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Inhibition profile of *Leishmania mexicana* arginase reveals differences with human arginase I

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Abstract

Arginase (ARG), the enzyme that catalyzes the conversion of arginine to ornithine and urea, is the first and committed step in polyamine biosynthesis in *Leishmania*. The creation of a conditionally lethal Δarg null mutant in *Leishmania mexicana* has established that ARG is an essential enzyme for the promastigote form of the parasite and that the enzyme provides an important defense mechanism for parasite survival in the eukaryotic host. Furthermore, human ARG1 (HsARG1) has also been implicated as a key factor in parasite proliferation. Thus, inhibitors of ARG offer a rational paradigm for drug design. To initiate a search for inhibitors of the *L. mexicana* ARG (LmARG), recombinant LmARG and HsARG1 enzymes were purified from *Escherichia coli*. Both LmARG and HsARG1 were specific for L-arginine and exhibited no activity with either D-arginine or agmatine as possible substrates. LmARG exhibited a K_m of 25 ± 4 mM for L-arginine, a pH optimum ~ 9.0 , and was dependent upon the presence of a divalent cation, preferentially manganese. A K_m of 13.5 ± 2 mM for L-arginine was calculated for the HsARG1. A collection of 37 compounds was evaluated against both enzymes. Twelve of these compounds were identified as being either strong inhibitors of both LmARG and HsARG1 or differential inhibitors between the two enzymes. Of the 12 compounds, six were selected for further analysis and the type and extent of inhibition determined.

Keywords

Leishmania; Arginase; Polyamines; Inhibitors; Amino acids

1. Introduction

Leishmania are protozoan parasites that cause a spectrum of disfiguring to deadly diseases in humans. The genus is digenetic with the extracellular, flagellated promastigote existing in the phlebotomine sandfly host, while the intracellular, non-motile amastigote resides within the phagolysosome of macrophages and other reticuloendothelial cells in the mammalian host. A key effector in mammalian macrophages that inhibits the extent of infection of a

variety of obligatory intracellular pathogens, including *Leishmania*, is nitric oxide, which is synthesized from L-arginine by the inducible nitrogen oxide synthase (iNOS) (Mauel et al., 1991; Wanasen and Soong, 2008). iNOS shares its amino acid substrate with arginase (L-arginine ureohydrolase; EC 3.5.3.1, ARG), a metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea. Ornithine is an intermediate in the urea cycle in mammalian cells and can also serve as a precursor for the synthesis of proline, glutamate and polyamines. Whether macrophages kill or tolerate *Leishmania* depends on the delicate balance between the two competing iNOS and ARG activities that are reciprocally regulated by cytokines secreted by Th1 and Th2 CD4⁺ T helper cells, respectively (Iniesta et al., 2001; Wanasen and Soong, 2008).

Human cells express two ARG enzymes; human ARG I (HsARGI) is a cytosolic enzyme that primarily functions in hepatocytes as a component of the urea cycle, while human arginase II (HsARGII) is broadly distributed among tissues and primarily found in the mitochondrial matrix. Interestingly, murine bone marrow and peritoneal macrophages express robust levels of HsARGI mRNA and protein after up-regulation by Th2 cytokines, although quiescent macrophages express negligible levels of HsARGI (Louis et al., 1999; Munder et al., 1999). Unstimulated macrophages also constitutively express HsARGII at levels that are unresponsive to Th2 cytokines (Louis et al., 1999; Munder et al., 1999). Both HsARGI and HsARGII have been extensively characterized at the biochemical level, and high resolution crystal structures of the two enzymes have been determined (Cox et al., 2001; Cama et al., 2003a, c; Shin et al., 2004; Di Costanzo et al., 2005). In contrast, *Leishmania* only express a single ARG enzyme. The availability of genetic Δarg knockouts of both *Leishmania mexicana* and *Leishmania major* have proven that the sole function of the leishmanial ARG, a glycosomal enzyme, is to serve as precursor for the biosynthesis of polyamines (Roberts et al., 2004; Reguera et al., 2009), ubiquitous aliphatic cations found in virtually every eukaryotic cell that play vital roles in such physiological processes as growth, differentiation and macromolecular biosynthesis (Pegg and McCann, 1982; Pegg, 2009).

Because robust activity of host ARG removes substrate available for nitric oxide synthesis via iNOS, ARG is widely viewed as a viable therapeutic target. Furthermore, it is well-documented in the murine infectivity model of *L. major* that an increased expression of host ARG I in susceptible Balb/c mice is associated with exacerbation of parasitemia in activated macrophages (Iniesta et al., 2001, 2002, 2005; Taylor-Robinson, 2001; Kropf et al., 2003, 2005). Employing inhibitors of ARG, several groups have independently demonstrated that ARG activity is important for the intracellular survival and growth of *L. major* in murine macrophages and mice (Iniesta et al., 2001, 2002; Kropf et al., 2005). N^ω-hydroxy-L-arginine (NOHA) dramatically reduces parasite loads in infected macrophages, a result that can be reversed by supplementation with ornithine (Iniesta et al., 2001). In addition, N^ω-hydroxy-nor-L-arginine (nor-NOHA) has been shown to diminish ARG activity, lesion size, and tissue parasite burden in infected mice (Iniesta et al., 2005; Kropf et al., 2005). While nor-NOHA does not reduce parasite ARG activity in intact parasites (Kropf et al., 2005), NOHA inhibits proliferation of *L. major* promastigotes by targeting ARG (Reguera et al., 2009).

