Introduction

Streptococcus pneumoniae is the major cause of community acquired pneumonia. In recent years, fluoroquinolones have been used to treat pneumococcal infection, resulting in the emergence of quinolone-resistant pneumococci. The main mechanism of quinolone resistance is due to mutations in the target enzymes, DNA gyrase and topoisomerase IV. Topoisomerase IV of S. pneumoniae is believed to be the primary target for most quinolones. Strains with low-level resistance contain parC mutations, whereas those with a high level of resistance have mutations in both gyrA and parC, which occurs by a two-step mutation. The increasing resistance observed in this species worldwide has led to the continued search for more active compounds.

Sitafoxacin (also known as DU-6859a;(-)-7-[(7S)-7-amino-5-azaspiro(2,4)heptan-5-yl]-8-chloro-6-fluoro-1-[(1R, 2S)-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxo-3-quino- linone-carboxylic acid sesquihydrate) is a novel quinolone antibacterial agent. It has activity against a wide range of bacteria and is particularly effective against Gram-positive bacteria. In order to clarify the mechanism of action of sitafoxacin, we purified S. pneumoniae DNA gyrase and topoisomerase IV and determined the inhibitory activities of sitafoxacin against the purified enzymes.

Materials and methods

Antibacterial agents and bacterial strains

All quinolones used in this study were synthesized at New Product Research Laboratories I, Daiichi Pharmaceutical Co., Ltd, Tokyo, Japan. Benzylpenicillin was purchased from Sigma-Aldrich Co., Ltd, St. Louis, MO, USA. The bacterial strain used in this study was quinolone- and penicillin-sensitive S. pneumoniae J24.

Determination of MIC

The MICS were determined by the standard agar dilution method with Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA) containing 5% horse blood. Drug-containing agar plates were inoculated with one loopful (5 μL) of an inoculum corresponding to approximately 10⁴ cfu/spot and were incubated for 18 h at 37°C. The MIC was defined as the lowest drug concentration that prevented visible growth of bacteria.

Construction of expression vectors

Four sets of oligonucleotide primers were designed for amplification of gyrA, gyrB, parC and parE genes and subsequent insertion into the pMAL-c2 fusion protein.

The in-vitro inhibitory activities of sitafoxacin (DU-6859a) and other quinolones against Streptococcus pneumoniae DNA gyrase and topoisomerase IV were measured. IC₅₀s of levofloxacin, ciprofloxacin, sparflaxin and tosufloxacin against DNA gyrase were almost three to 12 times higher than those against topoisomerase IV. On the other hand, sitafoxacin showed dual inhibitory activity against both enzymes and its IC₅₀s were the lowest among those of the quinolones tested. These results suggest that sitafoxacin is an effective agent against pneumococcal infections and that the incidence of drug-resistant mutants is low.
expression vector (New England Biolabs, Beverly, MA, USA). In each case, the sequence of the forward primer was chosen at the initiation codon. For reverse primers, an Xba I site was introduced for cloning purposes. For gyrA, the forward primer was 5'-ATGACAGAAGAAATCAAAAATCTG-3' (containing the 1–21 bp region of gyrA) and the reverse primer was 5'-AACATCTCTTCTAGAATTGAATT-3' (2572–98) for 5'-AAATCCT (2512–36). Primers for the forward and reverse primers were 5'-ATGACAGAAGAAATCAAAAATCTG-3' (1–24) and 5'-CATATTTTCCTAGAATTGAATT-3' (1964–86), 5'-ATGCTCTAATTCAAAATCTGCTACCTCTG-3' (1–27) and 5'-CCAATCTCTTCTAGAATTGAATT-3' (2572–98) for parC, 5'-GTGCTCAAAAAAGGAAATCAATATTAAC-3' (1–24) and 5'-TGCCTCTCTTTGTCAGC-3' (1–27) and 5'-GCCCTCTCTTTGTCAGC-3' (1–27) and 5'-GCCCTCTCTTTGTCAGC-3' (1–27) and 5'-TGCCTCTCTTTGTCAGC-3' (1–27) and 5'-GCCCTCTCTTTGTCAGC-3' (1–27) and 5'-GCCCTCTCTTTGTCAGC-3' (1–27). Purification of the enzymes

The GyrA and GyrB proteins of DNA gyrase, and ParC and ParE of topoisomerase IV, were purified separately as maltose-binding protein (MBP) fusion products from overproducing strains of E. coli. Each gene was amplified for 25 cycles, in which the conditions were 0.5 min at 94°C for denaturation, 0.5 min at 60°C for annealing and 2 min at 72°C for polymerization. The DNA fragments were digested with Xba I, ligated into the pMAL-c2 expression vector and transformed into E. coli MC1061.

Purification of the enzymes

The GyrA and GyrB proteins of DNA gyrase, and ParC and ParE of topoisomerase IV, were purified separately as maltose-binding protein (MBP) fusion products from overproducing strains of E. coli. Each gene was amplified for 25 cycles, in which the conditions were 0.5 min at 94°C for denaturation, 0.5 min at 60°C for annealing and 2 min at 72°C for polymerization. The DNA fragments were digested with Xba I, ligated into the pMAL-c2 expression vector and transformed into E. coli MC1061.

