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EXPERIMENTAL THERAPEUTICS

Antileishmanial Efficacy and Pharmacokinetics of DB766-Azole Combinations

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ABSTRACT Given the limitations of current antileishmanial drugs and the utility of oral combination therapy for other infections, developing an oral combination against visceral leishmaniasis should be a high priority. *In vitro* combination studies with DB766 and antifungal azoles against intracellular *Leishmania donovani* showed that posaconazole and ketoconazole, but not fluconazole, enhanced DB766 potency. Pharmacokinetic analysis of DB766-azole combinations in uninfected Swiss Webster mice revealed that DB766 exposure was increased by higher posaconazole and ketoconazole doses, while DB766 decreased ketoconazole exposure. In *L. donovani*-infected BALB/c mice, DB766-posaconazole combinations given orally for 5 days were more effective than DB766 or posaconazole alone. For example, 81% ± 1% (means ± standard errors) inhibition of liver parasite burden was observed for 37.5 mg/kg of body weight DB766 plus 15 mg/kg posaconazole, while 37.5 mg/kg DB766 and 15 mg/kg posaconazole administered as monotherapy gave 40% ± 5% and 21% ± 3% inhibition, respectively. Combination index (CI) analysis indicated that synergy or moderate synergy was observed in six of nine combined dose groups, while the other three were nearly additive. Liver concentrations of DB766 and posaconazole increased in almost all combination groups compared to monotherapy groups, although many increases were not statistically significant. For DB766-ketoconazole combinations evaluated in this model, two were antagonistic, one displayed synergy, and one was nearly additive. These data indicate that the efficacy of DB766-posaconazole and DB766-ketoconazole combinations *in vivo* is influenced in part by the pharmacokinetics of the combination, and that the former combination deserves further consideration in developing new treatment strategies against visceral leishmaniasis.

KEYWORDS DB766, *Leishmania*, chemotherapy, posaconazole

As the second-most deadly vector-borne parasitic disease, visceral leishmaniasis (VL) remains a major public health problem in several developing regions. The current treatment of VL with pentavalent antimonials, amphotericin B (either as a deoxycholate or liposomal formulation), paromomycin, and miltefosine is inadequate. These drugs are either parenteral (antimonials, amphotericin B formulations, and paromomycin), display significant toxicities (antimonials, amphotericin B deoxycholate, and miltefosine), or require long courses of administration (antimonials, amphotericin B deoxycholate, paromomycin, and miltefosine) (1). Combination therapy is a useful approach in treating infections because the doses of individual compounds can frequently be

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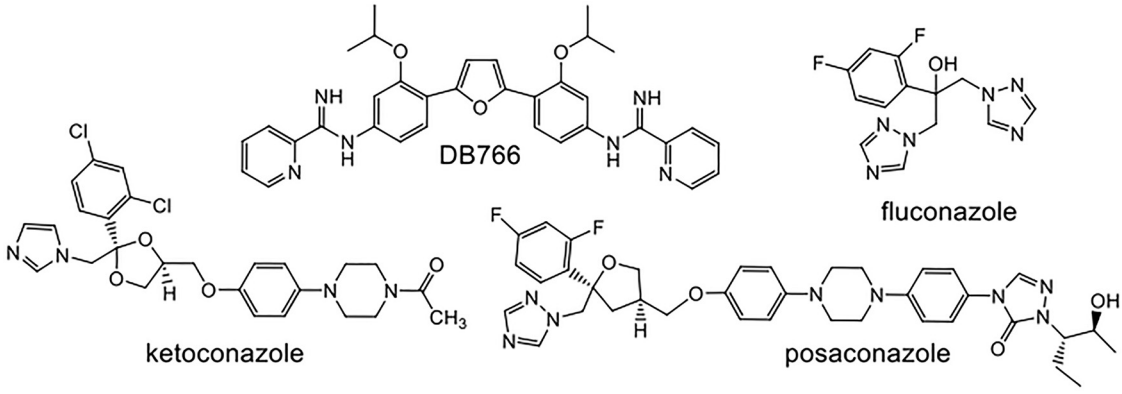

FIG 1 Structures of DB766, posaconazole, ketoconazole, and fluconazole.

lowered, resistance to individual components is minimized, and the duration of therapy is often shortened. Considering the success of oral drug combinations for other infectious diseases, such as tuberculosis and malaria, development of such a combination for VL would be a significant advance in VL treatment that would aid control/elimination efforts.

We previously showed that arylimidamides (AIAs) possess potent *in vitro* antileishmanial activity and good *in vivo* efficacy in rodent VL models. Through *in vitro* and *in vivo* testing of AIAs, 2,5-bis[2-(2-*i*-propoxy)-4-(2-pyridylimino) aminophenyl]furan hydrochloride (DB766; key chemical structures are given in Fig. 1) was identified as the most promising member of this class (2). DB766 exhibits 50% inhibitory concentrations (IC_{50}s) below 0.10 μM against intracellular *Leishmania donovani*, *L. amazonensis*, and *L. major*, is active in both murine and hamster models of *L. donovani* infection when given orally, is not mutagenic in an Ames assay, and does not increase liver or kidney enzyme levels when given at 100 mg/kg of body weight/day for five consecutive days in a repeat-dose toxicity study in mice (2). Although it possesses many favorable qualities as an antileishmanial candidate, DB766 does not display the therapeutic index needed to progress further for development as monotherapy against VL. While many other bis-AIAs have been synthesized, none are superior to DB766 (3–6). As part of an investigation into the mechanism of action of DB766, *L. donovani* axenic amastigotes resistant to this compound were raised through DB766 pressure in culture. The resultant DB766-resistant parasites were hypersensitive to the azole antifungal drugs posaconazole and ketoconazole, decreased expression of the protein CYP5122A1 was observed in the resistant parasites, and synergy was reported between DB766 and posaconazole against *L. donovani* β-lactamase-expressing parasites in an intracellular assay (7). CYP5122A1 is a novel cytochrome P450 essential for *L. donovani* survival that plays an unknown role in ergosterol biosynthesis in *Leishmania* (8), implicating sterol metabolism in the mechanism of action of DB766. Considering that these azole antifungals are orally available and have shown various degrees of antileishmanial efficacy when used in the clinic (9–11), *in vitro* interactions between DB766 and the oral azole antifungal agents posaconazole, ketoconazole, and fluconazole (Fig. 1 shows relevant chemical structures) have been investigated against intracellular *L. donovani* using a high-content assay. The *in vivo* pharmacokinetics and antileishmanial efficacy of DB766-posaconazole and DB766-ketoconazole combinations also have been determined in mouse models. The results presented here reveal the importance of pharmacokinetic considerations on the *in vivo* efficacy of these combinations.

