Reduced uptake and accumulation of norfloxacin in resistant strains of *Neisseria gonorrhoeae* isolated in Japan

M Tanaka, H Fukuda, K Hirai, M Hosaka, T Matsumoto, J Kumazawa

Abstract

***Objective*—To investigate the alteration of cell permeability toward fluoroquinolones in *Neisseria gonorrhoeae*, which is a major quinolone-resistance mechanism along with the alteration of DNA gyrase in gram-negative bacteria. The prevalence of fluoroquinolone-resistant *N gonorrhoeae* strains is rapidly increasing in Japan.**

***Materials and methods*—The uptake and accumulation of norfloxacin by gonococcal cells, including six clinical and five World Health Organization (WHO) reference strains, were measured. Of the six clinical strains, two were highly resistant to norfloxacin (MIC 8·0 and 4·0 μg/ml), two were moderately resistant (MIC 1·0 and 0·5 μg/ml), and two were sensitive (MIC 0·063 and 0·004 μg/ml). All five WHO reference strains were sensitive to norfloxacin (MIC ≤0·001 to 0·063 μg/ml).**

***Results*—Mean initial norfloxacin uptake in the four resistant strains (104 ng/mg of dry cells) was significantly lower than that in the seven sensitive strains (158 ng/mg of dry cells) ($p < 0·05$). The mean uptake after 20 minutes was also significantly lower in the four resistant strains (130 ng/mg of dry cells) than in the seven sensitive strains (194 ng/mg of dry cells) ($p < 0·05$). However, there was no significant difference in mean norfloxacin accumulation after 20 minutes between the four resistant strains (26 ng/mg of dry cells) and the seven sensitive strains (36 ng/mg of dry cells). The accumulation of norfloxacin after 20 minutes was almost zero in two of the four resistant strains, while the remaining two strains accumulated norfloxacin as well as the sensitive strains.**

***Conclusions*—These findings suggest that alteration of bacterial cell permeability is a quinolone-resistance mechanism in *N gonorrhoeae* isolated in Japan, and that this bacteria may exhibit other mechanisms such as alteration of DNA gyrase.**

(*Genitourin Med* 1994;70:253–255)

Department of Urology, Faculty of Medicine, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812, Japan
M Tanaka
T Matsumoto
J Kumazawa

Central Research Laboratories, Kyorin Pharmaceutical Co., Ltd., 2399-1 Mitarai, Nogi-machi, Shimotsuga-gun, Tochigi, 329-01, Japan
H Fukuda
K Hirai
M Hosaka

Address correspondence to:
Dr Masatoshi Tanaka

Accepted for publication
7 April 1994

Introduction

Fluoroquinolones such as norfloxacin, ofloxacin, and ciprofloxacin demonstrate excellent in vitro antimicrobial activity against *Neisseria gonorrhoeae*, including penicillin- and tetracycline-resistant strains, and are highly effective as oral single-dose treatment for gonococcal infections.[1–4] Treating for gonorrhoea with fluoroquinolones is recommended by the Centers for Disease Control and Prevention (CDC) in the United States[5] and by the World Health Organization (WHO).[6] In Japan, fluoroquinolones have frequently been used as first-line therapy for gonorrhoea for last few years.

However, reduced sensitivity to ciprofloxacin and treatment failure with this agent have recently been reported.[7 8] In addition, we have already reported a high prevalence of *N gonorrhoeae* strains with reduced sensitivity to fluoroquinolones in Japan.[9] The development of fluoroquinolone-resistance in *N gonorrhoeae* may be a new worldwide problem in treating gonococcal infections.

It has been reported that alterations in the cell permeability of the bacterial outer membrane and in DNA gyrase are common mechanisms of resistance to quinolones in gram-negative bacteria, including *Escherichia coli* and *Pesudomonas aeruginosa*.[10] Recently, an active efflux system has been also reported as the quinolone-resistance mechanism in gram-negative bacteria.[11] Less is known about quinolone-resistance mechanisms in *N gonorrhoeae*.[12–13] Therefore, to investigate alterations in membrane permeability to a fluoroquinolone in resistant strains of *N gonorrhoeae* isolated in Japan, we compared the uptake and accumulation of norfloxacin in resistant gonococcal cells with those of sensitive gonococcal cells.

