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The reformulation of Amphotericin B for oral administration to treat systemic fungal infections and visceral leishmaniasis

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Amphotericin B (AmB) is a parenterally administered broad-spectrum antifungal and leishmanicidal drug that has been on the market for over sixty years. Unfortunately, significant infusion-related side effects and renal toxicity often accompany treatment, limiting its clinical applications. Lipid-based formulations have somewhat ameliorated the associated toxicity, but the increased cost of formulations restricts widespread use. AmB is amphipathic and exhibits low solubility and permeability, resulting in negligible absorption when administered orally. Advances in drug delivery systems have overcome some of the solubility issues that prevent oral bioavailability and new formulations are currently in development. The existence of an effective, safe and inexpensive oral formulation of amphotericin B would have significant applications for the treatment of disseminated fungal infections and would dramatically expand access to treatment of visceral leishmaniasis by introducing a readily available highly tolerated oral formulation of a drug with known efficacy.

Keywords: amphotericin B, antifungal, leishmaniasis, oral formulation

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1. Introduction

In the pharmaceutical sector, the repositioning of a previously approved drug has become a more frequent occurrence. With the advent of novel drug delivery systems that provide alternative delivery routes, controlled delivery and focused targeting, an increase in drug efficacy with decreased toxicity profiles is possible. Drugs that exhibit low solubility and/or low permeability are often restricted to parenteral therapy. The reformulation of such drugs for oral administration would significantly lower the overall cost of treatment, improve access to treatment and increase patient satisfaction.

The antiparasitic fungicide Amphotericin B (AmB) has clinical limitations due to its toxicity and poor solubility, necessitating hospitalization and a parenteral route of administration. In developed countries, this results in an elevated risk of complications, cost, and inconvenience for the patient. Over the last decade, advances in healthcare have inadvertently led to an increase in the patient population infected with pathogenic and opportunistic fungi [1]. Transplant recipients, cancer patients and individuals exposed to sustained intensive care therapy all

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exhibit an elevated level of immunosuppression, placing them at greater risk for systemic fungal infections [2].

The use of AmB treatment for parasitic infections is also on the rise. *Leishmania* is a protozoan parasite that is responsible for several disease states, collectively known as leishmaniasis. On the Indian subcontinent alone, over 500,000 individuals are infected with the most severe form of this macrophagic infection, which rapidly infiltrates the vital organs and ultimately leads to severe infection of the visceral reticuloenothelial system (visceral leishmaniasis; VL) [3]. For more than seventy years, the conventional management of VL relied on parenteral administration of pentavalent antimonials. This class of drug is slow to act against VL, and frequently induces adverse toxic effects, including acute pancreatitis and cardiac arrhythmia [4]. In Bihar, India, home to more than half of the world's VL caseload, resistance to pentavalent antimonials has rendered the drug ineffective [5]. Parenteral AmB therapy, which is the most potent antileishmanial agent in clinical use, is now considered the first-line treatment [3]. However, therapy with the first-generation formulation (Fungizone®; Bristol-Myers Squibb, NY, USA) involves IV administration over a period of 30 to 40 days and is associated with infusion and drug-related side effects (infection of the indwelling catheter, patient chills and shaking due to RBC hemolysis, dose-dependent renal toxicity, fever, bone pain, thrombophlebitis). Although lipid-based formulations exist (i.e., Abelcet® [Enzon Inc, NJ, USA] and AmBisome® [Gilead Sciences and Astellas Pharma US, IL, USA]), which require a shorter course of therapy (3 – 5 days), are highly effective and exhibit lower toxicity when compared to Fungizone®, the cost of these formulations is a barrier to widespread use [6,7]. In light of an increasing requirement for AmB therapy, the development of an oral formulation would be of significant benefit to the patient populations in need.

Amphotericin B (AmB; **Figure 1**) is a polyene macrolide antifungal antibiotic produced by *Streptomyces nodosus*, a member of the largest genus of actinobacteria, with over 500 described species. Streptomycetes are gram-positive bacteria and the source of over two-thirds of the clinically useful antibiotics of natural origin. In 1950, the discovery and subsequent development of the antifungal polyene Nystatin prompted a broad screening of Streptomycete cultures for other compounds exhibiting antifungal activity. In January of 1953, culture M 4575, an isolate from soil obtained in the Orinoco basin in Venezuela, yielded considerable antifungal activity [8]. Purification and characterization revealed a new antifungal substance exhibiting substantially greater inhibitory activity than Nystatin. Amphotericin B (AmB) is comprised of a macrolactone ring, one side of which is a rigid nonpolar heptene unit while the other side is a more flexible polar polyol region, conferring amphipathic characteristics on the molecule. A mycosamine group and a carboxyl group located at one end of the molecule are both charged at neutral pH and account for its amphoteric characteristics. AmB is therefore poorly soluble in water, one of the important factors limiting its therapeutic applications [9].

2. Mechanism of action

Early on in the development of polyene antibiotics, it was recognized that ergosterol, a membrane bound sterol compound, was a critical component dictating AmB's efficacy [10]. The monomeric form of AmB damages cell membranes by binding to ergosterol or cholesterol, forming a pore that leads to K^+ leakage and cell death [11]. Ergosterol is found in the membranes of fungal species, as well as some flagellated protozoans, providing an opportunity for drug targeting in a number of disease states. As AmB's affinity for ergosterol is higher than for cholesterol, AmB exhibits selectivity for fungal and leishmanial cells over the cholesterol-containing membranes of the host. However, as AmB accumulates in organs, particularly the kidney, its affinity for cholesterol becomes deleterious to the organism through the destruction of host cells, resulting in moderate to severe toxicity [12].

2.1 Historical review of early oral amphotericin B research

In 1955, the initial *in vivo* studies using an oral AmB solution appeared quite promising [13]. AmB was solubilised in N,N-dimethylacetamide (DMA) and hydrochloric acid, then mixed with water to form a colloidal suspension. Oral dosages of 20 – 40 ug/mouse/day (approximately equivalent to 1 mg/kg), BID for two days resulted in 90 – 100% survivorship of *Candida albicans* infected mice. A second formulation using an oral solution prepared with 0.5% lecithin and both crystalline and amorphous forms of AmB yielded a similar dose dependent efficacy (Dosages of 4, 8, 16, 32 ug/mouse/day BID for two days; for amorphous only – dosage included 65 ug/mouse/day; [13]). In a separate study, AmB formulations were made by suspending known quantities of amorphous AmB in saline supplemented with penicillin and streptomycin at a concentration of 1000 units each per ml. Mice infected with *Coccidioides immitis* were dosed with 12 mg/mouse/day for 23 days via oral gavage. Results indicated a 100% survivorship; however, post mortem cultures indicated that 30% of animals exhibited positive cultures for lung, liver and kidney, and 90% exhibited positive cultures for spleen and omentum, indicating that incomplete eradication of the fungi occurred. In one mouse, no evidence of disease was observed either by gross autopsy examination or by culture [14].

