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In Vivo Evaluation of NM441, a New Thiazeto-Quinoline Derivative

MASAKUNI OZAKI,[1]* MASATO MATSUDA,[1] YOSHIFUMI TOMII,[1] KIYOSHI KIMURA,[1] JUN SEGAWA,[2] MASAHIKO KITANO,[2] MASAHIRO KISE,[2] KAZUO SHIBATA,[3] MASAKO OTSUKI,[3] AND TAKESHI NISHINO[3]

Biology Laboratories[1] and Chemistry Laboratories,[2] Nippon Shinyaku Co., Ltd., Nishiohji Hachijo, Minami-ku, Kyoto 601, and Department of Microbiology, Kyoto Pharmaceutical University, Kyoto 607,[3] Japan

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NM441 is a lipophilic prodrug of a new thiazeto-quinoline carboxylic acid derivative NM394, and when it is administered orally it is readily absorbed and hydrolyzed to its parent compound. After oral administration of NM441 at a dose of 20 mg/kg to dogs, the peak concentration of NM394 in plasma was 2.39 μg/ml, whereas it was 0.63 μg/ml for NM394 administered alone. The in vivo activity of NM441 was compared with those of ciprofloxacin, ofloxacin, and enoxacin in mouse protection studies. NM441 was as effective as ofloxacin and was twice as effective as ciprofloxacin against systemic infection with *Staphylococcus aureus*. Against infections with streptococci, NM441 was two to three times as effective as ofloxacin and five times as effective as ciprofloxacin. Against infection with *Escherichia coli*, NM441 was as effective as ciprofloxacin and ofloxacin, but against infections with *Klebsiella pneumoniae*, *Serratia marcescens*, and *Pseudomonas aeruginosa*, NM441 was two to four times as effective as ciprofloxacin and ofloxacin. NM441 was three to seven times as effective as enoxacin in systemic infections. Against urinary tract infections with *E. coli*, NM441 reduced the number of bacterial CFU per gram of kidney by 1 to 2 $\log_{10}$ more and, with *P. aeruginosa*, by 1 to 6 $\log_{10}$ more than did ciprofloxacin and ofloxacin. Against respiratory tract infections with *K. pneumoniae*, NM441 was as effective as ofloxacin and was twice as effective as ciprofloxacin.

In the course of our search for a new 6-fluoroquinolone antibacterial agent, thiazeto-quinoline carboxylic acid derivatives with a sulfur atom at the C-2 position were tested, and a new compound, NM394, 6-fluoro-1-methyl-7-(1-piperazinyl)-4-oxo-4*H*-[1,3]thiazeto[3,2-*a*]quinoline-3-carboxylic acid, was chosen as the most promising compound with potent antibacterial activity in vitro (7). However, despite its broad spectrum of activity, the protective effect of NM394 against experimental infections is poor when it is administered orally. In order to increase the absorption of NM394 from the intestinal tract, the prodrug of NM394, NM441, 6-fluoro-1-methyl-7-[4-(5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl-1-piperazinyl]-4-oxo-4*H*-[1,3]thiazeto[3,2-*a*]quinoline-3-carboxylic acid, was synthesized (Fig. 1). When NM441 was administered orally, it was readily absorbed and hydrolyzed to its parent compound, NM394, in blood and showed an excellent protective effect against experimental infections in mice. In this study, we compared the in vivo antibacterial activity and pharmacokinetic parameters of NM441 dosed orally compared with those of ciprofloxacin (11), ofloxacin (9) and enoxacin (4) and determined the pharmacokinetics of NM394 and NM441 in dogs.

(A part of this work was presented at the 29th Interscience Conference on Antimicrobial Agents and Chemotherapy [6].)

MATERIALS AND METHODS

Antimicrobial agents. NM394 and NM441 were synthesized in the chemistry laboratories of Nippon Shinyaku Co., Ltd. The other drugs used in this study were ofloxacin (Daiichi Seiyaku Co., Ltd., Tokyo, Japan), ciprofloxacin (Bayer Yakuhin Co., Ltd., Osaka, Japan), and enoxacin (Dainippon Seiyaku Co., Ltd., Osaka, Japan).

Bacterial strains. The bacterial strains used in this study were maintained in the Department of Microbiology, Kyoto Pharmaceutical University, Kyoto, Japan.

