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Synergic effect of metronidazole and pyrantel pamoate on *Giardia lamblia*

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ABSTRACT

Giardia lamblia is a pathogenic protozoan presenting as the main characteristic, the trophozoite capacity to adhere in host intestinal epithelium, infecting mammals, including humans. The clinical treatment of this disease is based on metronidazole (Mz) that acts as an alternative electron acceptor, and its reduction promotes DNA impairment. In veterinary treatment, one of the best options is pyrantel pamoate (Pm), which the mode of action has not elucidated yet. Different strategies for *Giardia* treatment have been explored to avoid side effects to the host. In this context, the efficiency of treatment combining drugs raise as an interesting alternative for protozoan diseases. Here, we evaluated *in vitro* synergic effect of Mz and Pm on trophozoites and on its adherence to IEC-6 cells. The treatment with Mz or Pm was effective on trophozoites, with IC_{50}/24 h values of 5.3 ± 0.9 μM and 13.8 ± 1.4 μM, respectively. The treatment of trophozoites with different combinations of Mz and Pm were also evaluated, as showed by fractional inhibitory concentration index (FICI) under 0.5 in all conditions tested, corresponding to the synergic effect. This synergic activity was also observed when the combinations of 5.3 μM Mz + 0.4 μM Pm and 13.8 μM Pm + 0.1 μM Mz induced a remarkable reduction in % adhesion (85–90% and 52–59%, respectively) and in number of adhered parasites per 100 cells. The low cytotoxicity to the host cells of the combinations, associated to the strong synergic potential of the combination, encourage us to further investigate its effect in *in vivo* models.

1. Introduction

Giardia spp. is a pathogenic protozoan considered the earliest branching eukaryote, presenting as the main characteristic, the capacity of trophozoite form to adhere in host intestinal epithelium [1,2]. *Giardia lamblia* infects different mammals including dogs, cats and humans, and the same strain can be responsible for the disease in domestic animals and humans [3]. The drugs susceptibility of these intestinal protozoa is common related to compounds previously tested on helminths. Despite the evolutionary distance between these organisms and *Giardia* sp., it was already reported that the most of those drugs share similar mechanisms of action and cellular targets such as anaerobic pathways and the microtubules organization in both organisms [4–6].

The current treatment of *G. lamblia* infection is based on metronidazole (Mz), a cytotoxic nitroimidazole that induces to several uncomfortable adverse effects such as headache, gastrointestinal tract disorders and hypersensivity reactions, accordingly to the U.S. National Institutes of Health. This compound is also employed for radiosensitisation of hypoxic tumours, being included as a potential human carcinogen by the International Agency for Research on Cancer (IARC) [6–8]. In *Giardia* sp., Mz acts as an alternative electron acceptor, and its reduction in the anaerobic metabolism, promotes DNA damage, blocking its segregation and the parasite division [9]. Morphological alterations were previously characterized on trophozoites treated with Mz such as bubble-like structures [10]. In veterinary treatment, the main options are the praziquantel and pyrantel pamoate (Pm), which are usually prescribed alone or combined [11]. The mode of action of Pm in *G. lamblia* has not elucidated yet, but the involvement of cholinesterases was described in helminths nervous system [12]. Ultrastructural studies showed that Pm induced a disorganization of protozoan lateral flange and an inhibition of the attachment and growth of trophozoites *in vitro* [13].

Extensive efforts are being directed to the development of different strategies for *Giardia* treatment, avoiding side effects to the host tissues and organs. The increase in the resistance of the parasite to the clinical compounds, together with the recognizable efficiency of combined treatment on many helminths, supports the employment of the combined treatment for protozoa diseases as an interesting alternative. It was also described that drugs combinations potentialize the effect of the compounds alone, therefore outcoming in more effective treatment in a variety of parasitic protozoa such as *Plasmodium falciparum* and *Trypanosoma cruzi* [14,15]. In *Entamoeba*

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hystolitica, the combinations of Mz and lactoferrin derivatives induced a higher effect when compared to the drugs alone [16]. In this work, we evaluated the synergic activity of Mz and Pm on *G. lamblia* trophozoite and on its adherence to intestinal epithelial cells *in vitro*.

