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Comparative Study of lipid bilayer membranes permeability with reconstituted five AmB-lipid dry powder formulations by Using chamber

Kajiram Adhikari

Department of Pharmacology, People`s Dental College and Hospital Pvt. Ltd., affiliated with Tribhuvan University, Naya Bazar, Kathmandu, Nepal

Abstract : The aim of this research study was to determine amphotericin B (AmB) permeation across lipid bilayer membranes mounted on Transwell® composed of two different lipid bilayers. The lipid bilayer membranes were prepared in the composition of phospholipid and ergosterol as well as phospholipid and cholesterol in a ratio (67:33 mol%). AmB-lipid formulations were prepared from AmB incorporated with five lipid derivatives (KDC, KC, SDC, SC and SDCS) during a lyophilization process. The lipid bilayer membranes and AmB-lipid formulations were successfully prepared. Permeation results were two to five fold higher than for pure AmB in the ergosterol containing lipid bilayer and two to four fold higher than AmB in the cholesterol containing compositions, both of which were enough to kill the fungi according to their MICs and MFCs. We suggest that these products especially the AmB-sodium deoxycholate sulfate are potential candidates for targeting AM cells for the treatment of invasive pulmonary aspergillosis.

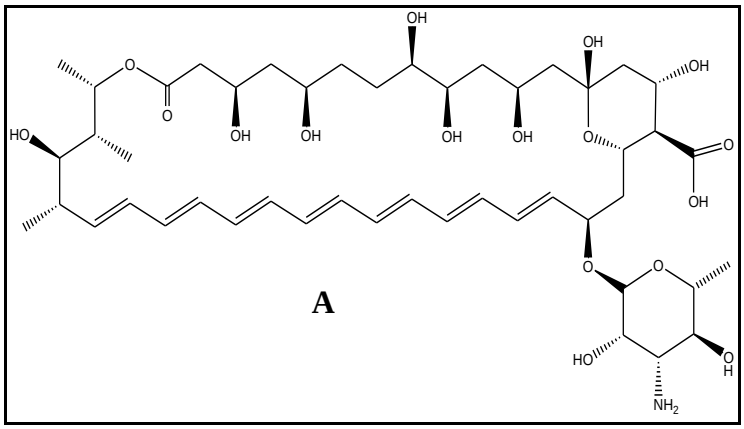
Key Words: Amphotericin B; Cholesterol; Ergosterol; Phospholipid, Lipid bilayer; Ussing chamber.

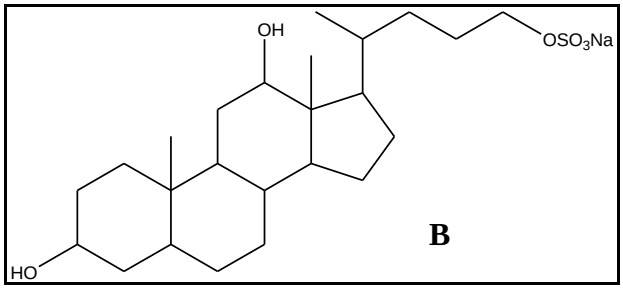
Introduction

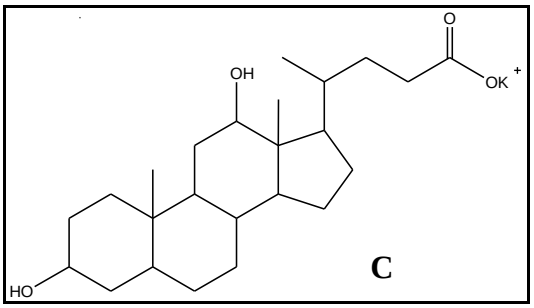
The incidence of pulmonary fungal infections has increased significantly due to the growing number of immune-compromised patients that are related to the number of patients with human immunodeficiency virus (HIV), organ transplantations, hematologic disorders and cancer during the period of the last decades[1]. *Aspergillus* species are ubiquitous and their spores are inevitably inhaled into the airways. The inhalation of spores is the first step in the pathogenesis of invasive pulmonary aspergillosis (IPA), followed by failure of the resident macrophages and neutrophils to inhibit their development into mycelia. In the market, there are many anti-fungal drugs available such as amphotericin B (AmB), itraconazole, voriconazole, and caspofungin[1]. Among them, Amphotericin B (AmB) is a polyene macrolide antibiotic that has been used as a gold standard drug to treat systemic fungal infections for more than 40 years. Although, hemolysis and nephrotoxicity are the major side effects, it is still the drug of choice used to treat advanced infections due to the lack of better alternatives[2-5]. It is most commonly used to treat life-threatening conditions such as cryptococcosis, histoplasmosis, and IPA. The chemical structure of AmB is shown in **Fig. 1(A)**. However, the nephrotoxicity of AmB is a major clinical problem that can often lead to discontinuation of treatment[6]. Systemic fungal infections are often observed as side effects in chemotherapy treatments which impair the immune system, and therefore, finding new anti-fungal drugs or improving old standards is an important emerging problem[5, 7-8]. Unlike other anti-fungal drugs, AmB is very rare generate drug resistance in fungal strains[4]. This desirable property further enhances AmB's medical importance the design of less toxic derivatives or formulations have important medical uses. Some successful results concerning lipid or liposome AmB formulations have been introduced recently[9]. But, such clinical applications are too expensive for a typical medical treatment, therefore limiting their usage in clinical practices. AmB's molecular mechanism of action is still not understood well

