Differential Antifungal Activity of Isomeric Forms of Nystatin

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When nystatin is placed in RPMI and other biological fluids, there is loss of pure nystatin, with the development of two distinguishable chromatographic peaks, 1 and 2. Peak 1 appears identical to commercially prepared nystatin. By nuclear magnetic resonance (NMR) and mass spectral analysis, peak 2 appears to be an isomer of peak 1. The isomers are quantitatively and fully interconvertible. Formation of peak 2 is accelerated at a pH of >7.0 and ultimately reaches a near 55:45 (peak 1/peak 2 ratio) mixture. We sought to determine the relative activities of peaks 1 and 2 against *Candida* spp. Peak 2 consistently showed higher MICs when it was the predominant form during the experiment. Time-kill analyses showed that peak 2 required $\geq 8\times$ the concentration of peak 1 to produce a modest and delayed killing effect, which was never of the same magnitude as that produced by peak 1. In both types of assays, the activity of peak 2 corresponded with intra-assay formation of peak 1. Both MIC measurements and time-kill analysis suggest that peak 2 has considerably less activity, if any at all, against *Candida* spp. Peak 2 may serve as a reservoir for peak 1.

Life-threatening fungal infections have become increasingly prevalent among patients with human immunodeficiency virus or AIDS, patients with cancer, transplant recipients, and patients in intensive care units (1, 2–4, 6, 10). Therapeutic options are often limited by the toxicity of currently available systemic antifungal agents and the emergence of resistance (9, 24, 25). This has prompted the development of new antifungal agents, as well as the “rediscovery” and “reengineering” of agents where use had been limited due to toxicity (16).

Nystatin is a polyene-macrolide antifungal antibiotic produced by *Streptomyces noursei* that was discovered and developed in the 1950s (18). It is now widely available for the topical treatment of localized fungal infections. Toxicity problems prevented its use as a systemic agent, but recently developed liposomal delivery technologies have made it an attractive candidate for the treatment of severe systemic fungal infections (12, 16, 17, 20, 26; C. J. Jessup, T. J. Wallace, and M. A. Ghannoum, 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. F-88, p. 161, 1997). This has prompted new investigations of its antifungal properties and spectrum as well as its physicochemical and pharmacokinetic characteristics (5, 7, 11, 15; S. Arikan, M. Lozano-Chiu, V. Paetznick, D. Gordon, T. Wallace, and J. H. Rex, Abstr. 98th Gen. Meet. Am. Society Microbiol., abstr. C-280, p. 178, 1998).

Commercially prepared nystatin appears as a single, highly pure chromatographic peak (hereinafter referred as peak 1) while in an organic solvent. However, when placed in a biological matrix, such as human plasma or culture medium, chromatographic analysis yields a second peak that elutes after the pure peak seen from nystatin stored in an organic solvent. Earlier work (data on file at Aronex Pharmaceuticals) sug-

gested that the appearance of this second peak, termed peak 2, is accelerated at a pH above 7.0 and relatively inhibited at a pH below 6.0. The purpose of this study was to determine the nature of nystatin peak 2, as well as to measure the relative antifungal activities of these two forms of nystatin.

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MATERIALS AND METHODS

Peak 2 isolation. Nystatin as received from the manufacturer (Gist-Brocades, Capua, Italy) generates a single peak on high-performance liquid chromatography (HPLC) that contains >99% of the loaded material. This peak is termed peak 1. Nystatin peak 2 was generated by adding 0.5 ml of nystatin stock solution (300 mg/ml in dimethyl sulfoxide [DMSO]) to 4.5 ml of RPMI 1640 tissue culture medium (pH 7.5) and vortexing for 1 min. This mixture was then extracted with 12.5 ml of methanol, vortexed for 1 to 5 min, and then allowed to incubate at 37°C overnight (16 to 18 h). After incubation, the sample was vortexed and then centrifuged at $1,800 \times g$ to pellet any precipitated material. The supernatant was then transferred to a 20-ml syringe for injection onto the preparative HPLC system.

Nystatin peaks 1 and 2 were separated by a preparative method in which nystatin peak 1 eluted with a retention time of approximately 8 to 11 min and peak 2 eluted with a retention time of approximately 11 to 18 min. A reversed-phase column (YMC-Pack ODS-AQ, AQ12S05–2520WT, 250 by 20 mm inside diameter, 5 μ m, 120 Å) was equilibrated and eluted with 40% water, 30% methanol, and 30% acetonitrile (pH 5.5). The flow rate was 30 ml/min, and the injection volume of the sample was 17.5 ml. The column eluent was connected in-line to a preparative flow detector with a fixed detection filter at 310 nm. The detector data were collected with the Millennium₃₂ Chromatography Manager software via an analog signal along a SAT/IN module. This manner of data collection allowed a real-time collection of peaks 1 and 2. The chromatograms were observed during each run, and the peaks were collected by hand.

