

Study of antifungal activity of amphotericin B-lipid formulations with five lipid carriers

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Abstract

Introduction: Invasive pulmonary aspergillosis (IPA) is a prime cause of morbidity and mortality in immunocompromised individuals, who is undergoing lung transplantation. Mortality among infected patients is high. Death rate in excess of 90% has been shown in study by Richardson. Most of these invasive mould infections are acquired through the respiratory tract. An increased incidence of invasive fungal infection has created major challenges for medical practitioners. Amphotericin B (AmB) has been the drug of choice for the treatment of many fungal infections, and it is still used as a gold standard therapy against invasive fungal infection and most commonly used to treat life-threatening conditions such as cryptococcosis, histoplasmosis, and IPA. Currently, lipid formulations are accepted as a less toxic alternative to the traditional colloidal dispersion. Drug toxicity, the nephrotoxicity of AmB is the major clinical problem, which could lead to treatment discontinuation. We designed five lipid formulations of AmB, and compared the dose-response effects of all lipid formulations in-vitro against three different fungal strains including *Cryptococcus neoformans*, *Candida albicans* and *Saccharomyces cerevisiae*.

Methods: Five lipid formulations amphotericin B were formulated by lyophilization process. The cylinder plate method performs the relative potency by determining the clear zone of AmB-lipid formulation in the inoculum medium as compared with a clear zone of AmB standard. Broth microdilution method can be used to measure the minimum inhibitory concentration (MIC). The MIC value is based upon predetermined end point, which may be interpreted as an absence of visible growth in a broth containing known concentration of AmB. The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined using *Cryptococcus neoformans* ATCC 90113 NS and *Candida albicans* ATCC 90028.

Results: All five formulations showed good responses against *C. neoformans* and *Candida albicans*, with the MIC and MFC values in the range between 0.16-0.32 µg/ml. The potency of these formulations was equivalent to pure AmB (100%).

Conclusions: The results indicate that the investigated potassium cholate, potassium deoxycholate, sodium deoxycholate sulfate, sodium cholate and sodium deoxycholate may be used as a promising alternative carrier system for amphotericin B.

Key words: Amphotericin B, *Cryptococcus neoformans*, *Candida albicans*, Potency, Lipid drug carriers.

Introduction

Invasive pulmonary aspergillosis (IPA) is a prime cause of morbidity and mortality in immunocompromised individuals, who is undergoing for lung transplantation^{1,2}. Mortality among infected patients is high. Death rate in excess of 90% has been shown in studies by Richardson³. Most of these invasive mould infections are acquired through the respiratory tract. An increased incidence of invasive fungal infection has created major challenges for medical practitioners. In the last decades, the incidence of

pulmonary fungal infections that has been increased due to the growing number of immunocompromised patients related to human immunodeficiency virus (HIV), organ transplantations, hematologic disorders and cancer⁴. Fungal infections of the lungs are less common than bacterial or viral infections but it is more difficult for diagnosis in early stage, because the clinical manifestations of invasive fungal and bacterial infections are similar, leading treatment to be delayed and more complicated. Antifungal drugs used for the treatment of pulmonary fungal infections can be classified into several groups such as the polyenes

(e.g. amphotericin B), the azoles (e.g. fluconazole, itraconazole, and ketoconazole) and pyrimidines (e.g. flucytosine)⁵. Over 40 years, AmB remains the drug of choice for treatment of life threatening conditions^{6, 7}. It is a broad spectrum and least resistance to the systemic fungal infection^{8,9}. The chemical structure of AmB (Fig. 1) shows a large lactone ring of 37 carbon atoms in which one side of the ring is a hydrophobic conjugated heptaene chain and the other side is hydrophilic due to the presence of seven hydroxyl groups. Amphotericin B (AmB) is a potent polyene antifungal and leishmanial agent. As with other polyene antifungals, AmB binds with ergosterol, a component of fungal cell membranes, resulting in the formation of pores that leads to monovalent ion (K^+ , Na^+ , H^+ and Cl^-) leakage, which is the primary effect leading to fungal cell death¹⁰. AmB has been the drug of choice for the treatment of many fungal infections, and it is still used as a gold standard therapy against invasive fungal infection¹¹ and most commonly used to treat life-threatening conditions such as cryptococcosis, histoplasmosis, and IPA. AmB is a amphiphilic drug composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic hydrocarbon chain on the other side with multiple conjugated double bonds, which has posed major hurdles for oral administration, despite the development of various carrier systems for oral delivery of AmB including lipid-based formulations¹², nano-suspension¹³, polymeric nanoparticles¹⁴, and cochleates¹⁵, the oral preparations of AmB is not yet commercially available because of a limited oral bioavailability due to low aqueous solubility, and low membrane permeability of AmB¹⁶. The various parenteral AmB products have been developed to overcome its low solubility, such as a liposomal formulation (Ambisone®), micellar dispersion with deoxycholate (Fungizone™) and lipid complex (Abelcet®), but currently, lipid formulations are accepted as a less toxic alternative to the traditional colloidal dispersion¹⁷. The nephrotoxicity of AmB is the major clinical problem, which could lead to treatment discontinuation^{18-19,9}. It is inevitable that there is a demand to develop less toxic product using new delivery system. In this study, we designed five lipid formulations of AmB, and compared the dose-response effects of all lipid formulations in-vitro against three different fungal strains including *Cryptococcus neoformans* and *Candida albicans*.

