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Antibacterial Properties of Ro 40-6890, a Broad-Spectrum Cephalosporin, and Its Novel Orally Absorbable Ester, Ro 41-3399

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Ro 41-3399 is a novel orally active ester of Ro 40-6890, an aminothiazolyl cephalosporin with potent in vitro activities against commonly encountered aerobic gram-positive bacteria (streptococci and methicillin-susceptible staphylococci) and gram-negative bacteria (members of the family *Enterobacteriaceae*, haemophili, meningococci, and gonococci). In terms of the MICs determined by the methods recommended by the National Committee for Clinical Laboratory Standards, for 50 and 90% of gram-positive organisms, the water-soluble free carboxylic acid Ro 40-6890 proved to be at least as active as or two- to fourfold more active than cefpodoxime, cefuroxime, cefaclor, amoxicillin, amoxicillin-clavulanic acid, and ceftriaxone; against aerobic gram-negative organisms, Ro 40-6890 was usually two- to fourfold more active than cefpodoxime, the next most potent of the oral drugs under comparison, but remained usually two- to fourfold weaker than ceftriaxone. Ro 40-6890 showed a high affinity for the essential penicillin-binding proteins of susceptible bacteria and was resistant to hydrolysis by a broad array of β-lactamases. Ro 41-3399 bopentil was well absorbed in mice when administered by oral gavage and proved effective in several experimental bacterial infections. Further studies with Ro 41-3399 and Ro 40-6890 are in progress.

Most potent cephalosporins are poorly absorbed from the human intestinal tract and are therefore suited only for parenteral use. However, some of these compounds can be rendered amenable to oral use by esterification of the C-4 carboxyl group of the dihydrothiazine ring. Examples are cefuroxime axetil (6, 14, 16), cefpodoxime proxetil (5, 8), and cefetamet pivoxil (1). Structure-bioavailability relationship studies indicate that the nature of the alcohol used for derivatization has a major impact on the oral bioavailability of the ester.

Ro 41-3399 {(*E*)-2-(isobutoxycarbonyl)-2-pentenyl (6*R*,7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-(azidomethyl)-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylate} (Fig. 1A) is a novel oral ester of the poorly absorbed broad-spectrum cephalosporin Ro 40-6890 {(6*R*, 7*R*)-7-[(*Z*)-2-(2-amino-4-thiazolyl)-2-(methoxyimino)acetamido]-3-(azidomethyl)-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid sodium salt} (Fig. 1B).

Ro 41-3399 was chosen for development from other esters of the free carboxylic acid Ro 40-6890 because of its higher oral bioavailability in rats (7). In this paper, we describe the evaluation of the free carboxylic acid Ro 40-6890 in vitro and the in vivo activities of Ro 41-3399 as a hydrochloride salt in comparison with those of other oral β-lactams or their respective free carboxylic acids and ceftriaxone, a widely used parenteral aminothiazolyl cephalosporin.

MATERIALS AND METHODS

Antibiotics. Ro 40-6890 and Ro 41-3399 were synthesized at the Pharmaceutical Research Laboratories, F. Hoffmann-La Roche Ltd., Basel, Switzerland. Cefpodoxime and ceftriaxone were obtained from in-house sources, and amoxicillin-clavulanic acid (ratio, 4:1), amoxicillin, cefaclor, and cephaloridine were purchased from commercial outlets.

Bacteria. The strains used in this study were recent single-patient isolates from clinical microbiology laboratories in various, mainly European and Southeast Asian, hospitals. They were identified by standard methods (11) and kept as stock cultures at −70°C or below until use.

The bacterial strains used as sources for β-lactamases were of the following origins: *Escherichia coli* SNO3(pAD7) was obtained from S. Normark, *Citrobacter freundii* 1203 was obtained from B. Wiedemann, *Enterobacter cloacae* 908R is a laboratory isolate selected by exposure to β-lactam antibiotics, *Bacillus licheniformis* 729/C was obtained from R. Ambler, *Staphylococcus aureus* PC1 was obtained from R. Pairs, *E. coli* DC2 (TEM-1) was obtained from M. Arisawa, *E. coli* CF 102 (TEM-3) was obtained from J. Sirot, and *Pseudomonas aeruginosa* 18SH was obtained from E. Zimmermann.

Susceptibility studies. MICs were determined for the most part by the agar dilution methods advocated by the National Committee for Clinical Laboratory Standards (NCCLS) (12). Nonfastidious aerobic organisms, including *Moraxella catarrhalis*, were grown on Mueller-Hinton agar (MHA) (Difco); haemophili were grown on HTM medium (MHA [Remel, Lenexa, Kans.] supplemented with 5 g of yeast extract per liter, 15 μg of hematin per ml, and 15 μg of β-NAD per ml); gonococci were grown on GC medium base (Difco) agar enriched with 2 μg of menadione per liter, 2% hemoglobin (Difco), and 1% IsoVitaleX (BBL); streptococci, corynebacteria, meningococci, and *Neisseria sicca* were grown on MHA with 5% sheep blood; and protein was grown on MHA with 2.4% agarose. Similarly, Iso-Sensitest agar (Oxoid), Diagnostic Sensitivity Test agar (Oxoid), Iso-Sensitest broth (Oxoid), and Mueller-Hinton broth (BBL)

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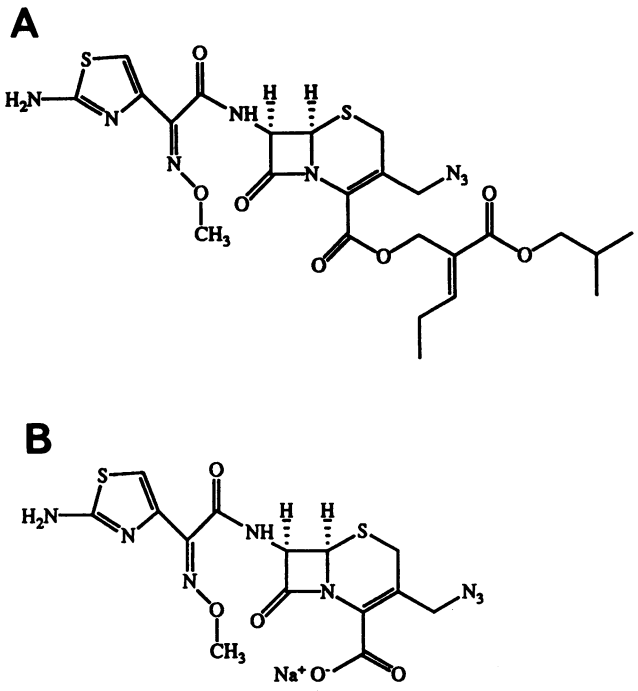

FIG. 1. Chemical structures of Ro 41-3399 (A) and Ro 40-6890 (B).

were used to study the effect of the medium on MICs. For broth tests, broth microdilution tests were performed as recommended by the NCCLS (12).