For analytical purposes, a slight modification of the method described above was used. Briefly, the peak 1 concentration was analyzed by an isocratic HPLC method in which it eluted with a retention time of approximately 6 ± 1.0 min, depending on the indwelling volume of the HPLC system. A reversed-phase column (Waters μ Bondapak C₁₈, 125 Å, 10 μ m, 3.9 by 300 mm) was equilibrated and run with 10 mM monobasic sodium phosphate, 1 mM EDTA, 30% methanol, and 30% acetonitrile (pH 6.0). A column heater set to 30°C was employed to minimize the retention time shifts of the peaks. The flow rate was 1.8 ml/min,

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FIG. 1. Typical analytical chromatogram of nystatin in RPMI. The first few peaks correspond to components of the media and impurities. Nystatin peak 1 elutes at a retention time of 6.47 min. Nystatin peak 2 elutes at a retention time of 7.32 min. Elution times are faster than those found in preparative HPLC. AU, absorbance units.

and the injection volume of the sample was 100 μ l. The autosampler sample chamber was set at 4°C to minimize drug profile changes.

Nystatin, as received from the manufacturer, chromatographically purified nystatin peak 1, and chromatographically purified nystatin peak 2, as obtained by the method described above, were prepared and stored in DMSO at -70°C by Aronex Pharmaceuticals (The Woodlands, Tex.). Testing of stored aliquots, as well as the testing done as part of the experiments described below, demonstrated that the materials so prepared and stored remained stable for the time periods covered by these investigations.

Analysis of the physical characteristics of peaks 1 and 2. Mass spectral analysis by fast atom bombardment was performed by M-Scan, Inc. (West Chester, Philadelphia, Pa.), with a VG Analytical ZAB 2-SE high-field mass spectrometer.

Nuclear magnetic resonance (NMR) spectra were obtained by H-1 (proton) correlation spectroscopy, which provides information on proton connectivities in the molecule, and C-13, two-dimensional, heteronuclear correlation, which provides information on proton-carbon connectivities. Analysis was performed by Philips Petroleum Company (Bartlesville, Okla.).

Isolates. Starting with a collection of *Candida* bloodstream isolates that had been previously tested against liposomal nystatin (Arikan et al., Abstr. 98th Gen. Meeting, Am. Soc. Microbiol.), preliminary testing was carried out to select four strains that showed similar growth in RPMI at pH 6.0 and 7.5. The selected

strains were ATCC 750 (*Candida tropicalis*), 5W31 (ATCC 200950, *Candida lusitanae*), 34028074 (*Candida albicans*), and 34028111 (*Candida glabrata*).

Drugs. The preparation of nystatin, chromatographically purified peak 1, and chromatographically purified peak 2 was carried out as described above. Amphotericin B powder was obtained from Bristol-Myers Squibb (New Brunswick, N.J.). All drug dilutions were prepared at 100 $\times$ final concentration in 100% DMSO and then diluted once into the final testing medium.

Susceptibility testing. Susceptibility testing was done according to the National Committee for Clinical Laboratory Standards M27-A microdilution procedure (19) with the following modifications. The test medium was RPMI 1640 supplemented with an additional 18 g of glucose per liter (producing a final concentration of 20 g/liter). So that there would be adequate buffering across the range of relevant pH values, the medium was buffered with 0.075 M MOPS [3-(*N*-morpholino)propanesulfonic acid; $pK_a = 7.2$; useful buffering range, 6.5 to 7.9] and 0.075 M MES [2-(*N*-morpholino)ethanesulfonic acid; $pK_a = 6.1$; useful buffering range, 5.5 to 6.7]. The final pH was adjusted to either 6.0 or 7.5, depending on the experimental conditions. This medium is referred to as RPMI-MOPS/MES. After growth on Sabouraud dextrose agar overnight, the fungal inocula were prepared by the M27-A procedure to yield a final $2 \times$ inoculum (1×10^3 to 5×10^3 CFU/ml). Then, 100 μ l of the standardized $2 \times$ inoculum was combined with 100 μ l of RPMI-MOPS/MES containing the drug at twice the

FIG. 2. Typical time course of interconversion of peaks 1 and 2 starting with pure peak 2. The proportion remains relatively stable at pH 6.0, and the peak mixture reaches an approximate 60:40 proportion after 24 h at pH 7.5 ($\diamond$, peak 1; $\blacksquare$, peak 2).

