Activity of Hydroxyurea against *Leishmania mexicana*  

Hugo Martinez-Rojano,1,2 Javier Mancilla-Ramirez,1,3 Laura Quiñonez-Diaz,1,4 and Norma Galindo-Sevilla3*  

Departamento de Posgrado, Escuela Superior de Medicina, Instituto Politecnico Nacional, Mexico City, Mexico;  
Departamento de Urgencias Pediatricas, Hospital de Gineco-Pediatrica 3A, IMSS, Mexico City, Mexico;  
Subdireccion de Investigacion Clinica, Instituto Nacional de Perinatologia, Mexico City, Mexico;  
and Division Academica de Ciencias de la Salud, Universidad Juarez Autonoma de Tabasco, Villahermosa, Tabasco, Mexico  

Received 28 January 2008/Returned for modification 26 March 2008/Accepted 28 July 2008

*Leishmania mexicana* is a protozoan parasite that causes a disease in humans with frequent relapses after treatment. It is also highly resistant to the currently available drugs. For this reason, there is an urgent need for more effective antileishmanial drugs. Hydroxyurea, an antineoplastic agent that is able to arrest the cell cycle at the interface of the G1 phase-S phase, is highly effective in killing intracellular amastigotes in vitro.

The choices of drug therapy for human leishmaniasis include pentavalent antimonials, namely, sodium stibogluconate and meglumine antimoniate, which have been widely used since the 1940s, and amphotericin B in its liposomal formulation. Pentamidine and paromomycin (2, 3, 8, 11) have also been used. The disadvantages of these drugs include their high cost, the need for long-term treatment, the lack of an oral formulation, and serious side effects that require intense patient monitoring (2). Furthermore, the emergence of antimony-resistant parasites has been reported (2, 16, 21); this has compelled a search for new antileishmanial agents. New therapeutic approaches with orally administered drugs are being investigated. The oral formulation of the alkylphosphocholine miltefosine has shown clinical efficacy against visceral leishmaniasis. However, it is not certain that miltefosine will be as useful in the treatment of diffuse cutaneous leishmaniasis or in immunocompromised patients coinfected with human immunodeficiency virus (HIV) (7); these patients may frequently have relapses of leishmaniasis after treatment. Leishmaniasis is caused by *Leishmania*, a eukaryotic parasite with a rapid in vitro replication rate. This characteristic makes it extremely susceptible to antitumor drugs, such as mitofosine and acridine compounds (11, 22). In light of this, hydroxyurea, an antineoplastic agent that is able to arrest the *Leishmania major* cell cycle (34), was investigated in the study described in this paper for its possible action against *Leishmania mexicana*. In the more than four decades since the ribonucleotide reductase inhibitor hydroxyurea was first evaluated clinically, a number of diverse applications of the drug in malignant and nonmalignant diseases have been identified (5, 12, 24, 26).

The primary site of action of hydroxyurea is the ribonucleotide reductase enzyme. This enzyme catalyzes the reductive conversion of ribonucleotides into deoxyribonucleotides; and when it is inactivated, the reduction of intracellular concentrations of the deoxynucleoside triphosphates, the inhibition of DNA synthesis, the inhibition of DNA repair in quiescent cells, cell cycle arrest, and apoptosis are induced (26). Hydroxyurea is a radical scavenger and inactivates the R-2 subunit of ribonucleotide reductase by reducing its tyrosyl radical to a normal tyrosine residue by the transfer of one electron. The drug is specific for the S phase of the cell cycle, in which large amounts of deoxyribonucleotides are required and which is also when the DNA of the R-2 subunit is transcribed. As a consequence, the arrest of the cell cycle at the interface of the G1 phase-S phase is seen (26).

Hydroxyurea is conventionally an orally administered drug. Oral administration has the definite advantage of offering patients convenience (24). Additionally, hydroxyurea may be helpful for patients with leishmaniasis and compromised T-cell function, such as those infected with HIV (32), in whom infections may become difficult to treat and drugs must be used at higher concentrations and for longer periods of time (10). The aim of this study was to investigate the possible effects that hydroxyurea has on the survival and cell cycle of *Leishmania mexicana*.

