IN VITRO AND *IN VIVO* ANTIFUNGAL ACTIVITIES OF BMS-181184

TOSHIKAZU OKI, MASATOSHI KAKUSHIMA, MINORU HIRANO, AKIYO TAKAHASHI, AKEMI OHTA, SHINJI MASUYOSHI, MASAMI HATORI and HIDEO KAMEI

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

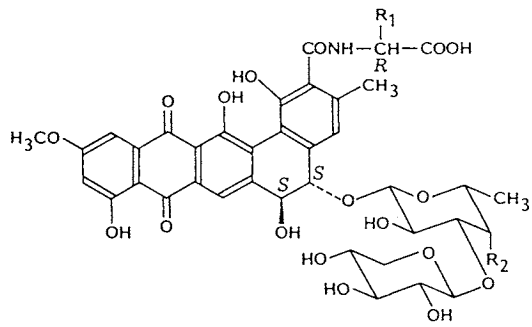
(Received for publication April 16, 1992)

BMS-181184 is a water-soluble pradimicin derivative with a broad antifungal spectrum *in vitro* and demonstrable efficacy against systemic infections with *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* in normal and cyclophosphamide-treated immunosuppressed mice.

The pradimicins[1~4] and benanomicins[5] are family of benzo[*a*]naphthacenequinone antifungal agents which have come into prominence in recent years because of their anticipated use for the treatment of fatal systemic fungal infections. Pradimicin A (PRM A) which was isolated from the cultured filtrates of *Actinomadura hibisca* P157-2 (ATCC 53557)[6] exhibited broad-spectrum fungicidal activity including activity against a variety of *Candida* species that were resistant to other antifungal agents[2,7]. Its mode of fungicidal action appears to be unique; PRM A binds to the surface of *Candida albicans* in the presence of Ca^{2+}, causing perturbation of the fungal membrane[8]. Although PRM A is relatively non-toxic when compared with amphotericin B (AMPH), its poor solubility in aqueous media has severely curtailed the usage of this compound. Nevertheless, a promise for a safe, broad-spectrum therapy[9~11] provided a formidable challenge to the drug industry.

Chemical modification of PRM A and PRM FA-2 focused on the substituent at C4′ afforded a number of active derivatives[12]. BMS-181184 is one of the semisynthetic compounds that were derived from PRM FA-2 (Fig. 1). It showed potent activity *in vitro* against *C. albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, and excellent water solubility at physiological pHs. These profiles prompted us to study and compare the efficacies of BMS-181184 with those of PRM A and AMPH *in vitro* against a wide variety of fungi as well as *in vivo* against experimental systemic *C. albicans*, *C. neoformans* and *A. fumigatus* infections in mice.

Fig. 1. Structures of PRM A, PRM FA-2 and BMS-181184.



	R_1	R_2
PRM A	CH_3	$NHCH_3$
PRM FA-2	CH_2OH	NH_2
BMS-181184	CH_2OH	OH

Materials and Methods

Antibiotics

BMS-181184 and PRM A were prepared at Bristol-Myers Squibb Research Institute, Tokyo, Japan. A 5-mg/ml solution of the antibiotic was prepared in 10% dimethyl sulfoxide (DMSO) at pH

7.5, and twofold dilutions were made with water and filter sterilized (Millex-GV, 0.22 μm). AMPH was formulated by dissolving Fungizone (AMPH - sodium deoxycholate complex) in 10% DMSO, providing a stock solution of 2 mg/ml and twofold dilutions made with water.

Fungi

A panel comprised of 18 strains of yeasts and fungi was used for the *in vitro* study. Yeasts were grown at 28°C in yeast extract - peptone - glucose broth (YPG; yeast extract 0.4%, Polypepton 1.0%, glucose 1.5%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%) for 18 hours with shaking. A 1-ml suspension was inoculated into fresh YPG (100 ml) and grown at 28°C for 5 hours with shaking. The others were grown at 28°C on YPG - 1.5% agar slants for 14 days, and spore suspensions were prepared by washing spores from the agar surface with 0.067 M phosphate buffer (pH 7.0) containing 0.2% (v/v) Tween 80. Inocula in each case were adjusted by hemacytometer counting.

Susceptibility Testing

MICs were determined on yeast morphology agar (YMA, Difco Laboratories, Detroit, Mich., U.S.A.) buffered with 0.067 M phosphate, pH 7.0. Nine parts of molten agar were combined with one part of antibiotic dilution in petri dishes. A 5-μl suspension containing 2×10^6 cells per ml (2×10^7 cells per ml for *Trichophyton mentagrophytes* 4329) was spotted on the surface of the agar plates. The plates were incubated at 28°C for 60 hours. MICs were recorded after 40 hours of incubation except the *T. mentagrophytes* isolate which required 60 hours to produce visually evaluable growth on the drug-free control plates. MICs were defined as the lowest antibiotic concentrations showing no growth or less than five discrete colonies per spot.

