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In Vitro Synergism Testing of Three Antimicrobial Agents against Multidrug-Resistant and Extensively Drug-Resistant *Mycobacterium tuberculosis* by Checkerboard Method

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Abstract

Objective: In view of the national key project for infectious diseases " the retreatment research of tuberculosis", we determined the in vitro anti-mycobacterial activity of four drugs in the treatment, pasiniazid (Pa), moxifloxacin (Mfx), Rifabutin (Rfb) and rifapentini (Rft) in combination to multidrug-resistant and extensively drug-resistant mycobacterium tuberculosis.

Method: Three-dimensional checkerboard in Middlebrook 7H9 broth microdilutions was used to detect the fractional inhibitory concentration index (FICI) of anti-tuberculosis drug combination (MfxPa, MfxPaRfb and MfxPaRft) to twenty clinical isolates of Mycobacterium tuberculosis, including ten multidrug-resistant isolates and ten extensively drug-resistant isolates. The test results were interpreted by calculating the FICI to judge the in vitro synergy, with FICI < 0. 5 and FICI < 0.75 as the basis of two drugs and three drugs have synergy.

Results: The FICI range of MfxPa combination for multidrug-resistant isolates and ten extensively drug-resistant isolates was 0.28-1, only three isolates <0.5, showed synergistic effect. The FICI range of MfxPaRfb combination for ten isolates of multidrug-resistant isolates was from 0.31 to 1.25, two isolates <0.75, showed synergistic effect and for ten isolates of extensively drug-resistant isolates was from 0.53to 1.25, five isolates <0.75, showed synergistic effect. The FICI range of MfxPaRft combination for ten isolates of multidrug-resistant isolates was from 0.16to 0.655, all showed synergistic effect and for ten isolates of extensively drug-resistant isolates was from 0.34 to 1.68, five isolates<0.75, showed synergistic effect.

Conclusion: The synergism of MfxPa combination was poor. When a third agent (Rfb or Rft) was added to the combination, the synergistic effect was better. The MfxPaRft combination showed better synergism than MfxPaRfb combination.

Keywords: Multidrug-resistant *M. tuberculosis*; Extensively drug-resistant *M. tuberculosis*; *In vitro* synergy; Fractional inhibitory concentration index; Broth microdilution checkerboard

Introduction

Drug-resistant TB (DR-TB) is a major threat worldwide today. The main source of drug-resistant pulmonary tuberculosis is retreatment patients [1]. According to the survey in 72 countries and territories around the world, the rate of DR-TB in retreatment pulmonary tuberculosis patients with sputum smear positive was 0.0%-85.9% and the rate of multidrug-resistant tuberculosis (MDR-TB) was 0.0%-62.5%. In China, the rate of DR-TB in retreatment pulmonary tuberculosis patients with sputum smear positive was 55.17% and the rate of MDR-TB was 25.64% [2]. While DR-TB is a formidable obstacle to effective TB care and prevention globally, the more effective therapeutic regimen for retreatment pulmonary tuberculosis is urgently needed. However, the synergistic effect is crucial for assessing the effectiveness of the anti-tuberculosis chemotherapy [3-5]. Moxifloxacin(Mfx), Pasiniazid(Pa), Rifabutin(Rfb) and Rifapentine(Rft) were core drugs of the national key project for infectious diseases (the retreatment research of tuberculosis). These drugs have been carried out in the clinical application and have appeared as promising new anti-TB therapies in patients with resistance to classical drugs. But there has not been report on the synergism of these drugs. To address this need, we conducted this study of *in vitro* synergism of these drugs on twenty DR-MTB clinical isolates including ten MDR-TB and ten XDR-TB by a three-dimensional checkerboard in Middlebrook 7H9 broth microdilutions. We calculated the fractional inhibitory concentration index (FICI) of anti-tuberculosis drug combinations (MfxPa, MfxPaRfb and MfxPaRft) for these isolates and judge the synergism of these drugs.

Materials and Methods

Test isolates

A total of twenty clinical isolates of M. tuberculosis including ten multidrug-resistant strains (isolate Nos.1-10) and ten extensively drug-resistant strains (isolate Nos.11-20) were included in this study from Shanghai Pulmonary Hospital, Tongji University School of Medicine. The isolates were obtained from sputum after culture with BACTEC MGIT 960 method and then were identified by biochemical tests. A strain of H37Rv (M. tuberculosis ATCC 27294), gift of the National Tuberculosis Reference Laboratory (Beijing, China) was used as control.

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Antimicrobial agents

The antimicrobial agents Pa, Rfb, Rft were purchased from Sigma Chemical Company (St Louis, MO). Mfx was purchased from Bayer Pharmaceutical Co. Ltd. Initial stock solutions of these antimicrobial agents were prepared according to manufacturers' instructions and stored at-70°C until use [6].

