Pharmacodynamics of Amphotericin B in a Neutropenic-Mouse Disseminated-Candidiasis Model

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In vivo pharmacodynamic parameters have been described for a variety of antibacterials. These parameters have been studied in correlation with in vivo outcomes in order to determine which dosing parameter is predictive of outcome and the magnitude of that parameter associated with efficacy. Very little is known about pharmacodynamics for antifungal agents. We utilized a neutropenic mouse model of disseminated candidiasis to correlate pharmacodynamic parameters (percent time above MIC [T > MIC], area under the concentration time curve [AUC/MIC ratio, and peak serum level/MIC ratio] for amphotericin B in vivo with efficacy, as measured by organism number in homogenized kidney cultures after 72 h of therapy. Amphotericin B was administered by the intraperitoneal route. Drug kinetics for amphotericin B in infected mice were nonlinear. Serum half-lives ranged from 13 to 27 h. Infection was achieved by intravenous inoculation with 108 CFU of yeast cells per ml via the lateral tail vein of neutropenic mice. Groups of mice were treated with fourfold escalating total doses of amphotericin B ranging from 0.08 to 20 mg/kg of body weight divided into 1, 3, or 6 doses over 72 h. Increasing doses produced concentration-dependent killing, ranging from 0 to 2 log10 CFU/kidney compared to the organism number at the start of therapy. Amphotericin B also produced prolonged dose-dependent suppression of growth after serum levels had fallen below the MIC. Nonlinear regression analysis was used to determine which pharmacodynamic parameter best correlated with efficacy. Peak serum level in relation to the MIC (peak serum level/MIC ratio) was the parameter best predictive of outcome, while the AUC/MIC ratio and T > MIC were only slightly less predictive (peak serum level/MIC ratio, coefficient of determination [R2] = 90 to 93%; AUC/MIC ratio, R2 = 49 to 69%; T > MIC, R2 = 67 to 85%). The total amount of drug necessary to achieve various microbiological outcomes over the treatment period was 4.8- to 7.6-fold smaller when the dosing schedule called for large single doses than when the same amount of total drug was administered in 2 to 6 doses. Given the narrow therapeutic window of amphotericin B and frequent treatment failures, these results suggest the need for a reevaluation of current dosing regimens.

The incidence of nosocomial candida infections has risen sharply, representing nearly 10% of hospital-acquired bloodstream infections (4). Currently available therapies result in unacceptably high failure rates (25). In addition, available antifungal therapies often produce significant toxicities (12). Although the discovery of new antifungal agents is promising, approaches to optimize efficacy and limit toxicity of currently available agents through rational pharmacodynamic dosing may offer more immediate impact (9, 11).

Amphotericin B is an intravenously administered polyene antibiotic that has been available for clinical use for more than 40 years. Studies in both experimental infection models and clinical trials have demonstrated the potency of amphotericin B against a variety of yeasts (2, 3, 10, 12, 14, 17, 24). In a recent consensus publication on the therapy of candidemia, nearly all of the participants would include amphotericin B for therapy of candidemia for patients with life-threatening illness (10). Despite the potency of this drug, many clinicians have grown reluctant to use amphotericin B because of the relatively narrow therapeutic or toxic window (12).

Pharmacodynamic characterization of amphotericin B should maximize dosing efficacy and perhaps limit toxicity. In the present experiments we have characterized the pharmacodynamic parameter predictive of efficacy of amphotericin B monotherapy in a neutropenic mouse model of disseminated candidiasis.

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MATERIALS AND METHODS

Organisms. Three clinical isolates of Candida albicans (K-1, 98-17, and 98-234) and single clinical isolates of Candida krusei 5810 and Candida dubliniensis 3588 were used in the experiments. Each Candida strain was a bloodstream isolate, with the exception of C. dubliniensis, which was an oropharyngeal isolate. The organisms were maintained, grown, subcultured, and quantified on Sabouraud dextrose agar (SDA) slants (Difco Laboratories, Detroit, Mich.). Twenty-four hours prior to study, organisms were subcultured at 35°C.

Antifungal. Amphotericin B desoxycholate was obtained as a powder from Bristol-Myers Squibb (Princeton, N.J.). The powder was stored at −70°C. Drug solutions were prepared on the day of study by dissolving the powder in sterile H2O. Subsequent drug dilutions were obtained with D5W.

In vitro susceptibility testing. MICs were determined using a microbroth modification of the NCCLS M27-A method with both RPMI buffered with 0.165 M morpholine propane sulfonic acid and antibiotic medium 3 (16, 19). Determinations were performed in duplicate on at least two separate occasions. Final results are expressed as the geometric means of these results.

