FULL PAPER *Toxicology*

Central and Peripheral Neurotoxic Effects of Ivermectin in Rats

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ABSTRACT. Ivermectin is considered a very safe drug; however, there are reports of toxic effects in particularly sensitive populations or due to accidental overdose. The aim of this study was (1) to further characterize the central and peripheral toxic effects of ivermectin in animals and (2) to determine possible therapeutic strategies for use in cases of ivermectin poisoning. We tested the effects of experimental doses of ivermectin previously reported to cause various intensities of CNS depression. However, in our study, ivermectin at 2.5, 5.0 and 7.5 mg/kg i.v. did not produce visible CNS depression in rats and 10 mg/kg resulted in sleepiness and staggering 10 to 40 min after application, while a dose of 15 mg/kg caused CNS depression very similar to general anesthesia. Ivermectin dose-dependently potentiates thiopentone-induced sleeping time in rats. Flumazenil (0.2 mg/kg), the benzodiazepine antagonist, did not affect the action of thiopentone; however, it significantly reduced sleeping time in rats treated with a combination of ivermectin (10 mg/kg) and thiopentone (25 mg/kg; from 189.86 ± 45.28 min to 83.13 ± 32.22 min; mean ± SD). Ivermectin causes an increase in the tonus (EC_{50}=50.18 μM) and contraction amplitude (EC_{50}=59.32 μM) of isolated guinea pig ileum, very similar to GABA, but without the initial relaxation period. These effects are dose-dependent and sensitive to atropine. Our results confirm the central and peripheral GABAergic properties of ivermectin in mammals and also indicate involvement of the cholinergic system in its toxicity. In addition, the results suggest that flumazenil and atropine have potential clinical roles in the treatment of ivermectin toxicity.

KEY WORDS: flumazenil, GABA, isolated ileum, ivermectin, thiopentone.

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Ivermectin (22,23-dihydroavermectin B1a) is a macrocyclic lactone widely used as an antiparasitic agent in veterinary and human medicine. Ivermectin belongs to the avermectins and is primarily isolated from fermentation products of the fungi *Streptomyces avermitilis* [6, 35]. The target of its antiparasitic action is an ivermectin-sensitive glutamate gated Cl^- channel receptor (GluCLR) that exists only in a number of invertebrates and the $GABA_A$ receptor. However, ivermectin and other avermectins can sometimes produce side effects in hosts. These effects can be related to GABAergic neurotransmission. Ivermectin is thought to promote the release of GABA and/or acts as a GABA receptor agonist, thus raising the GABA-induced chloride conductance [36, 37, 44].

The most dominant clinical symptoms of ivermectin poisoning in dogs and cats [24], sheep [5], pigs [38], horses [19] and other domestic and wild animals are CNS depression and sometimes coma, frequently resulting in death. There is no evidence of ivermectin neurotoxicity in humans, who are mostly treated against filariasis. This can be explained by the high efficacy of p-glycoprotein, a transmembrane protein highly conserved in the human population [22] that plays a central role in limiting drug uptake into the brain [14]. In domestic animals, the depressive effect of ivermectin is common and may include more than one mechanism. Certainly, ivermectin penetrates the brain capillary barrier easily and enters the brain tissue thereafter. Previously, it has been demonstrated that ivermectin produces a direct agonistic action on the $GABA_A$ receptor. There is also an opinion that avermectins in general act at sites recognized by benzodiazepines on the GABA receptor-ionophore complex chloride channel. We have demonstrated previously that ivermectin has important anticonvulsive properties in lidocaine and strychnine-induced convulsions, although these have different mechanisms [41]. Other authors have shown similar anticonvulsant actions of ivermectin in pentylenetetrazole-induced seizures [12]. Furthermore, some authors reported transient gastrointestinal upset either in humans or in animals poisoned by ivermectin, which may imply an action on neuronal tissue in the stomach and gut [3].

Ivermectin in the mammalian central nervous system interacts with at least three targets, a GABA-dependent chloride channel, a glycine-dependent chloride channel and a voltage-dependent chloride channel [1, 13, 15, 32]. Through these interactions, ivermectin realizes its central depressive effect, depending on the concentration in the CNS. All these findings are mainly derived *in vitro* from electrophysiological investigation or ligand binding assays. It is difficult to explain why proven *in vitro* antagonists of different types of chloride channel (picrotoxin, DIDS, strychnine, *etc.*) have no therapeutic value in treatment for poisoning with ivermectin [1, 39]. Alternatively, as we have noted previously, there is substantial evidence that ivermectin interacts with the binding sites of benzodiazepines on the GABA receptor-ionophore complex chloride channel [25,

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45].