Liquid culture medium

Liquid culture medium was Middlebrook 7H9 liquid culture containing 10% OADC enrichment ([Becton Dickinson Co., U.S.A.], the mixture of antimicrobial agents and growth indicator). Middlebrook 7H9 liquid culture was prepared according to the literature [7,8].

Inoculum preparation

M. tuberculosis suspensions in log-phase growth were adjusted to an optical density of 1.0 McFarland standard in sterile saline, corresponding to a cell density of approximately 10^7 colony forming units (cfu/ml). The cell suspensions were then subjected to ten-fold serial dilutions to give a final concentration of 10^5 cfu/ml at the time of inoculation.

Antimycobacterial susceptibility testing

Minimum inhibitory concentration (MIC) of Rfb and Rft as single agent was examined using the microwell plate method. Before use, aliquot of 20ul liquid culture medium contained Rfb or Rft dilutions was prepared and added to the sterile 96-well polystyrene U-bottom microdilution tray. The concentration range of Rfb or Rft was from 0.15 μg/ml to 320 μg/ml. When 200 μl suspension of M. tuberculosis was inoculated, the final concentration range was from 0.015 μg/ml to 32 μg/ml. Three drug-free controls were inoculated with the same suspensions diluted 1:1, 1:10 and 1:100 respectively. The MIC of Rfb or Rft is the lowest concentration causing visible white bacterial precipitation in the bottom of the well less than that of the 1:10 drug-free control.

Preparing micro dilution checkerboard panels and synergism testing

Liquid culture medium contained Pa or Mfx dilutions were prepared. The final concentration range was from 0.75 μg/ml to 160 μg/ml for Pa, and for Mfx was from 0.3 μg/ml to 40 μg/ml. Two-dimensional micro dilution checkerboard was prepared by dispensing the serially diluted Pa in the X-axis and Mfx in the Y-axis of the 96-well micro dilution tray except the first well on the left in the order from low concentration to high concentration. Pa test concentration range was from 0.075 μg/ml to 8 μg/ml and Mfx test concentration range was from 0.03 μg/ml to 2 μg/ml, as shown in (Table 1). Three-dimensional micro dilution checkerboard was based on the two-dimensional micro dilution checkerboard. Rfb or Rft was diluted five concentration gradients and then dispensed throughout the wells except the first well on the left at sub inhibitory concentrations ranging from 1/32 to 1/2 of the MIC. Add 200 μl of standardized suspension per well and at the same time add 10% the amount of bacteria to the first well on the left as growth control. After the inoculation of specimen suspension, the culture media was incubated at 36°C. The results were observed with the aid of a matching inverted magnifying glass after seven days, ten days and fourteen days respectively. The appearance of visible white bacterial precipitation in the bottom of the well indicates positive. The MIC of PaMfx synergy is the lowest concentration causing white bacterial precipitation in the first row/column well of the checkerboard less than that of the 1:10 drug-free control. The MIC of three drug synergy is the lowest concentration causing white bacterial precipitation in of the well in addition to the first row and the first column well of the checkerboard less than that of the 1:10 drug-free control.

The test results were interpreted by fractional inhibitory concentration index (FICI). For the standard two-dimensional checkerboard assay, the FICI was calculated and interpreted as: FICI = MIC [A] combination / MIC [A] alone+ MIC [B] combination / MIC [B] alone; where A and B were the two respective antimicrobial agents tested. The lowest FICI was used to interpret the test results as follows: synergism, ≤ 0.5; indifference, >0.5–4; and antagonism, >4 [9]. Calculation of the FICI for a three-dimensional checkerboard was modified as: FICI = MIC [A] combination / MIC [A] alone+ MIC [B] combination / MIC [B] alone + MIC [C] combination / MIC [C] alone, where A, B and C were the three respective antimicrobial agents tested. The lowest FICI was used to interpret the test results as follows: synergism, ≤0.75; indifference, >0.75–4; and antagonism, >4[8].

Results

Antimicrobial effect of Rfb and Rft

We developed susceptibility test to determine MICs of Rfb and Rft alone against twenty isolates. The result indicated that the antimicrobial effect of Rfb was dramatically better than Rft ($P<0.05$).

