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Antituberculosis Activity of Clarithromycin

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Antituberculosis activity of clarithromycin (CLA), a macrolide antibiotic, was investigated in vitro, in macrophages, and in C57BL/6 mice. CLA showed high in vitro MICs (4 to > 16 μg/ml) for several strains of *Mycobacterium tuberculosis* and caused slight enhancement of activity of rifampin (RIF) against H37Rv but failed to increase the activity of either RIF or isoniazid (INH) against other strains. However, inside J774A.1 macrophages, CLA showed high activity and was synergistic with RIF against some strains of tubercle bacilli susceptible or resistant to INH and RIF. In the in vivo studies with a drug-susceptible strain (H37Rv), CLA protected mice from mortality due to tuberculosis for up to 8 weeks of observation. The CFU data for lungs and spleens revealed that the antituberculosis activity of CLA is inferior to those of INH and streptomycin. However, the activity of CLA when used alone or in combination was comparable to that of thiacetazone, indicating its potential usefulness as a secondary drug for the treatment of tuberculosis.

Recent outbreaks of tuberculosis with multiple-drug-resistant (MDR) tubercle bacilli have indicated an urgent need for new drugs. New methods of establishing the antimycobacterial properties of the available drugs to the maximum extent are also being pursued. In this endeavor, enhancement of the susceptibility of the MDR bacilli to some drugs to which they were originally resistant has been investigated by us and others (1–3, 12, 15). Clarithromycin (CLA), a recently introduced macrolide antibiotic, was shown to have in vitro synergistic activity with isoniazid (INH), ethambutol, and rifampin (RIF) against MDR strains of tubercle bacilli (1, 3). This finding is interesting, since individually none of these drugs, including CLA, is active against such organisms. In fact, CLA, which is used for *Mycobacterium avium* complex (MAC) disease (4–6), has not been used for treatment of *Mycobacterium tuberculosis* because of its high MIC.

We have investigated the activity of CLA alone and in combination with INH and RIF against drug-susceptible and MDR tubercle bacilli. Besides extending the findings of earlier reports (1, 3) to more-detailed studies using the radiometric (BACTEC) technique and assessing synergism by quantitative methods, our studies involved assessments of the activity of CLA by itself and in combination against drug-susceptible and MDR strains of tubercle bacilli in the macrophage model and against drug-susceptible *M. tuberculosis* H37Rv infections in C57BL/6 mice.

MATERIALS AND METHODS

Mycobacterial strains. The *M. tuberculosis* strains used in this study consisted of two drug-susceptible strains, including H37Rv, and seven MDR isolates obtained from Centers for Disease Control and Prevention, Atlanta, Ga., and University of Illinois Hospital, Chicago. The organisms were grown in Middlebrook 7H9 broth, and the bacilli in log-phase growth were washed twice with Hanks' balanced salt solution (HBSS) and adjusted to a McFarland no. 1 standard.

Drugs. CLA was obtained from Abbott Laboratories, Abbott Park, Chicago, Ill.; INH, RIF, thiacetazone (TZ), and streptomycin (SM) were obtained from Sigma Chemical Company, St. Louis, Mo. For in vitro and macrophage studies, stock solutions of CLA were prepared according to the recommendations of the manufacturer. RIF was initially dissolved in dimethyl sulfoxide and INH was dissolved in distilled water, and subsequent dilutions were made in distilled water. For in vivo studies, INH and SM were dissolved in distilled water whereas CLA and TZ were suspended in saline containing 0.5% methylcellulose and 0.2% Tween 80.

Determination of MICs. MICs were determined by the radiometric method (14). Bacillary suspensions of the strains were prepared by growing the organisms in BACTEC 12B medium (pH 6.6 to 7.0) until the growth index (GI) reached 999 (GI is a scale in the BACTEC system which reflects the amount of growth). The BACTEC vials were dispensed with different concentrations of the drugs ranging from 2.0 to 0.06 μg/ml. All the drug-containing vials were inoculated with 0.1 ml of the bacterial suspensions as described above. With each test, two drug-free controls were included, one with 0.1 ml of the same suspension used for the drug-containing vials and the other with a 1/100 dilution of the suspension. The vials were incubated at 37°C, and growth was measured with a BACTEC 460 reader every day until the GI in the control inoculated with a 1/100 dilution of the suspension reached ≥30, with a daily increase in GI of ≥10 for 3 consecutive days. If the 1/100-diluted control reached a GI of ≥30 within 4 days or after 8 days of incubation, the test was repeated. The MIC was defined as the lowest concentration of the drug that caused an increase in GI equal to or less than that of the control inoculated with a 1/100 dilution of the suspension (9). Whenever the MICs fell out of the range used, the test was repeated with higher concentration of the drugs.

