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Determination of in vitro synergy when three antimicrobial agents are combined against *Mycobacterium tuberculosis*

Y. Bhusal *, C.M. Shiohira, N. Yamane

Department of Laboratory Medicine, Graduate School and Faculty of Medicine, University of the Ryukyus, 207 Uehara, Nishihara-Nakagami, Okinawa 903-0215, Japan

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Abstract

We determined the in vitro antimycobacterial activity of rifampicin, isoniazid and a third agent in combination using a three-dimensional chequerboard in Middlebrook 7H9 broth microdilutions. Of 28 agents screened, ethambutol, streptomycin, clarithromycin, minocycline, ciprofloxacin, levofloxacin, sparfloxacin, gatifloxacin and sitafloxacin were potentially synergistic. A further three-dimensional chequerboard assay quantitatively looked for synergy against ten clinical isolates of *Mycobacterium tuberculosis*, including seven multidrug-resistant isolates. Sitafloxacin, gatifloxacin and clarithromycin showed significant synergy, with fractional inhibitory concentration indices ranging from 0.41 to 0.79, 0.39 to 0.90 and 0.48 to 0.95, respectively. It is concluded that three-dimensional chequerboard assay can quantitatively determine antimycobacterial synergy, and that fluoroquinolones and antibacterial agents such as clarithromycin are effective against multidrug-resistant isolates of *M. tuberculosis* when combined with rifampicin and isoniazid.
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1. Introduction

Tuberculosis is a leading cause of death among those infections with a single aetiology [1]. Antituberculosis chemotherapy may induce drug resistance through the accumulation of spontaneous mutations in infecting organisms of *Mycobacterium tuberculosis*. To prevent the emergence of drug resistance, combinations of different antimycobacterial agents have been given. It has been demonstrated empirically that certain drug combinations are synergistic. Currently, the emergence of multidrug-resistant *M. tuberculosis* (MDR-TB) poses a special problem because most second-line drugs are either very toxic or very expensive [2]. Therefore, an alternative therapeutic regimen is urgently needed. To establish a new therapeutic regimen, it is necessary to develop quantitative and reproducible test procedures to estimate antimycobacterial activity when two or more agents are combined. We previously developed and described the test procedure based on the microdilution susceptibility test to determine minimal inhibitory concentrations (MICs) against *M. tuberculosis* and this is now commercially available in Japan as BrothMIC MTB (Kyokuto Pharmaceuticals, Tokyo, Japan) [3,4]. In this study, we used a three-dimensional broth microdilution chequerboard assay to determine in vitro synergy against *M. tuberculosis* when a third agent is added to the combination of rifampicin (RFP) and isoniazid (INH).

2. Materials and methods

2.1. Test isolates

A total of ten clinical isolates of *M. tuberculosis* from University Hospital of the Ryukyus, Okinawa, and Osaka Prefectural Habikino Hospital, Osaka, were included in this study. The isolates were first identified by the Accu-probe assay (Gen-Probe, San Diego, CA) and then biochemically differentiated from *Mycobacterium bovis* by niacin accumulation, inhibition by thiophene-2-carboxylic acid hydrazide

* Corresponding author. Tel.: +81 98 895 1317; fax: +81 98 895 1463.
E-mail address: f011144@med.u-ryukyu.ac.jp (Y. Bhusal).

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and nitrate reduction [5]. Of the isolates included, seven (isolate Nos. 1–7) were multidrug resistant (MDR), with MICs of more than 2 mg/L against both RFP and INH. Two isolates (Nos. 8 and 9) were monoresistant against RFP, and the remaining isolate (No. 10) was a susceptible wild-type isolate. A strain of H37Rv (*M. tuberculosis* ATCC 27294) was used as the control. Stock solutions of the isolates were kept in frozen Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) culture at −80 °C until assay.

2.2. Antimicrobial agents

The antimicrobial agents RFP, INH, ethambutol (EB), streptomycin (SM) and minocycline (MIN) were purchased from Sigma Chemical Company (St Louis, MO). Clarithromycin (CLR), sitafloxacin (STFX) and gatifloxacin (GFLX) were kindly provided by Taisho Pharmaceutical Co. Ltd. (Tokyo, Japan), Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) and Kyorin Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively. The remaining 20 antimicrobial agents were obtained from their respective manufacturers. Initial stock solutions of these 28 antimicrobial agents were prepared according to manufacturers' instructions. Further dilutions were made in 10% oleic acid bovine albumin dextrose catalase (OADC)-enriched Middlebrook 7H9 broth (Difco Laboratories).

