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MINIREVIEW

Amphotericin B: Current Understanding of Mechanisms of Action†

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INTRODUCTION

Amphotericin B (AmB) is an important drug widely used to treat serious systemic fungal infections.

Several strategies have been developed over the past few years in an effort to overcome the disadvantages associated with the clinical use of AmB. (i) Alternative antifungal agents have been developed (45). (ii) Derivatives of AmB have been synthesized and studied (27, 28). (iii) The cellular effects of AmB have been exploited to increase the antifungal action of the second agents (38). In particular, the combination of AmB and flucytosine has achieved antifungal synergism and has become clinically useful in the treatment of cryptococcal meningitis (2, 16). (iv) Other investigators have "packaged" AmB into lipid vesicles in an effort to increase antifungal specificity and decrease toxicity. Some of these preparations have been used clinically with promising results (33).

This minireview describes the problems associated with the use of AmB and what is known about the mechanism(s) of its action. The efforts to design a more efficient vehicle for AmB are summarized in the following minireview (8).

PROBLEMS WITH THE USE OF AmB

Toxicity. In spite of its proven track record, there has often been a reluctance to use AmB. The requirement for parenteral administration for long periods is inconvenient, frequently necessitating hospitalization and prolonged intravenous access. An additional problem occurs with fungal infections in severely immunocompromised patients, such as those with the acquired immunodeficiency syndrome; cure is unlikely, and lifelong maintenance therapy may therefore be required to prevent relapse. Clinical use of AmB is also limited by the frequent toxic reactions (35). Nephrotoxicity is ultimately the dose-limiting factor in many patients, particularly when AmB is used in combination with other potentially nephrotoxic agents (aminoglycosides, cyclosporins, etc.), or in situations in which any renal damage is of extreme concern (e.g., in kidney transplant recipients).

Resistance. Even after 30 years of clinical use, reports of fungi resistant to AmB are infrequent (15, 24, 39, 41). However, the true incidence is difficult to determine since susceptibility studies are not routinely done, and there are no standardized test procedures which can be used to compare the results of testing in different laboratories (20).

The mechanism(s) of AmB resistance among fungi varies. The primary target of AmB is ergosterol in the cell membrane of fungi, and to gain access to the membrane AmB must first traverse the rigid cell wall of the fungus which is composed of chitin and β-1,3-glucans. The exact role of the β-1,3-glucans in the cell wall in inhibiting AmB access to ergosterol and in contributing to significant resistance is poorly understood (19).

Most of the AmB-resistant fungi that have been characterized have quantitative or qualitative alterations in the lipid composition of their cell membranes (26, 51). Athar and Winner (1) found that AmB-resistant cells have reduced ergosterol content, whereas Hamilton-Miller (23) isolated resistant mutants of *Candida albicans* with increased ergosterol content. Others have shown that alteration in lipid composition alone may not be sufficient to account for AmB resistance (43). The comparison of the susceptibilities to AmB-induced permeability and killing of a laboratory-derived mutant and a clinical isolate of *C. albicans* led us to conclude that resistance of these strains to oxidation-dependent damage likely contributed to a diminished response to AmB-induced killing (49).

Acquired resistance of isolates of *C. albicans* (defined as resistance to 2 μg of AmB per ml) has been reported (14), but the frequency is very low. The problem has been seen more frequently with *Candida tropicalis*, *Candida parapsilosis*, and *Candida lusitaniae*. Most of these resistant yeasts have been recovered from immunocompromised patients who had received AmB for prolonged periods (15, 17, 39, 41). In a recent study (44), we found that there was a strong association between the in vitro decreased susceptibility to AmB of *Candida* species isolated from severely immunocompromised patients with fungemia and subsequent poor clinical outcome. Yeast isolates resistant to more than 0.8 μg of AmB per ml were associated with a fatal outcome in all cases.