enough to make rational design of new derivatives possible. It is known that AmB interacts with the components of cell membrane and forms ion channels as shown in **Fig**.2[10-13]. Chemotherapeutic application of AmB is based on the slightly higher affinity of the antibiotic towards ergosterol-containing membranes (fungal cells) than cholesterol-containing membranes (human cells)[14-16]. Fungi are eukaryotic organisms, and there are many similarities between the biochemistry of fungal cell and human cells. Fungal cell membrane consists of lipid particle which is known as sterol. The fungal sterol is different from the mammalian cell membrane. The ergosterol is the main constituent of sterol in fungal membrane; where as in mammalian cell membrane is cholesterol. There are only minor structural differences between cholesterol and ergosterol. The chemical structure of ergosterol is shown in **Fig.3(A)** and cholesterol as shown in **Fig.3(B).** Unfortunately, the affinity of AmB toward cholesterol molecules is not negligible and this interaction is responsible for the severe toxicity (nephrotoxicity) of the antibiotic. AmB exerts its antifungal activity on the cell membrane after binding to ergosterol, the most abundant sterol found in the cell membrane of sensitive fungi and then creates channels or pores. The consequent increase in cell membrane permeability leads to the leakage of sodium, potassium and hydrogen ions and eventually cell death [17-18]. For lung fungal infections, a nebulizer is an appropriate drug delivery system, and should provide a targeted therapy that will allow for effective concentrations of a drug to react at the disease site (lungs) without exposing other tissues (such as kidney) to toxicity. For this purpose, the alveolar macrophages (AM) are the target cells and parts of the immune system. AM engulf microorganisms and normally destroy them[19]. AM are mobile phagocytic cells located within the alveolar regions and small airways of the lungs. Nitric oxide (NO) is a free radical that is produced by various cells in the lungs. NO produced by iNOS plays an important role in defense against airborne pathogens or in tissue damage associated with inflammatory processes in the lungs[20]. AM are active producers of cytokines and leukotrienes, and have important pro-inflammatory roles in the alveolus. The use of delivery systems that directly targets the drugs to the AM is therefore an attractive approach for delivering drugs to the lungs of immunocompromised patients with fungal infections. This provides rapid access of the drug to the infected AM, to quickly combat aspergillosis infections[21]. Alveolar delivery systems have a lot of benefits such as being target oriented they have a low dose, with low side effects and require less frequent administration[22]. Aerosolized liposomal AmB has also been reported for treatment of pulmonary fungal infections with a jet-nebulization [23-25]. Other approaches have aimed to reduce the AmB toxicity by modifying the drug formulations as well as the lipid-based formulations of AmB that have been successfully developed. Three lipid formulations of AmB (AmBisome®, Amphocil®, Abelcet™) are available in the market. The major nephrotoxicity of AmB is reduced [26-27]. AmB-lipid formulations can be administered at higher doses because of their reduced toxicity and provide for a greater efficacy. However, these lipid formulations have a low rate of elimination and high doses of administered AmB may accumulate in the body[28]. Lipid bilayer membranes are key objects in drug research in relation to (i) interactions of drugs with membrane-bound receptors, (ii) drug targeting, penetration, and permeation of cell membranes and (iii) use of liposomes in micro-encapsulations technologies for drug delivery. The rational design of new drugs and drug-delivery systems therefore requires insight into the physical properties of lipid-bilayer membranes. Lipids play a major role in lipid membrane organization and functionality. Lipid bilayer permeability is vital for targeting the drug delivery system[29]. The permeation and transport characteristics of lipophilic and hydrophilic drugs across the lipid membrane part of bio-membrane are of crucial importance for the ability of drugs to reach their target and action sites. The different types of lipids constituting the lipid membrane as well as the external thermodynamic conditions such as temperature, pH, degree of hydration and ionic strength are all major determinants of the macroscopic phase behavior and the associated physical properties. The temperature, acyl chain length, cholesterol and drugs are the parameters which can affect lipid bilayer permeability. During this study, all parameters were kept constant except lipid carriers and lipid bilayer membranes. We determined AmB permeation across the lipid bilayer and expected that the amount of AmB was sufficient to kill fungi in both compositions (phospholipid and ergosterol as well as phospholipid and cholesterol).

In order to solve these problems, other lipid derivative carriers such as sodium deoxycholate sulfate (SDCS), potassium deoxycholate (KDC), potassium cholate (KC) have been introduced in this research work. Their chemical structures are shown in **Fig.1(B**), **Fig.1(C)** and **Fig.1(D),** respectively. Two other lipids, sodium deoxycholate (SDC) and sodium cholate (SC) have been chosen for use as AmB carriers **Fig.1(E)** and **Fig.1(F)**, respectively. SDC was used as a control. In the present work, we hypothesized that these AmB-lipid derivative formulations could enhance the drug permeability through lipid bilayer membrane and target the AM.