2. Materials and methods

2.1. Parasites and cell cultures

Experiments were performed with trophozoites of *G. lamblia* (WB strain, ATCC 30957) which were grown up to log phase in TYI-S-33 medium (pH 7.0) at 37 °C for 72 h, supplemented with 10% fetal bovine serum and 0.1 mg/mL of bovine bile, without added vitamins and antibiotics [17].

Monolayers of rat intestinal epithelial cells (IEC-6 line, ATCC CRL-1592) were grown in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, USA) added to 10% fetal bovine serum and 1 U/mL of regular human insulin. For all experiments IEC-6 cells were trypsinized, added to 24-well and 96-well plates (Nunc Inc., USA) at final concentrations of 1×10^5 cells/mL and $0{,}8\times10^4$ cells/mL, respectively, and maintained at 37 °C for 24 h in 5% CO_2 atmosphere.

2.2. Direct effect on G. lamblia

G. lamblia trophozoites (2×10^5 parasites/mL) were resuspended in TYI-S-33 medium containing desired final concentrations of Mz and Pm (Sigma-Aldrich, USA) for 24 h at 37 °C. Cell counts were performed in Neubauer chamber and the activity of the compounds and their combinations was expressed as IC_{50}, corresponding to the concentration that leads to 50% parasite lysis. The stock solutions of Mz and Pm were prepared in dimethyllsulfoxide (Sigma-Aldrich, USA) at the concentration of 60 mM and 20 mM, respectively.

For the determination of the synergic effect, parasites suspension was incubated with different combinations of Mz and Pm for 24 h in prior to define the optimal and sub-optimal concentrations of each compound. Fractional inhibitory concentration index (FICI) values were calculated by the equation $FICI = IC_{50}$ (optimal dose of Mz + sub-optimal dose of Pm)/IC_{50} (Mz alone) + IC_{50} (optimal dose of Pm + sub-optimal dose of Mz)/IC_{50} (Pm alone) [18]. Classical isobolograms were constructed by plotting drugs concentrations (alone and in combination) that inhibits 50% trophozoites growth. According to accepted guidelines: $FICI \leq 0.5$, synergic effect; $0.5 < FICI \leq 4.0$, no interaction; and $FICI > 4.0$, antagonic effect [19].

The IEC-6 cells achieved monolayers were washed and incubated with *G. lamblia* trophozoites (ratio 2:1 parasite/host cell) for 1 h at 37 °C in the interaction medium that consists in DMEM (pH 7.4), 20% foetal bovine serum, 0.95% trypticase peptone of casein (BBL Microbiology Systems, USA), 0.07% L-cystein (Sigma-Aldrich, USA) and 1 U/mL insulin [20]. After the removal of non-adhered parasites by washing, Mz, Pm and their combinations were added to the infected cultures for 24 h and the percentage of adhesion and the number of adhered parasites/100 cells were quantified using a Zeiss Axiophot microscope (Oberkochen, Germany).

2.3. Toxicity to mammalian cells

Non-infected IEC-6 cells cultured on 96-well plates (Nunc Inc., USA) were treated with the two drugs and their respective combinations for 24 h and their toxicity was evaluated by a dye-reduction assay [21]. The cells were incubated with 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h at 37 °C, then DMSO was added to stop the reaction and the absorbance was read at 490 nm in Molecular Devices VersaMax microplate reader (Sunnyvale, USA).

2.4. Statistical analysis

The comparison between control and treated groups was performed by the Mann–Whitney test. Differences with $p \leq 0.05$ were considered as statistically significant.

3. Results

The treatment with Mz or Pm was effective on trophozoites *in vitro*, with IC_{50}/24 h values of 5.3 ± 0.9 μM and 13.8 ± 1.4 μM, respectively (Fig. 1a,b). An increased activity was observed in parasites treated with different sub-optimal doses of Mz + 13.8 μM Pm in relation to the treatment with each compound alone, leading to the inhibition up to 66% (Fig. 1c). The combinations of 5.3 μM Mz + sub-optimal doses of Pm showed no significant increase effect in comparison to each IC_{50}/24 h drug alone (Fig. 1c).

