

ACTIVITY OF NATURAL AND SYNTHETIC NAPHTHOQUINONES AGAINST *TOXOPLASMA GONDII*, IN VITRO AND IN MURINE MODELS OF INFECTION

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Summary:

The effect of 14 natural and synthetic naphthoquinones in the replication of *Toxoplasma gondii* was evaluated. *In vitro* studies were accomplished in cultures of 2C4 fibroblasts infected with RH strain. Enzyme-linked immunosorbent assay was used to quantify parasite growth. For the studies *in vivo*, mice were infected with tachyzoites of the RH strain or cysts of the *T. gondii* EGS strain. *In vitro*, seven naphthoquinones demonstrated significant inhibition of intracellular *T. gondii* growth at concentrations of 1 and 5 µg/ml. Only three compounds were significantly protective when tested in animals: 2-hydroxy-3-(3'-pentenyl)-1,4-naphthoquinone (PHNQ4), 2-hydroxy-3-(1'-vinylphenyl)-1,4-naphthoquinone (PHNQ5) and 2-hydroxy-3-(1'-propen-3'-phenyl)-1,4-naphthoquinone (PHNQ6). In animals infected with the EGS strain and treated with PHNQ4 (50 mg/kg/day orally), a 7-day prolongation of the time to death was observed. Treatment with 100 mg/kg/day orally or 50 mg/kg/day i.p. of PHNQ5 resulted in a 5-day and 16-day prolongation of the time to death, respectively. Treatment with 50 mg/kg/day orally or 50 mg/kg/day i.p. of PHNQ6 resulted in a 4-day prolongation of the time to death or up to 30 days after treatment, respectively. Our results suggest that the naphthoquinones may be important therapeutic agents for the treatment of toxoplasmosis.

KEY WORDS : naphthoquinones, *Toxoplasma gondii*, strain, *in vitro*, murine models.

Résumé : ACTIVITÉ DE NAPHTHOQUINONES NATURELLES ET SYNTHÉTIQUES CONTRE *TOXOPLASMA GONDII* IN VITRO ET DANS UN MODÈLE MURIN

L'effet de 14 naphthoquinones naturelles et synthétiques a été évalué sur la multiplication de *Toxoplasma gondii*. Les études *in vitro* ont été effectuées avec des cultures de fibroblastes 2C4 infectés avec la souche RH. Un ELISA a été utilisé pour mesurer la croissance du parasite. Pour les études *in vivo*, les souris ont été infectées avec des tachyzoïtes de la souche RH ou des kystes de la souche EGS de *T. gondii*. Les essais *in vitro* avec sept naphthoquinones ont démontré une inhibition importante du développement intracellulaire de *T. gondii* aux concentrations de 1 et 5 µg/ml. Seulement trois composés ont démontré une activité protectrice *in vivo* : 2-hydroxy-3-(3'-pentenyl)-1,4-naphthoquinone (PHNQ4), 2-hydroxy-3-(1'-vinylphenyl)-1,4-naphthoquinone (PHNQ5) et 2-hydroxy-3-(1'-propen-3'-phenyl)-1,4-naphthoquinone (PHNQ6). Dans le groupe des animaux infectés avec la souche EGS et traités avec PHNQ4 (50 mg/kg/jour per os), une augmentation de sept jours de la survie des animaux a été observée. Le traitement avec 100 mg/kg/jour per os ou 50 mg/kg/jour i.p. de PHNQ5 a provoqué une augmentation de la survie de cinq et 16 jours respectivement. Le traitement avec 50 mg/kg/jour per os ou 50 mg/kg/jour i.p. de PHNQ6 a entraîné une augmentation de la survie de quatre et de plus de 30 jours après le traitement, respectivement. Ces résultats montrent donc que les naphthoquinones peuvent être des molécules d'avenir pour le traitement de la toxoplasmose.

MOTS CLÉS : naphthoquinones, *Toxoplasma gondii*, souche, *in vitro*, modèle murin.

