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UK-18,892: Resistance to Modification by Aminoglycoside-Inactivating Enzymes

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UK-18,892, a new semisynthetic aminoglycoside, was active against bacteria possessing aminoglycoside-inactivating enzymes, with the exception of some known to possess AAC(6′) or AAD(4′) enzymes. This activity has been rationalized by using cell-free extracts of bacteria containing known inactivating enzymes, where it was shown that UK-18,892 was not a substrate for the APH(3′), AAD(2″), AAC(3), and AAC(2′) enzymes. It was also demonstrated that UK-18,892 protected mice against lethal infections caused by organisms possessing aminoglycoside-inactivating enzymes.

Aminoglycoside antibiotics are widely used for the treatment of serious gram-negative bacterial infections. Their utility is being threatened, however, by the appearance of resistant bacteria which, in the majority of clinical situations, possess enzymes capable of inactivating one or more aminoglycosides (3, 5). Although the inactivating enzymes vary considerably in their substrate specificities, known modifications are restricted to acetylation of amino groups and adenylylation or phosphorylation of hydroxyl groups (3, 5, 7).

UK-18,892 (Fig. 1) is a new semisynthetic aminoglycoside derived from kanamycin A, which is active in vitro against both aminoglycoside-susceptible and aminoglycoside-resistant bacteria (11). In the present study we have examined the ability of enzymes in cell-free extracts of aminoglycoside-resistant bacteria to modify UK-18,892 and have compared the extent of modification with that obtained with other aminoglycosides. In addition, we report the efficacy of UK-18,892 against experimental infections in mice caused by bacteria possessing aminoglycoside-inactivating enzymes.

MATERIALS AND METHODS

Bacterial isolates. The sources of bacteria and the types of aminoglycoside-inactivating enzymes present in each strain are shown in Table 1. The enzymes present in those bacteria designated clinical isolates, together with *Pseudomonas* HK232 and *Proteus* HK 238, were deduced from the pattern of resistance exhibited by each isolate against a panel of nine aminoglycosides, as described by Price et al. (10). The inactivating enzymes present in the remaining aminoglycoside-resistant bacteria were identified by the workers who supplied these isolates (Table 1).

Aminoglycosides. Kanamycin B (Kanendomycin, Meiji) and gentamicin (gentamicin sulfate, Roussel-UCLAF) were obtained commercially. UK-18,892 was a research sample of the sulfate salt. Concentrations of antibiotics are expressed in terms of aminoglycoside base.

Cell-free enzyme extracts. Cell-free bacterial enzyme extracts were prepared by methods similar to published procedures (2, 8).

Bacteria were grown for 18 h at 37°C in brain heart infusion broth (Difco) containing 10 μg of an appropriate aminoglycoside per ml. i.e., one to which the isolate was resistant. This "starter broth" (5 ml) was inoculated into 500 ml of antibiotic-free brain heart infusion and grown at 37°C, with aeration, until the culture was in the late-log phase. The cells were harvested by centrifugation and washed twice in a cold buffer solution of 0.01 M tris(hydroxymethyl)methylamine (Tris)-hydrochloride, pH 7.4, containing 0.03 M NaCl.

In the case of *Escherichia coli*, the washed cells were resuspended in 30 ml of 0.033 M Tris-hydrochloride buffer, pH 7.3, containing 0.006 M ethylenediaminetetraacetic acid and 20% (wt/vol) sucrose. The suspension was stirred gently by means of a magnetic stirrer for 20 min at room temperature and then centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was discarded, and as much buffer as possible was removed by inverting the centrifuge tubes and subsequently wiping the walls of the tubes with cotton swabs. The cell pellet was suspended in ice-cold 0.5 mM $MgCl_2$ (5 ml), stirred for 15 min at 4°C, and then centrifuged at 20,000 × *g* for 15 min at 4°C. The resulting supernatant was used as the cell-free enzyme source. This preparation was stored in suitable portions at −20°C.