Systemic infections in mice. *Streptococcus pyogenes* C-203 and *Streptococcus pneumoniae* type III were cultured at 37°C for 20 h in heart infusion broth (Nissui, Tokyo, Japan) supplemented with 5% horse serum and diluted with nutrient broth (Nissui). The other organisms were cultured at 37°C for 20 h in nutrient broth and mixed with equal volumes of 6% mucin (Bacto Mucin Bacteriological; Difco Laboratories, Detroit, Mich.). Mice (Slc:ddY; male; weight, 19 to 22 g; Japan SLC. Inc., Shizuoka, Japan) in groups of 10 each were challenged intraperitoneally with 0.5 ml of the bacterial suspension. The organisms (challenge dose per mouse) were as follows: *S. pyogenes* C-203 (1.4×10^2 CFU, 316 times the 50% lethal dose [LD_{50}] in nutrient broth), *S. pneumoniae* type III (2.1×10^2, 5.4 times the LD_{50} in nutrient broth), *Staphylococcus aureus* Smith (2.1×10^6, 185 times the LD_{50} in mucin), *Escherichia coli* KC-14 (4.3×10^4, 54 times the LD_{50} in mucin), *Klebsiella pneumoniae* KC-1 (5.6×10^3, 280 times the LD_{50} in mucin), *Serratia marcescens* T-55 (4.6×10^6, 815 times the LD_{50} in mucin), and *Pseudomonas aeruginosa* E-2 (3.9×10^6, 578 times the LD_{50} in mucin).

Two hours after infection, graded doses of NM441, ciprofloxacin, ofloxacin, and enoxacin were administered orally to mice. All mice without drug treatment died within 3 days. Results were calculated as the 50% effective dose (ED_{50}),

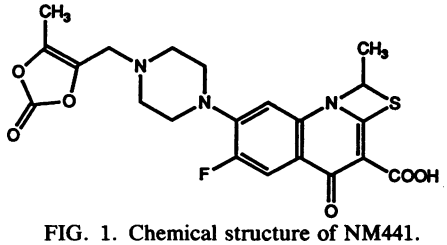

FIG. 1. Chemical structure of NM441.

* Corresponding author.

TABLE 1. Protective effects of NM441 and other quinolones against systemic infections in mice

Organism	Challenge dose (CFU/mouse [xLD_{50}])	Drug	MIC (μg/ml)	ED_{50} (mg/kg [95% confidence limits])[a]
Staphylococcus aureus Smith	2.1×10^6 (185)	NM441	0.20[b]	4.40 (3.80–5.65)
		Ciprofloxacin	0.10	11.00 (9.55–13.75)
		Ofloxacin	0.20	5.80 (5.00–7.45)
		Enoxacin	0.39	15.05 (12.35–17.10)
Streptococcus pyogenes C-203[c]	1.4×10^2 (316)	NM441	0.20[b]	22.00 (19.15–27.50)
		Ciprofloxacin	0.39	127.95 (106.2–144.45)
		Ofloxacin	0.78	70.70 (55.20–90.55)
		Enoxacin	3.13	>200
Streptococcus pneumoniae type III[c]	2.1×10^2 (5.4)	NM441	0.39[b]	23.00 (14.95–37.00)
		Ciprofloxacin	0.39	115.25 (75.65–236.25)
		Ofloxacin	0.78	48.85 (37.00–57.65)
		Enoxacin	3.13	>200
Escherichia coli KC-14	4.3×10^4 (54)	NM441	0.05[b]	0.35 (0.25–0.55)
		Ciprofloxacin	0.012	0.45 (0.35–0.60)
		Ofloxacin	0.05	0.55 (0.50–0.60)
		Enoxacin	0.20	2.20 (1.75–2.85)
Klebsiella pneumoniae KC-1	5.6×10^3 (280)	NM441	0.05[b]	0.55 (0.40–0.75)
		Ciprofloxacin	0.012	1.20 (0.90–1.55)
		Ofloxacin	0.05	1.10 (0.85–1.40)
		Enoxacin	0.20	4.05 (2.95–5.60)
Serratia marcescens T-55	4.6×10^6 (815)	NM441	0.39[b]	1.95 (1.25–3.00)
		Ciprofloxacin	0.10	5.55 (3.25–13.70)
		Ofloxacin	0.20	8.95 (6.20–13.40)
		Enoxacin	0.39	7.55 (5.05–11.45)
Pseudomonas aeruginosa E-2	3.9×10^6 (578)	NM441	0.39[b]	4.95 (19.10–32.75)
		Ciprofloxacin	0.39	46.50 (40.00–59.70)
		Ofloxacin	1.56	93.00 (79.95–119.40)
		Enoxacin	1.56	120.25 (98.75–136.75)

