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Scientific Research

Screening of Several Anti-Infectives for *in Vitro* Activity against *Mycobacterium smegmatis*

Grace Lovia Allotey-Babington[1,2], Henry Nettey[1], Philip Debrah[1], Ofosua Adi-Dako[1], Clement Sasu[1], Anastasia Antwi[1], Yvonne Darko[1], Newriza Nartey[1], Jida Asare[1]

[1]University of Ghana School of Pharmacy, Legon, Ghana
[2]Mercer University School of Pharmacy, Atlanta, GA, USA
Email: hnettey@msn.com, hnettey@ug.edu.gh

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Abstract

Aim: To evaluate *in vitro* the effectiveness of several anti-infective agents alone or in combination against *Mycobacterium smegmatis*. Method: A convenient stratified sampling method was used to obtain selected anti-infective agents. For individual drug samples, Minimum Inhibitory Concentrations (MIC) were obtained using the agar-well plate diffusion technique. Fractional Inhibitory Concentration Indices (FICI) were calculated for drug combinations using their MIC as obtained from the broth dilution method. Results: Of the thirty (30) anti-infective agents analyzed, ten (10) had MIC equivalent to or better than rifampicin (reference TB drug). Seven (7) drugs had MIC higher than rifampicin, while twelve (12) showed no growth inhibition of *M. smegmatis*. Analysis of the effect of drug combinations on *M. smegmatis* indicated that four (4) combinations, including rifampicin/ethambutol showed synergism. One (1) was additive, two (2) were indifferent and one (1) combination showed antagonism. Conclusion: Notable in the results obtained was the high effectiveness of the carbapenems in inhibiting the growth of *M. smegmatis*. Carbapenems, though not indicated for TB treatment, has a potential of playing a significant role in the treatment of tuberculosis. Also the drug combinations which showed synergism, especially those that involved the macrolide antibiotics, should further be investigated. These results have to be confirmed by *in vivo* clinical studies to define their roles in tuberculosis treatment.

Keywords

Antimicrobials, Minimum Inhibitory Concentration, *M. smegmatis*, Tuberculosis

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1. Introduction

Tuberculosis (TB) is an infection ultimately caused by the *Mycobacterium tuberculosis* (MTB) that is spread from person to person through airborne particles [1]. TB of the lungs, resulting in symptoms such as chronic bloody coughs, night sweats and weight loss, is the most common clinical manifestation of MTB infection. However, any organ in the body can be affected by spread of bacteria through the lymphatic system, causing disseminated or extra-pulmonary TB [2]. Extra-pulmonary TB can manifest itself as pericarditis, meningitis, or spinal TB [3].

Transmission of *Mycobacterium tuberculosis* occurs by inhalation of contaminated droplets released from the lungs of an infected individual, typically through coughing. Upon inhalation, the bacterium is ingested by means of phagocytosis by resident alveolar macrophages and tissue dendritic cells (DC), which are designed to kill pathogens but inside which MTB can subvert the killing mechanisms to allow replication [4].

MTB replicates very slowly, with a doubling time of about 24 hours. The bacterium measures around 0.5 μm in diameter and 1 - 4 μm in length, and is an aerobic intracellular pathogen [2]. There are other species which include *bovis*; the causative organism of TB in cows and rarely in humans. Others belonging to the *Mycobacterium* genus include *Mycobacterium avium* which causes a TB-like disease especially prevalent in patients in the advanced stages of Acquired Immune Deficiency Syndrome (AIDS), *Mycobacterium leprae*, the causative agent of leprosy as well as *Mycobacterium smegmatis* is non-pathogenic and very useful for the research analysis of other species in the genus Mycobacteria in cell culture laboratories among others [5].

Mycobacterium smegmatis, a soil dwelling saprophyte is distantly related to *M. tuberculosis*, is a mycobacterium model that is used to understand the pathogenesis of *M. tuberculosis* because of the disadvantages in the direct study of the mycobacterium. One disadvantage is that *M. tuberculosis* is a Category 3 human pathogen, requiring biosafety level III laboratory and animal facilities, substantial training before handling, and carries with it a risk of accidental exposure [6]. Secondly, *M. tuberculosis* grows slowly and colony formation requires two to three weeks, making its utilization for experimentation, time consuming [7]. Apart from its avirulent nature, allowing for the use of biosafety one laboratory, *M. smegmatis* is also fast growing and colony generation occurs in two to three days. According to Barry 2009 [8], 12 out of 19 *M. tuberculosis* virulence genes described to date share closely related homologues in *M. smegmatis*. Further, to determine the usefulness of *M. smegmatis* as an anti-tubercular drug discovery model, Altaf *et al*. 2010 [9] quantified the efficiency of *M. smegmatis* in detecting compounds that are inhibitory towards *M. tuberculosis* in compound library screening. From their results, *M. smegmatis* clearly illustrated usefulness in tuberculosis drug discovery.

