CALCIUM-DEPENDENT ANTICANDIDAL ACTION OF PRADIMICIN A

YOSUKE SAWADA, KEI-ICHI NUMATA, TSUTOMU MURAKAMI, HARUMI TANIMICHI, SATOSHI YAMAMOTO and TOSHIKAZU OKI

Bristol-Myers Research Institute, Ltd., Tokyo Research Center, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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Pradimicin A shows candicidal activity at 10 μg/ml *in vitro*. The action of pradimicin A on *Candida albicans* cells involves a set of specific cell surface interactions in a Ca^{2+}-dependent manner. These include binding to the mannan components on the cell surface and subsequent interactions at the level of the plasma membrane, causing K^+ leakage and cell death. The protoplasts prepared from *C. albicans* undergo lysis rapidly when treated with pradimicin A. These results suggest that pradimicin A acts primarily on the candidal plasma membrane, leading to a perturbation of membrane function.

Pradimicin A produced by *Actinomadura hibisca* sp. nov. Strain No. P157-2 (ATCC 53557) is a potent antifungal antibiotic possessing a benzo[*a*]naphthacenequinone nucleus substituted with D-alanine and two sugars.[1,2] It has a broad antifungal spectrum against a wide variety of pathogenic fungi *in vitro* and is highly effective against systemic infections with *Candida albicans*, *Cryptococcus neoformans* or *Aspergillus fumigatus* in mice.[3] The acute toxicity of pradimicin A in mice was much less than that of amphotericin B.[4] There is no cross-resistance to other antifungal agents such as amphotericin B, 5-fluorocytosine and ketoconazole.[3,4] Pradimicin A is thus chemically as well as functionally different from the other major classes of antifungal agents.

This report describes studies on the biochemical effects of pradimicin A on *C. albicans* in an attempt to provide a biochemical basis for its biological activity.

Materials and Methods

Microorganism

C. albicans A9540, a standard laboratory strain, was used throughout this study. It was grown overnight at 37°C on a slant of YPD agar medium (glucose 2%, Polypeptone 2%, yeast extract 1% and agar 1.6%, pH 7.2 before autoclaving) and maintained at 4°C until used.

Chemicals

Pradimicin A was isolated and purified as its monosodium salt.[1,2] It does not contain either K^+ or Ca^{2+} ion in quantities detectable by a Flame Spectrophotometer (Type 170-30, Hitachi Co., Tokyo). Amphotericin B and ethyleneglycol bis (β-aminoethylether)*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (St. Louis, MO.). L-[4,5-^{3}H]Leucine (52 Ci/mmol) and Zymolyase 100T were purchased from Amersham Japan Co. (Tokyo) and Kirin Brewery Co. (Tokyo), respectively. Deionized water was obtained by a water distiller (Still Ace SA-27E, Tokyo Rikakikai Co., Tokyo). Pradimicin A and amphotericin B were dissolved first in DMSO and then diluted with water to give a final DMSO concentration of 10% as the stock solutions. Other chemicals were dissolved in deionized water.

Media and Buffers

YNBG-PB medium was prepared by mixing equal parts of twice-concentrated YNBG (Difco yeast nitrogen base (YNB) 1.34% and glucose 2%) and 1/7.5 M phosphate buffer (pH 7.0). YNBG-PB contains

approximately 1 mM of Ca^{2+}. A Ca^{2+}-free YNBG-PB medium was prepared by mixing Ca^{2+}-free ingredients listed for YNB in Difco's manual and phosphate buffer. Glucose was sterilized separately. Sodium phosphate buffer (50 mM, pH 7.0) was used as the medium for the non-growing experiment.

Determination of Viable Cells

The number of viable cells was determined by counting the colonies grown on YPD agar plates which were incubated at 27°C for 48 hours after plating. The number of viable cells is expressed as cfu per ml. Cells were cultured in YPD medium for 18 hours at 27°C with rotary shaking (180 rpm), harvested by centrifugation (3,500 rpm, 5 minutes) and washed twice with sterilized saline (washed cells). For the growing experiments the washed cells were resuspended in YNBG-PB and adjusted to 1×10^4 cfu/ml. For the non-growing experiments the washed cells were resuspended in sodium phosphate buffer (pH 7.0) yielding a final inoculum of 1×10^7 cfu/ml.

