Amphotericin B Is Cytotoxic at Locally Delivered Concentrations

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Abstract

Background Orthopaedic fungal infections are commonly treated with systemic amphotericin, which has a narrow therapeutic index and is associated with systemic toxicities. Local delivery of amphotericin has been described yet is poorly understood. As with bacterial infections, fungal infections are associated with biofilm. However, it is unclear whether experience with local delivery of antibacterials can be applied to local antifungal delivery.

Questions/purposes We asked whether (1) 100 to 1000 µg amphotericin/mL caused osteoblast cell death; (2) 1 to 10 µg amphotericin/mL caused sublethal toxicity to osteoblasts and fibroblasts; and (3) sublethal amphotericin toxicity could be reversed.

Methods Mouse osteoblasts and fibroblasts were exposed in vitro to amphotericin concentrations of 0, 1, 10, 100, and 1000 µg/mL for 5 hours or 0, 1, 5, and 10 µg/mL for 7 days and then 3 days with no amphotericin. Cell morphology on light microscopy and proliferation assays

(alamarBlue® and MTT) were used as measures of toxicity.

Results Amphotericin concentrations of 100 µg/mL and above caused cell death; 5 to 10 µg/mL caused abnormal cell morphology and decreased proliferation. Cells regained normal morphology and resumed cell proliferation within 3 days after removal of amphotericin.

Conclusions In this in vitro study, amphotericin was cytotoxic to osteoblasts and fibroblasts at concentrations achievable by local delivery.

Clinical Relevance If local concentrations of 100 to 1000 times the minimum inhibitory concentration are necessary to treat biofilm-associated fungal infections as they are for bacterial infection, cell toxicity at the local depot site should be considered.

Introduction

Amphotericin B is a highly toxic parenteral antifungal agent used in the management of fungal infections. This drug complexes with cell membrane sterols of host cells, forming pores that leak electrolytes similar to its antifungal action. This drug's mechanism of action can lead to systemic toxicity, including renal, hepatic, cardiac, and blood dyscrasia and death [1, 18]. Concentrations of amphotericin in the blood and renal system are highly dependent on their formulation and delivery mechanism [6]. The recommended dosing regimen to minimize the risk of systemic toxicity is 1.0 mg/kg/day or less [7].

Orthopaedic fungal infections develop biofilms on implant and connective tissue surfaces [9]. Similar to bacteria in biofilm, susceptibility of fungi in biofilm to antifungal agents is severely suppressed [5] (as much as 100–1000 times) [11]. The typical minimum inhibitory

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concentration for amphotericin B is 0.5 to 1.0 $\mu\text{g/mL}$, leading to a concentration target of 1000 $\mu\text{g/mL}$ for biofilm-associated fungi. Local delivery is needed to achieve these concentrations, especially for antifungals as toxic as amphotericin B. While there is substantial literature on systemic toxicity [1, 18], blood toxicity [14], and adverse reactions to amphotericin B [1], there is relatively little literature concerned with its effects on local cells involved in the wound-healing response and fracture healing. Tissues adjacent to locally delivered amphotericin B may also be vulnerable to amphotericin B toxicity. Muscle, bone, connective tissue, and wound healing are all at risk. The concern is whether amphotericin B is toxic to local tissue at locally delivered concentrations.

We asked whether (1) 100 to 1000 μg amphotericin B/mL caused osteoblast cell death; (2) 1 to 10 μg amphotericin B/mL caused sublethal toxicity to osteoblasts and fibroblasts; and (3) sublethal amphotericin B toxicity could be reversed.

Materials and Methods

We studied amphotericin B toxicity *in vitro* using mouse osteoblasts. For each of five concentrations, 0, 1, 10, 100, and 1000 $\mu\text{g/mL}$, eight wells of 3000 osteoblasts in 96-well plates were exposed to amphotericin B for 1, 3, and 5 hours (120 wells). Viability of the osteoblasts was determined by observing morphology under light microscopy and by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (lethal) proliferation assay. Separately, six wells of both mouse osteoblast and fibroblast cells were exposed to four amphotericin B concentrations, 0, 1, 5, and 10 $\mu\text{g/mL}$, for 7 days (144 wells) and repeatedly analyzed using alamarBlue[®] reagent (nonlethal) and morphology to determine proliferation in the presence of amphotericin B. The effect of amphotericin concentration on cellular growth rate was determined using Friedman's test. Recovery from sublethal toxicity for 5 and 10 $\mu\text{g/mL}$ was determined by alamarBlue[®] and morphology 3 days after removal of the amphotericin B from the medium (24 wells) and assessed by paired sign test.

