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Development and Evaluation of Antifungal *in vivo* of Liposomal Amphotericin B

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ABSTRACT

Amphotericin B is a polyene antifungal drug used intravenously for systemic fungal infections. It has severe and potentially lethal side effects, therefore, it has been limited use in clinical. Liposomes are widely used as vehicles to target organ in pharmaceutical technology due to their ability to improve the delivery of drugs, increasing therapeutic efficacy and decreasing toxicity. The aim of this study is to prepare a liposomal amphotericin B by hydration of a thin lipid film and ethanol-injection methods and evaluate its antifungal activity *in vivo*. Prepared liposomal amphotericin B by both methods has particle size smaller than 150 nm, quite homogeneous and the entrapment drug was greater than 90%. The antifungal activity of liposomal amphotericin B was studied on three strains *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* fungal infection in mice models. Our results have shown that liposomal amphotericin B prepared by both methods have strong effect to prolong the survival in the infected mice and significantly reduced the Colony Forming Units (CFU) in target organ with similar effect of AmBisome.

Key words: Amphotericin B, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, liposome, drug targets

INTRODUCTION

Invasive fungal infection is the leading cause of death and long-term illness in patients with cancer and immunodeficiency. Amphotericin B is a polyene antifungal agent against a wide variety of fungal pathogens. Amphotericin B exerts its antifungal activity by disruption of fungal cell wall synthesis by its ability to bind to ergosterol, which leads to form pores and allow leakage of cellular components (Kaminski, 2014). However, this compound is almost insoluble in water, so it is difficult to prepare for injection dosage. Also amphotericin B has severe and potentially lethal side effects, especially in kidney (Ellis, 2002). Therefore, it is limited use in clinical practice. Some drug delivery system has been applied for amphotericin B, such as a cholesteryl sulfate complex (Amphotec) as a lipid complex (Abelcel) and as a liposomal formulation (Lemke *et al*., 2005). Liposome is an advanced drug delivery systems. It can increase the amount of drug to the target and simultaneously decrease drug's toxicity. Furthermore, liposome has high biocompatibility, biodegradability and ability to trap both hydrophilic and lipophilic drugs and simplify site-specific

drug delivery to tumor tissues. Liposomes are microscopic vesicles composed of a bilayer of phospholipids (Akbarzadeh *et al*., 2013). Liposomal amphotericin B (AmBisome) is a lipid-associated of amphotericin B. It is active against many fungal infections and is approved for the treatment of invasive fungal infections in many countries worldwide. AmBisome is a homogeneous suspension of unilamellar vesicles and after the administration, AmBisome remains intact in the blood and distributes to the tissues where fungal infections may occur (Takemoto *et al*., 2004). Liposomal amphotericin B was demonstrated more effective than amphotericin B deoxycholate in clinical treatment of invasive *Candida* spp. or *Aspergillus* spp. infections (Moen *et al*., 2009). There are some commercialized amphotericin B-lipid formulations present in vietnam but they vary in pharmacokinetic profiles. In formulating AmBisome, the ratio of amphotericin B: Lipid is value critical and must be carefully controlled to ensure that the decreased toxicity of the amphotericin B. This is important to the therapeutic index of the drug (Olson *et al*., 2008). The type of phospholipids such as phosphatidylcholine, phosphatidylglycerol or variations in the length of the fatty acid chain of the phosphatidylglycerol can significantly influence to efficacy and toxicity of AmBisome (Olson *et al*., 2008).

The present study was carried out to develop a liposomal amphotericin B formulation with the purpose of reducing toxicity and improving the antifungal activity of amphotericin B. Liposomal amphotericin B was prepared by the lipid film hydration and ethanol-injection method. Furthermore, *in vivo* antifungal effect on mice of liposomal amphotericin B prepared was also studied.

MATERIALS AND METHODS

Reagents: Amphotericin B (AMB) (China-USP Standards), distearoyl phosphatidylglycerol (Lipoid-manufacturer standard), hydrogenated soybean phosphatidylcholine (lipoid-manufacturer standard), cholesterol (Sigma-aldrich), sucrose (Fisher-bpsucrose), manitol (China-USP standard), N,N-dimethylacetamide (DMA) (Sigma aldrich). All other reagents and solvents used to meet requirements for pharmaceutical and analytical grade. AmBisome (Gilead sciences, USA) was used as reference drugs.