INTRODUCTION

Toxoplasma gondii is an intracellular protozoan capable of invading all types of nucleic cells. The immune systems of immunocompetent patients control tachyzoite multiplication, whereas infections in immunocompromised patients can result in fatal encephalitis. Pyrimethamine alone or in combination with

sulfadiazine has been the conventional treatment for acute toxoplasmosis and toxoplasmic encephalitis although adverse reactions are common (Katlama, 1991; Wong & Remington, 1994; Weller & Williams, 2001) which may lead to the interruption of chemotherapy (Leport *et al.*, 1988; Wong *et al.*, 1994).

It is recognized that there is an urgent need for new therapeutic approaches to treat toxoplasmosis, particularly for patients with AIDS (Luft & Remington, 1992; Khan *et al.*, 1998; Gherardi *et al.*, 2000). Several naphthoquinones have been reported to exhibit antitumor, anti-*Trypanosoma cruzi*, antimalarial and anti-*Leishmania chagasi* activities (Hammond *et al.*, 1985; Jernigan *et al.*, 1996; Dolabella, 1997; Gourelart *et al.*, 1997). Importantly, the naphthoquinone atovaquone has been proven to be effective against *Pneumocystis carini* and *Toxoplasma gondii* (Araujo *et al.*, 1991;

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Vilar *et al.*, 1996). The present study reports the evaluation of 14 naphthoquinones against *T. gondii* *in vitro* and in a murine model of infection.

MATERIALS AND METHODS

MICE

Outbred female Swiss mice from the Centro de Bioterismo of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil) were used and weighed roughly 20 g at the beginning of each experiment. Food and water were available to the animals throughout the experiment.

T. GONDII ISOLATES

Strains RH and EGS were used. *In vitro* and *in vivo* studies used tachyzoites obtained from the peritoneal cavities of mice infected with the RH strain (Sabin, 1941) for two days. The virulent EGS strain (Ferreira *et al.*, 2001) used in the *in vivo* analysis was isolated in our laboratory from the amniotic fluid of an infected human patient (Castro *et al.*, 2001). The EGS strain was maintained at a chronic stage by treating infected mice with sulfadiazine in drinking water. Mice were infected either *i.p.* with 10^3 tachyzoites of the RH strain or orally with 10 tissue cysts of the EGS strain.

COMPOUNDS

The *para*-hydroxynaphthoquinones (PHNQs), *ortho*-furanonaphthoquinones (OFNQs) and *para*-furanonaphthoquinones (PFNQs) used in the experiments were synthesized via a route starting with 2-hydroxy-1,4-naphthoquinone and aliphatic aldehydes, followed by oxidation of the condensation products (Ferreira *et al.*, 1989). These compounds were obtained at the Departamento de Produtos Farmacêuticos, Faculdade de Farmácia, Universidade Federal de Minas Gerais (Belo Horizonte, Brazil). In addition to the synthetic naphthoquinones, three natural naphthoquinones – lapachol, α -lapachone and β -lapachone (Fig. 1) – were tested. The Lapachol, α -lapachone and β -lapachone were extracted from plants belonging to the family Bignoniaceae, particularly to the genus *Tabebuia*. Sulfadiazine was used for comparison with the compounds tested in this study.

IN VITRO ACTIVITY

The compounds were dissolved in a small amount of dimethyl sulfoxide and Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, Mo.) containing penicillin, streptomycin and 10 % fetal bovine serum. The concentrations were prepared in

DMEM and varied between 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 $\mu\text{g/ml}$. The effect of each compound on intracellular *T. gondii* replication was evaluated using enzyme-linked immunosorbent assay (ELISA), with modifications (Romand *et al.*, 1993). Semiconfluent monolayers of 2C4 fibroblasts were prepared in 96-well tissue culture plates and inoculated with tachyzoites of *T. gondii* RH strain at a ratio of the three parasites/cell at 37°C in 5 % CO₂. Eight wells were used for each drug concentration. After four hours, the various concentrations of compounds were added to the medium and the cultures were incubated for 48 h. The ELISA was done directly on the fixed cultures.

