FORTIMICINS A AND B, NEW AMINOGLYCOSIDE ANTIBIOTICS

IV. *IN VITRO* STUDY OF FORTIMICIN A COMPARED WITH OTHER AMINOGLYCOSIDES

ROLAND L. GIROLAMI and JOHN M. STAMM

Abbott Laboratories, Division of Antibiotics and Natural Products
North Chicago, Illinois 60064, U.S.A.

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The *in vitro* antimicrobial activity of fortimicin A, the most active member of the fortimicin complex, was compared with that of amikacin, gentamicin, sagamicin and tobramycin against 352 strains of *Enterobacteriaceae* and other medically significant organisms. Against most of these organisms fortimicin and amikacin had comparable levels of antimicrobial activity, generally slightly less than that of gentamicin, sagamicin or tobramycin. Fortimicin had relatively weak activity against *Pseudomonas aeruginosa* strains. Fortimicin shows many of the characteristics of other aminoglycoside antibiotics: (i) improved activity at alkaline pH, (ii) rapid, bactericidal action, (iii) reduced activity with increasing inoculum levels, and (iv) synergistic activity with penicillin against enterococci. The activity of fortimicin was compared to that of gentamicin, tobramycin and amikacin against a group of 95 naturally occurring, antibiotic-resistant Gram-negative bacilli other than *Pseudomonas*. The organisms were isolated from clinical sources and selected primarily for gentamicin resistance by the sensitivity disc test. Fortimicin showed excellent activity against this group of organisms. At a concentration of 6.2 mcg/ml, fortimicin inhibited the most strains (92.6%) followed by amikacin (90.5%), gentamicin (23.2%) and tobramycin (8.4%).

Fortimicin A, the most active member of the fortimicin complex described thus far, is a new potent, broad-spectrum antibiotic of the aminoglycoside type.[1,2] This study was done in order to expand on the previous *in vitro* observations and to compare and contrast the properties of fortimicin A with clinically useful aminoglycosides.

Materials and Methods

MIC Determination

The minimum inhibitory concentration (MIC) of the antibiotics was determined by the agar dilution method,[3] using the inocula replicating device of STEERS *et al.*[4] Inoculum was adjusted so as to deposit approximately 10^4 CFU (colony forming units) per point of application. MUELLER-HINTON agar, pH 7.4, was used for most determinations. Whole or chocolatized sheep blood, 5%, was added for tests of *Streptococcus* and *Haemophilus* respectively. GC Agar Base plus supplement B (Difco) was used for *Neisseria*. *Haemophilus* and *Neisseria* were incubated in 5% CO_2.

One or more of the following organisms was included in every evaluation of sensitivity as a procedure control: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853.

MBC Determination

The minimum bactericidal concentration (MBC) of the antibiotics was determined by the broth dilution method[3] using MUELLER-HINTON Broth, pH 7.4, with an inoculum level of 10^5 CFU/ml. The MBC was the lowest concentration of antibiotic which resulted in a minimum 99.9% reduction in initial microbial count after 24 hours incubation.

Antibiotics

The antibiotics used were: amikacin base (Bristol Laboratories), tobramycin base (Eli Lilly & Co.), gentamicin sulfate (Schering Corp.), sagamicin sulfate (Abbott Laboratories) and fortimicin A sulfate (Abbott Laboratories). All concentrations are expressed in terms of free base. All references to fortimicin in this paper mean fortimicin A.

Microorganisms

Most of the organisms used in these studies were recent, random isolates from clinical material and were obtained from several hospital and public health laboratories.

The 95 aminoglycoside-resistant strains were obtained from 4 hospital laboratories. Included were strains of the following: *Escherichia coli*, *Enterobacter* sp., *Klebsiella pneumoniae*, *Serratia* sp., *Citrobacter* sp., *Providencia* sp., *Proteus* sp. and *Acinetobacter*. The majority of these organisms were selected for resistance on the basis of the antibiotic sensitivity disc test, primarily for resistance to gentamicin. *Pseudomonas* strains were not included in the study of antibiotic efficacy against resistant organisms because of the relatively weak activity of fortimicin against most strains of this organism.

