Association of oxamniquine praziquantel and clonazepam in experimental Schistosomiasis mansoni

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The antischistosomal activity of clonazepam, when administered alone or in association with oxamniquine and praziquantel, was experimentally evaluated in mice infected with Schistosoma mansoni. *The animals were treated 45 days post-infection with a single dose, by oral route, according to three treatment schedules: clonazepam 25 mg/kg and sacrificed 15 min, 1h or 4 h after treatment; clonazepam 1.0, 2.5 or 10.0 mg/kg and sacrificed 15 days post-treatment or with the dose of 10 mg/kg in association with oxamniquine 50 mg/kg or praziquantel 200 mg/kg, single dose, orally, every schedule with a control group. The efficacy of the drugs in vivo was assessed by means of worm counts and their distribution in mesentery and liver, mortality and oogram changes. In the chemotherapeutic schedules used, clonazepam did not present antischistosomal activity and the result of the association of this drug with oxamniquine or praziquantel was not significantly different from the one obtained when these two last drugs were administered alone. In the in vitro experiments, the worms exposed to 0.6 mg/mL clonazepam remained motionless throughout the 8-day-period of observation, without egg-laying, whereas the worms of the control group showed normal movements, egg-laying and hatching of miracidia on the last day of observation. The results obtained in the present study confirm the action of clonazepam on* S. mansoni *adult worm, in vitro, causing total paralysis of males and females. However, no additive or synergistic effects were observed when clonazepam were used in association with oxamniquine or praziquantel.*

Key words: *Schistosoma mansoni* - clonazepam - drug association

The most efficient measure for the morbidity control of schistosomiais is the treatment of infected patients. According to Savioli et al. (2004), the antischistosomal drugs play an important role for the treatment of infection and for the control of morbidity and transmission.

In spite of oxamniquine and praziquantel, that are the two available antischistosomal drugs for treatment of the disease, being efficient and presenting few side effects, some relevant aspects must be considered. Oxamniquine has a complicated manufacturing process, requiring big fermentation tanks for biological synthesis, resulting in a high cost, when compared to praziquantel. Due to this difficulty, praziquantel has been practically the unique drug utilized at the moment, and the use of a single drug in a mass scale and in recurrent treatments may result in the emergence of resistant strains.

For the reasons above, it is clear that there is the need for new and effective drugs, or new alternatives for the treatment of schistosomiasis. In our laboratories, a large project to find new antischistosomal drugs is being conducted. One attempt is to use known antischistosomal agents (mainly oxamniquine or praziquantel) in association with other drugs that may increase the activity of these known schistosomicides. In this context, we recently demonstrated that the concomitant use of lovastatine (potent inhibitor of the synthesis of cholesterol) produces in vitro and in vivo action against egg production and development (Araujo et al. 2008). Clonazepam belongs to the chemical group known as benzodiazepines, the main properties of which are mild inhibition of the functions of the central nervous system with anticonvulsive and sedative activity, muscular relaxant and tranquilizer effects, as its main proprieties. Clonazepam as an antischistosomal agent was quoted for the first time at Hoffman-La Roche Laboratories by Stohler (1978). Pax et al. (1978) revealed that *Schistosoma mansoni* worms incubated in clonazepam concentrations presented muscular contractions and remained motionless, thus resulting in the antischistosomal effect of the drug. Studies performed by Bennett (1980) suggested that the male worms of *S. mansoni* present binding sites for benzodiazepines in their tegument.

Based on these studies, this work investigates the antischistosomal activity of clonazepam in experimentally infected mice with *S. mansoni*, as well as its association with oxamniquine and praziquantel, well known antischistosomal medicines. In vitro studies were also carried out to observe the possible damages caused in *S. mansoni* worms by clonazepam.

