Inhibition of \textit{Leishmania} (\textit{Leishmania}) \textit{amazonensis} growth and infectivity by aureobasidin A

Ameria K. Tanaka, Valderez B. Valero, Helio K. Takahashi and Anita H. Straus*

Department of Biochemistry, Universidade Federal de São Paulo/Escola Paulista de Medicina,
Rua Botucatu 862, São Paulo, SP, 04023-900, Brazil

Received 17 July 2006; returned 27 August 2006; revised 6 October 2006; accepted 27 November 2006

Objectives: To study the effect of aureobasidin A, an inhibitor of inositol phosphorylceramide (IPC) synthase, on \textit{Leishmania} growth and infectivity.

Methods: Effects of aureobasidin A were determined for: (i) promastigote growth in axenic culture; (ii) promastigote infectivity in macrophage monolayers; (iii) development of footpad lesions in BALB/c mice; (iv) differentiation of amastigotes into promastigotes.

Results: Aureobasidin A (20 \mu M) inhibited 90% of \textit{Leishmania} (\textit{Leishmania}) \textit{amazonensis} promastigote growth in axenic culture, but the parasites remained viable, i.e. growth curves returned to normal after aureobasidin A was removed from culture medium. The aureobasidin A IC$_{50}$ was determined by MTT assay as 4.1 \mu M for \textit{L. (L.)} amazonensis promastigotes, 12.6 \mu M for \textit{Leishmania} (\textit{Leishmania}) major and 13.7 \mu M for \textit{Leishmania} (\textit{Viannia}) braziliensis. There was a significant delay in infection when \textit{L. (L.)} amazonensis promastigotes pre-treated with aureobasidin A were inoculated into BALB/c mouse footpads. When aureobasidin A was added to cultured macrophages infected with amastigotes, the number of infected macrophages was reduced by $>$90%.

Conclusions: Aureobasidin A is an interesting pharmacological tool to investigate the effect of lipid metabolism inhibition in \textit{Leishmania} spp.

Keywords: anti-\textit{Leishmania}, sphingolipids, amastigotes

Introduction

Studies during the past two decades have demonstrated important roles of sphingolipids in many biological processes in eukaryotic cells, including cell adhesion and recognition, signal transduction, membrane trafficking, and lipid raft formation.$^{1-5}$ The major sphingolipid present in \textit{Leishmania} is inositol phosphorylceramide (IPC),$^{6,7}$ which is also present in other parasites and fungi, but absent in mammalian cells (which contain predominantly sphingomyelin). The enzyme IPC synthase is responsible for IPC synthesis in parasites and fungi. More detailed knowledge of the biological role and biosynthetic pathway of IPC will facilitate the development of new drugs targeted specifically to parasites and fungi, and not presenting toxic side effects to mammalian hosts.

Aureobasidin A, a cyclic antibiotic produced by the fungus \textit{Aureobasidium pullulans}, is a potent inhibitor of IPC synthase, and was shown to be lethal to a broad range of pathogenic fungi without affecting synthesis of mammalian sphingolipids.$^{8,9}$ Aureobasidin A blocks \textit{in vitro} replication of the parasite \textit{Toxoplasma gondii}, inhibits conversion of its tachyzoite to bradyzoite stage without affecting host cell metabolism,$^{10}$ and impairs differentiation of \textit{Trypanosoma cruzi} trypomastigotes at acidic pH.$^{11}$

We now report the effects of aureobasidin A on amastigote and promastigote forms of the parasite \textit{Leishmania} (\textit{Leishmania}) \textit{amazonensis}, both \textit{in vitro} (infection of cultured peritoneal macrophages) and \textit{in vivo} (development of footpad lesions in BALB/c mice).