FIG. 3. Biochemical structure of nystatin and proposed structure of nystatin peak 2 based on the methylated region changes found in NMR analysis.

desired final concentration. Plates were incubated at 35°C for 24 h. MICs were read after 12 and 24 h of growth. Following agitation of the plate, the MIC was taken to be the lowest concentration of drug that produced an optically clear well (visual MIC) or that produced 95% reduction of the optical density (OD) at 570 nm relative to the OD of the drug-free growth control well. The experiments were repeated in duplicate, and when duplicate rows yielded different values, the higher MIC was recorded. The testing range for nystatin was 0.008 to 4 µg/ml, and for amphotericin B, it was 0.015 to 8 µg/ml.

Time-kill analysis. Time-kill analysis by the method of Klepser et al. (14) for chromatographically purified peaks 1 and 2 was performed at the two pH values with *C. albicans* strain 34028074. Briefly, a suspension of 1×10^6 to 5×10^6 CFU was prepared in pH-adjusted RPMI-MOPS/MES, and 1 ml was inoculated in a tube containing 9 ml of pH-adjusted RPMI-MOPS/MES. This dilution yielded a final suspension of 1×10^5 to 5×10^5 organisms per tube. Peak 2 at pH 6.0 was tested at 1 to 32 µg/ml, and all other drug pH concentrations were tested at 0.125 to 4 µg/ml. (These concentration ranges extended above and below the MICs obtained in the susceptibility testing experiments.) Tubes were incubated at 35°C and shaken and sampled at 0, 0.5, 1, 2, 4, 6, 12, and 24 h. At each interval, 10 µl of the solution was directly plated, and another 10 µl was subjected to two 10-fold dilutions and then plated. Viable colony counts for each test condition interval and dilution were read and recorded after 24 to 36 h. The minimal yield of detection with these dilutions was 100 CFU/ml.

Chromatographic analysis. Samples from both the MIC studies and the time-kill analysis for the different intervals were frozen at -70°C and shipped to Aronex Pharmaceuticals for chromatographic quantification of absolute and

relative concentrations of nystatin peaks 1 and 2 by the analytical method described above.

RESULTS

Chromatographic and spectroscopic analysis. Figure 1 shows the analytical chromatogram of nystatin peaks 1 and 2 as obtained from RPMI medium at the end of one of the pH 7.5 susceptibility testing experiments. As seen in Fig. 2, the pace of conversion was dependent on the pH of the test medium. Conversion was rapid at pH 7.5, but slower at pH 6.0. Conversion occurred in both directions, from peak 1 to peak 2 and vice versa (data not shown). Quantification of peaks 1 and 2 for both susceptibility testing and time-kill assays (see below) showed that, regardless of initial starting form, peak 1 and 2 interconversion at pH 7.5 reached an approximate 55:45 (peak 1/peak 2 ratio) proportion after approximately 24 h in all experiments.

Mass spectroscopy for both peaks showed that the predominant ion was observed at m/z 926, with a corresponding sodium adduct at m/z 948. These masses are consistent with the

TABLE 1. Activity of amphotericin B and the two isomers of nystatin at pH 6.0 and 7.5^a

Drug and pH	Mean % at:				MIC (μg/ml) for strain:			
	0 h		24 h		<i>C. tropicalis</i> ATCC 750	<i>C. lusitaniae</i> 5W31	<i>C. albicans</i> 34028074	<i>C. globata</i> 34028111
	Peak 1	Peak 2	Peak 1	Peak 2				
Amphotericin B								
6.0					0.125	0.062	0.031	0.062
7.5					0.062	0.031	0.015	0.062
Nystatin								
6.0	98	2	83	17	0.5	0.5	0.25	0.25
7.5	16	84	53	47	0.5	0.5	0.5	0.5
Peak 1								
6.0	94	6	80	20	0.5	0.5	0.25	0.5
7.5	79	21	54	46	0.5	0.5	0.5	0.25
Peak 2								
6.0	7	93	27	73	2	2	2	2
7.5	23	77	54	46	0.5	0.5	0.5	0.5

^a Mean relative percentages of peaks 1 and 2 and microdilution MICs of amphotericin B, nystatin, and nystatin peaks 1 and 2 under the two pH conditions are shown.

calculated molecular mass (926.1 Da) of nystatin. Other minor ion clusters were observed at *m/z* 846, 864, 881, and 908 (data not shown). These minor ion clusters can all be explained by fragmentation of the parent compound. NMR results showed great overlap in both the H-1 and C-13 spectra of both peaks. Although precise structural estimation or prediction of peak 2 was not possible, there was a significant variation in the methylated region of peak 2 at 0.80 to 1.20 ppm in the H-1 spectra and 8.7 to 17.8 ppm in the C-13 spectra. These data, plus the time course and quantitative interconversion data, suggest that

peaks 1 and 2 are isomers. A plausible reconstruction based on the idea of a rotational change in the environment of the methyl group is shown in Fig. 3.

