MICROBIOLOGICAL EVALUATION OF BB-K 8, A NEW SEMISYNTHETIC AMINOGLYCOSIDE

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BB-K 8 is a new semisynthetic aminoglycoside antibiotic that has an extremely broad spectrum of antimicrobial activity which is due, at least in part, to the compound's high degree of resistance to aminoglycoside-inactivating enzymes. The new derivative inhibited 99.7% of 308 clinical isolates of *Enterobacteriaceae*, 100% of 97 *Staphylococcus aureus* strains, and 94.5% of 110 *Pseudomonas* sp. in *in vitro* tests at concentrations achievable in serum following administration of safe doses to humans. Of 23 strains of *Pseudomonas* sp. found resistant to gentamicin, 17 were susceptible to BB-K 8 indicating an absence of complete cross-resistance between these antibiotics. BB-K 8's bactericidal potential, as well as its response to changes in inoculum size and variations in medium constituents, were similar to those of kanamycin and gentamicin. As has been reported for gentamicin, combinations of BB-K 8 with carbenicillin give a high incidence of synergistic responses against *Pseudomonas* strains. BB-K 8 was well absorbed by mice when administered by the intramuscular route and was less toxic than kanamycin. In experimental infections of mice, BB-K 8 was as active as kanamycin in infections caused by kanamycin-sensitive bacteria. In addition, it was highly efficacious in infections produced by kanamycin- and/or gentamicin-resistant organisms.

Aminoglycoside antibiotics, principally kanamycin A and gentamicin, have assumed an important place in the armamentarium of drugs utilized for treatment of serious gram-negative infections. Kanamycin A, although possessing a relatively low toxicity potential, is virtually devoid of inhibitory effects for *Pseudomonas* species and also has a low degree of activity against some strains of *Escherichia coli*, *Serratia*, *Klebsiella-Enterobacter*, and *Proteus* sp.[2,21,40,45]. Unfortunately, gentamicin, which is inhibitory for a broader spectrum of microbes (including *Pseudomonas* sp.), is more oto- and nephrotoxic than kanamycin[7,25,44,70]. Furthermore the incidence of gentamicin-resistant strains being encountered in the clinic is rising as the antibiotic's usage increases[39,52,55]. In view of these therapeutic limitations, a program was initiated at Bristol Banyu Research Institute in Tokyo, Japan, to prepare new and potentially superior semisynthetic derivatives of the kanamycins. The most interesting compound evolving from this series up to now has been given the designation, BB-K 8. Synthesis of the new antibiotic involves acylation of the 1-amino group of the 2-deoxystreptamine moiety of kanamycin A with L(−)-γ-amino-α-hydroxybutyric acid[30]. The present report describes results obtained with BB-K 8 in laboratory

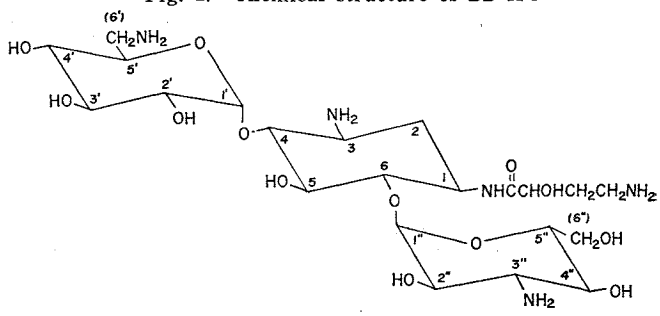
studies that were conducted in order to characterize its *in vitro* and *in vivo* antimicrobial activities as well as some of its pharmacological properties.

Experimental

Antibiotics.

BB-K 8 is a water-soluble aminoglycosidic antibotic whose chemical structure is shown in Fig. 1. Although most of the studies described in the present report were conducted with high purity samples of the base, a lot of the disulfate salt estimated to be approximately 70 % pure was utilized in specified experiments. Purity adjustments were not made in all of these cases due to the belated recognition that this sample which had been assumed to be relatively free of impurities was, in fact, contaminated with a closely related isomer of BB-K 8 that is virtually devoid of antibacterial activity. Subsequent testing has revealed that the contaminating isomer had a disproportionately large suppressive effect on BB-K 8's antibacterial potency. In direct comparative tests, pure materials have been found to possess approximately 2-fold more activity than impure ones, suggesting that the contaminant may actually have antagonized the activity of BB-K 8, perhaps through some competitive mechanism.

Fig. 1. Chemical structure of BB-K 8



Bulk kanamycin A sulfate (kanamycin) and gentamicin sulfate (gentamicin), the latter obtained through the courtesy of the Schering Corp., and composed of a mixture of gentamicins C_1, C_{1a}, and C_2[65], were utilized as control compounds for the studies with BB-K 8.

In vitro antibacterial activity against clinical isolates.

The comparative minimal inhibitory concentrations (MIC) of these antibiotics were determined in tests utilizing a large number of gram-positive and gram-negative bacteria that were predominantly of clinical origin. An agar dilution procedure was used in which inocula of the appropriate size were added by means of the multiple inoculator apparatus described by STEERS *et al.*[58]. Strains of *Diplococcus pneumoniae*, *Listeria monocytogenes*, *Streptococcus pyogenes*, and all enterococci were tested on plates containing MUELLER-HINTON Medium (Difco) +4 % defibrinated sheep blood with undiluted overnight cultures serving as the inoculm source. Strains of *Haemophilus influenzae* and *Neisseria gonorrhoeae* were plated on GC Base Medium (BBL)+1 % Hemoglobin (Difco)+1 % Isovitalex (BBL). The inoculum for each of the *H. influenzae* strains was a 10^{-3} dilution of a 48-hour broth culture. In the case of *N. gonorrhoeae*, 24-hour broth cultures were adjusted to give an optical density of 0.3 at 560 nm with a Bausch and Lomb Spectronic-20 Colorimeter using a 13×100 mm cuvette. A 10^{-1} dilution of this standardized suspension was then utilized as inoculum. All other organisms were tested on plates containing MUELLER-HINTON Medium using 10^{-2} dilutions of overnight cultures as the inoculum source.

In each case, the MIC, which was considered to be the lowest antibiotic concentration completely suppressing visible bacterial growth, was determined after overnight incubaction at 37°C.

BB-K 8 activity against enzyme-producing bacteria.

In addition to the agar dilution tests described above, the MIC of selected strains of microorganisms were determined by means of the standard 2-fold dilution technique utilizing MUELLER-HINTON Broth (Difco). Many of these strains are either known to produce or are

thought to produce aminoglycoside-inactivating enzymes. A 10^{-4} dilution of each culture was added to the antibiotic-containing tubes after which incubation was carried out at 37°C for 18 hours. The MIC was the minimal concentration that completely suppressed development of turbidity.

Synergy between BB-K 8 and carbenicillin.

Studies were conducted to determine whether BB-K 8 and carbenicillin combinations behave synergistically against 32 BB-K 8- and gentamicin-sensitive strains of *Pseudomonas aeruginosa*. A variation of the checkerboard procedure devised by SABATH *et al.*[49] was utilized. Varying concentrations (2-fold dose increments) of BB-K 8 or gentamicin were incorporated into plates of MUELLER-HINTON Medium containing 2-fold increments of disodium carbenicillin (Geopen, Chas. Pfizer & Co.). The antibiotic-containing plates were inoculated by means of the STEERS apparatus with 10^{-2} dilutions of overnight broth cultures of the pseudomonal strains and incubated at 37°C for 18 hours. MIC values were determined and interpreted as follows: strains inhibited by mixtures in which each antibiotic was present at one-fourth or less its inhibitory concentration when used alone were considered to have responded synergistically, while those whose growth was suppressed by one-fourfh the MIC of one of the antibiotics in combination with one-half the MIC of the other displayed a "greater-than" additive response. In cases where one-half the MIC of each was required, the effect was considered strictly additive.

Response to inoculum size and bactericidal potential.

Inoculum size studies utilized 10^{-2}, 10^{-4}, and 10^{-6} dilutions of overnight cultures (standardized prior to dilution so as to contain 1 to 3×10^8 cells/ml) in tubes of MUELLER-HINTON Broth containing 2-fold serial dilutions of the antibiotics. After 18-hour incubation at 37°C, MIC values were recorded.

Minimum bactericidal concentrations (MBC) of the antibiotics were determined for each of the organisms utilizing tubes that had received 10^{-4} culture dilutions as inoculum. The MBC was established by streaking 0.1-ml samples from all tubes that failed to show grossly visible turbidity onto the surface of plates containing antibiotic-free MUELLER-HINTON Medium. The minimum antibiotic concentration permitting development of 10 or less colonies/plate after 18-hour incubation at 37°C was considered to be the MBC.

Effect of the test medium on antibacterial activity.

