PRISTINAMYCIN ACCUMULATION BY *STAPHYLOCOCCUS AUREUS*

PATRICIA LACROIX, MARIE LOUISE CAPMAU and FRANÇOIS LE GOFFIC*

Centre National de la Recherche Scientifique, CERCOA
2 à 8, rue Henri Dunant, 94320 Thiais, France

(Received for publication April 5, 1984)

Pristinamycins I_A and II_A (PI_A and PII_A) accumulation by *Staphylococcus aureus* has been studied with two hydrogenated analogs, $(H_2)PI_A$ and $(H_2)PII_A$. Rapid accumulation of both antibiotics at 37°C is observed and internal concentrations can reach up to 58-fold the external concentration; this accumulation cannot be reduced by either metabolic inhibitors or tetracycline. The synergistic activity of pristinamycins I_A and II_A is not observed at the bacterial accumulation level. We propose that pristinamycins enter into bacteria by a passive diffusion process and that the internal concentration is maintained by binding of the antibiotic to the bacterial ribosomes.

The pristinamycins (P)[1] belong to the macrolide-lincosamide-streptogramine (MLS) group of antibiotics. They contain two major components: pristinamycin I_A (PI_A) which is a cyclic hexadepsipeptide and pristinamycin II_A (PII_A) which is a macrocyclic lactone. Both have bacteriostatic activity. A mixture of the two shows strong synergy and is bactericidal[2,3,4].

The pristinamycins bind to bacterial ribosomes and inhibit protein synthesis. The interaction of each component with its receptor site is enhanced by the presence of the other[5~12]. This may explain the observed synergism. However the mechanism of action of the pristinamycins as well as that of the synergistic effect are not fully understood.

Few studies have been undertaken on the transport of MLS antibiotics[13]. In the present paper, we report studies on the accumulation of the pristinamycins and the influence of one component on the uptake of the other. $(H_2)PI_A$ and $(H_2)PII_A$ were used in the present study as they are readily available in a radioactive form and have biological properties identical to the parent compounds[9,14].

Materials and Methods

Chemicals

All chemicals were of analytical grade whenever available. Reagent sources were: potassium cyanide and sodium azide from Prolabo; oxamic acid, sodium salt from Jansen Pharmaceutica; 2,4-dinitrophenol (DNP) from Merck; *N*-ethylmaleimide (NEM) from Fluka A.G.; carbonylcyanide *m*-chlorophenylhydrazone (CCCP) from Sigma Chemical Co.; polyethylene glycol (PEG) 6000 from Touzart et Matignon. Pristinamycins and dihydrostreptomycin were from Rhône-Poulenc; tetracycline was from Pfizer; lasalocid, nigericin, grisorixin, gramicidin D, calcimycin and valinomycin were gifts from Dr. G. JEMINET, University of Clermont-Ferrand, France. [^{3}H]Dihydropristinamycins I_A (N and A) and II_A (α and β) were synthesized as described previously[9,14]; [^{3}H]dihydrostreptomycin was purchased from Amersham.

Bacterial Strains and Growth

Staphylococcus aureus 209P strain was from our laboratory collection. Trypticase Soy Broth (Difco) was used as the culture medium. A 1 ml overnight culture was inoculated in a flask containing 100 ml fresh medium and shaken at 37°C until the turbidity reached 100 Klett units (Klett Summerson, blue filter 400~420 nm). The bacteria were harvested by centrifugation (1,500 × *g*, 10 minutes),

washed by suspension in 40 ml medium A (containing per liter $(NH_4)_2SO_4$ 0.7 g, KH_2PO_4 1 g, $Na_2HPO_4 \cdot 12H_2O$ 6 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, pH 7.2), collected by low-speed centrifugation, and suspended in the same medium at a concentration corresponding to 300 Klett units (7×10^8 bacteria/ml). Cell viability was followed by dilution and counting on Mueller-Hinton agar (Difco).