Materials and methods

Parasites and promastigote culture

Promastigote forms of \textit{L. (L.)} \textit{amazonensis} (MHOM/BR/1973/M2269) were kindly provided by Dr J. J. Shaw, Instituto Evandro Chagas, Belém, Pará, Brazil. \textit{Leishmania} (\textit{Leishmania}) \textit{major} (MRHO/SU/1959/P) were kindly provided by Dr A. Cruz,
Faculdade de Medicina de Ribeirão Preto, Brazil. Leishmania (Viannia) braziliensis (MHOM/BR/1987/M11272) were kindly provided by Dr T. G. V. Silveira, from Universidade Estadual de Maringá, PR, Brazil. Promastigotes were grown at 23°C in liver infusion (LIT) medium containing 10% fetal calf serum (FCS) (Cultilab, São Paulo, SP, Brazil). LIT medium is prepared by mixing 1 mL of 10 mg/mL haemin (bovine, type I) (Sigma, St Louis, MO, USA) with 900 mL of solution containing 4.0 g of NaCl, 0.4 g of KCl, 8.0 g of Na2HPO4, 2.0 g of glucose, 5.0 g of liver infusion broth (Difco Laboratories Inc., Detroit, MI, USA), 5.0 g of tryptose (Difco Laboratories Inc.) and 12.5 mg of folic acid (Sigma) at pH 7.0.12 Promastigotes were isolated from stationary growth phase (metacyclic promastigotes, at 5–6-day-old culture).

Preparation of amastigotes

L. (L.) amazonensis amastigotes were maintained by footpad infection of Golden hamsters and isolated as described previously.13 Amastigote forms (2 × 10^7) were inoculated into hamster footpads; after 5–6 weeks, the lesions were surgically removed and the tissue minced in phosphate-buffered saline (PBS). Debris was eliminated by nylon Nitex filtration (pore size, 80 μm; Tekto, Monterey Park, CA, USA). The cell suspension was centrifuged at 1800 g for 10 min. To lyse erythrocytes, the pellet was resuspended in ammonium chloride solution (8.29 g of NH4Cl, 1 g of KHCO3 and 37.3 mg of EDTA per litre) for 10 min, and the insoluble material containing infected macrophage cells was homogenized (20 strokes). The resulting suspension was centrifuged four times at 1800 g, and the final pellet was resuspended in RPMI 1640 (Sigma) in the presence of penicillin/streptomycin. The suspension was centrifuged four times at 1800 g, and the final pellet was resuspended in RPMI 1640 (Sigma) in the presence of penicillin/streptomycin. The suspension was shaken for 3 h and the amastigotes washed four times by centrifugation at 1800 g. The parasite yield was 5 × 10^6 amastigotes/hamster.

All animal experimental procedures were approved by the Institutional Ethics Committee.

Aureobasidin A

Aureobasidin A was purchased from Takara Biomedicals (Shiga, Japan). Stock solution of 19 mM aureobasidin A in methanol was maintained at −20°C (for a maximum of 1 month), and diluted to the appropriate concentration for each experiment.

Assays of cytotoxicity

Inhibition of cell growth was assayed by cultivating promastigotes or amastigotes (1 × 10^6) in 0.5 mL of LIT medium with increasing concentrations of aureobasidin A (1–80 μM) and amphotericin B (as control drug, 0.01–0.5 μM) (Sigma) at 23°C. Culture aliquots were collected every 24 h for counting of parasites. The reversibility of the aureobasidin A effect was tested after 72 h, by washing the cultures with PBS and resuspending the parasites in LIT medium without aureobasidin A. Promastigote viability was determined by flagellar movement.

To estimate the 50% inhibitory concentration (IC50), and the viability of amastigotes and macrophages, an MTT micromethod described previously14 was mainly used throughout the experiments. Parasites were resuspended in LIT medium supplemented with 10% FCS and gentamicin (0.5 μg/mL) (medium A) and plated in 96-well plates (1 × 10^6 promastigotes/well, or 5 × 10^3 amastigotes), and incubated with increasing concentration of aureobasidin A (1–80 μM) and amphotericin B (0.01–0.5 μM) in triplicate. After 24 h, for amastigotes incubated at 36°C, or after 72 h, for promastigotes incubated at 23°C, plates were centrifuged at 900 g for 15 min and the supernatant removed. The formation of formazan was measured by adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Molecular Probes, Eugene, OR, USA) (2.0 mg/mL in medium A, 100 μg/well) and incubating the wells for 4 h in the dark at 37°C. The plates were subsequently centrifuged at 900 g for 15 min and the supernatant was removed; the pellet was dissolved in 200 μL of dimethylsulphoxide and the absorbance measured in an ELISA reader at 492 nm ( Labsystems Multiskan). IC50 with 95% confidence limits was determined by GraphPad Prism, Version 4.0 software. The r^2 value obtained for each curve always exceeded 0.96.

