

In Vitro Efficacy of Azithromycin and Roxithromycin Against *Toxoplasma gondii**

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Abstract: Toxoplasmosis is a common problem, accounting for significant morbidity in developing countries. Conventional treatments are associated with frequent relapses and with additional problems including undesirable side effects. Better-tolerated and more effective drugs are clearly needed. The aim of this study is to evaluate the in vitro efficacy of azithromycin and roxithromycin against *Toxoplasma gondii* using acridine orange in the fluorescence microscopic assay. It was found that azithromycin and roxithromycin had significant microbicidal activity ($P < 0.05$). In conclusion, this observation should be assessed carefully in future clinical trials involving a large number of cases with toxoplasmosis.

Key Words: *Toxoplasma gondii*, azithromycin, roxithromycin, in vitro efficacy

*Toxoplasma gondii*ye Karşı Azitromisin ve Roksitromisinin In Vitro Etkinliği

Özet: Toksoplazmozis gelişmekte olan ülkelerde önemli derecede sıklığı olan yaygın bir sağlık sorunudur. Klasik tedaviler istenmeyen yan etkileri içeren sorunlar ve nöksler ile birliktedir. Daha iyi tolere edilen ve daha etkin ilaçlara gereksinim vardır. Bu çalışmanın amacı *Toxoplasma gondii*ye karşı fluoresan mikroskop yöntemiyle acridine orange kullanılarak azitromisin ve roksitromisinin in vitro etkinliğini değerlendirmektir. Azitromisin ve roksitromisinin anlamlı derecede mikrobisidal aktiviteye sahip olduğu bulundu ($P < 0,05$). Sonuçta, bu gözlem gelecekte toksoplazmozisli geniş bir grup olguyu içeren klinik çalışmalarda dikkatlice değerlendirilmelidir.

Anahtar Sözcükler: *Toxoplasma gondii*, azitromisin, roksitromisin, in vitro etkinlik

Introduction

Toxoplasma gondii, an intracellular coccidian protozoan, has a wide range of mammalian hosts, but the most important definitive host from the human point of view is the domestic cat (1). In the absence of drugs that can eradicate *T. gondii* tissue cysts, more reliable therapy

is needed to prevent this infection. Since the kinetic and action mechanisms of macrolide, ketolide and azalide compounds are similar, synergic effect and possible therapy with the available combination of drugs may be feasible (2,3). Azithromycin has a 15-membered ring structure and is classified as an azalide (4). Roxithromycin

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is a monobasic macrolide, which is largely in bound form in plasma and produces high plasma and tissue concentrations. Also, it is concentrated by phagocytes (5,6). The aim of this study was to determine the in vitro microbicidal activities of azithromycin and roxithromycin against *T. gondii* using different techniques such as acridine orange in the fluorescence microscopy assay.

Materials and Methods

Heparinized blood was drawn from healthy (6 males, 30-35 years old) volunteers who were negative for IgG and IgM antibodies to *T. gondii* determined by an enzyme linked fluorescence assay (ELFA) (VIDAS, Vitek Immuno Diagnostic System Assay, BioMerieux, France) as previously described by Hofgartner et al. (7). These samples were negative for rheumatoid factor and anti-nuclear antibodies, which have been reported to cause false positive results in VIDAS for IgM antibodies. Blood samples were collected in 10 ml evacuated EDTA tubes (Becton Dickinson, USA) for neutrophil isolation. Differential leukocyte counts were performed on blood films prepared on glass cover slips and stained with Wright stain. Complement was inactivated by heating at 56 °C for 30 min in serum. Isolation of neutrophils from EDTA anticoagulated blood was carried out according to the method described previously by McDonald and Pruul (8). Briefly, 10 ml of blood was gently mixed with 6% dextran (w/v) (Sigma, USA) in PBS, (pH 7.2) (5/1, v/v). The red blood cells (RBC) were allowed to settle for 60 min at 37 °C. The supernatant was layered over 10 ml of Histopaque 1077 (Sigma, USA) and centrifuged (Megafuged 1.0R, Heraeus Sepatech GmbH, Germany) at 700 g for 30 min. The supernatant and mononuclear cell layer were discarded and the resulting pellet was collected after hypotonic lysis of the RBC and resuspended in Medium 199 (Sigma, USA). Cell viability (Trypan Blue exclusion, Sigma, USA) was 94% or greater and purity was greater than 93%. Eosinophils, lymphocytes and monocytes comprised the remaining 7% of the cells. Cells were counted in a haemocytometer, and the concentration of live polymorphonuclear leukocyte (PMNL) was adjusted to 2×10^7 cells ml^{-1} . Azithromycin (Biosen-Fako, Turkey) and roxithromycin (Hoechst-Marion Roussel, Turkey) stock solutions were prepared in acetonitrile (1 mg ml^{-1}) and diluted to the desired concentrations in PBS. The virulent Ankara strain of *T. gondii* was maintained by intraperitoneal inoculation of 6-