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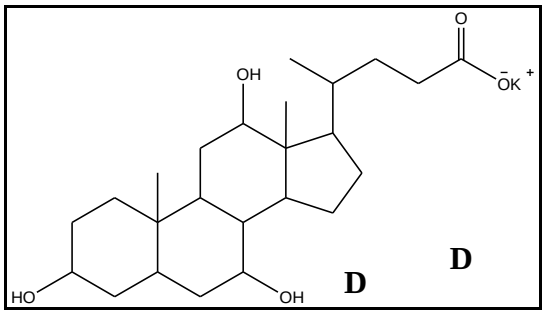

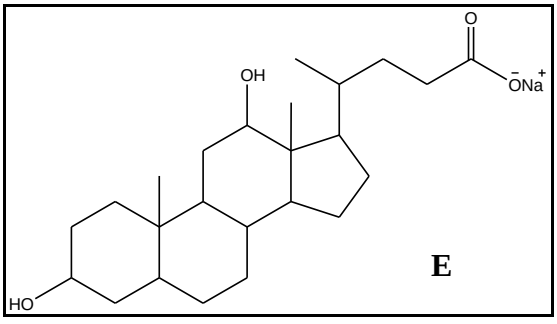

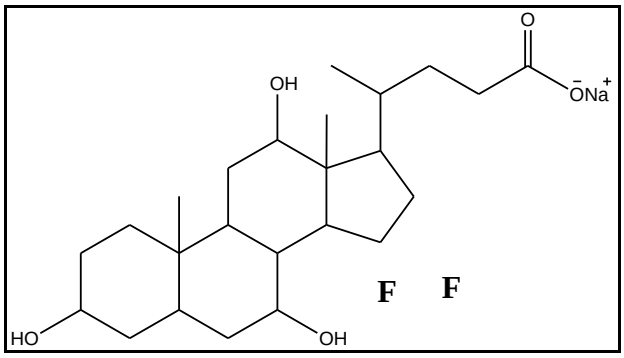

Figure1. The chemical structure of Amphotericin B (A), Sodium deoxycholate sulfate (B), Potassium deoxycholate (C), Potassium cholate (D), Sodium deoxycholate (E) and Sodium cholate (F).

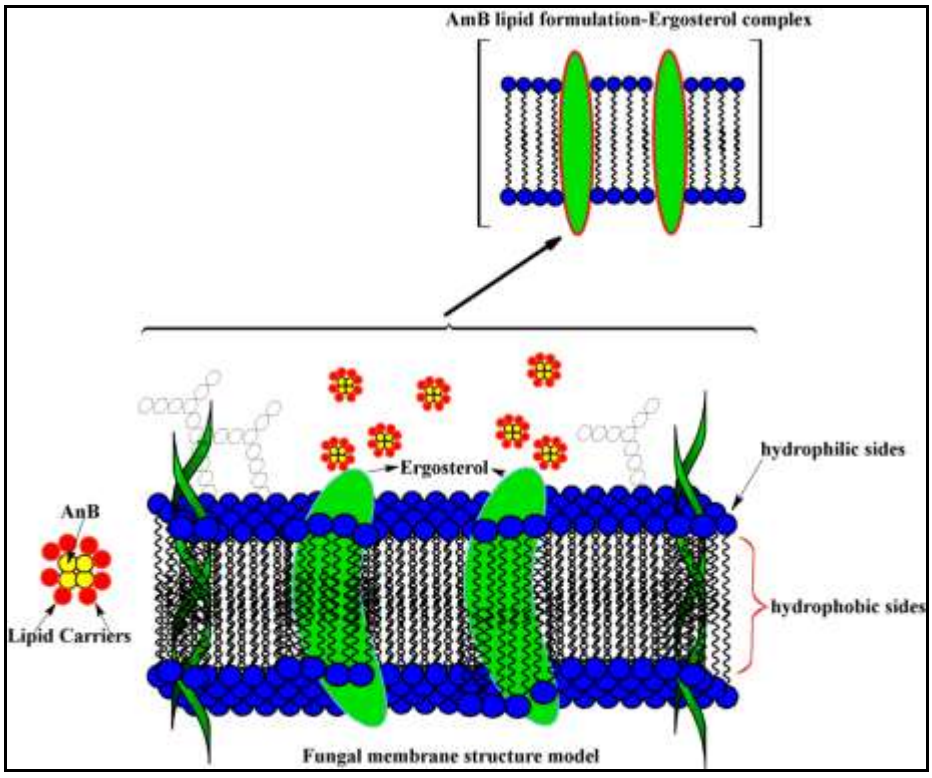

Figure 2. Fungal membrane structure model and ion-channel pore formation as well as permeation AmB from the AmB-lipid formulation by lipid bilayer.

