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In Vitro and In Vivo Antibacterial Activity of KW1070, a New Aminoglycoside Antibiotic

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KW1070, a new aminoglycoside antibiotic with the novel aminocyclitol, fortamine, has a broad spectrum of activity in vitro and in vivo against gram-positive and gram-negative bacteria. The minimum inhibitory concentrations of KW1070 are similar to those of kanamycin against aminoglycoside-susceptible strains, slightly less than those of gentamicin or 3',4'-dideoxykanamycin B. Minimal bactericidal concentrations were found to be near minimal inhibitory concentrations. KW1070 was active in vitro against many aminoglycoside-resistant bacterial strains that possess aminoglycoside-inactivating enzymes, particularly AAC(6'), AAC(2'), AAD(2''), and APH(3'). The activities of KW1070 in mice infected with *Staphylococcus aureus*, *Escherichia coli*, *Proteus* sp., and *Serratia marcescens* compared favorably with the activities of amikacin and kanamycin; KW1070 was also significantly active in mice infected with resistant strains bearing the aminoglycoside-inactivating enzymes listed above.

KW1070, a new aminoglycoside antibiotic isolated from *Micromonospora* species MK70(1), has a unique chemical structure with a novel aminocyclitol, fortamine, and a glycyl amide substituent at position C-4 of fortamine (1) (Fig. 1).

KW1070 is highly active in vitro against a broad spectrum of bacteria (2) and retains this activity against aminoglycoside-resistant strains that possess aminoglycoside-inactivating enzymes (5). The current report summarizes the results of both in vitro and in vivo evaluations of KW1070 against gram-positive and gram-negative bacteria and compares the antibacterial activity of this novel agent with the activities of other aminoglycosides.

MATERIALS AND METHODS

Antibiotics. KW1070 is the product of Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Gentamicin (Gm), 3'4'-dideoxykanamycin B (Dk), kanamycin (Km), ribostamycin, sisomicin, netilmicin, tobramycin, streptomycin, and amikacin (Ak), as sulfate salts, were used as reference materials.

Strains. These studies were based on evaluations with 1,091 strains of gram-positive and gram-negative bacteria, 69 Gm-resistant strains of *Serratia marcescens*, 97 Gm-resistant strains of *Pseudomonas aeruginosa*, and 21 strains identified by their aminoglycoside resistance mechanisms, all of which were clinical isolates. They were maintained among the stock cultures of the Laboratory of Bacterial Resistance, School of Medicine, Gunma University.

Media. The media used in these studies included: heart infusion agar and brain heart infusion broth, the products of Eiken Chemical Co., Ltd., peptone broth, consisting of 10 g of polypeptone, 5 g of NaCl, and 1,000 ml of distilled water; and normal broth, consisting of 10 g of beef extract, 10 g of polypeptone, and 2 g of NaCl in 1,000 ml of distilled water.

In vitro antibacterial activities. Minimal inhibitory concentrations (MICs) were determined by an agar dilution method, using heart infusion agar. Plates were inoculated with 1 loopful (about 0.005 ml) of 10^6 cells per ml of overnight culture in peptone broth. MICs were scored after 18 h of incubation at 37°C.

In assessing bactericidal activities, an overnight culture of each strain in brain heart infusion broth was diluted to a final concentration of about 10^4 cells per ml with broth containing serial twofold dilutions of antibiotics. After incubation at 37°C for 18 h, one loopful of each culture was spotted onto heart infusion agar plates to examine the growth of bacteria. Minimal bactericidal concentrations were scored after incubation at 37°C for 18 h.

Enzymatic assay. Cell-free extracts were prepared as described previously (3). The reaction mixture consisted of 0.15 ml of S-30 fraction (10 mg of protein per ml), 0.05 ml of 0.5 mM drug, 0.05 ml of 1 mM coenzyme A, 0.05 ml of 20 mM disodium adenosine 5'-triphosphate, 0.05 ml of 0.02 M magnesium acetate, and 0.15 ml of 0.2 M tris(hydroxymethyl)aminomethane-malate buffer (pH 6.0, 7.0, and 8.0). After incubation at 37°C for 6h, residual antibiotic activity in the reaction mixture was determined by bioassay using *Bacillus subtilis* ATCC6633.

