PRADIMICINS T1 AND T2, NEW ANTIFUNGAL ANTIBIOTICS PRODUCED BY AN ACTINOMYCETE

I. TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

TAMOTSU FURUMAI, TOSHIFUMI HASEGAWA, MASATOSHI KAKUSHIMA, KIYOSHI SUZUKI, HARUAKI YAMAMOTO, SATOSHI YAMAMOTO, MINORU HIRANO and TOSHIKAZU OKI

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

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Pradimicins T1 and T2, new members of the pradimicin family of antibiotics, were produced by an actinomycete strain AA3798. Pradimicin T1 exhibited potent activity against a wide spectrum of fungi *in vitro* and demonstrated efficacy against systemic *Candida albicans* and *Aspergillus fumigatus* infections in mice.

The pradimicins (PRMs)[1~4] and benanomicins (BNMs)[5,6] represent a novel family of dihydrobenzo[*a*]naphthacenequinone antibiotics. Their efficacies *in vivo* against systemic candida, cryptococcus and aspergillus infections in mice[7~9] as well as anti-HIV activities[10~12] have fueled an intense search for novel therapeutic agents from this family. Although a number of natural analogs[13~16] and their derivatives[17~22] emerged from these discovery efforts, we felt it important to modify the dihydrobenzo[*a*]naphthacenequinone nucleus and establish structure-activity relationships. For this purpose, we set up a screen designed to detect new members of this family[23]. Searching rare actinomycetes producing diffusible red pigments in agar medium, we have found the broth filtrate of an actinomycete strain AA3798 producing a large inhibition zone in the agar hole plate assay using *Candida albicans* A9540 as test microoraganism. Analysis of the active broth filtrate by HPLC indicated the presence of 2 new antibiotics which had UV-visible spectra indicative of a dihydrobenzo[*a*]naphthacenequinone. Herein, we describe the taxonomic studies of the producing strain, production, isolation, physico-chemical and biological properties of these antibiotics, designated pradimicin T1 (PRM T1) and pradimicin T2 (PRM T2). The accompanying paper describes the structures and biosynthesis of PRM T1 and PRM T2[24].

Materials and Methods

Taxonomic Studies

The producing strain, AA3798, was isolated from a soil sample collected at Nerima, Tokyo, Japan, on January 29, 1989 and has been deposited in the American Type Culture Collection under the accession number ATCC 55288. The cultural and physiological characteristics were observed on several media described by SHIRLING and GOTTLIEB[25], WAKSMAN[26] and ARAI[27]. Morphological observations were made with light and electron microscopes from cultures grown at 32°C for 2 to 4 weeks. The color names and hue numbers used in this study were taken from the Manual of Color Names (Japan Color Enterprise Co., Ltd., 1987). Whole cell hydrolysates were analyzed by the methods of BECKER and LECHEVALIER[28] and of STANECK and ROBERTS[29]. The composition of whole cell sugars was determined by HPLC as described by YOKOTA and HASEGAWA[30]. Mycolic acid analysis was based on the method described by MINNIKIN *et*

Correspondence should be addressed to JUN OKUMURA, Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan.

al.[31]. Menaquinones were analyzed by HPLC and mass using the method described by COLLINS[32]. Fatty acid analysis was performed by the method of SUZUKI[33]. Fatty acid methyl esters were prepared from lyophilized whole cells and analyzed by gas-liquid chromatography with a Hewlett-Packard 5890 series II gaschromatograph. DNAs were isolated by the procedure of FURUMAI *et al.*[34] and analyzed by HPLC for G+C content[35,36].

