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Research Article

AMPHOTERICIN-B – *SCLEROTIUM ROLFSII* LECTIN COMPLEX: IT'S SOLUBILITY IN WATER AND ANTIFUNGAL ACTIVITY

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ABSTRACT

An *in silico* and an *in vitro* analyses on the binding of Amphotericin B to *Sclerotium rolfsii* lectin (SRL) were carried out. The *in silico* analysis indicates that the drug binds on the outer surface of the lectin by interacting with six amino acids (Asp77, Gln98, Asn100, Arg101, Arg107 and Tyr112) at a distance of ~3Å and has an overall free energy of binding of -8.5kcal/mol. This complex was prepared and found to be water-soluble. The determination of *in vitro* antifungal activity suggests that the complex is fungistatic in nature.

Keywords: Amphotericin B; *Sclerotium rolfsii* lectin; *In silico* analysis, Antifungal activity.

INTRODUCTION

Amphotericin B (AmB), a macrocyclic polyene with broad spectrum antifungal activity has been used for more than 40 years in clinical practice. It has been a drug of choice for invasive fungal infections and has been licensed for a greater number of indications than any other antifungal agent[1,2]. By itself AmB is poorly soluble in water, and at high doses it is toxic for use because of its hemolytic activity. These limitations prevent AmB from being used above a threshold level, restricting the systemic use of the molecule as a drug. To circumvent these limitations, AmB is normally administered as a deoxycholate or as various other lipid formulations. However these strategies, though partially successful, narrow the therapeutic index of the molecule[3]. In this regard the development of a more versatile water-stable and well dispersed aqueous solution of AmB with low intrinsic toxicity and low manufacturing cost remains a noteworthy objective.

Lectins are among the well characterised proteinaceous agents that facilitate the aqueous dispersion of insoluble compounds. Lectins are known to bind carbohydrates with high specificity which involves both hydrophobic and hydrogen bond interactions[4]. The use of lectins as drug delivery agents has also been considered earlier[5]. A non-immunogenic lectin derived from the fruiting bodies of a soil borne plant pathogenic fungi, *Sclerotium rolfsii* termed SRL was studied for its role as drug carrier[6]. The crystallographic structure of *S. rolfsii* lectin has been reported by Swamy *et al*[7]. This 17 kDa lectin is known to possess specific antigen binding and immuno-modulatory properties and is also reported for its nematicidal activity[6,8].

Previously, we have reported *in silico* and *in vitro* studies on albumin-AmB complexes and demonstrated their water solubility and biological activities by broth dilution method[9]. In the present study we have explored interaction of AmB with this SRL lectin in order to form a water soluble complex and evaluated its antifungal property.

MATERIALS AND METHODS

Materials

Human serum albumin ≥96% (HSA) and Amphotericin B (AmB) were obtained from Sigma–Aldrich (St. Louis, MO, USA). AmB was dissolved in dimethyl sulphoxide (DMSO) as stock solutions of 1mg/ml and stored at -20°C until used. The lectin from *Sclerotium rolfsii* (SRL) was purified in-house and was single entity as determined by SDS-PAGE[7]. All other reagents were of analytical grade and MilliQ water was used throughout.

Culture preparation

Candida albicans (NCIM 3466) and *Candida glabrata* (NCIM 3236) were obtained from NCIM, National Chemical Laboratory, Pune, India. The cultures were grown overnight in Sabouraud dextrose broth (Himedia, India) at 37°C and used for the assays.

In silico analysis of SRL-AmB complex

AmB structure was obtained in 3D format from PubChem (http://pubchem.ncbi.nlm.nih.gov) and energy minimized using VEGA-22 program. The crystal structure of *Sclerotium rolfsii* lectin (SRL) was obtained from the Protein Data Bank (PDB ID-2OFC). The crystal structure indicates a stable dimer formation. In this study a monomer was taken and docked with AmB using AutoDock Vina-1.1.1 running on Windows-7.0, 64-bit, i3 processor-computing machine[10]. A grid box of 46, 36, 42 points in x, y and z directions was built, centred on the SRL protein. At the end of docking, ligand with the most favorable free energy of binding was selected as the resultant complex structure. The resultant structure was analyzed using UCSF Chimera and PyMOL visualization program[11,12].