Accumulation Experiments

The accumulation of [^{3}H]dihydropristinamycin I_A ([$^3H_2$]PI_A(N) and [$^3H_2$]PI_A(A)) (600 Ci/mol) and [^{3}H]dihydropristinamycin II_A ([$^3H_2$]$PII_A(\alpha)$ and [$^3H_2$]$PII_A(\beta)$) (400 Ci/mol) were studied as follows:

a) At different times after [^{3}H]dihydropristinamycin addition, a 500 μl sample of bacterial suspension was removed and rapidly filtered through a glass fiber filter (Whatman GF/F). The filters were washed with cold medium A until constant radioactivity (3×5 ml), dried, and counted in 5 ml scintillation fluid (Beckman NA). The background, due to binding of [^{3}H]dihydropristinamycins to filters, was determined under the same conditions in the absence of bacteria.

b) Aqueous Two Phase Partitioning[15]: The stock solutions were 40% PEG 6000 (w/w) in 20 mM Na_2HPO_4 and 40% $MgSO_4 \cdot 7H_2O$ (w/w) in 20 mM Na_2HPO_4. At different times after [^{3}H]-dihydropristinamycin addition, 500 μl of bacterial suspension was removed and added to a cold mixture containing 0.375 ml PEG stock solution and 1.125 ml $MgSO_4$ stock solution. After stirring, the mixture was left for 30 minutes at 0°C. The resulting volumes were 0.5 ml for the upper PEG rich phase and 1.5 ml for the lower salt rich phase. 100 μl of the upper phase and 400 μl of the lower phase were counted in 5 ml scintillation fluid (Beckman HP). *S. aureus* were present in the lower salt phase. The partition coefficients are 25.5 for [$^3H_2$]PI_A and 23.2 for [$^3H_2$]PII_A.

Results were the same whether accumulation of dihydropristinamycins II_A was measured by method a) or b); however, method b) was preferred for experiments with [$^3H_2$]PI_A because of high background due to binding on filters.

Background for accumulation was determined by adding [^{3}H]dihydropristinamycins to bacteria at 0°C and was measured by method a) or b).

The accumulation of [^{3}H]dihydrostreptomycin (250 Ci/mol) was determined by method a) with the following modification:

At different times after [^{3}H]dihydrostreptomycin addition, 500 μl of bacterial suspension was removed and rapidly filtered through Millipore filters (HA, 0.45 μm pore size). Membrane filters were pretreated by filtering 2.5 ml of a solution of dihydrostreptomycin (2,500 μg/ml). Filters were washed with 3×3 ml of LiCl (0.1 M).

Results and Discussion

No significant differences in accumulation were observed between [$^3H_2$]PI_A(N) and [$^3H_2$]PI_A(A) on one hand, and between [$^3H_2$]$PII_A(\alpha)$ and [$^3H_2$]$PII_A(\beta)$ on the other (results not shown).

As a consequence, one of the two dihydropristinamycins of each group was selected for the accumulation studies: [$^3H_2$]PI_A(N) and [$^3H_2$]$PII_A(\alpha)$ respectively*.

Effect of Temperature on [^{3}H]Dihydropristinamycin Accumulation

The effect of temperature on the kinetics of accumulation of [$^3H_2$]PI_A and [$^3H_2$]PII_A is shown on Fig. 1. The accumulation reaches a maximum at 37°C, 2 minutes after addition of [$^3H_2$]PI_A and 5 minutes after addition of [$^3H_2$]PII_A. Under these conditions (2 μM external antibiotic concentration) *S. aureus* accumulates 100 pmol [$^3H_2$]$PI_A/10^9$ bacteria and 53 pmol [$^3H_2$]$PII_A/10^9$ bacteria at maximal rate. At 25°C, the initial velocity of accumulation is reduced 2- and 4-fold for [$^3H_2$]PI_A and [$^3H_2$]PII_A respectively; the same maximum as at 37°C is reached. When the temperature is decreased (15°C and 4°C), the initial velocities are dramatically reduced and at 4°C the drugs are not, for all practical purposes, accumulated.

* In the text, those molecules are indicated [$^3H_2$]PI_A and [$^3H_2$]PII_A.

Fig. 1. Effect of temperature on dihydropristinamycin accumulation by *S. aureus* 209P.

(A) Resuspended cells were preincubated 10 minutes at 37°C (▲), at 25°C (△), at 15°C (○) or at 4°C (●). [$^3H_2$]PI_A was added to give a final concentration of 2 μM, and accumulation was assayed at those temperatures by aqueous two phase partitioning method.