Fermentation

A slant culture of strain AA3798 grown at 32°C for 20 days on an agar medium composed of soluble starch 1%, yeast extract (Difco Laboratories) 0.1%, soytone (Difco Laboratories) 0.1%, $CaCl_2 \cdot 2H_2O$ 0.05% and agar 1.6% , was inoculated into three 500-ml Erlenmeyer flasks each containing 100 ml of sterile seed medium composed of glucose 1.5%, peptone (Daigo Eiyo Co.) 0.2%, NZ-case (Scheffield Products) 0.2%, meat extract (Mikuni Kogyo Co.) 1.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $CaCl_2 \cdot 2H_2O$ 0.05%, and the flasks were incubated at 32°C for 5 days on a rotary shaker at 200 rpm. Five-ml aliquots were transferred into forty 500-ml Erlenmeyer flasks containing 100 ml of medium composed of soybean meal (Nikko Seiyu Co.) 3%, Pharmamedia (Trader's Protein) 0.5%, glucose 3%, yeast extract 0.1% and $CaCO_3$ 0.3%. The pH of the medium was adjusted to 7.0 before autoclaving. The flasks were incubated at 28°C for 11 days on a rotary shaker at 200 rpm. The progress of fermentation was monitored by UV-vis spectroscopy and HPLC using a column of Excelpak SIL-C18 5B (4.6 i.d. × 150 mm, Yokogawa Electronic Co., Ltd). The mobile phase was acetonitrile - 0.01 M phosphate buffer (19 : 81, pH 3.5) at a flow rate of 1 ml/minute. The detector wavelength was set at 460 nm. The sample for HPLC analysis was prepared as follows: The fermented broth was centrifuged at 10,000 rpm for 10 minutes, and the supernatant was diluted 10-fold with DMSO and filtered (0.45 μm).

Isolation

The fermentation broth was centrifuged at 3,000 rpm for 20 minutes. The supernatant (3.5 liters) was mixed with 2.2 liters of Diaion HP-20. The resin was washed successively with 7 liters of water and 5 liters of 20% aq MeOH and the antibiotics eluted with 5.3 liters of 40% aq MeOH. The eluate was concentrated *in vacuo* and the aq residue lyophilized to yield a crude mass (5.9 g). This material (4.9 g) was dissolved in 2 liters of water and the pH was adjusted to 2.0 with 1 N HCl. The product was extracted into 2 liters of *n*-butanol and back-extracted twice with 0.6 liter of alkaline water (pH 8.5).The aq layer was adjusted to pH 7.0 and concentrated *in vacuo*. The aq residue (0.2 liter) was dropwise added to 1.8 liters of acetone with stirring and the resulting dark red amorphous solid was centrifuged (3,000 rpm, 5°C), collected by filtration and dried. The solid (2.4 g) was then reconstituted in 240 ml of acetonitrile - 0.01 M phosphate buffer (14.5 : 85.5, pH 3.5) and applied to a column of YMC GEL ODS-A 60 (10 liters, YMC Co., Ltd.) using acetonitrile - 0.01 M phosphate buffer (14.5 : 85.5, pH 3.5) as eluent. The combined fractions containing the major product were concentrated *in vacuo* and the aq residue mixed with 100 ml of Diaion HP-20. The resin was washed with 1 liter of water and the product eluted with 200 ml of 50% aq acetone. The acetone was removed *in vacuo* and the aq residue lyophilized to yield 293 mg of PRM T1. The sample for analysis was prepared as follows: A portion of the lyophile (50 mg) in 2 ml of water was added dropwise to 20 ml of acetone and cooled. The solid formed was collected by centrifugation, washed with acetone and dried at 60°C for 2 days.

The fractions containing the minor product were combined and concentrated *in vacuo*. The residue was desalted with 50 ml of Diaion HP-20 as described above and concentrated *in vacuo*. The aq residue was lyophilized to yield 79 mg of PRM T2. The analytical sample was prepared as described above and dried at 60°C.

In Vitro Assays

Agar dilution MICs against 12 fungi were determined on yeast morphology agar (YMA, Difco Laboratories) buffered with 0.067 M phosphate, pH 7.0, after 40 hours of incubation at 28°C as described previously[7]. MICs were defined as the lowest antibiotic concentrations showing no growth or less than five discrete colonies per spot.

