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Liposomal amphotericin B—the past

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The discovery of amphotericin B, a polyene antifungal compound, in the 1950s, and the formulation of this compound in a liposomal drug delivery system, has resulted in decades of use in systemic fungal infections. The use of liposomal amphotericin B formulation is referenced in many international guidelines for the treatment of fungal infections such as *Aspergillus* and cryptococcal disease and *Candida* infections, as well as other less common infections such as visceral leishmaniasis. With the development of liposomal amphotericin B, an improved therapeutic index could be achieved that allowed the attainment of higher drug concentrations in both the plasma and tissue while simultaneously lowering the toxicity compared with amphotericin B deoxycholate. In over 30 years of experience with this drug, a vast amount of information has been collected on preclinical and clinical efficacy against a wide variety of pathogens, as well as evidence on its toxicity. This article explores the history and nature of the liposomal formulation, the key clinical studies that developed the pharmacokinetic, safety and efficacy profile of the liposomal formulation, and the available microbiological data.

Introduction

The period from 1950 to 1969 has been called the 'Advent of Antifungal and Immunosuppressive Therapies'.[1] During this period, increased opportunistic systemic fungal infections were noted, in part due to the greater recognition of these infections and increased use of antibiotics and immunosuppressive therapies. This period also featured significant antifungal drug discoveries. For example, the polyene antibiotic amphotericin B was discovered in 1953 when, in what can be thought of as 'Fleming in reverse', the bacterium *Streptomyces nodosus* was found to produce a broad-spectrum antifungal compound.[2] Serious impediments to broad medical use of this compound were that it is completely insoluble in aqueous media and that it has no useful oral bioavailability. Eventually, an IV formulation was developed using the bile acid deoxycholate, which provides solubilisation and a way to produce a sterile injectable formulation.

Amphotericin B forms pores or channels in biological membranes, principally anchored by the membrane sterol, resulting in compromise of barrier function and ultimately death of the target cell.[3] The result of this is that, in addition to antifungal activity, amphotericin B exhibits substantial toxicity to mammalian cells. The utility of the amphotericin B deoxycholate formulation derives from a net favourable therapeutic window, with efficacy in a favourable balance with toxicity. This is because the fungal membranes utilise ergosterol as a membrane sterol, whereas the mammalian membranes utilise cholesterol, and the binding avidity of amphotericin B for ergosterol is approximately 10-fold higher than for cholesterol.[4] However, this window of utility is limited to doses no more than approximately 1 mg/kg, and the deoxycholate formulation carries substantial risk, including potentially fatal cardiac or cardiorespiratory arrest on overdose (specifically in the setting of too high an infusion rate), fever and other infusion-related adverse events, decreased renal function and renal function abnormalities including substantial nephrotoxicity.[5] These manifestations of toxicity derive directly or indirectly from amphotericin B host membrane interactions.

The 1970s saw the birth of a scientific effort to use engineered versions of naturally occurring structures named liposomes, which are phospholipid bilayer-based structures with entrapped aqueous spaces, as carrier vehicles for drug delivery.[6] By the 1980s, several companies had been established to develop liposome-based therapeutics. One of these companies, Vestar Inc. in California, engineered liposomes that were small (less than 100 nm diameter) and featured single bilayer membranes, a neutral net particle charge, and a lipid bilayer motif of distearoylphosphatidylcholine and cholesterol in a 2:1 mole ratio.[7] This formulation, and associated high shear production methods, afforded a relatively 'solid' or 'rigid' state at human body temperature and exhibited extended stability post injection.[7] If active drug substances are stably incorporated in such liposomes, they can exhibit substantially reduced clearance by typical clearance pathways (e.g. liver, kidney, macrophage uptake) and thus show

extended plasma lifetime and tissue distribution. Vestar had an initial focus on anti-cancer compounds, but the technology was adapted by Professor Jill Adler-Moore and colleagues to develop a liposomal formulation of amphotericin B.[8] In this case, an additional phospholipid, distearoylphosphatidylglycerol, was added to afford a negative particle charge, and the drug itself was formulated in the phospholipid bilayer, taking advantage of the natural tendency of the drug to bind to cholesterol in the membrane.