Clinical isolates of *Pseudallescheria boydii* are usually resistant to AmB, with MICs greater than 2 μg/ml; infections with this organism frequently do not respond to AmB, and the azoles may be the agents of choice in treatment (34). The MICs of AmB for *Malassezia furfur*, a lipophilic yeastlike organism associated with catheter tip sepsis, fall between 0.3 and 2.5 μg/ml (36). It has been reported that some strains of *Trichosporon* may be susceptible to AmB and others may have partial or complete resistance (E. Haron, E. Anaissie, A. Espinel-Ingroff, K. Rolston, D. H. Ho, and G. P. Bodey, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1525, 1988). Both resistant and susceptible organisms of various clinically relevant species of *Fusarium* have been reported, and the results of in vitro susceptibility tests on *Fusarium chlamydosoporum* may depend on the test conditions used (29). Such studies further emphasize that susceptibility can be influenced greatly by the test used and indicate the need for standardized testing

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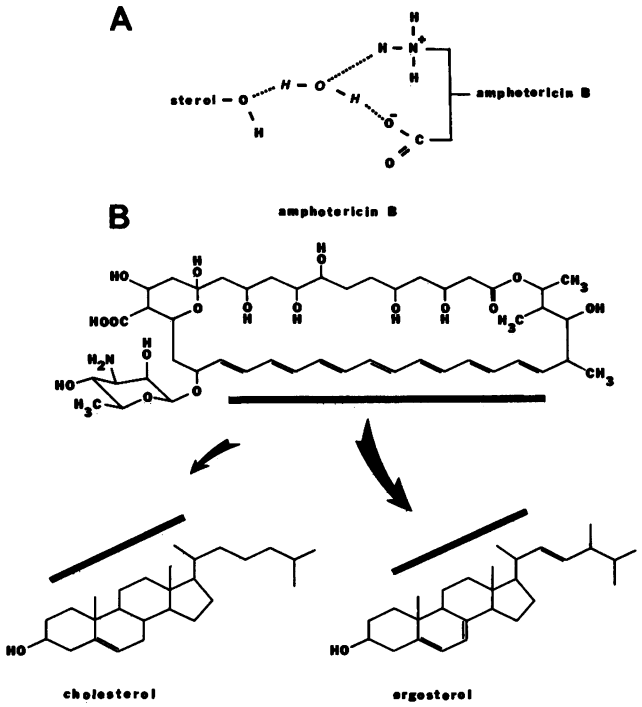

FIG. 1. Schematic representation of hydrogen bond formation (25) (A) and nonspecific interactions between AmB and sterols (B).

procedures for fungi and comparisons of the results with relevant in vivo models.

MOLECULAR BASIS OF STEROL SPECIFICITY OF POLYENES

It is generally accepted that the damaging action of AmB to cells originates from its binding to sterols incorporated in cellular membranes: ergosterol in the case of fungal cells and cholesterol in mammalian cells. More avid binding of AmB to ergosterol than to cholesterol (22) and to ergosterol-containing membranes than to cholesterol-containing membranes (54) has been demonstrated by spectrophotometry.

Although some studies question the role of sterol in the effects of AmB on artificial membranes (40) and no simple relationship between the binding and biological activity of AmB has been found (52), the assumption that the basis of the clinical usefulness of AmB is its greater avidity for ergosterol-containing membranes than for cholesterol-containing membranes most adequately fits the results of the studies found in the literature and discussed in this minireview.

Figure 1 presents two kinds of binding of AmB to ergosterol or cholesterol. Figure 1A (drawn according to a model in reference 25) shows how AmB and sterols, with the participation of H_2O, may form a "cage" resulting from hydrogen bonds. These bonds are regulated by proton donor-acceptor forces (specific forces). The functional groups involved in the hydrogen bonds are the hydroxyl groups of the sterols and the carboxyl group at C-18 of the AmB molecule. This binding is strengthened by participation of the amino group of the amino sugar. Both ergosterol and cholesterol are 3-β-hydroxy sterols, and it can be assumed that their reactions with AmB involving hydrogen bonds are equivalent.

The second type of interaction, involving the rigid chain of seven conjugated double bonds of AmB and the whole sterol molecule, is governed by van der Waals forces (nonspecific forces). This interaction is schematically indicated in Fig. 1B. Hervé and co-workers (25) concluded, on the basis of experiments comparing effects of polyenes on the permeability of liposomes containing different sterols, that the alkyl side chain of ergosterol with the double bond located at C-22 was responsible for the greater sensitivity to AmB of ergosterol-containing membranes compared with sensitivity to AmB of cholesterol-containing membranes. Conformational analysis of ergosterol and cholesterol was recently performed, and it was shown that the overall shape of most ergosterol conformers is flat. In contrast, a flat shape is only one of the possible conformations of cholesterol because the cholesterol side chain without the double bond at C-22 is more flexible (1a). The flat shape of the ergosterol molecule may facilitate intermolecular contacts with the polyene macrolide.

