ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1984, p. 479–482
0066-4804/84/040479-04$02.00/0

Vol. 25, No. 4

Correlation of Antibiotic Resistance with V_{max}/K_m Ratio of Enzymatic Modification of Aminoglycosides by Kanamycin Acetyltransferase

KESAVAN RADIKA† AND DEXTER B. NORTHROP*

Division of Pharmaceutical Biochemistry, University of Wisconsin School of Pharmacy, Madison, Wisconsin 53706

Received 22 August 1983/Accepted 23 January 1984

Kinetic data for the antibiotic-modifying enzyme kanamycin acetyltransferase AAC(6')-IV have been determined for five aminoglycoside antibiotics (amikacin, gentamicin C_{1a}, kanamycin A, sisomicin, and tobramycin) and compared with close-interval determinations of the minimal inhibitory concentrations of the same antibiotics against *Escherichia coli* W677 harboring the resistance plasmid pMH67. These minimal inhibitory concentrations for the resistant bacteria varied from 80 to 800 μg/ml. Of the kinetic parameters V_{max}, K_m, and V_{max}/K_m ratio only V_{max}/K_m ratio had a linear correlation with minimal inhibitory concentrations ($r = +0.818$) at pH 7.8, where all antibiotics produced substrate inhibition, but not at pH 6.0, where they did not. The correlation with only V_{max}/K_m ratio has implications regarding the expression of resistance within the dynamics of the bacterial cell (i.e., antibiotic uptake versus modification), whereas substrate inhibition presents an opportunity to search for new chemotherapeutic agents which will combat resistance directly.

Clinical resistance to aminoglycoside antibiotics is known to be mediated by specific enzymes which acylate, nucleotidylate, or phosphorylate these antibiotics (1). It is not altogether certain that catalytic turnover by the enzymes—and not, say, simple binding or a single turnover—is the cause for an expression of resistance. Davies and Kagan (4) raised this question because: "there are several modified aminoglycosides that retain substantial biological activity; when an R^+ strain is grown in the presence of drug no modified aminoglycoside can be detected free in the culture medium; finally, in a number of instances, bacterial strains are extremely sensitive to a drug even though the strain produces a potent modifying enzyme for the drug!" If, however, catalysis were the initiating action of resistance regardless of subsequent effects, then it seems reasonable that enzyme-mediated resistance should correlate with some aspect of the kinetics of the responsible enzyme.

The first attempt to link resistance to enzyme kinetics was made by Williams and Northrop (12), who compared kinetic parameters determined with homogeneous gentamicin acetyltransferase AAC(3)-I from *Escherichia coli* C600(pJR88) to literature values of minimal inhibitory concentrations (MICs) against the same organism (2). The latter were determined by the traditional method of serial twofold dilutions of antibiotics; hence, any link between kinetics and resistance would at best be qualitative. Nevertheless, an antibiotic which bound tightly to the enzyme as an inhibitor did not have an elevated MIC, and therefore it appeared that catalysis was at least a minimal requirement for resistance. Comparing antibiotics which displayed differences in MICs of greater than 10-fold, one pair had a negligible difference in V_{max} and one other pair had a negligible difference in K_m. But these same antibiotics differed more than 10-fold with respect to the V_{max}/K_m ratio (V/K). Williams and Northrop concluded that neither V_{max} nor K_m was the kinetic determinant of resistance, but V/K could be. The data were the absolute minimum on which to draw this conclusion, and thus the correlation could be coincidental. The present work was undertaken to elaborate in both number and precision the data upon which this link is based with the goal of replacing coincidence with direct proportionality. Moreover, this work extends the investigation to yet another enzyme.

