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# Inhibition of the Emergence of Ciprofloxacin Resistance in *Streptococcus pneumoniae* by the Multidrug Efflux Inhibitor Reserpine

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**Recent evidence supports the contribution of a multidrug efflux mechanism to fluoroquinolone resistance in *Streptococcus pneumoniae*. In this paper I show that reserpine, an inhibitor of multidrug transporters in gram-positive bacteria, dramatically suppresses the in vitro emergence of ciprofloxacin-resistant variants of *S. pneumoniae*, suggesting that the combination of a fluoroquinolone with an inhibitor of multidrug transport may help preserve the efficacy of this class of antibiotics.**

Many chromosomally encoded multidrug transporters have recently been identified in gram-positive bacteria, including Bmr and Blt in *Bacillus subtilis* (1, 10), NorA in *Staphylococcus aureus* (13), LmrA and LmrP in *Lactococcus lactis* (3, 12), and LfrA in *Mycobacterium smegmatis* (11). These proteins transport structurally diverse compounds, including fluoroquinolone antibiotics and toxic cations such as ethidium bromide and rhodamine, out of cells, thereby preventing their intracellular accumulation. The activities of many of these pumps can be inhibited by the alkaloid reserpine. Studies with *S. aureus* have demonstrated that reserpine potentiates fluoroquinolone bacteriotoxicity in wild-type cells, reverses NorA-mediated fluoroquinolone resistance in clinical isolates, and suppresses the emergence of fluoroquinolone-resistant variants (6, 7).

Recent evidence indicates the presence of a reserpine-sensitive multidrug transporter in the respiratory pathogen *Streptococcus pneumoniae* (2). Expression of this putative efflux transporter augments resistance to the fluoroquinolones ciprofloxacin and norfloxacin, as well as to ethidium bromide and acriflavine (2, 4, 14). To date, this efflux mechanism has been detected in the following: (i) wild-type strains, where it appears to contribute to the decreased intrinsic susceptibility of this pathogen to fluoroquinolones (2, 5); (ii) a strain selected for increased resistance to the multidrug transporter substrate ethidium bromide (2); and (iii) first-step spontaneous mutants selected in vitro for low-level fluoroquinolone resistance (4, 5, 14). Importantly, this efflux mechanism appears to be a prevalent cause of clinically significant fluoroquinolone resistance in *S. pneumoniae*. Brenwald et al. very recently reported that almost half of isolates showing a reduced susceptibility to the fluoroquinolones norfloxacin and ciprofloxacin exhibit a reserpine-sensitive drug resistance phenotype consistent with the expression of PmrA (5). I speculated that, similar to previous findings with *S. aureus* (7), reserpine would be found to inhibit the emergence of fluoroquinolone resistance in *S. pneumoniae*.

Single-step mutants of *S. pneumoniae* (ATCC 49619) resistant to three- or fourfold the MIC of ciprofloxacin (1.5 or 2 μg/ml) were selected in vitro, in either the absence or presence of reserpine at a concentration of 10 μg/ml. Approximately $2 \times 10^9$ cells were plated on Mueller-Hinton agar plates containing 10% lysed horse blood and were incubated for 72 h at 37°C in an oxygen-reduced atmosphere. Susceptibility testing of mutants was conducted by using broth microdilution assays (9). Sequence analysis of the amplified quinolone resistance determining region (QRDR) of *parC* was performed essentially as previously described (8). PCR products were purified with the Wizard PCR system (Promega), and sequencing was performed with the *fmol* DNA sequencing system (Promega).

The emergence of ciprofloxacin resistance was strikingly suppressed in the presence of reserpine. Whereas in the absence of reserpine 135 colonies of *S. pneumoniae* ATCC 49619 were obtained in medium containing three times the MIC of ciprofloxacin and 19 colonies were obtained in four times the MIC of ciprofloxacin, in the presence of reserpine only 3 colonies resistant to three times the MIC (a 45-fold reduction) were obtained, and no colonies resistant to four times the MIC were obtained. This dramatic effect could not be attributed to a toxic effect of reserpine. The MIC of reserpine for strain ATCC 49619 is greater than 40 μg/ml, the solubility limit for this drug in cation-adjusted Mueller-Hinton broth. Additionally, reserpine affected neither the colony-forming ability nor the colony size of *S. pneumoniae* plated in the absence of ciprofloxacin.

