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# Eagle Effect in Nonreplicating Persister Mycobacteria

**Mu-Lu Wu, Jasmie Tan,* Thomas Dick**

Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, National University Health System, Singapore

**We determined the microbicidal activities of antibacterials against nonreplicating *Mycobacterium smegmatis* grown in a starvation-based Loebel model for persistence. Whereas most drugs lost their activity, fluoroquinolones retained lethal potency. Dose-response characterizations showed a paradoxical more-drug-kills-less Eagle effect. Pretreatment of cultures with chloramphenicol blocked the lethal action of the gyrase inhibitors. These results suggest that fluoroquinolones at low concentrations trigger a protein synthesis-dependent cell death pathway and shut off this suicide pathway at elevated concentrations.**

The long treatment time required to cure tuberculosis is due to the ability of *Mycobacterium tuberculosis* to persist in its human host despite extensive chemotherapy. Persister bacilli are thought to be non-drug-susceptible nonreplicating organisms (1, 2). To study persister mycobacteria, a culture model based on nutrient deprivation, known as the Loebel model, was developed (3). When exponentially growing tubercle bacilli are transferred to phosphate-buffered saline (PBS), the bacteria stop replicating but maintain viability for extended periods (4, 5). Drug susceptibility studies revealed that antitubercular drugs show strongly reduced bactericidal activities against these nonreplicating starved bacilli (6–8).

To facilitate the analyses of the molecular mechanisms underlying this nutrient starvation-induced mycobacterial dormancy response, we demonstrated previously that the fast-growing and nonpathogenic relative of the tubercle bacillus *Mycobacterium smegmatis* mc$^2$155 is also capable of surviving starvation in PBS and thus may be a useful model for the dissection of this process (M.-L. Wu and T. Dick, unpublished data).

Here, we carried out a comparative analysis of the bactericidal activities of antimycobacterials against nongrowing *M. smegmatis* that were starved for 14 days in PBS versus exponentially growing bacteria to characterize the susceptibility of the nonreplicating form of the organism. To generate starved *M. smegmatis* culture, the saprophyte was grown in 7H9 liquid medium to an optical density at 600 nm of 0.5, and cells were collected by centrifugation, washed, and resuspended in PBS with 0.025% Tween80 at $10^7$ CFU/ml and incubated in roller bottles at 2 rpm for 14 days. The cultures were then diluted to $10^6$ CFU/ml and treated with 100 μM of various antimycobacterials for 1 day, and the effect on viability was determined via CFU enumeration on 7H10 agar (4). In parallel, exponentially growing cultures in 7H9 medium adjusted to $10^6$ CFU/ml were exposed to the same drugs at the same concentration and for the same time to determine the bactericidal effect on replicating cells. Table 1 shows that most drugs lost their bactericidal activity against nonreplicators, as expected. However, two drug classes, aminoglycosides and fluoroquinolones, retained a significant level of activity against the otherwise nonsusceptible bacilli.

To characterize the bactericidal activity of these two drug classes against nongrowing bacteria, comparative dose-response experiments were carried out in which exponentially growing and 14-day-old starved cultures were exposed to 1 to 100 μM streptomycin and ciprofloxacin, respectively.

Streptomycin (MIC = 0.6 μM) displayed monophasic kill curves; i.e., higher concentrations of the aminoglycoside drug killed more bacilli in exponentially growing and nonreplicating starved cultures. Exposure to 5 μM streptomycin resulted in a 3-log CFU reduction under both culture conditions. Other translation inhibitors, including linezolid, tetracycline, clarithromycin,

**TABLE 1** Drug susceptibility of 14-day-old starved versus exponentially growing *M. smegmatis* cultures

| Drug | Fold kill in[a]: | |
|---|---|---|
| | Growing culture | Nongrowing culture |
| Isoniazid | 60 | 1 |
| Rifampin | 40 | 1 |
| Ethambutol | 20 | 1 |
| Linezolid | 30 | 1 |
| Tetracycline | 22 | 1 |
| Clarithromycin | 14 | 1 |
| Chloramphenicol | 8 | 1 |
| Erythromycin | 6 | 1 |
| Ciprofloxacin[b] | >10,000 | 100 |
| Ofloxacin | >10,000 | 30 |
| Moxifloxacin[b] | >10,000 | 16 |
| Streptomycin | >10,000 | >10,000 |
| Kanamycin | >10,000 | >10,000 |
| Amikacin | >10,000 | >10,000 |