Materials and methods

Bacterial strains

Six clinical strains of *N gonorrhoeae* isolated in Japan between February and July 1992 from men with acute urethritis and 5 WHO *N gonorrhoeae* reference strains (kindly supplied by Dr. J. W. Tapsall, The Prince of Wales Hospital, Australia) were used for cell permeability testing. All six clinical isolates were negative for β-lactamase production. The minimum inhibitory concentrations (MICs) of norfloxacin, ofloxacin, and ciprofloxacin against the 11 tested strains of *N gonorrhoeae* are shown in table 1. The MICs were determined using an agar dilution technique with a GC agar base (Difco) containing 1% IsoVitaleX (BBL). Plates were inoculated with 5 μl of 10^6 colony-forming units (cfu)/ml of each isolate using a multipoint inoculator. The plates were incubated for 24 hours at

Table 1 MICs of norfloxacin, ofloxacin, and ciprofloxacin against the 11 tested strains of N gonorrhoeae

Strain	MIC (μg/ml)		
	Norfloxacin	Ofloxacin	Ciprofloxacin
A-10	8·0	1·0	0·5
A-22	4·0	1·0	0·5
A-55	1·0	0·5	0·125
A-161	0·5	0·25	0·031
A-37	0·063	0·063	0·016
A-69	0·004	0·002	≤0·001
WHO-A	0·004	0·004	≤0·001
WHO-B	0·031	0·016	0·016
WHO-C	≤0·001	≤0·001	≤0·001
WHO-D	0·063	0·063	0·004
WHO-E	0·031	0·016	0·008

35°C in a 5% CO_2 atmosphere. MICs were read as the lowest concentration of antibiotic that inhibited growth.

Of the six clinical strains, two (A-10: MIC, 8·0 μg/ml; A-22: MIC, 4·0 μg/ml) were highly resistant to norfloxacin, two (A-55: MIC, 1·0 μg/ml; A-161: MIC, 0·5 μg/ml) were moderately resistant, and two (A-37: MIC, 0·063 μg/ml; A-69: MIC, 0·004 μg/ml) were sensitive. All five WHO reference strains (A-E: MIC, ≤0·001-0·063 μg/ml) were sensitive to norfloxacin. The strains that showed resistance to norfloxacin were also resistant to ofloxacin and ciprofloxacin (table 1).

All of the strains were stored at −80°C in GDO medium™ (Nissui) containing gelatin with 10% skim milk, 2% activated charcoal, and 17% glucose until norfloxacin uptake testing was performed.

Uptake of norfloxacin by gonococcal cells

The uptake of norfloxacin by gonococcal cells was measured using a method previously described by Hirai *et al.*[14 15] All *N gonorrhoeae* strains were grown on GC agar (Difco) for 24 hours at 35°C in 5% CO_2. A bacterial cell suspension was prepared in pre-warmed antibiotic medium 3 (Difco) to an optical density of 0·7 at 570 nm. Norfloxacin was added to the bacterial suspension to a final concentration of 10 μg/ml, and the suspension was incubated at 37°C with shaking. After incubation for 20 minutes, 10 ml of the suspension was chilled and the cells were sedimented by centrifugation, then washed once in 2 ml of saline. The cells were then resuspended in 1 ml of saline. The suspension was immersed in boiling water for 7 minutes and then centrifuged. The concentration of norfloxacin in the supernatants was measured by bioassay with *Escherichia coli* NIHJ JC-2.[16] The amount of norfloxacin absorbed by the cells immediately after its addition to the bacterial suspension was also measured. The accumulation of norfloxacin was calculated by subtracting the absorbed norfloxacin at zero time from the total norfloxacin eluted.