Five commercially available broth media were used in these studies. They were Nutrient Broth (Difco), MUELLER-HINTON Broth, Antibiotic Assay Broth (BBL), Trypticase Soy Broth (BBL), and Heart Infusion Broth (Difco). BB-K 8 base (potency 940 μg/mg) and gentamicin were tested against 10 susceptible strains each of *Escherichia coli* and *P. aeruginosa*. Kanamycin was evaluated against the *E. coli*, but not against the *P. aeruginosa* strains since the antibiotic is virtually devoid of activity against members of the latter species. Comparative activity in the different media was determined by means of the standard 2-fold serial dilution technique in which 10^{-4} dilutions of overnight cultures were used as inocula.

Studies were also conducted to establish whether the type of agar utilized in agar dilution tests has any influence on MIC values of the aminoglycoside antibiotics. In these studies the inocula, which were 10^{-2} dilutions of overnight cultures (10 strains each of *E. coli* and *P. aeruginosa*), were added by means of the STEERS multiple inoculator apparatus to plates containing commercial MUELLER-HINTON Medium, MUELLER-HINTON Broth +1.5 % Ionagar (Colab), or MUELLER-HINTON Broth+1.5 % Bacto-Agar (Difco). MIC determinations were made after overnight incubation at 37°C. The lowest antibiotic concentration suppressing visible bacterial growth on the agar surface was considered to be the MIC.

Relationship between susceptibility-disc zone sizes and broth and agar dilution MIC values.

Discs containing 30 μg of BB-K 8 that had been prepared from a lot of the compound having a base potency of 940 μg/mg were utilized in these studies. After the sizes of the

zones around these discs were determined for 104 *Pseudomonas* strains by the standard method of BAUER *et al.*[5], they were compared with MIC values found for these organisms when they were tested by the broth dilution procedure described by ERICSSON and SHERRIS[22]. Zone sizes were also compared with agar dilution MIC values obtained for 103 *Pseudomonas* strains using plates containing MUELLER-HINTON Medium. The inoculum used for each strain was a 10^{-2} dilution of an overnight culture that was deposited on the agar surface by means of the STEERS multiple inoculator apparatus. The nature of the relationships between the different test systems was examined by calculating PEARSON correlation coefficients. The levels of significance of these coefficients were then estimated by use of the appropriate. "significance" tables.

Absorbability in rodents.

Mouse blood level tests were conducted utilizing male Swiss-Webster mice that averaged 20 g in weight. Concentrations of the aminoglycosides in blood were determined at various times after intramuscular (IM) administration of the drugs by collecting specimens from orbital sinuses. The procedure used for preparation of compounds, sample collection, and bioassay were identical to those previously described by PRICE *et al.*[48] except that plates of Antibiotic Assay Agar (BBL) adjusted to pH 8.0 and seeded with *Bacillus subtilis* ATCC 6633 were used to estimate concentrations of the antibiotics in blood.

The extent of urinary excretion of BB-K 8, kanamycin, and gentamicin was measured during 0~6 and 6~24 hour intervals following IM administration of a single 10 mg/kg dose. Five male Sprague-Dawley rats averaging 200 g in weight were utilized for each compound in each experiment. Individual urine samples were collected over dry ice during each post-administration time period. Samples were bioassayed in the same manner as mouse blood specimens except that standard lines were prepared by "spiking" normal urine with known quantities of the drugs. Urine volumes for each collection period were recorded and these values used to calculate total urinary excretion. Data were analyzed for significant differences by analysis of variance.

Therapeutic effectiveness of BB-K 8 in experimental infections.

A lot of BB-K 8, whose weight was adjusted to account for use of the 70 % pure disulfate salt, was compared with kanamycin and gentamicin in experimental bacterial infections of mice. In each of these studies, male Swiss-Webster mice were challenged via the intraperitoneal route with sufficient bacterial cells to kill all non-treated mice within 48 hours. Challenge doses of all of the organisms with the exception of *Klebsiella pneumoniae* A 9977 were given as suspensions in 5 % mucin. Antibiotic treatment was administered by the subcutaneous route at 1 and 4 hours post-challenge or at 0, 2, 4. and 6 hours post-challege. In all cases, dose-responses were conducted in which 6 ~ 8 different drug dosages were varied by 2-fold increments with 5 animals being utilized at each of the dose levels. At the conclusion of the experiments (usually day 4), total number of surviving mice was recorded and the PD_{50} (protective dose, 50 %) estimated by means of a log-probit plot.

Results and Discussion

In presenting results of *in vitro* antibacterial tests, cognizance must be given to the fact that there are marked differences in the toxicological properties of aminoglycoside antibiotics that significantly affect the dosage level at which each of the drugs can safely be administered to patients. Toxicological studies conducted at Bristol Laboratories[27] indicate that BB-K 8's oto- and nephrotoxic liability is less than that of kanamycin although blood and serum concentrations are essentially comparable for the 2 antibiotics after parenteral dosing in rodents (see Fig. 6), and in dog and man[12]. Thus, if the doses of BB-K 8 required for therapeutic success are no higher than those of kanamycin, treatment with the former antibiotic should

provide a somewhat greater margin of safety. The kanamycin dosage routinely employed in man is 7.5 mg/kg which produces an average serum level peak of about 20 μg/ml[31,36]. Gentamicin, on the other hand, because of its greater potential for producing toxicity, cannot be administered at this high dose level. The usual single dose of gentamicin is 0.8 mg/kg but it is sometimes given at 1.6 mg/kg in severe infections. The latter dose yields a serum level peak in man of about 8 μg/ml[7]. This closely approaches the 10 μg/ml level which is considered to be the absolute maximum serum concentration that is safe for man[14,24,38,47]. Thus, in designating whether bacterial strains are "resistant" or "susceptible" to a particular antibiotic, the average peak level of that compound attainable in human serum after an ordinary dose has been chosen as a guide. Since the serum level peak that would usually be achieved after kanamycin or BB-K 8 administration is 20 μg/ml, any strain that is inhibited *in vitro* at this concentration can be considered susceptible. On the other hand, since gentamicin's average peak serum level after a safe dose does not exceed 8 μg/ml, only those microorganisms inhibited at this concentration or lower can be considered susceptible. A similar basis for selection of kanamycin and gentamicin susceptibility end-points has been previously recommended by KIRBY and STANDIFORD[31]. However, these authors, who utilized MUELLER-HINTON Medium in their laboratory tests, chose a 5 rather than 8 μg/ml cut-off point for gentamicin. MUELLER-HINTON Medium was also used in the present studies because it has been designated as the medium of choice for antibiotic susceptibility testing by both clinical investigators[5,22] and the Food and Drug Administration[19].

Table 1 shows the cumulative percentages of a representative group of clinical isolates from the family *Enterobacteriaceae* whose growth was inhibited at the indicated concentrations of kanamycin, gentamicin, and BB-K 8. All compounds were tested on an equipotency basis.

These results show that each of the aminoglycosides was quite active against the majority strains from the 9 genera for which data are presented. However, BB-K 8 was found to be significantly more effective than its parent compound, kanamycin. BB-K8's superiority was particularly striking in tests against *E. coli*, *Serratia marcescens*, and *Klebsiella*, *Enterobacter*, and *Salmonella* sp. where at least 96 % of the strains examined proved to be susceptible to BB-K 8, whereas the precentage susceptible to kanamycin for this group ranged from a low of 81.0 (*Klebsiella* sp.) to high of 90.3 (*Salmonella* sp.). Strains considered susceptible were those inhibited by antibiotic concentrations of 20 μg/ml or less. On the basis of this cut-off point, of 305 strains tested, only one could be considered resistant to BB-K 8 as compared to 30 for kanamycin. When results obtained with 3 miscellaneous *Enterobacteriaceae* strains (*Edwardsiella tarda* 2, and *Arizona hinshawii* 1—all of which proved to be susceptible to BB-K 8 and kanamycin) are included, then 307 of 308 or 99.7 % of the organisms tested were susceptible to BB-K 8 as compared to 278 of 308 or 90.3 % to kanamycin.

Gentamicin's intrinsic activity (effectiveness against wild-type susceptible strains) against these same organisms was in some cases greater than that of BB-K 8 or kanamycin. However, despite this slightly higher degree of potency, the actual percentage of strains susceptible to gentamicin (those inhibited by 8 μg/ml or less) was appreciably

less than the percentage found for BB-K 8. Overall, 294 of 308 cultures (including the 3 miscellaneous strains not listed in Table 1) or 95.5% were susceptible to gentamicin as compared to 99.7% for BB-K 8.

MIC determinations were also made for 12 additional gram-negative strains of clinical origin, 10 *Alcaligenes faecalis* and 2 *Aeromonas liquefaciens*. An unusually high degree of resistance was demonstrated by the *A. faecalis* strains since 4 of 10 were completely refractory to the action of BB-K 8, kanamycin, and gentamicin. The remaining 6 strains and the 2 *A. liquefaciens* cultures, however, were fully susceptible to all 3 antibiotics.

