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The activity of 14-hydroxy clarithromycin, alone and in combination with clarithromycin, against penicillin- and erythromycin-resistant *Streptococcus pneumoniae*

Steven J. Martin*, Cory G. Garvin, Christopher R. McBurney and Eric G. Sahloff

The Infectious Diseases Research Laboratory at The University of Toledo, College of Pharmacy, 2801 W. Bancroft Street, Toledo, OH 43606, USA

There are no data regarding the activity of clarithromycin's active metabolite, 14-hydroxy clarithromycin, against penicillin-intermediate, penicillin-resistant or erythromycin-resistant *Streptococcus pneumoniae*. Agar dilution MICs were determined for clarithromycin, 14-hydroxy clarithromycin (henceforth called 'metabolite'), azithromycin, erythromycin and clarithromycin/metabolite (2:1 and 1:1 ratio) against 24 penicillin-intermediate and 14 penicillin-resistant strains, including 13 erythromycin-resistant clinical strains and one ATCC strain of *S. pneumoniae*. The interaction between clarithromycin and its metabolite was determined using an agar chequerboard assay against all isolates, and time–kill tests were performed against five penicillin-intermediate (macrolide-susceptible) and five penicillin-resistant (two macrolide-resistant) strains of *S. pneumoniae* using all antibiotics alone at simulated peak serum concentrations, and clarithromycin/metabolite in a 2:1 ratio (physiological). MICs were as follows: clarithromycin, 0.008–>64 mg/L; metabolite, 0.015–>64 mg/L; erythromycin, 0.015–>64 mg/L; azithromycin, 0.125–>64 mg/L; clarithromycin/metabolite (1:1 and 2:1 combinations), 0.001–>64 mg/L. The MIC of the clarithromycin/metabolite combination was one or more tube dilution lower than the MIC of clarithromycin in 28 of the isolates tested. In chequerboard testing, 13 strains (seven erythromycin susceptible and six erythromycin resistant) demonstrated synergy, 18 additivity and seven indifference. In time–kill testing, bacterial eradication below detection limits occurred with clarithromycin and metabolite in seven of 10 organisms. The combination of parent and metabolite was more rapidly bactericidal than clarithromycin alone in six of the seven isolates ($P = 0.026$). The metabolite has potent activity against *S. pneumoniae* and enhances the activity of the parent compound against this organism. The metabolite's activity must be considered in evaluating clarithromycin *in vitro* to avoid underestimation of clarithromycin's activity against the pneumococcus.

Introduction

Macrolide resistance in *Streptococcus pneumoniae* is increasing.[1] Macrolide resistance can be mediated by ribosomal modification or active drug efflux.[2,3] The former mechanism involves the *erm*(AM) gene and the latter involves the *mef*(E) gene.[2,4] Ribosomal modification is associated with high-level resistance to macrolides, lincosamides and streptogramins (MLS_B-type resistance pattern), often producing MICs of >64–128 mg/L.[2,5] Efflux is associated with low-level resistance to 14- and 15-membered ring macrolides only (M-type resistance pattern).[3,5] Both resistance mechanisms are acquired determinants.[5] It is difficult to predict macrolide susceptibility solely by penicillin resistance.

Clarithromycin is extensively metabolized in the liver by oxidative and hydrolytic mechanisms.[6] An active metabolite, 14-hydroxy clarithromycin (henceforth called 'metabolite', accounts for approximately 20% of the parent drug's metabolism.[6] Neither parent nor metabolite is extensively bound to plasma proteins.[6] The active metabolite provides the majority of the compound's antibacterial effects against *Haemophilus influenzae*.[7] Although few data are available, it appears that the metabolite is also active against

*Corresponding author. Tel: +1-419-530-1964; Fax: +1-419-530-1950; E-mail: smartin2@pop3.utoledo.edu

penicillin-susceptible, erythromycin-susceptible *S. pneumoniae*.[7–11] Its activity against penicillin-intermediate, penicillin-resistant or erythromycin-resistant *S. pneumoniae* is not known.

The objective of this work was to characterize more fully the potency of the metabolite against penicillin- and erythromycin-resistant *S. pneumoniae*, and to investigate whether this metabolite has a role in the parent compound's activity against these organisms *in vitro*. We investigated the *in vitro* activity of the metabolite alone and in combination with clarithromycin against several clinical strains of penicillin-intermediate, penicillin-resistant and erythromycin-resistant *S. pneumoniae*. Interaction was tested by investigating combination MICs, agar dilution chequerboards and time–kill assays.