Two-agent checkerboard assay of PaMfx combination

Mfx was tested in two-dimensional checkerboard plates in combination with Pa. The combination of Pa and Mfx was interpreted

	1	2	3	4	5	6	7	8	9	10	11	12
A	No drug	Pa0.0075	Pa0.015	Pa0.03	Pa0.06	Pa0.125	Pa0.25	Pa0.5	Pa 1	Pa2	Pa 4	Pa 8
B	Mfx 0.03	Pa0.0075 Mfx 0.03	Pa0.015 Mfx0.03	Pa0.03 Mfx0.03	Pa0.06 Mfx0.03	Pa0.125 Mfx0.03	Pa0.25 Mfx 0.03	Pa0.5 Mfx 0.03	Pa 1 Mfx 0.03	Pa2 Mfx0.03	Pa 4 Mfx0.03	Pa 8 Mfx 0.03
C	Mfx 0.06	Pa0.0075 Mfx0.06	Pa0.015 Mfx 0.06	Pa0.03 Mfx 0.06	Pa0.06 Mfx 0.06	Pa0.125 Mfx 0.06	Pa0.25 Mfx 0.06	Pa0.5 Mfx 0.06	Pa 1 Mfx 0.06	Pa2 Mfx 0.06	Pa 4 Mfx 0.06	Pa 8 Mfx 0.06
D	Mfx 0.12	Pa0.0075 Mfx 0.125	Pa0.015 Mfx0.125	Pa0.03 Mfx 0.125	Pa0.06 Mfx 0.125	Pa0.125 Mfx 0.125	Pa0.25 Mfx 0.125	Pa0.5 Mfx0.125	Pa 1 Mfx 0.125	Pa2 Mfx 0.125	Pa 4 Mfx 0.125	Pa 8 Mfx0.125
E	Mfx 0.25	Pa0.0075 Mfx 0.25	Pa0.015 Mfx 0.25	Pa0.03 Mfx 0.25	Pa0.06 Mfx 0.25	Pa0.125 Mfx 0.25	Pa0.25 Mfx 0.25	Pa0.5 Mfx 0.25	Pa 1 Mfx 0.25	Pa2 Mfx 0.25	Pa 4 Mfx 0.25	Pa 8 Mfx 0.25
F	Mfx 0.5	Pa0.5 Mfx0.5	Pa 1 Mfx0.5	Pa2 Mfx0.5	Pa 4 Mfx 0.5	Pa 8 Mfx0.5	Pa16 Mfx 0.5	Pa0.5 Mfx 0.5	Pa 1 Mfx 0.5	Pa2 Mfx0.5	Pa 4 Mfx 0.5	Pa 8 Mfx 0.5
G	Mfx 1.0	Pa0.0075 Mfx 1	Pa0.015 Mfx 1	Pa0.03 Mfx 1	Pa0.06 Mfx1	Pa0.125 Mfx 1	Pa0.25 Mfx1	Pa0.5 Mfx 1	Pa 1 Mfx1	Pa2 Mfx1	Pa 4 Mfx 1	Pa 8 Mfx1
H	Mfx 2.0	Pa0.0075 Mfx 2	Pa0.015 Mfx 2	Pa0.03 Mfx2	Pa0.06 Mfx2	Pa0.125 Mfx2	Pa0.25 Mfx 2	Pa0.5 Mfx2	Pa 1 Mfx 2	Pa2 Mfx 2	Pa 4 Mfx 2	Pa 8 Mfx 2

Table 1: Checkerboard layout pattern

as being indifferent, with FICI ranging from 0.5 to 1.0. Only two isolates revealed synergy with FICI <0.5, as shown in (Table 2).

Three-agent checkerboard assay of PaMfxRfb and PaMfxRft combination

As shown in Table 2, the combination of PaMfxRfb and PaMfxRft was interpreted as being synergy to most of the tested MDR-TB and XDR-TB isolates, especially PaMfxRft combination. PaMfxRfb combination showed synergism against two MDR isolates with FICI of 0.31and 0.53 and five XDR isolates with FICI ranging from 0.54 to 0.59; showed indifferent against eight MDR isolates with FICI ranging from 0.78 to 1.26 and four XDR isolates with FICI ranging from 0.76 to 1.25. Additionally for one XDR isolate, the synergy could not be determined because the MIC did not fall into the set range. As for the PaMfxRft combination, FICI of ten MDR isolates ranged from 0.16 to 0.655, all showing synergism. FICI of eight XDR isolates ranged from 0.34 to 0.75, showing synergism. FICI of one XDR isolate was 1.28, showing indifferent. Also for one XDR isolate, the synergy could not be determined because the MIC did not fall into the set range. Significantly statistical difference was detected between the FICI of PaMfxRfb combination and PaMfxRft combination (P=0.003).