ARG was found to be an indispensable enzyme for promastigote proliferation, since *L. mexicana* and *L. major* Δarg parasites rely on ornithine or polyamine supplementation for survival (Roberts et al., 2004; Reguera et al., 2009). The abilities of *L. mexicana* and *L. major* Δarg null mutants to retain their capacity to infect Balb/c mice implies both that amastigotes of these cutaneous species can salvage sufficient host ornithine or polyamines to at least partially meet their own polyamine requirements and that the parasite ARG by itself is not essential for maintenance of intracellular infection. However, the considerably

reduced infectivity phenotypes of the *L. mexicana* and *L. major* Δ arg mutants in mice also suggest that the parasite ARG is necessary for optimal infectivity.

Intriguingly, the reduced infectivity of the *L. mexicana* Δ arg parasites appears to correlate with an increased production of nitric oxide by the infected macrophages (Gaur et al., 2007). Similarly, immunohistochemistry of tissues from mice infected with *L. mexicana* Δ arg revealed higher levels of nitrosylated tyrosine residues compared with tissues from mice infected with wild type parasites (Gaur et al., 2007). The reduced infectivity phenotype of the *L. major* Δ arg parasites, in contrast, does not appear to correlate with increased nitric oxide production (Muleme et al., 2009).

Due to the relevance of both the host and parasite ARG activities in the maintenance of leishmanial virulence, we performed an initial pharmacological profile of the *L. mexicana* ARG (LmARG), with a particular focus on its comparative pharmacological features with HsARGI. We purified LmARG in large and replenishable quantities, determined its kinetic parameters and response to various divalent cations and pH changes, and compared its pharmacological profile with that of the purified HsARGI with respect to a battery of 37 potential inhibitors (<http://www.brenda-enzymes.org/>). Potent inhibitors of either LmARG or HsARGI were analyzed further for their mechanism of inhibition. This pharmacological profile provides a basis for differential or dual inhibition of the human and LmARG activities.

2. Materials and methods

2.1. Chemicals and reagents

Metal salts, isopropyl β -D-1-thiogalactopyranoside (IPTG), ARG substrates and commercially available ARG inhibitors were purchased from Sigma-Aldrich® (St. Louis, MO, USA) and Fisher Scientific (Pittsburgh, PA, USA). Ni²⁺-nitrilotriacetate (Ni-NTA) agarose beads were procured from Qiagen (Valencia, CA, USA). Complete Mini EDTA-free protease inhibitor and bovine pancreatic RNAase were bought from Roche Diagnostics Corp. (Indianapolis, IN, USA). The TOPO-TA and pET200/D-TOPO® expression kits, the HiFi Platinum Supermix for PCR, oligonucleotide primers, and the Qubit™ fluorometer and protein quantification kits were all obtained from Invitrogen (Carlsbad, CA, USA). The QuantiChrom™ Arginase Assay Kit was acquired from BioAssay Systems (Hayward, CA, USA) and Biosafe™ Coomassie from Bio-Rad Laboratories Life Science Research (Hercules, CA, USA). The two known inhibitors of the human ARGs, 2(S)-amino-6-boronohexanoic acid (ABH), and S-(2-boronoethyl)-L-cysteine (BEC), as well as a hexahistidine-(His₆)-tagged version of the *HsARGI* cDNA ligated into the pET11d vector were graciously supplied by Dr. David Christianson, University of Pennsylvania (Philadelphia, PA, USA). For the purposes of this article, the *HsARGI* cDNA expression plasmid is referred to as pETHsARGI.

2.2. Expression and purification of LmARG

The cloning of *LmARG* (GenBank™ accession number AF038409) has been reported (Roberts et al., 2004). The *LmARG* open reading frame (ORF) was amplified from a cosmid DNA template using PCR technology. Oligonucleotide sense and antisense primers were 5'-CACCATGGAGCACGTGCAGCAG-3' and 5'-CTACAGCTTGAGCTCGTATGCGGAGT-3', respectively. The amplified *LmARG* ORF was inserted into the pET200/D-TOPO® *Escherichia coli* expression vector, which automatically attaches a (His₆)-tag to the NH₂-terminus of the expressed protein and transformed into One Shot® TOP10 *E. coli* according to the manufacturer's directions. The chimeric plasmid was designated pETLmARG. Appropriate segments within pETLmARG

were sequenced in both directions in order to ensure sequence fidelity and correct insertion of the *LmARG* ORF within the expression plasmid. pET*LmARG* was then purified from the One Shot® TOP10 *E. coli* and transformed into BL21 Star™ (DE3) One Shot® *E. coli* for over-expression. *LmARG* was expressed using 1 mM IPTG according to the manufacturer's brochure except that the *E. coli* were grown at 30 °C and the growth media included 10% glycerol. Four liters of transformed *E. coli* that had been treated with IPTG were lysed in 200 ml of 50 mM sodium phosphate buffer, pH 8.0 containing 0.5 M NaCl, a dissolved protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA), and 5 µg ml⁻¹ units of DNAase (Sigma-Aldrich Corp., St. Louis, MO, USA) by means of two passages through a French press at 12,000 psi. The bacterial lysate was then clarified by centrifugation at 10,000 g for 20 min at 4 °C. Lysate was loaded onto a 3.0 ml Ni-NTA column and washed with 5 vol. of *E. coli* lysis buffer. Purified *LmARG* protein was eluted by the addition of 250 mM imidazole to the lysis buffer. Individual 0.5 ml elution fractions were collected, and eluted protein in each fraction was quantified on a Qubit® fluorometer using the Quant-iT™ Protein Assay Kit. The purity of the final eluted product was confirmed by fractionation on a 10% SDS-PAGE and subsequent staining with Biosafe™ Coomassie.