**MATERIALS AND METHODS**

**Compound formulation.** A stock solution of hydroxyurea (CH$_2$N$_2$O$_2$; catalogue number H 8627; Sigma-Aldrich, St. Louis, MO) was made fresh at 10 mg/ml in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY) without fetal calf serum (FCS; Gibco) and was diluted to the appropriate concentration according to the experimental conditions.

**Hydroxyurea concentration.** Hydroxyurea concentration was selected on the basis of the concentration of hydroxyurea in the plasma of patients treated with 10 mg/kg per day for 7 days, as recommended by the manufacturer (26). The concentration of 10 mg/ml was chosen for the first experiments, after which the concentration was decreased to 100 μg/ml and 1 μg/ml.

**Cell culture model.** We used a model of intracellular infection of murine macrophages by *Leishmania mexicana* to test the ability of hydroxyurea to eliminate intracellular parasites. The human monocyte-derived macrophage cell line N9 was provided by D. A. Warrell (National Institute for Medical Research, London, United Kingdom) and used as described (27). The culture medium was made up as follows: Ham's F12 medium (Gibco, Grand Island, NY) without fetal calf serum (FCS; Gibco) and was diluted to the appropriate concentration according to the experimental conditions.

**The cell cycle.** The cell cycle was analyzed by flow cytometry using a flow cytometer (Becton Dickinson, San Jose, CA). The samples were fixed in 3.7% formaldehyde in PBS and stained with 1 μg/ml of propidium iodide. The cell cycle distribution was determined by the modality and the percentage of the subpopulations of cells in the different phases of the cell cycle (26).

**In vitro experiments.** The experiment was performed on N9 cells infected with *Leishmania mexicana* by the procedure described previously (26). The cells were incubated at 37°C with 10% CO$_2$ and the experiment was performed as described (26).

**Trypan blue exclusion test.** The viability of the cultures was determined by the trypan blue exclusion test. The percentage of viable cells was calculated using a hemocytometer.

**DNA synthesis.** The DNA synthesis was determined by the method described previously (26).

**DNA content.** The DNA content was determined by the method described previously (26).

**Apoptosis.** The apoptosis was determined by the method described previously (26).

**Statistical analysis.** The statistical analysis was done with the Student t test or the analysis of variance (ANOVA) followed by the Tukey post-test. Differences were considered significant at a probability level of 0.05 (26).

**REFERENCES**


concentrations. Meglumine antimoniate (Rhone-Poulenc Rorer, Mexico) was used as the reference drug. Each ampoule of 5 ml contained 1.5 g of meglumine antimoniate, which corresponded to 0.405 g of pentavalent antimony at 81 mg/ml. The concentration of pentavalent antimonial compounds was calculated by weight over volume of pentavalent antimony.

Parasites. Reference strains *L. mexicana* MNYC/BZ/62/M379 and MHOM/MX/00/Tab3, isolated in Tabasco, Mexico, from a patient with diffuse cutaneous leishmaniasis (15), were used. The parasites were maintained as promastigotes at 26°C in DMEM (Gibco) containing 10% heat-inactivated FCS (Gibco) or were transformed to an amastigote-like form by setting the temperature at 32°C. Overnight incubation was enough to obtain complete transformation at neutral pH. The doubling time was about 12 h and was identical for both forms: promastigotes at 26°C and amastigote-like forms at 32°C. The parasites were also able to be transformed into promastigotes from amastigote-like forms just by changing the temperature from 32 to 26°C.

Effects of hydroxyurea on *Leishmania mexicana* in a cell-free system. The susceptibilities of the promastigotes or the amastigote-like forms of *Leishmania* to hydroxyurea were tested by culturing them in cell-free medium at 26 and 32°C, respectively. The efficacy of hydroxyurea was tested at 0.01, 0.1, 1, 10, and 100 μg/ml. Parasite density was counted with a hemacytometer every other day.