Overnight cultures were diluted to a concentration of about 5×10^7 CFU/ml. Agar plates containing serial twofold dilutions of antibiotics were inoculated with the help of a multipoint inoculator (Denley A400) to yield about 1×10^4 to 5×10^4 CFU per spot; broth microdilution wells were inoculated with a multipipettor to yield about 1×10^4 to 5×10^4 CFU per well. Three reference strains, *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and *S. aureus* ATCC 25923, were included in all parts of the study, as needed.

The inoculated plates were incubated for 18 h at 35°C in room air, except those for staphylococci, which were incubated for 24 h, and those for haemophili, gonococci, and pneumococci, which were incubated in a CO_2 incubator (10% CO_2).

The MICs were read with the naked eye and defined as the lowest concentration of antibiotic that prevented clearly visible growth (a barely visible haze and the growth of five or fewer colonies per spot were disregarded). The interpretive criteria used for the comparative agents were those advocated by the NCCLS (13).

The presence of bacterial β-lactamase was detected by the nitrocefin (Cefinase; BBL) colorimetric method. A change in color from yellow to red within 2 min was interpreted as hydrolysis of the β-lactam ring of the nitrocefin molecule by a β-lactamase(s).

Plasma protein binding. The plasma protein binding of Ro 40-6890 was evaluated in triplicate in murine plasma (Swiss albino mice; Jbm MoRo [specific pathogen free]; Biomedical Research Laboratories, Füllinsdorf, Switzerland) and pooled human plasma from healthy volunteers by a standard ultrafiltration technique (10 min at 2,000 × *g* and 37°C) with a Centrifree micropartition system (Amicon, Lexington, Mass.). The concentrations of Ro 40-6890 and comparative agents were 25, 50, and 250 μg/ml. Antibiotic and pooled plasma (2 ml) were allowed to equilibrate for 10 min at 37°C and pH 7.4 prior to centrifugation. The protein-free ultrafiltrate (0.1 ml) was assayed in triplicate by an agar well bioassay method. The indicator organism was *E. coli* 1346, and the medium was Antibiotic Medium no. 1 (Difco). A series of standards were prepared and incubated along with the unknowns. Inhibition diameters for the filtrate (Cf), the spiked plasma (Cp) and, as a control, the antibiotic solution used to spike the plasma were plotted on a graph that allowed extrapolation of the results for the unknown nonprotein-bound fraction of the antibacterial agent. The percentage of "free" drug in the filtrate was calculated as (Cf/Cp) × 100, and the percentage of plasma protein-bound drug was calculated as [1 − (Cf/Cp)] × 100.

Affinity for essential PBPs. The concentrations of several β-lactam antibiotics that decreased the binding of [^{14}C]benzylpenicillin (specific activity, 53 to 55 mCi/mmol; Amersham International, Amersham, United Kingdom) by 50% were determined for the essential penicillin-binding proteins (PBPs) of *E. coli* W3110 and *S. aureus* Schoch as described earlier (15).

Hydrolysis by β-lactamases. All enzymes used were purified by adaptions of established procedures and appeared homogeneous in Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels.

The spectral parameters used for Ro 40-6890 were $\lambda = 262$ nm and $\Delta\epsilon = 7{,}700\ M^{-1}\ cm^{-1}$. All kinetic measurements were performed at 37°C with 0.1 M sodium phosphate (0.06 1 M Na_2HPO_4, 0.039 M NaH_2PO_4) (pH 7.0). The rates of hydrolysis were determined spectrophotometrically with either a Kontron Uvikon 860 spectrophotometer or a Perkin Elmer λ2 spectrophotometer coupled to an Epson PC2Xe personal computer for data capture and analysis. Kinetic parameters were estimated from time courses recorded for reaction mixtures containing different initial concentrations of the substrate by use of direct, weighted fits to the Michaelis-Menten equation (10). The K_i was determined by competition with nitrocefin by use of various inhibitory concentrations and nitrocefin concentrations. The range of concentrations used was between 0.3 and 3 times the K_m (or K_i) when feasible. The highest inhibitory concentration tested was 500 μM.

Single-dose pharmacokinetics in animals. For single-dose trials, Ro 40-6890, Ro 41-3399, and comparative β-lactams were given to mice (Jbm MoRo [specific pathogen free]; weight, 30 to 40 g) per os (p.o.) or subcutaneously (s.c.) at a concentration of 20 mg/kg of body weight. Antibacterial agents were dissolved in 4% (vol/vol) Tween (Sigma) and administered by forced gavage or by s.c. injection (nuchal skinfold). Animals (four mice per time point) were rendered unconscious with CO_2 just prior to the sampling times, and heart blood was removed with potassium ammonium citrate (10 μl of a 25% aqueous solution per ml of blood) as an anticoagulant. Sampling times were 0, 5, 10, 15, 30, 45, 60, 90, 120, 180, 210, and 300 min after antibiotic administration. Plasma was separated by centrifugation, pooled for each time point, and stored at −70°C or below until further use. A bioassay with *E. coli* 1346 as an indicator strain, Antibiotic Medium no. 1 (Difco), and a standard agar well technique was performed, and inhibition zone diameters were recorded and compared with a series of standards obtained with spiked murine plasma.

