



Toxoplasma gondii: The effect of fluconazole combined with sulfadiazine and pyrimethamine against acute toxoplasmosis in murine model

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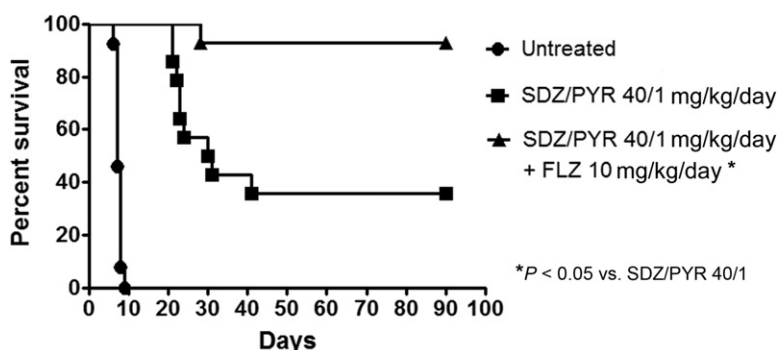
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HIGHLIGHTS

- ▶ The beneficial combination of fluconazole plus sulfadiazine and pyrimethamine was determined.
- ▶ Fluconazole enhanced the effect of sulfadiazine and pyrimethamine against acute toxoplasmosis.
- ▶ Fluconazole enhanced the effect of pyrimethamine alone against acute toxoplasmosis.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasma gondii is an important opportunistic pathogen for immunocompromised patients and responsible for toxoplasmic encephalitis, which is often lethal. Treatment for this infection is limited to a restricted therapeutic arsenal. In this work we tested the combination of fluconazole with the current treatment for acute toxoplasmosis on the murine model *in vivo*. Different experimental groups were treated with combinations of sulfadiazine plus pyrimethamine with fluconazole and pyrimethamine with fluconazole. Fluconazole is an important antifungal triazole used against others CNS related opportunistic pathogens such as *Cryptococcus neoformans* and *Candida* spp. The combinations of fluconazole plus sulfadiazine and pyrimethamine or fluconazole plus pyrimethamine were remarkably effective against *T. gondii* *in vivo*. The 10-day treatment with 10 mg/kg/day of fluconazole combined with 40/1 mg/kg/day sulfadiazine and pyrimethamine resulted in 93% survival of CF1 mice acutely infected with the highly virulent *T. gondii* RH strain, versus 36% of mice treated with just sulfadiazine and pyrimethamine. Combinations of fluconazole with lower doses of sulfadiazine and pyrimethamine or with just pyrimethamine were also efficient in reducing the mortality of mice compared with the treatment without fluconazole. The results obtained are promising for the treatment of human toxoplasmosis and point to the need to extend these studies to other murine models.

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1. Introduction

Toxoplasmosis is an important opportunistic disease in immunocompromised patients and is caused by the protozoan *Toxoplasma gondii* (Luft and Remington, 1988; Singer et al., 2010). In HIV patients, cerebral toxoplasmosis is the most frequent cause

of focal cerebral lesions and CNS disorders (Antinori et al., 2004; Vidal et al., 2005). The introduction of the highly active antiretroviral therapy (HAART) has reduced the incidence of toxoplasmic encephalitis (TE) and other opportunistic diseases in AIDS patients; however, TE is still responsible for extensive mortality and morbidity in immunocompromised patients. The lack of or inadequate HAART treatment and an ineffective program for HIV diagnosis in some geographical areas are acclaimed to be the main factors for TE incidence. Many HIV patients are hospitalized only when the CNS is affected, and in many cases, both TE and HIV are co-diagnosed (Antinori et al., 2004; Kiderlen et al., 2011; Nobre et al., 2003; Vidal et al., 2005). In addition, ocular toxoplasmosis is frequently found in AIDS patients presenting TE, which is usually very aggressive (Commodaro et al., 2009).

