Discrepancy between in vitro and in vivo antifungal activity of albendazole

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Albendazole has in vitro activity against Cryptococcus neoformans and reduced in vitro activity for albendazole when compared with Candida albicans. The major metabolite of albendazole, albendazole sulphoxide showed no in vitro activity against isolates of either fungus. Immunocompetent mice infected intravenously (IV) with C. albicans were treated with albendazole doses of 20–600 mg kg⁻¹ per day in noble agar or sesame oil for per oral (PO) administration, or 80 mg kg⁻¹ per day in DMSO for intraperitoneal (IP) and IV administration for 10 days, and were observed for survival. Mice infected with C. neoformans intracranially received albendazole in daily doses of 600 mg kg⁻¹ prepared in DMSO (IP) or peanut butter/rat chow (PO) for 10 days and were observed for survival. Mortality was not different between the treated and control animals in any study. Plasma samples from uninfected mice dosed with similar formulations and doses of albendazole were analysed by HPLC for albendazole and albendazole sulphoxide. No albendazole could be detected in any sample, while concentrations of albendazole sulphoxide (286–8697 ng ml⁻¹) were observed in all samples. These data suggest that the absence of in vivo activity for albendazole is due to rapid conversion to the inactive albendazole sulphoxide metabolite.

Keywords antifungal, albendazole, Candida albicans, Cryptococcus neoformans

Introduction

Benzimidazoles are agents used primarily as anthelmintics in veterinary medicine. At present, thiabendazole (Mintezol®), mebendazole (Vermox®), and albendazole (Albenza®, SmithKline Beecham) are the only benzimidazoles approved in the USA for therapeutic use in humans. The mechanism of activity for the benzimidazoles appears dependent on disruption of mitosis by blocking polymerization of microtubules in susceptible pathogens [1]. While antiparasitic activity is the principle therapeutic feature associated with the benzimidazoles, several of these agents have been reported to possess significant antifungal activity as well when tested in vitro [2,3]. Albendazole, a benzimidazole widely used in the management of cysticercosis and echinococcosis, has also been shown to possess inhibitory activity against Pneumocystis carinii [4,5]. In the light of these reports, we evaluated the in vitro and in vivo antifungal activity of albendazole and its primary metabolite, albendazole sulphoxide, against multiple clinical isolates of Cryptococcus neoformans and Candida albicans.

Materials and methods

In vitro susceptibility testing

The NCCLS Standard (M27-T) procedure for antifungal susceptibility testing was employed. Albendazole (Sigma Chemicals) and albendazole sulphoxide (courtesy of SmithKline Beecham) were prepared separately in concentrations ranging from 0.6 to 10 μg ml⁻¹. To solubilize both compounds, 50% DMSO in water was used, and then further diluted to 1:10 with the addition of broth medium. The broth medium was RPMI-1640 supplemented with glutamine and titrated to a pH of 7.0. To evaluate the effect of this amount of DMSO on the
growth of the organisms tested, a drug-free growth control composed of 0.1 ml of the solvent and 0.9 ml of the growth medium was used. Decreases in turbidity were measured against this growth control instead of the medium growth control. The 1% dilution scheme proposed in the NCCLS M27T was not sufficient to maintain the succin concentration in solution; therefore, a higher concentration of solvent was used. Multiple clinical isolates of C. neoformans (n = 2) and C. albicans (n = 6) with known susceptibility profiles to standard antifungal therapy were evaluated in inoculum sizes of 0.5 × 10^3 to 2.5 × 10^3 asexual propagules per ml. Inoculated tubes were incubated at 35°C. Minimum inhibitory concentration (MIC) was defined as the first concentration tube resulting in an 80% reduction in turbidity compared with the drug-free solvent growth control. MIC readings for C. albicans isolates were done at 24 and 48 h, and at 48 and 72 h for isolates of C. neoformans. The minimum lethal concentration (MLC) was determined 24 h following the initial MIC assessment. Quality control was achieved by using an isolate of Candida parapsilosis, ATCC 22019, in each run to ensure reproducibility and optimal growth conditions. The MIC (μg/ml) results for this control isolate were amphotericin B, 0.06, flucytosine, 0.25, fluconazole, 4-0 and albendazole, 5-0.

In vivo testing

Immunocompetent male ICR mice (Harlan Sprague Dawley) weighing approximately 30 g each were used for all survival studies. Mice were housed in bonneted cages with no more than five mice per cage. Single clinical isolates of C. neoformans (93-175) and C. albicans (R-1590) were used for in vivo testing. The MIC of albendazole for 93-175 was ≤ 0.6 μg/ml, while for R-1590 the MIC was 10 μg/ml.