MATERIAL AND METHODS

Experimental chemotherapy - Swiss mice (mean weight 20 g) were infected by subcutaneous route with 100 ± 10 cercariae of *S. mansoni* (LE strain). The Guidelines of the Ethical Committee for the use of experimental animals of the Fiocruz were followed. Forty-five days

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post-infection, groups of animals were orally treated, single dose, according to three schedules: 25 mg/kg clonazepam (Medley Industry) and sacrificed 15 min, 1h or 4 h after treatment; 1.0, 2.5, 5.0 or 10.0 mg/kg clonazepam and sacrificed 15 days after treatment; 10 mg/kg clonazepam associated to 50 mg/kg oxamniquine (Mansil®/Pfizer), or to 200 mg/kg praziquantel (Farmanguinhos/Fiocruz), simultaneously with the control groups. The animals were sacrificed by cervical fracture and perfusion was performed for collecting worms in the mesentery and liver. The same procedures were adopted for the control groups constituted by infected and untreated mice (Pellegrino & Katz 1968).

In vivo activity evaluation - Drug activity was assessed by means of perfusion of the animals pertaining to treated and control groups, considering the mean of the number of worms, rates of worm distribution in the mesentery and liver, dead worms in the liver and oogram changes (Pellegrino et al. 1962).

In vitro trials - Mice infected with *S. mansoni* cercariae (LE strain) were sacrificed using sodium pentobarbital 3% (30 µL/mice) and perfused according to the technique by Smithers and Terry (1965). The collected worms were distributed into plates of six wells each (4 pairs of worms per well and kept in culture medium RPMI-1640 supplemented with 5% fetal bovine serum, 100 µg/mL of antibiotics penicillin/streptomycin). In the experimental group, the worms were exposed to clonazepam at the dose of 0.6mg/mL (1mL of pediatric clonazepam, 2.5 mg/mL in 3 mL of culture medium) for 24 h and maintained in incubator at 37°C and 5% CO_2. In the control group, the worms were kept under the same conditions, except for the presence of the drug. Afterwards, the worms were washed with culture medium and maintained under the same previous conditions, but without drug addition throughout the rest of the trial. Observations under inverted microscope were documented 1 h after drug contact 5, 24, 48, 72 and 96 h after drug discontinuation and eight days after the beginning of the experiment. The culture medium was changed in alternate days. The experiments were performed in duplicate and repeated twice (2 experiments).

Statistical analysis - The results obtained were compared by means of the Student's *t* test, $p \geq 0.05$ being stipulated as significance level.

RESULTS

The results obtained with experimentally infected mice with *S. mansoni* (LE strain), treated with clonazepam 25 mg/kg/body weight, single dose, by oral route, and sacrificed 15 min, 1h or 4 h after treatment can be seen in Table I. In the treated animals, a discrete migration of the worms (*ca* 30%) to the liver could be observed, whereas in the animals of the control group this displacement was about 10% (not significant difference). No dead worms in the liver, as well as oogram changes were observed.

Table II shows the results obtained in mice treated with clonazepam 1.0, 2.5, 5.0 or 10.0 mg/kg and sacrificed 4 h or 15 days after treatment. No significant differences were presented by the animals of the treated groups when compared with the control group. It is worth highlighting that the animals treated with the highest dose (25 mg/kg body weight) remained sedated for at least 3 h, being recovered after this period. In none of the groups could be found a dead animal due to sedation.

The results of the trial with 10 mg/kg clonazepam in association with 50 mg/kg oxamniquine or with 200 mg/kg praziquantel are indicated in Table III. In the animals treated with clonazepam alone, a worm mortality rate of 6.8% could be observed, however, the other parameters did not show any change when compared with the control group. The results obtained in animals treated with clonazepam alone did not present significant differences, when compared with those obtained in animals treated with clonazepam in association with oxamniquine or praziquantel.

In vitro trials - The worms exposed to clonazepam could be seen motionless 1 h after contact with the drug, and so they remained throughout the rest of the experiment (Table IV). In the first trial, some worms could be seen contracted, when the observation was performed 48 h after the beginning of the experiment. In the second trial, the worms remained motionless and some blisters appeared in the tegument up to the 8th day of observation. *S. mansoni* eggs could not be seen in the experimental wells. The worms in the control groups appeared with normal movements and morphology, presenting eggs during the first 24 h, with the presence of newly hatched miracidia, when examined eight days after the beginning of the experiment (Figure).

