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Comparative Study of Ofloxacin Accumulation in *M. smegmatis, M. kansasii,* and *M. tuberculosis*

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Abstract

Fluoroquinolones (FQs) manifest bactericidal activity in a concentration-dependent manner as they inhibit DNA gyrase and topoisomerase activity that are crucial in the replication process. They are the key component of a multidrug-resistant tuberculosis treatment regimen. FQs are effective against bacterial infections that cause skin, urinary tract, and prostate-related infections. Ofloxacin is also being tried as part of modified Multidrug Treatment (MDT) regimens against leprosy. This study examined the accumulation of ofloxacin in *M. smegmatis, M. kansasii,* and *M. tuberculosis.* These mycobacteria were grown in Sauton's liquid medium up to the exponential phase. Finally, cells were harvested, cleaned by washing, and resuspended. Suspensions were incubated with ofloxacin at 37 °C, and ofloxacin concentration in supernatants was observed using spectrofluorimetry. Ofloxacin concentrations were also observed in the absence or presence of Carbonyl cyanide m-chlorophenylhydrazone (CCCP), which is a proton motive force inhibitor. Steady state concentration (SSC) of ofloxacin was achieved in *M. smegmatis* (03 minutes), *M. kansasii* (03 minutes), and *M. tuberculosis* (05 minutes), and the ofloxacin accumulation level was 102 ng/mg, 100 ng/mg, and 107 ng/mg (dry weight of the bacilli), respectively. Ofloxacin accumulation was found at 10 µg/ml concentrations; however, CCCP addition was unable to influence the ofloxacin accumulation. This study concludes that ofloxacin accumulation is a simple diffusion in *M. smegmatis, M. kansasii,* and *M. tuberculosis*. Through simple diffusion, ofloxacin is transported across the mycobacterial cell envelope, inhibiting type II DNA isomerase (gyrase), which is required for DNA replication. Thus, it is effective in the treatment of disease.

Keywords: *Mycobacteria, Ofloxacin, Fluoroquinolones* (FQ), Carbonyl Cyanide *m*-chlorophenyl-hydrazone

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INTRODUCTION

The reduced permeation of antimicrobials through the cell wall of mycobacteria is crucial in antibiotic resistance in different mycobacterial species.[1-3] Rare antimicrobial agents are efficiently active against *Mycobacterium tuberculosis* as well as atypical mycobacteria (*Mycobacterium avium* complex). Tuberculosis (TB) is the foremost contributor to the high mortality rate worldwide. Only a few antimycobacterial agents are clinically active against *Mycobacterium tuberculosis*,[4] which is frequently prescribed in the present era, specifically in combinations due to the emergence of multidrug-resistance (MDR). MDR is often viewed as a consequence of interrupted treatment of active TB during the longer course regimens, weak monitoring, poor patient compliance, healthcare facilities, and socioeconomic factors.[5,6] For the bacterial dormancy or antibiotic-resistant phase, antibiotic pressure is also required. Fluoroquinolones (FQs) in combination are used clinically both as first and second-line treatment, and thus are considered valuable weapons against tuberculosis. Due to the higher potential and broad-spectrum activity of FQs, they are widely used as antibacterial drugs.[7] FQs bind to DNA gyrase, topoisomerase II, and IV, leading to the inhibition of replication and transcription processes.[8] *M. tuberculosis, M. bovis, M. leprae, M. marinum, M. kansasii,* and *M. xenopi* growth was repressed in vitro by ofloxacin/ciprofloxacin 0.5-2.0 mg/L concentrations.[9-10] Broeck et al. reported the DNA gyrase structure of *E. coli* in the form of a nucleoprotein complex trapped by the gepotidacin antibiotic.[11] Disentanglement of the DNA-binding and cleavage domain leads to an enhanced insight into the enzyme complex's allosteric movements, which opens the avenue for targeting the conformational complexes or intermediates. As DNA gyrase is intracellular, FQs should reach inside the cell to exhibit the antibacterial effect. Therefore, permeability plays a crucial role in the FQs accumulation among the mycobacterial species.[12] Ofloxacin is extensively used as part of modified multidrug therapy regimens in the treatment of leprosy. A pilot study was done to check the accumulation of ofloxacin drug in *M. smegmatis, M. tuberculosis,* and *M. kansasii* by fluorometry. *M. smegmatis* was considered a model organism because of its fast-growing nature and ability to produce homogeneous suspensions as compared to other species.[13] The "TB Lipid Man", Minnikin, proposed a chemical model depicting structural elucidation of the cell envelope in mycobacteria, the presence of mycolic acid, a virulence factor,[14] and applied it to studies of *M. smegmatis* and other mycobacteria.[15] Furthermore, for exploring the mycobacterial genetics, *M. smegmatis* has been considered as a model strain.[16] *M. kansasii* is a non-tuberculosis mycobacterium (NTM), a slow grower, and shows the clinical pattern of infection in six different ways, as in other mycobacterial species. *M. kansasii* infection usually causes chronic pulmonary cavitary disease, and due to this, initially, the patients are misdiagnosed with pulmonary tuberculosis.[17] Apart from this, it also causes musculoskeletal infections, skin and soft tissue disease, lymphadenitis, sacroiliitis, skin lesions, and catheter-associated disease.[18] TB is the biggest killer among infectious diseases despite the availability of the BCG vaccine and chemotherapy. Emergence of the drug-resistant *M. tuberculosis*, co-infection with HIV, and SARS-CoV-2, is still considered the biggest public health issue in the 21st century.[19] An earlier study evaluated the different quinolone accumulation in other mycobacteria.[20] The present study aimed to speculate on the OFX accumulation in *M. smegmatis, M. kansasii,* and *M. tuberculosis* using fluorometric analysis.

