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ORIGINAL ARTICLE

Inhibition of the *Mycobacterium tuberculosis* reserpine-sensitive efflux pump augments intracellular concentrations of ciprofloxacin and enhances susceptibility of some clinical isolates

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Background/Purpose: Active efflux is known to play a major role in the resistance of many bacteria to antibiotics. To evaluate the possibility of overcoming resistance by suppressing the efflux, we determined the effect of reserpine, an efflux pump inhibitor.
Methods: Intracellular accumulations and the minimal inhibitory concentrations (MICs) of ciprofloxacin in *M. tuberculosis* H37Rv and 16 clinical isolates were determined, compared, and analyzed. Nine of the clinical isolates were resistant to isoniazid and rifampin (multiple-drug resistant MDR). Five of these were resistant to ciprofloxacin.
Results: A reserpine-inhibited efflux system was identified in the H37Rv control and 10:1 (90.9%) of ciprofloxacin-susceptible and 4:1 (80%) of ciprofloxacin-resistant clinical isolates. The MIC of ciprofloxacin decreased in the presence of reserpine in 3/10 (30%) of the ciprofloxacin-

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susceptible and 2/4 (50%) of the MDR ciprofloxacin-resistant strains that expressed efflux pumps. Two of the efflux-positive, ciprofloxacin-resistant strains in which the MIC of ciprofloxacin was not decreased by reserpine were found to carry a D94A *gyrA* mutation. In contrast, two strains with the D94G *gyrA* mutation were susceptible to ciprofloxacin in the presence of reserpine. An efflux-negative strain, highly resistant to multiple antibiotics, was found to have a novel G247S mutation that differs from known mutations in the QRDR region of the *gyrA* gene.
Conclusion: These findings indicate t hat reserpine can increase intracellular concentrations of ciprofloxacin, but is unable to overcome other mechanisms of resistance in clinical isolates.

Introduction

There were an estimated 9.4 million new cases diagnosed and 1.7 million people died from tuberculosis (TB) in 2009 in the world.[1] Although chemotherapy is highly effective for drug-susceptible strains in compliant patient populations, the world-wide emergence of multidrug resistant strains (MDR) to the first-line agents, isoniazid and rifampin, and often to second line agents has made treatment much more difficult. This drug resistance has resulted in increased use of fluoroquinolones in combination with other second-line agents to treat tuberculosis.

The *Mycobacterium tuberculosis* (*M. tuberculosis*) DNA gyrase type II topoisomerase is the unique target for fluoroquinolones.[2] Subunits A and B of the DNA gyrase are encoded by genes *gyrA* and *gyrB*. Acquisition of fluoroquinolone resistance is mainly due to mutations in specific quinolone resistance-determining regions (QRDRs) of the genetic targets.[3] We and others indicate that the genetic changes cannot completely account for the resistance of clinical isolates.[4–6] This has led to a search for additional mechanisms of fluoroquinolone resistance.

Several efflux proteins have been described in laboratory-derived fluoroquinolone resistant mutants of *M. tuberculosis*.[7–9] To our knowledge, only one study has reported the presence of efflux pumps in clinical isolates of *M. tuberculosis*.[10] A decrease in the MIC of fluoroquinolones was associated with the presence of efflux pump inhibitors. However, it is not clear whether efflux pumps are important determinants of resistance in clinical isolates of *M. tuberculosis*. The current study was designed to obtain additional information about the intracellular events that occur when efflux pump inhibitors are added. To accomplish this, we directly measured intracellular ciprofloxacin concentrations before and after the treatment with the efflux pump inhibitor reserpine and determined its effect on the susceptibility to ciprofloxacin.

We found that most of our clinical isolates, including MDR strains, possess a reserpine-responsive efflux pump and that some, but not all, became more susceptible to ciprofloxacin in the presence of reserpine. These findings are explained, in part, by mutations at the D94A *gyrA* and other sites in DNA.

