Inhibitory Activity on DNA Gyrase and Intracellular Accumulation of Quinolones: Structure–Activity Relationship of Q-35 Analogs

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Q-35, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methylamino-piperidine-1-yl)-4-oxoquinoline-3-carboxylic acid, has excellent activity against gram-positive bacteria and inhibits S. aureus gyrase at concentrations more than 10-fold lower than those of other quinolones. In this paper, the effect of the C-7 and C-8 substituents of Q-35 on the inhibitory activity of gyrase purified from S. aureus, M. luteus, E. coli, and P. aeruginosa are described. In addition, intracellular accumulation of Q-35 was examined.

The 50% inhibitory concentrations (IC 50 ) of Q-35, 8-fluoro- Q-35, and 8-hydro-Q-35 on DNA gyrase purified from S. aureus were 2.5, 7.8, and 68 μg/ml, respectively. The IC 50 on gyrase from P. aeruginosa were 11, 5.2, and 17 μg/ml, respectively. It is concluded that the introduction of a methoxy group into the 8 position of the quinolone leads to greater antibacterial activity against gram-positive bacteria.

The concentrations of Q-35 which accumulated in S. aureus and E. coli were almost equal to ciprofloxacin, but in P. aeruginosa, Q-35 was lower than ciprofloxacin.

Keywords DNA gyrase; quinolone; Q-35; intracellular accumulation; Staphylococcus aureus; Pseudomonas aeruginosa

Q-35, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methylaminopiperidine-1-yl)-4-oxoquinoline-3-carboxylic acid, which was developed with special attention to an advantage in terms of safety (for instance, phototoxicity), is a new fluoroquinolone in which the 8 position is substituted with a methoxy group. Its antibacterial spectrum is broad, ranging from gram-positive to gram-negative bacteria. Q-35 has demonstrated excellent activity against gram-positive bacteria, being 4-fold more active than ofloxacin (OFLX) and ciprofloxacin (CPFX), but is less active than two of them against Pseudomonas aeruginosa. An interesting characteristic of Q-35 is its improved activity against certain quinolone-resistant staphylococci, including methicillin-resistant Staphylococcus aureus.

The inhibitory activity of Q-35 on the supercoiling reaction of DNA gyrase was investigated. Against Escherichia coli and P. aeruginosa gyrase, the inhibitory activity of Q-35 is less active or equal to other new quinolones. It is interesting that Q-35 inhibited the S. aureus gyrase at concentrations more than 10-fold lower than those of other new quinolones. In this report, we describe the structure–activity relationship of the 7 and 8 position substituents of Q-35 for the purpose of elucidating the characteristics of both its antibacterial and anti-gyrase activity. In addition, the intracellular accumulation of Q-35 was investigated.

MATERIALS AND METHODS

Antibacterial Agents Q-35 and Q-35 analogs (Fig. 1) were synthesized at the Research Foundation of Chugai Pharmaceutical Co., Ltd. Norfloxacin (NFLX, Kyorin Pharmaceutical Co., Ltd.), OFLX (Daichi Pharmaceutical Co., Ltd.), CPFX (Bayer Pharmaceutical Co., Ltd.), tosufloxacin (TFLX, Toyama Chemical Co., Ltd.), lomefloxacin (LFLX, Hokuriku Pharmaceutical Co., Ltd.), and sparfloxacin (SPFX, Dainippon Pharmaceutical Co., Ltd.) were used as reference quinolones. Q-35, Q-35 analogs and the reference quinolones, except for ciprofloxacin chloride, were initially dissolved in 0.1 N NaOH, diluted with water and neutralized with 0.1 N HCl. CPFX was initially dissolved in water and neutralized with 0.1 N NaOH. These solutions were immediately used.

Bacterial Strains Staphylococcus aureus FDA 209P, Micrococcus luteus ATCC 9341, Escherichia coli NIHJ JC-2, and Pseudomonas aeruginosa PAO-1 were used. All strains were maintained as frozen (−80°C) stock cultures at Kyoto Pharmaceutical University.