The treatment of toxoplasmosis consists in the combination of pyrimethamine (PYR) and sulfadiazine (SDZ), or in cases of sulfa intolerance, pyrimethamine is combined with clindamycin. The co-administration of folinic acid is also included in order to minimize the toxic effects of pyrimethamine in patients. However, both managements are commonly linked to side effects in TE patients, which can lead to the interruption of the full 4–6 week therapy course and cause a relapse of the disease (Katlama et al., 1996).

The fungi *Cryptococcus neoformans* and *Candida* spp. are important opportunistic pathogens in immunocompromised patients (Nobre et al., 2003). *C. neoformans* is an important CNS related opportunistic pathogen and the most frequent cause of HIV-associated meningitis. Amphotericin B plus flucytosine is the choice therapy for cryptococcosis, but administration of fluconazole is also used for the consolidation therapy or the maintenance of the treatment in patients (Singer et al., 2010). For candidiasis treatment, fluconazole is the first line therapy (Thompson et al., 2010). Fluconazole is a first-generation triazole drug, which was introduced in the early 90s, and is known for its favorable pharmacokinetics and wide therapeutic index (Lass-Flörl, 2011).

Although co-infections of *T. gondii* and *C. neoformans* or *Candida* spp. are common in AIDS patients (Kiderlen et al., 2011; Luft and Remington, 1988), the effect of the combination of sulfadiazine and pyrimethamine with fluconazole has never been investigated for the treatment of toxoplasmosis. A previous study by our group showed that fluconazole treatment enhances the survival of mice acutely infected with *T. gondii* (Martins-Duarte et al., 2010). Thus, in this study we investigated the effect of the association of the antifungal fluconazole (FLZ) with sulfadiazine (SDZ) plus pyrimethamine (PYR) or just FLZ with PYR, in the treatment of mice acutely infected with *T. gondii*. The results obtained showed the greater efficacy of these combinations in comparison with the current therapy (SDZ + PYR) to enhance mouse survival in *T. gondii* infections.

2. Experimental methodology

2.1. Parasites

Tachyzoites of the RH strain were used for the *in vitro* and *in vivo* tests and were obtained from the peritoneal cavities of mice 2 days after infection.

2.2. Mice

Four to six week-old female CF1 mice weighing 18–22 g at the beginning of the experiment were used for the *in vivo* experiments. Drinking water and food were given *ad libitum*. The experimental protocols for animal use in this study were approved by the Institutional Ethics Committee for Animal Use (approval ID: CEUA/CCS/UFRRJ/IBCCF 100).

2.3. Drugs

As the *in vitro* and *in vivo* sets of experiments had a long gap (18 months) between them, two different purchases of fluconazole were made. For *in vivo* tests, 150 mg capsules of FLZ were purchased from EMS Genéricos lot # 065313 (EMS[®], São Paulo, Brazil). For *in vitro* assays 150 mg capsules of FLZ were purchased from Zydus lot # MG6351 (Cadila Healthcare Ltd., Ahmedabad, India). For obtaining the quantity of fluconazole required for the *in vivo* tests or stock solution preparation for the *in vitro* experiments, all the content of the capsule was weighted and a correction factor was calculated considering the excipient content (for example: factor = 400 mg (total content of capsule)/150 mg of fluconazole = 2.67; i.e. 2.67 mg of capsule content was equivalent to 1 mg of fluconazole). Sulfadiazine and pyrimethamine were purchased from Sigma (Sigma–Aldrich, St. Louis, USA). For *in vivo* tests the drugs were diluted in poly-ethylene glycol (PEG) mol. wt. 200 (Sigma–Aldrich, St. Louis, USA), and for the *in vitro* experiments, the drugs were diluted in DMSO (Merck KGaA, Darmstadt, Germany).