Although the initial results were positive, the development of an oral therapy was not to be. In contrast to the efficacy observed in mice and rats, early clinical trials in humans indicated that excessively large dosages were required to produce any noticeable effect, and significant gastrointestinal side effects were observed. In 1957, the LD50 of IV AmB was established for mice, rabbits, dogs and monkeys using a lyophilized AmB and sodium desoxycholate reconstituted with water or dextrose [15]. Concurrent with these animal studies, the first human clinical trials in patients with systemic fungal disease were undertaken. Four oral formulations of AmB were prepared and administered with maximum daily

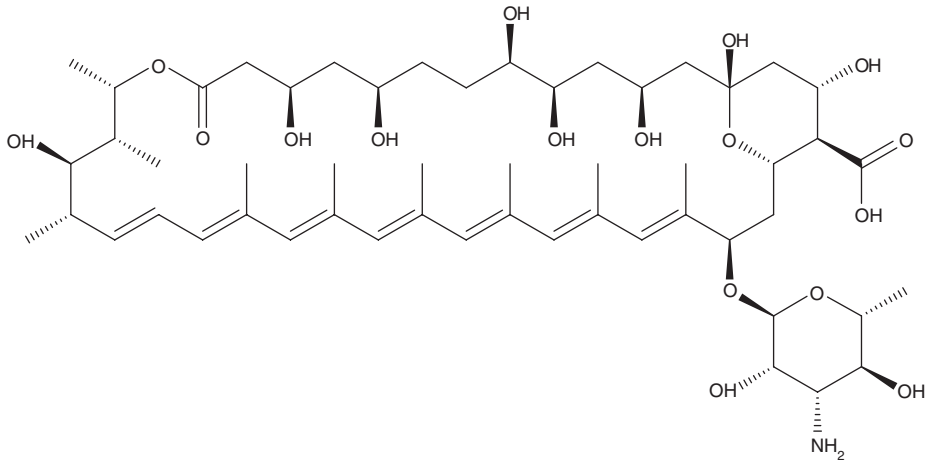

Figure 1. Structure of Amphotericin B. Molecular Weight: 924.079 g/mol. Molecular Formula: C47H73NO17

dosage of 5 g given in four to eight divided doses. The drug was administered for durations ranging from 5 to 350 days, and all formulations were reportedly poorly absorbed and resulted in low blood levels. A patient population with systemic fungal infections treated with IV AmB therapy also did not fare well, although the daily dosage range was low (10 – 60 mg) compared to the current recommended dosage of 0.75 to 1.0 mg/kg [16]. Further studies in man supported the finding that oral preparations were poorly absorbed and failed to produce assayable blood serum levels, therefore the oral formulations appeared to be of no clinical value [17-19]. Subsequent studies have moved forward to examine AmB efficacy when administered intravenously, intra-articularly, intrathecally, intrapulmonary, and intrathoracically and orally have concluded that IV therapy is the most efficacious against a variety of fungal infections and leishmaniasis [17,20,21]. In a review entitled "The Discovery and Development of Amphotericin B", James Dutcher (1968) provided the following summary: "Excitement ran high when the experimental studies showed that the oral administration of amphotericin B protected mice and rats with experimental fungal infections. Alas, the bugaboo of species variation soon reared its ugly head. Experiments with dogs, and eventually humans, showed that there was essentially no absorption from the gastrointestinal tract of these species. Nearly every chemical and physical trick imaginable was explored in an effort to overcome the lack of absorption; but without avail." [22]. With the legacy of failed clinical trials and physicochemical properties that precluded solubility, the possibility of an effective oral AmB was firmly renounced.

3. Challenges of oral formulation development

In the face of significant physicochemical barriers and a collection of failed attempts at oral reformulation, the pervasive attitude toward the impossibility of an oral AmB formulation emerged. In recent years, advances in drug delivery systems and a greater understanding of alternate delivery routes have led scientists to revisit and question such established paradigms. Solubility is a primary factor responsible for the low bioavailability of orally administered drugs; encapsulation in a drug delivery system that has the ability to shield a molecule's unfavorable physicochemical characteristics provides a possible solution. Stability in the acidic gastric environment and protection from enzyme degradation are also key requirements. Once the drug/delivery system complex enters the body, the drug must be released in an active form in order to achieve significant efficacy. The development of a successful oral formulation of AmB must balance the need to increase absorption and protect the acid-labile molecule from destruction in the gastric environment with the requisite release of the drug in a monomeric form at the site of action. As the patient population requiring AmB therapy is increasing, science must rise to meet the challenge.

3.1 Disease state-Leishmaniasis

The benefit of developing an oral AmB therapy is illustrated by the protozoan *Leishmania donovani*, an insidious parasite that is transmitted by the bite of an infected sandfly. The World Health Organization estimates that in India alone, over 500,000 people are infected by this parasite [23]. Visceral leishmaniasis (VL) is the most severe form of the infection; without treatment, this disease is invariably fatal [24]. The therapeutic arsenal against *Leishmania* is limited to a small number of parenterally administered agents. Due to the difficult route of drug administration, toxicity issues and cost, AmB is failing to reach the infected population and mortality continues to rise.

Members of the *Leishmania* genus are obligate intracellular parasites that infect cells of the macrophage-dendritic cell lineage. Leishmaniae spend part of their life cycle in the gut of the sandfly as extracellular flagellated promastigotes, but complete their life cycle in a vertebrate host (**Figure 2**).