Determination of in vitro synergistic activity. BACTEC 12B vials were inoculated with two drugs, CLA and RIF or INH. All the drugs were tested at the MIC or sub-MIC against each of the strains. Two concentrations of CLA (2.0 and 0.5 μg/ml) were used in conjunction with three concentrations of RIF or INH (0.125, 0.06, and 0.03 μg/ml for susceptible strains or 2.0, 1.0, and 0.5 μg/ml for resistant strains). The drug-containing vials were inoculated with 0.1 ml of mycobacterial suspensions. With each test, two drug-free controls were included as described above. All the vials were incubated at 37°C, and results were read daily up to 13 days (9).

Determination of intracellular activity and synergism. Intracellular activity of the drugs was determined with J774A.1 macrophages. Twenty-four-well tissue culture chambers were seeded with J774A.1 macrophages (10^6/ml per well) in Dulbecco's modified Eagle's medium with 1% fetal bovine serum, and the chambers were incubated at 37°C in a 5% CO_2 atmosphere for 2 h. At the end of the incubation, the macrophage monolayers were washed once with warm HBSS to remove nonadherent cells. The macrophages were infected with the required strains of *M. tuberculosis* in log-phase growth in 7H9 broth at a bacterium-to-macrophage multiplicity of infection of 10:1. After 2 h of incubation, the nonphagocytosed organisms were removed by three washings with warm HBSS. The cells were replaced with fresh tissue culture medium containing different concentrations of drugs. Control chambers contained only tissue culture medium. The tissue culture plates were incubated at 37°C in an atmosphere of 5% CO_2 for 4 days. Infected macrophages were exposed to different concentrations of drugs throughout the incubation period.

In the initial experiments, inhibitory concentrations of the drugs were established for each strain by testing different concentrations in the macrophages. In the subsequent studies of synergism, subinhibitory concentrations of CLA, RIF, and INH were used. At 0 and 4 days postinfection, macrophages in duplicate

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TABLE 1. Radiometrically determined MICs of different drugs for *M. tuberculosis* isolates

Isolate no.	MIC (μg/ml)		
	RIF	INH	CLA
20	>16.0	<2.0	16.0
33	2.0	0.50	4.0
40	<0.5	2.0	16.0
2218	0.062	4.0	4.0
2219	>16.0	>4.0	>16.0
2225	>16.0	>4.0	>16.0
2227	4.0	>4.0	16.0
2237	0.125	0.125	>16.0
H37Rv	0.125	0.125	>16.0

wells from each group were lysed with 0.25% sodium dodecyl sulfate. The antimycobacterial activity was determined by injecting aliquots of the macrophage lysates into BACTEC 12B vials and determining the GI after 24 or 48 h of incubation as described previously (17). Assessment of the synergy between the drugs was also made quantitatively by adopting the *x/y* quotient method of Hoffner et al. (10). In general, if the *x/y* quotient was <0.5, the combination was considered to be synergistic, whereas a quotient of >1 indicates no synergistic effect and a quotient of >2 indicates antagonism between the drugs.

Determination of in vivo activity. Four- to six-week-old C57BL/6 mice of either sex obtained from Jackson Laboratories, Bar Harbor, Maine, were used. They were caged in microisolator cages (five mice each), and food and water were given ad libitum.

In a preliminary study, mice were infected with about 5×10^7 CFU of the drug-susceptible *M. tuberculosis* H37Rv strain by intravenous route through a lateral caudal vein. The following day, five mice were killed to obtain the baseline counts. The rest of the infected mice were divided into three groups; group 1 (10 mice) was dosed with 200 mg of CLA per kg of body weight, group 2 (5 mice) was dosed with 25 mg of INH per kg, and group 3 (10 mice) served as drug-free controls. Both drugs were given by oral gavage 5 days a week. Since all the control mice died before 4 weeks, surviving animals in groups 2 and 3 were killed at week 4 or 8 to determine CFU in the organs. The lungs and spleens were collected aseptically and homogenized in 10 ml of saline, and 1/10-ml samples of three 10-fold dilutions of the homogenates were plated on 7H11 agar plates and incubated at 37°C. The CFU were counted at the end of 3 weeks of incubation.