Effects of hydroxyurea on intracellular macrophage infection by *Leishmania mexicana*. To test the effectiveness of hydroxyurea on intracellular parasites, a macrophage monolayer was prepared in 24-well plates with peritoneal resident macrophages harvested from BALB/c male mice weighing 20 to 25 g. Macrophages were collected after irrigation of the peritoneal cavity with 10 ml of DMEM. After pooling of the macrophages from several mice, three washes were performed with cold medium; the macrophages were counted in a hemacytometer and adjusted to 10⁶ mononuclear cells/ml. One milliliter of the cell suspension was placed on each sterile 24-well culture plate. The culture plates were incubated at 32°C under an atmosphere of 5% CO₂ for 24 h. Nonadherent cells were removed by washing the plates with prewarmed, sterile phosphate-buffered saline (PBS), and adherent cells were infected with 1 ml of 10⁷ mid-logarithmic phase amastigote-like forms in DMEM plus 10% FCS. The cultures were incubated for 48 h at 32°C to allow the parasites to be internalized. The cultures were then washed three times with prewarmed PBS to remove extracellular parasites, and 900 μl of fresh medium was added to each well. The ratio cells in the intracellular infection was usually 10 parasites to 1 macrophage. Various concentrations of hydroxyurea (0.01, 0.1, 1, 10, and 100 μg/ml) were added at 100 μl to each well, as was meglumine antimoniate as dosages of 0.01, 0.1, 1, 10, and 100 μg of pentavalent antimony/ml as a reference. The cultures were returned to 32°C under an atmosphere of 5% CO₂ for 3, 6, 9, or 12 days. After each of these times, the drug was removed and fresh medium was added. Subsequently, the plates were transferred to 26°C to promote the transformation of the parasites to the motile form and to cause their release from the macrophages. The parasites were counted with a hemacytometer on days 2, 4, 6, and 8 after drug removal. Each point was evaluated in triplicate. The percentage of growth inhibition was calculated by using the following formula: 100 \times \left( {T_0 - T_p} / T_0 \right), where *T₀* is the number of parasites/ml in the control wells and *Tₚ* is the average number of parasites/ml corresponding to each dosage on days 3, 6, 9, and 12 after drug exposure. The 50% effective dose (ED₅₀) was defined in this study as the drug concentration that reduced the survival of *Leishmania* parasites by 50%.

Viability of macrophages assays by trypan blue exclusion. Macrophage survival in medium containing hydroxyurea (0.01, 0.1, 1, 10, and 100 μg/ml) was tested after 6 days of incubation by staining with trypan blue at a final concentration of 0.2% in phosphate buffer. Macrophage viability was verified by microscopic quantification of the number of viable macrophages among 100 macrophages.

Viability of *Leishmania* under hydroxyurea exposure. *Leishmania* survival in hydroxyurea was evaluated with the dye propidium iodide (PI) after incubation for 48 h with 1, 10, and 100 μg/ml of hydroxyurea at room temperature. The parasites were then washed three times with PBS containing 0.02 M EDTA and were then resuspended in 0.5 ml of PBS containing PI (25 μg/ml. Sigma). The stained parasites were analyzed after 20 min in a fluorescence-activated cell sorter (EPICS-ALTRA flow cytometer; Beckman-Coulter, Fullerton, CA).

Cell cycle analysis. To demonstrate whether hydroxyurea affected the *Leishmania* cell cycle, parasites growing in mid-logarithmic phase were incubated for 48 h with 1, 10, or 100 μg/ml of hydroxyurea at 26°C. Afterward, the parasites were washed three times with PBS containing 0.02 M EDTA to avoid clumps and were then fixed with cold methanol for 24 h. The parasites were resuspended in 0.5 ml of PBS containing RNase I (50 μg/ml) and PI (25 μg/ml) and were then incubated at 25°C for 20 min. The material was kept on ice until analysis. The stained parasites were analyzed in a fluorescence-activated cell sorter.

Statistical analysis. Experiments were conducted four times, and the results of each experiment were analyzed individually. For each experiment, the data were recorded in triplicate and were analyzed for statistical significance by one-way analysis of variance (ANOVA). A probability (*P*) value of <0.05 was considered significant. The ED₅₀ were calculated by polynomial regression analysis.

**RESULTS**

Effect of hydroxyurea on growth of *Leishmania* promastigotes in vitro. The activity of hydroxyurea against promastigotes of *Leishmania* strains M379 and Tab3 was assessed in a cell-free system. A decrease in their growth was noticed when the parasites were treated for 48 h with 10 and 100 μg/ml of hydroxyurea. Six days of treatment resulted in promastigote survival rates of 11.6% for strain M379 and 3.1% for strain Tab3 (Fig. 1). An ED₅₀ of 0.05 μg/ml was observed for hydroxyurea for promastigotes when it was measured on day 6.