RESULTS

Development of a high-content assay for intracellular *L. donovani*. Previous work in our laboratory made use of both microscopic enumeration (2) and parasites expressing a β-lactamase gene (12, 13) for the evaluation of compounds for activity

against intracellular *L. donovani*. Since microscopic enumeration is too tedious for routine evaluation of compounds and their combinations and because our β-lactamase parasites no longer reliably express this reporter gene, a high-content assay for intracellular *L. donovani* (see Fig. S1 in the supplemental material) was developed based on previously reported image-based assays (14, 15). Employing overnight infection of macrophages as done previously to validate the assay, the Z′ factor (16) for the infected versus uninfected controls was 0.74 ± 0.03 (means ± standard errors; $n = 6$). In these assays, the IC_{50} for amphotericin B (AMB) was 0.046 ± 0.002 μM ($n = 6$), while the IC_{50} for DB766 was 0.019 ± 0.001 μM ($n = 3$). Infected and uninfected control values along with a dose-response curve for AMB from a representative experiment are shown in Fig. S2. These values, obtained by high-content image analysis, are comparable with our previously reported IC_{50}s for AMB and DB766 against intracellular *L. donovani*. For AMB, we previously reported IC_{50}s of 0.066 μM (2) and 0.041 μM (13), as determined by microscopy and with the β-lactamase-expressing strain, respectively, while for DB766, we reported an IC_{50} of 0.036 μM (2) as determined by microscopy. Intracellular parasite burden was also analyzed, and the number of parasites per macrophage in untreated controls was static for the duration of the experiment (Table S1).

Analysis of the interaction between DB766 and posaconazole using fixed-ratio dilutions. A synergistic interaction was previously reported between DB766 and posaconazole against intracellular *L. donovani* using parasites expressing a β-lactamase gene (7). The interaction between DB766 and posaconazole was examined using the high-content assay described above. While posaconazole decreased intracellular parasite burden as measured in the high-content assay, it did not clear intracellular *L. donovani* from the macrophages, and precise IC_{50}s were difficult to determine with posaconazole because of its low *in vitro* selectivity for the parasites. When macrophages were infected for 4 to 6 h prior to washing in an attempt to limit intracellular parasite burden, IC_{50}s for DB766 were 0.020 ± 0.006 μM in four such experiments (means ± standard errors). An antiparasitic effect was observed with posaconazole in each of these experiments, but precise IC_{50}s for this azole could be calculated in only two of the four experiments due to the toxicity of this azole on the host cells. Given the mean ∑FIC (sum of the fractional inhibitory concentration) values for the DB766-posaconazole combination in experiments where IC_{50}s could be determined for posaconazole (1.2 for both) and the FIC values for DB766 in the experiments in which IC_{50}s for posaconazole could not be calculated (ranging from 0.63 to 0.97 at different DB766/posaconazole ratios), the interaction between DB766 and posaconazole against *L. donovani* is characterized as indifferent by the fixed-ratio method using the intracellular high-content assay. The results of these individual experiments are summarized in tabular form in Table S2.

***In vitro* combinations of DB766 with posaconazole, ketoconazole, and fluconazole.** With the goal of identifying oral combinations for *in vivo* evaluation as antileishmanial candidates, the effect of three orally available azole antifungals was examined in combination with DB766. It was difficult to determine an IC_{50} for ketoconazole in the intracellular assay for the same reasons described above for posaconazole, while fluconazole displayed little activity against intracellular *L. donovani in vitro*. Thus, the effect of fixed concentrations of these azole drugs on the IC_{50} of DB766 was determined (Fig. 2). In each case, the fixed concentrations of the azole drugs posaconazole, ketoconazole, and fluconazole, included with serial dilutions of DB766, resulted in <50% inhibition of parasite burden when used alone (representative dose-response scatter plots for these azoles in the intracellular *L. donovani* assay are provided in Fig. S3). Inclusion of 4 μM posaconazole or ketoconazole at 20 μM, 15 μM, and 10 μM resulted in a significant reduction of the DB766 IC_{50} ($P < 0.01$), while addition of 2 μM posaconazole or fluconazole at 20 μM and 40 μM did not have a significant effect on the IC_{50} of DB766 against intracellular *L. donovani*.

Efficacy of posaconazole and ketoconazole in the murine visceral leishmaniasis model. Since both posaconazole and ketoconazole enhanced the activity of DB766 against intracellular *L. donovani in vitro*, combinations between these two azoles and

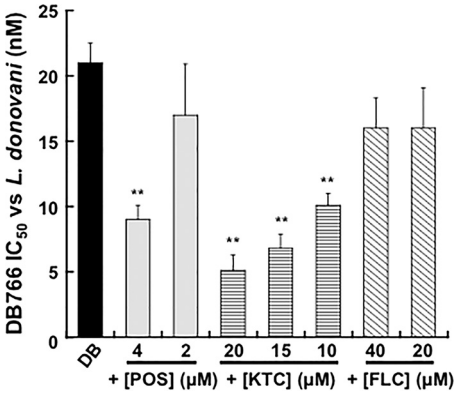

FIG 2 *In vitro* evaluation of DB766-azole combinations against intracellular *L. donovani*. Posaconazole (POS; gray bars), ketoconazole (KTC; horizontal striped bars), or fluconazole (FLC; diagonal striped bars) was added to a serial dilution of DB766, with the azole at a fixed concentration, in at least three independent experiments. The concentrations of azole drugs employed were below those required for 50% inhibition alone (parasite burden was reduced by 39% ± 3% at 6.3 μM posaconazole alone [$n = 3$], 23% ± 8% at 25 μM ketoconazole alone [$n = 3$], and 14% ± 5% at 50 μM fluconazole alone [$n = 4$]). Error bars and measurements represent the standard errors of the means. Two-sided Student's *t* test was used to compare the groups to the DB766-alone group. **, $P < 0.01$.

DB766 were evaluated *in vivo*. Prior to testing these combinations in mice, the maximum tolerated doses and the efficacy of posaconazole and ketoconazole were determined in the same murine models that were to be used to assess the combinations. Posaconazole and ketoconazole were first assessed at doses of up to 100 mg/kg by the oral route in groups of two uninfected mice per dose. Posaconazole was tolerated at an oral dose of 100 mg/kg/day for 5 days. One animal met euthanasia criteria in the group given ketoconazole at 100 mg/kg/day for 5 days, but this drug was tolerated by both mice at a dose of 50 mg/kg/day for 5 days.

