Amphiphilic Antimony(V) Complexes for Oral Treatment of Visceral Leishmaniasis

Flaviana R. Fernandes, Weverson A. Ferreira, Mariana A. Campos, Guilherme S. Ramos, Kelly C. Kato, Gregório G. Almeida, José D. Corrêa Junior, Maria N. Melo, Cynthia Demicheli, Frédéric Frézard

Departamento de Fisiologia e Biofísica, Instituto de Ciências Biológicas; Departamento de Química, Instituto de Ciências Exatas; Departamento de Parasitologia, Instituto de Ciências Biológicas, and Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Pampulha, Belo Horizonte, Minas Gerais, Brazil

The need for daily parenteral administration is an important limitation in the clinical use of pentavalent antimonial drugs against leishmaniasis. In this study, amphiphilic antimony(V) complexes were prepared from alkylmethylglucamides (L8 and L10, with carbon chain lengths of 8 and 10, respectively), and their potential for the oral treatment of visceral leishmaniasis (VL) was evaluated. Complexes of Sb and ligand at 1:3 (SbL8 and SbL10) were obtained from the reaction of antimony(V) with L8 and L10, as evidenced by elemental and electrospray ionization-tandem mass spectrometry (ESI-MS) analyses. Fluorescence probing of hydrophobic environment and negative-staining transmission electron microscopy showed that SbL8 forms kinetically stabilized nanoassemblies in water. Pharmacokinetic studies with mice in which the compound was administered by the oral route at 200 mg of Sb/kg of body weight indicated that the SbL8 complex promoted greater and more sustained Sb levels in serum and liver than the levels obtained for the conventional antimonial drug meglumine antimoniate (Glu). The efficacy of SbL8 and SbL10 administered by the oral route was evaluated in BALB/c mice infected with Leishmania infantum after a daily dose of 200 mg of Sb/kg for 20 days. Both complexes promoted significant reduction in the liver and spleen parasite burdens in relation to those in the saline-treated control group. The extent of parasite suppression (>99.96%) was similar to that achieved after Glu given intraperitoneally at 80 mg of Sb/kg/day. As expected, there was no significant reduction in the parasitic load in the group treated orally with Glu at 200 mg of Sb/(kg day). In conclusion, amphiphilic antimony(V) complexes emerge as an innovative and promising strategy for the oral treatment of VL.

Visceral leishmaniasis (VL) is a systemic parasitic disease which leads to high rates of morbidity and mortality in humans worldwide. Even with scientific advances related to diagnosis, treatment, and prevention over the past 10 years, VL still is a neglected disease leading to 60,000 human deaths/year (1, 2). The clinical manifestations of VL are attributed to obligate intracellular protozoa of the Leishmania donovani complex, and depending on the etiological agent, the disease presents two distinct forms: anthroponotic VL that is endemic in India and Central Africa, caused by L. donovani, and zoonotic VL that occurs in countries of the Mediterranean basin, Central Asia, and the Americas, caused by Leishmania infantum.

Even though pentavalent antimonials, including meglumine antimoniate (Glu) and sodium stibogluconate (Pentostam), are still the first-line drugs in several developing countries for treatment of all forms of leishmaniasis (2), they have several limitations (3, 4, 5). These drugs have to be given parenterally, daily, for at least 3 weeks (typically, 20 mg of Sb/kg of body weight/day for 20 to 30 days). Antimony therapy is often accompanied by local pain during intramuscular injections and by systemic side effects, requiring very careful medical supervision. Typical side effects include nausea, vomiting, weakness and myalgia, abdominal colic, diarrhea, skin rashes, and hepatotoxicity, together with the most severe pancreatitis and cardiotoxicity. All these factors contribute to compliance difficulties and, eventually, treatment failures.

At present, only one oral VL therapy exists. Miltefosine (hexadecylphosphocholine) was found to exhibit efficacy against VL. In 2002, miltefosine (Impavido; Zentaris GmBH) was registered and licensed in India for oral treatment of VL, but teratogenicity restricted its use in women of childbearing age (6, 7, 8). In addition, it has a long half-life (~150 h) and requires 28 days of treatment; these factors, when the drug is taken orally on an outpatient basis, make the drug critically prone to low compliance and increase the possibility of the development of drug resistance (9, 10).

In this context, new drugs must be developed that are capable of being administered orally with minimal medical supervision, at an affordable price.