[a] Cultures were exposed to the drugs at 100 μM for 1 day, and survival was determined via CFU enumeration. Fold kill was calculated as the ratio of initial CFU/ml ($10^6$)/surviving CFU/ml after 1 day of drug treatment. 1, no kill; >10,000, surviving CFU/ml for the particular drug was below the limit of detection ($10^2$ CFU/ml).
[b] See Fig. 1 for a higher resolution of lethal activities of ciprofloxacin and moxifloxacin.

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Address correspondence to Thomas Dick, thomas_dick@nuhs.edu.sg.
* Present address: Jasmie Tan, Laboratory of Virus Evolution, Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore.

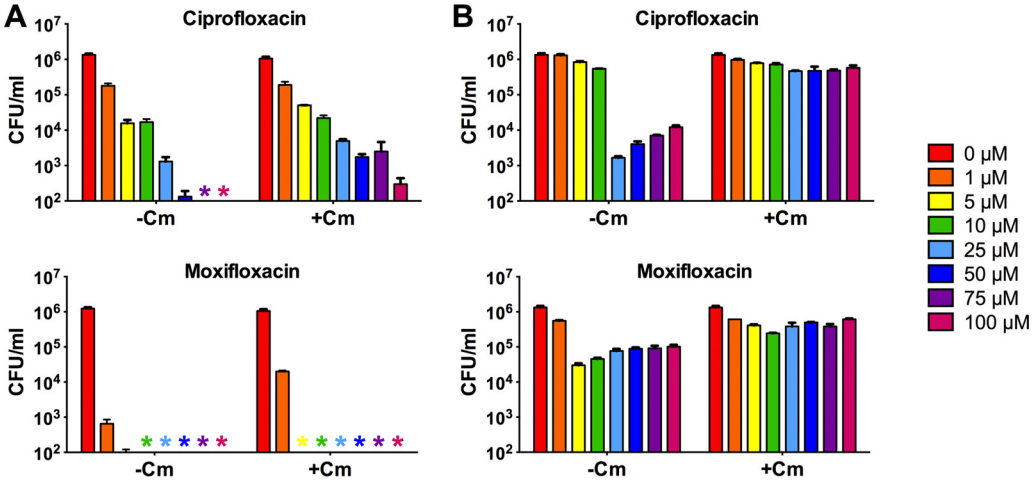


**FIG 1** Concentration-kill curves of ciprofloxacin and moxifloxacin for exponentially growing (A) and nutrient-starved (B) 14-day-old nongrowing *M. smegmatis* cultures. −Cm, without chloramphenicol pretreatment, cultures were exposed to the fluoroquinolones at 1 to 100 μM for 1 day, and survival was determined via CFU enumeration; +Cm, cultures were pretreated with a sublethal (growth-inhibitory) concentration of chloramphenicol (45 μM) for 1 h to halt growth and then exposed to the fluoroquinolones. Experiments were performed three times independently in triplicate, and representative results are shown as means and standard deviations. Colored asterisks on the *x* axis indicate that the CFU concentration for the particular drug concentration was below the limit of detection ($10^2$ CFU/ml).

and chloramphenicol, did not show this behavior (Table 1), indicating that this aminoglycoside effect might be due to their property of inducing mistranslation or might be protein synthesis independent.

We then characterized the microbicidal activities of ciprofloxacin. Figure 1A (−Cm) shows the concentration-kill curve of the drug (MIC = 0.6 μM) for growing *M. smegmatis*. Exposure to 5 μM fluoroquinolone resulted in a 2-log CFU reduction compared to the initial inoculum. Higher concentrations of ciprofloxacin (50 to 100 μM) killed the growing culture down to the limit of detection, i.e., >4 logs.