The key to successful elimination of tuberculosis (TB) is treatment of cases with optimum chemotherapy. Isoniazid (INH), rifampin (RIF), ethambutol (EMB), pyrazinamide (PZA) and streptomycin (STR) are the essential first-line anti-tuberculosis drugs. Second-line anti-tuberculosis drugs include Aminoglycosides (kanamycin), quinolones (ciprofloxacilin), ethionamide, among others. The major problem of global concern is the emergence of resistance to existing medications by the causative organism. There have however been urgent calls for the inclusion of antimicrobials with some *in vitro* anti-tuberculosis (anti-TB) activity as part of the recommended drugs for the treatment to help solve the problems of high cost of therapy, inaccessibility of anti-TB drugs as well as bacterial resistance to existing medications. This motivated our interest to screen, *in vitro*, various anti-infective agents for effect against *M. smegmatis*.

2. Materials and Methods

2.1. Test Organism

The test organism, *Mycobacterium smegmatis* (MC2 155) was a gift from Noguchi Memorial Institute, Legon, Ghana. Middlebrook 7H9 powder, nutrient agar, and all reagents used for experiments were purchased from VWR, U.S.A. A loop full of a 24 hr culture of *M. smegmatis* was transferred into sterile water and enumeration was done using the counting chamber method.

2.2. Drugs

Drug standards were obtained as gifts from the Centers for Disease Control, Atlanta, GA, USA. All other antimicrobials were purchased as tablets, capsules, or injectables from various pharmacies in Ghana and the United States of America. Drugs were prepared initially as 4 mg/ml stock in 10% Dimethyl sulfoxide (DMSO) solution.

Final drug working solutions were prepared in Middlebrook 7H9 with 1% DMSO.

2.3. Agar Diffusion Method-Individual Drug Solutions

25 ml nutrient agar portions were melted over boiling water at 100˚C and stabilized in a water bath kept at 45˚C prior to use. The stabilized agar was aseptically seeded with 100 µl inoculum, equivalent to 2.0×10^6 cells/ml of *Mycobacterium smegmatis* and transferred into a sterile petri dish. Four wells were made in agar using a sterile #7 cork borer.100µl of various concentrations (0.2 - 40 µg/ml) of each anti-infective agent was transferred to each well, except for the last well which contained media without drug. The plates were prepared in triplicates with each plate serving as its own control. Negative control plates were prepared with only sterile media in the wells. The zones of inhibition (ZOI) were measured as the diameter of clear area without bacterial lawn around the edges of the wells. The minimum inhibitory concentration (MIC) was obtained as zero intercept of a linear regression of the diameter of these inhibition zones, y, plotted against the natural logarithm of the antibiotic concentration, x (**Figure 1**).

2.4. Microdilution-Checkerboard Method

Double strength (D/S) Middlebrook 7H9 media was used for this test. 1000 µl of D/S Middlebrook broth was pipetted into each well of 24-wellmicrodilution plates. 200 µl of the first anti-infective of the combination was put, in decreasing order of dilutions, into wells along the ordinate, while 200 µl of the second drug was put into wells along the abscissa. 100 µl each of the two drugs was put in the corresponding wells to produce drug combination volume of 200 µl in varying concentrations. Each well was, then, filled with 700 µl of media with 1% DMSO solution and inoculated with 100 µl bacterial inoculum equivalent to 2.0×10^6 cells/ml. Wells for positive control without any drug content and negative control wells without any inoculum were included on the plates. The resulting checkerboard contained each combination of two anti-infectives, with wells that contained the highest concentration of each antibiotic at opposite corners. The plates were incubated at 37˚C for 24 hrs under aerobic conditions. The MIC was determined as the lowest concentration of anti-infective that completely inhibited the growth of the organism as detected with the naked eye. The Fractional Inhibitory Concentration Index (FICI) was then calculated from the MIC of the individual drugs and combinations. Checkerboard assays result in a number of FIC indices. The sum of a number of FIC indices divided by the number of indices is designated as average, ΣFIC. The ΣFICs were calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the

