UPTAKE OF (METHYL-^{14}C)-SISOMICIN AND (METHYL-^{14}C)-GENTAMICIN INTO BACTERIAL CELLS

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Eight sensitive strains (two *Staphylococcus aureus*, two *Escherichia coli*, two *Pseudomonas aeruginosa* and two *Klebsiella pneumoniae*) and four resistant *Pseudomonas aeruginosa* strains were used to study uptake of sisomicin and gentamicin by the bacterial cells. In eleven out of the twelve organisms studied employing (methyl-^{14}C)-sisomicin and (methyl-^{14}C)- gentamicin, uptake of the former was found higher than that of the latter. In one organism, the uptake of the two antibiotics was similar. This higher uptake of sisomicin may help explain the superior potency of the antibiotic in relation to gentamicin.

Sisomicin[1] is a double bonded ((4′-5′)-dehydro) analogue of gentamicin C_{1a}[2], and gentamicin[3] is a mixture of three components, C_{1a}, C_2, and C_1[4]. When sisomicin was compared to gentamicin and tobramycin, the *in vitro* activity of the antibiotic was greater than that of either of the two antibiotics[5]. YOUNG and HEWITT[6] compared sisomicin to gentamicin, tobramycin, amikacin, and butirosin, respectively, and showed that the *in vitro* activity of sisomicin was greatest among the five antibiotics tested, and also the killing rate of bacteria by sisomicin was the most rapid. Sisomicin in *in vivo* tests was more active than gentamicin or tobramycin[7]. In order to explain why sisomicin is more active than gentamicin despite their structural similarities, we undertook a series of studies on the relationship between sensitivity of the organisms to the two antibiotics and the rates of uptake of the drugs into the bacterial cells.

Aminoglycoside antibiotics are known to enter the bacterial cells, bind to the ribosomes, and consequently inhibit protein synthesis[8]. BRYAN *et al.*[9,10] studied the uptake of gentamicin in clinical isolates of *Escherichia coli* and *Pseudomonas aeruginosa* strains and observed three distinctive phases of the uptake; the energy-independent initial stage 1, and the subsequent energy-dependent stages 2 and 3, which are necessary for the killing of bacteria. BRYAN and his co-workers used a filtration method for their studies. However, in the experiments a centrifugation method was used to study the uptake of (methyl-^{14}C)-sisomicin[11] and (methyl-^{14}C)-gentamicin[12] in two stages; the energy-independent initial ionic binding stage 1, and the subsequent energy-dependent uptake stage 2, equivalent to BRYAN's stages 2 and 3. The centrifugation method has the disadvantage of requiring five minutes in the work-up. However, unlike the filtration method, it avoids the problem of adsorption of labeled antibiotics onto the filters.

Materials and Methods

Strains

Sensitive strains used for the study were *Escherichia coli* 1574–1 and ATCC 10536, *Staphylococcus aureus* FDA 209P (ATCC 6538P) and Wood, *Klebsiella pneumoniae* Ad 17 and Burstein, and *Pseudomonas aeruginosa* Stone 20 and Robi M. Resistant strains studied were *Pseudomonas aeruginosa*

Bryan 1136 (permeability strain)[10], 3-N-acetyltransferase (AAC(3)-1)[13]-carrying Travers, 6′-N-acetyltransferase (AAC(6′))[14]-carrying GN 315, and 2″-O-adenyltransferase (ANT(2″))[15]-carrying POW. All of the strains were clinical isolates with the exceptions of *Staphylococcus aureus* ATCC 6538P and *Escherichia coli* ATCC 10536.

Labeled Antibiotics

(Methyl-^{14}C)-gentamicin and (methyl-^{14}C)-sisomicin had specific radioactivity of 1,710 nCi/mg bioactivity (792 nCi/μ mole) and 2,650 nCi/mg bioactivity (1,185 nCi/μ mole) respectively. Because the specific radioactivity of the (methyl-^{14}C)-sisomicin was higher than that of the (methyl-^{14}C)-gentamicin, two parts of bioactivity of the labeled sisomicin was diluted with one part bioactivity of unlabeled sisomicin so that the specific radioactivities of the two labeled antibiotics were equalized.