Each culture well was washed three times with 100 μl of phosphate-buffered saline (PBS) containing 0.05 % Tween 20 (pH 7.6). The plates were then fixed with cold methanol for 15 min and filled with 5 % powdered skim milk in PBS-Tween for 60 min at 37°C. Then the plates were washed twice for five minutes each time with PBS-Tween 20 pH 7.6. Positive sera (100 μl) from mice experimentally infected with *T. gondii* were diluted 1:50 in powdered skim milk in 5 % PBS-Tween and incubated for 45 min at 37°C then added to each well. Plates were washed four times followed by the addition of 100 μl of peroxidase-labelled anti-mouse IgG conjugate (Sigma) diluted 1/15000 and incubated for 45 min at 37°C. Plates were washed four times and then 100 μl of the substrate (*ortho*-phenylenediamine) was added to each well. After 20 min the reaction was stopped by the addition of 100 μl of 4 N H₂SO₄ and the optical density of the supernatant was determined for each culture well by ELISA readings (A₄₉₀ nm). In each experiment, eight positive control wells were made with no compound but with DMEM and *T. gondii* tachyzoites while eight wells with no parasites and no compound were used as negative controls.

DRUG TOXICITY FOR FIBROBLASTS

The toxicity of the compounds for 2C4 fibroblasts was determined by colorimetric assay using methylene blue (Gomes *et al.*, 1995). Cells were seeded in four replicate wells, using 10^4 cells per well. After incubation for 24 h at 37°C in 5 % CO₂, the wells containing the test compounds were washed three times in a solution of PBS 0.05 %-Tween 20 and fixed with cold methanol for 15 min. Cells that remained in the wells were stained with 0.1 % methylene blue in 0.1 M borate buffer (pH 8.7) for 10 min. Excess stain was removed by washing three times with 0.01 M borate buffer (pH 8.7) and stain incorporated in the cells was extracted through the addition of 100 μl of 0.1 M HCl to each well. Cells that were not treated with the compound were used as a control. Color intensity was measured by optical density reading at 660 nm in an ELISA reader.

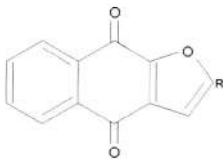
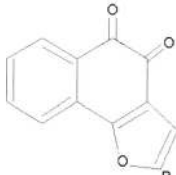
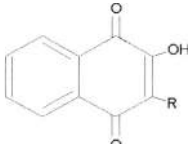
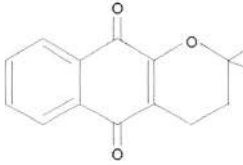
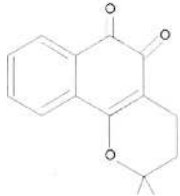
Compound No.	Chemical structure R	Quinone
1 (PFNQ1) 2 (PFNQ2) 3 (PFNQ3)	 Et CH ₂ CH ₂ CH ₃ CHMe ₂	2-ethyl-naphtho [2,3 <i>b</i>]-furan-4,9-quinone 2-n-propyl-naphtho[2,3 <i>b</i>]-furan-4,9-quinone 2-isopropyl-naphtho [2,3 <i>b</i>]-furan-4,9-quinone
1 (OFNQ1) 2 (OFNQ2) 3 (OFNQ3)	 CH ₂ CH ₂ CH ₃ Et CHMe ₂	2-n-propyl-naphtho [1,2 <i>b</i>]-furan-4,5-quinone 2-ethyl-naphtho [1,2 <i>b</i>]-furan-4,5-quinone 2-isopropyl-naphtho [1,2 <i>b</i>]-furan-4,5-quinone
1 (PHNQ1) 2 (PHNQ2) 3 (PHNQ3) 4 (PHNQ4) 5 (PHNQ5) 6 (PHNQ6)	 CH=CH CH ₂ CH ₃ CH ₂ CH=CMe ₂ CH=CH-CHMe ₂ CH=CH CH ₂ CH ₂ CH ₃ CH=CH C ₆ H ₅ CH=CHCH ₂ C ₆ H ₄	2-hydroxy-3-(1'-butenyl)-1,4-naphthoquinone 2-hydroxy-3-(3'-methyl-2'-butenyl)-1,4-naphthoquinone (Lapachol) 2-hydroxy-3-(1'-isopentenyl)-1,4-naphthoquinone 2-hydroxy-3-(3'-pentenyl)-1,4-naphthoquinone 2-hydroxy-3-(1'-vinylphenyl)-1,4-naphthoquinone 2-hydroxy-3-(1'-propen-3-phenyl)-1,4-naphthoquinone
PPNQ	 α -Lapachone	3,4-dihydro-2,2-dimethyl-2H-nafto-[2,3 <i>b</i>]-pyran-5,10-quinone
OPNQ	 β -Lapachone	3,4-dihydro-2,2-dimethyl-2H-nafto-[1,2 <i>b</i>]-pyran-5,6-quinone