[a] ED_{50}s were calculated by the probit method from the survival rates on day 7 after infection.
[b] MIC of NM394.
[c] Infected without mucin.

including 95% confidence limits, by the probit method from the survival rates on day 7 after challenge.

Urinary tract infection. Mice (Crj:CD-1; female; weight, 18 to 23 g; Charles River Japan, Kanagawa, Japan) in groups of six or seven each were infected transurethrally (5) with *E. coli* KC-1404 (1.05×10^4 per mouse in 0.1 ml of saline) or *P. aeruginosa* E-2 (1.55×10^4 per mouse in 0.1 ml of saline), and then the distal end of the urethra was clamped for 4 h. NM441, ciprofloxacin, and ofloxacin were administered orally 4 h after challenge and subsequently twice a day for the next 3 days. Four days after challenge, the kidneys were removed aseptically, homogenized, serially diluted in saline, and cultured on BTB lactose agar (Nissui) to determine the number of bacterial CFU per gram of kidney tissue.

Respiratory tract infection. Mice (Slc:ddY; male; weight, 23 to 26 g) were infected with a nebulized bacterial suspension (10 ml, 4.05×10^8 CFU/ml) of *K. pneumoniae* B-54 (2) in an aerosol exposure apparatus (Ikemoto Scientific Technology Co., Ltd., Tokyo, Japan). NM441, ciprofloxacin, and ofloxacin were administered orally twice a day for 3 days beginning 20 h after challenge. All mice without drug treatment died within 6 days. Results were calculated as the ED_{50}, including 95% confidence limits, by the probit method from the survival rates on day 8 after challenge.

Thin-layer chromatography–bioautography of blood. Blood samples were collected from the orbital sinus 30 min after the oral administration of NM441 at a dose of 20 mg/kg to mice, mixed immediately with acetonitrile (0.5 ml/ml of blood), and then treated with dichloromethane (10 ml/ml of blood). NM441 in blood, if any, is extracted in the dichloromethane layer and its metabolites remain in the water layer. The water layer and the dichloromethane layer were dried in vacuo, and the residue dissolved in methanol was applied to a thin-layer chromatograph (DC-Alufolien Kieselgel $60F_{245}$; Merck) (solvent, dioxane, water, and formic acid [3:1:0.1]), with NM441 and NM394 used as standards. Bioautography was carried out with *E. coli* Kp as the assay organism. R_f values of the active compound in blood were compared with those of NM441 and NM394.

Pharmacokinetic study in mice. Mice (Slc:ddY; male; weight, 24 to 26 g) in groups of 20 each were orally administered 20 mg of NM441, ciprofloxacin, ofloxacin, and enoxacin per kg of body weight. At 0.5, 1, 2, and 4 h after dosing, blood samples were collected from the orbital sinus of five mice in a group. Serum samples were assayed by the agar-well method, with *E. coli* Kp used as the assay organism.

Pharmacokinetic study in dogs. NM441 or NM394 at a dose of 20 mg/kg of body weight was administered orally to dogs (beagle; male; weight, 9.5 to 11 kg) in groups of four

TABLE 2. Effects of NM441, ciprofloxacin, and ofloxacin on experimental urinary tract infections