Amphotericin B in the liposomal formulation (liposomal amphotericin B), known as AmBisome®, forms functioning ion channels.[9] Non-clinical (mouse, rat, rabbit and dog) studies have revealed substantial increases in plasma and tissue drug concentrations and substantially reduced toxicity relative to the deoxycholate formulation.[8,10] In an *in vitro* demonstration,[11] the propensity of liposomal amphotericin B to transfer amphotericin B to a target membrane was evaluated by measuring potassium release from the latter, which indicates leakage from the formation of new intact and functioning amphotericin B channels in the target cell and acts as a surrogate for potential off-target toxicity. Much higher concentrations of liposomal amphotericin B than of the 'free drug' (deoxycholate) formulation were needed to achieve potassium release in target mammalian RBC membranes. This is in contrast to observations using *Candida albicans* cells in the same assay, in which all amphotericin B formulations release the drug at the same concentration.[11] This study illustrates the critical role of sterol–amphotericin B binding avidity; formulations with no cholesterol release the drug readily in the presence of a membrane containing cholesterol, including into RBC membranes, while a liposome with a stable phospholipid bilayer—and successfully entrapped drug and cholesterol—has no thermodynamic driving force to readily partition the drug to a mammalian membrane containing the same sterol, cholesterol. However, for an encounter of the liposomal particle with a fungal membrane containing ergosterol, the binding avidity difference results in a ready driving force for drug transfer. The same is true for ergosterol-containing *Leishmania* protozoa. Stable assembly of the liposomal structure of liposomal amphotericin B is as much a function of the manufacturing process as of the formulation;[12] products produced with the same formulation but by altered processes exhibit substantially different performance.[13] A number of other liposome formulations, including some with ostensibly identical formulations, have nevertheless revealed significant differences in particle size distribution and the fidelity of the lipid bilayer and drug entrapment. Consequently, there are substantial potential safety ramifications,[13] likely due to differences in raw material quality, testing and/or production process.

The liposomal formulation properly manufactured thus afforded the potential for an improved therapeutic index—drug retention in the engineered bilayer to reduce clearance and toxicity, while retaining the ability to deliver lethal consequences to a targeted pathogen. This formed the rationale for the first use of liposomal amphotericin B in clinical settings and has not been matched with other lipid-based formulations that have been used clinically.[13] Other elements of the formulation may play a role in antifungal activity and reveal an element of targeting. Microscopy, gold-label and *Candida* mutant studies[14,15] have revealed that the liposomal particle in liposomal amphotericin B, as confirmed with a drug-free placebo, will traverse the fungal cell wall, despite an ostensibly dense matrix of nominally narrow effective pore size, and bind to the surface of the fungal cell membrane. This may in part depend on the negative surface charge on the liposome binding to positively charged elements on the fungal membrane. However, when the drug is present, the liposome will decompose on or in the membrane, depending on the presence of ergosterol in the fungal membrane. Mechanistic elements of liposomal amphotericin B are summarised in Figure 1, which captures the essence of mechanistic concepts developed by the Gow laboratory.[15]

Pharmacokinetics and pharmacodynamics of liposomal amphotericin B

Adults

After the preclinical phase, clinical trials of liposomal amphotericin B started with Phase I/II dose-finding studies in healthy volunteers. These Phase I/II trials typically involved dose escalation, single ascending and multiple dose trials, and the information derived was then used to define the dosages used in Phase III clinical trials.

For liposomal amphotericin B, the first dose-finding trial in healthy volunteers was an open-label, sequential-dose-escalation, multiple-dose pharmacokinetic study in 36 adults with febrile neutropenia.[16] Each cohort consisted of 8–12 participants who received 1.0, 2.5, 5.0 or 7.5 mg/kg liposomal amphotericin B infused over 1 h. This study showed that liposomal amphotericin B exhibits non-linear pharmacokinetics (i.e. exposure increases disproportionally with increasing dose). These findings were consistent with saturation of the reticuloendothelial uptake as the major clearance pathway. In this small cohort, liposomal amphotericin B was well tolerated, and there was a limited number of infusion-related adverse events.