Medium

MUELLER-HINTON broth (pH 7.2 ~ 7.4) was used throughout.

Determination of Minimal Inhibitory Concentration (MIC) Values

Broth MIC values were determined by inoculating approximately 1×10^6 organisms from an overnight culture into 3 ml MUELLER-HINTON broth containing 2-fold dilutions of each antibiotic, and recording the lowest concentrations of the antibiotic which yielded clear tubes at 48 hour incubation at 37°C.

Growth Curves

A Coleman spectrophotometer (model 6/20) was used to follow growth of each organism at OD_{620nm} in the absence and presence of the antibiotics. For the OD-readings, culture broths were diluted with distilled water in Coleman round cuvettes (19 mm × 150 mm). In general, higher concentrations of the antibiotics were required to inhibit growth of the organisms during their logarithmically growing stages, as compared to the MIC values determined under static conditions (Table 1).

Determination of Uptake of Antibiotics

Inoculum for each organism was prepared by overnight growth in MUELLER-HINTON broth at 37°C, 0.05 ml of each inoculum was added to 50 ml of fresh MUELLER-HINTON broth in 250-ml flasks. In the cases of resistant strains, culture maintenance and inoculum preparations were carried out in the presence of 5 μg/ml gentamicin or sisomicin. For the uptake studies, flasks were shaken at 350 rpm at 37°C with a G-25 shaker (New Brunswick Scientific). Following 2 ~ 3-hour incubation (OD_{620} 0.5 ~ 0.7), labeled antibiotics were added to the respective flasks at various concentrations (Table 1). Five-ml samples were withdrawn at 1, 15, 30 and 60-minute intervals, and 1 mg each of the unlabeled antibiotics was immediately added to each sample in order to dilute the labeled antibiotics. Samples were immediately centrifuged for 5 minutes at 48,000 g at 4°C in a Sorval RC 2-B centrifuge. The pellets were suspended in water containing 200 μg/ml of the unlabeled antibiotics, and re-centrifuged under the same conditions. Each of the washed pellets was suspended in 1 ml water, the suspension was added to 15 ml Scintisol complete, and radioactivity was determined using an Intertechnique SL 4000 scintillation counter.

Results

BRYAN and his co-workers[9,10] used a filtration method, and studied three phases of the uptake of gentamicin. We have used a centrifugation method, and examined the uptake of sisomicin and gentamicin in two phases; the energy-independent ionic binding phase 1, and the energy-dependent uptake phase 2. Because each centrifugation step required a five-minute time period, we were unable to study the uptake of these antibiotics in three different phases as BRYAN and his co-workers did in their experiments using the filtration method.

As compared to the MIC values determined under static conditions, higher concentrations of the antibiotics were required to inhibit growth of the organisms in a short time period under shaking (aerobic) conditions. Sisomicin was more potent in 4 out of 8 sensitive strains growing aerobically,

equally potent in two strains, and less potent in the other two strains (Table 1). The initial ionic binding and the subsequent uptake of the (methyl-^{14}C)-sisomicin were higher than those of the (methyl-^{14}C)-gentamicin in all of the sensitive strains tested (Table 2, Fig. 1).

Table 1. Growth of organisms in the presence of antibiotic (gentamicin or sisomicin) added