2.3. Inoculum preparation

The test isolates were grown in Middlebrook 7H9 broth with OADC enrichment for 5–10 days at 36 °C to a turbidity of 1.0 McFarland standard. The cell suspension was then adjusted to give a final concentration of ca. 10^5 cells/mL at the time of inoculation [6].

2.4. Antimycobacterial susceptibility testing

Three-dimensional chequerboard microdilutions were principally based on the standard two-dimensional chequerboard assay. First, two-dimensional microdilution chequerboard plates were prepared by dispensing the serially diluted RFP in the *x*-axis and INH in the *y*-axis in a 96-well microtitre plate [7]. The third agent was then dispensed throughout the wells as an overlay at subinhibitory concentrations ranging from 1/32 to 1/2 of the MIC. In the initial screening study, the third agent was tested at two different concentrations, 10 mg/L and 100 mg/L, or at 1/2 of the MIC when complete growth inhibition was observed at 10 mg/L, against three clinical MDR isolates. After inoculation, microplates were incubated for 2–3 weeks at 36 °C in 7% CO_2 until adequate growth in a growth control well was visually read. The test results were interpreted by fractional inhibitory concentration (FIC) and were graphically represented as isobolograms using Statistica 5.5 (Statsoft Inc., Tulsa, OK).

For the standard two-dimensional chequerboard assay, the FIC was calculated and interpreted as previously described [7,8]. Calculation of the FIC index for a three-dimensional chequerboard was modified as:

$$\text{FIC index} = \frac{\text{MIC [A] combination}}{\text{MIC [A] alone}} + \frac{\text{MIC [B] combination}}{\text{MIC [B] alone}} + \frac{\text{MIC [C] combination}}{\text{MIC [C] alone}}$$

where A, B and C were the three respective antimicrobial agents tested. The lowest FIC index was used to interpret the test results as follows: synergism, ≤0.75; indifference, >0.75–4; and antagonism, >4.

2.5. Time–kill study

The killing kinetics were determined by incubation of the test isolate in Middlebrook 7H9 broth in the presence of antimicrobial agents. The isolate was first grown to a turbidity of 1.0 McFarland standard, and the cell suspension was then adjusted to give a final concentration of ca. 10^5 cells/mL in culture broth. Single antimicrobial agents and those in combinations were added to the cell suspension broth to achieve one-half of the MICs of the respective agents. During incubation at 36 °C, part of the culture broth was collected, and colony-forming units (CFUs) were determined on Middlebrook 7H11 agar plates. Synergy was defined as 2 or more decrease in $\log_{10}$ CFU/mL compared with those for the respective single agents [9,10].

3. Results

3.1. Antimicrobial combination screening for synergy

As shown in Table 1, a total of 28 antimicrobial agents were screened to determine whether they showed synergy when combined with RFP and INH. Of these, five fluoroquinolones, ciprofloxacin, levofloxacin, sparfloxacin, GFLX

Table 1
Fractional inhibitory concentration (FIC) indices of 28 antimicrobial agents when combined with rifampicin and isoniazid against three clinical isolates of multidrug-resistant *M. tuberculosis*

Antimicrobial agent	FIC range	Antimicrobial agent	FIC range
Ampicillin	0.99–1.19	Erythromycin	0.99–1.04
Ampicillin–sulbactam	1.08–1.19	Clindamycin	0.89–1.16
Oxacillin	0.89–1.08	Minocycline[a]	0.60–0.76
Piperacillin	1.08–1.12	Tetracycline	1.08–1.12
Cefaclor	1.10–1.19	Chloramphenicol	1.04–1.08
Cefazolin	1.08–1.09	Teicoplanin	1.04–1.14
Cefepime	0.82–1.16	Vancomycin	1.10–1.19
Cefotaxime	0.92–1.06	Fluconazole	0.68–1.10
Ceftizoxime	0.92–1.09	Ethambutol[a]	0.70–1.12
Imipenem	0.97–1.16	Ciprofloxacin[a]	0.43–0.68
Amikacin	0.53–0.93	Gatifloxacin[a]	0.39–0.65
Streptomycin[a]	0.55–0.91	Levofloxacin[a]	0.67–0.73
Azithromycin	0.69–1.04	Sitafloxacin[a]	0.44–0.50
Clarithromycin[a]	0.48–0.55	Sparfloxacin[a]	0.39–0.48

[a] Assayed at one-half of minimal inhibitory concentration.