Aureobasidin A cytotoxicity to macrophages was also evaluated for macrophages cultured in 96-well plates in the presence of increasing concentrations of aureobasidin A (1–40 μM). Viability was assessed after 48 h by MTT procedure, as described below, >95% of macrophages maintained their viability at all aureobasidin A concentrations tested.

Peritoneal macrophage culture

Resident peritoneal macrophages were harvested by washing the peritoneal cavity of BALB/c mice with PBS. Macrophages were washed three times with cold PBS by centrifugation at 400 g, and the final pellet was resuspended in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES and penicillin (100 U/mL)/streptomycin (100 μg/mL). Macrophages (~5 × 10^6) were placed on sterile glass coverslips in 24-well plates. Non-adherent cells were removed by several washes with RPMI and the plates were kept at 37°C in a CO2 incubator.

Macrophage infectivity by L. (L.) amazonensis amastigotes

Amastigotes were added (1 × 10^5 parasites/well, two parasites/macrophage) to macrophage monolayers and maintained for 2 h in RPMI 1640 without FCS at 37°C. Non-adherent parasites were removed by washing the monolayers with culture medium and then RPMI 1640 with 5% FCS was added.15 Two sets of experiments were carried out: (i) aureobasidin A at a final concentration of 1, 4 or 10 μM was added 2 h after infection, and cultures were maintained for 48 or 72 h (post-infection) in a CO2 incubator; (ii) aureobasidin A at 10 μM was added 24 h after macrophage infection and cultures were maintained for 48 or 72 h post-infection in a CO2 incubator. Following these procedures, cells were fixed with methanol, stained with Hema 3 Stain Set (Fisher Scientific) and the infectivity index was determined by multiplying the percentage of macrophages that had at least one intracellular parasite by the average number of intracellular parasites per infected macrophage (300 cells were examined).

L. (L.) amazonensis promastigote infectivity in BALB/c mice

BALB/c mice (five per group) were inoculated subcutaneously at the left hind footpad with 5 × 10^3 metacyclic parasites pre-treated or not with 10 μM aureobasidin A for 3 days. Parasite number and viability was checked before the infection. Infection was monitored by measuring the thickness of infected footpads with a caliper.
Aureobasidin A effect on Leishmania

$[3^H]$serine metabolic labelling and lipid analysis

Promastigotes of L. (L.) amazonensis ($5 \times 10^7$ parasites/mL) incubated with 10 $\mu$M aureobasidin A, were metabolically labelled with 20 nCi/mL $[3^H]$serine (Amersham; specific activity 33 Ci/mmol) for 8 h, in DMEM lacking serine, supplemented with 10% previously dialysed FCS. Parasite lipids were extracted with iso-propanol/hexane/water (55:50:25) and subjected to mild base previously dialysed FCS. Parasite lipids were extracted with iso-propanol/hexane/water (55:50:25) and subjected to mild base treatment. The samples were dissolved in 1 mL of ethanol/water/diethyl ether/pyridine (15:15:5:1), 1 mL of 0.2 M KOH in methanol was added, and the mixture was incubated for 1 h at room temperature. The pH of the reaction mixture was neutralized with 0.2 mL of 1 M acetic acid and the samples were dried under N$_2$. Sphingolipids were recovered by partition with 1-butanol/water (1:1), and the butanolic fraction was dried, resuspended in chloroform/methanol (2:1), and analysed by high performance thin layer chromatography (HPTLC) using Silica-Gel 60 (Merck, Darmstadt, Germany), developed in chloroform/methanol/40% methylene (63:35:10). Radiolabelled sphingolipids were detected by fluorography after spraying of HPTLC plates with EN'HANCE (Perkin-Elmer, Boston, MA, USA) and exposure to Kodak X-Omat film at $–70^\circ$C. Non-radioactive IPC standard was detected by spraying the plates with Dittmer–Lester reagent and IPC isolated from Saccharomyces cerevisiae was used as standard.$^{17}$

