Studies on the Antileishmanial Mechanism of Action of the Arylimidamide DB766: Azole Interactions and Role of CYP5122A1

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Arylimidamides (AIAs) are inspired by diamidine antimicrobials but show superior activity against intracellular parasites. The AIA DB766 {2,5-bis[2-(2-*i*-propoxy)-4-(2-pyridylimino)aminophenyl]furan hydrochloride} displays outstanding potency against intracellular *Leishmania* parasites and is effective in murine and hamster models of visceral leishmaniasis when given orally, but its mechanism of action is unknown. In this study, through the use of continuous DB766 pressure, we raised *Leishmania donovani* axenic amastigotes that displayed 12-fold resistance to this compound. These DB766-resistant (DB766R) parasites were 2-fold more sensitive to miltefosine than wild-type organisms and were hypersensitive to the sterol 14 α -demethylase (CYP51) inhibitors ketoconazole and posaconazole (2,000-fold more sensitive and over 12,000-fold more sensitive than the wild type, respectively). Western blot analysis of DB766R parasites indicated that while expression of CYP51 is slightly increased in these organisms, expression of CYP5122A1, a recently identified cytochrome P450 associated with ergosterol metabolism in *Leishmania*, is dramatically reduced in DB766R parasites. *In vitro* susceptibility assays demonstrated that CYP5122A1 half-knockout *L. donovani* promastigotes were significantly less susceptible to DB766 and more susceptible to ketoconazole than their wild-type counterparts, consistent with observations in DB766R parasites. Further, DB766-posaconazole combinations displayed synergistic activity in both axenic and intracellular *L. donovani* amastigotes. Taken together, these studies implicate CYP5122A1 in the antileishmanial action of the AIAs and suggest that DB766-azole combinations are potential candidates for the development of synergistic antileishmanial therapy.

Designated by the World Health Organization (WHO) as a neglected tropical disease, leishmaniasis is a diverse and complex vector-borne infection caused by over 20 different species of protozoan parasites of the genus *Leishmania*. Depending upon the causative species, the disease has four major clinical manifestations: (i) a self-healing cutaneous form resulting in skin lesions, (ii) a disseminated cutaneous manifestation that is more chronic in nature, (iii) a mucocutaneous form affecting the mucosal lining, and (iv) a fatal visceral form with spleen and liver involvement caused by parasites of the *Leishmania donovani*-*Leishmania infantum* complex (<http://www.who.int/tdr/diseases-topics/leishmaniasis/en/>). Leishmaniasis is endemic in 98 countries across five continents, and the estimated numbers of new cases of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL) are in the range of 300,000 and 1,000,000 per year, respectively (1). Although pentavalent antimonials have long served as the first line of treatment for both VL and CL, they are no longer effective against Indian VL because of the emergence of drug-resistant strains. Liposomal amphotericin B formulations, while very effective, are limited by the route of drug administration and costs associated with treatment. Miltefosine, the first oral antileishmanial drug, and paromomycin are effective and have been approved for the treatment of VL in India. However, the use of the former is limited due to its gastrointestinal toxicity, teratogenicity, and relatively high cost (2, 3), and the latter must be given by injection over a period of 3 weeks (4). Thus, while there have been some recent advances in VL chemotherapy, the need for new, inexpensive oral agents with improved efficacy against existing drug-resistant strains and reduced toxicity is urgent.

Arylimidamides (AIAs) are potent antiprotozoal agents that are members of a library of cationic diamidines and their analogs.

Although the design of AIAs was inspired by diamidine antimicrobials such as pentamidine, AIAs possess physicochemical properties that are distinct from those of diamidines (5). These differences are believed to translate into improved activity against intracellular pathogens, such as *Mycobacterium tuberculosis* (6), *Trypanosoma cruzi* (7), and *Leishmania* (8). The lead AIA in this series, DB766 {2,5-bis[2-(2-*i*-propoxy)-4-(2-pyridylimino)aminophenyl]furan hydrochloride}, displayed outstanding potency against *Leishmania donovani* intracellular amastigotes (50% inhibitory concentration [IC₅₀] = 0.036 μ M) *in vitro* as well as oral efficacy in murine and hamster models of visceral leishmaniasis (71% and 89% reductions in liver parasitemia when given orally at 100 mg/kg of body weight/day for 5 days, respectively) (5). Unfortunately, neither DB766 nor its corresponding mesylate salt, DB1960, possesses a sufficient therapeutic window to permit further development of this molecule as an antileishmanial drug (9), and none of the newer bis-AIAs that have been prepared are superior to DB766 as antileishmanial candidates (10, 11).

