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Enhancement of the In Vitro and In Vivo Activities of Clarithromycin against *Haemophilus influenzae* by 14-Hydroxy-Clarithromycin, Its Major Metabolite in Humans

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MICs of clarithromycin and its major human metabolite, 14-hydroxy-clarithromycin, for *Haemophilus influenzae* in combination were reduced two- to fourfold compared with the MICs of each compound alone. Serum reduced the MICs of the parent compound and metabolite two- to fourfold compared with the MICs in medium without serum. In serum spiked with clinically relevant concentrations of clarithromycin and 14-hydroxy-clarithromycin at a fixed ratio of 4:1, 15 of 16 strains (94%) were inhibited and killed by combinations containing 1.2 and 0.3 μg/ml, respectively. In time kill experiments, the combination of parent compound and metabolite at one-fourth and one-half of their individual MICs, respectively, reduced bacterial counts by >5 log CFU. The postantibiotic effect of clarithromycin combined with 14-hydroxy-clarithromycin was twice that of clarithromycin when tested alone. When orally administered to gerbils with *H. influenzae* otitis media, the 14-hydroxy metabolite was significantly more active than clarithromycin in reducing bacterial counts from the middle ear. The in vivo activity of the two compounds in combination was synergistic or additive, depending on the level of *H. influenzae* present at the time treatment was initiated. Significant reductions in bacterial counts and increases in cure rates were observed when clarithromycin at 50 or 100 mg/kg of body weight was combined with 14-hydroxy-clarithromycin at 12 mg/kg or higher. Results from in vitro and in vivo combinations suggest that routine susceptibility tests and animal efficacy studies with clarithromycin alone may underestimate its potential efficacy against *H. influenzae*.

Clarithromycin is an acid-stable 14-membered macrolide which achieves higher peak levels in human serum after oral dosing than does erythromycin and has a serum half-life which is twice that of erythromycin (L. T. Sennello, S.-Y. Chu, D. S. Wilson, K. S. Laws, S. T. Bunnell, L. L. Varga, and K. Snyder, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 419, 1986). The MICs of clarithromycin for organisms such as staphylococci, streptococci (including *Streptococcus pneumoniae* and *Streptococcus pyogenes*), *Listeria monocytogenes*, diphtheroids, and *Branhamella catarrhalis* are twofold lower than those of erythromycin, while the MICs of clarithromycin for *Legionella pneumophila* are generally fourfold lower (5, 11). The MICs of clarithromycin for *Haemophilus influenzae*, however, are generally twofold higher than those of erythromycin. After oral dosing in mouse protection tests, clarithromycin is 2 to 10 times more active than erythromycin against *S. pneumoniae*, *S. pyogenes*, and *Staphylococcus aureus* (5). In guinea pigs experimentally infected with *L. pneumophila*, clarithromycin is significantly more effective than erythromycin in reducing bacterial counts from lungs and spleens. In addition, the in vitro postantibiotic effects of clarithromycin for *S. pyogenes* and *Staphylococcus aureus* are three times those of erythromycin and correlate with results from mouse protection tests in which the optimal dosing intervals of clarithromycin were once a day compared with three times a day for erythromycin (8).

In humans, but not rodents, the major metabolite of clarithromycin is 14-hydroxy-clarithromycin (J. L. Ferrero, B. A. Bopp, K. C. Marsh, S. C. Quigley, M. K. Johnson, D. J. Anderson, J. E. Lamm, K. G. Tolman, S. W. Sanders, J. H. Cavanaugh, and R. C. Sonders, Drug Metab. Dispos., in press; T. Suwa, H. Yoshida, S. Yoshitomi, and K. Kamei, 26th ICAAC, abstr. no. 414, 1986). Following twice-daily dosing with 500 mg of clarithromycin, the peak concentrations of clarithromycin and 14-hydroxy-clarithromycin in sera of human volunteers were 2.4 and 0.66 μg/ml, respectively. 14-Hydroxy-clarithromycin is generally as active as clarithromycin in vitro against organisms implicated in community-acquired respiratory infections and is twofold more active than clarithromycin against *H. influenzae* (7, 11).

The presence of a major bioactive metabolite in humans may provide a therapeutic enhancement of antimicrobial activity over that predicted from MICs of clarithromycin alone or from mouse protection tests in which the active metabolite is not produced. Preliminary in vitro studies, in fact, have shown that in combination tests with clarithromycin and 14-hydroxy-clarithromycin, the MICs of the metabolite for *H. influenzae* are decreased 4- to 64-fold (P. B. Fernandes and L. A. Freiberg, 26th ICAAC, abstr. no. 408, 1986). In this report, we expand the preliminary observation and describe the individual and combined activities of clarithromycin and 14-hydroxy-clarithromycin against *H. influenzae* in vitro and in a gerbil model of otitis media.