This trial opened up the scene for the next one, which involved further dose escalation in 44 patients with neutropenia who received 10, 12.5 and 15 mg/kg liposomal amphotericin B,[17] with the goal of deriving the maximum tolerable dose. This study confirmed the previously identified non-linear pharmacokinetic profile of liposomal amphotericin B. Adverse events were more prevalent at these higher doses and typically included a syndrome of substernal chest tightness, dyspnoea and flank pain. In addition, a doubling of serum creatinine from baseline, reflecting a decline in renal function, was observed in 32% of patients. Strikingly, the pharmacokinetics revealed that maximum exposure values occurred at 10 mg/kg but exposure then declined at 12.5 and 15 mg/kg.

A key step in dose finding is performing mass-balance studies to determine the excretion pathways of the parent drug and its possible metabolites and to elucidate the metabolic fate of the drug. Two mass-balance studies were carried out with liposomal amphotericin B.[18,19] The first trial involved five healthy volunteers who received ^{14}C-cholesterol-labelled liposomal amphotericin B at 2 mg/kg (1 μCi/kg) infused over 2 h.[18] About 9.5% of the radioactive dose administered was recovered from faeces. Combined faecal and renal clearance was less than 18%, indicating a long residual time of liposomal amphotericin B in the body. Liposomal amphotericin B was indeed found to remain in the circulation for an extended period while releasing amphotericin