In an attempt to capitalize on the antileishmanial potency of

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FIG 1 Structures of arylimidamides and diamidines.

AIAs for the development of drug candidates with improved activity against leishmaniasis, a series of experiments was performed with the initial goal of obtaining an understanding of the antileishmanial mechanism of action of AIAs. These findings shed light on the action of DB766 in *Leishmania*, may provide further insight into the effects of azoles on these parasites, and point toward a new strategy for antileishmanial drug development. The structures of the arylimidamides and diamidines used in this study are given in Fig. 1.

MATERIALS AND METHODS

Parasites and culture conditions. *Leishmania donovani* MHOM/SD/62/1S-CL2_D promastigotes were adapted to axenic amastigote forms by culturing the former at 37°C in a humidified 5% CO₂ atmosphere in axenic amastigote medium as described previously (12). For intracellular assays, β-lactamase-expressing *L. donovani* promastigotes (provided by Frederick Buckner, University of Washington) were maintained as outlined earlier (13). Wild-type and CYP5122A1 half-knockout (HKO) promastigotes of *L. donovani* MHOM/IN/80/DD8 (14) were used in DB766 and ketoconazole susceptibility assays. Both wild-type and CYP5122A1 HKO *L. donovani* promastigotes were grown and cultured as described previously (15).

Drugs and reagents. The CellTiter reagent was obtained from Promega (Madison, WI), miltefosine was purchased from Cayman Chemical Company (Ann Arbor, MI), and DB1111, DB766, DB745, and DB1852 were synthesized according to known methods (5, 8, 16). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Selection of a DB766-resistant *Leishmania donovani* cell line. Axenically grown *Leishmania donovani* amastigotes were exposed to increasing DB766 pressure starting at a concentration of 0.05 μM and rising to a final concentration of 8 μM. A stepwise increase in the DB766 concentration was applied only when pressured cultures showed a growth rate equivalent to that of untreated cultures.

In vitro differentiation and growth curve. The transformation of *L. donovani* axenic amastigote forms to promastigotes was initiated by inoculating 5×10^6 parasites/ml in 4 ml of RPMI 1640 medium containing 20% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin at pH 6.88 and 23°C. The cell density and number of promastigote-like slender forms were determined by hemocytometer-based counting every 24 h for 72 h in three separate experiments.

In vitro susceptibility studies. The *in vitro* susceptibility of the DB766-resistant *L. donovani* cell line was evaluated after the cells were allowed to grow in the absence of DB766 for at least 3 days. Briefly, 10^6 DB766-sensitive or DB766-resistant axenic amastigotes per ml in a total volume of 60 μl were treated with a 2-fold dilution series of each compound in a 96-well plate at 37°C for 72 h. At the end of the treatment, cell viability was determined using the tetrazolium dye-based CellTiter reagent (Promega, Madison, WI). IC₅₀s were calculated using a four-parameter curve with SoftMax Pro software (Amersham Biosciences, Piscataway, NJ).

Each compound was tested in at least three separate experiments.

The nature of the interaction between DB766 and posaconazole was determined according to the modified fixed-ratio isobologram method (17). In assays employing *L. donovani* MHOM/SD/62/1S-CL2_D axenic amastigotes, a series of solutions was prepared by making 10 2-fold dilutions of fixed-ratio solutions of posaconazole and DB766 (5:0; 4:1; 3:2; 2:3; 1:4; and 0:5); the highest concentration of posaconazole and DB766 used in these assays was 25 μM each. This allowed determination of the IC₅₀ of each drug alone against wild-type axenic amastigotes from fixed-ratio solutions of 5:0 and 0:5, as well as the IC₅₀s of drug combinations from fixed-ratio solutions of 4:1, 3:2, 2:3, and 1:4. Each point was tested in triplicate. Endpoints were determined as described previously in drug susceptibility assays. The fractional inhibitory concentration (FIC) of DB766 was defined as the IC₅₀ of DB766 in combination/IC₅₀ of DB766 alone; the FIC of posaconazole was defined as the IC₅₀ of posaconazole in combination/IC₅₀ of posaconazole alone. FICs were used for constructing the isobolograms, with the sum of the FICs (ΣFICs) being equal to the FIC of DB766 plus the FIC of posaconazole, thus allowing the determination of the nature of the DB766-posaconazole interaction. The susceptibility of intracellular β-lactamase-expressing *L. donovani* to DB766, posaconazole, and fixed-ratio combinations of these two compounds was determined as outlined previously (13). FIC, ΣFIC, and mean ΣFIC values were calculated as described above for *L. donovani* axenic amastigotes. In both assays, the interaction between DB766 and posaconazole was classified as synergistic if the ΣFIC was ≤0.5, indifferent if the ΣFIC was >0.5 and <4, and antagonistic if the ΣFIC was >4 (17).