2.4. *In vitro* antiproliferative assay

Approximately $1.5\text{--}2.0 \times 10^6$ LLC-MK₂ cells (kidney, Rhesus monkey, *Macaca mulata* – ATCC CCL7, Rockville, MD, USA) placed in a 24-well tissue culture plate were infected at the ratio of 10:1 parasite/host cell with freshly obtained RH strain parasites resuspended in RPMI medium. The cells and the tachyzoites were allowed to interact for 1 h and then they were washed twice with RPMI to remove unattached parasites. Treatment started 6 h after infection. Different concentrations of FLZ (0.05, 0.1, 0.5, 1.0 and 3.0 μM) were added to the dose response curves of SDZ/PYR (0.1 μM /0.4 nM to 100 μM /0.4 μM) and to PYR (4 nM–4 μM). Dose response curves of FLZ plus SDZ/PYR and FLZ plus PYR were also made. After 24 h of treatment, the cells were fixed with Bouin, stained with Panotico kit solutions 2 and 3 (Laborclin Ltda, Paraná, Brazil) and observed under a light microscope. The antiproliferative effect was evaluated after examining at least 1200 cells in two different coverslips for each concentration. The parasite proliferation index was determined by multiplying the percentage of infected cells by the total number of intracellular parasites and divided by the total number of cells. The 50% inhibitory concentration (IC₅₀) was calculated using a sigmoidal dose response curve in Sigma Plot 8.0 software (Systat Software Inc., Chicago, IL, USA).

2.5. Drug interaction *in vitro*

The fractional inhibitory concentration (FICI) values were calculated according to the Loewe additive model, defined by the equation $a/A + b/B = 1$, where A and B are the IC₅₀ of individual drugs and a and b are the IC₅₀ of the drugs in combination (Tallarida, 2006). The combination effect was analyzed according to FICI values: ≤ 0.5 , synergism; $>0.5\text{--}4$, no interaction; and >4 , antagonism (Odds, 2003).

2.6. Cell toxicity experiments

The effect of the combinations of FLZ and SDZ/PYR or PYR on LLC-MK₂ cells was evaluated by the MTS assay. For that, 2×10^5 cells were placed in a 96-well tissue plate and treated for 24 h with the same combined doses of FLZ and SDZ/PYR or PYR utilized for the *in vitro* test against *T. gondii*. At the end of incubation time, cells were washed with PBS, each well was filled with 100 μL of 10 mM Glucose in PBS and 20 μL of MTS reagent (Promega, Madison, WI, USA) was added. The absorbance was read at 490 nm after 3 h of incubation and cytotoxicity was calculated as the percentage of viable cells versus untreated cells (control).

2.7. In vivo experiments

For the *in vivo* study, mice were infected intraperitoneally (i.p.) with tachyzoites of the RH strain of *T. gondii*. Treatment was initiated 24 h post infection and groups of three or four mice were housed per cage and arbitrarily assigned to administration with FLZ plus SDZ/PYR or FLZ plus PYR or left untreated as the control group. PEG (diluent) was administered to the untreated mice. The treatment lasted for 10 days and the drugs were administered orally by gavage once a day. The mice and the mortality rates were monitored for a period of 90 days. The survival curves and the median survival (day at which fractional survival equals 50%) were calculated using the product limit method of Kaplan and Meier, and the comparison of the survival curves was carried out using the log-rank (Mantel–Cox) test in Graphpad Prism 5.0 (GraphPad Software Inc.). $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Effect of the *in vitro* combination of FLZ with SDZ/PYR or FLZ with PYR

The *in vitro* interactions of FLZ plus SDZ/PYR and FLZ plus PYR were evaluated by the addition of fixed concentrations of FLZ (0.05, 0.1, 0.5, 1.0 and 3.0 μM) to the dose effect curves of SDZ/PYR or to the PYR curves (Table 1). FLZ, SDZ/PYR and PYR single curves were also performed in parallel to drug combined experiments and the IC_{50} and FICI values were calculated for each experiment and are the mean of three independent experiments. The FLZ IC_{50} obtained were 8.4 ± 1.2 and 8.7 ± 0.8 for combinations with SDZ/PYR and PYR, respectively. The majority of the different FLZ treatments with either SDZ/PYR or PYR did not exert a synergistic effect *in vitro*. The addition of 0.1 μM FLZ to SDZ/PYR was the only combination which resulted in a synergistic effect, as it lowered the SDZ/PYR IC_{50} by more than two-fold (15.0–7.1 μM) producing a FICI of 0.5. However, none of the other combinations evaluated caused a significant reduction of the SDZ/PYR IC_{50} or PYR IC_{50} and resulted in indexes close to 1.