His₆-tagged HsARGI was purified using the identical purification protocol as described above for *LmARG* except the expression plasmid contained the pET11d backbone and the enzyme was purified from 500 ml of transformed *E. coli*.

2.3. *LmARG* and HsARGI activity measurements

All *LmARG* and HsARGI assays were performed on freshly purified protein at 37 °C in 100 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer, pH 9.5, unless otherwise noted. Immediately prior to the assay, HsARGI and *LmARG* were activated in 25 mM maleic acid and 2 mM MnCl₂ (unless otherwise noted) at 37 °C for 15 min. ARG assays were carried out using three adjacent rows of 96-well microtiter plates (Becton-Dickinson Biosciences, Le Pont de Claix, France) in 200 µl vol.: the first row was employed for dilution of activated *LmARG* or HsARGI protein into the CHES buffer, the second row contained L-arginine in buffer to initiate the enzyme reaction, and the subsequent rows were pre-loaded with 20 µl glacial acetic acid to quench the ARG reaction. The reactions were initiated by the addition of an appropriate dilution of activated *LmARG* or HsARGI protein that gave linear kinetics to the corresponding well containing the L-arginine. The ARG reactions were terminated by removal of 20 µl aliquots from the reaction mixture and their addition to the glacial acetic acid. The urea product of the ARG reactions was quantified by removal of a 5 µl aliquot of the quenched reaction and measuring the urea colorimetrically using the Quantichrom™ urea assay kit. Appropriate amounts of standards, e.g., urea (0 – 10 mM) and arginine (0 – 200 mM), diluted in CHES buffer, were also quantified using the Quantichrom™ kit. Two types of measurements were obtained: i) the kinetics of ARG activity with respect to time were acquired over five to six time points that were generally spaced 30 s apart, and production of urea versus time calculated by linear regression; and ii) endpoint assays were performed under linear conditions in which a single aliquot was taken at a single time point and total urea product measured.

2.4. Determination of ARG kinetic parameters

K_m determinations were performed at L-arginine concentrations ranging from 7.8 – 50 mM for *LmARG* and 2.5 – 200 mM for HsARGI. Protein concentrations in the assay were calculated with the Qubit™ fluorometer in order to calculate the k_{cat} value.

2.5. pH optima

pH optima for LmARG were determined over a pH range of 7.5 – 11.0 in 0.5 pH unit increments in appropriate adjusted buffers containing 150 mM 3-(N-morpholino)propanesulfonic acid, 150 mM CHES, and 150 mM *N*-cyclohexyl-3-aminopropanesulfonic acid. The concentration of L-arginine in these assays was 250 mM. Velocity assays were used to determine the reaction rate for each pH value, and ARG activity calculated as a percentage of the average peak velocity of three independent measurements.

2.6. Ion dependence

To determine the ion dependence of LmARG, residual ions from the purified enzyme preparation were first chelated with 2 mM EDTA, followed by dialysis at 4 °C against 1 L of 25 mM maleic acid buffer, pH 7.0, to remove the EDTA. Divalent cations (BaCl₂, CaCl₂, CdCl₂, CoCl₂, CuSO₄, FeSO₄, HgCl₂, MgSO₄, MnCl₂, NiCl₂, Pb(NO₃)₂, SnCl₂, or ZnCl₂) at 2 mM concentrations were then added back to the dialyzed enzyme preparation, and the mixture heat-activated at 37 °C for 15 min. Control enzyme samples without any divalent cation or to which 2 mM EDTA was added were included in each experiment. The LmARG reaction in these experiments was performed in the presence of 250 mM L-arginine and was terminated after 5 min, a time point during which ureohydrolysis was linear.

2.7. Inhibitor profiles

A panel of 37 compounds bearing some structural relationship to L-arginine was tested for the ability to inhibit LmARG and HsARGI activities. These compounds are listed in Table 1. Each of these compounds was evaluated for its ability to inhibit LmARG and HsARGI at a concentration of 40 mM, and the L-arginine substrate concentration was fixed at 40 mM to establish the inhibitory effects of these compounds against the *L. mexicana* and human enzymes. Residual rates of ARG activity were calculated as a percentage of the uninhibited velocity for both enzymes. Selected inhibitors were subjected to additional kinetic analysis by either performing velocity assays under a variety of inhibitor and substrate concentrations or by calculating the K_i from the panel data.

Two of the compounds, D-arginine and agmatine, were also tested as possible substrates for both LmARG and HsARGI. These reactions were carried out with either 50 mM D-arginine or 50 mM agmatine in the absence of L-arginine and allowed to progress for 30 min. A separate tube containing 50 mM L-arginine was employed as a positive control in this substrate specificity analysis. Urea produced from D-arginine or agmatine was then quantified as above for L-arginine.

2.8. Data analysis

To determine initial velocities, data were analyzed by linear regression using Microsoft Excel. If the data were significantly non-linear ($r^2 < 0.85$), then the data point was eliminated. Non-linear regression, curve-fitting and transformation of data to create Dixon and Haynes-Woolf plots were performed in Prism4 for Macintosh (version 4.0b, GraphPad Software, San Diego California USA, www.graphpad.com). Prism4 was also employed for error analysis of the data. For calculation of K_i values, data presented in Fig. 5 were used with the following equations (Segel, 1975). Competitive inhibition:

$$K_i = \frac{[I]aK_m}{(1 + \frac{[S]}{K_m})(1 - a)} \quad (1)$$

Uncompetitive inhibition:

$$K_i = \frac{[I]aK_m}{(1 + \frac{K_m}{[S]})(1 - a)} \quad (2)$$

where $[I]$ is the concentration of inhibitor, $[S]$ is the concentration of substrate, and a is the relative velocity measured in the reaction.