Killing Curves

The microorganisms were grown in MUELLER-HINTON broth to a concentration of approximately 5×10^7 CFU/ml. An appropriate dilution of the culture was made in MUELLER-HINTON broth containing the desired amount of the antibiotic. The culture suspensions were held at 37°C and sampled at 0 and after 1, 2 and 4 hours incubation. The number of colony forming units (CFU) was determined by standard pour plate technique using soybean-casein digest agar, with incubation at 37°C for 18~22 hours.

Results

The *in vitro* activity of fortimicin compared to that of amikacin, gentamicin, sagamicin and tobramycin against a spectrum of 352 organisms is given in Table 1.

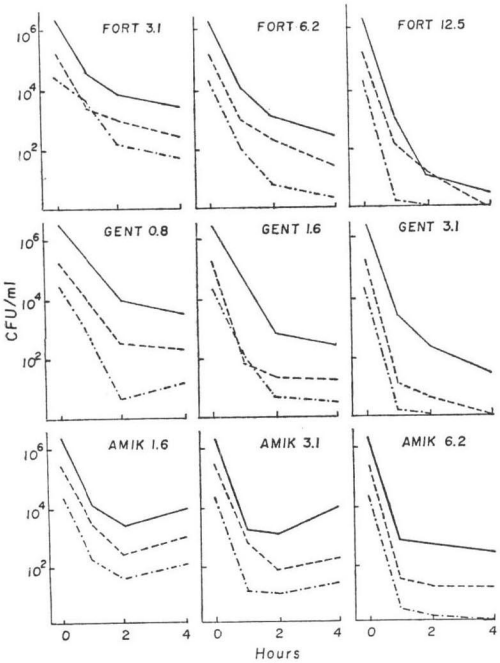
All five antibiotics had a high degree of activity against most species of *Enterobacteriaceae* and *S. aureus*. The activity of these antibiotics against strains of *Streptococcus, Neisseria* and *Haemophilus* was generally weak by comparison with agents currently in clinical use, *e.g.*, penicillin.

Table 1. Comparative *in vitro* activity of five aminoglycoside antibiotics against 352 strains of various bacteria as determined by the agar dilution method.*

Organism	No. of strains	Minimum inhibitory concentration (mcg/ml) obtained with:				
		Fortimicin	Amikacin	Gentamicin	Sagamicin	Tobramycin
Escherichia coli	58	3.1	3.1	1.6	1.6	1.6
Klebsiella pneumoniae	33	3.1	1.6	0.8	0.8	0.8
Proteus mirabilis	30	6.2	3.1	1.6	1.6	0.8
Proteus (indole+)	27	12.5	12.5	6.2	3.1	3.1
Enterobacter sp.	20	3.1	3.1	0.8	1.6	1.6
Serratia sp.	23	6.2	12.5	6.2	12.5	50
Salmonella sp.	19	3.1	3.1	1.6	1.6	1.6
Shigella sp.	19	6.2	12.5	3.1	3.1	3.1
Providencia sp.	11	6.2	12.5	>100	>100	100
Citrobacter sp.	8	6.2	3.1	1.6	1.6	1.6
Pseudomonas aeruginosa	44	100	12.5	6.2	6.2	1.6
Staphylococcus aureus	11	0.8	0.8	0.2	0.2	0.2
Streptococcus pyogenes	20	12.5	—	3.1	3.1	—
Neisseria gonorrhoeae	18	12.5	—	12.5	12.5	—
Haemophilus influenzae	11	3.1	—	3.1	3.1	—

