**In Vitro and In Vivo Trypanocidal Effects of the Cyclopalladated Compound 7a, a Drug Candidate for Treatment of Chagas’ Disease**

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Chagas’ disease, a neglected tropical infection, affects about 18 million people, and 100 million are at risk. The only drug available, benznidazole, is effective in the acute form and in the early chronic form, but its efficacy and tolerance are inversely related to the age of the patients. Side effects are frequent in elderly patients. The search for new drugs is thus warranted. In the present study we evaluated the *in vitro* and *in vivo* effect of a cyclopalladated compound (7a) against *Trypanosoma cruzi*, the agent of Chagas’ disease. The 7a compound inhibits trypanostagite cell invasion, decreases intracellular amastigote proliferation, and is very effective as a trypanocidal drug *in vivo*, even at very low dosages. It was 340-fold more cytotoxic to parasites than to mammalian cells and was more effective than benznidazole in all *in vitro* and *in vivo* experiments. The 7a cyclopalladate complex exerts an apoptosis-like death in *T. cruzi* trypanostagite forms and causes mitochondrial disruption seen by electron microscopy.

Chagas’ disease, also known as American trypanosomiasis, is an infection caused by the parasite *Trypanosoma cruzi*. Humans are infected by contaminated feces and urine of the insect vector, transfusion with blood from infected patients, oral transmission and contaminated food, and placental or birth canal transmission (5). Inside humans, the parasite is present as a circulating nonreplicative trypomastigote form and inside cells as the replicative amastigote stage. The disease is endemic from Mexico to Argentina, where 10% to 30% of chronically infected persons develop cardiomyopathy and gastrointestinal problems (25). This neglected tropical disease affects about 18 million people, and another 100 million are at risk. Mortality ranges from 8% to 12% (31), with predicted deaths of 50,000 people per year (10).

Nowadays, chemotherapy treatment of Chagas’ disease is limited, being successful in the acute form, less so in the chronic form, with severe side effects in elderly patients. No successful vaccine has been developed so far (15). Prevention is obtained by elimination of the insect vector and by increased control in blood banks and congenital transmission. Benznidazole, a nitroimidazole derivative, has been used for the treatment of acute and congenital *T. cruzi* infection. In a randomized, double-blind, placebo-controlled trial in a rural area in Brazil where Chagas’ disease is endemic, a 60-day course of benznidazole treatment of early chronic *T. cruzi* infection in children was safe and 55.8% effective, with negative seroconversion of specific antibodies (6). The 6-year follow-up of adolescents enrolled in that trial showed successful chemotherapy in 84.7% by seronegativity using chemiluminescent enzyme-linked immunosorbent assay (ELISA) with a purified trypomastigote mucin antigen (2). Benznidazole, however, does not have all the conditions of an ideal drug, with its efficacy and tolerance being inversely related to the age of the patient (29). The other drug clinically available was Nifurtimox, but it has been discontinued from Bayer’s production line. It is known that both drugs may increase the oxidative stress causing parasite death (14). Generally, it is accepted that new chemotherapeutic drugs should be developed for treatment of Chagas’ disease together with improved methods to determine a parasitological cure based on negative serology (1).

In our laboratory we have studied cyclopalladated complexes with biological activity. They are much more stable and less toxic than other derivatives of palladium (II) (18) and were able to inhibit cathepsin B (3). One of these compounds, called complex 7a, showed specific *in vitro* and *in vivo* activities against tumor cells, with no significant toxicity in animals, even at high doses (20). The 7a compound causes anokis and apoptosis of tumor cells (F. A. Serrano, D. C. Arruda, A. L. Matsuo, L. S. Silva, S. Smaili, A. C. F. Caires, L. R. Travassos, and E. G. Rodrigues, unpublished data), and its enantiomer is able to oxidize mitochondrial proteins leading to permeabilization and cytochrome c release (21).

