IRREVERSIBLE BINDING OF PRISTINAMYCIN II_A (STREPTOGRAMIN A) TO RIBOSOMES EXPLAINS ITS "LASTING DAMAGE" EFFECT

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In vitro and *in vivo* studies are presented to test the hypothesis that the synergistic action of the pristinamycins is not due to a catalytic effect of pristinamycin II_A (PII_A) on the bacterial ribosome. We demonstrate that there is a proportionality between the quantity of PII_A bound on the ribosome and pristinamycin I_A (PI_A) retained by it. Moreover *in vitro* and *in vivo* experiments correlated to biological effects (growth and protein synthesis) demonstrate that pristinamycin II_A is tightly bound on 70S ribosome, which satisfactory explains the so called "lasting damage effect".

The pristinamycins (PI_A and PII_A) act separately as bacteriostatic agents but in combination are synergistic and strongly bactericidal. The mechanism by which these molecules act synergistically has been investigated. COCITO and colleagues have proposed the following model[1]: virginiamycin M, a molecule identical to pristinamycin II_A[2], acts catalytically by inducing an irreversible conformational modification of the 50S subunit of the bacterial ribosome. As a result of this conformational change (called "lasting damage") there is an increase in virginisamycin S (identical in structure to pristinamycin I_A) binding to the 50S subunit which could explain the synergistic effect noticed.

We have studied the interactions of reduced pristinamycins II_A with ribosomes (H_2PII_A (α) and (β))[3] and reached the conclusion that these molecules behave like PII_A in terms of their bacteriological effect as well as synergism with PI_A. We have demonstrated that H_2PII_A (α) binds so tightly to *Escherichia coli* ribosomes that neither gel filtration nor analytical centrifugation are able to dissociate the complex. This finding encouraged us to reinvestigate the synergistic effect of pristinamycin II_A; both *in vivo* or *in vitro* experiments clearly show that PII_A binds stoichiometrically and irreversibly to the bacterial ribosome which explains the so called "lasting damage" effect.

Materials and Methods

Materials

Tris was obtained from Merck, $Mg(OAc)_2$ and NH_4Cl from Prolabo.

The pristinamycins were a gift from Rhône-Poulenc. The tritiated pristinamycins, [^{3}H]H_2PII_A (α), specific activity of 1.17 Ci/mmol or 2.18 Ci/mmol, were synthethized as described earlier[3].

Norit was from Sigma.

Buffers used are: Buffer A: Tris-HCl 30 mM (pH 7.4), $Mg(OAc)_2$ 10 mM, KCl 100 mM. Buffer B: Tris-HCl 10 mM (pH 7.6), $MgCl_2$ 0.1 mM to which is added sucrose (BDH) 20% and lysostaphin (Sigma) 0.02 mg/ml and 5 μg/ml DNAse (Boehringer) RNAse free. Buffer C: Tris-HCl 10 mM (pH 7.6), $MgCl_2$ 10 mM.

List of abbreviations: PI_A; pristinamycin I_A, PII_A; pristinamycin II_A, [^{3}H]H_2PI_A; tritiated dihydropristinamycin I_A, [^{3}H]H_2PII_A; tritiated dihydropristinamycin II_A, H_2PII_A; reduced pristinamycin II_A.

Staphylococcus aureus 209P strain sensitive to both pristinamycins, was from our collection. The Tryptic Soy Broth medium was from Merieux.

The "tight" 70S ribosomes were obtained following NOLL's method[4] from *Escherichia coli* MRE 600 strain (Microbial Research Establishment Paton England). Gel filtrations were achieved either with Sephadex G-25 or Sephacryl S-200.

