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TECHNICAL NOTE

Self-assembled Amphotericin B Pharmacosome-like Vesicles Derived from Lipid-based Microtubes: A Model Carrier to Further Explore

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Abstract: ***Background****:* Self-assembled drug delivery systems are of much interest since they can be produced by simple low cost and solvent-free procedures. Pharmacosomes are supramolecular-structured nanocarriers with benefits for drug stability and targeting delivery. Amphotericin B (AmB) still remains an important agent for the treatment of invasive mold infections, *e.g* invasive aspergillosis, although the challenge for new formulations is still prevailing due to high rates of toxicity.

Objective*:* We have previously reported the incorporation of AmB into 12-hydroxystearic acid lipid-based microtubes (MTs) for topical use, herein we report the ability of AmB-MTs to self-assemble into vesicles upon dilution.

Methods: AmB-MTs with different drug concentrations (1, 3, 5 mg/ml) were prepared, and size determination was carried out for different dilutions. Morphology was evaluated by microscopy. *In vitro* cytotoxicity was evaluated in Vero cells and *in vitro* activity against *Aspergillus fumigatus* and *Aspergillus flavus* was assessed.

Results*:* AmB-MTs closed upon dilution to form vesicles ranging from 200 nm to 1µm. AmB MIC (Minimum inhibitory concentration) for both *Aspergillus* species was 0.0625 and 0.125 µg/ml for dispersion and reconstituted lyophilized, respectively.

Conclusion: AmB pharmacosome-like vesicles are smaller structures than MTs may thus be favourable for other delivery routes. We assume that this kind of pharmacosomes-like carrier is a promising model for the obtention of new vesicular carriers based on lipid MTs.

Keywords: Self-assembly, lipid microtubes, pharmacosome-like, Amphotericin B, vesicles, nanotechnology, drug delivery.

1. INTRODUCTION

Nanotechnology has provided novel opportunities to improve the therapy of several diseases with the ultimate goal of a better quality of life. Several nanomaterials are being investigated for targeting drug delivery to disease sites. Lipid-based microtubes (MTs) are supramolecular structures usually self-assembled from amphiphilic molecules. MTs constitute promising biomaterials because they are simple to produce in aqueous solutions without using hazardous organic solvents. Three-dimensional lipid tubes are formed by molecular association through intermolecular forces forming concentrically packed bilayers where amphiphilic drug molecules can be packed during the process of tube formation [1-4]. Additionally, pharmacosomes are defined as colloidal dispersions of drugs covalently bound to lipids, which may exist as vesicular, micellar, or hexagonal aggregates. Active compounds possessing a free carboxyl group or an active hydrogen atom can bind to the hydroxyl group of a lipid molecule and form an amphiphilic complex, which then converts into pharmacosomes by dilution with water. Pharmacosomes enhance solubility and stability of loaded drugs and also serve as a targeting and controlled release carrier [5-7].

Amphotericin B (AmB) is a polyene antifungal that has already been investigated for inclusion into vesicles such as liposomes and niosomes [8, 9]. AmB is used to treat a number of fungal infections, including invasive aspergillosis (IA) a severe form of the disease in immunosuppressed patients, with mortality rates greater than 50% [10, 11]. Currently, intravenous AmB deoxycholate (D-AmB) and liposomal AmB are important therapeutic tools for the treatment of invasive fungal infections, however the investigation of safer

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and more affordable medicines, as well as an alternative route of administration is still of great interest [12, 13]. Consequently, nebulized AmB was proposed for IA treatment in order to deliver high drug concentrations directly to the disease site to maximize effectiveness and limit systemic toxicity [14-16]. The development of an appropriate drug-loaded formulation is, however, a major challenge in inhalation therapy since drug carriers should have adequate aerodynamic characteristics to be deposited in the lung, so particles in the range 0.5–5 microns would be the most appropriate. Liposomes have been studied for pulmonary delivery, also niosomes and pharmacosomes have been proposed [17-19].

In earlier studies, we have reported the incorporation of AmB into 12-hydroxystearic acid (12HSA) MTs for topical use. Freshly prepared AmB-MTs are twisted tubular structures 4.5–12.5 μm in length with drug loaded within the MT wall. Nonetheless, dispersions had short time stability [20]. A preliminary lyophilization study showed the possibility to extend the long- term stability of AmB both at 25 and 4 °C (data not shown), while AmB-MTs were easily reconstituted and kept their structure after reconstitution. Herein we introduce AmB-MTs as a vesicle-forming system; *in vitro* antifungal activity against *Aspergillus fumigatus and Aspergillus flavus*, and *in vitro* cytotoxicity are reported.