The increase in antibacterial spectrum of kanamycin achieved by modifying it chemically to yield BB-K 8 can be noted not only when one examines strains of *Enterobacteriaceae*, but is also strikingly apparent in tests against strains of *Pseudomonas* sp. A summary of results obtained with 110 strains (106 of which were *P. aeruginosa*) is presented in Table 2.

Table 1. Susceptibility of 305 *Enterobacteriaceae* strains to BB-K 8, kanamycin and gentamicin

Organism (No. of strains)	Antibiotic	Antibiotic concentration (μg/ml)									
		0.32	0.63	1.25	2.5	5	8	10	20	40	>80
Escherichia coli (90)	BB-K 8	5.6[a]	33.2	73.3	88.9	95.6	NT[b]	97.8	100[c]	—	—
	Kanamycin	2.2	13.3	26.7	62.2	77.8	NT	86.7	87.8	87.8	100
	Gentamicin	21.1	80.0	93.3	94.4	95.6	95.6	95.6	98.9	100	—
Klebsiella species (42)	BB-K 8	2.4	28.6	73.8	85.7	95.2	NT	97.6	100	—	—
	Kanamycin	2.4	26.2	66.7	76.2	76.2	NT	78.6	81.0	81.0	100
	Gentamicin	23.8	78.6	78.6	81.0	83.3	83.3	83.3	97.6	97.6	100
Enterobacter species (27)	BB-K 8	0	17.6	59.3	92.6	96.3	NT	96.3	96.3	96.3	100
	Kanamycin	0	17.6	77.8	88.9	88.9	NT	88.9	88.9	92.6	100
	Gentamicin	48.4	96.3	96.3	96.3	96.3	96.3	96.3	100	—	—
Proteus mirabilis (32)	BB-K 8	0	3.1	37.5	84.4	96.9	NT	100	—	—	—
	Kanamycin	0	34.4	78.1	90.6	100	NT	—	—	—	—
	Gentamicin	31.3	78.1	96.9	100	—	—	—	—	—	—
Proteus species indol-positive (32)	BB-K 8	6.3	50.0	71.9	93.8	100	NT	—	—	—	—
	Kanamycin	18.8	65.6	78.1	100	—	NT	—	—	—	—
	Gentamicin	62.5	87.5	96.9	96.9	96.9	100	—	—	—	—
Serratia marcescens (27)	BB-K 8	0	22.2	85.2	92.6	92.6	NT	100	—	—	—
	Kanamycin	22.2	59.3	70.4	70.4	74.1	NT	74.1	81.5	81.5	100
	Gentamicin	22.2	77.8	100	—	—	—	—	—	—	—
Salmonella species (31)	BB-K 8	9.7	29.0	64.5	100	—	NT	—	—	—	—
	Kanamycin	12.9	32.2	64.5	90.3	90.3	NT	90.3	90.3	90.3	100
	Gentamicin	51.6	98.6	100	—	—	—	—	—	—	—
Shigella species (13)	BB-K 8	0	7.7	7.7	46.2	92.3	NT	100	—	—	—
	Kanamycin	0	7.7	15.4	15.4	92.3	NT	100	—	—	—
	Gentamicin	0	30.8	69.2	100	—	—	—	—	—	—
Providencia species (6)	BB-K 8	50	83.3	100	—	—	NT	—	—	—	—
	Kanamycin	33.3	83.3	100	—	—	NT	—	—	—	—
	Gentamicin	33.3	50	66.7	66.7	66.7	66.7	83.3	100	—	—
Citrobacter freundii (5)	BB-K 8	0	40	100	—	—	NT	—	—	—	—
	Kanamycin	20	80	100	—	—	NT	—	—	—	—
	Gentamicin	0	0	40	100	—	—	—	—	—	—

[a] Cumulative percentage of strains inhibited at indicated antibiotic concentration.
[b] NT=Not tested.
[c] Organisms inhibited by antibiotic concentrations to the left of the bold-faced line are considered susceptible; those on the right, resistant. Tests conducted on MUELLER-HINTON Medium (Difco).

It can be seen that BB-K 8 possesses an antipseudomonal spectrum that is significantly better than that of gentamicin since the percentages of strains susceptible to the 2 antibiotics were 94.5 and 79.1, respectively. Furthermore, the purified BB-K 8 preparation used produced a level of antipseudomonal activity that was only slightly lower (≤2-fold) than that of gentamicin. This is indicated by the fact that inhibition of 50% of the strains was achieved with 2.5 μg/ml of gentamicin and somewhat less than 5 μg/ml of BB-K 8. Kanamycin was virtually devoid of activity against pseudomonads since only 6.4% of the strains were susceptible to a concentration of 20 μg/ml.

The relative activity of BB-K 8, kanamycin, and gentamicin was also determined for a large number of strains of *Staphylococcus aureus*, a gram-positive species against which aminoglycoside antibiotics have significant clinical utility. Results obtained with 97 strains are presented in Table 3. All 97 strains proved to be susceptible to BB-K 8 and gentamicin as compared to only 91 of 97 or 93.8% for kanamycin. As had been found with gram-negative organisms, the intrinsic potency of gentamicin

Table 2. Susceptibility of 110 *Pseudomonas* strains[a] to BB-K 8, kanamycin and gentamicin

Antibiotic	Antibiotic concentration (μg/ml)								
	0.63	1.25	2.5	5	8	10	20	40	⩾80
BB-K 8	0.9[b]	2.7	20.9	66.4	NT[c]	90.0	94.5[d]	96.4	100
Kanamycin	0	0	0	0	NT	2.7	6.4	17.3	100
Gentamicin	1.8	7.3	51.8	76.4	79.1	80.9	83.6	85.5	100

[a] 106 strains were classified as *Pseudomonas aeruginosa*.
[b] Cumulative percentage of strains inhibited at indicated antibiotic concentration.
[c] NT=Not tested.
[d] Organisms inhibited by antibiotic concentrations to the left of the bold-faced line are considered susceptible; those to the right, resistant. Tests conducted on MUELLER-HINTON Medium (Difco).

Table 3. Susceptibility of 97 *Staphylococcus aureus* strains to BB-K 8, kanamycin and gentamicin

Antibiotic	Antibiotic concentration (μg/ml)									
	0.32	0.63	1.25	2.5	5	8	10	20	40	⩾80
BB-K 8	18.6[a]	88.7	96.9	99.0	100	NT[b]	—	—	[c] —	—
Kanamycin	43.3	85.6	93.8	93.8	93.8	NT	93.8	93.8	93.8	100
Gentamicin	86.6	100	—	—	—	—	—	—	—	—

[a] Cumulative percentage of strains inhibited at indicated antibiotic concentration.
[b] NT=Not tested.
[c] Organisms inhibited by antibiotic concentrations to the left of the bold-faced line are considered susceptible; those to the right, resistant. Tests conducted on MUELLER-HINTON Medium (Difco).

Table 4. Susceptibility of several gram-positive and gram-negative bacterial species to BB-K 8, kanamycin and gentamicin

Organism* (No. of strains)	Gram-stain reaction	Percentage strains susceptible at indicated antibiotic concentrations		
		BB-K 8 20 μg/ml	Kanamycin 20 μg/ml	Gentamicin 8 μg/ml
Streptococcus pyogenes (20)	+	50.0	25.0	95
Streptococcus sp. type D (14)	+	14.3	21.4	35.7
Diplococcus pneumoniae (17)	+	76.5	58.8	88.2
Listeria monocytogenes (7)	+	100	100	100
Haemophilus influenzae (17)	−	100	100	100
Neisseria gonorrhoeae (12)	−	83.3	83.3	100

* The *H. influenzae* and *N. gonorrhoeae* strains were tested on GC Base Medium (BBL)+1% Hemoglobin (Difco) +1% Isovitalex (BBL), while streptococci, *L. monocytogenes*, and *D. pneumoniae* were tested on MUELLER-HINTON Medium (Difco)+4% sheep blood.

was slightly greater than that of the other antibiotics.

Among the remaining species of bacteria subjected to susceptibility testing were strains of *S. pyogenes*, type D *Streptococcus* sp. (enterococci), *D. pneumoniae*, *L. monocytogenes*, *H. influenzae*, and *N. gonorrhoeae*. Results obtained with these organisms are summarized in Table 4.

The gram-positive species listed here, with the exception of *L. monocytogenes*, have long been known to possess a low order of susceptibility to aminoglycosidic antibiotics, and as expected, generally responded poorly to the present test compounds.

All 17 *H. influenzae* strains were susceptible to each of the antibiotics with gentamicin being 2- to 4-fold more active than the other compounds. All *N. gonorrhoeae* strains were also inhibited by a clinically achievable level (8 μg/ml) of gentamicin; however, the growth of 2 of 12 strains failed to be suppressed by a 20 μg/ml concentration of BB-K 8 or kanamycin.

