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Imbuing Aqueous Solubility to Amphotericin B and Nystatin with a Vitamin

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Abstract: Aqueous solubilities of many drugs in current clinical use are very low, necessitating formulations that often present problems for parenteral administration, including toxicities due to the excipients used. Recognizing that pharmacologically active compounds frequently possess amines, we asked whether pyridoxal phosphate (PLP), an inoccuous, water-soluble vitamin, could be utilized to form prodrug-like complexes via the formation of imine or iminium adducts and whether the vitamin would impart solubilizing properties to such complexes. Direct spectroscopic and crystallographic data obtained using model primary and secondary amines showed that PLP forms stable imine adducts with primary amines under entirely aqueous conditions and at physiologic pH, while no reaction was observed for secondary amines; the basis of the exceptional stability appears to be a consequence of favorable H-bond interactions of the imine nitrogen with the 5-OH group of PLP. Amphotericin B and nystatin in their native forms display marked aqueous insolubility and possess lone primary amines. We were able to utilize PLP in achieving excellent solubilization of both of these antifungal agents, surpassing aqueous solubilities of 100 mg/mL. In in vitro bioassays, both polyenes in their PLP-adducted form display attenuated antifungal potencies which are attributable to "prodrug-like" complexes. These results point to the utility of excipient-free, entirely aqueous formulations of amphotericin B for parenteral use, and may also be extended to other primary amine-bearing compounds exhibiting poor aqueous solubility.

Keywords: Amphotericin B; nystatin; pyridoxal phosphate; aqueous solubilization

Introduction

Several drugs in current clinical use display very poor aqueous solubility. Parenteral formulations of drugs classified as Class II (high permeability, low solubility) or Class IV (low permeability, low solubility) in the Biopharmaceutics Classification System[1,2] are often challenging, and occasionally insuperable. Although we had earlier examined the issue of solubilizing amphotericin B (AmB) by covalent modification of the mycosamine residue,[3] our interest in developing

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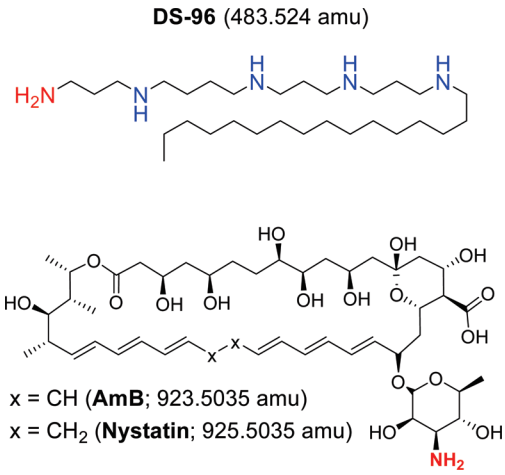

Figure 1. Structures of DS-96, AmB, and nystatin. Primary amines are colored red, while secondary amines are in blue.

general approaches toward overcoming problems with solubility of drugs perhaps began almost three decades ago when, as a medical student, one of the authors had noted but not understood at the time why nurses in the ICU had held that dextrose solutions were better than any other commonly used fluids at "clearing" intravenous lines and preventing thrombophlebitis.

Recent experiences with formulating DS-96 (Figure 1), an *N*-alkylhomospermine lipopolysaccharide-sequestering agent[4] for preclinical evaluation in animal models, were beset by unanticipated problems with aqueous solubility; although the polyamine compound would be expected to be very soluble, several salt forms, including the pharmaceutically acceptable hydrochloride salt, were not. We wondered whether the "dextrose solution" that the ICU nurses had relied upon could be brought to bear on this problem, reasoning that the highly water-soluble aldose could reversibly react with the primary and secondary amines of the polyamine, forming transient imine and iminium species, respectively. We were pleased when we found that the compound was indeed exceptionally soluble with dextrose.