Toxicity studies of the different combinations on LLC-MK₂ proliferation after 24 h of treatment showed that combined concentrations of FLZ with SDZ/PYR or PYR did not affect significantly host cell proliferation (Table 2).

3.2. Combination of FLZ plus SDZ/PYR *in vivo*

In a preliminary test with only one experimental group (3 mice per experiment), the effect of different doses of FLZ alone against mice infected with 10^3 tachyzoites of RH strain was evaluated. The treatment with doses of 20, 40 or 80 mg/kg/day of FLZ alone

Table 1
 IC_{50} and FICI obtained for the *in vitro* combination of different concentrations of FLZ plus SDZ/PYR and FLZ plus PYR against *T. gondii*.

FLZ ^a (μM)	SDZ/PYR		PYR	
	IC_{50} ($\mu\text{M}/\text{nM}$)	FICI ^b	IC_{50} (μM)	FICI ^b
0.0	$15.0 \pm 2.8/60.0 \pm 11.2$	–	0.23 ± 0.10	–
0.05	$14.4 \pm 2.0/57.6 \pm 8.0$	1.0	0.19 ± 0.06	0.8
0.1	$7.1 \pm 0.5/28.4 \pm 2.0$	0.5	0.23 ± 0.08	1.0
0.5	$12.3 \pm 2.1/49.2 \pm 8.4$	0.9	0.34 ± 0.13	1.5
1.0	$9.2 \pm 1.9/36.8 \pm 7.6$	0.7	0.14 ± 0.07	0.7
3.0	$13.3 \pm 3.6/53.2 \pm 14.4$	1.2	0.19 ± 0.05	1.2

Results are the mean \pm standard deviation of three different experiments.

^a FLZ IC_{50} were 8.4 ± 1.2 and 8.7 ± 0.8 for combinations with SDZ/PYR and PYR, respectively.

^b FICI values of ≤ 0.5 ; >0.5 –4; and >4 , indicate synergism, no interaction or antagonism, respectively.

did not prolong mouse survival compared with untreated, and animals died after 7–9 days (data not shown).

The combination of FLZ plus SDZ/PYR achieved significant results in survival. Protection from death increased significantly for mice treated with 10 mg/kg/day of FLZ combined to 40/1 mg/kg/day of SDZ/PYR ($P = 0.0015$). At the end of 90 days 93% of mice treated with this combination were alive versus 36% (median survival of 31 days) of mice treated only with 40/1 mg of SDZ/PYR (Fig. 1A). In a separate experiment, the treatment with 5 or 20 mg of FLZ combined to 40/1 mg SDZ/PYR also increased mouse survival compared to treatment with SDZ/PYR alone (Fig. 1B). At the end of 90 days, 73% and 91% of mice treated, with combinations of 5 mg and 20 mg of FLZ, respectively, continued alive, versus 33% (median survival of 43 days) of mice treated with SDZ/PYR alone. However, only the combination with 20 mg of FLZ was statistically significant ($P = 0.008$).

Mice treated with a lower dose of SDZ/PYR (20/0.5 mg) and different doses of FLZ also increased mouse survival compared to the treatment with only SDZ/PYR (Fig. 2). The combination with 20 mg of FLZ achieved the best result in mouse survival compared to SDZ/PYR alone and the combinations of 10 or 40 mg of FLZ, protecting 25% of mice from death with a median survival of 25 days ($P < 0.05$). Mice only treated with SDZ/PYR died after 43 days and had a median survival of 15 days. Combinations of 10 or 40 mg of FLZ did not protect mice from death, and resulted in a median survival of 21 and 22 days, respectively. Although these combinations had enhanced mouse survival compared to treatment with only SDZ/PYR, the differences between these groups were not statistically significant ($P > 0.05$).

