Pyrronamycin A and B, Novel Antitumor Antibiotics Containing Pyrrole-amide Repeating Unit, Produced by *Streptomyces* sp.

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DNA and DNA topoisomerases have been shown to be the principal cellular target for a number of clinically important antitumor agents[1~3]. We developed a new assay using *Bacillus stearothermophilus* NUB3620 and bacteriophage TP-68 to detect potential antitumor compounds acting on DNA or topoisomerases[4]. In order to discover the new lead compounds targeting on DNA or DNA topoisomerases, we have screened cultures of actinomycetes and fungi for their ability to inhibit selectively the growth of bacteriophage TP-68 at the concentration that shows no apparent antibacterial activity against NUB3620. In the course of microbial screening, strain KY11768 was identified to produce active metabolites. We described here production including fermentation and isolation, and biological activity of novel pyrrole-amide antitumor antibiotics, pyrronamycin A and B, produced by *Streptomyces* sp.

The producing organism KY11768 was isolated from a soil sample collected in Okazaki city, Aichi prefecture, Japan and assigned to the *Streptomyces* sp. Fermentation was carried out at 25°C for 42 hours with appropriate aeration and agitation in 30-liter jar fermenter containing the 15 liters of culture medium consisting of glycerol 5%, dry yeast 1.5%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $Mg_3(PO_4)_2 \cdot 8H_2O$ 0.05%, pH 7.0. Pyrronamycin A and B were accumulated in the culture medium. The culture filtrate was applied to a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited). The column was washed with deionized water - MeOH (7 : 3) and eluted with deionized water - MeOH (6 : 4). The active eluate was concentrated and applied to a column of Diaion HP-20SS. The column was washed with deionized water - MeOH (7 : 3) and eluted with deionized water - MeOH (6 : 4). The active fraction was concentrated, and then purified through HPLC using a packed ODS column (HG-5, Nomura Chemical Co.) under the conditions, in which the fraction was developed with developers of 5 to 10% CH_3CN/0.5% TFA. Thus a fraction containing pyrronamycin B (retention time 26 minutes) and a fraction containing pyrronamycin A (retention time 30 minutes) were obtained separately. Each fraction was concentrated and further fractionated through HPLC, and resulting active fraction was concentrated and then lyophilized. Thus 2.5 mg of pyrronamycin A and 10 mg of pyrronamycin B were obtained as water-soluble white solids. Large scale preparation with 2k-liter tank fermenter afforded 604 mg of pyrronamycin B. Pyrronamycin A and B were readily soluble in H_2O, MeOH and DMSO but insoluble in $CHCl_3$ and *n*-hexane. ^1H, ^{13}C NMR and further ^{15}N NMR spectroscopic studies with ^{15}N labeled pyrronamycin B elucidated that both products were novel antibiotics containing pyrrole-amide repeating unit that is present in the antiviral antibiotic distamycin A[5] and its related compounds (Figure 1). Details of structure determination with preparation of ^{15}N labeled pyrronamycin B will be reported elsewhere[6].

Antibacteriophage activity[4] of pyrronamycin A was comparable to that of pyrronamycin B on the growth of *Bacillus stearothermophilus*-infective phage (Table 1), while distamycin A (Sigma) did not show activity at amounts up to 10 μg/disc. Adriamycin and m-AMSA, both of which act on topoisomerase II, showed antibacteriophage activity in this assay system[4], while pyrronamycin A and B did not show the effect on mammalian topoisomerase I or II *in vitro* and in cells (data not shown).

In the absence of topoisomerases, pyrronamycin B induced a band shift of supercoiled pBR322 in a dose dependent manner as shown in Figure 2. Since the mobility of retarded band (S′ in Figure 2) was different from that of relaxed or linear form of DNA, the shifted band seemed to be composed of drug-DNA complex. Structurally related antibiotic distamycin A is known to bind to minor groove of AT-rich sequence on DNA[7]. The pyrrole-amide skeleton of distamycin A have been utilized for synthesizing the small

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Fig. 1. Structures of pyrronamycin A and B and related antibiotic distamycin A.

