recorded. The anxiolytic effect of the drug was presented as a % increase of tolerated shocks. Mean number of tolerated shocks, standard deviation of the mean, % increase and statistical comparison of groups were calculated by ANOVA and Dunnett-test (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA).

Marble burying test

Marble burying behaviour of mice was tested in 10 Plexiglas boxes with a 5 cm thick layer of sawdust, according to the method of Broekkamp (Broekkamp et al., 1986). 24 marbles of 1 cm in diameter were placed on the top of the sawdust layer. The doses of drugs tested were: deramciclane: 0.3, 1.0, 3.0, 10.0, 30.0 mg/kg p.o.; diazepam 1.0, 2.0, 4.0, 8.0 mg/kg p.o.; ritanserin: 1.0, 3.0, 10.0 mg/kg p.o.

60 min after receiving drug or vehicle the animals were placed into the boxes individually and were left there for 15 min. The number of marbles covered (i.e. buried) by sawdust at least two-thirds was recorded. ID_{50} values were calculated by linear regression analysis using % inhibition data. Statistical difference between groups was calculated by ANOVA and Dunnett-test (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA).

Light-dark test

Light-dark activity was measured in a 6-box Animal Activity Collecting System (model no. 2012, Rhema Labortechnik, Germany) apparatus. Boxes were divided into a small, closed, dark part and a bigger, open-topped, brightly lit part. The light and the dark parts were connected by a 5x5 cm opening. Activity was measured by the changes in the magnetic field induced by locomotion. Drugs and their doses tested were: deramciclane 0.3, 1.0, 3.0 mg/kg, diazepam 0.3, 1.0, 3.0 mg/kg. The animals treated with drug or vehicle sc. (0.4% methyl-cellulose suspension) were placed individually in the boxes 20 min after drug administration. Behaviour was recorded for 8 min. Mean, standard deviation, standard error of the mean and statistical difference between groups (ANOVA and Dunnett-test) were calculated by GraphPad Prism (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA). A significant increase in the number of light-dark transitions was considered as an anxiolytic effect.

Effects on the spontaneous motor activity of mice

Spontaneous motor activity was measured in a 10-channel “Digitál moti-méter” constructed at EGIS Pharmaceuticals. The doses of drugs tested were: deramciclane:12.5, 25.0, 50.0, 100.0, 150.0 mg/kg p.o., diazepam: 1.0, 3.0, 10.0, 30.0 mg/kg p.o., ritanserin 12.5, 25.0, 50.0 mg/kg p.o. 0.4% methyl-cellulose served as vehicle suspension.

Activity was measured by the interruption of 3 parallel infrared beams in each box. The animals were placed individually in the boxes 60 min after p.o. administration of a drug or vehicle, and the number of infrared beam interruptions was registered for 30 min. ID_{50} values were calculated by linear regression analysis from the % effects of the doses. Statistical

comparison of groups was made by ANOVA and Dunnett-test (GraphPad Prism 4.0, GraphPad Software Incorporation, San Diego, USA).

RESULTS

Determination of receptor binding profile

Deramciclane showed the highest affinity for the 5-HT_{2A} (11nM) and the 5-HT_{2C} (8.7 nM) receptors from the 5-HT receptor family. Affinity for the σ_1 receptors (52 nM), the 5-HT_6 (70 nM), 5-HT_7 (105 nM) and D_2 receptors (113 nM) was moderate (table 1.). Affinity for the α_1, α_2, β_1, D_1, 5-HT_{1A}, benzodiazepine, $GABA_A$, CCK_A, CCK_B and H_1 receptors was very low, inconsiderable (K_i>1000 nM, not presented in table 1.).

Table 1. Affinity of deramciclane for different receptors in the brain

Receptor	**K_i (nM)±SE**
5-HT_{2C}	8,7±0,7
5-HT_{2A}	11±1,5
σ_1	52±8,0
5-HT_6	70±0,9
5-HT_7	105±3,16
D_2	113±10,0

Effects on the peripheral 5-HT system

Investigation of the 5-HT_{2A} receptor antagonist effect in the rabbit aortic strip

All three compounds inhibited the contractions evoked by the submaximal dose of serotonin. The IC_{50} value of deramciclane was about ten-fold higher than that of mianserin and 100-fold higher than those of ketanserin and ritanserin (table 2.).