Mouse fibroblasts (BALB/3T3 A31; American Type Culture Collection, Manassas, VA) and mouse osteoblasts (MC3T3; American Type Culture Collection) were grown in Dulbecco's Modified Eagle's Medium with 2 mmol glutamine/L, 5% bovine serum; 10 U penicillin/mL, and 10 μg streptomycin/mL (all from Fisher Scientific, Pittsburgh, PA). Cells used in this study were of moderate passage number and were cultured with antibacterials to prevent any bacterial contamination. Neither of these lines is present on the lists of likely contaminated cells [10], and the cultures do not involve feeder layers or genetic

modification. Although we did not independently verify the cell lines, their behavior in culture was typical for osteoblast and fibroblast cell lines.

Osteoblasts plated at 3000 cells per well in 96-well plates were exposed to amphotericin B at concentrations of 0, 1, 10, 100, and 1000 $\mu\text{g/mL}$ for 5 hours ($n = 8$ wells/concentration), observed under light microscopy for changes in cell morphology, and assayed for cell viability using a MTT proliferation assay (Promega, Madison, WI) at 1, 3, and 5 hours. Absorbance was measured on a FLUOstar Omega Multiplate Reader (BMG Labtech, Cary, NC).

Fibroblasts and osteoblasts plated at 10,000 cells per well in 24-well plates were exposed to amphotericin B (Xgen Pharmaceuticals, Big Flatts, NY) at concentrations of 0, 1, 5, or 10 $\mu\text{g/mL}$ ($n = 6$ wells/concentration). At 1, 4, and 7 days, the amphotericin-containing medium was removed and replaced with 0.5 mL fresh medium containing 50 μL alamarBlue[®] (Invitrogen, Carlsbad, CA). The alamarBlue[®] fluorescence was measured on a FLUOstar Omega Multiplate Reader to determine cell proliferation. Cell proliferation results are reported in fluorescence rather than cell counts in accordance with literature provided by Invitrogen [8]. After measuring the fluorescence, the medium with alamarBlue[®] was removed, and fresh medium with the proper concentration of amphotericin was reapplied. At 7 days, the amphotericin-containing medium was removed and replaced with fresh medium without amphotericin.

Morphology was considered "normal" for both cell lines if cells were adhered to the polystyrene, spread, and had a normal variable shape. Morphology was considered "abnormal" if cells were rounded or shriveled.

We determined whether amphotericin was cytotoxic (cell viability and proliferation) to osteoblasts and fibroblasts using Friedman's test. Recovery was assessed with paired sign tests comparing Day 7 fluorescence to Day 10 fluorescence for the 5- and 10- $\mu\text{g/mL}$ samples. Data were analyzed with the use of MINITAB[®] (Minitab Inc, State College, PA).

Results

Widespread osteoblast cell death was caused by exposure to amphotericin B at concentrations of 100 and 1000 $\mu\text{g/mL}$ for 1 hour, as indicated by MTT assay and grossly abnormal morphology on light microscopy (Fig. 1; Table 1).

Exposure to amphotericin B at concentrations of 1 $\mu\text{g/mL}$ for 7 days was not lethal to osteoblasts or fibroblasts as indicated by the alamarBlue[®] assay. The increase in fluorescence was similar to control. There was no evidence of toxicity with normal morphology on light

Fig. 1A–C Images summarize the experimental results of exposure of mouse osteoblasts to varying levels of amphotericin B (bright field, grayscale, no stain; original magnification, $\times 100$). **(A)** At 1 $\mu\text{g/mL}$, the cells show normal morphology and density. **(B)** At 10 $\mu\text{g/mL}$, many of the cells show rounded and other abnormal shapes and reduced density. **(C)** At 100 and 1000 $\mu\text{g/mL}$, there was evidence of amphotericin precipitation, shrinkage, irregularity, and widespread cell death.

microscopy or from the alamarBlue® assay (Table 1). There were, however, some osteoblasts and fibroblasts that died with exposure to 5 and 10 μg amphotericin B/mL and some that did not, as indicated by the alamarBlue® assay (Figs. 2, 3; Table 2). Fluorescence decreased ($p = 0.042$) with increasing amphotericin B concentration. Many of the cells showed abnormal rounded morphology on light microscopy.