Our success, however, could not be applied to more recalcitrant drugs such as AmB and nystatin (Figure 1). The antifungal agent AmB and its closely related analogue nystatin are notoriously insoluble and, unlike DS-96 AmB and nystatin would not yield at all to glucose; insignificant solubility was observed even in the presence of other cosolvents. We surmised that a pharmacologically innocuous, charged, polar, aldehyde would confer auxiliary solvation that neutral glucose could not. Pyridoxal phosphate (PLP; vitamin B_6) seemed eminently suitable, and indeed DS-96 was freely soluble when formulated with PLP. Both AmB and nystatin possess a lone primary amine on the mycosaminyl residue, whereas DS-96 has a single terminal primary amine and four secondary amines (Figure 1),[5] and it was of interest to examine whether primary or secondary amines could interact with PLP to form imine or iminium adducts, respectively, with sufficient stability as to confer aqueous solubility. We began with spectroscopic and crystallization studies with simple model primary and secondary amines which unequivocally showed that PLP reacts with primary but not secondary amines, leading to the formation of stable imine adducts which are stabilized by favorable H-bond interactions with the 5-OH group of PLP. Noting, as mentioned earlier, that AmB and nystatin have a single primary amine, we asked whether the complexation with PLP would allow aqueous formulations of these otherwise insoluble antifungal agents to be obtained. We found that both AmB and nystatin are easily solubilized in water, in the presence of PLP, achieving concentrations of >100 mg/mL (the highest concentration tested). The development of aqueous-soluble formulations of AmB, in particular, may have significant clinical implications.

Materials and Methods

Ethylamine, diethylamine, PLP, AmB, and nystatin were procured from Sigma-Aldrich (St. Louis, MO). Multiplicity-edited $^1H-^{13}C$ HSQC was acquired on a 500 MHz Bruker AVII spectrometer equipped with a cryogenically cooled probe. This experiment was collected with four transients per increment and an interscan delay of 2 s for a total acquistion time of 36 min. A total of 256 and 1024 data points with spectral widths of 27 672.80 and 8012.82 Hz were acquired in t1 and t2, respectively. The proton and carbon carrier were set to 4.703 and 100 ppm, respectively. Crystals of a 1:1 (molar equiv) mixture of N^1-methylpropane-1,3-diamine and PLP were obtained from a 1:1 methanol−water mixture. The structure of this model diamine−PLP complex was solved at the University of Kansas Small-Molecule X-ray Crystallography Laboratory. All hydrogen atoms were located from a difference Fourier and included in the structural model as individual isotropic atoms whose parameters were allowed to vary in least-squares refinement cycles.

Synthesis of Pyridoxal Phosphate Adduct of Ethylamine. The ethylamine−PLP adduct was prepared by the addition of PLP monohydrate (32.52 mg, 0.123 mmol) to ethylamine hydrochloride (5 mg, 0.61 mmol) in 2:1 molar ratio in 0.7 mL of D_2O. Anhydrous sodium carbonate was thereafter added to the clear solution of D_2O to adjust its pH to 7. The diethyamine−PLP adduct was prepared by the addition of PLP monohydrate (24.19 mg, 0.091 mmol) to diethylamine hydrochloride (5 mg, 0.046 mmol) in 2:1 molar ratio in 1 mL of formic acid. The reaction was stirred for 15 min, and the formic acid was thereafter completely removed

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under reduced pressure to obtain a yellow solid, which was solubilized in 0.7 mL of D_2O and pH adjusted to 7 by adding anhydrous sodium carbonate to the clear solution of D_2O.