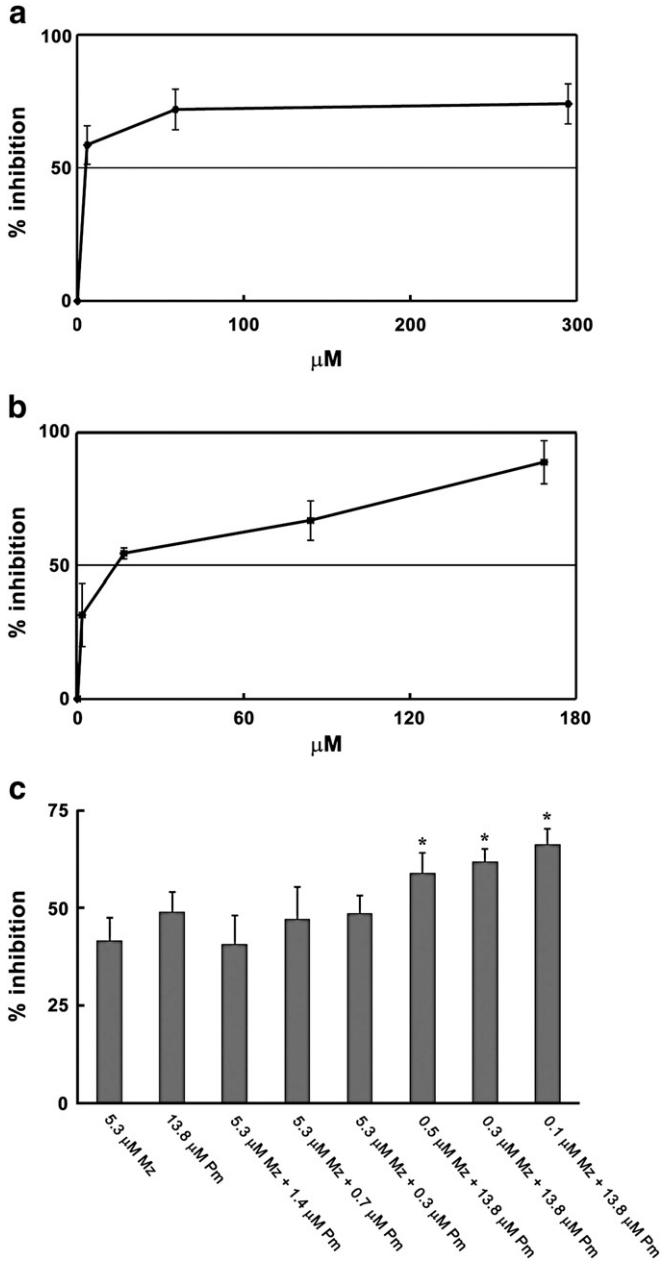

Fig. 1. Effect of (a) Mz, (b) Pm and (c) their combinations on *G. lamblia* trophozoites in TYI-S-33 medium at 37 °C for 24 h. The asterisks indicate significant differences in relation to 5.3 μM Mz and 13.8 μM Pm groups ($p \leq 0.05$).

In prior to analyze the synergic effect of Mz and Pm on trophozoites, different combinations were employed to construct isobolograms: 5.3 μM Mz + 1.4 μM Pm and 0.5 μM Mz + 13.8 μM Pm; 5.3 μM Mz + 0.7 μM Pm and 0.3 μM Mz + 13.8 μM Pm; and 5.3 μM Mz + 0.4 μM Pm and 0.1 μM Mz + 13.8 μM Pm, being FICI = 0.31 ± 0.02, 0.15 ± 0.03 and 0.07 ± 0.01, respectively (Table 1). As described above, FICI values under 0.5, correspond a synergic activity, and all the combinations tested are in this range. It was also observed that the strongest synergic effect at combinations containing lower sub-optimal doses of both compounds (Table 1).