The oral bioavailability of meglumine antimoniate was found to be enhanced through complexation with cyclodextrin (11), presumably as a result of the formation of a ternary meglumine-Sb-cyclodextrin complex, which maintains the antimonial compound depolymerized and sustainedly releases the low-molecular-weight 1:1 Sb-meglumine (12, 13). However, the advance achieved is still limited, because of the insufficient solubility of the ternary complex for application in large animals and the highly hydrophilic character of Sb-meglumine complex (14, 15).

In the present study, a novel oral delivery strategy for pentavalent antimonials was investigated, based on the formation of an amphiphilic antimony(V) complex. Such a complex was obtained...
after reaction of antimony(V) with nonionic surfactants from the N-alkyl-N-methylglucamide series (Fig. 1). Improved oral bioavailability and pharmacokinetics of Sb in mice were achieved from such a complex, compared to meglumine antimoniate. The resulting amphiphilic complexes were found to be active by the oral route in a murine model of VL.

MATERIALS AND METHODS

Materials and drugs. N-Decanoyl-N-methylglucamide (MEGA-10 or L10; 100% ultrapure grade) was purchased from Anresco (SOLON, OH). N-Octanoyl-N-methylglucamide (MEGA-8 or L8; 98%), potassium hexahydroxoantimonate [KSb(OH)6], and 1,6-diphenylhexatriene (DPH) were obtained from Sigma-Aldrich Chemical Co. (USA). Glucan-time (Glu) was obtained from Sanofi-Aventis (São Paulo, Brazil). All other reagents were of at least reagent grade. Double-distilled deionized water was used throughout all the experiments.

Animals. Swiss and BALB/c mice (female, 4 to 6 weeks old) were obtained from CEBIO of the Institute of Biological Sciences, Federal University of Minas Gerais (UFMG; Belo Horizonte, Brazil). Free access to a standard diet was allowed, and tap water was supplied ad libitum. The studies involving animals were approved by the Ethical Committee for Animal Experimentation of the UFMG with protocol numbers 215/09 and 199/2011.

Parasites. Leishmania infantum (MHOM/BR/70/BH46) parasites were routinely maintained and isolated from golden (Syrian) hamsters (Mesocricetus auratus) and were grown as promastigotes at 26°C in Dulbecco modified Eagle medium (DMEM; Sigma) supplemented with 20% fetal bovine serum (FBS; CultiLab, Brazil), 2 mM L-glutamine, 25 mM HEPES, 50 μM 2-mercaptoethanol, and 20 μg/mL of gentamicin at pH 7.0.

Preparation and characterization of amphiphilic antimony(V) complexes. The SbL8 and SbL10 compounds were prepared by mixing KSb(OH)6 and L8 or L10 surfactant in water at a 1:3 Sb/surfactant molar ratio and a final surfactant concentration of 0.08 M. The mixture was heated at 60°C under agitation until complete solvent evaporation. The resulting film was redissolved in water at 25°C, and the dispersion was heated at 60°C under agitation until complete solvent evaporation. The freeze-dried compounds were first characterized by C, H, and N elemental analysis, inductively coupled plasma optical emission spectrometry (Sb and K), and thermogravimetry. In the case of the SbL8 compound, analysis found the following: C, 47.28; H, 7.85; N, 3.75; K, 3.6; Sb, 10.69%. The calculation for C116H212K13N15O42Sb (1:3 Sb/L8 complex) is as follows: C, 48.3; H, 7.84; N, 3.76; K, 3.5; Sb, 10.88%. In the case of the SbL10 compound, analysis found the following: C, 49.48; H, 8.19; N, 3.62; K, 3.3; Sb, 10.24%. The calculation for C192H344K26N30O64Sb (1:3 Sb/L10 complex) is as follows: C, 50.91; H, 8.29; N, 3.49; K, 3.2; Sb, 10.12%.

Electrospary ionization-tandem mass spectrometry (ESI-MS) spectra were collected on a Thermo-Scientific QCLFleet mass spectrometer operating in both the negative and positive modes. Samples were prepared in 1:1 acidic water-methanol solutions, and measurements used the following main instrument parameters: capillary voltage, 3 kV, and cone voltage, 30 kV.