Determination of Growth Inhibition

Growth inhibition of *C. albicans* was determined by the microdilution turbidimetric method using flat-bottom microtiter plates (96 wells). Two hundred μl of the washed cells resuspended in Ca^{2+}-free YNBG-PB (1.25×10^4 cfu/ml), 25 μl of varying concentrations of $CaCl_2$ and 25 μl of pradimicin A solution (250 μg/ml) were mixed. The microtiter plate was covered with a lid and incubated for 20 hours at 37°C. The OD at 620 nm of each well was read by an automatic reader (Titertek Multiskan MCC, Labsystem and Flow Lab.). On the basis of OD value of the pradimicin A-treated cultures relative to those of the untreated control cultures (0% inhibition), the percent inhibition by pradimicin A was calculated.

Measurement of K^+ Release

The washed-cell suspension (1×10^7 cfu/ml) in sodium phosphate buffer (pH 7.0) was mixed with $CaCl_2$ or EGTA and pradimicin A in a total volume of 1.0 ml, and incubated with occasional shaking at 37°C. At intervals samples were taken and centrifuged at 15,000 rpm for 30 seconds at 4°C. The supernatant was collected and its K^+ level was measured by a Flame Spectrophotometer. The relative content of K^+ is expressed as percent of K^+ concentration in the supernatant from the cells heated at 95°C for 10 minutes. Each point represents the average of two determinations.

Preparation of *C. albicans* Protoplasts

The washed cells were further rinsed twice with saline and once with 67 mM phosphate buffer (pH 7.4) containing 0.2% 2-mercaptoethanol and 60 mM EDTA. The cells were resuspended in 67 mM phosphate buffer (pH 7.4) containing 0.2% 2-mercaptoethanol and 0.6 M KCl to give approximately 1×10^8 cfu/ml. To the cell suspension Zymolyase 100T suspended in the same buffer was added to give a concentration of 100 μg/ml; the mixture was incubated with gentle shaking at 37°C for 2 hours. The formation of protoplasts was followed roughly by diluting a sample of the incubation mixture 10-times with water and checking the number of remaining cells. The resulting protoplasts were spun down for 5 minutes at 3,500 rpm at room temperature. The pellet was washed 3 times by centrifugation in 0.8 M sorbitol and resuspended in YNBG-PB supplemented with 0.8 M sorbitol. The number of protoplasts was determined by counting on a hemocytometer slide (Kayagaki Co., Tokyo).

Incorporation of Radioactivity

The washed cells (or protoplasts) were resuspended in YNBG-PB (or YNBG-PB plus 0.8 M sorbitol) at 1×10^7 cells (or protoplasts)/ml. To the suspension with or without EGTA pradimicin A was added in a total volume of 1.0 ml, mixed, and 40 μl of [^{3}H]leucine (final 0.4 μCi/2 nmol) was added. The mixture was incubated for 60 minutes at 37°C with occasional shaking. One ml of 10% TCA was added to the reaction mixture. The mixture was filtered through a glass-microfilter GF/A (Whatman Co., Maidstone, England). The filter was extensively washed with 5% TCA and dried under air. Radioactivity incorporated into TCA-insoluble materials was measured in a toluene scintillation cocktail using a liquid scintillation counter (LSC-700, Aloka Co., Tokyo).

Binding of Pradimicin A

The washed cells (or protoplasts) were resuspended in YNBG-PB (or YNBG-PB plus 0.8 M sorbitol)

yielding an inoculum of 1×10^7 cfu (or protoplasts)/ml. The suspension was treated with varying concentrations of pradimicin A, left to stand for 5 minutes at room temperature, and then centrifuged at 10,000 rpm for 1 minute. The concentration of pradimicin A in the supernatant was determined by measuring the OD at 490 nm. The amount of binding to cells (or protoplasts) was estimated by subtracting the concentration of pradimicin A in the supernatant from the initial concentration. EGTA at a final concentration of 2 mM was added to the suspension to neutralize Ca^{2+} ion before addition of pradimicin A.

Results

Effect on Macromolecular Biosynthesis and Interaction with DNA

To determine the effect of pradimicin A on macromolecular synthesis, we measured the incorporation of radiolabeled precursors by *C. albicans* into DNA ([^{14}C]thymidine), RNA ([^{14}C]uridine), and protein ([^{3}H]leucine). DNA synthesis was inhibited non-specifically in comparison to RNA and protein syntheses. It was also found that pradimicin A does not intercalate to calf thymus double stranded DNA (Type I, Sigma Co.) as determined by the thermal denaturation curves. Furthermore, pradimicin A at 500 μg/ml was negative in the Ames test (data not shown).