Table 2. The effect of deramciclane and the reference compounds on the 5-HT-induced contractions in the aorta strip-preparations

Compound	IC_{50} (M)	n
Deramciclane	$4{,}2x10^{-7}$	3
Ketanserin	$3{,}5x10^{-9}$	3
Ritanserin	$5{,}5x10^{-9}$	3
Mianserine	$2{,}1x10^{-8}$	3

Inhibition of the serotonin-induced paw oedema

Both deramciclane and ritanserin inhibited the serotonin-induced paw edema in rats in a dose-dependent manner (fig. 1.). In the case of ritanserin, the statistically significant effect (minimum effective dose) was observed at a 100-fold lower dose (1.0 mg/kg p.o.) than that of deramciclane (30.0 mg/kg p.o., fig.1.).

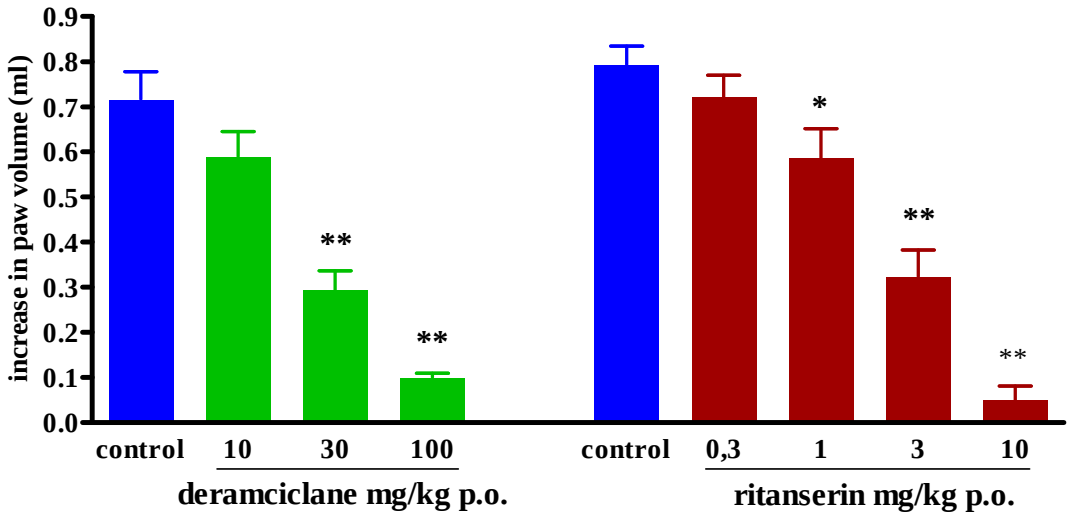

Fig. 1. The effect of deramciclane and the reference compound ritanserin on the serotonin-induced paw edema in the rat

Effects on the central nervous system

Inhibition of the DOI-induced head-twitch in the rat

Both deramciclane and ritanserin dose-dependently inhibited the DOI-induced head twitch at the same order of magnitude of concentrations. The effects of both compounds were statistically significant at all of the tested doses ($p<0.05$, Dunnett-test, fig. 2.).