Experimental Infections in Normal Mice

Groups of 5 male ICR mice weighing 20~24 g at each dose level received 10 LD_{50} of *C. albicans* A9540, *C. neoformans* IAM 4514 or *A. fumigatus* IAM 2034 intravenously (iv) and test compounds given iv once immediately after the infection. The 50% protective dose (PD_{50}) was calculated by the method of LITCHFIELD and WILCOXON[13] from the survival rate 20 days after the fungal challenge.

Experimental Infections in Cyclophosphamide (CY)-treated Mice

Groups of 5 male ICR mice at each dose level were treated intraperitoneally (ip) with 200 mg/kg of CY (Shionogi, Osaka, Japan) 4 days prior to an iv infection of *C. albicans* A9540, *C. neoformans* IAM 4514 or *A. fumigatus* IAM 2034 (10 LD_{50}). Test compounds were given iv once immediately after the infection or once daily for 2 consecutive days beginning immediately after the infection. The PD_{50} was calculated from the survival rate 20 and 40 days after the fungal challenge.

Acute Toxicity

Groups of 5 male ICR mice weighing 20~24 g at each dose level were treated iv with test compounds in 10% DMSO (0.2 ml per 10 g body weight) and observed for 10 days.

Results

Antifungal Activities *In Vitro* and *In Vivo*

Susceptibilities of 18 strains to BMS-181184, PRM A and AMPH were determined by the standard agar dilution method on YMA. As summarized in Table 1, BMS-181184 had potent and broad-spectrum activity against a wide variety of fungi. It inhibited the growth of most strains including those less susceptible to PRM A at concentrations of 12.5 μg/ml, while *Fusarium moniliforme* A2284 and *Mucor spinosus* IFO 5317 were not susceptible. The MIC of BMS-181184 against a polyene-resistant strain of *C. albicans* (ATCC 38247) indicates that there is no cross resistance between the pradimicin derivative and AMPH.

The *in vivo* efficacies of BMS-181184, PRM A and AMPH were comparatively evaluated in lethal

Table 1. *In vitro* antifungal activities of BMS-181184, PRM A and AMPH.

Organism	MIC (μg/ml)		
	BMS-181184	PRM A	AMPH
Saccharomyces cerevisiae ATCC 9763	3.1	6.3	0.8
Candida albicans IAM 4888	6.3	6.3	0.8
C. albicans A9540	6.3	12.5	0.8
C. albicans ATCC 38247	1.6	1.6	6.3
C. albicans ATCC 32354 (B311)	3.1	3.1	0.4
C. albicans 83-2-14 (Juntendo)	12.5	25	0.4
C. tropicalis 85-8 (Kitasato)	12.5	>100	0.8
C. tropicalis IFO 10241	3.1	>100	1.6
Cryptococcus neoformans D49	3.1	3.1	0.8
C. neoformans IAM 4514	6.3	1.6	0.8
Aspergillus fumigatus IAM 2034	6.3	0.8	1.6
A. fumigatus IFM 4442	6.3	0.8	1.6
A. flavus FA21436, NRRL 484	12.5	6.3	12.5
Trichophyton mentagrophytes D155	6.3	6.3	3.1
T. mentagrophytes 4329	6.3	6.3	3.1
Fusarium moniliforme A2284	100	3.1	6.3
Sporothrix schenckii IFO 8158	6.3	0.8	6.3
Mucor spinosus IFO 5317	>100	>100	1.6

Abbreviations: AMPH, Amphotericin B; PRM A, Pradimicin A. MICs were determined on YMA buffered with 0.067 M phosphate, pH 7.0.

Table 2. *In vivo* efficacies of BMS-181184, PRM A and AMPH against systemic *Candida*, *Cryptococcus* and *Aspergillus* infections in normal mice ($n=5$).

Treatment (dose, mg/kg)	No. of 20-day survivors/treated		
	C. albicans A9540	*C. neoformans* IAM 4514	*A. fumigatus* IAM 2034
BMS-181184			
50	5/5	4/5	4/5
25	5/5	3/5	2/5
12.5	4/5	2/5	0/5
6.3	1/5	2/5	0.5
3.1	0/5	0.5	—
PD_{50} (mg/kg)	8.8	18	31
PRM A			
50	5/5	5/5	4/5
25	5/5	5/5	3/5
12.5	4/5	4/5	0/5
6.3	1/5	2/5	1/5
3.1	0/5	1/5	0/5
PD_{50} (mg/kg)	8.8	6.7	23
AMPH			
2.0	5/5	5/5	5/5
1.0	5/5	4/5	4/5
0.5	5/5	3/5	3/5
0.25	2/5	1/5	2/5
0.13	0/5	0/5	0/5
PD_{50} (mg/kg)	0.27	0.54	0.41

Male ICR mice (20~24 g) received an iv infection of 10 LD_{50} of fungi and compounds given iv once immediately after the fungal challenge.