Animals. Six-week-old ICR Swiss-specific-pathogen-free female mice weighing 23 to 27 g were used for all studies (Harlan Sprague Dawley, Madison, Wis.). All animal studies were approved by the Animal Research Committee of the William S. Middleton Memorial VA Hospital.
Infection model. Mice were rendered neutropenic (<100 polymorphonuclear leukocytes per mm³) by injection with cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, Ind.) intraperitoneally 4 days (150 mg/kg of body weight) and 1 day (100 mg/kg) before infection. Organisms were subcultured on SDA 24 h prior to infection. Inoculum was prepared by placing six colonies into 5 ml of sterile pyrogen-free 0.9% saline warmed to 35°C. Fungal counts of the inoculum determined by viable counts on SDA were 10⁶ CFU/ml.

Disseminated infection with the Candida organisms was achieved by injection of 0.1 ml of inoculum via the lateral tail vein 2 h prior to the start of drug therapy. At the end of the study period, animals were sacrificed by CO₂ asphyxiation. After sacrifice, the kidneys of each mouse were immediately removed and placed in sterile 0.9% saline at 4°C. The homogenate was then serially diluted 1:10, and aliquots were plated on SDA for viable fungal colony counts after incubation for 24 h at 35°C. The lower limit of detection was 100 CFU/ml. Results were expressed as the mean CFU per kidney for two mice (four kidneys).

Pharmacokinetics. Single-dose pharmacokinetics of amphotericin B were determined in individual neutropenic-infected ICR-Swiss mice following intraperitoneal doses of 0.625, 2.5, 5.0, 10.0, and 20 mg/kg administered in 0.2-ml volumes. At each dose examined, groups of three mice under light halothane anesthesia were sampled three or four times by retro-orbital puncture. Samples were collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, Pa.) at 5- to 18-h intervals. Tubes were centrifuged (model MB; International Equipment Co.) at 10,000 × g for 5 min. The serum was subsequently removed, and drug levels were determined by standard drug diffusion bioassay using Pseudocymes variotii as the assay organism in antibiotic medium 12 (20). Assays of serum samples and standard curves prepared for mouse serum were performed on the same day. Intraday coefficient of variation ranged from 2.3 to 9.6%. The lower level of detection for this assay was 0.15 mg/liter. Pharmacokinetic constants, including elimination half-life and the concentration of drug in serum at time zero (C₀), were calculated via nonlinear least-squares techniques (MINSQ; MicroMath, Inc., Salt Lake City, Utah). The area under the concentration-time curve (AUC) was calculated by the trapezoidal rule. For doses that had no kinetics determined, pharmacokinetic parameters were extrapolated from the values obtained in the actual studies.

In vivo PAE. Infection in neutropenic mice was produced as described above. Two hours after infection with C. albicans K-1, mice were treated with single intraperitoneal doses of amphotericin B (0.25, 1.0, and 4.0 mg/kg). Groups of two treated and control mice were sacrificed at each sampling time interval, ranging from 2 to 12 h. Control growth was determined over 24 h at five sampling times. The treated groups were sampled six to eight times over 56 h. Kidneys were removed at each time point and processed immediately for CFU determination, as outlined above. The time that levels of amphotericin B in serum remained above the MIC (T > MIC) for the organism following the three doses was calculated from the pharmacokinetic data. The postantibiotic effect (PAE) was calculated by determining the time it took for controls to increase 1 log₁₀ CFU/kidney (C) and subtracting this from the amount of time it took organisms from the treated animals to grow 1 log₁₀ CFU/kidney (T) after serum levels fell below the MIC for the organism (PAE = T – C) (22).

Pharmacodynamic parameter determination. Neutropenic mice were infected with each of the Candida species 2 h prior to the start of therapy. Fifteen dosing regimens were chosen to minimize the interdependence among the three pharmacodynamic parameters studied and also to describe the complete dose-response relationship. Groups of two mice were treated for 72 h with dosing regimens of amphotericin B using fourfold increasing total doses administered at 12-, 24-, or 72-h dosing intervals. Total doses ranged from 0.078 to 20 mg/kg/72 h. The drug was administered in 0.2-ml volumes. Mice were sacrificed after 72 h of therapy, and kidneys were removed for CFU determination as described above. Untreated control mice were sacrificed just before treatment and at the end of the experiment. Efficacy was defined as the change in log₁₀ CFU/kidney over the 72-h treatment period and was calculated by subtracting the mean log₁₀ CFU/kidney in untreated control mice after 72 h from the mean number of CFU from kidneys of two mice at the end of therapy.

Data analysis. A sigmoid dose-effect model was used to measure the in vivo potency of amphotericin B. The model is described by the Hill equation: E = E_max/(ED5₀^N + D^N), where E is the observed effect (change in log₁₀ CFU/kidney compared with untreated controls at 72 h), D is the cumulative 72-h dose, E_max is the maximum effect, ED₅₀ is the dose required to achieve 50% of E_max, and N is the slope of the dose-effect relationship. The correlation between efficacy and each of the three parameters studied was determined by nonlinear least-squares multivariate regression analysis (Sigmat: Jandel Scientific Software, San Rafael, Calif.). The coefficient of determination (R²) was used to estimate the percent variance in the change of log₁₀ CFU/kidney over the treatment period for the different dosing regimens that could be attributed to each of the pharmacodynamic parameters.