Statistical analysis

Differences in the uptake and accumulation of norfloxacin between norfloxacin-resistant and norfloxacin-sensitive gonococcal cells were determined using the Student's *t* test.

Results

Comparison of the uptake and accumulation of norfloxacin between the four norfloxacin-resistant and the seven norfloxacin-sensitive gonococcal cell strains is shown in table 2. Mean initial uptake in the four resistant strains (104 ng/mg of dry cells) was significantly lower than that in the seven sensitive strains (158 ng/mg of dry cells) ($p < 0·05$). Mean uptake after 20 minutes was also significantly lower in the four resistant strains (130 ng/mg of dry cells) than in the seven sensitive strains (194 ng/mg of dry cells) ($p < 0·05$). Moreover, lower mean norfloxacin accumulation was observed in the four resistant strains (26 ng/mg of dry cells) as compared with the seven sensitive strains (36 ng/mg of dry cells). However, this difference was not statistically significant.

The values for the uptake and accumulation of norfloxacin by cells of each *N gonorrhoeae* strain are shown in table 3. The accumulation of norfloxacin after 20 minutes was ranged from 20 to 87 ng/mg of dry cells in the seven sensitive strains. There was a large difference in norfloxacin accumulation among the four resistant strains. The accumulation of norfloxacin after 20 minutes was almost zero in strain A-10 (high-level resistance) and strain A-161 (moderate-level resistance) but was 48 ng/mg of dry cells in strain A-22 (high-level resistance) and 54 ng/mg of dry cells in strain A-55 (moderate-level resistance). Therefore, reduced membrane permeability appears to be the main quinolone-resistance mechanism in strains A-10 and A-161, while another quinolone-resistance mechanism such as a change in DNA gyrase may occur in strains A-22 and A-55.

Table 2 Comparison of mean norfloxacin uptake and accumulation between resistant and sensitive N gonorrhoeae strains

Strain	Uptake of NFLX (ng/mg of dry cells)		Accumulation of NFLX (ng/mg of dry cells)
	0 min.	20 min.	20–0 min.
Norfloxacin-resistant (n = 4)	104*	130†	26‡
Norfloxacin-sensitive (n = 7)	158*	194†	36‡

*p < 0·05; †p < 0·05; ‡ns by t-test; NFLX: norfloxacin.

Table 3 Uptake and accumulation of norfloxacin by the 11 tested strains of N gonorrhoeae

Strain	MIC of NFLX (μg/ml)	Uptake of NFLX (ng/mg of dry cells)		Accumulation of NFLX (ng/mg of dry cells)
		0 min.	20 min.	20–0 min.
A-10	8·0	105	105	0
A-22	4·0	76	124	48
A-55	1·0	139	193	54
A-161	0·5	97	97	0
A-37	0·063	130	150	20
A-69	0·004	224	253	29
WHO-A	0·004	141	163	22
WHO-B	0·031	153	240	87
WHO-C	≤0·001	187	212	25
WHO-D	0·063	132	180	48
WHO-E	0·031	136	158	22

NFLX: norfloxacin.

Discussion

Gonococcal resistance to antimicrobial agents is an increasing worldwide problem in the treatment of gonorrhoea. A high prevalence of plasmid-mediated high-level or chromosomally mediated low-level resistance to penicillin or tetracycline has been reported in East Asia[17-20] and in African countries.[21-23] The recently developed fluoroquinolones have strong antimicrobial activity against *N gonorrhoeae*, including isolates with plasmid- or chromosomally-mediated resistance to penicillin and tetracycline. However, the appearance of strains showing reduced sensitivity to ciprofloxacin in vitro[7 8] and treatment failure with this antibiotic have recently been reported,[7] although the prevalence of resistant strains to ciprofloxacin is not yet a major problem.[24 25] We have already demonstrated that there was a significantly higher prevalence of reduced sensitivity to ciprofloxacin, norfloxacin, and ofloxacin in current strains compared with that in control strains isolated between 1981 and 1984.[9] The increase in fluoroquinolone-resistant *N gonorrhoeae* strains will become more and more of a problem in Japan if fluoroquinolones are frequently used as first-line treatment for gonococcal infections.

