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Further evidence that naphthoquinone inhibits *Toxoplasma gondii* growth *in vitro*

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ABSTRACT

Toxoplasmosis is a widely disseminated disease caused by *Toxoplasma gondii*, an intracellular protozoan parasite. Standard treatment causes many side effects, such as depletion of bone marrow cells, skin rashes and gastrointestinal implications. Therefore, it is necessary to find chemotherapeutic alternatives for the treatment of this disease. It was shown that a naphthoquinone derivative compound is active against *T. gondii*, RH strain, with an IC_{50} around 2.5 μM. Here, three different naphthoquinone derivative compounds with activity against leukemia cells and breast carcinoma cell were tested against *T. gondii* (RH strain) infected LLC-MK2 cell line. All the compounds were able to inhibit parasite growth *in vitro*, but one of them showed an IC_{50} activity below 1 μM after 48 h of treatment. The compounds showed low toxicity to the host cell. In addition, these compounds were able to induce tachyzoite-bradyzoite conversion confirmed by morphological changes, *Dolichus biflorus* lectin cyst wall labeling and characterization of amylopectin granules in the parasites by electron microscopy analysis using the Thierry technique. Furthermore, the compounds induced alterations on the ultrastructure of the parasite. Taken together, our results point to the naphthoquinone derivative (LQB 151) as a potential compound for the development of new drugs for the treatment of toxoplasmosis.

1. Introduction

Toxoplasmosis is a disease caused by *Toxoplasma gondii*. The life cycle of this parasite is characterized by two phases: sexual and asexual. The sexual phase occurs in the intestine of the definitive host, felines, resulting in the shedding of resistant oocysts containing sporozoites [1–6]. Tachyzoites and bradyzoites are the asexual forms of this parasite; the former replicates rapidly and is associated with acute infection in the host; the latter represents the latent form of infection inside tissue cysts [1–6]. The tachyzoite conversion to bradyzoite occurs when the host immune system is triggered; patients with an immune-deficient system are unable to contain the acute infection and thus are susceptible to damage in various organs such as the brain [7]. The uncontrolled replication of tachyzoites causes a severe lytic cell cycle and host tissue damage [8].

Chemotherapeutic treatment for toxoplasmosis causes various side effects in patients, such as depletion of bone marrow cells, skin rashes and gastrointestinal implications [9]. Some of the available medications to control parasite infection include pyrimethamine, sulfadiazine, clindamycin, atovaquone and trimethoprim sulfamethoxazole [9]. The treatment of pregnant women and patients suffering from acquired immunodeficiency syndrome can be challenging due to adverse side effects such as teratogenicity and the toxicity to the host [10]. Therefore, the search for new chemotherapeutic agents is essential to find an alternative treatment that may reduce or even eliminate the side effects caused by the conventional treatment.

A compound that has been widely studied is lapachol, one of the main representatives of naphthoquinone compounds found in plants of the family Bignoniaceae, particularly in the genus *Tabebuia* [11]. Lapachol and derivatives have several activities such as antimicrobial

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and antifungal [12,13]; cercaricidal by preventing the penetration into the skin of *Schistosoma mansoni* cercariae [14]; molluscicidal by killing *Biomphalaria glabrata* snails, the intermediate host of *S. mansoni* [15]; leishmanicidal with intracellular activity against amastigotes of *Leishmania braziliensis* [16,17]; trypanocidal with activity against *Trypanosoma cruzi* [18]; antiplasmodial with activity against *Plasmodium falciparum* parasitizing erythrocytes [19,20]; and antitumoral [21].

Naphthoquinone derivatives (LQB 150 and LQB151) were tested on human leukemia cells and showed to be effective at eliminating these cells with low toxicity to peripheral blood mononuclear cells activated with hemagglutinin A [22]. Furthermore, LQB 94 presented high activity against *P. falciparum* and moderated activity against the breast cancer cell line, MCF-7, but presented low activity against *Leishmania amazonensis* [23]. In addition, Portes et al. [24] showed that LQB 118 (another naphthoquinone compound derivative) was able to inhibit the rate of growth of tachyzoites of *T. gondii* in the LLC-MK2 cell line with a 50% inhibitory concentration (IC_{50}) of 2.5 μM; this is the scaffold from which LQB150 and LQB151 derived. Furthermore, treatment with this compound induced ultrastructural changes at the plasma membrane of the parasites and was also able to induce tachyzoite-bradyzoite stage conversion. Thus, in search of a compound that may inhibit intracellular growth of *T. gondii*, the objective of this work was to verify if the compounds LQB 94, 150 and 151 (Fig. 1) were able to control *T. gondii* growth in LLC-MK2 cell line.

2. Materials and methods

2.1. LLC-MK2 cells

The LLC-MK2 cell line (kidney epithelial of the rhesus monkey) was maintained in culture flasks of 25 cm^2 with Dulbecco's Modified Eagle Medium (DMEM-Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS-Life Technologies) at 37 °C in a 5% CO_2 atmosphere. For the experiments, the cells were seeded over glass coverslips in 24-well plates (TPP), in 96-well plates (TPP) or in 25 cm^2 culture flasks (TPP) and cultured for 24 h.