Fungal isolates were incubated at 37°C overnight in brain-heart infusion broth, washed three times in phosphate buffered saline, and counted in a haemacytometer. Infecting inoculum was cultured, and the number of propagules reported as CFU per mouse. After inoculation, mice were assessed daily for survival for up to 30 days after infection. Inoculum for the C. albicans studies were based on a desire to achieve 100% mortality in untreated control mice by day 7. Previous experience in this laboratory indicated that an inocula of 10^6 to 10^7 CFU would be needed. For the C. neoformans studies, the necessary inoculum was unclear. Initially, a low inoculum was administered, but when the untreated controls survived for most of the study period, a larger inoculum was used in the follow-up study. Even at this higher inoculum, several of the untreated controls survived for longer periods.

Cryptococcus neoformans study no. 1

Twenty mice were anesthetized by inhalation of methoxyflurane, and isopropyl alcohol was used to clean the mouse heads. Intracranial injection of C. neoformans (0-06 ml) was performed 6 mm posterior to the orbits in the midline using a 27 gauge needle attached to a tuberculin syringe. A hub was used to prevent the needle from penetrating more than 1 mm. Ten mice served as untreated controls, and the remaining mice were treated orally with albendazole 600 mg kg^-1 per day in pellets of a mixture of peanut butter and rodent chow for 10 days beginning the day after inoculation.

Cryptococcus neoformans study no. 2

Four groups of ten mice were infected intracranially with C. neoformans (0-06 ml) as described above. Group 1 served as untreated controls. Group 2 served as controls fed pellets of peanut butter and rodent chow without albendazole. Group 3 was treated with oral albendazole (600 mg kg^-1 per day) in pellets of a mixture of peanut butter and rodent chow for 10 days beginning the day after inoculation. Group 4 mice were treated with 600 mg kg^-1 per day of albendazole in 100% DMSO administered intraperitoneally for 10 days starting the day after infection. The intraperitoneal injection volume was 0.2 ml per dose. Other food was not available to the mice until all drug containing pellets were consumed.

Candida albicans study no. 1

Forty mice were infected intravenously (0.2 ml) with C. albicans and divided into four equal groups. Group 1 served as untreated controls. Group 2 mice were treated orally with albendazole suspended in 0.3% noble agar (80 mg kg^-1 per day) for 10 days. The mice in groups 3 and 4 were treated orally with similarly prepared albendazole in doses of 40 mg kg^-1 per day and 20 mg kg^-1 per day, respectively.

Candida albicans study no. 2

Three groups of 10 mice were infected intravenously (0.2 ml) with C. albicans. Group 1 served as untreated controls. The other mice were treated orally with albendazole formulated in sesame oil in doses of 600 mg kg^-1 per day (group 2) or 300 mg kg^-1 per day (group 3) for 10 days.

Candida albicans study no. 3

Twenty mice were infected intravenously (0.2 ml) with C. albicans and divided equally into two groups. Group 1 served as untreated controls and group 2 mice were...
treated with 80 mg kg\(^{-1}\) per day of albendazole in 100% DMSO by intraperitoneal injection for 8 days. The intraperitoneal injection volume was 0·2 ml per dose.

*Candida albicans* study no. 4

Two groups of 5 mice were infected intravenously (0·2 ml) with *C. albicans*. Group 1 served as untreated controls. Group 2 was treated with intravenous albendazole in 100% DMSO in a dose of 80 mg kg\(^{-1}\) per day for 6 days. The intravenous injection volume was 0·1 ml per dose.

**Vehicles for drug delivery**

Owing to the poor solubility of many compounds, including albendazole, evaluated for antifungal activity in these models a number of drug delivery vehicles can be used. Historically, we have started with 0·3% noble agar, then progressed to sesame oil to facilitate oral administration of the drug. Based on work published by Bartlett evaluating albendazole in the treatment of *Pneumocystis carinii* infections, we also used the peanut butter and rodent chow mixture pellets for delivery of similar doses of albendazole [5]. Mice were not provided additional chow until all drug containing pellets were consumed. During the course of these studies, the peanut butter/chow pellets were consumed readily by all mice, even the ill appearing mice. At no time did we detect any preferential feedings that would suggest that the less sick mice were eating larger quantities of the pellets. For IP an IV administration of albendazole, 100% DMSO was used as the drug delivery vehicle.