Since the existing drugs against *T. cruzi* lead to oxidative stress, we considered testing the 7a compound against the parasite. *T. cruzi*, as well as other kinetoplastid protozoa, has a single and large mitochondrion containing multiple copies of DNA, forming a structure called a kinetoplast. The kinetoplas-
tid mitochondrion also presents unique features regarding the electron transport chain. For example, they contain a cytochrome-independent alternative oxidase and have unique proteins for kinetoplast platination, as topoisomerase, and also an essential fatty acid pathway (22). Here we show that compound 7a targets the parasite mitochondrion, inhibits amastigote intracellular proliferation, and reduces experimental parasitemia and mortality with very low and nontoxic doses.

MATERIALS AND METHODS

Mice, cell line, parasites, and drugs. BALB/c female mice (8 to 10 weeks old) were obtained from the CEDEME animal facility at UNIFESP. All animal experiments and procedures were approved by the institution’s committee on the ethical handling of laboratory animals. Trypomastigote forms (Y strain) were obtained from the supernatant of infected LLCMK2 cells (ATCC CCL7) maintained in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% of fetal bovine serum at 37°C in a humidified atmosphere with 5% CO2. For mouse infections, trypomastigotes were routinely maintained by weekly intraperitoneal (i.p.) injection in BALB/c mice with 500 parasites, as previously described (26). Benznidazole was a gift from Renato Mortara, Department of Parasitology, UNIFESP.

Cyclopalladated complex 7a. The palladacycle compound 7a (Fig. 1) was obtained from N,N-dimethyl-1-phenethylamine (DMPA), complexed to 1,2-ethane-bis (diphenylphosphine) (DPPE) ligand. Binucleate complex 7a was highly characterized as described by Rodrigues et al. (20) and shown to have antitumor activity. Binucleate complex 7a was fully characterized as described by Rodrigues et al. (20) and shown to have antitumor activity.

Parasite and mammalian cell drug cytotoxicity. Parasite killing levels for the tested drugs were determined as described by Muñoz-Serrano et al. (17). Briefly, parasites recovered from the supernatants of disrupted infected cells were counted in a Neubauer chamber and distributed in 96-well flat-bottom plates (5 × 10⁴ parasites per well). Drug 7a or benznidazole at 50 pmol · 100 µM · 1.1 well−1 · well−1 (500 nM) or 12.5 nmol · 100 µM · 1.1 well−1 (125 µM), respectively, was added, and the time viability of parasites was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay in a period up to 24 h.

Alternatively, for 50% inhibitory concentration (IC₅₀) determination, wells containing 2 × 10⁴ parasites or LLCMK2 cells were incubated for 24 h with different concentrations of 7a ranging from 5 pmol · 100 µM · 1.1 well−1 · well−1 (50 nM) to 100 fmol · 100 µM · 1.1 well−1 (1 nM) or 1 nmol · 100 µM · 1.1 well−1 (10 µM) to 50 pmol · 100 µM · 1.1 well−1 (500 nM), respectively. Viable parasites were counted in a Neubauer chamber, and viable cells were measured by the MTT assay.

Cell invasion experiments. LLCMK2 cells (4 × 10⁴) were plated on 13-mm glass coverslips inside a 24-well plate. After 24 h, cells were washed twice with phosphate-buffered saline (PBS) and incubated with 5 × 10⁴ trypomastigotes in the presence of 7a ranging from 500 pmol · 100 µM · 1.1 well−1 · well−1 (500 nM) to 500 pmol · 100 µM · 1.1 well−1 · well−1 (500 nM) or 100 pmol · 100 µM · 1.1 well−1 (100 µM) of benznidazole in DMEM supplemented with 10% fetal bovine serum (FBS) for 2 h at 37°C with 5% CO2. Cells were then washed five times with PBS to remove extracellular trypomastigotes and further incubated in DMEM/10% FBS at 37°C for two additional hours. Infected cells were examined for the presence of internalized parasites by optical microscopy using standard Giemsa staining (9).

Drug effect on the proliferation of intracellular amastigotes. After invasion, as described above, noninternalized parasites were washed away with phosphate-buffered saline, and cells were incubated in the presence of 500 pmol · 100 µM · 1.1 well−1 · well−1 (500 nM) of 7a or 500 pmol · 100 µM · 1.1 well−1 · well−1 (500 µM) of benznidazole in DMEM containing 10% FBS. Fresh medium containing each compound was replaced every 24 h. After 48 h, cells were fixed and stained with Giemsa, and intracellular parasites counted by observation using an optical microscope.