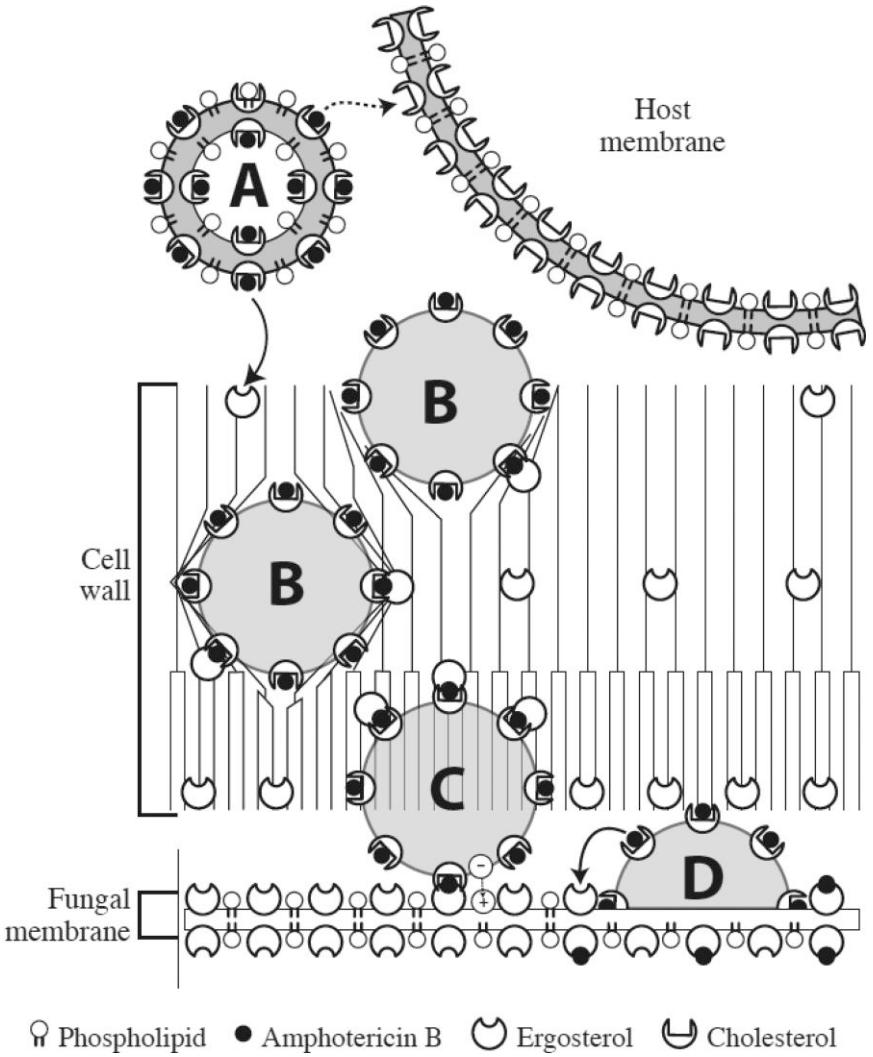

Figure 1. Mechanistic elements of liposomal AMB (AmBisome). The liposomes are rendered as spheres, with AMB as black dots (representing membrane-spanning aggregates of AMB as formed channels), and cholesterol or ergosterol as symbols as indicated. (A) The liposome has a relatively low propensity to transfer drug to a mammalian membrane; (B) the liposome formulation is able to pass through the fungal cell wall and bind to the fungal membrane surface (C); the latter may be facilitated by the complementary charged surfaces of the liposome and membrane; (D) driven by the presence of ergosterol and reflecting the ability of AMB to bind to ergosterol in preference to cholesterol, the liposome delivers the drug to the fungal membrane and is degraded. The structures of any transition states between AMB in the liposome bound to cholesterol and AMB in the fungal membrane bound to ergosterol are not known. AMB, amphotericin B.

B. The second trial was a pharmacokinetic and mass-balance study in healthy volunteers who received 2 mg/kg liposomal amphotericin B infused over 2 h.[19] This study found that less than 10% of the liposomal amphotericin B was excreted unchanged and that no metabolites were observed. This second study also investigated the degree of liposomal amphotericin B versus non-liposomal amphotericin B. The latter was further subdivided into protein-bound and free drug. The authors reported that over 97% of amphotericin B was liposome incorporated at 4 h after administration. This declined to 55% at 168 h after administration. The urinary clearance of the unbound (or free) drug was equal to the glomerular filtration rate.

The biggest driver for defining the dose of liposomal amphotericin B for *Aspergillus* disease in the past 30 years has been the results of the AmBiLoad study.[20] Until the results of this study were reported, the dose recommended in guidelines was 5 mg/kg. The AmBiLoad study was a double-blind trial comparing a 3 mg/kg dose with a 10 mg/kg dose until Day 14 in 201 haematology patients. After Day 14, the dose was de-escalated to 3 mg/kg for all participants. The primary endpoint was complete or partial response at the end of treatment. Safety and survival were secondary outcome measures.

Contrary to expectations, the 3 mg/kg dose performed equally well as the 10 mg/kg dose. In addition, there was a substantially higher rate of adverse events with the 10 mg/kg dose, typically nephrotoxicity, but without additional clinical benefit. At this point, all international guidelines adopted 3 mg/kg as the recommended dose for treatment of *Aspergillus*

disease.[21,22] This evidence was also translated to paediatric patients.[23] The AmBiLoad trial can still be considered a pivotal dose-finding trial and heralded a paradigm shift in balancing optimal efficacy while minimising toxicity.

Special populations

Today, the pharmaceutical industry typically conducts pharmacokinetic studies in patients with renal and hepatic impairment. Liposomal amphotericin B was licensed prior to these requirements, and thus there are limited data on its pharmacokinetics in these two special populations. The Summary of Product Characteristics indicates that liposomal amphotericin B has been given successfully to patients with pre-existing renal failure but acknowledges the absence of these data.[24] The same applies to hepatic impairment; liposomal amphotericin B has not been studied in patients with mild, moderate or severe hepatic impairment, and thus it remains unclear if the pharmacokinetics are altered in this setting. No formal recommendation is given. Finally, it remains to be investigated if sex or ethnicity has an impact on the pharmacokinetics of liposomal amphotericin B.

