Evaluation of α,β-Unsaturated Ketones as Antileishmanial Agents

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In this study, we assessed the antileishmanial activity of 126 α,β-unaturated ketones. The compounds NC901, NC884, and NC2459 showed high leishmanicidal activity for both the extracellular (50% effective concentration [EC50], 456 nM, 1,122 nM, and 20 nM, respectively) and intracellular (EC50, 1,870 nM, 937 nM, and 625 nM, respectively) forms of Leishmania major propagated in macrophages, with little or no toxicity to mammalian cells. Bioluminescent imaging of parasite replication showed that all three compounds reduced the parasite burden in the murine model, with no apparent toxicity.

Leishmania major is the causative agent of cutaneous leishmaniasis (CL) disease (1). Worldwide, the incidence of CL is estimated to be 0.7 to 1.2 million new cases per year (2). Other than the drug miltefosine, which was approved by the FDA in 2014, most of the currently used antileishmanial drugs were developed in the 1940s (3, 4). However, drug-resistant strains of the parasite have emerged. Furthermore, toxicity to the host and the high costs of these drugs limit their wider application and use (4, 5). Therefore, the burden of leishmaniasis and limited effective treatments clearly point to the need for new drugs.

In view of the current interest in examining different antineoplastic agents for their antiprotozoal properties (6–8), we assessed the antileishmanial activity of α,β-unaturated ketones (enones) provided by J. R. Dimmock from the University of Saskatchewan in Canada (1, 9). Enones react preferentially with cellular thiols, in contrast to amino or hydroxyl functional groups present in protein and DNA (10, 11); therefore, interactions with nucleic acids, which can lead to adverse genotoxic effects, should be absent in enones (12). Since thiol-dependent metabolism is the main detoxifying mechanism in trypanosomatids, we hypothesized that enones are attractive candidates for examination as potential antileishmanial agents.

Hence, in this study, we screened the enone library for antiparasitic and cytotoxic activity. The compounds were dissolved in dimethyl sulfoxide (DMSO) and tested at concentrations ranging from 500 μM to 1 nM (1, 9). The parasites tested were a firefly luciferase-expressing line of L. major promastigotes described previously (Lmj-FV1-LUC-TK [L. major strain Friedlin [MHOM/JL/80/Friedlin]], clone V1) and cultured as previously described (13, 14). The 126 enones were incubated with 10^6 L. major promastigotes or rhesus monkey kidney epithelial cells (LLC-MK₂)/well for 96 h and were analyzed for toxicity and parasite survival as measured by luciferase activity with the substrate 5’-fluoroluciferin (ONE-Glo luciferase assay system; Promega) using a luminometer (Luminoskan; Thermo Scientific). Sixty-four compounds inhibited the survival of L. major promastigotes at >75%. At the same concentrations tested, 20 out of 126 screened compounds displayed minimal to no toxicity (>75% survival) against LLC-MK₂ cells. Only six compounds met the criteria for both antiparasitic activity (<25%) and low cytotoxicity to LLC-MK₂ cells (>75%) (see Fig. S1 in the supplemental material). These six compounds were further tested in the following mammalian cell lines: Hs27 human fibroblasts, RAW 264.7 murine macrophages (American Type Culture Collection [ATCC], Manassas, VA), and peritoneal BALB/c mice macrophages, obtained as described previously (15).

The in vitro infectivity experiments were carried out to determine the activity of NC901, NC884, and NC2459 against L. major intracellular amastigotes. Peritoneal macrophages isolated from BALB/c mice were infected with L. major metacyclic promastigotes for 24 h, followed by treatment with the NC lead compounds for an additional 48 h (Table 1). The antileishmanial activity exhibited by the compounds was evaluated using BD Pathway Biomaster high-content imaging assay (HCIA) analysis (16–18). In comparison to the 1% DMSO control, all three compounds showed a significant decrease in the percentage of infected cells. NC2459 was the most effective at a concentration of 1.25 μM (Table 1).

Additionally, the activity of the compounds against L. major was investigated in a murine model of CL. The first set of experiments (Fig. 1A) consisted of 12 female BALB/c mice organized in four groups of three, each infected with 10^7 L. major metacyclic promastigotes (Table 2). As shown in Fig. 1A, at 46 days postinfection, all experimental groups exhibited a lesion size smaller...
than or equal to that of the group treated with amphotericin B. Further support was obtained by determining the relative amount of luminescence emitted from the luciferase-expressing parasites in the infected footpad at the endpoint of the study, namely, 46 days postinfection (Fig. 1C). All mice in the NC2459 group did not develop measurable lesions until 2 to 3 weeks after the last treatment (total of 6 to 7 weeks postinfection). Furthermore, one out of the three mice did not develop a lesion up to 12 weeks postinfection.

To determine the relative toxicity of the compounds, the weights of the mice were measured twice a week (data not shown). There was no significant weight loss for any of the groups except those of the control group. The procedures were performed to minimize distress and pain for the animals according to NIH guidance and the animal protocol approved by The University of Texas at El Paso (UTEP) Institutional Animal Care and Use Committee (IACUC).