2. MATERIALS AND METHODS

MTs were prepared as previously reported [20]. Briefly, 1% 12HSA (CASTOROIL S.A.C.I.A.F., Argentina) was mixed with distilled water and a 1M monoethanolamine (ETA; Sigma-Aldrich) solution to get 1:1 lipid: ETA molar ratio. The mixture was heated at 75-80° C in a water bath for 15 minutes until the melting of the lipid, and then vortexed. MTs form spontaneously upon cooling of the clear isotropic solution. AmB (Alpharma, Unifarma SA, Argentina) was added after heating the mixtures 12HSA-ETA to obtain AmB-MTs with final drug concentrations 1, 3 and 5 mg/ml. After cooling samples were filtered by 1.2 μm membrane (GL Microfiber, Titan 2). Size determination was carried out for different dilutions in distilled water (1/5, 1/10 and 1/100) at 25 ºC using a NanoZetasizer -zs (Malvern Instrument, Malvern, UK). The reported values are the average of at least 3 measurements.

For lyophilization AmB-MTs dispersions (AmB 1mg/ml) were poured into glass vials, frozen and lyophilized (freeze-dryer FIC-L05, FIC, Scientific Instrumental Manufacturing, Argentina). The temperature of the freeze-dryer shelf and the condenser were -14°C and -40°C, respectively, and the pressure was 0.03 mbar. Samples were resuspended by adding the corresponding volume of distilled water (1ml) to the dry powder and it was observed if they were easily redispersed.

Phase-contrast microscopy was used to observe diluted and reconstituted samples after lyophilization. Observations were performed at 100X magnification using a Zeiss Axioskop 2 Plus (Germany) equipped with a Sony Exwave HAD video camera to collect digital images (768 x 494 pixels). A drop of sample was placed on the glass-slide and covered with a cover slip without any additional treatment.

In vitro activity against *A. fumigatus* (ATCC 15560), *A. flavus* (ATCC 073167), and *A. fumigatus* recovered from a clinical sample isolated from a patient with IA was assessed using the broth microdilution method according to the CLSI M38A2 document for AmB. MIC was determined in freshly prepared AmB-MTs dispersion and after lyophilization. AmB raw material and D-AmB marketed formulation were used as control and reference, respectively. The final AmB concentration in samples ranged 8.0 to 0.015 μg/ml. Visual readings were performed using a microtiter reading mirror after 24 h and 48 h of incubation at 37 °C. Samples were analyzed at least twice on different days. Blank-MTs was used as negative control; media sterility and media growth controls were also performed.

Cytotoxicity was tested in the mammalian continuous cell line derived from the kidney of an African green monkey, Vero cells (ATCC N°: CCL-81). Cells were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies Corp.) at 37°C in a humidified atmosphere of 5% CO_2. Cells were seeded in 96-well plates ($2.0x10^4$ cells/well), grown overnight and treated with AmB-MTs freshly prepared and reconstituted after lyophilization; D-AmB was tested as a reference. Samples were assayed for different AmB concentrations (0-1000 μg/ml). Cytotoxic activity was determined after 24 h using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS) assay (Promega, Madison, WI). The absorbance at λ=495 nm was determined using a FlexStation 3 (Molecular Devices, LLC, Sunnyvale, CA, USA).

3. RESULTS AND DISCUSSION

Size determination by DLS (Dynamic Light Scattering) showed that upon 1/5, 1/10 and 1/100 dilution, AmB-MTs closed to form vesicles in the range of 1μm- 200 nm. The size was not only defined by dilution but also by AmB concentration in the sample (Fig. **1**). For 1/5 to 1/100 diluted samples size ranges were as follows: 4.25 μm -258. 35 nm (PDI : polydispersity index 0.265-0.421) for blank-MTs, 1.53 μm - 265.60 nm (PDI 0.155-0.354) for AmB-MTs 1mg/ml; 409.17 - 269.37 nm (PDI 0.243-0.303) for AmB-MTs 3 mg/ml, and 339.03 - 284.93 nm (PDI 0.229 -0.245) for AmB-MTs 5 mg/ml. The presence of vesicular-like structures was observed by phase-contrast microscopy (Fig. **2**). As reported for other chiral molecules, 12HSA is expected to have the observed behaviour in order to reduce the system free energy. Thus, the inclusion of an amphiphilic drug molecule into the carrier wall led to smaller structures in comparison to blank-MTs [21-23]. Given that AmB-MTs closed into a small round pharmacosomes-like structure by proper dilution this type of drug delivery system gains interest and is potentially useful for alternative administration routes such as inhaled therapy; as above mentioned particle size in the range of 0.5-5 μm is necessary for effective nebulization and lung deposition and only drug-loaded particles less than 2 μm will be able to penetrate the alveolar region.