MATERIALS AND METHODS

Bacterial strains. In vitro studies were conducted with 17 strains of *H. influenzae* that were received either from several hospitals in the United States (and now are part of the Abbott culture collection) or from the American Type

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Culture Collection, Rockville, Md. Eight of these strains were capsular serotype b (three β-lactamase positive), and nine strains were non-type b (one β-lactamase positive). All β-lactamase-negative strains were susceptible to ampicillin. In vivo studies were also conducted with *H. influenzae* ATCC 43095, which is non-type b and β-lactamase negative. All strains were identified by standard procedures and maintained frozen at −60°C. β-Lactamase production was determined by using nitrocefin as a chromogenic substrate. The capsular serotype was determined as b or non-b by agglutination with specific antisera (Difco Laboratories, Detroit, Mich.).

Antibacterial agents. Clarithromycin, 14-hydroxy-clarithromycin, erythromycin, and azithromycin were prepared at Abbott Laboratories, Abbott Park, Ill. Ampicillin was purchased from Parke-Davis, Morris Plains, N.J. Stock solutions of clarithromycin for in vitro tests were prepared by dissolving 1 mg of compound in 140 μl of methanol and bringing the solution to volume in 0.1 M phosphate buffer (pH 6.8); stock solutions of other macrolides were prepared in methanol. For in vivo tests, clarithromycin and 14-hydroxy-clarithromycin were prepared in phosphate-buffered saline (pH 6.8) and the other macrolides were prepared in phosphate-buffered saline (pH 7.2). Ampicillin was dissolved in sterile water.

Inoculum preparation for susceptibility tests. Inocula for all in vitro tests were prepared by suspending colonies from aerobically incubated 20- to 24-h chocolate agar cultures into sterile saline. Suspensions of organisms in saline were adjusted to match the turbidity of a 0.5 McFarland standard by using an A-just turbidity meter (Abbott Diagnostics, Irving, Tex.) (1, 6). Adjusted bacterial suspensions were further diluted for in vitro tests as indicated.

MIC determinations. MICs were determined by the twofold broth microdilution method described by the National Committee for Clinical Laboratory Standards (16) in haemophilus test medium (HTM) (13) and HTM supplemented with 50% (vol/vol) heat-inactivated (56°C, 30 min) pooled human serum (serum-HTM) (pH 7.3). Bacterial suspensions adjusted to a 0.5 McFarland standard were further diluted in growth media to deliver 5×10^5 CFU/ml in microdilution tests.

The MICs of clarithromycin and 14-hydroxy-clarithromycin were also separately determined by a broth microdilution two-dimensional checkerboard technique to assess activities at concentrations between standard twofold dilutions (14). The two-dimensional dilution of a compound in microtiter plates produced a series of twofold concentrations ranging from 0.125 to 16 μg/ml. In addition, a nonexponential series of concentrations was obtained from the two-dimensional dilutions, in which the twofold concentrations were increased by 0.03 μg/ml and its doubling increments. HTM and serum-HTM were used as media for these tests. All tests were incubated in ambient air at 35°C for 20 to 24 h.

In vitro combination tests. The effect of combining clarithromycin and 14-hydroxy-clarithromycin in vitro against *H. influenzae* was determined by using a microdilution checkerboard technique (14). HTM and serum-HTM were used as media for these tests. Bacterial suspensions adjusted to a 0.5 McFarland standard were further diluted in growth medium to deliver 5×10^5 CFU/ml. All tests were incubated in ambient air at 35°C for 20 to 24 h.

Fractional inhibitory concentrations (FICs) were calculated for each agent by dividing the MIC of the compound in combination by the MIC of the compound alone. The FIC index is the sum of the FICs of the individual compounds at the most effective concentrations and was used to define the following drug interactions: synergism (FIC index, ≤ 0.5) and antagonism (FIC index, ≥ 4.0).

The effect of combining clarithromycin and 14-hydroxy-clarithromycin against *H. influenzae* Dillard (type b and β-lactamase positive) was also determined by the time kill curve technique in brain heart infusion broth supplemented with 5% Fildes enrichment (Difco Laboratories). Bacterial suspensions adjusted to a 0.5 McFarland standard were further diluted in growth medium and added to 50-ml flasks containing 10 ml of broth to yield final cell densities of approximately 5×10^5 CFU/ml. Individual agents were added to separate flasks before inoculation to achieve final concentrations equal to the MIC, 0.5 times the MIC, and 0.25 times the MIC; combinations of the two agents at these concentrations were also tested. Separate flasks without compound served as controls for growth. Flasks were incubated at 37°C with rotary shaking. At designated time intervals, portions (0.5 ml) of cultures were aseptically removed, serially diluted in 10-fold increments in saline, and plated (0.1 ml) onto drug-free chocolate agar (BBL Microbiology Systems, Cockeysville, Md.). To eliminate the effects of drug carry-over, a minimum dilution of 1:100 was used through 6 h. At 24 h, 0.1-ml samples of test cultures were diluted 1:10 and plated without further dilution to detect viable counts of $<10^3$ CFU/ml. Plates were incubated in ambient air at 35°C for 24 h.