Table 2
Fractional inhibitory concentration indices of antimicrobial agents determined by a two-dimensional chequerboard assay against ten clinical isolates of *M. tuberculosis* when combined with rifampicin (RFP) and isoniazid (INH)

Isolate No.	RFP combined with:							INH combined with:					
	INH	STFX	GFLX	CLR	MIN	SM	EB	STFX	GFLX	CLR	MIN	SM	EB
1	1.10	1.19	0.79	1.16	1.19	1.08	1.16	1.16	1.04	1.10	1.19	1.01	1.09
2	1.00	1.08	0.94	0.74	0.95	0.91	1.08	1.23	1.19	0.98	1.09	1.09	1.23
3	1.09	1.19	1.04	1.16	1.09	1.07	1.19	1.19	1.10	1.19	1.19	0.59	1.19
4	1.12	1.16	0.98	1.16	1.09	1.08	1.18	0.99	1.12	0.98	0.94	1.03	1.16
5	1.19	1.16	1.19	1.14	1.23	0.91	1.16	0.85	1.10	0.59	1.23	1.19	1.19
6	1.10	1.12	1.10	1.10	1.12	1.19	1.10	0.95	1.12	1.19	1.12	1.19	1.10
7	0.94	0.81	1.12	0.87	0.89	0.68	1.08	1.16	1.29	1.23	1.09	0.99	1.23
8	1.08	0.94	1.10	0.22	1.19	0.76	1.16	1.23	1.10	0.98	1.09	1.50	1.12
9	1.08	1.08	1.09	1.09	0.92	1.07	0.85	1.16	1.04	0.98	0.92	1.08	0.99
10	1.01	1.23	1.10	1.23	1.23	0.91	1.08	1.19	1.23	1.19	1.19	1.30	1.10
Min.	0.94	0.81	0.79	0.22	0.89	0.68	0.85	0.85	1.04	0.59	0.92	0.59	0.99
Max.	1.19	1.23	1.19	1.23	1.23	1.19	1.19	1.23	1.29	1.23	1.23	1.50	1.23
H37Rv	0.94	1.10	0.79	1.08	0.99	1.09	0.84	1.16	0.79	0.85	1.09	1.23	1.08

STFX, sitafloxacin; GFLX, gatifloxacin; CLR, clarithromycin; MIN, minocycline; SM, streptomycin; EB, ethambutol.

and STFX, consistently showed synergy, with FIC indices ranging from 0.39 to 0.73. Additionally, CLR, MIN, SM and EB had FIC indices ≤0.75 for two or three of the isolates tested. The remaining 19 agents were interpreted as being mostly indifferent. Following these results, STFX, GFLX, CLR and MIN, along with the first-line agents SM and EB, were further tested.

3.2. Two-agent chequerboard assay

A total of six antimicrobial agents selected were tested in two-dimensional chequerboard plates in combination with RFP or INH. However, none of the agents tested demonstrated significant synergism or antagonism (Table 2). Only one isolate (No. 8) revealed synergy when the combination of RFP and CLR was tested. Also, the combination of RFP and INH was interpreted as being indifferent, with FIC indices ranging from 0.94 to 1.19.

3.3. Three-agent chequerboard assay

Table 3 indicates the FIC indices when a total of ten clinical isolates were tested by the three-dimensional chequerboard assay. All the antimicrobial agents tested in combination with RFP and INH revealed synergy for one to ten isolates. The newer fluoroquinolones, STFX and GFLX, showed marked synergism against nine isolates, including MDR isolates, with FIC indices ranging from 0.39 to 0.74. The concentrations of STFX and GFLX at which the FIC indices were the lowest against the individual isolates ranged between 0.015 and 0.125 mg/L and 0.0075 and 0.25 mg/L, respectively. Also, CLR revealed synergism against nine of the ten isolates, and the FIC indices ranged from 0.48 to 0.74. MIN and SM were partially synergistic by the isolates tested, and FIC indices were close to the indifference interpretation. EB was mostly indifferent, but two isolates were interpreted to be synergistic, with FIC indices of 0.70 and 0.72.

