ORIGINAL ARTICLE

In vitro Evaluation of Various Drugs Against *Toxoplasma gondii*

G H Tee, BSc*, A A Khairul, PhD**, M Rohela, MPHTM**, N Rahmah, PhD***, *Division of Epidemiology & Disease Control, Public Health Institute, Jalan Bangsar, 50590 Kuala Lumpur, **Department of Parasitology, Faculty of Medicine, University Malaya, 50603 Kuala Lumpur, ***Department of Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan

Summary

The *in vitro* activities of three macrolides (erythromycin, azithromycin and roxithromycin) and two aminoglycosides (gentamicin and streptomycin) against tachyzoites of *Toxoplasma gondii* were evaluated by using a microscopic counting method. The virulent RH strain of *T. gondii* was used to infect confluent monolayers of Vero cells grown in 96-well tissue culture microplates. Various concentrations of erythromycin, azithromycin, gentamicin and streptomycin (0, 10, 20, 30 and 40μg/ml) and roxithromycin (0, 10, 20, 30, 40, 50 and 60μg/ml) were then added to the monolayers. After incubation, free tachyzoites in the culture suspension were counted by using 0.4% trypan blue and IC_{50} and IC_{90} of each drug were then determined. The IC_{50}s were calculated at 9.5, 19, 21.5, 26.8 and 30μg/ml for erythromycin, azithromycin, roxithromycin, streptomycin and gentamicin respectively; whereas the IC_{90}s were calculated at 32.7μg/ml for azithromycin; 37.7μg/ml for erythromycin; 57.3μg/ml for roxithromycin and above 40μg/ml for streptomycin and gentamicin. Erythromycin and azithromycin were found to be effective in inhibiting the growth of *T. gondii* tachyzoites, roxithromycin only had moderate activity; whereas streptomycin and gentamicin were found to be ineffective against *T. gondii* infection.

Key Words: *Toxoplasma gondii*, Macrolides, Aminoglycosides, Erythromycin, Azithromycin, Roxithromycin, Gentamicin, Streptomycin

Introduction

Toxoplasma gondii is an ubiquitous obligate intracellular protozoan parasite[1]. It is estimated that up to 30% to 60% of the American adult population have been infected; while as many as 90% of adults in European countries are seropositive for *T. gondii*[2]. Epidemiological surveys in Malaysia indicated that *Toxoplasma* antibody is common among all ethnic groups of all ages, with the highest prevalence rate found in Malays (33%); followed by Indians (29%) and Chinese (18%)[3]. The age group of 15-26 years old was found to have the highest rate of antibody production, thus sero-negative pregnant women in Malaysia may be at risk of contracting *T. gondii* infection. Foetuses infected with *T. gondii* often develop serious sequelae such as hydrocephalus, microcephalus, deafness, blindness or foetal death[4].

Even though the current therapy for toxoplasmosis is effective, there are many side effects[4]. Therefore there have been active research into newer drugs with fewer or no side effects, such as azithromycin and roxithromycin[2,5,6,7,8,9].

The aim of this study was to evaluate the *in vitro* activities of three macrolides (erythromycin, azithromycin and roxithromycin) and two aminoglycosides (streptomycin and gentamicin) against *T. gondii*. The drugs tested were selected on the basis of suggested efficacy against *T. gondii* or other microorganisms. The macrolides azithromycin and

roxithromycin were studied because of their known *in vitro* as well as *in vivo* activities against *T. gondii*[2,5,7,9]. Erythromycin is the parent compound of both azithromycin and roxithromycin, therefore a similar effect was expected[10]. Streptomycin and gentamicin were chosen for the study because streptomycin is one of the drugs used for the treatment of intracellular bacteria, *Mycobacterium tuberculosis*[11], whereas gentamicin with its broad spectrum activity against both gram positive and gram negative bacteria has been used in serious infections caused by gram negative bacteria non-susceptible to other drugs[12].