Modified serum bactericidal tests. The activities of clarithromycin and 14-hydroxy-clarithromycin against *H. influenzae* at fixed ratios in 50% (vol/vol) heat-inactivated (56°C, 30 min) pooled human serum (pH 7.3) were determined by a microdilution method analogous to the serum bactericidal test (17). Serum was spiked with the two agents such that after twofold dilution in serum and inoculation with test organisms in HTM broth, the final composition of the medium was 50% serum-HTM broth. Cell densities in these tests were confirmed by plating serial dilutions of inocula onto chocolate agar. After incubation for 20 to 24 h, duplicate 10-μl samples from wells containing the MIC, one-half the MIC, and multiples of the MIC were plated by being spread onto chocolate agar and were incubated overnight. Minimum concentrations which killed 99.9% of the original inoculum, i.e., MBCs, were determined as previously described (19).

The fixed ratios (and highest concentrations) of clarithromycin and 14-hydroxy-clarithromycin in these tests were 4:1 (4.8 and 1.2 μg/ml, respectively), 3:1 (4.5 and 1.5 μg/ml), and 2:1 (4.0 and 2.0 μg/ml). When diluted twofold, these concentrations represent approximations of the maximum concentrations in serum of clarithromycin (2.4 μg/ml) and 14-hydroxy-clarithromycin (0.66 μg/ml) at steady state in human serum following 500 mg twice daily oral dosing with clarithromycin (S.-Y. Chu, L. T. Sennello, S. T. Bunnell, L. L. Varga, D. S. Wilson, R. L. Deaton, K. E. Rice, S. D. Gupta, M. J. Klepper, D. M. Moyse, and K. Tolman, 28th ICAAC, abstr. no. 136, 1988).

Postantibiotic effect. Suspensions of *H. influenzae* adjusted to match the turbidity of a McFarland 0.5 standard were diluted in prewarmed HTM broth to yield approximately 10^6 CFU/ml. Clarithromycin, 14-hydroxy-clarithromycin, and erythromycin were added to separate culture flasks at their respective MICs; clarithromycin and 14-hydroxy-clarithromycin were also tested in combination at their respective MICs. A culture without drug served as a control for growth. The flasks were incubated for 2 h at 37°C with rotary shaking; viable bacterium counts were determined at 1-h

intervals by plating dilutions of the cultures onto drug-free chocolate agar. After 2 h of incubation, the cultures were diluted 1:100 into fresh prewarmed broth without antibiotic and reincubated (3). Viable counts were determined at 1-h intervals.

Experimental otitis media. A 5-h log-phase culture of *H. influenzae* ATCC 43095 was prepared in brain heart infusion broth (BBL) supplemented with 4% Fildes enrichment (BBL) and 0.001% NAD (Sigma Chemical Co., St. Louis, Mo.). Female Mongolian gerbils weighing 40 to 50 g (Tumblebrook Farms, West Brookfield, Mass.) were anesthetized with ether and injected percutaneously in the superior posterior chamber of the left middle-ear bulla with a 0.02-ml inoculum containing approximately 10^6 bacteria (9). Immediately prior to treatment, middle-ear aspirates from five gerbils were obtained as described below and cultured to determine the levels of infection at the onset of therapy. Antibiotics were administered by gavage in a 0.5-ml volume at 17 h postinfection and then three times daily for 2 days to groups of five gerbils. Eighteen hours after the final treatment, gerbils were euthanized with T-61 euthanasia solution (Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.), and 0.02 ml of brain heart infusion broth was injected into the middle-ear bulla through the tympanic membrane. Middle-ear aspirates were collected before the needle was removed. The aspirates were diluted in brain heart infusion broth and plated in duplicate onto chocolate agar (BBL). Colonies were counted with a colony counter (Artek Systems Corp., Farmingdale, N.Y.) after overnight incubation. Mean log CFU per middle ear were determined for each treatment group. The minimum number of bacteria detectable by this method was 100 CFU. Bacterial counts from the treated animals were compared with those from untreated controls. Gerbils were considered cured of infection if no bacteria were recovered from undiluted middle-ear aspirates.

A one-way (fixed-effects) analysis of variance model was used to compare the mean log CFU values in treated and untreated gerbils. The Fisher exact test was used to compare the cure rate of each treatment group with that of controls.

Response surface methodology was employed to evaluate the efficacy of the clarithromycin-14-hydroxy-clarithromycin combinations (4, 15). For this analysis, both mean log CFU and cure rate were used as measures of in vivo potency. Linear and logistic regression techniques were used in the analysis of the response surface for mean log CFU and cure rate, respectively. A full quadratic model was used in each of these analyses to assess the underlying relationship between the two test compounds. In particular, the type of response exhibited by combinations of the two test compounds (e.g., synergistic or antagonistic) was determined from a statistical test of the quadratic term in the model.