Instruments: The evaporation system Rovapor R-210; Spectra/Por® 4 dialysis Membrane, MWCO: 12,000-14,000 Daltons, Analyzer size system Zetasizer ZS90, Ultrasound machines, UV-VIS Spectrophotometer, pH InoLab meter, Tangential Flow MicroKros Filter Modules® (Spectrum Labs) with membrane polysulfone 10 kD, 28 cm^2 (USP), Centrifuge Hettich Universal 320R (Germany), High Pressure Homogenizers EmulsiFlex-c5 (Avestin-Canada), magnetic stirrer and other common tools.

Strains: *Candida albicans* (code ATCC 90028), *Cryptococcus neoformans* (code ATCC 90113) and *Aspergillus fumigatus* (code ATCC 1022) were bought from the USA, preserved in 10% glycerol solution and stored at -80°C. For each experiment, the fungi were grown at room temperature on Sabouraud dextrose agar or MEA and 3 or 4 days old conidia were harvested with sterile saline. It was then centrifuged at 2000 g for 5 min at room temperature. The conidia obtained were re-suspended in sterile saline. The number of conidia was counted on a haemocytometer and adjusted to get 10^6 conidia mL^{-1}. For *Aspergillus fumigatus*, the suspension needed to filter to remove hyphae.

Animals: Swiss white mice, those weighing 18-20 g were provided from Laboratory of Animal, Vietnam Military Medical University. All animal experiments were performed in accordance with the guidelines of Vietnam Military Medical University. Mice were kept under pathogen free conditions, under a 12 h light/dark cycle, controlled temperature (28±0.5°C) and humidity 55±5%. All animals were maintained accordingly to a protocol approved by the Ethical Committee of the Vietnam Military Medical University and following the international rules for animal research. They were fed *ad libitum* (Zeigler, USA) with a standard diet be sterilized before use. Mice were maintained for 5 days before randomly divided into 4 groups, 10 animals per group. The cage was located in the system with good ventilation and filter membrane to ensure the free of pathogens.

Preparation of liposomal amphotericin B: Liposomal amphotericin B was prepared by two methods: hydration of a thin lipid film and ethanol-injection (Singodia *et al*., 2012). Phospholipid using for the preparation of liposomes are HSPC and DSPG is the percentage of cholesterol is 40% of lipid total.

Method of hydration of a thin lipid film (LipoB): Dissolve DSPG in a mixture of methanol: chloroform (1:1), adjusted pH to 1.0-1.2 with HCl 2.5 N in methanol at 60°C. Disperse amphotericin B in methanol at 60°C add to above solution, stirring until a clear solution was obtained (solution A).

Dissolve HSPC in a mixture of methanol: chloroform (1:1), add to solution A. Then, the organic solvent was removed by evaporation using the Rovapor R-210 system at 40-45°C, rotational speed 150 rev min^{-1}. Vacuum pressure was regulated to evaporate slowly the solvent. After 30 min, lipidic film is formed, then decrease the rotational speed to 100 rev min^{-1}. Continue rotating 15 h to remove completely organic solvents.

Hydrate the thin lipid film by using a buffer citrate solution pH 5, 0-5, 5 at 50°C, rotational speed 250 rev min^{-1} during 15 min.

Homogenize liposome size by using membrane filter 400 nm in High Pressure Homogenizers EmulsiFlex-C5 with nitrogen gases at pressure 500 psi.

Method of ethanol-injection (LipoI) was performed as indicated by Domazou and Luisi (2002).

Prepare water phase: The 10 mM disodium succinate solution, pH 5.0-5.5 (pH adjusted by HCl 2.5 N).

Prepare ethanol phase: Disperse amphotericin B in N,N-Dimethylacetamide (DMA), acidified with 2.5 N HCl and shake for amphotericin B completely dissolved. Dissolve DSPG, HSPC and cholesterol in ethanol at 60-65°C and incorporate to amphotericin B solution.

