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MINIREVIEW

In Vitro Models for Studying Toxicity of Antifungal Agents

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Systemic fungal infections are an important cause of morbidity and mortality among immunocompromised patients (57, 58). Amphotericin B (AmB) is the "gold standard" for their treatment (19), but triazole compounds, such as fluconazole or itraconazole, are less toxic than AmB and have proven to be effective in several types of infections (13, 36, 47, 49). AmB was recently accorded renewed interest because of the use of liposomal formulations. Despite these improvements, some mycoses, such as invasive apergillosis in neutropenic patients (15) and bone marrow transplant recipients (12), remain a real therapeutic problem.

The in vivo toxicity of antifungal agents results partly from direct interactions with mammalian cells, since most of these agents have the same type of effect on mammalian and fungal cells. Therefore, the use of antifungal drugs as therapeutic agents relies on the extent of selectivity of these effects against fungi. In vitro studies of the toxicity of these compounds against mammalian cells help us better understand the mechanisms involved in in vivo toxicity and compare the deleterious effects of new antifungal agents or new formulations of a defined compound.

We review herein the contribution of in vitro models to the study of the toxicity of polyenes, imidazoles, and triazoles in mammals.

POLYENES

Polyene antibiotics are produced by several different species of *Streptomyces* and have in common a macrocyclic lactone ring with a series of conjugated double bonds. About 100 polyene antibiotics have been described; they differ in the number of double bonds in the molecule and the substituents on the ring. AmB is the only polyene available for systemic administration, but its use is limited by systemic side effects (fever, chills) and renal toxicity. Indeed, its nephrotoxicity often limits the duration of therapy.

All polyenes alter the membrane permeability of eukaryotic cells and lead to cell lysis (16). Although some studies question the role of sterol in the effects of AmB, especially on artificial membranes, there is an extensive amount of data supporting the sterol hypothesis: it is generally admitted that cell sensitivity to polyene antibiotics is determined by the presence of sterol in the membrane and that the greater activity of AmB against fungi than against mammalian cells results from the drug's greater avidity for ergosterol-containing membranes than for cholesterol-containing membranes (52, 53).

In vitro models of toxicity might help elucidate the still-debated relationship between permeability alterations and cell death (for a review, see reference 3) and might also help in the study of other parameters that might account for the toxicity of polyenes. Comparison of the cellular effects of AmB with those of other polyenes with different properties (size, solubility) may reveal some of the mechanisms responsible for the antibiotic's cellular toxicity.

Models. The effects of polyenes on membrane properties can be studied with artificial membranes or intact cells.

(i) **Artificial membranes.** The model of phospholipid vesicles has been the most commonly used over the last few years. This model allows evaluation of the functional leakage of encapsulated components and measurement of AmB binding by using spectroscopic methods, such as circular dichroism (that is, the difference between the absorption of left and right circularly polarized light), which is highly sensitive to conformational alterations of the drug resulting from environmental changes (28). Large unilamellar vesicles may be preferred to other vesicles for cell membrane models, since such vesicles have the highest membrane curvature. Membrane composition as regards phospholipid properties (transition temperature, charge, length) and the presence or absence of sterol can be controlled. Therefore, vesicles enable a comparison of AmB binding and side effects with or without the presence of sterol.

(ii) **Intact cells.** Experiments performed on intact cells enable analysis of the role of membrane permeability alterations in cell mortality, study of the effects of AmB on enzyme activities, and investigation of the changes resulting from AmB binding that are not related to membrane permeability modifications. The cells used vary from model to model, from erythrocytes (25, 27) or macrophages (48), with which simple and nonspecific parameters can be evaluated, to more complex systems, such as renal tubular cells in culture (23, 31), or immunocompetent cells (2, 46), with which the actions of polyenes on more-specific functions can be assessed.