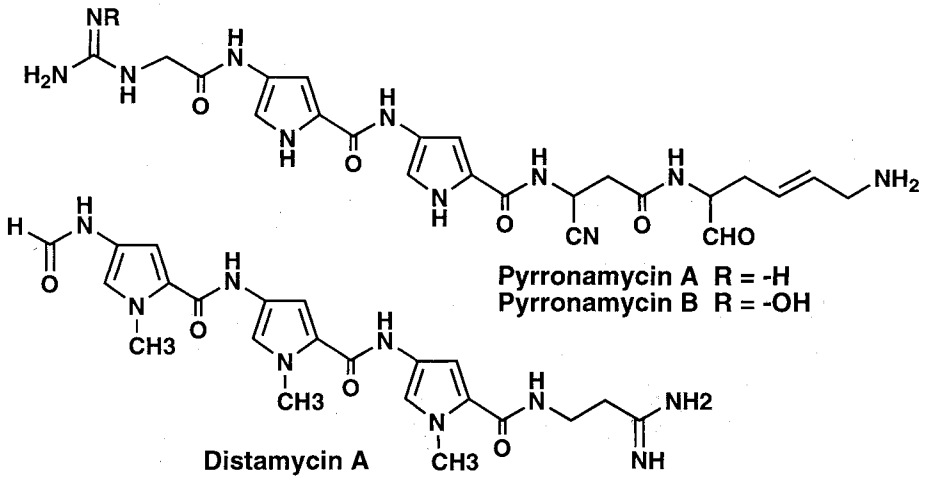


Table 1. Antibacteriophage activity of pyrronamycin A and B and distamycin A.

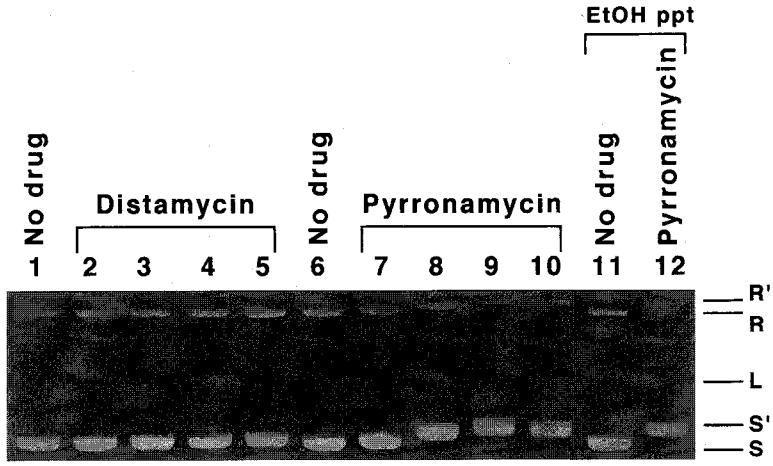
Compounds	Activity (diameter mm)	
	2 μg / disc	10 μg / disc
Pyrronamycin A	20	23
Pyrronamycin B	21	24

molecule that recognize specific sequence of DNA[8]. The structures of pyrronamycin A and B strongly suggest that these compounds belong to this class of DNA minor groove binder that recognize specific DNA sequence. Our preliminary results indicate that pyrronamycin B preferentially binds to AT-rich sequence as distamycin A does. On the basis of these results, it is likely that antibacteriophage activity of pyrronamycins is due to the interaction of drug with DNA.