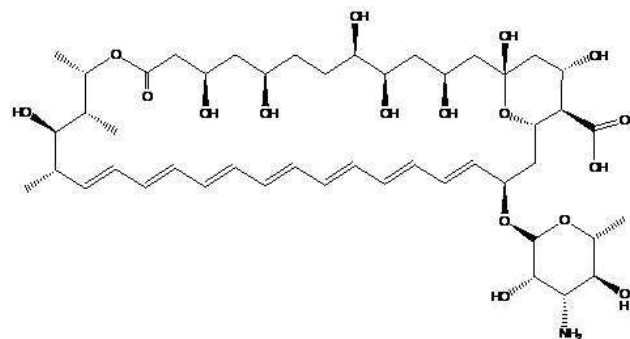


Figure 1. The chemical structure of Amphotericin B (AmB)

Methods

Pure AmB was obtained from Ambalal Sharabhai Enterprises Pvt. Ltd., Vadodara, India. Deoxycholic acid, cholic acid, sodium cholate, and sodium deoxycholate were purchased from Sigma-Aldrich, St. Louis, USA. Sodium deoxycholate sulfate, potassium cholate and potassium deoxycholate were synthesized in a laboratory. Dimethylsulfoxide was purchased from Riedel-de Haën, Seelze, Germany. Polyamide membranes with a pore size of 0.22 μm and 0.45 μm were obtained from Sartorius, Gottingen, Germany. All chemicals were used as received without further purification except tetrahydrofuran (THF). All other reagents and chemicals are analytical grade.

Preparation of Amphotericin B-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS)

Sodium deoxycholate sulfate (SDCS, 245 mg) was taken in beaker (100 mL) containing 30 mL distilled water and added sodium hydroxide (2.7 mL, 0.2M) to this solution with constant stirring (500 rpm) in a magnetic stirrer Heidolph MR Hei-Mix L (Heidolph Instrument, Schwabach, Germany). After obtaining clear solution, amphotericin B powder was slowly added (AmB, 250 mg) in part wise. When AmB was dissolved completely, it formed a clear yellowish color solution at room temperature. The pH of the solution was adjusted by adding phosphoric acid (0.2 M) to obtain a pH of 7.4 for an in situ phosphate buffer using pH meter (Precisa pH 900, Dietikon, Switzerland). The final volume of the solution was made to 50 mL by adding distilled water. The solution was filled into 10 mL on each vial and lyophilization by a freeze dryer (Dura Dry™ MP, FTS Systems Inc., NY, USA). The yellowish caked powder was formed. A similar methodology was employed to prepare the sodium deoxycholate (AmB-SDC), potassium deoxycholate (AmB-KDC), potassium cholate (AmB-KC) and sodium cholate (AmB-SC) formulation as that of deoxycholic acid as well as cholic acid and AmB.

Assay for anti-fungal activity

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms (USP 29-NF 24, 2006)^[20]. The cylinder plate method and broth microdilution were employed for microbial assay of AmB in lipid derivatives. The cylinder plate method performs the relative potency by determining the clear zone of AmB-lipid formulation in the inoculum medium as compared with a clear zone of AmB standard. Broth microdilution method can be used to measure the minimum inhibitory concentration (MIC). The MIC is that concentration of antifungal agents which inhibits the growth of a fungus under standardized test condition. The MIC value is based upon predetermined end point, which may be interpreted as an absence of visible growth in a broth containing known concentration of AmB^[21]. For

1-level assay with a standard curve, dilutions were prepared representing five test levels of the standard (S1-S5) were 0.64, 0.8, 1.0, 1.25, 1.56 µg/mL, respectively and a single test level of the unknown (U3) 1.0 µg/mL corresponding S3 of the standard curve. For deriving the standard curve, alternative cylinder were filled on each of five plates with the mean test dilution (S3) of the standard and each of remaining nine cylinders with one of the other four dilutions of the standard. The process was repeated for the five dilutions of the standard.