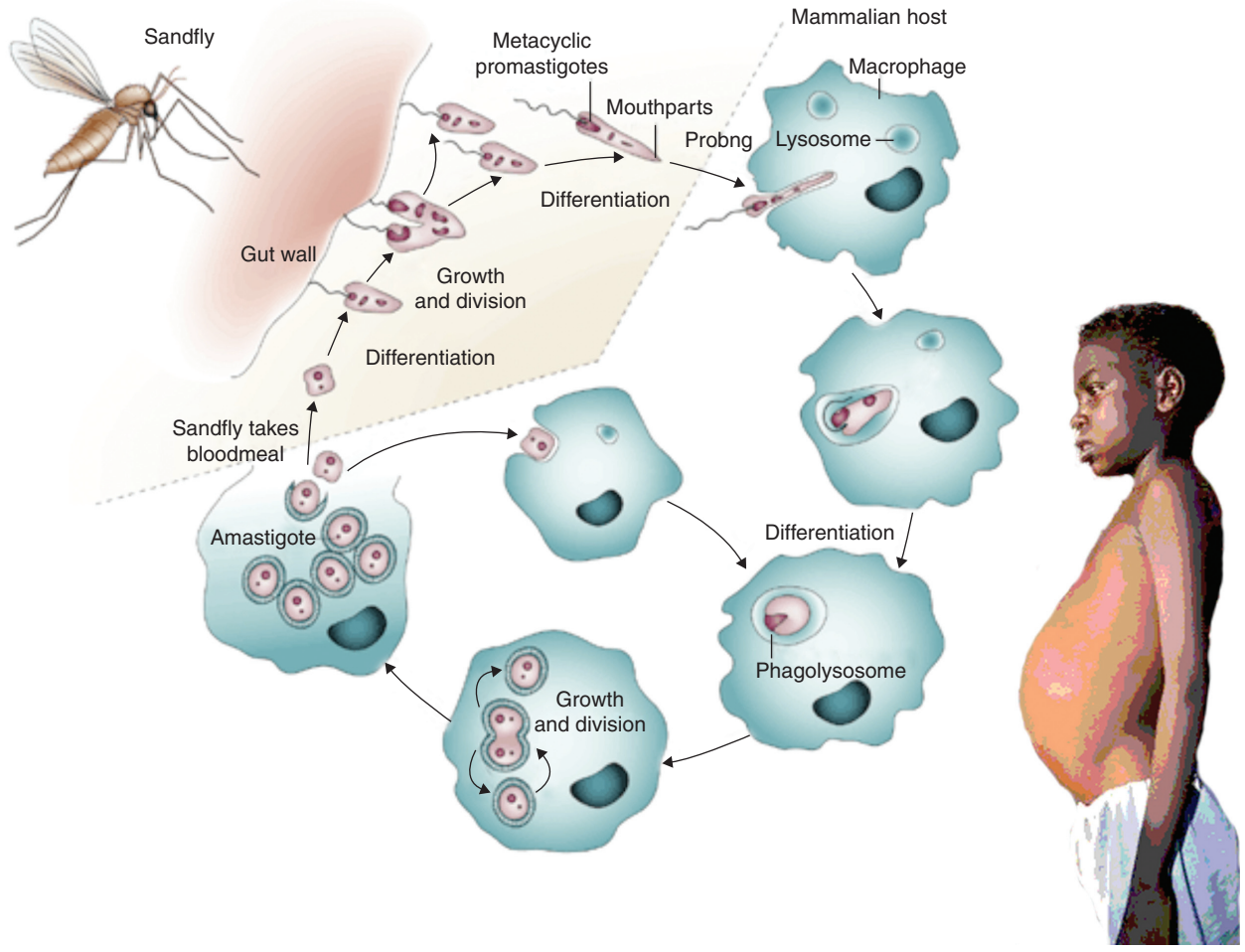

Figure 2. Leishmania parasites are transmitted to the human host via the bite of a female sandfly. A small number of infectious-stage promastigotes enter into the skin and are taken up by the macrophages, where they transform into replicating amastigotes. The macrophages swell and rupture, allowing the parasite to spread to other macrophages and infiltrate the reticuloendothelial system. The cycle continues when a female sandfly feeds on an infected host and ingests amastigotes, which transform rapidly to rapidly dividing non-infectious stage promastigotes. Differentiation into infective non-dividing promastigotes occurs, and repositioning in the alimentary tract prepares the organism for transmission to the next host. Modified from Sacks *et al*, 2002 [25].

The cycle begins when a sandfly feeds on a vertebrate host, infiltrating the wound with saliva containing *Leishmania* promastigotes. These promastigotes are then phagocytosed by macrophages, where they undergo transformation to an amastigote (aflagellar form). The amastigotes multiply through binary fission within the macrophage, causing the cells to rupture and allowing the parasite to spread to other macrophages. The released amastigotes are taken up by additional macrophages and ultimately, all organs are infected, especially the spleen, liver and bone marrow [25]. The cycle begins again when a sandfly feeds on an infected individual, ingesting amastigotes. Almost immediately the amastigotes transform in to the motile, elongated (10 – 20 micrometers), flagellated promastigote form, which migrate to the alimentary tract and infiltrate the salivary glands of the insect [26]. Although the *Leishmania* species differ clinically and biologically, their characteristics overlap and each clinical syndrome can be produced by multiple species [27].

Visceral leishmaniasis is the most severe form of leishmanial infection and involves amastigote dissemination throughout the reticuloendothelial system [28]. Symptoms of affected patients include fever, hepatosplenomegaly, pancytopenia, weakness, and progressive emaciation [29]. Without treatment, the disease is inevitably fatal, but survivors have been observed to develop immunity [30]. Typically, visceral leishmaniasis incubates for weeks to months before becoming clinically apparent and can manifest in immunocompromised patients years after they have left endemic region.

3.1.1 *Parenteral therapies – visceral leishmaniasis*

In the 1940s, sodium antimony gluconate (SAG), was introduced in Bihar, India as a first line drug for the treatment of VL [31].

For the next six decades, parenteral therapy with pentavalent antimonials was considered the gold standard for treatment of leishmaniasis. Two preparations are currently available: sodium stibogluconate (Pentostam; GlaxoSmithKline, Middlesex, UK) for IV therapy and meglumine antimoniate via IM injection (Glucantime; Specia Rhone Poulenc, Paris, France) [32]. However, pentavalent antimonials are slow to act against VL, and frequently induce adverse toxic effects, including acute pancreatitis and cardiac arrhythmia [24]. In addition, misuse of this class of drug has led to substantial antimony resistance in parts of India, especially northern Bihar, the site of more than half of the world's VL caseload [33]. The initial dosage of 10 mg/kg for 6 – 10 days has been subjected to successive upward revisions, leading to a 10-fold increase in drug dosage for current therapies [3]. Widespread resistance has led to a significant reduction in the efficacy and use of pentavalent antimonials [5].

Amphotericin B (AmB) and pentamidine were considered second line to the antimony compounds for VL, primarily based on the toxicity associated with systemic administration of these drugs. Pentamidine, an aromatic diamidine, initially proved to be useful in areas of antimony-resistant VL, but is limited by significant side effects and cost. Pentamidine frequently induces nephrotoxicity and/or life-threatening hyperkalemia [34]. In addition, a significant number of patients exhibit irreversible insulin dependent diabetes mellitus [5]. The unacceptable toxicity, combined with a decline in efficacy has removed this drug as a treatment option in India [3].