The similar band shift of pBR322 was seen (A. ASAI *et al.*, unpublished results) by treatment with DNA-alkylating agent duocarmycin A[9]. Ethanol precipitation of the DNA treated with pyrronamycin B did not affect to the band shift, indicating that pyrronamycin B induces the complex with DNA mediating the stable bond such as covalent one. On the other hand, distamycin A did not induce the band shift under these conditions. Distamycin A is known as a non-alkylating DNA minor groove binder, and possesses the reversible DNA binding property[7]. These results indicate that mode of DNA binding of pyrronamycin B is distinct from that of distamycin A. In addition, pyrronamycin A and B exhibited more potent antimicrobial activity than distamycin A (Table 2). Both of them showed potent antimicrobial activity against not only Gram-positive but also Gram-negative bacteria. It is noteworthy that pyrronamycin A and B showed potent activity against several Gram-negative bacteria because these bacteria are usually insensitive to known antitumor antibiotics. Regarding the effect on cell proliferation, pyrronamycin A (IC_{50}: 1.2 μM) and B (IC_{50}: 1.0 μM) showed the comparable cytotoxic activity against HeLa S3 cells on the experiments of 72 hours exposure. In contrast, distamycin A showed no apparent cytotoxic activity (IC_{50}: >100 μM) under the same conditions.

Evaluation on murine tumor models *in vivo* revealed that pyrronamycin B showed antitumor activity against sarcoma 180 (T/C 0.44) at a single dose of 0.38 mg/kg and also induced marginal regression of human lung carcinoma A549 (T/C 0.51) inoculated into nude mice at a single dose of 0.25 mg/kg. Distamycin A is known to be inactive as antitumor drug presumably due to the non-alkylating property. However, it has been reported that a synthetic distamycin A analog bearing DNA-alkylating moiety, FCE24517, showed prominent antitumor activity in murine tumor models and is under clinical evaluation[10,11]. These results imply that a stable complex formation of pyrronamycin A and B with DNA could contribute to the superior biological activity of these antibiotics to that of

Fig. 2. Pyrronamycin B induces a band shift of pBR322.



pBR322 (0.05 mM base pair) was incubated with or without drug in 10 mM phosphate buffer at 37°C for 30 minutes. After incubation, 1% SDS was added to the reaction mixture (final 0.1%) and then applied to agarose gel electrophoresis.

Lanes 1, 2, 3, 4 and 5-pBR322 with 0, 0.32, 1.6, 8.0 and 40 μM of distamycin A; Lanes 6, 7, 8, 9 and 10-pBR322 with 0, 0.32, 1.6, 8.0 and 40 μM of pyrronamycin B; Lanes 11 and 12-EtOH-precipitated pBR322 after incubation with or without 40 μM of pyrronamycin B.

Relax, shifted relax, linear, supercoil and shifted supercoil form of DNA were referred as R, R', L, S, and S', respectively.

Table 2. Antimicrobial activity of pyrronamycin A and B and distamycin A.

Test microorganisms	MIC (μg / ml)		
	Pyrronamycin		Distamycin
	A	B	
Bacillus subtilis No.10707	0.08	0.33	10.4
Staphylococcus aureus subsp. *aureus* ATCC6538P	2.6	5.2	>100
Enterococcus hirae ATCC10541	2.6	10.4	>100
Escherichia coli ATCC26	0.16	0.08	83.3
Klebsiella pneumoniae subsp. *pneumoniae* ATCC10031	0.04	0.04	83.3
Pseudomonas aeruginosa BMH No.1	>100	>100	>100
Proteus vulgaris ATCC6897	0.16	0.33	>100
Shigella sonnei ATCC9290	0.16	0.16	>100
Salmonella choleraesuis subsp. *choleraesuis* ATCC9992	0.16	0.33	>100
Candida albicans ATCC10231	10.4	41.7	>100

distamycin A. We have not yet proved whether the mode of stable complex formation is attributable to the DNA-alkylation. Further examination on the mode of DNA-binding of the pyrronamycin B and evaluation for their *in vivo* antitumor activity are underway.

Table 3. Antitumor activity of pyrronamycin B on murine sarcoma 180.

Compounds	Schedule	Dose (mg/kg)	T/C (%)
Control		0	100
Pyrronamycin B	Day 1	0.19	60
		0.38	44
		0.75	22 (1/5)*
Mitomycin C	Day 1	6	21

Sarcoma 180 cells (5×10^6 cells/mouse) were inoculated sc on day 0. Drugs were administrated iv on each day and the tumor volume was measured on day 7.

* Number of toxic mouse death.

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