Posaconazole and ketoconazole were evaluated in the murine visceral leishmaniasis model reported on several occasions to test various compounds/formulations for antileishmanial activity (4, 17, 18), including DB766 (2). According to the results of the tolerability study outlined above, the highest dose of posaconazole administered was 100 mg/kg/day for 5 days, while the highest dose of ketoconazole given was 50 mg/kg/day for 5 days. Both azoles showed good activity in this model, with posaconazole and ketoconazole exhibiting ED_{50} values (the dose of the compound providing 50% reduction of the liver parasite burden compared to the positive-control group) of 21 mg/kg and 13 mg/kg, respectively (Fig. 3). No apparent drug-related adverse effects

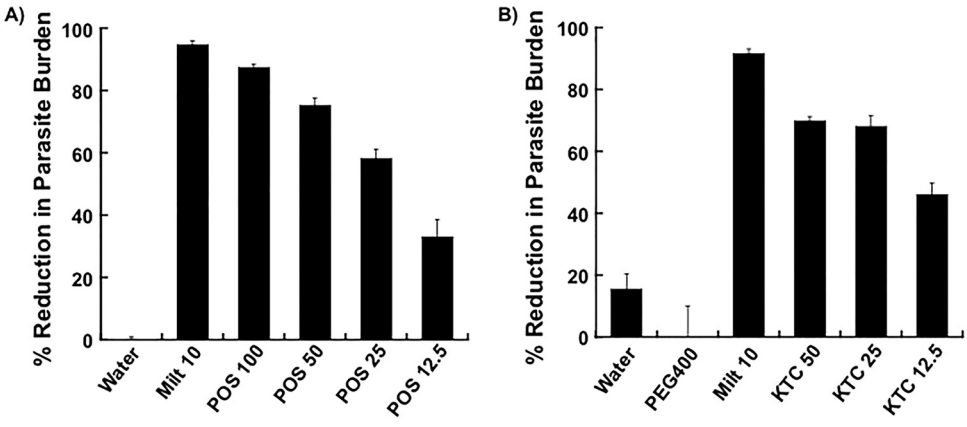

FIG 3 *In vivo* analysis of posaconazole and ketoconazole against *L. donovani*-infected BALB/c mice. Posaconazole (POS) (A) and ketoconazole (KTC) (B) were given p.o. daily for 5 consecutive days to infected mice at the doses indicated (in mg/kg). Results are shown as the percent reduction in LDU (Leishman-Donovan units) in liver tissue versus untreated controls, with error bars representing the standard errors of the means.

TABLE 1 Pharmacokinetic parameters of oral posaconazole and DB766 when administered alone and in combination to healthy mice

	Value(s) for:											
	Posaconazole						DB766					
Parameter,[a] unit	Alone			Combined with DB766			Alone			Combined with posaconazole		
Posaconazole dose, mg/kg (μmol/kg)	25 (36)	50 (71)	100 (143)	25 (36)	50 (71)	100 (143)	0	0	0	100 (143)	50 (71)	25 (36)
DB766 dose, mg/kg (μmol/kg)	0	0	0	100 (174)	50 (87)	25 (44)	25 (44)	50 (87)	100 (174)	25 (44)	50 (87)	100 (174)
C_{max}, μM	13 ± 1	19 ± 4	29 ± 5	13 ± 2	21 ± 6	32 ± 3	0.26 ± 0.06	1.9 ± 1.8	2.1 ± 1.2	0.26 ± 0.15	4.7 ± 2	1.6 ± 0.2
T_{max}, h	12	12	12	12	24	24	2.0	0.5	2	1.0	1.0	2
$AUC_{0-\infty}$, μM · h	392	698	858	437	802	1285	6.6	22	59	11	44	36
$t_{1/2}$, h	11	9.6	8.4	11	9.3	11	30	25	26	33	36	29
CL/F, liter/h/kg	0.092	0.10	0.17	0.082	0.089	0.11	6.6	4.0	2.9	4.1	2.0	4.8

[a]Abbreviations: C_{max}, maximum plasma concentration ± standard deviations; T_{max}, time to reach C_{max}; $AUC_{0-\infty}$, area under the plasma concentration-time curve from time zero to infinite time; $t_{1/2}$, terminal elimination half-life; CL/F, apparent clearance without adjusting for bioavailability (F).

were observed in these studies, with the exception of one mouse in the 100-mg/kg posaconazole group that met early removal criteria. At an oral dose of 10 mg/kg/day, the miltefosine standard showed efficacy consistent with that observed previously (4, 6).

Pharmacokinetics of DB766-posaconazole and DB766-ketoconazole combinations. Effects of the partner drug on the pharmacokinetics of individual components of DB766-posaconazole combinations were determined using healthy mice. The two higher doses (50 and 100 mg/kg) of DB766 had little effect (<15%) on the plasma exposure (maximum plasma drug concentration [C_{max}] and area under the concentration-time curve [AUC]) of posaconazole (Table 1 and Fig. S4). Although the low dose (25 mg/kg) of DB766 appears to have increased the plasma exposure (by 50% for AUC) of posaconazole, such an increase was presumed to be a result of intrinsic variability of posaconazole pharmacokinetics at the high dose (100 mg/kg). In contrast, the two higher doses (50 and 100 mg/kg) of posaconazole markedly increased the plasma exposure (by up to 150% for C_{max} and up to 100% for AUC) of DB766 (Table 1 and Fig. S5). An *in vitro* metabolic stability study showed that microsomal half-lives of DB766 increased in the presence of posaconazole (see Table 3), suggesting an inhibitory effect of posaconazole on DB766 metabolism. Hence, inhibition of DB766 metabolism by posaconazole likely contributed to the decreased clearance and increased exposure to DB766 observed in the mouse pharmacokinetic study.