In the second in vivo study, the chemotherapeutic activity of CLA alone and in combination was investigated. One hundred C57BL/6 mice were infected with 4.8×10^6 CFU of *M. tuberculosis* H37Rv as described above. The following day, five mice were killed to determine baseline CFU in the lungs and spleens, and the remaining mice were divided into nine groups. The animals in groups 1 through 4 were dosed with CLA (200 mg/kg), INH (25 mg/kg), TZ (60 mg/kg), and SM (200 mg/kg), respectively. Group 5 animals were dosed with a combination of CLA and INH (200 and 25 mg/kg, respectively), group 6 was given CLA plus TZ (200 and 60 mg/kg, respectively), group 7 was given CLA plus SM (200 and 200 mg/kg, respectively), and group 8 was given INH plus TZ (25 and 60 mg/kg, respectively). Group 9 served as drug-free controls. Except for SM, which was given subcutaneously, all drugs were given by oral gavage. In combination chemotherapy, all the drugs were dosed separately. The control group had 15 mice, and the rest of the groups had 10 mice each. Infected mice were treated with the drugs as described above, five days a week for 8 weeks. The mice were observed for mortality. The CFU of organisms recoverable from the spleens and lungs of five mice in each group, killed at 4 and 8 weeks postchallenge, were determined as described above.

RESULTS

Drug susceptibility patterns of the mycobacterial strains used (radiometric method). The MICs of CLA varied significantly, ranging from 4 μg/ml for strains 33 and 2218 to ≥16 μg/ml for the rest of the strains tested (Table 1). There was no consistent pattern of susceptibility to CLA for the RIF- and INH-susceptible strains (H37Rv and 2237) or the MDR strains. In general, the MICs of CLA were very high for all the strains of *M. tuberculosis*, in contrast to those for MAC strains. These concentrations (2 to 4.4 μg/ml) are much higher than those attained in the sera of patients following treatment with conventional doses (13).

Combined effect of CLA with other antituberculosis drugs in vitro (radiometric studies). Different combinations and concentrations of CLA and RIF or INH were tested against four different strains of *M. tuberculosis* (H37Rv, 20, 2218, and 2219). On the basis of *x/y* quotient analysis, with none of the combinations of drugs was there any evidence of synergism, with *x/y* quotients of >0.5 in all cases (data not shown). However, for H37Rv, CLA enhanced the activity of RIF, but the enhancement was not significant enough to give an *x/y* quotient of ≤0.5 (Fig. 1A). Representative growth curves showing a lack of synergism of CLA with INH are presented in Fig. 1B.

Activity of CLA against tubercle bacilli inside macrophages. The intracellular antimycobacterial activity of CLA against five *M. tuberculosis* strains in J774A.1 cells was assessed. Despite high in vitro MICs, the drug showed considerable inhibition of intracellular growth at concentrations ranging from 0.06 to 2.0 μg/ml for the five strains tested (Fig. 2).

Intracellular synergistic activity of CLA with other drugs. Quantitative analysis of the intracellular synergism of the drugs was determined by adopting the *x/y* quotient analysis of Hoffner et al. (10), where *x* represents the GI of lysates of