To evaluate the susceptibility of wild-type and CYP5122A1 HKO *L.*

FIG 2 Generation of a DB766-resistant *L. donovani* cell line. *Leishmania donovani* axenic amastigotes were cultured under increasing DB766 pressure starting at a concentration of 0.05 μM and rising to a final concentration of 8 μM.

FIG 3 *In vitro* differentiation efficacy and growth curve. (A) Growth curve of slender forms adapted from wild-type (WT) and DB766-resistant (766R) axenic amastigotes over a period of 5 days in culture. A total of 5×10^6 axenic amastigote forms/ml were cultured in promastigote medium in the absence of DB766. The number of slender forms arising from transformation of DB766-resistant and wild-type axenic amastigotes was determined by hemocytometer-based counting every 24 h for 5 days. Results indicate the mean $\pm$ SE of three separate measurements. (B) Efficiency of transformation of axenic amastigotes to promastigotes adapted from wild-type and DB766-resistant *L. donovani*. A total of 5×10^6 axenic amastigotes/ml were cultured in promastigote medium in the absence of DB766. Total cell density and the number of slender forms arising from DB766-resistant and wild-type axenic amastigotes were determined by hemocytometer-based counting every 24 h for 72 h. Values are expressed as the percentage of slender forms relative to the total cell density. Results indicate the mean $\pm$ SE of three separate measurements. *, $P < 0.01$; **, $P < 0.005$.

donovani MHOM/IN/80/DD8 promastigotes to DB766 or ketoconazole, these parasites were incubated with or without DB766 (50 to 750 nM) or ketoconazole (10 and 30 μ M) at a seeding density of 10^6 cells/ml at 23°C for 24 h. For assessment of cell viability, parasites were harvested by centrifugation at $1,100 \times g$ for 5 min, followed by resuspension in phosphate-buffered saline. Propidium iodide was added at a final concentration of 2 μ g/ml, and the mixture was incubated for 5 min before analyzing the fluorescence on the FL2 channel of a BD FACSCalibur flow cytometer.

Western blotting. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer (Pierce), and protein determinations were performed using a bicinchoninic acid protein assay kit (Pierce) according to the manufacturer's instructions. Proteins were electrophoresed on 10% poly-

acrylamide gels by standard denaturing SDS-PAGE using precast gels from Bio-Rad. For Western blotting, proteins were transferred to a polyvinylidene difluoride membrane (GE Life Sciences) at a constant voltage of 80 kV for 2 h. Tris-buffered saline containing 5% nonfat milk in 0.1% Tween was used for blocking and probing the membrane with a 1:20,000 dilution of anti-CYP5122A1 antibody, a 1:500 dilution of anti-CYP51 antibody (provided by Frederick Buckner, University of Washington, Seattle, WA), or a 1:1,000 dilution of anti- α -enolase antibody (provided by Paul Michels, Catholic University of Louvain, Brussels, Belgium). To visualize the bands, enhanced chemiluminescence was performed according to the manufacturer's instructions (Cell Signaling Technologies, Danvers, MA).

RESULTS

The trypanosomatid mitochondrion has been shown to be the main subcellular target of pentamidine and other diamidines (18). Since AIAs contain amidine functional groups, the ultrastructural effects caused by the lead AIA DB766 were compared with those caused by the diamidine DB1111 in *Leishmania donovani* axenic amastigotes. While DB1111 caused dilation of the *L. donovani* mitochondrion, as observed previously (16), no changes in mitochondrial morphology were observed upon DB766 treatment. Instead, other ultrastructural alterations were noted in other organelles, including an increased number of vesicles in the flagellar pocket, damage to the flagellar membrane, and increased cytoplasmic vacuolization (data not shown).