Table 1. Summary table of experimental results

Concentration ($\mu\text{g/mL}$)	Experiment		
	5-hour viability	7-day proliferation	3-day recovery
1000	Dead		
100	Dead		
10	Decreased	Decreased	Recovered
5		Decreased	Recovered
1	Normal	Normal	Normal
0	Normal	Normal	Normal

The sublethal toxicity seen in both the osteoblasts and fibroblasts that survived exposure to both 5 μg and 10 μg amphotericin B/mL was reversible. The number of visible cells detected by fluorescence on alamarBlue® assay increased ($p = 0.031$) from Day 7 to Day 10 after removal of the amphotericin B from the culture medium on Day 7 (Table 2). Cell morphology seen on light microscopy was normal on Day 10.

Discussion

Amphotericin B can be delivered locally to clinically manage infection. We questioned whether high concentrations of amphotericin B that are possible during local delivery are toxic to fibroblasts and osteoblasts. We subsequently examined whether growth of those cells was inhibited by intermediate concentrations of amphotericin and finally whether cells exposed to sublethal toxicity recovered their morphology and replication once the amphotericin was removed.

We note a number of limitations to our study. First, the in vitro design of our study precludes direct application of the results to clinical practice. Confluent cell colonies or living human tissues may be more resilient to amphotericin B than the cell suspensions initially exposed to amphotericin in this study. Although we were unable to locate any correlative vivo data in the literature related to amphotericin B, cell toxicity to aminoglycosides has been documented in cell culture [12, 17] and there is a notable lack of reports that any local tissue injury is seen in animals or patients. Histologic and imaging data in a canine model [2] and second-stage procedures in clinical reports lack data associating the use of local aminoglycosides with local wound complications or delay in bone healing [16]. Second, we do not know whether or how other cell lines (macrophages, white blood cells, vascular endothelial progenitor cells) in a surgical wound would respond to high concentrations of amphotericin B. Third, we tested mouse cells, not human cells, and it is possible human cells have

Fig. 2 Mouse fibroblast (BALB/3T3 A31) fluorescence increases over time as a function of amphotericin B concentration. The cells were exposed to 0, 1, 5, or 10 µg amphotericin B/mL. The wells with 5 and 10 µg/mL were grown for a further 3 days without amphotericin to determine whether the cells could proliferate once the drug was removed. Black bars = 1-day exposure; hatched bars = 4-day exposure; white bars = 7-day exposure; striped bars = recovery period measured on Day 10; error bars = SDs.

Fig. 3 Mouse osteoblast (MC3T3) fluorescence increases as a function of amphotericin B concentration. The cells were exposed to 0, 1, 5, or 10 µg amphotericin B/mL. The wells with 5 and 10 µg/mL were grown for a further 3 days without amphotericin to determine whether the cells could proliferate once the drug was removed. Black bars = 1-day exposure; hatched bars = 4-day exposure; white bars = 7-day exposure; striped bars = recovery period measured on Day 10; error bars = SDs.

Table 2. Fluorescence measurements for osteoblast and fibroblast cell viability

Cell type	Exposure time (days)	Fluorescence measurements							
		0 µg/mL amphotericin B		1 µg/mL amphotericin B		5 µg/mL amphotericin B		10 µg/mL amphotericin B	
		Fluorescence	Change	Fluorescence	Change	Fluorescence	Change	Fluorescence	Change
Osteoblasts	0	11,512		11,512		11,512		11,512	
	1	26,393	2.29	27,465	2.39	14,209	1.23	12,267	1.07
	4	77,974	6.77	83,562	7.26	44,037	3.82	10,771	0.94
	7	106,984	9.29	113,227	9.83	96,868	8.41	20,705	1.80
	10					137,675	1.42	36,294	1.75
Fibroblasts	0	18,663		18,663		18,663		18,663	
	1	46,827	2.51	47,965	2.57	28,288	1.52	20,087	1.08
	4	83,284	4.46	86,324	4.63	38,686	2.07	19,244	1.03
	7	225,998	12.11	196,409	10.52	50,835	2.72	18,037	0.97
	10					169,345	3.33	32,701	1.81

Cell viability is expressed as a fluorescence value from the alamarBlue[®] assay in the fluorescence columns; Day 10 represents values obtained 3 days after removal of amphotericin B from the culture medium on Day 7; the change in fluorescence value in the change columns is expressed as the multiple of the value at Day 0, or for Day 10, as a multiple of the value on Day 7, after the removal of amphotericin B from the culture medium on Day 7.