With *Providencia stuartii* the washed cell pellets were frozen (−20°C), and ground in the frozen state with alumina (2.5 times their own weight) with a previously cooled pestle and mortar. A 5-ml amount of ice-cold buffer solution containing 0.2 M Tris, 32 mM $MgCl_2$ and 40 mM 2-mercaptoethanol at pH 7 was added, and the suspension was ground for a further 15 min.

Staphylococcus epidermidis was treated in a similar manner to *P. stuartii* except that ethylenediaminetetraacetic acid (0.5 mM), lysozyme (4 mg/ml; Sigma), and deoxyribonuclease (4 μg/ml; Sigma) were included in the Tris buffer used in the final grinding process.

Enzymatic modification of aminoglycosides. Aminoglycoside phosphorylating, adenylylating, and acetylating assays were carried out with appropriate radiolabeled substrates by methods similar to published procedures (1, 9).

The final assay volume was 100 μl and contained 0.1 mg of enzyme preparation (estimated by absorbance at 280 nm), 50 mM Tris-maleate buffer (pH 6, 7, or 8), 8 mM magnesium chloride, 10 mM mercaptoethanol, 5 μg of aminoglycoside, and 40 nmol of either [^{14}C]ATP (3 μCi/mol), or [^{14}C]acetylcoenzyme A (3 μCi/mol) or 310 nmol of [^{32}P]ATP (5 μCi/mol).

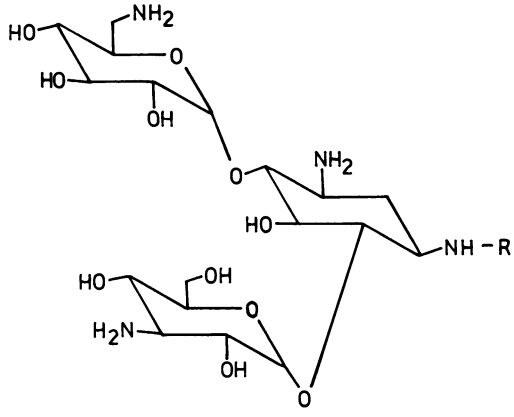

FIG. 1. *Structure of UK-18,892.*

After incubation for 2 h at 35°C, 75 μl of the reaction mixture was pipetted on to a square (2.5 cm^2) of phosphocellulose paper (Whatman P81). The paper was washed three times in distilled water, dried in a current of air, and finally placed in 10 ml of toluene-based scintillation fluid (Omnifluor, New England Nuclear). Radionuclides were obtained from the Radiochemical Centre, Amersham, and radioactivity was determined with a Nuclear Chicago liquid scintillation spectrometer.

Determination of MIC. Minimum inhibitory concentrations (MICs) were determined by a standard agar plate technique. Organisms were grown in brain heart infusion broth at 37°C for 18 h. Cultures were diluted 100-fold in brain heart infusion broth and inoculated onto diagnostic sensitivity test agar (Oxoid) containing serial doubling dilutions of antibiotic by using a multipoint inoculator (Denley Instruments), giving an inoculum of approximately 10^4 colony-forming units. After incubation at 37°C for 18 h the MIC was recorded as the lowest concentration of antibiotic which completely inhibited visible growth of the organism.

Experimental infections in mice. Acute systemic infections in mice were produced by intraperitoneal inoculation of standardized bacterial cultures suspended in 5% hog gastric mucin. The challenge dose varied between 1 and 10 lethal doses, depending on the organism, i.e., 1 to 10 times the number of organisms needed to kill 100% of the mice within 48 h. The dosage regimen for all experimental infections was 0.5 and 4 h postinfection, and all compounds were administered subcutaneously. After 72 h, a 50% protective dose value (PD_{50}), expressed in milligrams per kilogram of body weight, was calculated by a probit method. The PD_{50} values reported are expressed in terms of the dose of compound given at each administration.

All mice used were females, strain CD-1, from the Charles River Co., with an average weight of 20 g.