DNA degradation. Trypomastigotes released from LLCMK2 cultures (1 × 10⁴) were treated with 10 nmol · 100 µM · 1.1 well−1 · well−1 (10 µM) of 7a in DMEM supplemented with FBS at 37°C for 6 h. Parasites were centrifuged, resuspended in 500 µl of 62.5 mM EDTA, 4% Triton X-100, 2.5 M LiCl, and 50 mM Tris-HCl, pH 8.0 (TELT), and gently homogenized for 5 min. The same volume of chloroform phenol (1:1) equilibrated in 0.1 M Tris-HCl, pH 8.0, was added, and phases were separated by centrifugation. The aqueous phase was recovered, and the DNA was precipitated with 2 volumes of ethanol and 0.1 M sodium acetate. The DNA was recovered by centrifugation, washed with 70% ethanol, resuspended in Milli-Q water with 20 µg · 1.1 ml−1 · 37°C, and incubated at 37°C for 1 h. DNA integrity was observed by electrophoresis using 0.8% agarose gel in standard Tris-acetate EDTA buffer.

Indirect immunofluorescence. Trypomastigotes (5 × 10⁴ per ml) were treated with 500 pmol · 100 µM · 1.1 well−1 (500 nM) of 7a in DMEM supplemented with FBS at 37°C for 30 min, then attached onto a glass slide for 5 min and subsequently fixed with 4% p-formaldehyde in PBS for 20 min. The slides were washed three times with PBS, treated with Triton X-100, 0.1% in PBS for 5 min, and then incubated with 1% bovine serum albumin in PBS for 30 min. The slides were washed three times with PBS, incubated with anti-dihydropyridine dehydrogenase (a mitochondria matrix protein) antiserum (27) (kindly provided by Kevin Taylor, University of East Anglia, United Kingdom) diluted in PBS for 1 h. Labeling was detected with anti-rabbit IgG-Alexa Fluor 594 (Invitrogen) in the presence of 0.01 mg/ml 4,6-diamidino-2-phenylindole (DAPI), Vectashield (Vector Laboratories) was used for mounting, and the slides were visualized with a 100× oil immersion objective (1.41 numerical aperture) on a Olympus BX61 microscope. The images were acquired using cell software and processed and in line with the manufacturer’s instructions. Data acquisition was performed using a FACScalibur flow cytometer (Becton Dickinson, CA), and data were analyzed with CellQuest software (Joseph Trotter, CA).

Transmission electron microscopy (TEM). Trypomastigotes (5 × 10⁴) submitted to treatment with 1 nmol · 100 µM · 1.1 well−1 (1 µM) of 7a in DMEM supplemented with FBS at 37°C for 1 h were fixed for 1 h at room temperature with 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. The fixed parasites were then decanted overnight on an alar film precoated with 0.01% poly-L-lysine. The alar film was washed three times with 0.1 M cacodylate, pH 7.2 (10 min each), incubated with 1% osmium tetroxide in the same buffer for 30 min at room temperature, washed with cacodylate buffer, and washed three times with water. Subsequently the alar was treated for 30 min with 0.4% uranyl acetate for membrane contrast enhancement, gradually dehydrated in a series of ethanol solutions, and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate, mounted on Formvar grids, and observed in a JEOL 1200 EX II transmission electron microscope at 80 kV.

Animal infection. Blood parasitemia in the infected animal was quantified by counting motile parasites in 10 µl of fresh blood sample drawn from lateral tail veins (26). Each mouse received a single dose i.p. of 200 nmol · 1.1 kg−1 · 4,6-diamidino-2-phenylindole (DAPI) antiserum in PBS at 37°C for 20 min. The 4,6-diamidino-2-phenylindole was subsequently fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2. The fixed parasites were then incubated with 1% osmium tetroxide in the same buffer for 30 min at room temperature, washed with cacodylate buffer, and washed three times with water. Subsequently the alar was treated for 30 min with 0.4% uranyl acetate for membrane contrast enhancement, gradually dehydrated in a series of ethanol solutions, and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate, mounted on Formvar grids, and observed in a JEOL 1200 EX II transmission electron microscope at 80 kV.