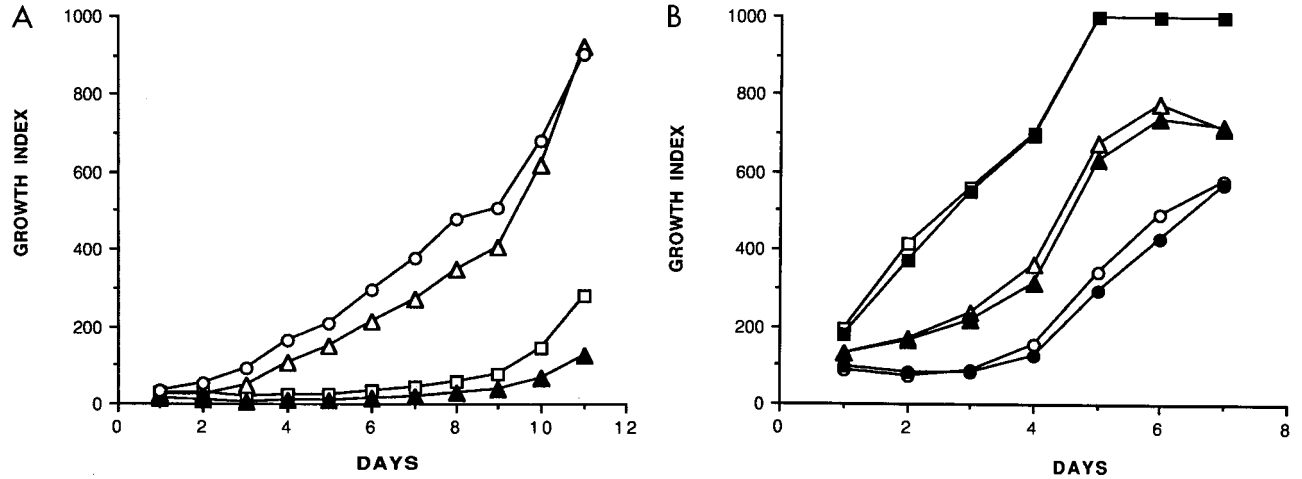

FIG. 1. In vitro radiometric growth curves showing the activity of CLA alone or in combination with RIF or INH. (A) Activity against *M. tuberculosis* H37Rv. □, RIF (0.125 μg/ml); △, CLA (0.5 μg/ml); ▲, RIF plus CLA (0.125 and 0.5 μg/ml, respectively); ○, control. (B) Activity against *M. tuberculosis* 2218. □, INH (1.0 μg/ml); ○ and △, CLA (2.0 and 0.5 μg/ml, respectively); ● and ▲, INH plus CLA (1.0 and 2.0 μg/ml and 1.0 and 0.5 μg/ml, respectively); ■, control.

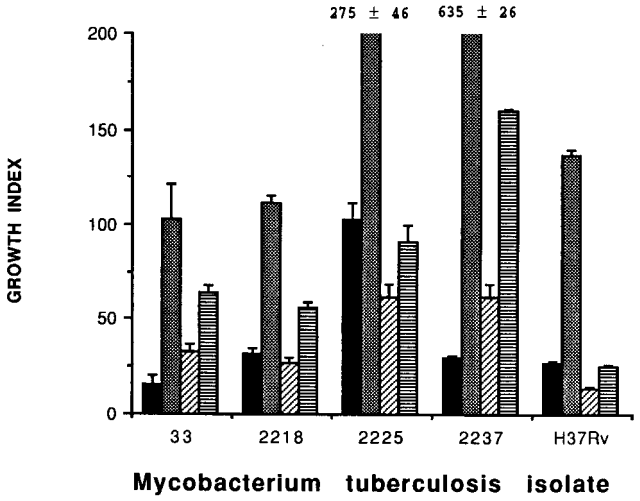

FIG. 2. Intracellular activity of CLA against *M. tuberculosis* isolates. The 1× concentrations of CLA were 0.06 μg/ml for isolate 33, 0.125 μg/ml for isolate 2218, 0.25 μg/ml for isolates 2225 and 2237, and 0.5 μg/ml for strain H37Rv. ■, day 0; ▩, day 4; ▨ and ▤, CLA at 4× and 1× concentrations, respectively.

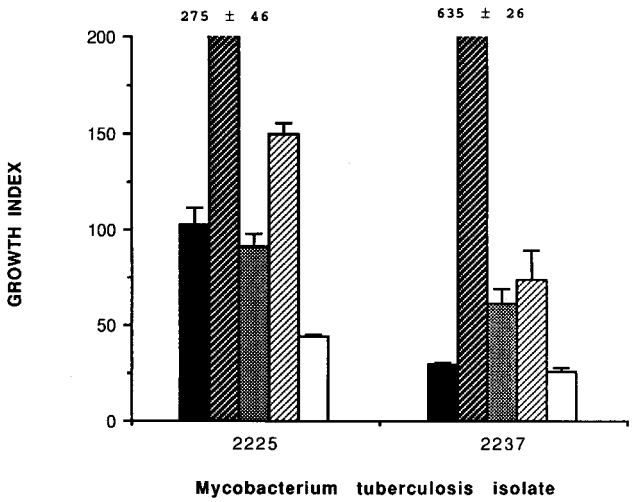

FIG. 3. Intracellular synergistic activity of CLA with RIF. The concentrations of CLA and RIF used for isolate 2237 were 1.0 and 0.125 μg/ml, respectively; for isolate 2225, the respective concentrations were 0.25 and 2.0 μg/ml. ■, day 0 control; ▨, day 4 control; ▩, CLA; ▨, RIF; □, CLA plus RIF.