Results

Dose-dependent inhibitory effect of aureobasidin A on L. (L.) amazonensis promastigote growth

Three days incubation with 4 $\mu$M aureobasidin A caused significant (~85%) growth inhibition of L. (L.) amazonensis promastigotes (Figure 1a) and growth was completely blocked by 20 $\mu$M aureobasidin A. In order to test reversibility of the effect, parasites were cultured with aureobasidin A (10 or 20 $\mu$M) for 3 days under the same conditions as in Figure 1(a), then washed with PBS and placed in LIT medium without aureobasidin A. Growth resumed after 24 h and approached a stationary level similar to that of the control experiment at day 8 (5 days after aureobasidin A removal) (Figure 1b). Promastigote viability during these experiments, as monitored by flagellar movement, was always >98%.

Effect of aureobasidin A on promastigotes of other Leishmania species

The effect of aureobasidin A on different species of Leishmania was also analysed by MTT analysis, based on the ability of viable parasites to reduce the tetrazolium salt to an insoluble formazan product. The aureobasidin A IC$_{50}$ (i.e. the concentration able to inhibit 50% of growth over 96 h compared with controls) was determined for promastigotes of L. (L.) amazonensis (IC$_{50}$, 4.1 $\mu$M; 95% confidence limits, 2.5–6.7 $\mu$M), L. (V.) braziliensis (IC$_{50}$, 13.7 $\mu$M; 95% confidence limits, 9.1–20.5 $\mu$M) and L. (L.) major (IC$_{50}$, 12.6 $\mu$M; 95% confidence limits, 7.8–20.3 $\mu$M). For L. (L.) amazonensis aureobasidin A IC$_{50}$ is 24.2 $\mu$M, while for L. (V.) braziliensis and L. (L.) major aureobasidin A at 80 $\mu$M inhibited 76% and 70% of parasite growth, respectively. These results indicate that L. (L.) amazonensis is more susceptible to aureobasidin A than L. (V.) braziliensis and L. (L.) major. For MTT assays, amphotericin B was used as a control drug, and the IC$_{50}$ determined for L. (L.) amazonensis (0.09 $\mu$M, 95% confidence limits 0.04–0.18 $\mu$M), L. (V.) braziliensis (0.12 $\mu$M; 95% confidence limits 0.03–0.19 $\mu$M) and L. (L.) major (0.07 $\mu$M; 95% confidence limits 0.03–0.17 $\mu$M). For the three Leishmania species amphotericin B at 0.25 $\mu$M completely inhibited the parasite growth.

Effect of aureobasidin A on L. (L.) amazonensis promastigote infectivity in BALB/c mice

Promastigotes at stationary phase (metacyclic form) were cultured with 10 $\mu$M aureobasidin A for 3 days. Next, $\sim 5 \times 10^5$ parasites (viability >98%) were inoculated subcutaneously into footpads of BALB/c mice and progression of lesions was checked weekly. It was observed that mice infected with parasites pre-treated with 10 $\mu$M aureobasidin A developed significantly smaller lesions than mice infected with non-treated parasites. At week 7 the lesion size was 2.16 ± 0.27 mm for animals infected with aureobasidin A pre-treated parasites, in contrast with controls which presented lesions measuring 4.06 ± 0.67 mm. The lesions were significantly smaller in mice infected with parasites pre-incubated with aureobasidin A than...
controls until week 8 of infection ($P < 0.05$). It is worth mentioning that no mice were cured.

**Effect of aureobasidin A on *L. (L.) amazonensis* amastigotes**

Amastigote forms isolated from hamster footpad lesions were incubated with aureobasidin A at 37°C for 24 h to assess the effect on their viability by MTT assay. Under these conditions it was verified that aureobasidin A is toxic to amastigotes; at 5.6 μM (3.6–8.7 μM, 95% confidence limits) only 50% of parasites remained viable after 24 h, compared with the control experiment. Under similar conditions, for amphotericin B it was determined that the concentration for which 50% of parasites remained viable after 24 h was 0.31 μM (confidence limits 0.22–0.43). Also, the effect of aureobasidin A (10 μM) on differentiation into promastigotes was analysed in amastigotes incubated at 23°C, as shown in Figure 2. For control cultures at 23°C, 24% of amastigotes differentiated into promastigotes by day 3, and by day 6 the number of promastigotes had increased 8-fold and no amastigotes were present (Figure 2a). When amastigotes were cultured with 10 μM aureobasidin A at 23°C, ~50% were killed within the first 24 h (Figure 2b). Some promastigotes were detectable at day 4, but they were not able to divide. At day 7, only promastigotes were detected, at a level that was 7% of control (see Figure 2a).