2.9. Parasite proliferation assays

Leishmania mexicana (MNYC/BZ/62/M379) promastigotes were cultivated in DME-L medium, a completely defined Dulbecco's modified Eagle-based medium that was specifically designed for growing *Leishmania* promastigotes (Iovannisci and Ullman, 1983). To determine toxicity of select compounds against *L. mexicana*, 5×10^4 promastigotes were seeded in 100 μ l vol. with serial two-fold dilutions of inhibitor. After 5 days, alamarBlue™ (Biosource International, Camarillo, CA, USA) was added and reduction of alamarBlue™ to resorufin quantified after 16 h at 570 and 600 nm on a Multiskan Ascent plate reader (Thermo Labsystems, Vantaa, Finland). Results of experiments are expressed as a percentage of alamarBlue™ reduction in inhibitor-treated cultures compared with untreated control cultures and are the mean values and S.D.s of triplicate experiments.

3. Results

3.1. Expression and purification of LmARG

LmARG expression in *E. coli* was induced with IPTG from the pETLmARG expression construct and purified to effective homogeneity over a Ni-NTA column (Fig. 1). Fractionation of the purified protein on a denaturing gel revealed a single polypeptide that migrated slightly more slowly than its predicted ~39 kDa molecular mass (Fig. 1). The overall yield of the pure recombinant LmARG was ~10 mg per liter. HsARGI was also purified to homogeneity, as previously reported (Di Costanzo et al., 2007), from an extract of the pETHsARGI *E. coli* transformant with similar yields (data not shown).

3.2. Kinetic analysis of LmARG

In order to evaluate the pharmacophore profile of LmARG, an assessment of ideal assay conditions and kinetic parameters was an essential prerequisite. LmARG displayed a broad pH optimum peak centered at pH 9.0 (Fig. 2). Activity dropped off steeply below pH 8.5, and more gradually at pH values greater than 9.5. At these pH values, the α -carboxylate and guanidino groups of the L-arginine substrate are fully charged, while the α -NH₂ moiety is partially protonated. The basic pH optimum for LmARG is similar to that previously determined for HsARGI (Beruter et al., 1978). Based on these pH optimum data, all subsequent ARG activity measurements were performed at pH 9.0.

Steady state kinetic analysis showed that LmARG displayed classic Michaelis-Menten kinetics as a function of L-arginine concentration. Non-linear regression revealed a K_m of 25 ± 4 mM for L-arginine (Fig. 3A), a value virtually identical to that (21.5 ± 0.90 mM) previously obtained for the *Leishmania amazonensis* enzyme (da Silva et al., 2008). The k_{cat} for LmARG was calculated to be ~ 1.7 s⁻¹. The HsARGI also exhibited Michaelis-Menten kinetics with a K_m of 13 ± 2 mM for L-arginine (Fig. 3B). LmARG did not recognize either D-arginine or L-arginine (decarboxylated L-arginine) as substrate (data not shown).

Manganese is the customary divalent cation required by members of the ureohydrolase family (Cama et al., 2003b). LmARG was inactive in the absence of added cation or in the

presence of EDTA (Fig. 4). The enzyme exhibited the most robust activity in the presence of manganese, but cobalt and nickel were also able to support LmARG activity at ~48% and ~39%, respectively, of the maximal levels obtained with manganese (Fig. 4). Other metal ions did not support ARG activity. Because the LmARG amino acid sequence (Roberts et al., 2004) contains a potential zing finger motif (residues 289–320), the effect of zinc on manganese activation of LmARG was examined. Co-activation with manganese and zinc reduced activity to zero (data not shown).

3.3. Inhibitor profiles of LmARG and HsARGI

A collection of 37 compounds that are either known inhibitors of HsARGI activity or structural analogs of L-arginine (<http://www.brenda-enzymes.org/>) was evaluated for the ability to inhibit both LmARG and HsARGI activities. Each compound was screened at a 40 mM concentration in the presence of 40 mM L-arginine substrate. The majority of these chemicals did not significantly (<25%) inhibit either LmARG or HsARGI under these conditions (Fig. 5). Nine compounds, ABH, BEC, nor-NOHA, NOHA, boric acid, 8-aminoguanine, L-cysteine, trypanothione, and DL-homocysteine inhibited the LmARG enzyme by >50% under these conditions, while nine chemicals, ABH, BEC, nor-NOHA, NOHA, boric acid, trypanothione, L-leucine, L-ornithine, and L-lysine inhibited the human counterpart by >50% (Figs. 5 and 6). However, four compounds, ABH, BEC, nor-NOHA, and NOHA, completely blocked both HsARGI and LmARG activities under the assay conditions (Fig. 5). Furthermore, selective inhibition was observed among several of the inhibitors. Five compounds, boric acid, trypanothione, L-leucine, L-ornithine and L-lysine, and 8-aminoguanine, preferentially inhibited the HsARGI activity, whereas three compounds, 8-aminoguanine, L-cysteine, DL-homocysteine, demonstrated greater potency toward LmARG (Fig. 5).