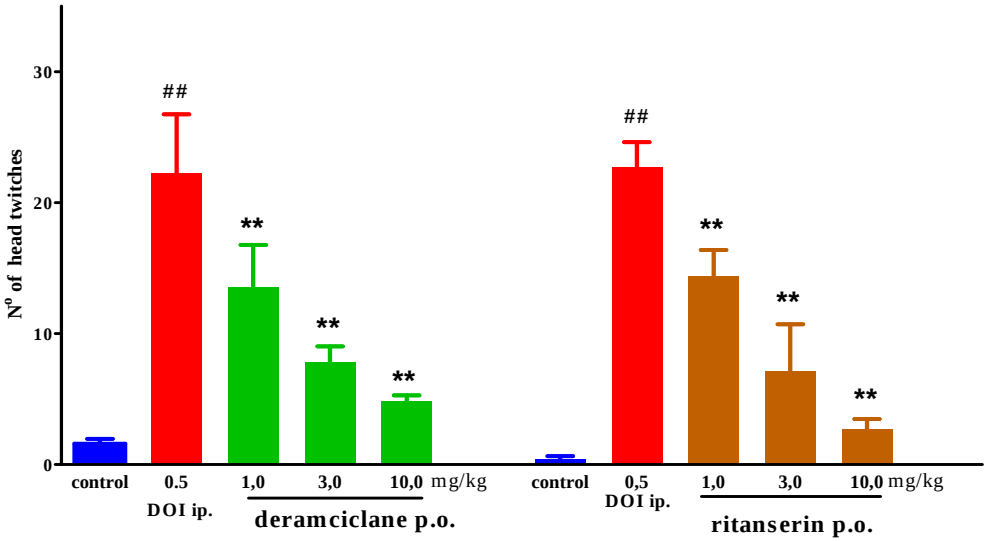

Fig. 2. Effect of deramciclane and the reference compound ritanserin on the DOI-induced head-twitch in the rat

Anxiolytic activity

Vogel lick-conflict test

Deramciclane significantly inhibited the decrease in the number of licks at doses of 1.0 mg/kg and 10.0 mg/kg p.o. (fig. 3.). Diazepam was effective at 0.3, 1.0 and 3.0 mg/kg (fig. 4.). Ritanserin inhibited the decrease in the number of accepted shocks at 0.3 mg/kg and 3.0 mg/kg, but the effect missed significance (fig 4.).

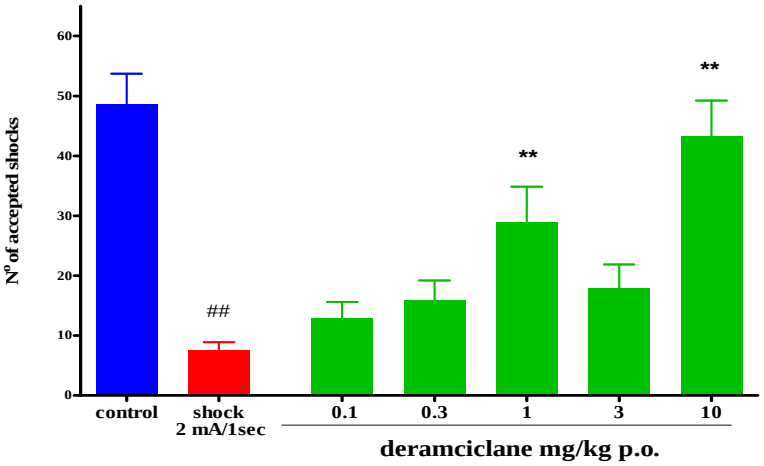

Fig. 3. The effect of deramciclane on the number of licks in the Vogel lick-conflict test.