Another set of experiments was performed to evaluate the effect of Mz, Pm and their combinations on the interaction of trophozoites and IEC-6 cells. Based on the IC_{50}/24 h values for the axenic parasites, it was observed that 5.3 μM Mz led to an important decrease in % adhesion of trophozoites on cell surface (Fig. 2a) and in the number of adhered parasites per 100 cells (Fig. 2b), inhibiting 70% of adhesion in both parameters while 13.8 μM Pm not presented a significant difference in relation to control (Fig. 2a,b). The combinations of 5.3 μM Mz + 0.4 μM Pm and 13.8 μM Pm + 0.1 μM Mz also induced a remarkable reduction in % adhesion and number of adhered parasites per 100 cells, being the % inhibition in range of 85–90% and 52–59%, respectively. MTT assays carried out with non-infected IEC-6 cells showed that the preservation of Mz-treated cells viability up to 84.8 μM, however high doses of Pm (220.8 μM) led to lysis of 50% cells. It was also observed that the combinations presented the same viability of untreated cells, except to a strong combination of 5.3 μM Mz + 138 μM Pm which the toxicity was similar to the higher dose of Pm alone (Table 2).

4. Discussion

Many efforts have been made for substitute the current treatment of *Giardia* infection, due to the increasing number of refractory cases after use of nitroimidazoles or other chemotherapic agents (i.e. praquizantel, benzimidazoles and pamoate salts). In order to improve the efficacy and reduce treatment course, drug combinations have been explored for different bacterial, viral and parasitic infections [22]. On axenic trophozoites, Mz presented a higher activity (IC_{50}/24 h = 5.3 ± 0.9 μM) in relation to Pm (IC_{50}/24 h = 13.8 ± 1.4 μM), being similar to IC_{50}/24 h values of Mz and Pm obtained for other *G. lamblia* strains and isolates [10,13,23,24]. Recently, structural modifications in Mz molecule were made, and four analogues presented higher giardicidal activity in comparison to Mz, without relevant cytotoxic effects to the host cells [25]. The employment of Mz analogues alone or in combination with other drugs such as Pm, could represent an interesting opportunity to increase even more the anti-giardia potential.

The combination of different concentrations of Mz and Pm showed that sub-optimal doses of Mz + 13.8 μM Pm led to an increase in the inhibition of the parasite proliferation. It suggests a potencialization of the compounds effect, as already described for *P. falciparum* and *T. cruzi* treated with clotrimazole + amphotericin B and mevinolin + ergosterol biosynthesis inhibitors, respectively [14,26]. Isobolograms analysis in the present study indicated a synergic activity in all experimental conditions performed in the *Giardia* sp. model employing Mz and Pm. A dose-dependent decrease in FICI values, points to the higher synergy in combinations of 5.3 μM Mz + 0.3 μM Pm and 0.1 μM Mz + 13.8 μM Pm on trophozoites, open promising possibilities of doses reduction during the giardiasis treatment, maintaining similar proliferation inhibition. The synergic effect of drugs on *G. lamblia* was already reported for phenyl-carbamate derivatives and albendazole, being suggested the further potential application in the giardiasis therapy [27]. Previous studies pointed to potent *in vivo* synergic effect of miltefosine + amphotericin B and mevinolin + ketoconazole on *Leishmania* sp. and *T. cruzi*, respectively [22,26]. The treatment of experimental *Giardia* sp. infected kittens with combinations of febantel, pyrantel and praziquantel demonstrated potential results, increasing the percentage of apparent cure [11]. Therefore, our *in vitro* results motivate the investigation of the combined treatment of Mz and Pm in murine models.