Table 3. *In vivo* efficacies of BMS-181184, PRM A and AMPH against systemic *Candida*, *Cryptococcus* and *Aspergillus* infections in CY-treated mice ($n=5$).

Treatment (dose, mg/kg)		No. of 20-day survivors/treated		
		C. albicans A9540	*C. neoformans* IAM 4514	*A. fumigatus* IAM 2034
BMS-181184				
100		5/5	5/5	—
50		4/5	3/5	2/5
25		2/5	1/5	2/5
12.5		0/5	1/5	0/5
6.3		1/5	0/5	0/5
	PD_{50} (mg/kg)	31	41	>50
PRM A				
50		5/5	5/5	4/5
25		4/5	5/5	2/5
12.5		3/5	2/5	2/5
6.3		0/5	0/5	0/5
	PD_{50} (mg/kg)	13	13	27
AMPH				
2.0		5/5	5/5	5/5
1.0		5/5	4/5	4/5
0.5		2/5	3/5	2/5
0.25		0/5	0/5	2/5
0.13		0/5	—	0/5
	PD_{50} (mg/kg)	0.54	0.47	0.41

Male ICR mice (20~24 g) were treated ip with 200 mg/kg of cyclophosphamide 4 days prior to an iv infection of 10 LD_{50} of fungi, and compounds given iv once immediately after the fungal challenge.

Table 4. Effect of 2 days of treatment on a systemic *Aspergillus* infection in CY-treated mice ($n=5$).

Treatment (dose, mg/kg/day)		No. of mice infected	No. of surviving animals	
			After 20 days	After 40 days
Control		10	0	0
BMS-181184				
100		5	4	3
50		5	4	2
25		5	3	1
12.5		5	0	0
	PD_{50} (mg/kg)		23	54
PRM A				
50		5	5	4
25		5	4	2
12.5		5	4	1
6.3		5	0	0
	PD_{50} (mg/kg)		10	27
AMPH				
2.0		5	5	5
1.0		5	5	4
0.5		5	3	2
0.25		5	0	0
	PD_{50} (mg/kg)		0.47	0.62

Male ICR mice (20~24 g) were treated ip with 200 mg/kg of cyclophosphamide 4 days prior to an iv infection of 10 LD_{50} of *A. fumigatus* IAM 2034, and compounds were given iv once daily for 2 consecutive days beginning immediately after the fungal challenge.

systemic infections in normal and CY-treated mice by a single iv administration. By day 20, all untreated control mice died. In the infection experiments in normal mice (Table 2), BMS-181184 therapy at single iv doses of 50 and 25 mg/kg significantly prolonged the survival of *C. albicans* A9540-, *C. neoformans* IAM 4514- or *A. fumigatus* IAM 2034-infected mice. The PD_{50}s were 8.8, 18 and 31 mg/kg, respectively. In the experiments in CY-treated mice, BMS-181184 given at a single iv dose of 50 mg/kg resulted in the survival of 80% and 60% of *C. albicans* A9540- and *C. neoformans* IAM 4514-infected mice, respectively. The PD_{50}s were 31 and 41 mg/kg, respectively (Table 3). Against an *A. fumigatus* IAM 2034 infection in CY-treated mice, BMS-181184 therapy at a single dose of 50 or 25 mg/kg resulted in the survival of only 40% of the infected mice (Table 3). However, when the treatment was given twice, BMS-181184 at daily doses of 50 and 25 mg/kg was found effective, resulting in the survival of 80 and 60% of *A. fumigatus*-infected mice, respectively (Table 4).

Acute Toxicity

BMS-181184 was well tolerated in mice. No acute lethal or apparent side effects were noted during 10 days of observation following an iv administration of up to 600 mg of BMS-181184 per kg body weight, while the LD_{50}s of PRM A and AMPH were 120 and 4.2 mg/kg, respectively.