2.2. Parasites

T. gondii, RH strain, was maintained by passages in the peritoneal cavity of Swiss mice over 2–3 days. Peritoneal lavage was performed with Hank's solution and centrifuged at 100 *g* for 5 min at 4 °C to remove cell debris and peritoneal leukocytes. The supernatant containing the parasites was collected and centrifuged at 1000 *g* for 10 min at 4 °C. The parasites were resuspended and counted in a Neubauer® chamber.

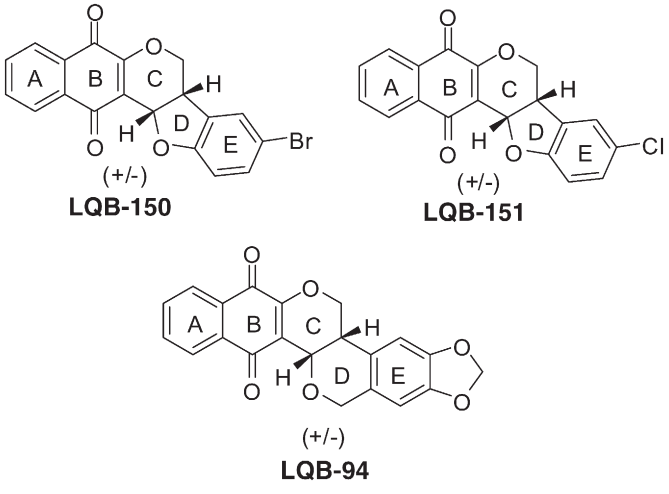

Fig. 1. Molecular structures of the naphthoquinone compound derivatives LQB 94, LQB 150 and LQB 151.

This study was carried out in strict accordance with the animal experimentation Brazilian Law #11794/08. The protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (Permit Number: 259). Mice were euthanized with CO_2 in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH.

2.3. Compounds

The naphthoquinone derivative compounds used were synthesized in the Bioorganic Chemistry Laboratory, Research Institute of Natural Products, Center for Health Sciences, Federal University of Rio de Janeiro and Chemistry Laboratory of Federal University of Rio de Janeiro, Macaé. Briefly, LQB-94 was obtained through an oxa-Heck reaction between chromenequinone and 2-chloromercurio-4,5-methylenedioxybenzylalcohol as a yellow solid in 30% yield [23]. LQB-150 was obtained through an electrophilic aromatic substitution reaction of LQB-118 with *N*-bromosuccinimide as a yellow solid in 57% yield [22]. LQB-151 was obtained through an electrophilic aromatic substitution reaction of LQB-118 with *N*-chlorosuccinimide as a yellow solid in 55% yield [22].The compounds LQB 94, LQB 150 and LQB 151 were diluted in dimethyl sulfoxide (Sigma-Aldrich), further diluted in DMEM at a concentration of 5 mM (final concentration of 4.25% dimethyl sulfoxide) and stored in a freezer (−20 °C). The compounds were tested at the following concentrations: 1.0, 2.5, 5.0 and 10.0 μM. Dimethyl sulfoxide at 0.05% did not interfere with the cell viability or intracellular growth of the parasites.

2.4. Cell viability assays

Three assays were used to check the viability of LLC-MK2 cells after treatment with the compounds: exclusion of ethidium bromide, reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT-Sigma-Aldrich) and lactate dehydrogenase (LDH-Doles) release. For the ethidium bromide (Sigma-Aldrich) essay LLC-MK2 cells cultured over coverslips in 24-well plates were incubated with the compounds at a concentration of 20 μM for 48 h. After this time, the coverslips with cells were collected and inverted over 10 μl of 10 mg/ml of ethidium bromide in DMEM. After 5 min cell viability was assessed under a Zeiss Axioplan microscope equipped with differential interference contrast microscopy, epifluorescent illumination and an HBO100 mercury lamp. Dead cells were differentiated from live ones by the intense red labeling of their nucleus; at least 200 cells per coverslip in triplicate were quantified.

A detailed analysis of the viability of LLC-MK2 cells treated with LQB151 was performed by the MTT [25,26] and the LDH assays. For these assays, 1×10^5 cells were seeded per well in 96-well plates and cultured with DMEM supplemented with 5% FBS. After 24 h, the cells were washed and LQB151 compound was added to the cells at serially diluted concentrations in DMEM supplemented with 5% FBS starting at 0.2 μM until 100 μM. For the negative control cells were cultured with 10% Triton X-100 in DMEM. For the positive controls, cells were cultured without LQB151 in DMEM supplemented with 5% FBS. After 24 h of treatment, the culture supernatant was removed and 15 μl of MTT DMEM solution (5 mg/ml) was added to each well for 4 h. After this period of incubation, the formazan crystals were solubilized with 100 μl of dimethyl sulfoxide. The plate was centrifuged at 400 *g* for 7 min, 100 μL of the supernatant was collected and transferred to a new 96-well plate that was read in a Versamax microplate reader (Molecular Devices) at 570 nm using the 6.0 SoftMax Pro®. Cells treated without LQB151 in DMEM supplemented with 5% FBS were considered 100% viable. The LDH measurement [27,28] was performed in the stored culture supernatant of the LLC-MK2 cells using the Doles® kit. For quantification of LDH, 50 μl of the cultured supernatant was transferred to a new 96-well plate, followed by solution A for 3 min and solution B for