Materials and Methods

T. gondii strain

The virulent RH strain of *T. gondii* was maintained by intraperitoneal inoculation of six week old inbred Balb/c mice. Tachyzoites were harvested from mice asceptically (after 3 days of infection) by lavage of the peritoneal cavity with 5 ml phosphate buffered saline (PBS), pH 7.2. The lavage fluid was centrifuged at 500 rpm for 5 mins to pellet the host cells and other debris. The supernatant was collected and the number of tachyzoites was counted on a haemocytometer; the viability of tachyzoites was then evaluated by using 0.4% trypan blue (Gibco) exclusion test. The viability of tachyzoites must be above 95% before they could be used for subsequent drug testing. The tachyzoites suspension was diluted to the desired concentration with RPMI-1640 (Flow Laboratories), pH 7.2 with 10% heat inactivated foetal calf serum and 0.06 g/l (RPMI-FCS) gentamicin sulphate (Gibco).

Cell culture

Vero cells (African green monkey kidney: strain ATCC CCL81) were maintained in 25 cm^2 tissue culture flasks (Costar) in RPMI-FCS at 37°C, 95% air and 5% CO_2 atmosphere. Each well of a 96-well tissue culture microplate was seeded with 100µl of 2 x 10^5 cells/ml and allowed to grow to confluence (72h) before being challenged with *T. gondii* tachyzoites and drug susceptibility testing.

Drugs

Five drugs were tested, namely azithromycin (powder form); erythromycin (powder form); roxithromycin (powder form); gentamicin (injectable form) and streptomycin (powder form). Azithromycin – a fifteen-membered macrolide and a derivative of erythromycin (mw 749), was supplied by Pfizer Malaysia. Roxithromycin – a fourteen-membered macrolide and an ether oxime derivative of erythromycin (mw 837.04) was supplied by Hoechst-Malaysia Pharmaceuticals. Erythromycin, a fourteen-membered macrolides (mw 734); gentamicin, an aminoglycoside (mw 1546) in the form of gentamicin sulphate; and streptomycin, an aminoglycoside (mw 1457) in the form of streptomycin sulphate, were purchased from Sigma, USA.

Drug susceptibility testing

100µl of 1 x 10^6 tachyzoites/ml were added to the confluent Vero cells in microtitre plates. Plates were incubated for 4h at 37°C before the addition of the drugs. Azithromycin, erythromycin and roxithromycin were prepared in 95% ethanol at final concentrations of 1mg/ml; whereas streptomycin was diluted in PBS, pH 7.2. They were then diluted to the desired concentrations by using RPMI-1640 (without gentamicin) containing 10% FCS. The drug concentrations used in the experiment were as follows: 0µg/ml, 10µg/ml, 20µg/ml, 30µg/ml and 40µg/ml; for roxithromycin, additional concentrations of 50µg/ml and 60µg/ml were also used.

25µl of the various dilutions of each drug were added to the monolayers infected with *T. gondii* tachyzoites, this was repeated four times for each dilution. The microtitre plates were then incubated for a further 72h. Free tachyzoites in the culture supernatant of each drug concentration (five wells per concentration) were pooled in a tube. The number of free tachyzoites in the suspension was counted using 0.4% trypan blue.

Relative percentages of inhibition against each concentration of the five drugs were calculated as follows:-

$$100 \times 1 - \frac{\text{number of free tachyzoites/well with antimicrobial agent}}{\text{number of free tachyzoites/well without antimicrobial agent}}$$

Graphs of the relative percentages of inhibitions of intracellular *T. gondii* growth versus various concentrations of drugs were plotted and the IC_{50} and

IC_{90} of each drug were determined. IC_{50} and IC_{90} are the concentrations required to produce 50% and 90% reduction (respectively) of the number of tachyzoites as compared with the control wells (without drugs).