(B) Resuspended cells were preincubated 10 minutes at 37°C (▲), at 25°C (△), at 15°C (○) or at 4°C (●). [$^3H_2$]PII_A was added to give a final concentration of 2 μM. Accumulation was assayed at those temperatures by filtration method.

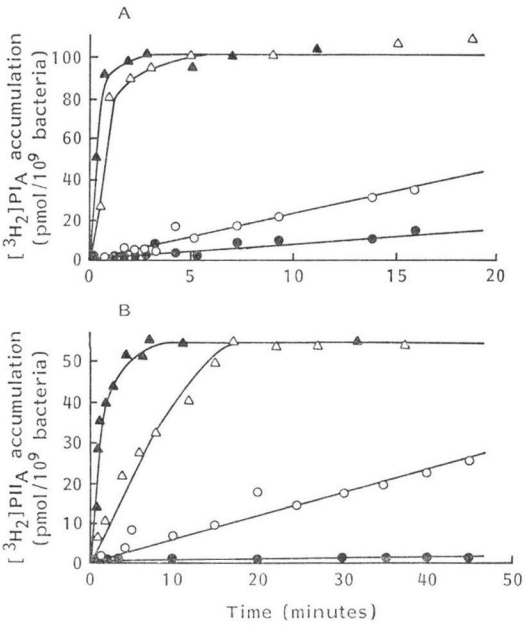
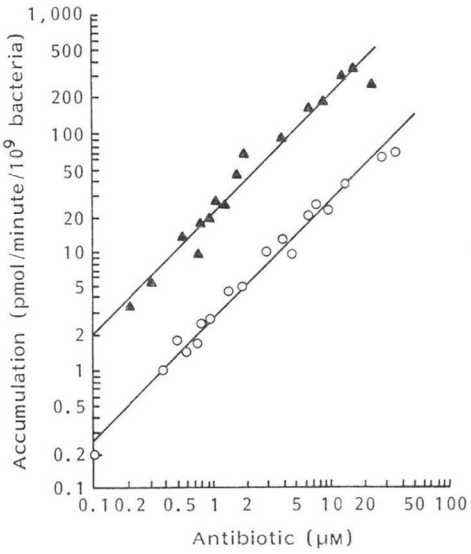


Fig. 2. Initial velocity of accumulation of dihydropristinamycins by *S. aureus* 209P as a function of antibiotic concentration in the medium.

[$^3H_2$]PI_A (▲) or [$^3H_2$]PII_A (○) at different specific activities was added to a cell suspension at 25°C. Initial rate of accumulation was determined at 30 seconds or 1 minute. The initial velocity of accumulation is expressed in pmol/minute/10^9 bacteria and is plotted as a function of antibiotic concentration in the medium using log-log coordinates.



Kinetics of [^{3}H]Dihydropristinamycin Accumulation

The effect of increasing [$^3H_2$]PI_A and [$^3H_2$]PII_A concentrations on their initial velocity of accumulation is shown on Fig. 2; these experiments are performed at 25°C. For both compounds, the initial velocity of accumulation increases linearly as a function of antibiotic concentration without reaching a plateau. The transport of [$^3H_2$]PI_A and [$^3H_2$]PII_A appears to be non-saturable in the concentration range tested (up to 20 and 30 μM respectively).

The maximum amount of [^{3}H]dihydropristinamycin accumulated by *S. aureus* is dependent on the external concentration (Table 1). For an extracellular concentration of [^{3}H]dihydropristinamycin ranging between 0.2 μM and 2 μM, the antibiotic is concentrated about 50-fold the extracellular concentration for [$^3H_2$]PI_A, and about 30-fold the extracellular concentration for [$^3H_2$]PII_A. These values are calculated assuming a *S. aureus* cell volume of about 1.14×10^{-12} ml[16]. At higher external concentrations, the ratio of internal concentration to external concentration decreases to 3- and 5-fold.