TABLE I

Results obtained in mice experimentally infected with 100 ± 10 cercariae of *Schistosoma mansoni* (LE strain) treated with clonazepam 25 mg/kg body weight, single dose, orally, 45 days after infection

Time after treatment	Number of animals examined	Mean of worms	Distribution of worms %		Dead worms in the liver %	Oogram changes %
			Mesentery	Liver		
15 min	3	13.3	80.0	20.0	0.0	0.0
1 h	3	7.5	73.3	26.7	0.0	0.0
4 h	3	14.0	69.0	31.0	0.0	0.0
Control	3	10.6	90.6	9.4	0.0	0.0

TABLE II

Results obtained in mice experimentally infected with 100 ± 10 cercariae of *Schistosoma mansoni* (LE strain), treated with clonazepam, single dose, orally, 45 days after infection

Treatment schedule mg/kg	Time after treatment	Number of animals[a]		Mean of worms	Distribution of worms %		Dead worms in the liver %	Oogram changes %
		Treated	Examined		Mesentery	Liver		
1.0	4 h	3	3	6.7	85.0	15.0	0.0	0.0
1.0	15 days	5	5	14.0	97.1	2.9	0.0	0.0
2.5	4 h	3	3	9.3	85.7	14.3	0.0	0.0
2.5	15 days	5	5	14.8	86.5	13.5	0.0	0.0
5.0	4 h	3	3	11.0	90.9	9.1	0.0	0.0
5.0	15 days	5	3	8.3	84.0	16.0	0.0	0.0
10.0	4 h	3	3	8.0	95.8	4.2	0.0	0.0
10.0	15 days	5	1	7.0	100.0	0.0	0.0	0.0
Control	-	-	5	15.6	88.5	11.5	0.0	0.0

a: the difference between the number of treated animals and examined animals means the number of dead animals.

TABLE III

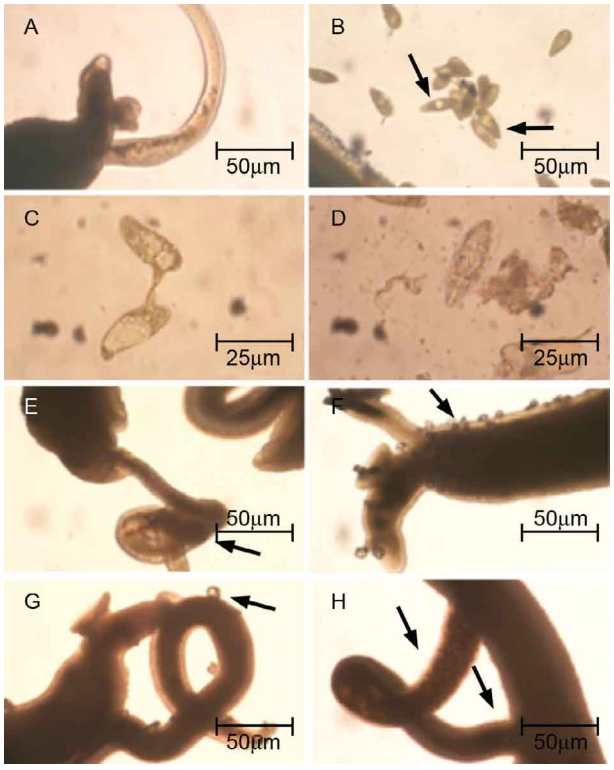
Results obtained in experimentally infected mice with 100 ± 10 *Schistosoma mansoni* cercariae treated 45 days after infection with 10 mg/kg clonazepam (CLO) alone or in association with 50 mg/kg oxamniquine (OXA) or 200 mg/kg praziquantel (PZQ), single dose, orally, and sacrificed 15 days post-treatment. There was no significant difference between the results obtained with oxamniquine and praziquantel when administered alone and those ones obtained with these two drugs in association with clonazepam.