In an effort to help determine the basis for BB-K 8's broad antibacterial spectrum, a number of microorganisms with known or suspected mechanisms of resistance to one or more of the aminoglycoside antibiotics was selected for *in vitro* testing. Minimum inhibitory concentrations were determined in MUELLER-HINTON Broth using a lot of BB-K 8 that was free of contaminating isomers and estimated to have a potency of 940 μg/mg as the free base. Results of the study are shown in Table 5.

Kanamycin phosphotransferase is known to react with a number of aminoglycosidic antibiotics to yield biologically-inactive, phosphorylated derivatives[16]. The site of enzyme activity, which is mediated by a transferable resistance factor in *Entero-*

Table 5. Relative activity of BB-K 8, kanamycin and gentamicin against bacterial strains resistant to one or more aminoglycoside antibiotics

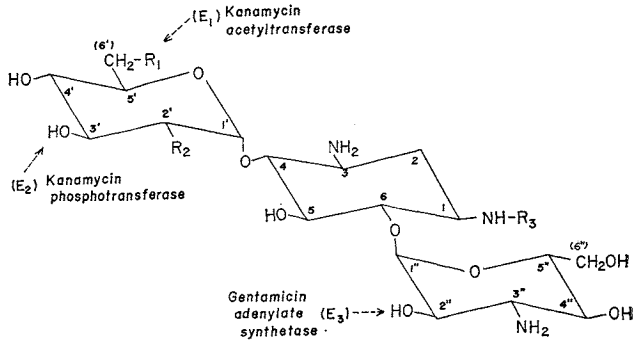
Bacterial strain	Resistance mechansm*	Geometric mean MIC** (μg/ml)		
		BB-K 8	Kanamycin	Gentamicin
Staphylococcus aureus Smith	None	1.4	1.4	0.2
S. aureus A 20240	Kanamycin phosphorylation ?	2.5	>125	0.2
Enterobacter cloacae A 9656	None	1	1.4	0.2
E. cloacae A 20364	Kanamycin phosphorylation ?	1	>125	0.2
Klebsiella pneumoniae A 9662	None	1	1.4	0.1
K. pneumoniae A 20680	Gentamicin adenylylation+ kanamycin phosphorylation	1.4	>125	11.3
Escherichia coli K-12	None	1	0.6	0.3
E. coli A 20664	Kanamysin acetylation	2	11.4	0.1
E. coli A 20665	Kanamycin phosphorylation	0.6	>125	0.4
E. coli A 20683	Gentamicin adenylylation+ kanamycin phosphorylation	2	>125	16
Pseudomonas aeruginosa A 20229	None ?	0.4	5.7	0.4
P. aeruginosa A 9843	Kanamycin phosphorylation ?	1.4	45	0.8
P. aeruginosa A 20718	Gentamicin acetylation+ kanamycin phosphorylation ?	3.6	63	32

* All strains with a *known* mechanism of resistance were obtained through the courtesy of Dr. JULIAN DAVIES, University of Wisconsin.

** MIC values are geometric means of two independent tests. Values determined in 2-ford serial dilution tests using MUELLER-HINTON Broth Difco).

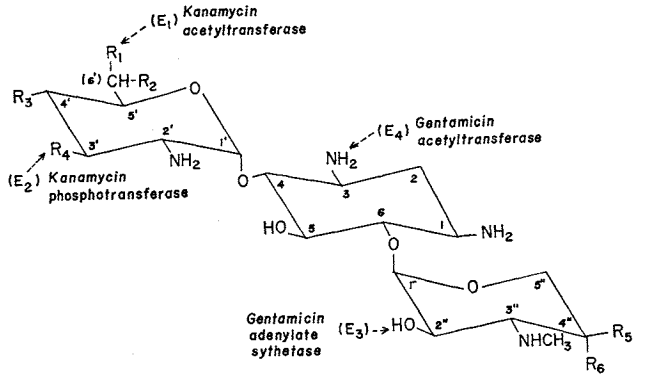
bacteriaceae and some *P. aeruginosa* strains, is the 3′-hydroxyl group[61], a substituent present in antibiotics such as kanamycins A, B, and C (Fig. 2), the neomycins, and gentamicin A. These antibiotics may also be inactivated by phosphotransferases from *P. aeruginosa* strains that act at this same site but do not appear to be controlled by a transferable factor[15]. Gentamicins C_1, C_{1a}, and C_2 (Fig. 3), 3′-deoxykanamycin A[64], 3′, 4′-dideoxykanamycin B[63], and tobramycin[35], as one might expect, do not serve as substrates for the phosphorylating enzyme since all lack a hydroxyl group on the 3′-carbon. BB-K 8, on the other hand, as is readily evident from results shown in Table 5, is not inactivated by kanamycin phosphotransferase despite the fact that it possesses a 3′-hydroxyl group. It is now known that phosphorylation of the antibiotic does not take place (J. DAVIES, University of Wisconsin, personal communication) due to the blocking effect of the 1-amino group substituent (Fig. 2). This property of BB-K 8 is an extremely important one, since phosphorylation is thought to be the major mechanism of resistance to kanamycin of *P. aeruginosa*[18] and many other clinically significant organisms[17,61].

Fig. 2. The structures of the kanamycins and BB-K 8 are shown. The sites of attack and substrates of the kanamycin-gentamicin inactivating enzymes are identified.



Antibiotic	R_1	R_2	R_3	Substrate for
Kanamycin A	$-NH_2$	$-OH$	$-H$	E_1, E_2, E_3
Kanamycin B	$-NH_2$	$-NH_2$	$-H$	E_1, E_2, E_3
Kanamycin C	$-OH$	$-NH_2$	$-H$	E_2, E_3
BB-K8	$-NH_2$	$-OH$	$-COCHOHCH_2CH_2NH_2$	E_1

Fig. 3. The structures of the gentamicins are shown. The sites of attack and substrates of the kanamycin-gentamicin inactivating enzymes are identified.



Antibiotic	R_1	R_2	R_3	R_4	R_5	R_6	Substrate for
Gentamicin A	$-OH$	$-H$	$-OH$	$-OH$	$-OH$	$-H$	E_2, E_3
Gentamicin C_1	$-NHCH_3$	$-CH_3$	$-H$	$-H$	$-CH_3$	$-OH$	E_3, E_4
Gentamicin C_{1a}	$-NH_2$	$-H$	$-H$	$-H$	$-CH_3$	$-OH$	E_1, E_3, E_4
Gentamicin C_2	$-NH_2$	$-CH_3$	$-H$	$-H$	$-CH_3$	$-OH$	E_1, E_3, E_4

Another mechanism of kanamycin resistance is also enzymatic in origin[62], but differs from phosphorylation of kanamycin in that it causes only partial inactivation of susceptible antibiotics and is only rarely found among clinical strains.

This enzyme, which has as its site of attack the 6′-amino group (Figs. 2 and 3), has been shown to be an R-factor-mediated acetyltransferase[46]. It acts on antibiotics

such as kanamycins A and B, neomycin B, gentamicins C_{1a} and C_2, and tobramycin, while kanamycin C, paromomycin, and gentamicins A and C_1, which lack a primary amine at the 6′-carbon, are resistant[16]. As can be seen in Table 5, *E. coli* A 20664, the strain that possesses this resistance mechanism is 16-fold less susceptible to kanamycin than the parent K-12 strain. However, in the case of BB-K 8 and gentamicin, this strain's degree of sensitivity is about the same as that of the parent which lacks this enzyme. Nevertheless, it has recently been shown by J. DAVIES (personal communicaton) that BB-K 8 is acetylated by *E. coli* A 20664.

Gentamicin adenylate synthetase is another enzyme that antagonizes the activity of certain aminoglycoside antibiotics. The site of attack of this enzyme, which is also mediated by a transferable resistance factor[6,34], has not been unequivocally established in the case of gentamicin. However, recent studies[48,74], showing that 3′,4′-dideoxykanamycin B is adenylylated at the 2″-hydroxyl group of 3-aminoglucose, offers a compelling suggestion that adenylylation of gentamicin occurs at the 2″-hydroxyl of gentamicin's garosamine moiety. The kanamycins and tobramycin also appear to be substrates for this enzyme while antibiotics having a pentose in glycosidic linkage with 2-deoxystreptamine (neomycins, butirosins[73], and lividomycins) are not susceptible. It is apparent from results shown in Table 5 that *K. pneumoniae* A 20680 and *E. coli* A 20683, the latter having received from the former R-factors that mediate gentamicin adenylylation and kanamycin phosphorylation, are both markedly resistant to kanamycin and gentamicin, but fully susceptible to BB-K 8. Thus, acylation of kanamycin's 1-amino group with γ-amino-α-hydroxybutyric acid (to give BB-K 8) blocks the adenylylation reaction (J. DAVIES, personal communication).