responses to amphotericin different from those of mouse cells. Fourth, cell viability staining is not necessarily a complete measurement of cell health, and there may be effects of amphotericin exposure on protein production or other cell functions not evaluated in this study. Since toxicity of amphotericin B is dependent on its disruption of the cell membrane [4], it is expected the results observed with these cell lines will be grossly similar across different mammalian cells. Amphotericin B is insoluble in water, forming micelles above the critical micelle concentration (6.3×10^{-7} mol/L, ~ 1 µg/mL) [20]. It was in micellar form in the culture medium at the lethal concentrations. It is

unknown in this study whether toxicity is caused by the monomeric or micellar form of amphotericin B. Previous studies have reported the monomeric form as being more toxic to erythrocytes than the micellar form [13]. Even if this effect can be determined for this cell type and controlled in vitro, it is unlikely micelle formation can be controlled in vivo and will primarily be determined by the concentration of amphotericin B that could be experienced with local delivery, accompanying salts or lipids of the formulation, and the surrounding tissue.

Initially, we questioned the immediate toxicity of amphotericin B. Local delivery of amphotericin B used to

treat orthopaedic fungal infection has been reported to produce a concentration of 3.2 µg/mL in surgical drain fluid [15]. This concentration is below the lowest level that produced toxicity in our study and far below the lethal levels in our study but may not represent the tissue concentration adjacent to the local depot. Collette et al. [3] reported tissue concentrations of amphotericin B from intravenous infusion assessed with high-performance liquid chromatography of as high as 147 µg/g. The authors observed a correlation between increased amphotericin B delivery and increased fungistatic and fungicidal titer; however, the local toxicity of the amphotericin B was not evaluated as samples were taken from deceased subjects. Legrand et al. [14] evaluated the toxicity of amphotericin B to erythrocytes, showing substantial toxicity between concentrations of 1 and 10 µmol/L. Although their procedure began with a solvent extraction in dimethyl formamide or dimethyl sulfoxide and was performed on a different cell line with different methodologies, their toxic concentrations are lower than ours. Janoff et al. [13] noted the potential to reduce the toxicity of amphotericin B through the use of lipids to form aggregates and determined the lethal dose 50% for intravenous injection in mice. While the literature suggests general agreement in regard to the systemic toxicity and toxicity to erythrocytes, Sealy et al. [19] published the only study investigating toxicity of antifungals (amphotericin B, micafungin, fluconazole) to musculoskeletal tissue. They detected no toxicity to osteoblast-like cells in cell culture from any of these agents; however, they did not note the concentration of drug to which the osteoblasts were exposed. The cytotoxicity we observed from 100 µg/mL and above raises a concern that clinically important toxicity could occur if local delivery produces levels this high.

Secondly, we questioned whether the local cells were inhibited by the presence of 5- and 10-µg/mL concentrations of amphotericin B. We found no literature reporting longitudinal cell culture studies with amphotericin B. Toxicity studies were generally performed in mice or other in vivo models [13]. While these studies provide outcome data, they do not examine in detail the local effects of the drug. Additionally, most methods for measuring cell proliferation, such as MTT assays, involve killing the cells before counting, which makes longitudinal experiments difficult and highly variable. AlamarBlue® does not kill the cells and therefore does not prevent longitudinal studies. The data reported by Sealy et al. [19] on cytotoxicity of amphotericin B to human osteoblast-like cells cannot be related to our data for concentrations in the 5- to 10-µg/mL range either because the concentration in that study is unknown. Our data are consistent with clinical experience that bone and fibrous tissue can sustain levels of less than 5 µg/mL typically delivered systemically. In vivo

investigation may be appropriate to further evaluate toxicity to muscle, cartilage, nerve, and the local immune and wound-healing response to concentrations at these levels.

Finally, we evaluated cells for their ability to increase replication once the amphotericin was removed. We are unaware of any studies that report the recovery of cells in culture after amphotericin B is removed. Many of the cellular toxicity tests were performed with erythrocytes at dosages that kill the cells [14]. Since erythrocytes do not undergo mitosis, effects on cellular proliferation could not be assessed. While our experiments do not evaluate protein production or other indices of cell function, the resumption of cell growth is an indication that tissues might recover after the local concentration of amphotericin falls below toxic levels.

In conclusion, from our in vitro study, amphotericin B is lethal to osteoblasts and fibroblasts at concentrations of 100 µg/mL and above. This antifungal drug causes sublethal cytotoxicity at 5 and 10 µg/mL. Local delivery of amphotericin B may be necessary to generate the high local concentrations needed to control fungal infections in biofilm on orthopaedic implants or bone, but high concentrations could be locally toxic and impact surgical wound site healing.

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