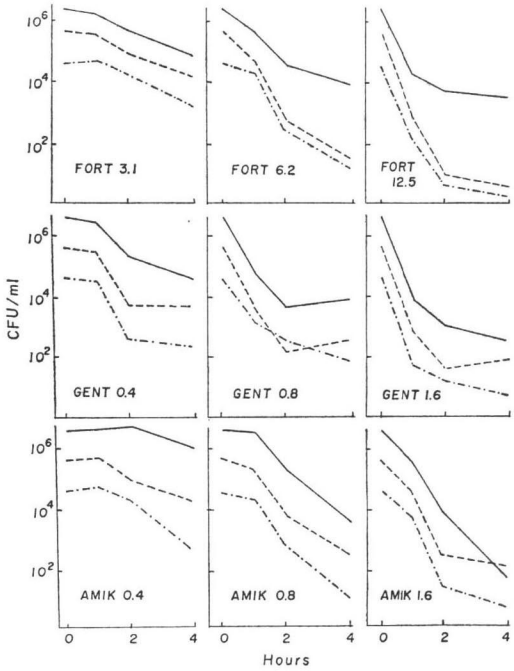
* Minimum inhibitory concentration (MIC) is the lowest concentration of antibiotic required to inhibit approximately 90% or more of the strains of each species of organism.

Fig. 1. The comparative rate of kill of *Escherichia coli* ATCC 25922 by fortimicin (FORT.), gentamicin (GENT.) and amikacin (AMIK.) at 1, 2 and 4 times the minimum inhibitory concentration (MIC)* at three inoculum levels.



* MIC determined by the broth dilution method in MUELLER-HINTON broth, inoculum level 10^5 CFU/ml.

Fig. 2. The comparative rate of kill of *Pseudomonas aeruginosa* U566-1 by fortimicin (FORT.), gentamicin (GENT.) and amikacin (AMIK.) at 1, 2 and 4 times the minimum inhibitory concentration (MIC)* at three inoculum levels.



* MIC determined by the broth dilution method in MUELLER-HINTON broth, inoculum level 10^5 CFU/ml.

Against most species in this spectrum, gentamicin, sagamicin and tobramycin were slightly more active on a weight basis than were fortimicin or amikacin. Tobramycin was more effective against *Pseudomonas* but was less active against *Serratia* than the other antibiotics. Fortimicin and amikacin were considerably more active against *Providencia* than the other agents.

Fortimicin is a bactericidal antibiotic as demonstrated by the data in Table 2. These data show that the MIC and MBC endpoints for a group of 8 organisms are essentially the same for either of the two levels of inoculum used.

The rates at which sensitive organisms are killed by fortimicin, gentamicin and amikacin are shown in Fig. 1 for *E. coli* ATCC 25922 and in Fig. 2 for a clinical isolate of *P. aeruginosa*, strain U566-1. The effect of each anti-

Table 2. Comparison of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of fortimicin at two inoculum levels.*

Organism		Inoculum level (CFU/ml)			
		10^5		10^7	
		MIC	MBC	MIC	MBC
Escherichia coli	4041	3.1	3.1	6.2	6.2
Escherichia coli	C-1	3.1	3.1	6.2	6.2
Klebsiella pneumoniae	52809	0.8	0.8	3.1	3.1
Enterobacter cloacae	28	3.1	3.1	6.2	6.2
Serratia sp.	23	3.1	3.1	12.5	12.5
Proteus mirabilis	C-39	1.6	6.2	12.5	12.5
Proteus morganii	49822	3.1	3.1	6.2	6.2
Providencia sp.	444-5	12.5	25	25	25

* MIC values (mcg/ml) were determined by the twofold broth dilution method in MUELLER-HINTON broth. The MBC was the lowest antibiotic concentration causing a minimum 99.9% reduction in microbial count after 24 hours incubation.

biotic at 1, 2 and 4 times the MIC concentration was determined at 3 levels of inoculum. Fortimicin, gentamicin and amikacin all show prompt and rapid, lethal activity against both microorganisms under most test conditions. The parallelism of the curves for the 3 inoculum levels for each test condition indicates that the rate of kill is independent of the cell concentration, at least over this 2 $\log_{10}$ range in count. In addition, it can be seen that the rate of kill in the early stages of incubation becomes more rapid as the concentration of each antibiotic is increased.