Although hemolysis does not complicate the treatment of patients with AmB, the nature of the interaction of erythrocytes with polyene antibiotics has been widely addressed. This is because these cells provide a handy tool for many reasons: they are readily available, their intracellular metabolism is reduced, their membrane properties are well known, and cell lysis is easy to monitor. On the other hand, renal tubular cells in culture are representative of the target of AmB toxicity in vivo. Proximal tubular cells, derived from cell lines (31) or in primary culture (23), retain specific functions, such as sodium-dependent uptake of phosphate, amino acid, and hexose, which enable specific investigation of the early toxic effects of polyene antibiotics on the functional activity of these cells.

AmB binding to membranes. (i) **Methods.** AmB binding to membranes is difficult to determine because this polyene is not fluorescent and no radiolabeled compound retaining the

biophysical properties of AmB is available. Experimental procedures used to separate free from membrane-bound AmB are limited by the cosedimentation of free-drug aggregates and the membrane-bound drug during centrifugation. So far, other methods, such as equilibrium dialysis and gel filtration, have been unsuccessful because the drug binds to the dialysis membrane and the gel.

However, AmB binding to model membranes can be assessed spectroscopically, for example, by electronic absorption or circular dichroism, because of the strong extinction coefficient of the drug (ε = 150,000 for monomeric AmB in water) and the fact that AmB absorbs at 400 nm, a wavelength far from the absorption spectra of most cellular components. Because they provide information for low concentrations of the antibiotic in the range of 10^{-7} M, at which in vitro activity begins to be observed, these spectroscopic methods are particularly useful. Circular dichroism is useful because, for instance, when the drug is bound to membranes, a reduction of the intensity of the dichroic signal due to the reduction of self-associated free drug is observed, thereby enabling the percentages of free and bound drug to be calculated. Isolated cell membranes (e.g., erythrocyte ghosts) may be preferred to intact cells to avoid the problem of light scattering when AmB-membrane interaction is measured.

More recently, AmB binding to intact cell membrane has been studied by using the fluorescent compound 1-[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH). TMA-DPH fluoresces when it is incorporated into the cell's plasma membrane, whereas the probe's fluorescence in the aqueous medium is negligible (38). Since the absorption spectrum of AmB and the emission spectrum of TMA-DPH overlap, AmB binding to the membrane is responsible for the extinction of the probe's fluorescence. The amount of polyene bound can be calculated from the experimental energy transfer efficiency between the two molecules in the membrane. This method has been used to measure the binding of AmB and *N*-fructosyl-AmB to murine thymocytes (17) and renal tubular cells (24).

(iii) Results. Many studies have demonstrated that the binding of AmB to membranes parallels the occurrence of changes in membrane permeability. Circular dichroism revealed that AmB bound to membranes can adopt many different conformations depending on its concentration, the drug/lipid concentration ratio, the amount and nature of membrane sterol, the physical nature (gel or liquid state) of the phospholipids, and the length of incubation (for a review, see reference 3).

In the field of toxicity, i.e., interactions between AmB and mammalian cell membrane, few data concerning the role of cholesterol are available, and some of those available are contradictory. The cholesterol content was low in AmB-resistant cell lines isolated from cultured Chinese hamster cells (18), but the AmB sensitivity of four cultured cell lines did not correlate with the level of cholesterol in their membranes (14).

We demonstrated that cholesterol mediated the binding and toxicity of AmB against renal tubular cells in primary culture (24). In that study, AmB toxicity was assessed on the basis of inhibition of Na^+-dependent uptake of phosphate secondary to the increased membrane permeability to monovalent cations. Incubation of cells with ketoconazole, an inhibitor of membrane cholesterol synthesis, reduced by more than 80% the cellular cholesterol content as well as the membrane binding and cell toxicity of AmB. AmB binding and toxicity were restored in the presence of exogenous exchangeable cholesterol, thereby confirming that the protective effect of ketoconazole was due to cholesterol depletion of the membrane. These results show that cholesterol increased the binding of AmB to tubular cell membrane and that early AmB toxicity paralleled the binding of AmB to membrane. However, when the cells were extensively depleted of cholesterol, AmB binding and toxicity were reduced by only 50%, in agreement with data obtained on phospholipid vesicles, which indicated that AmB can bind to sterol-free membranes.