Hyperinfection with *L. major* was tested. Twenty-six female BALB/c mice organized in four groups of five and one group of six mice were infected with $10^6$ *L. major* metacyclic promastigotes (Table 1). All experimental groups were given higher doses than the control group ($P < 0.05$). However, at 29 days postinfection, the mice in the diluent control group had to be euthanized to avoid physical distress caused by a large lesion; all other groups were monitored for an additional 4 days. For this reason, the luminescence from the infected footpads for each group was analyzed at 29 days postinfection and not at the endpoint of the experiment (Fig. 1D). After 1 week of treatment with NC2459, three of the mice from this group were given double the daily dose of drug (8 mg/kg of body weight/day), while the other three remained on the same daily dose (4 mg/kg/day). In addition, the three mice given the higher dose were treated with four additional doses. Those given NC2459 (extended treatment) at 33 days postinfection showed a smaller lesion than that of all the other groups ($P < 0.05$). The increase in dosage for NC2459 (extended treatment) did not result in any additional weight loss or toxic side effects in comparison to those of the control group. The procedures were performed to minimize distress and pain for the animals according to NIH guidance and the animal protocol approved by The University of Texas at El Paso (UTEP) Institutional Animal Care and Use Committee (IACUC).

Our findings demonstrate the ability of these compounds to reduce *L. major* replication in vivo without any obvious toxic side effects. NC2459 evidently exhibited a much smaller amount of swelling than that in all other groups by decreasing the footpad swelling than that in all other groups by decreasing the footpad

### TABLE 1 Summary of EC₅₀ and IC₅₀ for NC901, NC884, and NC2459 tested against *L. major* and mammalian cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mammalian cell EC₅₀ (µM) ± SD for:</th>
<th>L. major cells (µM) (TI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peritoneal murine macrophages</td>
<td>LLC-MK₂</td>
</tr>
<tr>
<td>NC901</td>
<td>7.03 ± 0.42</td>
<td>8.02 ± 0.25</td>
</tr>
<tr>
<td>NC884</td>
<td>7.67 ± 0.31</td>
<td>15.1 ± 0.33</td>
</tr>
<tr>
<td>NC2459</td>
<td>5.45 ± 0.36</td>
<td>2.0 ± 0.093</td>
</tr>
</tbody>
</table>

*EC₅₀, half-maximal effective concentration calculated with 95% confidence; IC₅₀, 50% inhibitory concentration; TI, therapeutic index, calculated as (EC₅₀ peritoneal murine macrophages)/(EC₅₀/IC₅₀ parasites). The EC₅₀ was obtained as the exponent of the negative ratio of the y-intercept and the slope of the fitted regression line (version 9.2; SAS Software). P value, <0.0001 for all concentrations. The Z-factor calculated for the HCIA is in the range of 0.5 to 0.91, indicating that the quality of the assay is excellent.*
ACKNOWLEDGMENTS

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REFERENCES


**TABLE 2** Treatment regimens of the in vivo experiments

<table>
<thead>
<tr>
<th>Compound</th>
<th>Treatment (mg/kg/day) after infection with:</th>
<th>10⁵ promastigotes</th>
<th>10⁶ promastigotes</th>
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<tbody>
<tr>
<td>DMSO (100 μl)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AmpB</td>
<td></td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>NC901</td>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>NC884</td>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>NC2459</td>
<td></td>
<td>1</td>
<td>4 (3 mice/14 days)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8 (3 mice/days 8–17)</td>
</tr>
</tbody>
</table>

*The mice infected with 10⁵ *L. major* metacyclic promastigotes were treated for 14 consecutive days at the stated dosages.

*bThe mice infected with 10⁶ *L. major* metacyclic promastigotes (hyperinfection experiment) were split into two groups of three mice after 7 days of treatment with NC2459. One group remained on the same treatment, 4 mg/kg/day, for seven more days (14 total days of treatment), and the other group was given double the daily dosage, 8 mg/kg/day, for 10 more days (17 total days of treatment).

*a AmpB, amphotericin B.

**FIG 1** *In vivo* activity of compounds NC901, NC884, and NC2459 in the murine model of CL. (A and B) Footpad size of BALB/c mice infected with 10⁵ and 10⁶ *L. major* metacyclic promastigotes, respectively. The Rx1 line refers to the time period in which treatment was administered daily, and the Rx2 line indicates that the daily dosage of NC2459 was doubled for that time period. (C) Luminescence of BALB/c mice footpads at 46 days postinfection with 10⁵ metacyclic promastigotes. *, *P* < 0.05. (D) Luminescence of BALB/c mice footpads at 29 days postinfection with 10⁶ metacyclic promastigotes. C, group treated with vehicle control; CD, group treated with amphotericin B. A statistical analysis of the net intensity (luminescence) of both the 10⁵ and 10⁶ experiments was carried out using the two-sided unpaired *t* test. *, *P* ≤ 0.05; **, *P* ≤ 0.01.