Likewise, effects of the partner drug on the pharmacokinetics of individual components of DB766-ketoconazole combinations were determined using healthy mice. DB766 dose-dependently decreased the plasma C_{max} (by 1.3- to 4.9-fold) of ketoconazole, and it also markedly decreased the plasma AUC (by 1.3- to 2.4-fold) of ketoconazole (Table 2 and Fig. S6). In contrast, the two higher doses (15 and 30 mg/kg) of ketoconazole increased the plasma exposure (by up to 88% for C_{max} and up to 68% for AUC) of DB766 (Table 2 and Fig. S7). Metabolic stability study showed that microsomal half-lives of DB766 increased in the presence of ketoconazole (Table 3), suggesting an inhibitory effect of ketoconazole on DB766 metabolism. Hence, like posaconazole, inhibition of DB766 metabolism by ketoconazole likely contributed to the decreased

TABLE 2 Pharmacokinetic parameters of oral ketoconazole and DB766 when administered alone and in combination to healthy mice

	Value(s) for:											
	Ketoconazole						DB766					
Parameter,[a] unit	Alone			Combined with DB766			Alone			Combined with ketoconazole		
Ketoconazole dose, mg/kg (μmol/kg)	7.5 (14)	15 (28)	30 (56)	7.5 (14)	15 (28)	30 (56)	0	0	0	30 (56)	15 (28)	7.5 (14)
DB766 dose, mg/kg (μmol/kg)	0	0	0	50 (87)	25 (44)	12.5 (22)	12.5 (22)	25 (44)	50 (87)	12.5 (22)	25 (44)	50 (87)
C_{max}, μM	0.74 ± 0.41	1.8 ± 0.8	4.5 ± 1.7	0.15 ± 0.03	1.2 ± 0.8	3.5 ± 1.4	0.08 ± 0.04	0.25 ± 0.1	1.2 ± 0.9	0.15 ± 0.04	0.37 ± 0.3	0.82 ± 0.3
T_{max}, h	0.50	0.50	1.0	1.0	1.0	0.5	2.0	2.0	0.5	12	2.0	2
$AUC_{0-\infty}$, μM · h	1.9	5.8	30	0.8	4.4	19	3.7	6.8	19	6.2	7.0	21
$t_{1/2}$, h	1.7	1.8	3.6	1.7	1.7	2.8	25	24	26	24	25	29
CL/F, liter/h/kg	7.3	4.8	1.9	17	6.3	3.0	5.9	6.4	4.7	3.6	6.3	4.1

[a]Abbreviations: C_{max}, maximum plasma concentration ± standard deviations; T_{max}, time to reach C_{max}; $AUC_{0-\infty}$, area under the plasma concentration-time curve from time zero to infinite time; $t_{1/2}$, terminal elimination half-life; CL/F, apparent clearance without adjusting for bioavailability (F).

TABLE 3 Metabolic stability of DB766 in mouse liver microsomes in the presence of an azole as inhibitor[a]

		Inhibitor					
		Posaconazole			Ketoconazole		
DB766 substrate (μM)	No inhibitor	3 μM	10 μM	30 μM	0.1 μM	1 μM	10 μM
0.2	33	48	60	67	37	47	63
2	69	68	91	111	70	88	113

[a]Metabolic stability is measured as half-life, in minutes.

clearance and increased exposure of DB766 observed in the mouse pharmacokinetic study.

Efficacy of DB766-posaconazole and DB766-ketoconazole combinations in a murine visceral leishmaniasis model. A checkerboard approach was used to evaluate the efficacy of DB766-posaconazole combinations in *L. donovani*-infected BALB/c mice in which three dose groups of the two compounds alone (DB766 at 75, 37.5, and 18.8 mg/kg/day for 5 days; posaconazole at 30, 15, and 7.5 mg/kg/day for 5 days) were included to assess the efficacy of the monotherapies along with the nine different combinations of these doses given to other groups of animals. Including the control group, which received the polyethylene glycol 400 (PEG400) vehicle, 16 groups containing four mice in each group were used in this combination study. The results of this study are shown in Fig. 4. The ED_{50} value for DB766 alone in this experiment is 45 mg/kg, as calculated by Compusyn software, while the ED_{50} value of posaconazole determined in this experiment is 27 mg/kg/day. In all cases, the DB766-posaconazole combinations were more effective at reducing liver parasite burden than an equivalent dose of either DB766 alone or posaconazole alone. Combination indexes (CIs) for each combination group were also calculated with the aid of Compusyn. This method has been used on numerous occasions to assess synergy between drug combinations (19–22). CI values calculated for each combination enable a determination of the interaction between individual components at each dose. For DB766-posaconazole combinations, four of the groups showed synergism (CI, 0.30 to 0.70), two were moderately synergistic (CI, 0.70 to 0.85), and three were nearly additive (CI, 0.90 to 1.10) in the murine visceral leishmaniasis model according to the definitions for these effects provided in earlier papers (19, 20).

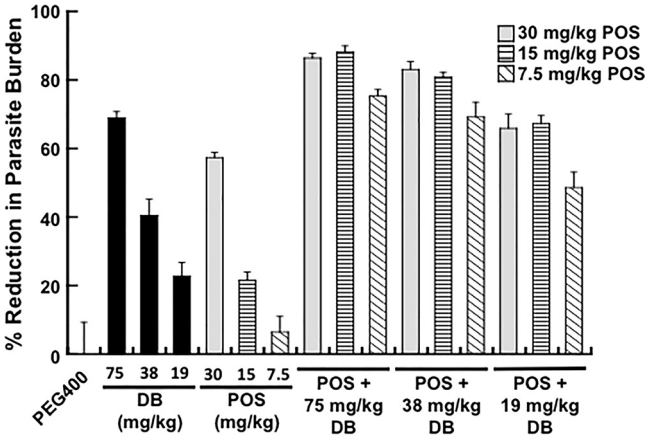

FIG 4 *In vivo* analysis of posaconazole-DB766 combinations in *L. donovani*-infected female BALB/c mice. Compounds were given p.o. daily for five consecutive days to infected mice starting 1 week postinfection in the presence of the indicated doses (in mg/kg/day) of DB766 (DB) and/or posaconazole (POS). Liver smears were prepared 2 weeks postinfection, and parasite burden was determined microscopically. Results are shown as the percent reduction of liver parasite burden versus the PEG400 control with error bars representing the standard errors of the means.

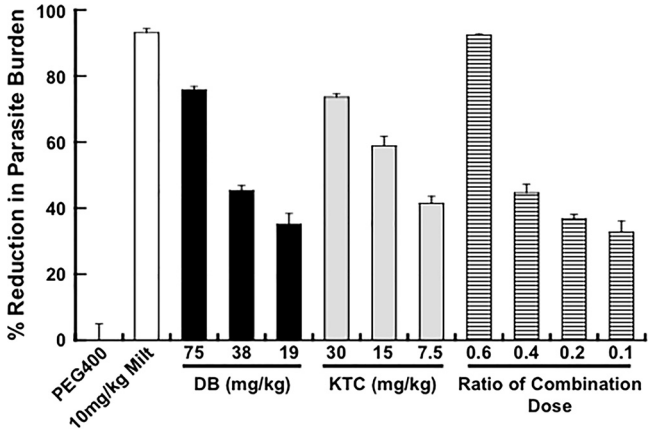

FIG 5 *In vivo* analysis of ketoconazole-DB766 combinations in *L. donovani*-infected female BALB/c mice. Compounds were given p.o. daily for five consecutive days to infected mice starting 1 week postinfection in the presence of the indicated doses (mg/kg/day) of DB766 (DB; black bars) and/or ketoconazole (KTC; gray bars). The combinations (horizontal striped bars) are given as a ratio of each of the highest doses of ketoconazole and DB766. The miltefosine control group (white bar) received this compound at a dose of 10 mg/kg/day for 5 days by the oral route. Liver parasite burden was determined, and results are presented as described in the text.