Isolation of enzymatically acetylated KW1070. Enzymatic acetylation of KW1070 was carried out at 37°C for 18 h in a reaction mixture containing 140 mg of KW1070, 936 mg of disodium adenosine 5'-triphosphate, 40.5 mg of disodium coenzyme A, 139 mg of $Mg(CH_3COO)_2$, 120 ml of the S-105 fraction from *P.*

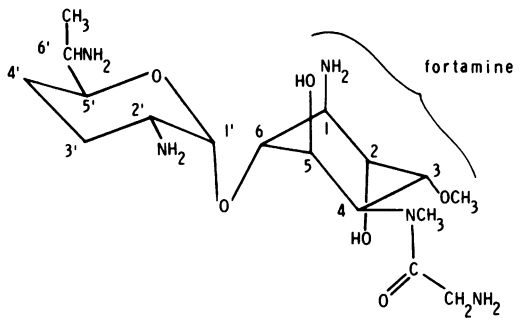

FIG. 1. *Structure of KW1070.*

Aeruginosa GN3054 (12.6 mg of protein per ml), and 60 ml of 0.1 M acetate buffer (pH 6.2) in a total volume of 200 ml. The reaction was stopped by heating at 100°C for 10 min. The supernatant fluid obtained by centrifugation at 13,000 rpm for 40 min was passed through a column of Amberlite CG-50 (NH_4^+ form, 60 ml), and the column was washed with distilled water. The inactivated KW1070 was then eluted with 0.5 N NH_4OH. The eluted fractions that gave a positive ninhydrin reaction were collected, further subjected to chromatography on CM-Sephadex C-25 (NH_4^+ form, 50 ml), and washed with 0.05 N NH_4OH. The acetylated KW1070 was eluted with 0.5 N NH_4OH.

Partial purification of the inactivating enzyme. Aminoglycoside 3-acetyltransferase [AAC(3)-I] was purified by affinity chromatography. Sagamicin-Sepharose 4B was prepared by the procedure described previously (3) and was washed with 20 mM tris(hydroxymethyl)aminomethane-malate buffer (pH 7.5)–20% glycerin containing 5 mM magnesium acetate, and packed in a column (1 by 10 cm). The S-105 fraction (12.8 mg of protein per ml) from *Enterobacter cloacae* GN8282 was passed through the column and eluted with a linear gradient elution with NaCl from 0 to 0.8 M in the same buffer.

Thin-layer chromatography. Thin-layer chromatography was carried out on silica gel (Tokyo Kasei Kogyo Co.) using isopropanol–$CHCl_3$–25% NH_4OH (2:1:1), methanol–$CHCl_3$–25% NH_4OH (1:2:1), or *n*-butanol–ethanol–$CHCl_3$–25% NH_4OH (4:5:5:2) as the solvent system. The spot on a chromatogram was detected by the ninhydrin reaction.

In vivo antimicrobial activity. Mouse protection tests were performed with male ICR mice weighing approximately 18 g. Mice were inoculated intraperitoneally, and 2 h later they were injected with antibiotics. In each case the challenge dose constituted ca. 100 50% lethal doses. The numbers of surviving mice were recorded 1 week after infection, and the amount of a drug that provided 50% protection at that time was estimated by the log-probit method (4).