The mechanisms by which cholesterol influences AmB binding remain to be elucidated, and it should be emphasized that there is no proof of the existence of cholesterol-AmB complexes in membranes, at least in the low concentration range sufficient for the onset of toxicity. Cholesterol could alternatively modify membrane fluidity and thereby allow better insertion of the drug (4) into the membrane.

The study of AmB binding to cell membranes allows us to understand the first step involved in AmB toxicity. Although it has been shown that AmB binding to fungi is clinically relevant, as demonstrated by the existence of strains resistant to AmB which exhibit a low sterol content, no relationship between AmB binding to mammalian cells and in vivo toxicity has been reported. However, the avidity of the drug for cell membrane, as seen in vitro, might be responsible for its strong binding to tissue samples obtained from patients (11).

Toxic effects of AmB and other polyenes. (i) Induction of permeability to monovalent cations. Intact cells release K^+ following a short exposure to low doses of polyene antibiotics. K^+ leakage is easy to measure, and this method enables determination of the sensitivities of different cell types to the drug's toxicity (3). For limited concentrations of AmB, a repair mechanism ensures that the initial intracellular K^+ level is more or less restored (1, 7), probably through disruption of the channels and reactivation of Na^+/K^+ ATPase. The permeabilizing effects of AmB and its methylester derivative have also been demonstrated with brush border membrane vesicles (9).

Using artificial membranes, it has been shown that the permeability induced by AmB depends on the phospholipid fluidity. Most studies have used phospholipid in the liquid crystalline state, which corresponds to their in vivo conformation in the plasma membrane of intact cells. In this case, AmB binding increased membrane permeability, first, selectively, to monovalent cations. When the AmB/lipid concentration ratio increased, ion selectivity was much weaker and leakage of divalent cations was observed. For an equivalent amount of AmB, the leakage was greater when sterol was present in the membrane (52). When the phospholipids were in the gel state, sterols did not increase the permeabilizing effects of AmB.

A proposed model for AmB-sterol interactions is based on a comparison of dose-response curves for induction of K^+ release from cholesterol- and ergosterol-containing vesicles on the one hand and from erythrocytes and *Saccharomyces cerevisiae* cells on the other (4). This model takes into account the fact that AmB in an aqueous medium is a mixture of different forms in equilibrium: monomers, water-soluble oligomers, and water-insoluble aggregates. Only self-associated species (oligomers or aggregates) seem to be active on any type of membrane, causing K^+ leakage. In contrast, the monomers do not seem to form channels in erythrocytes and cholesterol-containing vesicles, whereas they do so in fungi and ergosterol-containing vesicles, probably by binding directly to ergosterol. AmB selectivity for

fungal cells might result from this difference, and this mechanism might account for the lower toxicity of formulations containing low concentrations of self-associated AmB, such as liposomal preparations, than that of free AmB or the deoxycholate formulation (Fungizone). This study shows that appropriate models of artificial membranes are useful in predicting the alterations in membrane permeability induced by AmB on whole fungal or mammalian cells.

(ii) Alteration of membrane-related functions. AmB toxicity in LLC-PK1 cells, a kidney cell line with properties of proximal cells (31) and proximal tubular cells in primary culture (23), has been studied. In both cases, alterations in membrane integrity had important consequences for cell functional activity at the membrane level. Nonspecific toxicity, expressed as lactate dehydrogenase release, cell detachments from plastic, or protein loss, was described after acute exposure (1 or 2 h) to AmB concentrations of about 40 μM. Acute exposure to lower concentrations of the drug inhibited Na^+-dependent uptakes of phosphate and hexose in the absence of cell lysis. This alteration of Na^+-dependent uptakes, which were inversely correlated to K^+ release, might result from an alteration of the sodium gradient due to an increase in membrane permeability to monovalent cations and/or the concomitant inhibition of the Na^+/K^+ ATPase, as previously described for erythrocytes (55).