The 1H and 13C nuclear magnetic resonance (NMR) spectra were acquired at 25°C on a Bruker DRX400-ADVANCE spectrometer operating at 400.129 MHz under standard conditions using a direct-detection 5-mm 1H/13C dual probe with 90° pulse lengths of 7.2 μs for 1H. TMS [3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt] was used as an internal reference. Samples were prepared in CDC13 or d4 dimethyl sulfoxide (DMSO) at 25 g/liter of L8. Two-dimensional 1H homonuclear correlation spectroscopy (COSY) and heteronuclear multiple quantum coherence (HMBC) analyses allowed for the assignment of hydrogen and carbon signals.

The formation of hydrophobic environment in aqueous dispersions of L8 and SbL8 was investigated using the lipophilic fluorescent probe DPH, as described previously (16). This assay is based on the principle that DPH is not fluorescent in water due to molecular aggregation but fluoresces after incorporation into a hydrophobic environment, such as those provided by micelles, allowing for determination of the critical micellar concentration (CMC). Fluorescence measurements were carried out using a Cary Eclipse fluorescence spectrometer (Varian Inc.). Temperature was controlled at 25°C through a jacketed cuvette holder from a refrigerated circulating water bath. To evaluate the dependence of DPH fluorescence upon L8 concentration, DPH was added from a tetrahydrofuran stock solution at a final concentration of 5 × 10−7 M to dispersions of L8 or SbL8 in water at various concentrations. After a 24-h incubation at 25°C under light protection, the fluorescence intensity was measured at excitation and emission wavelengths of 360 and 428 nm, respectively. To evaluate the kinetic stability of SbL8 and L8 nanoassemblies, dispersions of SbL8 and L8 were prepared in water at 50 mM and 100 mM, respectively, and were diluted to 7.5 mM. After different times of incubation at 25°C, the interaction of DPH was assessed through measurement of fluorescence intensity.

Dynamic light scattering (DLS) measurements of L8 and SbL8 dispersions were performed using a Zetasizer (Nano ZS90; Malvern Instruments, United Kingdom). Dispersions were determined according to the Nanoparticle Tracking Analysis (NTA) technique and using NTA 2.3 software (17). L8 and SbL8 were analyzed using the same camera gain. This technique is based on the tracking of Brownian motion of individual particles and determination of size according to the Stokes–Einstein equation.

Negative-staining transmission electron microscopy (TEM) analysis was also performed. A drop of SbL8 aqueous dispersion (0.001 M Sb) was placed onto a 300-mesh Formvar copper grid (TABB Laboratories Equipment, Berks, United Kingdom) and allowed to adsorb, and the surplus was removed by filter paper. A drop of 5% (wt/vol) aqueous solution of uranyl acetate was added. The surplus water was removed by filter paper, and the sample was dried at room temperature before the nanostructures were imaged. Representative electron micrographs were taken at magnifications of ×22,000 to ×30,000.

Pharmacokinetic studies of antimonial compounds in mice. Swiss mice received through oral inoculum, with the aid of a needle gauge, 0.2 ml of an aqueous solution of either commercial antimonial drug (Glu) or SbL8 complex at 200 mg of Sb/kg of body weight. Mice from each group (n = 5/time) were sacrificed at the following times: 15 and 30 min and 1, 2, 3, 6, 12, and 24 h after administration. The pharmacokinetics of Glu was also evaluated after administration to mice by the intravenous (i.v.) route at 300 mg of Sb/kg. Animals (n = 5 to 7/time) received by the tail vein 0.1 ml of Glu and were sacrificed at the following time points: 10, 20, 40, 60, and 90 min and 2 and 3 h. Animal sacrifice was done by cervical disloca-
tion after ketamine-xylazine anesthesia. Blood samples were then collected from the brachial plexus, and the serum was recovered and frozen at −20°C. The livers were also recovered, homogenized, and subjected to digestion with nitric acid in a dry block (MA 4004; Marconi, São Paulo, Brazil), as described previously (18). Antimony was determined in diluted serum and digested liver by electrothermal atomic absorption spectrometry (ETAAS) using a Perkin-Elmer AA600 graphite furnace atomic absorption spectrometer, as described previously (15, 18). The analytical methods for determination of Sb in the serum and liver were validated and showed suitable levels of precision (coefficient of variation [CV] < 5%), accuracy (80 to 120% analyte recovery), and linearity (range of 10 to 180 μg of Sb/liter). The quantification limits of the analytical methods were 240 μg of Sb/liter and 0.93 μg of Sb/g for the serum and liver, respectively. Pharmacokinetic parameters were determined using the R-STRIP 4.03 computer program. Experimental Sb concentration-time data were subjected to iterative weighted nonlinear least-squares regression (19). A biexponential model with oral bolus input was chosen. Fitted parameters included maximum concentration of Sb (Cmax), the time for reaching the peak Sb concentration (Tmax), the mean residence time of Sb in the 0- to 24-h interval (MRT), the area under the concentration–time curve in the 0- to 24-h interval (AUC), and the coefficient of partition of Sb to the liver (Kp = AUC in liver/AUC in serum).