Table 3
Fractional inhibitory concentration indices of antimicrobial agents determined by a three-dimensional chequerboard assay against ten clinical isolates of *M. tuberculosis* when combined with rifampicin (RFP) and isoniazid (INH)

Isolate No.	RFP plus INH combined with:					
	STFX	GFLX	CLR	MIN	SM	EB
1	0.79	0.39	0.58	0.73	0.58	1.10
2	0.47	0.65	0.66	0.75	0.92	1.16
3	0.50	0.47	0.67	0.80	0.62	1.10
4	0.55	0.42	0.48	0.98	0.46	1.13
5	0.51	0.57	0.58	0.94	0.97	1.04
6	0.43	0.44	0.74	0.79	0.98	0.97
7	0.41	0.68	0.68	0.60	0.87	0.70
8	0.45	0.90	0.65	0.74	0.91	0.84
9	0.49	0.72	0.95	0.74	0.71	1.12
10	0.44	0.74	0.69	0.73	0.55	0.72
Range	0.41–0.79	0.39–0.90	0.48–0.95	0.60–0.98	0.46–0.98	0.70–1.16
H37Rv	0.42	0.39	0.62	0.70	0.72	0.62

STFX, sitafloxacin; GFLX, gatifloxacin; CLR, clarithromycin; MIN, minocycline; SM, streptomycin; EB, ethambutol.

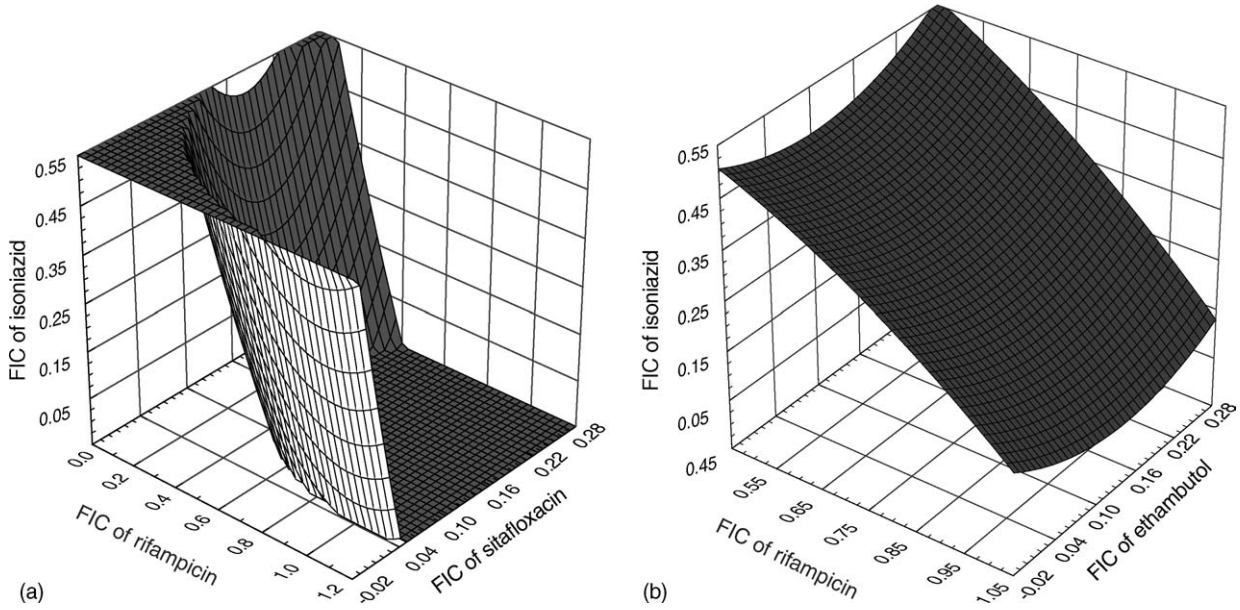

Fig. 1. Three-dimensional isobologram of fractional inhibitory concentration (FIC) indices when three-agent combinations were tested against a multidrug-resistant isolate of *M. tuberculosis* (isolate No. 2). The combinations of (a) sitafloxacin, rifampicin and isoniazid and (b) ethambutol, rifampicin and isoniazid were tested, and the lowest FIC indices were calculated to be 0.47 and 1.16, respectively.