To allow a more meaningful comparison of potency among the dosing regimens studied, we calculated the dose required to produce a fungistatic dose or no net growth over 72 h and the dose required to achieve a 1 log₁₀ reduction in colony counts compared to numbers at the start of therapy. If the doses needed to achieve these benchmarks increased significantly as the dosing interval was lengthened from every 12 h to every 72 h, T > MIC was the parameter predictive of efficacy. On the other hand, if the doses necessary to reach these outcomes decreased with the lengthening of the dosing interval, then the parameter associated with these outcomes would be the peak serum level. If the doses remained similar independent of changes in the dosing interval, then the AUC would be predictive of efficacy.

RESULTS

In vitro susceptibility testing. There was no difference in MICs determined with both the accepted M27 methodology and antibiotic medium 3. MICs of amphotericin B for the Candida species were 0.25 mg/liter for each of the organisms tested, with the exception of C. dubliniensis, for which the MIC of amphotericin B was 0.5 mg/liter.

Pharmacokinetics. The time courses of amphotericin B in serum of infected neutropenic mice following intraperitoneal doses of 0.625, 2.5, 5.0, 10.0, and 20 mg/kg are shown in Fig. 1. Peak serum levels and the AUC did not increase in a linear fashion with dose escalation. Peak levels were achieved within 6 h for each of the doses and ranged from 2.51 ± 0.40 to 0.28 ± 0.03 mg/liter. The elimination half-life ranged from 13.7 to 27 h, similar to that previously described in a murine model (13). The AUC, as determined by the trapezoidal rule, ranged from 10 to 83 mg·h/liter with the lowest and highest doses, respectively.

In vivo PAE. Following tail vein inoculation of 10⁶ CFU/ml, growth of Candida organisms in the kidneys of untreated mice increased (3.31 ± 0.20) log₁₀ CFU/kidney over 24 h. Control growth of 1 log₁₀ CFU/kidney in untreated mice was achieved in 5.2 h. No drug carryover was observed in treatment groups. Based upon the above pharmacokinetics, the three doses of amphotericin B studied (0.25, 1.0, and 4.0 mg/kg) would produce serum levels above the MIC for the Candida organism (0.25 mg/liter) for 0, 14, and 46 h, respectively. Only treatment

FIG. 1. Concentrations of amphotericin B in serum after subcutaneous doses of 20, 10, 5, 2.5, and 0.625 mg/kg in neutropenic infected mice. Each symbol represents the geometric mean ± standard deviation of serum levels from three mice.

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with the highest single dose resulted in any significant reduction in colony counts compared with numbers at the start of therapy. Growth curves for both the control group and experimental groups following the single doses of amphotericin B are shown in Fig. 2. Amphotericin B suppressed regrowth of organisms at each of the three doses studied in a dose-dependent fashion. PAEs increased from 23 to 30 h with escalation of dosage from 0.25 to 1.0 mg/kg. We were unable to calculate a PAE for the highest dose studied, as organisms did not regrow during the period of study. The growth suppression due to the 0.25 mg/kg dose was due entirely to sub-MIC effects, as the peak concentration with this dose never reached the MIC of amphotericin B for the organism.

**Pharmacodynamic parameter determination.** At the start of therapy, each kidney had \((3.81 \pm 0.12)\) log_{10} CFU. After 72 h, the organisms grew \((3.11 \pm 0.39)\) log_{10} CFU/kidney in untreated mice and resulted in the death of each of the control mice. Drug carryover was not observed in any of the samples. Escalating doses of amphotericin B produced significant net killing compared to the inoculum in control animals at the start of therapy. Highest total doses for the different regimens resulted in a mean reduction in colony count compared with numbers at the start of therapy of \((1.84 \pm 0.26)\) log_{10} CFU/kidney for the 72-h dosing interval and \((0.69 \pm 0.84)\) log_{10} CFU/kidney with the shortest dosing interval (Fig. 3).