Synthesis of ^{15}N-Alanine Methyl Ester and *N*-Ethyl ^{15}N-Alanine Methyl Ester. ^{15}N-Alanine methyl ester (model primary amine) was synthesized by reaction of ^{15}N-alanine (Sigma-Aldrich) in methanol using 1.5 equiv of thionyl chloride under anhydrous conditions; the reaction was stirred for 24 h, and then the solvent was removed under vacuum and dried to obtain the product in quantitative yields. ^{15}N-Ethyl alanine methylester (model secondary amine) was obtained by reductive amination of ^{15}N-alanine methylester and acetaldehyde (0.7 equiv) using sodium cyanoborohydride and four to five drops of acetic acid. The reaction mixture was stirred for 4 h, and the solvent was then removed under vacuum to obtain the crude residue. In order to facilitate isolation by conventional flash chromatography, the crude residue was first *N*-Boc protected using $(Boc)_2O$ and triethylamine. The *N*-Boc group was then deprotected using HCl/dioxane solution by stirring for 12 h. The solvent was then removed under vacuum, and the residue was dried to obtain the ^{15}N-ethyl alanine methylester. Both of the above model amines were dissolved in 85% H_2O/D_2O with or without 2.0 equiv of PLP, and the pH was adjusted to 7.4 with anhydrous Na_2CO_3. 2D-$^{15}N-^{1}H$ HSQC NMR spectra were acquired using a Bruker Avance 800 MHz NMR instrument equipped with a TCI cryo probe. The spectra were recorded with 1024 complex points in the ^1H (t2, acquisition) dimension and 32 complex points in the ^{15}N (t1, indirect) dimension with sweep widths of 12 820 Hz in ^1H and 2595 Hz in ^{15}N and 32 scans per t1 increment. The sample temperature was maintained at 298 K (25 °C). Identical acquisition parameters were used for all samples.

Synthesis of Pyridoxal Phosphate Adducts of AmB and Nystatin. To a solution of 20.0 mg of AmB or nystatin in 2 mL of dimethylformamide (DMF) [10.8 mM] was added a solution of 20 mg of PLP in 2 mL of water (adjusted to pH 7.4 with Na_2CO_3) [40.4 mM]. Both solutions were flash-frozen in liquid nitrogen and lyophilized overnight using a VirTis AdVantage bench top freeze-dryer. The resultant solids were all found to be soluble upon addition of water; both AmB− and nystatin−PLP adducts were found to be soluble at 100 mg/mL (the highest concentration tested). The presence of the polyene antifungals in aqueous solution was confirmed by LC-MS.

LC-MS Characterization. The PLP adducts were analyzed by reverse-phase LC-ESI-MS performed on a Shimadzu LC system (LC-10AD binary pumps, SCL-10A diode array detector) using a Zorbax 3.0 mm × 150 mm 3.5 μm stable-bond C_{18} reverse-phase column with a 40 min binary gradient (10−100% linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (TFA)/0.1% TFA in H_2O). ESI-MS data was acquired on an Agilent LC/MSD-TOF instrument with a mass accuracy of 3 ppm and a range of 100−3500 Da. Calibration drift was minimized on a scan-by-scan basis to less than 10 ppm by using internal standards corresponding to 922.0001 and 2721.0201 marker ions