TABLE 1. *Origin and enzyme content of bacterial strains*

Organism	Source	Aminoglycoside-inactivating enzyme
E. coli NR79/W677	J. Davies (Wisconsin)	AAC(6′)
E. coli RIP135	J. Witchitz (Paris)	AAC(3)
E. coli RIP55	J. Witchitz (Paris)	AAD(2″)
E. coli JR35/25	J. Davies (Wisconsin)	APH(3′)-I
S. epidermidis 109	F. H. Kayser (Zurich)	AAD(4′)
Providencia sp. 164	J. Davies (Wisconsin)	AAC(2′)
Providencia sp. A20894	K. E. Price (Syracuse, N.Y.)	AAC(2′)
Klebsiella spp. A20636	K. E. Price (Syracuse, N.Y.)	AAD(2″), APH(3′)-I
Proteus spp. HK238	F. H. Kayser (Zurich)	AAC(3), APH(3′)
Pseudomonas sp. HK232	F. H. Kayser (Zurich)	AAD(2″)
E. coli 172	Clinical isolate	None
S. aureus 246	Clinical isolate	APH(3′)-I, II
S. aureus 223	Clinical isolate	None
Klebsiella sp. 33	Clinical isolate	None
Proteus sp. 8	Clinical isolate	None
Pseudomonas sp. 48	Clinical isolate	None

RESULTS

Enzymatic modification of aminoglycosides. The relative degrees of modification of UK-18,892, gentamicin, and kanamycin B by cell-free enzyme preparations of APH(3')-I, AAD(2"), AAC(3), AAC(2'), AAC(6'), and AAD(4'), together with the MIC of the three aminoglycosides against the bacteria from which the enzymes were isolated, are shown in Table 2.

The degree of enzymatic modification was determined at pH 6, 7, and 8 because different enzymes and aminoglycosides have been shown to have varying pH optima in such systems (1, 4, 8). Accordingly, the highest radioactivity value obtained was used in calculating the extent of modification. For each enzyme, an arbitrary value of 100 was assigned to the aminoglycoside with the highest associated radioactivity, and the extent of modification of the other two was expressed relative to this.

UK-18,892 was a substrate for only AAC(6') and AAD(4'), and the organisms containing these enzymes (*E. coli* NR79/W677 and *S. epidermidis* 109) were resistant to this antibiotic. The remaining four aminoglycoside-resistant bacteria were susceptible to UK-18,892.

Kanamycin B was a substrate for all six enzymes examined, whereas gentamicin was not modified significantly by APH(3')-I or AAD(4'). The bacteria containing these latter two enzymes, *E. coli* JR35/25 and *S. epidermidis* 109, were characteristically susceptible to gentamicin, whereas the other organisms examined were resistant to this aminoglycoside. Kanamycin B was inactive against four of the organisms tested (*E. coli* RIP55, *E. coli* JR35/25, *S. epidermidis* 109, and *Providencia* 164), but had weak activity against *E. coli* NR79/W677 and was typically active against *E. coli* RIP135.

Lethal infections in mice. Table 3 shows the PD_{50} values of UK-18,892, gentamicin, and kanamycin B against lethal infections in mice caused by both aminoglycoside-susceptible and aminoglycoside-resistant bacteria. The PD_{50} values of UK-18,892 against aminoglycoside-resistant bacteria (*E. coli* RIP135, *Staphylococcus aureus* 246, *Klebsiella* A20636, *Proteus* HK238, and *Pseudomonas* HK232) were similar to those obtained against aminoglycoside-susceptible organisms of the same genera (*E. coli* 172, *S. aureus* 223, *Klebsiella* 33, *Proteus* 8, *Pseudomonas* 48). Kanamycin B, however, was considerably less active against the aminoglycoside-resistant isolates, with the exception of *E. coli*