Methods

Binding Experiments

a: The "tight" 70S ribosomes (8.6×10^{-7} M) were incubated for 25 minutes at 37°C in the presence of [^{3}H]H_2PII_A (α) in buffer A. To each 200 μl was added 30 μl of a 2.5% (w/v) suspension of Norit. Each sample was then centrifuged for 15 minutes at 4,500 rpm. Radioactivity was evaluated on an aliquot of 100 μl of supernatant in a TM HP/b scintillation liquid (Beckman).

b: The "tight" 70S ribosomes 10^{-6} M were incubated in buffer A with PII_A (4×10^{-6} and 2×10^{-8} M) respectively and [^{3}H]H_2PI_A (2×10^{-6} M). An aliquot of this solution 300 μl was placed on a Sephadex G-25 column which was eluted with buffer A. Elution was followed at 260 nm. Radioactivity eluted was determined on an aliquot of each fraction as established earlier.

Cell Growth and Viability

A preculture of *S. aureus* 209P was introduced into a Tryptic Soy Broth medium (TSB). At a concentration of 2×10^8 cells per ml, the PI_A, PII_A, PI_A+PII_A antibiotics, at chosen concentrations, were then added. The total incubation time was 4 hours at 37°C during which growth was measured by turbidimetry (Klett filter absorbtion max 500~550 nm) and viability was estimated as usual. These measurements were made at various times during incubation.

In Vivo Protein Synthesis

S. aureus bacteria growth was started, at a Klett index corresponding to 2×10^8 cells per ml and the various antibiotics were added to the concentrations specified. Incubation lasted 30 minutes at 37°C.

The bacteria were centrifuged 10 minutes at 5,000 rpm, the pellets were washed twice with 20 ml of TSB. Each test had a volume of 6 ml and contained 100 μl of [^{3}H]lysine (50 mCi/mmol, 100 μCi/ml) and 100 μl of [^{3}H]phenylalanine (50 mCi/mmol, 100 μCi/ml). The kinetics of synthesis were observed by measuring the incorporation of radioactive amino acids in a 1 ml aliquots to which was added at 0°C 1 ml of a 10% TCA solution. The precipitate was collected on a GF/C filter impregnated with 1% TCA, and washed with 5×3 ml 1% TCA. The filter was dried and radioactivity determined by liquid scintillation.

Role of PII_A on the Retention of [^{3}H]H_2PII_A (α) by Ribosomes

In Vitro: The experiment was composed of three tests treated in a strictly identical way. The 70S "tight" ribosomes of *E. coli* MRE 600 8.6×10^{-7} M were incubated for 25 minutes at 37°C in buffer A;

(I) in the presence of 2×10^{-8} M of [^{3}H]H_2PII_A (α) (2.18 Ci/mmol)

(II) in the presence of PII_A

(III) a blank was made with [^{3}H]H_2PII_A (α) 2×10^{-8} M alone.

The volume of assay test was 1 ml. After incubation the ribosomes were dialyzed in 100 ml of buffer A for 16 hours at 4°C.

The ribosomes of test (II) were reincubated in the presence of [^{3}H]H_2PII_A (α) at 37°C for 30 minutes. This preparation was dialyzed under the same conditions as before.

The radioactivity incorporated into the ribosome in each case was measured and compared to optical density.

In Vivo: Antibiotics were added to *S. aureus* 209P cultures containing 2×10^8 cells/ml (see Fig. 6) and incubated for 30 minutes at 37°C; the bacteria were washed as before and resuspended in 20 ml of TSB. The culture was poured onto 20 ml of frozen buffer B. The mixture was left to thaw at room temp and centrifuged for 15 minutes at 5,000 rpm. The pellet was frozen at −80°C and 2 ml of buffer C added; the mixture was left to thaw at room temp and centrifuged for 30 minutes at 16,000 rpm. The supernatant recovered constitutes the S-30 fraction; 30 OD units were placed on a column of Sephacryl S-200, eluted with buffer A and followed by measuring the optical density at 260 nm. Radioactivity was

measured on an aliquot of each fraction by liquid scintillation.