The microbiological method consisted of a cylinder-plate agar diffusion assay using *Saccharomyces cerevisiae* (ATCC 9763, Rockville, MD, USA) was the test microorganism as follows.

S. cerevisiae with approximately 10^8 colony-forming units (CFU)/mL was cultivated at 30°C on Sabouraud dextrose agar (Difco, NJ, USA) for 48 h. The yeast was then suspended in 0.9% NaCl and diluted to obtain a turbidity of $25 \pm 2\%$ at 530 nm. One milliliter portion of this suspension was added to 100 mL sterile antibiotic medium 19 at 50°C and used as the pre-inoculated layer. Six stainless steel cylinders of uniform size [8 mm (o.d.)x6 mm (i.d.)x10 mm] were placed on the surface of the inoculated medium using a mechanical guide. The cylinders were filled with standard AmB or the AmB in the lipid derivatives reconstituted dry powder formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS). All plates were incubated at 30°C for 16-18 h. These experiments were carried out in triplicate. The inhibition zone diameters were measured and the concentration of AmB calculated from the standard curve[20].

Microdilution method for determination of MIC and MFC

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined using

Cryptococcus neoformans ATCC 90113 NS and *Candida albicans* ATCC 90028 (Department of Pathology, Faculty of Medicine, Songklanagarind Hospital, Prince of Songkla University, Thailand) as described in previous work^[21]. Before testing, fungi were subcultured on Sabouraud dextrose agar (SDA) (Difco, NJ, USA) at 35°C for 24-48 h to ensure cultures for inoculation were in an active phase of growth. Stock suspensions were prepared in a sterile 0.9% sodium chloride solution and adjusted to give a final concentration of 1×10^6 - 5×10^6 CFU/mL (90% transmittance, 530 nm). For the standard AmB and AmB-lipid formulations reconstituted dry powders preparations, these were dissolved in sterile DMSO and sterile water at a concentration 5000 µg/mL, respectively. Stock solutions were stored at -70°C. Subsequently, the stock solutions were diluted with antibiotic medium 3 broth (Merck, Darmstadt, Germany) to a concentration of between 0.004 and 10 µg/mL. 10 microliter of the inoculum was added to the 96-well plate and 100 µL of a two-fold serial dilutions of standard AmB or the reconstituted AmB-lipid dry powder derivatives (AmB-SDCS, AmB-SDC, AmB-KDC, AmB-KC and AmB-SC) at concentrations of 0.004-10 µg/mL. The sterile medium was used as a negative control (sterility of medium) while the sterile medium with the inoculum was used as a positive control (growth of inoculums without AmB). The plates were incubated at 30°C for 48 h, and the absorption of the sample was recorded at a wavelength of 570 nm. All experiments were carried out in triplicate.

Results

A comparison of the potency of the five AmB-lipid formulations (AmB-KC, AmB-KDC, AmB-SC, AmB-SDC and AmB-SDCS) with those of the same concentrations of standard AmB are shown in Table 1.

Table1 Potency, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of AmB in lipid derivatives reconstituted dry powder (n=3)

Material	Lipid :AmB (mole ratio)	Potency %	<i>C. albicans</i>		<i>C. neoformans</i>	
			MIC (µg/mL)	MFC (µg/mL)	MIC (µg/mL)	MFC (µg/mL)
Pure AmB	0:1	100	0.32	0.63	0.32	0.63
AmB-SDC	2:1	102	0.16	0.32	0.16	0.32
AmB-SDCS	2:1	103	0.16	0.32	0.16	0.32
AmB-KDC	2:1	102	0.16	0.32	0.16	0.32
AmB-KC	2:1	104	0.16	0.32	0.16	0.32
AmB-SC	2:1	98	0.16	0.32	0.16	0.32

MIC and MFC were evaluated of five formulations (AmB-KDC, AmB-KC, AmB-SC, AmB-SDC and AmB-SDCS) and pure AmB against *C. albicans* and *C. neoformans*. The MIC and MFC values for the AmB are shown in Table 1

Discussion

Anti-fungal studies

The lipid derivatives carriers (KC, KDC, SC SDC and SDCS) without AmB did not inhibit the growth of yeast (this data is not presented). The lipid carriers did not inhibit the growth of fungi in culture plate, which was similar as an inoculum media contained the culture plate. AmB-lipid formulations of AmB-SDC, AmB-SDCS, AmB-KDC, AmB-KC and AmB-SC in the reconstituted dry powders had potency ranges of 102%, 103%, 102%, 104% and 98%, respectively. These values were equivalent to that of the standard AmB (100%). These results indicated that the lipid did not affect the potency of the AmB when compared to the standard. It also did not contribute to enhancing the potency of AmB by transporting it into fungal cells and / or facilitating a greater penetration into the fungus membrane¹¹. However, the lipid derivatives do play important roles for dissolving the poorly soluble drug AmB in water.