Broth dilution MICs against *C. albicans* A9540 were determined in yeast nitrogen base-glucose (YNBG) after 20 hours of incubation at 37°C as described previously[19].

The syncytium formation inhibition assay was performed as described previously[37]. Briefly, HeLa-T4 cells expressing CD4 antigen and BSC-1 cells expressing gp-120 were incubated in the presence of 2-fold dilutions of antibiotic, and the number of syncytia formed was compared with the number of those formed in the absence of antibiotic.

Experimental Infections in Mice

Groups of 5 male ICR mice weighing 20~24 g at each dose level received 10 LD_{50} of *C. albicans* A9540 or *Aspergillus fumigatus* IAM 2034 iv and test compounds given iv once daily for 2 consecutive days beginning immediately after the infection. The 50% protective dose (PD_{50}) was calculated by the method of LITCHFIELD and WILCOXON[38] from the survival rate 20 days after the fungal challenge.

Acute Toxicity in Mice

Test compounds were dissolved in 10% DMSO and the pH adjusted to 7.5. Two-fold dilutions were made with 10% DMSO and filter sterilized. Groups of 3 ICR mice weighing 20~24 g at each dose level were treated iv with test solutions (0.2 ml per 10 g of body weight) and observed for 10 days.

Serum Levels and Urinary Recovery of PRM T1 in Mice

A group of 48 mice were used to determine the serum levels of PRM T1. A single dose of PRM T1 at 20 mg/kg of body weight was administered iv and the blood samples were collected, via cardiac puncture, from 6 mice at 0.17, 0.5, 1, 2, 3, 5, 7 and 24 hours postdose. The concentrations of PRM T1 were determined by HPLC using a column of Waters μ Bondasphere (5μm, C18-100A, 3.9 × 150 mm, Nihon Millipore Ltd). The detector wavelength was set at 290 nm. The lowest concentration detectable by HPLC was 0.5 μg/ml. For urinary analysis, a single dose of PRM T1 at 20 mg/kg of body weight was given to 48 mice and the urinary samples were collected at 0.17, 0.5, 1, 2, 3, 5, 7 and 24 hours postdose.

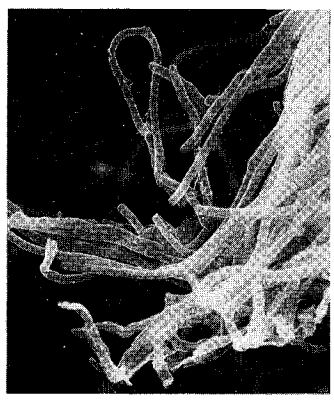
Results and Discussion

Taxonomy

Strain AA3798 grew well at 32°C on media such as yeast extract-malt extract agar (ISP-2), inorganic salt-starch agar (ISP-4), yeast-starch agar and BENNETT's agar. The colonies were thickly covered with white powdery to velvety aerial mycelia that were abundant, long, moderately branched, straight or irregularly curved. The mycelia became pinkish upon prolonged incubation. Fig. 1 is a scanning electron micrograph showing the spore chains of aerial mycelia. The mycelia fragmented completely into spores that were bacillar-shaped, nonmotile, 0.3 × 1.0~2.0 μm in size, with smooth surface. The substrate mycelia were well developed and branched irregularly. They were zig-zagged irregularly and fragmented into short rod elements on agar media such as water agar. Strain AA3798 produced soft pink to dark red vegetative and diffusible pigments on organic media. The diffusible pigments were pH sensitive, changing from pale reddish yellow to strong yellowish orange upon addition of 0.1 N HCl. These cultural characteristics are shown in Table 1.

Fig. 1. Scanning electron micrograph of spore chains of strain AA3798 grown on yeast-starch agar 32°C for 14 days.

Bar represents 5 μm.