Parenteral AmB therapy, which is the most potent antileishmanial agent in clinical use, is now considered a first-line treatment. AmB exhibits excellent leishmanicidal activity, with positive results for cutaneous, mucocutaneous and visceral leishmaniasis associated with IV courses of AmB deoxycholate (Fungizone®). However, the infusion and drug-related side effects associated with a 30 to 40 day course of therapy are significant and include infection of the indwelling catheter, RBC haemolysis, dose-dependent renal toxicity, fever and bone pain. [35]. The risk of substantial side effects restricts AmB administration to facilities capable of prolonged monitoring and intervention in case of a serious toxic response, thus precluding its use at peripheral health posts. However, lipid-based formulations are quite successful in reducing the well-recognized drawbacks of the traditional deoxycholate formulation [6]. The development of AmB formulations as a lipid complex (Abelcet®, Enzon Pharmaceuticals), a cholesterol dispersion (Amphotec® [Three Rivers Pharmaceuticals, PA, USA], InterMune) and a liposomal AmB (AmBisome®, Gilead Sciences) all resulted in a profound decrease in nephrotoxicity. It is believed that the association of AmB with the lipid drug carrier reduces the interaction of monomeric AmB with cholesterol found in the host membranes. The drug is likely released from the lipid package through the action of phospholipases, which are present on the surface of fungal cells [12,36,37] These enzymes break down the lipid delivery system and allow the monomeric form of AmB to come in contact with the ergosterol-containing fungal membrane. Lipid formulations have significant clinical appeal for the treatment of VL, as they are remarkably well-tolerated and maintain substantial clinical activity, allowing for an increase in dosage and decrease in treatment period [38]. However, despite a 90% price reduction of AmBisome® for use in VL endemic countries negotiated by the World Health Organization, it is still five times the cost of conventional AmB, significantly limiting its use in developing countries [39].

Intramuscular injections of paramomycin have shown promise in addressing the antimony-resistant population in India. An aminoglycoside derived from Streptomycete bacteria, paramomycin was found to exhibit elevated efficacy in resistant cases of VL when compared to the declining drug effect of antimonials [40]. A Phase III trial comparing IM injections of paramomycin (21 mg/kg IM for 21 days) to IV AmB (Fungizone® 1 mg/kg every other day for 30 days) demonstrated similar efficacy, but reported an increase in paramomycin-induced adverse effects when compared to AmB [41].

As the majority of treatment options require at a minimum significant involvement of healthcare personnel and some degree of parenteral treatment ranging from 5 to 30 days, access to treatment in developing countries is somewhat uncertain. Socioeconomic barriers such as the lack of infrastructure required for drug administration, substantial treatment-associated costs and loss of income during infusion therapy also play a significant role, and the populations requiring treatment are unable or unwilling to place themselves into treatment programs, thus continuing the cycle of disease. As an infected individual is an ongoing reservoir for parasites, any hope of diminishing the incidence of infection lies in both effective treatment and prevention regimes. An accessible, affordable and efficacious treatment would provide a possible break in the cycle and a step toward eradication of this deadly disease.

3.1.2 *Oral therapies-visceral leishmaniasis*

A new oral treatment option has recently been approved for use in Columbia, Germany and India for treatment of visceral leishmaniasis. Miltefosine (Impavido®; Aeterna Zentaris, Quebec, Canada), a phosphorylcholine ester was originally developed as a therapeutic agent for breast cancer, but was found to have substantial antileishmanial activity [42-44]. Miltefosine is reasonably well tolerated, with mild to moderate transient GI side effects [45]. A major drawback to treatment is that miltefosine has been shown to be teratogenic in animals, placing limitations on its use in women of childbearing age. The Phase IV trial results indicate substantial efficacy, reporting an intention-to-treat analysis initial cure rate of 93.2%. However, data obtained from a subpopulation at a 6-month follow-up indicated a final cure rate of 81%, suggesting a significant rate of relapse or reinfection occurs [46]. Concerns have been

raised regarding the likelihood of emergence of resistant strains of Leishmania, as the half-life of miltefosine is prolonged (150 – 200 h), and drug resistance is easily induced *in vitro* [47,48]. Responsible administration of miltefosine is paramount, with the implementation of necessary safeguards to protect female patients in child-bearing age, exploration of combination therapy options, and directly observed therapy as a means for optimizing adherence to protect against the development of miltefosine-resistant strains [49]. Miltefosine has recently been launched in the European market for canine leishmaniasis treatment in Cyprus, Greece, Italy, Portugal and Spain. Given the long half-life of the drug and the fact that dogs are never cured parasitologically, this policy may contribute to the emergence of miltefosine-resistant parasites [50].

Sitamaquine is an orally administered 8-aminoquinoline currently in Phase IIb trials for the treatment of VL in India. Pre-clinical and subsequent clinical investigations have demonstrated oral efficacy against *Leishmania donovani* [51]. Side effects include methemoglobinemia and transient renal adverse effects, although it is difficult to definitively attribute the side effects to treatment, as no comparator or placebo arm was used in the Phase II clinical trials [52]. However, as with miltefosine, a sitamaquine-resistant strain of Leishmania has been selected for by stepwise increases in *in vitro* drug pressure, and placing it at risk for antileishmanial resistance *in vivo* [53].

3.2 Disease state – systemic fungal infections

Over the past decade, the introduction of novel immunosuppressive agents, improvements in organ transplant surgical outcomes, and increased incidence of cancer treatments have all contributed to the escalation of the immunocompromised patient population. Within this vulnerable group, systemic fungal infections may account for as many as 30% of deaths [54]. Invasive mycoses now pose the primary infectious challenge in the fields of oncology, hematology and intensive care practice. The rising number of invasive hospital procedures has also led to an increase in opportunities for fungal cells to breach the anatomical barriers that normally provide a first line of defence against infection. Surgeries, indwelling catheters, skin damage via radiation treatment and other invasive therapies all facilitate the entry of fungal cells into the body [1]. In contrast to the escalation of patient numbers, the pharmaceutical arsenal of treatment options against mycotic pathogens remains limited. Unlike bacteria, fungal cells are eukaryotic and share many similar biochemical characteristics with their mammalian host. The similarity in biochemical pathways makes it difficult to exclusively target drug action to the fungal cell without collateral damage. The lack of pharmaceutical options for the treatment of fungal infections reflects the scarcity of unique components in the biochemical pathways of fungi. The systemic fungicides currently in use are broadly classified into three main groups: polyenes (e.g., AmB), azoles (e.g., fluconazole) and most recently, echinocandins (e.g., caspofungin).

3.2.1 *Current therapies – systemic fungal infections*

For decades, the treatment of systemic fungal infections was restricted to intravenous therapy with AmB Fungizone®. Although AmB therapy possesses a broad spectrum of antifungal activity against systemic mycoses, the associated renal toxicity and infusion-related side effects are significant. As with VL therapy, the advent of lipid-based AmB formulations in the form of Ambisome®, Abelcet® and Amphocil/Amphotec® represented an important advance in the management of invasive mycoses.