Table 1. Dihydropristinamycin intracellular concentration in *S. aureus* at various antibiotic concentrations in the medium.[a)]

Antibiotic	Extracellular concentration C_e (μM)	Intracellular concentration C_i (μM)[a)]	C_i/C_e	Antibiotic	Extracellular concentration C_e (μM)	Intracellular concentration C_i (μM)[a)]	C_i/C_e
$[^3H_2]PI_A$	0.2	11.4	57	$[^3H_2]PII_A$	0.4	9.6	21
	0.3	17.5	58		1.0	39.4	40
	1.0	57.9	58		1.5	42.9	31
	1.3	65.7	51		2.0	46.5	23
	1.7	83.3	56		6.0	30.7	5
	2.0	87.7	44		9.8	31.6	3
	7.3	100.0	14		15.0	38.6	3
	9.5	108.7	11		18.0	46.5	3
	14.0	106.8	7		25.0	58.8	2
	19.0	95.6	5		35.0	93.8	3

[a)] Intracellular concentration is calculated at the maximal amount of antibiotic accumulation at 37°C, assuming a cell volume of 1.14×10^{-12} ml[16].

Table 2. The effect of metabolic inhibitors on dihydropristinamycin and dihydrostreptomycin accumulation by *S. aureus* 209P.

Inhibitors	Accumulation (pmol/10^9 bacteria)				
	$[^3H_2][PI_A$[a)]		$[^3H_2]PII_A$[a)]		[^{3}H]Dihydro-streptomycin[b)]
	1 minute	25 minutes	1 minute	25 minutes	25 minutes
None	50	100	3.9	53	233
KCN (10 mM)	50	93	3.7	52	67
NaN_3 (50 mM)	48	93	3.9	49	98
Oxamic acid (10 mM)	50	100	3.9	53	154
CCCP (5 μM)	47	92	3.8	49	103
DNP (5 mM)	50	100	3.9	53	72
NEM (30 mM)	50	100	3.9	53	98

[a)] Cells were preincubated at 25°C, 10 minutes. Inhibitor was added 5 minutes before antibiotic (2 μM).
[b)] Cells were preincubated at 37°C in medium A with glucose (2 g/liter), 10 minutes. Inhibitor was added 5 minutes before antibiotic (8 μM).

Effect of Glucose, Metabolic Inhibitors and Protein Synthesis on [^{3}H]Dihydropristinamycin Accumulation

The accumulation of $[^3H_2]PI_A$ (2 μM) and $[^3H_2]PII_A$ (4.5 μM) by *S. aureus* in buffer containing glucose (2 g/liter) or not was studied. The kinetics of accumulation of the two molecules were essentially identical in both media. The maximum accumulation in the presence and in absence of glucose were, respectively, 97 pmol and 100 pmol per 10^9 bacteria for $[^3H_2]PI_A$, 42 pmol and 57 pmol per 10^9 bacteria for $[^3H_2]PII_A$.

The effect of metabolic inhibitors on the initial rate and the maximum accumulation of $[^3H_2]PI_A$ and $[^3H_2]PII_A$ was next studied. To verify that in those experiments metabolic inhibitors were actually blocking active uptake, their action on dihydrostreptomycin accumulation was tested. The results are shown in Table 2. The accumulation of dihydrostreptomycin was affected by those inhibitors as was previously demonstrated[17,18]. But neither electron transport inhibitors (oxamic acid, KCN, NaN_3), nor agents which uncouple oxidative phosphorylation and electron transport (2~4 DNP, CCCP), nor sulfhydryl group inhibitors (NEM) inhibit [^{3}H]dihydropristinamycin accumulation at the

initial rate or at the maximum accumulation. Identical results were obtained either by incubating bacteria with inhibitor for a period 5 minutes before antibiotic addition, or by simultaneous addition. These results suggest that pristinamycin accumulation by *S. aureus* is not energy dependent.

The effect of inhibition of protein synthesis on accumulation of [$^3H_2$]PI_A (1.2 μM) and [$^3H_2$]PII_A (1.6 μM) was examinated. Cells were incubated in buffer containing glucose (2 g/liter) and tetracycline (0.4 μg/ml) at 37°C. After 30 minutes, when protein synthesis was inhibited[19], dihydropristinamycin was added and uptake followed. The kinetics of accumulation in the control cells and the cells incubated in presence of tetracycline were essentially identical for the two dihydropristinamycins (results not shown). These results suggest that protein synthesis is not required for dihydropristinamycin accumulation.