With respect to carbon sources, strain AA3798 utilized the following carbohydrates; arabinose,

Table 1. Cultural characteristics of strain AA3798.

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (Waksman med. No. 1)	Pale reddish yellow (130)	Soft yellowish brown (75)	White (388) powdery	Soft yellowish pink (28)
Glycerol-nitrate agar	Pale reddish	Light reddish yellow (126)	White (388)~yellow (131)	None
Glucose-asparagine agar (Waksman med. No. 2)	Light orange (64)	Light orange (64)	White (388) powdery	None
Yeast extract-malt extract agar (ISP No. 2)	Dark red (57)	Dark red (57)	Pinkish white (391) powdery, good	Dark red (57)
Oatmeal agar (ISP No. 3)	Colorless~grayish pink (33)	Soft yellowish pink (27)	White (388) powdery	Soft pink (25)
Inorganic salts-starch agar (ISP No. 4)	Yellowish brown (101)	Soft orange (83)	White (388) powdery, good	None~biege white (392)
Glycerol-asparagine agar (ISP No. 5)	Light orange (64)	Light orange (65)	White (388) powdery	None
Tyrosine agar (ISP No. 7)	Light orange (65)	Light orange (65)	Beige white (392) powdery	None
Nutrient agar (Waksman med. No. 14)	Pale reddish yellow (125)	Pale reddish yellow (125)	None	None
Yeast-starch agar	Dark red (57)	Dark red (57)	White (388) powdery, good	Strong purplish red (44)
BENNETT's agar (Waksman med. No. 30)	Dark red (57)	Dark red (57)	White (389) powdery, good	Strong purplish red (44)
GAUZE's agar	Pale reddish yellow (125)	Light orange (64)	White (388) powdery, good	Pinkish white (391)
Peptone corn agar	Grayish pink (32)	Grayish pink (32)	White (389) powdery, scant	Soft pink (25)

Table 2. Physiological characteristics of strain AA3798.

Test	Results
Starch hydrolysis (on ISP No. 7)	Positive
Nitrate reduction (Nitrate broth, Difco)	Negative
Milk (10% Skimmed milk, Difco)	
Coagulation	Positive
Peptonization	Positive
Cellulose decomposition (sucrose nitrate solution with a paper strip as the sole carbon source)	Negative
Gelatin liquefaction	
On plain gelatin	No growth
On glucose peptone gelatin	No growth
Melanin formation (on ISP No. 7)	Negative
Temperature range for growth	19~40°C
Optimum temperature on yeast starch agar	29~24°C
pH range for growth	6~8
Optimum pH on trypticase soy broth (BBL)	7~8
Growth in/at	
Lysozyme 0.01%	Positive
0.1%	Negative
NaCl 2.0%	Positive
8.0%	Negative
G+C content	65%

inositol, D-mannitol, sucrose, L-rhamnose, raffinose, D-xylose and D-glucose. Moderate growth was observed on fructose. The physiological characteristics are shown in Table 2.

Analysis of the whole cell hydrolysate showed the presence of *meso*-diaminopimeric acid, glucose, glucosamine, galactose, ribose, mannose and xylose, but did not reveal madurose, arabinose or rhamnose. Mycolic acids were absent. The major menaquinones were 69% MK-9(H_8), 18% MK-9(H_6), 6% MK-9(H_4) and 6% MK-9(H_{10}). The major fatty acids were 14-methylpentadecanoic acid (16:0 *iso*), 14-methylhexadecanoic acid (17:0 *anteiso*), 15-methylhexadecanoic acid (17:0 *iso*) and 10-methyloctadecanoic acid (10-Me 18:0) as shown in Table 3.