Children

After completion of the pharmacokinetic dose-finding and mass-balance studies in adults, a formal Phase II trial was designed in paediatric patients.[25] Forty paediatric patients aged 1–17 years participated in a multi-dose escalation trial. By that time, independent researchers had also investigated the pharmacokinetics of liposomal amphotericin B in paediatric patients.[26,27] In the first study, participants received dosages of 2.5, 5, 7.5 and 10 mg/kg liposomal amphotericin B once daily as a 1 h infusion.[26] The authors found that the pharmacokinetics of liposomal amphotericin B followed similar patterns as in adult patients and it could be given at identical dosages. Declines in renal function and other adverse events such as hypokalaemia were found to be dose dependent. The pharmacokinetics observed in these two independent studies were best described by a linear two-compartment model with weight as a relevant covariate for clearance. Weight was a relevant covariate in one study, whereas the other study used an exponential decay function to describe the volume of distribution. Explanations on differences observed on the impact of weight and age on clearance including non-linearity remain unresolved. The role of the macrophage function as driver for clearance and whether this system is saturable over time, weight and age are currently unknown and warrant further investigations.

Other studies

In addition to the studies performed for the regulatory approval of liposomal amphotericin B, numerous other pharmacokinetic studies have been carried out. These include a large study in adult haematology patients,[28] as well as a study in obese patients.[29]

Beyond the specific pharmacokinetic studies, many clinical trials have been conducted and demonstrated the added value of liposomal amphotericin B. Unfortunately, none of these studies have investigated the pharmacokinetics or exposure–response and exposure–toxicity relationships. So to date, there has been no clinical study to confirm the preclinically derived pharmacokinetic–pharmacodynamic indices for liposomal amphotericin B or establish a relationship between concentration, cumulative exposure and the occurrence of adverse events such as impaired renal function.

Microbiology

As discussed, liposomal amphotericin B is a unique liposomal formulation of amphotericin B and is approved for use in adults and children aged 1 month to 18 years for the treatment of severe systemic and/or deep mycoses, visceral leishmaniasis in immunocompetent patients including both adults and children, and the empirical treatment of presumed fungal infections in patients with febrile neutropenia, whose fever has failed to respond to broad-spectrum antibiotics, and appropriate investigations have failed to define a bacterial or viral cause. Infections successfully treated with liposomal amphotericin B include disseminated candidiasis, aspergillosis, mucormycosis, chronic mycetoma, cryptococcal meningitis and visceral leishmaniasis.[24] Amphotericin B has a very broad spectrum of action, with only a few fungal pathogens displaying primary or acquired resistance.[30,31]

CLSI and EUCAST established reference methods for antifungal susceptibility testing, epidemiological cut-off values (ECOFFs or ECVs), clinical breakpoints (CBPs) and interpretative categories for antifungals. Both techniques are based on similar formats but with differences such as media and inocula;[32–34] amphotericin B endpoints for CLSI M27-A4 and EUCAST E.DEF.7.3.1 are defined as 100% and ≥90% decrease in growth, respectively.[32] However, comparable results for amphotericin B MICs[33] are also provided with commercially available testing methods.[35] Both CLSI and EUCAST have defined CBPs for several antifungal drug–fungal species combinations. Due to the paucity of clinical outcome data, CLSI has not yet established CBPs for any moulds, with the exception of voriconazole against *Aspergillus fumigatus*.[32] EUCAST released CBPs for amphotericin B against *C. albicans*, *Candida glabrata*, *Candida krusei* and *Candida tropicalis* and established CBPs for amphotericin B against *A. fumigatus* and *Aspergillus niger* (Table 1). ECVs facilitate the identification of strains harbouring acquired or innate resistance by defining the upper limit of the WT MIC distribution. However, an ECV does not necessarily predict clinical success or failure;[32] so far, for most fungi, especially rare fungal pathogens, interpretive criteria for amphotericin B are missing. In the absence of ECVs and CBPs for amphotericin B, strains displaying amphotericin B MICs >2 mg/L are generally interpreted as 'resistant'.[33,36] However, there is no clinical validation for this recommendation. In addition, clinical interpretation of amphotericin B MICs is challenging because it remains unclear how precisely the current methods distinguish between susceptible and resistant strains. Most MICs fall within a narrow range of dilutions (0.25–1 mg/L) and hence overlap with accepted error ranges of quality control strains.[30,35] Overall, amphotericin B resistance tends to be species dependent and emerges uncommonly and slowly; the development of resistance to amphotericin B has not been a major factor in the treatment of patients.[37] The importance of susceptibility testing for liposomal amphotericin B is not known, but comparable *in vitro* results (within one dilution) have been observed for liposomal amphotericin B and amphotericin B.[30,33]