The concentration results for plasma were analyzed by a standard nonlinear least-squares computer program (NON

TABLE 1. Comparative in vitro activities of Ro 40-6890 and six standard β-lactams against aerobic gram-positive cocci

Organism (no. tested)	Antimicrobial agent[a]	MIC (μg/ml)		
		50%	90%	Range
Streptococcus pyogenes (35)	Ro 40-6890	0.015	0.015	0.015–0.03
	Cefpodoxime	0.015	0.015	0.015–0.03
	Amoxicillin-clavulanic acid	0.03	0.03	0.03–0.06
	Amoxicillin	0.03	0.03	0.03
	Cefuroxime	0.015	0.03	0.015–0.125
	Cefaclor	0.12	0.25	0.06–0.25
	Ceftriaxone	0.03	0.06	≤0.015–0.06
Streptococcus pneumoniae, penicillin susceptible[b] (17)	Ro 40-6890	≤0.03	≤0.03	≤0.03
	Cefpodoxime	≤0.03	≤0.03	≤0.03–0.06
	Amoxicillin-clavulanic acid	≤0.03	≤0.03	≤0.03–0.06
	Amoxicillin	≤0.03	≤0.03	≤0.03
	Cefuroxime	≤0.03	≤0.03	≤0.03–0.06
	Cefaclor	0.5	0.5	0.25–0.5
	Ceftriaxone	0.03	0.03	≤0.015–0.25
Streptococcus pneumoniae, penicillin resistant[c] (18)	Ro 40-6890	1	2	0.12–2
	Cefpodoxime	1	4	0.12–4
	Amoxicillin-clavulanic acid	0.25	2	≤0.03–2
	Amoxicillin	0.25	2	≤0.03–2
	Cefuroxime	1	4	0.12–4
	Cefaclor	2	32	0.5–>32
	Ceftriaxone	1	2	0.06–2
Staphylococcus aureus, methicillin susceptible (18)	Ro 40-6890	1	1	0.12–1
	Cefpodoxime	2	4	1–4
	Amoxicillin-clavulanic acid	0.5	1	0.25–1
	Amoxicillin	1	32	0.12–32
	Cefuroxime	1	1	0.25–2
	Cefaclor	1	4	0.5–8
	Ceftriaxone	2	2	0.25–2
Staphylococcus epidermidis (14)	Ro 40-6890	0.5	0.5	0.12–0.5
	Cefpodoxime	1	1	0.5–1
	Amoxicillin-clavulanic acid	0.25	0.5	0.12–1
	Amoxicillin	0.5	1	0.06–4
	Cefuroxime	0.5	0.5	0.12–1
	Cefaclor	1	1	0.5–2
	Ceftriaxone	1	2	0.5–2
Staphylococcus saprophyticus (18)	Ro 40-6890	2	4	0.5–4
	Cefpodoxime	4	8	1–8
	Amoxicillin-clavulanic acid	1	1	0.25–1
	Amoxicillin	0.5	1	0.25–1
	Cefuroxime	2	4	0.5–8
	Cefaclor	4	8	1–8
	Ceftriaxone	NA[e]	NA	NA
Corynebacterium spp.[d] (8)	Ro 40-6890			≤0.03–0.5
	Cefpodoxime			0.25–2
	Amoxicillin-clavulanic acid			0.06–4
	Amoxicillin			0.06–2
	Cefuroxime			0.06–2
	Cefaclor			0.06–16
	Ceftriaxone			0.25–2
Corynebacterium jeikeium (10)	Ro 40-6890	8	≥64	4–≥64
	Cefpodoxime	≥64	≥64	32–≥64
	Amoxicillin-clavulanic acid	≥64	≥64	8–≥64
	Amoxicillin	≥64	≥64	8–≥64
	Cefuroxime	8	≥64	8–≥64
	Cefaclor	≥64	≥64	16–≥64
	Ceftriaxone	16	≥64	1–≥64

[a] The MIC of amoxicillin-clavulanic acid is expressed as the value for the amoxicillin component of the combination.
[b] Penicillin MIC, ≤0.06 μg/ml.
[c] Penicillin MIC, ≥0.12 μg/ml.
[d] *C. diphtheriae* (n = 1), *C. pseudodiphtheriticum* (n = 3), *C. equi* (n = 1), and *C. haemolyticum* (n = 3).
[e] NA, not available.

TABLE 2. Comparative in vitro activities of Ro 40-6890 and six standard β-lactams against aerobic gram-negative bacteria