The aim of the study was to further characterize the central and peripheral toxic effects of ivermectin in mammals and to determine possible therapeutic strategies for use in cases of ivermectin poisoning. Selection of the ivermectin doses used in the study was based on the available literature on experimental doses able to cause CNS depression as well as poisoning in different animal species [5, 12, 24]. We were interested in whether ivermectin prolonged thiopentone-induced sleeping behaviors and whether this effect was dose-dependent. Also, we chose to investigate the efficacy of flumazenil (clinically effective benzodiazepine-receptor antagonist) in neutralizing the central depressive action of ivermectin in a animal model. The central depressive effect of ivermectin in rats was evaluated on the basis of duration of sleeping time after application of increasing doses of the drug. The depressive effect on the CNS was also evaluated through the influence of ivermectin on the sleeping time in rats caused by thiopentone. Changes in barbiturate-induced sleep time can be a useful tool for examining stimulatory or inhibitory effects on the CNS, in particular for investigating influences on the GABAergic system. Barbiturates interact with GABA receptors and modulate GABAergic effects, while many hypnotic, antianxiety and anti-epilepsy drugs prolong barbiturate-induced sleeping time [30]. To elucidate the peripheral toxic action of ivermectin, we tested the effects of high concentrations on the basal tonus and amplitude of isolated guinea pig ileum. We chose supratherapeutic doses of ivermectin to mimic situations where overdose or accidental ingestion happened [18, 26]. In the enteric nervous system, there is a well-described direct link between GABAergic and cholinergic function [40]; therefore, it was important to examine whether the effect of ivermectin was sensitive to atropine, a broad-spectrum muscarinic antagonist.

The present study was carried out to clarify the mechanism of the toxic effects of ivermectin in animals and to examine possible approaches for therapy after ivermectin poisoning.

MATERIALS AND METHODS

Animals: White male Wistar rats weighing 150–200 g were housed under standard conditions for laboratory animals in groups of five with a controlled 12-hr light/dark cycle, temperature of 21 to 24ºC and "*ad libitum*" access to standard diet and water. All procedures in the study conformed to EEC Directive 86/609 and were approved by the Ethics Committee, Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Belgrade. At the end of the experiments, all rats were humanely euthanized by overdose with pentobarbitone in accordance with the Home Office Code of Practice (1997).

Male Dunkin-Hartley guinea pigs (300–350 g) were anesthetized with pentobarbitone (40 mg/kg i.p.) and then exsanguinated. The abdomen was opened immediately, and segments (10–15 cm) of intestine proximal to the ileocecal valve were removed. Gut contents were removed by flushing the lumen with saline solution (NaCl 0.9%), and the gut was placed in bubbled (95% O_2/5% CO_2) Krebs solution containing (mM) NaCl 139.9, KCl 2.7, CaCl 1.8, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5. at 37ºC.

Drugs and method of administration: Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied ivermectin, GABA, acetylcholine and atropine. Flumazenil (Anexate®) was obtained from Roche Pharma (Reinach, Switzerland), and thiopentone sodium (Trapanal® was obtained from Byk Gulden (Germany). Ivermectin was dissolved in propylene glycol + glycerol mixture (60:40 v/v), thiopentone sodium was dissolved in water for injections and flumazenil (Anexate®) was administrated in the original formulation. All drugs were dissolved immediately prior to application.

All drugs were administered by slow intravenous injection (*via* the lateral tail vein) using an intravenous cannula (Optiva™ 2, Johnson & Johnson, Arlington, TX, U.S.A.). In the case of multiple applications, the time between 2 injections was 3 min, and total volumes did not exceed 0.1 m*l*/100 g body weight.

Experimental design

Investigation of ivermectin depressive effect on the rat CNS: To study the potential depressive effect of ivermectin on the CNS, rats were treated with increasing intravenous doses of this drug. Animals were divided into 6 groups (n=15), one control group and 5 ivermectin-treated groups (2.5, 5, 7.5, 10 and 15 mg/kg). Ivermectin, or solvent for control rats, was injected intravenously, whereby application always lasted for 30 sec.

Thiopentone-induced sleeping time: To determine the dose-dependent sleeping time caused by thiopentone, investigations were done on a total of 105 rats divided in 7 equal groups (n=15 per dose), one control and 6 thiopentone-treated groups (10, 15, 20, 25, 30 and 35 mg/kg).

Influence of ivermectin (7.5 mg/kg) on thiopentone sleeping time: This part of the research was aimed at determining whether ivermectin (in a dose that by itself does not cause visible CNS depression) potentiates sleeping time caused by increasing doses of thiopentone. After 10 days of rest, the same rats from the previous study divided into 7 equal groups (n=15) were treated with 7.5 mg/kg of ivermectin and after 3 min with increasing doses of thiopentone sodium (10, 15, 20, 25, 30 and 35 mg/kg). Control rats, after administration of ivermectin, were treated with water for injections (0.1 m*l*/100 g body weight).

Influence of increasing doses of ivermectin on thiopentone (25 mg/kg) sleeping time: In order to determine whether ivermectin exhibits a dose-dependent effect on sleeping time induced by thiopentone, rats were divided into 5 equal groups (n=15) and pretreated with increasing doses of ivermectin (2.5, 5.0, 7.5 and 10 mg/kg). After 3 min, all rats were treated with thiopentone sodium (25 mg/kg). Control rats were pretreated with the solvent for ivermectin (propylene glycol + glycerol formal) in a volume of 0.1 m*l*/100 g body weight, followed by the treatment with thiopentone sodium (25 mg/kg) after 3 min. In this study

(after 14 days of rest), we used the same rats that were treated with ivermectin in the first investigation.