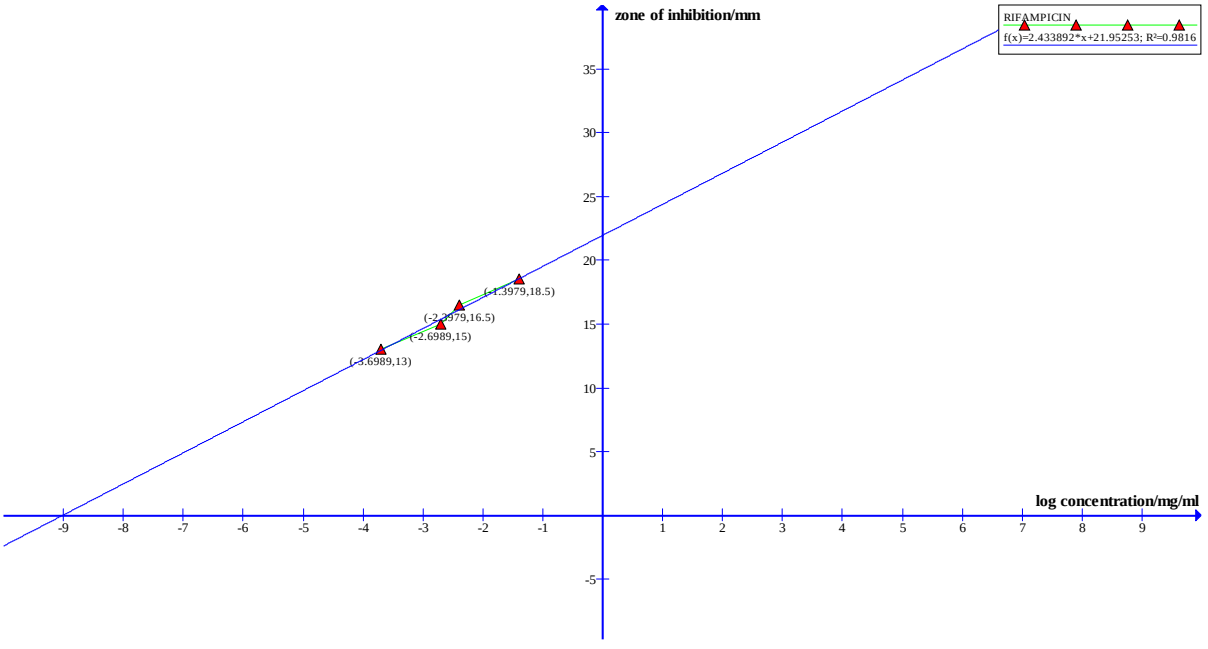

Figure 1. Sample Plot for MIC calculations.

MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination is considered synergistic when the ΣFIC is ≤0.5, additive when 0.5 < ΣFIC < 1, indifferent when the 1 < ΣFIC < 2, and antagonistic when the ΣFIC is ≥2 [10] [11].

3. Results and Discussion

3.1. Results

3.1.1. Minimum Inhibitory Concentrations of Individual Anti-Infectives

Agar diffusion method is a susceptibility test which gives an indication of the effectiveness of anti-infective agents against infections. In determining whether microbes are susceptible or not, the zones of inhibition are compared with that of reference ranges and an inference made. In the absence of reference ranges for the drug the MIC calculated for each drug is compared with the MIC of a standard anti-tuberculosis drug(s) used. For drug combinations, the MIC is compared with standard anti-TB drug combinations used in clinical practice. As such in the susceptibility determination a drug combination can be said to be effective against the mycobacterium if its MIC is equal to or lower than that of the standard combinations.

The MIC obtained for rifampicin, a first-line anti-TB drug was 4.16×10^{-3} µg/ml per the agar diffusion method. Analysis of all other individual antimicrobials showed that meropenem, doripenem, ciprofloxacin, clarithromycin, erythromycin and clindamycin had considerably lower MIC than rifampicin (**Table 1**). Gentamicin, levofloxacin, tetracycline and piperacillin/tazobactam had MICs comparable to that of rifampicin. Imipenem, amikacin, cefaclor, doxycycline, dicloxacillin, azithromycin, and chloramphenicol had MICs much higher than that of rifampicin (**Table 1**). All the other first-line TB drugs, isoniazid, ethambutol and pyrazinamide were not active agents the strain of *M. smegmatis* used.