The approach described by Chou (20) was employed to examine the antileishmanial efficacy of the DB766-ketoconazole combination (Fig. 5). According to this strategy, the highest-combination dose contains a fraction of the maximum dose of each drug administered alone (in this case, 0.6 + 0.6). Since the highest doses of DB766 and ketoconazole administered alone were 75 mg/kg/day and 30 mg/kg/day, respectively, the highest combination dose examined in this experiment was 45 mg/kg DB766 plus 18 mg/kg ketoconazole. The remaining doses for the combinations are also fractions of the highest single doses of each drug at 0.4 + 0.4, 0.2 + 0.2, and 0.1 + 0.1. Similar ED_{50} values for both DB766 and ketoconazole were determined in this experiment compared to those observed earlier (ED_{50} values of 34 mg/kg and 11 mg/kg, respectively) (Fig. 5). However, only one of the DB766-ketoconazole combination groups showed synergism (45 mg/kg DB766 plus 18.0 mg/kg ketoconazole; CI, 0.32), while the two intermediate-dose groups displayed antagonism (30 mg/kg DB766 plus 12.0 mg/kg ketoconazole; 15.0 mg/kg DB766 plus 6.0 mg/kg ketoconazole; CI, 2.5 and 1.7, respectively). The lowest-dose DB766-ketoconazole combination was nearly additive (CI, 0.97).

Liver concentrations of DB766 and posaconazole in *L. donovani*-infected BALB/c mice. In an attempt to correlate the effectiveness of the DB766-posaconazole combinations with the levels of compound in the target organ, liver concentrations of both DB766 and posaconazole were determined at the end of the efficacy experiment in *L. donovani*-infected mice. High concentrations of DB766 were observed in the liver (Fig. 6A), consistent with earlier published results (2). There was a slight, but insignificant, decrease in the liver concentration of DB766 when given at 75 mg/kg/day in the presence of posaconazole at 7.5 or 15 mg/kg/day. For all other DB766-posaconazole combinations, there was an increase in the DB766 concentration in the liver when DB766 was administered with posaconazole that was statistically significant in two dose groups. There were no decreases in posaconazole liver concentrations when this drug was given together with DB766 compared to when this azole was given alone (Fig. 6B). On the contrary, in all cases, posaconazole concentrations in the liver increased when the drug was given together with DB766, although most were not statistically significant due to the high variability (Fig. 6B). Table 4 provides a listing of efficacy, CI values, and liver concentrations for each group receiving DB766 and/or posaconazole in this experiment.

DISCUSSION

The interaction between azole drugs and other classes of compounds has been examined in fungi and in kinetoplastid parasites. Several studies have demonstrated

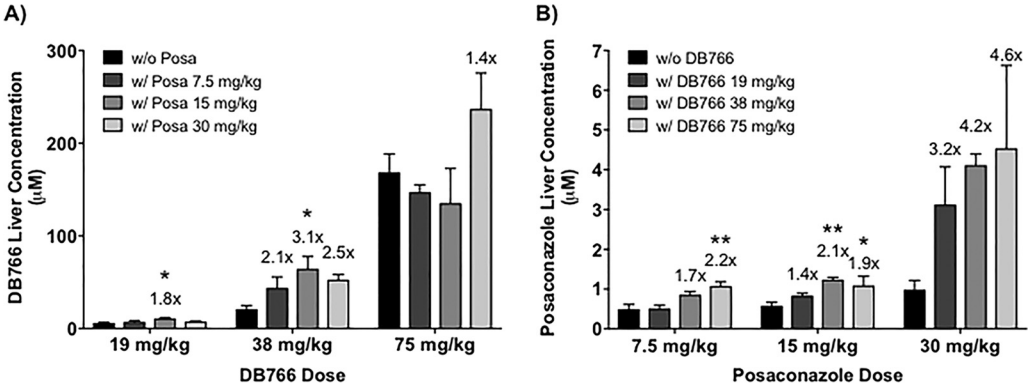

FIG 6 Tissue levels of DB766 (A) and posaconazole (B) in liver samples obtained from *L. donovani*-infected BALB/c mice. Infected mice were treated with DB766, posaconazole, or DB766-posaconazole combinations as indicated in Fig. 4. At 2 weeks postinfection, liver samples from each animal were obtained and stored at −20°C or below prior to determination of drug concentrations by UPLC-MS/MS. Symbols and error bars represent the means and standard errors of quadruplicate determinations. One-way analysis of variance was used to compare treatments with combinations to drug alone (*, $P < 0.05$; **, $P < 0.01$).

synergy between azoles and calcineurin inhibitors in fungi (23–26), while most of the *in vitro* interactions between approved systemic antifungal drugs were indifferent when assessed in three *Candida* species and two strains of *Aspergillus fumigatus* (27). In *Trypanosoma cruzi*, combinations meeting the definition of *in vitro* synergy were not always the most effective pairs *in vivo* (28). Interestingly, two combinations containing posaconazole had superior efficacy compared to these drugs when administered alone in a mouse model of *T. cruzi* infection (28). *In vitro* synergy has been described between the squalene synthase inhibitor E5700 and both posaconazole and itraconazole against intracellular *L. amazonensis* (29). We earlier reported *in vitro* synergy between DB766 and posaconazole in an intracellular assay using β-lactamase-expressing *L. donovani* parasites (7). However, the results described here, obtained from a high-content assay using wild-type *L. donovani* parasites, indicates an additive or indifferent interaction between these compounds. Despite the differences in the interactions observed between DB766 and posaconazole in the two intracellular studies, IC_{50}s for DB766 and for posaconazole were similar, although the current high-content assay more clearly

TABLE 4 Efficacy, CI values, and liver concentrations for groups in the DB766-posaconazole combination efficacy study in *L. donovani*-infected mice