TABLE 2. *Enzymatic modification of UK-18,892, kanamycin B, and gentamicin*

Organism	Enzyme	Aminoglycoside	MIC (μg/ml)	Radioactivity (cpm) incorporated at:			Relative degree of modification[a] at optimum pH
				pH 6	pH 7	pH 8	
E. coli RIP135	AAC(3)	UK-18,892	3.1	37	138	0	2.5
		Kanamycin B	1.6	363	2,423	1,418	43.6
		Gentamicin	50.0	5,267	5,549	5,558	100.0
E. coli RIP55	AAD(2")	UK-18,892	3.1	37	71	108	4.3
		Kanamycin B	25.0	306	2,514	2,420	99.0
		Gentamicin	50.0	2,200	2,540	1,407	100.0
E. coli JR35/25	APH(3')-I	UK-18,892	6.3	0	0	87	5.4
		Kanamycin B	>100.0	3,126	1,615	1,533	100.0
		Gentamicin	1.6	ND[b]	130	135	8.4
E. coli NR79/W677	AAC(6')	UK-18,892	50.0	3,287	3,150	3,664	100.0
		Kanamycin B	12.5	2,878	3,289	3,426	93.5
		Gentamicin	1.6	1,476	2,181	2,301	62.8
S. epidermidis 109	AAD(4')	UK-18,892	50.0	700	1,121	612	72.3
		Kanamycin B	>100.0	1,550	1,369	1,342	100.0
		Gentamicin	0.4	86	92	44	5.9
Providencia 164	AAC(2')	UK-18,892	12.5	15	0	7	0
		Kanamycin B	50.0	2,357	2,028	2,109	71.2
		Gentamicin	25.0	1,561	2,524	3,312	100.0

[a] A value of 100 was assigned to aminoglycoside associated with highest radioactivity.
[b] ND, Not determined.

TABLE 3. *Activity of UK-18,892, kanamycin B, and gentamicin against lethal infections in mice*

Organism	Aminoglycoside-inactivating enzyme	PD_{50} (mg/kg)		
		UK-18,892	Kanamycin B	Gentamicin
E. coli RIP135	AAC(3)	2.9	1.7	22.7
		(3.1)[a]	(1.6)	(50.0)
E. coli RIP55	AAD(2″)	3.4	30.9	32.0
		(3.1)	(25.0)	(50.0)
E. coli JR35/25	APH(3′)-I	4.5	>50.0	1.3
		(6.3)	(>100)	(1.6)
E. coli 172	None	1.9	1.9	0.5
		(1.6)	(1.6)	(1.0)
S. aureus 246	APH(3′)I, II	5.9	>50.0	0.8
		(3.1)	(>100)	(0.4)
S. aureus 223	None	1.2	0.7	0.3
		(0.4)	(0.4)	(0.2)
Klebsiella A20636	AAD(2″), APH(3′)	1.3	>50.0	21.8
		(3.1)	(>100)	(12.5)
Klebsiella 33	None	0.5	0.4	0.2
		(1.6)	(0.8)	(0.6)
Proteus HK238	AAC(3), APH(3′)	5.2	>50.0	24.0
		(3.1)	(100)	(25.0)
Proteus 8	None	1.8	1.4	0.2
		(1.6)	(1.6)	(0.8)
Pseudomonas HK232	AAD(2″)	10.8	>50.0	>50.0
		(0.8)	(12.5)	(100)
Pseudomonas 48	None	4.8	31.9	4.8
		(0.8)	(12.5)	(0.8)
Providencia A20894	AAC(2′)	5.5	>25	9.4
		(12.5)	(50.0)	(25.0)

[a] Data in parentheses indicate MIC (micrograms per milliliter).

RIP135, than against the same genera of aminoglycoside-susceptible bacteria. Similarly, gentamicin was less active against the aminoglycoside-resistant bacteria, with the exception of *E. coli* JR35/25 and *S. aureus* 246.