Effect of Combinations of Pristinamycin on Accumulation

To determine whether PI_A would influence PII_A accumulation, and *vice versa*, we studied the effect of increasing concentrations of non-radioactive PI_A on the accumulation of [$^3H_2$]PII_A (1.9 μM) as well as the effect of increasing PII_A concentration on [$^3H_2$]PI_A (1.8 μM) accumulation. The results presented on Fig. 3 clearly show that the non-radioactive partner has no effect on the accumulation of the other, even at a ratio which has been shown to have synergistic effect *in vivo* (PI_A 25%, PII_A 75%). Therefore, the transport of the two antibiotics seems to be unrelated.

As PI_A is known to be a proton and cation carrier[20], we have also studied the activity of PII_A on *S. aureus* 209P in the presence of other ionophores (valinomycin, gramicidin D, monensin, nigericin, grisorixin, lasalocid and calcimycin) (results not shown). None of these carriers enhanced the antibiotic activity of PII_A.

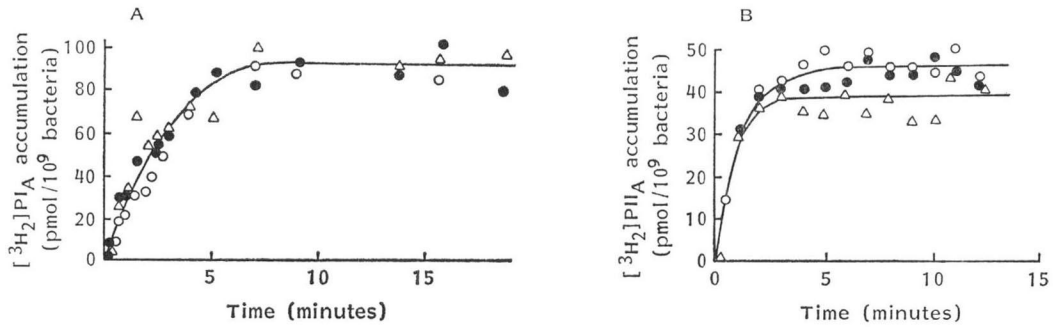
Conclusion

The activity of pristinamycins on *S. aureus* is associated with a rapid accumulation of these mo-

Fig. 3. Effect of unlabelled pristinamycin on radioactive labelled drug accumulation by *S. aureus* 209P.

(A) Resuspended cells were preincubated 10 minutes, at 25°C. [$^3H_2$]PI_A was added to give a final concentration of 1.8 μM without or with PII_A. The ratios between the two antibiotics are: [$^3H_2$]PI_A 100% - PII_A 0% (△), [$^3H_2$]PI_A 50% - PII_A 50% (○) or [$^3H_2$]PI_A 25% - PII_A 75% (●). Accumulation was assayed at 25°C by two phase partitioning method.

(B) Resuspended cells were preincubated 10 minutes, at 37°C. [$^3H_2$]PII_A was added to a final concentration of 1.9 μM without or with PI_A. The ratios between the two antibiotics are: [$^3H_2$]PII_A 100% - PI_A 0% (△), [$^3H_2$]PII_A 50% - PI_A 50% (○) or [$^3H_2$]PII_A 75% - PI_A 25% (●). Accumulation was assayed at 37°C by filtration method.



lecules at 37°C. However, their strong synergistic activity is probably unrelated to antibiotic uptake.

Although the intracellular concentration is higher than that found in the surrounding medium, active transport appears not to be involved: metabolic inhibitors have no effect on their accumulation and increased extracellular concentrations do not lead to a saturation. Accumulation is, in addition, insensitive to inhibition of protein synthesis and sulfhydryl poisons. These data indicate that transport of pristinamycins does not require newly synthesized protein and functional sulfhydryl groups. The actual mechanism by which pristinamycins enter cells remains unclear, but our results suggest for pristinamycin accumulation, a passive diffusion way.

The total cell accumulations after 25 minutes of incubation at 37°C are 11.4 μM for [$^3H_2$]PI_A and 9.6 μM for [$^3H_2$]PII_A, compared to the medium concentration which is 0.2 and 0.4 μM respectively. This difference between external and internal concentrations, could be satisfactorily explained by strong binding of this group of antibiotics to the bacterial ribosome, as has been previously demonstrated[5~12].

Acknowledgments

This research was supported for grants from SPECIA Laboratories.