Determination of Minimum Inhibitory Concentrations (MICs) MICs were determined by the two-fold agar dilution method, recommended by the Japan Society of Chemotherapy, with Sensitivity Test agar (Eiken Chemical Co., Ltd., Tokyo, Japan). The overnight broth cultures of the bacterial strains were diluted with broth corresponding to final concentrations of about 10^6 CFU/ml. Five microliters of each bacterial suspension was applied. The MIC was defined as the lowest concentration of the compound that prevented visible growth on the agar.

Inhibition of DNA Gyrase S. aureus FDA 209P, E. coli NIHJ JC-2 and P. aeruginosa PAO-1 gyrase were purified by a novobiocin affinity column, as described previously. M. luteus gyrase was purchased from Gibco/BRL.

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Fig. 1. Structure of Q-35 and Its Analogs Used in This Study
Gyrase activity was measured in terms of supercoiling activity. The enzyme catalyzed the conversion from the relaxed form of the plasmid pBR 322 to the supercoiled form. Approximately 2 units of the gyrase holoenzyme of *E. coli* and *M. luteus* were used. Approximately 2 units each of gyrase A and B subunits of *P. aeruginosa* and *S. aureus* was used per reaction.

Ten microliters of an enzyme solution, 5 μl of quinolone solution and 5 μl of an assay buffer containing the relaxed form plasmid were mixed on a 96 well microtiterplate to a final volume of 20 μl. Each gyrase reaction was assayed under optimized conditions (Table I).

After 1 h of incubation at 37°C, the reaction was stopped by the addition of sodium dodecyl sulfate at a final concentration of 1.3%, and then subjected to 0.8% agarose gel electrophoresis.

The gel was stained with ethidium bromide (2 μg/ml) and photographed with a UV transilluminator. Results were expressed as 50% inhibitory concentrations (IC50).

**Intracellular Accumulation** The method described by Celesk and Robillard, with slight modifications, was used to measure the intracellular accumulation of quinolones. Cells were grown in Sensitivity Test broth (Eiken Chemical Co., Ltd.) and the turbidity was adjusted at optical density (OD) 1.0 (550 nm). Accumulation was assayed at 37°C in broth upon the addition of 10 μg of the quinolone per milliliter. At 10 min, a carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma) methanol solution or methanol was added. At 20 min, a 900 μl sample was placed onto the surface of 500 μl of silicon oil (Towar Silicon Co., Ltd., SH500; SH556 = 5:6) in a 1.5 ml Eppendorf centrifuge tube. The tubes were centrifuged for 7 min at 12000 × g, frozen at −80°C and then cut in the middle of the oil layer and inverted to eliminate excess oil from the pellet.

The cell was suspended in 300 μl of phosphate buffer (pH 8.0), boiled for 5 min and centrifuged to remove cell debris. The concentration of quinolones in the supernatant was measured by bioassay using *B. subtilis* ATCC6633.

**RESULTS**

**Antibacterial Activity and Inhibitory Activity on DNA Gyrase** The results of testing the drug inhibitory activity on *S. aureus*, *M. luteus*, *E. coli*, and *P. aeruginosa* are given in Table II. Figure 2 shows the relationship between the antibacterial activity (MIC) and inhibitory activity on DNA gyrase (IC50).

The inhibitory concentrations of gram-positive bacterial gyrase ranged widely, but for gram-negative bacteria, the range was narrow. For *M. luteus*, there was a high correlation between MIC and IC50 (r = 0.957), however for another strains, there was low correlation.

In comparison with other referenced quinolones, Q-35 exhibited the strongest inhibition on *S. aureus* and *M. luteus* gyrase, in spite of the same level of antibacterial activity with TFLX and SFPX. The antibacterial activities of Q-35 against *E. coli* and *P. aeruginosa* were 1- to 16-fold less active than the referenced quinolones, but Q-35 inhibited these gyrase at the same level as the other quinolones.

**Q-1**, a demethyl analog of Q-35, exhibited the strongest inhibition on gram-positive bacterial gyrase. The antibacterial and anti-gyrase activity of Q-1 were greater than Q-35 on both gram-positive and -negative bacteria.

The antibacterial activity of Q-4, a substituent of an 8-methoxy group to hydrogen, was 1- to 16-fold less active than that of Q-35. The inhibitory activity on all bacterial gyrase of Q-4 was 1.5- to 27-fold less active than that of Q-35.