Pharmacokinetics. Concentrations of clarithromycin and 14-hydroxy-clarithromycin in sera and middle-ear fluids of *H. influenzae*-infected gerbils were determined by high-pressure liquid chromatography. Gerbils received a single oral dose containing 100 mg of clarithromycin per kg of body weight and 12 mg of 14-hydroxy-clarithromycin per kg. Sera and middle-ear aspirates were collected at 1, 3, 6, 8, and 24 h after drug administration. Samples were pooled from groups of six gerbils, and antibiotic concentrations were determined by high-pressure liquid chromatography methods reported previously (S.-Y. Chu and L. T. Sennello, Program Abstr. 4th Jpn.-Am. Conf. Pharm. Biopharm., abstr. no. 023, 1988).

TABLE 1. MICs of clarithromycin and 14-hydroxy-clarithromycin for *H. influenzae*

Medium (no. of strains), dilution, and compound	MIC (μg/ml)			
	Range	50%[a]	90%	Geometric mean
HTM (17)				
Twofold				
Clarithromycin	2–8	4	8	3.54
14-Hydroxy-clarithromycin	1–8	2	4	1.92
Non-twofold				
Clarithromycin	1.5–8	2.5	4	2.86
14-Hydroxy-clarithromycin	0.75–4	1.5	4	1.64
50% serum-HTM (16)				
Twofold				
Clarithromycin	0.5–4	2	4	1.76
14-Hydroxy-clarithromycin	0.25–2	1	2	0.96
Non-twofold				
Clarithromycin	0.31–4	2	2.5	1.43
14-Hydroxy-clarithromycin	0.25–2	1	2	0.81

[a] 50%, MIC for 50% of the strains tested.

RESULTS

MIC determinations. The MICs of clarithromycin and its metabolite for 17 strains of *H. influenzae* are presented in Table 1. 14-Hydroxy-clarithromycin was twofold more active than clarithromycin against these strains. The MICs for 90% of the strains tested (MIC_{90}s) of clarithromycin and 14-hydroxy-clarithromycin as determined by standard twofold dilution method in HTM were 8 and 4 μg/ml, respectively. Geometric mean MICs of each compound when determined in serum-HTM were twofold lower than those determined in HTM without serum.

The mean MICs of clarithromycin and 14-hydroxy-clarithromycin determined by non-twofold dilution methods were 0.2 to 0.7 μg/ml lower than the MICs determined by standard twofold dilution methods.

Combined in vitro activities. MICs of clarithromycin and 14-hydroxy-clarithromycin at their most active combinations in HTM broth were determined. For 11 of 17 strains, the MICs of one of the compounds in the combination were reduced at least fourfold while the MICs of the other compound were reduced twofold, i.e., the FIC indices were ≤0.75, compared with MICs of each compound determined alone. For 6 of 17 strains, the MICs of each compound in combination were reduced twofold, i.e., the FIC indices were 1.0. The MIC_{90}s of clarithromycin and 14-hydroxy-clarithromycin at their most active combinations were 2 μg/ml.

The combined activities of clarithromycin and 14-hydroxy-clarithromycin in serum-HTM were also determined. For 7 of 16 strains, the MICs of one of the compounds in the combination were reduced at least fourfold while the MICs of the other compound were reduced twofold, and for 8 of 16 strains, the MICs of each compound in the combination were reduced twofold. For one strain, the MICs of the two compounds in combination were unchanged from the MICs determined alone. The MIC_{90}s of clarithromycin and 14-hydroxy-clarithromycin at their most active combinations were 1 and 0.5 μg/ml, respectively.

In time kill experiments after 24 h of incubation, cultures of *H. influenzae* Dillard exposed to either compound alone at 0.5 or 0.25 times their MICs had increased approximately 3 log CFU. The viability of a culture exposed to a combination of clarithromycin and 14-hydroxy-clarithromycin at 0.25 and