MATERIALS AND METHODS

Antibiotics and chemicals

FQs (Ofloxacin, Sigma-Aldrich), carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) were acquired from Merck (USA), 50 mM Sodium Phosphate Buffer (pH 7), and 0.1 M Glycine-HCl were procured from Merck (USA), and double-distilled water was purchased from Central Drug House (Delhi). Other chemicals were procured from local vendors.

Ethical consideration

The study deals with a mycobacterial strain, which has been taken from the repository

of our Institute. This study does not deal with human subjects; it is exempt from the human ethical committee.

Type of sampling and reasons for selection

Mycobacteria were used in this study. The reason for the selection of Mycobacteria for the study was that ofloxacin transports across the cell envelope through simple diffusion.

Inclusion criteria

To compare the accumulation of ofloxacin in non-tuberculous mycobacteria and tuberculous mycobacteria.

Exclusion criteria

Mycobacteria other than *M. tuberculosis,* which causes infection in humans, were excluded.

Bacterial strain

Standard strains of *Mycobacterium smegmatis* (ATCC-607), *Mycobacterium kansasii,* and *Mycobacterium tuberculosis,* which are sensitive to ofloxacin, were obtained from the repository of ICMR- National JALMA Institute for Leprosy and Other Mycobacterial Diseases, India.

Ofloxacin accumulation in *M. kansasii, M tuberculosis,* and *M. smegmatis*

M. kansasii, M tuberculosis, and *M. smegmatis* were cultured separately in Sauton's liquid medium until the exponential phase. Cell pellets, centrifuged and suspended in buffer (0.1 M phosphate buffer, pH 7.2, O.D. of 0.4-0.5) and incubated in the presence of ofloxacin (10 µg/ml) at 37 °C. One ml of each aliquot was taken at various time intervals (minutes) for twenty minutes, and centrifuged, the ofloxacin concentration was tested in the supernatants using spectrofluorimetry. A similar set of experiments was also done with Carbonyl cyanide m-chlorophenylhydrazone (CCCP), at 100 µM, 10 minutes before and after ofloxacin addition.[21]

Dry cell weight

To proceed with every set of experiments, 1 mL aliquots of each *M. smegmatis, M. kansasii,*