Discussion

Current research for combined anti-TB drugs sensitivity *in vitro* mostly contains only two drugs. The reason is that with the number of drugs in combined sensitivity test increases, the times of configuration of drug concentrations increases exponentially, but the accuracy reduces

Isolate No.	MfxPa	MfxPaRfb	MfxPaRft
1	0.75	1.03	0.655
2	0.625	1.26	0.53
3	0.51	1.25	0.545
4	1	0.31	0.53
5	0.515	0.53	0.575
6	0.25	0.78	0.28
7	0.56	0.875	0.16
8	0.75	1.03	0.53
9	1.0	0.78	0.655
10	0.28	0.81	0.28
11	ND	ND	ND
12	0.53	0.59	0.53
13	0.74	0.56	0.655
14	0.98	1.25	1.28
15	0.15	0.76	0.75
16	1.0	0.56	0.34
17	0.75	0.54	0.65
18	0.75	0.53	0.56
19	0.515	0.78	0.54
20	0.5	1.25	0.37
Range	0.15-1.0	0.53-1.26	0.16-1.28
H37Rv	no growth		

ND: not determined

Table 3: FICI of the twenty clinical isolates

[4]. However, study on combined drug sensitivity of three drugs is more practical since most of the Anti-TB therapeutic regimens contain at least three drugs [2]. Chemotherapy for retreatment tuberculosis in the national key project for infectious diseases is consisted of five drugs, but we are more concerned about the synergism of Rfb or Rft, Mfx and Pa, especially combinations of MfxPaRfb or MfxPaRft in vitro. We accordingly conducted the quantitative three-dimensional checkerboard study on these four drugs.

The new generation of fluoroquinolones has been shown to be highly useful as second-line anti-TB agents against MDR-TB, especially Levofloxacin (Lfx), Moxifloxacin (Mfx) and Gatifloxacin (Gfx), which are expected to shorten the course of treatment [8]. Studies *in vitro* and animal experiment have demonstrated that Moxifloxacin has a high intracellular concentration, therefore has a good anti-TB activity [10]. Pa is a compound which is composed of aminosalicylic acid and isoniazid. It is well known that INH is potentially capable of altering cell wall permeability. Aminosalicylic acid increases the bactericidal effect of Isoniazid by delaying acetylation of Isoniazid in vivo. Pa is soluble and easily absorbed. It also has the characteristics of light irritation to the gastrointestinal tract and light injury to liver [11]. Some of the isolates which are resistant to aminosalicylic acid and isoniazid are susceptible to Pa [12]. Consequently, for a part of drug-resistant PTB, Mfx and Pa are still effective. To our knowledge, however, interaction of the two antimicrobial agents against M. tuberculosis has not been systematically evaluated. Our results indicate that most FICI of the two-agent combination was from 0.5 to 1.0, indicating indifferent. FICI of only three isolates was less than 0.5, indicating synergism. At that time, the concentration of Pa and Mfx is 0.06-1 μg/ml and 0.03-0.25 μg/ml respectively. Therefore, we draw the conclusion that there is no clear synergism of Pa and Mfx except in a condition of certain concentration.

Rfb and Rft are derivatives of rifampicin (Rfp). Antibacterial mechanism of them is to inhibit DNA dependent RNA polymerase, like Rfp. Moreover, Rfb has a higher affinity with beta subunit of RNA polymerase than Rfp and it can inhibit RNA dependent DNA synthesis directly. So the *in vitro* antibacterial effect of Pfb is 2-4 times of Rfp [13]. The half-time of Rft is much longer than Rfp. The intracellular mobility of Rft is 10 times of Rfp. The MIC of Rft is significantly lower than Rfp [14-16]. Although Rfb and Rfp have been used to treat drug-resistant tuberculosis as first-line agents, there has not been report on the *in vitro* synergism of the two agents with other drugs. Our study found that the combination of MfxPaRfb showed synergism only to two MDR isolates and five XDR isolates. However, the combination of MfxPaRft showed synergism to ten MDR isolates and eight XDR isolates. It is apparent that MfxPa demonstrated marked synergism against MDR and XDR isolates when combined with Rfb or Rft. But the synergistic effect of MfxPaRft is stronger than that of MfxPaRfb (P=0.003). This is not consistent with the results of single drug MIC testing. The possible reasons are not necessarily explainable at present. Therefore, it is necessary to carry out *in vitro* checkerboard assay to test the synergism of different combination of drugs before formulating new chemotherapy regimens.

In China, this research is the first report of using the checkerboard method to analyze the *in vitro* combined effect of three kinds of anti-tuberculosis drugs. We successfully obtained the result of synergistic effect of three combinations of MfxPa, MfxPaRfb and MfxPaRft. The result can provide reference for the formulation of drug-resistant tuberculosis chemotherapy in clinic. Due to small sample size, it

is necessary to do more research on this method to investigate the application value.

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