Fig. 1. – Chemical structures of the assayed compounds.

IN VIVO ACTIVITY

The compounds that showed activity *in vitro* against *T. gondii* were selected for *in vivo* analysis. Evaluation was done with compounds that were in the neutral or sodium salt forms and administered orally or i.p., respectively. All the compounds in the salt form were dissolved in PBS, pH 7.2. Concentrations of the compounds that were insoluble in PBS (e.g. neutral forms) were prepared using a small amount of 20 % ethanol and the desired concentrations were obtained by the addition of 0.25 % carboxymethylcellulose. Prior to the *in vivo* experiments, toxicity tests were done using five uninfected mice per group that were treated orally or i.p. for 10 days with 10, 50 and 100 mg/kg/day of the compounds. Signs of toxicity such as piloerection, lethargy, weight loss or death were observed daily. Doses that were determined not to be toxic to the uninfected mice were tested in mice infected with the RH or EGS strains.

Treatment of mice infected with RH-strain tachyzoites was initiated 24 h after parasite inoculation. In mice infected with EGS-strain cysts, treatment was started two days post infection. In both groups treatment lasted 10 consecutive days. All experiments included 10 infected mice per group. The mice were observed for 30 days of infection for mortality and time to death.

STATISTICAL ANALYSIS

Data plotting was used to describe the effect of compounds *in vitro*. Optical density was plotted as a logarithmic function of concentration. For this, regression analysis was calculated using MicroCal Origin Version 2.24. The 50 % inhibitory concentrations were calculated from the regression curves. The toxic effect of the compounds was analyzed using the Student's t-test comparing cells exposed and not exposed to the drugs. Survival curves were evaluated with the Kaplan-Meier log-rank test (Kleinbaum, 1999).

RESULTS

IN VITRO ACTIVITY

AGAINST INTRACELLULAR *T. GONDII* REPLICATION

A significant inhibitory effect on *Toxoplasma* growth was seen with the *para*-hydroxynaphthoquinones. PHNQ1, PHNQ2 (lapachol), PHNQ3, PHNQ4, PHNQ5, PHNQ6 and PPNQ (α -lapachone) presented *in vitro* activity against *T. gondii* beginning at a concentration of 5 μ g/ml for the first two compounds and 1 mg/ml for the others ($P < 0.05$). Sulfadiazine presented activity *in vitro* against *T. gondii* at

Compound	Inhibition of <i>T. gondii</i> growth IC ₅₀ (μ g/mL)	Cell toxicity (μ g/mL)
PFNQ1	*	10
PFNQ2	*	5
PFNQ3	*	1
OFNQ1	ND	0,01
OFNQ2	ND	0,01
OFNQ3	ND	0,01
PHNQ1	25,45	> 50
PHNQ2 (Lapachol)	4,88	10
PHNQ3	8,31	> 50
PHNQ4	37,20	> 50
PHNQ5	6,31	> 50
PHNQ6	3,01	> 50
α -Lapachona	5,75	20
β -Lapachona	ND	0,01

* The compound was not inhibitory for *Toxoplasma gondii*.
ND = not done.