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

For AmB-lipid formulations (AmB-KDC, AmB-KC, AmB-SC, AmB-SDC and AmB-SDCS), the MIC and MFC exhibited a lower value than the pure AmB against *C. neoformans* and *C. albicans* when compared to that of the pure AmB. The obtained MIC and MFC results were almost similar to that of previous reported studies against *C. albicans* and *C. neoformans* in the range of 0.015 to 2 µg/mL^{22-23, 11, 24-27}. They were essential for the solubility of the AmB.

Conclusion

Five lipid derivatives such as KC, KDC, SC, SDC and SDCS, were chosen as lipid drug carriers. These carriers were applied to develop as lipid drug carriers system as a reconstituted dry powder AmB-lipid formulations.

AmB-lipid formulations were successfully prepared by lyophilization process (freeze drying) in molar ratio 1:2 (AmB: lipid carrier), formed solid caked, which was a very light, free flowing, hygroscopic in nature. These formulations were highly water soluble and stable in solution form with negatively charge developed. The potency and susceptibility testing against yeasts, *S. cerevisiae*, *C. albicans* and *C. neoformans*, the MICs and MFCs of all formulations was 2 fold less than that of standard AmB. The potency these formulations were an equivalent to pure AmB (100%). Although, these lipid drug carriers play vital role to dissolve the poorly soluble AmB into highly soluble and stable solution form.

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References

1. Segal BH. Aspergillosis. N. Engl. J. Med. 2009; 360: 1870–1884.
2. Mihara T, Takeya H, Izumikawa K, Obata Y, Nishino T, Takazono T, Kosai K, Morinaga, Kurihara S, Nakamura S, Imamura Y, Miyazaki T, Tsukamoto M, Yamamoto Y, Yanagihara K, Tashiro T, Kohno S. Efficacy of aerosolized liposomal amphotericin B against murine invasive pulmonary mucormycosis. J. Infect. Chemother. 2014; 20: 104–108.
3. Richardson MD. Changing patterns and trends in systemic fungal infection. J. Antimicrob. Chemother. 2005; 56 (suppl 1): i5–i11.
4. Limper AH, Knox KS, Sarosi GA, Ampel NM, Bennett JE, Catanzaro A, Davies SF, Dismukes WE, Hage CA, Marr KA, Mody CH, Perfect JR, Stevens DA, ATSF Group. An official American Thoracic Society statement: treatment of fungal infections in adult pulmonary and critical care patients. Am. J. Respir. Crit. Care Med. 2011; 183: 96–128.
5. Arnold TM, Dotson E, Sarosi GA, Hage CA. Proc Am Thorac Soc 2010; 7: 222–228.
6. Matsumori N, Eiraku N, Matsuo S, Oishi T, Murata M, Aoki T, Ide T. An amphotericin B–ergosterol covalent conjugate with powerful membrane permeabilizing activity. Chem. Biol. 2004; 11: 673–679.
7. Chudzick B, Tracz IB, Czernel G, Fiolka MJ, Borsuk G, Gagos M. Amphotericin B–copper (II) complex as a potential agent with higher antifungal activity against *Candida albicans*. Eur. J. Pharm. Sci. 2013; 49: 850–857.
8. Butani D, Yewale C, Misra A. Amphotericin B topical microemulsion: formulation, characterization and evaluation. Colloids Surf. B 2014; 116: 351–358.
9. Ruiz HK, Serrano DR, Dea-Ayuela MA, Bilbao-Ramos PE, Bolas-Fernandez F, Torrado JJ, Molero G. New amphotericin B–gamma cyclodextrin formulation for topical use with synergistic activity against diverse fungal species and *Leishmania* spp. Int. J. Pharm. 2014; 473: 148–157.
10. Cohen BE. Amphotericin B membrane action: role for two types of ion channels in eliciting cell survival and lethal effects. J. Membr. Biol. 2010; 238: 1–20.
11. Chuealee R, Aramwit P, Noipha K, Srichana, T. Bioactivity and toxicity studies of amphotericin B incorporated in liquid crystals. Eur. J. Pharm. Sci. 2011; 43: 38–46.
12. Wasan EK, Gershovich P, Zhao J, Zhu X, Werbovets