Fig. 1 shows the three-dimensional isobologram indicating the individual FIC indices plotted at five subinhibitory concentrations for isolate No. 2. The isobologram plotted against STFX-RFP-INH combination (Fig. 1a) sank toward the origin, i.e. FIC indices drew near to zero, indicating significant synergy; whereas the isobologram of EB-RFP-INH combination (Fig. 1b) did not show significant depression, with the results indicating indifference. The lowest FIC indices of STFX-RFP-INH and EB-RFP-INH were calculated to be 0.47 and 1.16, respectively.

3.4. Time–kill assay

The antimicrobial combinations that were interpreted as being synergistic by the three-agent chequerboard assay were further studied by time–kill assay. Fig. 2 shows the results obtained for the MDR isolate No. 3. During the incubation in Middlebrook 7H9 broth, the concentrations of viable cells gradually decreased when the test broth contained RFP, INH and either STFX or GFLX at concentrations of the respective one-half of the MICs. On the eighth day of incubation, a $\geq 2 \log_{10}$ CFU/mL decrease by the three-drug combination compared with the respective single agents was demonstrated.

4. Discussion

All the wild strains of *M. tuberculosis* that have never come into contact with antituberculosis agents have a high and uniform degree of susceptibility. However, in contrast to acute bacterial infections, antituberculosis chemotherapy continues for several months and therefore induces and accumulates drug-resistant mutants. Mutations for resistance against individual antituberculosis agents are independent, and the frequency of a resistant mutant against two or more agents usually ranges from 10^{-14} to 10^{-20} or less. This

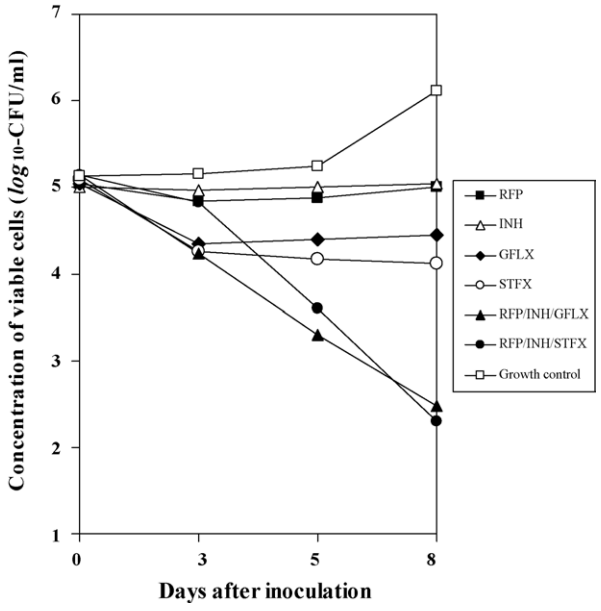

Fig. 2. Time–kill experiments for a multidrug-resistant isolate of *M. tuberculosis* (isolate No. 3) using one-half the minimal inhibitory concentration of each agent (16 mg/L of rifampicin (RFP), 4 mg/L of isoniazid (INH), 0.25 mg/L of sitafloxacin (STFX) and 0.5 mg/L of gatifloxacin (GFLX)) alone and in combinations. Viable cell concentrations in culture broth at the indicated time were determined on Middlebrook 7H11 agar. CFU, colony-forming units.

theoretical basis has led to the development of multidrug regimens as a principle of antituberculosis chemotherapy. The emergence of MDR-TB is a result of insufficient or inappropriate chemotherapy, and the patient subsequently exhales resistant mutants to the public. The study aimed first to determine whether two or more antimicrobial agent combinations were synergistic against *M. tuberculosis* in vitro, and second to find an alternative regimen against MDR-TB, particularly including antimicrobial agents not well recognised as antituberculosis agents.

