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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