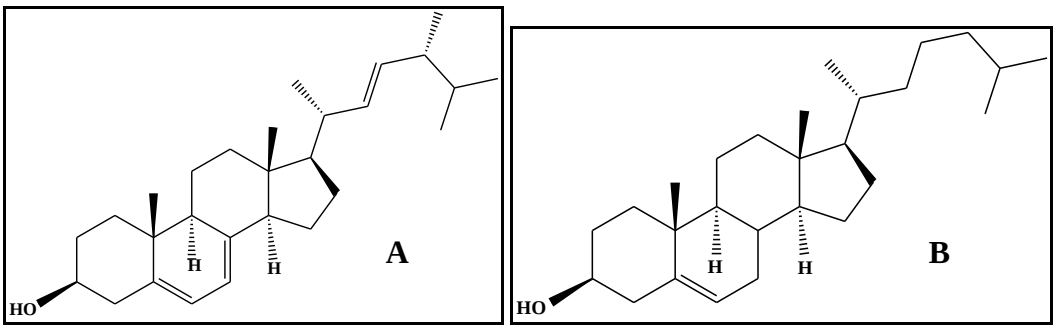

Figure 3. The chemical structures of ergosterol (A) and cholesterol (B).

Experimental

Materials

AmB was obtained from Ambalal Sharabhai Enterprises Pvt. Ltd., Vadodara, India. Deoxycholic acid, cholic acid, sodium cholate, sodium deoxycholate and ergosterol were purchased from Sigma-Aldrich, St. Louis, USA. Sodium deoxycholate sulfate, potassium cholate and potassium deoxycholate were synthesized in a laboratory. Sodium dihydrogen phosphate dihydrate and disodium hydrogen phosphate dihydrate were purchased from Ajax Finechem Pty Ltd, NSW, Australia. Acetonitrile and methanol were purchased from Labscan Asia, Bangkok, Thailand. 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 Dry Powder AmB-Lipid Formulations (AmB-KC, AmB-KDC, AmB-SDC, AmB-SDCS, and AmB-SC)

A mixture of AmB (250 mg) and KC (235 mg) in a 1:2 (mol ratio) was prepared in distilled water (30 mL) and stirred for a few minutes. Potassium hydroxide solution (2.6 mL, 0.2 M) was then added slowly dropwise, at room temperature to obtain a clear yellowish-colored solution. 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. The final volume of the solution was adjusted to 50 mL by adding distilled water. The solution was lyophilized in a freeze dryer (Dura-Dry™ MP, FTS Systems Inc., NY, USA), and a caked yellowish dry powder was obtained (**Fig. 4A & 4B**). A similar methodology to that used for deoxycholic acid and AmB was employed to prepare the sodium deoxycholate (AmB-SDC), sodium deoxycholate sulfate (AmB-SDCS), potassium deoxycholate (AmB-KDC) formulations. A similar methodology to that used for cholic acid and AmB was employed to prepare sodium cholate (AmB-SC) formulation.

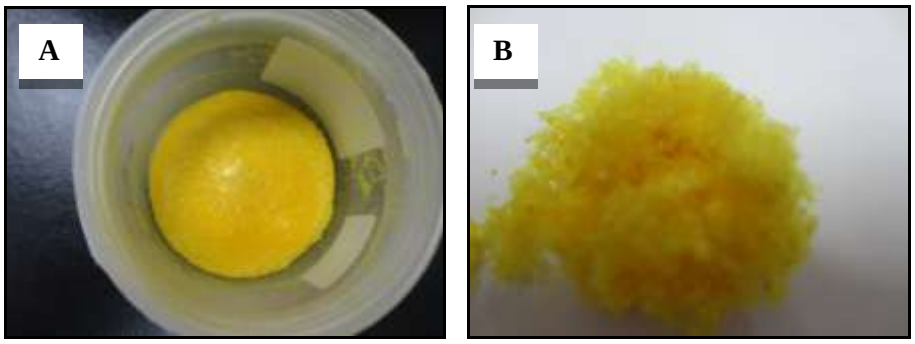

Figure 4. Reconstituted AmB-lipid dry powder caked formed (A), after breaking caked, it formed free flowing powder (B).

Determination of AmB permeation across lipid bilayer

Lipid bilayer and permeability

The lipid bilayer is a universal component of all cell membranes and its structural components provide the barrier that marks the cell boundaries. The cell membrane is a lipid bilayer of between 5-10 nm thick consisting of two external, a hydrophilic region (polar head groups) and an internal hydrophobic (nonpolar tail) region. Phospholipids are the major form of lipid involved. The phospholipids organize themselves into a bilayer to hide their hydrophobic tail regions and expose their hydrophilic regions to water. This organization is spontaneous, a natural process that occurs without any energy requirement. The most important properties of the lipid bilayer is the formation of a highly selective semipermeable structure and its fluidity at normal temperatures. This fluidity will provide mobility within the lipid bilayer, which is biologically important, as it influences membrane transport. The lipid bilayer component of the cell membrane helps to sustain osmotic gradients across the membrane, but it is not a perfect chemical seal. Ions, water, and other molecular compounds will invariably passively cross the membrane. Crossing the permeability barrier of the lipid bilayer is vital for targeting any drug delivery system[29]. The permeation and transport characteristics of lipophilic and hydrophilic drugs across the lipid membrane are of crucial importance for allowing drugs to reach their target and action sites. During our study, all parameters were kept constant, such as the temperature of 37 ºC and the acyl chain length of the lipid bilayer membrane was the same for all experiments (phospholipid and ergosterol concentration was a constant 67:33 mol%). The AmB or AmB-lipid formulations were also fixed at 50 µg of AmB in 5 mL phosphate buffer solution. We determined the relationship between the AmB and the AmB-lipid derivatives formulations by examining their permeation across the lipid bilayer membranes.

