AMPHOTERICIN B 2-MORPHOLINOETHYLAMIDE DIASPARTATE, A NEW WATER SOLUBLE DERIVATIVE OF THE ANTIBIOTIC SYNTHESIS AND BIOLOGICAL PROPERTIES

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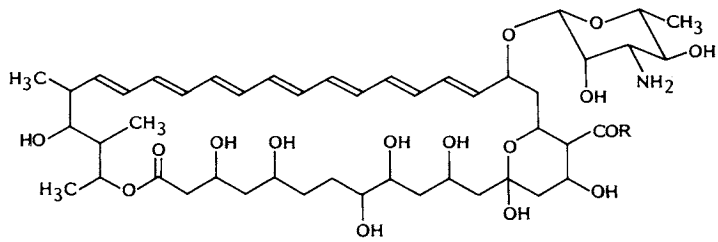
The synthesis of amphotericin B 2-morpholinoethylamide diaspartate, a new water soluble antibiotic derivative with improved selective toxicity is described and *in vitro* biological data are presented.

Amphotericin B (AMB), Fig. 1, a polyene macrolide of the non-aromatic heptaene group is the most effective antibiotic currently available for the treatment of a wide variety of deep-seated systemic fungal infections in humans[1]. However, AMB cannot provide optimal therapy due to its severe toxicity characterized by a number of undesirable side effects, the most serious being the impairment of renal function. This property, also common to other polyene macrolides, is a consequence of its poor selectivity of interaction with the animal and fungal cellular targets, membrane cholesterol and ergosterol, respectively[2].

In recent years a substantial effort has gone into the synthesis of water soluble and less toxic derivatives of the antibiotic. A variety of AMB modification products has been obtained including esters, amides, *N*-alkyl, *N*-acyl and guanidino derivatives (for a review see ref 3). However, none of these compounds have achieved clinical importance.

We have recently demonstrated that improvement of selective toxicity of AMB may be attained by

Fig. 1. Structure of AMB and its derivatives.



AMB R=OH

AMBM R= NHCH$_2$CH$_2$N(morpholino)O

AMA R=NHCH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$

blocking the carboxyl group, resulting in the absence of a carboxylate anion[4~6]. From the practical point of view, AMB amides[7] are easily available and give unambiguous products, in contrast to the antibiotic ester obtained with diazomethane[8].

It has been shown that a strong net charge on the molecule is an essential factor in allowing good water solubility of AMB derivatives[9]. This can be achieved by AMB amide formation in a reaction of the antibiotic with an amine bearing an additional basic nitrogen atom. For example, amphotericin B 3-dimethylamino propylamide diaspartate (AMA), the optimal compound of this type, exhibited *in vitro* and *in vivo* biological properties much improved as compared to the parent antibiotic[7,10,11].

In the search for further water soluble and less toxic AMB amides we synthesized amphotericin B 2-morpholinoethylamide diaspartate (AMBM). In this paper the preparation of AMBM and its biological properties determined in comparison with AMB and AMA are presented.

Materials and Methods

General

IR spectra were taken on a UR-10 (Carl Zeiss, Jena) spectrometer in KBr. UV spectra were determined with a Beckman Model 3600 spectrophotometer in MeOH. The reaction was monitored by TLC on Silica gel 60 plates (Merck).

Biological Assays

Stock cultures were from Polish Collection of Microorganisms. Clinical isolates were obtained from several hospitals in Gdańsk.

Sabouraud dextrose agar or broth were used for the determination of MICs. Broth consisted of Bacto-peptone (Difco) 1% and glucose 2%. For solid medium it was supplemented with Bacto-agar (Difco) 1.5%. Media were sterilized by autoclaving at 121°C for 15 minutes.

Cultures of yeasts were grown at 30°C for 48 hours in Sabouraud dextrose broth with shaking and cultures of filamentous or dimorphic fungi were grown statically on Sabouraud dextrose agar slants until mature. The final inoculum level was 10^4 cfu. Microconidia in dermatophytes and conidia in molds were diluted with sterile saline to provide 10^4 conidia per ml (direct count in a hemacytometer). Viable cell counts were determined by plating on Sabouraud dextrose agar plates. The MIC was defined as the lowest compound concentration that inhibited development of visible growth in broth (37°C, 24 hours) or on agar (37°C, 48 hours).

Hemolytic activity (EH_{50}) and potassium release from erythrocytes (EK_{50}) were determined as described previously[12].