The alterations of membrane-associated tubular functions seen in vitro can be related to the urinary electrolyte loss observed in vivo. Since proximal tubular cells are a target of AmB toxicity in vivo (10, 22), this model appeared appropriate for studying the drug's renal tubular toxicity and for clarifying the mechanisms involved in its cellular toxicity. However, these experiments did not test the possibility that the drug's harmful effects occur through the basolateral pole of the cells. Toxicity against cells from other parts of the nephron which are more sensitive to AmB in vivo, such as Henle's loop, has not been studied in vitro. Why some parts of the nephron are more specifically injured by AmB remains unexplained. Simultaneous in vitro study of AmB toxicity against different types of tubular cells might be helpful in attempting to clarify this point.

(iii) Other manifestations of toxicity. The traditional indicators of membrane damage are prominent only for high doses of AmB or long exposures to the drug, and it is not possible to correlate the initial loss of viability to a specific extent of membrane damage. Erythrocytes have proved to be a model of choice to better characterize AmB action in this field. The increase in cell permeability to monovalent cations leads to cell swelling and hemolysis (7). The presence of carbohydrates in the incubation medium protects against hemolysis, although cell membrane permeability alterations are still observed. By contrast, small-polyene (pentaene) toxicity occurs in only one irreversible stage, and carbohydrates only transiently protect against hemolysis (7). This suggests that a colloidosmotic mechanism might be involved in cell lysis and that the longer the polyene, the easier a transmembrane channel could be formed early, before cell lysis.

AmB-induced hemolysis also seems to result from oxidative damage to the membrane. Evidence for the role of active oxygen species in the lytic activity of AmB was obtained from experiments which showed that AmB injury to cells could be reduced by hypoxia or extracellular catalase (6). AKR mouse erythrocytes that have high levels of catalase activity are less sensitive to lysis by AmB than are C57BL/6 mouse erythrocytes, which have lower levels of this enzyme activity. These in vitro results agree with those of in vivo experiments, which show that AKR mice are more resistant to the toxic effects of AmB than are C57BL/6 mice (5). Oxidative damage may result from the auto-oxidation of AmB (32), although other mechanisms cannot be excluded. Whether a relationship between the ionophoric and oxidative effects of AmB exists remains unknown. However, lipid peroxidation may render the membrane more fragile, thereby making the cell more sensitive to osmotic shock.

Finally, disruption of the membrane due to a detergent effect is another possible mechanism of cell toxicity, which has been described in the case of small polyenes (7). The lack of hemolysis observed in vivo in patients treated with AmB might be related to the low level of unbound, potentially toxic AmB in serum.

The existence of modulating effects of AmB on the host immune system, whether inhibitory or stimulatory, have also been reported (33). Some in vitro studies have shown an inhibitory effect of AmB on monocytes, T lymphocytes, or B lymphocytes independent of any alteration of cellular viability (2, 35, 46, 56). Whether inhibition results from the direct toxicity of AmB against the target cell or is mediated by active suppression is not always clear, and beneficial immunostimulant effects of AmB have also been reported (44). The in vivo significance of these in vitro results remains to be determined since the immune system and the interaction between the different mechanisms involved in immune response are complex.

In summary, AmB toxicity towards mammalian cells is probably a multifaceted phenomenon in which the role played by the induction of membrane permeability to monovalent cations is not yet fully understood, although this parameter remains a widely used method of measuring AmB side effects.

Although in vitro models help greatly in elucidating the mechanisms involved in AmB toxicity, they do not explain why the major manifestation of this toxicity in vivo is nephrotoxicity. Among the wide variety of mammalian cell types evaluated in vitro, including renal epithelial cells, the AmB threshold concentrations responsible for cell toxicity did not appear to differ markedly from one cell type to another. Several parameters could explain the AmB toxicity observed in vivo. Major factors include the pharmacokinetics of the drug, as shown for AmB methylester, which is neurotoxic in vivo although 10-fold less toxic than AmB against glial cells in culture (40); the environmental medium, which influences the amount of free toxic drug (free AmB in serum is reduced by binding to lipoproteins, whereas all drug molecules are free in urine); the specialization of cells, like renal tubular cells, in membrane-related functions; and finally, renal vasoconstriction and reduction of the glomerular filtration rate, which occurs independently of any tubular injury (41).

