

The in-vitro anti-rickettsial activity of macrolides

Avi Keysary^{a*}, Avi Itzhaki^b, Ethan Rubinstein^c, Chaya Oron^c and Gershon Keren^c

^aDepartment of Infectious Diseases, Israel Institute for Biological Research, POB 19 Ness-Ziona; ^bAsaf-Harofe Medical Center, Zrifin; ^cInfectious Diseases Unit, Sheba Medical Center, Tel-Hashomer, Tel-Aviv University, School of Medicine, Israel

The anti-rickettsial activity of azithromycin and clarithromycin was studied in Vero cells. The rate of rickettsial inhibition-growth caused by both macrolides was determined using rickettsial counts and ELISA. Both macrolides inhibited > 50% the growth of *Rickettsia conorii* and *Rickettsia typhi* at concentrations of 1.0 and 0.1 mg/L, respectively. The growth of *Coxiella burnetii* was inhibited to a rate of $\geq 50\%$ at the concentrations of 0.01 and 1.0 mg/L of azithromycin and clarithromycin, respectively.

Introduction

Rickettsia conorii, *Rickettsia typhi* and *Coxiella burnetii* are intracellular bacteria which cause Mediterranean spotted-fever, endemic-typhus and Q-fever, respectively. Since macrolides reach effective concentrations in many tissues and possess preferential penetration into cells (Hand & King-Thompson, 1990; Lambert & O'Grady, 1992) their activity against intracellular pathogens is of great interest. The activity of two novel macrolides—azithromycin and clarithromycin—against *R. conorii*, *R. typhi* and *C. burnetii* was studied in a Vero cell line. The growth of rickettsiae in the cells was measured in the presence of both antibiotics using rickettsial counts (Turco & Winkler, 1982) and ELISA (Keren *et al.*, 1995).

Materials and methods

Antibiotics

The antibiotics used were: azithromycin (Pfizer Labs, USA); clarithromycin (Abbott Laboratories, USA); and minocycline hydrochloride (Lederle, USA). Minocycline served as a reference antibiotic known to inhibit rickettsial growth. Azithromycin and clarithromycin were dissolved in dimethyl sulphoxide (DMSO) to final concentrations of 10.0 and 1.0 mg/mL, and stored at -20°C . The presence of azithromycin and clarithromycin at the concentrations used in this study, did not cause toxic effect in the cells as shown by trypan-blue cell-counts.

*Tel: +972-8-381542; Fax: +972-8-401094; Email: xiibr@weizmann.weizmann.ac.il

Rickettsiae

R. conorii (Moroccan strain), *R. typhi* (Wilmington strain) and *C. burnetii* (phase I, Ohio 314 strain) were propagated in yolk-sacs of embryonated hen eggs and partially purified by differential centrifugation as described previously (Keren *et al.*, 1995). Rickettsial suspensions were kept at -70°C until used.

Tissue Cultures

Vero C1008 (ATCC no. CRL 1586) cell-line was used. Cells were grown in Dulbecco's modified minimal essential medium (Biological Industries, Beth-Haemek, Israel) supplemented with 10% fetal calf serum, 2 mM glutamine and 1:100 dilution of non-essential amino-acid solution (Biological Industries, Beth-Haemek, Israel). Cells were grown at 34°C in a CO_2 (3.5%) humidified incubator.