Beyond fungal infections, liposomal amphotericin B is recommended for the treatment of visceral leishmaniasis, occurring

Table 1. Overview of the spectrum of activity of amphotericin B and liposomal amphotericin B (adapted from[2,30–39,41])

The pathogen			CLSI		EUCAST		Comments (the spectrum of activity of L-AMB is similar to AMB)
			CBP	ECV	CBP	ECV	
Fungi	Yeasts	*C. albicans*, *C. glabrata*, *Candida parapsilosis*, *C. tropicalis*, *C. krusei*		≥2	>1	≤1	*Candida* species are usually considered to be AMB susceptible; some reports show resistance for *C. tropicalis* and *C. krusei*; *C. glabrata*, *C. parapsilosis*, *Candida kefyr*, *Candida famata*, *Candida lusitaniae* and *Candida guilliermondii* are usually considered to be AMB susceptible, although occasional resistant strains may exist.
		Candida dubliniensis			>1	≤0.25	
		Other yeasts					*Candida auris* and species of the *Candida haemulonii* complex are frequently observed to be AMB resistant (intrinsic/acquired); *Saccharomyces cerevisiae*[a] are usually considered to be AMB susceptible; *Trichosporon* species are usually AMB susceptible; *Trichosporon asahii* may demonstrate resistance; *Malassezia* species are difficult to culture but are considered to be AMB susceptible; *Malassezia furfur* may demonstrate AMB resistance.
		Cryptococcus neoformans			>1	(1)[a]	
		Cryptococcus gattii[a]				(0.5)[a]	
	Moulds	*A. fumigatus*			>1	≤1	Overall, reports show a low incidence of AMB resistance in *Aspergillus* species; for *A. fumigatus*, the most common cause of invasive aspergillosis, AMB resistance is rarely described; intrinsic AMB resistance is well known for *Aspergillus terreus*; however, AMB-susceptible variants do exist; intrinsic AMB-resistant isolates exist within *Aspergillus flavus* and *Aspergillus lentulus*.
		A. flavus				≤4	
		A. niger			>1	(0.5)[a]	
		A. terreus				8	
		Other *Aspergillus* species				(4)[a]	*Aspergillus nidulans* and *A. niger*[a] are, in principle, susceptible to AMB.
		Mucorales	No cut-off values defined				Generally considered to be susceptible to AMB; some *Rhizopus* and *Cunninghamella* strains show decreased AMB susceptibility.
		Fusarium species				(8)[a]	AMB is recommended to treat fusariosis; members of the genus *Fusarium* are generally susceptible to AMB; primary resistance may be seen in *Fusarium fujikuroi*[a] and *Fusarium solani SC*[a], yet is quite variable.
		Scedosporium species	No cut-off values defined				Members of the genus *Scedosporium* are generally susceptible to AMB; primary resistance may be seen in *Pseudallescheria boydii*, *Scedosporium apiospermum* and *Lomentospora prolificans*, the latter representatives are, in general, MDR pathogens.
		Other moulds	No cut-off values defined				*Talaromyces marneffei* is considered to be susceptible to AMB; *Paecilomyces* species, *Bipolaris* species, *Exophiala* species and *Cladophialophora* species demonstrate intermediate susceptibility to AMB, depending upon the species.
	Dimorphic	*Histoplasma capsulatum* *Blastomyces dermatitidis* *Coccidioides immitis* *Paracoccidioides brasiliensis*	No cut-off values defined				Dimorphic fungi are usually susceptible to AMB; some strains of *Sporothrix schenckii* may show primary resistance.