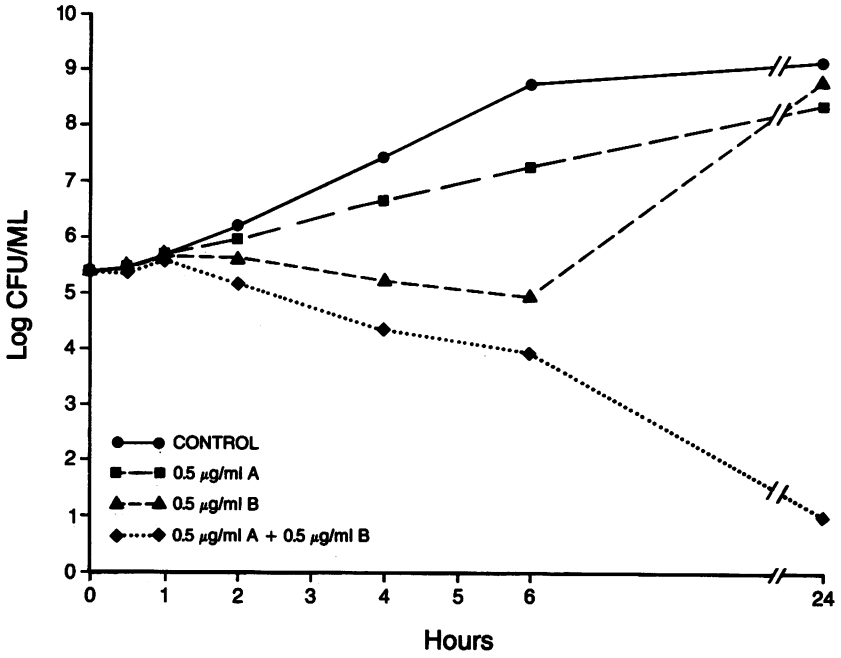

FIG. 1. Effect of combining clarithromycin (A) with 14-hydroxy-clarithromycin (B) on viability of *H. influenzae* Dillard.

0.5 times the individual MICs, respectively, was reduced >5 log CFU after 24 h of incubation (Fig. 1). This combination was bactericidal and synergistic. Combinations of clarithromycin and its metabolite at 0.5 plus 0.5 and 0.5 plus 0.25 times the individual MICs, respectively, produced similar reductions in bacterial counts (data not shown). A combination of the two compounds at 0.25 times their individual MICs was not bactericidal for this strain.

Modified serum bactericidal tests. The combined activities of clarithromycin and 14-hydroxy-clarithromycin in serum-HTM spiked with these compounds at a fixed ratio of 4:1 (highest concentrations, 4.8 and 1.2 μg/ml, respectively) are presented in Table 2. Cell densities in these tests ranged from 6×10^5 to 1×10^6 CFU/ml. For 7 of 16 strains, the combination MICs of both agents were reduced at least twofold compared with the MICs determined alone. For 9 of 16 strains, the combination MICs of the metabolite were reduced at least twofold while that of the parent compound was unchanged. The MIC_{90}s of clarithromycin and 14-hydroxy-clarithromycin in these combination tests were 1.2 and 0.3 μg/ml, respectively. In these tests, the MBCs of clarithromycin and 14-hydroxy-clarithromycin in combination were equal to (in 14 of 16 strains) or twofold higher than (in 2 of 16 strains) their MICs. Similar results were observed in serum-HTM for combinations of clarithromycin and 14-hydroxy-clarithromycin with fixed ratios of 3:1 and 2:1 (data not shown).

Postantibiotic effect. The time required for the density of drug-free control cultures of *H. influenzae* Dillard to increase 1 log CFU after a 1:100 dilution was 2 h. The times required for cultures exposed (for 2 h) to MICs of clarithromycin (2 μg/ml), 14-hydroxy-clarithromycin (1 μg/ml), and erythromycin (2 μg/ml) to increase 1 log CFU after dilution were 2.9, 2.9, and 2.8 h, respectively. The postantibiotic effects of clarithromycin, 14-hydroxy-clarithromycin, and erythromycin when tested alone were, therefore, 0.9, 0.9, and 0.8 h, respectively. The postantibiotic effects of clarithromycin and 14-hydroxy-clarithromycin when tested in combination at their MICs was 1.8 h.

In vivo efficacy. Efficacies of 14-hydroxy-clarithromycin, clarithromycin, and ampicillin administered to experimentally infected gerbils are shown in Table 3. 14-Hydroxy-clarithromycin was significantly more active than clarithromycin in reducing bacterial counts. *H. influenzae* ATCC 43095 was undetectable in middle-ear aspirates of gerbils receiving 100 mg of 14-hydroxy-clarithromycin per kg three times per day, while bacterial counts in gerbils receiving the same dose of clarithromycin were not significantly different

TABLE 2. Activities of clarithromycin and 14-hydroxy-clarithromycin at a fixed ratio of 4:1 in serum-HTM against 16 strains of *H. influenzae*

Compound	MIC (μg/ml)						MBC[a] (μg/ml)					
	Alone			In combination			Alone			In combination		
	50%[b]	90%	GM[c]	50%	90%	GM	50%	90%	GM	50%	90%	GM
Clarithromycin	1.2	2.4	1.30	1.2	1.2	0.97	1.2	4.8	1.43	1.2	1.2	1.05
14-Hydroxy-clarithromycin	0.6	1.2	0.71	0.3	0.3	0.24	0.6	1.2	1.05	0.3	0.3	0.26

[a] 50% and 90%, MBC for 50 and 90% of strains tested, respectively.
[b] 50%, MIC for 50% of strains tested.
[c] GM, Geometric mean.