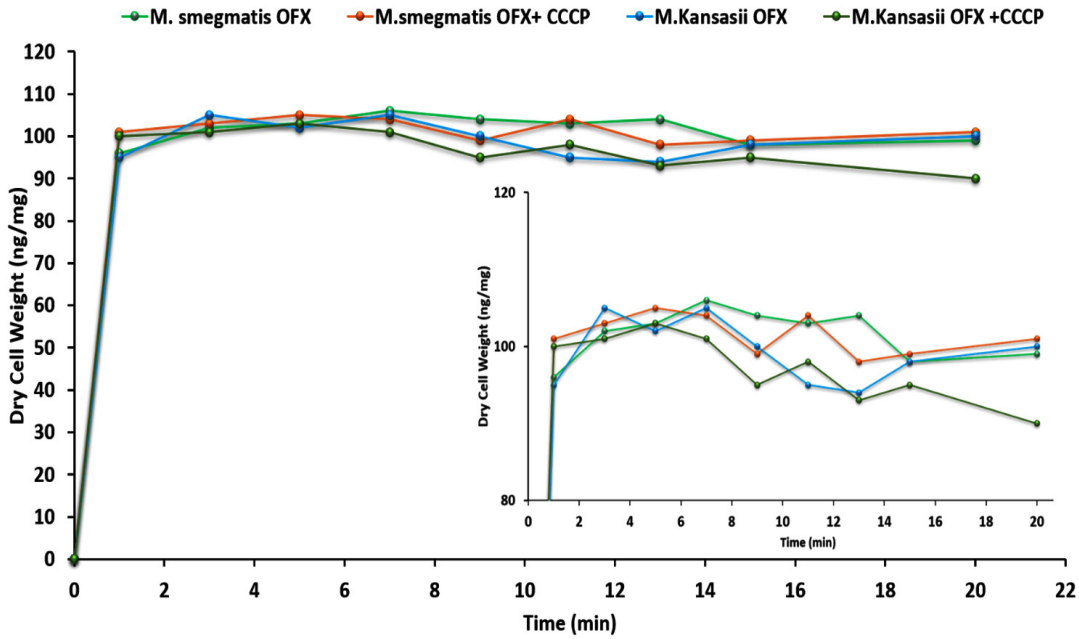

Figure 1. Accumulation of ofloxacin (OFX) in *M. smegmatis* and *M. kansasii* without and with CCCP (ng/mg dry cell weight; *Mean of 3 values)

Table 1. Accumulation of ofloxacin (OFX) in *M. smegmatis* and *M. kansasii* without and with CCCP (ng/mg dry cell weight; *Mean of 3 values)

Time (Minutes)	*M. smegmatis* OFX	*M. smegmatis* OFX + CCCP	*M. kansasii* OFX	*M. kansasii* OFX + CCCP
0	0	0	0	0
1	96	101	95	100
3	102	103	105	101
5	103	105	102	103
7	106	104	105	101
9	104	99	100	95
11	103	104	95	98
13	104	98	94	93
15	98	99	98	95
20	99	101	100	90

and *M. tuberculosis* suspension were taken in 1.5 mL tubes separately. Then, cells were centrifuged at 3000 x g for 15 minutes at 4 °C. The cell pellet was again washed with HPLC-grade water (10 mL) and then centrifuged at 3003 x g for 15 minutes at 4 °C. Finally, all pellets were air-dried at 60 °C for approximately twelve hours, and weights were calculated. All the tests were performed in triplicate.

RESULTS

Ofloxacin accumulation

The method employed to determine ofloxacin accumulation was initially developed by Williams et al. for norfloxacin accumulation in *M. aurum* and *M. smegmatis.*[21] To achieve a better fluorescence, the optimum concentration was set at 10 µg/ml for accumulation studies. The ofloxacin accumulation kinetics for *M. smegmatis* and *M.*

