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Assessment of novel oral lipid-based formulations of amphotericin B using an *in vitro* lipolysis model

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ABSTRACT

The purpose of this study was to investigate the intraluminal processing of novel oral lipid-based formulations of amphotericin B using an *in vitro* lipolysis model. Amphotericin B (AmB) was formulated in three lipid-based formulations consisting of different lipid components: iCo-009, iCo-010 and iCo-011. Various lipid loads (0.25, 0.5, 1 and 2 g) were digested using the lipolysis model to assess AmB distribution among the lipolysis phases. The duration of lipolysis was comparable among the three formulations except for 2 g load of iCo-009 which had a significantly longer lipolysis than iCo-010 and iCo-011. The lipid components of iCo-009 experienced lower extent of lipolysis as compared to other formulations. Amphotericin B concentration in the aqueous phases was the highest with iCo-010 which also had the lowest sediment recovery. Amphotericin B levels in the undigested lipid layers were comparable between iCo-009 and iCo-010 and were higher than with iCo-011. Given the observation that iCo-010 had the highest aqueous micellar solubilization and the lowest sediment recovery of AmB among the tested formulations, these results could potentially be used to interpret and predict the *in vivo* performance of AmB- SEDDS formulations in future studies.

1. Introduction

Amphotericin B (AmB) is a fungicidal and leishmanicidal agent that is currently only administered parenterally (Baginski et al., 2005). Amphotericin B is a Biopharmaceutical Classification System (BCS) class IV compound (Menez et al., 2007) with limited solubility and permeability properties resulting in low oral bioavailability (Ouellette et al., 2004). Various parenteral AmB products have been developed to overcome its low solubility, such as a liposomal formulation (Ambisome®), a micellar dispersion with deoxycholate (Fungizone™) and lipid complex (Abelecet®) (Thornton and Wasan, 2009). However, the high cost of the liposomal formulation, the need of hospitalization for parenteral infusion administration and the acute side effects of parenteral AmB (infection of the indwelling catheter, hemolysis, fever, bone pain, thrombophlebitis and dose dependent nephrotoxicity) limit the safety and the widespread use of these treatments (Ostrosky-Zeichner et al., 2003). Development of an oral formulation of AmB is important to overcome the drawbacks of safety and high cost of the parenteral formulations, especially in developing countries.

Different groups (including ours) have incorporated AmB into various carrier systems for oral delivery including lipid-based formulations (self-emulsifying drug delivery systems (SEDDS)) (Wasan et al., 2009a, 2010), nanosuspensions (Golenser, 2006), polymeric nanoparticles (Verma et al., 2011) and cochleates (Delmas et al., 2002). Oral lipid-based formulations drew attention to their ability to improve oral bioavailability of drugs with poor water solubility using low-cost ingredients (Wasan et al., 2009a). Moreover, the nephrotoxicity associated with AmB from the oral lipid-based formulations was significantly lower than those of the commercial products Ambisome® and Fungizone™ (Leon et al., 2011; Sivak et al., 2011; Wasan et al., 2009b). Three oral lipid-based formulations were developed in order to enhance the absorption and efficacy of AmB, namely iCo-009, iCo-010 and iCo-011. These formulations are efficacious in the treatment of leishmanial and fungal infections (Wasan et al., 2009a, b, 2010). The enhanced efficacy of oral AmB lipid-based formulations is attributed to improved absorption profiles of the formulated AmB (Gershkovich et al., 2009). Both iCo-010 and iCo-011 contain lipid (Peceol), surfactant (Gelucire 44/14) and VitE-TPGS as a co-surfactant. Both formulations could be considered as SEDDS formulations and are able to form instant submicron sized emulsion upon mixing with simulated gastrointestinal fluids. On the other

Abbreviations: AmB, amphotericin B; BCS, Biopharmaceutical Classification System; VitE-TPGS, D-Alpha-tocopherol; DSPE-PEG-2000, polyethylene-glycol-succinate-distearoylphosphatidylamine polyethyleneglycol-2000; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; PC, phosphatidylcholine; PPT, precipitate.

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hand, iCo-009 which contains 100% Peceol® as the lipid component forms translucent emulsion upon mixing with simulated intestinal fluids (Kastantin et al., 2010; Wasan et al., 2009a). The dispersed AmB was solubilized within the formed emulsion particles after mixing with gastrointestinal fluids which resulted in improvement of its oral absorption (Larsen et al., 2008; Wasan et al., 2009a). Other mechanisms that could be involved in enhancing oral absorption of AmB from SEDDS include prolongation of gastrointestinal transit time and increase of intestinal wall permeability (Dahan and Hoffman, 2007).

The evaluation of intraluminal processing of poorly water soluble drugs formulated in lipid-based formulations is complicated due to underlying lipid digestion process. Simple dissolution and dispersion tests will not be suitable to assess the digestion of lipid-based formulations (Larsen et al., 2011; Porter et al., 2007). Thus, a suitable model should be implemented to account for the complex colloidal system of intestinal lipolysis. An *in vitro* lipolysis model was previously adapted to simulate the intestinal digestion process and was used to evaluate the distribution of drugs among lipolysis phases (aqueous, undigested lipids and sediment layers) (Larsen et al., 2011; Porter and Charman, 2001). The adapted model accounts for the presence of phospholipids, bile salts and lipase enzyme in a digestion buffer at 37 °C and constant pH levels. Pancreatic lipase enzyme digests the lipid-based formulation after being vigorously stirred to ensure equilibration and homogeneity of the digestion process. Accordingly, the dispersed drug is either solubilized in vesicular structures formed by the association of bile salts, lipid digestion products and phospholipids; precipitated; or dispersed in the undigested lipids (Larsen et al., 2011; Porter and Charman, 2001). The lipolysis phases can be separated using ultracentrifugation and the content of AmB in each phase can be quantified.