References

1) Preud'Homme, J.; P. Tarridec & A. Belloc: Pristinamycine: isolement, caracterisation et identification des constituants. Bull. Soc. Chim. Paris 2: 585~591, 1968
2) Vazquez, D.: Streptogramin family of antibiotics. *In* Antibiotics. I. *Ed.*, D. Gottlieb & P. D. Shaw, pp. 387~403, Springer-Verlag, Berlin, 1967
3) Tanaka, N.: Mikamycin. Antibiotics. III. *Ed.*, J.W. Corcoran & F.E. Hahn, pp. 487~497, Springer-Verlag, Berlin, 1975
4) Cocito, C.: Antibiotics of the virginiamycin family, inhibitors which contain synergistic components. Microbiol. Rev. 43: 145~198, 1979
5) Ennis, H. L.: Interaction of vernamycin A with *E. coli* ribosomes. Biochemistry 10: 1265~1270, 1971
6) Ennis, H. L.: Binding of antibiotic vernamycin B to *E. coli* ribosomes. Arch. Biochem. Biophys. 160: 394~401, 1974
7) Parfait, R.; M. P. De Bethune & C. Cocito: A spectrofluorometric study of the interaction between virginiamycin S and bacterial ribosomes. Mol. Gen. Genet. 166: 45~51, 1978
8) Cocito, C. & M. Di Giambattista: *In vitro* binding of virginiamycin M to bacterial ribosomes and ribosomal subunits. Mol. Gen. Genet. 166: 53~59, 1978
9) Abbe, J.; M. L. Capmau, E. Vindimian & F. Le Goffic: Contribution à l'étude du mécanisme d'action des pristinamycines. Eur. J. Med. Chem. 17: 542~546, 1982
10) Moureau, P.; Y. Engelborghs, M. Di Giambasttista & C. Cocito: Fluorescence stopped flow analysis of the interaction of virginiamycin components and erythromycin with bacterial ribosomes. J. Biol. Chem. 258: 14233~14238, 1983
11) Cocito, C.: Properties of virginiamycin-like antibiotics (synergimycins). Inhibitors containing synergistic components. Antibiotics. VI. *Ed.*, J. W. Corcoran & F. E. Hahn, pp. 296~332, Springer-Verlag, Berlin, 1983
12) Contreras, A. & D. Vazquez: Synergistic interaction of streptogramins with the ribosomes. Eur. J. Biochem. 74: 549~551, 1977
13) Mao, J. C. H. & M. Putterman: Accumulation in Gram-positive and Gram-negative bacteria as a mechanism of resistance to erythromycin. J. Bacteriol. 95: 1111~1117, 1968
14) Le Goffic, F.; M. L. Capmau, J. Abbe, J. Charles & J. Montastier: Transformations chimiques de la pristinamycine II en vue de l'étude de son mécanisme d'action. Eur. J. Med. Chem. 16: 69~72, 1981
15) Moreau, N.; P. Lacroix & L. Fournel: Antibiotic uptake by bacteria as measured by partition in polymer aqueous phase systems. Anal. Biochem., in press., 1984
16) Coolins, S. H. & N. A. Hamilton: Magnitude of the protonmotive force in respiring *Staphylococcus aureus* and *Escherichia coli*. J. Bacteriol. 126: 1224~1231, 1976
17) Bryan, L. E. & H. M. Van Den Elzen: Streptomycin accumulation in susceptible and resistant strains of *Escherichia coli* and *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 9: 928~938, 1976
18) Bryan, L. E. & H. M. Van Den Elzen: Effects on membrane-energy mutations and cations on strepto-

mycin and gentamicin accumulation by bacteria: a model for entry of streptomycin and gentamicin in susceptible and resistant bacteria. Antimicrob. Agents Chemother. 12: 163~177, 1977

19) HUTCHINGS, B. L.: Tetracycline transport in *Staphylococcus aureus* H. Biochem. Biophys. Acta 174: 734~748, 1969

20) GRELL, E.; I. OBERBAUMER, H. RUF & H. P. ZINGSHEIM: Elementary steps and dynamic aspects of carrier-mediated cation transport through membranes: the streptogramin antibiotics (group B). Proceedings in Life Sciences, Biochemistry of Membrane Transport, FEBS Symposium No. 42, *Ed.*, G. SEMENZA & E. CARAFOLI, pp. 147~178, Heidelberg, Berlin, N.Y., 1977