Preparation of the lipid bilayer membrane

To conduct an *in-vitro* permeability test, the lipid bilayer membranes were prepared from phospholipid (L-α-phophatidylcholine) and ergosterol in a ratio of (67:33 mol %) as previously reported by Ostroumova[30] (**Fig. 5**). These were prepared by dissolving L-α-phosphatidylcholine 67 mol% and ergosterol 33 mol% in 30 mL of chloroform in a 100 mL round bottomed flask for uniform mixing. The solvent was removed under

reduced pressure using a rotary evaporator (Eyela, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 55 °C until it formed a dried cake. The dried cake was transferred to a petri-dish and further heated at 40 °C for 48 h on a hotplate to completely remove the solvent and a dry powder mixture was obtained. The dried powder was transferred into a vial with 6 mL of distilled water and kept in a water bath (50 °C) for 1 h for hydration. The hydrated suspension (1000µL) was loaded onto each Transwell® (Corning Inc., NY, 14831, USA) and dried at 40 °C for 48 h (**Fig.5B&5C**). The dried lipid membrane on Transwell® was equipped in an Ussing chamber system (Insert for 2300 Easy Mount Diffusion Chambers, Physiologic Instruments, Harvard Apparatus Companies, USA) (**Fig.8**). Similar, methodology was applied for preparing cholesterol containing lipid bilayer membrane from phospholipid (L-α-phophatidylcholine) and cholesterol in a ratio of (67:33 mol %) as mentioned above.

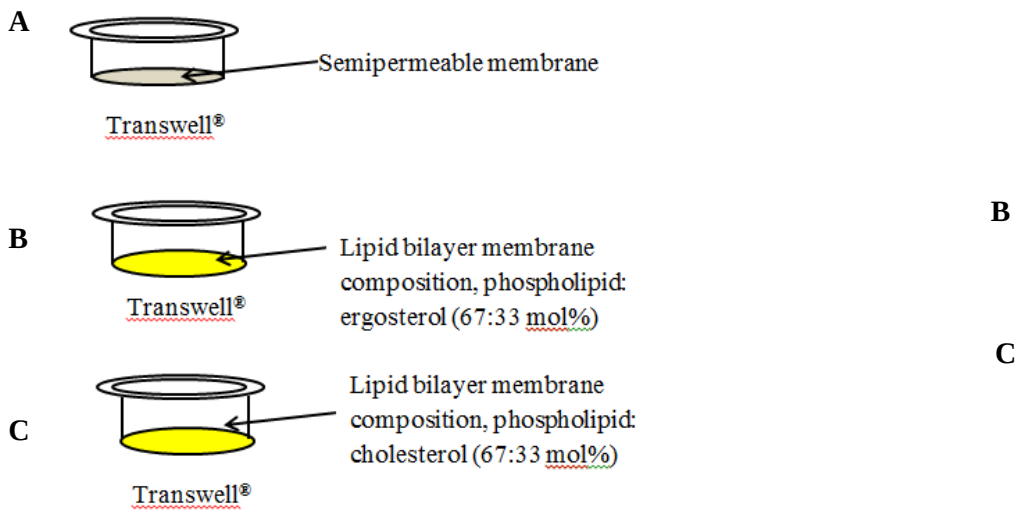

Figure 5. Picture of Transwell® semipermeable membrane (A), In Transwell® ergosterol containing lipid bilayer membrane prepared (B) and In Transwell® cholesterol containing lipid bilayer membrane preparation (C).

Preparation of the electrodes

The electrode set consisted of two pairs of electrodes, one pair for the passing current and the other for sensing the voltage. The voltage-sensing electrode (black housing) was a small pellet formed by compressing AgCl about an Ag wire. AgCl was used for sensing the voltage because it provided a relatively stable electrode potential and was reversible. The current electrode (white housing) was a Ag wire that had been plated with chloride ions to lower its resistance. Each type of electrode was inserted into a pipette tip and a connection was made to the phosphate buffered solution in the chamber via a KCl-filled agar bridge **(Fig.6).** Ag/AgCl electrodes with agarose/2M KCl bridges were used to apply the transmembrane voltage (V) and to measure the transmembrane current (I). The electrodes were prepared by dissolving 10 mL of 2M KCl and 3% agar in a beaker and kept on water bath at 100 °C for 5 min with continuously stirring until it was well mixed and should be free from trapped air bubbles. When the agar was completely dissolved and formed a transparent solution it was then transferred inside an electrode (5 mm of length) with the help of a 2 mL syringe from the tip of the electrode and the rest of the space was filled with KCl (2M) solution. The filled pipette was placed in KCl (2M) solution and the container was sealed until used. KCl was chosen as the filling solution because K^+ and Cl^- have nearly equal free solution mobilities and, therefore, will not cause a significant diffusion potential even though the concentration gradient from the pipette to the chamber was very large.

Formation of lipid bilayer membrane in the Ussing chamber

The lipid bilayer Transwell® was fitted into the Ussing chamber. Phosphate buffer solution (10 mM, pH 7.4) 5 mL was added to both chambers and left for 1 h to completely hydrate and stabilize the membrane on the Transwell® in the presence of the phosphate buffer solution (**Fig.6**).