Table I. – *In vitro* effect of 14 naphthoquinones on *Toxoplasma gondii* growth in human fibroblast 2C4.

concentration of 5 μ g/ml (Fig. 2). These results were confirmed in a second experiment (data not shown). From the regression curve analysis, IC₅₀ % was 25,45 μ g/ml for PHNQ1, 4,88 μ g/ml for PHNQ2, 8,31 μ g/ml for PHNQ3, 37,20 μ g/ml for PHNQ4, 6,31 μ g/ml for PHNQ5, 3,01 μ g/ml for PHNQ6 and 5,75 μ g/ml for PPNQ (α -lapachone). All the *para*-naphthoquinones were selected for *in vivo* assays. PFNQ1, PFNQ2 and PFNQ3 did not present anti-*T. gondii* activity *in vitro*. All the compounds classified as OFNQs and OPNQ (β -lapachone) were high toxic to 2C4 fibroblasts (100 % of cells were dead by 24hs after addition of compound at a concentration of 0.01 μ g/ml.), as evidenced by the methylene blue assay and alterations in cell morphology. The *para*-hydroxynaphthoquinones (PHNQs) and *para*-furanonaphthoquinones (PFNQs) had low (no toxicity at a much higher concentration of the drug ≥ 50 μ g/ml) and medium (a toxic effect was noted with ≥ 1 μ g/ml of compound) toxicity, respectively. Table I shows the IC₅₀ for *T. gondii* growth in 2C4 fibroblasts cultures and the minimum concentration that was found toxic (microscopic examination and methylene blue assay) for the monolayers.

IN VIVO EXPERIMENTS

Doses of 10, 50 and 100 mg/kg/day of lapachol (PHNQ2), PHNQ1, PHNQ5 and PPNQ (α -lapachone) were used for i.p. (sodium salt of the compound) treatment. Due to its toxicity, the PHNQ3 compound was used in doses of 10 and 25 mg/kg/day. Dosages of PHNQ4 were 10 and 50 mg/kg/day and for PHNQ6 they were 25, 50 and 100 mg/kg/day. For treatment via the oral route, all compounds were used in concentrations of 10, 50 and 100 mg/kg/day. All experiments included normal mice treated only with the diluent and