Determination of inhibitory activity of drugs

The supercoiling activity of DNA gyrase and the decatenation activity of topoisomerase IV were measured by methods described previously.9 One unit of supercoiling activity was defined as the amount of GyrA and GyrB proteins required to supercoil 50% of 0.2 μg of relaxed pBR322 plasmid DNA. One unit of decatenation activity was defined as the amount of ParC and ParE proteins required to fully decatenate 0.4 μg of kDNA. The IC50 was defined as the drug concentration that reduced the enzymatic activity observed with drug-free controls by 50%.

Results

Purification and characterization of topoisomerase IV and DNA gyrase subunit proteins

The ParC and ParE proteins of topoisomerase IV of S. pneumoniae J24 were purified separately as MBP fusion proteins and single bands for each protein were observed by SDS-PAGE at about 130 kDa and 110 kDa for MBP–ParC and MBP–ParE, respectively (Figure, a). A factor Xa recognition site was introduced into the fusion proteins and, after factor Xa digestion, the ParC and ParE proteins migrated at 93 and 72 kDa, respectively (Figure, a). Although no single protein had enzymatic activity, ParC and ParE together showed decatenation activity (Figure, b). Because these activities were not detected in the absence of ATP and Mg2+, these enzymes were ATP- and Mg2+-dependent (Figure, b). The optimum concentration range for the potassium cation was 10–40 mM, and that for the magnesium cation was >5 mM (data not shown). From these results, the conditions for the decatenation assay were determined as described in Materials and methods. The GyrA and GyrB proteins of DNA gyrase were purified in a similar fashion, and reconstituted enzyme showed ATP-dependent supercoiling activity (Figure, c).

Comparison of inhibitory activities of antibacterial agents against topoisomerase IV and DNA gyrase

The quinolones inhibited the activities of the enzymes in a concentration-dependent manner (Figure, d). In contrast, benzylpenicillin, which does not inhibit either enzyme, had no effect on their activity (data not shown). The IC50s of the quinolones were calculated from the quantified bands, which corresponded to fully decatenated substrate or supercoiled DNA (Table). The IC50 values of the quinolones against type II topoisomerases compared with their MICs had correlation coefficients of 0.88 for topoisomerase IV and 0.87 for DNA gyrase. Of the quinolones tested, sitafloxacin showed the highest inhibitory activity against the enzymes. Moreover, unlike the other quinolones, the IC50 of sitafloxacin against DNA gyrase and topoisomerase IV were almost equal.

Discussion

We expressed and purified the S. pneumoniae DNA gyrase and topoisomerase IV subunit proteins. The GyrA and GyrB proteins together, and the ParC and ParE proteins...
Dual inhibitory activity of sitafloxacin together, reconstituted ATP-dependent supercoiling activity characteristic of a DNA gyrase and ATP-dependent decatenation activity characteristic of a topoisomerase IV. The quinolones inhibited both enzymes, and the inhibitory activity of sitafloxacin was especially high. In addition, the ratios of the IC\textsubscript{50}s of the new quinolones tested for type II topoisomerase (IC\textsubscript{50} for DNA gyrase/IC\textsubscript{50} for topoisomerase IV) were all greater than 2.8, with the exception of sitafloxacin with a ratio of almost 1. This indicates that sitafloxacin has relatively equivalent inhibitory activity against both enzymes.

Against S. pneumoniae, the first target of quinolones, sitafloxacin exhibited the highest inhibitory activity. The IC\textsubscript{50}s of sitafloxacin for DNA gyrase and topoisomerase IV were 0.05 mg/L and 1.88 mg/L, respectively, indicating a high inhibitory effect against both enzymes. This makes sitafloxacin a promising candidate for the treatment of infections caused by S. pneumoniae, especially against those that are resistant to other quinolones.
differs between the drugs. The target of levofloxacin and ciprofloxacin is thought to be topoisomerase IV,4,5 whereas that of sparfl oxacin is reported to be DNA gyrase.10 When resistant mutants were selected stepwise with increasing ciprofloxacin concentrations, a parC mutation was found in low-level resistant mutants, and parC and gyrA double mutations were detected in high-level resistant strains.5 S. pneumoniae acquires greater resistance to quinolones step-by-step. However, as sitafloxacin showed similar and the lowest IC50 values against the two target enzymes, the incidence of sitafloxacin-resistant strains should be very low, for the acquisition of sitafloxacin resistance would necessitate mutation of both enzymes at the same time. Furthermore, this drug would be effective against parC mutants and gyrA mutants, since it has the ability to inhibit the wild-type enzymes. From these results, sitafloxacin should be an effective agent against pneumococcal infections. The activity of sitafloxacin against the mutated enzymes and the role of other quinolone-resistant factors, such as the efflux pump, will be clarified by further study.

References

...