**Antileishmanial activity in murine model of visceral leishmaniasis.** Groups of BALB/c mice (n = 7 to 10) were infected in the tail vein with 1 × 10⁶ late-log-phase *L. infantum* promastigotes. The treatment was started 8 days after infection, with daily doses for 20 days. The first experiment included 5 groups that received the following formulations: SbL8 by the oral route (46 mg in 200 μl of water, equivalent to 200 mg of Sb/kg, for each daily dose), L8 alone by the oral route (same dose as in the group treated with SbL8), Glu by the oral route (58 μl of Glu plus 142 μl of saline, equivalent to 200 mg of Sb/kg, for each daily dose), Glu by the intraperitoneal route (24 μl of Glu plus 77 μl of saline, equivalent to 80 mg of Sb/kg, for each daily dose), and saline by the oral route.

Twenty-one days after the beginning of treatment, the animals were sacrificed and the spleen and liver were harvested for determination of parasite burdens by limiting dilution, as previously described (20). Briefly, organs were weighed and fragmented, and a tissue homogenate was obtained in 1 ml of DMEM plus 20% FBS at pH 7.0. Each tissue homogenate was serially diluted (10-fold) in a 96-well flat-bottom microtiter plates (Nunc; Nunclon). Samples, in duplicate, were incubated at 23°C. The wells containing motile promastigotes were identified with a microscope (Axiocvert 25; Zeiss), and the parasite burden was determined from the highest dilution at which promastigotes had grown after 12 days of incubation, as follows: parasite burden = 10(log parasite burden/mg of organ + 10). Data are presented as log parasite burden/mg of organ. The resulting transformed data (normally distributed) were statistically analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni posttest.

A parasite suppression index (SI) was also calculated by the following formula: SI = 100 − [100 × (median of parasite burden/mg of organ of treated mice)/(median of parasite burden/mg of organ of control mice)]. In the former experiment, a fragment of the liver was also used for determination of Sb, as described above.

**RESULTS**

**Physicochemical characterization of amphiphilic antimony complexes.** Complexation of Sb(V) with the surfactants L8 (Fig. 1) and L10 was achieved through reaction of KSb(OH)₆ with the surfactant in water at a 1:3 molar ratio. According to elemental, thermogravimetry, and conductimetry analyses as well as K and Sb determinations, the resulting product was characterized as a potassium salt containing Sb and the surfactant at a 1:3 molar ratio. Interestingly, the resulting products dissolved at high concentration in nonpolar solvents, such as chloroform and DMSO, and also in water.

*Figure 2* presents the ESI-MS spectrum of SbL8 in the negative mode, and *Table 1* displays the main species identified in SbL8 and SbL10.
TABLE 1 ESI-MS characterization of SbL8 and SbL10 complexes

<table>
<thead>
<tr>
<th>Anionic species</th>
<th>m/z</th>
<th>SbL8</th>
<th>SbL10</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(2L-6H)SbV]−</td>
<td>757.3</td>
<td>813.8</td>
<td></td>
</tr>
<tr>
<td>[(2L-4H)SbV(O)]−</td>
<td>775.3</td>
<td>831.9</td>
<td></td>
</tr>
<tr>
<td>[(2L-4H)SbV(OH)2]−</td>
<td>793.3</td>
<td>845.8</td>
<td></td>
</tr>
<tr>
<td>[(2L-4H)SbV(O)2]−</td>
<td>877.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[(5L-12H)(SbV)2]3−</td>
<td>917.9</td>
<td>988.0</td>
<td></td>
</tr>
<tr>
<td>[(3L-6H)SbV]−</td>
<td>1,078.5a</td>
<td>1,162.6a</td>
<td></td>
</tr>
<tr>
<td>[(5L-12H+K)(SbV)2]3−</td>
<td>1,874.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Peak of higher intensity.