Preparation of tissues for histopathological analysis. The hematoxylin and eosin (H&E) staining of parasitized sections was performed with heart, lung, bladder, spleen, intestine, and muscle tissues. The sections obtained from the experimental and control groups were fixed in neutral buffered formalin (10% formalin in PBS), dehydrated, and embedded in paraffin by a routine technique, and semiserial sections of 5 µm were stained with H&E. The results are expressed as the mean number of amastigote nests per microscope field. The data presented...
for each group are the mean ± standard deviation (SD) of results for three animals; 20 fields in 4 sections were counted in each tissue.

**Statistical analyses.** The Student t test was used throughout, except for with the parasitemia and the survival curves, for which one-way analysis of variance (ANOVA) and the Mantel-Cox test were employed, respectively.

**RESULTS**

**Cyclopalladated 7a is highly toxic for T. cruzi trypomastigotes compared to mammalian cells in culture.** To evaluate the relative toxicity of the cyclopalladated compound 7a, we measured the IC_{50} for mammalian cells and trypomastigote forms of the parasite. Cell culture-derived trypomastigotes (2 × 10^6) were incubated for 24 h with different concentrations of 7a, and the number of moving, refringent parasites was counted in a Neubauer chamber. An IC_{50} of 15 nM was obtained (data not shown). It is known that some metallic drugs have a nephrotoxic effect. For this reason we used the mammalian kidney cell lineage LLCMK2 for mammalian cell cytotoxic evaluation. About 2 × 10^6 cells were distributed into a 96-well plate and incubated with different concentrations of 7a. After 24 h, 7a showed an IC_{50} of 5.1 μM, measured by the MTT method (data not shown). Thus, 7a drug affects preferentially the parasite, being effective at concentrations 340 times less than the cytotoxic concentration for mammalian cells.

A trypanocide effect of drug 7a was observed at a much lower concentration than that of benznidazole. Benznidazole is the drug of choice for treatment of Chagas’ disease. To compare the efficacies of drug 7a and benznidazole, cell-derived trypomastigotes (5 × 10^6) were treated for 0, 2, 6, and 24 h with 7a or benznidazole at 37°C in DMEM supplemented with 10% FBS. As shown in Fig. 2, drug 7a at 50 pmol · μl⁻¹ · well⁻¹ (500 nM) or benznidazole (12.5 nmol · 100 μl⁻¹ · well⁻¹; 125 μM) during 24 h. *P < 0.05.

**FIG. 2.** Time-dependent viability (MTT method) of 5 × 10^6 trypomastigotes treated with compound 7a (50 pmol · 100 μl⁻¹ · well⁻¹; 500 nM) or benznidazole (12.5 nmol · 100 μl⁻¹ · well⁻¹; 125 μM) during 24 h. *P < 0.05.

**Inhibition of T. cruzi cell invasion by 7a.** Cell invasion by trypomastigotes is a key step for establishment of T. cruzi infection in the mammalian host. Therefore, we evaluated whether 7a could prevent mammalian cell invasion in culture by trypomastigotes. As shown in Fig. 3, drug 7a inhibited cell invasion (P < 0.05) at concentration as low as 5 pmol · μl⁻¹ · well⁻¹ (5 nM) with 90% inhibition at 500 pmol · μl⁻¹ · well⁻¹ (500 nM). This inhibitory effect cannot be attributed to a direct parasite killing, since about 50% of parasites kept motility at 50 pmol · 100 μl⁻¹ · well⁻¹ (500 nM) of 7a after 2 h of incubation. In contrast, benznidazole had no effect on trypomastigote invasion, even at high concentrations, probably due to its slow action.