Organism (challenge dose)[a]	Drug	MIC (μg/ml)	Dose (mg/kg)[b]	Log CFU/g (mean ± SE)[c]
Escherichia coli KC-1404 (1.11×10^4)	NM441	0.012[d]	0.123	4.67 ± 0.56
			0.37	3.80 ± 0.57
			1.1	2.61 ± 0.43
	Ciprofloxacin	0.025	0.123	6.40 ± 0.27
			0.37	4.30 ± 0.71
			1.1	3.24 ± 0.71
	Ofloxacin	0.05	0.123	6.10 ± 0.69
			0.37	4.93 ± 0.66
			1.1	3.46 ± 0.79
	Control			7.52 ± 0.27
Pseudomonas aeruginosa E-2 (1.55×10^4)	NM441	0.20[d]	3.0	3.46 ± 0.79
			9.0	2.21 ± 0.47
			27.0	1.10 ± 0.10
	Ciprofloxacin	0.20	3.0	5.32 ± 0.70
			9.0	3.87 ± 0.70
			27.0	2.20 ± 0.41
	Ofloxacin	1.56	3.0	9.92 ± 0.24
			9.0	4.15 ± 1.05
			27.0	3.27 ± 0.74
	Control			8.45 ± 0.36

[a] Mice were infected transurethrally with the test organism in 0.1 ml of saline.
[b] Drugs were administered orally 4 h after infection and then twice a day for the next 3 days.
[c] Numbers of bacterial CFU per gram in the kidneys were determined on day 4 after infection.
[d] MIC of NM394.

(NM441) and three (NM394) animals each. Blood samples were taken at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after dosing, and urine samples were collected over 24 h. The concentrations of active NM394 in plasma and urine were measured by the agar-well method, with *E. coli* Kp used as the assay organism.

TABLE 3. Effects of NM441, ciprofloxacin, and ofloxacin on experimental *K. pneumoniae* B-54 respiratory tract infections in mice[a]

Drug[b]	MIC (μg/ml)	ED_{50} (mg/kg [95% confidence limits])[c]
NM441	0.012[d]	0.981 (0.395–2.154)
Ciprofloxacin	0.025	2.243 (0.907–5.910)
Ofloxacin	0.10	1.180 (0.540–2.396)

[a] Mice were infected with a nebulized bacterial suspension (4×10^6 CFU/ml, 10 ml) in an aerosol exposure apparatus.
[b] Drugs were administered orally 20 h after infection and twice a day for the next 3 days.
[c] ED_{50}s were calculated by the probit method from the survival rates on day 8 after infection.
[d] MIC of NM394.

TABLE 4. Pharmacokinetic parameters of NM441, ciprofloxacin, ofloxacin, and enoxacin in mice[a]

Drug	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2\beta}$ (h)	$AUC_{0-\infty}$ (μg · h/ml)
NM441	0.83[b]	0.5	2.19	1.687
Ciprofloxacin	0.60	1	2.85	1.926
Ofloxacin	2.39[c]	0.5	1.26	4.637
Enoxacin	1.21	0.5	1.24	3.331

[a] Drugs were administered to mice at a dose of 20 mg/kg. NM441 was administered at a dose equivalent to that of NM394. C_{max}, peak concentration in serum; T_{max}, peak time in serum; $t_{1/2\beta}$, elimination half-life in serum; $AUC_{0-\infty}$, area under the serum concentration-time curve from 0 h to infinity.
[b] Concentration of NM394 in serum.
[c] Statistically significantly difference from NM441 ($P < 0.05$).

RESULTS

Protection against systemic infection. The protective effect of NM441 against systemic infections in mice was compared with those of ciprofloxacin, ofloxacin, and enoxacin (Table 1). NM441 was as effective as ofloxacin against infection with *S. aureus* Smith and was as effective as ofloxacin and ciprofloxacin against infection with *E. coli* KC-14. NM441 was the most effective compound against infections with streptococci. The ED_{50}s for *S. pyogenes* C-203 and *S. pneumoniae* type III were 22.0 and 23.0 mg/kg, respectively; these values were two to three times lower than those of ofloxacin and five to six times lower than those of ciprofloxacin. Against infections with *K. pneumoniae* KC-1, *S. marcescens* T-55, and *P. aeruginosa* E-2, the ED_{50}s of NM441 were 0.55, 1.95, and 24.95 mg/kg, respectively, which were two to three times lower than those of ciprofloxacin, two to four times lower than those of ofloxacin, and four to seven times lower than those of enoxacin.