Organism	MIC* (μg/ml)	Antibiotic added (μg/ml)	Growth (OD_{620nm})		
			1 min	30 min	60 min
Staphylococcus aureus 6538	0.25	G–20**	0.43	0.60	0.65
Staphylococcus aureus 6538	0.25	S–20***	0.43	0.60	0.64
Staphylococcus aureus Wood	0.125	G–10	0.93	1.23	1.45
Staphylococcus aureus Wood	0.25	S–10	0.75	1.05	1.20
Escherichia coli 1574–1	0.5	G–7	0.73	1.00	1.01
Escherichia coli 1574–1	0.5	S–7	0.75	0.96	1.01
Escherichia coli 10536	0.5	G–7	0.55	0.57	0.52
Escherichia coli 10536	0.25	S–7	0.55	0.50	0.51
Pseudomonas aeruginosa Stone	2	G–5	0.53	0.72	0.68
Pseudomonas aeruginosa Stone	2	S–5	0.51	0.68	0.67
Pseudomonas aeruginosa Robi M.	1	G–5	0.57	0.68	0.78
Pseudomonas aeruginosa Robi M.	0.5	S–5	0.67	0.78	0.87
Klebsiella pneumoniae Ad 17	2	G–7	0.70	1.25	1.47
Klebsiella pneumoniae Ad 17	1	S–7	0.74	1.40	1.70
Klebsiella pneumoniae Burstein	0.5	G–7	0.74	0.96	0.85
Klebsiella pneumoniae Burstein	0.25	S–7	0.71	0.92	0.80
Pseudomonas aeruginosa Bryan 1136	>32	G–80	0.66	0.93	1.12
Pseudomonas aeruginosa Bryan 1136	16	S–80	0.68	0.90	1.01
Pseudomonas aeruginosa POW	>32	G–40	0.80	1.15	1.28
Pseudomonas aeruginosa POW	>32	S–39	0.92	0.95	1.02
Pseudomonas aeruginosa Travers	>32	G–50	0.74	1.16	1.40
Pseudomonas aeruginosa Travers	>32	S–50	0.66	0.92	1.01
Pseudomonas aeruginosa GN 315	2	G–40	0.90	1.55	2.03
Pseudomonas aeruginosa GN 315	>32	S–39	0.88	1.53	2.30

* MIC=Determined during the growth of the organism under static conditions.

** G–20=20 μg (methyl-^{14}C)-gentamicin containing 77,504 dpm.

*** S–20=20 μg mixture of two parts of (methyl-^{14}C)-sisomicin containing 77,854 dpm and one part of unlabeled sisomicin.

Table 2. Uptake of labeled antibiotics

Organism	Antibiotic added/ml		Uptake (dpm/ml culture broth)			
	μg	dpm	1 min	15 min	30 min	60 min
Staphylococcus aureus 6538	G–20	77,504	341	516	580	920
Staphylococcus aureus 6538	S–20	77,854	813	829	980	1530
Escherichia coli 10536	G–7	27,240	182	448	646	1018
Escherichia coli 10536	S–7	27,120	248	520	656	964
Pseudomonas aeruginosa Robi M.	G–5	19,376	120	144	177	403
Pseudomonas aeruginosa Robi M.	S–5	18,874	281	328	429	756
Klebsiella pneumoniae Burstein	G–7	27,240	128	321	527	1014
Klebsiella pneumoniae Burstein	S–7	27,120	196	477	737	1097

The microbiological potency of sisomicin was greater than that of gentamicin in the resistant *Pseudomonas aeruginosa* strains; Bryan 1136, POW and Travers (Table 1).

In the case of *Pseudomonas aeruginosa* Bryan 1136, described as a resistant permeability strain, only the initial ionic binding of the (methyl-^{14}C)-gentamicin was observable. Other than this ionic binding, there was little uptake of the (methyl-^{14}C)-gentamicin. Not only was the initial ionic binding of the (methyl-^{14}C)-sisomicin higher than that of the (methyl-^{14}C)-gentamicin, but there was a significant uptake of the labeled sisomicin in samples incubated for 15 minutes or longer (Fig. 2).

In the 2″-O-adenylating *Pseudomonas aeruginosa* POW strain and the 3-N-acetylating *Pseudomonas aeruginosa* Travers strain, both the ionic binding and the uptake of the (methyl-^{14}C)-sisomicin were higher than those of the (methyl-^{14}C)-gentamicin as seen in the sensitive strains (Fig. 2).