Potential modulatory effect of flumazenil on thiopentone sleeping time prolonged by ivermectin: Flumazenil, a specific antagonist of benzodiazepine receptors, was tested for any influence on the potentiating effect of ivermectin on barbiturate sleeping time. The investigation was performed on 60 rats randomly divided into 4 groups (n=15). The first group of rats was treated with thiopentone sodium (25 mg/kg), and served as a control. Rats from the second group were pretreated with ivermectin (10 mg/kg) and after 3 min were treated with thiopentone sodium (25 mg/kg), to determine the effect of ivermectin on the barbiturate sleeping time (this result was used from the previous examination - ivermectin 10 mg/kg + thiopentone 25 mg/kg). The third group of rats was pretreated with flumazenil (0.2 mg/kg) and after 3 min was treated with thiopentone sodium (25 mg/kg) in order to confirm that flumazenil does not affect the depressive effect of barbiturates [20]. Rats from the fourth group were pretreated with flumazenil 0.2 mg/kg and was subsequently treated with ivermectin (10 mg/kg) and thiopentone sodium (25 mg/kg) at 3-min intervals to determine whether flumazenil modulates the effect of ivermectin.

In all experiments, the number of sleeping animals and duration of sleeping time were recorded. The sleeping time was defined as the time interval between loss and restoration of the righting reflex measured with a chronometer and expressed in minutes. For sleeping time measurements, rats were placed on their backs on a warmed (35°C) pad in an acrylic box immediately after final application of the drug. The criterion for determining the beginning of sleeping time was loss of three successive righting reflexes. The criterion for restoration of the righting reflex was that the rats had to regain their normal posture three consecutive times.

Investigation of the ivermectin effects on isolated guinea pig ileum: Isolated whole segments from the ileum, 2–3 cm long, were cut and placed in the longitudinal direction in a 25-m*l* organ bath filled with bubbled (95% O_2/5% CO_2) Krebs solution at 37ºC. The upper end of the preparation was tied to an isometric transducer (Ugo Basile, Italy, load 0.5 g) connected to chart recorder (Gemini 7070, Ugo Basile, Italy). The preparations were allowed to equilibrate for 30 min (with intervening washing) before drugs were added. The effects of ivermectin and GABA on basal amplitude and tonus of spontaneous ileal contractions were observed during the 5 min after addition of the drugs, and the maximal response was measured. Acetylcholine (1 μM) contractions were measured at the beginning of each experiment as a viability control.

Ivermectin was dissolved in ethanol (95%), while atropine, acetylcholine and GABA were dissolved in distilled water. When tested, ethanol did not alter the resting activity of preparations and did not alter the drug responses.

Statistical analysis: Values are expressed as means and standard deviation of the mean (mean ± SD). Unless stated otherwise, all results were tested using the Student's *t*-test, and differences were considered significant at $p<0.05$.

Thiopentone sleeping time was tested by linear regression [Y (Effect) = a (y-intercept ± SEM) + b (slope ± SEM) • X (dose)]. Regression lines of thiopentone alone and the combination of the same doses of thiopentone and ivermectin (7.5 mg/kg) were then compared by F-tests.

Nonlinear regression analysis was used to determine the EC_{50} values of ivermectin in experiments with isolated guinea pig ileum (mean ± SD).

All statistical analyses were performed using commercial statistical software, GraphPad Prism, Version 4.0 (San Diego, Ca, U.S.A.). The linear regression lines and sigmoidal dose-response curves shown in the figures are original graphics obtained by GraphPad Prism.

RESULTS

The increasing doses of ivermectin, 2.5, 5.0 and 7.5 mg/kg i.v., did not produce visible CNS depression in rats. Rats treated with 10 mg/kg of ivermectin demonstrated sleepiness and staggering during the 10 to 40 min after drug administration. However, the highest tested dose of ivermectin, 15 mg/kg, caused CNS depression very similar to general anesthesia. Mean sleeping time in treated rats (n=15) was longer than 4 hr, but three rats died. The solvent for ivermectin (propylene glycol + glycerol formal) had no effect on righting reflex or on the other functions of the CNS.