In the 1980s, a new class of antifungals emerged. Azoles were found to inhibit the cytochrome P450 dependent enzyme lanosterol demethylase (14α-sterol demethylase or P450DM), an enzyme in the sterol biosynthesis pathway that leads from lanosterol to ergosterol. Exposure of fungi to azoles results in depletion of ergosterol and a subsequent disruption of the cell membrane. Lanosterol demethylase is also present in the mammalian cholesterol biosynthetic pathway; however, at required therapeutic concentrations, azoles exhibit a greater affinity for fungal P450DM. Ketoconazole (Nizoral®; Janssen Pharmaceutica, NJ, USA) is an imidazole antifungal agent comprised of five-membered ring structures containing two nitrogen atoms. Ketoconazole is the only member of the imidazole class that is currently used for treatment of systemic infections. Prior to the development of newer, less toxic, and more effective triazole compounds, ketoconazole was widely used for systemic fungal infections. It had now largely been replaced by Fluconazole (Diflucan®; Pfizer Pharmaceuticals, NY, USA), a bis-triazole antifungal agent. As with other triazoles, Fluconazole has five-membered ring structures containing three nitrogen atoms. Both oral and intravenous formulations of fluconazole are available. However, fluconazole has no meaningful activity against *Aspergillus sp*. or most other mould fungi [55,56]. Itraconazole, another triazole antifungal agent, is also available in oral capsule form and intravenous therapy. It has a major advantage over fluconazole in that it exhibits activity against most *Aspergillus* isolates and a subset of fluconazole resistant *Candida* strains [57]. Two second-generation triazole molecules have recently been approved for the treatment of systemic fungal infections. Voriconazole and Posaconazole both exhibit significant oral bioavailability and are efficacious against acute invasive aspergillosis and candidiasis [2,58]. Unfortunately, these second-generation azoles interact with other drugs that are substrates of cytochrome P450 3A4. The majority of patients with systemic fungal infections are on multiple medicines, leading to complications with co-administration. Other therapy-limiting adverse effects of azoles include gastrointestinal symptoms (nausea, vomiting) as well as hepatotoxicity [59].

The echinocandin caspofungin (Cancidas®; Merck Research Laboratories, NJ, USA) is a new semisynthetic lipopeptide drug of the candin family exhibiting fungicidal activity against yeasts and moulds. Caspofungin inhibits the biosynthesis of beta-(1,3) D-glucan, an essential component of fungal

cell wall. As the primary mechanism of other antifungals involves interaction with ergosterol function or biosynthesis, the echinocandins offer an alternate mechanism of action and hold promise for combination therapies [60]. The lack of glucan synthesis enzymes in mammalian tissue makes this an attractive target for antifungal activity, as the risk of collateral damage to the host cells is minimized [58].

Although a number of other options exist for the treatment of systemic fungal infections, limitations in efficacy, unfavorable drug interactions and/or toxicity issues are complicating factors. The development of an effective, safe and inexpensive oral formulation of amphotericin B would have significant applications for the treatment of disseminated fungal infections and would dramatically expand access to treatment of visceral leishmaniasis by introducing a readily available highly tolerated oral formulation of a drug with known efficacy.

4. Recent oral formulations of Amphotericin B

In an attempt to facilitate AmB uptake across the gastrointestinal tract, a nanosuspension of AmB was developed by high-pressure homogenization technique. Nanosuspensions have been shown to adhere to the gastrointestinal mucosa, increasing drug contact time and potentially enhancing uptake [61]. The nanosuspensions were produced by subjecting a 0.4% m/m AmB suspension in an aqueous solution of Tween 80, Pluronic F68 and sodium cholate to high-pressure homogenization. The suspension was then dispersed and re-homogenized 3 times at increasing pressures. The resulting particle size was characterized by photon correlation spectroscopy and laser diffractometry, and deemed to be in the nanometer range [62]. Oral treatment of *L. donovani* infected Balb/c mice indicated that the nanosuspension of AmB showed superior drug uptake and some reduction in parasite numbers when compared to oral administration of micronised AmB, Ambisome® or Fungizone® [62]. Although no curative effect was observed, the study identifies a method of increasing AmB solubility that has potential for further development.

Another attempt at the preparation of AmB nanoparticles was undertaken using a melt dispersion technique to form lipid nanospheres [63]. Methanolic AmB was combined with one of three lipidic mixtures consisting of glyceryl tristearate and/or glyceryl monstearate as the main constituents and 20% w/w egg phosphatidyl choline as a stabilizer. The lipid phase and the aqueous phase were emulsified by heating to 65°C and dispersing the mixture at 1500, 2000, 2500 and 3000 rpm using a homogenizer. The mixture was then probe sonicated for 5 min at a 60W output to form lipid nanospheres. The formulation that resulted in the smallest particle size (0.341 ± 0.15 µm) was a mixture of AmB, glyceryl trisearate and egg phosphatidyl choline, stirred at 2500 rpm for 45 min. AmB serum levels from rats after oral administration of the lipid nanosphere formulations indicated that absorption of the lipid nanosphere formulations was substantially increased over aqueous AmB suspension alone. The greatest absorption correlated with the formulation of the smallest particle size [63]. Although the mechanism of absorption of nanoparticles is not well understood, possible routes of absorption include enterocytic endocytosis, paracellular transport, lymphatic transport, and uptake via Peyer's patches in the small intestine.

Polymeric nanoparticles have been investigated for use as an oral delivery vehicle for a variety of water insoluble drugs. These particles are thought to adsorb to the apical membranes of the enterocyte with a size-dependent uptake of particles influencing drug absorption. AmB nanoparticles were prepared by a nanoprecipitation method. Briefly, AmB was dissolved in DMSO; the co-polymer PLGA was added, followed by Vitamin E-TPGS solution. The resulting solution was stirred at 1200 rpm to form the nanosuspension with a particle size of 165 nm [64]. Although tissue distribution and basic PK parameters were not reported, the AUC for AmB nanoparticles indicates an 8-fold increase over orally administered Fungizone®. The nanoparticle formulation also demonstrated some improvement in toxicity factors when compared to Fungizone®.

An oral cochleate formulation of AmB (Bioral® Amphotericin B; BioDelivery Sciences International (BDSI), NC, USA) has been developed to facilitate absorption and provide protection for AmB from gastric acid degradation. Cochleates are the precipitate that forms as a result of a chemical interaction between negatively charged phosphatidylserine and calcium cations, and were originally investigated as a delivery system in vaccine and gene therapy [65]. The use of cochleates in drug delivery favors hydrophobic or amphipathic molecules that can easily insert into membrane bilayers, therefore AmB represents an ideal model for cochleate preparation. The AmB cochleate formulation is derived from a liposomal suspension in an aqueous two-phase polymer solution. Due to phase separation, the polar molecules undergo differential partitioning. When the two-phase polymer solution is treated with positively charged molecules such as Ca^{2+}, a cochleate precipitate is formed [65]. They have a defined multilayered structure that consists of a continuous spiral sheet of solid lipid bilayer with no internal aqueous compartment. The differential partitioning of the polar molecule results in stable, nontoxic lipid particles of a particle size less than one micron that facilitate systemic delivery and encourage interaction with biological membranes [66]. It is hypothesized that AmB localizes in the rigid lipid bilayers of the cochleate and is therefore protected from degradation in the acidic gastric environment.