TABLE 3. Efficacies of 14-hydroxy-clarithromycin, clarithromycin, and ampicillin in treating *H. influenzae* otitis media in gerbils[a]

Antibiotic	MIC (μg/ml)	Dose[b] (mg/kg)	Mean log CFU/ear ± SD	Mean log reduction in CFU	% of gerbils cured
14-Hydroxy-clarithromycin	4	100.0	0.00 ± 0.00	7.48[c]	100[c]
		25.0	7.08 ± 0.48	0.40	0
		6.2	7.38 ± 0.32	0.10	0
Clarithromycin	8	100.0	7.45 ± 0.31	0.03	0
Ampicillin	0.25	33.3	0.45 ± 0.90	7.48[c]	100[c]
		11.0	7.48 ± 0.10	0.00	0
		3.7	7.53 ± 0.17	−0.05	0
Untreated controls		0	7.48 ± 0.82		0

[a] Middle ears of infected control gerbils contained 4.6 log CFU at the onset of treatment.
[b] Antibiotics were administered orally three times daily for 2 days.
[c] Significantly different from result for untreated controls at $P = 0.05$.

from those of untreated control animals. 14-Hydroxy-clarithromycin was less active than ampicillin. In a separate experiment (data not shown), erythromycin at oral doses up to 100 mg/kg was not effective in reducing bacterial counts from the middle ears of gerbils with a mean of 5.55 log CFU at the onset of treatment.

Data from combination studies are shown in Tables 4 and 5. In the first experiment, untreated control gerbils had a mean of 4.6 log CFU of bacteria per middle ear at the onset of treatment and a mean of 5.28 log CFU at the time of sacrifice (Table 4). Oral treatment with 50 or 100 mg of clarithromycin per kg reduced bacterial counts by a mean of 3.26 and 1.68 log CFU, respectively; this difference was not statistically significant. Therapy with 32 and 24 mg of 14-hydroxy-clarithromycin per kg lowered bacterial counts by a mean of 4.26 and 4.00 log CFU, respectively, with both groups having an 80% cure rate. Lower doses of 14-hydroxy-clarithromycin did not produce statistically significant reductions in bacterial counts compared with counts for control gerbils. Combinations containing 100 mg of clarithromycin per kg and 16, 24, or 32 mg of 14-hydroxy-clarithromycin per kg reduced bacterial counts to undetectable levels in middle-ear aspirates from all gerbils (100% cure) and were as effective as either azithromycin (MIC, 1 μg/ml) or ampicillin at 32 mg/kg. Combinations of 14-hydroxy-clarithromycin with 50 mg of clarithromycin per kg appeared additive, as determined by reductions in bacterial counts and cure rates. Isobolograms obtained from the response surface analysis of results in Table 4 confirmed that the combination of clarithromycin and 14-hydroxy-clarithromycin was additive.

In the second combination experiment, untreated control gerbils had a 10-fold greater number of bacteria (mean of 5.6 log CFU) per middle ear at the onset of treatment and a mean of 7.12 log CFU at sacrifice (Table 5). Clarithromycin and 14-hydroxy-clarithromycin were ineffective as single agents when measured by CFU reduction or cure rate. In five of six combinations, however, significant reductions in CFU were seen. The maximum effect seen in this experiment, a mean 5.98 log reduction in CFU, was observed when 32 mg of metabolite per kg was combined with 100 mg of parent compound per kg. Mean 3.10 and 2.88 log CFU reductions in bacterial counts were observed when 16 mg of metabolite per kg was coadministered with 50 or 100 mg, respectively, of parent compound per kg. Isobolograms for results in Table 5 indicated that the combination of clarithromycin and 14-hydroxy-clarithromycin was synergistic. In a separate experiment (data not shown), bacterial counts from infected middle ears were reduced by a mean of 3.09 log CFU (significant at $P = 0.05$) when 12 mg of metabolite per kg was coadministered with 100 mg of parent compound per kg, but neither agent alone at these levels produced a significant reduction in bacterial counts.

TABLE 4. Efficacies of 14-hydroxy-clarithromycin alone and in combination with clarithromycin in treating *H. influenzae* otitis media in gerbils[a]