Six of these inhibitors were selected for further investigation by Dixon plot analysis to determine the mode of inhibition and inhibition constants (K_i) of the LmARG-inhibitor complexes (Fig. 7 and Table 2). The Dixon plots revealed that three of the four most potent inhibitors, ABH, BEC and NOHA, all inhibited LmARG in a competitive fashion with K_i values ranging from 1.3 – 85 μ M (Fig. 7A–C). Nor-NOHA was not evaluated further due to its structural similarity to NOHA. None of the four compounds, however, was specific for LmARG (Table 2). The type and extent of inhibition of the four most potent inhibitors against HsARGI have already been established (Di Costanzo et al., 2010). 8-aminoguanine, trypanothione and L-cysteine were much less potent inhibitors with K_i values in the mM range for LmARG and HsAGI (Fig. 7 and Table 2). 8-aminoguanine and trypanothione inhibited LmARG in an uncompetitive fashion, while L-cysteine was a competitive inhibitor (Fig. 7D–E). The inhibition of AHB against the HsARGI was tested experimentally by Dixon plot analysis in order to verify the accuracy of our assay method (Fig. 7F).

3.4. Effect of LmARG inhibitors on cultured promastigotes

To determine the toxicity of the LmARG inhibitors toward *L. mexicana* promastigotes, parasites were incubated with various concentrations of the compounds that inhibited LmARG activity by >50% and growth was assessed after 5 days. The only compounds that exhibited any toxicity toward the parasites were NOHA and nor-NOHA with effective concentrations of compound that inhibited growth by 50% (EC_{50} values) of 6 mM and 3 mM, respectively (data not shown).

4. Discussion

A variety of studies have implicated ARG activity in ameliorating *Leishmania* infections in mammalian macrophages via the provision of carbon skeletons for polyamine biosynthesis

and/or through the depletion of substrate for nitric oxide synthesis (Iniasta et al., 2001, 2002, 2005; Taylor-Robinson, 2001; Kropf et al., 2003, 2005). Thus, inhibitors of ARG offer a rational therapeutic paradigm for the treatment of leishmaniasis. This study was undertaken to assess the effects of known inhibitors of the HsARGI (Iniasta et al., 2001, 2002; Di Costanzo et al., 2005, 2010; Kropf et al., 2005), as well as amino acids, polyamines and a variety of other compounds, on LmARG activity. The recombinant LmARG exhibited similar kinetics, e.g., K_m value and pH and ion dependence, to the previously characterized ARG enzyme from *L. amazonensis* (da Silva et al., 2008), as well as to the human ARG (Ash et al., 2000; Ash, 2004). At equimolar concentrations of inhibitor and substrate, four compounds, ABH, BEC, nor-NOHA and NOHA, obliterated both LmARG and HsArgI activity, with K_i values in the μM range. ABH and BEC are synthetic boronic acid analogs that form tetrahedral intermediates within the active site of human ARG enzymes (Di Costanzo et al., 2005), whereas NOHA, a hydroxy-arginine analog, is an intermediate of nitric oxide synthesis and nor-NOHA an analog of NOHA. All four are well-characterized inhibitors of mammalian ARG enzymes (Di Costanzo et al., 2005, 2010) and inhibited both LmARG and HsARGI in a competitive manner. Several other compounds tested (Fig. 6) appeared to be selective for either the leishmanial or human enzymes. Dixon plot analysis revealed that 8-aminoguanine and L-cysteine, both of which affect LmARG to a much more significant extent than HsARGI, inhibited LmARG in an uncompetitive and competitive mode, respectively.

Although NOHA and nor-NOHA are both potent inhibitors of the recombinant LmARG enzyme, their effects on ARG activity in intact parasites is disparate. While nor-NOHA does not inhibit the ability of intact *L. major* promastigotes to convert arginine to ornithine (Kropf et al., 2005), another study showed that NOHA has an EC_{50} of 40 μM against *L. major* promastigotes, while *L. major* ARG over-producers were resistant to the drug (Reguera et al., 2009). Our own studies indicate that only millimolar concentrations of NOHA or nor-NOHA even slightly impaired growth of *L. mexicana* promastigotes, although this modest inhibition could be overcome by putrescine supplementation (data not shown). It remains to be determined whether the lack of efficacy of NOHA and nor-NOHA against intact *L. mexicana* is due to limited uptake into the parasite or delivery to the glycosome, the organelle in which ARG resides (Roberts et al., 2004).

HsARGI has emerged as a key factor for parasite virulence (Iniasta et al., 2001, 2002, 2005; Taylor-Robinson, 2001; Kropf et al., 2003, 2005; Wanasen and Soong, 2008). Numerous studies have found an augmented activity of HsARGI associated with increased parasitemia (Iniasta et al., 2001, 2002, 2005; Taylor-Robinson, 2001; Kropf et al., 2003, 2005). Furthermore, pharmacological inhibition of host ARG with either NOHA or nor-NOHA causes significant reduction of parasite numbers in macrophages or tissue parasite burden and lesion size in infected mice, respectively (Iniasta et al., 2001, 2002; Kropf et al., 2005). Because the selective inhibition host ARG by nor-NOHA is not sufficient to clear parasitemia in mice, it is reasonable to presume that the parasite ARG also plays a significant role in infectivity. However, it cannot be ruled out in these studies with nor-NOHA that the host ARG is not completely inhibited or that polyamines are being produced by HsARGII or via a newly described alternative pathway using arginine decarboxylase and agmatinase (Sastre et al., 1998).