Organism (no. tested)	Antimicrobial agent[a]	MIC (μg/ml)		
		50%	90%	Range
Escherichia coli (19)	Ro 40-6890	0.12	0.12	0.06–0.25
	Cefpodoxime	0.5	1	0.5–1
	Amoxicillin-clavulanic acid	8	8	4–16
	Amoxicillin	8	≥64	4–≥64
	Cefuroxime	4	8	2–8
	Cefaclor	2	4	1–4
	Ceftriaxone	0.06	0.06	≤0.03–0.06
Citrobacter freundii (29)	Ro 40-6890	16	≥64	0.06–≥64
	Cefpodoxime	≥64	≥64	1–≥64
	Amoxicillin-clavulanic acid	≥64	≥64	2–≥64
	Amoxicillin	≥64	≥64	≥64
	Cefuroxime	≥64	≥64	1–≥64
	Cefaclor	≥64	≥64	≤8–≥64
	Ceftriaxone	≥64	≥64	
Enterobacter cloacae (42)	Ro 40-6890	1	≥64	0.012–≥64
	Cefpodoxime	8	≥64	0.5–≥64
	Amoxicillin-clavulanic acid	≥64	≥64	32–≥64
	Amoxicillin	≥64	≥64	≤8–≥64
	Cefuroxime	16	≥64	2–≥64
	Cefaclor	≥64	≥64	16–≥64
	Ceftriaxone	4	128	0.12–128
Klebsiella oxytoca (26)	Ro 40-6890	≤0.03	1	≤0.03–1
	Cefpodoxime	0.06	4	0.06–4
	Amoxicillin-clavulanic acid	4	≥64	2–≥64
	Amoxicillin	≥64	≥64	16–≥64
	Cefuroxime	2	≥64	1–≥64
	Cefaclor[b]	1	1	0.5–1
	Ceftriaxone	0.03	0.5	0.015–8
Klebsiella pneumoniae (18)	Ro 40-6890	0.06	0.5	≤0.03–0.5
	Cefpodoxime	0.25	1	0.03–2
	Amoxicillin-clavulanic acid	16	≥64	8–≥64
	Amoxicillin	≥64	≥64	8–≥64
	Cefuroxime	4	32	0.06–32
	Cefaclor	1	32	0.5–32
	Ceftriaxone	0.06	0.25	0.03–0.25
Morganella morganii (18)	Ro 40-6890	1	2	0.5–4
	Cefpodoxime	8	16	8–16
	Amoxicillin-clavulanic acid	≥64	≥64	≥64
	Amoxicillin	≥64	≥64	≥64
	Cefuroxime	≥64	≥64	32–≥64
	Cefaclor	≥64	≥64	≥64
	Ceftriaxone	≤0.015	0.06	≤0.015–8
Proteus mirabilis (18)	Ro 40-6890	≤0.03	≤0.03	≤0.03–0.06
	Cefpodoxime	0.06	0.12	≤0.03–0.12
	Amoxicillin-clavulanic acid	2	2	1–8
	Amoxicillin	1	2	1–≥64
	Cefuroxime	2	2	1–4
	Cefaclor	2	2	0.5–2
	Ceftriaxone	≤0.015	≤0.015	≤0.015
Proteus vulgaris (18)	Ro 40-6890	0.06	0.12	≤0.03–0.12
	Cefpodoxime	0.25	0.5	0.06–0.5
	Amoxicillin-clavulanic acid	16	16	2–16
	Amoxicillin	≥64	≥64	1–≥64
	Cefuroxime	≥64	≥64	2–≥64
	Cefaclor	≥64	≥64	4–≥64
	Ceftriaxone	0.12	0.12	0.015–0.25

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TABLE 2–*Continued*

Organism (no. tested)	Antimicrobial agent[a]	MIC (μg/ml)		
		50%	90%	Range
Serratia liquefaciens (8)	Ro 40-6890			0.25–0.5
	Cefpodoxime			0.5–1
	Amoxicillin-clavulanic acid			8–32
	Amoxicillin			8–32
	Cefuroxime			8–32
	Cefaclor			16–≥64
	Ceftriaxone			0.12–0.25
Serratia marcescens (18)	Ro 40-6890	0.5	2	0.25–32
	Cefpodoxime	2	8	1–≥64
	Amoxicillin-clavulanic acid	≥64	≥64	≥64
	Amoxicillin	≥64	≥64	≥64
	Cefuroxime	≥64	≥64	32–≥64
	Cefaclor	≥64	≥64	≥64
	Ceftriaxone	0.5	1	0.25–16
Plesiomonas shigelloides (18)	Ro 40-6890	≤0.03	≤0.03	≤0.03
	Cefpodoxime	≤0.03	≤0.03	≤0.03
	Amoxicillin-clavulanic acid	8	8	4–8
	Amoxicillin	16	32	8–32
	Cefuroxime	0.03	0.06	≤0.03–0.06
	Cefaclor	0.25	0.5	0.25–0.5
	Ceftriaxone	≤0.015	≤0.015	≤0.015
Vibrio cholerae[b] (17)	Ro 40-6890	≤0.03	≤0.03	≤0.03–0.12
	Cefpodoxime	0.12	0.12	0.06–0.12
	Amoxicillin-clavulanic acid	≥32	≥32	≥32
	Amoxicillin	≥32	≥32	≥32
	Cefuroxime	0.5	0.5	0.25–1
	Cefaclor	2	2	2–4
	Ceftriaxone	0.03	0.03	0.03
Acinetobacter anitratus (17)	Ro 40-6890	32	≥64	1–≥64
	Cefpodoxime	16	≥64	4–≥64
	Amoxicillin-clavulanic acid	≥64	≥64	16–≥64
	Amoxicillin	≥64	≥64	16–≥64
	Cefuroxime	32	≥64	8–≥64
	Cefaclor	≥64	≥64	≥64
	Ceftriaxone	32	64	4–64
Haemophilus influenzae, β-lactamase negative (18)	Ro 40-6890	0.03	0.03	0.015–0.06
	Cefpodoxime	0.06	0.12	0.06–0.12
	Amoxicillin-clavulanic acid	0.5	1	0.5–1
	Amoxicillin	0.5	1	0.5–1
	Cefuroxime	0.5	1	0.5–1
	Cefaclor	4	8	2–8
	Ceftriaxone	≤0.008	≤0.008	≤0.008
Haemophilus parainfluenzae, β-lactamase negative (18)	Ro 40-6890	0.03	0.12	0.03–0.12
	Cefpodoxime	0.12	0.12	0.03–0.12
	Amoxicillin-clavulanic acid	0.5	1	0.25–1
	Amoxicillin	1	1	0.25–1
	Cefuroxime	1	1	0.25–2
	Cefaclor	4	8	1–16
	Ceftriaxone	0.004	0.008	0.001–0.008
Moraxella catarrhalis (16)	Ro 40-6890	0.25	0.5	≤0.03–1
	Cefpodoxime	0.5	1	0.06–2
	Amoxicillin-clavulanic acid	0.12	0.5	≤0.03–0.5
	Amoxicillin	1	4	≤0.03–4
	Cefuroxime	1	2	0.12–2
	Cefaclor	1	1	0.12–1
	Ceftriaxone	0.03	0.5	≤0.015–1