RESULTS

Antibacterial activity. The MICs of KW1070 were compared with those of Gm, Dk, Km, and ribostamycin against 1,091 clinical isolates of gram-positive and gram-negative organisms. Table 1 shows the concentrations of drugs

TABLE 1. *Antibacterial activity of KW1070 against clinical isolates*

Species	No. of strains	MIC_{50} (μg/ml)[a]					MIC_{80} (μg/ml)[a]				
		KW1070	Gm	Dk	Km	Rm[b]	KW1070	Gm	Dk	Km	Rm
Staphylococcus aureus	100	0.3	0.1	0.1	0.8	1.7	0.8	0.2	0.2	3.6	6.3
Streptococcus pyogenes	100	15	4.2	17	47	14	37	12	50	96	30
Escherichia coli	100	2.4	0.6	1.0	3.0	4.3	4.9	1.2	1.3	>100	>100
Klebsiella pneumoniae	100	1.4	0.3	0.5	1.7	2.2	2.3	0.5	0.7	>100	>100
Proteus mirabilis	100	2.8	0.9	0.8	2.4	3.6	5.4	1.6	2.0	>100	>100
P. vulgaris	55	2.2	0.8	1.2	2.3	4.6	6.4	1.9	3.2	6.5	28
P. morganii	55	2.6	0.5	0.7	2.4	4.2	4.0	1.6	1.4	>100	>100
P. rettgeri	31	2.5	0.7	0.9	4.0	6.8	6.3	2.7	8.4	>100	>100
P. inconstans	16	1.9	3.2	8.5	2.0	>100	5.2	8.0	21	>100	>100
Serratia marcescens	175	1.4	0.6	4.8	4.4	50	3.0	2.9	23	>100	>100
Enterobacter cloacae	159	1.1	0.3	0.5	2.4	2.6	2.0	0.6	1.2	>100	>100
Pseudomonas aeruginosa	100	11	1.4	0.7	53	>200	22	3.1	1.6	>200	>200

[a] MIC_{50} and MIC_{80} represent the concentrations required to inhibit the growth of 50 and 90%, respectively, of the total number of strains used.
[b] Rm, Ribostamycin.

required to inhibit the growth of 50% and 90% of the total number of tested strains (MIC_{50} and MIC_{90}, respectively). KW1070 was slightly less active than Gm and Dk against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus* sp., *E. cloacae*, and *P. aeruginosa*. KW1070 was more active than Dk and Km against *S. marcescens*, and similar to Gm in activity against *S. marcescens*. Minimal bactericidal concentrations of KW1070 against *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *Proteus morganii*, *S. marcescens*, *E. cloacae*, and *P. aeruginosa* were found to be the same or only twofold higher than the MICs. Bactericidal activity of KW1070 was similar to those of Gm, Dk, Ak, and Km.

Antibacterial activity against Gm-, Km-, and streptomycin-resistant strains. Examination of the activity of KW1070 against 69 Gm-resistant strains of *S. marcescens* indicated that KW1070 was more active than Dk, Km, Ak, and netilmicin (Fig. 2). Against 97 Gm-resistant strains of *P. aeruginosa*, KW1070 was less active than Ak, equal to tobramycin in activity, and more active than sisomicin and Dk (Fig. 3).

KW1070 was active against aminoglycoside-resistant strains that possess the following aminoglycoside-inactivating enzymes: (i) aminoglycoside phosphorylation enzymes APH(3′) and APH(6′); (ii) aminoglycoside-adenylylating enzymes AAD(2″), AAD(4′), and AAD(3″); and (iii) aminoglycoside-acetylating enzymes AAC(2′), AAC(6′), and AAC(3)-III (Table 2). KW1070 was not active against strains that possess acetylating enzyme AAC(3)-I, indicating that it has a unique spectrum of antibacterial activity against aminoglycoside-resistant strains.