Discussion

BMS-181184 is a water-soluble semisynthetic derivative of PRM FA-2. The present study demonstrated that it is a safe broad-spectrum antifungal agent and that the *in vitro* activity was clearly translated into *in vivo* results. When compared to PRM A, BMS-181184 was as efficacious as PRM A in preventing deaths of normal mice from lethal systemic fungal infections, and was 2 to 3-fold less efficacious than PRM A in CY-treated mice. When the efficacies and toxicity of BMS-181184 were compared to those of AMPH, BMS-181184 was 40 to 50-fold less efficacious, but at least 130-fold safer than AMPH on a milligram-per-kilogram basis. It is important to note that BMS-181184 was effective against *C. albicans*, *C. neoformans* and *A. fumigatus* infections in CY-treated mice by a single or 2 days of iv administration. Since the incidence of systemic infections brought about by these fungi is more common and increasing in patients with suppressed immune systems, the results with BMS-181184 in the infection experiments in CY-treated mice are encouraging and further evaluation of BMS-181184 is warranted.

Acknowledgment

We thank Dr. T. FURUMAI and associates for the supply of the fermentation products, and Dr. K. IMAE and Mr. H. HOSHI for the supply of BMS-181184. We acknowledge the technical assistance of Ms. M. SHINODA.

References

1) OKI, T.; M. KONISHI, K. TOMATSU, K. TOMITA, K. SAITOH, M. TSUNAKAWA, M. NISHIO, T. MIYAKI & H. KAWAGUCHI: Pradimicin, a novel class of potent antifungal antibiotics. J. Antibiotics 41: 1701～1704, 1988
2) OKI, T.; O. TENMYO, M. HIRANO, K. TOMATSU & H. KAMEI: Pradimicins A, B and C: New antifungal antibiotics. II. *In vitro* and *in vivo* biological activities. J. Antibiotics 43: 763～770, 1990
3) SAWADA, Y.; M. NISHIO, H. YAMAMOTO, M. HATORI, T. MIYAKI, M. KONISHI & T. OKI: New antifungal antibiotics, pradimicins D and E. Glycine analogs of pradimicins A and C. J. Antibiotics 43: 771～777, 1990
4) SAWADA, Y.; M. HATORI, H. YAMAMOTO, M. NISHIO, T. MIYAKI & T. OKI: New antifungal antibiotics pradimicins FA-1 and FA-2: D-Serine analogs of pradimicins A and C. J. Antibiotics 43: 1223～1229, 1990
5) GOMI, S.; M. SEZAKI, S. KONDO, T. HARA, H. NAGANAWA & T. TAKEUCHI: The structures of new antifungal antibiotics, benanomicins A and B. J. Antibiotics 41: 1019～1028, 1988
6) TOMITA, K.; M. NISHIO, K. SAITOH, H. YAMAMOTO, Y. HOSHINO, H. OHKUMA, M. KONISHI, T. MIYAKI & T. OKI: Pradimicins A, B and C: New antifungal antibiotics. I. Taxonomy, production, isolation and physico-chemical

properties. J. Antibiotics 43: 755~762, 1990

7) DESIDERIO, J.; G. LEONARD, L. LAMB, R. BRUTKIEWICZ, J. HIBBARD, B. BEAUDOIN & R. E. KESSLER: Program and Abstracts of the 28th Intersci. Conf. on Antimicrob. Agents Chemother., No. 1002, New York, Oct. 23~26, 1988
8) SAWADA, Y.; K. NUMATA, T.MURAKAMI, H. TANIMICHI, S. YAMAMOTO & T. OKI: Calcium-dependent anticandidal action of pradimicin A. J. Antibiotics 43: 715~721, 1990
9) OKI, T.; M. KAKUSHIMA, M. NISHIO, H. KAMEI, M. HIRANO, Y. SAWADA & M. KONISHI: Water-soluble pradimicin derivatives, synthesis and antifungal evaluation of *N,N*-dimethyl pradimicins. J. Antibiotics 43: 1230~1235, 1990
10) OKI, T.: A new family of antibiotics: benzo[*a*]naphthacenequinones, a water-soluble pradimcin derivative, BMY-28864. *In* Recent Progress in Antifungal Chemotherapy. *Ed.*, H. YAMAGUCHI *et al.*, pp. 381~391, Marcel Dekker Inc., 1991
11) KAKUSHIMA, M.; S. MASUYOSHI, M. HIRANO, M. SHINODA, A. OHTA, H. KAMEI & T. OKI: In vitro and in vivo antifungal activities of BMY-28864, a water-soluble pradimicin derivative. Antimicrob. Agents Chemother. 35: 2185~2190, 1991
12) KAMACHI, H.; S. IIMURA, S. OKUYAMA, H. HOSHI, S. TAMURA, M. SHINODA, K. SAITOH, M. KONISHI & T. OKI: Synthesis and antifungal activities of pradimicin derivatives. Modification at C4'-position. J. Antibiotics 45: 1518~1525, 1992
13) LITCHFIELD, J. T. & F. WILCOXON: A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99~113, 1949