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# An investigation of the antimicrobial impact of drug combinations against *Mycobacterium tuberculosis* strains*

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**Aim:** To investigate the in vitro activity of linezolid and ofloxacin in combination with first-line antituberculosis agents (isoniazid and rifampicin) against *M. tuberculosis* strains. The use of combinations that include fluoroquinolones and oxazolidinone is now being considered for the treatment of resistant *Mycobacterium tuberculosis* strains.

**Materials and methods:** The minimum inhibitory concentrations of the test drugs were determined by the standard agar dilution method. The interaction of drug combinations was investigated by time-kill method.

**Results:** A total of 9 *M. tuberculosis* strains were used in this study, 5 of which were multidrug-resistant (MDR) and 4 of which were not. The studied 2-agent drug combinations were indifferent in 4 MDR and 2 non-MDR *M. tuberculosis* strains, respectively. Additive interaction was observed between isoniazid and ofloxacin in one MDR *M. tuberculosis* strain on the eighth day and in one non-MDR strain on the third, fifth, and eighth days, respectively, while rifampicin and linezolid exhibited additive interaction in another non-MDR strain on the eighth day.

**Conclusion:** Although many previous studies have found a synergistic activity with similar drug combinations, only additive interaction was observed in some of the *M. tuberculosis* strains involved in this study.

**Key words:** *Mycobacterium tuberculosis*, drug combinations, ofloxacin, linezolid

## *Mycobacterium tuberculosis* kökenleri üzerine ilaç kombinasyonlarının antimikrobiyal etkisinin araştırılması

**Amaç:** Son yıllarda dirençli *Mycobacterium tuberculosis* kökenlerinin tedavisinde florokinolon ve oksazolidinon grubu ilaçların da bulunduğu kombinasyonların kullanımı gündeme gelmiştir. Bu çalışmada, *M. tuberculosis* kökenleri üzerine birinci seçenek antitüberküloz ilaçlardan izoniazid ve rifampisinin, linezolid ve ofloksasin ile kombinasyonunun etkinliğinin araştırılması amaçlandı.

**Yöntem ve gereç:** Antimikrobiyallerin minimal inhibitör konsantrasyon değerleri agar proporsiyon yöntemi ile belirlendi. İlaç kombinasyonlarının etkileşimleri zamana bağlı öldürme eğrisi yöntemi ile araştırıldı.

**Bulgular:** Beşi "çok ilaca dirençli" (ÇİD), dördü ÇİD olmayan toplam dokuz *M. tuberculosis* kökeni incelendi. ÇİD *M. tuberculosis* kökenlerinden dördünde, ÇİD olmayan kökenlerin ise ikisinde, incelenen antibiyotik kombinasyonları arasında etkileşim saptanmadı. Bir ÇİD *M. tuberculosis* kökeninde, sekizinci günde izoniazid-ofloksasin arasında aditif etki belirlendi. ÇİD olmayan kökenlerin birinde, üçüncü, beşinci ve sekizinci günlerde izoniazid-ofloksasin arasında, diğer bir duyarlı kökende ise sekizinci günde rifampisin-linezolid arasında aditif etki görüldü.

**Sonuç:** Bu konuda sınırlı sayıdaki çalışmaların çoğunda benzer ilaç kombinasyonlarında sinerjistik etki saptanmasına rağmen, bu çalışmada bazı kökenlerde sadece aditif etkileşim gözlenmiştir.

**Anahtar sözcükler:** *Mycobacterium tuberculosis*, ilaç kombinasyonları, ofloksasin, linezolid

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## Introduction

Despite being one of the oldest diseases, tuberculosis is still a global epidemic. The mortality rate of tuberculosis is more than 90% in developing countries. Factors such as the high prevalence of human immunodeficiency virus (HIV)-infected patients, the emergence of multidrug-resistant (MDR) cases, and outbreaks involving MDR strains in hospitals have decreased the efficacy of antituberculosis drugs. Isoniazid (INH), rifampicin (RIF), ethambutol (ETB), streptomycin (SM), and pyrazinamide (PZA) are first-line antituberculosis drugs. Recently, however, the number of *Mycobacterium tuberculosis* strains resistant to at least INH and RIF, a resistance which is known as MDR, has increased. Second-line drugs may be used as alternatives for the treatment of infections caused by MDR strains, but these drugs tend to be neither very effective nor cheap. Moreover, many have toxic side effects (1-5).