In our previous studies AmB-MTs activity against Candida sp. has been tested [24]. We considered interesting to further evaluate AmB-MTs activity against other fungi and we found that MICs results for pathogenic species of

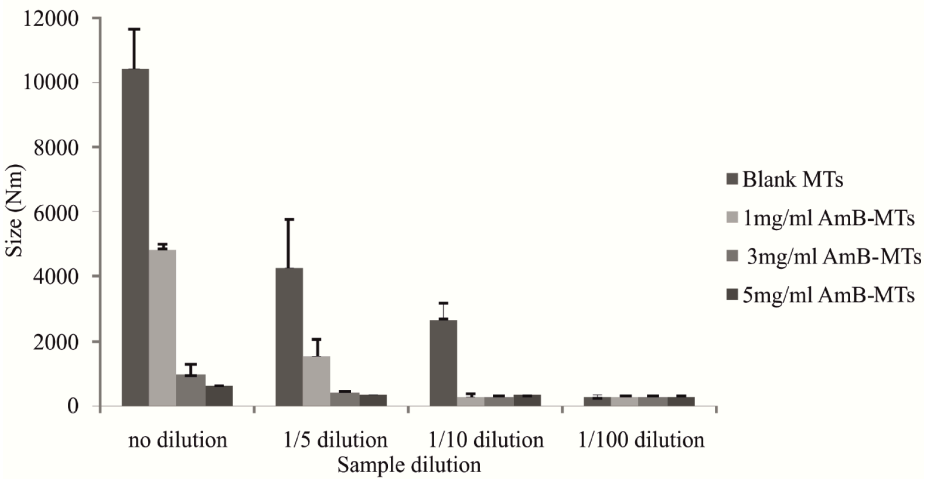

Fig. (1). MTs size upon dilution. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

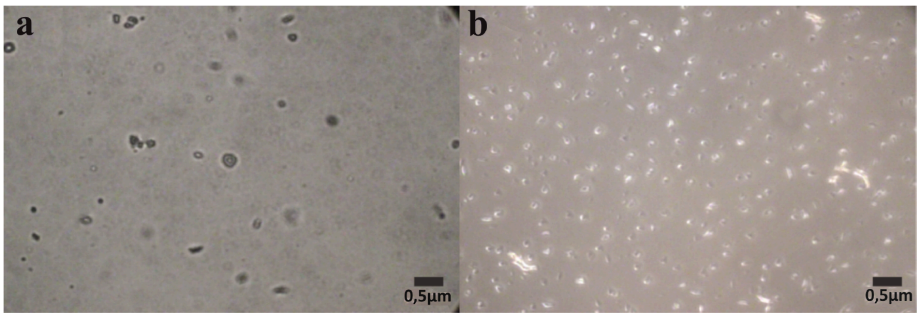

Fig. (2). 1/10 dilution of 1mg/ml AmB-MTs dispersion **a:** conventional **b:** phase-contrast microscopy 100X. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Minimum inhibitory concentration (MIC) ***for Aspergillus spp.***

Sample	MIC (µg/ ml)		
	A.fumigatus **ATCC 15560**	***A.fumigatus*** **(IA patient)**	***A.flavus*** **ATCC 073167**
AmB raw material	0.125	0.125	0.5
D-AmB	0.5	0.5	1.0
Blank-MTs dispersion	No inhibition	No inhibition	No inhibition
AmB – MTs 1mg/ml dispersion	0.0625	0.0625	0.25
Blank-MTs dispersion after lyophilization	No inhibition	No inhibition	No inhibition
AmB – MTs 1mg/ml after lyophilization	0.125	0.0625	0.25