Infection of cells with rickettsiae in the presence of antibiotics

Vero cells were seeded in 96 well-plates (Costar, Cambridge, MA, USA) 5×10^4 cells per well in 0.1 mL medium, 1 day before infection. On the day of infection, cells were washed once with Hank's balanced salt solution supplemented with 5.0 mM L-glutamic acid-monopotassium salt and 0.1% gelatin. Wells were thereafter inoculated with suspensions of *R. conorii*, *R. typhi*, and *C. burnetii* at the concentrations of 2×10^4 , 3×10^3 and 5×10^5 EID₅₀ (egg infective doses 50%) per mL, respectively. *R. conorii* and *R. typhi* infected well-plates were centrifuged at 600 g for 15 min at room temperature and then incubated for additional 45 min at 34°C in a humidified CO_2 incubator. *C. burnetii* inoculated plates were centrifuged for 60 min without further incubation. Then plates were washed three times with growth-medium containing 1 mg/L cycloheximide, which inhibits DNA and protein synthesis of eucaryotic cells but does not affect procaryotic cells (Ripa & Mardh, 1977). The two last washes were made with medium containing the tested antibiotic in the relevant concentrations. Each concentration was tested in duplicate. Every experiment contained wells with sham-infected cells, rickettsiae-infected cells without antibiotics and rickettsiae-infected cells treated with minocycline (10 mg/L). *R. conorii* and *R. typhi* inoculated cells were incubated at 34°C in a humidified CO_2 incubator for 3 days. *C. burnetii* inoculated cells were incubated at 37°C in a humidified CO_2 incubator for 6 days.

Rickettsial counts

Rickettsial growth was determined by rickettsial counts as described previously (Turco & Winkler, 1982). Suspensions of the infected cells were stained by the modification of Gimenez stain as described by Wisseman, Waddel & Walsh (1974). The number of intracellular rickettsiae at each treatment was counted from duplicate wells. Fifty cells were counted from each well. When there were more than 30 rickettsiae/cell, a value of 30 organisms/cell was assigned.

ELISA

ELISA was performed as described previously (Keren *et al.*, 1995). Four wells from each treatment were tested. The growth-medium in the wells was aspirated and the cells

were washed with PBS. The cells within each well were fixed with 0.1 mL solution of acetone:water 4:1 (v:v) for 15 min at room temperature. Then the solution was aspirated and the dried plates were kept at 4°C until assayed. The wells were washed three times with TST (0.05 M Tris; 0.85% NaCl; 0.05% Tween 20; pH 7.6). Then the cells were incubated 1 h at 37°C with anti-rickettsial guinea-pig high titre homologous sera (1:3000 in an immunofluorescence assay) diluted 1:100 (0.1 mL per well). Wells were washed three times with TST solution and anti-guinea-IgG:peroxidase conjugate (Sigma) diluted, was added (1:400, 0.05 mL per well). After similar incubation and four washing steps, ABTS reagent (Sigma) was added to the wells (0.05 mL per well). Following incubation of 20 min at room temperature, the plates were read at 405 nm. Wells containing sham-infected cells served as blanks.

Evaluation of antibiotic activity

Inhibition of rickettsial growth was studied in several concentrations of the studied antibiotics. Since all wells contained the same number of cells, rickettsial growth rates were derived from the average number of rickettsiae per cell or from the average ELISA values per well rickettsial counts and ELISA. Values from control wells (no antibiotics) and rickettsiae-infected cells treated with minocycline were considered as 0% and 100% inhibition rates, respectively.

Results and discussion

The growth of *R. conorii*, *R. typhi* and *C. burnetii* in Vero cells was inhibited by both antibiotics as shown by the two methods (Table). Rickettsial counts determinations showed that *R. conorii* and *R. typhi* multiplied over a 3-day period 18- and 17-fold, respectively, and *C. burnetii* multiplied 27-fold over a 6-day period. The presence of 10 mg/L minocycline in the medium inhibited rickettsial growth completely. Clarithromycin exerted a high anti-rickettsial activity against the tested species at a concentration of 1.0 mg/L. Azithromycin inhibited the growth of *R. conorii*, *R. typhi* and *C. burnetii* at concentrations of 1.0, 0.01 and 10.0 mg/L, respectively. The maximal inhibitory effect of the antibiotics studied was expressed over a range of two to three decimal dilutions of the antibiotics, except in the case of azithromycin and *C. burnetii*. Azithromycin inhibited the growth of *C. burnetii* to the extent of 56–81% (as measured by rickettsial counts) over a range of four decimal antibiotic dilutions (0.01 to 10 mg/L). The ELISA values in the parallel wells didn't show significant growth-inhibition (<30%), indicating that coxiellae produced specific antigens in the presence of azithromycin. These results suggest that azithromycin may be less effective than the other antibiotics in the treatment of patients with Q-fever.