A fourth and only recently recognized enzymatic mechanism of aminoglycoside resistance has been found in *P. aeruginosa*[42]. The enzyme produced is an acetyltransferase and since it affects activity of gentamicins but not kanamycins, it can appropriately be designated gentamicin acetyltransferase. Its site of action as indicated in Fig. 3 is the 3-amino group of 2-deoxystreptamine[9]. Those aminoglycoside antibiotics which lack a 4′-hydroxyl group such as gentamicins C_1, C_{1a}, and C_2 are good substrates while gentamicin A, kanamycin B, and tobramycin are poor ones. Kanamycin A and BB-K 8 do not serve as substrates for this enzyme. MIC data presented in Table 5 for *P. aeruginosa* A 20718 indicate that this strain is resistant to gentamicin and kanamycin (the latter probably by virtue of kanamycin phosphotransferase) but is susceptible to BB-K 8.

Thus the broad spectrum of BB-K 8 can be attributed, at least in part, to its resistance to bacterial enzymes that cause inactivation of kanamycin and/or gentamicin. Its activity appears to be virtually fully expressed in MIC tests against bacterial strains known to produce kanamycin phosphotransferase, kanamycin acetyltransferase, gentamicin acetyltransferase, and/or gentamicin adenylate synthetase.

Despite its resistance to inactivating enzymes, one still finds some organisms, particularly strains of *Alcaligenes* and *Pseudomonas*, that are resistant to BB-K 8. Since all of these strains are also resistant to gentamicin, kanamycin, and various other aminoglycosides, it is possible that resistance mechanisms other than those of

the enzymic type are operative here. One example of this type of mechanism would be that caused by a non-specific reduction in permeability of cells to this class of antibiotics while still another mechanism which probably involves a somewhat greater degree of specificity is that attributable to mutants present in the population which are significantly altered at the ribosomal target site of a particular aminoglycosidic antibiotic. The latter type of mechanism has been described primarily in strains that have laboratory-developed antibiotic resistance[15]. However, *Pseudomonas* isolates that contain altered ribosomes endowing them with resistance to gentamicin have been cultured from burn wounds in patients[59]

Since cross-resistance to aminoglycoside antibiotics among strains having altered ribosomes is usually absent or incomplete, it is apparent that laboratory-induced resistance may also stem from other mechanisms. For example, recently completed studies in our laboratories show that all sensitive strains of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* tested became resistant after 4~5 transfers in sublethal concentrations of BB-K 8, kanamycin, or gentamicin, and that a resistance increase of approximately the same order (8- to 63-fold) occurred for each strain with derived resistance to a given antibiotic when tested against the other 2. This finding is in agreement with that of WEINSTEIN *et al.*[67] who observed that strains possessing laboratory-induced gentamicin resistance were completely cross-resistant with other aminoglycoside antibiotics. These investigators further noted that such strains "rapidly revert to sensitivity, grow less vigorously, are nutritionally dependent, and lack virulence". The nature of these strains was in marked contrast to that of resistant isolates of clinical origin which did not differ from sensitive strains in regard to the above listed characteristics. They further observed that absolute cross-resistance between gentamicin and tobramycin did not occur among gentamicin-resistant and tobramycin-resistant clinical isolates of *P. aeruginosa* and *K. pneumoniae*. This is in agreement with findings made in the present investigation where MIC tests were conducted with BB-K 8 and gentamicin against 110 clinical isolates of *Pseudomonas* sp. Among strains found resistant to one or both aminoglycosides, 6 failed to respond to a 20 μg/ml concentration of BB-K 8 and 23 to an 8 μg/ml concentration of gentamicin. Table 6 shows the distribution of gentamicin and BB-K 8 MIC values for these resistant strains.

It is apparent from the data in this table that cross-resistance occurred only among 6 of the 23 resistant strains. Seventeen were resistant to gentamicin but not BB-K 8, while none showed exclusive resistance to BB-K 8. Thus it appears that gentamicin-resistant *Pseudomonas* strains may be sensitive or resistant to BB-K 8, while all BB-K 8-resistant strains are also gentamicin-resistant.

Table 6. Distribution of gentamicin and BB-K 8 MIC values for *Pseudomonas* strains found resistant to one or both aminoglycosides*

BB-K 8 susceptibility status	BB-K 8 MIC μg/ml	Gentamicin MIC μg/ml					Total strains
		15	20	40	80	>80	
Sensitive	2.5	0**	0	1	0	0	17
	5	0	0	0	2	3	
	10	1	1	0	0	6	
	20	1	1	0	0	1	
Resistant	40	0	1	1	0	0	6
	80	0	0	0	0	1	
	>80	0	0	0	2	1	

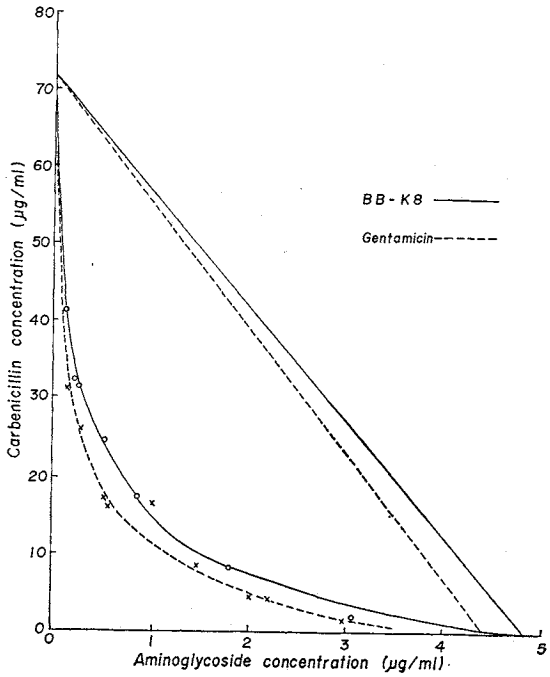
* Tests were conducted on MUELLER-HINTON Medium.
** Number of strains having indicated susceptibility pattern.

There is an ever-increasing incidence of gentamicin-resistant strains in the hospital environment which is probably directly attributable to the more extensive use of gentamicin[39,52,55,56]. It seems likely this problem will become of even greater magnitude in the future since in several instances[18,29,39,72] such strains have been shown to possess transmissible R-factors that mediate resistance to gentamicin. Thus the fact that 17 of 23 gentamicin-resistant clinical isolates of *Pseudomonas* are susceptible to BB-K 8 suggests that this antibiotic will play a very important therapeutic role in the future.

One approach presently being evaluated[8,32,50,75] in the clinic in an effort to circumvent the increasing emergence of gentamicin-resistant *P. aeruginosa* strains is the use of this antibiotic in combination with carbenicillin, a semisynthetic penicillin that possesses antipseudomonal activity[1]. These investigations have been prompted by *in vitro* studies[20,53,57] which have demonstrated a high incidence of synergistic action between these antibiotics. It was deemed of interest, therefore, to determine whether similar synergistic effects occurred when BB-K 8 was utilized in combination with carbenicillin. Geometric mean MIC values obtained with BB-K 8-carbenicillin and gentamicin-carbenicillin combinations for 32 *P. aeruginosa* strains have been calculated and are presented in graphic form in Fig. 4.

The straight lines in this isobologram show the theoretical values one would obtain if the effects of the combinations were strictly additive. However, the significant inward bowing observed when the actual means are plotted indicates that the responses were much greater than additive. It is readily apparent that the BB-K 8-carbenicillin and the gentamicin-carbenicillin combinations produced almost identical effects. Actually in the former case, the response of 28 of 32 strains met the requirements for synergism (inhibition caused by one-fourth the minimal inhibitory dosage of each antibiotic), while the other 4 strains gave a "greater-than" additive effect. Twenty-nine of 32 strains responded synergistically to the gentamicin-carbenicillin combination with the remaining 3 strains all showing a "greater-than" additive response.

Fig. 4. Synergistic antibacterial activity of carbenicillin and two aminoglycosides against *Pseudomonas aeruginosa* strains. Each point is the geometric mean of the MIC values obtained for 32 strains on MUELLER-HINTON Medium.