Thus there was a correlation between the antibiotic potency and the uptake of the two labeled antibiotics in most of the cases studied. In one instance, however, the opposite relationship was observed: sisomicin was less potent against 6′-N-acetylating *Pseudomonas aeruginosa* GN 315 strain than gentamicin, but the uptake

Fig. 1. Uptake of labeled antibiotics by the four representative sensitive strains.
Staph. aureus Wood
(Methyl-^{14}C)-sisomicin (38,927 dpm/ml added).
(Methyl-^{14}C)-gentamicin (38,752 dpm/ml added).
E. coli 1574–1
(Methyl-^{14}C)-sisomicin (27,120 dpm/ml added).
(Methyl-^{14}C)-gentamicin (27,240 dpm/ml added).
K. pneumoniae Ad 17
(Methyl-^{14}C)-sisomicin (27,120 dpm/ml added).
(Methyl-^{14}C)-gentamicin (27,240 dpm/ml added).
P. aeruginosa Stone 20
(Methyl-^{14}C)-sisomicin (18,874 dpm/ml added).
(Methyl-^{14}C)-gentamicin (19,376 dpm/ml added).

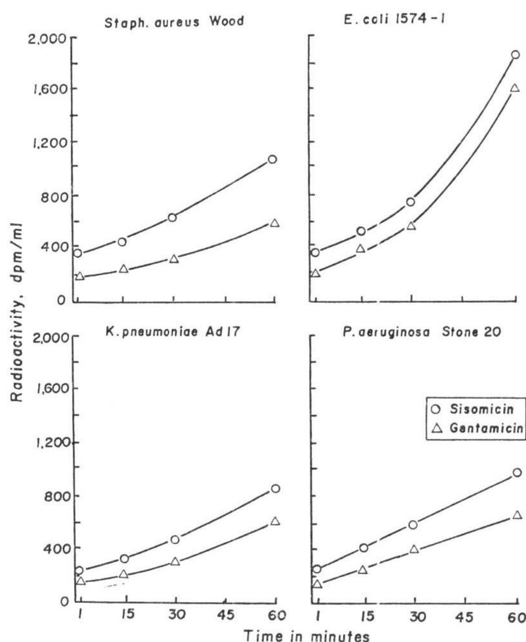


Fig. 2. Uptake of labeled antibiotics by resistant *P. aeruginosa* strains.

Bryan 1136
(Methyl-^{14}C)-sisomicin (311,400 dpm/ml added).
(Methyl-^{14}C)-gentamicin (310,000 dpm/ml added).
POW
(Methyl-^{14}C)-sisomicin (155,700 dpm/ml added).
(Methyl-^{14}C)-gentamicin (155,000 dpm/ml added).
Travers
(Methyl-^{14}C)-sisomicin (194,630 dpm/ml added).
(Methyl-^{14}C)-gentamicin (193,760 dpm/ml added).

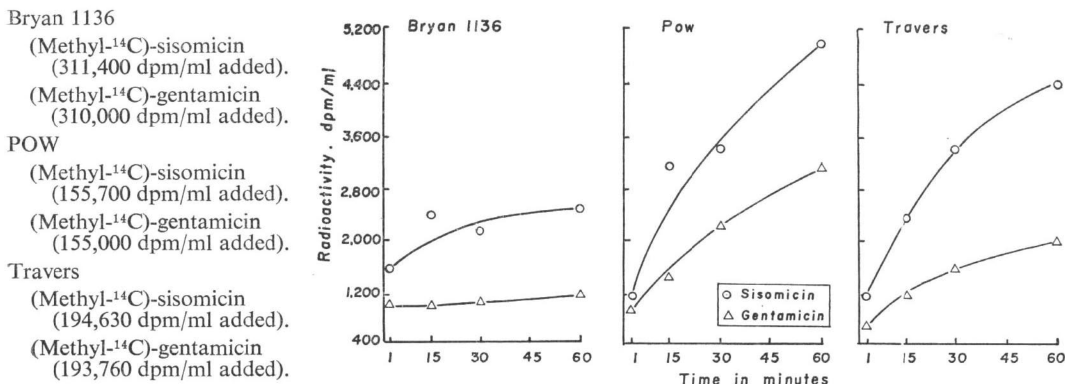


Fig. 3. Growth curves of resistant *P. aeruginosa* GN 315 strain, and uptake of labeled antibiotics by the strain.