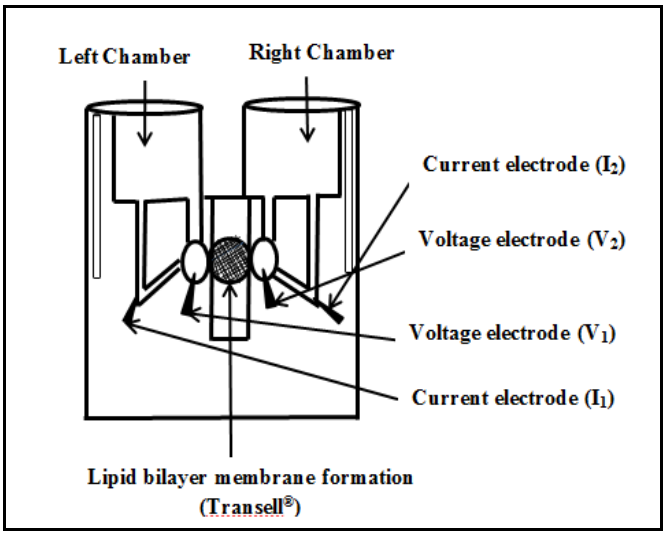

Fig. 7 Picture of Ussing chamber

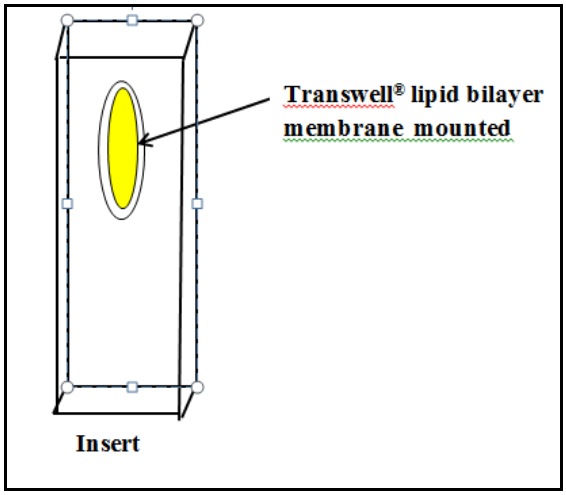

Fig. 8 Insert hole was fitted with lipid-bilayer membrane prepared.

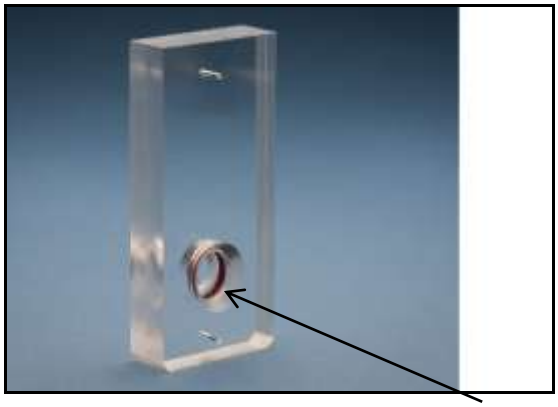

Fig. 9 picture of insert

Insert hole fitted with Transwell® lipid bilayer membrane

Ussing chambers permeation across lipid bilayer model

The Ussing chamber (**Fig.7**) is suitable for our experimental design in shape and size, with a charge induced by an AmB-lipid formulations in a solution form. Its basic principle was applied to measure the short-circuit current as an indicator of net ion transport taking place across the lipid bilayer membrane (from left chamber to right chamber) (**Fig.6**). In the Ussing chamber, electrodes are placed on both sides of the lipid bilayer membrane to record the potential differences (PD) across the lipid bilayer. A set of two other electrodes allows for the injection of a current (short-circuit current or Isc) to nullify the PD. The intensity of the current to be injected was monitored by an electric clamp apparatus. The Ussing chamber system was filled with the experimental phosphate buffer (10 mM, pH: 7.4) 5 mL solution in both chambers and the experiment was performed at room temperature (37 ºC). To perform our study, some modifications were made to this instrument. Instead of a biological membrane, we applied a preformed lipid bilayer membrane in the Transwell® to act as a membrane that supported for permeability of AmB or AmB-lipid formulations in the Ussing chamber [31].

Collection of samples from the Ussing chamber

Before addition of the AmB suspension into the Ussing chamber system, first, we confirmed that there was no leakage from the chambers. This was done by applying grease in the two half chambers, when the

electrodes were inserted into the Transwell® holes (**Fig.9**) to assist with making them air tight and leak proof. After confirming this we started the test for permeability. First of all, 50 µL of the drug solution or the AmB-lipid formulations (concentration of AmB 1000 µg/mL) was added to the left chamber and the initial current was noted, then after every 15 min the current was noted and 1 mL of sample was withdrawn from the right chamber and the reduced volume was compensated with 1 mL of phosphate buffer solution in the right chamber until the 2 h period for collecting samples were completed. All experiments were performed at 37 ºC and in triplicate. The collected sample solutions were quantified for AmB by the HPLC method to determine the percentage cumulative AmB permeation from the left chamber to right chamber. The drug contents assay was carried out using a standard HPLC method (Waters, Singapore). For the HPLC conditions, acetate buffer (20 mM, pH at 7.2) and acetonitrile (60:40 v/v) at a flow rate of 1 mL/min were used as the mobile phase. The microbondapak C_{18} column (Phenomenex®, USA) (150×4.6 mm i.d., 5 µm) was the stationary phase. UV detection was at a wavelength of 405 nm[32].