Preparation of SRL- Amphotericin B complex

To a solution of SRL (100mg) in 200ml phosphate buffered saline (PBS), pH 7.4, AmB (5mg dissolved in 500µl DMSO) was added and stirred well. The mixture was incubated at 37°C for 30min with constant stirring. The complex was diluted to 300ml by addition of (100ml) PBS and subjected to diafiltration (Amicon-stirred cell setup) under nitrogen pressure until the volume reduced to 100ml. The process of dilution and concentration was repeated twice (until barely detectable range of antibiotics was found by spectral analysis of the flow through). The final clear coloured retantate was lyophilized upon clarification by centrifugation.

Preparation of HSA- Amphotericin B complex

To a solution of HSA (100mg) in 200ml PBS (pH 7.0), AmB (5mg dissolved in 500µl DMSO) was added and stirred well. The mixture was incubated at 37°C for 30min with constant stirring. The complex was diluted to 300ml by addition of (100ml) PBS and subjected to diafiltration as above.

Spectroscopic measurements

A UV-visible spectrum of the protein, drug-protein complex in MilliQ water with DMSO (1% v/v) was recorded. AmB dissolved in DMSO was also recorded in the range of 250-600nm at 37°C with 1cm path-length using Shimadzu UV-Visible spectrophotometer (UV 2450). The concentration of AmB in the complex was estimated by extracting the drug from the complex using DMSO and estimating the levels using extinction coefficient at 416nm (ε=1.214 X 10^5 $M^{-1}cm^{-1}$)[13].

Determination of antifungal activity

The Minimum inhibitory concentration (MIC) of the drug and its complexes were determined by Broth dilution method according to NCCLS guidelines[14].

Determination of post-antifungal effect

The protein and its complexes were solubilized in water and filter sterilized using 0.45µ sterile syringe filters. Different concentrations, namely 2µg and 5µg of AmB were aliquoted into sterile micro-centrifuge tubes and made up to 1ml using sterile water. These were in turn transferred aseptically to 5ml Sabouraud dextrose broth (the final conc. of AmB was 0.33µg/ml and 0.83µg/ml). Inoculums of *Candida albicans / Candida glabrata* (overnight culture) were added such that the final $O.D_{600nm}$ was 0.1. Also AmB and DMSO were taken as controls. The culture tubes were incubated for 16h at 37°C and $O.D_{600nm}$ was recorded.

The overnight incubated media from culture tubes (600µl) was aliquoted into sterile micro-centrifuge tubes and centrifuged at 8000rpm for 10min. The supernatant discarded and re-suspended in fresh sterile Sabouraud dextrose broth (600µl) and plated (50µl) in triplicates on Sabouraud dextrose agar. Plates were incubated at 37°C for 24h and the number of colony forming unit (CFU) determined. A total of five independent trials were conducted and the data was presented as mean ± standard deviation.

RESULTS

In silico analysis of SRL-AmB complex

AmB was successfully docked on to SRL according to the protocol mentioned above (Figure-1). The free energy of binding and the region of binding were determined using these computational studies. The study predicts that the polar groups of the drug binds on to the surface of the protein by formation of Hydrogen bonds. The analysis of inter-model H-bonds indicates that there is a possible formation of one or more hydrogen bonds by interacting with amino acids namely Arg101 (either of $-NH_2$ groups), Arg107 ($-NH_2$) and Tyr112 (either of –OH groups). The overall free energy of binding was estimated to be -8.5 kcal /mol.

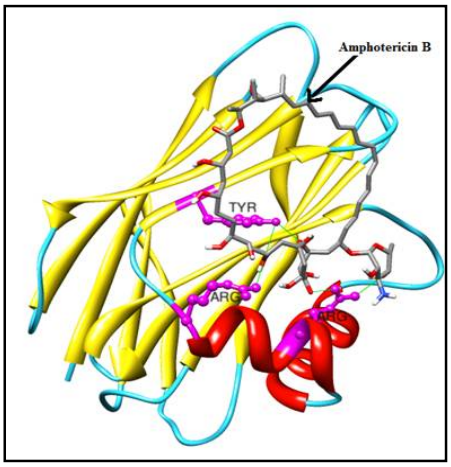

Fig. 1: *In silico* analysis- binding of Amphotericin-B to SRL.

Analysis of SRL-AmB complex by UV-visible spectroscopy

The UV-visible absorption spectrum of AmB in DMSO showed sharp peaks at 416, 391, 372 and 353nm. However when the spectrum was recorded in PBS to which AmB solubilized in DMSO (final conc. 1% v/v) was added, the spectrum showed peaks at 408, 384, 364 and 345nm. There was an overall blue-shift of about 8nm with reduced peak intensity (Figure 2). This observation is similar to the one reported earlier by Vandermeulen *et al*[15].