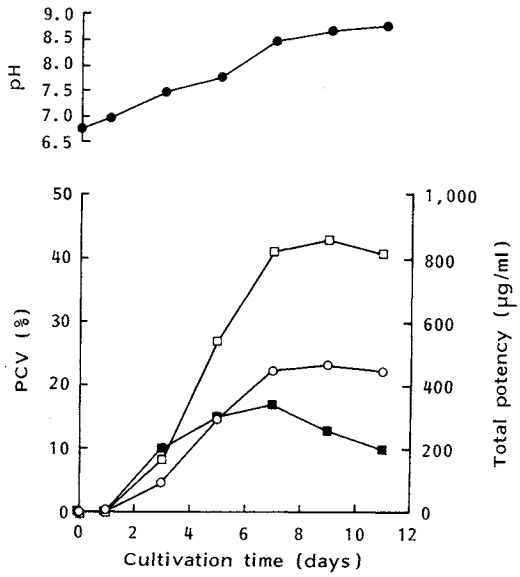
The observed cultural characteristics and the cell wall analysis indicate that strain AA3798 belongs to the genus *Nocardiopsis* Meyer 1976[39] as the only generic possibility. However, the menaqui-

Table 3. Cellular fatty acid composition of strain AA3798.

Fatty acid		Composition (%)
Normal acid	16:0	1
	17:0	2
	18:1^{9}	1
Iso acid	16:0	34
	17:0	14
	18:0	4
	18:1	1
Anteiso acid	17:0	21
10-Methyl acid	17:0	3
	18:0	10

Fig. 2. Time course of pradimicins T1 and T2 fermentation.

pH (●), packed cell volume (PCV, ■) and pradimicin T1 (□) and T2 (○).



none and fatty acid compositions of strain AA3798 are inconsistent with the genus *Nocardiopsis*. Consequently we have concluded that strain AA3798 is a hitherto undescribed actinomycete.

Fermentation

Fig. 2 presents time course data from a typical 500-ml flask fermentation showing the production of PRM T1 and PRM T2, pH and packed cell volume. Production reached to a maximum level after 9 days of incubation, yielding 855 μg/ml of PRM T1 and 460 μg/ml of PRM T2 on day 9. Neither pradimicin A nor benanomicin A could be detected during the course of fermentation.

Physico-chemical Properties

The physico-chemical properties of PRM T1 and PRM T2 are summarized in Table 4. The molecular formulas of PRM T1 and PRM T2 were determined as $C_{42}H_{45}NO_{23}$ and $C_{37}H_{37}NO_{19}$ from the FAB-MS (positive) peaks at *m/z* 932 and 800 $(M+H)^+$, respectively, and elemental analysis. The UV-visible and IR spectra of PRM T1 and PRM T2 are similar to those of pradimicin A (PRM A)[2], suggesting a dihydrobenzo[*a*]naphthacenequinone structure. Both PRM T1 and PRM T2 are acidic substances, readily soluble in aqueous media (>pH 5), dimethyl sulfoxide and dimethylformamide, slightly soluble in acidic aqueous media and methanol, but practically insoluble in other common organic solvents such as benzene, acetone, EtOAc and *n*-BuOH.

Biological properties

Antifungal Activities

Table 5 summarizes MICs of PRM T1, PRM T2, a pradimicin derivative (BMY-28864)[18,19] and amphotericin B (AMPH) against 12 fungi determined by the agar dilution method on buffered YMA. PRM T1 had an antifungal spectrum similar to that of BMY-28864, giving MICs ranging from 1.6 to 25 μg/ml against the strains tested. PRM T2 had a somewhat narrow spectrum of activity. MICs against *C. albicans* A9540 (2×10^5 cfu/ml in YNBG) determined by the broth dilution method were 6.3 μg/ml for PRM T1, PRM T2 and BMY-28864, and 0.4 μg/ml for AMPH. Addition of fetal bovine serum at concentrations of up to 20% did not affect the activity of PRM T1 or PRM T2, suggesting low binding

Table 4. Physico-chemical properties of pradimicins T1 and T2.