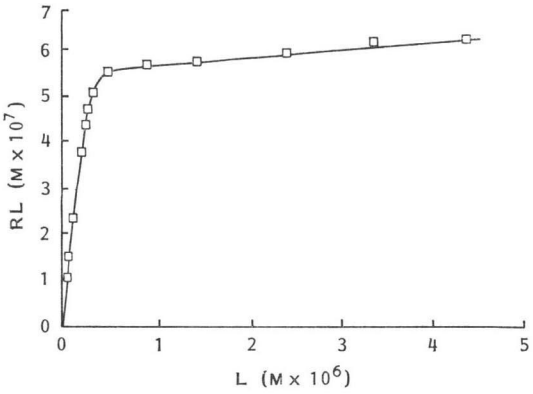
Analysis by centrifugation in a 5~30% sucrose gradient for 16 hours at 20,000 rpm in a SW41 Beckman rotor shows this S-30 fraction similar to S-30 obtained by grinding and contains the ribosomal entities 30, 50 and 70S.

Direct Proof that PII_A Remains Bound to 70S Ribosomes

a: 1 ml of a solution of PII_A 1.08×10^{-6} M was dialyzed at 4°C in 100 ml of buffer A for 16 hours.

b: 1 ml of a mixture of PII_A 1.08×10^{-6} M and ribosomes 1.08×10^{-5} M was incubated 30 minutes at 37°C and dialyzed at 4°C in 100 ml of buffer A for 16 hours.

Fig. 1. Binding of $[^3H]H_2PII_A$ (α) to *E. coli* MRE 600 70S ribosomes (8.6×10^{-7} M) as a function of free $[^3H]H_2PII_A$ (α) concentration.



The two solutions were then lyophilized to reduce their volumes to 4~5 ml, extracted by 4×6 ml of methylene chloride which was then dried with sodium sulfate. The residue obtained after removed of sodium sulfate and solvent was dissolved in 200 μl of EtOH.

The amount of extracted pristinamycin was determined according to DUBOST's method[5].

Results

The Action of Pristinamycin II_A is Stoichiometric and not Catalytic

Fig. 1 shows the saturation curve of *E. coli* MRE 600 70S "tight" ribosomes in relation to increasing quantities of free $[^3H]H_2PII_A$ (α).

Fig. 2. Filtration of $[^3H]H_2PI_A$ (all) *E. coli* MRE 600 70S ribosomes complex on Sephadex G-25 gel. For incubation condition see Materials and Methods.

a; UV absorbance at 260 nm. b; Radioactivity in dpm in the presence of PII_A 2×10^{-8} M. c; Radioactivity in dpm in the presence of PII_A 4×10^{-6} M.

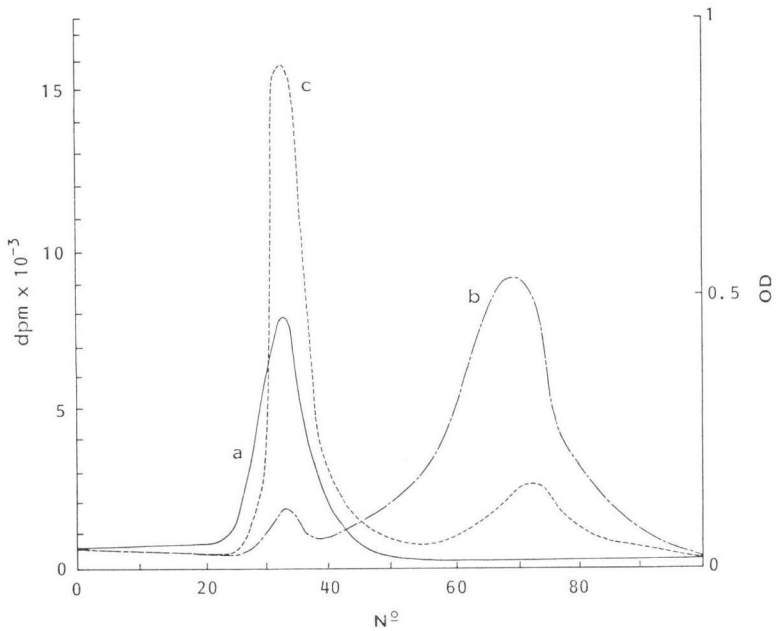


Fig. 3. *S. aureus* 209P growth.