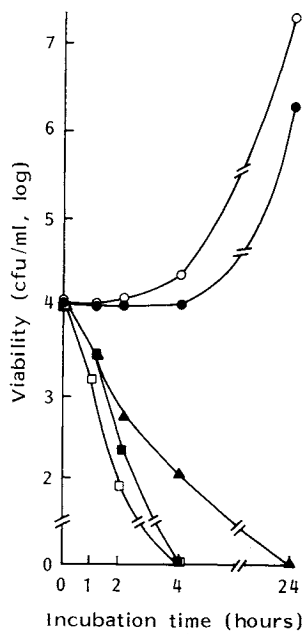
Effect on the Growth of *C. albicans* on a Ca^{2+}-Dependency

To investigate the mode of action of pradimicin A, it is important to determine whether the antibiotic is fungicidal or fungistatic. Fig. 1 shows the effect of pradimicin A on the growth of *C. albicans* A9540. The minimum candicidal concentration of pradimicin A was around 10 μg/ml. Concentrations of pradimicin A below 10 μg/ml caused retardation of the growth. Pradimicin A at 60 and 30 μg/ml totally killed the cells within 4 hours.

Our preliminary results suggested that the Ca^{2+}-chelator EGTA was effective in neutralizing pradimicin A activity. We therefore examined the

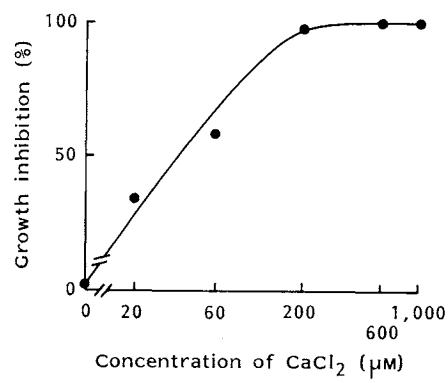
Fig. 1. Effect of pradimicin A on the growth of *Candida albicans* A9540.

○ Untreated control, ● pradimicin A (3 μg/ml), ▲ pradimicin A (10 μg/ml), ■ pradimicin A (30 μg/ml), □ pradimicin A (60 μg/ml).



The cell suspension (1×10^4 cfu/ml in YNBG-PB, pH 7.0) was treated with varying concentrations of pradimicin A for indicated times at 37°C. An aliquot was taken, diluted serially 10-times with deionized water and the diluted cell suspension (0.1 ml) was spread on YPD agar plates.

Fig. 2. Effect of $CaCl_2$ on the antifungal activity of pradimicin A (25 μg/ml) against *Candida albicans* A9540.



Ca^{2+}-Free YNBG-PB, pH 7.0, was used as the test medium. Experimental conditions are described in the Materials and Methods.

Table 1. Viable cells of pradimicin A-treated *Candida albicans* A9540 in sodium phosphate buffer (resting condition).

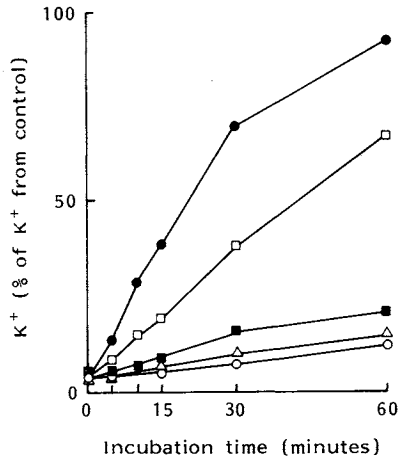
Compound	Concentration (μg/ml)	Additive	Viable cells (cfu/ml)
None	—	—	1.2×10^{7}[a]
Pradimicin A	60	—	9.0×10^{6}
Pradimicin A	60	$CaCl_2$ (200 μM)	1.8×10^{4}
Pradimicin A	60	$CaCl_2$ (200 μM) + EGTA (2 mM)	1.0×10^{7}
Amphotericin B	1	—	5.0×10^{2}

Washed cells at 1×10^{7} cells/ml in sodium phosphate buffer (pH 7.0) were exposed to each of drugs for 4 hours, diluted with deionized water and spread on YPD agar plates. Viable cells were counted followed by incubation for 48 hours at 27°C.

[a] Viable cells at 0 time of treatment was 1.2×10^{7} cells/ml.

Fig. 3. K^+ Leakage from pradimicin A-treated *Candida albicans* A9540 in sodium phosphate buffer (non-growing condition).