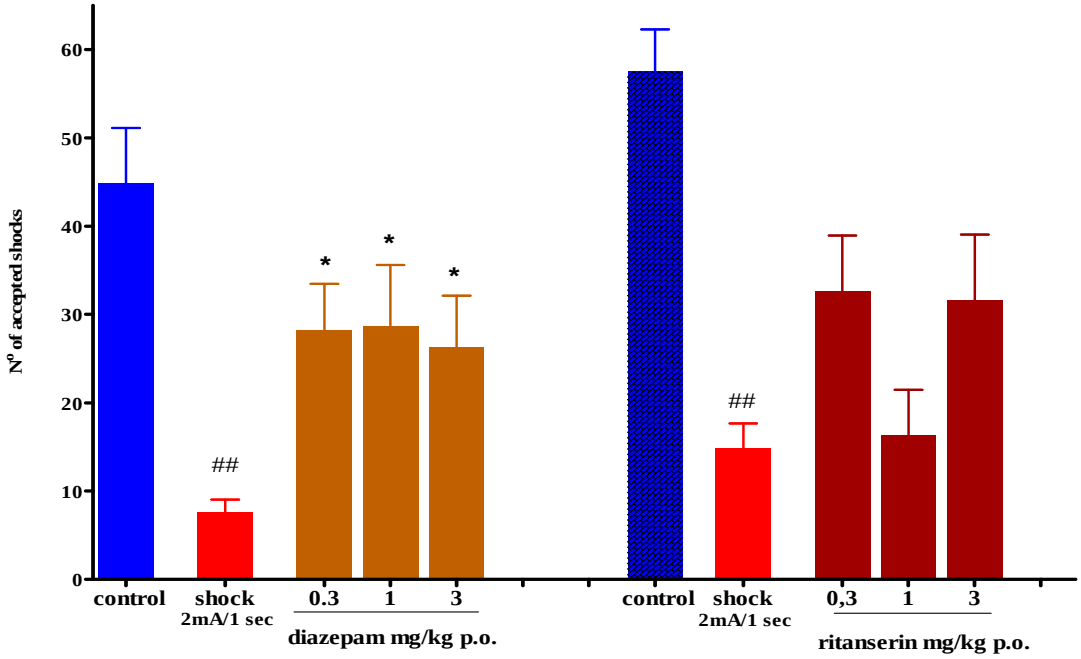

Fig. 4. The effect of diazepam and ritanserin on the number of licks in the Vogel lick-conflict test.

Marble burying test

All three anxiolytics, which were tested, dose-dependently inhibited the marble burying behaviour of mice. The effect of deramciclane at doses of 10.0 mg/kg and 30.0 mg/kg p.o. was statistically significant (fig. 5.). The ID_{50} value of deramciclane was similar to those of the reference compounds diazepam and ritanserin (table 3.).

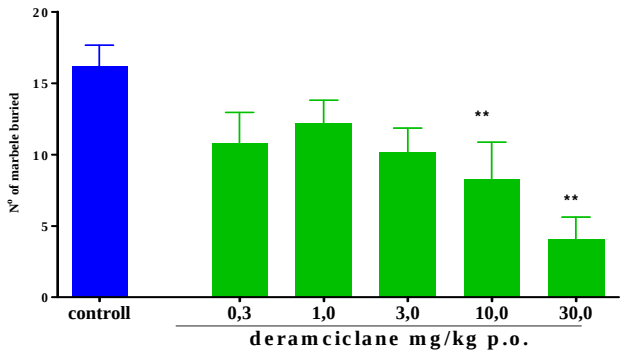

Fig. 5. The effect of deramciclane on the marble burying behavior of mice.

Table 3. ID_{50} values of different anxiolytic drugs in the marble burying test.

Compound	ID_{50} mg/kg p.o.	Maximum inhibition (%)	N° of doses
Deramciclane	**7,1**	**74,7**	**5**
Diazepam	**3,3**	**78,1**	**4**
Ritanserin	**3,7**	**76,0**	**3**

Light-dark test

Deramciclane could significantly increase the number of light-dark transitions at 3 mg/kg sc. (fig. 6.). The reference compound diazepam increased the number of transitions at the dose of 1.0 mg/kg but decreased it at 3.0 mg/kg. (fig. 6.). Deramciclane did not affect the general activity of the animals at any of the doses tested (fig. 7.). Diazepam significantly decreased general activity at 3 mg/kg (fig. 7.).

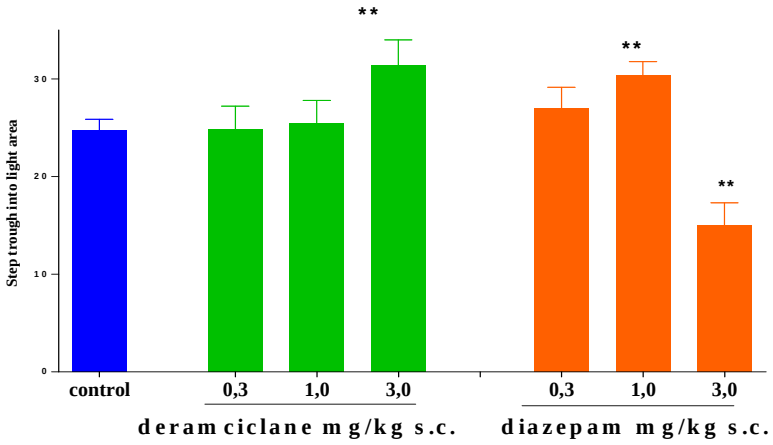

Fig. 6. The effects of deramciclane and diazepam on the light-dark transitions in the light-dark test.