Materials and methods

The pneumococci tested included 38 clinical isolates and one ATCC control strain (*S. pneumoniae* 49619). These consisted of 24 penicillin-intermediate (MIC 0.1–1.0 mg/L) and 14 penicillin-resistant *S. pneumoniae* (MIC $\geqslant$ 2.0 mg/L). Twenty five of these isolates were erythromycin susceptible (MIC $\leqslant$ 0.25 mg/L) and 13 erythromycin resistant (MIC $\geqslant$ 1 mg/L). MICs of erythromycin, azithromycin and penicillin were also determined, and erythromycin and azithromycin were included in chequerboard and time–kill testing for comparison. Clarithromycin and metabolite were obtained from Abbott Laboratories (Abbott Park, IL, USA). Erythromycin, azithromycin and penicillin V potassium were obtained from the United States Pharmacopoeia (Rockville, MD, USA).

Agar dilution MICs were determined according to the standards of the NCCLS with incubation in 5% CO_2. Final antibiotic concentrations tested were 0.001–64 mg/L. *S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 29213 were included for quality control. All procedures were performed in duplicate. MICs were determined for each agent individually and for clarithromycin and metabolite in a 2:1 and 1:1 ratio.

Isolates that were not susceptible to erythromycin (MIC $\geqslant$ 1 mg/L) were screened for the presence of known MLS_B resistance genes by polymerase chain reaction (PCR) amplification with the following gene-specific primers: *erm*(AM): upper primer (5′ position 974), TCAACCAAATAAT AAAACAA, lower (3′ position 1311), AATCCTTCTT CAACAATCAG; *mef*(E): upper (5′ position 206), ATGCAGACCAAAAGCCACCAT, lower (3′ position 439), GCCATAGACAAGACCATCGC.[12] Crude lysates of the strains were prepared and stored at 4°C until tested. One microlitre of lysate was used in a 25 μL amplification reaction (PCR Supermix; Gibco-BRL, Bethesda, MD, USA). The presence of the *erm*(AM) and *mef*(E) genes was identified in 13 organisms.

Agar chequerboard testing was performed using the 25 erythromycin-susceptible and 13 erythromycin-resistant isolates by combining the parent compound and metabolite, each at concentrations of 0.001–64 mg/L.[13] Interaction between the two compounds was determined by calculating the fractional inhibitory concentration (FIC) index.[13] FIC indices were interpreted as follows: $\leqslant$0.5, synergy; >0.5–1, additive; >1–4, indifference; and >4, antagonism.[14]

Standardized time–kill assays were performed in a CO_2 environment using 10 representative pneumococcal strains (two erythromycin resistant). The macrolides were each tested alone at simulated physiological peak serum concentrations: clarithromycin, 2.6 mg/L; metabolite, 0.8 mg/L; azithromycin, 0.4 mg/L; and erythromycin, 2.0 mg/L.[15–17] Clarithromycin and metabolite were also tested in combination. All work was performed in duplicate. There was excellent correlation between duplicate colony counts, so results presented are mean values.

Results

Metabolite alone was as potent as the parent compound against *S. pneumoniae* (Tables I and II). For erythromycin-susceptible strains, the MIC_{90}s of clarithromycin, metabolite and erythromycin were 0.125 mg/L. The MIC_{90} of azithromycin was 0.5 mg/L. The MIC_{90}s of the clarithromycin/metabolite 1:1 and 2:1 ratio were 0.06 mg/L; this was one tube dilution lower than the MIC of the parent compound alone.

For 13 erythromycin-resistant strains, the MIC_{90}s of all the macrolides were >64 mg/L, but MIC_{90}s for organisms expressing the *mef*(E) gene (erythromycin, 8 mg/L; azithromycin, 32 mg/L; clarithromycin, 4 mg/L; metabolite, 8 mg/L; clarithromycin/metabolite 1:1, 4 mg/L; and clarithromycin/metabolite 2:1, 4 mg/L) were lower than MICs for those expressing the *erm*(AM) gene (>64 mg/L for all).

The clarithromycin/metabolite combination in a 2:1 ratio was more potent than clarithromycin (having a lower MIC) for 25 strains of pneumococcus tested (19 with a decrease of one tube dilution and six with a decrease of two or more tube dilutions). The combination in a 1:1 ratio was more potent than clarithromycin alone (having a lower MIC) for 28 strains of pneumococcus tested (17 with a decrease of one tube dilution and 11 with a decrease of two tube dilutions). In five of the 13 erythromycin-resistant isolates the clarithromycin MIC was reduced by one or more tube dilutions by combination with the metabolite (Table II).