Left (growth curves)

Control

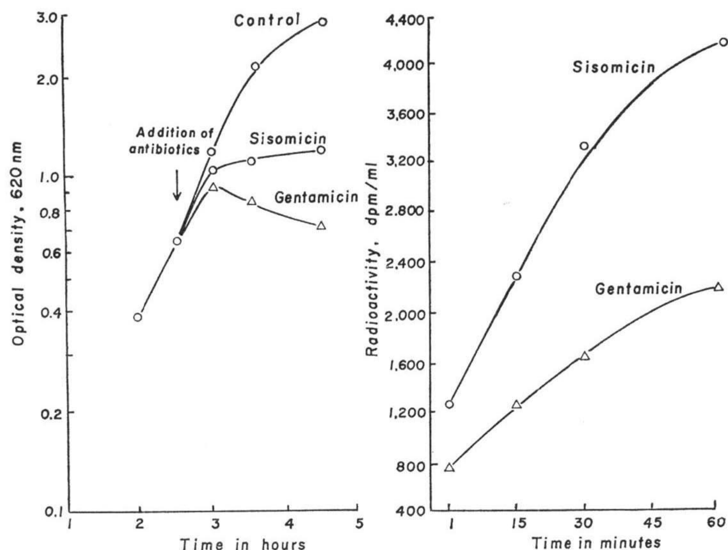
Mixture of 26 μg/ml (methyl-^{14}C)-sisomicin and 13 μg/ml unlabeled sisomicin.

40 μg/ml (methyl-^{14}C)-gentamicin.

Right (uptake)

(Methyl-^{14}C)-sisomicin (155,700 dpm/ml added).

(Methyl-^{14}C)-gentamicin (155,000 dpm/ml added).



of the former was much higher than that of the latter (Fig. 3). The ionic binding of the (methyl-^{14}C)-sisomicin in this strain was also higher as in all other organisms. The lower antibacterial activity of sisomicin may be attributable to the susceptibility of the antibiotic to the 6′-N-acetylation by the organism, while gentamicin complex is not susceptible to the acetylation due to the presence of the C_1 component which is not a substrate for the 6′-N-acetylating enzyme.

Discussion

A higher uptake of sisomicin in general may account for a higher microbiological potency of the antibiotic than gentamicin. Among the resistant *Pseudomonas aeruginosa* strains tested, sisomicin showed a higher antibacterial potency, a higher ionic binding, and a higher uptake in the Bryan 1136 (permeability strain), the 2′-N-adenylating POW strain and the 3-N-acetylating Travers strain.

In the permeability strain (Bryan 1136), substantial amounts of sisomicin were taken up at 15 minutes. But the increase of the uptake in samples incubated longer than 15 minutes was not noticeable. Other than the initial ionic binding (at 1 minute), little uptake of gentamicin was observed in samples incubated for 15 minutes or longer. In contrast to gentamicin, certain amounts of sisomicin at high concentrations seem to enter the cells of the permeability strain during the early exposure (15 minutes) of the organism to the drug. It may be possible that permeability barrier of this strain may be altered at high concentrations of sisomicin, resulting in the entry of the antibiotic molecules into the cells. This action could explain why sisomicin at high concentrations is more active than gentamicin against the permeability strain.

In the 2″-O-adenylating *Pseudomonas aeruginosa* POW and the 3-N-acetylating *Pseudomonas aeruginosa* Travers strain, sisomicin showed a higher ionic binding (at 1 minute) and a higher uptake in

samples incubated for 15 minutes or longer. Thus it appears obvious that more molecules of sisomicin than gentamicin enter the cells of these strains. But it is not clear that the radioactivity measured for the uptake was that of each antibiotic as it is, or as the enzymatically inactivated (adenylated or acetylated) form of the antibiotic, or a mixture of the two.

In the 6′-N-acetylating *Pseudomonas aeruginosa* GN 315 strain, the microbiological potency of gentamicin was shown to be much higher than that of sisomicin. Gentamicin is a mixture of the C_{1a}, C_2 (6′-C-methyl-C_{1a}) and C_1 (6′-C,N-dimethyl-C_{1a}) components, and sisomicin is a single component, (4′-5′)-dehydrogentamicin C_{1a}. The gentamicin C_1 component, mixed in the gentamicin complex, does not possess 6′-primary amino group, therefore, it is not a substrate for the 6′-N-acetylation. For this reason the gentamicin mixture should be more potent than sisomicin against the 6′-N-acetylating strain. Contrary to the potency, sisomicin showed a higher uptake than gentamicin. This may be explained: more molecules of sisomicin than gentamicin may enter the cells of the *Pseudomonas* strain, but they become immediately inactivated (6′-N-acetylated). Viable cell counts and cellular fractionation to determine the distribution of radioactivity would be required to answer these questions.