Other *in vitro* studies have been conducted with a diverse group of microorganisms in order (i) to investigate the effect of inoculum size on the MIC and (ii) to determine the relative

Table 7. Studies on inoculum size effects and bactericidal potential of BB-K 8, kanamycin and gentamicin

Organism	MIC*, (MBC)** in μg/ml at indicated inoculum level-cell/ml								
	BB-K 8			Kanamycin			Gentamicin		
	~2×10^2	~2×10^4	~2×10^6	~2×10^2	~2×10^4	~2×10^6	~2×10^2	~2×10^4	~2×10^6
Staphylococcus aureus A 9537	0.5	1 (4)	16	0.25	1 (4)	4	00.3	0.25(.25)	1
S. aureus A 9606	0.5	8 (63)	16	0.25	4 (125)	16	0.06	0.5 (63)	2
Escherichia coli A 9675	0.25	0.25 (2)	0.5	0.25	0.25 (2)	1	0.06	0.13 (2)	0.25
E coli A 15119	1	2 (32)	8	1	2 (32)	8	0.25	1 (32)	2
Klebsiella pneumoniae A 9977	1	2 (16)	8	1	4 (16)	8	0.25	1 (8)	2
Pseudomonas sp. A 9931	2	2 (63)	8	16	16 (>250)	125	0.5	1 (32)	4

* MIC=minimum inhibitory concentration. Tests conducted in MUELLER-HINTON Broth (Difco).
** MBC=minimum bactericidal concentration. Number of surviving cells determined on MUELLER-HINTON Medium (Difco).

bactericidal potential of BB-K 8, kanamycin, and gentamicin.

Table 7 shows results obtained when the disulfate salt of BB-K 8 (70 % pure), kanamycin, and gentamicin were tested against a representative group of microoganisms. With the exception of the *S. aureus* strains, increasing the inoculum size from 2×10^2 to 2×10^4 cells/ml had only a minimal effect on the MIC of the antibiotics. In the case of *S. aureus* A 9537, the increase in the MIC of BB-K 8 was also minimal (2-fold), whereas the MIC of kanamycin and gentamicin at the higher inoculum level was 4- and 8-fold greater, respectively. *S. aureus* A 9606, on the other hand, proved to be markedly more resistant to all 3 antibiotics when the inoculum size was increased from 2×10^2 to 2×10^4 cells/ml.

Raising the cell concentration in the inoculum still further (from 2×10^4 to 2×10^6 cells/ml resulted in an MIC for all 3 antibiotics that was on the average some 4-fold higher against both gram-positive and gram-negative species. Results found for gentamicin and kanamycin in this study are consistent with those previously reported by others[4,26].

The differences between MBC and MIC for BB-K 8, kanamycin, and gentamicin, although generally of the same order for all of the antibiotics, varied widely depending upon the test organism. For example, the ratio between MBC and MIC rarely exceeded 8 for any of the antibiotics in tests against *S. aureus* A 9537, *E. coli* A 9675, and *K. pneumoniae* A 9977. However, this ratio was almost invariably exceeded in tests against the remaining organisms. This same wide degree of variability in MBC/MIC ratios has previously been observed for both kanamycin[69] and gentamicin[8].

In view of the well-known effects that medium composition has antibacterial activity of aminoglycosidic antibiotics, an experiment was conducted to determine whether MIC values of BB-K 8 were also influenced by the type of medium employed. This compound as well as kanamycin and gentamicin were tested against selected microorganisms in 5 commercially available broth media. Results are summarized in Table 8.

It is apparent that all 3 antibiotics vary widely in their inhibitory action against bacteria depending on the type of medium utilized. On the basis of MIC values, the media appear to fall into 3 general groups. The first group contains Nutrient Broth,

Table 8. Influence of the type of test broth on geometric mean MIC values for BB-K 8, kanamycin and gentamicn

Organism* (No. of strains)	Antibiotic	Geometric mean MIC in μg/ml and (fold-increase over Nurient Broth MIC) in indicated broth medium				
		Nutrient	Mueller-Hinton	Heart Infusion	Antibiotic Assay	Trypticase Soy
Escherichia coli (10)	BB-K 8	0.1	3.5 (35)	7.0 (70)	19.7(197)	14.9(149)
	Kanamycin	0.2	6.1 (31)	6.5 (33)	22.6(226)	16.0(160)
	Gentamictn	0.03	0.5 (17)	2.1 (70)	4.6(153)	5.7(190)
Pseudomonas aeruginosa (10)	BB-K 8	0.08	1.4 (17.5)	1.7 (21.3)	2.8 (35)	4.3(54.3)
	Gentamicin	0.06	0.3 (5)	0.6 (10)	0.8 (13.3)	2.5(41.7)

* The inocula utilized were 10^{-4} dilutions of overnight cultures.

in which the antibiotics act with maximum efficiency; the second, Mueller-Hinton and Heart Infusion Broth, where intermediate effectiveness is found; and the third, Antibiotic Assay and Trypticase Soy Broths, where activity is even further suppressed. This same type of ranking has also been observed by Medeiros *et al.*[41], who tested gentamicin and kanamycin against several species of bacteria in Nutrient, Mueller-Hinton, and Trypticase Soy Broths. In the present study, the fold-increase in MIC values obtained in the various media relative to that of Nutrient Broth appears very similar for the 3 antibiotics in tests against *E. coli*. However, where *P. aeruginosa* strains are involved, it is clear that, with the possible exception of Trypticase Soy Broth, BB-K 8's activity relative to that found in Nutrient Broth is affected to a greater extent than is gentamicin's. Particularly relevant to the present report, where the bulk of the work has been done in Mueller-Hinton Broth or Mueller-Hinton Medium, is the finding that the effectiveness of both BB-K 8 and kanamycin in this type of medium, relative to that of Nutrient Broth, is reduced to a greater extent (2- to 3-fold) than that of gentamicin.

It seems probable that the basis for BB-K 8's varying responses, as has been shown for kanamycin and gentamicin, is the level in the medium of metallic cations (particularly sodium, calcium, and magnesium), the pH and the overall electrolyte concentration[23,41]. *P. aeruginosa* presents somewhat of a special case, since its response to gentamicin, for example, is influenced to a remarkable degree by the cation concentration of the test medium, with the Mg^{++} content playing the most significant role[23]. This same high degree of sensitivity of gentamicin to cation content was not seen in tests with *E. coli* and *S. marcescens*[41].

The type of agar utilized in susceptibility test media also contributes to the degree of effectiveness of the aminoglycosides. Waitz and Weinstein[65], for example, showed that zone sizes in disc diffusion tests were significantly larger when highly purified agar (Ionagar) was used with Nutrient Broth than when commercial Nutrient Agar (containing an agar of lesser purity) was the test medium. Because of this, agar dilution studies were conducted with BB-K 8, kanamycin, and gentamicin to investigate the relative effect of various agars on antimicrobial activity of these antibiotics. Results of these experiments indicated that differences in MIC values, while usually small (≤2-fold), were consistently higher in tests utilizing commercial Mueller-Hinton Medium than in those where Mueller-Hinton Broth was supplemented with a

similar amount of Bacto-Agar or Ionagar. This was true for all 3 antibiotics against both *E. coli* and *P. aeruginosa* strains.

The final *in vitro* experiments to be reported were conducted in order to investigate the relationship between BB-K 8 broth and agar dilution MIC values for *Pseudomonas* strains and the corresponding zone sizes obtained by the standardized method of BAUER *et al.*[5] Studies were conducted with the hope that the data would provide guidelines for interpretation of susceptibility determinations in the clinical setting. Results are presented in Figs. 5 and 6.

Fig. 5. Relationship between zone size and broth dilution MIC values of BB-K 8 for 104 strains of *Pseudomonas* species.

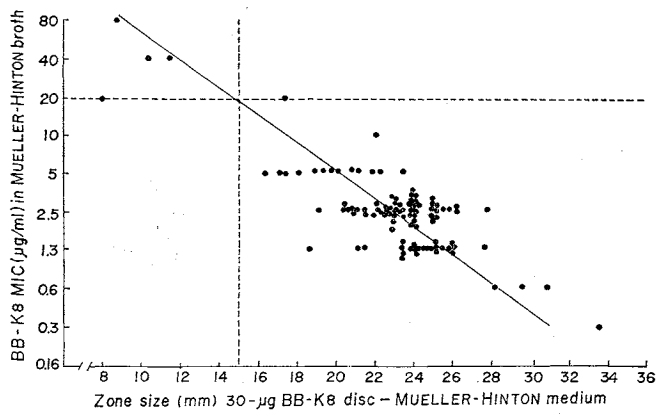
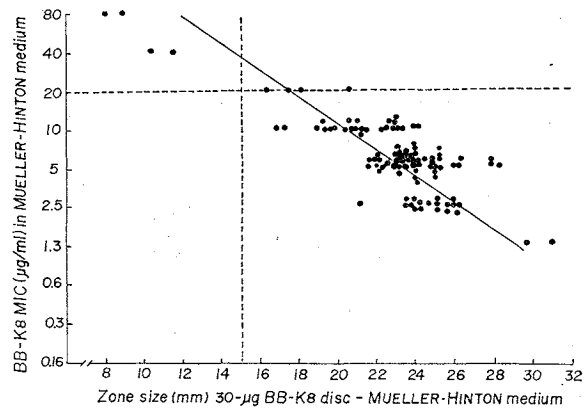


Fig. 6. Relationship between zone size and agar dilution MIC values of BB-K 8 for 103 strains of *Pseudomonas* species.