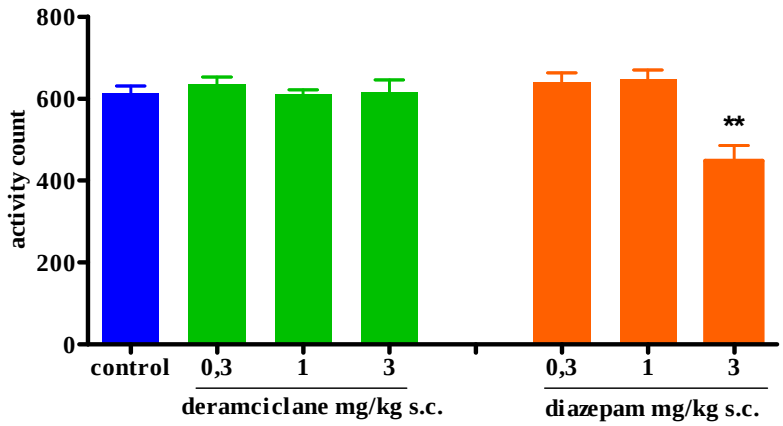

Fig. 7. The effects of deramciclane and diazepam on the activity measured during the 8-min test.

Effect on the spontaneous motor activity of mice

Deramciclane dose-dependently inhibited the spontaneous activity of mice. The inhibitory effect was significant at doses of 25 mg/kg p.o. and above (fig 8.). The reference compound ritanserin showed a comparable inhibitory effect at similar doses (table 4.).

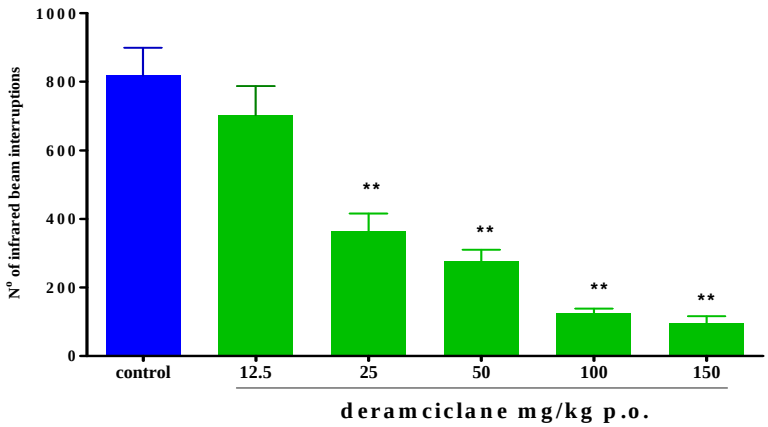

Fig. 8. The effect of deramciclane on the spontaneous motor activity of mice.

Table 4. ID_{50} values of different anxiolytic drugs in the spontaneous motor activity test

Compound	ID_{50} mg/kg p.o.	Largest inhibition observed (%)	Number of doses
Deramciclane	7,1	74,7	5
Diazepam	3,3	78,1	4
Ritanserin	3,7	76,0	3

CONCLUSIONS

- Deramciclane has high affinity for the 5-HT_{2A} and 5-HT_{2C} receptors in the brain and only moderate affinity for the 5-HT_6, 5-HT_7, σ_1 and D_2 receptors.
- Deramciclane shows no affinity for α_1, α_2, β_1, D_1, 5-HT_{1A}, benzodiazepine, $GABA_A$, CCK_A, CCK_B and H_1 receptors.
- Deramciclane has an antagonistic effect on the peripheral and central 5-HT system.
- Deramciclane can efficiently inhibit the *in vivo* behavioural and physiological responses induced by DOI, which confirms the results of the receptor binding studies.
- The effects of deramciclane on the serotonin system appear in the central nervous system at an at least 10-fold lower dose or concentration range than in the periphery.
- Deramciclane shows anxiolytic activity in three experimental models of anxiety.
- Deramciclane may have a therapeutic utility in the treatment of obsessive-compulsive disorder.
- Deramciclane has no effects on the spontaneous locomotion at anxiolytic doses.
- The mechanism of anxiolytic activity of deramciclane is most likely due to its antagonistic effect on the 5-$HT_{2A/2C}$ receptors.
- It cannot be excluded that modulation of 5-HT_6, 5-HT_7 and σ_1 receptors has a role in the anxiolytic activity of deramciclane.
- The dopamine antagonist effect of deramciclane, which can be detected at high doses, will presumably cause no side-effects at anxiolytic doses.

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