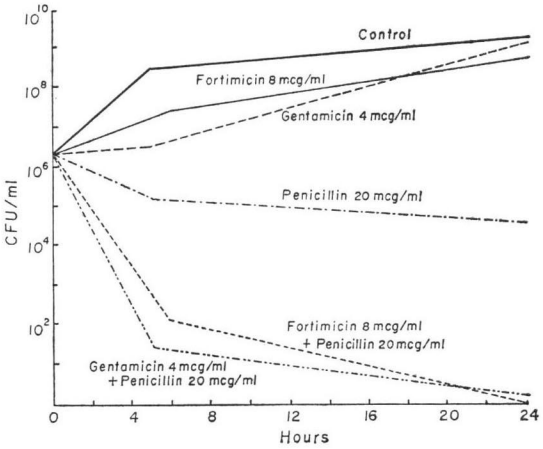
The shape of many of the killing curves shows an interesting pattern. After an initial, almost logarithmic reduction in count for the first 1 to 2 hours the rate of kill levels off, and in a few cases the viable cell count increases slightly. These data suggest that the population is heterogeneous with regard to antibiotic sensitivity. The effect of sublethal concentrations of fortimicin or gentamicin in

Table 3. Effect of inoculum size on the antimicrobial activity of fortimicin and gentamicin.*

Organism	Inoculum size (No. CFU/ml)	Minimum inhibitory concentration (mcg/ml) obtained with:	
		Fortimicin	Gentamicin
Escherichia coli ATCC 25922	10^3	0.8	0.2
	10^5	1.6	0.8
	10^7	6.2	—
Klebsiella pneumoniae 11339	10^3	0.4	0.1
	10^5	0.8	0.2
	10^7	3.1	0.4
Proteus mirabilis 48575	10^3	3.1	0.4
	10^5	6.2	0.8
	10^7	12.5	1.6
Proteus rettgeri 7566	10^3	0.4	0.2
	10^5	3.1	0.8
	10^7	—	1.6
Serratia sp. 23	10^3	0.8	0.8
	10^5	3.1	1.6
	10^7	6.2	3.1
Enterobacter cloacae 28	10^3	0.8	0.1
	10^5	3.1	0.2
	10^7	6.2	0.8
Pseudomonas aeruginosa ATCC 27853	10^3	6.2	0.4
	10^5	12.5	0.4
	10^7	25	0.8

* The minimum inhibitory concentrations (MIC) were determined by the broth dilution method in MUELLER-HINTON broth. An overnight culture was diluted to yield inoculum sizes as indicated.

Fig. 3. The synergistic effect of fortimicin-penicillin and gentamicin-penicillin compared with the effect of fortimicin, gentamicin and penicillin alone against a representative strain of enterococcus 93.



Tests for synergism were performed in tubes of MUELLER-HINTON broth containing the appropriate antibiotic concentration. Tubes were inoculated with a dilution of a overnight broth culture so as to give a concentration of $2\sim3\times10^6$ CFU/ml.

Table 4. Effect of pH on the antimicrobial activity of fortimicin.*

Organism	Minimum inhibitory concentration (mcg/ml) at pH of:		
	6.4	7.4	8.4
Escherichia coli ATCC 25922	6.2	1.6	1.6
Klebsiella pneumoniae C31	6.2	3.1	1.6
Proteus mirabilis 48575	12.5	6.2	3.1
Pseudomonas aeruginosa ATCC 27853	50	25	6.2
Proteus rettgeri 7566	3.1	0.8	0.8
Enterobacter cloacae 28	12.5	1.6	1.6
Staphylococcus aureus ATCC 25923	1.6	0.8	0.8

* The minimum inhibitory concentrations (MIC) were determined by the agar dilution method on MUELLER-HINTON agar adjusted to pH 6.4, 7.4 or 8.4.

combination with penicillin against a representative strain of enterococcus was examined by the killing curve technique. These results are shown in Fig. 3.