● Without pristinamycins, △ in the presence of PI_A (8 μg/ml), □ in the presence of PII_A (2 μg/ml), ○ in the presence of PI_A+PII_A (0.5 μg/ml).

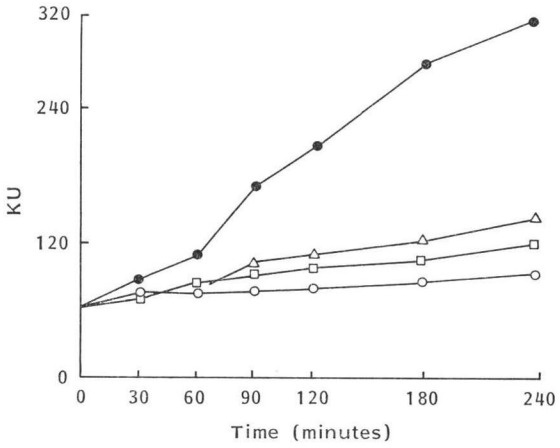


Fig. 4. Viability of *S. aureus* 209P as function of time.

Time zero is determined by the addition of the antibiotics. The cells were counted on drug free agar.

● Without antibiotic, △ with PI_A (8 μg/ml), □ with PII_A (2 μg/ml), ○ with PI_A+PII_A (0.5 μg/ml).

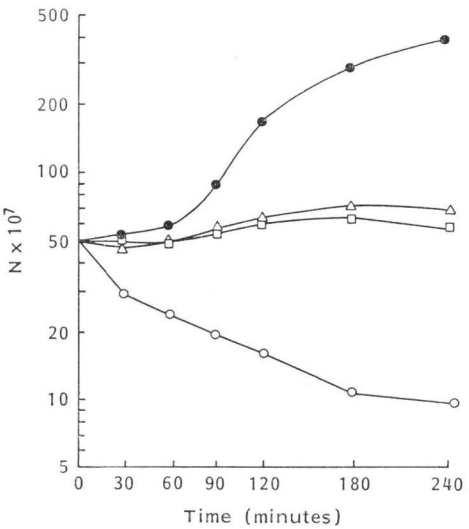
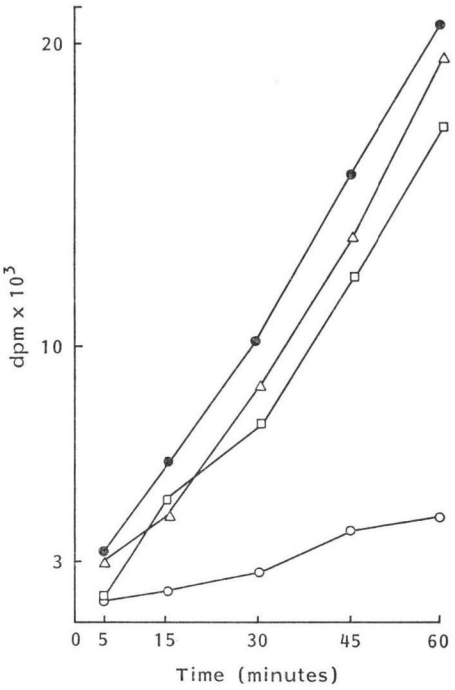


Fig. 5. Effect of the pristinamycins on protein synthesis.

● Without antibiotic, △ with PI_A (0.8 μg/ml), □ with PII_A (0.08 μg/ml), ○ with PI_A+PII_A (0.88 μg/ml).

Experimental conditions are described in Materials and Methods.



The saturation of a single ribosome site is obtained with 1.6×10^{-6} M [^{3}H]H_2PII_A.

Fig. 2 represents the exclusion profile of ribosomes incubated with [^{3}H]H_2PI_A (all)[3] in the presence of two concentrations of PII_A: A clear-cut correlation between the amount of PII_A present and that of [^{3}H]H_2PI_A (all) retained is observed; 1 mol of PII_A retained on the receptor site corresponds to 1 mol of [^{3}H]H_2PI_A. Similar elution profiles were obtained with dihydrogenated PII_A (results not shown).