Aspergillus were lower than those for D-AmB and AmB raw material. In this regard, AmB loaded in MTs increased antifungal activity, 2 and 8 times for A. fumigatus, and 2 and 4 times for A. flavus, in comparison with D-AmB and AmB raw material, respectively (Table **1**). Lyophilization process did not affect drug activity. As reported elsewhere MIC values for AmB nanoparticles ranged 0.75 - 0.125 µg/ml for A. fumigates whereas MIC values for liposomal AmB ranged 0.06 - >16 µg/ml for A. flavus, hence our results are encouraging and highlight the role of the carrier [14, 24]. Recent studies have shown that prophylaxis against IA following lung transplantation using local therapy with aerosolized AmB from liposomal, lipid complex or nanoparticles formulations, was effective and with fewer adverse events than D-AmB. Inhalation therapy may help to achieve the therapeutic effect using low dose of the drug and prevent systemic side effects. The development of new alternatives for this non-invasive, targeted route of administration is of relevance [14, 16, 25-27].

Cytotoxicity was determined for the following AmB concentrations 1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.95, 0.98 0.49 and 0 µg/ml. Cell viability was > 80% at any concentration for D-AmB whereas AmB-MTs showed

Table 2. Cell viability.

CC (μg/ml)	D-AMB	AMB-RM	AMB-MTs	AMB-MT $_L$	Blank-MTs
0 (control)	1	1	1	1	1
0.49	0.978 ± 0.136	0.962 ± 0.040	0.908 ± 0.136	0.901 ± 0.141	0.970 ± 0.130
0.98	0.885 ± 0.076	0.927 ± 0.020	0.890 ± 0.185	0.872 ± 0.026	0.893 ± 0.085
1.95	0.857 ± 0.035	0.841 ± 0.059	0.888 ± 0.289	0.821 ± 0.044	0.878 ± 0.209
3.90	0.846 ± 0.023	0.829 ± 0.063	0.831 ± 0.096	0.803 ± 0.058	0.852 ± 0.190
7.81	0.822 ± 0.071	0.811 ± 0.059	0.764 ± 0.001	0.765 ± 0.076	0.774 ± 0.060
15,62	0.817 ± 0.063	0.806 ± 0.063	0.752 ± 0.061	0,726 ± 0,008	0.769 ± 0.060
31.25	0.803± 0.021	0.788± 0.042	0.678 ± 0.033	0,705 ± 0,004	0.608 ± 0.053
65.50	0.792 ± 0.033	0.679 ± 0.063	0.550 ± 0.254	0.578 ± 0.154	0.559 ± 0.204
125	0.741 ± 0.078	0.627 ± 0.120	0.522 ± 0.125	0.530 ± 0.125	0.510 ± 0.025
250	0.719 ± 0.099	0.512 ± 0.026	0.510 ± 0.082	0.520± 0.090	0.500 ± 0.182
500	0.71 ± 0.131	0,508 ± 0,044	0.497 ± 0.042	0.483 ± 0.142	0.499 ± 0.022
1000	0.683 ± 0.120	0,493 ± 0,013	0.413 ± 0.142	0.472 ± 0.083	0.453 ± 0.161

AMB-RM: raw material; AMB-MT $_L$: after lyophilization

greater cell growth inhibition; viability was near 50% for concentrations > 31.25 μg/ml and only for the range 0.49 - 31.25 μg/ml viability was > 80%. Blank-MTs showed similar results as those obtained with AmB loaded samples. On the contrary AmB raw material exhibited approximately 80 - 95% of viability for samples < 62.5 μg/ml and approximately 50% for those > 125 μg/ml, which shows that the carrier may be responsible for such toxicity (Table **2**). Despite these results, in some clinical settings the dose of medication to be applied would be rather low, for instance a pharmacokinetic-pharmacodynamic model showed complete *A fumigatus* and *A flavus* growth inhibition at Cmax ≥ 0.3 mg/L and ≥ 0.8 mg/L, respectively, and the susceptibility breakpoints was reported as 2 μg/ml for *A. fumigatus* and 1μg/ml for *A. flavus*. Moreover in the case nebulization and pulmonary administration only 20-30% of the administered medication is deposited. Herein, AmB pharmacosomes-like is presented as a model system; similar lipids should be further studied for a safer behaviour and the for obtention of suitable dosage forms according to the intended administration route.