**Evaluation of DMSO as delivery vehicle**

To evaluate the influence of using 100% DMSO as a drug delivery vehicle in these studies, two studies were performed to mimic the administration of DMSO in the above described studies. Ten immunocompetent male ICR mice were administered IV 0·1 ml daily of 100% DMSO using a tail vein. Drug administration was discontinued on day 4 because the tails were necrotic and falling off. All the mice were alive on day 4 and remained alive throughout the 30-day observation period. Another ten immunocompetent male ICR mice were administered IP 0·2 ml of 100% DMSO daily for 10 days. Two mice died on day 8, and the remaining eight mice survived for the entire 30-day observation period, giving a mean survival of 25·6 days for the group.

**Assessment of plasma concentrations of albendazole and albendazole sulphoxide**

Non-infected male immunocompetent ICR mice (approximately 30 g each) were administered oral doses of albendazole ranging from 80 to 600 mg kg\(^{-1}\) formulated in pellets of the peanut butter/rodent chow mixture. Additional non-infected mice were administered similar doses of albendazole in 100% DMSO (0·2 ml IP). Mice were sacrificed at variable times after dosing, and blood was collected by intracardiac puncture. Blood collected from two to three mice was pooled, centrifuged and plasma separated and frozen until assay.

**Determination of albendazole and albendazole sulphoxide concentrations**

Albendazole and albendazole sulphoxide concentrations in plasma were determined by high performance liquid chromatography (HPLC) using mebendazole (Sigma) as the internal standard. Twenty-five microlitres of mebendazole (5 μg ml\(^{-1}\) in methanol) were added to glass vials and evaporated to dryness under nitrogen. Following the addition of 250 μl of murine plasma to be analysed and 25 μl of 0·8 N KOH to each glass vial, the contents were vortexed for 20 s.

Methanol:chloroform in ratio of 1:9 (2·5 μl) was then added to each vial. Following 20 min of mixing utilizing a rotary mixer, the vials were centrifuged at 3000 rev min\(^{-1}\) for 10 min. The vials were then removed from the centrifuge and allowed to stand for 30 min. The methanol: chloroform layer (bottom) was then removed, placed in another labelled glass vial, and evaporated to dryness under nitrogen. Samples were reconstituted and vortexed with 200 μl of mobil phase and analysed. The HPLC system included a solvent pump (Waters 510), an autosampler (Waters 712 B WISP), an absorbance detector (Waters 486) set at a wavelength of 290 nm, precolumn gland (Waters Guardpak, Novapak C\(_{18}\)), and a reverse phase column (Varian C\(_{18}\), 4 mm × 30 cm, 10 μ). The system was connected to a computerized chromatography manager program (Millenium 2010, Millipore Corp.). The mobile phase used was 0·017 M H\(_3\)PO\(_4\) (in H\(_2\)O):acetonitrile (60:40). The injection volume was 50 μl, and the flow rate was 1 ml min\(^{-1}\). Under these conditions, the retention times observed were albendazole sulphoxide: 3·3 min; mebendazole: 6·5 min, and albendazole: 8·5 min. On each assay day, a standard curve was prepared using spiked stock murine plasma assayed under similar conditions. Plasma concentrations were determined by plotting the peak area ratio against concentration using a weighted 1/concentration least squares regression. The standard curve was linear from 50 ng ml\(^{-1}\) to 4000 ng ml\(^{-1}\) for albendazole sulphoxide.

**Statistical analyses**

Statistical comparisons of treatment regimens of survival for the various treatment regimens within each study...
were performed by the nonparametric log-rank test. Significance was defined as $P < 0.05$.

**Results**

Albendazole demonstrated good *in vitro* activity against both clinical isolates of *C. neoformans* tested. The MIC$_{48}$, MIC$_{72}$ and MLC of albendazole against each isolate was $\leq 0.6 \mu g \text{ml}^{-1}$. Against *C. neoformans*, the albendazole sulphoxide metabolite had little activity, as evidenced by the MIC$_{48}$ and MIC$_{72}$ being 5 and $> 10 \mu g \text{ml}^{-1}$ for one isolate and $> 10$ and $> 10 \mu g \text{ml}^{-1}$ for the other isolate. Poor antifungal activity was observed for albendazole against the six clinical isolates of *C. albicans* tested. The MIC$_{24}$, MIC$_{48}$ and MLC for five isolates were $5$, $> 10$ and $> 10 \mu g \text{ml}^{-1}$, respectively, while the sixth isolate had an MIC$_{24}$ of $10 \mu g \text{ml}^{-1}$. For albendazole sulphoxide, no antifungal activity was observed against any of the six of the *C. albicans* isolates tested with all MIC$_{24}$s $> 10 \mu g \text{ml}^{-1}$. These data are summarized in Table 1 with the antifungal susceptibility profiles for each clinical isolate to standard antifungal agents.