A failure to effectively treat MDR tuberculosis can lead to the generation of extensively drug-resistant (XDR) *M. tuberculosis* strains. Recent outbreaks of MDR and XDR tuberculosis have led to a demand for the discovery of new antituberculosis drugs (4,6). Fluoroquinolones have emerged as alternative antituberculosis drugs, especially for the treatment of infections due to MDR *M. tuberculosis* strains. Fluoroquinolones inhibit topoisomerase II (DNA gyrase) in *M. tuberculosis*. A notable property of fluoroquinolones relates to their ability to penetrate into macrophages and to exert intracellular mycobactericidal activity. Among the drugs that have shown promising activity in the treatment of MDR tuberculosis is linezolid (LIN). It is a member of the oxazolidinone class and its mechanism of action involves the inhibition of protein synthesis (4,5,7-9).

To prevent the emergence of drug resistance and to lower the toxic side effects, combinations of different antimycobacterial agents are used. Some of the in vitro studies investigating the efficacy of the new drugs use only one antibiotic, but the activity of these new compounds must also be studied in combination with classic drugs (10,11). Hence, we aimed to evaluate the in vitro activity of ofloxacin (OFX), as a member of the fluoroquinolones, and LIN in combination with first-line antituberculosis agents (INH and RIF) against *M. tuberculosis* strains.

## Materials and methods

### Strains and determination of MIC values

After being isolated from various clinical samples (1 strain per patient), 9 *M. tuberculosis* strains were investigated in the Tuberculosis Laboratory of the Department of Microbiology and Clinical Microbiology at Ege University's Faculty of Medicine. Using the agar dilution method, the susceptibility patterns of all isolates were studied with regard to first-line antituberculosis drugs (12). Out of a total of 9 strains, 5 were found to be MDR strains.

OFX (Koçak Ltd. Şti.), LIN (Pfizer Inc.), INH, and RIF (Sigma) stock solutions were prepared in distilled water at a concentration of 1 mg/mL. Aliquots of these solutions were frozen at –20 °C until used.

The agar dilution method was performed as described by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (12), and H37Rv (ATCC 27294) was used as the control strain. Briefly, after sterilization, Middlebrook (MB) 7H10 agar medium (BD & Difco) was cooled to 50-55 °C and supplemented with MB OADC (oleic acid, albumin, dextrose, and catalase) (BD) (10%, vol/vol). The appropriate volumes of diluted LIN, OFX, INH, and RIF stock solutions were incorporated into aliquots of 7H10 agar medium in order to achieve the desired final concentrations of LIN (0.03-4 mg/L), OFX (0.06-4 mg/L), INH (0.025-128 mg/L), and RIF (0.03-128 mg/L). Once the drugs were introduced to the media, the solutions were dispensed quickly into sterile plastic bipartite petri dishes, allowed to solidify, and either used immediately or stored at 4 °C until used. For each strain, one agar medium without drug was also prepared as the growth control. The standard inoculum of each isolate was adjusted to an equal density of 1 McFarland standard by diluting the initial inoculum with MB 7H9 broth (BD & Difco). Final suspensions were prepared using $10^{-2}$ and $10^{-4}$ dilutions of the standardized suspensions with MB 7H9 broth. On each part of the agar plates, 100 µL of the diluted inoculum was placed, both with and without drugs. All plates were sealed and incubated at 37 °C in 5%-10% $CO_2$ for 3 weeks. The MIC of each isolate was determined to be the lowest concentration of an antibiotic that inhibited >99% of the colonies growing on the drug-free control (12).

**Time-kill study**

The in vitro activity of OFX and LIN in combination with first-line antituberculosis agents (INH and RIF) against *M. tuberculosis* strains was determined by use of the time-kill method (6,13). The isolates were first grown to a turbidity of 1 McFarland standard in MB 7H9 broth supplemented with OADC for 7-10 days at 37 °C. The cell suspensions were then adjusted to give a final concentration of $10^5$ CFU (colony forming unit)/mL in the culture broth. The actual number of CFUs per milliliter in each tube at time zero was determined by plating a 0.1 mL fraction of dilutions prepared in MB 7H9 broth. Solutions of single antimicrobial agents and those in combinations were prepared to achieve the MICs of the respective agents. Cell suspensions were added to both the test and control tubes and incubated at 37 °C in 5%-10% $CO_2$ atmosphere. During incubation, a 0.1-mL sample was removed from these tubes on the initial day, as well as on the third, fifth, and eighth days, and spread onto MB 7H10 agar containing OADC. The plates were incubated at 37 °C for 21 days and the colonies were counted, a procedure that was repeated twice. Synergy or antagonism was defined as a decrease or increase of 2 or more in $\log_{10}$ CFU/mL when compared with the figures for the respective single agents. A decrease in CFU/mL between 2 $\log_{10}$ and 1 $\log_{10}$ was evaluated as additive interaction (6,13).