	PRM T1	PRM T2
Appearance	Dark red amorphous solid	Dark red amorphous solid
Melting point	190~195°C (dec)	185~190°C (dec)
UV λ_{max} nm (ε)		
in 0.02 N HCl-MeOH (1:1)	232 (33,100), 290 (25,300), 457 (11,000)	233 (29,600), 290 (22,600), 456 (9,800)
in 0.02 N NaOH-MeOH (1:1)	243 (33,600), 316 (14,000), 500 (14,200)	242 (29,500), 316 (12,200), 499 (12,200)
IR ν_{max} (KBr) cm^{-1}	3600~2500, 1730, 1630, 1610, 1295, 1080, 1045, 1000	3600~2500, 1730, 1630, 1610, 1295, 1075, 1040, 1000
Molecular formula	$C_{42}H_{45}NO_{23}$	$C_{37}H_{37}NO_{19}$
FAB-MS (positive) *m/z*	932 $(M+H)^+$	800 $(M+H)^+$
Elemental analysis		
Calcd for:	$C_{42}H_{45}NO_{23}\cdot 2H_2O$	$C_{37}H_{37}NO_{19}\cdot 2H_2O$
	C 52.12, H 5.10, N 1.45	C 53.18, H 4.94, N 1.68
Found:	C 52.41, H 4.84, N 1.41	C 52.75, H 5.00, N 1.95
Solubility		
Soluble:	DMSO, DMF water (>pH 5)	DMSO, DMF water (>pH 5)
Slightly soluble:	MeOH, water (<pH 5)	MeOH, water (<pH 5)
Insoluble:	Acetone, EtOAc, *n*-BuOH	Acetone, EtOAc, *n*-BuOH
Rf value (TLC, SiO_2)	0.48[a]	0.50[a]
	0.28[b]	0.38[b]
Rt (HPLC, ODS[c])	7.5 minutes	4.1 minutes

[a] *n*-BuOH-AcOH-H_2O (2:1:1).
[b] *n*-BuOH-AcOH-pyridine-H_2O (6:1:4:3).
[c] YMC ODS A-301-3 (4.6 mm i.d. × 100 mm); CH_3CN-0.01 M phosphate buffer (12:88, pH 7.0); 1 ml/minute; detection, UV 254 nm.

Table 5. *In vitro* antifungal activities of pradimicins T1 and T2, BMY-28864 and amphotericin B.

Organism	MIC (μg/ml)			
	PRM T1	PRM T2	BMY-28864	AMPH
Saccharomyces cerevisiae ATCC 9763	3.1	1.6	3.1	0.4
Candida albicans IAM 4888	6.3	6.3	6.3	0.4
C. albicans A9540	6.3	6.3	6.3	0.4
C. albicans ATCC 38247	1.6	3.1	1.6	12.5
C. albicans ATCC 32354 (B311)	3.1	3.1	6.3	0.4
C. albicans 83-2-14 (Juntendo)	6.3	3.1	6.3	0.4
C. tropicalis 85-8 (Kitasato)	25	6.3	12.5	0.8
C. tropicalis IFO 10241	25	12.5	12.5	0.8
Cryptococcus neoformans D49	1.6	3.1	1.6	0.4
C. neoformans IAM 4514	1.6	6.3	1.6	0.4
Aspergillus fumigatus IAM 2034	6.3	>100	3.1	0.8
Trichophyton mentagrophytes 4329	12.5	>100	6.3	0.8

Medium: YMA + 0.067 M phosphate buffer, pH 7.0.
Incubation: 28°C, 40 hours.
Inoculum size: 1×10^4 cells/5-μl spot (*T. mentagrophytes*: 1×10^5 cells/5-μl spot).

of these antibiotics to the serum protein. However, as was observed with BMY-28864[19], addition of ethylene glycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) at a final concentration of 2 mM to the test medium at or 2 hours after the initiation of the incubation, led to total inactivation of these antibiotics, indicating that both PRM T1 and PRM T2 require Ca^{2+} for manifestation of antifungal activity.