Susceptibility testing. Growth at 12 h was too limited to allow analysis. After 24 h of incubation, visual and spectrophotometric results were virtually identical, so spectrophotometric MICs were used for all results. Table 1 shows the final relative average concentration of peaks 1 and 2 in the testing wells, as well as the visual and spectrophotometric MIC results at 24 h. Amphotericin B showed either no changes or a 1-dilution

FIG. 4. Time-kill curves for nystatin peaks 1 and 2 under pH 6.0 and 7.5 conditions. For each condition, the time-kill results at a series of drug concentrations (in micrograms per milliliter) are shown plotted against the left-hand y-axis. The percentage of drug found to be peak 1 at each time point is plotted against the right-hand y-axis. The percentage of drug found to be peak 2 is equal to 100 – the percentage of peak 1. Note that all conditions use the same range of drug concentrations, with the exception of peak 2 at pH 6.0. GC, growth control.

increase in the MIC as the pH fell from 7.5 to 6.0. The MICs of nystatin and chromatographically purified nystatin peak 1 were virtually identical at the two pH values. On the other hand, the MICs of nystatin peak 2 at pH 6.0 were consistently fourfold higher than those for any other condition. Under this condition, peak 2 was the predominant form during the entire experiment, with peak 1 never representing more than 27% of the total drug.

Time-kill analysis. The standard susceptibility results suggested, but did not prove that peak 1 was more active than peak 2. The interconversion of the peaks, with the mixture tending towards a 55:45 mixture of the two peaks over time, complicated interpretation of the results. To better assess the effect of each isomer alone, a time-kill analysis was performed with *C. albicans* strain 34028074. Because the effect of polyenes occurs rapidly (8), early results from a time-kill assay should be a stronger measure of the effect of the starting isomer. As seen in Fig. 4, peak 2 required much higher concentrations than peak 1 to produce a modest killing effect that was not of the same magnitude as the others. The two peaks did not remain pure in any experiment, although testing at pH 6.0 provided >90% peak 1 at the end of the experiment when starting with peak 1 and >75% peak 2 at the end of the experiment when starting with peak 2. As seen in the same figure (and supported by data in Table 1), at pH 7.5, an approximate 55:45 mixture of both peaks was found after 12 h.

DISCUSSION

The formation of peaks 1 and 2 had been observed since early chromatograms, but had never been fully investigated (18, 21, 22). The biochemical data show that peaks 1 and 2 are very similar by mass spectrometry and NMR analysis. These data, plus the minor change in the methylated region seen on NMR and the quantitative interconversion of the two peaks, suggest that they are structural isomers. In the microbiological portion of the study, nystatin peak 1 proved to be more potent than nystatin peak 2. It is even possible that, if one takes into account the interconversion of these two isomers and the absence of pure peak 2 in any of the experiments, the antifungal activity seen when beginning with purified nystatin peak 2 is related entirely to its conversion to nystatin peak 1. Both the susceptibility testing and the time-kill analysis provide data to support this conclusion. For example, antifungal susceptibility testing at 24 h showed MICs of 2 µg/ml for peak 2, while peak 1 and pure nystatin produced MICs of 0.5 µg/ml. This is roughly fourfold less antifungal activity, and this activity may be due to the fact that the nystatin was 25% peak 1 at the end of the experiment. Likewise, the time-kill analyses suggest a difference in potency of at least eightfold.

The effects of pH on in vitro testing conditions are well known (13). Our susceptibility testing assay showed a onefold dilution variation for the different pH conditions when amphotericin B was used as a control. In assays with nystatin in which the initial isomer was principally peak 1, little effect of pH was seen. However, when the isomer was principally peak 2, there was a dramatic effect on activity. This effect is noticeable at both pH 6.0 and 7.5, although it is more evident at pH 6.0, due presumably to the reduced conversion to peak 1.

Looking at the pH 7.5 peak proportion data in Fig. 2 and 4,

we find a near 55:45 mixture of the peaks starting at 12 h and maintained up to our 24-h endpoint. Taking into account the bidirectional nature of this phenomenon, one could theoretically argue that formation of peak 2 acts as a reservoir for peak 1. Peak 1-peak 2 interconversion appears to be an unavoidable occurrence of little relevance, since the potency of the drug is unchanged. The in vivo significance of these findings, as well as their impact on pharmacodynamic parameters such as MIC/drug concentration ratios in vivo, remains to be studied. Further studies should focus on the final molecular characterization of both peaks, as well as traditional and basic pharmacokinetic properties such as differential protein binding (23) and toxicity issues for the two isomers, since these are largely unknown. Furthermore, the effect that liposome encapsulation may have on this phenomenon should be studied as the drug is further developed in that kind of formulation.

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