DISCUSSION

Resistance to aminoglycosides in staphylococci and gram-negative bacilli, when encountered clinically, is almost entirely attributable to the presence of antibiotic-inactivating enzymes (3, 5). Many different enzymes distinguishable by their substrate specificities have been described (5, 7), but the frequency of occurrence of the different types is less well documented. From a study of 152 aminoglycoside-resistant clinical isolates, Price et al. (10) concluded that phosphorylating [APH(3′)] and/or adenylylating [AAD(2″)] enzymes were most frequently present, and that acetylating [AAC(3)-I, AAC(2′)] enzymes were the next most commonly encountered. Only one 6′-acetylating enzyme [AAC(6′)] was detected in the isolates examined. We also found, in a study of 280 aminoglycoside-resistant clinical isolates (unpublished data), that phosphorylating [APH(3′)-I,II] and adenylylating [AAD(2″)] enzymes were present with greatest frequency, followed by 3-acetylating [AAC(3)] enzymes. No examples of AAC(6′) were identified, and only one 4′-adenylylating [AAD(4′)] enzyme was found. UK-18,892 inhibited 93% of these isolates at 12.5 μg/ml, and more than half of the remaining UK-18,892-resistant isolates showed high-level resistance to all other aminoglycosides studied, possibly indicating resistance by mechanisms other than enzymatic inactivation. We concluded that UK-18,892 was not inactivated by the most commonly encountered inactiving enzymes found in aminoglycoside-resistant bacteria isolated in the clinics.

In the present study we have attempted to confirm this conclusion by using cell-free extracts of bacteria containing known inactivating enzymes. Gentamicin and kanamycin B were chosen for comparison, because together they possess most of the amino and hydroxyl groups of aminoglycosides which are susceptible to enzymatic modification. UK-18,892 is not a substrate for APH(3′)-I, AAD(2″), AAC(3), or AAC(2′) in cell-free enzyme preparations obtained from aminoglycoside-resistant bacteria (Table 2), and organisms which contain these enzymes are susceptible to this aminoglycoside. 2′-Acetylation does not occur because UK-18,

892 does not possess a 2'-amino group. However, because UK-18,892 possesses 3'-hydroxyl, 2"-hydroxyl, and 3-amino groups, it would appear that substitution at the 1-amino group of kanamycin A by a 4-amino-2-hydroxybutyl moiety prevents inactivation by APH(3')-I, AAD(2"), and AAC(3). Both AAC(6') and AAD(4') enzymes inactivate UK-18,892. Similar findings have been reported for amikacin (6, 8, 12).

Although kanamycin B is a substrate for all six enzymes examined, it is not completely inactivated by bacteria containing AAC(3) and AAC(6'), as evidenced by its MIC against *E. coli* NR79/W677 and *E. coli* RUP135. It is possible that the rate of modification of kanamycin B by intact bacteria is too slow to prevent its antibacterial action. Because gentamicin does not possess 3'- or 4'-hydroxyl groups, it is not modified by APH(3')-I or AAD(4') and is therefore active against organisms containing these enzymes. It is, however, a substrate for AAD(2"), AAC(2'), and AAC(6'), although *E. coli* NR79/W677, which possesses AAC(6'), is susceptible to gentamicin. This is because two of the three components of commercial gentamicin (gentamicin C_{1A} and C_2) are still antibacterially active after modification by AAC(6') (1). The third active component, gentamicin C_1, is not a substrate for AAC(6') (1).

In mice, UK-18,892 is effective against infections caused by bacteria possessing APH(3')-I, AAD(2"), AAC(3), and AAC(2') enzymes, including three isolates possessing more than one enzyme. The degree of protection is consistent with the MIC of UK-18,892 against the infecting organism. Although we have not evaluated UK-18,892 against infections caused by bacteria possessing either AAC(6') or AAD(4'), it is unlikely that it would afford useful protection against such organisms in the absence of adequate in vitro activity.

The in vivo protection afforded by gentamicin and kanamycin B is also readily correlated with their activity in vitro. Gentamicin is considerably less effective against organisms possessing AAD(2") and AAC(3) enzymes than against the same species of aminoglycoside-susceptible bacteria. Similarly, kanamycin B does not afford useful protection against organisms possessing APH(3')-I, AAD(2"), or AAC(2').

In conclusion, the assumption made earlier (11; unpublished data) that the potent in vitro activity of UK-18,892 against aminoglycoside-resistant bacteria is due to its resistance to modification by most of the known aminoglycoside-inactivating enzymes has been verified. In addition, it is demonstrated that UK-18,892 protects against lethal infections in mice caused by these organisms.

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