Generation of DB766-resistant *L. donovani*. In an attempt to obtain mechanistic information concerning AIAs, we generated *L. donovani* axenic amastigotes that were approximately 12-fold resistant to DB766 by culturing parasites in the presence of increasing DB766 concentrations. As indicated in Fig. 2, the development of resistance to DB766 occurred with difficulty in culture. The time required to induce $\sim$ 12-fold resistance to DB766 through increasing pressure (assessed by comparing the IC_{50} s of resistant parasites with those of wild-type parasites) was about 18 months. Further, this resistance was maintained for at least 5 months in the absence of DB766 pressure, indicating a stable chemoresistant phenotype.

DB766-resistant parasites show defects in amastigote-to-promastigote differentiation. Wild-type and DB766-resistant axenic amastigotes had comparable growth rates (doubling time,

TABLE 1 Susceptibility profiles of wild-type and DB766-resistant *L. donovani* axenic amastigotes at 72 h posttreatment

Compound	IC_{50}^a (μ M)		Fold difference
	Wild type	DB766R	
DB766	0.66 ± 0.15	7.7 ± 1.4	$+11.7^b$
DB745	0.67 ± 0.15	5.5 ± 1.3	$+8.2^b$
DB1852	1.3 ± 0.3	6.5 ± 0.0	$+5.0^c$
Pentamidine	1.3 ± 0.2	1.2 ± 0.0	1.1
Verapamil	>100	>100	
DB766 + 50 μ M verapamil	1.1 ± 0.7	11 ± 2	$+9.8^b$
Amphotericin B	0.15 ± 0.04	0.14 ± 0.04	1.1
Miltefosine	2.7 ± 0.1	1.2 ± 0.3	-2.3^b
Ketoconazole	45 ± 1	0.016 ± 0.005	$-2,800^c$
Fluconazole	140 ± 50	120 ± 30	1.2
Posaconazole	12 ± 0	0.0010 ± 0.0005	$-12,000^c$
Terbinafine	99 ± 25	77 ± 5	1.3

^a Data are means $\pm$ standard deviations from ≥ 3 separate determinations. DB766R, DB766-resistant strain.

^b $P < 0.005$.

^c $P < 0.0005$.

FIG 4 Expression profile of CYP5122A1 and CYP51 in wild-type and DB766-resistant *L. donovani* axenic amastigotes. (A) A Western blot of 10 μ g of total protein from wild-type and DB766-resistant *L. donovani* axenic amastigotes probed with anti-CYP51 (top) and anti-CYP5122A1 (middle) antibodies is shown. α -Enolase was used as a loading control (bottom). The figure is representative of three separate experiments. (B) Histograms representing normalized means from densitometric analysis of the immunoblots shown in panel A and in two other experiments, as quantified using ImageJ software (public domain; National Institutes of Health). *, $P < 0.05$; **, $P < 0.001$.

~12 h). To test their ability to differentiate into promastigotes, axenically grown amastigotes were cultured in promastigote medium in the absence of DB766 at 23°C. The growth rate (doubling time, ~24 h) and the maximum cell density of promastigotes adapted from DB766-resistant axenic amastigotes were significantly lower than those of the wild-type cells (doubling time, ~12 h; Fig. 3A), and these cells were much smaller and less motile than their wild-type counterparts. About 80% of the population of wild-type axenic amastigotes transformed into promastigotes within 48 h; the transformation was complete at 72 h. Under the same experimental conditions, the transformation of DB766-resistant amastigotes was incomplete, with these cultures containing only about 60% promastigotes at the end of 72 h (Fig. 3B).

Resistance to DB766 alters drug susceptibility in *L. donovani* axenic amastigotes. The susceptibility profile of the DB766-resistant cell line to other structurally related and unrelated drugs is summarized in Table 1. There was no statistically significant difference between resistant and wild-type axenic amastigotes in susceptibility to pentamidine, amphotericin B, fluconazole, or terbinafine. Resistance to DB766 was not reversed by verapamil, a calcium channel blocker known to reverse multidrug resistance associated with overexpression of P glycoprotein (PgP)-type efflux pumps. Further, DB766-resistant parasites are cross resistant to the bis-AIAs DB745 (~8-fold) and DB1852 (~5-fold). DB766-resistant parasites were twice as sensitive to miltefosine and, re-

markably, were more than 2,000-fold more sensitive to ketoconazole and over 12,000-fold more sensitive to posaconazole than wild-type organisms.