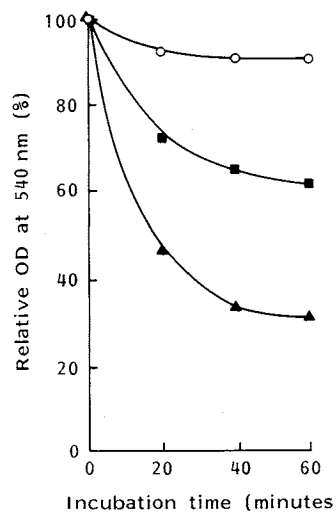
● Amphotericin B (1 μg/ml), □ pradimicin A (60 μg/ml) + $CaCl_2$ (200 μM), ■ pradimicin A (60 μg/ml), △ pradimicin A (60 μg/ml) + $CaCl_2$ (200 μM) + EGTA (2 mM), ○ untreated control.



The washed cells (1×10^{7} cells/ml) were resuspended in sodium phosphate buffer (pH 7.0) and treated with each of drugs at 37°C. At indicated times sample was taken and K^+ level was determined as described in Materials and Methods.

Fig. 4. Effect of pradimicin A on the lysis of *Candida albicans* A9540 protoplasts in the presence of Ca^{2+}.

○ Untreated control, ■ pradimicin A (10 μg/ml), ▲ pradimicin A (40 μg/ml).



Protoplasts (1×10^{8} protoplasts/ml in YNBG-PB supplemented with 0.8 M sorbitol, pH 7.0) were treated with varying concentrations of pradimicin A. At indicated times the OD at 540 nm of the test samples was read (100% = 0.60).

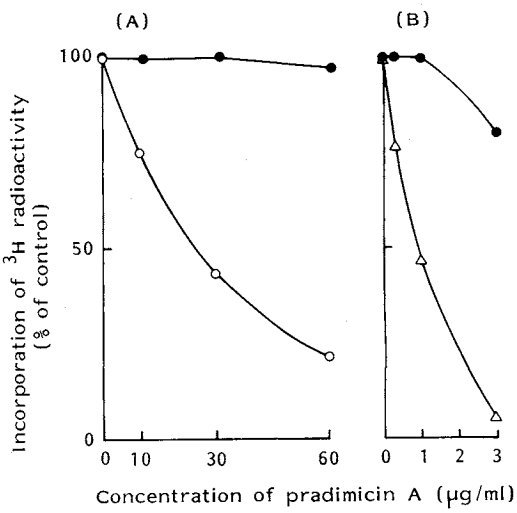
effect of Ca^{2+} ion on the anticandidal activity of pradimicin A. Fig. 2 shows that the growth-inhibiting activity of pradimicin A at 25 μg/ml in Ca^{2+}-free YNBG-PB against *C. albicans* clearly depends on the concentration of Ca^{2+} in the medium. Ca^{2+} at 200 μM maximized the activity of pradimicin A.

Effect on Membrane Permeability and Viability under Non-Growing Condition

The degree of change of membrane permeability in *C. albicans* cells was estimated by measuring K^+ leakage from the cells treated with pradimicin A under non-growing conditions. Fig. 3 shows that the effect of pradimicin A on membrane permeability is Ca^{2+}-dependent and that addition of EGTA at 2 mM

Fig. 5. Effect of pradimicin A on the incorporation of [^{3}H]leucine into intact cells and protoplasts of *Candida albicans* A9540.

(A) Intact cells, (B) protoplasts. ● +EGTA (2 mM), ○ IC_{50} 24 μg/ml, △ IC_{50} 0.9 μg/ml.



The washed cells (or protoplasts) were resuspended in YNBG-PB (or YNBG-PB plus 0.8 M sorbitol, pH 7.0) at 1×10^7 cells (or protoplasts)/ml. To the suspension with or without EGTA pradimicin A and [^{3}H]leucine was added. Incorporation of ^{3}H radioactivity into TCA insoluble materials was obtained as described in Materials and Methods.

Incorporation of ^{3}H: A, 100% = 69,900 dpm; B, 100% = 58,000 dpm.

Table 2. Binding characteristics of pradimicin A to *Candida albicans* A9540 and its protoplasts.

Concentration of pradimicin A (μg/ml)	(Binding μg/1 × 10^7 cells)			
	Intact cells		Protoplasts	
	−EGTA	+EGTA	−EGTA	+EGTA
10	10	1	4	1
30	30	1	8	1
40	31	2	9	0
60	32	2	10	0

The washed cells (or protoplasts) were resuspended in YNBG-PB (or YNBG-PB plus 0.8 M sorbitol, pH 7.0) yielding an inocula of 1×10^7 cfu (or protoplasts)/ml. To the suspension with or without EGTA (2 mM) pradimicin A was added. Binding of pradimicin A was obtained as described in Materials and Methods.

totally abolished the effect.