MATERIALS AND METHODS

Materials. Sisomicin sulfate (batch SCM-6-N-6) and gentamicin C_{1a} sulfate (batch 7017-119-I) were gifts from J. A. Waitz of Schering Corp.; tobramycin (lot 8JY55) was a gift from M. Gorman of Eli Lilly & Co.; F. Leitner and R. P. Elander, both of Bristol Laboratories, donated kanamycin A sulfate (lot 79F235) and amikacin (lot 75F110), respectively. *E. coli* W677(pMH67) was provided by M. J. Haas of J. Davies' laboratory. Acetylcoenzyme A, tris(hydroxymethyl)aminopropanesulfonic acid (TAPS), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma Chemical Co. Aldrithiol-4 (4,4'-dithiodipyridine) was from Aldrich Chemical Co., Inc. Tryptic soy was from Becton Dickinson and Co.; yeast extract, Bacto-Peptone, and Bacto-Agar were from Difco Laboratories. Kanamycin acetyltransferase AAC(6')-4 was purified 112-fold from *E. coli* W677(pMH67) by a modification (8) of the procedures described by Scarbrough et al. (10).

Growth of bacteria. Liquid medium contained 12 g of Bacto-Peptone, 5 g of yeast extract, 10 g of glycerol, and 100 mmol each of dibasic and monobasic potassium phosphate per liter and was autoclaved for 60 min. Solid medium contained 30 g of tryptic soy in place of Bacto-Peptone plus 15 g of Bacto-Agar and was autoclaved for 30 min. Cultures of *E. coli* W677(pMH67) were maintained on slants of solid medium. These were transferred weekly to plates which were incubated overnight at 37°C. Single colonies of bacteria were selected from the plates and streaked onto new slants. MICs were determined in the liquid medium containing

* Corresponding author.
† Present address: Department of Obstetrics and Gynaecology, The University of Western Ontario, London, Ontario, Canada, N6A 5A5.

TABLE 1. MICs

Antibiotic	MIC in μg/ml for:	
	E. coli K-12[a]	*E. coli* W677(pMH67)
Sisomicin	0.25	80
Gentamicin C_{1a}	0.5	100
Amikacin	1.0	480
Tobramycin	1.0	640
Kanamycin A	1.0	800

[a] From reference 6.

various amounts of antibiotic. Stock solutions of antibiotics were sterilized by filtration and diluted into 5 ml of medium to achieve a range of concentration bracketing the MIC under study with a 20% difference between consecutive concentrations. The inoculating bacterial cultures were grown for 6 h at 37°C in 50 ml of liquid medium containing no antibiotic. Cell populations of 1.25×10^7, as determined by plate count, were added to each tube containing medium plus various amounts of antibiotic. The bacteria were allowed to grow for 20 h at 37°C, and their growth was verified by observing turbidity. The MIC for each antibiotic was assessed as the lowest concentration of antibiotic at and above which there was no turbidity.

Enzyme assays. The coenzyme A released from acetylcoenzyme A upon acetylation of antibiotic was detected by its reaction with the sulfhydryl reagent Aldrithiol-4, as described previously (10, 11). Full-scale settings of 0.04 to 0.2 absorbance units on a Gilford model 240 spectrophotometer were used. Cuvette chambers were maintained at 25°C by circulating water baths. Enzyme samples were diluted with 0.025 M TAPS buffer (pH 8.5) which had been filtered and degassed. Assay mixtures contained 25 mM potassium phosphate buffer (pH 7.8) plus 60 μM each of acetylcoenzyme A and Aldrithiol-4 in a volume of 1 ml in 10-mm-pathlength quartz cuvettes. At pH 6, the assay mixture contained 0.1 M MES buffer, 0.1 M potassium chloride, and 0.1 mM EDTA plus 200 μM acetylcoenzyme A and 120 μM Aldrithiol-4 in a volume of 1.5 ml in 50-mm-pathlength optical-glass cuvettes. The latter were necessary for the determination of very low Michaelis constants and required a specially constructed cuvette chamber used with suitable pinholes to prevent light from falling on the sides of the long, narrow cuvettes. Reactions were monitored at 324 nm and were initiated with 0.01 ml of enzyme which was added and mixed with a paddle.