Methods

Antibiotics and chemicals

Ciprofloxacin (Bayer, Wuppertal, Germany) and reserpine (Sigma-Aldrich Co., St. Louis, MO) were prepared according to the manufacturers' instructions.

Bacterial strains

Clinical isolates of *M. tuberculosis* were obtained from patients with active tuberculosis at the Kaohsiung Veterans General Hospital located in southern Taiwan. Ciprofloxacin susceptibility was determined by standard agar dilution method as described previously.[11] Resistance was defined as an MIC of $\geq$ 2 mg/L. The antimicrobial susceptibility to a panel of commonly used anti-tuberculosis drugs was determined by standard laboratory methods.[12] *M. tuberculosis* strain H37Rv (MIC to ciprofloxacin 0.5 mg/L) was used for quality control.

All experiments were performed in a class II type B2 biosafety cabinet within a biosafety level III facility.

DNA sequencing

DNA was extracted with the Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. DNA segments of *gyrA* and *gyrB* were amplified by polymerase chain reaction (PCR) with the forward and reverse primers described by Siddiqi et al.[13] The amplification was carried out with a GeneAmp System 9600 thermocycler (Perkin-Elmer Corp., Foster City, CA, USA) with the following parameters: 5 minutes at 94°C followed by 30 cycles of 60 seconds at 94°C, 30 seconds at 58°C for *gyrA* and 56.5°C for *gyrB*, and 60 seconds at 72°C and terminated with a final extension step at 72°C for 10 minutes. The PCR products were purified and sequenced with an automated sequencer, ABI PRISM 310 Genetic Analyzer (ABI, Applied Biosystems, Foster City, CA, USA). The sequence data were compared with those previously published.

Measurement of fluoroquinolone accumulation

A modified fluorometric method was adapted to accommodate the growth characteristics of mycobacteria.[14] *M. tuberculosis* colonies were suspended in 7H9 broth. The mixture was vortexed and allowed to settle for 20 minutes. The supernatant fluid was then transferred to a second tube. After the process was repeated twice, the cells were diluted in 10 mL of 50 mM sodium phosphate buffer (pH 7.0) and adjusted with a colorimeter (Vitek) to yield 10% transmittance. The suspension contained approximately 10^8 CFU/mL.

The suspension was incubated at 37°C in a water bath for 10 min. Ciprofloxacin was added at a final concentration of 10 mg/L. The suspension was then divided into two equal portions. Reserpine was added at a final concentration of

20 mg/L at 5 minutes to one portion while buffer was added to the other. 1-mL samples were removed from each at 5, 8, 11, 15, and 20 minutes. The cells were immediately centrifuged at 12,000 rpm in an Eppendorf microfuge (model 5403) for 3 minutes at 4°C. The pellets were washed once with ice-cold sodium phosphate buffer (50 mM, pH 7) and resuspended in 1 mL of 0.1 M glycine hydrochloride (pH 3). The samples were left overnight at room temperature with agitation. They were then centrifuged again and the supernatants were filtered through a 0.45 μm filter to remove debris of lysed bacteria. The intracellular concentration of ciprofloxacin was determined by a fluorescence spectrophotometer atrst author ta would not go to the trash can, ve told her so. As to credits, we should have an open mind to share es not ha the excitation wavelengths of 275 nm and emission wavelengths of 440 nm.[15] The presence of an efflux system was defined as a >30% increase in the intracellular concentrations of ciprofloxacin over a 20-minute period in the presence of reserpine compared to paired untreated controls. Efflux positive strains exhibited reproducible increases of 55 to 154%.

Statistical analysis

Statistical significance of differences between or among groups was analyzed by the Kruskal-Wallis test. The Eta-value was used as a measure of nonlinear correlation in the crosstab in the SPSS version 12.0 software program.