Both the fortimicin-penicillin and the gentamicin-penicillin combinations show marked synergistic activity as demonstrated by the substantial potentiation of bactericidal action by the combinations in contrast to the action of the antibiotics individually. Essentially similar results were obtained with 3 other strains of enterococci.

Table 5. Distribution of 95 resistant organisms according to antibiotic sensitivity.

Antibiotic	No. of strains susceptible to (mcg/ml)*			
	≤6.2	12.5	25	≥50
Fortimicin	88	2	5	0
Amikacin	86	4	5	0
Gentamicin	22	22	24	27
Tobramycin	8	27	18	42

* Minimum inhibitory concentration (MIC) by agar dilution
Species were distributed as follows: *Escherichia coli* (6), *Enterobacter* sp. (7), *Klebsiella pneumoniae* (28), *Serratia* sp. (35), *Citrobacter* sp. (3), *Providencia* sp. (10), *Proteus* sp. (2) and *Acinetobacter* (4).

Table 3 summarizes the effect of inoculum levels on the MIC's obtained for a group of 8 organisms. Generally, an increase of 100-fold in inoculum resulted in a two-fold increase in the MIC for both fortimicin and gentamicin. Similar results have been obtained for amikacin[5] and tobramycin[6].

Fortimicin is most active at alkaline pH. Table 4 shows the 2~4 fold reduction in MIC for all seven organisms examined with a change from pH 6.4 to 7.4. A further reduction in MIC was observed for only 3 of the test organisms as the pH was raised to 8.4. Other aminoglycosides also show maximum activity at alkaline pH[5,7,8].

The activity of fortimicin compared to that of gentamicin, tobramycin and amikacin against a group of 95 Gram-negative bacilli other than *Pseudomonas* is given in Table 5. The organisms were isolated from clinical sources and selected primarily for gentamicin resistance by the sensitivity disc test. Fortimicin and amikacin were highly active against this group of organisms. The distribution of strains susceptible to 6.2 mcg/ml or less of each antibiotic is as follows: fortimicin 88 (92.6%), amikacin 86 (90.5%), gentamicin 22 (23.2%) and tobramycin 8 (8.4%). There was no information for any of these strains regarding the presence or absence of aminoglycoside inactivating enzymes.

Discussion

The spectrum and levels of activity reported here for the four 2-deoxystreptamine containing antibiotics are in general agreement with published reports on these agents[5,7,9~12]. Gentamicin, sagamicin and tobramycin had very similar levels of activity, with tobramycin showing the most *Pseudomonas* activity and the least *Serratia* activity. Against other organisms differences were minor.

Fortimicin and amikacin resembled one another in spectrum and level of activity. *P. aeruginosa* was the major exception with amikacin showing good activity and fortimicin comparatively weak intrinsic activity against this species; however, a number of strains were sensitive to low levels of fortimicin (see strain U566-1, Fig. 2).

The killing curve technique was used in an attempt to compare the lethality of fortimicin, gentamicin and amikacin at equivalent levels of antibiotic activity by using multiples of the MIC. It is recognized that this value is quite imprecise and can be profoundly influenced by levels of inoculum, pH and medium composition. Thus, to compare or rank the lethality of these agents on the basis of these data is somewhat tenuous. There is a suggestion in these data, however, that amikacin is less rapidly lethal than fortimicin or gentamicin against *P. aeruginosa* U556-1 and against *E. coli* ATCC 25922, especially during the first two hours of exposure. All three antibiotics show greater efficacy

against *E. coli* than against *P. aeruginosa*. Because only one strain of each species was examined it cannot be assumed that this is a general phenomenon. However, YOUNG and HEWITT[8] also found that gentamicin and amikacin killed *E. coli* more rapidly than *P. aeruginosa* (2 strains of each).

The heterogeneity of the microbial population with respect to antibiotic sensitivity suggested by the slope of the kill curves was not unexpected based on reports with other organisms. WILSON and SANDERS[18] studied a number of *S. aureus* strains by means of killing curves and reported the presence of a small subpopulation of cells more resistant to aminoglycosides than the parent strains. The frequency of resistance suggested spontaneous mutation.