weeks-old BALB/C female mice (The animals were provided by Gülhane Military Medical Academy Research and Development Center, Ankara, Turkey). The collected cells were suspended in PBS and centrifuged at 200 g for five min. The supernatant fluid was discarded and the pellet was washed twice with PBS and resuspended in Medium 199. The viability test was performed by using 0.2 mmol l^{-1} methylene blue (Merck, Germany). Cell viability was 98% or greater. The cells were counted in a hemocytometer and resuspended to a final concentration of 1×10^8 cells ml^{-1} . Five groups were set up (Group I [control], Group II, III, IV and V) for in vitro experiments. Azithromycin and roxithromycin were tested at in vitro concentrations of 0.05, 0.1, 0.3 and 0.6 mg ml^{-1} in incubation medium, respectively. The first incubation mixtures included 0.5 ml of neutrophil suspension (1×10^7 cells), 0.5 ml of the *T. gondii* tachyzoites (5×10^7 cells) in Medium 199, 0.2 ml inactivated serum (10% of total volume of incubation mixtures) and 0.3 ml Medium 199. This mixture was allowed to incubate at 37 °C for 60 min, followed by rocking (4 rpm) for 60 min (Gerhardt, Schüttelwalsserbad SW 20, Bonn, Germany). After the incubation, 0.5 ml azithromycin or roxithromycin solutions (or 0.5 ml PBS in control) were added. Subsequently, these second incubation mixtures were allowed to incubate for 60 min under rotation (4 rpm) at 37 °C. Neutrophils were added to produce a final concentration of 5×10^6 cells ml^{-1} . *T. gondii* was added to yield a 5:1 ratio of microorganism to neutrophils in all incubation tubes. The ingestion and killing activities of the *T. gondii* tachyzoites by neutrophils were observed by the fluorescent microscopy (Zeiss HBO 100 W/2, Germany), using the fluorochrome dye, acridine orange (Sigma, USA) (9). At the end of the second incubation period, 0.1 ml aliquots were removed from the incubation tubes and stained with 0.025 ml acridine orange (14 mg in 100 ml Medium 199) for 60 seconds. The sample was then mounted on a plain 25 x 75 mm glass microscope slide and examined with an oil immersion objective at a magnification of x 1000 on a microscope with an incident light, and vertical fluorescence illuminator (50 W mercury vapor lamp, dichronic 510 nm, barrier 520 nm). The total number of intracellular microorganisms, including live (green) and dead (red) was recorded for 200 randomly selected neutrophils for each slide preparation. Each slide was assigned a random number and scored blindly. The percentage phagocytic activity was calculated as [number of phagocytic neutrophils/number of total

selected neutrophils] x100. The percentage killing activity was determined as [number of dead phagocytosed *T. gondii* tachyzoites/number of live + number of dead phagocytosed *T. gondii* tachyzoites] x 100. For statistical analysis, Duncan's test was used to compare the differences between the means of five groups by using Minitab (Release 9.2, 1993). Differences of $P < 0.05$ were considered significant. All data in this study were tabulated as mean \pm SE.

Results

In our study, in order to control contamination and prevent contamination related discrepancies, all incubation materials were also cultured for possible contaminating bacteria. It was also determined that acetonitrile, which was used to prepare the stock solution of azithromycin and roxithromycin, had no phagocytic and microbicidal effect alone. As shown in Table 1,

azithromycin had no effect on phagocytic activity at any of the tested concentrations ($P > 0.05$). When we compared control and Group IV (0.3 mg ml^{-1}), there was a significant difference ($P < 0.05$) in the microbicidal activity. As shown in Table 2, however, roxithromycin had no effect on phagocytic activity; it had a significant effect on microbicidal activity at all concentrations when compared to control. At same doses of these two drugs, roxithromycin consistently had a stronger effect than azithromycin.

Discussion

There is a need for new, effective and safe antitoxoplasmal drugs, because of the lack of therapy for this common worldwide infection. Azithromycin, as an azalide (4), and roxithromycin, a monobasic macrolide, are important to the understanding of the clinical activity of these agents are findings from in vivo and in vitro

Table 1. Phagocytic and microbicidal activity values of azithromycin in neutrophils (Mean \pm SE, n = 6).

Study Groups	Phagocytic Activity (%)	Microbicidal activity (%)
Group I (control)	70.0 \pm 6.2 ^a	48.3 \pm 8.0 ^a
Group II ($0.05 \text{ } \mu\text{g ml}^{-1}$)	73.5 \pm 7.3 ^a	44.6 \pm 6.5 ^{ab}
Group III ($0.1 \text{ } \mu\text{g ml}^{-1}$)	71.4 \pm 9.0 ^a	51.6 \pm 5.5 ^{ab}
Group IV ($0.3 \text{ } \mu\text{g ml}^{-1}$)	72.0 \pm 8.0 ^a	61.6 \pm 4.0 ^b
Group V ($0.6 \text{ } \mu\text{g ml}^{-1}$)	69.3 \pm 6.8 ^a	49.4 \pm 3.2 ^{ab}

^{a,b} Different letters in the same column indicate significant differences.