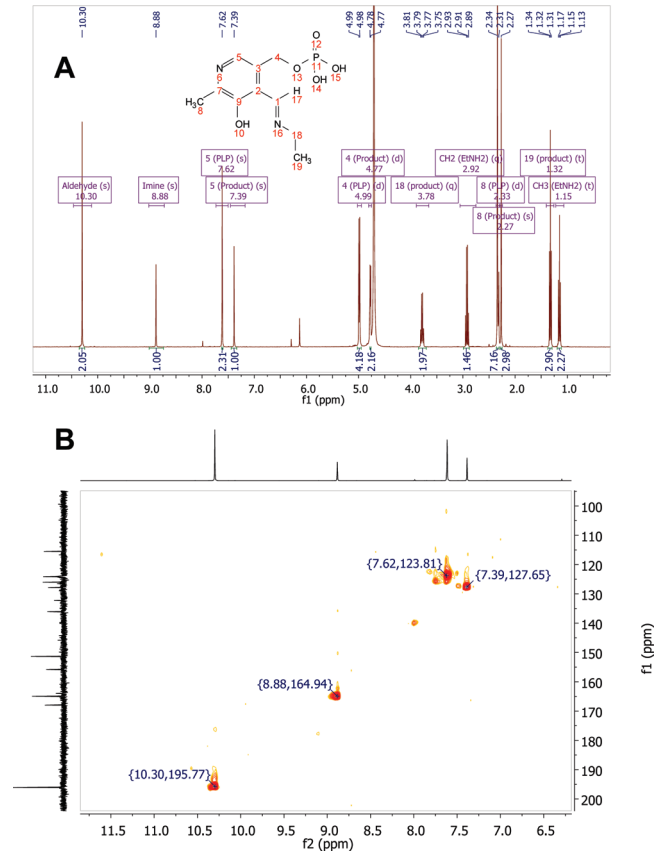

Figure 2. (A)^1H NMR spectrum of a 1:2 molar ratio mixture of ethylamine and PLP in D_2O, showing the imine proton at 8.88 ppm and two distinct sets of methyl and methylene proton resonances indicating the coexistence of complexed and free amine. (B) Partial $^{13}C-^{1}H$ HSQC NMR spectrum of ethylamine−PLP in D_2O. The imine cross-peak at 8.88 ppm (^1H dimension) and 164.94 ppm (^{13}C dimension) is clearly evident.

infused concurrently through a second nebulizer in the ionization chamber. MS acquisition was commenced 5−7 min after injection to avoid contamination of the ionization chamber (as well as ion suppression) by excess PLP and inorganic solutes.

Determination of Minimum Inhibitory Concentration (MIC). Minimum inhibitory concentrations of reference AmB and nystatin (stock solutions at 100 μg/mL in dimethyl sulfoxide (DMSO)) and the AmB−PLP and nystatin−PLP adducts (100 μg/mL in H_2O) were determined by appropriate broth microdilution methods as described previously.[3,6] Compounds were serially twofold diluted in quadruplicate in a 384-well microtiter plate in Sabouraud's dextrose broth using a Biotek Precision 2000 automated microplate pipetting system. A single colony of *C. albicans* ATCC 26278 was dispersed in the broth and was added to the wells with

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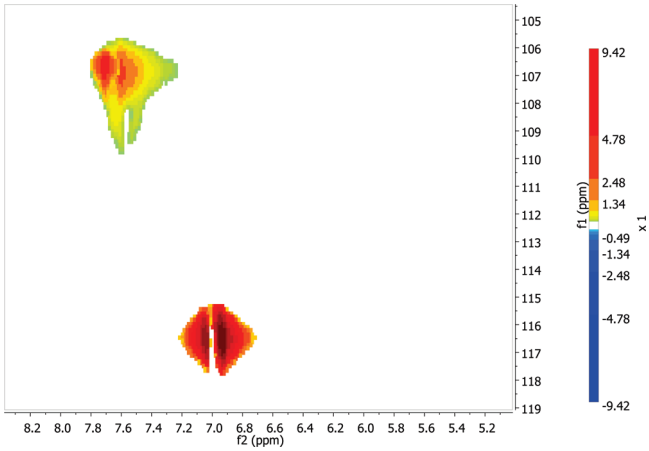

Figure 3. Overlay of $^{15}N-^{1}H$ HSQC NMR of ^{15}N-alanine methyl ester and reaction of ^{15}N alanine-methyl ester with pyridoxal phosphate. Samples were prepared in 85% H_2O/D_2O. The cross-peak centered at 107 ppm (^{15}N dimension) and 7.7 ppm (^{1}H dimension) corresponds to the free primary amine, while the cross-peak at 117 ppm (^{15}N dimension) and 7 ppm (^{1}H dimension) appears after the addition of PLP and is attributable to the imine.

appropriate vehicle-only controls. The microtiter plates were incubated overnight at 37 °C and then read at an absorbance of 600 nm.