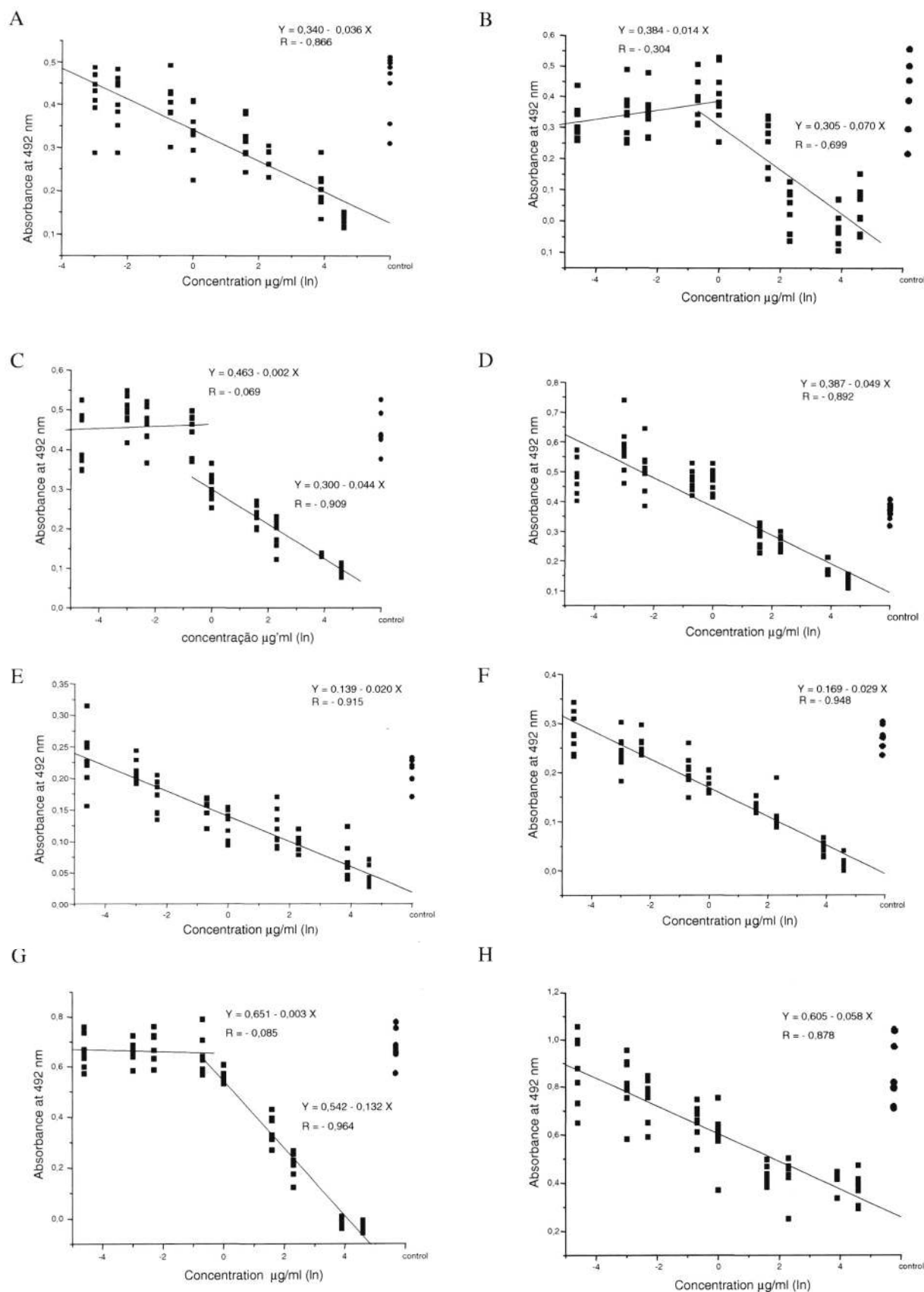
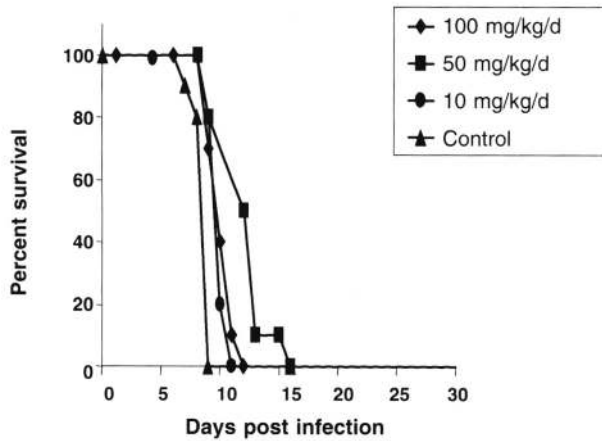
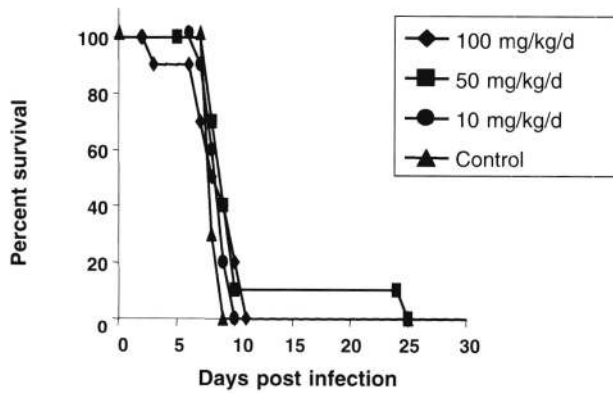


Fig. 2 – *In vitro* effect of *para*-hydroxynaphthoquinones PHNQ1 (A), PHNQ2 (B), PHNQ3 (C), PHNQ4 (D), PHNQ5 (E), PHNQ6 (F), PPNQ (G), on *Toxoplasma* growth. Absorbance values in the enzyme-linked immunosorbent assay of infected cultures were plotted versus the *ln* of the concentrations of drugs. (H) = Reference drug: Sulfadiazine. Control = cultures without drug.