**Results**

The MIC values for INH, RIF, LIN, and OFX are shown in the Table, as are the origins of the *M. tuberculosis* strains. The studied 2-agent antibiotic drug combinations were indifferent in 4 of the MDR *M. tuberculosis* strains and in 2 of the non-MDR strains. Additive interaction was observed between INH and OFX in one MDR *M. tuberculosis* strain on the eighth day (Figure 1), while the same effect was found between INH and OFX in one non-MDR strain on the third, fifth, and eighth days, respectively. A similar additive interaction was also observed between RIF and LIN in another non-MDR strain on the eighth day (Figure 2).

Table. The origins of *M. tuberculosis* strains and MIC values for INH, RIF, OFX, and LIN.

| Strains | Origin | MIC (mg/L) | | | |
|---|---|---|---|---|---|
| | | INH | RIF | OFX | LIN |
| MDR *M. tuberculosis* strains | | | | | |
| 1 | Sputum | 2 | 128 | 0.5 | 0.06 |
| 2 | Urine | 8 | 16 | 0.5 | 0.5 |
| 3 | Sputum | 8 | 8 | 1 | 0.5 |
| 4 | Cerebrospinal fluid | 64 | 32 | 0.5 | 0.5 |
| 5 | Bronchial aspirate | 8 | 4 | 0.5 | 0.5 |
| Non-MDR *M. tuberculosis* strains | | | | | |
| 1 | Abscess | 0.5 | 0.125 | 0.5 | 0.5 |
| 2 | Sputum | 0.05 | 0.125 | 1 | 0.06 |
| 3 | Sputum | 0.5 | 0.06 | 1 | 0.5 |
| 4 | Bronchial aspirate | 0.5 | 0.25 | 0.5 | 0.25 |

Multidrug-resistant: MDR; minimum inhibitory concentration: MIC;
isoniazid: INH; rifampicin: RIF; ofloxacin: OFX; linezolid: LIN.

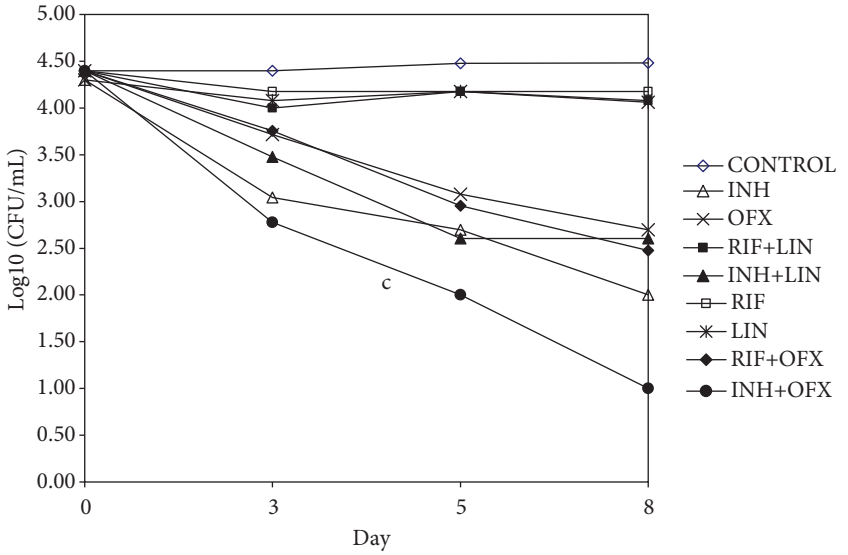


Figure 1. Time-kill curve results of MDR strain 4 at the MIC values of antibiotics. Isoniazid: INH; rifampicin: RIF; ofloxacin: OFX; linezolid: LIN; RIF + LIN: no interaction; INH + LIN: no interaction; RIF + OFX: no interaction; INH + OFX: additive.

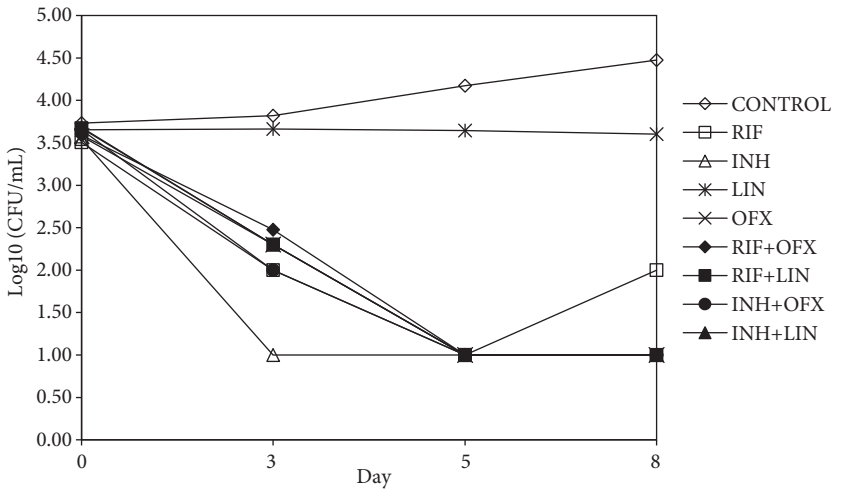


Figure 2. Time-kill curve results of non-MDR strain 2 at the MIC values of antibiotics. Isoniazid: INH; rifampicin: RIF; ofloxacin: OFX; linezolid: LIN; RIF + LIN: additive; INH + LIN: no interaction; RIF + OFX: no interaction; INH + OFX: no interaction.