Synergy was demonstrated against 13 strains and additive effects against 18 strains of *S. pneumoniae*. Against erythromycin-resistant organisms, three of eight *erm*(AM)-producing strains demonstrated synergy, as compared with three of five *mef*(E)-producing strains. Against erythromycin-susceptible isolates, seven of 25 strains demonstrated synergy, 16 additive effects and two indifference.

Table I. Agar dilution MICs and clarithromycin/metabolite chequerboard FICs for 25 erythromycin-susceptible strains of *S. pneumoniae*

	MIC (mg/L)							
					clarithromycin + metabolite			
Organism	erythromycin	azithromycin	clarithromycin	metabolite	1:1 ratio	2:1 ratio	penicillin	FIC[a]
P10	0.015	0.5	0.008	0.015	0.004	0.008	0.25	0.75
P11	0.06	0.25	0.06	0.06	0.03	0.03	2	0.75
C10	0.06	0.5	0.06	0.125	0.03	0.03	0.015	0.625
C11	0.06	0.5	0.06	0.125	0.03	0.03	0.015	0.75
C12	0.06	0.5	0.125	0.125	0.03	0.03	0.015	0.5
U10	0.06	0.125	0.125	0.125	0.03	0.06	1	0.75
C13	0.125	0.5	0.06	0.125	0.03	0.03	1	0.625
I10	0.125	0.5	0.06	0.125	0.03	0.03	0.25	0.75
P12	0.125	0.25	0.06	0.125	0.03	0.03	1	0.75
I11	0.125	0.5	0.125	0.125	0.03	0.03	1	0.5
C14	0.125	0.5	0.06	0.125	0.03	0.06	2	0.75
C15	0.125	0.125	0.06	0.125	0.03	0.06	0.125	0.75
T10	0.125	0.5	0.06	0.125	0.03	0.06	0.25	0.75
C16	0.125	0.5	0.06	0.125	0.03	0.06	0.06	1
I12	0.125	0.5	0.125	0.125	0.03	0.06	0.5	0.5
P13	0.125	0.25	0.125	0.125	0.03	0.06	2	0.5
P14	0.125	0.5	0.125	0.125	0.03	0.06	4	0.5
U11	0.125	0.5	0.125	0.125	0.03	0.06	1	0.5
I13	0.125	0.5	0.125	0.125	0.03	0.06	4	0.75
C17	0.125	0.25	0.125	0.125	0.06	0.06	2	0.75
I14	0.125	0.5	0.125	0.125	0.06	0.06	2	0.75
P15	0.125	0.5	0.125	0.125	0.06	0.06	0.125	0.75
P16	0.125	0.25	0.125	0.125	0.06	0.06	0.125	0.75
C18	0.125	0.5	0.125	0.125	0.06	0.06	2	1
I15	0.125	0.25	0.06	0.125	0.001	0.001	0.002	0.025

[a]Fractional inhibitory index for clarithromycin/metabolite chequerboard, calculated as $A/MIC_A + B/MIC_B$, where A is the concentration of drug A in a tube that is the lowest inhibitory concentration in its row; MIC_A is the MIC of drug A alone for the organism; B and MIC_B are the equivalent data for drug B.[10]

Representative time–kill curves for macrolide-resistant isolates are shown in Figures 1 and 2. For the macrolide-susceptible isolates (data not shown), clarithromycin and its 14-hydroxy metabolite alone and in combination were bactericidal and produced colony counts below the lower limit of detection at 24 h. Against the macrolide-resistant [*mef*(E)], penicillin-resistant isolate (Figure 1), clarithromycin and the combination of parent and metabolite reduced the colony count by approximately 2 log cfu/mL at 24 h, whereas growth in the presence of all other agents resembled that of the control. For the macrolide-resistant [*erm*(AM)], penicillin-intermediate *S. pneumoniae* (Figure 2), an initial decrease in colony counts was observed over the first 12 h in the presence of clarithromycin, its metabolite, the combination and erythromycin, but in all tubes there was regrowth above baseline at 24 h.

Clarithromycin and its metabolite completely eradicated seven of the 10 organisms (seven of the eight erythromycin-susceptible strains and neither of the two erythromycin-resistant ones). The time required for bactericidal activity to be manifested was determined for these seven isolates. The combination of parent and metabolite was more rapidly bactericidal than clarithromycin alone in six of the seven isolates ($P = 0.026$). Metabolite was more rapidly bactericidal than clarithromycin in five of the seven isolates, but the cumulative difference did not reach statistical significance ($P = 0.222$).