Therefore, it appears that van der Waals forces are decisive for the specificity of AmB for ergosterol, whose conformational state is most favorable for this kind of interaction. Recently, Góralski et al. (P. Góralski, J. Brajtburg, and M. Tkanyk, submitted for publication) noted the importance of nonspecific (van der Waals) forces compared with specific forces (hydrogen bonds) in cholesterol interactions by calorimetric measurements of the heat of dissolution of cholesterol in simple chemical compounds used as solvents.

It is reasonable to assume that other heptaene antibiotics with a free carboxyl and amino group would interact with sterols in a fashion similar to that of AmB. For example, vacidin A (Fig. 2), an aromatic heptaene antibiotic, induced in lipid vesicles the same type of permeability to cations as

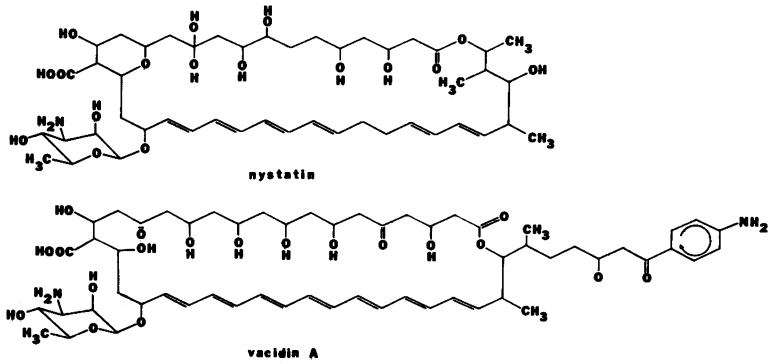

FIG. 2. Structures of polyenes.

AmB (12, 25). The hemolytic activities of AmB and vacidin A on erythrocytes were also similar (13). In another study, fungi were more susceptible than erythrocytes to the toxic effects of several other heptaene antibiotics (30). One can therefore assume that heptaene antibiotics are good candidates for further investigations on clinical utility.

An alternative method of improving the therapeutic index of AmB is to use semisynthetic derivatives of the drug. According to the hypothetical formation of the cage (shown in Fig. 1A) by the hydroxyl group of the sterol and carboxyl and amino groups of AmB, hydrogen bonding between sterols and heptaenes with an esterified carboxyl group would occur under less favorable conditions than with the parent antibiotic because of lack of the hydrogen bond between the hydroxyl group of the sterol and the carboxyl group of the polyene. Therefore, under these circumstances, the difference in binding to sterols through nonspecific forces would be accentuated. Hervé et al (25) showed that semisynthetic derivatives of AmB and vacidin A with esterified carboxyl groups differed more than the parent compounds in the extent of permeability induced in vesicles containing ergosterol compared with that in vesicles containing cholesterol. This observation on artificial membranes correlated with the cellular data which demonstrated a substantial decrease in toxic effects of the methyl ester derivative of AmB (AmE) compared with those of AmB on mammalian cells, with an insignificant decrease in toxicity to fungal cells (11). This decreased toxicity to mammalian cells suggested that AmE might be useful clinically (4), and limited trials were done (27, 42). However, an unanticipated neurotoxicity has been attributed to AmE and its *N*-aminoacyl derivative, necessitating further investigation (18, 27).

According to the model discussed above (Fig. 1), the amino group of the polyene also participates in the formation of the hydrogen bond between the polyene and the sterol. When polyene effects on ionic permeability of large unilamellar vesicles were compared (11, 25), the acylation of the amino group did not affect the sterol selectivity of the derivative but resulted in an increase in the polyene concentration which was necessary to induce permeability. Consistent with these results, the acylation of the amino group in AmB resulted in decreased activity against both fungal cells and erythrocytes (11, 30).

Nystatin is structurally very similar to AmB, except that one of the conjugated double bonds is saturated. It is therefore a diene-tetraene combination (Fig. 2). The carboxyl and amino groups of AmB and nystatin are in the same position, and one should expect that the hydrogen bond between nystatin and sterols would be formed with the same strength as the AmB-sterol hydrogen bonds. In contrast, the nonspecific binding to sterol of a polyene with a chain composed of four and two double bonds can be expected to be weaker than the binding of a polyene with a chain of seven conjugated bonds. Consistent with this reasoning, it was observed that nystatin resembles AmB in the dose-dependent separation of its permeabilizing and lethal effects. However, nystatin differs greatly from AmB in relative potency toward fungal and mammalian cells; nystatin is as potent as or less potent than AmB in inducing K^+ leakage from yeast cells but much less potent in inducing K^+ leakage from erythrocytes (30). These results suggest that the decreased ability to bind nonspecifically to sterols impairs the interaction with cholesterol (the weaker partner) more than that with ergosterol (the stronger partner). Because of this selectivity in action, despite its decrease in antifungal effects compared with those of AmB, nystatin deserves attention as a candidate for further clinical studies on utility for therapy for systemic fungal infections.