Results and Discussion

All five lipids compounds were white amorphous powders and highly water soluble. AmB-lipid formulations were prepared from lyophilization process, which were yellowish powders very light and free flowing as shown in **Fig.4(A&B).** These products were highly water soluble and stable in solution form. These are hygroscopic in nature and sensitive to light. Therefore, they should be stored in airtight amber bottles below 8 °C in a refrigerator. During the AmB-lipid formulations, AmB and lipid carriers mole ratio were 1:2 and four moles of AmB combined with eight moles of lipid carriers formed complex compound. AmB was solubilized by lipid carrier due to the formation of micelle and this micelle stabilized the AmB to prevent the aggregation of AmB in water and existed in monomeric form, which was less toxic than dimeric or tetramer or hexamer forms. In between AmB and lipid carriers such as SDCS, KDC, SDC, SC and KC were formed hydrogen bonding interaction different types of cations and anions make into potential with rationale design of lipids. Therefore, these materials are chosen as carrier to formulate AmB micro-particulate powders of reconstituted of nebulization for the treatment of lung fungal infections.

Permeation of AmB and AmB-lipid formulations across lipid bilayer

The permeability of AmB transported through ion-permeable channels was studied using the Ussing chambers in a phosphate buffer solution (10 mM, pH 7.4) with different AmB-lipid formulations (AmB-KDC, AmB-KC, AmB-SC, AmB-SDC and AmB-SDCS) and AmB. The percentage of the cumulative AmB that was transferred from the left chamber to right chamber in a 2 h period are shown in **Fig.10** and **Fig.11**. The cumulative drug transferred from the left to the right chamber using the AmB-lipid formulations were 10.6, 4.3, 3.6, 5.6 and 4.0 %, respectively. Whereas, AmB only was cumulatively transferred 2.0%. This was in lipid bilayer containing ergosterol and phospholipid bilayer membrane. Similarly, cholesterol and phospholipid containing lipid bilayer membrane, the cumulative drug transferred from the left to the right chamber using the AmB-lipid formulations (AmB-SDCS, AmB-SDC, AmB-SC, AmB-KDC, and AmB-KC) were 8.9, 4.8, 4.6, 5.5 and 4.9% respectively. Whereas, AmB only was cumulatively transferred 2.0%. Only, AmB-SDCS lipid formulation was highest permeability in both lipid bilayer membranes and others were almost similar permeability.

During permeability study period, we had measured the current. At the initial period (before addition of sample) it was 0.6 µA. After an addition of 50 µL AmB suspension into the left chamber, the current was increased to 1.1 µA at 15 min. At every 15 min interval, current was gradually increased with time, reached 1.9 µA at the end of sampling time period (2 h). Similarly, the current of AmB-lipid formulations was found to be 0.3-1.5 µA. This indicated that AmB, and AmB-lipid formulations permeated across lipid bilayer membrane from the left chamber to the right chamber. AmB has a very low water solubility (<1 µg/mL at pH 6-7) and the AmB was in an aggregated form in solution and the lowest amount of AmB was able to cross the membrane. On the other hand the AmB-lipid formulations have counter ions that interacted with the lipid bilayer and facilitated the permeation of AmB. In the case of AmB, there were no charges available to counteract the lipid bilayer membrane. AmB may form a complex with the lipid bilayer resulting in low permeability. This suggestion has been supported by the work of Herec[33] who proposed that hydrogen bonding between the horizontally oriented AmB and the polar groups of the lipids makes the membrane more compact and less permeable to ions. AmB-SDCS had the highest zeta potential (-45.53 mV),[32] and it produced the highest

permeability (10.6%) is shown in **Fig.10** and (8.9%) is shown in **Fig.11** in ergosterol and cholesterol containing lipid bilayer membranes respectively. This was due to the replacement of a carbonyl group of the deoxycholic acid ring with the sulfate group, which developed a highly negative charge. This sulfate group has a vital role to interact with the lipid bilayer to increase the pore size and facilitate the penetration of more AmB. Similar phenomena have also occurred with other formulations. These negatively charged ions facilitated 2-5-fold more AmB from the formulations to cross the lipid bilayer as compared with pure AmB in ergosterol containing lipid bilayer, similarly, 2-4 fold more AmB in cholesterol containing lipid bilayer was reported by Adhikari[34]. Although, only a small amount of AmB was transferred from the left to the right chamber. It was postulated that this amount was enough to kill the fungi as the MIC was 0.16 µg/mL and the MFC was 0.32 µg/mL was reported by Gangadhar[33]. It is possible that the AmB and lipid bilayer might form a complex channel with the lipid bilayer and therefore reduce the amount of AmB available for permeation.