In both the disseminated candidiasis and the cryptococcal meningitis murine models of infection there was no improvement in survival with any of the albendazole treatment regimens employed. In fact, there was significantly increased mortality observed in mice receiving albendazole formulated in 100% DMSO for either IP or IV administration. These survival studies are summarized in Table 2.

As shown in Table 3, albendazole was not detected in any of the murine plasma samples analysed. While albendazole sulphoxide was present in all samples taken from the non-infected mice treated with a variety of albendazole dosages and routes of administration. This would suggest that albendazole is indeed reaching the systemic circulation, but is being converted to inactive metabolites very rapidly.

**Discussion**

*In vitro* screening of new potentially active antifungal compounds against common fungal pathogens has proven to be useful in the identification of agents to be tested *in vivo*. Such *in vitro* screening controls the exposure of the fungal isolate to known concentrations of the compound being evaluated and the environmental conditions that may influence any observed antifungal activity. When tested *in vivo*, however, these conditions are not controlled and results can be very different from those seen with *in vitro* testing. This report describes such a discrepancy.

Cruz and colleagues reported the *in vitro* activity of ten benzimidazoles against clinical isolates of *C. neoformans* [2]. The albendazole concentrations inhibiting 50% of growth (IC$_{50}$) of 13 isolates were $\leq 0.45 \mu g \text{ml}^{-1}$, consistent with the observed MIC of $\leq 0.6 \mu g \text{ml}^{-1}$ for our two isolates. Based on these *in vitro* results, albendazole appeared to have sufficient activity against *C. neoformans* to warrant further evaluation in a murine model of cryptococcal meningitis. We were disappointed with the absence of improvement in the initial survival study, so we tried an alternative dosing formulation and route of administration as described by Bartlett and coworkers in the second study in an attempt to optimize systemic availability of albendazole [5]. Again, there was no improvement in survival noted with the orally administered albendazole in these mice, while the mice who were treated with IP injections of albendazole dissolved in DMSO had greatly increased mortality, possibly related to the DMSO.

The poor *in vitro* activity of albendazole against *C. albicans* was consistent with that reported by others [2,3]. Nevertheless, we pursued investigation of *in vivo* activity of albendazole in a murine model of disseminated *C. albicans* infection. We observed no change in survival with albendazole administered in a variety of dosages orally. We observed significantly reduced survival in animals receiving albendazole in 100% DMSO by

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**Table 1 Antifungal susceptibility results for clinical isolates evaluated reported as MIC**

<table>
<thead>
<tr>
<th>Clinical fungal isolate</th>
<th>Amphotericin B</th>
<th>5FC</th>
<th>Fluconazole</th>
<th>Albendazole</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> 92-343</td>
<td>$\leq 0.14$</td>
<td>40.3</td>
<td>$&gt; 80$</td>
<td>5</td>
</tr>
<tr>
<td><em>C. albicans</em> 92-824</td>
<td>0.58</td>
<td>$&gt; 322$</td>
<td>$\leq 1.25$</td>
<td>5</td>
</tr>
<tr>
<td><em>C. albicans</em> 92-889</td>
<td>$\leq 0.14$</td>
<td>$\leq 10.9$</td>
<td>$&gt; 80$</td>
<td>5</td>
</tr>
<tr>
<td><em>C. albicans</em> 92-1341</td>
<td>0.29</td>
<td>40.3</td>
<td>$\leq 1.25$</td>
<td>5</td>
</tr>
<tr>
<td><em>C. albicans</em> 92-1729</td>
<td>0.29</td>
<td>$\leq 10.9$</td>
<td>$\leq 1.25$</td>
<td>5</td>
</tr>
<tr>
<td><em>C. albicans</em> R-1590</td>
<td>$\leq 0.14$</td>
<td>ND</td>
<td>$\leq 1.25$</td>
<td>10</td>
</tr>
<tr>
<td><em>C. neoformans</em> 93-175</td>
<td>$\leq 0.14$</td>
<td>40.3</td>
<td>40</td>
<td>$\leq 0.6$</td>
</tr>
<tr>
<td><em>C. neoformans</em> 93-219</td>
<td>0.29</td>
<td>20.2</td>
<td>2.5</td>
<td>$\leq 0.6$</td>
</tr>
</tbody>
</table>

†MIC read at 24 h for *C. albicans* and at 48 h for *C. neoformans*. ND, not done.
Table 2 Summary of murine survival studies of antifungal activity of albendazole