SbL8 and their proposed structures. Accordingly, the main peak was attributed to a 1:3 ratio of Sb to ligand complex. ESI-MS analysis of the 1,078.5 species identified in SbL8 led to the appearance of m/z 757.5 ions corresponding to 1:2 complexes of Sb to L8, indicating the release of one L8 molecule.

To get insight into the site of Sb(V) binding to the surfactant molecule, SbL8 was further characterized using 1H and 13C NMR in CDCl3. No significant change of the chemical shift of L8 hydrogens was observed upon complexation (data not shown). On the other hand, as illustrated in Table 2, comparative 13C NMR analyses of L8 and its complex showed changes of the resonance of C1, C1′, C4, and C5, supporting the complexation of Sb(V) with the polar head of the surfactant. According to 1H NMR spectra obtained in d6 DMSO, integration of the proton signals related to hydroxyls (5- to 4-ppm range) gave values of 4.9 and 2.5 for L8 and its complex, respectively. These data strongly indicate that Sb(V) coordinates between 2 and 3 hydroxyls in L8.

To address the changes in the physicochemical behavior of L8 upon complexation with Sb(V), the nanoassemblies formed in aqueous solution were characterized through DPH fluorescence probing of hydrophobic environment, as well as size and concentration analyses of nanoparticles by DLS, TEM, and NTA.

As shown in Fig. 3A, the lipophilic fluorescent probe DPH exhibited an increase of the fluorescence intensity when incubated with L8 dispersion in water, from a concentration of about 50 mM. This property was expected from the reported value of critical micellar concentration (CMC) of L8 (21). Interestingly, the SbL8 complex formed a hydrophobic environment from a lower CMC value of about 10 mM, indicating an enhanced thermodynamic stability of the nanoassemblies.

The dispersions of SbL8 in water were found to be thermodynamically stable for at least 1 month upon storage at room temperature. This long-term stability was evidenced through macroscopic evaluation and DPH incorporation (data not shown). On the other hand, upon dilution of a concentrated SbL8 solution (from 50 mM to 7.5 mM in water), a slow decrease of DPH incorporation was observed (Fig. 3B), indicating that SbL8 nanoassemblies dissociate slowly after dilution. Indeed, micelle nanoassemblies are expected to dissociate upon dilution below the CMC; however, surfactant micelles usually show immediate dissociation, as observed in this study in the case of noncomplexed L8 micelles (Fig. 3B). Thus, SbL8 nanoassemblies exhibited unusual kinetic stabilization upon dilution, supporting the model that these nanoparticles do not readily dissociate following administration and may act as a sustained release system of Sb.

The size distribution of nanoparticles in L8 and SbL8 aqueous dispersions could not be fully assessed by DLS because of high polydispersity (L8 and SbL8) and low count (L8). DLS analysis of SbL8 suggests the coexistence of micelle-type particles with a diameter of a few nanometers, together with much larger nanoassemblies with a mean diameter in the 100-nm range (data not shown). This interpretation was supported by transmission electron microscopy analysis of SbL8 dispersion in water after negative staining. As illustrated in Fig. 4, two main populations of nanoparticles were identified: one population with mean diameter in the 20- to 30-nm range and the other one showing a mean diameter of 80 nm. NTA, with expected sensitivity for nanoparticles with diameter higher than 30 nm (17), revealed the presence of nanoparticles with mean hydrodynamic diameter in the range of 115 to 140 nm in both L8 and SbL8 (Table 3). Inter-

FIG 3 Interaction of DPH fluorescent probe with L8 and SbL8 aqueous dispersions at 25°C. (A) Fluorescence intensity of 0.5 μM DPH after 24 h of incubation with L8 or SbL8 dispersions, as a function of L8 concentration. (B) Fluorescence intensity of 0.5 μM DPH in SbL8 or L8 dispersion at different times after dilution of concentrated aqueous solution (50 mM for SbL8 and 100 mM for L8) to 7.5 mM L8. a.u., arbitrary units.