3.3. Combination of FLZ and PYR *in vivo*

PYR is considered the drug more active to treat *T. gondii* infections, thus a combination of just PYR with FLZ was also evaluated. The effect of different doses (20, 40 or 80 mg/kg/day) of FLZ combined with 1, 5 or 10 mg/kg/day of just PYR was evaluated. The combination with 1 mg of PYR, which was the highest dose used in the tests with SDZ/PYR, did not result in any improvement in mouse survival compared to the treatment with PYR alone or to untreated mice (data not shown). On the other hand, mice treated with combinations of FLZ plus 5 or 10 mg of PYR prolonged mouse survival (Fig. 3A and B). The treatment with all doses of FLZ combined to 5 mg of PYR resulted in a significant enhancement in mouse survival compared to PYR monotherapy (Fig. 3A). Mice treated with only 5 mg of PYR had a median of survival of 9 days, a slight difference compared to the median survival of 8 days of untreated ones. However, mice treated with combinations of 20 mg, 40 mg and 80 mg of FLZ and 5 mg of PYR had a median of survival of 23 days, 12 days and 16.5 days, respectively ($P < 0.05$ versus PYR 5 mg). The combination with 80 mg of FLZ plus 5 mg of PYR protected only 1 mouse (8.3%) from death.

The combinations of FLZ plus 10 mg of PYR (Fig. 3B) also significantly enhanced mouse survival, and results were better than the combination with 5 mg of PYR. The different dose of FLZ combined to 10 mg of PYR had a dose dependent effect on mouse survival, as 20 mg, 40 mg and 80 mg of FLZ protected 42%, 54% and 64% of mice from death, respectively ($P < 0.05$ versus PYR 10 mg). Mice treated only with 10 mg of PYR survived up to 32 days and had a median survival of 21 days ($P < 0.05$).

4. Discussion

The evaluation of drugs available on the market is a good strategy for the discovery of new potential therapies, as they have been previously approved for human use. FLZ is a drug that is well-tolerated

Table 2

Percentage of host cell proliferation after 24 h of treatment with different combined concentrations of FLZ and SDZ/PYR or PYR.

FLZ (μM)	SDZ/PYR (μM)				PYR (μM)			
	0.1/0.0004	1.0/0.004	10/0.04	100/0.4	0.004	0.04	0.4	4.0
0.0	99 \pm 1	94 \pm 10	97 \pm 5	99 \pm 2	96 \pm 8	100	97 \pm 5	97 \pm 5
0.05	100	95 \pm 8	96 \pm 4	91 \pm 11	93 \pm 7	96 \pm 7	95 \pm 2	88 \pm 12
0.1	97 \pm 6	99 \pm 1	99 \pm 2	93 \pm 6	90 \pm 9	90 \pm 2	96 \pm 5	94 \pm 11
0.5	90 \pm 15	95 \pm 8	95 \pm 7	88 \pm 14	92 \pm 8	95 \pm 5	89 \pm 5	86 \pm 5
1.0	97 \pm 5	99 \pm 2	95 \pm 2	97 \pm 4	94 \pm 10	92 \pm 3	96 \pm 8	93 \pm 11
3.0	95 \pm 9	98 \pm 3	97 \pm 5	91 \pm 10	93 \pm 8	92 \pm 6	83 \pm 6	89 \pm 7