ARG-deficient strains of *L. mexicana* (Roberts et al., 2004; Gaur et al., 2007) and *L. major* (Muleme et al., 2009; Reguera et al., 2009) that were created via double targeted gene replacement are still capable of eliciting cutaneous disease in Balb/c mice, although the virulence phenotypes were clearly attenuated. Because the Δarg *Leishmania* are auxotrophic for polyamines, the diminished virulence phenotype of these knockouts implies that the cutaneous macrophages in which these parasites reside have residual supplies of either

polyamines or polyamine precursors to sustain amastigote growth. The diminished virulence of the Δarg *L. mexicana* null mutant is associated with an increased amount of nitric oxide production, a phenomenon that has been observed in vitro and in vivo (Gaur et al., 2007). This increased capacity of the host to produce nitric oxide may be due to higher levels of available arginine caused by decreased arginine scavenge from the Δarg *L. mexicana* parasites. The conjecture that the parasite may counteract the accumulation of intracellular arginine by reducing arginine import reasonable, since it has been demonstrated that expression and activity of the *L. donovani* LdAAP3 arginine transporter is regulated by intracellular levels of arginine (Darlyuk et al., 2009). Thus, it remains unclear whether the reduced infectivity of the Δarg *L. mexicana* is due to polyamine scavenge being less efficient than endogenous polyamine biosynthesis and/or due to the ability of the host to synthesize more nitric oxide. Regardless, it is evident that while parasite ARG activity is not essential for parasite survival, it is important for disease pathogenesis.

Taken together, previously published reports intimate that both host and parasite ARG activities contribute to the pathogenesis of *Leishmania* parasites. It is apparent that inhibition of leishmanial ARG activity by itself, at least in cutaneous strains, is insufficient to eliminate infection in vivo, and has also been shown that inhibition of the host ARG alone appears to be inadequate as a therapeutic strategy (Iniesta et al., 2001; Kropf et al., 2005). The hypothesis that inhibition of host parasite ARG alone is insufficient to prevent *Leishmania* infections can presumably be tested in recently developed mice strains in which the ARG1 gene has been specifically eliminated in murine macrophages by gene targeting strategies (El Kasmi et al., 2008). Mice harboring the conditional gene deletion at the HsARG1 locus were considerably less susceptible to experimental infections of both *Mycobacterium tuberculosis* and *Toxoplasma gondii* (El Kasmi et al., 2008).

Whether dual inhibition of both enzymes is a superior therapeutic strategy merits further investigation. To this end, we have provided evidence that LmARG and HsARG1 are similar enough in their kinetic behavior to be targeted by an inhibitor. Furthermore, it should be noted that the ARG activity assay established in this work can easily be scaled for high throughput in screening assays for discovery of novel inhibitors of the LmARG and/or HsARG1 enzymes.

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References

- Ash DE. Structure and function of arginases. *J Nutr.* 2004; 134:2760S–2764S. [PubMed: 15465781]
- Ash DE, Cox JD, Christianson DW. Arginase: a binuclear manganese metalloenzyme. *Met Ions Biol Syst.* 2000; 37:407–428. [PubMed: 10693141]
- Beruter J, Colombo JP, Bachmann C. Purification and properties of arginase from human liver and erythrocytes. *Biochem J.* 1978; 175:449–454. [PubMed: 743206]
- Cama E, Colletuori DM, Emig FA, Shin H, Kim SW, Kim NN, Traish AM, Ash DE, Christianson DW. Human arginase II: crystal structure and physiological role in male and female sexual arousal. *Biochemistry.* 2003a; 42:8445–8451. [PubMed: 12859189]
- Cama E, Emig FA, Ash DE, Christianson DW. Structural and functional importance of first-shell metal ligands in the binuclear manganese cluster of arginase I. *Biochemistry.* 2003b; 42:7748–7758. [PubMed: 12820884]
- Cama E, Shin H, Christianson DW. Design of amino acid sulfonamides as transition-state analogue inhibitors of arginase. *J Am Chem Soc.* 2003c; 125:13052–13057. [PubMed: 14570477]