Drug	Treatment schedule mg/kg	Number of animals		Mean of worms	Worm distribution %		Dead worms in the liver %	Oogram changes %
		Treated	Examined		Mesentery	Liver		
OXA	50	14	14	6.2	62.1	37.9	32.2	50.0
PZQ	200	14	14	9.7	36.8	63.2	56.6	42.9
CLO	10	14	14	10.2	79.5	20.5	6.8	0.0
CLO + OXA	10 + 50	14	14	9.3	40.0	60.0	56.2	64.3
CLO + PZQ	10 + 200	14	14	13.6	32.6	67.4	61.0	42.9
Control	-	-	14	12.6	86.4	13.6	0.0	0.0

TABLE IV

Results obtained in in vitro experiments using adult *Schistosoma mansoni* worms exposed to clonazepam at the dose of 0.6 mg/mL

Group	Period of observation	Observations (worms)
Experimental	1 h of contact	motionless, paired, without contraction, normal morphology, absence of eggs
Control		live, paired, normal movements and morphology, absence of eggs
Experimental	24 h of contact	motionless, paired, absence of eggs
Control		live, paired, normal movements and morphology, ± 100 eggs at the first stage and dead
Experimental	5 h after contact	motionless, paired, absence of eggs
Control		live, paired, normal movements and morphology, ± 100 eggs a\t the first stage and dead
Experimental	24 h after contact	motionless, 2 pairs of mated worms and 3 unmated other ones, contracted, absence of eggs
Control		live, paired, normal movements and morphology, ± 150 eggs at 1st and 2nd stages and dead
Experimental	48 h after contact	motionless, absence of eggs
Control		live, paired, normal movements and morphology, eggs at the 1st, 2nd, 3rd and 4th stages and dead
Experimental	7 days after contact	motionless, apparently without damages in the tegument, absence of eggs
Control		live, paired, normal movements and morphology, eggs at all developmental stages, presence of hatched miracidia but dead



Evaluation of the in vitro activity of clonazepam on *Schistosoma mansoni* worms. Control group not exposed to the drug. A: paired adult worms without apparent morphological change, two days of culture; B: presence of eggs of 1st (arrow) and 2nd (head of the arrow) stages, two days of culture; C: presence of eggs at the 4th stage, eight days of culture; D: presence of hatched miracidium, eight days of culture. Evaluation of the in vitro activity of clonazepam on *S. mansoni* worms. Group exposed to 0.6 mg/mL of the drug; E: adult worms: paired, motionless and contracted (female/arrow), two days of culture; F, G: adult worms: paired, motionless and contracted (2 and 3 days of culture, respectively), presence of crystals adhered to the worm tegument (arrow); H: paired female and male presenting separation of the tegument (arrow), eight days of culture. In none of the observations eggs in the groups exposed to clonazepam could be detected.

DISCUSSION

The results obtained show that clonazepam did not present antischistosomal activity in vivo when administered alone, nor addictive or synergistic action, when used in association with antischistosomal drugs (oxamniquine and praziquantel).