Surprisingly, starved nonreplicating mycobacterial cultures showed a triphasic concentration-kill curve (Fig. 1B, −Cm). Ciprofloxacin concentrations of up to 10 μM showed only a mild effect on *M. smegmatis* viability, while the same concentrations caused a loss of viability of 2 logs in growing cultures. At 25 μM, a 3-log kill was observed. A further increase in ciprofloxacin concentration to 50 μM resulted in less kill. Exposure to 100 μM ciprofloxacin further increased survival by 1 log relative to the 25 μM exposure experiment; i.e., more drug killed less. This paradoxical concentration-kill effect was not observed for exponentially growing cultures (Fig. 1A, −Cm).

Previously, Malik and colleagues (9, 10) studied the effect of fluoroquinolones on nonreplicating mycobacteria employing the protein synthesis inhibitor chloramphenicol at static concentrations to halt growth. To determine whether the paradoxical kill effect of ciprofloxacin observed for starvation-induced nonreplicating *M. smegmatis* can also be observed for drug-induced nonreplicating bacilli, we exposed exponentially growing cultures to a growth-inhibitory (nonmicrobicidal) concentration of 45 μM chloramphenicol for 1 h to halt growth (10) and then treated the nongrowing culture with 1 to 100 μM ciprofloxacin. Figure 1A (+Cm) shows a monophasic kill curve of the fluoroquinolone for the growth-arrested cultures: higher concentrations of ciprofloxacin killed more; i.e., the paradoxical kill effect observed for starvation-induced nonreplicating bacilli was not seen against chloramphenicol-pretreated log-phase cultures. Consistent with data reported by Malik and colleagues (9, 10), inhibition of protein synthesis had a strong attenuating effect on the lethal activity of ciprofloxacin; i.e., 5 μM of fluoroquinolone, the concentration that killed 2 logs of growing culture, reduced viability of the chloramphenicol-halted culture by merely 1 log (Fig. 1A, +Cm).

To determine whether inhibition of protein synthesis also affects the ciprofloxacin-induced killing of starvation-induced nongrowing culture, we pretreated 14-day-old starved cultures with 45 μM chloramphenicol (nonmicrobicidal) for 1 h and then added 1 to 100 μM ciprofloxacin. Figure 1B (+Cm) shows that inhibition of protein synthesis almost completely abolished the lethal activity of ciprofloxacin against starved cultures, suggesting that the 3-log kill observed with 25 μM ciprofloxacin in starved (chloramphenicol-free) cultures required protein synthesis.

Malik et al. (10) reported intriguing differences between the cell death mechanism of ciprofloxacin (a C-8-H fluoroquinolone) and moxifloxacin (a C-8-methoxy fluoroquinolone). Whereas inhibition of protein synthesis of growing *M. smegmatis* cultures via pretreatment with chloramphenicol had a strong attenuating effect on the microbicidal activity of ciprofloxacin, the lethal effect of moxifloxacin was almost unaffected by the translation inhibitor. Figure 1A (−Cm/+Cm) shows that this behavior was reproduced under our culture assay conditions. Pretreatment of growing cultures with chloramphenicol had only a minor attenuating effect on the lethal activity of moxifloxacin (MIC = 0.1 μM), thus supporting previous reports that moxifloxacin, in contrast to ciprofloxacin (and gatifloxacin with *M. tuberculosis* [9]), kills mostly via a protein synthesis-independent pathway (10).

To determine whether the triphasic concentration-kill curve observed for ciprofloxacin in starvation-induced cultures can also be observed for moxifloxacin, we treated starved cultures with the C-8-methoxy fluoroquinolone and measured CFU. Figure 1B (−Cm) shows that moxifloxacin treatment also generated a triphasic kill curve. However, the concentration-kill curve was somewhat compressed along the *y* axis (CFU/ml) compared to the

curve generated by ciprofloxacin. Although the optimum microbicidal concentration for moxifloxacin was lower than that for ciprofloxacin (5 μM versus 25 μM), the drug killed fewer bacilli at this concentration (1 log versus 3 logs). Figure 1B (+Cm) shows that pretreatment of nutrient-starved cultures with chloramphenicol reduced the lethal effect of moxifloxacin. However, the impact of protein synthesis inhibition on the lethal activity of moxifloxacin appeared to be less pronounced than that of ciprofloxacin.