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TABLE 2—*Continued*

Organism (no. tested)	Antimicrobial agent[a]	MIC (μg/ml)		
		50%	90%	Range
Neisseria[c] spp. (14)	Ro 40-6890	≤0.03	0.5	≤0.03–2
	Cefpodoxime	≤0.03	0.5	≤0.03–1
	Amoxicillin-clavulanic acid	0.25	2	0.12–2
	Amoxicillin	0.12	2	0.06–2
	Cefuroxime	≤0.03	16	≤0.03–16
	Cefaclor	0.12	8	0.06–8
	Ceftriaxone	≤0.015	0.25	≤0.015–0.25
Neisseria gonorrhoeae, β-lactamase negative (18)	Ro 40-6890	0.015	0.12	0.001–0.5
	Cefpodoxime	0.015	0.03	0.001–0.5
	Amoxicillin-clavulanic acid	0.5	1	0.06–2
	Amoxicillin	0.5	4	0.06–16
	Cefuroxime	0.03	0.5	0.008–1
	Cefaclor	2	4	0.25–≥16
	Ceftriaxone	0.004	0.015	0.001–0.06
Neisseria gonorrhoeae, penicillinase producing (10)	Ro 40-6890	0.03	0.06	0.008–0.06
	Cefpodoxime	0.03	0.06	0.008–0.06
	Amoxicillin-clavulanic acid	2	2	1–2
	Amoxicillin	≥64	≥64	16–≥64
	Cefuroxime	0.25	0.5	0.06–0.5
	Cefaclor	4	8	2–8
	Ceftriaxone	0.008	0.015	0.004–0.015
Neisseria gonorrhoeae, chromosomally mediated penicillin resistant (8)	Ro 40-6890			0.03–0.25
	Cefpodoxime			0.015–0.12
	Amoxicillin-clavulanic acid			1–2
	Amoxicillin			1–4
	Cefuroxime			0.25–2
	Cefaclor			4–≥16
	Ceftriaxone			0.015–0.06

[a] See Table 1, footnote *a*.
[b] Only nine strains tested.
[c] *V. cholerae* 01, El Tor, subtype Inaba (n = 10); *V. cholerae* 01, El Tor, subtype Ogawa (n = 7).
[d] *N. meningitidis* (n = 9) and *N. sicca* (n = 5).

LIN) that is based on a two-compartment open model. The area under the plasma concentration-time curve from 0 h to infinity was obtained by conventional trapezoidal summation and extrapolation methods. The presence and the biological activity of metabolites were not investigated. Any biological inhibitory activity found in pooled murine plasma was expressed as inhibitory activity of unchanged parent compound.

Experimental septicemia in mice. Septicemia was induced in outbred Swiss albino mice (Jbm MoRo [specific pathogen free]; weight, 16 to 20 g). Mice were infected by intraperitoneal injection of diluted overnight cultures of the test organisms. *S. aureus* Schoch and *S. aureus* 887 were injected as suspensions in 4% hog gastric mucin (American Laboratories, Omaha, Nebr.), and *Haemophilus influenzae* 1 was injected as a suspension in fresh egg yolk. No virulence enhancers were used for any of the other strains. Bacterial challenge doses were 8 to 20 times the number of organisms required to kill 50% of untreated animals within 72 h. The test compounds were administered p.o. 1 and 3 h after the bacterial challenge. Control and treatment groups at each dose were composed of five mice each. The 50% effective dose (ED_{50}, in milligrams per kilogram) was calculated by probit analysis as described by Finney (4) from the survival rates on day 4 after infection.

RESULTS

Susceptibility testing. The results of testing of the activities of Ro 40-6890 against 138 gram-positive bacterial isolates are summarized in Table 1. Ro 40-6890 inhibited *Streptococcus pyogenes* and penicillin-susceptible pneumococci at a concentration of ≤0.03 μg/ml. Penicillin-resistant pneumococci, however, were at least 30-fold less susceptible than penicillin-susceptible pneumococci to all of the newer β-lactams tested, except for cefaclor. Along with cefuroxime and amoxicillin-clavulanic acid, Ro 40-6890 was also inhibitory at a concentration of ≤1 μg/ml against methicillin-susceptible *S. aureus*, *Staphylococcus epidermidis*, and *Corynebacterium* spp., with an MIC_{90} (MIC for 90% of isolates) of 2 μg/ml against *S. aureus*, and had an MIC_{90} of 0.5 μg/ml against *Staphylococcus saprophyticus*. For all methicillin-resistant staphylococci (data not shown) and all but one of the *Corynebacterium jeikeium* strains, MICs were ≥16 μg/ml. Ro 40-6890 displayed low activities (MICs, ≥16 μg/ml) like that of the comparative cephalosporins against representative isolates of enterococci (*Enterococcus faecalis* and *Enterococcus faecium*) and *Listeria* spp. (data not shown).

The activities of Ro 40-6890 against 368 aerobic gram-negative bacterial isolates (Table 2) were comparable to those of ceftriaxone (with MICs usually 1 to 2 dilution steps lower than those of Ro 40-6890) and cefpodoxime (with

TABLE 3. Influence of medium on the activities of Ro 40-6890 against 21 strains[a]

Organism	Ro 40-6890 MIC (mg/liter) with the following medium[b]:				
	Solid			Liquid	
	MHA	IA	DSTA	MHB	IB
Staphylococcus aureus Schoch	1	0.5	0.5	1	1
Edwardsiella tarda QR	0.03	0.25	0.5	≤0.03	≤0.03
Escherichia coli BFP	0.12	0.25	0.25	0.25	0.12
Escherichia coli UB 1005	0.06	0.06	0.06	0.06	0.06
Escherichia coli J44	0.06	0.06	0.06	≤0.03	0.12
Escherichia coli ATCC 35218	0.12	0.06	0.12	0.12	0.12
Escherichia coli ATCC 25922	0.25	0.25	0.12	0.25	0.25
Klebsiella pneumoniae NCTC 418	0.12	0.12	0.06	0.12	0.12
Klebsiella pneumoniae 1020	≤0.03	0.06	≤0.03	≤0.03	≤0.03
Klebsiella oxytoca 1082E	32	32	≥64	≥64	≥64
Escherichia coli 43	0.12	0.5	0.12	1	0.25
Serratia marcescens 69438	8	1	1	1	1
Enterobacter cloacae 908 Ssi	1	0.5	0.5	0.5	0.5
Citrobacter freundii 902	0.5	0.5	0.25	0.5	1
Citrobacter diversus 10/90	0.12	0.5	0.12	0.12	0.12
Proteus mirabilis 2117	0.5	0.5	0.5	0.06	0.06
Proteus vulgaris 1028	1	1	2	≥64	≥64
Morganella morganii 64137	0.12	0.5	0.25	0.25	0.25
Salmonella typhimurium	0.06	0.25	0.06	0.06	0.06
Staphylococcus aureus ATCC 25923	0.5	0.5	0.5	0.5	1
Aeromonas hydrophila	0.03	0.12	≤0.03	0.06	≤0.03

[a] The inoculum was 10^4 CFU per spot or well.
[b] IA, Iso-Sensitest agar; DSTA, Diagnostic Sensitivity Test agar; MHB, Mueller-Hinton broth; IB, Iso-Sensitest broth.