macrophages exposed to a combination of two drugs and *y* represents the lower of the two GIs of the lysates of macrophages exposed to a single drug. With an *x/y* quotient of <0.5 considered indicative of synergism, considerable synergism of CLA with RIF was seen with three of the strains tested, though such activity was less pronounced with INH (Table 2). Histograms depicting the synergism of CLA with RIF for two strains are shown in Fig. 3.

In vivo chemotherapeutic activity. (i) Mortality. None of the mice treated with CLA or any other antituberculosis drug died by 8 weeks, whereas all the drug-free control mice died by that time; in fact, in the preliminary study in which mice were infected with 5×10^7 CFU, all the control mice died by the fourth week.

(ii) CFU data. In the preliminary experiment, in CLA-treated mice, the numbers of viable organisms in the lungs and spleens increased from 7.71 and 6.91 to 9.14 and 7.62 log CFU/g of tissue, respectively, by the end of 8 weeks of treatment, an increase of 1.43 log CFU in lungs and 0.71 log CFU in spleens (Table 3). On the other hand, INH-treated mice showed a significant reduction in CFU in lungs as well as spleens. Lungs showed a reduction of 2.62 log CFU and spleens showed a reduction of 2.14 log CFU after 8 weeks of treatment with INH.

In the second experiment, on the basis of the numbers of viable organisms recovered from both lungs and spleens of treated mice, INH showed superior activity, followed by SM, CLA, and TZ (Table 4). No significant difference in CFU was observed for the organs of the mice treated with INH alone or with a combination of INH plus CLA or INH plus TZ. Similarly, the number of viable organisms recovered from the mice treated with either SM alone or SM plus CLA did not differ significantly, indicating that addition of a less active drug to more powerful drugs does not enhance the activity significantly. The activity of the combination of CLA plus TZ was slightly greater than the activities of the individual drugs.

TABLE 2. Intracellular synergistic activity of CLA with INH and RIF[a]

M. tuberculosis strain	INH		RIF	
	Synergy according to *x/y* quotient analysis[b]	Concn at synergy[c]	Synergy according to *x/y* quotient analysis	Concn at synergy
33	Yes	0.06 + 0.5	No	
2218	No		ND[d]	
2225	No		Yes	0.25 + 2.0
2237	No		Yes	1.0 + 0.125
H37Rv	No		Yes	2.0 + 0.06
				0.5 + 0.06

[a] J774A.1 cells were infected with *M. tuberculosis* isolates and treated for 4 days with single or combined drugs.
[b] Synergism was considered when the *x/y* quotient was <0.5.
[c] Concentrations of CLA plus INH or RIF, in micrograms per milliliter.
[d] ND, not done.

DISCUSSION

CLA, which is now used in the treatment of MAC disease (3–5), has not been considered as a potential antituberculosis drug, mainly because of its high MIC in vitro as tested by either conventional or radiometric (BACTEC) methods (8). In most cases, the MIC was much higher than concentrations achievable in serum. In our search for new drugs, our attention was caught by the reports of Biehle and Cavalieri (1) and Cavalieri et al. (3) of the in vitro synergistic activity of CLA with other antituberculosis drugs against MDR tubercle bacilli. Unlike

TABLE 3. In vivo activity of CLA against *M. tuberculosis* H37Rv in C57BL/6 mice

Treatment group (dose [mg/kg])	Organ	log CFU/g of tissue ± SD		
		Day 1	4th wk	8th wk
CLA (200)	Lung		8.48 ± 0.21	9.14 ± 0.14
	Spleen		7.45 ± 0.04	7.62 ± 0.18
INH (25)	Lung		5.45 ± 0.32	5.09 ± 0.57
	Spleen		5.51 ± 0.09	4.77 ± 0.12
Control (0)	Lung	7.71 ± 0.22	—[a]	—
	Spleen	6.91 ± 0.03	—	—

[a] —, all mice died.