Table 1 shows the effect of pradimicin A on cell viability under non-growing conditions. Again the effect is Ca^{2+}-dependent and was inhibited by addition of EGTA. Amphotericin B at 1 μg/ml showed a marked decrease in numbers of viable cells.

Effect on the Protoplast Lysis

Fig. 4 shows the effect of pradimicin A on protoplasts under hypertonic conditions. Decrease of OD values represents protoplasts lysis. Pradimicin A at 40 μg/ml rapidly decreased the OD value by approximately 70% within 60 minutes. This rate was equivalent to approximately 90% lysis of protoplasts as determined by direct counting under microscope.

Effect on [^{3}H]Leucine Incorporation into *C. albicans*

Fig. 5 shows that pradimicin A inhibited the incorporation of [^{3}H]leucine into both intact cells and protoplasts; an IC_{50} of pradimicin A in intact cells was 24 μg/ml, while that in protoplasts was 0.9 μg/ml. Particularly, protoplasts has an increased susceptibility to pradimicin A, though the incorporation of ^{3}H radioactivity into pradimicin A-untreated cells and protoplasts was almost comparable. EGTA at 2 mM in both intact cells and protoplasts reduced the inhibitory activity of pradimicin A.

Binding of Pradimicin A to the Cells

Table 2 shows the amount of pradimicin A bound to either intact cells or protoplasts in the presence or absence of EGTA at 2 mM. Upon treatment with pradimicin A at 60 μg/ml intact cells (1×10^7 cfu) can adsorb as much as 32 μg of pradimicin A, while protoplasts (1×10^7 protoplasts) can adsorb 10 μg of pradimicin A in the absence of EGTA. In the presence of EGTA, pradimicin A was not significantly bound to either intact cells or protoplasts. Pradimicin A was found to bind rapidly and substantially to

yeast mannan (preparations M7504 and M3640, Sigma Co.) to give an insoluble complex in the presence of Ca^{2+} (data not shown).

Contrary to *C. albicans* cells, human erythrocytes or other cultured mammalian cells did not induce K^+ leakage when they were treated with pradimicin A at 60 μg/ml in the presence of Ca^{2+}. In a parallel experiment, binding of pradimicin A to these cells was found to be insignificant in the presence of Ca^{2+} (data not shown).

Discussion

Pradimicin A at 10 μg/ml or more showed fungicidal activity against *C. albicans* both under growing and non-growing conditions. The candicidal activity in the non-growing cells suggests that pradimicin A acts on a cellular component rather than inhibiting the biosynthesis of cellular components. This is compatible with the fact that there is no cross-resistance to other antifungal agents such as 5-fluorocytosine and azoles.[4]

Pradimicin A causes rapid induction of K^+ leakage from the cells dependent on Ca^{2+} concentration. Also a significant observation was the substantial binding of pradimicin A to the outer surface of *C. albicans* cells in a Ca^+-dependent manner. Therefore, these two findings must be closely linked to the mode of anticandidal action of pradimicin A. The action of pradimicin A is associated with alteration of the fungal membrane permeability as determined by induction of K^+ leakage (Fig. 3). Polyene antibiotics such as amphotericin B and nystatin are well-known to cause changes in membrane integrity.[5] The results of the present study, however, indicate that the action of pradimicin A is differentiated from these antibiotics. The activity of pradimicin A is Ca^{2+}-dependent and thus antagonized by addition of EGTA. Other cell wall (or membrane)-acting agents such as iturin,[6] polygodial,[7] and killer toxins[8] have not been reported to require Ca^{2+} for the activity. Pradimicin A preferentially binds to the cell wall because pradimicin A binding in intact cells is much greater than with protoplasts (Table 2). Pradimicin A can bind rapidly to yeast mannan to yield an insoluble complex in the presence of Ca^{2+}. This result indicates that pradimicin A binds to a mannan component in the cell wall or plasma membrane of *C. albicans*.[9] The nature of the binding to the candidal cell wall and plasma membrane or the target molecule to which pradimicin A binds is not well understood. Nor is it clear how the binding relates to membrane perturbation in *C. albicans*.

Pradimicin A showed an insignificant membrane damage of erythrocytes or other cultured mammalian cells in the presence of Ca^{2+}. Insights into how pradimicin A works on both fungal and mammalian cells have important clinical ramifications.

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