- K, Tidwell RR, Clement JG, Thornton SJ, Wasan KM. A Novel Tropically Stable Oral Amphotericin B Formulation (iCo-010) Exhibits Efficacy against Visceral Leishmaniasis in a Murine Model. *PLOS Negl Trop. Dis* 2010; 4:913.
13. Delmas G, Park S, Chen ZW, Tan F, Kashiwazaki R, Zarif L, Perlin DS. Efficacy of Orally Delivered Cochleates Containing Amphotericin B in a Murine Model of Aspergillosis. *Antimicrob Agents Chemother*. 2002; 46: 2704-2707.
 14. Verma RK, Pandya S, Misra A. Loading and release of amphotericin-B from biodegradable poly(lactic-co-glycolic acid) nanoparticles. *J. Biomed. Nanotechnol*. 2011; 7: 118-120.
 15. Golenser J, Domb A. New Formulations and Derivatives of Amphotericin B for Treatment of Leishmaniasis. *AMini Rev Med Chem*. 2006; 6: 153-162.
 16. Menez C, Legrand P, Rosilio V, Lesieur S, Barratt G. Physicochemical characterization of molecular assemblies of miltefosine and amphotericin B. *Mol. Pharm.* 2007; 4(2) :281-288.
 17. Thornton SJ, Wasan KM. The reformulation of amphotericin B for oral administration to treat systemic fungal infections and visceral leishmaniasis. *Expert Opin Drug Deliv*. 2009; 6: 271-284.
 18. Laniado-Laborín R, Cabrales-Vargas MN. Amphotericin B: side effects and toxicity. *Rev. Iberoam Micol*. 2009; 26: 223-227.
 19. Malone ME, Corrigan OI, Kavanagh PV, Gowing C, Donnelly M, D'Arcy DM. Pharmacokinetics of amphotericin B lipid complex in critically ill patients undergoing continuous venovenous haemodiafiltration. *Int. J. Antimicrob. Agents* 2013; 42: 335-342.
 20. U.S. Pharmacopeial Convention, USP29-NF24, 2006. Guideline No. 66: Antibiotics, Microbial Assays. USP, Rockville, MD.
 21. McGinnis MR, Rinaldi GM. Antifungal drugs: mechanisms of action, drug resistance, susceptibility testing, and assays of activity in biological fluids, In: Lorian, V. (Ed.), *Antibiotics in Laboratory Medicine*. Lippincott Williams & Wilkins, Baltimore, Maryland 21202, USA. 1991; p. 198-257.
 22. Aller AI, Martin-Mazuelos E, Gutierrez MJ, Bernal S, Chavez M, Recio FJ. Comparison of the Etest and microdilution method for antifungal susceptibility testing of *Cryptococcus neoformans* to four antifungal agents. *J. Antimicrob. Chemother*. 2000; 46: 997-1000.
 23. Archibald LK, Tuohy MJ, Wilson DA, Nwanyanwu O, Kazembe PN, Tansuphasawadikul S, Eampokalap B, Chaovavanich A, Reller LB, Jarvis WR, Hall GS, Procop GW. Antifungal susceptibility of *Cryptococcus neoformans*. *Emerg. Infect. Dis*. 2004; 10 :143-145.
 24. Lopez-Jodra O, Torres-Rodriguez JM, Mendez-Vasquez R, Ribas-Forcadell E, Morera-Lopez Y, Baro-Tomas T, Alia-Aponte C. In vitro susceptibility of *Cryptococcus neoformans* isolated to five antifungal drugs using a colorimetric system and the reference microbroth methods. *J. Antimicrob. Chemother*. 2000; 45: 645-649.
 25. Lozano-Chiu M, Paetznick VL, Ghannoum MA, Rex JH. Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: Performances of three different media assessed by using E-test and national committee for clinical laboratory standards M27-A methodologies. *J. Clin. Microbiol*. 1998; 36: 2817-2822.
 26. Park BJ, Arthington-Skaggs BA, Hajjeh RA, Iqbal N, Ciblak MA, Lee-Yang W, Hairston MD, Phelan M, Plikaytis BD, Sofair AN, Harrison LH, Fridkin SK, Warnock DW. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. *Antimicrob. Agents Chemother*. 2006; 50: 1287-1292.
 27. Van Eldere J, Joosten L, Verhaeghe V, Surmont I. Fluconazole and amphotericin B antifungal susceptibility testing by National Committee for Clinical Laboratory Standards broth macrodilution method compared with E-test and semiautomated broth microdilution test. *J. Clin. Microbiol*. 1996; 34: 842-847.