TABLE 4. In vivo activity of CLA alone and in combination with other antituberculosis drugs against *M. tuberculosis* H37Rv in C57BL/6 mice

Treatment group (dose [mg/kg])	Organ	log CFU/g of tissue ± SD		
		Day 1	4th wk	8th wk
CLA (200)	Lung		7.24 ± 0.20	7.36 ± 0.18
	Spleen		6.47 ± 0.13	6.68 ± 0.08
INH (25)	Lung		5.19 ± 0.10	4.44 ± 0.03
	Spleen		4.97 ± 0.07	4.30 ± 0.12
TZ (60)	Lung		7.39 ± 0.15	7.61 ± 0.13
	Spleen		6.59 ± 0.14	7.11 ± 0.14
SM (200)	Lung		5.87 ± 0.21	5.58 ± 0.12
	Spleen		5.94 ± 0.13	5.55 ± 0.29
CLA + INH (200 + 25)	Lung		4.57 ± 0.13	4.03 ± 0.14
	Spleen		4.76 ± 0.18	4.62 ± 0.22
CLA + TZ (200 + 60)	Lung		6.29 ± 0.21	7.07 ± 0.35
	Spleen		6.31 ± 0.12	6.37 ± 0.28
CLA + SM (200 + 200)	Lung		5.85 ± 0.15	5.95 ± 0.17
	Spleen		5.65 ± 0.14	5.56 ± 0.01
INH + TZ (25 + 60)	Lung		5.05 ± 0.23	4.18 ± 0.11
	Spleen		4.90 ± 0.06	4.50 ± 0.08
Control	Lung	6.94 ± 0.10	8.65 ± 0.27	—[a]
	Spleen	6.57 ± 0.17	7.16 ± 0.18	—

[a] —, all mice died.

their findings (1, 3), our studies using the radiometric (BACTEC) method showed that CLA caused only slight enhancement of the activity of RIF but not INH against *M. tuberculosis* H37Rv (Fig. 1); however, no enhancement was observed with any of the MDR strains tested. A lack of in vitro synergism between CLA and other antituberculosis drugs has also been reported by others (2). In contrast, in macrophages, the activity of the combination of CLA with either INH or RIF was better than that of the individual drugs. The current data are in agreement with earlier observations with MAC (16) that a CLA-plus-RIF combination showed greater synergism in macrophages than in vitro.

A valuable fringe benefit came from our studies in the finding that CLA by itself, at concentrations much below the MIC, is capable of inhibition of intracellular tubercle bacilli. This is evident with all the strains of tubercle bacilli tested, including those resistant to INH or RIF. This finding is all the more important since CLA was active in the macrophages at concentrations of 0.06 to 2.0 μg/ml, which are much lower than the attainable levels in serum.

Earlier, it has been shown that CLA penetrates phagocytic cells efficiently, resulting in high ratios of intracellular to extracellular concentrations (11). Our data for intracellular activity of CLA are consistent with these findings. In addition, the drug has also been shown to concentrate more in the lung tissues than in other tissues (7). These findings prompted us to explore whether CLA has any antituberculosis activity alone and in combination with other antituberculosis drugs in animals. Initial experiments were done with combinations of INH and CLA or TZ; further in vivo studies of the combination of CLA and RIF, which showed synergism in macrophages, are being planned. The present chemotherapeutic studies of C57BL/6 mice with RIF- and INH-susceptible *M. tuberculosis* H37Rv infection have shown that CLA at a dose of 200 mg/kg, which is the conventional dose of this drug used against MAC disease, has demonstrated complete protection from death due to tuberculosis, while all (100%) of the untreated control animals died within 8 weeks after challenge. CLA also caused reduction of CFU in both lungs and spleens compared with the CFU in controls, with an activity slightly superior to that of TZ, a widely used antituberculosis drug. However, both CLA and TZ were inferior to INH or SM. The combination of CLA and INH showed slightly better activity than the combination of INH and TZ. Our data thus indicate that CLA, besides having high in vitro MICs, shows some antituberculosis activity, which is slightly better than that of TZ, and therefore could be considered as an alternative secondary drug for the treatment of tuberculosis.

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