Stohler (1978) identified the benzodiazepines clonazepam and RO 11-3128 (methylclonazepam) as being antischistosomal drugs. That author showed that RO 11-3128 was highly effective against *S. mansoni* infection, similar to niridazole, hycanthone and oxamniquine. In vitro, when added at a concentration of 6.25 μg/mL to the medium containing *S. mansoni* male worms, contraction of these worms could be observed after some seconds. In hamsters, the activity against *Schistosoma haematobium* was similar to that showed for *S. mansoni*. However, infection due to *Schistosoma japonicum* was resistant to treatment when using hamsters treated with doses up to 150 mg/kg administered in five times. Pax et al. (1978) studied the activity of benzodiazepine RO 11-3128 and of praziquantel in the musculature of *S. mansoni* and *S. japonicum* worms and showed that at low concentrations this compound causes tension, paralysis and lack of movement in male worms. Bennett (1980), in vitro studies using 20 *S. mansoni* male worms and 20 also of *S. japonicum,* incubated at 37°C for 10 min in 2.0 mL of Earl's solution and varying the concentration of benzodiazepine RO 11-3128, suggested that *S. mansoni* male worm is endowed with a specific binding site, when this worm presents an intact tegument. These binding sites were clearly changed in presence of agents that, as it is well known, are able to destroy the membrane integrity. Boyle et al. (1985) evaluated the effect of meclonazepam (3-methylclonazepam) in volunteers, using single doses of 1, 2 and 4 mg, orally, and reported that the doses higher than 1 mg caused dose-dependent damages in the cognitive and psychomotor functions of patients, as well as mood changes and ataxia. These effects could be markedly observed at the first 3 h after drug administration and a mild sedation up to 6 h after drug administration of 4 mg. The authors suggest, as a result of the findings, that the use of benzodiazepines as antischistosomal drugs needs further investigation. Leite and Monteiro (2007) studied the participation of the binding sites in the contractive activity of clonazepam and 3-methylclonazepam on *S. mansoni* worms. Male adult worms were placed on glass slides containing saline solution and the muscular contraction effect of benzodiazepines was evaluated through the reduction of the body area, measured by analysis of the images captured by cameras. They observed that the utilized benzodiazepines at the concentration of 10 μM led to worm contraction in a time-dependent manner, similar to that of praziquantel, when used at the concentration of 1 μM. Based on these results, they concluded that the effect of contraction caused by both benzodiazepines is not due either to a direct effect in the worm muscle or as a result of binding to one of the two benzodiazepinic receptors present in *S. mansoni*. Noel et al. (2007) investigated the presence of gama-aminobutyric acid (GABA) in *S. mansoni* adult worms, using [3H]-flunitrazepam to label the alosteric binding sites of the benzodiazepinic receptors and detected a large number of binding sites to [3H]-flunitrazepam in the great majority of the worm population. The authors describe the pharmacological proprieties of some diazepine receptors as possible targets for the development of new antischistosomal drugs. The presence of benzodiazepinic peripheral receptors in the parasite was found for the first time by Noel et al. (2007). Pica-Mattoccia et al. (2008) studied the mode of action of RO11-3128 (methyl-clonazepam) and praziquantel, in vitro and in vivo, on *Schistosoma* worms. Both drugs cause paralysis, influx of the calcium ducts and damages in the parasites´ tegument. Since it is well known that RO 11-3128 is active against immature

worms of *S. mansoni* and praziquantel is ineffective at this phase, praziquantel being active against *S. japonicum*, whereas RO 11-3128 is inactive, the excess of one drug on another one was studied. It was verified that the excess of praziquantel does not inhibit the activity of RO 11-3128 on *S. mansoni* immature worms and an excess of RO 11-3128 does not inhibit the efficacy of praziquantel on *S. japonicum*, suggesting that the binding site of the worm with both drugs is rather different. On the other hand, when cytochalasin D, an agent that blocks the calcium channel, was used it inhibited the activity of both agents. Association of these results suggests that both drugs, although binding themselves at different receptor sites in the parasite, present the same schistosomicidal mechanism (Picca-Mattoccia et al. 2008).

It must be highlighted that in the majority of the studies above mentioned, the activity of clonazepam and RO 11-3128 was observed in male worms of *S. mansoni* proceeding from unisexual infections. Pica-Mattocia et al. (2008) showed the activity of praziquantel and RO 11-3128 on *S. japonicum* adult worms, males and females, and reported that praziquantel was found to be more active against male worms. Pre-incubation with RO 11-3128 for 1 h before addition of praziquantel did not affect the activity of this last drug (Picca-Mattoccia et al. 2008).

The results obtained in the present study confirm the action of clonazepam on *S. mansoni* adult worm, in vitro, causing total paralysis of males and females. However, with the therapeutic schedules used in experimentally infected mice, it was not possible to detect any activity against *S. mansoni*, when clonazepam was administered alone or in association with oxamniquine or praziquantel. No addictive or synergistic effects were observed when clonazepam was used in association with these two known antischistosomal drugs.

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