Coordinate two phases: Inject rapid ethanol phase into water phase at 60-65°C with ratio 1:10 through the needle 27 G, homogenize with rotational speed 3900 rev min^{-1} for 10 min.

Use tangential flow filtration to concentrate liposome, remove DMA and ethanol.

Lyophilized liposome: Sucrose is added to liposome suspension with ratio of sucrose:lipid (4:1), move into glass bottle with a content of 50 mg/vial, cover loosely. Lyophilized as following:

- **Freezing:** Temperature was reduced to -50°C and maintained for 8 h
- **Primary drying:** Increased temperatures from -50 to -35°C with the heating rate is 0.25°C min^{-1} and maintained for 20 h
- **Secondary drying:** Increased temperatures from -35 to 25°C with the heating rate is 0.25°C min^{-1} and maintained for 8 h
- Sealed caps of the vials, stored at 2-8°C, protected from light
- **When testing:** Shake lyophilized powder with 10 mL of water to form liposome suspension

Evaluation of liposomal amphotericin B: Quantification of AMB: using a HPLC method:

- **Column:** Phenomenex-Gemini 5 μm C18-110A column, 250×4,60 mm, 5 μm particle size
- **Detector:** PDA, 407 nm
- **Flow rate:** 1.0 mL min^{-1}
- **Injection volume:** 10 μL

Mobil phase: Mixture of acetonitrile with 10 mM sodium acetate buffer solution, pH 4.0 with gradient elution as shown in Table 1.

Entrapment efficiency: To evaluate the amount of AMB bounded into lipids, firstly needed to remove free-AMB. Free-AMB is not water insoluble and precipitate in water and can be removed by filter the suspension through membranes 100 nm. Quantify the AMB after filter to calculate the entrapment efficiency.

Morphology and structure of liposome: Using the method of negative staining Transmission Electron Microscopy (TEM).

Liposome size and their distribution of particles: Using the method of Dynamic Light Scattering (DLS) with instrument Zetasizer ZS90. Dilute suspension of liposome 200 times with deionized water.

Evaluation *in vivo* antifungal effects of liposomal amphotericin B
Find LD10 and LD90 of strains in mice:

- First find the minimum lethal dose mice infected by infect 2 mice/group
- **With strain *Candida albicans*, *Aspergillus fumigatus*:** Infected mice by tail vein injection with dose 0.2 mL/mouse, with strain *Cryptococcus neoformans*: Infected mice by brain injection with dose 0.2 mL/mouse
- **Infected mice with different doses to find the LD10 and LD90:** The lowest dose is the minimum lethal dose, then increasing doses. Infecting at least 5 groups, each group has 12 mice, tracking mouse dead in 14 days
- Calculate LD10 and LD90

Table 1: Gradient elution of mobile phase

Time (min)	Sodium acetate buffer (%)	Acetonitrile (%)
0.00	70.0	30.0
4.00	40.0	60.0
8.00	20.0	80.0
9.00	60.0	40.0

Evaluate the survival rate of liposomal amphotericin B:

- Infect 60 mice with LD90 dose
- After 24 h infection, starts the treatment: Divide animals into four groups: Control, REF, LipoB and LipoI, each group has 15 mice. Control group received 5% glucose solution, REF group received AmBisome, LipoB and LipoI received liposome B and liposome I, respectively. The freeze-drying powder of the products was reconstituted and dispersed in 5% glucose to get concentration 0.1 mg mL^{-1} and was injected intraperitoneally in mice with 10 mL g^{-1} b.wt., during 7 days
- After finish the treatment (7 days), record the number of mice died everyday
- Calculate the survival rate in each group by using Kaplan and Meier log rank analysis

Evaluate the effect on target organs of liposomal amphotericin B:

- Infect 40 mice with LD 10 dose
- After 24 h infection, starts the treatment: Divide animals into four groups: Control, REF, LipoB and LipoI, each group has 15 mice. Control group received 5% glucose solution, REF group received AmBisome, LipoB and LipoI received liposome B and liposome I, respectively. The amphotericin B liposomes was dispersed in 5% glucose to get concentration 0,1 mg mL^{-1} and was injected intraperitoneally in mice with 10 mL g^{-1} b.wt., during 5 days
- After one day of treatment, sacrifice the mice, weight brain and kidney
- Homogenize brain and kidney in 2 mL physiological saline. Dilute the suspension to 1/10 and 1/100
- The strains were inoculated (100 μL suspension 1/1, 1/10 and 1/100) on Sabouraud's dextrose agar plate
- After 24 h, count the colonies
- Calculate colony forming unit CFU/g of tissue, evaluated by nonparametric test (Mann-Whitney U test)

Statistical analysis: All data are shown as the Mean±Standard Error (SD). One-way analysis of variance (ANOVA) was used to determine significance among groups. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Preparation and characterization of liposome amphotericin B: Amphotericin B liposomes were prepared with different ratio of HSPC: amphotericin B (mol:mol) as 1:1 (Lipo B1/Lipo I1), 1, 5:1 (Lipo B2/Lipo I2), 2:1 (Lipo B3/Lipo I3), 2,5:1 (Lipo B4/Lipo I4) to find out which is the best the ratio of HSPC: amphotericin B (mol:mol). After the liposomal amphotericin B were obtained, we analyzed some properties including mean particle size, polydispersity index (PDI) and drug entrapment efficiency. The results were shown in Table 2, 3 and Fig. 1.

The results showed that the liposomal amphotericin B have size smaller than 150 nm. This is one index important for evaluation quality of the liposomes because the size of particles plays important role due to their interaction with the biological environment. Liposomes with PDI value between 0.1 and 0.25 show high uniformity and physical stability. Our liposomes have PDI values smaller than 0, 25, which indicated they are uniform and homogeneous. Also obtained liposomes have high entrapment efficiency, over than 80%.

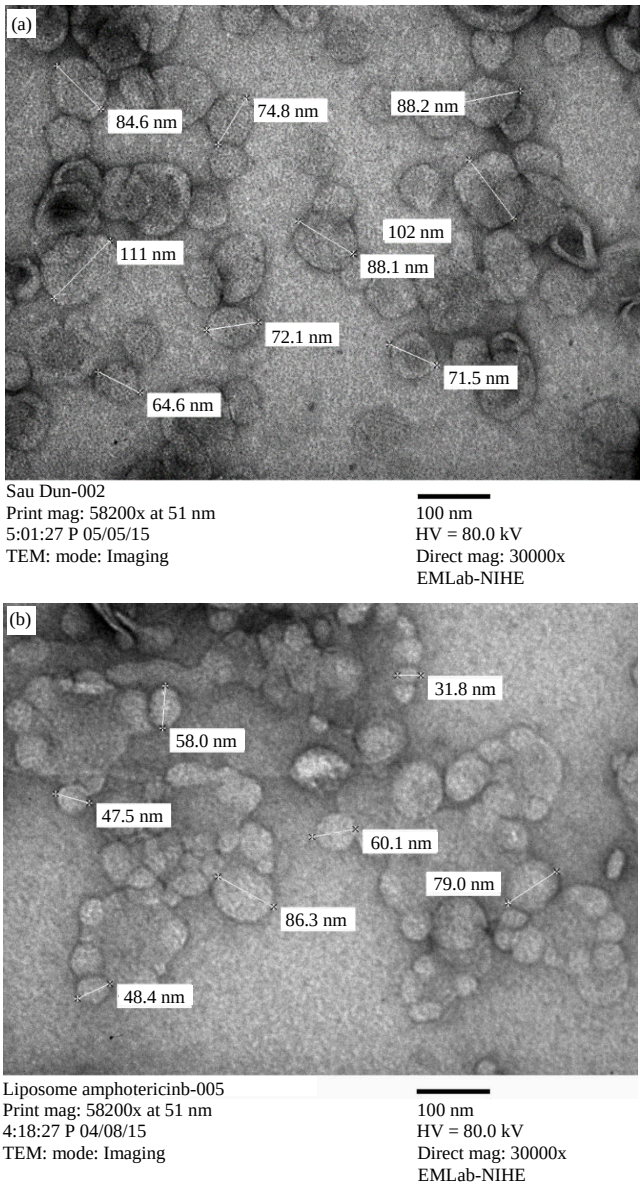

Fig. 1(a-b): Liposomal amphotericin B was taken by TEM, (a) By film hydration method and (b) By ethanol-injection method