Discussion

We speculated that clarithromycin's active metabolite, 14-hydroxy clarithromycin, may have significant activity against penicillin- and erythromycin-intermediate and

Table II. Agar dilution MICs (mg/L) and clarithromycin/metabolite chequerboard FICs for 13 erythromycin-resistant strains of *S. pneumoniae* and controls

		MIC (mg/L)							
						clarithromycin + metabolite			
Organism[a]	Gene	erythromycin	azithromycin	clarithromycin	metabolite	1:1 ratio	2:1 ratio	penicillin	FIC
T-C	*mef*(E)	4	32[b]	4	4	0.5	0.5	2	0.06
U-A	*erm*(AM)	4	>64	2	2	2	1	0.25	0.06
T-B	*mef*(E)	4	16[b]	2	4	2	1	4	1
T-A	*mef*(E)	4	8[b]	2	4	2	2	2	2
P-A	*mef*(E)	4	16[b]	2	2	2	2	1	0.06
I-A	*mef*(E)	8	32[b]	4	8	4	4	4	0.5
I-C	*erm*(AM)	32	64	16	32	16	16	0.002	0.5
U-B	*erm*(AM)	>64	>64	>64	>64	16	16	1	0.375
I-B	*erm*(AM)	>64	>64	>64	>64	32	32	0.125	1
I-D	*erm*(AM)	>64	>64	>64	>64	>64	>64	0.125	2
P-B	*erm*(AM)	>64	>64	>64	>64	>64	>64	1	2
P-C	*erm*(AM)	>64	>64	>64	>64	>64	>64	4	2
C-A	*erm*(AM) and *mef*(E)	>64	>64[b]	>64	>64	>64	>64	4	2
ATCC 49619 *S. aureus*	N/A	0.12	0.25	0.12	0.12	0.06	0.06	0.5	N/A
ATCC 29213	N/A	0.5	1	0.25	0.25	0.125	0.25	0.5	N/A

[a]Macrolide-resistance gene detected by PCR.
[b]MICs in room air were: T-C, 8 mg/L; T-B, 8 mg/L; T-A, 4 mg/L; P-A, 4 mg/L; I-A, 8 mg/L; C-A, >64 mg/L.

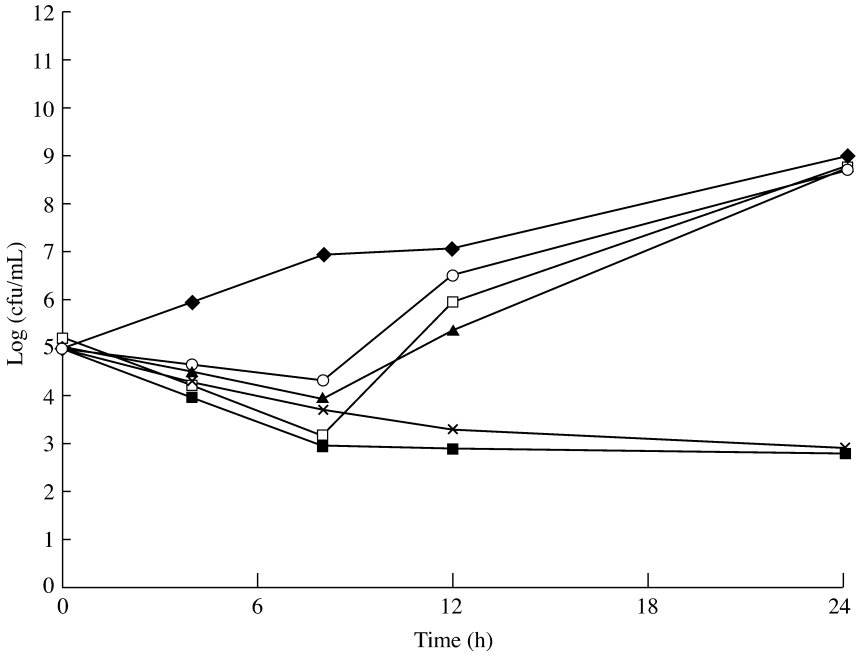

Figure 1. Time–kill curve for macrolide-resistant [*mef*(E)], penicillin-resistant *S. pneumoniae* (P–A). ◆, Control; ■, clarithromycin; ▲, metabolite; ×, clarithromycin/metabolite; □, erythromycin; ○, azithromycin.