TABLE 2 13C NMR data for L8 and SbL8 in CDCl3

<table>
<thead>
<tr>
<th>Carbon(s)</th>
<th>L8</th>
<th>SbL8</th>
<th>Δδ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2′</td>
<td>33.08/33.66</td>
<td>33.14/33.69</td>
<td>−0.06/−0.03</td>
</tr>
<tr>
<td>3′</td>
<td>25.25/25.66</td>
<td>25.08/25.61</td>
<td>−0.03/0.05</td>
</tr>
<tr>
<td>4′–7′</td>
<td>22.65/29.12/29.49/51.79/31.82</td>
<td>22.66/29.14/29.49/31.79/31.82</td>
<td>−0.01/−0.02/−0.05/−0.03</td>
</tr>
<tr>
<td>8′</td>
<td>14.09</td>
<td>14.09</td>
<td>0.01</td>
</tr>
<tr>
<td>1′</td>
<td>34.57/37.55</td>
<td>34.38/37.28</td>
<td>0.19/0.27</td>
</tr>
<tr>
<td>2</td>
<td>72.05</td>
<td>72.05</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>51.74/52.82</td>
<td>51.48/52.65</td>
<td>0.26/0.17</td>
</tr>
<tr>
<td>3</td>
<td>70.21</td>
<td>70.29</td>
<td>0.08</td>
</tr>
<tr>
<td>4 and 5</td>
<td>71.85 or 73.13</td>
<td>71.67 or 73.23</td>
<td>0.18 or −0.1</td>
</tr>
<tr>
<td>6</td>
<td>63.89</td>
<td>63.84</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Downloaded from http://aac.asm.org on August 15, 2017 by guest
Interestingly, the concentration of nanoparticles was much higher in SbL8 than L8 dispersion, in accordance with the higher CMC of L8.

Pharmacokinetic studies of SbL8 in mice by the oral route. SbL8 given by the oral route was evaluated in mice and compared to the hydrophilic commercial antimonial drug Glu, regarding pharmacokinetic profiles of Sb in the serum and liver. Figure 5 shows the level of Sb in the serum and liver in Swiss mice after administration of SbL8 and Glu at the same dose of Sb (200 mg of Sb/kg). Table 4 displays the serum pharmacokinetic parameters. Both compounds were found to be rapidly absorbed; however, SbL8 promoted more sustained serum Sb levels compared to serum Glu levels, with significantly greater serum Sb concentrations after 6 and 24 h (Fig. 5A). Accordingly, the area under the curve (AUC) and mean residence time (MRT) were about 4-fold greater for SbL8 than for Glu (Table 4). It noteworthy that Sb reached the liver more rapidly from SbL8 than Glu and that SbL8 promoted significantly higher Sb levels in the 0.5- to 3-h period (Fig. 5B), resulting in a 2.4-fold-greater AUC in this tissue (Table 4). These data suggest that part of SbL8 is retained in the liver after absorption into the portal system before being released into the bloodstream. This is in contrast with Glu, whose pharmacokinetic data suggest an accumulation of Sb in the liver after entrance in the blood circulation. The lower MRT of Glu is also consistent with the fast renal excretion of this water-soluble drug (4).

The pharmacokinetic parameters of SbL8 and Glu by the oral route were also compared to those of Glu given intravenously at 300 mg of Sb/kg (Table 4). Even though oral antimonial compounds exhibited much lower values for serum $C_{\text{max}}$ and AUC than does intravenous Glu, these oral drugs showed much longer MRTs. Interestingly, the AUC determined in the liver for oral SbL8 was close to that for intravenous Glu. Furthermore, the coefficient of partition of the drug to the liver ($K_{\text{p, H11005}} = AUC\text{liver}/AUC\text{serum}$) was more favorable for the oral drugs than for intravenous Glu (Table 4). These results suggest that a higher targeting of Sb to the liver was achieved when the drugs were given by the oral route.

Efficacies of SbL8 and SbL10 in a murine model of visceral leishmaniasis. The observation that the amphiphilic antimonial drug promoted greater concentrations of Sb in the liver compared to Glu led us to further evaluate the antileishmanial efficacies of SbL8 and SbL10 in BALB/c mice infected with *Leishmania infantum*, as an experimental model of VL. In the first experiment, the efficacy of oral SbL8 at 200 mg of Sb/kg/day was compared to that of Glu given orally and i.p. routes at 200 and 80 mg of Sb/kg/day, respectively. Control groups received saline and L8 orally. However, treatment of mice with L8, given at the same dose as in SbL8 group, had to be interrupted because of acute toxicity, with 50% animal loss after 10 days of treatment. The fact that no animal died during the treatment with SbL8 strongly suggests that complexation of L8 with Sb(V) reduced its acute toxicity. Figure 6 shows the parasite burdens in the livers and spleens of mice after 20 days of treatment. Oral SbL8 promoted a significant reduction in the liver and spleen parasite burdens compared to those in the control group (saline). These data represent 99.999% and 99.987% parasite suppression in the liver and spleen, respectively. In contrast, Glu given orally did not significantly reduce the liver