Table 2: Characterization of liposomal amphotericin B by film hydration method

Samples	Mean particle size (nm)	PDI	Drug entrapment efficiency (%)
LipoB1	128.50±3.46	0.215±0.014	92.37±2.31
LipoB2	135.40±2.84	0.197±0.007	96.78±1.05
LipoB3	132.90±3.71	0.255±0.009	94.63±2.18
LipoB4	121.78±3.34	0.252±0.017	90.61±2.57

Table 3: Characterization of liposomal amphotericin B by ethanol-injection method

Samples	Mean particle size (nm)	PDI	Drug entrapment efficiency (%)
LipoI1	92.90±4.34	0.237±0.0126	80.51±0.67
LipoI2	84.40±11.24	0.247±0.0136	93.68±0.71
LipoI3	84.94±3.96	0.228±0.0071	96.61±2.89
LipoI4	92.53±4.34	0.225±0.0137	90.97±1.66

Table 4: LD10 and LD90 of three strains used in this study

Strain (cell/mouse)	LD10	LD90
Aspergillus fumigatus	50.000	500.000
Candida albicans	40.000	750.000
Cryptococcus neoformans	1.602	4.500

The ratio of HSPC/amphotericin B do not influenced significantly to the liposome's size in the both methods. However, the ratio of HSPC/amphotericin B as 1.5:1 results the highest entrapment efficiency in film hydration method, while in the ethanol-injection method, the ratio is needed to give the best entrapment efficiency is 2:1. Each method has advantages and disadvantages. The film hydration method creates large-sized liposome, so it is necessary to reduce the size. In this method we had to apply combination of two techniques: Homogeneous particles by high-pressure and pressed through membranes to produce liposome-size less than 150 nm. Ethanol-injection method has advantage that it can produce small liposome-size and homogeneous liposomes. However, ethanol-injection method produce diluted suspension liposome, then it is necessary apply tangential filtration process to remove the solvent and it is difficult to remove all residual solvent.

LD10 and LD90 of strains in infected mice: Low dose represents the dose at which no individuals are expected to die. The LD10 and LD90 refer to the dose at which 10 and 90%, respectively, of the individuals will die. We used the LD10 dose to determine the effect on target organs of liposomal amphotericin B in mice and LD90 dose to determine the effect of liposomal amphotericin B related to survival rate of mice. The results were shown on Table 4.

The data are in line with previous reports. *Aspergillus fumigatus* is a fungus of the genus Aspergillus, is the most common cause of invasive fungal infections in severely immunocompromised patients. The virulence of *Aspergillus fumigatus in vivo* is very different, depending on the strains and animals. Mavridou *et al*. (2010) reports that LD90 of *Aspergillus fumigatus* is 2.4×10^7 in outbred CD-1 female mice. Mirkov *et al*. (2012) have found the dose from 6.2×10^5 to $3\text{-}5\times10^7$ CFU/mouse caused almost 100% mice died.

Candida albicans has high virulence, infected mice at a dose of $5\text{-}6{,}8\times10^5$ CFU results to 7 days of the median survival time (Wiederhold *et al*., 2011). Gondal *et al*. (1989) showed that at dose $3{,}5\times10^5$ CFU/mouse of *Candida albicans* induced 100% of mice died after 7 days.

Cryptococcus neoformans is an opportunistic fungal pathogen that may cause meningitis in immunocompromised individuals. Velez *et al*. (1993) have studied twenty clinical isolated cryptococcal meningitis in mouse model. Eight high-virulence isolates had an LD_{50} of < or = 252 CFU of *Cryptococcus neoformans* and twelve low-virulence isolates had an LD_{50} of >252 CFU, in which 7 low-virulence isolates, the LD_{50} was >20,000 CFU. Other author have shown the lethal dose of *Cryptococcus neoformans* are in range from 300-20.000 CFU/mouse (Graybill, 2000).