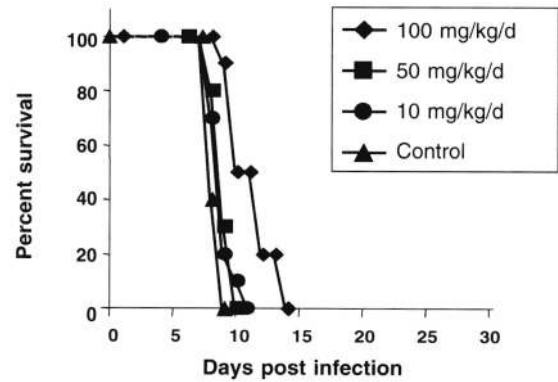
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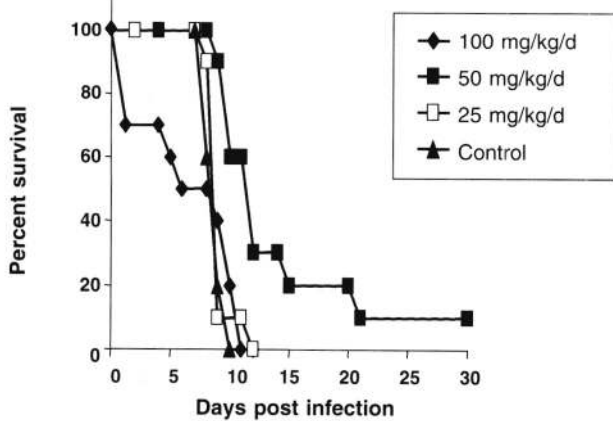
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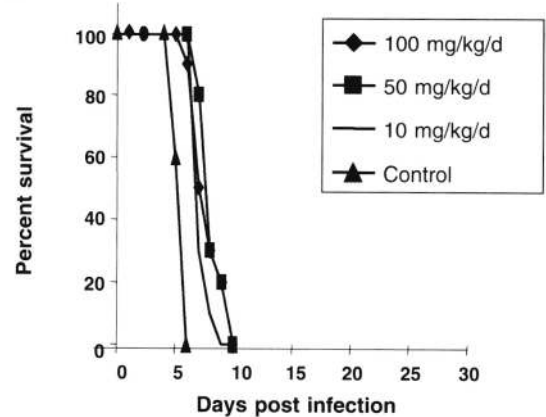


Fig. 3 – Activities of different doses of PHN4 (A), PHN5 (B and C), PHN6 (D and E) in disseminated acute murine toxoplasmosis oral infection with cysts of the EGS strain. Treatment was initiated 48 h after infection and was continued for 10 days. For panel A, C and E the drug was administered orally by gavage and for panel B and D the drug was administered by injection i.p. There were 10 mice in each experimental and control group.

untreated infected mice as controls. There were 10 mice in each experimental and control group.

All the infected untreated control animals died within a period of five to seven days when infected with the RH strain and seven to 10 days when infected with the EGS strain.

None of the compounds administered either orally or i.p. had an effect on mice infected with the RH strain (data not shown). Oral or i.p. treatment with PHNQ1, PHNQ2 (lapachol) and PPNQ (α -lapachone) at 10, 50 or 100 mg/kg/day did not provide significant protection against death in animals infected with EGS cysts (data not shown). The PHNQ3 compound administered i.p. in mice infected with the EGS strain prolonged survival in only one (10 %) of the animals treated with a dose of 10 mg/kg/day ($P = 0.0567$) (data not shown). Retarded mortality was observed when PHNQ4 was given orally in a dose of 50 mg/kg/day ($P = 0.0004$) to mice infected with the EGS strain (Fig. 3A). Survival was also prolonged in animals that received 50 mg/kg/day ($P = 0.0014$) i.p. or 100 mg/kg/day ($P < 0.001$) of PHNQ5 administered as a single oral dose daily for 10 days. (Fig. 3B and 3C). The results obtained using PHNQ6 administered i.p. resulted in prolongation of the time to death. In animals infected with the EGS strain, treatment with 50 mg/kg/day of PHNQ6 i.p., for 10 consecutive days, resulted in a prolongation up to 30 days ($P = 0.0004$) after the conclusion of treatment (Fig. 3D). An oral dose of ≥ 50 mg/kg/day of PHNQ6 resulted in additional four days ($P < 0.0015$) in the time to death (Fig. 3E). A 100 mg/kg/day dose of PHNQ6 administered i.p. appeared to be toxic, since 20 % of the treated mice died earlier than the untreated mice (controls).