MICs usually 1 to 2 dilution steps higher than those of Ro 40-6890) and distinctly higher than those of the other agents tested. Only members of the family *Enterobacteriaceae* that overproduce group 1 β-lactamases, e.g., *C. freundii*, *E. cloacae*, and *Serratia marcescens*, were highly resistant to all compounds tested, whereas bacterial isolates that did not express these enzymes were highly susceptible to Ro 40-6890 and ceftriaxone. Representative isolates of *Citrobacter diversus* (data not shown), *Klebsiella oxytoca*, *Morganella morganii*, *Proteus vulgaris*, and *Serratia liquefaciens* were readily inhibited by the new oral aminothiazolyl cephalosporin and its congeners but not by most of the older compounds. There were some exceptions; for example, cefaclor had good activity against *K. oxytoca*. For representative

TABLE 4. MICs of Ro 40-6890 and other drugs for quality control strains

Organism (no. tested)	Antimicrobial agent	MIC (mg/liter)		
		Modal	Range	Control limit[a]
Escherichia coli ATCC 25922 (39)	Ro 40-6890	0.12	0.12–0.5	NA[b]
	Cefpodoxime[c]	0.5	0.5	NA
	Amoxicillin-clavulanic acid[d]	8,16	8–32	2–8
	Amoxicillin[e]	8	4–16	2–8
	Cefuroxime	4	4	2–8
	Cefaclor	2	1–4	1–4
Staphylococcus aureus ATCC 25923 (37)	Ro 40-6890	0.5	0.25–0.5	NA
	Cefpodoxime	1	0.5–2	NA
	Amoxicillin-clavulanic acid	0.25,0.5	0.25–2	NA
	Amoxicillin	0.25	0.12–0.25	NA
	Cefuroxime	0.5	0.25–1	NA
	Cefaclor	1	0.5–2	NA
Staphylococcus aureus ATCC 29213 (44)	Ro 40-6890	1	0.5–2	NA
	Cefpodoxime	2	1–4	NA
	Amoxicillin-clavulanic acid	0.5,1	0.5–2	0.25–1
	Amoxicillin	1,2	0.5–2	0.5–2
	Cefuroxime	1,2	0.5–2	1–4
	Cefaclor	2	1–2	1–8

[a] Acceptable ranges of MICs for control strains in the reference dilution method on MHA without blood or other supplements (13).
[b] NA, not available.
[c] No quality control recommendations have been published by NCCLS to date.
[d] Expressed in terms of the amoxicillin component only.
[e] Interpreted as ampicillin.

TABLE 5. Affinities of Ro 40-6890 for the PBPs of *E. coli* and *S. aureus*

Organism	PBP	IC_{50} (μg/ml) of:					
		Ro 40-6890	Ceftriaxone	Cefpodoxime	Cefuroxime	Cefaclor	Ampicillin
Escherichia coli W3110	1a	0.03	0.06	0.25	0.1	24	0.1
	1b	0.9	1.3	4	5	44	1.6
	2	3.5	0.9	6.2	25	50	0.3
	3	0.03	0.02	0.25	0.5	2.8	0.2
	4	30	40	100	10	31	0.5
	5	>100	>100	>100	>100	>100	>100
	6	67	52	>100	>100	>100	5
Staphylococcus aureus Schoch	1	0.4	0.4	0.5	0.5	0.4	≤0.01
	2	0.1	0.06	1	0.06	>100	0.1
	3	6.1	0.4	1	1.3	0.03	0.02
	4	44	>100	>100	25	>100	67

clinical isolates of salmonellae (including *Salmonella typhi*), shigellae (data not shown), and species of the family *Vibrionaceae* other than *Vibrio cholerae*, Ro 40-6890 MIC_{90}s were ≤0.5 μg/ml. In contrast, the activities of Ro 40-6890 were poor (MIC_{90}s, ≥64 μg/ml) against non-glucose-fermenting gram-negative rods and notably uniformly poor against *Acinetobacter* spp. and *P. aeruginosa* (data not shown).

The common gram-negative pathogenic agents of respiratory tract infections, *H. influenzae*, *Haemophilus parainfluenzae*, *M. catarrhalis*, and *Neisseria* spp., were universally susceptible to Ro 40-6890, including β-lactamase-producing fresh clinical isolates (data not shown). In keeping with the high resistance of Ro 40-6890 to hydrolysis by TEM-1 and TEM-2 β-lactamases (see below) was the high potency of this compound (MIC_{90}, 0.06 μg/ml) against penicillinase-producing *Neisseria gonorrhoeae*, which readily hydrolyzed amoxicillin (MIC_{90}, ≥64 μg/ml). Chromosomally mediated penicillin-resistant *N. gonorrhoeae* demonstrated decreased susceptibility to all β-lactams tested, including Ro 40-6890.

Standard susceptibility test media, liquid or solid, had only a minor influence on the MIC results (Table 3) for common pathogens. MIC test results obtained on MHA were highly reproducible with the standard reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, more so than with *S. aureus* ATCC 25923 (Table 4). This result was equally true for cefuroxime, a compound for which there are published quality control reference values (12).