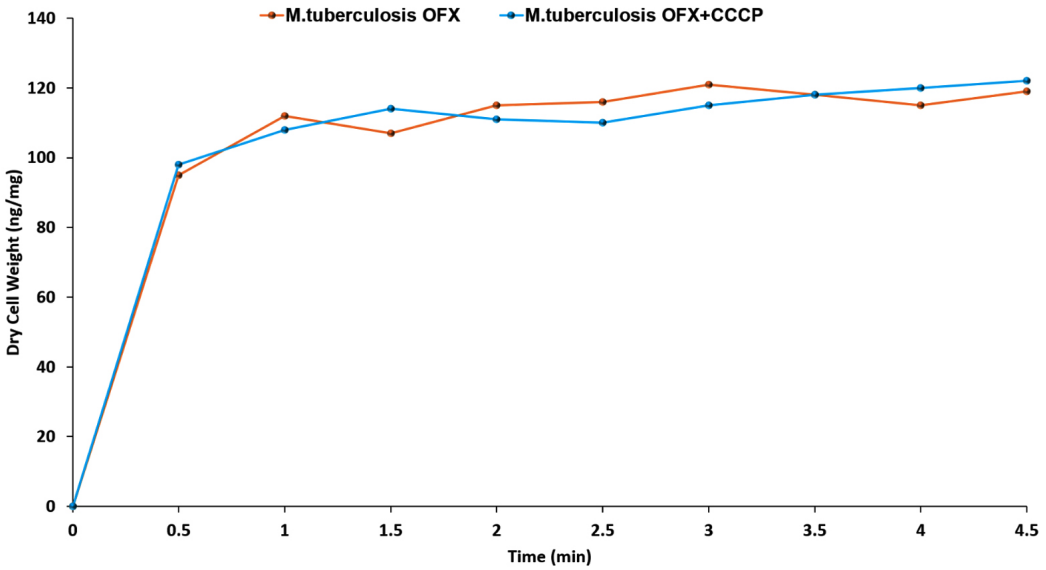

Figure 2. Accumulation of ofloxacin (OFX) in *M. tuberculosis* without and with CCCP (ng/mg dry cell weight; *Mean of 3 values)

Table 2. Accumulation of ofloxacin (OFX) in *M. tuberculosis* without and with CCCP (ng/mg dry cell weight; *Mean of 3 values)

Time (Minutes)	*M. tuberculosis* OFX	*M. tuberculosis* OFX + CCCP
0	0	0
0.5	95	98
1	112	108
1.5	107	114
2	115	111
2.5	116	110
3	121	115
3.5	118	118
4	115	120
4.5	119	122

kansasii have been shown (Figure 1) and (Table 1). The accumulation kinetics in *M. tuberculosis* are tabulated in Table 2 and shown in Figure 2.

Steady State Concentration (SSC) of ofloxacin in *M. smegmatis* was achieved within 3 minutes, and the ofloxacin accumulation level was 102 ng/mg of the bacilli dry weight. For *M. kansasii* and *M. tuberculosis,* the time span for SSC was 5 minutes, and the levels of ofloxacin accumulation were 100 ng/mg and 107 ng/mg, respectively. The accumulated ofloxacin concentration was approximately 10 µg/ml of exposed concentrations, which indicated that CCCP was unable to influence the ofloxacin accumulation. The addition of 100 µM CCCP for 10 minutes before the drug exposure did not influence the accumulation kinetics.

DISCUSSION

FQs belong to the family of broad-spectrum antibiotics, are greatly effective towards Gram-negative bacteria, and tremendously impart bactericidal action.[22-25] Bove et al.[26] mentioned that due to their broad-spectrum activity of FQs and their physicochemical properties are mostly used as antimicrobials in human and animal medicine, which has led to great attraction in clinical situations.[24,26] Over the last two decades, FQs have become popular due to their promising group of antimicrobial agents to treat the infection caused by MDR bacterial strains, such as aminoglycosides, β-lactam, and macrolide-resistant microbes.[26,27] The addition of a fluorine atom to nalidixic acid leads to the derivation of FQs. Due to this, the activity against bacteria increases and leads to improved absorption, metabolism, and excretion, including high tissue and intracellular distribution.[28-30] Compared to other groups of antibacterial agents, FQs are more effective at lower concentrations.