Our results are in accordance with those obtained for other new-generation macrolides such as roxithromycin, josamycin and pristinamycin which inhibited *R. rickettsii* and *R. conorii* growth in tissue cultures in concentrations of 1, 1 and 2 mg/L, respectively (Raoult & Drancourt, 1991). In another study it was found that MIC of clarithromycin for three spotted-fever group rickettsiae ranged from 1–2 mg/L and for four *Coxiella* strains from 1–4 mg/L (Maurin & Raoult, 1993).

The effective concentrations of azithromycin and clarithromycin against rickettsiae in the cell-cultures system are the same as those achieved in humans after conventional dosages which are known to be therapeutic for other infections. Thus, the

Table The effect of azithromycin and clarithromycin on the growth of rickettsiae in Vero cells^a

mg/L	<i>R. conorii</i> ^b			Azithromycin			<i>C. burnetii</i> ^d			<i>R. typhi</i> ^c			Clarithromycin			<i>C. burnetii</i> ^d			
	count	ELISA	<i>R. typhi</i> ^c count	ELISA	<i>R. typhi</i> ^c count	ELISA	count	ELISA	<i>R. typhi</i> ^c count	ELISA	<i>R. conorii</i> ^b count	ELISA	count	ELISA	<i>R. typhi</i> ^c count	ELISA	count	ELISA	
0.0001			0 ± 4	11 ± 7					0 ± 0					0 ± 0					
0.001	3 ± 4	1 ± 1	32 ± 34	57 ± 20	24 ± 34	4 ± 1	0 ± 0	0 ± 0	7 ± 9	0 ± 0	0 ± 0	3 ± 4	0 ± 0	7 ± 9	3 ± 4	0 ± 0	0 ± 0	0 ± 0	0 ± 0
0.01	7 ± 9	3 ± 4	100 ± 0	100 ± 0	56 ± 11	1 ± 0	23 ± 32	14 ± 9	15 ± 1	14 ± 9	15 ± 1	2 ± 2	23 ± 32	15 ± 1	2 ± 2	23 ± 32	20 ± 6	23 ± 32	20 ± 6
0.1	22 ± 9	45 ± 72			58 ± 21	6 ± 1	89 ± 14	26 ± 32	96 ± 6	89 ± 14	26 ± 32	84 ± 4	76 ± 4	96 ± 6	84 ± 4	76 ± 4	53 ± 13	76 ± 4	53 ± 13
1.0	100 ± 0	92 ± 11			63 ± 18	0 ± 1	100 ± 0	86 ± 10	100 ± 0	100 ± 0	86 ± 10	90 ± 7	100 ± 3	100 ± 0	90 ± 7	100 ± 3	96 ± 30	100 ± 3	96 ± 30
10.0					81 ± 2	26 ± 3													

^aValues are percents of growth-inhibition as determined by rickettsial count or ELISA. Result expressed by average ± s.d.

^b*R. conorii* counts values were 1.5 ± 0.3 and 27.3 ± 3.2 rickettsial per cell on day 0 and day 3, respectively. Cells treated with minocycline contained 0.3 ± 0.2 rickettsiae per cell on day 3. ELISA values on day 3 were 0.25 ± 0.07 and 0.07 ± 0.03 for control infected cells and minocycline-treated infected cells, respectively.

^c*R. typhi* counts were 1.5 ± 0.6 and 26.6 ± 3.2 rickettsiae per cell on day 0 and day 3, respectively. Cells treated with minocycline contained 0.3 ± 0.1 rickettsiae per cell on day 3. ELISA values on day 3 were 0.33 ± 0.09 and 0.07 ± 0.03 for control infected cells and minocycline-treated infected cells, respectively.

^d*C. burnetii* counts values were 0.5 ± 0.2 and 13.7 ± 2.3 rickettsiae per cell on day 0 and day 6, respectively. Cells treated with minocycline contained 0.2 ± 0.1 rickettsiae per cell on day 6. ELISA values on day 6 were 0.24 ± 0.04 and 0.03 ± 0.02 for control infected cells and minocycline-treated infected cells, respectively.

demonstration of the anti-rickettsial activity in this study encourages their clinical evaluation.

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