DB766 dose[a]	Posaconazole dose[a]	% Reduction in parasite burden	CI value	Liver [DB766],[b] in μM (fold change)	Liver [POS],[b] in μM (fold change)
75	—[c]	69	—	168	—
37.5	—	40	—	21	—
18.8	—	23	—	6.3	—
—	30	57	—	—	0.99
—	15	21	—	—	0.59
—	7.5	6.5	—	—	0.50
75	30	86	0.94	237 (1.4)	4.5 (4.6)
75	15	88	0.64	135 (0.80)	1.1 (1.9)*
75	7.5	75	0.94	147 (0.88)	1.1 (2.2)**
37.5	30	83	0.81	53 (2.5)	4.1 (4.2)
37.5	15	81	0.60	65 (3.1)*	1.2 (2.1)**
37.5	7.5	69	0.67	44 (2.1)	0.86 (1.7)
18.8	30	66	1.09	7.9 (1.3)	3.1 (3.2)
18.8	15	67	0.66	11 (1.8)*	0.84 (1.4)
18.8	7.5	49	0.72	7.3 (1.2)	0.52 (1.0)

[a]Doses are given in mg/kg/day administered by oral gavage.
[b]Calculated as the tissue concentration of the compound when given at the specified dose in combination divided by the tissue concentration of the compound when administered alone at the same dose. *, $P < 0.05$; **, $P < 0.01$.
[c]—, no value (e.g., when only one compound is given or when no CI value can be calculated).

reveals the *in vitro* toxicity of posaconazole to peritoneal macrophages, as reflected by a reduction in macrophage nuclei at higher azole concentrations. Ren et al. reported that agreement rates for antifungal synergy testing were higher when MIC_{100} values were used as endpoints rather than MIC_{50} values (27). Complete inhibition cannot be used as an endpoint in these posaconazole/DB766 interaction experiments due to the inability of posaconazole to clear parasites from macrophages in our hands. Differences between the β-lactamase and high-content assays and between the β-lactamase-expressing and wild-type parasites also may account for the difference in the interaction between DB766 and posaconazole reported in the two studies. Unfortunately, the original data for the β-lactamase assays examining the DB766-posaconazole interaction (7) are not available for comparison.

Both posaconazole and ketoconazole showed *in vitro* antileishmanial activity in the present work as well as in other studies (29–31), while fluconazole had little effect on *Leishmania in vitro* either in our hands or in previous reports (30, 32). Thus, the latter drug was not considered for *in vivo* combination experiments. Results reported here are consistent with the work of Al-Abdely et al., showing that posaconazole reduced liver parasite burden by 0.5 to 1 log units in *L. donovani*-infected mice at an oral dose of 30 mg/kg given daily for 21 days (33). In female BALB/c mice infected with *L. infantum*, ketoconazole had no observable effect on liver parasite burden but cleared the infection from the spleen when given orally at 100 mg/kg/day for 11 days (34). The calculated ED_{50} values in the present murine VL model for posaconazole (21 mg/kg/day in Fig. 3A and 27 mg/kg/day in Fig. 4) and for ketoconazole (13 mg/kg/day in Fig. 3B and 11 mg/kg/day in Fig. 5) are lower than the ED_{50} values for DB766 (Fig. 4 and 5 and Wang et al. [2]). This is surprising given that DB766 is much more potent in the *in vitro* antileishmanial assay in our hands than these azoles and also accumulates in the liver (the target organ examined in our murine visceral leishmaniasis model) to a greater extent than posaconazole in *L. donovani*-infected mice (Fig. 6). The reason for the disconnect between *in vitro* and *in vivo* efficacy is unknown but may relate to the mechanisms of action of these compounds, their possible effects on the host immune system, and/or their potential distribution to different portions of the infected liver. The effect exerted by posaconazole and ketoconazole on the parasites may provide an explanation for the disconnect between the *in vitro* and *in vivo* antileishmanial activity of these azoles observed in the present study. Azoles generally are regarded as having a static antifungal effect on yeasts (35). In our intracellular *L. donovani* high-content assay, there is little to no multiplication of the untreated control parasites within the macrophages over the 72-h course of incubation with compounds (see Table S1 in the supplemental material). This is generally consistent with previous observations in our intracellular assay using β-lactamase-expressing *L. donovani*, where modest proliferation was observed over the course of the infection (see Fig. 1 in reference 36). Thus, if the azoles have a static effect on *Leishmania*, they may show modest activity in such an assay. In the infected BALB/c mouse, however, *L. donovani* liver parasite burden increases over the first month of infection (37), so a static azole compound may be effective in this murine model.

Given that posaconazole is a known inhibitor of hepatic CYP3A4 (38) and increases the microsomal half-life of DB766 (Table 3), inhibition of DB766 metabolism in mouse liver may be responsible for the increased exposure of DB766 in the presence of this azole. Ketoconazole is also a potent inhibitor of CYP3A4 but possesses activity against other CYPs as well (39) and also prolongs the microsomal half-life of DB766 (Table 3). While the highest dose of ketoconazole increased DB766 exposure, DB766 reduced ketoconazole exposure (AUC and C_{max}) by 22 to 80% (Table 2), perhaps due to reduction of the oral absorption of ketoconazole by DB766. Based solely on these initial pharmacokinetic experiments in uninfected mice, the posaconazole-DB766 combination would be predicted to be more effective than the ketoconazole-DB766 combination.

In the DB766-posaconazole combination study in the murine visceral leishmaniasis model, the lowest CI value (0.60) was observed in mice receiving 37.5 mg/kg/day DB766

and 15 mg/kg/day posaconazole. For this combination, there was an approximately 3-fold increase in the concentration of DB766 in the liver and about a 2-fold increase in the liver concentration of posaconazole compared to levels for the corresponding monotherapy dose groups; both of these increases were statistically significant ($P < 0.05$ and $P < 0.01$, respectively). Other levels of synergy/moderate synergy (CI values of 0.64, 0.66, 0.67, 0.72, and 0.81) or indifference/additivity (CI values of 0.94, 0.94, and 1.09) were accompanied by either increases in the liver concentration of one or both of the compounds compared to the levels for corresponding monotherapy groups or little to no change in these liver concentrations. Thus, we hypothesize that the favorable pharmacokinetics of the DB766/posaconazole combination, as observed in both uninfected animals (Table 1 and Fig. S3 and S4) and infected animals (Fig. 6, Table 4), contributes to the antileishmanial synergy observed for this combination in the murine visceral leishmaniasis model. In the DB766-ketoconazole study, only the highest dose of the DB766-ketoconazole combination (DB766 at 45 mg/kg/day, ketoconazole at 18 mg/kg/day) showed a synergistic CI value (0.32), while the two intermediate doses of the DB766-ketoconazole combination were antagonistic (CI, >1.5) and the lowest dose was indifferent. At the high dose of ketoconazole administered to uninfected animals (30 mg/kg), the AUC for DB766 increases by 68%, while lower doses of ketoconazole do not result in comparable increases in DB766 AUC (Table 2). However, coadministration of DB766 lowers the AUC and C_{max} of ketoconazole (Table 2). Thus, these pharmacokinetic studies in uninfected animals may help to explain the favorable interaction seen between DB766 and ketoconazole at the highest dose of ketoconazole and the less favorable interactions observed at lower doses. Unfortunately, the liver samples from the DB766-ketoconazole combination efficacy study were unavailable for PK analysis due to sample loss caused by a freezer failure.