As expected, thiopentone induced dose-dependent [$Y= -8.242 (\pm 2.075) + 1.248 (\pm 0.079) \bullet X$, $R^2 = 0.987$] sleeping in rats after intravenous administration of 15, 20, 25, 30 and 35 mg/kg. The sleeping times were 9.81 ± 6.0, 16.46 ± 9.87, 24.20 ± 8.59, 30.20 ± 5.92 and 34.13 ± 12.55 min, respectively (mean ± SD, n=15; Fig. 1A). The lowest tested dose of thiopentone (10 mg/kg) did not cause sleeping in the treated rats, and the only observed clinical symptom after drug application was short, transient CNS excitation. When ivermectin (7.5 mg/kg) was administered intravenously 3 min before thiopentone, barbiturate-sleeping time was significantly prolonged. Thus, the mean sleeping time induced by the same doses of thiopentone in the ivermectin-pretreated rats was significantly longer ($P<0.0001$), being 34.26 ± 14.18, 56.66 ± 26.99, 122.80 ± 42.68, 178.90 ± 31.84 and 232.10 ± 36.91 min (mean ± SD, n=15), respectively and dose-dependent [$Y= -100.1 (\pm 20.720) + 9.163 (\pm 0.860) \bullet X$, $R^2=0.965$] (Fig. 1A). Regression lines of those two effects were significantly different (test of parallelism and F-test; $P=0.00015$) (Fig. 1A). It is interesting to note that 10 mg/kg of thiopentone was not able to produce sleeping or any other kind of visible CNS depression in the treated rats. However, the same dose of thiopentone applied after ivermectin (7.5 mg/kg) pretreatment caused sleeping in rats that lasted 11.46 ± 8.40 min.

The mean sleeping time in the rats injected intravenously with 25 mg/kg of thiopentone sodium was 24.20 ± 8.59 min (mean ± SD, n=15), whereas the increasing intravenous doses of ivermectin, 2.5, 5.0, 7.5 and 10 mg/kg, administered 3 min before 25 mg/kg of thiopentone significantly

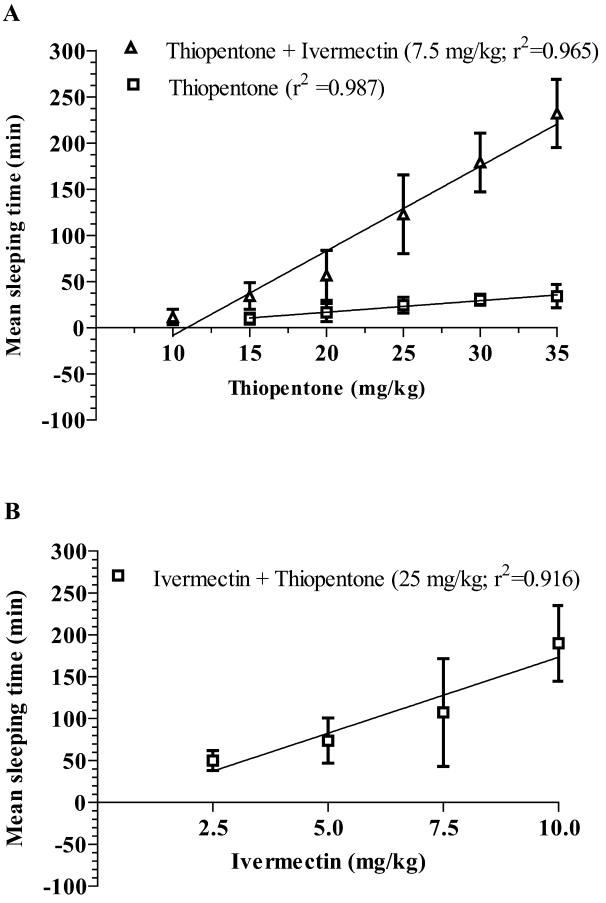

Fig. 1. A) Regression lines of the thiopentone induced sleeping time in rats and the sleeping time induced by the combination of ivermectin and thiopentone. Each point represents a group to 15 rats (mean ± SD). B) Regression line of the thiopentone induced sleeping time in rats (25 mg/kg) previously treated with increasing doses of ivermectin (2.5, 5.0, 7.5 and 10 mg/kg). Each point represents a group to 15 rats (mean ± SD).

(P<0.0001) and dose-dependently [Y= –8.065 (± 26.560) + 18.130 (± 3.879) • X, R^2 =0.916] prolonged barbiturate sleeping time (Fig. 1B). The mean sleeping times in the ivermectin-pretreated rats were 50.00 ± 11.89, 73.7 3± 26.92, 107.33 ± 64.49 and 189.86 ± 45.28 min (mean ± SD, n=15), respectively.

We have shown previously that the sleeping time in rats induced by 25 mg/kg of thiopentone lasted 24.20 ± 8.59 min. We have also demonstrated that ivermectin potentiates thiopentone sleeping time significantly and dose-dependently. Therefore, it was of interest to study whether flumazenil, an antagonist of benzodiazepine receptors, possesses the ability to modulate the depressive effects of ivermectin on the CNS. According to the results obtained, flumazenil alone (0.2 mg/kg) had no effect on thiopentone-induced sleeping time. Rats pretreated with flumazenil (0.2 mg/kg) and then treated with thiopentone (25 mg/kg) after 3 min, slept 25.66 ± 11.04 min, respectively. Ivermectin (10 mg/kg) significantly potentiated thiopentone-induced sleeping time in the previous experiments, from 24.20 ± 8.59 to 189.86 ± 45.28 min (P<0.0001). In animals pretreated with 0.2 mg/kg of flumazenil and then treated with ivermectin (10 mg/kg) and thiopentone (25 mg/kg) at 3-min intervals thereafter , the sleeping time was significantly less, 83.13 ± 32.22 min (P<0.0001; Fig. 2).