DB766-resistant and DB766-treated parasites have significantly reduced expression of CYP5122A1. Miltefosine and antifungal azoles are known to alter lipid and sterol metabolism in *Leishmania* (19, 20). It is conceivable that reduced expression of key sterol biosynthetic enzymes, such as CYP51 (sterol 14 α -demethylase, an antifungal azole target), would hypersensitize these organisms to the lethal effects of azoles, consistent with the earlier observations of enhanced sensitivity to antifungal azoles in CYP51 knockout fungi (21, 22). Recently, a novel cytochrome P450, CYP5122A1, essential for survival, virulence, drug response, and ergosterol metabolism in *Leishmania*, was identified (14). Although strains with double knockouts were not viable in culture, knockout of a single allele of CYP5122A1 in *L. donovani* (half knockout [HKO]) resulted in a 3.5-fold decrease in ergosterol levels and significant growth defects. The observed growth defects of CYP5122A1 HKOs were partially rectified upon supplementation with ergosterol in the growth medium and also upon complementation with episomally expressed CYP5122A1 in HKO parasites. These observations provide strong evidence for the role of CYP5122A1 in ergosterol metabolism in *Leishmania*. On the basis of the observations described above, modulation of ergosterol-metabolizing enzymes could occur as a consequence of ac-

FIG 5 Expression of CYP5122A1 in wild-type *L. donovani* axenic amastigotes treated with DB766. (A) A Western blot of 10 μ g of total protein from *L. donovani* axenic amastigotes treated with 0.1 μ M and 0.2 μ M DB766 for 48 h and 72 h is shown. α -Enolase was used as a loading control. The figure is representative of three separate experiments. (B) Histograms representing normalized CYP5122A1 expression levels from densitometric analysis of immunoblots shown in panel A and from two additional experiments, as quantified using ImageJ software (public domain; National Institutes of Health). *, $P < 0.05$.

FIG 6 Profile of susceptibility of wild-type and CYP5122A1 HKO *Leishmania donovani* promastigotes to DB766 and ketoconazole. A total of 10^6 parasites/ml from wild-type and CYP5122A1 HKO *Leishmania donovani* promastigotes were exposed to various concentrations of DB766 (50 to 750 nM) (A) or ketoconazole (10 or 30 μ M) (B) for 24 h. Cell viability was determined by propidium iodide staining by flow cytometry. The values represent percentage cell death relative to untreated controls. Results indicate the mean $\pm$ SE of three separate measurements. *, $P < 0.05$; #, $P < 0.001$. VT, vehicle treatment.

quired resistance to DB766. CYP51 and CYP5122A1 were chosen for investigation on the basis of the hypersensitivity of the DB766-resistant parasites to ketoconazole and posaconazole (Table 1). There were no significant differences in the transcript levels of CYP51 and CYP5122A1 in wild-type versus DB766-resistant *Leishmania* parasites, as assessed by real-time quantitative PCR (data not shown). While there was a slight increase in expression of the CYP51 protein in the resistant parasites (1.35-fold, $P < 0.05$), CYP5122A1 protein levels were dramatically reduced in the resistant parasites (3.80-fold, $P < 0.001$) compared to those in their wild-type counterparts (Fig. 4). To obtain further informa-

tion about CYP5122A1 expression in DB766-treated parasites, *L. donovani* axenic amastigotes were exposed to different concentrations of DB766 for different time periods, and then CYP5122A1 expression levels were measured as described above. CYP5122A1 levels were significantly reduced (1.80-fold, $P < 0.05$) in parasites treated with 0.2 μ M DB766 for 72 h (Fig. 5).