Examination of Fig. 5, where BB-K 8 tube dilution MIC values were plotted *versus* zone sizes produced by 30 μg BB-K 8 discs, shows that there are 2 distinct populations of *Pseudomonas* strains within the group of 104 tested. One population containing 4 strains had zone sizes of 12 mm or less with all but one of them giving MIC values in excess of 20 μg/ml. The other group of 100 strains had zone diameters ranging from 16 to 34 mm and MIC values of 20 μg/ml or less. Analysis of the relationship of zones sizes to tube dilution MIC values revealed that the correlation coefficient (−0.836) is sufficiently high to remove all doubt that there is indeed a true relationship (P=<0.001) between results obtained in the 2 systems against this population of pseudomonads.

This finding contrasts rather sharply with results reported for *P. aeruginosa* strains by TRAUB[60] and WEINSTEIN *et al.*[67] who found in tests with gentamicin that there was a rather poor correlation between tube dilution MIC values and agar diffusion zone sizes.

The relationship between BB-K 8 MIC values obtained by an agar dilution test (STEERS) and zone sizes was also examined (Fig. 6). Here, it can be seen that the 4 strains designated as resistant by virtue of the fact that their MIC values were in excess of 20 μg/ml, all produced zone sizes of <12 mm. The 99 sensitive strains tested (MIC of ≤20 μg/ml) had zone sizes ranging from 16 to 32 mm. The clearcut

relationship observed between these 2 test systems resulted in a correlation coefficient of -0.845 ($P=<0.001$).

The slope of the regression line of zone sizes to tube dilution MIC did not differ significantly from that found for zone sizes to agar dilution MIC, although MIC values obtained in the tube dilution test were approximately 2-fold lower than those found by the agar dilution method. The greater activity of BB-K 8 in MUELLER-HINTON Broth as compared to MUELLER-HINTON Medium has also been noted for gentamicin, presumably as a result of the higher Mg^{++} content contributed to the latter medium by the presence of agar[23].

On the basis of the above results, the suggested zone diameter for separation of BB-K 8-susceptible from BB-K 8-resistant pseudomonads when 30-μg discs are utilized is 15 mm. It should be pointed out, however, that continuing studies indicate that 10-μg discs may also be satisfatory for evaluating the susceptibility of *Pseudomonas* strains if one uses a 13 mm cut-off point for susceptibility. In addition, the lower concentration discs can be utilized for *Enterobacteriaceae* species with the recommended susceptibility cut-off point also being 13 mm.

The absorbability of BB-K 8 following intramuscular (IM) administration to mice has been compared with that of kanamycin and gentamicin. In the first experiment, each of the compounds was administered by the IM route to 8 mice. As all animals were bled at each of the designated time periods, each value shown in Table 9 is the average of 8 individual determinations.

These data show that all 3 aminoglycosides are well absorbed in the mouse after IM adminstration. The maximum serum level of BB-K 8 achieved at the 10 mg/kg dose was slightly higher than kanamycin's, a finding which is in agreement with the observations of KAWAGUCHI *et al.*[30] The concentration of gentamicin in serum at 15 minutes post-injecton was found to be still higher than that of BB-K 8. However, blood levels of all 3 drugs appeared to decline from the maximum concentration at virtually the same rate.

A pair of dose-response experiments was conducted in an effort to determine the dosages of the aminoglycosides that produce peak blood concentrations in mice equivalent to those considered to be the maximum acceptable ones in man. These peaks as previously noted are 8 μg/ml for gentamicin and 20 μg/ml for kanamycin and BB-K 8 with the estimate for the last compound having been extrapolated from animal toxicity[27] and human absorption[12] data.

Fig. 7 shows that the IM dose of BB-K 8 giving an antibiotic blood concentration

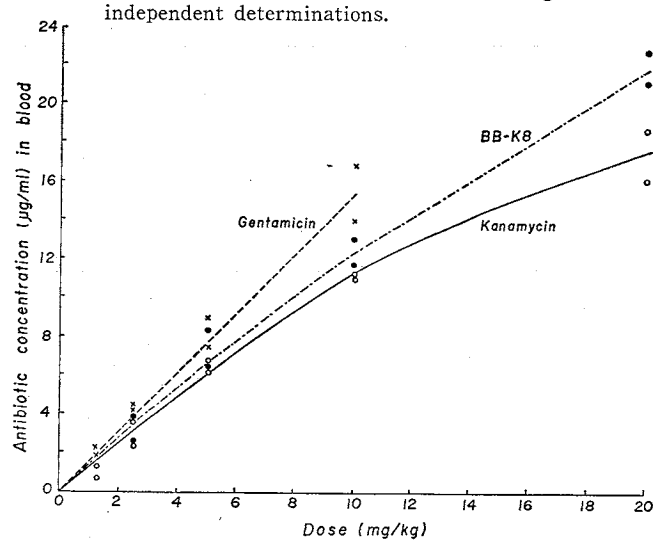
Table 9. Average blood level values found for BB-K 8, kanamycin and gentamicin at various time periods following intramuscular administration of a single 10 mg/kg dose

Antibiotic	Average blood levels in μg/ml found at indicated time in hours post-administration			
	0.25 hour	0.5 hour	1 hour	1.5 hours
BB-K 8	12.0(10.0~13.4)*	11.6(9.5~14.1)	3.1(2.3~4.1)	1.3(0.4~3.9)
Kanamycin	11.2(10.0~12.4)	7.5(6.7~8.4)	2.6(1.7~4.1)	0.6(0.2~1.9)
Gentamicin	16.7(12.9~21.5)	11.8(8.9~15.6)	4.7(2.9~7.7)	1.6(1.2~2.2)

* Figures in parentheses represent 95% confidence limits of the average value found for 8 mice.

of 20 μg/ml at 15 minutes post-administration (presumed to be the time where the peak serum concentration occurs) is about 19 mg/kg. The plot for kanamycin shows that its peak levels were somewhat lower than those of BB-K 8 at the higher doses, and suggests (by extrapolation) that a 24-mg/kg dose would give a peak blood level of 20 μg/ml. Gentamicin, with still higher peak levels, reached its maximum acceptable average blood concentration of 8 μg/ml following a 5- to 6-mg/kg dose. The estimated peak concentration achieved in mouse blood with a 5 mg/kg gentamicin dose in the present study corresponds precisely with the value previously found for this particular dose[65], while the peak levels of kanamycin and BB-K 8 obtained after administration of a 20 mg/kg dose were somewhat higher than those reported by other investigators[30,70].

Fig. 7. Antibiotic levels in mouse blood 15 minutes after intramuscular administration of the indicated dose of BB-K 8, kanamycin or gentamicin. The value represented by each symbol is the average of 8 independent determinations.



If one can extrapolate from results obtained in tests utilizing human serum, it is likely that high percentages of all 3 aminoglycosidic antibiotics are present in mouse blood in unbound form. Laboratory studies employing the ultrafiltration method described by BUCK *et al.*[10] indicate that the extent of binding of BB-K 8 to human serum is 20 % or less. This value is consistent with the low degree of binding (about 30 %) reported for gentamicin[7,11], and with the virtual lack of binding found for kanamycin by SCHOLTAN and SCHMID[51].

The excellent absorbability demonstrated for all 3 antibiotics in mice was also observed in rat studies where 2 separate experiments were conducted to determine the percentages of BB-K 8, kanamycin, and gentamicin recoverable in urine following administration of single 10 mg/kg IM doses. Results are summarized in Table 10.

These data show that the percentages of BB-K 8 and kanamycin excreted into the urine following their IM administration to rats are quite high as has been reported to be the case when they were administered to this species by the subcutaneous route[30]. Recovery of gentamicin after a 10 mg/kg IM dose was found to be lower than that of BB-K 8 and kanamycin, although all 3 antibiotics behave similarly in that the bulk of each was present in the 0 to 6-hour specimens. The finding that the total percentage recovery of gentamicin was significantly lower ($P=0.05$) than that of the other 2 compounds is somewhat in contrast to what one might have expected on the basis of mouse blood level results where gentamicin gave the highest peak values. No explanation is available at present to account for this observation.