Protection against urinary tract infection. As shown in Table 2, against urinary tract infections with *E. coli* KC-1404, NM441 reduced the number of bacterial CFU per gram of kidney by 1 to 2 $\log_{10}$ more and, with *P. aeruginosa* E-2, 1 to 6 $\log_{10}$ more than did ciprofloxacin and ofloxacin. NM441 was more potent than ciprofloxacin and ofloxacin against the urinary tract infection models used in this study.

Protection against respiratory tract infection. The ED_{50} of NM441 against respiratory tract infections was 0.98 mg/kg, which was equal to that of ofloxacin and two times lower than that of ciprofloxacin (Table 3).

Thin-layer chromatography–bioautography. Only one active compound with an R_f value on thin-layer chromatography which was identical to that of authentic NM394 was detected in the blood samples.

TABLE 5. Pharmacokinetic parameters of NM441 and NM394 in dogs[a]

Drug	C_{max} (μg/ml)	T_{max} (h)	$t_{1/2\beta}$ (h)	$AUC_{0-\infty}$ (μg · h/ml)	% Urinary recovery[b]
NM394	0.63	1.7	6.28	6.18	3.65
NM441	2.39[c]	2.0	6.19	22.15[c]	11.71[c]

[a] Drugs were administered at doses of 20 mg/kg to fasting dogs. NM441, $n = 4$; NM394, $n = 3$. NM441 was administered at a dose equivalent to that of NM394. C_{max}, peak concentration of NM394 in plasma after oral administration of NM441 and NM394; T_{max} peak time in plasma; $t_{1/2\beta}$, elimination half-life in plasma; $AUC_{0-\infty}$, area under the plasma concentration curve from 0 h to infinity.
[b] Recovery from 0 to 24 h.
[c] Statistically significantly difference from NM394 ($P < 0.01$).

Pharmacokinetics in mice. The peak concentrations of NM441, ciprofloxacin, ofloxacin, and enoxacin in serum were 0.83, 0.60, 2.39, and 1.21 μg/ml, respectively, and the areas under the curve (AUC) were 1.687, 1.926, 4.637, and 3.331 μg · h/ml, respectively (Table 4).

Pharmacokinetics in dogs. The pharmacokinetic parameters of NM394 and NM441 are given in Table 5. The peak concentration in plasma, AUC, and percent recovery from urine of NM394 after oral administration of NM441 were significantly increased compared with those of NM394 administered orally.

DISCUSSION

Among the thiazeto-quinoline carboxylic acid derivatives synthesized, NM394 showed the most potent antibacterial activity, but its oral absorption was poor. To improve the oral absorption, a prodrug of NM394, NM441, a *N*-(5-methyl-2-oxo-1,3-dioxolen-4-yl)methyl derivative of NM394, was synthesized. NM441 had potent protective activity against experimental systemic infections, urinary tract infections, and respiratory tract infections in mice. The ED_{50}s of NM441 against infections with gram-positive and gram-negative organisms, but not against infections with *E. coli* KC-14, were two to five times lower than those of ciprofloxacin. The MICs of NM394 against the organisms used in the protection tests were the same or higher than those of ciprofloxacin. The ED_{50}s observed for NM441 were much lower than those expected from its MICs in in vitro tests and compared with the results for the other reference drugs, ciprofloxacin, ofloxacin, and enoxacin. Such lower than expected ED_{50}s of NM441 could not be explained from the pharmacokinetic studies in mice, since the level of NM394 in serum was similar to those of ciprofloxacin and enoxacin and since all reference drugs had AUCs greater than that of NM441. These results suggest that factors other than in vitro antibacterial activities and peak levels in blood are responsible for the in vivo activity of NM441.

Thin-layer chromatography–bioautography of blood samples suggests that orally administered NM441 is readily absorbed and easily hydrolyzed to its parent compound, NM394. The hydrolysis of NM441 to NM394 probably occurs in blood, as demonstrated previously (1, 8).

When NM441 was administered orally, the level of NM394 in plasma was about 3.6 times higher than that when NM394 was administered alone. The AUC and recovery of NM394 from urine in dogs after oral administration of NM441 were also significantly greater than those observed after oral administration of NM394. The elimination half-life of NM394 in plasma was 6.19 h, which was longer than those of enoxacin (3) and ofloxacin (10).

The in vivo potencies of NM441 in mice and the longer half-life in dogs suggest that it should be evaluated further for treating infections in humans.

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