Results

Relative inhibitory activities of various drugs against *T. gondii*

Table I shows inhibitions of growth of *T. gondii* by the various drug concentrations. The number of free tachyzoites per well decreased as the concentrations of the drugs increased. Thus there was an increase in relative percentages of inhibition of *T. gondii* growth with increasing drug concentrations. Dose-response curves for each drug tested in the microassay are shown in Figure 1. Dose-response curves established for each drug were used to calculate the IC_{50} and IC_{90}. The IC_{50}s were 9.5µg/ml, 19µg/ml, 21.5µg/ml, 26.8µg/ml and 30µg/ml for erythromycin, azithromycin, roxithromycin, streptomycin and gentamicin respectively. The values for IC_{90}s were 32.7µg/ml, 37.7µg/ml, 57.3µg/ml for azithromycin, erythromycin and roxithromycin respectively. Streptomycin and gentamicin had their IC_{90}s above 40µg/ml. No further dilutions were done for these two aminoglycosides because at 40µg/ml, host cells showed toxicity towards the drugs; the morphology of the Vero cells changed from spindle shape to round.

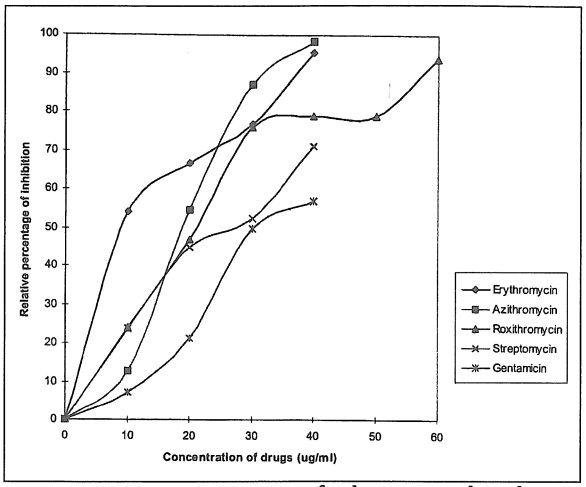

Fig. 1: Concentration of drugs and relative percentage of inhibition by microscopic method

Even though azithromycin had a higher IC_{50} than erythromycin, its IC_{90} was lower. The range between

Table I
Concentration of various drugs, number of free tachyzoites per well and relative percentage of inhibition by microscopic method

Conc of drug (µg/ml)	Erythromycin		Azithromycin		Roxithromycin		Streptomycin		Gentamicin	
	No. of free tachyzoites/well	Relative % of inhibition	No. of free tachyzoites/well	Relative % of inhibition	No. of free tachyzoites/well	Relative % of inhibition	No. of free tachyzoites/well	Relative % of inhibition	No. of free tachyzoites/well	Relative % of inhibition
0	10.8×10^5	0	3.1×10^5	0	4.0×10^5	0	3.8×10^5	0	1.4×10^5	0
10	4.95×10^5	54.2	2.7×10^5	12.9	2.6×10^5	24	2.9×10^5	23.7	1.3×10^5	7.1
20	3.6×10^5	66.7	1.4×10^5	54.8	1.5×10^5	47	2.1×10^5	44.7	1.1×10^5	21.4
30	2.5×10^5	76.9	0.4×10^5	87.1	0.8×10^5	76	1.8×10^5	52.6	0.7×10^5	50.0
40	0.5×10^5	95.4	0.05×10^5	98.4	0.7×10^5	79	1.1×10^5	71.1	0.6×10^5	57.1
50	-	-	-	-	0.7×10^5	79	-	-	-	-
60	-	-	-	-	0.2×10^5	94	-	-	-	-

IC_{50} and IC_{90} was very much lower for azithromycin (13.7μg/ml) than erythromycin (28.2μg/ml), azithromycin was thus considered to be a better drug than erythromycin.

This microassay showed that erythromycin and azithromycin caused inhibition towards growth of *T. gondii*. Roxithromycin had moderate inhibitory activity; while streptomycin and gentamicin exhibited limited growth inhibition towards this parasite.