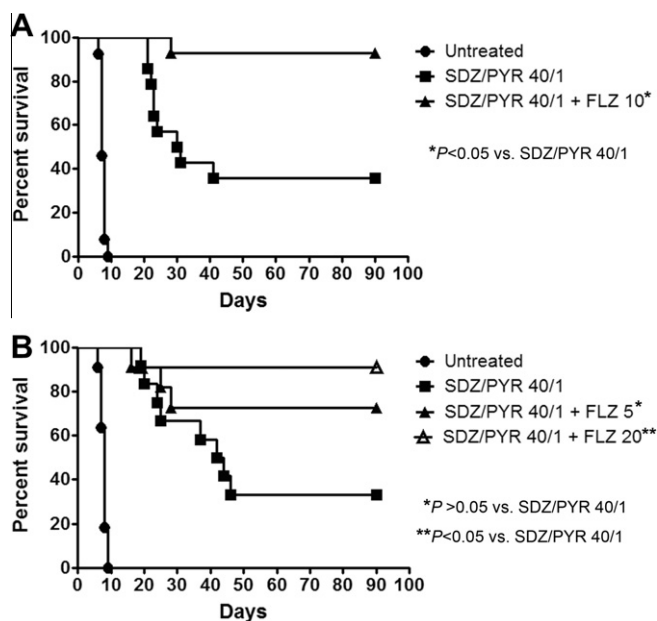
Results are the mean \pm standard deviation of three different experiments.

Fig. 1. Effect of a 10 day treatment with a combination of 10 mg/kg/day of FLZ + 40/1.0 mg/kg/day of SDZ /PYR (A) or 5 or 20 mg/kg/day of FLZ + 40/1.0 mg/kg/day of SDZ/PYR (B) on the survival of Swiss mice acutely infected with 10^3 tachyzoites of *T. gondii* RH strain. The results were evaluated by Kaplan–Meier product limit method. $P < 0.05$ for the combination of 10 and 20 mg of FLZ versus SDZ/PYR; $P > 0.05$ for the combination of 5 mg of FLZ versus SDZ/PYR. (A) Untreated ($n = 14$; four groups); SDZ 40/PYR 1 ($n = 14$; four groups); SDZ 40/PYR 1 + FLZ 10 ($n = 14$; four groups). (B) Untreated ($n = 11$, three groups); SDZ 40/PYR 1 ($n = 12$; three groups); SDZ 40/PYR 1 + FLZ 5 ($n = 11$; three groups); SDZ 40/PYR 1 + FLZ 20 ($n = 11$; three groups).

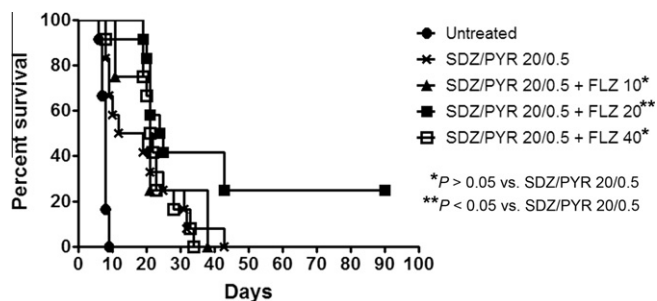


Fig. 2. Effect of a 10 day treatment with a combination of 10 or 20 or 40 mg/kg/day FLZ + 20/0.5 mg/kg/day SDZ /PYR on the survival of Swiss mice acutely infected with 10^3 tachyzoites of *T. gondii* RH strain. The results were evaluated by Kaplan–Meier product limit method. $P < 0.05$ for the combination of 20 mg FLZ versus SDZ/PYR; $P > 0.05$ for the combinations of 10 or 40 mg FLZ versus SDZ/PYR. Untreated ($n = 12$; three groups); SDZ 20/PYR 0.5 ($n = 12$; three groups); SDZ 20/PYR 0.5 + FLZ 10 ($n = 4$; one group); SDZ 20/PYR 0.5 + FLZ 20 ($n = 12$; three groups); SDZ 20/PYR 0.5 + FLZ 40 ($n = 12$; three groups).