Combinations of known antileishmanial drugs have shown excellent efficacy against visceral leishmaniasis in clinical trials (40–42), but all of these combinations include at least one injectable agent. Promising results also have been observed in rodent models of visceral leishmaniasis with combinations of diminazene and chloroquine (43), miltefosine plus a liposomally encapsulated immunostimulatory oligonucleotide (44), and antimony plus atovaquone (45), but these experimental combinations likewise contain an injectable component. The work reported here explores oral combinations of the frontrunner AIA DB766 with two orally available antifungal azoles possessing antileishmanial activity and highlights the influence of pharmacokinetics on the efficacy of these combinations. Given the efficacy of the DB766-posaconazole combination in the murine visceral leishmaniasis model, further evaluation of this pair of agents in the hamster model, which more closely mimics the progression of human VL, is warranted. The use of CYP inhibitors in combination therapy in both HIV and HCV therapy argues for the exploration of this approach against other infections; the CYP inhibitor in this case (posaconazole) has the advantage of possessing *in vivo* antileishmanial activity in its own right (Fig. 3A and 4). Furthermore, the present study contrasts the antileishmanial properties of the frontrunner AIA DB766 with those of posaconazole and ketoconazole. DB766 exhibits potent *in vitro* antileishmanial activity but lesser *in vivo* efficacy than ketoconazole and posaconazole, which display lower antileishmanial potency than DB766 *in vitro*. Thus, it may be useful to identify structural features that are responsible for antileishmanial potency and favorable pharmacokinetics from both classes of molecules as a strategy to discover new antileishmanial drug candidates.

MATERIALS AND METHODS

Parasites and culture conditions. *Leishmania donovani* LV82 promastigotes were cultured at 26°C in Schneider's insect medium (Life Technologies, Grand Island, NY) containing 25% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO), 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies). Experiments with these parasites were performed in accord with protocols approved by The Ohio State University Institutional Biosafety Committee.

Drugs and reagents. Miltefosine was purchased from Cayman Chemical Company (Ann Arbor, MI). Azoles were obtained from the following sources: ketoconazole was from TCI (Portland, OR), posaconazole was from Carbosynth (San Diego, CA), and fluconazole was from Cayman Chemical (Ann Arbor, MI).

DB766 was synthesized as described previously (2). All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

***In vitro* susceptibility assays.** An intracellular *L. donovani* high-content imaging assay was developed based on previously published methods (14, 15). Briefly, starch-elicited peritoneal macrophages harvested from CD-1 mice were added to 96-well plates (Corning, Corning, NY) at a density of 1×10^5 macrophages/well in a total volume of 100 μl macrophage medium (RPMI 1640 with GlutaMAX [Life Technologies, Grand Island, NY], 10% heat-inactivated FBS [Sigma-Aldrich], 100 U/ml penicillin, and 100 μg/ml streptomycin [Life Technologies], pH 7.4) and allowed to adhere overnight. The host cells then were infected with stationary-phase *L. donovani* LV82 promastigotes at a parasite/macrophage ratio of 5:1 at 37°C in a 5% CO_2 atmosphere. Extracellular parasites were removed by washing with Hanks' balanced salt solution (HBSS; Life Technologies) after either 4 to 6 h or overnight incubation. In the case where washing was performed after 4 to 6 h, fresh medium was added and incubation was continued overnight. After the overnight incubation period in both cases, cells were washed with HBSS and fresh medium containing either the compound(s) or the standard drug amphotericin B was added. Plates again were placed at 37°C in a 5% CO_2 atmosphere for an additional 72 h. Following this incubation, plates were washed with phosphate-buffered saline (PBS) and fixed for 30 min in 10% formalin in PBS (RICCA Chemical Company, Arlington, TX). After fixation, cells were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed again in PBS, and stained with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies) in PBS for 10 min. After a final wash in PBS, 100 μl of PBS was added and plates were stored at 4°C prior to analysis. Images were acquired and analyzed using a Thermo Scientific ArrayScan XTI live high-content platform (Thermo Fisher Scientific, Pittsburgh, PA).

In order to analyze these images, a standard DAPI filter set and the 20× lens standard on the Thermo Scientific ArrayScan XTI was used to image 15 central fields of view from each well of stained samples (each field, 485 by 485 μm [1,104 by 1,104 pixels at 440 nm/pixel]) using an exposure time approximately constraining pixel intensities to 4,000 gray levels (25% of the 14-bit range on the X1 charge-coupled-device camera). Images were analyzed by the integrated SpotDetector V4 BioApplication algorithm. Parasite nuclei (validated as 2 to 22 μm^2) were identified in annular (ring) regions of interest (ROIs) originating one pixel outside the borders of a host cell nuclei (identified by a higher intensity threshold than that for parasite nuclei and validated as 32 to 220 μm^2) and dilated isodiametrically to 50 pixels or to collision with adjacently dilating ROIs (no ROI overlap). In sum, DNA puncta within 22 μm of presumptive host cell nuclei are ascribed to the nearest cell as an infection. Additional SpotDetector parameters are available upon request.

IC_{50}s (the concentration of the compound required to reduce the parasite burden by 50% compared to infected controls) were calculated from the number of parasites per macrophage using the four-parameter equation $y = m1 + (m2 - m1)/[1 + (x/m3)^{m4}]$, determined by Kaleidagraph software (Synergy Software, Reading, PA) to provide the relative IC_{50}, where $m1$ is lower asymptote, $m2$ is upper asymptote, $m3$ is relative IC_{50}, and $m4$ is slope. This relative IC_{50} then was used to calculate the absolute IC_{50} using the following equation: absolute $IC_{50} = \exp(\log(m3) + \log((m2 - m1)/(50 - m1) - 1)/m4)$. Absolute IC_{50}s are provided throughout the manuscript.