Affinity for PBPs. The affinities measured for the *E. coli* PBPs (Table 5) were nearly identical for Ro 40-6890 and ceftriaxone, and this result is paralleled by very similar in vitro susceptibility test results (Tables 1 and 2). PBP 3 and PBP 1a were the targets inhibited by the lowest concentrations. Cefpodoxime had 50% inhibitory concentrations (IC_{50}s) slightly higher than those of Ro 40-6890, as did cefuroxime. Cefaclor had the highest IC_{50}s and distinctly the lowest affinities of the β-lactams tested. For the *S. aureus* PBPs (Table 5), the largest difference in affinities between Ro 40-6890 and ceftriaxone was found for PBP 3. Ro 40-6890 had a 15-fold higher IC_{50}. Cefaclor and ampicillin had distinctly lower IC_{50}s for PBP 1 and PBP 3, respectively.

Hydrolysis by β-lactamases. Ro 40-6890 was relatively resistant to hydrolysis by group 1 β-lactamases produced by gram-negative bacteria, such as members of the family *Enterobacteriaceae* and *P. aeruginosa* (Table 6). It was recognized with a high affinity (K_m, 0.5 to 5 nM) but was hydrolyzed slowly (k_{cat}, 0.008 to 0.02 s^{-1}). This stability was comparable to that of ceftriaxone (k_{cat}, ~0.01 to 0.05 s^{-1}) in the presence of these enzymes. The group 2a enzymes produced by gram-positive bacteria, such as *B. licheniformis* and *S. aureus*, hydrolyzed Ro 40-6890 more rapidly (k_{cat}, 2.0 s^{-1}) but had a much lower affinity for it (K_m, >500 μM). This behavior was similar to those of these enzymes with ceftriaxone, although the latter cephalosporin was hydrolyzed more rapidly. The extended broad-spectrum β-lactamases produced by *E. coli* (for example, TEM-3) actively hydrolyzed Ro 40-6890 (k_{cat}, 1.5 s^{-1}), while standard broad-spectrum β-lactamases (for example, TEM-1) were less active (k_{cat}, 0.07 s^{-1}).

Single-dose pharmacokinetics in mice. Preliminary pharmacokinetic parameters were determined for Ro 40-6890 after the administration of Ro 41-3399 by forced gavage and for Ro

TABLE 6. Kinetic parameters for the hydrolysis of Ro 40-6890 and ceftriaxone by β-lactamases

Enzyme source	β-Lactamases group[a]	Ro 40-6890			Ceftriaxone	
		k_{cat} (s^{-1})	K_m (μM)	K_i (μM)	k_{cat} (s^{-1})	K_m (μM)
Enterobacter cloacae 908R	1	0.020	0.0005	0.001	0.060	0.33
Escherichia coli SNO3(pAD7)	1	0.022	0.011	0.026	0.069	0.81
Citrobacter freundii 1203	1	0.008	0.004	0.006	0.018	0.11
Pseudomonas aeruginosa 18SH	1	0.021	0.15	0.45	0.18	0.65
Bacillus licheniformis 729/C	2a	2.0	530		18	1,080
Staphylococcus aureus PC1	2a	0.8	105		10	650
Escherichia coli (TEM-1)	2b	0.07	85		>1[b]	>500
Escherichia coli (TEM-3)	2b′	1.50	15		>5[b]	>500

[a] According to Bush (2).
[b] Complex kinetics not obeying Michaelis-Menten equations.

TABLE 7. Efficacies against experimental septicemia in mice[a]

Organism	Infective dose (CFU/mouse)	Compound	MIC[b] (μg/ml)	p.o. ED_{50}[c]
Staphylococcus aureus Schoch	3×10^5	Ro 41-3399	1	2.2 (1.6–3.0)
		Cefpodoxime proxetil	4	3.1 (2.1–4.6)
		Cefixime	32	>25
		Cefuroxime axetil	2	3.5 (2.2–5.7)
		Cefaclor	2	<0.8
Staphylococcus aureus 887	1×10^7	Ro 41-3399	2	4.2 (3.0–5.8)
		Cefpodoxime proxetil	4	14 (8.8–23)
		Cefixime	32	>25
		Cefuroxime axetil	2	4.2 (3.0–5.8)
		Cefaclor	8	11 (6.5–18)
Streptococcus pyogenes β15	1×10^5	Ro 41-3399	≤0.06	0.057 (0.035–0.092)
		Cefpodoxime proxetil	≤0.06	<0.1
		Cefixime	0.12	2.1 (1.5–2.9)
		Cefuroxime axetil	≤0.06	<0.1
		Cefaclor	0.5	0.28 (0.20–0.41)
Streptococcus pneumoniae BA	1×10^1	Ro 41-3399	≤0.06	0.22 (0.13–0.36)
		Cefpodoxime proxetil	<0.4	<0.4
		Cefixime	0.25	1.8 (1.1–2.9)
		Cefuroxime axetil	≤0.06	0.22 (0.095–0.53)
		Cefaclor	1	4.2 (3.0–5.8)
Escherichia coli ATCC 25922	5×10^3	Ro 41-3399	0.12	0.22 (0.13–0.36)
		Ro 40-6890	0.12	1.1 (0.76–1.6)
		Cefpodoxime proxetil	0.25	0.89 (0.55–1.5)
		Cefixime	0.25	1.1 (0.81–1.5)
		Cefuroxime axetil	1	4.2 (3.0–5.8)
		Cefaclor	4	1.3 (0.8–2.0)
Klebsiella pneumoniae 418	1×10^3	Ro 41-3399	0.12	0.55 (0.41–0.75)
		Cefpodoxime proxetil	0.12	2.1 (1.5–2.9)
		Cefixime	0.12	0.90 (0.38–2.2)
		Cefuroxime axetil	8	8.8 (6.1–12)
		Cefaclor	2	2.7 (1.8–3.7)
Proteus vulgaris 1028	1×10^6	Ro 41-3399	0.12	<0.7
		Cefpodoxime proxetil	0.12	2.2 (1.6–3.0)
		Cefixime	≤0.06	<0.7
		Cefuroxime axetil	>16	>12
		Cefaclor	>16	>12
Haemophilus influenzae 1	5×10^4	Ro 41-3399	≤0.06	<0.3
		Cefpodoxime proxetil	0.25	2.4 (1.6–3.3)
		Cefixime	0.12	<0.3
		Cefuroxime axetil	2	1.1 (0.36–3.3)
		Cefaclor	2	2.9 (2.0–4.1)

[a] Compounds were administered p.o. 1 and 3 h (for *S. pneumoniae*, 1, 3, and 24 h) after bacterial challenge.
[b] For the free acid; determined on MHA (inoculum, 10^4 CFU per spot).
[c] Milligrams per kilogram (95% confidence limits).