Various lipid components can be recruited for the formulation of oral lipid-based formulations. The lipids in the formulation affect the rate and extent of the digestion process due to the differences in lipase enzyme affinity to the type and the amount of digestible lipids. *In vitro* lipolysis model can be used to rank the formulations according to the level of AmB distribution among the lipolysis phases. Thus, formulations with the least AmB recovery in the sediment along with high levels in the aqueous and lipid phases should be considered for further development and *in vivo* investigation (Dahan and Hoffman, 2007; Larsen et al., 2008).

All the previous studies that had been conducted on AmB-lipid-based formulations were to assess their efficacy and biodistribution (Gershkovich et al., 2010, 2009; Leon et al., 2011; Sivak et al., 2011; Wasan et al., 2009a, 2010, 2009b). However, to date, no studies have been conducted to elucidate the impact of the oral lipid-based formulation on intraluminal processing of AmB. The focus of this work was to investigate the simulated intraluminal processing of AmB and its subsequent distribution among the lipolysis phases using *in vitro* lipolysis model. These results could be used in the future studies to interpret the *in vivo* performance of AmB-lipid-based formulations.

2. Materials and methods

2.1. Materials

Amphotericin B powder, D-alpha-tocopherol polyethylene-glycol-succinate (VitE-TPGS), egg phosphatidyl choline, sodium taurocholate, porcine pancreatin powder, tris maleate powder and 1-amino-4-nitronaphthalene were purchased from Sigma–Aldrich (St Louis, MO, USA). Peceol® (Glycerol Monooleate) and Gelucire 44/14® were a gift from Gattefossé Canada (Mississauga, Ontario, Canada). Gelucire 44/14 is a well-characterized lipid excipient comprised of PEG 1500 esters of long chain fatty acids, glycerol and free PEG. Distearoylphosphatidylamine polyethyleneglycol-2000 (DSPE-PEG-2000) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). All solvents and other chemicals were HPLC grade.

2.2. Formulation

The composition and the preparation of lipid-based formulations (iCo-009, iCo-010 and iCo-011) was previously reported (Wasan et al., 2009a, 2010). Briefly, AmB and DSPE-PEG-2000, suspended in ethanol, were mixed with Peceol®, to give a final AmB concentration of 5 mg/ml (iCo-009). The mixture was stirred for 1 h with gentle warming and the solvent was subsequently removed using a rotary evaporator. Both iCo-010 and iCo-011 also contained Peceol®, but with lauroyl polyoxyl-32 glycerides NF (Gelucire 44/14®) at ratios of 50/50 (v/v) and 60/40 (v/v), respectively. In addition, both iCo-010 and iCo-011 contained 5% (v/v) vitamin E D-alpha tocopheryl polyethylene-glycol-succinate (VitE-TPGS) (Wasan et al., 2009a, 2010).

2.3. Preparation of pancreatic lipase/colipase solution

One gram of porcine pancreatin was added to 5 ml of a lipolysis buffer which was composed of 50 mM tris maleate, 150 mM NaCl and 5 mM $CaCl_2$. The mixture was stirred for 15 min followed by centrifugation for 15 min at 5 °C at 4500 rpm. The supernatant was collected and kept on ice for less than 5 min.

2.4. In vitro lipolysis

The procedures of lipolysis were followed as described previously with minor modifications (Dahan and Hoffman, 2007; Gershkovich et al., 2012). Either AmB-SEDDS formulations (0.25, 0.5, 1 or 2 g of iCo-009, iCo-010 or iCo-011) or aqueous suspension (equivalent to 10 mg AmB) were added to the lipolysis buffer in a thermostated water bath at 37 °C. After equilibration, 3.5 ml of lipase enzyme (equivalent to 1000 IU/ml) was added to the lipolysis medium in order to start the digestion process. The pH was kept at 7.4 by titrating the medium against NaOH using a pH-stat titrator unit (T50 Graphics, Mettler Toledo Inc.). The digestion process was considered finished when the addition rate of NaOH was less than 0.005 ml/min. An aliquot of the medium was ultracentrifuged (L8-55 Ultracentrifuge, SW-41rotor, Beckman Co., Palo Alto, CA) at 40,000 rpm for 1.5 h at 37 °C. The lipid phase was separated by pipetting the lipid layer from the surface. Then, the aqueous phase was decanted away from the sediment which is then separated and dissolved in DMSO. The separated phases were analyzed for AmB quantification using HPLC. The concentration of AmB in the separated layers after ultracentrifugation was used to calculate the corresponding amount of AmB in each layer in the whole lipolysis medium. The remaining undigested lipids were calculated by subtracting the number of moles of NaOH consumed in the titration after correction for the background lipolysis from the initial number of fatty acid moles that were introduced into the medium (Cuine et al., 2008).

2.5. Measurement of the particle size of the formulations after dispersion in lipolysis buffer

The particle size of the dispersed formulations in the lipolysis buffer was determined before the addition of lipase enzyme. Briefly, at the end of 15 min equilibration of the formulation with the lipolysis buffer, a sample of the medium was withdrawn and diluted (1:1000) with formulation-free buffer. The particle size of the formed emulsions was determined by using a Malvern NanoZS particle size analyzer (Malvern, UK).