Table 2. Phagocytic and microbicidal activity values of roxithromycin in neutrophils (Mean \pm SE, n = 6).

Study Groups	Phagocytic Activity (%)	Microbicidal activity (%)
Group I (control)	59.0 \pm 3.6 ^a	53.4 \pm 9.7 ^a
Group II ($0.05 \text{ } \mu\text{g ml}^{-1}$)	61.0 \pm 5.6 ^a	58.8 \pm 10.7 ^{ab}
Group III ($0.1 \text{ } \mu\text{g ml}^{-1}$)	64.6 \pm 3.9 ^a	69.2 \pm 5.3 ^{cd}
Group IV ($0.3 \text{ } \mu\text{g ml}^{-1}$)	61.4 \pm 3.1 ^a	66.7 \pm 7.9 ^{cd}
Group V ($0.6 \text{ } \mu\text{g ml}^{-1}$)	63.8 \pm 4.2 ^a	72.6 \pm 5.6 ^d

^{a,b,c,d} Different letters in the same column indicate significant differences.

studies, which show that they rapidly become highly concentrated in phagocytic cells (5,6,10) and can easily penetrate into brain tissue, cerebrospinal fluid and aqueous humor of the eye (11). In the present study, in vitro model for antimicrobial activity is based upon the ability of the antibiotic to penetrate and kill *T. gondii* tachyzoites ingested by neutrophils. Therefore, this model is different from previously performed in vitro models, which investigated the activity of the antibiotic dilutions to study *T. gondii*. In order to evaluate the role of azithromycin and roxithromycin as a determinant of having microbicidal activity in the neutrophils, we used the viability test as a model system. From the results of this experiment it can be concluded that the use of the neutrophil-mediated acridine orange technique allows the period of microbicidal activity afforded by both macrolides (azithromycin and roxithromycin) to be extended significantly, even under conditions of heavy tachyzoites infection challenge. In this study, no significant reduction was observed for phagocytic activity in the presence of azithromycin and roxithromycin. Assessment of microbicidal activity is important in vitro bioactivity assays of a drug. Azithromycin concentration of 0.3 mg/ml had resulted in a statistically significant increase in microbicidal activity compared to the control. However, at the dose of 0.6 mg/ml azithromycin had no statistically significant effect on microbicidal activity ($P > 0.05$). Although roxithromycin had no statistically significant ($P > 0.05$) effect on phagocytic activity, it had statistically significant effect on microbicidal activity at all doses, and this effect was observed in a dose dependent manner. These results demonstrated that azithromycin and roxithromycin could become concentrated in the neutrophils and increase microbicidal activity. Higher drug concentrations within neutrophils might also be beneficial in overcoming therapy of intracellular infections of *T. gondii*. Not only the intracellular penetration of antimicrobials guarantee therapy of intracellular infection but also antimicrobials must be delivered to the

intracellular location of the bacteria. In this point of view, these results may show that azithromycin and roxithromycin were concentrated to the intracellular location of the parasite. In animal model studies, *T. gondii*-infected host cells were treated with clindamycin for various periods, and clindamycin had no effect on parasite invasion (12). Rothova et al. (13) reported that although azithromycin may be an effective alternative drug for seven of 11 patients with ocular toxoplasmosis which exhibited prompt resolution of the retinal lesion and no side effects of azithromycin. Brun-Pascaud et al. (14) showed that the combination of roxithromycin with dapsone or atovaquone reduced the burdens of *T. gondii* in tissues of the rat model of concurrent toxoplasmosis. In in vitro studies, roxithromycin inhibited toxoplasma growth in *T. gondii* infected fibroblast tissue culture (15). The antibacterial activities of the macrolides are known to result from their ability to inhibit protein synthesis by binding to the transpeptidation site of the larger ribosomal subunit. Thus all that display likely kinetics of exploit against parasite act by inhibiting protein synthesis in *T. gondii* as well (12). The mechanism of action of macrolides may be the ribosomes encoded by the plastid-like ribosomal genes can be a potential target for protein synthesis in parasite (16,17). Azithromycin and roxithromycin are probably acting by inhibition of protein synthesis (18). In conclusion, these data suggest that azithromycin and roxithromycin may reinforce host cellular immune defense for treating toxoplasmosis, and roxithromycin has a stronger effect than azithromycin. These results are very promising, and the observed significant microbicidal activity suggests that additional investigations should be performed.

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