To determine the effect of combining DB766 with posaconazole in fixed-ratio dilutions (46), each drug in a dilution series (drug A:drug B, 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5) was used. The fractional inhibitory concentration (FIC) for DB766 is defined as the IC_{50} of DB766 in combination/IC_{50} of DB766 alone. This analysis was then repeated for posaconazole when an IC_{50} could be determined and the average sum of the FICs was calculated. A mean ΣFIC of ≤0.5 indicates a synergistic *in vitro* interaction, a mean ΣFIC between 0.5 and 4 indicates an indifferent interaction, and a mean ΣFIC of >4 indicates antagonism (47). To determine the effect of azoles on the DB766 IC_{50}, the activity enhancement index (AEI) approach was used (48). Serial dilutions of DB766 (the active compound) were plated with fixed concentrations of the antifungal azole (the less active or inactive compound). IC_{50}s for DB766 alone and DB766 in combination were calculated as described above.

***In vivo* toxicity.** *In vivo* toxicity and efficacy studies were performed in accord with protocols approved by The Ohio State University IACUC. Six- to 8-week-old female BALB/c mice were given the appropriate doses of single agent or combination therapy via oral gavage once daily for 5 days. The overall appearance, weight, and activity of the animals were monitored, and any animals exhibiting adverse effects due to compound administration were euthanized. All animals were euthanized 24 h after the final dose and a gross necropsy was performed.

***In vivo* efficacy.** Experiments in the murine visceral leishmaniasis model were performed as described previously (17), with minor modifications. Briefly, BALB/c mice were inoculated with 5×10^7 *L. donovani* promastigotes intravenously via the tail vein. After infection, mice were randomly sorted (www.randomization.com; subjects randomized into 1 block) into groups of four and marked for individual identification. Treatment began 7 days postinfection and continued once daily for 5 days. DB766, posaconazole, and ketoconazole were suspended in PEG400 and given via oral gavage (p.o.). Control groups received either PEG400 vehicle or miltefosine (10 mg/kg/day) dissolved in water. Mice were euthanized on day 14 postinfection, livers were removed, and liver smear slides were prepared and stained with Giemsa. The number of amastigotes per 100 cell nuclei were determined microscopically and provided as Leishman-Donovan units (LDU). The following equation was used to calculate LDUs: LDU = number of amastigotes per 1,000 cell nuclei × total liver weight in grams. To determine the interactions between compounds used in combination, combination indexes were calculated using the program Compusyn (ComboSyn, Inc., Paramus, NJ) (19).

Metabolic stability, pharmacokinetics, and tissue concentration. Metabolic stability of DB766 was determined using mouse liver microsomes in the presence of different concentrations of posaconazole

or ketoconazole. Microsomal incubations were carried out as described previously (2, 49). The mouse liver microsomal protein concentration was 0.5 mg/ml. DB766 (as substrate) concentrations (0.2 and 2 μM) and azole (as inhibitor) concentrations (3, 10, and 30 μM for posaconazole; 0.1, 1, and 10 μM for ketoconazole) were selected to represent their maximal plasma concentrations in mice achieved with doses tested in this study. Reactions were allowed to proceed for up to 60 min at 37°C and were stopped with 2 volumes of 7:1 methanol-water containing 0.1% trifluoroacetic acid (vol/vol) and internal standard. After centrifugation, the supernatant fractions were analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to quantify the amount of DB766 remaining (2). Microsomal half-life ($t_{1/2}$) values were obtained by fitting the one-phase exponential decay equation ($C = C_0^{e-kt}$; $t_{1/2} = 0.693/k$) to percent substrate remaining versus time curves.

To determine the effects of drug combination on the pharmacokinetics of the individual drug, the single-dose pharmacokinetics of DB766 alone, azoles alone, and DB766-azole combinations were evaluated in uninfected male Swiss-Webster mice (in triplicate at each time point) after oral gavage. *In vivo* pharmacokinetic studies were performed in accord with protocols approved by the University of Kansas IACUC. DB766, posaconazole, and ketoconazole were dissolved in PEG400 as in the *in vivo* efficacy study. The DB766 doses were 25, 50, and 100 mg/kg (or 43.5, 87.1, and 174 μmol/kg). The posaconazole doses were 25, 50, and 100 mg/kg (or 35.7, 71.3, and 143 μmol/kg). The ketoconazole doses were 7.5, 15, and 30 mg/kg (or 14.1, 28.3, and 56.6 μmol/kg). These doses were similar to those used in the efficacy studies in BALB/c mice. The dose volume was 5 ml/kg, and no overt adverse effects were observed in mice at these dose levels and in combinations tested. Blood sampling (~40 μl per bleed) occurred 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 h postdose as described previously (50). Plasma was obtained by centrifugation. Excised mouse liver samples were quickly rinsed with distilled water, blotted dry with tissue paper, and weighed. All samples were stored at −20°C. Plasma and liver tissues were processed as described previously (50) for quantification by HPLC-MS/MS.

The quantification of DB766, posaconazole, and ketoconazole was performed on a Waters Xevo TQ-S triple-quadrupole mass spectrometer (Foster City, CA) coupled with a Waters Acquity ultraperformance liquid chromatography (UPLC) I-class system. Analytical conditions for DB766 were described previously in detail (2). The characteristic multiple reaction monitoring (MRM) transitions for posaconazole and ketoconazole were *m/z* 701.3→683.4 and 531.1→82.1, respectively, under positive electrospray ionization mode. Calibration curves for posaconazole and ketoconazole ranged from 0.025 to 25 μM for plasma samples and from 0.1 to 50 μM for liver homogenate samples. The interday coefficient of variation and accuracy were determined by measuring the same preparation of three standards three times on three different days; deviations for all azoles were within ±15%.

The area under the plasma concentration-time curve from time zero to the last time point (AUC_{last}), the area under the plasma concentration-time curve from time zero to time infinity ($AUC_{0-\infty}$), terminal elimination half-life ($t_{1/2}$), maximum plasma drug concentration (C_{max}), and time to reach C_{max} (T_{max}) were calculated using the trapezoidal rule extrapolation method and noncompartmental methods (WinNonlin version 6.4; Pharsight, Mountain View, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01129-17.

SUPPLEMENTAL FILE 1, PDF file, 8.8 MB.

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A.C.J., S.Y., M.Z.W., and K.A.W. conceived and designed the experiments. A.C.J., S.Y., H.M., and M.F. performed the experiments and analyzed the data. J.L. provided statistical analysis of the antileishmanial efficacy data. A.A.F. and D.W.B. synthesized DB766. A.C.J., S.Y., M.Z.W., and K.A.W. wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

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