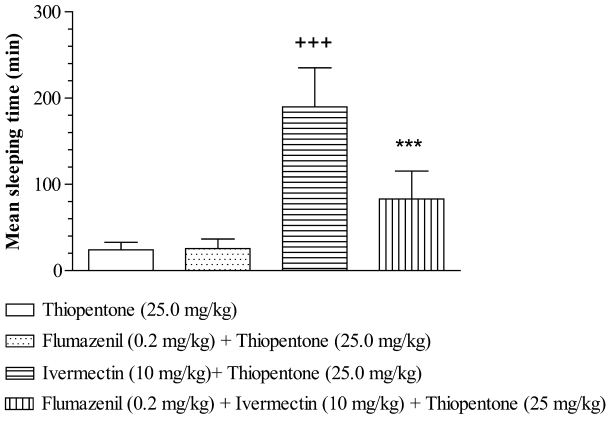

Fig. 2. The effect of flumazenil on barbiturate sleeping time in rats induced by thiopentone or the combination of thiopentone and ivermectin. $^{+++}$ P<0.0001 compared with mean sleeping time caused by thiopentone alone; *** P<0.0001 compared with mean sleeping time caused by ivermectin and thiopentone.

The addition of ivermectin to the organ bath fluid caused a gradual increase of the ileal basal tonus as well as the amplitude of spontaneous ileal contractions in a concentration-dependent manner (Table 1). The ivermectin effect began immediately after application and gradually increased until the end of the observation period of 5 min, at which point the ileum was washed out. We have produced a similar effect with GABA, but GABA exerted a biphasic relaxation-contractile action on the guinea pig ileum (Fig. 3a). Unlike ivermectin, GABA caused relaxation before the increase in tonus and amplitude of the ileum activity. Immediately after application of the drug to the organ bath fluid, basal tonus and the amplitude of the ileal mechanical activity appeared markedly decreased and lasted 1 to 2 min. After that, the tonus and amplitude of spontaneous ileal contractions began to increase, and in the final minute of observation, they were higher than the initial values. Furthermore, both observed effects are completely reversible after washing; hence, the tonus and amplitude of the ileal activity gradually returned to the predrug baseline values. As we have previously pointed out, the effect of ivermectin on isolated guinea pig ileum is concentration-dependent; therefore, according to our results, the EC_{50} of ivermectin for increasing the basal ileal tonus was 50.18 μM with a 95% CI of 44.79–56.22 μM, respectively (Fig. 4). Similarly, ivermectin significantly increased the amplitude of ileal mechanical activity, and the EC_{50} of ivermectin for increasing the amplitude of spontaneous ileal contractions was very similar at 59.32 μM with a 95% CI of 38.00–92.60

Table 1. The effects of increasing concentrations of ivermectin on the tonus and amplitude of spontaneous mechanical activities of isolated guinea pig ileum

Ivermectin (μM)	Mean tonus of ileum (g) ± SD	Mean amplitude of ileal contractions (g) ± SD before ivermectin addition	Mean amplitude of ileal contractions (g) ± SD after ivermectin addition	n
25.0	1.26 ± 0.402	0.205 ± 0.136	0.435 ± 0.192 *** (P<0.0001)	10
50.0	2.18 ± 0.710	0.280 ± 0.163	0.625 ± 0.243 ** (P=0.0016)	10
75.0	3.13 ± 1.079	0.330 ± 0.183	0.750 ± 0.189 *** (P<0.0001)	10
100.0	3.34 ± 0.939	0.380 ± 0.158	0.920 ± 0.284 *** (P<0.0001)	10
125.0	2.96 ± 0.723	0.316 ± 0.186	0.894 ± 0.324 *** (P=0.0003)	9

*– Compared with the mean amplitude before addition of ivermectin.

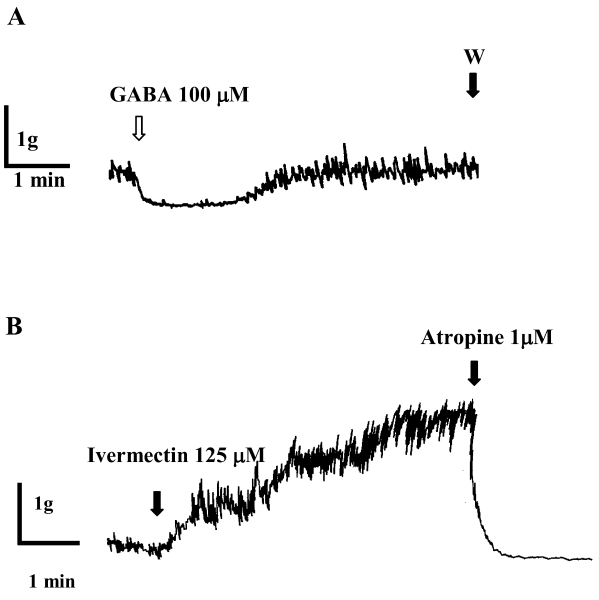

Fig. 3. A) Biphasic relaxation-contractile action of GABA (100 μM) on guinea pig ileum mechanical activity. B) Atropine (1 μM) effect on the increased tonus and amplitude of ileal contractions caused by ivermectin (125 μM).