with a low risk of hepatic toxicity (Wang et al., 2010) and is commonly administered for the treatment of fungal infections in HIV patients. The fungistatic effects of azoles involve the inhibition of ergosterol synthesis at the level of the C-14 α -lanosterol demethylase (Koltin and Hitchcock, 1997), and although this pathway is absent in Apicomplexa parasites, previous studies have shown that azoles displayed a strong effect against *T. gondii* and *Plasmodium falciparum* (Martins-Duarte et al., 2008, 2010; Penna-Coutinho et al., 2011). The anti-*Plasmodium* effect of itraconazole and posaconazole was suggested to be due to a competition of the NADH for the lactate dehydrogenase (Penna-Coutinho et al., 2011). The low *in vitro* IC₅₀ obtained for FLZ and itraconazole (Martins-Duarte et al., 2008, 2010), as well as, the *in vivo* effect of FLZ against *T. gondii* indicate the presence of a selective molecular target in *T. gondii*.

The use of a combined therapy is recognized as the most effective treatment for TE (Fung and Kirschenbaum, 1996; Katlama et al., 1996). Human treatment with a PYR monotherapy is not recommended, as patients in maintenance therapy with PYR alone were more susceptible to relapses of the disease (Foppa et al., 1991). Besides, the combined administration of drugs might lead to a decrease in drug dosages, while maintaining the therapeutic efficacy and reducing toxicity related to the treatment. In addition, it may reach multiple targets or multiple diseases simultaneously (Chou, 2006). Thus the study of new drug combinations is a promising strategy for discovering therapies for the treatment of toxoplasmosis.

For the combined tests with SDZ/PYR we decided to use a dose relation similar to the clinical management for the treatment of human patients presenting TE: 50–100 mg/day of PYR and 4–8 g/day of SDZ (Fung and Kirschenbaum, 1996). Although the relation of the SDZ/PYR dose can vary from 40–160/1, we chose a relation dose of 40/1.

The results obtained indicate that both combinations of FLZ plus SDZ/PYR and FLZ plus PYR are highly effective against *T. gondii* *in vivo*. The anti-*T. gondii* activity *in vivo* of FLZ against mice infected with the Me49 strain was previously reported by our group (Martins-Duarte et al., 2010), and the lack of effect against mice infected with the RH strain is possibly due to the highly virulent nature of this strain for mice. However, when FLZ in combination with SDZ/PYR or with PYR was administered against mice infected with the RH strain, a significant difference in survival and in protection was obtained against relapses and death from the disease, compared with the administration of SDZ/PYR or PYR without FLZ. The combination of 10 or 20 mg of FLZ with 40/1 mg/kg/day of SDZ/PYR had similar results in mouse survival and the combination of 20 mg of FLZ to a reduced dose of SDZ/PYR (20/0.5 mg) also improved mouse survival significantly compared to treatment with only SDZ/PYR. This observation is very interesting, since a reduction in SDZ/PYR doses will also diminish the side effects. However, the decrease in mouse survival when 40 mg of FLZ was used might suggest an antagonism effect with high doses of FLZ and SDZ/PYR. Differently to the treatment with SDZ/PYR plus FLZ, the combination of FLZ with PYR showed a dose dependent effect; the increase of the FLZ dose enhanced mouse protection from death.