Data analysis. Enzyme kinetic data were fitted to equations 1 and 2, using computer programs SUBINHO and HYPERO representing the presence and absence of substrate inhibition, respectively, as described by Cleland (3). Linear correlation analyses were performed by fitting data to equation 3, using Madison Academic Computing Center's program BMDP. The equations are as follows:

$$v = VS/(K_m + S + S^2/K_i) \quad (1)$$

$$v = VS/(K_m + S) \quad (2)$$

$$y = mx + c \quad (3)$$

where v represents a measured velocity, V is the maximal velocity, S is the concentration of antibiotic substrate, K_m is the Michaelis-Menten constant, K_i is the substrate inhibition constant, x and y are independent variables, and m and c are slope and intercept values, respectively.

TABLE 3. Apparent kinetic constants at pH 6.0

Substrate	K_m (μM)	V_{max} (μmol/min per mg)	V/K
Sisomicin	2.62 ± 0.63	0.59 ± 0.04	0.023 ± 0.04
Gentamicin C_{1a}	0.46 ± 0.04	0.64 ± 0.01	1.39 ± 0.12
Amikacin	1.73 ± 0.24	0.59 ± 0.02	0.35 ± 0.04
Tobramycin	0.70 ± 0.07	0.70 ± 0.13	1.00 ± 0.08
Kanamycin A	0.65 ± 0.09	0.35 ± 0.01	0.54 ± 0.06

RESULTS

The MICs of antibiotics against *E. coli* W677(pMH67) were determined and compared with literature values for *E. coli* K-12 (6). The results (Table 1) confirm that the plasmid conferred a very high level of resistance to all these clinically useful antibiotics. Also, the MIC data for resistant bacteria cover a 10-fold range of variation with an intermediate distribution suitable for correlation analysis.

The results from kinetic analyses at pH 7.8 are shown in Table 2. All antibiotics (including seven aminoglycosides which are not shown) exhibited significant substrate inhibition, as noted by the significant K_i values, obtained from computer fittings of data to equation 1. As a result of a search for conditions free of substrate inhibition, data were also collected at pH 6 (Table 3). Data for all antibiotics tested (eight in all) fit equation 2 better than equation 1, indicating a lack of significant substrate inhibition at the more acidic pH.

The results of linear correlation analyses of the kinetic and resistance data from Tables 1, 2, and 3 are shown in Table 4. To accommodate substrate inhibition, expected velocities were calculated from equation 1, given S = MIC, and included in the analyses. The V/K values at pH 7.8 gave the only positive correlation with the MICs. Furthermore, V/K values at pH 7.8 gave the only correlation at a 90% confidence level. A plot of these data is shown in Fig. 1; similarly, a representative plot of the other data (e.g., the next best correlation, V/K at pH 6) is shown in Fig. 2.

DISCUSSION

Only V/K gave a linear correlation which was positive and significant (at a 90% confidence level) with antibiotic resist-

TABLE 2. Apparent kinetic constants at pH 7.8

Substrate	K_m (μM)	V_{max} (μmol/min per mg)	V/K	K_i (μM)
Sisomicin	5.56 ± 0.86	3.33 ± 0.25	0.60 ± 0.05	81.1 ± 13.7
Gentamicin C_{1a}	0.51 ± 0.06	1.35 ± 0.06	2.65 ± 0.22	20.6 ± 2.6
Amikacin[a]	0.16 ± 0.06	0.46 ± 0.04	2.88 ± 0.92	11.4 ± 4.3
Tobramycin	0.32 ± 0.07	0.93 ± 0.05	2.91 ± 0.51	24.7 ± 3.4
Kanamycin A	0.13 ± 0.03	0.81 ± 0.004	6.23 ± 1.25	24.7 ± 5.7

[a] Data were obtained below 11 μM. At higher concentrations, anomolous kinetics were observed, indicating a second active site or a reversal of substrate inhibition.