Evaluate the survival rate of liposomal amphotericin B: We used Kaplan-Meyer analysis and log-rank test to compare the effect of different treatment.

Study with *Aspergillus fumigatus*: The efficacy of liposomal amphotericin B in infected mice with strain *Aspergillus fumigatus* was showed in Table 5.

The results of Kaplan-Meyer analysis and Log-rank test were showed in Table 6 and Fig. 2a.

Table 5: Results of the survival rate in infected mice with strain *Aspergillus fumigatus*

	Days														
Treatments	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
LipoB															
Death	0	0	1	2	2	3	4	4	4	4	7	9	9	10	10
Live	12	12	11	10	10	9	8	8	8	8	5	3	3	2	2
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
LipoI															
Death	0	0	0	2	3	4	4	5	7	9	9	9	9	9	10
Live	12	12	12	10	9	8	8	7	5	3	3	3	3	3	2
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
AmBisome															
Death	0	0	0	0	0	1	3	4	5	6	8	9	9	10	11
Live	12	12	12	12	12	11	9	8	7	6	4	3	3	2	1
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Control															
Death	0	0	1	3	5	7	9	11	13	14	14	14	14	14	14
Live	14	14	13	11	9	7	5	3	1	0	0	0	0	0	0
Total	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14

Table 6: Analysis by Log-rank test of different treatment in infected mice with *Aspergillus fumigatus*

	LipoB		LipoI		Ambisome		Control	
	Chi-Square	Significant	Chi-Square	Significant	Chi-Square	Significant	Chi-Square	Significant
Log rank (Mantel-Cox)								
LipoB			0.576	0.448	0.092	0.762	15.895	0.000
LipoI	0.576	0.448			1.144	0.285	10.235	0.001
AmBisome	0.092	0.762	1.144	0.285			18.786	0.000
Control	15.895	0.000	10.235	0.001	18.786	0.000		

Table 7: Results of the survival rate in infected mice with strain *Candida albicans*

	Days														
Treatments	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
LipoB															
Death	0	0	0	1	2	3	3	5	5	5	5	5	7	7	7
Live	14	14	14	13	12	11	11	9	9	9	9	9	7	7	7
Total	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
LipoI															
Death	0	0	0	1	2	3	4	5	5	5	5	5	5	5	5
Live	14	14	14	13	12	11	10	9	9	9	9	9	9	9	9
Total	14	14	14	14	14	14	14	14	14	14	14	14	14	14	14
AmBisome															
Death	0	0	0	1	2	3	4	5	5	5	5	6	6	6	6
Live	14	14	14	13	12	11	10	9	9	9	9	8	8	8	8
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Control															
Death	0	0	0	1	2	3	3	7	8	9	9	10	10	10	10
Live	10	10	10	9	8	7	7	3	2	1	1	0	0	0	0
Total	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

Present data have shown that there is no different significantly among the treatment with the LipoB, LipoI and AmBisome related to survival rate. The median survival time in infected mice with *Aspergillus fumigatus* of treatment with LipoB, LipoI and AmBisome have been significantly increased as compared with the control group using 5% of glucose ($p<0.05$).

Study with *Candida albicans*: The efficacy of liposomal amphotericin B in infected mice with strain *Candida albicans* was showed in Table 7 and 8.