40-6890 after s.c. administration in Swiss albino mice. The concentrations of Ro 40-6890 observed following p.o. administration of Ro 41-3399 were highest, 25 to 28 μg/ml, between 15 and 30 min. The corresponding areas under the concentration-time curves and apparent terminal half-lives were 38 to 42 mg · h/liter and 1.2 to 1.8 h following p.o. administration of Ro 41-3399 and 36 mg · h/liter and 0.7 h following s.c. administration of Ro 40-6890, respectively. Favorable pharmacokinetic results for Ro 41-3399 in rats have been reported (7).

Plasma protein binding. The protein binding of Ro 40-6890 in human plasma was 50% up to 50 μg/ml and about 18% at 250 μg/ml; in murine plasma, the corresponding values were also 50% up to 50 μg/ml and about 40% at 250 μg/ml. Murine values for amoxicillin were 15 and 24% at concentrations of 50 and 250 μg/ml, respectively.

Efficacy in experimental murine septicemia. Ro 41-3399 showed consistent pronounced in vivo activities upon p.o. administration in experimental infections against gram-positive (ED_{50}, <5 mg/kg) and gram-negative (ED_{50}, <0.7 mg/kg) pathogens that are susceptible in vitro to Ro 40-6890 (Table 7). The activities of Ro 41-3399 against staphylococci were of the same magnitude as those of cefuroxime axetil. Cefpodoxime proxetil was significantly less efficacious than Ro 41-3399 and cefuroxime axetil against the β-lactamase-positive strain *S. aureus* 887. Cefaclor was the most potent

compound against the β-lactamase-negative strain *S. aureus* Schoch but was less active than Ro 41-3399 and cefuroxime axetil against *S. aureus* 887. Ro 41-3399, cefuroxime axetil, and cefpodoxime proxetil showed high efficacy at <0.1 mg/kg against *S. pyogenes*. Cefixime was about 20-fold less active (ED_{50}, 2.1 mg/kg) against streptococci and inactive against staphylococci. Ro 41-3399 was the most potent compound against *E. coli* and equivalent to cefixime against *P. vulgaris* and *H. influenzae*. The remainder of the compounds showed activities severalfold lower than those of Ro 41-3399 against the gram-negative strains. Ro 40-6890 administered p.o. was considerably less active than Ro 41-3399, as indicated by the fivefold higher ED_{50} against *E. coli* 25922. This result reflects the lower oral bioavailability of Ro 40-6890 than of Ro 41-3399 bopentil.

DISCUSSION

Ro 41-3399 is a new type of oral ester derivative of the aminothiazolyl cephalosporin Ro 40-6890, which in its free carboxylic acid form has oral bioavailability too low to be considered for oral use. After absorption from the gastrointestinal tract, Ro 41-3399 is readily cleaved by esterases to form Ro 40-6890.

Ro 40-6890 showed high in vitro activities against a wide range of gram-positive and gram-negative pathogens. Ro 40-6890 inhibited over 90% of isolates of *E. coli*, *Klebsiella pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, *P. vulgaris*, *M. morganii*, *S. marcescens*, *V. cholerae*, and *M. catarrhalis* at a concentration of ≤2 μg/ml. It was consistently more active than cefaclor, cefuroxime, and the aminothiazolyl cephalosporin cefpodoxime against these isolates. Amoxicillin-clavulanic acid-resistant isolates of the family *Enterobacteriaceae*, except for *C. freundii* and *E. cloacae* isolates, which harbor chromosomally mediated group 1 β-lactamases, were susceptible to Ro 40-6890. The MIC_{50}s of 1 and 0.5 μg/ml for methicillin-susceptible isolates of *S. aureus* and coagulase-negative staphylococci suggest that methicillin-susceptible staphylococci may be included in the antibacterial spectrum of Ro 41-3399. The compound is also very active against streptococci and other respiratory tract pathogens, except for the highly penicillin-resistant pneumococci and the enterococci (data not shown).

Ro 40-6890 is similar to other aminothiazolyl cephalosporins with respect to inhibitory properties, affinities for PBPs, and resistance to the hydrolytic action of β-lactamases of groups 2b and 2e, based on the classification by Bush (2). It was bactericidal at concentrations equal to or slightly higher than the MIC, in accordance with the fact that PBP 3 is the preferential target in *E. coli*. Like other aminothiazolyl cephalosporins (3, 9), Ro 40-6890 was stable against the action of different types of β-lactamases, with the exception of group 1 β-lactamases and extended broad-spectrum (group 2b′) β-lactamases. The oral activities of Ro 41-3399 against experimental septicemia with strains of *S. aureus*, *Streptococcus pneumoniae*, *S. pyogenes*, *E. coli*, *P. vulgaris*, and *H. influenzae* reflect the in vitro activities of Ro 40-6890. These therapeutic experimental findings and the existing pharmacokinetic and metabolic data for mice, rats, and dogs (data not shown) indicate that the new ester moiety incorporated in Ro 41-3399 yields satisfactory oral absorption and experimentally useful levels of Ro 40-6890 in plasma.

In conclusion, the present in vitro and in vivo evaluations of Ro 40-6890 and Ro 41-3399, respectively, revealed that Ro 41-3399 is an oral broad-spectrum cephalosporin with advantages in terms of spectrum and potency over existing oral β-lactams. Further studies with Ro 41-3399 and Ro 40-6890 are in progress.

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