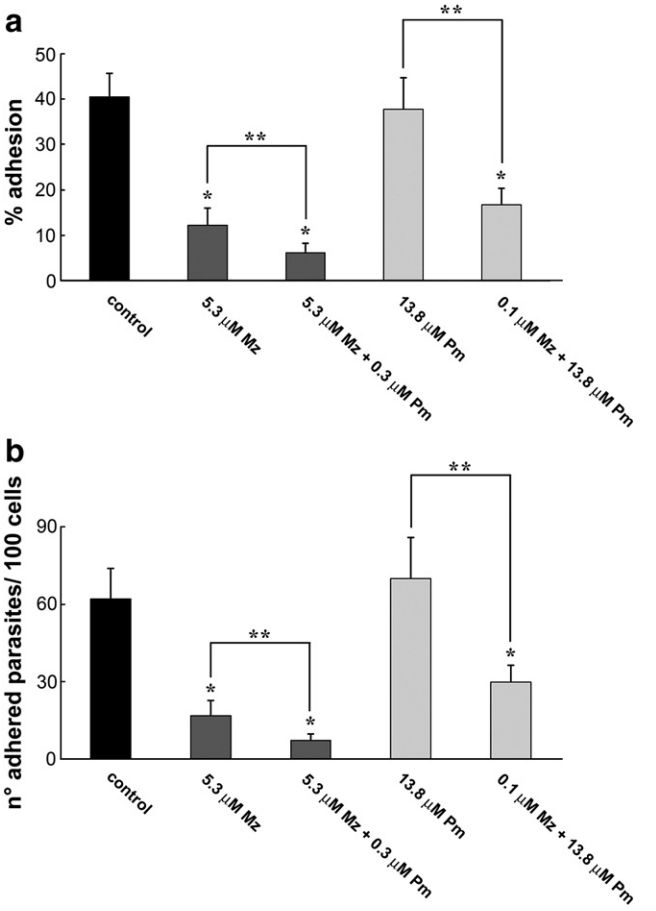

Fig. 2. Effect of Mz, Pm and their combinations on the attachment of *G. lamblia* trophozoites on IEC-6 cells. (a) The percent of adhesion and (b) the number of adhered parasites per 100 cells. The treatment with the combinations led to the increase in the inhibition in relation to Mz and Pm alone. One asterisk represents the significant difference in relation to control ($p \leq 0.02$), and two asterisks indicate differences in comparison to 5.3 μM Mz and 13.8 μM Pm groups ($p \leq 0.05$).

Table 1
Synergic effect of Mz and Pm on *G. lamblia* trophozoites.

Drugs combinations[a]	FICI[b]	Activity[c]
IC_{50} Mz + 10% IC_{50} Pm and 10% IC_{50} Mz + IC_{50} Pm	0.31 ± 0.02[d]	S
IC_{50} Mz + 5% IC_{50} Pm and 5% IC_{50} Mz + IC_{50} Pm	0.15 ± 0.03	S
IC_{50} Mz + 2% IC_{50} Pm and 2% IC_{50} Mz + IC_{50} Pm	0.07 ± 0.01	S

[a] IC_{50} drug "A" + sub-optimal dose drug "B" and sub-optimal dose drug "A" + IC_{50} drug "B."
[b] Fractional inhibitory concentration index.
[c] S, synergic effect; NI, no interaction; A, antagonic effect.
[d] Mean ± standard deviation for 4 independent experiments.

Table 2
Cytotoxic effect of Mz, Pm and their combinations on IEC-6 cells.

Drugs combinations	% of control
5.3 μM Mz	97.3[a] ± 8.8
84.8 μM Mz	92.4 ± 6.8
13.8 μM Pm	83.1 ± 12.9
220.8 μM Pm	43.1 ± 7.1
0.1 μM Mz + 13.8 μM Pm	86.0 ± 9.5
5.3 μM Mz + 0.3 μM Pm	98.1 ± 5.0
5.3 μM Mz + 13.8 μM Pm	85.8 ± 10.1
53 μM Mz + 13.8 μM Pm	81.1 ± 9.5
5.3 μM Mz + 138 μM Pm	49.9 ± 2.6

[a] Mean ± standard deviation for 4 independent experiments.