Growth and Viability of *S. aureus* 209P in the Presence of the Pristinamycins

Although such experiments have been described by others[8] our results are shown to allow comparisons under our experimental conditions.

Fig. 3 represents the growth curve of the pristinamycin-sensitive *S. aureus* 209P strain and Fig. 4 the corresponding viability curves. The bacteriostatic activity of each antibiotic as well as the strong bactericidal effect of PI_A, PII_A combinations is readily seen.

Table 1. Summary of experimental conditions and results which proof that PII_A compete with $[^3H]H_2PII_A$ for binding to ribosomes and remains bound to it even after dialysis.

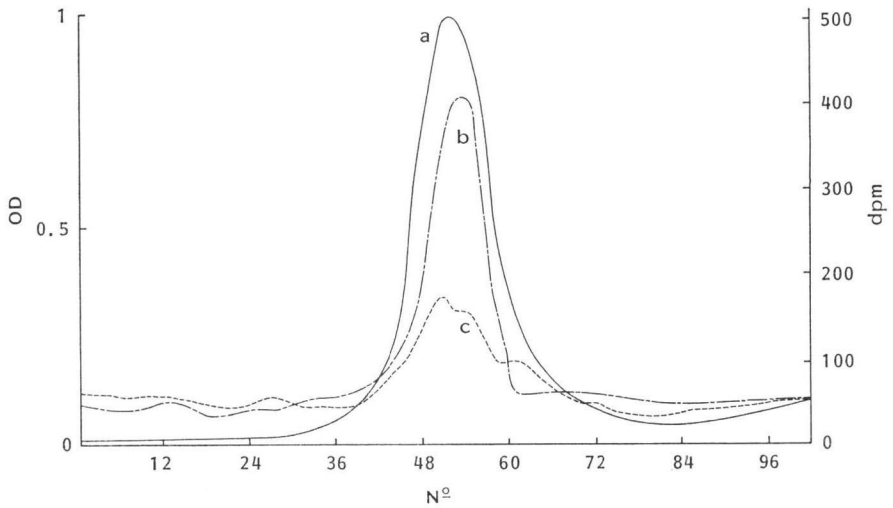
Experiment	Ribosome molarity (Y)	Radioactivity retained on ribosome after dialysis (%)	Corresponding molarity of $[^3H]H_2PII_A$ (α) (X)	X/Y
A	—	0.7	—	—
B	1.137×10^{-6}	87.5	1.75×10^{-8}	1.54×10^{-2}
C	1.05×10^{-6}	30.3	0.6×10^{-8}	0.57×10^{-2}

Experimental conditions: Experiment A; $[^3H]H_2PII_A$ (α) (2×10^{-8} M) alone dialysis, experiment B; $[^3H]H_2PII_A$ (α) (2×10^{-8} M)+ribosomes dialysis, experiment C; PII_A (9×10^{-6} M)+ribosomes dialysis+ $[^3H]H_2PII_A$ (α) (2×10^{-8} M) dialysis.

Fig. 6. *In vivo* retention of PII_A by *S. aureus* 209P ribosomes.

Extracted ribosomes were filtered through Sephacryl S-200 gel.

a; UV absorbance at 260 nm. b; Radioactivity on the ribosome when *S. aureus* is grown in the presence of $[^3H]H_2PII_A$ (α) (0.16 μg/ml). c; Radioactivity on the ribosomes when *S. aureus* is grown in the presence of PII_A (0.16 μg/ml)+$[^3H]H_2PII_A$ (α) (0.16 μg/ml).



In Vivo Protein Synthesis: An Expression of Synergy

Fig. 5 represents the incorporation of [^{3}H]lysine and of [^{3}H]phenylalanine by growing bacteria treated with sub-inhibitory quantities of PI_A, PII_A and PI_A+PII_A.