3.1.2. Effect of Various Drug Combinations on *M. smegmatis*

The standard anti-tuberculosis drug combination used was Rifampicin/Ethambutol. The MIC obtained for the combination was 8.37×10^{-2} µg/ml as obtained from an agar diffusion method (data not included).The MIC's of each drug alone and in combination with a second drug were obtained (**Table 2**). In all the drug combinations, the MIC of drug A in combination with drug B was either approximately equal to or lower than the MIC of drug A alone. Likewise, the MIC of drug B in each combination was close to or lower than the MIC of drug B alone, except the clindamycin/amikacin combination where amikacin in the combination had a higher MIC than alone (**Table 2**).

3.2. Discussion

The mean zones of inhibition exhibited by the anti-infective agents used in the study indicate some level of susceptibility of *Mycobacterium smegmatis* to these agents even at concentrations lower than 2×10^{-4} mg/ml, which was the lowest concentration used. For a method such as broth dilution the lowest concentration at which the isolate is completely inhibited (as evidenced by the absence of visible bacterial growth) is recorded as MIC.

The lower MIC of the carbapenems, ciprofloxacin, clarithromycin, erythromycin and clindamycin as compared to that of rifampicin indicate that *Mycobacterium smegmatis* is susceptible to these agents and hence the agents can be described as effective against this specie. Ciprofloxacin is a 2nd line TB drug, whereas clarithromycin and erythromycin are 3rd line TB drugs; however the carbapenems are not indicated for the infection. The low MIC indicated for the carbapenems show that at much lower concentrations than rifampicin, they can be more effective in inhibiting the growth of *Mycobacterium smegmatis* and hence have promising efficacy against *Mycobacterium species.*

According to the broth dilution method, each individual drug was to some extent effective against *M. smegmatis* as observed from their MIC; however, the aim of drug combination of individual drugs is to achieve susceptibility with synergy. This is indicative of a good combination which can be developed for clinical use. The checkerboard method further tests the drug combinations and characterizes their susceptibility as synergistic, additive, indifferent or antagonistic. An indifferent effect of a combination of antibiotics is one that is equal to the effects of the most active component. The additive effect of a combination of antibiotics is one in which the effect of the combination is equal to that of the sum of the effects of the individual components. Synergistic effect of a combination of antibiotics is present if the effect of the combination exceeds the additive effects of

Table 1. Minimum Inhibitory Concentrations (MIC) of various anti-infectives obtained by the Agar diffusion method as compared with the reference anti-TB drug-Rifampicin. NA = No Activity.

Anti-Infective Agents	MIC (mg/ml)	MIC (μg/ml)
Rifampicin (Reference)	4.16E−06	4.16E−03
Meropenem	1.58E−10	1.58E−07
Dorepenem	2.23E−07	2.23E−04
Imipenem	1.07E−04	1.07E−01
Piperacillin/Tazobactam	3.47E−06	3.47E−03
Amikacin	5.13E−05	5.13E−02
Gentamicin	3.24E−06	3.24E−03
Cefaclor	8.12E−03	8.12
Ciprofloxacin	6.17E−07	6.17E−04
Levofloxacin	1.41E−06	1.41E−03
Doxycycline	4.17E−05	4.17E−02
Dicloxacillin	2.45E−05	2.45E−02
Tetracycline	6.87E−06	6.87E−03
Azithromycin	8.71E−05	8.71E−02
Clarithromycin	7.24E−09	7.24E−06
Erythromycin	1.99E−07	1.99E−04
Clindamycin	9.55E−07	9.55E−04
Chloramphenicol	1.25E−05	1.25E−02
Amoxicillin	NA	NA
Amoxicillin/Clavulanate	NA	NA
Ceftriaxone	NA	NA
Flucloxacillin	NA	NA
Penicillin G Sodium	NA	NA
Nitrofurantoin	NA	NA
Pentamidine	NA	NA
Oseltamivir	NA	NA
Quinine	NA	NA
Isoniazid	NA	NA
Ethambutol	NA	NA
Pyrazinamide	NA	NA

Table 2. Minimum Inhibitory Concentrations (MIC) of Drug Combinations from broth dilution and the checkerboard method. A_a-MIC of drug A alone by broth dilution method, A_c-MIC of drug A in combination by checkerboard method, B_a-MIC of drug B alone by broth dilution method, B_c-MIC of drug B in combination by checkerboard method.