The combined treatment also interferes with % adhesion of parasites in the intestinal epithelial cells. The combination of 0.1 μM Mz + 13.8 μM Pm presented the highest synergic activity on axenic trophozoites, and also induced an important reduction in the % adhesion (about 80%). However, the highest inhibition of the attachment of *G. lamblia* on IEC-6 cells was determined after the treatment with 5.3 μM Mz + 0.3 μM Pm (in range of 90%). Previous studies with thiazolide showed a significant inhibitory effect on the adherence of the parasite in Caco-2 cultures, despite the moderate inhibition of *G. lamblia* proliferation in axenic condition [23]. In the present work, the intense synergic effect of Mz and Pm was observed on both experimental situations, pointing to the effect of the combinations on free and adhered parasites. Our data also demonstrated the damage to the host cells detected only in combined concentrations at least 10-fold higher than those doses employed to reduce the infection, an important indicative of low cytotoxicity, as already reported for IEC-6 cell line treated with natural products [28].

Pm belongs to the tetrahydropyrimidine family which mode of action involves different metabolic changes in the oxidative pathways, however in *Giardia* sp. and other anaerobic pathogens the mechanism is unclear. Previous pharmacokinetic studies reported a significant absorption of Pm in large intestine of pigs, decreasing the compound bioavailability in the infection site [29]. So far, the Pm incorporation by IEC-6 cells could explain the more prominent effect on the trophozoites adhesion of high concentration of Mz + sub-optimal doses of Pm. On the other hand, Mz degradation rate is constant in different experimental conditions such as buffer and pH variation [30]. Furthermore, it was demonstrated that intestinal epithelia of *G. lamblia* cronically infected animals secrete high rate of anions, mainly chloride salts [31]. So far, this active anion secretion was observed in other infections such as in HIV, resulting in the cytokines release from sub-epithelial immune cells [32]. For the effective removal of *G. lamblia*, it is well-described that mechanisms of host defense have to be activated in the intestinal lumen. Several components of the natural immune system, such as defensins and lactoferrin, possibly contribute in this process [33,34]. One of those strategies involves nitric oxide (NO) production by the intestinal epithelium. In order to evade the immune system, *G. lamblia* consumes arginine, the crucial substrate used by epithelial NO synthase to form NO [35]. Our hypothesis involves that the treatment with Mz + Pm could exacerbate a potent host defense, preventing the protozoan to escape. These secreted salts, cytokines and/or other molecules produced by IEC-6 cells could have an additional activity to the effect of the combined drugs. Studies performed with Caco-2 cells raise the possibility of an additional intestinal component provided by these intestinal cells could modulate the action of the drugs [23]. Complementary assays must be performed to answer these important questions.

Our light microscopy data showed that the treatment induced an accentuated decrease in % adhesion of parasites to IEC-6 cells but no morphological injury in host cells in any drug combination performed was observed. Potent cytoskeleton inhibitors (cytochalasin, colchicine and nocodazole) induced a completely misshapen, detachment from the parasite substract, cell division impairment and alterations in flagella number [38,39]. One possibility for this interference is that Mz and Pm interfered in the ventral disk functionality, as described for anti-cytoskeleton agents. This structure is a crucial in trophozoite cell biology, involved in the nutrients uptake, besides the attachment of the parasite to host cell [38,40]. The combined treatment of Mz and Pm could induce alterations in this structure, reducing the % adhesion and also leading to the trophozoites death, as demonstrated for thiazolides-treated parasites [23]. Flagellar and body damages were observed in trophozoites under treatment with other classes of drugs [23,36,37]. Our preliminary results indicate similar alterations in trophozoites treated with both combinations tested (0.1 μM Mz + 13.8 μM Pm and 5.3 μM Mz + 0.3 μM Pm) (data not shown). Interestingly, neither of its morphological alterations were previously observed in parasites treated with Mz or Pm alone [10,13], suggesting a specific mode of action of the combination of both compounds.

Giardiasis still poses critical challenges, including an efficient chemotherapy. The current severe side effects and questionable activity in chronic phase and an increased resistance are the main difficulties in the clinical administration of Mz [41]. Clinically, Pm is usually administrated in combination with other anti-helminthic drugs, and presented absence, or mild side effects for dogs and humans [42,43]. The strong activity of the combined treatment of Mz and Pm, increasing the inhibition of trophozoites proliferation and its attachment on the IEC-6 cultures, associated to the high preservation of the host cells morphology and metabolic viability, points to the combination of these two compounds, as an excellent alternative for the treatment assays of *Giardia* infection *in vivo*.

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