Inactivation of KW1070 by various aminoglycoside-inactivating enzymes. The inactivation of KW1070 by various aminoglycoside-inactivating enzymes was studied. KW1070 was resistant to APH(3′), APH(3″), APH(6), AAD(2″), AAD(3″), AAC(3)-III, AAC(2′), and AAC(6′). KW1070 is structurally quite different from Gm and sisomicin, which are inactivated by AAC(3)-I. We compared an KW1070-inactivating enzyme with a Gm-inactivating one, using affinity chromatography. The enzyme that inactivated Gm-C_1 and KW1070 appeared in the elution with 0.6 M NaCl, and the inactivation curves of two drugs using eluted fractions were almost the same. An AAC(3)-I was purified approximately 80-fold from the S-105 fraction. The optimal pH for both Gm-C_1 and KW1070 inactivation varied between 7.5 and 8.0, and the pH curves for inactivation of the two drugs were almost the same. These results indicated that an AAC(3)-I from GN8282 could inactivate both KW1070 and Gm-C_1.

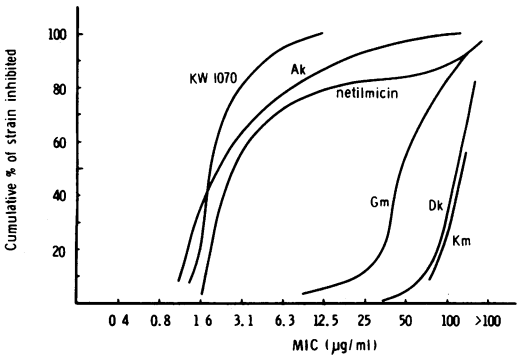

FIG. 2. *In vitro antibacterial activity of KW1070 against 69 Gm-resistant S. marcescens strains.*

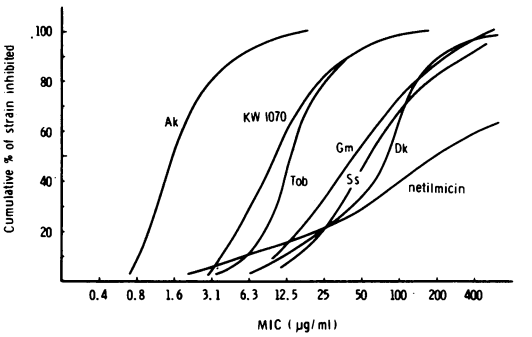

FIG. 3. *In vitro antibacterial activity of KW1070 against 97 Gm-resistant P. aeruginosa strains.*

Identification of the acetyl product. Inactivated KW1070, prepared as described in Materials and Methods, was purified and obtained as a white powder (99 mg). Its structure was determined with infrared and mass spectroscopy. Infrared spectrum showed a band at 1,640 cm^{-1}. This band was absent in the infrared spectrum of KW1070. High-resolution mass spectroscopy gave the following data. The signals of *m/e* 143 and *m/e* 334 showed that the amino groups of purprosamine moiety were not acetylated. The signal of *m/e* 417 showed that the amino group of glycine moiety was not acetylated; this signal was observed in the fragmentation of KW1070. Inactivated KW1070 and 1-*N*-acetyl KW1070 were developed by thin-layer chromatography and examined by ninhydrin reagent. R_f values of the inactivated KW1070 and the 1-*N*-acetyl KW1070 were 0.65 by solvent A, 0.46 by solvent B, and 0.44 by solvent C, respectively. In view of these experimental results, the inactivated KW1070 was concluded to be 1-*N*-acetyl KW1070.

In vivo antibacterial activity against susceptible strains. The therapeutic activities exhibited by KW1070 in mice infected with gram-

TABLE 2. *In vitro antibacterial activity of KW1070 against aminoglycoside-resistant bacteria*