The AmB cochleate formulation has been tested in mouse models of both candidiasis [66,67] and aspergillosis [68]. Treatment with cochleate AmB (0.1, 1, 2.5, 5, 10 and 20 mg/kg PO) in the mouse model of candidiasis was well tolerated for the duration of the 15-day trial and resulted in 100% survivorship of the animals. Although a dose-dependent reduction in organ fungal load (measured as Colony Forming Units or CFUs/g tissue) was reported for kidney, spleen and lung after 15 days of treatment, complete eradication was only achieved in the lung (**Figure 3**) [67]. In an immunosuppressed mouse

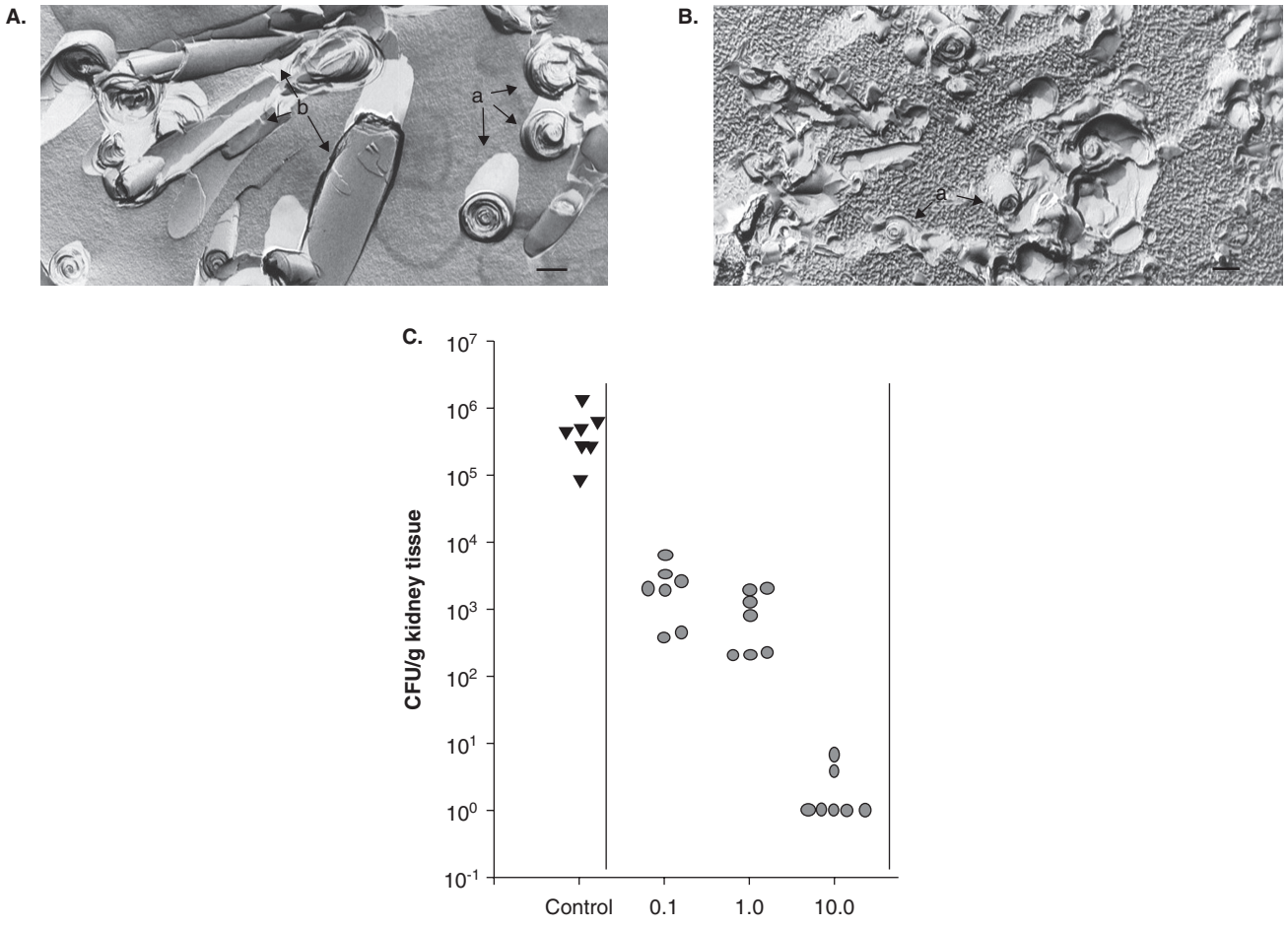

Figure 3. Oral administration of a cochleate AmB formulation for 15 days resulted in a significant decrease in colony-forming units (CFUs) in the kidney of a *Candida albicans*-infected BALB/c mouse model. Trapping film method freeze-fracture electron micrographs of A) empty cochleates and B) cochleate AmB formulation. Arrows indicate rolled-up cochleate structures. Bar = 275 nm; modified from Zarif et al., 2000 [66]. C) Kidney tissue burden of mice infected with *C. albicans* treated with oral gavage of cochleate AmB formulation for 15 days at 5, 10 or 20 mg/kg compared with control (empty cochleate PO). AmBisome® administered orally at a dose of 10 mg/kg was 10-fold less effective relative to the oral cochleate formulation. Data are presented as mean ± SD; n=10 in each group. *P<0.05 vs. untreated control. Modified from Santangelo et al, 2000 [67].

model of aspergillosis, treatment with the cochleate formulation of AmB significantly reduced the level of mortality and decreased fungal tissue burden after 15 days of administration. While the formulation did not achieve the 100% rate of survivability observed in the candidiasis study, the untreated immunosuppressed aspergillosis model experiences 100% mortality by four days post-infection, therefore elevated survivability over the 15 day administration period suggests that cochleates are promising vehicles for the oral delivery of AmB [68]. BDSI released preliminary results of its Phase I clinical Bioral® AmB trial on the company website and identified doses that were well tolerated with no meaningful changes in values associated with renal function. At this time, testing of the cochleate formulation in a VL model has not been explored, but a research collaboration and licensing agreement with the Drugs for Neglected Diseases initiative (DNDi), a not-for-profit foundation focused on the development of drugs for the treatment of neglected communicable diseases, has been announced and evaluation of oral AmB cochleates against VL is expected.