μM, respectively (Fig. 5).

In a separate series of experiments, we tested the possible effect of atropine, a muscarinic antagonist, on the ileum contractions caused by ivermectin. Addition of ivermectin (125 μM) to the organ bath fluid resulted in an increase in the tonus and amplitude of spontaneous ileal contractions (Fig. 3B). During the period of 5 min, tonus gradually increased from 0.5 g to 2.62 ± 0.34 g (mean ± SD, n=6). The amplitude of contractions also increased, from 0.320 ± 0.102 g to 0.880 ± 0.240 g (mean ± SD, n=6). Application of atropine (1 μM) in the organ bath fluid (without prior washing of ivermectin) antagonized the effect of ivermectin. Complete relaxation of the ileum was observed, tonus returned to the basic level and the amplitude of spontaneous contractions was equal to 0. The effect of atropine was completely reversible, and after washing, the amplitude of spontaneous ileal contractions returned to the value before ivermectin application.

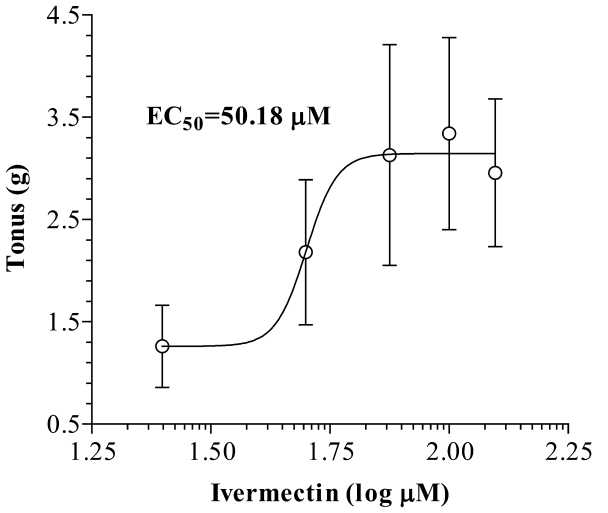

Fig. 4. Concentration-response curve of ivermectin effects on ileal basal tonus. The EC_{50} is 50.18 μM with a 95% CI of 44.79–56.22 μM.

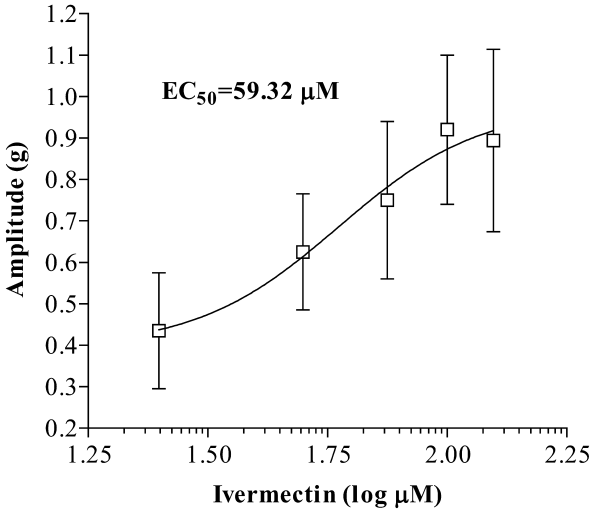

Fig. 5. Concentration-response curve of ivermectin effects on amplitude of ileal mechanical activity. The EC_{50} is 59.32 μM with a 95% CI of 39.88–70.62 μM.

DISCUSSION

The depressive effect of ivermectin in the mammalian CNS has been attributed to its GABAergic properties. In our study, increasing intravenous doses 2.5, 5.0, and 7.5 mg/kg of ivermectin did not cause clinically visible CNS depression in rats, but a dose of 10 mg/kg led to movement disorder, which disappeared spontaneously within 10 to 40 min. The highest tested dose of ivermectin (15 mg/kg) caused CNS depression, which was very similar to general anesthesia. However, it should be noted that these doses are significantly higher than the normal therapeutic doses of ivermectin (0.2–0.3 mg/kg), but still lower than the intravenous LD_{50} of 18 mg/kg [41]. There is little experimental data available on the CNS effects of high doses of ivermectin. However, observations on the CNS depressive effects of ivermectin after an accidental overdose in different animal species are frequently published [6]. A subpopulation of collie dogs, as well as some other breeds, is extremely sensitive to neurotoxicity induced by ivermectin. In these dogs, CNS depression, increased salivation, tremor, ataxia, temporary blindness and sometimes coma and death can occur after application of therapeutic doses of ivermectin. The described effects are attributed to a mutation in the multidrug-resistance gene (mdr1) causing a functional defect in P-glycoprotein (P-gp). P-gp is an integral part of the blood-brain barrier and acts as a drug-transport pump, transporting a variety of drugs from the brain back into the blood [27, 28]. However, this type of ivermectin toxicity was also described in dogs without a functional disorder in the drug-transport pump after administration of doses ranging from 0.2 to 2.5 mg/kg [29].