The theoretical approach to the three-dimensional chequerboard procedure was first described by Berenbaum [11], and was then applied to MDR Gram-negative nosocomial pathogens, *Pseudomonas maltophilia* [12] and *Acinetobacter baumannii* [13]. To our knowledge, however, interaction of two or more antimicrobial agents against *M. tuberculosis* has not been systematically evaluated. Thus, we intended to devise a practical three-dimensional microdilution chequerboard method based on our previously developed microdilution susceptibility test, BrothMIC MTB [3,4]. The interpretation of FIC index for a two-agent combination has been well established and a value of $\leq$0.5, indicating a four-fold decrease in MICs, is considered to be synergistic [7,8]. When three agents were combined, a FIC index <1, which denotes a three-fold decrease in MICs, has been used to define synergy [11–13]. However, in our study, a FIC index of $\leq$0.75, indicating a four-fold decrease in MICs, was used in order to eliminate possible technical errors due to the assay on different microplates for a single three-agent combination.

The results of three-dimensional broth microdilution tests employed were highly consistent and correlated with those of the time–kill assay. Of 28 antimicrobial agents first screened at fixed concentrations, five fluoroquinolones, in addition to CLR, MIN, SM and EB, showed synergistic activity, although almost all of the two-agent combinations were interpreted as being indifferent. Through the quantitative three-dimensional chequerboard studies, it became apparent that three agents, STFX, GFLX and CLR, demonstrated marked synergism against MDR isolates when combined with RFP and INH.

Several quinolones developed as broad-spectrum antibacterial agents have been used to treat MDR-TB [14,15]. The newer compounds, C-8-methoxyl fluoroquinolone (GFLX) and C-8-chloro fluoroquinolone (STFX) have significantly lower MICs for *M. tuberculosis* as well as better pharmacodynamic correlates [16,17]. Although if used alone easy development of drug resistance is likely, they have not been studied in combination with RFP and INH against MDR isolates. In our study, STFX and GFLX in three-agent combinations were highly synergistic for the test isolates, including MDR isolates, with GFLX having slightly higher FIC indices. Moreover, the MICs of the respective agents were at clinically achievable serum concentrations.

In addition, significant synergism was demonstrated when CLR or MIN was combined with RFP plus INH. CLR is frequently employed for the treatment of *Mycobacterium avium* complex infection [18], and MIN is an effective agent for *Mycobacterium leprae* [19]. However, the MICs of CLR and MIN against *M. tuberculosis* are far greater than those ordinarily achievable in serum [20]. The possible reasons for synergism when CLR or MIN was combined with RFP and INH are not necessarily explainable at present, however it was reported that cell wall inhibitors such as vancomycin and bacitracin could convert *M. tuberculosis* to susceptible against CLR [21]. Also, the combinations of cell wall inhibitor, amphotericin B, and several antibacterial agents including RFP, MIN and erythromycin were highly synergistic against yeast [22]. These reports indicate that access of drugs to their target molecules appears to be a key factor in determining susceptibility. Among the antimicrobial agents included in our study, all the agents except INH act on intracellular protein synthesis or nucleic acids. INH is potentially capable of altering cell wall permeability. Although the effect of INH at sub-MIC on cell wall permeability is not well examined, the initial effect on mycolic acid synthesis by INH may change the permeability barrier. Resistant clinical isolates of *M. tuberculosis* are always a mixture of resistant mutants and susceptible wild-cell populations. It is well known that INH susceptibility is dependent on the catalase–peroxidase enzyme that may convert the drug to an activated intermediate. Thus, it is possible that a population of wild, non-mutant cells will provide the necessary enzymatic activity to resistant mutants against INH, resulting in an alteration of cell wall permeability and a higher intracellular penetration of the other agents.

In conclusion, using a microdilution technique based on the chequerboard titration method, we have shown that fluoroquinolones and several non-antituberculosis agents including CLR and MIN are synergistic in vitro against *M. tuberculosis* combined with RFP and INH. The method employed in this study will provide quantitative and reproducible test results and will enable us to evaluate antimicrobial combinations including second-line agents for tuberculosis as well as newly developed agents such as linezolid [23]. Antituberculosis synergy may be promising for more effective chemotherapy, particularly against MDR-TB. However, testing of the combinations in animal models or in actual clinical situations is warranted.

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