Taken together, our results suggest that the motto "the more, the better" does not apply to fluoroquinolones against starvation-induced nonreplicating *M. smegmatis*; i.e., increasing the concentration of fluoroquinolones beyond an optimum bactericidal concentration did not increase the kill but actually increased survival. This phenomenon appears to be drug class specific, as it was not observed for aminoglycosides.

The paradoxical less-kills-more bactericidal phenomenon is termed the Eagle effect, according to Harry Eagle, who first described this phenomenon in 1948 (11). Eagle found that penicillin showed an optimum bactericidal concentration beyond which the rate of bacterial death was reduced against many strains of streptococci and staphylococci (11, 12). The observation Eagle made was later expanded to various Gram-negative bacteria, such as *Haemophilus influenzae* and *Proteus* species (13). Furthermore, drugs other than β-lactams, including colistin, exhibit this paradoxical effect (14). Interestingly, fluoroquinolones were shown to have an Eagle effect against staphylococci and *Escherichia coli* (15–18).

It is important to note that Drlica et al. (19) and Dong et al. (20) observed a fluoroquinolone-induced Eagle effect in growing cultures of *Mycobacterium bovis* BCG. We did not observe this effect in our fluoroquinolone treatments of growing *M. smegmatis*. Furthermore, it is interesting to note that Malik et al. (17) observed an Eagle effect of quinolones for chloramphenicol-treated *E. coli* cultures. We did not observe this effect in chloramphenicol-halted *M. smegmatis* cultures (Fig. 1A, +Cm). The reason for these discrepancies is not clear; differences in incubation time may be involved, since they are known to affect the mechanism of quinolone-mediated killing (21). The fact that we did not see an Eagle effect in growing and chloramphenicol-halted *M. smegmatis* cultures does not necessarily mean that this phenomenon does not occur under these culture conditions. The extensive killing we observed may have obscured such an effect.

What is known about the cell death mechanisms triggered by the fluoroquinolones in bacteria? The quinolones trap type II topoisomerases on DNA as a complex in which DNA is broken but constrained by protein. A consequence of drug-enzyme-DNA complex formation is a reversible inhibition of DNA replication and growth arrest, not death. Cell death arises from poorly understood subsequent protein synthesis-dependent and -independent events in which bacterial chromosomes are fragmented and toxic reactive oxygen species may be generated (22–29).

The observed killing of starved nonreplicating *M. smegmatis* reported here confirms previous observations that fluoroquinolones can cause cell death without concurrent DNA replication (23, 25). This appears to be true across bacterial species. Zhao et al. (30) used a temperature-sensitive *dnaB* mutant of *E. coli* to show that stopping replication had little effect on the lethal activity of quinolones.

How the fluoroquinolones precisely kill starved nonreplicating mycobacteria and how they block this apparently protein synthesis-dependent cell death pathway at elevated concentrations are under investigation. It appears that induction of efflux pumps as an explanation for the drug-induced nonsusceptibility can be excluded, as the pump inhibitors reserpine and verapamil (31, 32) did not eliminate the observed Eagle effect (data not shown). In contrast to most bacteria, which possess two type II topoisomerases (topoisomerase IV and DNA gyrase, both targets for fluoroquinolones), mycobacteria possess only DNA gyrase (33), which should simplify the molecular dissection of the fluoroquinolone-induced kill-and-rescue phenomenon.

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M.-L.W. and T.D. conceived the project, M.-L.W. and J.T. carried out the experiments, and M.-L.W. and T.D. analyzed the data and wrote the manuscript.

We declare that we have no conflicts of interest.

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