CNS depression caused by ivermectin, which is very similar to that induced by general anesthesia can be explained by an interaction with GABA receptors in the brain. Briefly, stimulation of GABA receptors, by ivermectin leads to the opening of chloride channels. Chloride ions then enter the nerve cells and hyperpolarize the postsynaptic membrane, which results in CNS depression. However, the CNS depression in rats observed in our study cannot be explained by this simple mechanism alone because, for example, the effect was not dose-dependent. The mechanism of CNS depression observed in our study is likely to be more complex and may involve multiple drug interactions and/or receptors. One possible explanation is that ivermectin stimulates the release of GABA at low concentrations, while high concentrations allow ivermectin binding to specific sites on the $GABA_A$ receptor-chloride channel complex. A 2nd explanation may lie in the limited ability of the p-glycoprotein pump to remove the drug from the CNS. Consequently, when ivermectin is present in high concentrations (in our study, i.v. doses more than 10 mg/kg), it accumulates in the CNS leading to a higher degree of depression. The depressive effects of ivermectin on the CNS cannot be explained by its action on $GABA_A$ receptors alone: picrotoxin, a potent $GABA_A$ receptor antagonist, is only partially effective in the treatment of poisoning and adverse effects of ivermectin in dogs [39]. Ivermectin has also been demonstrated to have effects on other vertebrate receptors. Avermectin B1a *in vitro* opens the $GABA_A$-receptor Cl channel by binding to the GABA recognition site and acting as a partial receptor agonist, but also opens a voltage-dependent Cl channel [1]. Additionally, the fact that ivermectin dose-dependently blocks convulsions caused by strychnine indicates a potential action on the chloride channels opened by glycine [41]. Finally, it is possible that different types of GABA-dependent ion channels with different receptor stoichiometry exhibit different sensitivities to ivermectin [42].

The nature of avermectins interaction with different types of $GABA_A$ receptors is still not clearly defined, but our results on the effect of ivermectin in the CNS of rats indicate a similarity to the effects of benzodiazepines. Most of the benzodiazepines have sedative-hypnotic, muscle-relaxant, anxiolytic and anticonvulsant properties [8, 42], but they do not cause dose-dependent general anesthesia. Benzodiazepines exhibit synergistic effects with barbiturates [7, 43] therefore, we tested the interaction of ivermectin with thiopentone. In our study, ivermectin potentiated thiopentone effects dose-dependently (Fig. 1B) and caused sleeping in rats at a concentration of thiopentone that does not cause CNS depression when used alone (thiopentone 10 mg/kg + ivermectin 7.5 mg/kg; Fig. 1A).

Given the similarities of the effects of ivermectin and benzodiazepines, it was important to examine the effect of flumazenil, a benzodiazepine-receptor antagonist. Previously, we have shown that flumazenil is able to significantly antagonize the anticonvulsive effects of ivermectin [41]. Our results demonstrate that flumazenil by itself did not affect the action of thiopentone; however, it significantly reduced sleeping (by an average of 106.93 min) in rats treated by the combination of ivermectin and thiopentone (Fig. 2). Flumazenil is a specific antagonist of benzodiazepine sites on the GABA-receptor chloride channel complex. It competitively antagonizes the binding and allosteric effects of benzodiazepines, but does not affect the effects of barbiturates [42], which was confirmed in our study (Fig. 2). Barbiturate action on the $GABA_A$ receptor requires the presence of 2 receptor subunits, α and β, while the effect of benzodiazepines and consequently flumazenil requires the α, β and $\gamma 2$ subunits [2, 42]. In our investigation, flumazenil achieved its activity by acting on components of the effect for which ivermectin is responsible. Our results indicate that ivermectin achieves its potentiation of barbiturate sleeping time by interacting with specific benzodiazepine binding sites on the $GABA_A$-receptor in the mammalian CNS, and we hypothesize that this interaction plays a significant role in ivermectin-induced CNS depression.

There is evidence that avermectins can also cause some peripheral nervous system effects. The peripheral nervous system and therefore multiple subtypes of chloride channel may also be important sites of action of avermectins toxicity [4]. The enteric nervous system is one possible target for avermectin action. Ivermectin has agonistic properties on $GABA_A$ receptors in the myenteric plexus of the guinea pig

intestine [10]. Rather than the therapeutic dose, we chose concentrations of ivermectin that would be likely to occur after overdose or accidental ingestion [18, 26]. In our study, ivermectin at high concentrations (10 times therapeutic), dose-dependently increased the tonus and amplitude of spontaneous mechanical activity of isolated guinea pig ileum (Table 1, Figs. 4 and 5). Coccini *et al*. [10] describe the dose-dependent contractions of isolated guinea pig ileum, caused by increasing concentrations of ivermectin that were sensitive to tetrodotoxin and hyoscine. Compared with the results of Coccini *et al*. [10], where ivermectin caused contractions, our study showed that ivermectin led to an increase in tonus and amplitude of the mechanical activity of the ileum, without causing classical contractions. Our results are in complete agreement with the results of Kerr and Ong [20], who reported that avermectin B1a caused neurally mediated rhythmic longitudinal mechanical activity in the isolated guinea pig ileum. On the other hand, the effect of GABA that Coccini *et al.* [10] described also differs from our results. They described only the contractile effect of GABA, while in most of the research about the effects of GABA on the contractions of isolated intestine, authors have always described a double effect, contraction and relaxation, as a result of the activation of $GABA_A$ and $GABA_B$ receptors [16, 31]. The reason for the described differences may be different experimental procedures. Coccini *et al.* [10] measured ileum contractions under isotonic conditions and used much higher concentrations of ivermectin (up to 300 μM) than we used. However, for us, it was particularly important that these disorders of gut contractility were sensitive to cholinergic antagonists, which gives the possibility of their clinical application in the case of ivermectin poisoning.