Antibiotic(s)[b]	Dose[c] (mg/kg)	Mean log CFU/ear ± SD	Mean log reduction in CFU	% of gerbils cured
14-OH	32	1.02[d] ± 2.28	4.26	80[d]
Clari	100	3.60 ± 2.25	1.68	20
14-OH–Clari	32/100	0.00[d] ± 0.00	5.28	100[d]
14-OH	24	1.28[d] ± 2.86	4.00	80[d]
Clari	100	3.60 ± 2.25	1.68	20
14-OH–Clari	24/100	0.00[d] ± 0.00	5.28	100[d]
14-OH	16	4.28 ± 3.32	1.00	20
Clari	100	3.60 ± 2.25	1.68	20
14-OH–Clari	16/100	0.00[d] ± 0.00	5.28	100[d]
14-OH	8	2.80 ± 2.59	2.48	20
Clari	100	3.60 ± 2.25	1.68	20
14-OH–Clari	8/100	2.64 ± 2.41	2.64	40
14-OH	32	1.02[d] ± 2.28	4.26	80[d]
Clari	50	2.02[d] ± 2.39	3.26	40
14-OH–Clari	32/50	0.00[d] ± 0.00	5.28	100[d]
14-OH	24	1.28 ± 2.86	4.00	80[d]
Clari	50	2.02[d] ± 2.39	3.26	40
14-OH–Clari	24/50	0.00[d] ± 0.00	5.28	100[d]
14-OH	16	4.28 ± 3.32	1.00	20
Clari	50	2.02[d] ± 2.39	3.26	40
14-OH–Clari	16/50	1.14[d] ± 2.55	4.14	80[d]
14-OH	8	2.80 ± 2.59	2.48	20
Clari	50	2.02[d] ± 2.39	3.26	40
14-OH–Clari	8/50	3.42 ± 2.92	1.86	20
Azithromycin	32	1.10[d] ± 2.46	4.18	80[d]
	16	2.96 ± 2.75	2.32	40
	8	4.38 ± 2.49	0.90	20
Ampicillin	32	0.00[d] ± 0.00	5.28	100[d]
	16	3.22 ± 2.95	2.06	40
	8	5.90 ± 0.70	−0.62	0
None (controls)	0	5.28 ± 1.66		0

[a] Middle ears of infected control gerbils contained 4.6 log CFU at the onset of treatment.
[b] 14-OH, 14-Hydroxy-clarithromycin; Clari, clarithromycin.
[c] Antibiotics were administered orally three times daily for 2 days.
[d] Significantly different from result for untreated controls at $P = 0.05$.

Pharmacokinetics in gerbils. After a single oral dose containing 100 mg of clarithromycin per kg and 12 mg of 14-hydroxy-clarithromycin per kg, the maximum concentration of clarithromycin in serum was 2.09 μg/ml while that of 14-hydroxy-clarithromycin was 0.76 μg/ml (Table 6). The maximum concentrations of clarithromycin and 14-hydroxy-clarithromycin in middle-ear aspirates were 1.16 and 0.25 μg/ml, respectively. The time to peak concentrations for both compounds was approximately 3 h.

TABLE 5. Efficacies of 14-hydroxy-clarithromycin alone and in combination with clarithromycin in treating *H. influenzae* otitis media in gerbils[a]

Antibiotic(s)[b]	Dose[c] (mg/kg)	Mean log CFU/ear ± SD	Mean log reduction in CFU	% of gerbils cured
14-OH	32	7.28 ± 0.11	−0.16	0
Clari	100	7.05 ± 0.44	0.07	0
14-OH–Clari	32/100	1.14[d] ± 1.73	5.98	60
14-OH	16	7.30 ± 0.14	−0.18	0
Clari	100	7.05 ± 0.44	0.07	0
14-OH–Clari	16/100	4.24[d] ± 3.87	2.88	40
14-OH	8	7.40 ± 0.12	−0.28	0
Clari	100	7.05 ± 0.44	0.07	0
14-OH–Clari	8/100	7.06 ± 0.72	0.06	0
14-OH	32	7.28 ± 0.11	−0.16	0
Clari	50	7.10 ± 0.31	0.02	0
14-OH–Clari	32/50	3.78[d] ± 3.50	3.34	40
14-OH	16	7.30 ± 0.14	−0.18	0
Clari	50	7.10 ± 0.31	0.02	0
14-OH–Clari	16/50	4.02[d] ± 3.67	3.10	40
14-OH	8	7.40 ± 0.12	−0.28	0
Clari	50	7.10 ± 0.31	0.02	0
14-OH–Clari	8/50	4.56[d] ± 2.22	2.56	0
Ampicillin	33	0.00[d] ± 0.00	7.12	100[d]
	11	3.38[d] ± 3.21	3.74	20
	3.7	7.42 ± 0.18	−0.30	0
None (controls)	0	7.12 ± 0.29		0

[a] Middle ears of infected control gerbils contained 5.6 log CFU at the onset of treatment.
[b] 14-OH, 14-Hydroxy-clarithromycin; Clari, clarithromycin.
[c] Antibiotics were administered orally three times daily for 2 days.
[d] Significantly different from result for untreated controls at $P = 0.05$.

DISCUSSION

The significance of metabolites of antimicrobial agents in effecting clinical cures has been recently reviewed (12, 18). The antimicrobial activities of metabolites alone and in combination with the parent compounds in some cases have been shown to have biological significance. In the case of metabolites with antimicrobial activity, routine in vitro susceptibility determinations which test only parental compounds can underestimate the potential efficacy of a drug.