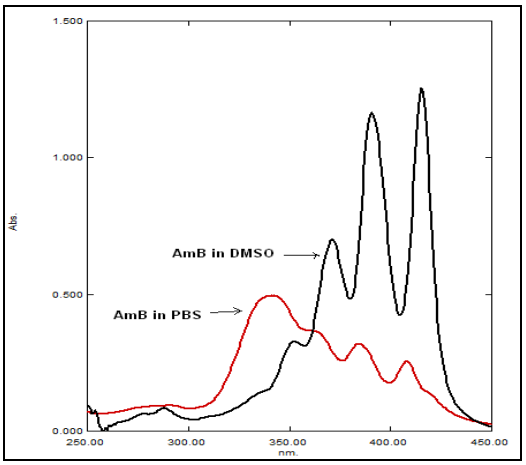

Fig. 2: UV-Visible spectral analysis of AmB dissolved in DMSO (100%) and in PBS containing 1% DMSO.

The protein, SRL in PBS containing 1% v/v DMSO showed a lone peak around 280 nm. When AmB was added to SRL in PBS with DMSO 1% v/v, the spectral property of AmB showed significant changes (Figure 3). The complex formed between AmB-SRL showed a peak at 408, 384, 364 and 335nm. Though there was blue-shift of 345nm peak to 335nm, the absorbance change showed hypochromicity. Similarly the peak at 364nm also showed decreased absorbance and the 408nm peak remained unaffected. The hypochromicity observed here for AmB binding to SRL is in direct contrast to the hyperchromicity noted for the same ligand bound to albumins[9,16]. This suggests that environment in the binding pocket is quite different in these proteins.

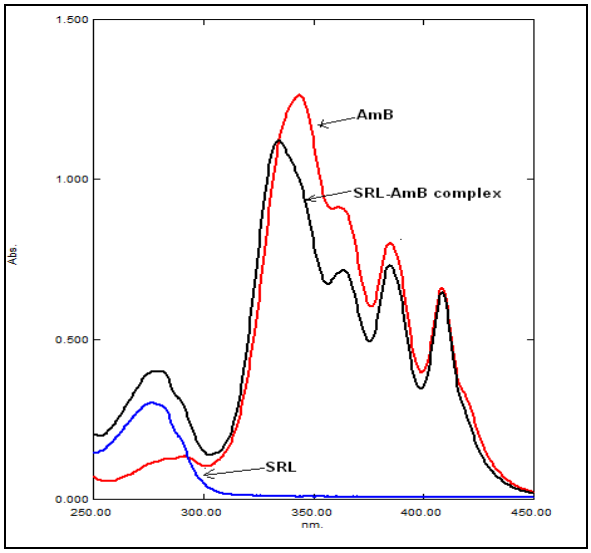

Fig. 3: UV-Visible spectral analysis of AmB and SRL-AmB complex.

Table 1: *In vitro* post-antifungal assay for free and complexed amphotericin B

Culture used/ Sample tested	Conc. of AmB/ Log_{10} CFU per ml [a]	
	0.33 µg/ml	0.83 µg/ml
Candida albicans (NCIM 3466)		
AmB	4.01 ± 0.15	3.14 ± 0.08
HSA-AmB complex	3.14 ± 0.06	2.38 ± 0.09
SRL-AmB complex	Dense	Dense
Candida glabrata (NCIM 3236)		
AmB	4.41 ± 0.04	3.30 ± 0.12
HSA-AmB complex	3.21 ± 0.05	2.43 ± 0.06
SRL-AmB complex	Dense	Dense

[a] Values are representative of mean ± SD (N=5).

AmB when dissolved in (100%) DMSO gives a ratio of (A_{345nm}/A_{408nm}) ~0.25 (Figure 2) suggesting that the drug is in monomeric form[17]. However in presence of PBS (with 1% DMSO) this ratio increases to ~1.9, suggesting an increase in self association (aggregation). Furthermore, in the presence of SRL it is noted that this value is ~1.7 (Figure 3). This would suggest that in the presence of protein the associative state of the antibiotic has slightly decreased.