- Cox JD, Cama E, Colletuori DM, Pethe S, Boucher JL, Mansuy D, Ash DE, Christianson DW. Mechanistic and metabolic inferences from the binding of substrate analogues and products to arginase. *Biochemistry*. 2001; 40:2689–2701. [PubMed: 11258880]
- da Silva ER, da Silva MF, Fischer H, Mortara RA, Mayer MG, Framesqui K, Silber AM, Floeter-Winter LM. Biochemical and biophysical properties of a highly active recombinant arginase from *Leishmania (Leishmania) amazonensis* and subcellular localization of native enzyme. *Mol Biochem Parasitol*. 2008; 159:104–111. [PubMed: 18400316]
- Darlyuk I, Goldman A, Roberts SC, Ullman B, Rentsch D, Zilberstein D. Arginine homeostasis and transport in the human pathogen *Leishmania donovani*. *J Biol Chem*. 2009; 284:19800–19807. [PubMed: 19439418]
- Di Costanzo L, Ilies M, Thorn KJ, Christianson DW. Inhibition of human arginase I by substrate and product analogues. *Arch Biochem Biophys*. 2010; 496:101–108. [PubMed: 20153713]
- Di Costanzo L, Moulin M, Haertlein M, Meilleur F, Christianson DW. Expression, purification, assay, and crystal structure of perdeuterated human arginase I. *Arch Biochem Biophys*. 2007; 465:82–89. [PubMed: 17562323]
- Di Costanzo L, Sabio G, Mora A, Rodriguez PC, Ochoa AC, Centeno F, Christianson DW. Crystal structure of human arginase I at 1.29-Å resolution and exploration of inhibition in the immune response. *Proc Natl Acad Sci U S A*. 2005; 102:13058–13063. [PubMed: 16141327]
- El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, Basaraba RJ, Konig T, Schleicher U, Koo MS, Kaplan G, Fitzgerald KA, Tuomanen EI, Orme IM, Kanneganti TD, Bogdan C, Wynn TA, Murray PJ. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. *Nat Immunol*. 2008; 9:1399–1406. [PubMed: 18978793]
- Gaur U, Roberts SC, Dalvi RP, Corraliza I, Ullman B, Wilson ME. An effect of parasite-encoded arginase on the outcome of murine cutaneous leishmaniasis. *J Immunol*. 2007; 179:8446–8453. [PubMed: 18056391]
- Iniesta V, Carcelen J, Molano I, Peixoto PM, Redondo E, Parra P, Mangas M, Monroy I, Campo ML, Nieto CG, Corraliza I. Arginase I induction during *Leishmania major* infection mediates the development of disease. *Infect Immun*. 2005; 73:6085–6090. [PubMed: 16113329]
- Iniesta V, Gomez-Nieto LC, Corraliza I. The inhibition of arginase by N(omega)-hydroxyl-arginine controls the growth of *Leishmania* inside macrophages. *J Exp Med*. 2001; 193:777–784. [PubMed: 11257143]
- Iniesta V, Gomez-Nieto LC, Molano I, Mohedano A, Carcelen J, Miron C, Alonso C, Corraliza I. Arginase I induction in macrophages, triggered by Th2-type cytokines, supports the growth of intracellular *Leishmania* parasites. *Parasite Immunol*. 2002; 24:113–118. [PubMed: 11982856]
- Iovannisci DM, Ullman B. High efficiency plating method for *Leishmania* promastigotes in semidefined or completely-defined medium. *J Parasitol*. 1983; 69:633–636. [PubMed: 6631633]
- Kropf P, Fuentes JM, Fahnrich E, Arpa L, Herath S, Weber V, Soler G, Celada A, Modolell M, Muller I. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *FASEB J*. 2005; 19:1000–1002. [PubMed: 15811879]
- Kropf P, Herath S, Weber V, Modolell M, Muller I. Factors influencing *Leishmania major* infection in IL-4-deficient BALB/c mice. *Parasite Immunol*. 2003; 25:439–447. [PubMed: 14651591]
- Louis CA, Mody V, Henry WL Jr, Reichner JS, Albina JE. Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. *Am J Physiol*. 1999; 276:R237–R242. [PubMed: 9887201]
- Mauel J, Ransijn A, Buchmuller-Rouiller Y. Killing of *Leishmania* parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen derivatives. *J Leukoc Biol*. 1991; 49:73–82. [PubMed: 1845812]
- Muleme HM, Reguera RM, Berard A, Azinwi R, Jia P, Okwor IB, Beverley S, Uzonna JE. Infection with arginase-deficient *Leishmania major* reveals a parasite number-dependent and cytokine-independent regulation of host cellular arginase activity and disease pathogenesis. *J Immunol*. 2009; 183:8068–8076. [PubMed: 19923451]

- Munder M, Eichmann K, Moran JM, Centeno F, Soler G, Modolell M. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J Immunol.* 1999; 163:3771–3777. [PubMed: 10490974]
- Pegg AE. Mammalian polyamine metabolism and function. *IUBMB Life.* 2009; 61:880–894. [PubMed: 19603518]
- Pegg AE, McCann PP. Polyamine metabolism and function. *Am J Physiol.* 1982; 243:C212–C221. [PubMed: 6814260]
- Reguera RM, Balana-Fouce R, Showalter M, Hickerson S, Beverley SM. *Leishmania major* lacking arginase (ARG) are auxotrophic for polyamines but retain infectivity to susceptible BALB/c mice. *Mol Biochem Parasitol.* 2009; 165:48–56. [PubMed: 19393161]
- Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase plays a pivotal role in polyamine precursor metabolism in *Leishmania*. Characterization of gene deletion mutants. *J Biol Chem.* 2004; 279:23668–23678. [PubMed: 15023992]
- Sastre M, Galea E, Feinstein D, Reis DJ, Regunathan S. Metabolism of agmatine in macrophages: modulation by lipopolysaccharide and inhibitory cytokines. *Biochem J.* 1998; 330(Pt 3):1405–1409. [PubMed: 9494113]
- Segel, I. Enzyme kinetics; behaviour and analysis of rapid equilibrium and steady state enzyme systems. New York: Wiley, John and Sons; 1975.
- Shin H, Cama E, Christianson DW. Design of amino acid aldehydes as transition-state analogue inhibitors of arginase. *J Am Chem Soc.* 2004; 126:10278–10284. [PubMed: 15315440]
- Taylor-Robinson A. Th1/Th2-regulated arginase availability modulates *Leishmania* infection. *Trends Parasitol.* 2001; 17:262. [PubMed: 11378016]
- Wanasen N, Soong L. L-arginine metabolism and its impact on host immunity against *Leishmania* infection. *Immunol Res.* 2008; 41:15–25. [PubMed: 18040886]

Fig. 1. *Leishmania mexicana* arginase (LmARG) expression and purification. Recombinant LmARG was over-expressed and purified from *Escherichia coli* as described in Section 2.2. Shown are the crude lysates from *E. coli* transformed with *LmARG* following a 4 h induction with isopropyl β -D-1-thiogalactopyranoside (IPTG) (lane 1), without induction (lane 2), and the first elution fraction from a Ni^{2+} -nitrilotriacetate (Ni-NTA) agarose column (lane 3), as fractionated by SDS-PAGE. The migration and size in kDa of molecular mass markers is indicated to the left of the figure.

Fig. 2.

Effect of pH on *Leishmania mexicana* arginase (LmARG) activity. LmARG-catalyzed urea production from L-arginine was measured over a pH range of 7.5 – 10.5. Data shown are the mean $\pm$ S.E.M. of three independent experiments.

Fig. 3.