The presence of both types of GABA receptors in the gut has been proved by immuno-histochemical methods [11]; generally, it is thought that $GABA_A$ is an excitatory receptor and that $GABA_B$ is an inhibitory receptor [9]. In order to compare the effects of ivermectin and GABA on isolated guinea pig ileum, we treated ileum preparations with increasing concentrations of gamma-aminobutyric acid. GABA exerted a biphasic effect, initially decreasing (relaxation) and then increasing the tonus and amplitude of ileal mechanical activity, while by the end of the observation period (5 min), the activity exceeded the baseline levels. This effect of GABA on isolated ileum differed from the effects of ivermectin (Fig. 3). Ivermectin immediately caused an increase in tonus and amplitude, without relaxation. The observed differences may be explained by the fact that GABA acts on both types of GABA receptor. Activation of the $GABA_B$ receptor is responsible for the observed relaxation, while the increase in tonus and amplitude of ileal mechanical activity takes place through the activation of $GABA_A$ receptors [16, 20, 33]. Baclofen, a $GABA_B$ agonist, induces dose-dependent relaxations of guinea pig ileum longitudinal muscle. $GABA_B$ receptor causes relaxation through an inhibitory presynaptic action on cholinergic postganglionic neurones [16]. On the other hand, baclofen elicited dose-dependent depression of cholinergic twitch contractions in the guinea-pig isolated ileum, which is sensitive to the $GABA_B$ receptor antagonists phaclofen and 2-hydroxysaclofen [21]. Our results suggest that ivermectin acts as an agonist exclusively on $GABA_A$ receptor in the gut myenteric plexus. In order to explain in detail the nature of ivermectin effects on spontaneous rhythmic activity of the guinea pig ileum, it was important to study the role of the cholinergic system. Tanaka [40] assumes that the $GABA_A$ receptor is mainly located in cholinergic motor neurons that innervate longitudinal intestinal muscles and that their activation causes contractions that are cholinergic in nature, but sensitive to bicuculline and picrotoxin, which are antagonists of GABAergic chloride channels. Our results in this regard confirm such assumptions. Ivermectin caused an increase in tonus and amplitude of spontaneous rhythmic activity of the ileum that was completely blocked by atropine, a muscarinic receptor antagonist. These results suggest that the myenteric plexus of the ileum is a site of action of ivermectin toxicity in the mammalian body. The dose-dependence of ivermectin in potentiating ileal mechanical activity indicates a relationship between the GABAergic and cholinergic systems in the gut and that the effect of ivermectin differs from the effects of GABA.

On the other hand, the available data relating to the effects of benzodiazepines on ileum contractions are contradictory. For example, Luzzi *et al.* [23] reported that diazepam by itself does not affect the ileum, but dose-dependently increases ileal contractions induced by activation of $GABA_A$ receptors, which effectively antagonizes flumazenil. However, diazepam does not affect the contractions induced by acetylcholine, 5-HT, histamine and electrical stimulation. Contrary to these results, Hullihan *et al.* [17] found that diazepam produced a dose-dependent decrease in the electrically induced contractions of a longitudinal ileal muscle strip and antagonized the contractions induced by K^+, Ca^{2+}, histamine and carbachol. Another benzodiazepine, tetrazepam, produced concentration-dependent and complete relaxation of muscle contractions induced by KCl (80 mM) in the guinea pig ileum, and this relaxant action was not antagonized by pretreatment with hexamethonium (0.1 mM), an antagonist for nicotinic receptors, atropine (1 μM), an antagonist for muscarinic receptors, or PK 11195 (1 μM) antagonist for peripheral-type benzodiazepine receptors [34].

It is obvious that the peripheral effects of benzodiazepines are different from the effect of ivermectin, which in our study, cause an increase in the tonus and amplitude of spontaneous ileal contractions. In addition, atropine does not influence the effect of benzodiazepines on the ileum mechanical activity, but completely inhibits the enhancing effect of ivermectin on the tonus and amplitude of spontaneous ileal mechanical activity.

Our results confirm the central and peripheral GABAergic properties of ivermectin in mammals. In addition, we describe the ability of flumazenil to antagonize CNS depres-

sion in animals caused by ivermectin. We also demonstrate that atropine can neutralize the gastrointestinal effects of high concentrations of ivermectin. The results suggest a potential clinical application of these drugs for the treatment of side and toxic effects of ivermectin.

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