To understand the nature of the complex further, SRL-AmB mixture was subjected to diafiltration with 10kDa cut off membrane with repeated addition of buffer (PBS) until there was barely detectable level of antibiotic in the filtrate. The UV-Visible spectrum for the retantate remained identical to the spectrum of the undialyzed mixture. This confirmed that the changes observed are presumably due to the complex formation.

Molar ratio and solubility of the complexes

The concentration of the AmB bound to SRL was calculated using UV-visible spectral data as mentioned in methods. The amount of AmB bound to SRL was 50µg/mg of protein (molar ratio ligand/protein is 0.86). The solubility of the SRL complex was uniformly at ~10mg/ml in water, similar to the HSA-AmB complex reported earlier[9].

Antifungal activity and post antifungal effects

The antifungal activity was based on minimal concentration of the drug (MIC) required to completely inhibit *Candida* spp. over a fixed period of time by standard broth dilution assays. Both *C. albicans* and *C. glabrata* isolates had MICs of 0.33µg/ml for AmB and HSA-AmB complex whereas, it was 0.4µg/ml for SRL-AmB complex. The test indicates that the antifungal activity of AmB was well preserved in albumin-AmB and SRL-AmB complexes. It was also observed that at the same concentrations, HSA and SRL proteins by themselves exhibit no antifungal activities.

The post-antifungal effect of AmB and its complexes were studied to understand the suppression of fungal growth that persists for a limited period of exposure to AmB-protein complex. A concentration equivalent to MIC and 2.5x MIC (0.33 and 0.83µg/ml) was taken in order to understand the effect of the drug and the complex. The *Candida spp*. when exposed to free AmB showed complete inhibition in the broth culture. But post exposure they showed Log_{10} CFU/ml in the order of ~4.0 (for 0.33µg/ml AmB) and ~3.0 (for 0.83µg/ml AmB) respectively, thus indicating that there is a partial fungicidal activity. As shown in Table 1, post exposure HSA-AmB complex show marginally better inhibition of *Candida* cultures when compared to the drug alone. The *C. glabrata* cultures exposed to HSA-AmB showed fewer colonies than AmB alone. Interestingly, SRL-AmB

complex did not exhibit post exposure inhibition of fungal growth, perhaps suggesting that it is fungistatic in nature. Also controls like SRL and HSA did not inhibit fungal growth (data not shown).

DISCUSSION

Amphotericin B has been used in the treatment of fungal infections for over four decades. Besides fungal infections, it has also been used for treating visceral leishmaniasis (Kala-azar) [3]. Though AmB exhibits broad antifungal activity, its adverse side effects such as nausea, electrolyte imbalance and nephrotoxicity are of great concern. It is thought that the aggregated form of AmB, which is largely responsible for membrane damage, brings about these side-effects[18]. In order to mitigate these side effects several drug delivery systems such as detergent (deoxycholate) dispersions and liposomal preparations of AmB have been used[3].

The ability of proteins such as albumin and lectins to bind diverse ligands and still remain soluble in water has been investigated earlier for their potential use in protein based delivery systems[3,5,19]. Cytotoxic agents like doxorubicin and fluorouracil form complexes with serum albumin and show greater bio efficacy than the free drug[4]. Interestingly, AmB has also been shown to interact with serum albumins and Polyvinylpyrrolidone (PVP) rendering it water soluble[3,17].

In the present study, a lectin from *Sclerotium rolfsii* (SRL) has been used as a carrier protein for AmB. We report that AmB appears to form a complex with SRL but seems to be distinctly different from its interaction with albumin. In the *in silico* studies the drug was not in a groove or pocket in the lectin, but a surface association was discernible. The complex however was stable and water-soluble. The spectral perturbations observed in the SRL-AmB complex were very different from the one noted earlier for AmB-albumin complex. Although the SRL associated AmB shows a 5nm blue-shift of 340nm peak, there was no hyper-chromicity, as observed in the case of the AmB-albumin complex.

The antifungal activity was determined by broth dilution assay and found to be 0.33µg/ml for the AmB and HSA-AmB complex whereas it was 0.4µg/ml for the SRL-AmB complex. Post-antifungal assay was conducted inorder to determine whether the prepared complexes were fungicidal or static in nature. The HSA-AmB complex showed activity equal to or better than the drug alone, showing a concentration dependent fungicidal activity. However the cultures exposed to SRL-AmB complex showed dense growth indicating that it has only fungistatic activity. This may be due to an insufficient release of the drug into the solution.

However further investigation is necessary to understand the interaction of AmB with lectin so as to discern its biological effects.

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