**Effect of aureobasidin A on IPC synthesis**

Promastigotes were metabolically labelled with [3H]serine for 8 h. Serine, by action of serine palmitoyltransferase, is incorporated in sphinganine, which by action of acyl transferase forms ceramide, the substrate for IPC synthase. After [3H]serine incorporation, lipids were extracted from the parasites with isopropanol/hexane/water (55:20:25), phosphoglycerolipids were hydrolysed by mild alkaline treatment and resistant sphingolipids were analysed by HPTLC. [3H]Serine was incorporated in the alkaline-resistant lipid fraction (Figure 4). In control promastigote culture (lane 1) [3H]serine was incorporated in components that co-migrate with IPC isolated from *S. cerevisiae*, and in standard ceramide. In promastigotes incubated with 10 μM aureobasidin A, the radiolabelled component that co-migrates with ceramide was reduced by 66%.

**Discussion**

Aureobasidin A showed a dose-dependent inhibitory effect on the growth of promastigotes. For *L. (L.) amazonensis* although 90% of growth was inhibited by 20 μM aureobasidin A, the parasites remained viable. Growth was restored after cultures were washed with PBS, showing that the aureobasidin A effect is reversible. Aureobasidin A also inhibited *L. (L.) major* and *L. (V.) braziliensis* promastigote growth. These results were confirmed by MTT assay. A significant difference in aureobasidin A IC50 was detected for *L. (L.) amazonensis* (IC50, 4.1 μM) and for *L. (L.) major* and *L. (V.) braziliensis* (IC50 of 12.6 and 13.7 μM, respectively). For *L. (L.) major* and *L. (V.) braziliensis* aureobasidin A at 80 μM inhibited at maximum 75% parasite growth. Promastigote sphingolipids metabolically labelled with [3H]serine were isolated, subjected to alkaline hydrolysis and analysed by HPTLC. The sphingolipid profile was altered when *L. (L.) amazonensis* was incubated with aureobasidin A. A component with chromatographic migration similar to IPC from *S. cerevisiae* was isolated. GC/MS analysis revealed peaks characteristic of inositol, sphingosine (d18:1), and fatty acids C18:0 and C16:0, indicating that the component corresponds to an IPC (data not shown). In promastigotes incubated with aureobasidin A, the radiolabelled component that co-migrates with IPC...
IPC was down-regulated, but no ceramide accumulation was observed. It is attractive to speculate that no accumulation of ceramide may explain the sustained promastigote viability, since the apoptotic effect of ceramide is well known. On the other hand, the inhibitory effect of aureobasidin A on cell division of \textit{L. (L.) amazonensis} promastigotes could be due to the fact that IPC is critical for membrane trafficking events in promastigotes\textsuperscript{5,18} and interferes with cell proliferation.

When \textit{L. (L.) amazonensis} promastigotes were pre-treated with aureobasidin A and then injected in BALB/c mice footpads, a significant delay in the infection was observed, similar to that seen in \textit{L. (L.) major} null mutants defective in sphingosine synthesis\textsuperscript{19}.