As with the other naphthoquinones, PHNQ6 did not have an effect on the animals infected with the RH strain even though the mice treated with this compound showed signals of murine toxicity (e.g. weight loss, lethargy and piloerection) later than the controls.

DISCUSSION

Our results indicate that the PHNQ4, PHNQ5 and PHNQ6 compounds presented *in vitro* and *in vivo* activity against *T. gondii*. These compounds, termed as *para*-hydroxynaphthoquinones (PHNQs), were not significantly toxic to 2C4 fibroblasts or mice in a concentration that inhibited parasite multiplication. On the other hand, the *para*-furanonaphthoquinones (PFNQs) did not show anti-*T. gondii* activity *in vitro* even though they presented medium toxicity to 2C4 fibroblasts. The *ortho*-furanonaphthoquinones (OFNQs) were significantly toxic to host cells.

The results of toxicity tests in mice indicate that the administration route is an important factor in determining toxicity. All the compounds that were tested via the oral route during 10 consecutive days did not demonstrate any toxic effect in normal mice. However, concentrations of 50 and 100 mg/kg/day of PHNQ3 and 100 mg/kg/day of PHNQ4 administered i.p. were extremely toxic to uninfected mice, killing them immediately after administration.

The administration route not only affected the toxicity of the compounds, it also altered their activity in protecting mice against death from *T. gondii* infection. PHNQ5 in a dose of 50 mg/kg/day i.p., inhibited replication of EGS-strain tachyzoites ($P = 0.0014$) but the same amount administered orally did not have any effect on the same strain. As previously reported, the pH of gastric juices and sites within the mucosa may be an important factor that potentially affects naphthoquinone activity (Kumiko *et al.*, 1998). In one study that tested the anti-*T. gondii* activity of two naphthoquinones (NSC52 and NSC55), both compounds demonstrated significant protective activity in mice infected i.p. or orally with *T. gondii*. However, protection was noted only when the compounds were administered i.p., oral administration was not effective (Khan *et al.*, 1998).

Of further interest is the difference that exists between the pathogenesis of the infection produced by inoculation of tachyzoites or cysts. The i.p. inoculation of 10^3 tachyzoites of the RH strain resulted in a fulminant infection with a large number of tachyzoites being produced in the peritoneal cavity of all the positive control animals. In contrast, mice infected by using an oral inoculum of 10 cysts of the EGS strain produced an infection that progressed more slowly. However, oral infection with cysts or oocysts may be more appropriate to simulate a natural infection.

The results demonstrate significant differences in susceptibility to infection with *Toxoplasma* between the two *T. gondii* strains analyzed. It appears that each strain has intrinsic characteristics that contribute to its virulence. The *T. gondii* RH strain cause significantly higher levels of parasitemia (Derouin & Garin, 1991). This high parasitemia may increase the severity of infection and may be crucial in influencing the efficacy of our anti-*Toxoplasma* therapy.

The mechanism of action of PHNQ4, PHNQ5 and PHNQ6 against *Toxoplasma* has not yet been investigated. However, the hydroxynaphthoquinones appear to act as potent inhibitors of the redox processes in the respiratory chain. It has been shown that several natural and synthetic naphthoquinones exhibit anti-protozoal activity by the generation of active oxygen species such as hydroxyl radical ($\cdot\text{OH}$) and superoxide anion (O_2^-) that cause the lipid peroxidation and alterations in the electron transport with inhibition of the

parasite respiration (Morello, 1988; Docampo *et al.*, 1978).

In light of the problems caused by toxoplasmic infection, particularly among AIDS patients, a continued search for new compounds and new therapeutic approaches for treatment of opportunistic infections caused by *T. gondii* is clear. The present study demonstrates that *para*-naphthoquinones are effective in inhibiting replication of the RH strain of *T. gondii* in cell cultures and suggest that *para*-hydroxynaphthoquinones may also be effective against the parasite *in vivo*. Further research should focus on evaluation of these *para*-hydroxynaphthoquinones compounds. Additionally, studies must be conducted to determine whether synergic effects are observed when these compounds are administered with other effective compounds.

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