CONCLUSION

Bearing in mind that self-assembled structures are of current interest as drug delivery candidates, the ability of AmB-MTs formed from an amphiphilic lipid to spontaneously become into pharmacosome-like vesicles upon appropriate dilution showed a promising base for designing new biocompatible cost-effective carriers.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Kumar, B.; Jajodia, K. Kumar, P.: Gautam, H.K. Recent advances in nanoparticle-mediated drug delivery. *J. Drug Deliv. Sci. Technol.,* **2017**, *41*, 260-268. http://dx.doi.org/10.1016/j.jddst.2017.07.019

[2] Branco, M.C.; Schneider, J.P. Self-assembling materials for therapeutic delivery. *Acta Biomater.,* **2009**, *5*(3), 817-831. http://dx.doi.org/10.1016/j.actbio.2008.09.018 PMID: 19010748

[3] Menzenski, M.Z.; Banerjee, I.A. Self-assembly of supramolecular nanostructures from phenylalanine derived bolaamphiphiles. *New J. Chem.,* **2007**, *31*, 1674-1680. http://dx.doi.org/10.1039/b702586f

[4] Douliez, J.P.; Gaillard, C.; Navailles, L.; Nallet, F. Novel lipid system forming hollow microtubes at high yields and concentration. *Langmuir,* **2006**, *22*(7), 2942-2945. http://dx.doi.org/10.1021/la053262t PMID: 16548537

[5] Bhingare, U.; Khadabadi, S.; Shinde, N. Pharmacosomes: A Novel Drug Delivery System. *Int J of Pharmaceutical Research & Allied Sciences,* **2014**, *3*(1), 14-20.

[6] Goyal, T.; Rawat, A.; Chauhan, M. Pharmacosomes: Opening new doors for drug delivery. *Int. J. Pharm. Pharm. Sci.,* **2012**, *4*(Suppl. 3), 25-29.

[7] Charanjeet, K.; Vaishali, G.; Rainu, D.; Aman, K.; Ujjwal, N.A. Review on current scenario of Pharmacosomes. *Int. J. Pharm. Med. Res.,* **2017**, *5*(1), 412-419.

[8] Adler-Moore, J.P.; Proffitt, R.T. Amphotericin B lipid preparations: what are the differences? *Clin. Microbiol. Infect.,* **2008**, *14*(Suppl. 4), 25-36.

http://dx.doi.org/10.1111/j.1469-0691.2008.01979.x PMID: 18430127

[9] Salerno, C.; Chiappetta, D.; Monteagudo, E.; Bregni, C. Influence of Surfactant Structure in the Encapsulation and Stability of Amphotericin B in Niosomes. *Lat. Am. J. Pharm.,* **2011**, *30*(9), 1728-1736.

[10] Verweij, P.E.; Chowdhary, A.; Melchers, W.J.; Meis, J.F. Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? *Clin. Infect. Dis.,* **2016**, *62*(3), 362-368.
http://dx.doi.org/10.1093/cid/civ885 PMID: 26486705

[11] Hadrich, I.; Makni, F.; Neji, S.; Abbes, S.; Cheikhrouhou, F.; Trabelsi, H.; Sellami, H.; Ayadi, A. Invasive aspergillosis: resistance to antifungal drugs. *Mycopathologia,* **2012**, *174*(2), 131-141.
http://dx.doi.org/10.1007/s11046-012-9526-y PMID: 22327841

[12] Patel, T.S.; Eschenauer, G.A.; Stuckey, L.J.; Carver, P.L. Antifungal Prophylaxis in Lung Transplant Recipients. *Transplantation,* **2016**, *100*(9), 1815-1826.
http://dx.doi.org/10.1097/TP.0000000000001050 PMID: 26950711

[13] Ishaqa, Z-A; Ahmed, N.; Anwar, N.M. Development and *in vitro* evaluation of cost effective amphotericin B polymeric emulsion *J Drug Deliv Sci Technol,* **2018**, *46*, 66-73.