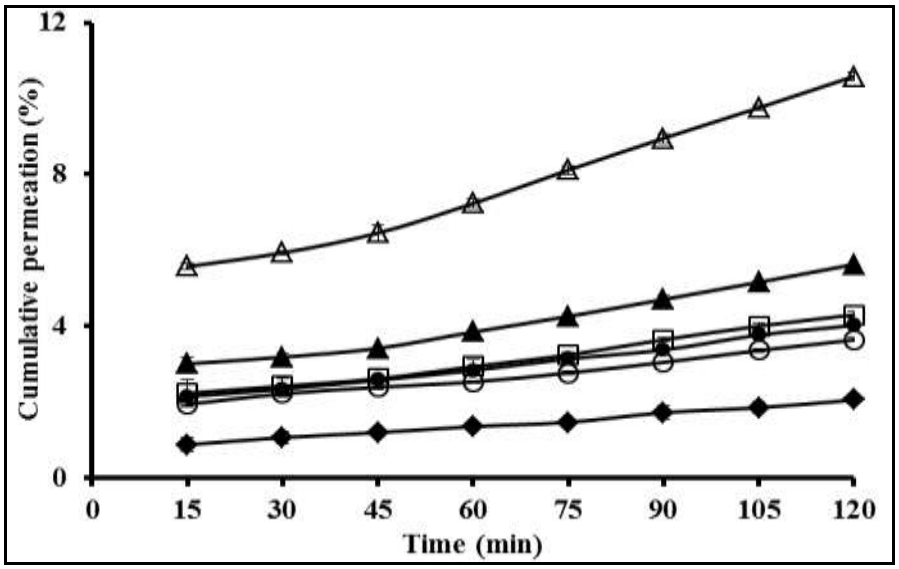

Figure 10. Permeability determination of AmB (◆), AmB-KDC (▲), AmB-SDC (□), AmB-SDCS (△), AmB-KC (●) and AmB-SC (O) from 15 min to 120 min, respectively (Mean ± SD, n=3), which contains ergosterol and phospholipid containing lipid bilayer membrane.

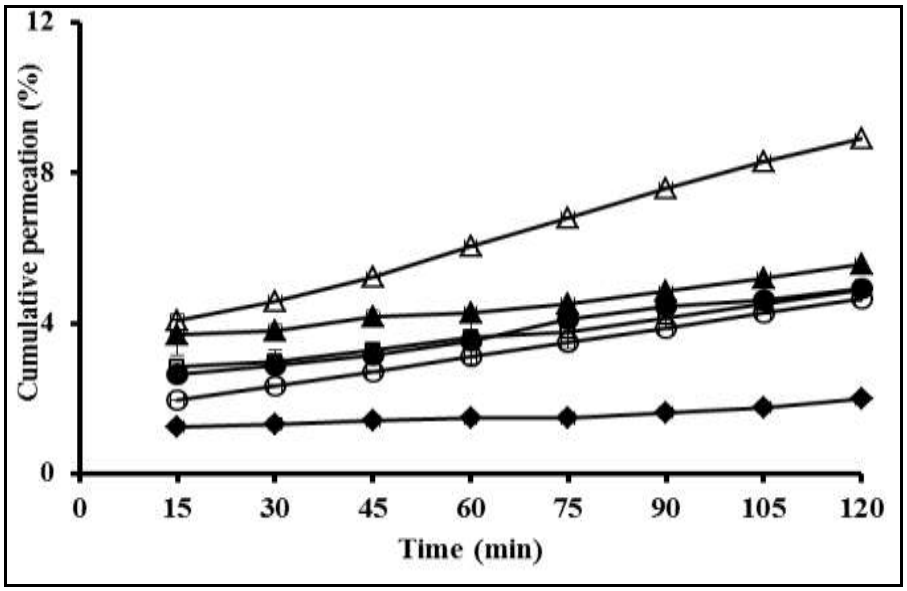

Figure 11. Permeability determination of AmB (◆), AmB-KDC (▲), AmB-SDC (□), AmB-SDCS (△), AmB-KC (●) and AmB-SC (O) from 15 min to 120 min, respectively (Mean ± SD, n=3), which contains cholesterol and phospholipid containing lipid bilayer membrane.

Conclusion:

Five 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 mole ratio 1:2 (AmB:lipid carrier), formed solid caked, which was a very light, free flowing, hygroscopic in nature **Fig.4(A&B**). These formulations were highly water soluble and stable in solution form with negatively charge developed. The particle sizes were found between 17 to 74 nm and zeta potential was above -30 mV for all formulations and among these, AmB-SDCS was the highest -45 mV. Although, these lipid drug carriers are vital role play to dissolve the poorly soluble AmB into highly soluble and stable solution form. The AM NR 8383 cells were phagocyted with AmB-lipid formulation was observed under fluorescence microscope was report by Adhikari[34]. This was possible for targeting the AM in lung fungal infections. The permeation across the lipid bilayer was determined using the Ussing chamber. AmB-lipid formulations were obtained 2-5 fold higher than AmB in ergosterol and phospholipid containing lipid bilayer and similarly, 2-4 fold higher than AmB in cholesterol and phospholipid containing lipid bilayer. From the experiment, it was postulated that cholesterol containing lipid bilayer membrane had less permeability than ergosterol containing lipid bilayer membrane in the AmB-SDCS formulation.

Conflict of interest:

The author states no conflicts of interest.

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