-resistant pneumococci, and contribute to the parent drug's clinical effectiveness. There are no reports of *in vitro* activity of the metabolite against these pathogens. The few data describing the compound's activity against *S. pneumoniae* isolates suggest that its potency is equal to or greater than that of its parent compound.[7,9] Clarithromycin and its metabolite have demonstrated synergy in combination against other bacteria, and this interaction may be responsible for the seemingly few clinical failures of this drug against *S. pneumoniae* strains that are not susceptible to penicillin.[8,18,19]

In MIC testing, the MIC of clarithromycin was reduced in >70% of the isolates when clarithromycin was combined with metabolite in a serum physiological ratio. Jones *et al.*[18] demonstrated similar findings for the combination against *Legionella* spp. The results of agar chequerboard studies confirmed this interaction, as 31 of 38 isolates demonstrated synergy or additive effects. To investigate the pharmacodynamic nature of the interaction, we performed time–kill assays against a variety of penicillin-intermediate, -resistant and macrolide-resistant isolates. Simulated serum concentrations were chosen for testing to simulate bacteraemia *in vivo*. We chose a 2:1 ratio of parent to metabolite to allow comparison of our results with those of other investigators.[7,18] Chu *et al.*[15] demonstrated that in healthy young and elderly volunteers the ratio of parent to metabolite C_{max} and C_{min} serum concentrations after five doses of clarithromycin (500 mg bd) ranged from 2.46:1 to 3.65:1 and from 1.92:1 to 2.28:1, respectively. One would expect that higher concentrations of these agents, as might be present in epithelial lining fluid, would produce similar or greater activity against pneumococcal strains. In all but the high-level macrolide-resistant isolate tested [I-B, *erm*(AM)], the clarithromycin/metabolite combination was more potent than the parent compound. In seven of the 10 time–kill assays, bactericidal activity was demonstrated for all of the macrolides. Thus, macrolides may not be effective against all pneumococcal isolates, but *in vitro* MICs may not be an accurate predictor of pharmacodynamic effect.

The *erm* gene probably encodes high-level macrolide resistance; our work confirms that of others in this regard.[20] In time–kill testing against an *erm*(AM)-expressing strain, erythromycin, and clarithromycin and metabolite either alone or in combination reduced colony counts over the first 12 h of the assay. Clarithromycin alone and in combination with the metabolite produced an approximately 2 log cfu/mL reduction in the macrolide-resistant strain encoded by the *mef*(E) gene. Erythromycin and azithromycin appeared to have little activity against this strain. This efflux mechanism produces low- to moderate-level macrolide resistance, and organisms expressing this gene may not be uniformly affected by the macrolides.

We have demonstrated that 14-hydroxy clarithromycin has activity against *S. pneumoniae in vitro* and that it contributes to the activity of clarithromycin in MIC and time–kill studies. Further work on the activity of this combination against *mef*(E)- and *erm*(AM)-expressing macrolide-resistant pneumococci is warranted. This work supports the argument that the activity of active metabolites must be taken into consideration in determining the antimicrobial activity of parent drugs.

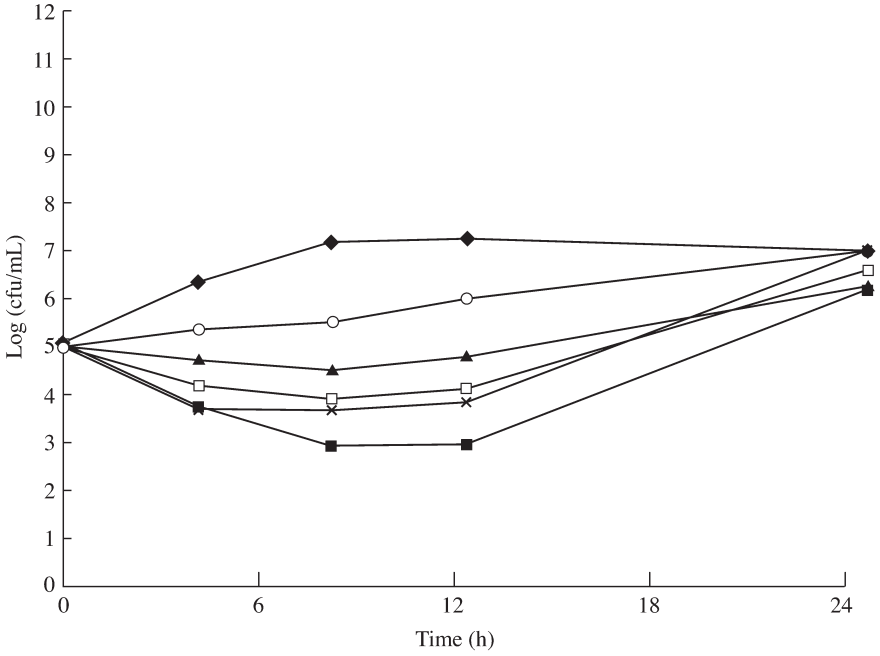

Figure 2. Time–kill curve for macrolide-resistant [*erm*(AM)], penicillin-intermediate *S. pneumoniae* (I-B). Symbols as in Figure 1.

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