TABLE 4. Linear correlation analyses

pH	Kinetic constant	Correlation coefficient[a]
6.0	K_m	−0.4497
	V_{max}	−0.4842
	V/K	−0.1234
7.8	K_m	−0.6316
	V_{max}	−0.6992
	V/K	+0.8184
	v^b	−0.6767

[a] Calculated with three degrees of freedom.
[b] Velocity calculated from equation 1 for antibiotics at concentrations equal to MIC.

ance. All the other kinetic constants gave negative correlations whose coefficients were too small to be significant. Davies and Kagan (4) have suggested that the expression of microbial resistance is the outcome of the competition between the rates of two processes—transport and modification. If so, one would not expect a correlation between resistance and K_m, because K_m is reflective of an equilibrium, not a rate process. Both V_{max} and V/K are rate constants. However, V_{max} is the rate constant which governs the rate of catalytic turnover at saturating antibiotic concentrations, which is the concentration range of antibiotic one would expect to find in a cell if the enzyme were failing in the competition with transport and if the cell were overwhelmed by the antibiotic. On the other hand, V/K is the rate constant which governs the rate of catalytic turnover at substrate concentrations below K_m levels, which is the concentration range of antibiotic one would expect to find in a cell if it were successfully "resisting" the antibiotic. Therefore, a linear and positive correlation between V/K and antibiotic resistance is reflective of the reality of a resistant bacterium which is successfully withstanding the antibiotic. This correlation has several important implications for antibiotic resistance.

First, antibiotic resistance is often described in an all-or-none manner, supported by MIC determinations which do not exceed measurements above 16 or 32 μg/ml. As a rough guide, bacteria inhibited by aminoglycoside antibiotics at microgram levels are considered "sensitive," and those not inhibited at above 10 μg are dubbed "resistant." But the

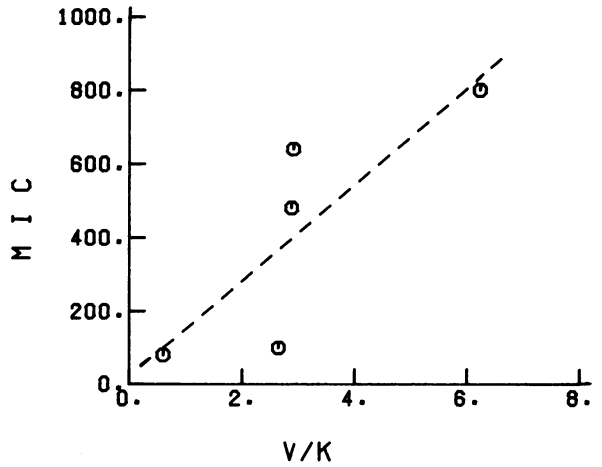

FIG. 1. MICs of antibiotics against R^+ *E. coli* W677 versus V/K values for kanamycin acetyltransferase at pH 7.8. Data points are from Tables 1 and 2. The dotted line was determined by a least-squares regression analysis.

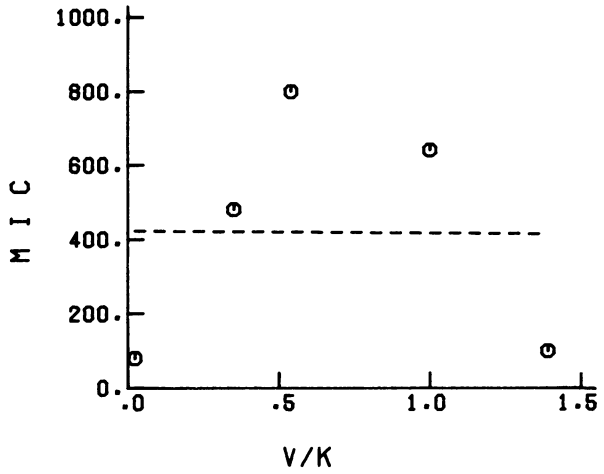

FIG. 2. MICs of antibiotics against R^+ *E. coli* W677 versus V/K values for kanamycin acetyltransferase at pH 6. Data points are from Tables 1 and 2. The dotted line was determined by a least-squares regression analysis.