Examples of the relationship between microbiological effect and each of the pharmacodynamic parameters, including \(T > \text{MIC}\), the AUC/MIC ratio, and the peak serum level/MIC.
TABLE 1. Relationship between the pharmacodynamic parameters and efficacy of amphotericin B against Candida organisms

<table>
<thead>
<tr>
<th>Organism</th>
<th>$R^2$ (%)</th>
<th>Peak serum level/MIC</th>
<th>AUC/MIC</th>
<th>$T &gt;$ MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans K-1</td>
<td>93</td>
<td>61</td>
<td>82</td>
<td>57</td>
</tr>
<tr>
<td>C. albicans 98-17</td>
<td>92</td>
<td>65</td>
<td>85</td>
<td>66</td>
</tr>
<tr>
<td>C. albicans 98-234</td>
<td>90</td>
<td>49</td>
<td>78</td>
<td>91</td>
</tr>
<tr>
<td>C. krusei 5810</td>
<td>90</td>
<td>69</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>C. dubliniensis 3588</td>
<td>93</td>
<td>67</td>
<td>69</td>
<td>91</td>
</tr>
</tbody>
</table>

TABLE 2. Effect of amphotericin B dosing interval on treatment outcome

<table>
<thead>
<tr>
<th>Organism</th>
<th>Dose (95% confidence interval)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>q72h</td>
</tr>
<tr>
<td></td>
<td>FD</td>
</tr>
<tr>
<td>C. albicans K-1</td>
<td>2.33 (1.63–3.03)</td>
</tr>
<tr>
<td>C. albicans 98-17</td>
<td>1.52 (1.51–1.53)</td>
</tr>
<tr>
<td>C. albicans 98-234</td>
<td>0.83 (0.77–0.89)</td>
</tr>
<tr>
<td>C. krusei 5810</td>
<td>0.47 (0.43–0.51)</td>
</tr>
<tr>
<td>C. dubliniensis 3588</td>
<td>1.18 (0.20–2.16)</td>
</tr>
</tbody>
</table>

$^a$ q72h, every 72 h; FD, fungistatic dose (in milligrams per kilogram per 72 h); $1 \log_{10}$, $1 \log_{10}$ kill (in milligrams per kilogram per 72 h).

centration of amphotericin B and the duration of exposure, ranging from 0.5 to 10 h (21). The PAEs we demonstrated in vivo were much longer than those found in vitro (8). This discrepancy in PAE duration is similar to that observed with several classes of antimicrobials (9). These in vivo studies are unable to determine what degree of growth suppression could be due to the antimicrobial effect of concentrations that are below the MIC with the two other doses. Previous studies have demonstrated a variety of sub-MIC effects on both C. albicans and Cryptococcus neoformans (1, 14, 18, 21). In addition, although serum drug concentrations have been shown to be a relatively good surrogate of tissue concentrations, the magnitude of the PAE in these experiments may have been different if we were able to accurately measure amphotericin B concentrations at the site of infection (the kidney). The duration of the PAE induced by amphotericin B may be related to the time necessary for yeast cell wall damage to be repaired and subsequent organism multiplication to resume.

Previous animal infection models have demonstrated the potency of amphotericin B against several Candida species (2, 3). In addition, several studies have demonstrated the concordance between in vitro susceptibility and in vivo endpoints (2, 17). These studies have, however, utilized only a single dosing interval, limiting one’s ability to determine which pharmacodynamic parameter best predicts efficacy. With only a single dosing interval, escalating doses increase all three parameters. The interdependence between the parameters with single-dosing-interval studies is too great to determine if one is more important than another.

Our in vivo studies demonstrated concentration-dependent activity in neutropenic animals with doses that covered a 250-fold range in total doses and an effect that varied by more than 3$\log_{10}$. These studies demonstrated that the peak serum level/MIC ratio was the pharmacokinetic and pharmacodynamic parameter that most strongly correlated with the outcome of amphotericin B. When the same total amount of drug was given over the 72-h treatment period, less total drug was needed to achieve a given effect (static dose or 1$\log_{10}$) when larger doses were administered infrequently, maximizing the concentration-dependent parameter, the peak serum level/MIC ratio. More than fivefold more drug was required to produce a net static effect when administered using the most frequent dosing in this model, compared to only a single dose during the 72-h study. Thus, these data suggest that high, infrequent doses of amphotericin B can be as effective as lower-dose, more frequently administered regimens of amphotericin B.

The most frequently recommended amphotericin B dosing,
every 24 h at doses ranging from 0.5 to 1.0 mg/kg, often results in unacceptable toxicities. If one were able to administer the drug to maximize peak serum levels but decrease the frequency of administrations to every 48 or even 72 h, not only might the efficacy of the drug be equivalent or improved, but toxicities may also be less likely. Very few studies have compared the kinetics of amphotericin B in humans using varying dosing intervals. Bindschadler and Bennett (5) demonstrated that higher peak serum levels were achieved when twice the daily dose was administered every other day than when administered daily. This dosing schedule was often recommended in earlier antifungal dosing guidelines but has been replaced more recently by daily dosing. These studies would support clinical studies reexamining administration of larger doses given less frequently, perhaps only two or three times per week. Future in vivo studies comparing the pharmacodynamics of administrations to every 48 or even 72 h, not only might the peak serum levels be higher, but toxicities may also be less likely.

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REFERENCES