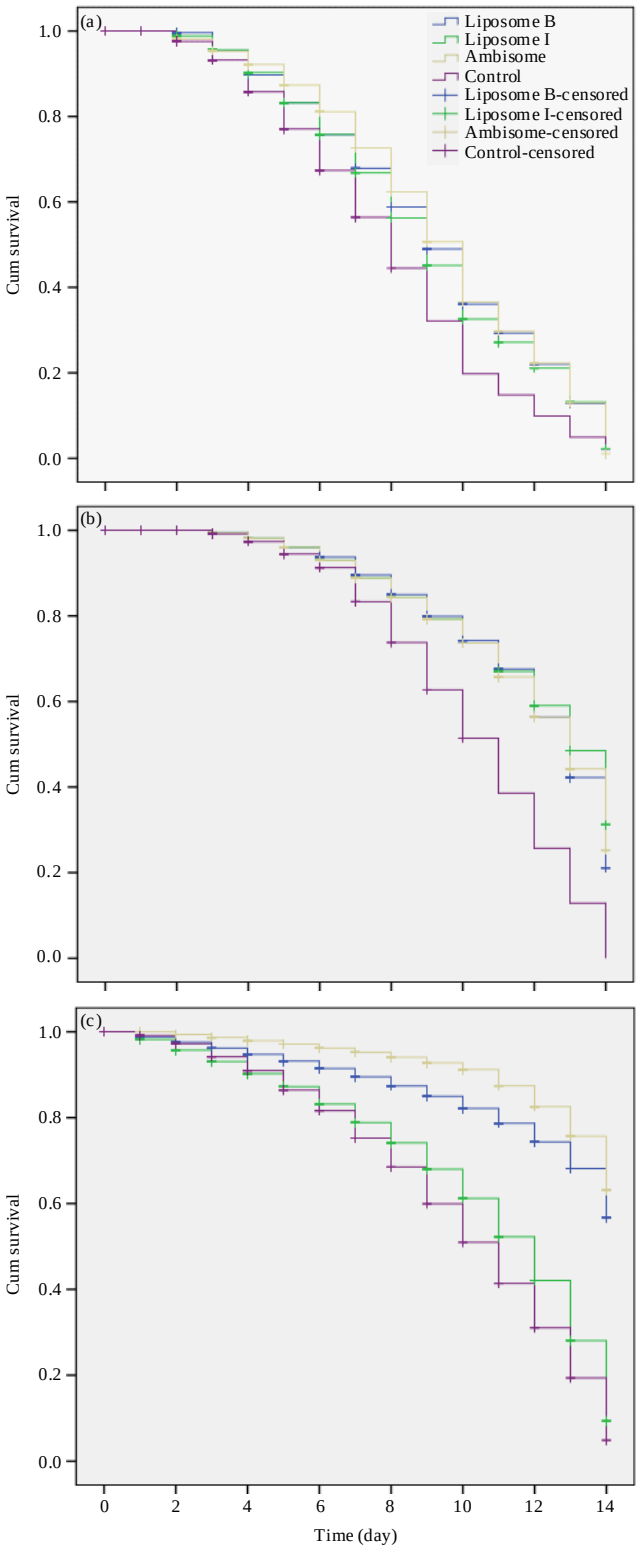

Fig. 2(a-c): Kaplan-Meier survival curve of different treatment in infected mice with (a) *Aspergillus fumigatus*, (b) *Candida albicans* and (c) *Cryptococcus neoformans*

Table 8: Analysis by log-rank test of different treatment in infected mice with *Candida albicans*

	LipoB		LipoI		Ambisome		Control	
	Chi-Square	Significant	Chi-Square	Significant	Chi-Square	Significant	Chi-Square	Significant
Log rank (Mantel-Cox)								
LipoB			0.269	0.604	0.011	0.918	23.520	0.000
LipoI	0.269	0.604			0.172	0.679	27.661	0.000
AmBisome	0.011	0.918	0.172	0.679			24.059	0.000
Control	23.520	0.000	27.661	0.000	24.059	0.000		

Table 9: Results of the survival rate in infected mice with strain *Cryptococcus neoformans*

	Days														
Treatments	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
LipoB															
Death	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Live	12	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
LipoI															
Death	0	3	4	4	4	4	5	5	5	6	6	7	7	8	8
Live	12	9	8	8	8	8	7	7	7	6	6	5	5	4	4
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
AmBisome															
Death	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2
Live	12	12	11	11	11	11	11	11	11	11	11	10	10	10	10
Total	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Control															
Death	0	1	2	3	3	4	4	5	5	6	6	6	6	6	6
Live	8	7	6	5	5	4	4	3	3	2	2	2	2	2	2
Total	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8