---

TABLE 3 NTA analyses of L8 and SbL8 at 30 mM in water

<table>
<thead>
<tr>
<th>Compound</th>
<th>Diam, mean diam ± SD* (nm)</th>
<th>Particle concn (10^8/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L8</td>
<td>138 ± 61</td>
<td>2.9</td>
</tr>
<tr>
<td>SbL8</td>
<td>114 ± 47</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* Data are the means and standard deviations of 2,297 and 455 tracks for SbL8 and L8, respectively.
or spleen parasite load. Furthermore, oral Glu was significantly less effective than oral SbL8 in reducing the liver parasite load. The group treated intraperitoneally with Glu, which served as a positive control, showed a significant parasite suppression in both the liver and spleen. The concentration of Sb in the liver was also determined just after treatment, as an attempt to correlate the antileishmanial activity to the liver Sb concentration. As shown in Fig. 7, the Sb concentration in the liver was significantly greater after oral SbL8 and intraperitoneal Glu than after oral Glu. Interestingly, the concentration of Sb did not differ significantly between the groups that received oral SbL8 and intraperitoneal Glu.

In a second experiment, the efficacy of SbL10 given orally at 200 mg of Sb/kg/day was evaluated in the same experimental model and was compared to that of Glu given intraperitoneally at 80 mg of Sb/kg/day. Figure 8 shows the parasite burdens in the livers and spleens of mice after 20 days of treatment. Importantly, oral SbL10 promoted a significant reduction in both the liver and spleen parasite burdens, compared to the values for the saline group, showing suppression levels (99.984% in liver and 99.969% in spleen) close to those achieved after intraperitoneal Glu (99.963% in liver and 99.995% in spleen).

These data, taken together, established for the first time the oral efficacy of amphiphilic antimony(V) complexes in a murine model of VL.

**DISCUSSION**

The present work reports for the first time the preparation of amphiphilic Sb(V) compounds from two nonionic surfactants of the N-alkyl-N-methylglucamide series (L8 and L10). These compounds consist essentially of 1:3 complexes of Sb to ligand. The amphiphilic character of the resulting complexes is evidenced by their abilities to dissolve in both organic solvent and water and to self-associate in aqueous solution, forming nanoassemblies. As major results of pharmacological evaluations, SbL8 exhibited an improved oral bioavailability of Sb and more favorable pharmacokinetic parameters in the serum and liver of mice than obtained with Glu. Importantly, the resulting amphiphilic complexes showed efficacy by the oral route in a murine model of VL.

Conventional pentavalent antimonials are considered inactive when given by the oral route. A previous pharmacokinetic study of meglumine antimoniate with dogs indicated an oral bioavailability of about 10% (15). Such a low bioavailability can be attributed to the highly hydrophilic character of meglumine antimoniate and also to its tendency to polymerize in concentrated aqueous solutions (22). As an attempt to improve the oral bioavailability of pentavalent antimony, a strategy based on the formation of amphiphilic antimony(V) complex was investigated. This strategy is supported by previous works showing improved delivery of metal across biological membranes through their complexation with li-

**TABLE 4 PK parameters of SbL8 and Glu in Swiss mice**

<table>
<thead>
<tr>
<th>Drug (route)</th>
<th>Dose (mg/kg)</th>
<th>Serum PK parameters</th>
<th>Liver PK parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C&lt;sub&gt;max&lt;/sub&gt; (mg/liter)</td>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>AUC (mg · h/liter)</td>
</tr>
<tr>
<td></td>
<td>K&lt;sub&gt;p&lt;/sub&gt; (AUC liver/AUC serum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SbL8, oral</td>
<td>200</td>
<td>2.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu, oral</td>
<td>200</td>
<td>2.9</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu, i.v.</td>
<td>300</td>
<td>1,652</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**FIG 6** Parasite load in the liver (A) and spleen (B) in BALB/c mice infected with *Leishmania infantum*, after 20 days of treatment with oral SbL8 (200 mg of Sb/kg/day), oral Glu (200 mg of Sb/kg/day), intraperitoneal Glu (80 mg of Sb/kg/day), or saline. Data are shown as means ± SEMs (n = 7). ***, P < 0.01; ****, P < 0.001 (one-way ANOVA followed by Bonferroni posttest).