It has been reported that alterations in the cell permeability of the bacterial outer membrane and in DNA gyrase are common mechanisms of resistance to quinolones in gram-negative bacteria.[10] Recently, an active efflux system has been reported as the quinolone-resistance mechanism in gram-negative bacteria.[11] Inactivation of quinolones by enzymatic degradation has not been demonstrated as a mechanism of bacterial resistance, and plasmid-mediated resistance to quinolones has not been proved. Research on the mechanisms of quinolone resistance in gonococci is very sparse.[12 13] One study[12] showed that the initial uptake of ciprofloxacin was reduced for a ciprofloxacin-resistant strain. However, in that study[12] only one ciprofloxacin-resistant *N gonorrhoeae* strain was tested and all of the ciprofloxacin-resistant and ciprofloxacin-sensitive isolates accumulated ciprofloxacin regardless of MIC and at similar rates.

In the present study, we measured the uptake and accumulation of norfloxacin by gonococcal cells to investigate alterations in cell permeability toward fluoroquinolones in *N gonorrhoeae*, because the strains with reduced sensitivity to norfloxacin also showed low susceptibility to ciprofloxacin and ofloxacin. Our investigation demonstrated that both initial uptake and uptake after 20 minutes was significantly lower in the resistant strains than in the sensitive strains. However, of the four norfloxacin-resistant strains, two did not accumulate norfloxacin, while two accumulated norfloxacin to the same extent as the sensitive strains.

These results suggest that low accumulation of quinolones in the cell is one of the quinolone-resistance mechanisms in *N gonorrhoeae* isolated in Japan, and that this bacteria may exhibit other quinolone-resistance mechanisms such as the alteration of DNA gyrase. Further investigations of fluoroquinolone-resistance mechanisms in *N gonorrhoeae*, including changes in porin protein and the lipopolysaccharide structure associated with outer membrane permeability,[14 15] an active efflux system,[11] and alteration in DNA gyrase are necessary.