**Compound 7a prevents intracellular growth of amastigotes.** After cell invasion, trypomastigote forms transform into amastigotes, which represent the intracellular replicative form of T. cruzi. To verify whether 7a could have any effect against amastigotes, preinfected cells were incubated with pmol · μl⁻¹ · well⁻¹ (500 nM) of 7a or 50 nmol · μl⁻¹ · well⁻¹ (50 μM) of benznidazole for 48 h. After treatment, cells were stained with Giemsa and observed by optical microscopy in order to evaluate the relative number of infected cells and amastigotes per infected cell. As shown in Fig. 4, both drugs at much different concentrations significantly reduced (P < 0.05) the relative number of infected cells (Fig. 4A) with ~50% parasites per infected cell (Fig. 4B).

**Compound 7a induces an apoptosis-like parasite death.** To understand the mechanism by which compound 7a promotes parasite death we compared the observed killing at 37°C with the effect at 4°C (Fig. 5). We found that at 4°C much less parasite lysis was observed, suggesting that metabolically active parasites are required and that the effect of 7a does not occur as a consequence of simple membrane destruction. Previous work from our group suggests that compound 7a interacts with mitochondria, causing a collapse in the cell extrusion of protons (20). Therefore, the parasites were stained by immunofluorescence with a mitochondrial matrix marker after incubation with 7a. As shown in Fig. 5A to C, the mitochondrial structure remains intact in control cells or parasites treated at 4°C, whereas parasites incubated with compound 7a at 37°C...
for 5 min showed morphological alterations in the mitochondrial structure (Fig. 5D). On prolonged incubation (>30 min), only those parasites incubated at 37°C died.

As death involving mitochondria is found in apoptosis, we looked for other features of apoptotic death in the parasite. DNA was extracted from 7a-treated and -untreated parasites, and its integrity was analyzed by agarose gel electrophoresis. Differently from the control, treated parasites showed DNA degradation after trypomastigote treatment with compound 7a for 6 h. A clear ladder pattern of degradation as found in apoptosis was observed with treated parasites.

FIG. 4. Inhibition of amastigote growth. After 48 h treatment with 7a (500 pmol·ml⁻¹·well⁻¹ concentration at 500 nM) or benznidazole (50 nmol·ml⁻¹·well⁻¹; 50 μM), infected cells were stained with Giemsa and analyzed. (A) Percentage of infected cells after treatment; (B) relative number of amastigotes per infected cell. *, P < 0.05.

FIG. 5. Effects of compound 7a on parasite mitochondrion at 4°C or 37°C. DNA staining was performed with DAPI (blue) and the mitochondrial matrix marker with Alexa fluor 594 (red). (A) Nontreated trypomastigotes at 4°C; (B) 7a-treated trypomastigotes at 4°C; (C) nontreated trypomastigotes at 37°C; (D) 7a-treated trypomastigotes at 37°C.

FIG. 6. DNA degradation after trypomastigote treatment with compound 7a for 6 h. A clear ladder pattern of degradation as found in apoptosis was observed with treated parasites.
degradation in a step pattern, suggestive of apoptosis (Fig. 6). We then labeled parasites treated with 7a for a short period, to avoid lysis, with annexin V to stain eventual phosphatidylserine flipping to the membrane surface, which is another common biochemical alteration observed in the apoptotic process. As shown in Fig. 7, there is a clear increase in annexin V labeling but no change in propidium iodide uptake in treated parasites compared to control parasites as detected by fluorescence-activated cell sorter (FACS), suggesting that labeling occurs owing to the surface expression of the phospholipid.

Ultrastructural changes in the 7a-treated parasites. We determined the ultrastructural disturbances in trypomastigote forms caused by compound 7a using electron microscopy. Figure 8A shows a typical mitochondrion (M), nucleus (N), kinetoplast (K), and flagellum (F). In contrast, as shown in Fig. 8B and C, treated parasites showed mitochondrion swelling (asterisk), formation of abnormal membrane structures inside the organelle (black arrowhead), and atypical vacuoles (triangle). This result corroborates the fluorescence microscopy in which the morphological alterations of mitochondria are shown.

Preinjection of compound 7a protects mice from acute infection. To verify the potential use of 7a compound for Chagas’ disease therapy, a single dose i.p. of 200 nmol kg⁻¹ body weight of compound 7a or benznidazole 30 min before infection with 500 trypomastigote forms. Percent survival is shown; n = 10. *, P < 0.01.