One possible explanation for the observed effect of reserpine is that the augmented fluoroquinolone resistance in the majority of first-step selected mutants is reserpine sensitive. Indeed, this proved to be the case. The MIC of ciprofloxacin was at least eightfold higher than that for the wild-type strain (4 to 8 versus 0.5 μg/ml) (Table 1) for 10 mutants evaluated. Importantly, the MIC of ciprofloxacin for eight of these mutants was reduced to 1 μg/ml or less in the presence of 10 μg of reserpine per ml. Consistent with the expression of a multidrug efflux mechanism, resistance to the multidrug transporter substrate ethidium bromide was also augmented (fourfold) in a reserpine-sensitive manner. Interestingly, two different phenotypes were distinguishable by the sensitivity of these efflux mutants to reserpine; the ciprofloxacin resistance of seven of the eight mutants was reversed fourfold by reserpine at a concentration of 2.5 to 5 μg/ml, while as little as 0.15 μg/ml, a 16- to 32-fold-lower concentration, had the same effect on the eighth mutant. This finding strongly suggests that two different multidrug transporters can contribute to ciprofloxacin resistance in *S. pneumoniae*.

Although reserpine, in a manner similar to its effect on the susceptible strain, increased the ciprofloxacin susceptibility of the remaining (nonefflux) mutants only twofold (MIC of cipro-

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TABLE 1. Susceptibility patterns of ciprofloxacin-resistant mutants compared to wild type[a]

| Strain (no. of isolates) | MIC (μg/ml) of: | | | |
|---|---|---|---|---|
| | Cip | Cip + R | EtBr | EtBr + R |
| Wild type (ATCC 49619) | 0.5 | 0.25 | 4 | 0.25 |
| Efflux (8) | 4–8 | 0.25–1 | 16 | 0.125–0.25 |
| Nonefflux (2) | 4 | 2 | 4 | 0.125–0.25 |
| R insensitive (3) | 4–8 | 4–8 | 2–4 | 0.125–0.5 |

[a] R, reserpine; EtBr, ethidium bromide; Cip, ciprofloxacin.

floxacin, 4 μg/ml), this effect is sufficient to prevent their emergence upon selection with 2 μg of ciprofloxacin per ml. Since ethidium bromide susceptibility was unchanged in these mutants, it appeared that they possessed alterations in a different fluoroquinolone target, most likely ParC, the primary target of ciprofloxacin in *S. pneumoniae* (8). Indeed, sequence analysis of the amplified QRDR of *parC* in these mutants (8) revealed a mutation altering S-79 to Y (TCT-TAT) in one mutant and D-83 to Y (GAT-TAT) in the other mutant, residues previously reported to be altered in pneumococcal isolates resistant to low levels of ciprofloxacin (8).

The three mutants resistant to three times the MIC of ciprofloxacin in the presence of reserpine also appeared to be ParC mutants; analysis of two of these mutants revealed a mutation of S-79 to F (TCT-TTT) in the QRDR of ParC. Apparently, the slightly lower selecting concentration of ciprofloxacin (1.5 versus 2 μg/ml) allows these mutants to emerge even in the presence of reserpine.

These results indicate that the combination of a fluoroquinolone, such as ciprofloxacin, with a multidrug efflux inhibitor would substantially improve the clinical efficacy of this class of antibiotics against *S. pneumoniae*. Such a combination drug not only would suppress the emergence of fluoroquinone-resistant bacteria but also would be effective in a large percentage of existing resistant strains. Since similar findings have been previously reported for *S. aureus*, it is likely that such a combination which would be effective against a spectrum of gram-positive pathogens could be identified. With this in mind, Influx, Inc., is currently developing clinically useful inhibitors of multidrug transporters.

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