In conclusion, in vitro models are most useful in estimating the balance between activity (antifungal effect) and cellular toxicity of AmB, which determines the therapeutic index. This is particularly helpful in the screening of new AmB formulations.

(iv) In vitro evaluation of new AmB formulations. Incorporation of AmB into liposomes decreases its in vivo toxicity and allows higher doses of the drug to be given to patients. The therapeutic efficacy is thereby increased, as has been shown by experimental and clinical studies (8). The reduced toxicity of AmB observed in vivo has been confirmed by in vitro studies using mammalian cells. The extent of the beneficial effect of liposomes depends on several factors: presence and type of sterol, phospholipid properties (electric

charge, length, presence of double bonds), and the lipid/AmB concentration ratio (21). Saturated phospholipids afford the greatest protection against mammalian cell damage (25). In light of their experiments, Juliano et al. (25) suggested that the reduced toxicity of liposomal AmB is not due to slow or limited release of the drug from its carrier but rather to a selective transfer of the drug from the liposomes to fungal- but not mammalian-cell membranes. They suggested that this selective transfer process probably occurs by diffusion rather than by a collisional process and would be regulated by the physical characteristics of donor and target membranes. AmB would induce pore formation once an AmB threshold concentration is reached in the membrane. The higher level required for mammalian cells than for fungal cells could explain the selectivity of liposomal AmB for antifungal effects. According to Bolard et al. (4), the low concentrations of unbound and water-soluble AmB present in the liposomal formulation (26) might be sufficient for antifungal activity but inadequate for forming aggregates and thus deleterious to mammalian cells.

In vitro screening might help select lipid-AmB formulations before more-extensive in vivo studies are undertaken. However, extrapolation to the in vivo situation is not straightforward, since many other factors, such as AmB binding to serum proteins and rapid dissociation of lipid-AmB complexes following administration, interfere with the activity and toxicity of lipid-AmB formulations. Small unilamellar vesicles prepared with dipalmitoyl phosphatidylcholine completely protected renal tubular cells exposed in vitro to a high dose of AmB (10-fold the lowest toxic dose of free-AmB) for 1 h but were protective in vivo only against the toxicity of low daily doses (1.5 mg/kg of body weight) of the drug (34). However, Krause and Juliano reported that liposomal AmB was toxic against LLC-PK1 cells provided that the incubation was long (24 h) (31). These last results argue for the predictive value of in vitro experiments.

In vitro experiments also contribute to the study of AmB derivatives either before in vivo experiments are performed or in experiments designed to increase understanding of the mechanisms of interaction of these compounds with cells. It has been shown that the low toxicity of an *N*-fructosyl-AmB derivative against thymocytes did not result from a cell membrane affinity lower than that of AmB but was the consequence of the lower intrinsic tendency of the derivative to disorganize membrane structure and fluidity (17). Highly hydrosoluble compounds with a low degree of self-association might be expected to be less toxic in vitro than AmB.

(v) Drug interactions. AmB can amplify the anticellular effects of some antitumoral agents (54): AmB has been shown to augment the activity of actinomycin D by increasing its intracellular penetration, and the oxidative damage due to AmB is enhanced by 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea.

AZOLES

Imidazoles and triazoles belong to the class of 14 α-demethylase inhibitors. Since its development in the late 1970s, ketoconazole has become the most important imidazole antifungal agent. More recently, two new azole compounds, itraconazole and fluconazole, have shown promise as systemic antifungal agents. Some side effects of azoles have been reported: gastrointestinal, hepatic, and endocrinological disorders and interference with oxidative drug metabolism in the liver.

The basic structural unit of all of the antifungal azoles is a five-member azole ring which is attached by a carbon-nitrogen bond to a side chain comprising other aromatic rings. Imidazole drugs contain two nitrogen atoms in the azole ring. The triazole class of agents contains a third nitrogen atom in the azole ring.