Results

Effect of reserpine on intracellular concentrations of ciprofloxacin

The study strains consisted of 16 clinical isolates of *M. tuberculosis*. A laboratory strain of H37Rv served as a quality control. Among the clinical isolates, 11 were ciprofloxacin-susceptible and 5 were ciprofloxacin-resistant. Nine were MDR strains (resistant to at least isoniazid and rifampin). Four of these were susceptible to ciprofloxacin and five were resistant (Fig. 1).

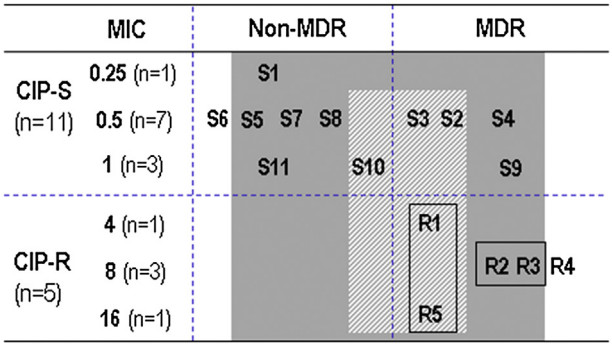

Figure 1 The effect of reserpine on 16 clinical isolates of *M. tuberculosis*. The shaded area represents the efflux positive strains (intracellular ciprofloxacin accumulation increased in the presence of reserpine); the hatched area represents the strains whose MIC was reduced in the presence of reserpine. Strains R2 and R3 have a D94A mutation in the *gyrA* gene; strains R1 and R5 have a D94G mutation in the *gyrA* gene; strain R4 has G247S mutation in the *gyrA* gene.

A reserpine-sensitive efflux system was observed in 10/11 (90.9%) of the ciprofloxacin-susceptible strains and 4/5 (80%) of ciprofloxacin-resistant strains (Fig. 1). Strains S6 and R4 lacked a reserpine-sensitive efflux system. Strain S6 was susceptible to all antimicrobials tested. R4, an MDR strain, was resistant to ciprofloxacin, ofloxacin, levofloxacin, and moxifloxacin. It was also resistant to para-amino-salicylic acid (PAS) and borderline-susceptible to kanamycin (MIC 6 mg/L). This strain was found to have a novel G247S *gyrA* mutation that differs from known mutations in the QRDR region of the *gyrA*.

Intracellular accumulation of ciprofloxacin in the presence or absence of reserpine

All strains of *M. tuberculosis* rapidly accumulated ciprofloxacin, reaching a steady-state within 3–5 minutes as reported by other investigators.[16] The intensity of fluorescence at 5 minutes in the 14 efflux-positive isolates is shown in Fig. 2. There was a significant relationship between the intracellular concentrations of ciprofloxacin and susceptibility to the drug ($p = 0.011$). The four non-MDR ciprofloxacin susceptible strains with MICs of 0.25 to 0.5 mg/L exhibited significantly higher levels of fluorescence than all other strains. All eight MDR strains (Fig. 2) and two non-MDR strains with MICs of 1 mg/L were found to have relatively low levels of fluorescence. The non-MDR strains tended to accumulate more ciprofloxacin than MDR strains ($p = 0.02$).

Effect of reserpine on the MIC of ciprofloxacin

Reserpine reduced the MIC of ciprofloxacin in 3/11 (27.2%) reserpine-responsive ciprofloxacin-susceptible clinical isolates (Fig. 1) and H37Rv. There was a 2-fold reduction in the MIC of ciprofloxacin for strains S2 and H37Rv, 4-fold reduction for strain S10, and >16-fold reduction for strain S3. There was a greater than 3-fold reduction in the MIC of ciprofloxacin for strains R1 and greater than 64-fold for strain R5. Both strains carried a D94G *gyrA* mutation.