Filipin is a pentaene antibiotic which does not contain a carboxyl group or mycosamine sugar moiety. The experiments indicating that the affinity of filipin to cholesterol is stronger than to ergosterol have been summarized by Bolard (3). Etruscomycin is a tetraene antibiotic which contains a carboxyl group and a mycosamine sugar moiety. In contrast to filipin, its binding to sterols is governed by two types of forces. Capuozzo and Bolard (10) demonstrated that etruscomycin, at a low antibiotic-lipid ratio, was more strongly bound to ergosterol-containing vesicles than to cholesterol-containing vesicles whereas at higher ratios the selectivity of binding was different.

On the basis of the evidence that is available, we predict that nonheptaenes which do not bind preferentially to ergosterol are not good candidates for further evaluation as systemic antifungal agents.

OXIDATION-DEPENDENT EVENTS IN STIMULATORY AND LYTIC OR LETHAL EFFECTS OF AmB ON CELLS

The binding of AmB to sterols incorporated in artificial or cellular membranes results in disorganization of the membrane (for a review, see reference 3), possibly by formation of specific pores composed of small aggregates of AmB and sterol (53). These defects cause depolarization of the membrane and an increase in membrane permeability to protons and monovalent cations.

The cellular effects of AmB are complex and depend on a variety of factors, such as the growth phase of the cells, dose, and mode of AmB administration (e.g., one or fractionated doses; 48). We have proposed that the effects of AmB on cultured L cells can be divided into separate dose-dependent stages: stimulation, permeabilization, and lethality (6). The stimulatory effect was observed at concentrations of AmB lower than those required for induction of permeability changes, and for this reason it could not be associated with changes in cell membrane permeability. The lethal effect occurred at AmB concentrations higher than those inducing increased cell membrane permeability. Our data and those from other laboratories have suggested that lethality is not a simple consequence of changes in permeability of cell membranes.

There are several reports indicating that oxidation-dependent events are involved in AmB-induced stimulation of cells of the immune system. AmB markedly augmented the polymorphonuclear leukocyte immunoglobulin G-mediated ingestion of opsonized sheep erythrocytes; this effect was inhibited by superoxide dismutase. Additionally, catalase inhibited AmB-stimulated phagocytosis of sheep erythrocytes (21). Catalase also inhibited, in a dose-dependent manner, polyclonal B-cell activation induced by AmE in cultures of spleen cells from AKR mice (50).

The evidence for the role of active oxygen species in the lytic or lethal actions of AmB was obtained from experiments which showed that AmB injury to cells could be modulated by extracellular scavengers, hypoxia, or prooxidants. In these experiments, hemolysis of erythrocytes (7) and killing of *C. albicans* or lysis of protoplasts caused by AmB could be inhibited by extracellular catalase (47) and potentiated in the presence of ascorbic acid and other prooxidants (5a). In addition, exposure of erythrocytes (7) or protoplasts of *C. albicans* (47) to AmB under hypoxic conditions reduced AmB-induced lysis compared with incubation in air. In contrast, extracellular scavengers, hypoxia,

and prooxidants did not affect prelytic or prelethal AmB-induced K^+ leakage from cells.

In vivo administration of AmB produced an increase in the ratio of oxidized to nonoxidized glutathione in lung tissue and in the plasma of animals and also in isolated perfused lungs. The increase in this ratio in tissues taken from perfused lungs was attenuated by addition of catalase or 1-phenyl-3-pyrazolidinone (Phenidone), a scavenger of oxygen radicals (37).

If AmB-induced cell damage is linked to the generation of reactive forms of oxygen, the ability to decompose them should affect cell resistance to damage. Two observations support this idea. Erythrocytes from AKR mice with higher levels of catalase activity were less sensitive to lysis by AmB than erythrocytes from C57BL/6 mice which had lower levels of catalase activity. These in vitro results correlated very well with the in vivo characterization. AKR mice with higher intracellular catalase levels were more resistant to the toxic effects of AmB than were C57BL/6 mice (5). Cultured HL-60 cells, whose levels of glutathione were lowered by incubation with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, were more sensitive to AmB-induced lysis than were control HL-60 cells (55).