TABLE 6. Pharmacokinetics of clarithromycin and 14-hydroxy-clarithromycin after a single oral dose of 100 mg of clarithromycin and 12 mg of 14-hydroxy-clarithromycin per kg

Site	Compound[a]	C_{max}[b] (μg/ml)	T_{max}[c] (h)	$t_{1/2}$[d] (h)	AUC[e] (μg · h/ml)
Serum	Clari	2.09	3	1.5	9.08
	14-OH	0.76	3	1.1	3.28
Middle ear	Clari	1.16	3	1.7	5.72
	14-OH	0.25	3	1.4	1.29

[a] Clari, Clarithromycin; 14-OH, 14-hydroxy-clarithromycin.
[b] Peak concentration of drug.
[c] Time to peak concentration.
[d] Estimated elimination half-life.
[e] Area under the curve.

Alternatively, routine in vitro susceptibility tests can overestimate the potential efficacy of a drug that is significantly metabolized to an inactive compound in vivo.

It has been documented that MICs of clarithromycin for *H. influenzae* are method and technique dependent (1, 6). Depending on the growth medium, method for inoculum preparation, and conditions of incubation, MIC_{90}s of clarithromycin vary from 2 to 16 μg/ml. In a collaborative study (1), the MIC_{90} of clarithromycin for *H. influenzae* in HTM broth was 8 μg/ml, which is in agreement with the present study. According to proposed interpretive criteria for dilution susceptibility tests with clarithromycin (2, 10), *H. influenzae* could be susceptible (MIC, ≤2 μg/ml), moderately susceptible (MIC, 4 μg/ml), or resistant (MIC, ≥8 μg/ml) to clarithromycin.

In the present study, we have shown that the MIC_{90} of 14-hydroxy-clarithromycin in HTM was twofold lower than that of clarithromycin and that the mean MICs of both compounds were reduced twofold in the presence of serum (Table 1). These data confirm the greater potency of the metabolite against *H. influenzae* previously reported (7, 11) and suggest that standard susceptibility tests in HTM may underestimate the potential efficacy of clarithromycin because of the activity of the metabolite and the effect of serum.

Since clarithromycin is not metabolized to 14-hydroxy-clarithromycin in gerbils, it was necessary to administer 14-hydroxy-clarithromycin to these animals to determine its activity. When administered separately to gerbils with *H. influenzae* otitis media, the 14-hydroxy metabolite was significantly more effective than the parent compound (Table 3). This observation can be explained in part by the greater in vitro potency of 14-hydroxy-clarithromycin against this organism. Other studies have shown that 14-hydroxy-clarithromycin is more effective than clarithromycin in treating *H. influenzae* pneumonia in mice (E. Azoulay-Dupuis, personal communication).

In vivo combination results have confirmed in vitro observations and further characterized the enhanced activity of the parent compound when the compound was combined with its major human metabolite. The combinations employed in the present study were designed to evaluate therapeutically relevant doses of the parent compound and its metabolite. Pharmacokinetic studies with gerbils receiving 100 mg of clarithromycin and 12 mg of 14-hydroxy-clarithromycin per kg (Table 6) demonstrated that peak levels of both compounds in serum, i.e., 2.09 μg/ml for clarithromycin and 0.76 μg/ml for 14-hydroxy-clarithromycin, were within range of concentrations achievable in humans. Significant reductions in bacterial counts occurred when clarithromycin at 50 or 100 mg/kg was combined with 14-hydroxy-clarithromycin at 12 mg/kg or higher. In these studies, the in vivo activity of the two compounds in combination was greater than the activity of either alone, as measured by reduction in bacterial counts or increase in cure rates. By using isobolograms obtained from a response surface analysis, the interaction between the two compounds was characterized as synergistic in one experiment and additive in another. The difference between these two experiments was in the level of *H. influenzae* infection at the onset of treatment. These observations of an inoculum effect in vivo confirm in vitro observations (C. Thornsberry, Antimicrob. Newsl. 2:62, 1985).

Results from this study suggest that routine in vitro susceptibility tests and animal efficacy studies with clarithromycin alone may underestimate the potential efficacy of

clarithromycin against *H. influenzae* in humans. The influence of a more active metabolite and additive or synergistic interactions between this metabolite and the parent compound might enhance the efficacy of the parent compound. In vitro combination tests with the parent compound and metabolite in the presence of serum and at ratios selected to mimic human pharmacokinetics may more accurately predict the efficacy of clarithromycin against *H. influenzae* in vivo than do tests which use standard methods with the parent compound alone. In addition, the bactericidal activity of clarithromycin and 14-hydroxy-clarithromycin when combined at levels achievable in humans suggests that the combination may be bactericidal in vivo. These factors could explain how *H. influenzae* which is moderately susceptible or resistant to clarithromycin by standard in vitro methods and interpretation might, in fact, be susceptible in vivo.

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