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References

1) Weinstein, M. J.; J. A. Marquez, R. T. Testa, G. H. Wagman, E. M. Oden & J. A. Waitz: Antibiotic 66-40, a new *Micromonospora*-produced aminoglycoside antibiotic. J. Antibiotics 23: 551~554, 1970
2) Cooper, D. J.; R. S. Jaret & H. Reiman: Structure of sisomicin, a novel unsaturated aminoglycoside antibiotic from *Micromonospora inyoensis*. J. Chem. Soc., Chem. Comm. 1971: 285~286, 1971
3) Weinstein, M. J.; G. H. Luedemann, E. M. Oden, G. H. Wagman, J. P. Rosselet, J. A. Marquez, C. T. Coniglio, W. Charney, H. L. Herzog & J. Black: Gentamicin, a new antibiotic complex from *Micromonospora*. J. Med. Chem. 6: 463~464, 1963
4) Cooper, D. J.; H. M. Marigliano, M. D. Yudis & T. Traubel: Recent developments in the chemistry of gentamicin. J. Inf. Dis. 119: 342~344, 1969
5) Growe, C. C. & E. Sanders: Sisomicin: Evaluation *in vitro* and comparison with gentamicin and tobramycin. Antimicr. Agents & Chemoth. 3: 24~28, 1973
6) Young, L. S. & W. L. Hewitt: Activity of five aminoglycoside antibiotics *in vitro* against gram-negative bacilli and *Staphylococcus*. Antimicr. Agents & Chemoth. 4: 617~625, 1973
7) Waitz, J. A.; E. L. Moss, C. G. Drube & M. J. Weinstein: Comparative activity of sisomicin, gentamicin, kanamycin, and tobramycin. Antimicr. Agents & Chemoth. 2: 431~437, 1972
8) Davies, J. & B. Davis: Misreading of ribonucleic acid code words induced by aminoglycoside antibiotics. J. Biol. Chem. 243: 3312~3316, 1968
9) Bryan, L. E. & H. M. Van Den Elzen: Gentamicin accumulation by sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. J. Antibiotics 28: 696~703, 1975
10) Bryan, L. E.; R. Haraphongse & H. M. Van Den Elzen: Gentamicin resistance in clinical-isolates of *Pseudomonas aeruginosa* associated with diminished gentamicin accumulation and no detectable enzymatic modification. J. Antibiotics 29: 743~753, 1976
11) Lee, B. K.; R. G. Condon, G. H. Wagman & M. J. Weinstein: *Micromonospora*-produced sisomicin components. J. Antibiotics 29: 677~684, 1976
12) Lee, B. K.; R. G. Condon, G. H. Wagman, K. Byrne & C. Schaffner: Incorporation of L-methionine-methyl-^{14}C into gentamicins. II. Large-scale preparation of methyl-^{14}C-gentamicins. J. Antibiotics 27: 822~825, 1974
13) Biddlecome, S.; M. Hass, J. Davies, G. H. Miller, D. F. Rane & P. J. L. Daniels: Enzymatic modification of aminoglycoside antibiotics: A new 3-N-acetylating enzyme from a *Pseudomonas aeruginosa* isolate. Antimicr. Agents & Chemoth. 9: 951~955, 1976
14) Yagisawa, M.; S. Kondo, T. Takeuchi & H. Umezawa: Aminoglycoside 6′-N-acetyltransferase of *Pseudomonas aeruginosa*: Structural requirements of substrate. J. Antibiotics 28: 486~489, 1975
15) Kabins, S.; C. Nathan & S. Cohen: Gentamicin-adenyltransferase activity as a cause of gentamicin resistance in clinical isolates of *Pseudomonas aeruginosa*. Antimicr. Agents & Chemoth. 5: 565~570, 1974