Results and Discussion

The aldehyde of PLP is well-known to form imines with the ε-amino group of lysine in the active site of PLP-dependent transaminases as well as with the amines of amino acid substrates.[7−9] Given that the otherwise aqueous insoluble DS-96 was found to be freely soluble in the presence of PLP, we were not sure if secondary amines could also interact with PLP to form quasi-stable iminium adducts. We therefore set out to examine the reactivity of primary and secondary amines with PLP under aqueous conditions using ethylamine and diethylamine as simple model amines.

In ^{1}H NMR experiments, the addition of PLP to ethylamine (2:1 molar ratio) in D_2O (adjusted to pH 7 with Na_2CO_3) resulted in the appearance of a new imine proton at 8.88 ppm; since an excess of PLP was used, the aldehydic proton at 10.30 ppm was, as expected, also observed (Figure 2A). Importantly, evidence for the coexistence of complexed and free ethylamine was clearly evident by the presence of two distinct sets of the methyl and methylene proton resonances of the $EtNH_2$ (Figure 2A). In $^{13}C-^{1}H$ HSQC experiments, all resonances (other than quaternary carbons) corresponding to the expected imine adduct were observed; the imine cross-peak at 8.88 ppm in the ^{1}H dimension corresponded to 164.94 ppm in the ^{13}C spectrum (Figure 2B). Consistent with these observations, we also observed the appearance of an imine cross-peak in $^{15}N-^{1}H$ HSQC experiments with ^{15}N-labeled alanine methyl ester (Figure 3). No evidence for stable adducts of PLP with either diethylamine (or *N*-ethyl ^{15}N-alanine methyl ester) were, however, observed under a variety of conditions (data not shown), indicating clearly that only primary but not secondary amines form stable adducts with PLP. We were at first surprised that the half-life of the imine adducts would be so long (relative to NMR time scales) at physiologic pH in pure aqueous conditions as to yield distinct spectroscopic signatures, but we noted that this unexpectedly large stability could be a consequence of favorable H-bonds as had been noted earlier in crystal structures.[7]

We wanted not only to explore the structural basis of the stability of the adduct but also to obtain definitive evidence for the participation of primary but not secondary amines with PLP. A crystal structure of PLP adducted to a model diamine (N^1-methylpropane-1,3-diamine) showed the formation of the anticipated H-bond-stabilized imine with the terminal primary amine, while the secondary amine (N3) was found to be free (Figure 4).

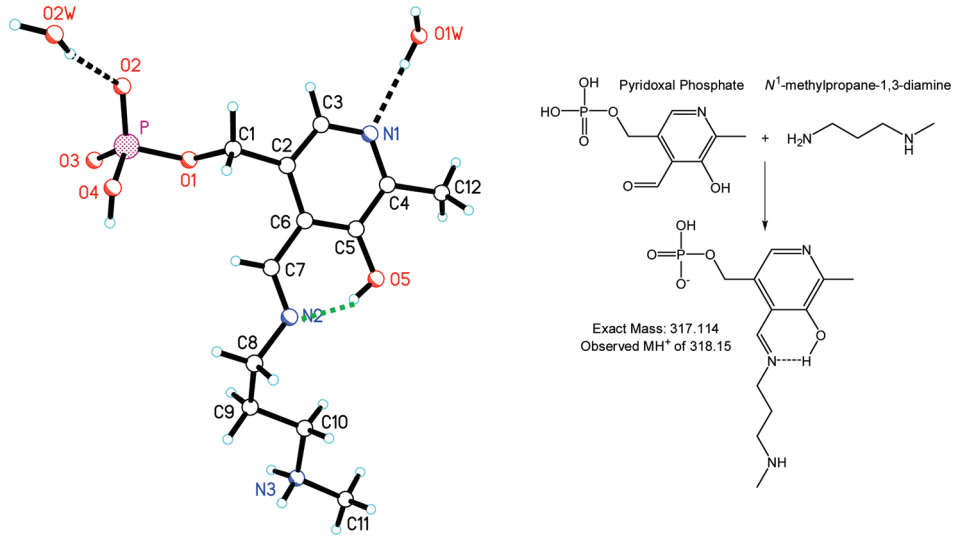

Figure 4. Crystal structure of N^1-methylpropane-1,3-diamine−PLP adduct showing the participation of the primary amine, and H-bond mediated stabilization of the resultant imine.