Table 10. Percentage recovery of BB-K 8, kanamycin and gentamicin from rat urine following IM administration of a single 10 mg/kg dose of each

Antibiotic	Experiment number	Average percentage of antibiotic recovered during indicated time interval in hours post-administration		
		0～6 hr.	6～24 hr.	Total (0～24 hr.)
BB-K 8	1	59.0(52.1～72.0)*	1.8(0.8～3.0)	60.8(52.9～73.5)
	2	51.2(36.2～59.3)	3.9(2.8～5.0)	55.1(41.2～62.1)
	Average	55.1	2.9	58.0
Kanamycin	1	53.1(39.0～62.5)	2.7(0.7～4.5)	58.8(43.5～64.7)
	2	66.7(46.6～81.1)	2.6(1.9～3.3)	69.2(49.4～84.3)
	Average	59.9	2.7	64.0
Gentamicin	1	43.8(38.5～54.9)	3.1(0.7～5.2)	46.9(42.5～58.4)
	2	41.1(34.0～55.3)	3.4(1.3～7.0)	44.5(41.0～56.6)
	Average	42.5	3.3	45.7**

* Figures in parentheses represent 95% confidence limits of the average value found for 5 rats.
** The average recovery value for gentamicin is significantly lower($P=0.05$)than those of BB-K 8 and kanamycin as determined by analysis of variance.

Urine specimens were examined in a thin-layer chromatography study in which plates were developed in a solvent mixture of $CH_3OH - NH_4OH - CHCl_3 - H_2O$ (4 : 2 : 1 : 1), then dried, and overlayed with *B. subtilis* ATCC 6633-seeded Streptomycin Assay Agar (Difco). After incubation overnight at 37°C, examination of the plates revealed that the only bioactive substances present were the parent compounds. Average concentrations of the antibiotics in the 0 to 6-hour urine samples were similar, with 127, 151, and 134 μg/ml, respectively, being found for BB-K 8, kanamycin and gentamicin.

The therapeutic effectiveness of BB-K 8 in experimental infections of mice was compared with that of kanamycin and gentamicin. Organisms used in the *in vivo* studies were divided on the basis of their *in vitro* antibiotic susceptibilities into the following 4 categories: sensitive to all 3 antibiotics, resistant to kanamycin only, resistant to gentamicin only, and resistant to both kanamycin and gentamicin. All strains were considered to be susceptible to BB-K 8. The susceptibility end-points used were the same as those previously employed, *i.e.*, organisms inhibited by 20 μg/ml or less of BB-K 8 or kanamycin were designated as sensitive to these antibiotics, while those organisms inhibited by 8 μg/ml or less were considered to be susceptible to gentamicin. Results are summarized in Table 11.

The first group of organisms was susceptible to all 3 of the antibiotics. The experimental infections produced in mice by these microorganisms responded in near identical fashion to BB-K 8 and kanamycin therapy. There was only one infection in this first group that was not controlled at doses well below those capable of producing peak antibiotic concentrations of 20 μg/ml in mouse blood. The exception was the *S. marcescens* A 20019 infection where PD_{50} values were at least 2-fold in excess of the BB-K 8 (19 mg/kg) and kanamycin (24 mg/kg) doses required to give the critical blood level peak. This infection must therefore be considered therapeutically unresponsive to both antibiotics.

Gentamicin, with the exception of its remarkable effectiveness in the *S. aureus* A 9537 infection, was found to induce a therapeutic response pattern similar to that

Table 11. Therapeutic efficacy of kanamycin, gentamicin and BB-K 8 in experimental infections of mice

Organism	Displays *in vitro* resistance to:	PD_{50}* in mg/kg/treatment		
		Kanamycin	Gentamicin	BB-K 8
Staphylococcus aureus A 9537	—	2	0.1	3
S. aureus A 9606	—	2	1	2
Klebsiella pneumoniae A 9977	—	4	1	4
Proteus mirabilis A 9900	—	6	2	7
Proteus morganii A 15153	—	3	2	3
Escherichia coli A 15119	—	6	0.4	3
Serratia marcescens A 20019	—	50	13	46
K. pneumoniae A 20328	Kanamycin	>200	0.8	3
E. coli A 20520	Kanamycin	240	2	4
E. coli A 20682	Kanamycin	200	0.2	1
Enterobacter sp. A 20364	Kanamycin	>400	5	19
Pseudomonas aeruginosa A 9843	Kanamycin	70	15	8
S. marcescens A 20460	Kanamycin	>200	2	4
Providencia sp. A 20615	Gentamicin	5	26	10
P. aeruginosa A 20717	Kanamycin & gentamicin	160 110***	75 44***	16 11***
K. pneumoniae A 20680	Kanamycin & gentamicin	>400	14	10

* Organisms for which kanamycin and BB-K 8 have MIC values of >20 μg/ml are considered resistant, while those with gentamicin MIC value of >8 μg/ml are considered resistant.
** PD_{50} is the dose in mg/kg/treatment that protected 50% of the mice. Mice were treated subcutaneously at both 1 and 4 hours post-challenge.
*** In this experiment, treatments were administered SC at 0, 2, 4, and 6 hours postchallenge.

of BB-K 8 and kanamycin. However, its overall degree of activity against this series of organisms was about 4 times greater than that of the other antibiotics. As was the case with BB-K 8 and kanamycin, gentamicin failed to protect mice infected with *S marcescens* A 20019 at a therapeutically acceptable dosage. Its PD_{50} of 13 mg/kg/treatment was at least 2 times higher than the 5 to 6 mg/kg dosage previously shown (Fig. 7) to produce an antibiotic blood level peak of 8 μg/ml, the average peak concentration achievable in man after administration of the maximum recommended dose.

The failure of all 3 aminoglycosidic antibiotics to control infections produced by the presumably susceptible strain of *S. marcescens* was possibly attributable to the heavy organism challenge utilized in the experimental model.

Generally speaking, PD_{50} values obtained for gentamicin and kanamycin in this study against susceptible strains of *S. aureus*, *E. coli*, *Klebsiella*, and *Proteus* sp. were in excellent agreement with those reported by other investigators[28,65,70].

The second group of organisms evaluated in mouse protection tests had all demonstrated *in vitro* resistance to kanamycin, but were susceptible to BB-K 8 and gentamicin. In general, the *in vitro* test results accurately predicted *in vivo* efficacy since all infections were refractory to kanamycin treatment and fully responsive to therapy with BB-K 8. Mice infected with species from the *Enterobacteriaceae* family were uniformly responsive to gentamicin therapy at doses approximately one-third those required for BB-K 8. On the other hand, the infection produced by *P. aeruginosa* strain A 9843 failed to respond to therapeutically acceptable doses of gentamicin. This

finding contrasts with results reported by other investigators[65,70] who have reported that infections caused by gentamicin-sensitive *P. aeruginosa* strains were several-fold more responsive to gentamicin than was the present *P. aeruginosa* A 9843-induced infection.

The third group contained only one organism, a strain of *Providencia* sp. resistant to gentamicin but sensitive to BB-K 8 and kanamycin. *In vivo* test results were in complete agreement with the predictions given by *in vitro* susceptibility tests.

The 2 organisms in the last group displayed *in vitro* resistance to both kanamycin and gentamicin, but were susceptible to BB-K 8. Mice infected with *P. aeruginosa* A 20717 were placed on both 2- and 4-dose treatment schedules. Although each compound was more effecive in terms of the size of the individual dose required when the 4-dose regimen was used, only BB-K 8 protected infected mice at doses likely to be devoid of potential toxic effects. This was true for both 2- and 4-treatment schedules. In contrast, even with the 4-treatment regimen, the individual kanamycin and gentamicin doses required for protection were 4 to 6 times higher than those considered to be safe. The infection produced by the final organism listed, *K. pneumoniae* A 20680, responded to BB-K 8 but not to gentamicin or kanamycin. These results along with those obtained in the previously-discussed *in vivo* tests, indicate that MIC values in MUELLER-HINTON Medium of 20 μg/ml for BB-K 8 and kanamycin and 8 μg/ml for gentamicin were good choices as susceptibility cut-off points. Overall, *in vitro* predictions of therapeutic response were accurate for about 94 % of the experimental infections.

The data in Table 11 clearly demonstrate that BB-K 8 was efficacious not only in infections caused by bacterial strains susceptible to other aminoglycosidic antibiotics, but also in those caused by certain kanamycin- and gentamicin-resistant microorganisms. BB-K 8 would therefore appear to have great potential as a replacement drug for kanamycin since the percentage of strains currently found resistant to the latter agent in a given hospital environment may be as high as 20~35 % among *Escherichia*[40], *Serratia*[45,71], *Klebsiella-Enterobacter*[21], indol-positive *Proteus*[2], and *Alcaligenes*[66] species. The same situation may ultimately prevail with gentamicin, although thus far gentamicin-resistant strains have been found in significant numbers only among species of *Providencia*[4,13,31,33,68,72], *Alcaligenes*[55], *Klebsiella*[72], and *Pseudomonas*[4,13,52,55,72]. Nevertheless, in view of the many recent reports[13,29,39,42,54,72] which describe the isolation of clinically-important strains of bacteria possessing transferable R-factors that mediate gentamicin resistance, there is a strong suggestion that continued usage of this antibiotic will exert selective pressure of sufficient intensity to cause a sharp rise in the incidence of infections caused by such resistant strains.

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