The antifungal activity of azoles originates mainly from their binding to a cytochrome P-450 molecule (14 α-demethylase) involved in the synthesis of ergosterol (51). Azoles bind by their basic nitrogen to the heme iron and by their N-1 substituent to the protein moiety of fungal P-450s and compete in this way with oxygen binding and activation, resulting in the inhibition of the P-450-mediated reactions. Cytochromes P-450 are enzymes belonging, for the most part, to the class of monooxygenases. In general, they catalyze the synthesis and metabolism of a long list of key compounds, such as sterols (ergosterol for fungal cells and cholesterol for mammalian cells), steroids, bile acids, thromboxane-A_2, prostacyclin, and leukotrienes.

In vitro models help identify P-450 inhibitors that are highly selective for fungal enzymes rather than for the various cytochrome P-450 enzymes present in mammalian organs and for which a high therapeutic index could be expected in vivo. Azole toxicity against mammalian cells can be evaluated by studying the inhibition of P-450-dependent reactions in intact cells or by analyzing interactions between the drug and subcellular fractions.

Intact cells. Ketoconazole reduces bile acid synthesis by hepatocytes in culture by inhibiting the rate-limiting enzyme, cholesterol 7 α-hydroxylase (39). These in vitro results were confirmed by in vivo experiments. Different studies have shown the inhibitory effect of azoles, especially ketoconazole, on the synthesis of cholesterol in various cells: hepatoma cells (29), normal human fibroblasts (30), and renal tubular cells (24). Itraconazole has less effect on cholesterol synthesis than ketoconazole (50).

The effects of azoles on mammalian steroid hormone synthesis have been studied in vitro (42). Ketoconazole at pharmacological levels as measured in patients inhibited testosterone release by rat testis cells in suspensions under basal and stimulated conditions. In the same model, fluconazole, a triazole, had little effect. These results appear predictive of the results of in vivo experiments, which demonstrated that ketoconazole, but not fluconazole, depressed serum levels of testosterone in male volunteers. Inhibitory effects of azoles on the synthesis of other hormones have been observed by using adrenal cells to investigate glucocorticoid synthesis (37). Here again, the triazole compound fluconazole seemed to be less able to inhibit hormone synthesis than ketoconazole (42).

Subcellular fractions. Microsomes prepared from liver (45) and ovaries (42) have been used to study the inhibitory effect of azoles on 14 α-demethylase and estrogen synthesis, respectively. Cytochrome P-450 interaction with nitrogen-containing compounds, including azole drugs, has been shown to produce a characteristic absorption spectrum (20), which enables determination of the affinity of the compound for the enzyme. Titration curves have been obtained with rat hepatic microsomes. The binding of fluconazole or ketoconazole to the rat microsomes was markedly greater for ketoconazole than for the triazole, suggesting a lower affinity of the triazole compound for mammalian P-450. Influence of azoles on the hepatic metabolism of other drugs or hormones can be studied in vitro by using rat hepatic microsomes (43).

In summary, azole antifungal agents not only interact with the fungal P-450 but also inhibit cholesterol synthesis, though at concentrations much higher than that required for

inhibiting ergosterol synthesis. The difference between fungal and mammalian enzymes supports the selective antifungal effect of these compounds. As for polyenes, in vitro models are more useful for estimating the selectivity of new compounds for fungal cells than for identifying a given toxic effect and its relevance to in vivo observations. Itraconazole and saperconazole, two potent, broad-spectrum, antifungal triazoles that are, up to 10 μM, devoid of effects on mammalian cytochromes P-450 involved in steroid synthesis and metabolism (50), were selected with the help of such in vitro tests.

CONCLUSION

In conclusion, in vitro models contribute to the screening of new drugs and to the better understanding of the mechanisms involved in the cellular toxicity of antifungal agents, thus making possible the design of compounds with increased selectivity. Cellular models corresponding to the clinical targets of toxicity might be useful in predicting a patient's in vivo tolerance to such treatment.

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