Discussion

Current therapy for toxoplasmosis is the combination of pyrimethamine and sulfadiazine[13]. Although these two drugs are active against *T. gondii* tachyzoites and act synergistically by blocking the metabolic pathway involving folic-folinic acid and p-aminobenzoic acid cycles respectively; the combination has many adverse and even toxic effects. Bone marrow depression, thrombocytopenia, leukopenia and anaemia are some of the many adverse and or toxic effects[4].

Spiramycin, a macrolide antibiotic, has proven efficacy against *Toxoplasma* infection during pregnancy[4,14] without showing toxicity to the foetus. However, it does not kill the parasite efficiently[4,15]. Clindamycin, a lincosamide antibiotic which concentrates in the choroid had proved effective in the treatment of ocular toxoplasmosis[16]. High rates of relapse and toxicity appeared to preclude the use of clindamycin in maintenance therapy for toxoplasmosis.

The inhibitory effects of five drugs (erythromycin, azithromycin, roxithromycin, gentamicin and streptomycin) on *T. gondii* tachyzoites were determined by microsopically counting free tachyzoites in the RPMI-1640 medium 72h after the addition of various concentrations of drugs. Assays were performed five times for each drug and drug concentrations and the potency of each drug was determined by comparing their IC_{50} and IC_{90}.

Erythromycin had the lowest IC_{50} (9.5μg/ml), followed by azithromycin (19μg/ml), roxithromycin (21.5μg/ml), streptomycin (26.8μg/ml) and gentamicin (30μg/ml). For values of IC_{90}, azithromycin had the lowest value (32.7μg/ml), followed by erythromycin (37.7μg/ml) and roxithromycin (57.3μg/ml); streptomycin and gentamicin had the IC_{90} values above 40μg/ml.

Among the three macrolides, azithromycin accumulates most readily inside *T. gondii* infected cells thereby interfering with the growth of the parasites[10]. Roxithromycin exerts its action by disrupting the parasite protein synthesis and is concentrated in human polymorphonuclear leucocytes and macrophages[17]. The more lysosomotropic activity of azithromycin compared to the other two macrolides is essentially due to the presence of two basic amine groups on the molecule. Azithromycin was shown to be the most active macrolide against *T. gondii in vitro* by Chang *et al*[9]. Chamberland *et al*[2] found the IC_{50} of azithromycin (8.61 μg/ml) to be lower than IC_{50} of erythromycin (14.38 μg/ml). This is in contrast to the results obtained in this study whereby erythromycin showed a lower IC_{50} value than azithromycin. The incubation conditions by Chamberland *et al* were similar to that used in this study but the culture medium and cell lines were different namely Dulbecco's Modified Eagle medium (DMEM) (Gibco), pH 7.2 and BT cells (ATCC CRL 1390) in 150 cm^2 culture flasks. Barry *et al*[18] also showed that the potency of azithromycin and erythromycin against *Haemophilus influenzae* is affected by the growth medium. Some studies indicated that azithromycin's entry into extracellular *T. gondii* and thus its potency is effected by pH, temperature and concentration[19,20]. The drug concentrations in this study differ from that employed by Chamberland *et al*, this could have also contributed to the different results obtained in the two studies.

Addition of inactivated serum to the growth medium has been shown to enhance the *in vitro* potency of erythromycin[21]; this might explained the low IC_{50} of erythromycin. Even though erythromycin had a much lower IC_{50} than azithromycin, its IC_{90} was slightly higher than azithromycin. Therefore, azithromycin may be considered a better drug over erythromycin because it takes a lower concentration of the drug to inhibit 90% of *T. gondii* growth. Azithromycin, a 15-membered macrolide derived from erythromycin inhibits the intracellular pathogen by preventing the fusion of phagosomes and lysosomes and acidification of the parasitophorous compartment[2]. Azithromycin may be regarded as alternate drug for the lifelong treatment

needed to prevent toxoplasma encephalitis in AIDS patients[6]. It has been found to have antitoxoplasmic activity in mice[5] as well as in *in vitro* models[2].