The relative ease with which cultures can be selected *in vitro* for resistance to aminoglycosides such as gentamicin is well known[14] and the observation of heterogeneity in both the *E. coli* and *Pseudomonas* strains to all three aminoglycosides is consistent with that property. Although this type of antibiotic resistance is of doubtful clinical significance,[14,15] it is of interest in evaluating different members of this class of antimicrobial agent.

There are many reports of synergism between aminoglycosides and beta-lactam antibiotics against enterococci[5,16,17] and against some strains of Gram-negative bacilli[18,19]. The mechanism by which these two classes of antibiotics act synergistically probably involves some action by the beta-lactam on the microbial cell wall which allows the aminoglycoside improved access to specific targets.

The results of the survey of antibiotic effectiveness against a variety of organisms with naturally occurring resistance to other aminoglycosides show that fortimicin has an outstanding degree of activity. The close, parallel activity of fortimicin and amikacin against this group of organisms is noteworthy.

It was anticipated that the majority of organisms in this group would be resistant to gentamicin because most, although not all, strains were selected for this property. In spite of the exclusion of *Pseudomonas* strains it was somewhat unexpected to find more of the test strains resistant to tobramycin than to gentamicin. Whether this is the result of a skewed microbial population or is instead a reasonably accurate reflection of resistance in the clinical situation has not been established.

A high degree of co-resistance between gentamicin and tobramycin, especially for organisms other than *Pseudomonas*, was reported by PRICE *et al.*[20] who found that the great majority of gentamicin and tobramycin resistant organisms were sensitive to amikacin. Many of the organisms they studied produced aminoglycoside inactivating enzymes for which amikacin proved to be a poor substrate. In the present study we also found amikacin active against most gentamicin and tobramycin resistant organisms. The mechanism of resistance for these strains is unknown but it is probable that some produce inactivating enzymes. The excellent activity of fortimicin against this group may be explained, at least partially, by a unique structure[21], which also makes fortimicin a poor substrate for common inactivating enzymes[1]. The explanation for fortimicin activity against organisms which do not inactivate aminoglycosides will require further study.

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References

1) NARA, T.; M. YAMAMOTO, I. KAWAMOTO, K. TAKAYAMA, R. OKACHI, S. TAKASAWA, T. SATO & S. SATO: Fortimicins A and B, new aminoglycoside antibiotics. I. Producing organism, fermentation and biological properties of fortimicins. J. Antibiotics 30: 533~540, 1977
2) OKACHI, R.; S. TAKASAWA, T. SATO, S. SATO, M. YAMAMOTO, I. KAWAMOTO & T. NARA: Fortimicins A and B, new aminoglycoside antibiotics. II. Isolation, physico-chemical and chromatographic properties. J. Antibiotics 30: 541~551, 1977
3) WASHINGTON, J. A. & A. L. BARRY: Dilution test procedures. *In* Manual of Clinical Microbiology, 2nd Edition, pp. 410~417 (Editors E. H. LENNETTE: E. H. SPAULDING & J. P. TRUANT), American Society for Microbiology, 1974
4) STEERS, E.; E. L. FOLTZ & B. S. GRAVES: An inocula replicating appratus for routine testing of bacterial susceptibility to antibiotics. Antibiot. & Chemoth. 9: 307~311, 1959