The difference in amino acid incorporation between the control and the bacteria treated with PI_A is small. Only a slight decrease in the incorporation of radioactivity is observed in bacteria treated by PII_A alone, whereas 78% inhibition of protein synthesis occurs in the presence of both agents.

Role of PII_A on the Retention of $[^3H]H_2PII_A$ (α) on Bacterial Ribosome

In Vitro

Table 1 gives the results of experiments which indirectly show that pristinamycin II_A is not removed from the bacterial ribosome by simple dialysis. Ribosomes incubated with PII_A, dialyzed, and then incubated with $[^3H]H_2PII_A$ (α) do not bind as much radioactive material as do ribosomes directly incubated with $[^3H]H_2PII_A$ (α) (compare the ratio X/Y in the experiments B and C).

In Vivo

Fig. 6 represents gel exclusion profiles of *S. aureus* 209P ribosomes isolated from the strains cultured:

— in the presence of [^{3}H]H_2PII_A (α) alone

— in the presence of PII_A+[^{3}H]H_2PII_A as specified in Materials and Methods.

The absorption at 260 nm represents a mixture of 30S, 50S and 70S particles. These particles cannot be separated by Sephacryl S-200 gel. However the amount of 70S ribosome can be assessed by centrifugation (not shown); the radioactivity observed in the ribosomal peak is most likely due to binding to 70S[1].

This also demonstrates that PII_A and [^{3}H]H_2PII_A (α) compete for the same binding site on the ribosome as expressed by the decrease in radioactivity when bacteria are incubated with both molecules. This competition reinforces the similarity of the two compounds[3].

Direct Proof that PII_A Remains Bound to 70S Ribosomes

In the absence of ribosomes, on dialysis, 34% of PII_A is recovered from the bag; in the presence of ribosomes the quantity of PII_A found outside the bag decreases to 2.8% of the initial radioactive material.

We conclude that, considering the pristinamycin lost during the experiment, approximately 90% of PII_A remains bound to ribosomes after dialysis.

Discussion

An important question concerning the mechanism of action of the pristinamycins is the following: Does pristinamycin II_A have a catalytic effect on the bacterial ribosome or does it bind tightly to its receptor site to induce a conformational modification of the ribosome with a consequent effect on protein synthesis?

It has been shown previously[3,7] that the affinity of pristinamycin I_A for the ribosome is increased by a factor of 3 to 6 in the presence of PII_A without any increase of the number of molecules bound. However the ratio PI_A/PII_A bound to the particle was not indicated. From the present experiments it appears that one molecule of [^{3}H]H_2PI_A, a compound closely related to PI_A, is retained on the ribosome when it is saturated with PII_A; this observation is not consistent with the proposal of a catalytic effect.

The *in vivo* experiments concerning bacterial growth and cell viability confirm our previous findings and allow us to relate the synergistic effect produced by PI_A+PII_A and the decrease in cells viability; such experiments also provide information on antibiotic interactions with the ribosome. A strong synergistic effect is noticed after the cells have been incubated with PII_A+PI_A, washed and cultured in an antibiotic free medium. Analysis of the ribosomes isolated from cells treated with [^{3}H]H_2PII_A shows that they retain radioactive antibiotic and that there is a strong competitive effect of PII_A versus [^{3}H]H_2PII_A (α). We also note that this type of competition is seen *in vitro*. In addition we show clearly that PII_A is irreversibly bound to the ribosome.

There is a strong contradiction between the experiments reported here and those previously described, which indicate that, the PII_A ribosome interaction has a K_D of 10^{-7} M[3]. This binding, which requires 30 minutes at 37°C[7], is probably a multistep process, the experiment measures only the final binding level.

We conclude that *in vivo* and *in vitro* there is a strong relationship between the stoichiometric binding of PII_A and the potentiated binding of PI_A. It is obvious that the so called "lasting damage" is the biochemical expression of the tight binding of PII_A to its receptor site which irreversibly modifies ribosome conformation.

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