The stimulatory effect of AmB on any cell type is biphasic. For example, the initial concentration-dependent increases in production of DNA or RNA by L cells (6) or oxidative bursts in macrophages (32) were followed by a decrease below the control level as the AmB concentrations were increased. It is obvious that stimulatory effects cannot be manifested by seriously damaged cells. This reasoning is supported by evidence that AmB stimulated a stronger respiratory burst in macrophages from AKR mice than in C57BL/6 mouse macrophages (which are less resistant to AmB damage). The implication of these results is that it should be possible to affect the stimulatory cell response by modulating the endogenous level of cell resistance to oxidative stress. There is some support for this hypothesis. AmE-induced stimulation of polyclonal B-cell activation in AKR mouse spleen cells was abolished when these cells were incubated with aminotriazol, a selective catalase inhibitor. The explanation proposed was that the aminotriazol induced a decrease in cellular catalase levels and made cells more sensitive to AmB-induced toxicity (50).

What is the mechanism of the AmB-induced oxidative events? The literature suggests two processes by which AmB could affect cells. The first is auto-oxidation of AmB and the formation of free radicals (31). The free radicals formed in this process may have several different effects on cells, among them stimulation. Stimulation, as well as other effects, may thus originate from the auto-oxidation of AmB bound to membrane components. In this case, oxidative events are not linked to membrane permeability and are not dependent on the kind of sterol. The second mode of action may result from AmB-induced increase of membrane permeability, especially to monovalent cations. This would be dependent on sterol structure (ergosterol-containing membranes are more sensitive than cholesterol-containing membranes). Some unpublished results from our laboratory suggest that there is a connection between the ionophoric and oxidative effects of AmB.

In addition, it has been demonstrated in isolated perfused rat kidneys that lethal cell injury induced by AmB depends on transport activity, which is associated with a rise in oxygen demand (9). In this case, AmB-induced cell injury might be associated with both ion movement and oxidative effects.

IMPROVING THERAPY BY UNDERSTANDING HOW POLYENES WORK

Improved therapy for certain fungal infections (*Cryptococcus neoformans*, *C. albicans*) has been achieved by combining AmB with flucytosine. The rationale for the clinical use of this drug combination was based on the observations that AmB potentiated the uptake and effects of flucytosine by increasing fungal cell membrane permeability (38).

We have shown that the lethal effects of AmB on fungi mediated by oxidation-dependent events can be potentiated by certain prooxidants. If the enhancement of antifungal effects by combining AmB with prooxidants occurs in vivo in animal models of infection, these kinds of drug combinations represent promising new antifungal regimens for clinical studies.

It should also be possible to augment the stimulatory effects of polyenes on cells by using derivatives with decreased toxicity. AmB derivatives without a free amino group are less toxic to cells than AmB (see above). Thus, an N-substituted derivative of AmB should have stimulatory action which would not be limited by toxicity. One nontoxic derivative of AmB, *N*-thiopropionyl AmB, induced a strong proliferative response of murine B cells under conditions in which AmB was weakly efficient or toxic (46). Another derivative, *N*-(1-deoxy-D-fructose-1-yl)AmB, which was less toxic toward murine thymocytes than AmB, was a potent immunostimulator (24a). These or similar derivatives should be tested for immunoadjuvant properties in animals and also for effects on host response to infection, particularly when used in combination with other drugs which have direct antimicrobial properties.

CONCLUDING REMARKS

In this review, we have tried to develop a perspective in evaluating the present and future role of AmB in the treatment of systemic fungal infections. Despite the many problems associated with its use, AmB remains a very effective agent. Recent insights into the basis of its specificity for fungi relative to that for host cells may lead to strategies for improving its efficacy. Molecular alterations which do not disturb this specificity or even increase it will be useful to pursue. Based on the model of AmB action, one can conclude that other polyenes with seven double bonds (heptaenes) are likely to be clinically useful and should be extensively studied. The evidence for involvement of oxidation-dependent events in the stimulatory and lethal effects of AmB opens up new areas to pursue to improve therapy. All of these efforts involve strategies which will potentiate the effects of these agents against fungi and/or protect the host from their toxic effects. In the second part of this minireview (8), we describe how alternative delivery systems for AmB might also achieve these goals.

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