Table 6. Effect of 2 days of treatment on systemic candida and aspergillus infections in mice.

Treatment (dose, mg/kg/day)	No. of survivors/treated after 20 days		Treatment (dose, mg/kg/day)	No. of survivors/treated after 20 days	
	C. albicans A9540	*A. fumigatus* IAM 2034		*C. albicans* A9540	*A. fumigatus* IAM 2034
Control	0/10	0/10	BMY-28864		
PRM T1			50	5/5	5/5
50	5/5	2/5	25	5/5	5/5
25	4/5	1/5	12.5	1/5	3/5
12.5	2/5	0/5	6.3	0/5	0/5
6.3	1/5	0.5	PD_{50}	15 (13~18)	12 (9.8~14)
PD_{50}	15 (12~19)	>50	AMPH		
PRM T2			1.0	5/5	5/5
100	4/5	—	0.5	3/5	5/5
50	2/5	—	0.25	4/5	4/5
25	1/5	—	0.13	1/5	2/5
12.5	0/5	—	PD_{50}	0.23 (0.18~0.30)	0.20 (0.18~0.23)
PD_{50}	54 (45~64)				

Mice were infected with 10 LD_{50} of *C. albicans* A9540 or *A. fumigatus* IAM 2034, and compounds were given iv once daily for 2 consecutive days beginning immediately after the infection. PD_{50}s were calculated from the survival rate 20 days after the infection. Figures in parentheses denote 95% confidence limits.

The Table 6 summarizes the efficacies of PRM T1 and PRM T2 in comparison with those of BMY-28864 and AMPH in the mouse models of systemic *C. albicans* A9540 and *A. fumigatus* IAM 2034 infections. In the candida infection model, untreated control mice ($n=10$) died from 9 to 14 days postinfection. PRM T1 and BMY-28864 at a daily dose of 50 mg/kg were equally efficacious against *C. albicans*, both giving 100% survival of the infected mice. The calculated PD_{50}s of PRM T1 and BMY-28864 from the survival rate were 15 mg/kg/day. PRM T2 was less efficacious, giving 80 and 40% survival of the infected mice at daily doses of 100 and 50 mg/kg/day, respectively. In the aspergillus infection model, untreated control mice ($n=10$) died from 4 to 12 days postinfection. PRM T1 was less efficacious than BMY-28864 against *A. fumigatus*, giving 40% survival of the infected mice at a daily dose of 50 mg/kg.

Antiviral Activity

Co-culture of HeLa-T4 cells expressing CD4 antigen and BSC-1 cells expressing gp-120 produced a multi-nucleated giant cell (syncytium) formation in 3 to 5 hours[38]. PRM T1 at a concentration of 25 μg/ml inhibited the syncytium formation almost completely with an ID_{50} of 11.6 μg/ml, which is favorably compared with dextran sulfate (ID_{50}: 19 μg/ml). PRM T2 at a concentration of 100 μg/ml was marginally active in this assay.

Other Biological Activities

PRM T1 and PRM T2 at concentrations of up to 100 μg/ml did not show any effect on the growth of *Staphylococcus aureus*, *Bacillus subtilis* or *Escherichia coli* as determined by the agar dilution method on nutrient agar.

No drug-related deaths were observed with PRM T1 at iv doses of up to 300 mg/kg body weight or with PRM T2 at iv doses of up to 600 mg/kg body weight.

PRM T1 was metabolically stable and greater than 90% of total dose (20 mg/kg body weight) excreted intact in urine in the first 2 hours output. The pharmacokinetic parameters calculated from the serum levels

were $T_{1/2}$ of 18.9 minutes (α phase 15.7 and β phase 33.2 minutes), AUC of 12.7 μg·hours/ml and V_{SS} of 0.81 liter/kg.

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