**Q-5** was a substituent of an 8-methoxy group to fluorine. Q-5 exhibited stronger antibacterial and anti-gyrase activity on gram-negative bacteria than Q-35, in spite of being less active on gram-positive bacteria.

Comparisons between 3- and 4-methylaminopiperidine/aminopiperidine were examined. The result was that the 3-group had slightly better activity on gram-positive bacterial gyrase while the 4-group had slightly better activity on gram-negative bacterial gyrase.

**Intracellular Accumulation of Q-35** The accumulation of Q-35 and CPFX in *S. aureus*, *E. coli*, and *P. aeruginosa* cells was examined in the absence or presence of an energy inhibitor, CCCP (Table III). Without CCCP, the levels of Q-35 which accumulated in *S. aureus* and *E. coli* cells were as same as CPFX. The level of Q-35 which accumulated in *P. aeruginosa* cells was 2-fold lower than CPFX.

CCCP inhibits the energy-dependent efflux pump. By the addition of CCCP, the levels of fluoroquinolone accumulation in all cells increased significantly. With CCCP, the levels of Q-35 which accumulated in *S. aureus* and *P. aeruginosa* were lower than CPFX, but the levels were equal in *E. coli*.
The inhibitory concentration of each quinolone on the gyrase purified from *E. coli* and *P. aeruginosa* ranged narrowly and scattered in low correlation, though those of *S. aureus* and *M. luteus* had a wide range. These gram-negative strains have an outer membrane which may play a role in the selective permeability of quinolone. It was thought that antibacterial activity against gram-negative bacteria was determined by membrane permeability rather than by the inhibitory activity of gyrase.

Intracellular accumulation of Q-35 and CPFX were measured for the purpose of explaining the contradiction between antibacterial activity and gyrase inhibition. By this method, it was hard to avoid the influence of drug adsorbed to the bacterial surface. Therefore, with or without CCCP, the level of accumulation includes not only intracellular concentration but also the amount of drug adsorbed to the bacterial surface. The difference between accumulation levels with or without CCCP was thought to occur when the influence of drug adsorbed to the bacterial surface was avoided. The difference of CPFX was larger than that of Q-35 in *S. aureus* and *P. aeruginosa*.

In conclusion, the greater antibacterial activity of Q-35 against gram-positive bacteria might be caused by the strong inhibition of gyrase. The lower antibacterial activity of Q-35 against *P. aeruginosa* may be caused by low membrane permeability.

QR-4 has the same structure as CPFX except at the 7-position. CPFX has a piperadine group at the 7-position, whereas QR-4 has methylaminopiperidine. In comparing the two agents, the former is excellent in antibacterial activity. The antibacterial and anti-gyrase activity of QR-1, a substituent of 7-methylaminopiperidine to aminopiperidine, exhibited the top levels of the tested quinolones.
From these data, it was thought that the structure of the 7-position amine plays an important role in a quinolone's interaction with gyrase.

The introduction of a methoxy group into the 8 position of quinolones causes an 8-fold increase in antibacterial activity against gram-positive bacteria. The introduction of a fluorine group increased antibacterial activity, but antibacterial activity against gram-positive bacteria was lower than with the methoxy group.

Shen et al. reported the action of quinolone on a gyrase–DNA cleavable complex. In this model, they reported that 2 molecules of quinolone formed a stack in 2 pairs, so a total of 4 molecules of quinolone interact with a quinolone pocket. The π electron of quinolone aromatic rings interacted with paired quinolones. The carboxy and carbonyl groups of the 3 and 4 positions of quinolone interacted with DNA. The amino group of the 7 position interacted with gyrase. A substituent of the 1 position had a tail-to-tail interaction with another pair of quinolones. They did not indicate the effect of substitution at the 8-position of quinolone.

We propose a hypothesis about the influence of the 8-position of quinolone in Shen's model. A substituent of the 8-position influences the π electron of the aromatic ring of quinolone. The methoxy group, an electron-donating group, interacts with the π electron and changes the distance of quinolone stacking. It was thought that the optimum distance of quinolone stacking was differed by bacterial strain. For these reasons, Q-35, 8-methoxy quinolone exhibited improved activity against gram-positive bacteria.

REFERENCES