Continued

Table 1. *Continued*

The pathogen		CLSI CBP	CLSI ECV	EUCAST CBP	EUCAST ECV	Comments (the spectrum of activity of L-AMB is similar to AMB)
Protozoa	*Leishmania species* *Leishmania donovani* complex *Leishmania tropica* *Leishmania major* *Leishmania mexicana*	No cut-off values defined				L-AMB is an alternative for the treatment of cutaneous and mucosal leishmaniasis, including old- and new-world *Leishmania* strains; MIC values obtained show a parasitical concentration of 0.5 mg/L for AMB and the various *Leishmania* species; resistance seems to be variable; an altered membrane composition, ATP-binding cassette transporters and an up-regulated thiol metabolic pathway have a role in AMB resistance in clinical isolates of *L. donovani*.

CBPs and ECVs expressed as mg/L. AMB, amphotericin B deoxycholate; L-AMB, liposomal amphotericin B; SC, species complex.
[a]Proposed ECVs.

mainly in India, South America and the Mediterranean area.[38,39] A total dose of 20 mg/kg appears to be effective in immunocompetent patients, but regional variations in the susceptibility of the parasite exist. Recently, the Sensititre™ YeastOne™ YO9 plate (Thermo Fisher Scientific, Waltham, MA, USA) was successfully used to study the susceptibility profiles of *Leishmania* spp. promastigotes in log phase with amphotericin B and fluconazole. New-world strains demonstrated reduced susceptibility to amphotericin B (0.25–0.50 mg/L) compared with old-world strains (0.12 mg/L). However, breakpoints for interpretative criteria are lacking.[40]

Conclusions

In this article, the history of the discovery and initial use of amphotericin B is reviewed, including the major toxicity-related limitations on use of a detergent suspension formulation of the drug in systemic fungal infections. Liposome formulation technology was leveraged to create a substantially altered form of injectable amphotericin B: liposomal amphotericin B, wherein amphotericin B is entrapped in a stable, cholesterol-containing phospholipid bilayer that forms a small (<100 nm) unilamellar liposome. In preclinical studies, this formulation was observed to afford substantial increases in plasma and tissue drug concentrations, and yet substantially reduced amphotericin B-associated toxicity relative to the non-liposomal formulation. Nevertheless, the amphotericin B in liposomal amphotericin B partitions readily through the fungal cell wall to the fungal membrane, with transfer of drug and hence fungicidal action. This is in part due to the higher binding avidity of the drug for fungal ergosterol versus the cholesterol present in the liposomal amphotericin B formulation and in host tissues.

As clinical study of liposomal amphotericin B began, data revealed non-linear pharmacokinetics and an opportunity to substantially escalate the dose relative to the non-liposomal formulation. The clearance profile and mass-balance studies confirmed high levels of association of amphotericin B with the liposome after injection. It was discovered that the highest tolerated dose may not have the most favourable balance between efficacy and toxicity; for example, a 3 mg/kg/day dosage was superior overall to a 10 mg/kg/day dosage in haematology patients, and determined the dose for pulmonary aspergillosis. Studies have been and are continuing to be conducted in special populations.

Liposomal amphotericin B has been shown to be effective in a wide range of patients with infections due to diverse fungal pathogens, including empirical treatment of presumed fungal infections in patients with febrile neutropenia. A wide range of infections are successfully treated, and liposomal amphotericin B shows a very low propensity to elicit acquired resistance, in part perhaps because of the rudimentary chemical nature of fungicidal activity (the formation of membrane-spanning ion channels). Susceptibility data have been generated on a wide range of moulds, yeasts and dimorphic fungi. Lastly, liposomal amphotericin B has shown particular value in the treatment of visceral leishmaniasis, leveraging the ability to provide high doses to patients with adequate safety, and susceptibility profiles across regional strains have been developed.

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Author contributions

Jill Adler-Moore agreed to write the introduction covering the development of liposomal amphotericin B but passed away before this could be achieved; consequently, Gerard Jensen, an employee of Gilead Sciences Inc., is now the author of this section. All authors contributed to the design of the review, undertook literature research, wrote sections of the review, reviewed the other sections and approved the final version.

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