Intracellular amastigotes were more susceptible to aureobasidin A than amastigotes maintained axenically in culture; 10 \textmu M aureobasidin A reduced the number of intracellular amastigotes by 95\% and that of axenic amastigotes by 50\%; it was determined by MTT assay that the aureobasidin A concentration for which only 50\% of parasites remained viable after 24 h was 5.6 \textmu M (95\% confidence limits 3.6–8.7 \textmu M). The significant decrease in \textit{Leishmania} infectivity index in macrophages caused by aureobasidin A was due mainly to a reduction in the number of intracellular amastigotes. \textit{L. (L.) amazonensis} amastigotes were susceptible to aureobasidin A regardless of whether the drug was added to culture medium 2 or 24 h after macrophage infection, indicating that aureobasidin A is toxic to amastigotes even when they are inside parasitophorous vacuoles. Aureobasidin A at doses up to 20 \textmu M did not increase macrophage NO production (data not shown), indicating that aureobasidin A toxicity

### Table 1. Effect of aureobasidin A on intracellular amastigotes

<table>
<thead>
<tr>
<th>Assay(^*)</th>
<th>Percentage of macrophages with \textit{Leishmania} after 48 h of infection</th>
<th>Percentage of macrophages with \textit{Leishmania} after 72 h of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47.00 ± 2.83</td>
<td>57.00 ± 4.38</td>
</tr>
<tr>
<td>AbA 2 h, 1 \textmu M</td>
<td>55.00 ± 7.07</td>
<td>57.00 ± 4.24</td>
</tr>
<tr>
<td>AbA 2 h, 4 \textmu M</td>
<td>31.50 ± 2.12(^*)</td>
<td>30.25 ± 2.47(^*)</td>
</tr>
<tr>
<td>AbA 2 h, 10 \textmu M</td>
<td>4.55 ± 0.07(^*)</td>
<td>1.90 ± 1.56(^*)</td>
</tr>
<tr>
<td>AbA 24 h, 10 \textmu M</td>
<td>39.00 ± 4.24</td>
<td>30.00 ± 1.41(^*)</td>
</tr>
</tbody>
</table>

\(^*\)Aureobasidin A was added to the culture 2 h after infection (AbA 2 h) or 24 h after infection (AbA 24 h).

\(^*\)Statistical analysis (Student’s t-test) indicates a significant difference (\(P < 0.05\)), compared with control.

**Figure 3.** Effect of aureobasidin A (AbA) on intracellular \textit{L. (L.) amazonensis} amastigotes. Macrophages were infected with amastigotes of \textit{L. (L.) amazonensis} for 2 h at 37 °C, and macrophage monolayers were washed with PBS. Two protocols were followed: aureobasidin A treatment (1, 4 or 10 \textmu M) started after 2 h of infection (a), and aureobasidin A treatment (10 \textmu M) started after 24 h of infection (b). Infectivity index was calculated after 48 and 72 h of infection, as indicated. \(^*\)Significant difference (\(P < 0.05\)), compared with control.

**Figure 4.** HPTLC profile of lipids of \textit{L. (L.) amazonensis} promastigotes labelled with \[^{3}H\]serine. Promastigotes (5 × 10\(^7\)) were metabolically labelled for 8 h with \[^{3}H\]serine in the presence of 10 \textmu M aureobasidin A. Lipids were extracted with isopropanol/hexane/water, subjected to mild base treatment and analysed by HPTLC in chloroform/methanol/40\% methylamine (63:35:10). Labelled sphingolipids were detected by fluorography as described in the Materials and methods section. Lane 1, non-treated promastigotes (control). Lane 2, promastigotes treated with aureobasidin A. IPC, inositol phosphorlyceramide; CER, ceramide; OR, origin.
to intracellular amastigotes was not due to its effect on macrophage viability or activation.

Although preliminary studies did not show expression of IPC in *L. (L.) amazonensis* amastigotes, this form of parasite is more susceptible to aureobasidin A. Perhaps amastigotes contain IPC at a very low concentration which is necessary as an intermediary metabolite for parasite survival in mammalian host cells. Since sphingolipid precursors and/or metabolites have been implicated in mammalian signalling, apoptosis and stress response, an interesting possibility is that aureobasidin A may alter intracellular parasite sphingolipid metabolites that have apoptotic effects.

The present results suggest that the sphingolipid biosynthetic pathway, particularly steps correlated with IPC synthesis, will provide a promising approach for the development of new drugs directed to *Leishmania* and other parasites.

**Acknowledgements**

The authors thank Dr Stephen Anderson for editing of the manuscript and Maria Vieira Seles for technical assistance. The studies were supported by FAPESP, CNPq, and CAPES.

**Transparency declarations**

None to declare.

**References**