FIG. 8. Ultrastructural changes in 7a-treated trypomastigotes. (A) Control parasite: normal mitochondrion (M), nucleus (N), kinetoplast (K), and flagellum (F); (B and C) treated parasites, showing mitochondrion swelling (asterisk), formation of abnormal membrane structures inside the organelle (black arrowhead), and atypical vacuoles (triangle).

FIG. 9. Survival of pretreated mice. To evaluate a possible in vivo drug effect, we treated female BALB/c mice with a single dose of 240 nmol kg⁻¹ body weight of compound 7a or benznidazole 30 min before infection with 500 trypomastigote forms. Percent survival is shown; n = 10. *, P < 0.01.
TABLE 1. Number of parasite nests in tissues from infected mice pre-treated with 7a*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control (n = 3)</th>
<th>7a (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>28 (± 8.5)</td>
<td>4.8 (± 9)*</td>
</tr>
<tr>
<td>Bladder</td>
<td>56 (± 9.1)</td>
<td>8 (± 10)*</td>
</tr>
<tr>
<td>Lung</td>
<td>3 (± 4.4)</td>
<td>0.2 (± 0.45)</td>
</tr>
<tr>
<td>Intestine</td>
<td>1.7 (± 2.1)</td>
<td>0.2 (0.45)</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.67 (± 1.2)</td>
<td>0.2 (± 0.45)</td>
</tr>
</tbody>
</table>

a The sections of hematoxylin/cosin-stained tissues from mice injected i.p. with 7a at 200 nmol · kg⁻¹ body weight 30 min before infection with 500 bloodstream trypomastigotes (Y strain) were scored for determination of the number of parasite nests on the indicated tissues. *, P < 0.05.

b Values are means ± standard deviations from 20 fields; magnification, ×400.

(P < 0.01). Specimens of heart, skeletal muscle, spleen, intestine, lungs, bladder, and kidney collected on day 13 postinfection were examined for the presence of parasite nests by regular H&E histopathology. The numbers of parasite nests decreased for all observed tissues of treated animals, but only for the bladder and heart were the differences statistically significant (P < 0.05). No parasites were seen in the spleen (Table 1).

**Compound 7a is effective in the treatment of acute Chagas’ disease.** As compound 7a proved to be effective against T. cruzi infection when injected in mice prior to infection, we tested whether it could eliminate the parasite from infected animals. Three i.p. daily doses of 40 nmol of compound 7a or benznidazole per kg of body weight within 4-h intervals were administered in mice 1 day after mouse infection with 500 bloodstream trypomastigotes, until the 14th day. Maximal parasitemia and heart were the differences statistically significant (P < 0.05).

No parasites were seen in the spleen (Table 1).

**DISCUSSION**

In the present work we demonstrate the potential of the cyclopalladated compound 7a against T. cruzi infection. This compound was previously shown to be effective against syngeneic murine melanoma in vivo (20). It seems to target mitochondria of eukaryotic cells (F. A. Serrano et al., unpublished data). Our experiments provide clear evidence that T. cruzi invasive and intracellular proliferative forms are about 300 times more sensitive to compound 7a than mammalian cells. Compound 7a destroys trypomastigotes probably by targeting the mitochondrion, followed by DNA fragmentation and phosphatidylserine surface exposure, in a process that resembles apoptosis of mammalian cells. It is well known that mitochondrial damage can initiate a series of events that lead cells to apoptosis characterized by nuclear condensation and fragmentations, caspase activation, and phosphatidyl serine cell surface exposure, in an active process (28). The reason for this apoptosis-like process in a unicellular organism is still controversial. In parasites like Leishmania, it has been proposed that a few dead cells in the population stimulate infection (30). It has also been proposed that T. cruzi insect forms are killed by complement through extensive free radical production by the mitochondrion and induction of a similar apoptotic-like mechanism (12). Reports using venom from Bothrops jararaca (7) and

![FIG. 10. Survival of acutely infected mice. One day after infection, animals were treated with three doses of 40 nmol · kg⁻¹ body weight of each drug per day until the 14th day. (A) Measurement of peak parasitemia; (B) percent mouse survival in the control, benznidazole, and 7a groups (n = 10). *, P < 0.05.](http://aac.asm.org/)