CYP5122A1 HKO *L. donovani* promastigotes display reduced susceptibility to DB766 and heightened sensitivity to ketoconazole compared to their wild-type counterparts. To test whether a reduction in CYP5122A1 expression alone caused reduced susceptibility to DB766 and increased sensitivity to keto-

TABLE 2 Mean Σ FICs for the interaction between DB766 and posaconazole in *L. donovani* at the IC₅₀

Expt no.	<i>L. donovani</i> axenic amastigotes			Intracellular <i>L. donovani</i>		
	IC ₅₀ ^a (μM)		Mean Σ FIC $\pm$ SD at IC ₅₀	IC ₅₀ (μM)		Mean Σ FIC $\pm$ SD at IC ₅₀
	DB766	Posaconazole		DB766	Posaconazole	
1	0.46	9.8	0.61 $\pm$ 0.22	0.046	6.1	0.34 $\pm$ 0.08
2	0.49	13	0.41 $\pm$ 0.04	0.036	6.0	0.47 $\pm$ 0.14

^a The IC₅₀ of each compound alone was used to calculate the FICs in the individual experiments.

conazole, the susceptibility of CYP5122A1 HKO *L. donovani* promastigotes to both of these agents was measured. As shown in Fig. 6A, CYP5122A1 HKO parasites exhibited significantly less cell death (fewer propidium iodide [PI]-positive cells) than wild-type organisms at all the tested concentrations of DB766 (50 nM to 750 nM). Figure 6B shows that these half-knockout cells undergo significantly more cell death (more PI-positive cells) when incubated with 10 μM and 30 μM ketoconazole. The CYP5122A1 HKO cell line is thus less susceptible to DB766 and more susceptible to ketoconazole than wild-type *L. donovani*, similar to the DB766-resistant *L. donovani* axenic amastigotes.

DB766 is synergistic with posaconazole against *L. donovani* *in vitro*. Since DB766-resistant parasites were hypersensitive to ketoconazole and posaconazole, the *in vitro* interaction between DB766 and posaconazole was assessed in *L. donovani* axenic amastigotes and intracellular amastigotes using the fixed-ratio isobologram method (17). On the basis of the mean Σ FIC values of 0.51 against axenic amastigotes and 0.41 against intracellular amastigotes (Table 2) and the concave isobolograms observed (Fig. 7), the DB766-posaconazole interaction was classified as borderline synergistic for axenic amastigotes and synergistic for intracellular amastigotes.

DISCUSSION

While kinetoplast DNA (kDNA) binding and disruption of mitochondrial function are among the likely mechanisms of action of diamidines in kinetoplastids (23), targets for the antiparasitic action of AIAs have not been clearly defined. The activity of AIAs against *T. cruzi* does not correlate with the ability of these compounds to bind kDNA (24), and treatment of *T. cruzi* intracellular amastigotes with AIAs resulted in not only swelling of the mitochondrion and disorganization of kDNA but also vacuolization

and the appearance of electron-dense bodies in the cytoplasm, the development of vesicles in the flagellar pocket, and disorganization of subpellicular microtubules (7). In terms of apicomplexan parasites, exposure to AIAs compromised the viability of intracellular forms of both *Neospora caninum* and *Toxoplasma gondii* specifically through the modulation of host cell processes (25). Considering that few mechanistic studies have been conducted with AIAs and no target proteins or pathways have been identified, the antiprotozoal mechanism of action of these compounds is poorly understood. In the present investigation, we show that (i) the mechanism of action of DB766 is distinct from that of diamidines in *Leishmania*, (ii) CYP5122A1, a novel P450 enzyme involved in *Leishmania* ergosterol metabolism, plays an important role in susceptibility and resistance to DB766 and azoles in *L. donovani*, and (iii) DB766 synergizes the antileishmanial potency of azoles, CYP51 inhibitors that disrupt sterol biosynthesis in fungi and trypanosomatids.

In vitro susceptibility assays with the DB766-resistant cell line (Table 1) showed that (i) there is no significant difference in susceptibility to pentamidine between wild-type and DB766-resistant *Leishmania* parasites, (ii) resistance to DB766 is not reversed by verapamil, indicating that the overexpression of PgP-type efflux pumps is unlikely to be responsible for resistance, (iii) DB766-resistant parasites are cross resistant to other AIAs, indicating that AIAs share a target or targets, and (iv) DB766-resistant parasites are twice as sensitive as wild-type axenic amastigotes to miltefosine and over 3 orders of magnitude more sensitive to ketoconazole and posaconazole than wild-type axenic amastigotes. These susceptibility data are consistent with the findings of ultrastructural studies, suggesting that the target of AIAs is different from that of diamidines in *L. donovani*. In addition, the hypersensitivity of the DB766-resistant cell line to ketoconazole and po-

FIG 7 Isobolograms showing *in vitro* interactions between DB766 and posaconazole at the IC₅₀ in *L. donovani*. (A) Analysis of *L. donovani* axenic amastigotes. (B) Analysis of intracellular *L. donovani* amastigotes using mouse peritoneal macrophages as host cells. The data shown in these panels are from representative experiments performed on two separate occasions (Table 2).

saconazole led to an investigation of the role of sterol biosynthesis enzymes, particularly sterol 14 α -demethylase, in the mechanism of action of and resistance to DB766 in *L. donovani*.