Table 10: Analysis by log-rank test of different treatment in infected mice with *Cryptococcus neoformans*

	LipoB		LipoI		AmBisome		Control	
	Chi-Square	Significant	Chi-Square	Significant	Chi-Square	Significant	Chi-Square	Significant
Log Rank (Mantel-Cox)								
LipoB			24.414	0.000	2.772	0.096	35.608	0.000
LipoI	24.414	0.000			41.457	0.000	1.979	0.159
AmBisome	2.772	0.096	41.457	0.000			55.278	0.000
Control	35.608	0.000	1.979	0.159	55.278	0.000		

The data have shown that there is no different significantly among the treatment with the LipoB, LipoI and AmBisome related to survival rate. The median survival time in infected mice with *Candida albicans* of treatment with LipoB, LipoI and AmBisome have been significantly increased as compared with the control group using 5% of glucose ($p<0.05$) (Fig. 2b).

Study with *Cryptococcus neoformans*: The efficacy of liposomal amphotericin B in infected mice with strain *Cryptococcus neoformans* was showed in Table 9 and 10.

The data have shown that there is no different significantly among the treatment with the LipoB and AmBisome related to survival rate ($p>0.05$), while effect of the liposome I on survival rate is less and significantly difference than other treatment ($p<0.05$). The median survival time in infected mice with *Aspergillus fumigatus* of treatment with liposome B and AmBisome have been significantly increased as compared with the control group using 5% of glucose ($p<0.05$). However, this is not happen with the LipoI ($p>0.05$) (Fig. 2c).

Table 11: Colony forming unit in target organ in mice treated with liposomal amphotericin B after infected with fungal strains

	Strain/target organ		
Treatments	*Aspergillus fumigatus*/kidney	*Candida albicans*/kidney	*Cryptococcus neoformans*/brain
LipoB	3.8525±0.2859 [a]	3.1280±0.4701[a]	4.1625±0.5519[a]
LipoI	3.876±0.27153[b]	3.5700±0.4681[b]	4.6320±0.5649[b]
Ambisome	3.7100±0.3395[c]	3.5360±0.32455[c]	4.2180±0.3645[c]
Control	4.4060±0.1080	4.5220±0.35337	5.0380±0.2058

Data expression as Average±SD (log_{10} CFU/gram protein) and [a]Significantly different between LipoB vs. Control, [b]LipoI vs. Control, [c]AmBisome vs. Control

Effect on target organs of liposomal amphotericin B: The results of liposomal amphotericin B reducing CFU strains the on target organs were shown on Table 11.

The results showed that the LipoB, LipoI and AmBisome are equivalent effective to reduce the CFU fungus *Aspergillus fumigatus* and *Candida albicans* density in kidney ($p>0.05$). The effect of LipoB, LipoI are significantly different as compared with control group ($p<0.05$). Also LipoB and LipoI have effect to reduce the CFU fungus *Cryptococcus neoformans* in brain similar AmBisome's effect ($p>0.05$) and significantly different compared to control group ($p<0.05$). AmBisome is a lipid-associated formulation of amphotericin B, a broad-spectrum polyene antifungal agent. It has been approved for the treatment of invasive fungal infections (Moen *et al*., 2009). Our prepared liposomal LipoB and LipoI have equivalent antifungal activity *in vivo* of AmBisome. Present data are in line with report of Zarif *et al*. (2000) which the author have shown AmBisome is highly effective in treating murine candidiasis in kidney.

CONCLUSION

In conclusion, liposomal amphotericin B (LipoB and LipoI) was prepared successfully by hydration of a thin lipid film and ethanol-injection method. Prepared liposomal amphotericin B has size smaller than 150 nm, PDI values smaller than 0.25 and the entrapment drug greater than 90%. Antifungal activity assay showed that both LipoB and LipoI have strong effect to prolong the survival in the infected mice with three fungal strains and significantly reduced the CFU in target organ with similar effect of AmBisome.

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