1 Loo PS, Ridgway GL, Oriel JD. Single dose ciprofloxacin for treating gonococcal infections in men. *Genitourin Med* 1985;**61**:302–5.
2 Panikabutra K, Lee CT, Hoo B, Bamberg P. Single dose oral norfloxacin or intramuscular spectionomycin to treat gonorrhoea (PPNG and non-PPNG infections): analysis of efficacy and patient preference. *Genitourin Med* 1988;**64**:235–40.
3 Black JR, Long JM, Zwickl BE, *et al.* Multicenter randomized study of single-dose ofloxacin versus amoxicillin-probenecid for treatment of uncomplicated gonococcal infection. *Antimicrob Agents Chemother* 1989;**33**:167–70.
4 Pabst KM, Siegel NA, Smith S, *et al.* Multicenter, comparative study of enoxacin and ceftriaxone for treatment of uncomplicated gonorrhea. *Sex Transm Dis* 1989;**16**: 148–51.
5 Centers for Disease Control. 1989 sexually transmitted diseases treatment guidelines. *MMWR* 1989;**38**(Suppl 8):1–43.
6 World Health Organization. *STD Treatment Strategies.* Geneva. World Health Organization 1989;WHO/VDT/ 89.447.
7 Gransden WR, Warren CA, Phillips I, Hodges M, Barlow D. Decreased susceptibility of *Neisseria gonorrhoeae* to ciprofloxacin. *Lancet* 1990;**335**:51.
8 Jephcott AE, Turner A. Ciprofloxacin resistance in gonococci. *Lancet* 1990;**335**:165.
9 Tanaka M, Kumazawa J, Matsumoto T, Kobayashi I. High prevalence of *Neisseria gonorrhoeae* strains with reduced susceptibility to fluoroquinolones in Japan. *Genitourin Med* 1994;**70**:90–3
10 Wolfson JS, Hooper DC. Bacterial resistance to quinolones: mechanisms and clinical importance. *Rev Infect Dis* 1989;**11**(Suppl 5):960–8.
11 Ishii H, Sato K, Hoshino K, *et al.* Active efflux of ofloxacin by a high quinolone-resistant strain of *Proteus vulgaris*. *J Antimicrob Chemother* 1991;**28**:827–36.
12 Corkill JE, Percival A, Lind M. Reduced uptake of ciprofloxacin in a resistant strain of *Neisseria gonorrhoeae* and transformation of resistance to other strains. *J Antimicrob Chemother* 1991;**28**:601–4.
13 Tapsall JW, Shultz TR, Phillips EA. Characteristics of *Neisseria gonorrhoeae* isolated in Australia showing decreased sensitivity to quinolone antibiotics. *Pathology* 1992;**24**:27–31.
14 Hirai K, Aoyama H, Irikura T, Iyobe S, Mitsuhashi S. Differences in susceptibilities to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. *Antimicrob Agents Chemother* 1986;**29**: 535–8.
15 Hirai K, Aoyama H, Suzue S, *et al.* Isolation and characterization of norfloxacin-resistant mutants of *Escherichia coli* K-12. *Antimicrob Agents Chemother* 1986;**30**:248–53.
16 Oomori Y, Murayama S, Abe Y, Irikura T. The studies on the bioassay method of AM-715 in body fluids. *Chemotherapy (Tokyo)* 1981;**29**(Suppl 4):91–7. (In Japanese).
17 Clendennen III TE, Hames CS, Kees ES, *et al.* In vitro antibiotic susceptibilities of *Neisseria gonorrhoeae* isolates in the Philippines. *Antimicrob Agents Chemother* 1992;**36**: 277–82.
18 Clendennen TE, Echeverria P, Saengeur S, *et al.* Antibiotic susceptibility survey of *Neisseria gonorrhoeae* in Thailand. *Antimicrob Agents Chemother* 1992;**36**:1682–7.
19 Chu M-L, Ho L-J, Lin H-C, Wu Y-C. Epidemiology of penicillin-resistant *Neisseria gonorrhoeae* isolated in Taiwan, 1960–1990. *Clin Infect Dis* 1992;**14**:450–7.
20 Kam K-M, Lai C-F, Egglestone S, Chan C-B B. Patterns of antibiotic susceptibility of gonococci isolated in Hong Kong, 1987–1990. *Sex Transm Dis* 1992;**19**:284–7.
21 Van Dyck E, Rossau R, Duhamel M, *et al.* Antimicrobial susceptibility of *Neisseria gonorrhoeae* in Zaire: high level plasmid-mediated tetracycline resistance in Central Africa. *Genitourin Med* 1992;**68**:111–6.
22 Ison CA, Pepin J, Roope NS, Demba E, Secka O, Easmon CSF. The dominance of a multiresistant strain of *Neisseria gonorrhoeae* among prostitutes and STD patients in The Gambia. *Genitourin Med* 1992;**68**: 356–60.
23 Bogaerts J, Tello WM, Akingeneye J, Mukantabana V, Van Dyck E, Piot P. Effectiveness of norfloxacin and ofloxacin for treatment of gonorrhoea and decrease of in vitro susceptibility to quinolones over time in Rwanda. *Genitourin Med* 1993;**69**:196–200.
24 Gransden WR, Warren C, Phillips I. 4-quinolone-resistant *Neisseria gonorrhoeae* in the United Kingdom. *J Med Microbiol* 1991;**34**:23–7.
25 Ison CA, Branley NS, Kirtland K, Easmon CSF. Surveillance of antibiotic resistance in clinical isolates of *Neisseria gonorrhoeae*. *BMJ* 1991;**303**:1307.