Our laboratory has recently developed a novel lipid-based oral formulation of AmB, (licensed to iCo Therapeutics Inc., Vancouver, BC, Canada) which has significant antifungal and leishmanicidal activity without renal toxicity. This formulation uses proprietary mixture of mono- and diglycerides with phospholipids (all FDA approved excipients) that enhance AmB solubility by 50-fold above its aqueous solubility. The lipid

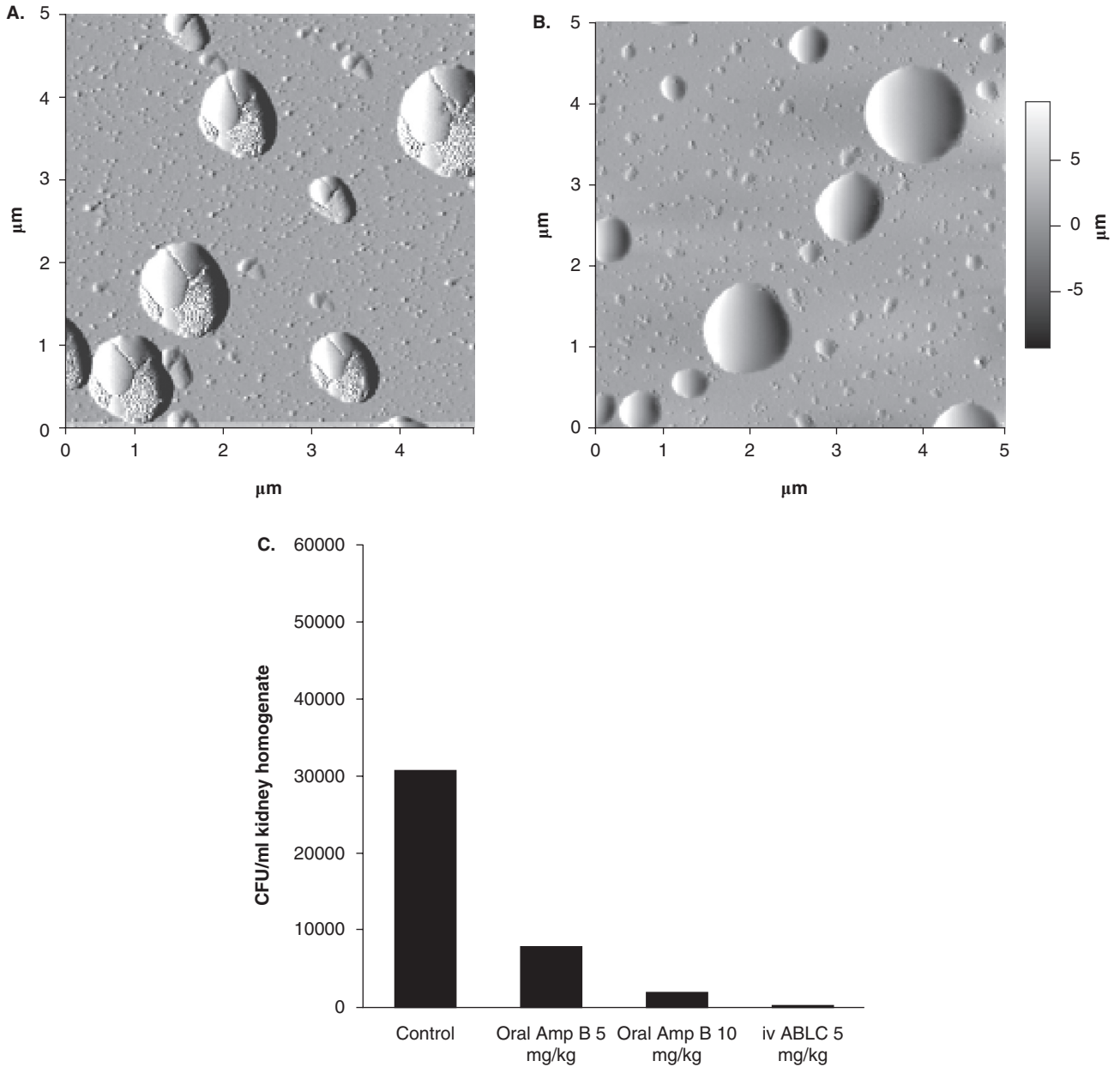

Figure 4. Oral administration of a novel lipid-based AmB formulation for two days significantly reduced the colony-forming units (CFUs) in the kidney of a *Candida albicans*-infected rat model. Atomic force microscopy images of Amphotericin B prepared in A) Peceol alone; B) Peceol/DSPEPEG2000. Droplets applied to the substrate exhibit phase separation of components in A but not in B, demonstrating superior homogeneity of the Peceol/DSPE-PEG2000 formulation. C) Kidney fungal analysis of *Candida albicans*-infected male Sprague Dawley rats treated with oral gavage doses of normal saline (non-treated control; n=9); oral AmB formulation of 5 mg/kg (n=5) or 10 mg/kg (n=7) BID x 2 days; or a single intravenous dose of Abelcet® (ABLC; 5 mg/kg (n=3) once daily x 2 days. Data are presented as mean ± SEM; *P<0.05 vs. untreated control. Modified from Wasan et al, 2009 [70].

nature of the formulation protects the acid labile AmB from destruction in the stomach and thus presents more AmB to the intestinal mucosa for absorption [69]. The lipid-based oral formulation comprised of AmB, DSPE-PEG, monoglycerides and diglycerides and demonstrates a 50-fold increase in solubility over conventional formulations, resulting in sustained plasma concentrations that approximate conventional AmB intravenous therapy (Fungizone®) without the significant drug and infusion-related side effects [70]. The success of the oral AmB formulation is likely due to the ability of the lipid carrier to restrict AmB's interaction with the host cells, yet allow exposure to occur in the presence of the parasite, thus protecting the host from

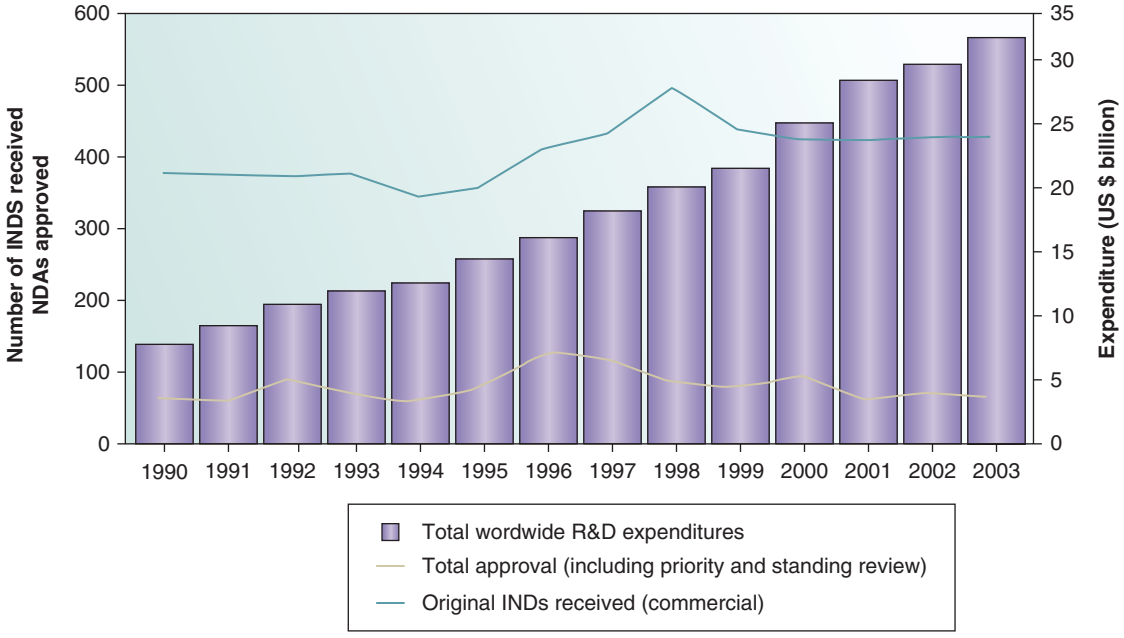

Figure 5. The field of Pharmaceutics has observed an unprecedented increase in R&D spending in the last decade; however, productivity in the form of new drug entities or IND applications has dropped substantially {{209 Ashburn,T.T. 2004;}}.
IND: Investigational new drug; NDA: New drug application.
Modified from Ashburn and Thor 2004 [72].