Roxithromycin, a macrolide which accumulated more readily than erythromycin in cultured J744 mouse macrophages[22] failed to demonstrate to be a more potent drug than erythromycin. The moderate activity of roxithromycin in this microassay was similar to that reported in previous *in vitro* studies[2,9]. On the contrary, this drug was shown to be effective in treating mice with acute toxoplasmosis[17] and murine encephalitis[8]. The moderate activity of roxithromycin in *in vitro* microassay against *T. gondii* might be partly due to the addition of heat inactivated serum since this drug had been shown to bind strongly to serum proteins[21]. Such discrepancies between animal models and *in vitro* assay cannot be easily explained, but should not be used to discredit the utility of *in vitro* assays in large-scale screening for active compounds. Drugs that are active *in vitro* may very well turn out to be active *in vivo* and would be more likely to have a direct activity against the parasite. For instance, pyrimethamine has a high level of parasite-specific activity in many *in vitro* assays and is also known to exert its inhibitory activity by inhibiting dihydrofolate reductase of *T. gondii*[22].

Whatever their mode of activity, macrolides need to penetrate the host cells to exert their effect on the intracellular *T. gondii.* Carlier *et al*[23] have shown that all macrolides are not similar in this respect. In macrophages of human and animal origin, roxithromycin accumulated more consistently and significantly than erythromycin; the former thus reaching considerably higher intracellular-extracellular concentration ratios. As a result, differences in the inhibitory effects among the three macrolides found in this study did not necessarily reflect corresponding differences in intrinsic activity. Indeed, different intracellular pharmacokinetics may also have to be considered.

The mode of action of two aminoglycosides, streptomycin and gentamicin is by inhibition of protein synthesis. Their IC_{50}s were 26.8 µg/ml and 30 µg/ml respectively and their IC_{90}s were more than 40 µg/ml. At such high concentration, host cells showed toxicity towards the drugs. Therefore they were considered not effective against *T. gondii* in *in vitro* microassay. However, the effect of combining aminoglycosides and macrolides in the microassay needs to be evaluated.

Conclusion

The evaluation of *in vitro* activity of the five drugs showed erythromycin and azithromycin to be effective in inhibiting the growth of *T. gondii* tachyzoites. Roxithromycin only had moderate activity, whereas gentamicin and streptomycin were found to be ineffective against *T. gondii* tachyzoites.

Acknowledgements

The authors thank Mr. P.L. Yap and En. Abdul Aziz Ahmad for their assistance in maintaining the *Toxoplasma gondii* tachyzoites in Balb/c mice. The authors also thank the Director General of Health, Ministry of Health for his kind permission to publish this paper. This project was supported by IRPA Toxoplasma grant : R & D 06-02-03-0318.

References

1. Karen BH. Clinical toxoplasmosis – 50 years. Trans R Soc Trop Med Hyg 1992;66 : 549-71.

2. Chamberland S, Kirst HA, Current WL. Compartive activity of macrolides against *Toxoplasma gondii* demonstrating utility of an *in vitro* microassay. Antimicob. Agents Chemother 1991;35 : 903-9.

3. Khairul Anuar A, Afifi AB, Dighe VC, Roslani AMM. *Toxoplasma* antibody in pregnant women in Northern Peninsula Malaysia. Diagnosa 1991;5(1) : 18-23.

4. Van De Ven ES, Melchers W, Camps W *et al.* Effectiveness of spiramycin for treatment of congenital *Toxoplasma gondii* infection in rhesus monkey. Antimicrob. Agents Chemother. 1994;38 : 1930-6.