**FIG 7** Sb concentration in the liver of BALB/c mice infected with *Leishmania infantum*, after 20 days of treatment with oral SbL8 (200 mg of Sb/kg/day), oral Glu (200 mg of Sb/kg/day), or intraperitoneal Glu (80 mg of Sb/kg/day). Data are shown as means ± SEMs (n = 7). *, P < 0.05; **, P < 0.01 (Kruskal-Wallis followed by Dunn’s multiple-comparison posttest).
pohilic ligand, as, for example, in Tc-99m HMPAO (99mTc-labeled hexamethylpropyleneamine oxime) (23) and satraplatin (24). N-Alkyl-N-methylglucamides were chosen as ligands for this purpose, because their polar head moiety is N-methylglucamine, which is the ligand of Sb(V) in meglumine antimoniate. Thus, these new complexes may be seen as amphiphilic meglumine antimoniate derivatives.

Regarding the pharmacokinetic data (Table 4), SbL8 showed a higher AUC than oral Glu, which may be explained by its amphiphilic character and its more effective permeation across the gastrointestinal tract. The aggregated state of SbL8 and the low rate of dissociation of these nanoassemblies probably contribute to the more sustained serum Sb levels, also evidenced by the greater MRT. Another contributing factor may be the different mechanisms and rates of Sb elimination after absorption of SbL8 and Glu. It is also noteworthy that SbL8 promoted higher levels of Sb in the liver in mice, presumably because of its improved retention in the liver during the first-pass removal and the longer half-life of SbL8 in the liver during the first-pass removal and the longer half-life of SbL8 in the liver, in mice, presumably because of its improved retention in the liver similarly to Glu. Nevertheless, oral SbL8 and i.p. Glu cannot be considered equally effective, since SbL8 was given at a dose that yielded 2.5-fold more Sb than Glu. Furthermore, when SbL8 was given orally at 80 mg of Sb/kg/day for 20 days, no significant reduction of parasite load could be detected in the same murine model of VL (data not shown).

Compared to our previous cyclodextrin-based oral formulation of meglumine antimoniate (11, 15), the most significant advantages of the present amphiphilic formulation are its much greater solubility in water (SbL8 aqueous dispersions were prepared at concentrations as high as 0.7 M Sb) and its superior bioavailability and sustained drug release property. Indeed, when evaluated in dogs, cyclodextrin-based oral formulation showed limited increases of bioavailability (15 versus 10%) and MRT (6.8 versus 4.1 h) compared to those of noncomplexed meglumine antimoniate (15).

The present work indicates that the formation of nanoassemblies not only allows for the application of very high doses of amphiphilic Sb with reduced acute toxicity but also contributes to the sustained action of the metal. Regarding the structure of these nanoassemblies, TEM and DLS analyses support the formation of micelles in both L8 and SbL8; however, NTA evidenced the formation of large nanostructures to a greater extent in SbL8. Nevertheless, the exact structure of SbL8 nanoaggregates requires further investigation. The kinetic stability of SbL8 nanoassemblies indicates that they do not readily dissociate following dilution in biological fluids. This property may be attributed to the low rate of dissociation of the Sb(V)—O bond (22, 25). This property may be explored in the future through the use of these nanostructures as carrier systems(275,718),(705,742) of other lipophilic drugs. Indeed, polymeric micelles or micelles formed from polymeric surfactants are being intensively studied as drug carrier systems, taking advantage of their kinetic stability (26, 27).

From the point of view of drug development, the present work represents promising preliminary evidence that the amphiphilic Sb(V) complex is a feasible strategy to achieve oral efficacy of pentavalent antimonials (4). The fact that treatment with oral SbL8 and that with i.p. Glu promoted about the same hepatic levels of Sb and also reduced the liver parasite burden to similar extents further supports the model that SbL8 action is mediated by the metal, similarly to Glu. Nevertheless, oral SbL8 and i.p. Glu cannot be considered equally effective, since SbL8 was given at a dose that yielded 2.5-fold more Sb than Glu. Furthermore, when SbL8 was given orally at 80 mg of Sb/kg/day for 20 days, no significant reduction of parasite load could be detected in the same murine model of VL (data not shown).

ACKNOWLEDGMENTS

We acknowledge the Brazilian agencies CNPq, FAPEMIG, and CAPES for financial support. F.F., C.D., and M.N.M. are recipients of a research fellowship from CNPq.
REFERENCES