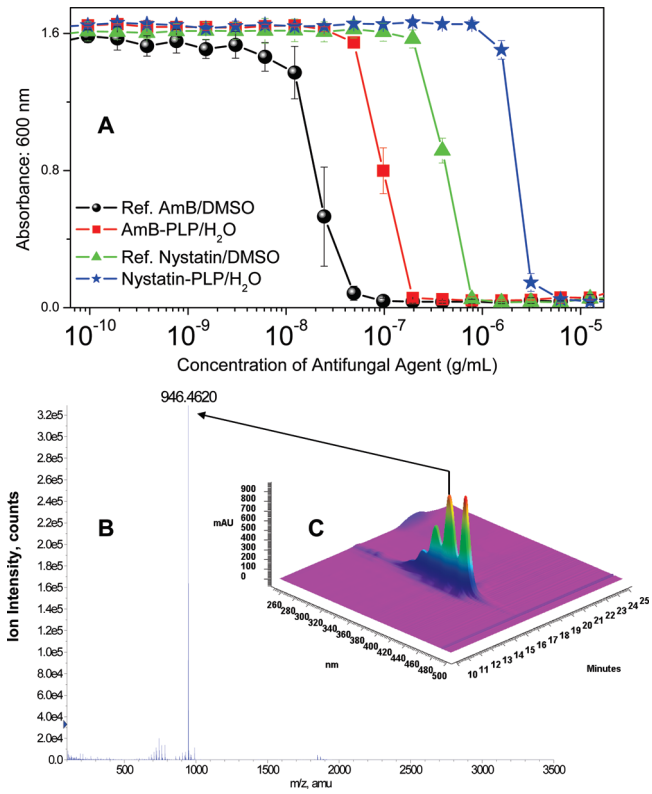

Figure 5. (A) Inhibition of growth of C. albicans by reference AmB and nystatin (in DMSO) and the AmB−PLP and nystatin−PLP adducts (in water) determined by microdilution method. Data represent means and SD obtained on quadruplicates. (B) LC-MS of an aqueous solution of AmB−PLP showing the expected M + Na^+ ion (946.46 amu). (C) Inset indicates the retention time and real-time absorption spectra (photodiode array detector output) of AmB.

We next asked whether the stable adducts that are formed with primary amines and PLP could be applied to clinically relevant, more complex molecules. Adducts of the antifungals, AmB and nystatin, obtained by lyophilization of the polyene−vitamin mixtures in DMF/H_2O, were found to be highly water-soluble (>100 mg/mL). The PLP adducts display lowered antifungal potencies in vitro; this is attributable to the expected preponderance of the prodrug-like adduct owing to the deliberate excess of PLP employed to increase mass-action-driven adduction (Figure 5A). Examination of aqueous solutions of the AmB−PLP adduct by LC-MS demonstrated unequivocally the presence of AmB (M + Na^+: 946.46 amu; Figure 5B) with its distinct absorption spectrum (Figure 5C). Control experiments with reference AmB dissolved in DMSO showed identical retention times, absorption, and mass spectra. Similar data were obtained for nystatin (data not shown).

The pharmaceutical applications of pyridoxal prodrug-like adducts are self-evident. Specifically, the toxic deoxycholate formulation of AmB can now be eliminated and replaced with purely aqueous formulations for parenteral use. These results may perhaps also be extended to other insoluble primary amine-bearing drugs.

Abbreviations Used

AmB, amphotericin B; ESI, electrospray ionization; LC-MS, liquid chromatography−mass spectrometry; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; PLP, pyridoxal phosphate; TFA, trifluoroacetic acid.

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