Model of intracellular infection of *Leishmania*. We sought to determine whether hydroxyurea was able to reach intracellular parasites in an in vitro model of intracellular infection on macrophage monolayers. By infesting macrophages with different numbers of amastigote-like forms grown at 32°C, different rates of infection were observed. To test the efficacy of hydroxyurea, a density of 10⁷ parasites/ml was used to infect the monolayer because we expected that 90% of the adherent macrophages may become infected and that each cell would harbor approximately 10 intracellular parasites after 48 h of incubation (Table 1).

**TABLE 1. Infectivity of axenic mid-log-phase amastigote-like forms of *Leishmania mexicana* to mouse macrophages**

<table>
<thead>
<tr>
<th>No. of amastigote-like forms (10⁶/ml)</th>
<th>Ratio of no. of intracellular parasites to one host cell</th>
<th>% Infected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.5:1</td>
<td>23</td>
</tr>
<tr>
<td>1</td>
<td>1:1</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>1:5</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>10:1</td>
<td>90</td>
</tr>
<tr>
<td>100</td>
<td>&gt;10:1</td>
<td>92</td>
</tr>
</tbody>
</table>

FIG. 1. In vitro effect of hydroxyurea on *L. mexicana* promastigotes. Data represent the mean parasite density ± standard error of the mean measured over 8 days. *P* < 0.001, ANOVA. •, control; ■, 1 μg/ml of hydroxyurea; ▲, 100 μg/ml of hydroxyurea.
Effect of hydroxyurea on intracellular infection with *Leishmania* in *vitro*. The effects of different concentrations of hydroxyurea on intracellular amastigotes treated for 3, 6, 9, and 12 days are shown in Fig. 2. A notable inhibitory effect of hydroxyurea on *Leishmania* growth was confirmed in the intracellular model of parasitism by reading the promastigote density at the mid-log phase of growth on day 6 after hydroxyurea removal and a shift of the temperature to 26°C. For the lowest hydroxyurea dosage of 0.01 μg/ml, 44% inhibition was observed after 3 days of incubation, with a maximum of 67% inhibition observed after 12 days. With 0.1 μg/ml of hydroxyurea, 72% inhibition was seen after 3 days of exposure and a maximum of 85% inhibition was reached after 12 days. When 1 μg/ml was tested, 86% inhibition was observed after 3 days and a maximum of 94% inhibition was reached after 12 days of hydroxyurea incubation. With 10 μg/ml, the level of inhibition began at 93% after 3 days and went up to 98% after 12 days. Intracellular amastigotes could not survive a hydroxyurea dosage of 100 μg/ml, even when the parasites were exposed for only 3 days (ANOVA, *P* < 0.001). For strain M379, annihilation of the parasites was effectively achieved at a minimal dose of 10 μg/ml of hydroxyurea after 6 days of exposure. In the absence of parasites, macrophages were viable after 6 days of incubation, even in the presence of 100 μg/ml hydroxyurea.

Antileishmanial activity of hydroxyurea compared to that of meglumine antimoniate. Macrophages with intracellular amastigote infection were incubated with 0.01, 0.1, 1, 10, and 100 μg/ml of either hydroxyurea or meglumine antimoniate. Both drugs reduced the level of multiplication of *Leishmania*; however, the level of multiplication of *Leishmania* Tab3 parasites was reduced more by hydroxyurea, since more than 85% parasite growth inhibition was achieved with hydroxyurea at a dosage of 1 μg/ml on day 6 of exposure, whereas with meglumine antimoniate the level of reduction was 50% at the same dosage on day 6 of exposure (Table 2). The data show that maximum inhibition of both *Leishmania* strains was reached at 100 μg/ml of hydroxyurea. On day 6, ED₅₀ for intracellular amastigotes of 0.015 μg/ml for hydroxyurea (Fig. 3) and 0.95 μg/ml for meglumine antimoniate were observed. The results were similar for both *Leishmania* strains, with minimal differences. Strain M379 was more susceptible to hydroxyurea than strain Tab3.
Effect of hydroxyurea on viability of *Leishmania* promastigotes. Parasite viability after hydroxyurea treatment was evaluated by flow cytometry after 48 h of drug exposure. After staining of the parasites with PI, it was determined that viability was dose dependent. Increases in the percentages of parasites with permeable membranes in the presence of higher concentrations of hydroxyurea were observed and were 1% for the control parasites (no treatment), 12% for parasites treated with 1 μg/ml of hydroxyurea, 26% for those treated with 10 μg/ml, and 55% for those treated with 100 μg/ml (Fig. 4). In parallel with this experiment, a reduction in the parasite density was also observed as the dosages of hydroxyurea increased. Furthermore, hydroxyurea treatment of the parasites was carried out for 48 h. After this length of exposure, 100 μl of the cell culture was moved to 8 ml of fresh medium. After 8 days, the parasite densities were $6.2 \times 10^6$ parasites/ml for the control and $8.5 \times 10^6$ parasites/ml, $4.3 \times 10^6$ parasites/ml, and 0 parasites/ml for the parasites treated with hydroxyurea at doses of 1, 10, and 100 μg/ml, respectively. The level of parasite growth was less than expected, as judged from their viabilities.