adverse reaction while maintaining efficacy. AmB's mechanism of action is due to its affinity with ergosterol, a cholesterol analogue found in the cell membranes of fungal and parasitic species. In its monomeric form, AmB associates with ergosterol, forming a disruptive pore or channel in the cell's membrane. Loss of membrane integrity results in cell lysis and forms the basis for AmB's efficacy against both fungal and parasitic infections. However, when monomeric AmB is found in high concentrations, it also has the ability to associate with cholesterol, damaging the cells of the host and resulting in red blood cell hemolysis, thrombophlebitis and renal toxicity.

We hypothesise that the oral AmB formulation is transported into the systemic circulation as a drug-glyceride-phospholipid delivery system. In this form, the monomeric AmB is unavailable for binding with either ergosterol or cholesterol, thus protecting the host from adverse effects (e.g., RBC haemolysis, nephrotoxicity). When the oral AmB delivery system is engulfed by macrophages, intracellular phospholipases are secreted which would break down the lipid carrier, releasing the monomeric AmB [71]. In VL infections, *Leishmania* amastigotes that are reproducing within the macrophage are directly exposed to the monomeric AmB, resulting in membrane disruption and cell death. There is growing evidence that both fungal and *Leishmania* species secrete surface phospholipases, which also serve to release the drug at the site of infection. In the human host, cells do not contain surface phospholipases, thus reducing the likelihood of cell/AmB interaction and decreasing or eliminating toxicity. Further studies are required to lend support to these hypotheses. We have tested our formulation in both fungal [70] (**Figure 4**) and VL disease models (data not shown) and have demonstrated efficacy against *Aspergillus*, *Candidiasis* and *Leishmania sp.* with no significant toxicity.

The ability to administer oral formulations of AmB would have significant impact on the treatment of systemic fungal infections and infectious parasitic diseases (leishmaniasis and possibly other trypanosomic disease states). This drug delivery platform also holds promise for oral formulations of other hard-to-deliver drugs (such as anticancer treatments) through the protection from the effects of the gastric environment and the reduction of toxic profiles.

5. Expert opinion and conclusion

Over the last decade, the development of novel drug therapies has reached an asymptote. In spite of substantial increases in pharma R&D spending and high-throughput's promise of massive returns, drug development has reached an all-time low (**Figure 5**). Drug repositioning (the redevelopment and expansion of previously approved drugs) has become an attractive method for elevating productivity in the pharmaceutical sector [72]. Repositioning requires substantial input from various disciplines, an approach that big pharma has not traditionally embraced. This hesitation to re-examine

and explore additional indications for existing compounds may be partly due to a general reluctance to question the existing paradigm surrounding drug pharmacodynamics and kinetics. However, advances in the field of drug delivery and a greater understanding of the nutritional effects on absorption have fostered the development of novel drug delivery formulations that seemingly fly in the face of traditional biopharmaceutical paradigms. Insoluble acid labile drugs are transported unscathed to the brush border and beyond, poor permeability is overcome, and improvements in drug targeting lead to increased efficacy with a complimentary decrease in toxicity. One prominent example of drug repositioning is the development of an oral formulation of the antifungal agent Amphotericin B.

AmB, the gold standard for treatment of systemic antifungal infections, is a well established, highly efficacious systemic fungicide that has a 50 year history of intravenous (IV) therapy. AmB was originally formulated as a micellar dispersion (Fungizone®; FZ). In the past 15 years, other lipid based formulations (Abelcet®, Amphotec®, Ambisome®) for IV therapy have been developed, yet to date, no oral formulations for systemic use are currently commercially available. The ability to allow patients to self administer treatment for systemic fungal infections would significantly increase the quality of life in developed nations, where the rates of opportunistic fungal infections such as candidiasis, histoplasmosis and aspergillosis are climbing, particularly within patients with cancer, organ transplant recipients, diabetics and HIV/AIDS. It has been widely believed that a formulation of AmB for oral administration is not a viable therapeutic strategy. This was based on the knowledge that AmB had low aqueous solubility (~ 0.1mg/ml), was subject to chemical degradation at acidic pH (i.e., stomach) and due to its large molecular weight (> 900). AmB suffered from both low solubility and low gastrointestinal permeability thus making it seemly impossible to develop a therapeutically acceptable oral drug delivery approach. However, advancement in our understanding of the gastrointestinal tract and in particular how lipids are digested and processed within the GI lumen has led to new approaches for the enhancement of solubility and GI permeability of drugs with the physicochemical characteristics exhibited by AmB.

The shift of an established paradigm is a difficult task in any discipline. When challenged with physicochemical barriers, physiological environment and the hostile acidic milieu of the stomach, the development of an oral therapy is daunting. However, with advances in drug delivery vehicles and methodologies designed to increase solubility and absorption, re-examining current drug therapies has become a feasible option [73]. One approach was recently developed by our laboratory. Our oral AmB formulation uses a novel, proprietary mixture of mono- and diglycerides with phospholipids (all FDA approved excipients) that enhances the solubility of AmB by 50-fold over traditional aqueous formulations [69]. The lipid nature of the formulation protects the acid labile AmB from destruction in the stomach and thus presents more AmB to the intestinal mucosa for absorption. This formulation has significant antifungal activity without renal toxicity. Another oral AmB formulation with underlying cochleate technology is currently in development by Biodelivery Sciences, Inc. (BDSI) and has promising data in mouse models of aspergillosis and candidiasis. A recent press release regarding the favorable outcome of the Phase I trial was released in February 2009 and plans to test the cochleate formulation in a VL model are underway.

So anything is possible! The social and economic impact of an oral AmB formulation is far reaching and has the potential to dramatically increase the delivery of healthcare to the patients who are in greatest need, offering substantial benefits to patients in both the developed and developing world. As science continues to move forward and we learn more about the complexities of the human body, the "repositioning" of old drugs will continue. The drug delivery platforms used to reformulate AmB and the principles used in developing them could be applied to a variety of other compounds, substantially expanding the current pharmaceutical arsenal. We should embrace this approach, as the possibilities appear to be endless.

Declaration of interest

The authors state no conflict of interest and no payment has been received in preparation of this manuscript.

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