Michaelis-Menten kinetics of *Leishmania mexicana* arginase (LmARG) and human ARG1 (HsARGI). The velocities of the LmARG (A) and HsARGI (B) were determined as a function of L-arginine concentration of substrate. The data were plotted by non-linear regression with r^2 values of 0.9188 and 0.8913 for LmARG and HsARGI, respectively. Hanes-Woolf plots of the Michaelis-Menten are presented in the insets of each panel and verify the results obtained by non-linear regression. The line in the Hanes-Woolf plot is the standard linear regression line with an r^2 value of 0.9857 for LmARG and 0.9399 for HsARGI, respectively. The data are the mean $\pm$ S.D. of three independent measurements for each enzyme.

Fig. 4.

Effects of divalent cation on *Leishmania mexicana* arginase (LmARG) activity. LmARG activity was measured in the absence or presence of a variety of different cations at 2 mM and/or 2 mM EDTA. Data are shown as a percentage of LmARG activity in the presence of Mn²⁺ and are the mean $\pm$ S.D. of two separate experiments.

Fig. 5.

Inhibition profiles for *Leishmania mexicana* arginase (LmARG) (A) and human ARG1 (HsARG1) (B). The inhibitor effects of a variety of different chemicals on the LmARG (A) and HsARG1 (B) catalyzed hydrolysis of L-arginine were measured in the presence of 40 mM of each chemical. Data are expressed as a percentage of no-inhibitor control. Presented are the mean $\pm$ S.D. of two separate experiments. ABH, 2(S)-amino-6-boronohexanoic acid; BEC, S-(2-boronoethyl)-L-cysteine; Nor-NOHA, N^ω-Hydroxy-nor-L-arginine; NOHA, N^ω-hydroxy-L-arginine; MGBG, methylglyoxal bis-guanylhydrazone; DFMO, α -difluoromethylornithine; GABA, γ -aminobutyric acid; and MGSD, monogluthathionyl-spermidine disulphide.

Fig. 6.

Comparison of selected arginase (ARG) inhibitors. The inhibitory effects of the most potent inhibitors of ARG in Fig. 1 were compared for *Leishmania mexicana* ARG (LmARG) and human ARG1 (HsARG1). The data presented are the means $\pm$ S.D. of two independent experiments. ABH, 2(S)-amino-6-borono-hexanoic acid; BEC, S-(2-boronoethyl)-L-cysteine; Nor-NOHA, N^ω-Hydroxy-nor-L-arginine; NOHA, N^ω-hydroxy-L-arginine.

Fig. 7.

Dixon plots for select inhibitors. 2(S)-amino-6-borono-hexanoic acid (ABH) (A), S-(2-boronoethyl)-L-cysteine (BEC) (B), N^ω-hydroxy-L-arginine (NOHA) (C), 8-aminoguanine (D) and Lcysteine (E) inhibition of *Leishmania mexicana* arginase (LmARG) is shown. ABH (F) inhibition of human ARG1 (HsARG1) is also exhibited. Data points were obtained by regression of velocity assay data with $r^2 > 0.85$, and linear regression performed on the reciprocals of velocity versus concentration of inhibitor in the reaction for indicated concentrations of L-arginine present as substrate. The units of velocity⁻¹ are given in sec mg protein nmol⁻¹ for all six panels. The selected concentrations of L-arginine are: 10 mM (▼), 20 mM (◆), 25 mM (■), 50 mM (▽), 100 mM (◇), and 150 mM (□).

Table 1Compounds tested for inhibition of *Leishmania mexicana* arginase (LmARG) and human ARG1 (HsARG1).

Adenosine	Guanidinoacetic acid
Agmatine	L-Homoarginine
2(S)-amino-6-boronohaxanoic acid (ABH)	DL-Homocysteine
γ -Amino-butyric acid (GABA)	L-Leucine
8-Aminoguanine	L-Lysine
D-Arginine	L-Methionine
Blasticidin S	N ^G -Methyl-L-arginine
Boric acid	Mitoguazone (MGBG)
S-(2-boronoethyl)-L-cysteine (BEC)	α -Methyl-ornithine
8-Bromoguanine	Monogluthionyl-spermidine disulphide
Cadaverine	N ^o -Hydroxy-nor-L-arginine (nor-NOHA)
Creatine	N ^o -Hydroxy-L-arginine (NOHA)
L-Cysteine	Ornithine
Difluoromethylornithine	L-Proline
Ethylenediamine	Putrescine
L-Glutamate	Spermidine
L-Glutamine	Trypanothione
Glutathione	Urea
Guanidine thiocyanate	

Table 2

Summary of type and extent of inhibition for selected compounds against *Leishmania mexicana* arginase (LmARG) and human ARG1 (HsARG1). The extent and type of inhibition for LmARG and HsARG1 were determined by Dixon plot analysis or (for those marked with 'a') by calculation from panel data assuming the type of inhibition shown in the final column and using equations (1) and (2) (Section 2.8).

Inhibitor	K _i vs. LmARG	K _i vs. HsARG1	Type of Inhibition
ABH	1.3 μ M	3.5 μ M	Competitive
BEC	~10 μ M	14 μ M ^a	Competitive
nor-NOHA	~50 μ M ^a	~10 μ M ^a	Competitive
NOHA	85 μ M	~70 μ M ^a	Competitive
8-Aminoguanine	< 2 mM	N/A	Uncompetitive
L-Cysteine	11 mM	> 30 mM ^a	Competitive
Trypanothione	< 5 mM	< 5 mM ^a	Uncompetitive

ABH, 2(S)-amino-6-boronohaxanoic acid; BEC, S-(2-boronoethyl)-L-cysteine; nor-NOHA, N⁶-Hydroxy-nor-L-arginine; NOHA, N⁶-Hydroxy-L-arginine.