Cell cycle arrest of *Leishmania* by hydroxyurea. Hydroxyurea at 10 and 100 μg/ml induced the arrest of the G2 phase-M phase of the cell cycle in *Leishmania mexicana* after 48 h of incubation; no effect on the cell cycle was observed with 1 μg/ml (Fig. 5). The dosages that caused disruption of the cell cycle were considered quite high compared to the concentrations used to arrest the cell cycle of the parasite at G1 phase-S phase, and 48 h was considered a long exposure time (34).

**DISCUSSION**

Hydroxyurea has in vitro activity against both the promastigote and intracellular amastigote forms of *L. mexicana* and at a dosage of 10 or 100 μg/ml is able to completely eliminate the parasite within a relatively short period of treatment. The reductions in the survival rates for both amastigotes and promastigotes were significant after day 3 of exposure for all dosages tested. The effect of hydroxyurea against amastigotes and promastigotes was dose and time dependent; however, 6 days of treatment of both forms of the parasite proved to be...
Hydroxyurea is readily absorbed from the gastrointestinal tract. The concentrations in plasma then peak at 0.8 mM (14), 0.26 mM (17), and 0.135 mM (30) at 1 to 2 h after the administration of oral doses of 2,000, 1,200, and 500 mg, respectively. The half-life in plasma is about 2 h. Approximately 80% of the drug is recovered in the urine within 12 h following oral or intravenous administration (17, 24). These dosages are used for actual clinical practice in the treatment of HIV (17, 30), glioblastoma multiforme (12), and myeloproliferative disorders (5).

Perhaps the most significant recent advance in the treatment of leishmaniasis has been the effective oral treatment of visceral leishmaniasis through the use of miltefosine, an alkylphosphocholine originally developed as an anticancer drug. A major limitation of miltefosine is perhaps the various therapeutic responses of Leishmania species from the New World to the drug that have been reported both in vitro and in vivo (28, 33). An L. mexicana isolate from Peru was insensitive to miltefosine in a macrophage-amastigote model (33), as well as clinical cases of cutaneous leishmaniasis in Guatemala, where L. mexicana and L. braziliensis are common; these clinical cases were, however, less responsive than clinical cases in Colombia (28). Observations from India suggest that patients with relapses showed resistance to miltefosine (29). Furthermore, miltefosine offers limited efficacy for the treatment of diffuse cutaneous leishmaniasis (7). In addition, laboratory studies have predicted that multidrug resistance may affect sensitivity to miltefosine and its analogs (9). For these reasons, other drugs, such as hydroxyurea, might be favored for testing in additional preclinical studies in order to evaluate their possible use as alternatives to miltefosine.

Meglumine antimoniate was used as a reference control during the development of this study because it was previously reported to be effective against L. mexicana, L. infantum, L. tropica, and L. donovani infections in human monocyte-derived macrophage cultures (6, 23, 25). The concentration used to treat the infection in this study is based on the findings of previous studies as well as the approximate levels in the plasma of humans treated with this drug (6). When the efficacy of hydroxyurea was compared with that of meglumine antimoniate, the former was shown to be more effective in eliminating parasites.

In conclusion, hydroxyurea is a good candidate for drug therapy for leishmaniasis because it induces parasite death and cell cycle arrest in the G2 phase-M phase when it is used at concentrations ranging from 10 to 100 μg/ml.
This work was supported by CONACyT-SIGLOF grant 00-02-006-T. We are grateful to Omar Hernandez for kindly providing us with glucantime.

REFERENCES