5. Araujo FG, Guptill DR, Remington JS. Azithromycin, a macrolide antibiotic with potent activity against *Toxoplasma gondii.* Antimicrob. Agents Chemother. 1998;32 : 755-7.

6. Godofsky EW. Treatment of presumed cerebral toxoplasmosis with azithromycin. N Engl J Med 1994;330 : 575-6.

7. Chang HR, Pechere JCF. Effect of roxithromycin on acute toxoplasmosis in mice. Antimicrob. Agents Chemother. 1987;31 : 1147-9.

8. Hofflin JM, Remington JS. *In vivo* synergism of roxithromycin (RU965) and interferon against *Toxoplasma gondii.* Antimicrob. Agents Chemother. 1987;31 : 346-8.

9. Chang HR, Pechere JCF. *In vitro* effects of four macrolides (Roxithromycin, Spiramycin, Azithromycin [CP-62993] and A-56268) on *Toxoplasma gondii.* Antimicrob. Agents Chemother. 1988;32 : 524-9.

10. Blais J, Beauchamp D, Chamberland S. Azithromycin uptake and intracellular accumulation by *Toxoplasma gondii* infected macrophages. J Antimicrob. Chemother. 1994;34 : 371-82.

11. Snider DE Jr, Cohn DL, Davidson PT *et al.* Standard therapy for tuberculosis 1985. Chest 1985;87(2 Suppl) : 117S-24S.

12. Antibacterial or antifungal chemotherapy. In Brooks GF, Butel JS, Ornston LN (eds), Jawetz, Melnick and Adelberg's Medical Microbiology (19th edition). Prentice Hall International Inc;pp 149-79.

13. Luft BJ, Remington JS. Toxoplasmosis. In Hoeprich PD, Jorden MC, Ronald AR (eds), Infectious Diseases. A Treatise of Infectious Processes, 5th ed. Philadelphia : JB Lippincott Co 1994;1201-13.

14. Desmonts GS, Couvseur J. Congenital toxoplasmosis. A prospective study of 378 pregnancies. N Engl J Med 1974;290 : 1110-6.

15. Chang HR, Pechere JCF. Activity of spiramycin against *Toxoplasma gondii in vitro,* in experimental infections and in human infections. Antimicrob. Agents Chemother. 1988;22(supp B) : 87-92.

16. Engstorm RE *et al* : Current pratices in the management of ocular toxoplasmosis. Am J Opthalmol. 1991;11 : 601-5.

17. Markham A, Faulds D. Roxithromycin. An uptake of its antimicrobial activity, pharmacokinetic properties and therapeutic use. Drugs 1994;48 : 297-326.

18. Barry AL, Jones RN, Thornsberry C. *In vitro* activities of azithromycin (CP 62993), clarithromycin (A-56268; TE-031), erythromycin, roxithromycin and clindamycin. Antimicrob. Agents Chemother. 1988;32 : 752-4.

19. Retsema J, Brennan L. Significance of environmental conditions on the *in vitro* potency of azithromycin. Central Research, Pfizer Inc; Groton, CT 06340.

20. Conrad J, Yang C, Slowik MR, Joiner KA. Localization of azithromycin in *Toxoplasma gondii* infected cells. Antimicrob. Agents Chemother. 1994;38 : 1620-7.

21. Jones RN, Barry AL. Unpredictable influence of human serum on antimicrobial activity of erythromycin and three oxime ether macrolides. European J Clin Microb 1987;6 : 81-2.

22. Kolvacs JA, Allerga CJ, Beaver D *et al.* Characterization of de novo folate synthesis in *Pneumocystic carinii* and *Toxoplasma gondii* : potential for screening therapeutic agents. J Infect Dis 1989;160 : 312-20.

23. Carlier MB, Zenebergh A, Tulkens PM. Cellular uptake and subcellular distribution of roxithromycin and erythromycin in phagocytic cells. J Antimicrob Chemother. 1987;20(Suppl B) : 47-56.