5) YU, P. K. W. & J. A. WASHINGTON II: Comparative *in vitro* activity of three aminoglycosidic antibiotics: BB-K8, kanamycin and gentamicin. Antimicr. Agents & Chemoth. 4: 133~139, 1973
6) BRITT, M. R.; R. A. GARIBALDI, J. N. WILFERT & C. B. SMITH: *In vitro* activity of tobramycin and gentamicin. Antimicr. Agents & Chemoth. 2: 236~241, 1972
7) BODEY, G. P. & D. STEWART: *In vitro* studies of BB-K8, a new aminoglycoside antibiotic. Antimicr. Agents & Chemoth. 4: 186~192, 1973
8) YOUNG, L. S. & W. L. HEWITT: Activity of five aminoglycoside antibiotics against gram-negative bacilli and *Staphylococcus aureus*. Antimicr. Agents & Chemoth. 4: 617~625, 1973
9) OKACHI, R.; I. KAWAMOTO, S. TAKASAWA, M. YAMAMOTO, S. SATO, T. SATO & T. NARA: A new antibiotic XK-62-2. I. Isolation, physicochemical and antibacterial properties. J. Antibiotics 27: 793~800, 1974
10) KARNEY, W.; K. K. HOLMES & M. TURCK: Comparison of five aminocyclitol antibiotics *in vitro* against *Enterobacteriaceae* and *Pseudomonas*. Antimicr. Agents & Chemoth. 3: 338~342, 1973
11) REYNOLDS, A. V.; J. M. T. HAMILTON-MILLER & W. BRUMFITT: Newer aminoglycoside—amikacin and tobramycin: An *in vitro* comparison with kanamycin and gentamicin. Br. Med. J. 1974- 3: 778~780, 1974
12) WAITZ, J. A.; E. L. MOSS, Jr., C. G. DRUBE & M. J. WEINSTEIN: Comparative activity of sisomicin, gentamicin, kanamycin and tobramycin. Antimicr. Agents & Chemoth. 2: 431~437, 1972
13) WILSON, S. G. & C. C. SANDERS: Selection and characterization of strains of *Staphylococcus aureus* displaying unusual resistance to aminoglycosides. Antimicr. Agents & Chemoth. 10: 519~525, 1976
14) WEINSTEIN, M. J.; C. G. DRUBE, E. L. MOSS, Jr. & J. A. WAITZ: Microbiologic studies related to bacterial resistance to gentamicin. J. Infect. Dis. 124 (Suppl.): 11~17, 1971
15) MITSUHASHI, S: Epidemiology and genetics of aminoglycoside antibiotic resistance in pathogenic bacteria. *In* Drug Action and Drug Resistance in Bacteria. 2. Aminoglycoside Antibiotics. pp. 179~207 (*Ed.* S. MITSUHASHI) University of Tokyo Press, 1975
16) LIBKE, R. D.; C. REGAMEY, J. T. CLARK & W. M. M. KIRBY: Synergism of carbenicillin and gentamicin against enterococci. Antimicr. Agents & Chemoth. 4: 564~568, 1973
17) WATANAKUNAKORN, C.: Penicillin combined with gentamicin or streptomycin: Synergism against enterococci. J. Infect. Dis. 124: 581~586, 1971
18) KLASTERSKY, J.; B. NYAMUBEYA & L. VANDENBORRE: Antimicrobial effectiveness of kanamycin, aminosidin, BB-K8, sisomicin, gentamicin and tobramycin combined with carbenicillin or cephalothin against gram-negative rods. J. Med. Microbiol. 7: 465~472, 1974
19) KLASTERSKY, J.; F. MEUNIER-CARPENTIER, J. M. PREVOST & M. STAQUET: Synergism between amikacin and cefazolin against *Klebsiella*: *In vitro* studies and effect on the bactericidal activity of serum. J. Infect. Dis. 134: 271~276, 1976
20) PRICE, K. E.; T. A. PURSIANO, M. D. DE FURIA & G. E. WRIGHT: Activity of BB-K8 (amikacin) against clinical isolates resistant to one or more aminoglycoside antibiotics. Antimicr. Agents & Chemoth. 5: 143~152, 1974
21) EGAN, R. S.; R. S. STANASZEK, M. CIROVIC, S. L. MUELLER, J. TADANIER, J. R. MARTIN, P. COLLUM, A.W. GOLDSTEIN, R. L. DE VAULT, A. C. SINCLAIR, E. E. FAGER & L. A. MITSCHER: Fortimicins A and B, new aminoglycoside antibiotics. III. Structural identification. J. Antibiotics 30: 552~563, 1977