[14] Shirkhani, K.; Teo, I.; Armstrong-James, D.; Shaunak, S. Nebulised amphotericin B-polymethacrylic acid nanoparticle prophylaxis prevents invasive aspergillosis. *Nanomedicine (Lond.),* **2015**, *11*(5), 1217-1226.
http://dx.doi.org/10.1016/j.nano.2015.02.012 PMID: 25791815

[15] Smola, M.; Vandamme, T.; Sokolowski, A. Nanocarriers as pulmonary drug delivery systems to treat and to diagnose respiratory and non respiratory diseases. Review Int. *Journal of Nanomedicine,* **2008**, *3*(1), 1-19.
PMID: 18201941

[16] Solé, A. Invasive fungal infections in lung transplantation: role of aerosolised amphotericin B. *Int. J. Antimicrob. Agents,* **2008**, *32*(2)(Suppl. 2), S161-S165.
http://dx.doi.org/10.1016/S0924-8579(08)70019-0 PMID: 19013342

[17] Pilcer, G.; Amighi, K. Formulation strategy and use of excipients in pulmonary drug delivery. *Int. J. Pharm.,* **2010**, *392*(1-2), 1-19.
http://dx.doi.org/10.1016/j.ijpharm.2010.03.017 PMID: 20223286

[18] El-Sherbiny, I.M.; Smyth, H.D. Biodegradable nano-micro carrier systems for sustained pulmonary drug delivery: (I) self-assembled nanoparticles encapsulated in respirable/swellable semi-IPN microspheres. *Int. J. Pharm.,* **2010**, *395*(1-2), 132-141.
http://dx.doi.org/10.1016/j.ijpharm.2010.05.032 PMID: 20580794

[19] Kelly, C.; Jefferies, C.; Cryan, S-A. Targeted Liposomal Drug Delivery to Monocytes and Macrophages *J Drug Del,* **2011**.

[20] Salerno, C.; Chiappetta, D.A.; Arechavala, A.; Gorzalczany, S.; Scioscia, S.L.; Bregni, C. Lipid-based microtubes for topical delivery of amphotericin B. *Colloids Surf. B Biointerfaces,* **2013**, *107*, 160-166.
http://dx.doi.org/10.1016/j.colsurfb.2013.02.001 PMID: 23500726

[21] Mohanty, A.; Dey, J. Spontaneous formation of vesicles and chiral self-assemblies of sodium N-(4-dodecyloxybenzoyl)-L-valinate in water. *Langmuir,* **2004**, *20*(20), 8452-8459.
http://dx.doi.org/10.1021/la035958x PMID: 15379460

[22] Li, G.; Feng, L.; Zhao, P.; Xu, W.; Wang, Y.; Song, A.; Hao, J. Lysine-based chiral vesicles. *J. Colloid Interface Sci.,* **2014**, *431*, 233-240.
http://dx.doi.org/10.1016/j.jcis.2014.05.069 PMID: 25016167

[23] Sakamoto, S.; Fujii, S.; Yoshida, K.; Sakurai, K. Supramolecular Chirality: Vesicle-to-Chiral Helix Transition of the Micelles Consisting of a Sugar-Bearing Calix[4]arene Surfactant. *Langmuir,* **2016**, *32*(47), 12434-12441.
http://dx.doi.org/10.1021/acs.langmuir.6b01671 PMID: 27389732

[24] Elefanti, A.; Mouton, J.W.; Verweij, P.E.; Zerva, L.; Meletiadis, J. Susceptibility breakpoints for amphotericin B and Aspergillus species in an *in vitro* pharmacokinetic-pharmacodynamic model simulating free-drug concentrations in human serum. *Antimicrob. Agents Chemother.,* **2014**, *58*(4), 2356-2362.
http://dx.doi.org/10.1128/AAC.02661-13 PMID: 24514094

[25] Chishimba, L.; Langridge, P.; Powell, G.; Niven, R.M., Denning, D.W. Efficacy and safety of nebulised amphotericin B in severe asthma with fungal sensitization and allergic bronchopulmonary aspergillosis. *J Asthma*, **2015**; *52*(3): 289-295.
http://dx.doi.org/10.3109/02770903.2014.958853 PMID: 25158109

[26] Neoh, C.F.; Snell, G.I.; Kotsimbos, T.; Levvey, B.; Morrissey, C.O.; Slavin, M.A.; Stewart, K.; Kong, D.C. Antifungal prophylaxis in lung transplantation--a world-wide survey. *Am. J. Transplant.,* **2011**, *11*(2), 361-366.
http://dx.doi.org/10.1111/j.1600-6143.2010.03375.x PMID: 21272239

[27] Canetti, D.; Cazzadori, A.; Adami, I.; Lifrieri, F.; Cristino, S.; Concia, E. Aerosolized amphotericin B lipid complex and invasive pulmonary aspergillosis: a case report. *Infez. Med.,* **2015**, *23*(1), 44-47.
PMID: 25819050