Strain	Biochemical mechanism of resistance due to the formation of:	MIC (μg/ml)					
		KW1070	Ak	Gm	Dk	Km	Sm
Staphylococcus aureus							
ML4845	AAD(4′)	0.2	6.3	<0.1	0.8	50	—
MS27	APH(6′)	0.8	—	—	—	—	100
Staphylococcus epidermidis							
ML4843	AAD(4′)	0.8	3.1	<0.2	0.8	50	—
Escherichia coli							
ML1629	APH(3′)	1.6	1.6	0.8	0.8	>400	—
GN3684	APH(3″)	1.6	—	—	—	—	100
GN3644	APH(3″)	3.1	—	—	—	—	200
GN3451	AAD(3″)	1.6	—	—	—	—	100
GN4669	AAD(3″)	3.1	—	—	—	—	200
ML4846	AAC(3)-I	400	0.8	6.3	0.4	0.8	—
R5/K-12	AAC(6′)-IV	0.8	6.3	0.4	12.5	50	—
JR66/W677	APH(3′) AAD(2″)	0.8	0.8	6.3	12.5	>400	—
Klebsiella pneumoniae							
GN3056	AAD(2″)	3.1	1.6	50	100	>400	—
GN3058	AAD(2″)	1.6	1.6	100	200	>400	—
Serratia marcescens							
GN7979	AAD(2″)	1.6	3.1	50	200	>400	—
GN6944	AAD(2″)	0.8	1.6	50	200	>400	—
Proteus inconstans							
GN1554	AAC(2′)	3.1	3.1	25	25	1.6	—
GN626	AAC(2′)	3.1	1.6	25	100	1.6	—
Enterobacter cloacae							
GN8282	AAC(3)-I	400	0.8	25	0.4	1.6	—
Pseudomonas aeruginosa							
ML4847	AAC(3)-III	25	6.3	>200	>200	>200	—
GN315	AAC(6′)-IV	12.5	50	3.1	100	200	—
GN3054	AAC(3)-I	400	6.3	200	1.6	200	—

positive and gram-negative bacteria are shown in Table 3. KW1070 was as active as Ak in mice infected with *S. aureus* Smith, *E. coli* GN2411-5, *K. pneumoniae* no. 8045, *P. mirabilis* 1287, *Proteus vulgaris* 6897, and *P. inconstans* no. 12. Its effectiveness was much greater than that of Km against infections with both *E. coli* ML4707 and *S. marcescens* GN7641. The MICs of KW1070 and Ak against *S. marcescens* no. 3 and *S. marcescens* KYF293 were almost the same, but KW1070 showed much higher in vivo effectiveness than did Ak.

In vivo antibacterial activity against aminoglycoside-resistant strains. The results of protection tests against a variety of aminoglycoside-resistant strains are shown in Table 4. In general, the in vivo antibacterial activity of KW1070 was consistent with the in vitro antibacterial activity. In particular, KW1070 showed a high antibacterial activity against the bacterial strains that possess aminoglycoside-acetylating enzymes AAC(6) and AAC(2′) and aminoglycoside-adenylylating enzyme AAD(2″).

DISCUSSION

KW1070, a new aminoglycoside antibiotic, has a broad antibacterial spectrum against gram-positive and gram-negative bacteria (2). KW1070 also showed a high activity against many clinical isolates, including both gram-positive and gram-negative bacteria. KW1070 has appreciably great activity against aminoglycoside-resistant strains of gram-positive and -negative bacteria, which can produce APH(3′), APH(3″), AAC(4′), AAD(2″), AAD(6), AAC(6′), AAC(2′), and AAC(3)-III. KW1070 was found to be acetylated only by AAC(3)-I, and the struc-

TABLE 3. *In vivo antibacterial activity of KW1070 against systemic infection of mice*