Against most of the susceptible microbes, FQs' minimum inhibitory concentrations (MICs) range from 0.001-1.0 µg/ml,[25] which was due to the prolonged post-antibiotic effect. Molecularly, Intracellular enzymes such as DNA gyrase and topoisomerase IV are the major targets of FQs. While in the microbe, FQs enter via pores and quickly accumulate with specific steady state concentrations.[31,32] The enzyme-DNA complex was stabilized by FQs' covalent binding, where the DNA is usually cleaved into two strands.[33] The binding is in a cooperative manner by DNA gyrase with the help of electrostatic binding. This proposes the binding kinetics of FQs, which can fit into the deep pocket and lead to gyrase inhibition.[34] Type II isomerase unwinds, cuts, and consecutively reseals the DNA; however, FQs inhibit the resealing and lead to fragment generation, which are later destroyed by exonucleases.[35,36] FQs' resistance does not emerge quickly because it is usually due to a mutation in the intrinsic genes of microbes, and it is rarely reported in plasmids.[23] Plasmid-mediated FQs resistance has not been documented.[37]

In vitro and in vivo studies showed FQs as potent anti-mycobacterial agents.[38,39] There is a tremendous effort to inculcate the FQs into frontline antibiotics like ofloxacin, moxifloxacin, and ciprofloxacin.[40-43] Industrial efforts have also been seen in the discovery and development of newer FQs, which might have potential in TB treatment. However, FQs' selection for TB treatment is based on their efficacy in murine models; therefore, few FQs are tested in these murine models. The cell wall of mycobacteria comprises 60% of lipids by dry weight, giving it extreme hydrophobicity, and has been used in chemotaxonomy and pathogenesis.[44-46] For maintaining cellular integrity and virulence, *M. tuberculosis* has a special structure of cell envelope (peptidoglycan layer) and composition.[47]

Jacobo-Delgado explored the convolution of the *M. tuberculosis* cell wall and analyzed the antimicrobial peptides' efficacy to clear the bacilli.[48] In *M. chelonae,* permeability for β-lactams is three times less than *E. coli* and 10-times higher than *P. aeruginosa*.[1] FQs are usually hydrophobic, and passive diffusion via lipid bilayer or porin is the existing mechanism in Gram-negative bacteria[49]; however, in bacteria (Gram-positive), it is simply diffusion across the membrane.[50] Due to the hydrophilic nature of norfloxacin, its accumulation was non-saturable in *M. aurum* up to the 0-100 mg/ml concentration range, and its transportation was not affected in CCCP presence.[51] Therefore, they suggested that noroxacin is transported through a non-saturable porin pathway. Porin efficiency in *M. smegmatis* and *M. chelonae* was lower than that of other bacterial porins.[52,53] Ofloxacin is a broader-spectrum analog of norfloxacin, a second-generation fluoroquinolone, and its uptake could be due to the amino acid transporter.[54] Newer generation FQs have an extended spectrum and improved activity against mycobacteria and other bacteria compared to older generation FQs. For ofloxacin accumulation, the cell wall does not serve as a barrier.

Earlier, Deo et al. reported the level of OFX, NFX, LFX, and CFX accumulation in *M. smegmatis* by simple diffusion.[55] The SSC observed in the present study is similar to NFX-SSCs in *M. smegmatis* mc2155, as shown in Liu et al.[56] Another previous report showed that the SSC of NFX was 35 ng/mg for *M. smegmatis* (strain CNCM 7326). Kocagoz et al.[57] also found that NFX-SSC was 110 pmol/mg of cells (35 ng/mg of cells) for avirulent strain *M. tuberculosis*, which suggests that NFX is less permeable compared to other mycobacteria.[58,59] The present study shows the OFX accumulation in *M. smegmatis, M. kansasii,* and *M. tuberculosis.* The SSC of OFX in *M. smegmatis* was 3 minutes, and the 102 ng/mg dry weight was the level of accumulation level. In *M. kansasii,* SSC time was five minutes, and 100 ng/mg was the accumulation level; however, in *M. tuberculosis,* SSC was 107 ng/mg in five minutes. The permeability of mycobacteria to ofloxacin varies between mycobacterial species.

CONCLUSION

In this study, the transportation of ofloxacin across the cell envelopes of *M. smegmatis, M. tuberculosis,* and *M. kansasii* was studied by diffusion. The intracellular level of drug in the presence of CCCP inhibitor was not affected, which suggests that FQs are transported by other means, not by efflux pumps, in these mycobacterial drug-susceptible clinical isolates. For validation, more studies are needed with FQ-resistant clinical isolates.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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