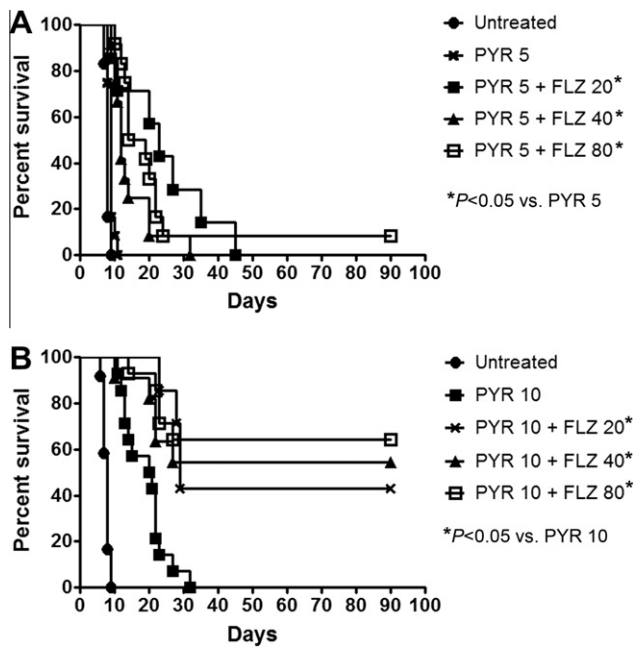


Fig. 3. Effect of a 10 day treatment with a combination of PYR 5.0 mg/kg/day + 20 or 40 or 80 mg/kg/day FLZ (A) and PYR 10 mg/kg/day + 20 or 40 or 80 mg/kg/day FLZ (B) on the survival of Swiss mice acutely infected with 10^3 tachyzoites of *T. gondii* RH strain. The results were evaluated by Kaplan–Meier product limit method $P < 0.05$ for all combinations versus PYR. (A) Untreated ($n = 12$; three groups); PYR 5 ($n = 12$; three groups); PYR 5 + FLZ 20 ($n = 7$; two groups); PYR 5 + FLZ 40 ($n = 12$; three groups); PYR 5 + FLZ 80 ($n = 12$). (B) Untreated ($n = 14$; four groups); PYR 10 ($n = 14$; four groups); PYR 10 + FLZ 20 ($n = 7$; two groups); PYR 10 + FLZ 40 ($n = 11$; three groups); PYR 10 + FLZ 80 ($n = 14$; four groups).

These results are very interesting, as the dose of 10 mg/kg/day FLZ is lower than the highest dose (800 mg/day, ~ 12 mg/kg) administered to treat patients with fungus infections (Hope et al., 2008), and currently the administration of doses up to 1200 mg/day (~ 18 mg/kg) have been considered for the treatment of cryptococcal meningitis (Nussbaum et al., 2010). The treatment with doses of FLZ up to 1600 mg/day (~ 24 mg/kg) is well tolerated, and although the treatment with 2000 mg/day (30 mg/kg) led to central nervous system side effects in some patients, this dose possess a safe profile (Anaissie et al., 1995; Voss and De Pauw, 1999). The *in vivo* effect suggests a synergistic interaction of FLZ plus SDZ/PYR and FLZ plus PYR, but when the combination effect was evaluated *in vitro* the drugs only had an additive effect. The *in vitro* combination of clindamycin and PYR did not result in synergic interaction (Harris et al., 1988), but the administration of clindamycin and PYR is effective for the treatment of TE patients. Thus, the lack of *in vitro* synergism does not preclude *in vivo* efficacy. However, a pharmacokinetic interaction cannot be excluded as FLZ is a known inhibitor of cytochrome P450 (CYP) oxidative metabolism. FLZ is a strong non-competitive inhibitor of CYP isoform 2C9, and can affect the biotransformation of other drugs (Gubbins and Amsden, 2005). CYP2C9 was shown to metabolize SDZ to its hydroxylamine intermediate in experiments utilizing human liver microsomes *in vitro* (Winter and Unadkat, 2005), thus the enhancement of the SDZ/PYR effect when combined to FLZ may be due to an increase of SDZ blood concentrations caused by the reduction on SDZ metabolism by FLZ. There is little information about the metabolism of PYR and although it is believed to be hepatic (Cavallito et al., 1978), the PYR metabolism by CYP oxidative enzymes was not detectable *in vitro* (Li et al., 2003).

In conclusion, the potential combinations of FLZ and SDZ/PYR or FLZ and PYR point to the need to extend these studies in other murine models aiming for a new treatment of human toxoplasmo-

sis. The administration of FLZ should be considered as a possibility to improve SDZ/PYR efficacy for the treatment of toxoplasmosis, leading to faster recovery and less relapses of the disease. Also such combinations are an opportunity, to decrease SDZ/PYR doses and their related side effects. The combination of FLZ and PYR may be also considered as an option to substitute the administration of SDZ/PYR in cases of intolerance to SDZ.

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