Lack of correlation between accumulation of ciprofloxacin in the presence of reserpine among ciprofloxacin-susceptible and resistant strains

In order to determine whether mechanisms other than a reserpine-sensitive efflux might account for differences in the intracellular accumulation of ciprofloxacin, we compared the percent increase in ciprofloxacin fluorescence in the presence of 20 mg/L of reserpine to controls at 20 minutes incubation. The effect of reserpine on accumulation of ciprofloxacin in ciprofloxacin-susceptible non-MDR strains, ciprofloxacin-susceptible MDR strains, and ciprofloxacin-resistant MDR strains is shown in Fig. 3. There were no significant differences in the effect of reserpine on accumulation of ciprofloxacin among the groups (p values of 0.78 and 1.07, respectively). We examined the correlations of MICs of ciprofloxacin with percent increase of ciprofloxacin accumulation in the presence of reserpine as

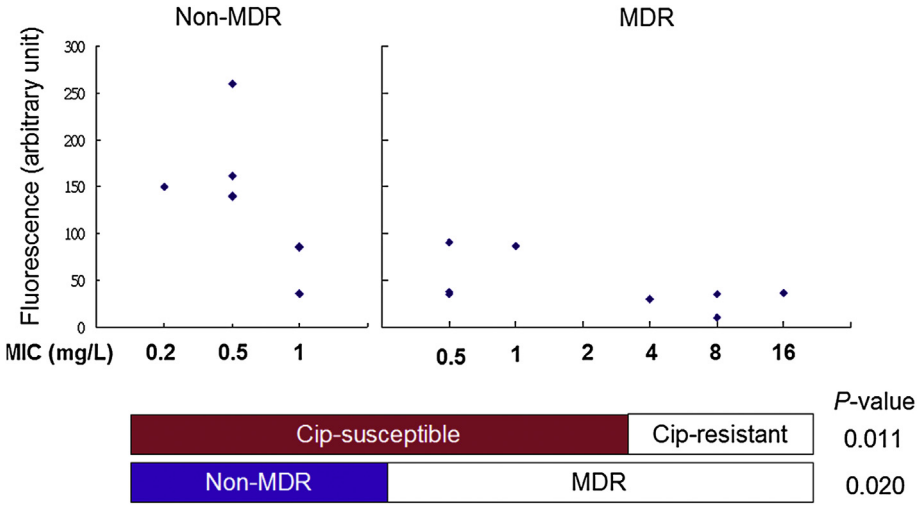

Figure 2 Accumulation of ciprofloxacin in 14 clinical isolates of *M. tuberculosis* shown to have reserpine-sensitive efflux pumps. Ciprofloxacin was added to the bacterial suspension and the intracellular concentration of ciprofloxacin was determined by fluorescence spectrometry (see text). Each dot represents the mean of three measurements from an individual strain. The coefficient of variation over the three measurements for each point averaged for all strains is 0.17. The corresponding MIC is shown on the horizontal axis. A ciprofloxacin MIC of $\geq$2 mg/L is defined as resistant.

an independent variable by calculating the Eta value. With this measure, there was a modest correlation between MICs of ciprofloxacin and increase accumulation of ciprofloxacin in the presence of reserpine in ciprofloxacin-susceptible strains, with an Eta-value 0.571. There was no significant difference between CIP-S non-MDR and CIP-S MDR groups ($p = 0.085$).

Gene mutation

The *gyrA* and *gyrB* genes of all 16 clinical isolates and H37Rv were amplified by PCR and examined by sequencing. None had mutations in *gyrB*. There were no mutations in *gyrA* in the ciprofloxacin-susceptible group. In contrast, all five ciprofloxacin-resistant strains exhibited a *gyrA* mutation.