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Inoculum</th>
<th>Treatment groups (10 mice per group unless otherwise shown)</th>
<th>Mean survival (days)</th>
<th>Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>8.4 × 10^6 CFU intravenously</td>
<td>1—untreated 2—80 mg kg⁻¹ per day PO 3—40 mg kg⁻¹ per day PO 4—20 mg kg⁻¹ per day PO (albendazole suspended in noble agar)</td>
<td>4.9</td>
<td>NS</td>
</tr>
<tr>
<td>C. albicans</td>
<td>1.5 × 10^7 CFU intravenously</td>
<td>1—untreated 2—600 mg kg⁻¹ per day PO 3—300 mg kg⁻¹ per day PO (albendazole formulated in sesame oil)</td>
<td>6.8</td>
<td>NS</td>
</tr>
<tr>
<td>C. albicans</td>
<td>3.7 × 10^6 CFU intravenously</td>
<td>1—untreated 2—80 mg kg⁻¹ per day IP (albendazole formulated in DMSO)</td>
<td>6.5</td>
<td>P &lt; 0.04</td>
</tr>
<tr>
<td>C. albicans</td>
<td>2.2 × 10^6 CFU intravenously</td>
<td>1—untreated (n = 5) 2—80 mg kg⁻¹ per day IV (n = 5) (albendazole formulated in DMSO)</td>
<td>10.2</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>34 CFU intracranially</td>
<td>1—untreated 2—600 mg kg⁻¹ per day PO (albendazole in PB/RC pellets)</td>
<td>20.5</td>
<td>NS</td>
</tr>
<tr>
<td>C. neoformans</td>
<td>2.8 × 10^3 CFU intracranially</td>
<td>1—untreated 2—PB/RC only PO 3—600 mg kg⁻¹ per day PO 4—600 mg kg⁻¹ per day IP (in DMSO)</td>
<td>16.8</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

NS, not significant.

Table 3 Observed plasma concentrations of albendazole and albendazole sulphoxide (µg ml⁻¹)

<table>
<thead>
<tr>
<th>Dosage (mg kg⁻¹)</th>
<th>Route</th>
<th>Time (h)</th>
<th>Albendazole conc.</th>
<th>Albendazole sulphoxide conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>IP</td>
<td>1</td>
<td>None detected</td>
<td>2.10</td>
</tr>
<tr>
<td>80</td>
<td>IP</td>
<td>2</td>
<td>None detected</td>
<td>5.12</td>
</tr>
<tr>
<td>80</td>
<td>IP</td>
<td>4</td>
<td>None detected</td>
<td>8.70</td>
</tr>
<tr>
<td>600</td>
<td>IP</td>
<td>1</td>
<td>None detected</td>
<td>8.69</td>
</tr>
<tr>
<td>600</td>
<td>PO</td>
<td>1</td>
<td>None detected</td>
<td>0.25</td>
</tr>
<tr>
<td>300</td>
<td>PO</td>
<td>1</td>
<td>None detected</td>
<td>0.32</td>
</tr>
<tr>
<td>600</td>
<td>PO</td>
<td>1</td>
<td>None detected</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Parenteral administration, and control studies demonstrated that this was not due to the DMSO.

The lack of effect of albendazole in our survival studies raised the question of whether albendazole was being absorbed systemically in the dosages and routes of administration employed in the studies. To assess this issue, we administered similar regimens to uninfected mice and sacrificed them at variable times to measure plasma albendazole and albendazole sulphoxide concentrations. Regardless of the dose and route of albendazole administered in these studies, there was no evidence of parent albendazole in any plasma sample evaluated. Albendazole sulphoxide, however, was present in variable concentrations in each sample analysed. A probable explanation for this observation would be a very rapid conversion of albendazole to the inactive albendazole sulphoxide in vivo, consistent with our measurement of this metabolite in each of the murine plasma samples tested. This is supported by reports in the literature of an inability to measure parent albendazole in biological samples taken from animals or humans treated with albendazole, while albendazole sulphoxide concentrations are consistently observed [6–8].

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The absence of in vivo antifungal activity of albendazole observed in our studies illustrates an example of the possible discrepancy that can exist between in vitro and in vivo observations.

Clearly, albendazole has in vitro antifungal activity against *C. neoformans*, while its main metabolite, albendazole sulphoxide, has none. The very rapid metabolic conversion of absorbed albendazole to albendazole sulphoxide prevents the in vivo accumulation of sufficient concentrations of albendazole to have any observable antifungal activity. On the other hand, the albendazole sulphoxide metabolite does have significant antiparasitic activity, thus explaining the utility of systemically administered albendazole in the management of both human and veterinary helminth infections. In addition, it would appear that the albendazole sulphoxide metabolite is probably responsible for the reported activity of albendazole against *P. carinii* [5].

Acknowledgements

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References