data in Tables 1 and 4 clearly show that resistance is not all or none, that it is relative, and that it is determined by a kinetic feature of the resistance-mediating enzyme which is knowable.

Second, just because an antibiotic is a substrate for a modifying enzyme, it does not necessarily follow that the bacterium producing the enzyme will be resistant to it. Stated conversely, a knowledge of the antibiotic-resistance spectrum of a bacterium is not sufficient to identify the substrate specificity of the modifying enzyme or to infer the identity of the enzyme itself. For resistance to be expressed, the antibiotic must be a "good" substrate, and as the results in Table 4 indicate, in the context of microbial resistance, the goodness of a substrate is determined neither by a low K_m nor by a high V_{max} alone but by their ratio.

Third, the correlation of resistance with a rate process clearly supports the hypothesis that continuing catalytic turnovers are necessary for resistance to be expressed, despite the absence of a buildup of modified aminoglycoside in the medium, unlike the cases of penicillin and chloramphenicol in which modified antibiotics accumulate. It is quite likely that there are three different rate processes engaged in the competition which determines resistance to aminoglycosides: in addition to transport and modification, there may be the involvement of hydrolytic enzymes such as peptidases, phosphatases, and diesterases which unmodify the modified aminoglycoside. (For example, an *N*-acetylkanamycin hydrolase has been found in a kanamycin-producing organism along with kanamycin acetyltransferase [9].) Hydrolytic enzymes are periplasmic or extracellular in occurrence, and therefore would encounter the modified antibiotic as it leaves the cell. Such a process may not only explain the lack of buildup of the modified aminoglycosides, but, if involved in the transport of modified antibiotic out of the bacterial cell, may also provide yet another target for combatting clinical resistance to this important group of antibiotics.

This study was made considerably more difficult by the presence of substrate inhibition. At neutral pH, the presence of substrate inhibition necessitated more careful measurements of velocities at very low concentrations of antibiotic than would have been necessary otherwise. Repeated experiments were performed at different pH values to obviate the problem; but although successful in escaping substrate

inhibition at acid pH, this tactic did not yield data which correlated with resistance. Nevertheless, this pH-dependent substrate inhibition may be an important finding. Substrate inhibition can be an inherent part of an enzymatic mechanism, in which case it may be a useful tool for proving the mechanism, but sometimes it has no mechanistic significance (7). Universal substrate inhibition at pH 7.8 and none at pH 6.0 suggests it has none. Nevertheless, the pH dependence may hold the key for separating out and identifying the pK_a values of specific groups on both the enzyme and antibiotic which are responsible for the inhibition. Once the mechanism of substrate inhibition is known, it may then be possible to modify the aminoglycoside antibiotics to make them better inhibitors and worse substrates.

The lack of a perfect one-to-one linear correlation between *V/K* and MICs also has several important implications. First, Davies and Kagan (4) as well as Williams and Northrop (12) assumed that all the different aminoglycosides are transported into the bacterial cell at equal rates. This assumption, based on the structural similarity of these compounds, is also implicit in the present expectation of a linear correlation. If the rate of transport of all aminoglycosides is indeed the same, then, for example, Fig. 1 predicts that gentamicin C_{1a} should have had a much higher MIC than was actually found in Table 1. However, if gentamicin C_{1a} were transported into the cell at a faster rate than the rest of the antibiotics, then a lower extracellular concentration of antibiotic (i.e., MIC) would be sufficient to attain a lethal intracellular concentration. Therefore, the deviation of the points from the regression line in Fig. 1 may be indicative of differences in the rates of transport of different aminoglycosides. Moreover, the regression lines and deviations from them may be different again if the same enzyme is expressed in different bacteria. For example, if resistance were measured in a pseudomonad carrying the pMH67 plasmid, then different antibiotics might fall above or below the regression line because membranes and transport systems are different.