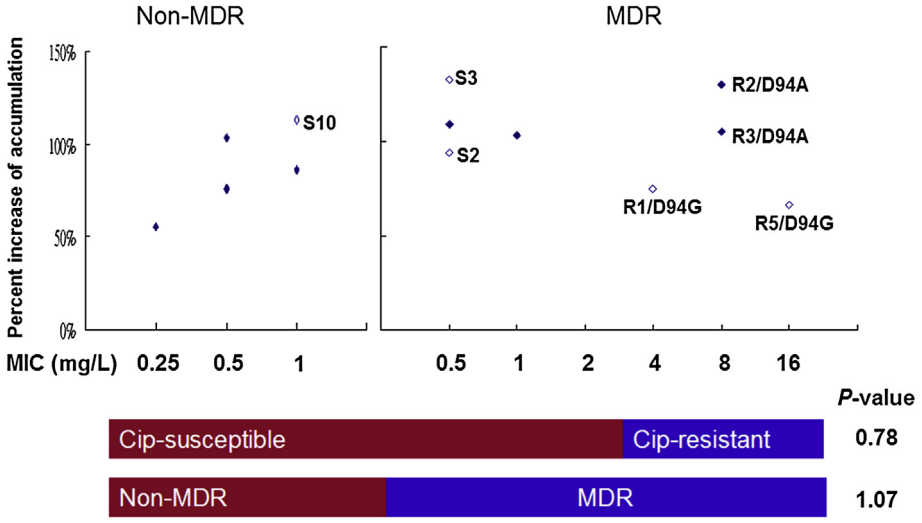

Figure 3 The effect of reserpine on ciprofloxacin accumulation in 14 efflux-positive strains of *M. tuberculosis*. In the presence and absence of 20 mg/L of reserpine, ciprofloxacin was added to the bacterial suspension and the amount of ciprofloxacin retained within the bacteria at 20 minutes was determined by a fluorescence spectrophotometer. The percent increase in accumulation of ciprofloxacin was calculated as the increased amount of ciprofloxacin in the presence of reserpine in relative to the amount in the absence of reserpine. The coefficient of variations over the three measurements for each point, averaged for all strains, are 0.08 and 0.24 for the intensity of fluorescence measured at 20 minutes in the absence and presence of reserpine, respectively. The corresponding MIC in the absence of reserpine is shown in the horizontal axis, and the MIC of ciprofloxacin of $\geq$2 mg/L is defined as "Resistant." The strains in which the MIC of ciprofloxacin decreased in the presence of reserpine are shown in open circles. Reserpine reduced the MIC of S2 from 0.5 mg/L to 0.25 mg/L; S3: 0.5 mg/L to $<$0.06 mg/L; S10: 1 mg/L to 0.25 mg/L; R1: 4 mg/L to $<$2 mg/L; R5: 16 mg/L to $<$0.125 mg/L. The *gyrA* gene mutations are also labeled.

Strains R2 and R3 had a D94A mutation and strains R1 and R5 had a D94G mutation in the QRDR region. Strain R4 had a novel G247S mutation in *gyrA*.

All isolates tested, regardless of susceptibility to ciprofloxacin, carried an S95T substitution in the *gyrA* gene. This is consistent with the previously reported finding that S95T is a marker for evolutionary genetics and does not correlate with drug resistance.[17]

Discussion

Antimicrobial drug resistance in *M. tuberculosis* can occur by several independent mechanisms. These include mutations at target genes or unidentified loci, modif ication of the target(s), decreased permeability and efflux pumps.[18]

Efflux pump-mediated antibiotic resistance has been described in many bacterial pathogens.[19] Drug efflux transporters from all known families except the MATE family (multidrug and toxic compound extrusion family) are found in the sequenced genome of *M. tuberculosis* strain H37Rv (http://www.membranetransport.org). Some have been characterized and shown to be involved in fluoroquinolone resistance. They all belonged to the ATP-binding cassette (ABC) superfamily (DrrABC, PstB, and Rv 2686c-Rv2687c-Rv2688c operon). These transporters are sensitive to reserpine, an ATP-dependent efflux pump inhibitor. Genes *drrAB* expressed in *M. smegmatis* confer resistance to a broad range of clinically relevant antibiotics, including norfloxacin. The resistance phenotype is reversed by treatment with efflux pump inhibitors reserpine and verapamil.[20] On the other hand, a laboratory-generated *M. smegmatis* mutant with high-level transcription and chromosomal amplification of the *pstB* gene increases phosphate uptake. Disruption of *pstB* and thus the phosphate-specific protein in *M. smegmatis* was correlated with a decrease in ciprofloxacin efflux and resulted in an increasing susceptibility to ciprofloxacin.[15] The *M. tuberculosis* Rv2686c-Rv2687c-Rv2688c operon confers resistance to ciprofloxacin (8× MIC) and, to a lesser extent, to norfloxacin (2× MIC) when over-expressed in *M. smegmatis*. The levels of resistance decreased in the presence of efflux pump inhibitors: reserpine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and verapamil.[9]