**TABLE 2. Histopathological analysis of tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Control (n = 5)</th>
<th>Bz (n = 5)</th>
<th>7a (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>15.7 (± 4.6)</td>
<td>12.3 (± 5.0)</td>
<td>5.3 (± 4.6)*</td>
</tr>
<tr>
<td>Bladder</td>
<td>54.7 (± 17.9)</td>
<td>36.7 (± 5.5)</td>
<td>4.7 (± 4.5)*</td>
</tr>
<tr>
<td>Lung</td>
<td>1.3 (± 1.5)</td>
<td>0</td>
<td>0.3 (± 0.58)</td>
</tr>
<tr>
<td>Intestine</td>
<td>21.5 (± 0.7)</td>
<td>0.67 (± 1.2)*</td>
<td>0*</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.7 (± 3.2)</td>
<td>2.3 (± 3.2)</td>
<td>0.67 (± 1.2)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a The sections of hematoxylin/cosin-stained tissues from mice treated i.p. with three doses per day of 7a at 40 nmol · kg⁻¹ body weight during 13 days after infection with 500 bloodstream trypomastigotes (Y strain) were scored for the number of parasite nests on the indicated tissues. *, P < 0.05.

b Values are means ± standard deviations from 20 fields; magnification, ×400. Bz, benznidazole.
The distinct mitochondrial of the parasite could explain its increased susceptibility, with an IC$_{50}$ 340-fold less than that in mammalian cells. Moreover, compound 7a acts very quickly on trypomastigotes, unlike benznidazole, which, even at high concentrations, may take more than 24 h to kill the parasite. The inefficiency of benznidazole with the Y strain infection may be explained by the partial resistance of this strain (16) and was confirmed here, as even a single dose of 400 nmol · kg$^{-1}$ body weight of benznidazole had minimal effect. As it has been proposed that benznidazole toxicity for T. cruzi is due to oxidative stress generated preferentially in the parasite (19), compound 7a could easily circumvent the resistance mechanism.

Recently, NO-liberating drugs have been tested against T. cruzi with successful in vivo results, leading to 60 to 80% survival and even 100% survival of infected mice in combination with benznidazole (11, 23, 24). In vitro, all these drugs were active, with IC$_{50}$ of >52 µM. In comparison, compound 7a was even more effective with a very low IC$_{50}$. Probably, using other treatment protocols or drug combinations, the effectiveness of compound 7a could be further improved to achieve 100% survival of infected animals.

The inhibition of cell invasion by 7a does not appear to occur as a consequence of trypomastigote death. The most probable explanation for this effect is that cyclopalладated compounds may directly inhibit cytoine proteases by direct oxidation of the catalytic domain (4). One of these enzymes is T. cruzi cruzain, which is important for parasite invasion (13).

Previous studies have shown that compound 7a was used in cancer therapy and was not toxic in mice, even at 240 nmol · kg$^{-1}$ body weight, injected three times a week. We performed preliminary analysis of acute administration of a 4,000-nmol · kg$^{-1}$ single dose, i.p., and found that it was not lethal to mice (data not shown). Presently, the therapeutic in vivo test showed that compound 7a was able to significantly reduce the parasitemia and the number of parasite nests in the bladder, heart, and intestine (P < 0.05), with 80% animal survival after 60 days of infection (P < 0.01). It is important to note that the treatment terminated on day 14 and that the first 7a-treated mouse died after 34 days of infection, while both PBS- and benznidazole-treated groups of mice were all dead on day 22. We report here on a promising chemotherapeutic approach against T. cruzi that might possibly be extended to all kine plastidae because the cyclopalладated compound rapidly disrupts the mitochondrion. This compound was demonstrated to have a fast antiparasite effect, decreasing its viability and invasion capacity and leading to an apoptosis-like death. In preclinical assays, it was very effective in protecting 80% of treated animals. Due to its high efficacy in vivo, it could be an alternative treatment for Chagas' disease. Further studies on the treatment protocols may help us to improve the compound efficacy in the treatment of experimental trypanosomiasis.

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