Second, some plasmids may code for more than one aminoglycoside-modifying enzyme (pMH67 does not, however [9]), and a second enzyme, acting on some but not all of the substrates of the first enzyme, could distort the correlation. For example, the *V/K* for tobramycin would anticipate a much lower MIC than observed. The higher value, representing greater-than-expected tolerance, may be indicative of yet another mechanism of resistance coming into play.

These implications are all part of a larger view, namely, that additional phenomena contributing to resistance will be expressed by data points not lying along the regression line between MICs and *V/K* values. This assertion invites new research into the biological controls of resistance, such as the role of other genes on a plasmid, by telling us when and where to look for additional mechanisms or modulators of antibiotic resistance.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI 1108 and Career Development award GM00254 (to D.B.N.) from the National Institutes of Health.

LITERATURE CITED

1. **Benveniste, R., and J. Davies.** 1973. Mechanisms of antibiotic resistance in bacteria. Ann. Rev. Biochem. **42**:471–506.
2. **Biddlecome, S., M. Haas, J. Davies, G. H. Miller, D. F. Rane, and P. J. L. Daniels.** 1976. Enzymatic modification of aminoglycoside antibiotics: a new 3-*N*-acetylating enzyme from a *Pseudomonas aeruginosa* isolate. Antimicrob. Agents Chemother. **9**:951–955.
3. **Cleland, W. W.** 1979. Statistical analysis of enzyme kinetic data. Methods Enzymol. **63**:103–138.
4. **Davies, J., and S. A. Kagan.** 1977. What is the mechanism for plasmid-determined resistance to aminoglycoside antibiotics? p. 207–219. *In* J. Drews and G. Honenauer (ed.), Topics in infectious diseases, vol. 2. R-factors: their properties and possible control. Springer-Verlag, New York.
5. **Haas, M., and J. Davies.** 1980. Characterization of the plasmids comprising the "R factor" R5 and their relationship to other R plasmids. Plasmid **3**:260–277.
6. **Le Goffic, F., A. Martel, M. L. Capmau, B. Beca, P. Goebel, H. Chardon, C. J. Soussy, J. Duval, and D. H. Bouvanchand.** 1968. New plasmid-mediated nucleotidylation of aminoglycoside antibiotics in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **10**:258–264.
7. **Northrop, D. B., and W. W. Cleland.** 1974. The kinetics of pig heart triphosphopyridine nucleotide-isocitrate dehydrogenase. II. Dead-end and multiple inhibition studies. J. Biol. Chem. **249**:2928–2931.
8. **Radika, K., and D. B. Northrop.** 1981. Purification and properties of kanamycin acetyltransferase. Fed. Proc. **40**:1583.
9. **Satoh, A., H. Ogawa, and Y. Satomura.** 1975. Effect of sclerin on production of the aminoglycoside antibiotics accompanied by salvage function in *Streptomyces*. Agric. Biol. Chem. **39**:1593–1598.
10. **Scarbrough, E., J. W. Williams, and D. B. Northrop.** 1979. Spectrophotometric assay for amikacin using purified kanamycin acetyltransferase. Antimicrob. Agents Chemother. **16**:221–224.
11. **Williams, J. W., and D. B. Northrop.** 1976. Purification and properties of gentamicin acetyltransferase I. Biochemistry **15**:125–131.
12. **Williams, J. W., and D. B. Northrop.** 1978. Substrate specificity and structure activity relationships of gentamicin acetyltransferase I. J. Biol. Chem. **253**:5908–5914.