Drug pumps belonging to the RND family and MATE family and known to confer fluoroquinolone resistance are found in numerous species of Gram-negative bacteria, other than mycobacteria.[19]

It is generally accepted that increases in accumulation of fluoroquinolones or reduction of the MIC in the presence of reserpine can serve as surrogate markers for the presence of an active efflux system.[21] Fluorescence spectroscopy, employed in the current study, has been shown to be a reliable method to determine the intracellular accumulation of ciprofloxacin in the clinical isolates of *M. tuberculosis*.[16] Reserpine increased the accumulation of ciprofloxacin in 14 of 16 (87.5%) clinical isolates and H37Rv regardless of their susceptibility to ciprofloxacin. These findings are consistent with a recent report by Escribano et al.[10] It has been found that resistances to ofloxacin and ciprofloxacin of clinical isolates of *M. tuberculosis* were reduced by the efflux pump inhibitors MC207.110. And addition of reserpine led to reduction of the MIC of 6/6 ciprofloxacin resistant strains and 11/12 ciprofloxacin-susceptible strains. A major difference from their findings is that our addition of reserpine decreased the MIC of ciprofloxacin in only 2/5 (40%) of ciprofloxacin-resistant strains and 3/11 (27.3%) of susceptible strains. It is not entirely clear why the high rate of increase in accumulation of ciprofloxacin in the presence of reserpine did not lead to a reduction of MICs to ciprofloxacin for all clinical isolates. This difference could be explained by strain selection (both sampling of small numbers) and geographic differences in the relative abundance of resistant strains. Our finding of a dissociation between inhibition of efflux pumps and antimicrobial susceptibility of clinical isolates of *M. tuberculosis* are similar to those reported for fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae* (45.4%)[22] and *Pseudomonas aeruginosa* (37.8%).[23]

We found no significant differences in the percent increase of intracellular concentrations of ciprofloxacin in the presence of reserpine among ciprofloxacin-susceptible non-MDR strains, ciprofloxacin-susceptible MDR strains, and ciprofloxacin-resistant MDR strains. This raises the possibility that another independent mechanism, unrelated to efflux pump activity, might account for differences among the strains in accumulation of ciprofloxacin. We are currently exploring whether decreased permeability could account for this phenomenon.

In summary, we found that most clinical isolates of *M. tuberculosis* are able to accumulate ciprofloxacin and express a reserpine-sensitive efflux pump. Reserpine augments susceptibility to ciprofloxacin in some, but not all, ciprofloxacin-susceptible strains. MDR strains were found to be less susceptible to ciprofloxacin than non-MDR strains and to accumulate lower intracellular concentrations of the drug; this phenomenon is probably due to the characteristics of MDR strains themselves because fluoroquinolone-resistant MDR-TB was not necessarily from a patient previously treated with fluoroquinolones.[6] Addition of reserpine markedly increased the susceptibility of ciprofloxacin-resistant strains carrying the D94G *gyrA* mutation. However, reduction of ciprofloxacin MICs in the presence of reserpine varied independently of *gyrA* mutation. Several independent mechanisms appear to be responsible for differences in susceptibility to ciprofloxacin in MDR strains. In addition to mutations in *gyrA*, other mechanisms such as decreased uptake and variations in intracellular distribution need to be explored.

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