Challenge organism	Challenge dose (no. of cells)	No. of mice in 1 group	Drug	MIC (μg/ml)	ED_{50}[a] (mg/kg)	Confidence limit (95%)	
S. aureus Smith	1.6×10^8	10	KW1070	0.2	2.7	1.8–4.0	
			Ak	0.4	2.5	1.6–4.0	
E. coli GN2411-5	1.3×10^7	10	KW1070	1.6	8.6	6.4–11.0	$P > 0.05$
			Ak	1.6	8.0	5.6–11.4	
E. coli ML4707	1.0×10^7	25	KW1070	3.1	5.97	4.22–8.54	$P < 0.05$
			Km	3.1	9.54	8.82–10.4	
K. pneumoniae no. 8045	9.5×10^6	10	KW1070	0.8	1.2	0.9–1.5	$P > 0.05$
			Ak	0.4	0.97	0.8–1.1	
P. mirabilis 1287	1.5×10^6, containing 5% mucin	10	KW1070	6.3	15.5	11.3–21.2	$P > 0.05$
			Ak	6.3	12.2	8.1–18.3	
P. vulgaris 6897	5.3×10^6, containing 5% mucin	10	KW1070	3.1	3.0	2.2–4.1	$P > 0.05$
			Ak	3.1	2.1	1.8–2.5	
P. inconstans no. 12	7.6×10^8, containing 5% mucin	10	KW1070	3.1	70.4	53.8–91.5	$P > 0.05$
			Ak	6.3	83.2	66.6–103	
P. inconstans KYF 437	1.0×10^8, containing 2.5% mucin	25	KW1070	3.1	17.2	10.6–21.1	$P > 0.05$
			Ak	1.6	25.6	20.8–31.4	
S. marcescens no. 3	6.5×10^7	10	KW1070	1.6	21.0	12.7–33.8	$P < 0.05$
			Ak	1.6	57.1	37.7–83.2	
S. marcescens KYF 293	1.9×10^7, containing 2.5% mucin	20	KW1070	3.1	6.0	5.1–7.0	$P < 0.05$
			Ak	3.1	9.3	8.0–9.8	
S. marcescens GN7641	1.0×10^8	25	KW1070	0.8	8.8	7.9–9.5	$P < 0.05$
			Km	1.6	12.4	11.2–14.1	

[a] ED_{50}, 50% effective dose.

TABLE 4. *In vivo antibacterial activity of KW1070 against systemic infection of mice with aminoglycoside-resistant strains*[a]

Challenge organism	Biochemical mechanisms of resistance due to the formation of:	Challenge dose[b] (no. of cells)	Drug	MIC (μg/ml)	ED_{50}[c] (mg/kg)	Confidence limit (95%)	
E. coli GN10260	AAD(2″)	1.5×10^7	KW1070	1.6	4.7	3.5–6.1	$P < 0.05$
			Gm	100	103	86–122	
K. pneumoniae GN3057	AAD(2″)	6.5×10^7	KW1070	3.1	127	95–164	$P < 0.05$
			Dk	25	>265	>265	
P. inconstans GN626	AAC(2′)	8.5×10^7	KW1070	3.1	10.8	7.4–16.7	$P < 0.05$
			Gm	25	45.6	26.5–85.6	
P. aeruginosa GN315	AAC(6′)-4	3.5×10^6	KW1070	12.5	34.9	24.5–55.0	$P > 0.05$
			Ak	25	68.9	36.2–97.3	
P. aeruginosa GN4496	APH(3′)	6.0×10^6	KW1070	6.3	276	—	—
			Km	100	424	—	
S. marcescens KYF 266	Unknown	2.3×10^7	KW1070	6.25	10.3	8.72–12.1	$P < 0.05$
			Ak	100	53.9	44.9–64.7	

[a] Ten mice were used for each group of the experiment.
[b] Bacterial cells were suspended in saline containing 5% of mucin.
[c] ED_{50}, 50% effective dose.

ture of inactivated product was determined to be 1-*N*-acetyl-KW1070 (6, 7). Therefore, KW1070 was effective against bacteria capable of producing aminoglycoside-inactivating enzymes except for AAC(3)-I. KW1070 has a unique chemical structure which contains a novel aminocyclitol, fortamine, and a glycyl amide at the C-4 position of fortamine, and has no 3′-hydroxyl group. Furthermore, KW1070 lacks a sugar corresponding to the 3-amino-glucose of tobramycin and garasamine of Gm. KW1070 is structurally quite different from Gm and sisomicin; its effectiveness against Gm-resistant strains may be accounted for by its unique structure.

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