## Discussion

The treatment of multidrug-resistant tuberculosis strains is extremely difficult, and a well-equipped laboratory is needed to determine drug resistance in these strains. Second-line antituberculosis drugs are used in the treatment of infections caused by strains that are resistant to first-line drugs. However, the effectiveness of these drugs is low, they are not cost effective, and they may also have toxic side effects. Therefore, tuberculosis chemotherapy requires new therapy options that have both different action mechanisms and bactericidal effects (7,10). LIN,

which inhibits the protein synthesis in bacteria, and OFX, a DNA gyrase enzyme inhibitor, are extremely effective against resistant tuberculosis strains. They have therefore recently acquired considerable importance in the treatment of MDR *M. tuberculosis* strains (1,5,14-16).

In recent years, a variety of in vitro studies have been conducted that have demonstrated the effectiveness of both LIN and OFX against tuberculosis. However, these drugs have to be used in combination with other antituberculosis drugs in order to prevent resistance development and to control the tuberculosis epidemic. These combinations do not only shorten the duration of treatment of the active disease but also destroy the latent *M. tuberculosis* strains in asymptomatic patients (10,17,18).

Rodriguez Diaz et al. (10) examined 16 *M. tuberculosis* strains and determined the synergism between INH and fluoroquinolones in 9 of the 10 INH-sensitive strains and in only 1 of the 6 INH-resistant strains. A synergistic effect between INH and LIN was observed in 3 of the 10 INH-sensitive strains while no interaction was seen in the INH-resistant strains. Synergistic activity was observed between RIF and LIN in 5 and between RIF and fluoroquinolones in 4 of the 15 RIF-sensitive strains. Such an interaction was not determined in the RIF-resistant strains, however. In the study conducted with strains in which the MDR characteristic was not specified, it was observed that the above mentioned combinations led to a synergistic effect primarily in the INH- and RIF-sensitive strains. A similar effect was observed between INH and fluoroquinolones in the 1996 study of Rastogi et al. (7), performed on INH-sensitive strains. Although synergism was not determined in this study, additive interaction was detected between INH and OFX in 3 strains, including 1 MDR strain.

In a study conducted in 2005 (11), the interaction between first-line antituberculosis drugs and 28 different agents was investigated using the 3-dimensional checkerboard method. The study made use of 10 different *M. tuberculosis* strains, including 7 that were MDR strains. Using the checkerboard method, it was concluded that a potential synergism exists between INH and RIF and a group of antimicrobials including agents such as fluoroquinolones and clarithromycin. This finding was confirmed by the time-kill curve method. Subsequently, the interaction of these selected agents with RIF or INH was investigated using the 2-dimensional checkerboard technique and no synergism or antagonistic effect was observed. These results are consistent with the time-kill curve obtained for this study.

In a study conducted by Lu and Drlica (19) to investigate the interaction between moxifloxacin, a C-8 methoxy-substituted fluoroquinolone, and first-line antituberculosis drugs, they reported that an additive effect was present between INH and moxifloxacin and that the same interaction was also observed between lower concentrations of RIF and moxifloxacin. Generally, C-8 methoxy- or chloro-substituted agents from the fluoroquinolone group, such as moxifloxacin, gatifloxacin, and sitafloxacin, have been used in studies in which a synergistic effect was found between fluoroquinolones and first-line antituberculosis drugs (11,19). In this study, however, OFX, an effective alternative antituberculosis drug, was used and only additive interactions were observed in some strains. It has been reported in some studies that the C-8 methoxy- and chloro-fluoroquinolones demonstrate a better antituberculosis activity compared to classical fluoroquinolones (11).

In a study investigating the effectiveness of the combination of LIN with first-line antituberculosis drugs, synergy was determined in INH- and RIF-susceptible strains, while no interaction was observed in RIF- or INH-resistant strains (10). Although the synergistic activity of OFX and LIN in combination with first-line antituberculosis agents was reported in most studies, indifferent or additive interactions were detected in this study. Because antagonism was not found, it was concluded that the studied 2-agent drug combinations could be used in the therapy of clinically suitable cases.

Since the data about this topic is limited, further studies investigating the antituberculosis effectiveness of combinations containing LIN and fluoroquinolones are needed. Additionally, an increase in the number of studies investigating the in vivo effectiveness of antituberculosis drug combinations would add clarity to the general knowledge in this field.

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