While reduced expression of CYP51 has been shown to enhance the susceptibility to antifungal azoles in fungi (21, 22), reduced expression of CYP5122A1 increased the sensitivity of *L. donovani* to miltefosine (14). Consistent with the hypothesis that resistance to DB766 was caused by altered expression of CYP5122A1 in *L. donovani*, Western blot analysis of these proteins indicated that expression of CYP5122A1 was dramatically reduced in the resistant organisms compared to that in their wild-type counterparts (Fig. 4). Further, CYP5122A1 HKO *L. donovani* promastigotes are less susceptible to DB766 (Fig. 6A) and more susceptible to ketoconazole (Fig. 6B) than the corresponding wild-type promastigotes, consistent with observations made with CYP5122A1-deficient DB766-resistant parasites (Table 1). Besides the similarities noted between CYP5122A1-deficient, DB766-resistant *L. donovani* parasites and CYP5122A1 HKO *L. donovani* parasites in their susceptibilities to DB766 and azoles, these two parasite lines also display significantly reduced growth rates compared with those of their wild-type counterparts and fail to differentiate completely to promastigotes (14) (Fig. 3). Taken together, these data indicate that CYP5122A1 plays a critical role in determining the susceptibility of *L. donovani* to both DB766 and antifungal azoles. On the basis of the available data, two mechanistic possibilities for the antileishmanial effects of DB766 involving CYP5122A1 appear to be plausible: (i) DB766 disrupts sterol metabolism in *L. donovani* by interfering with the action of CYP5122A1, or (ii) CYP5122A1 metabolizes DB766 to a more active form, resulting in toxicity to the parasite through an unknown mechanism. Preliminary experiments revealed minor differences in sterol composition between DB766-treated and untreated parasites (data not shown). These differences were not as dramatic as those observed when *Leishmania* parasites were exposed to azoles, where synthesis of 14-demethylated sterols could be almost completely blocked (20). Given its sequence similarity to mammalian CYP4A10 (25%), a CYP450 involved in drug metabolism, it is possible that CYP5122A1 may also play a role in *Leishmania* xenobiotic metabolism. Decreased expression of CYP5122A1 may also force the parasite to depend more heavily on CYP51 for sterol biosynthesis, providing a possible explanation for the synergism observed between DB766 and posaconazole (Fig. 7). While the data shown here provide support for the proposed mechanism responsible for the azole hypersensitivity seen in DB766-resistant *Leishmania* parasites and the posaconazole-DB766 synergy seen in wild-type parasites, extensive sterol analysis in DB766- and DB766-azole-treated *L. donovani* parasites, as well as expression and biochemical characterization of CYP5122A1, will be required to distinguish different mechanistic hypotheses for the antileishmanial action of DB766.

Aside from CYP51 and CYP5122A1, the expression of other enzymes as well as the modulation of genes other than those of the sterol biosynthetic pathway could also contribute to azole hypersensitivity and resistance to DB766 in *Leishmania* and warrant further investigation. However, given the important role of the CYP5122A1 protein in survival, virulence, drug response, and ergosterol metabolism in *Leishmania*, the dramatic downregulation of CYP5122A1 in DB766-resistant parasites at least partially explains their resistance to DB766 and hypersensitivity to antifungal azoles. The present studies suggest that CYP5122A1 is a critical

modulator of DB766 activity, downregulation of which offers a survival advantage during the acquisition of resistance to AIA in *L. donovani*, and provide additional evidence of a role for CYP5122A1 in ergosterol biosynthesis and drug metabolism in these parasites. Reduced expression of CYP5122A1 may also be responsible for the synergism observed between DB766 and posaconazole in this organism. In addition, this work further highlights the importance of sterol metabolism in the action of antileishmanial drugs and drug candidates and suggests that AIA-azole combinations could have therapeutic potential against *Leishmania*.

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