Results and Discussion

Synthesis

The antibiotic derivative was obtained by the azide method[13]. The synthesis was carried out according to the general procedure for amphotericin B amides described by JARZĘBSKI *et al.*[7]. The crude derivative was purified by counter-current distribution in a Craig apparatus ($CHCl_3$-MeOH-0.5% NaCl aq solution, 2:2:1; 250 transfers, partition coefficient K=1.03). Yield 1.203 g (38.7%); $E^{1\%}_{1\,cm}$=1,400 at 382 nm in MeOH. Chromatographic properties of the product and the parent antibiotic are given in

Table 1. Rf values of AMB and AMBM[a] on silica gel.

Compound	A	B	C
AMB	0.51	0.51	0.18
AMBM	0.72	0.69	0.08

[a] Free base.
A: 1-Butanol-ethanol-acetone-25% ammonia (2:5:1:3).
B: Ethanol-1-butanol-25% ammonia (5:3:3).
C: Ethyl acetate-acetic acid-water (4:1:1).

Table 1. The product exhibited UV absorption maxima at the same wavelengths as amphotericin B. The IR spectrum of the derivative showed a band at 1650 cm^{-1}, characteristic of a secondary amide.

The FAB-MS (positive ion mode) showed quasi molecular ions at *m/z* 1,036 and 1,058 ($(M+H)^+$ and $(M+Na)^+$, respectively) and some elimination ions, at *m/z* 1,018, 873, 855 and 837 ($(M+H-H_2O)^+$, $(M+H-S)^+$, $(M+H-S-H_2O)^+$ and $(M+H-S-2H_2O)^+$, respectively, where S is the mycosamine sugar moiety). An identical elimination pattern has been previously reported[8] for the positive ion FAB-MS of pimaricin, representing the tetraene macrolide antibiotics. In the mass spectrum of amphotericin B 2-morpholinoethylamide, an ion at *m/z* 131, corresponding to protonated 4-(2-aminoethyl)morpholine, was also found, thus confirming the assumed type of antibiotic derivatization.

The derivative has two basic amino groups enabling the formation of water soluble salts[9]. AMBM was prepared as follows: A solution of L-aspartic acid (0.293 g, 2.2 mmol) in H_2O (13 ml) was added to amphotericin B 2-morpholinoethylamide (free base, 1.138 g, 1.1 mmol) suspended in H_2O (3 ml). After addition of acetone (500 ml) the precipitate formed was separated by centrifugation, washed with acetone and ethyl ether and dried *in vacuo* to yield 1.303 g (91%) of the desired derivative salt ($E^{1\%}_{1cm}$ = 1,050 at 382 nm in MeOH). The product obtained was readily soluble in water up to a concentration of 50 mg/ml.

Biological Activity

The MICs of AMB, AMBM and AMA against various fungi are shown in Table 2. AMBM exhibits a spectrum of activity against yeasts, molds and a dermatophyte that is identical to that of the parent antibiotic. Its antifungal activity is somewhat lower than that of AMB, but the selectivity is markedly improved. AMBM, retaining most of the antifungal activity of AMB, exhibits a decreased ability to permeabilize human erythrocytes. This is shown by the decrease in hemolytic activity (EH_{50}: AMB, 2.0 μg/ml; AMBM, 11 μg/ml; AMA, 11 μg/ml) and also by the decrease in the ability to induce K^+ leakage from erythrocytes (EK_{50}: AMB, 0.4 μg/ml; AMBM, 4.3 μg/ml; AMA, 4.5 μg/ml). These results confirm our earlier findings concerning the improvement of selective toxicity of polyene macrolides upon substitution at the carboxyl group. AMBM and AMA, closely related AMB derivatives, exhibit practically identical

Table 2. The antibiotic spectra of AMB, AMBM and AMA.

Test organisms	MIC (μg/ml)		
	AMB	AMBM	AMA
Saccharomyces cerevisiae ATCC 9763	0.15	0.25	0.20
Candida albicans ATCC 26278	0.08	0.10	0.10
C. albicans[a] (3)	0.18	0.38~0.50	0.50
C. arborea PCM 1427	0.38	0.75	0.75
C. mycoderma Łock 2	0.25	0.50	0.50
C. tropicalis[a]	0.25	0.50	0.50
Geotrichum candidum[a]	0.15	0.25	0.25
Torulopsis candida PCM 282	0.75	1.25	1.50
Trichophyton nanum[a]	0.15	0.25	0.25
Aspergillus niger	60	75	75
A. nidulans 590[a]	50	50	50
Penicillium cytrinum	55	75	75
Mucor mucedo[a]	35	50	50

[a] Clinical isolates.
( ): No. of strains.

biological properties and are equally well water soluble. Nevertheless, according to our experience, AMBM is distinctly superior to AMA with regard to the synthetic procedure. It is also superior, with respect to water solubility, to other previously described AMB amides[7] which due the absence of the basic nitrogen atom in an amide moiety are significantly less soluble at physiological pH. The data obtained point to AMBM as a new promising antifungal agent.

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