Drug combinations (A/B)	MIC (mg/ml) A_a	MIC (mg/ml) A_c	MIC (mg/ml) B_a	MIC (mg/ml) B_c
Rif/Eth	4×10^{-2}	1.0×10^{-2}	4×10^{-1}	5.0×10^{-3}
Cip/Flu	4×10^{-3}	2.5×10^{-4}	4×10^{-1}	1.0×10^{-2}
Ery/Dox	4×10^{-2}	2.0×10^{-2}	4×10^{-3}	2.5×10^{-4}
Cla/Nit	4×10^{-2}	5.0×10^{-3}	4×10^{-1}	1.0×10^{-2}
Azi/Amo	4×10^{-3}	2.5×10^{-4}	4×10^{-1}	1.0×10^{-1}
Lev/Dic	4×10^{-4}	4.0×10^{-4}	4×10^{-1}	2.0×10^{-1}
Gen/Cef	4×10^{-2}	4.0×10^{-2}	4×10^{-3}	2.5×10^{-4}
Ami/Cli	4×10^{-2}	8.0×10^{-3}	4×10^{-3}	2.0×10^{-2}

Table 3. Minimum Inhibitory Concentrations (MIC) of Drug Combinations using the checkerboard method and their interpretation according to the reference ranges. Synergism when ΣFIC is ≤0.5, additive when ΣFIC is >0.5 - 1, indifferent when the ΣFIC is >1 to <2, and antagonistic when the ΣFIC is ≥2 [10] [11].

Drug Combinations	ΣFIC	Interpretation
Rif/Eth	0.26	Synergy
Cip/Flu	0.30	Synergy
Ery/Dox	0.56	Additive
Cla/Nit	0.38	Synergy
Azi/Amo	0.30	Synergy
Lev/Dic	1.50	Indifference
Gen/Cef	1.06	Indifference
Ami/Cli	5.20	Antagonism

the individual components whereas antagonism is present if a reduced effect of a combination of antibiotics is observed in comparison with the effect of the most effective individual substance [10].

From the characterization by the checkerboard method, combinations of Rifampicin/Ethambutol, Ciprofloxacin/Flucloxacillin, Clarithromycin/Nitrofurantoin and Azithromycin/Amoxicillin showed synergism. Erythromycin/Doxycycline showed an additive effect, combinations of Levofloxacin/Dicloxacillin and Gentamicin/Cefaclor showed indifference whereas the combination of Amikacin/Clindamycin showed antagonism (**Table 3**).

Antibiotics exert their effects as either bacteriostatic agents or bactericidal agents. By definition, a "bacteriostatic" agent is one that prevents the growth of bacteria. In other words, it keeps them in the stationary phase of growth. On the other hand, "bactericidal" agents kill actively growing bacteria [12]. In such light, the drug combinations were chosen such that drugs of similar effectiveness, to the extent possible, would be combined to potentiate their effects.

However from the results of the checkerboard method, it is observed that combinations of Clarithromycin/Nitrofurantoin and Azithromycin/Amoxicillin produced synergistic effects though they possessed different antibacterial effectiveness. Both Nitrofurantoin and Amoxicillin are considered bactericidal agents whereas the macrolides, such as Azithromycin and Clarithromycin are considered classic bacteriostatic drug classes [13]. Specific macrolides—Erythromycin, Azithromycin and Clarithromycin have been shown to have bactericidal activity, *in vitro*, against *Streptococcus pyogenes* and *Streptococcus pneumonia* [14]-[16]. As a result Clarithromycin and Azithromycin could have in combination exhibited bactericidal activity against *M. smegmatis* and thus producing the synergistic effect.

Notably the combination of Amikacin/Clindamycin showed a significant antagonistic effect with FICI of 5.20. An evaluation of Amikacin indicated that it was highly bactericidal for *Mycobacterium tuberculosis* [17]. Clindamycin, on the other hand, may be bactericidal *in vitro* depending on microorganism and growth factors though it is a known bacteriostatic agent [18] [19].

In terms of their mechanism of antibacterial action, Amikacin irreversibly binds to 30 S and 50 S ribosomal subunits to interfere with the initiation of bacterial protein synthesis [20]. Clindamycin blocks peptide bond formation and also inhibits bacterial protein synthesis by binding to the 50 S ribosomal units [21]. Both drugs exert their antibiotic effect via a similar mechanism of action-inhibition of 50 S ribosomal enzymes. Such similarity could have accounted for the drugs producing an antagonistic effect when in combination.

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