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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-unsaturated ketones. The compounds NC901, NC884, and NC2459 showed high leishmanicidal activity for both the extracellular (50% effective concentration [EC_{50}], 456 nM, 1,122 nM, and 20 nM, respectively) and intracellular (EC_{50}, 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-unsaturated 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-MK_2)/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-MK_2 cells. Only six compounds met the criteria for both antiparasitic activity (<25%) and low cytotoxicity to LLC-MK_2 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). Only NC901, NC884, and NC2459 showed effective activity against *L. major*. NC2459 showed a 100-fold difference between the 50% effective concentration (EC_{50}) of the parasite and that for all three mammalian cell lines (Table 1).

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 Bioimager 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 10^5 *L. major* metacyclic promastigotes (Table 2). As shown in Fig. 1A, at 46 days postinfection, all experimental groups exhibited a lesion size smaller

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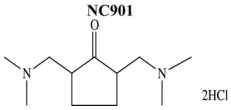
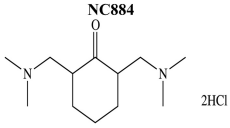
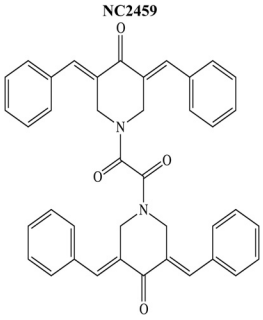
TABLE 1 Summary of EC_{50} and IC_{50} for NC901, NC884, and NC2459 tested against *L. major* and mammalian cells[a]

Compound	Mammalian cell EC_{50} (μM) ± SD for:			*L. major* cells (μM) (TI)	
	Peritoneal murine macrophages	LLC-MK_2	Hs27 fibroblasts	Promastigote EC_{50}	Intracellular amastigote IC_{50}
NC901 	7.03 ± 0.42	8.02 ± 0.25	16.0 ± 0.12	0.45 (15.62)	1.87 ± 0.20 (3.76)
NC884 	7.67 ± 0.31	15.1 ± 0.33	16.3 ± 0.17	1.12 (6.55)	0.937 ± 0.13 (8.18)
NC2459 	5.45 ± 0.36	2.0 ± 0.093	10.01 ± 0.09	0.020 (272.5)	0.625 ± 0.11 (8.72)

[a] EC_{50}, half-maximal effective concentration calculated with 95% confidence; IC_{50}, 50% inhibitory concentration; TI, therapeutic index, calculated as (EC_{50} peritoneal murine macrophages)/(EC_{50}/IC_{50} parasites). The EC_{50} 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.

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 for the mice receiving amphotericin B. Additionally, no mice died as a result of the toxicity of the compounds.

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 (Fig. 1B). All experimental groups were given higher doses than the groups receiving 10^5 *L. major* metacyclic promastigotes (Table 2). At 4 weeks postinfection, the lesion size in all three NC compounds significantly reduced in comparison to that of the diluent control group ($P \leq 0.05$). 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 ($P \leq 0.05$). 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 lesion by 99.99% (Fig. 1A) in comparison with that of the diluent-treated group. In summary, the data obtained in this study suggest that our lead compound, NC2459, is an excellent antiparasitic agent and might be useful as a potential alternative to the current clinical drugs used to treat leishmaniasis. Clearly, NC2459 is an important lead molecule, which is structurally divergent from contemporary medications employed to treat parasitic diseases. It is a prototypic molecule, and further molecular modifications are required, followed by the relevant bioassays, to increase potency and especially to increase the differential in toxicities between parasites and normal cells.

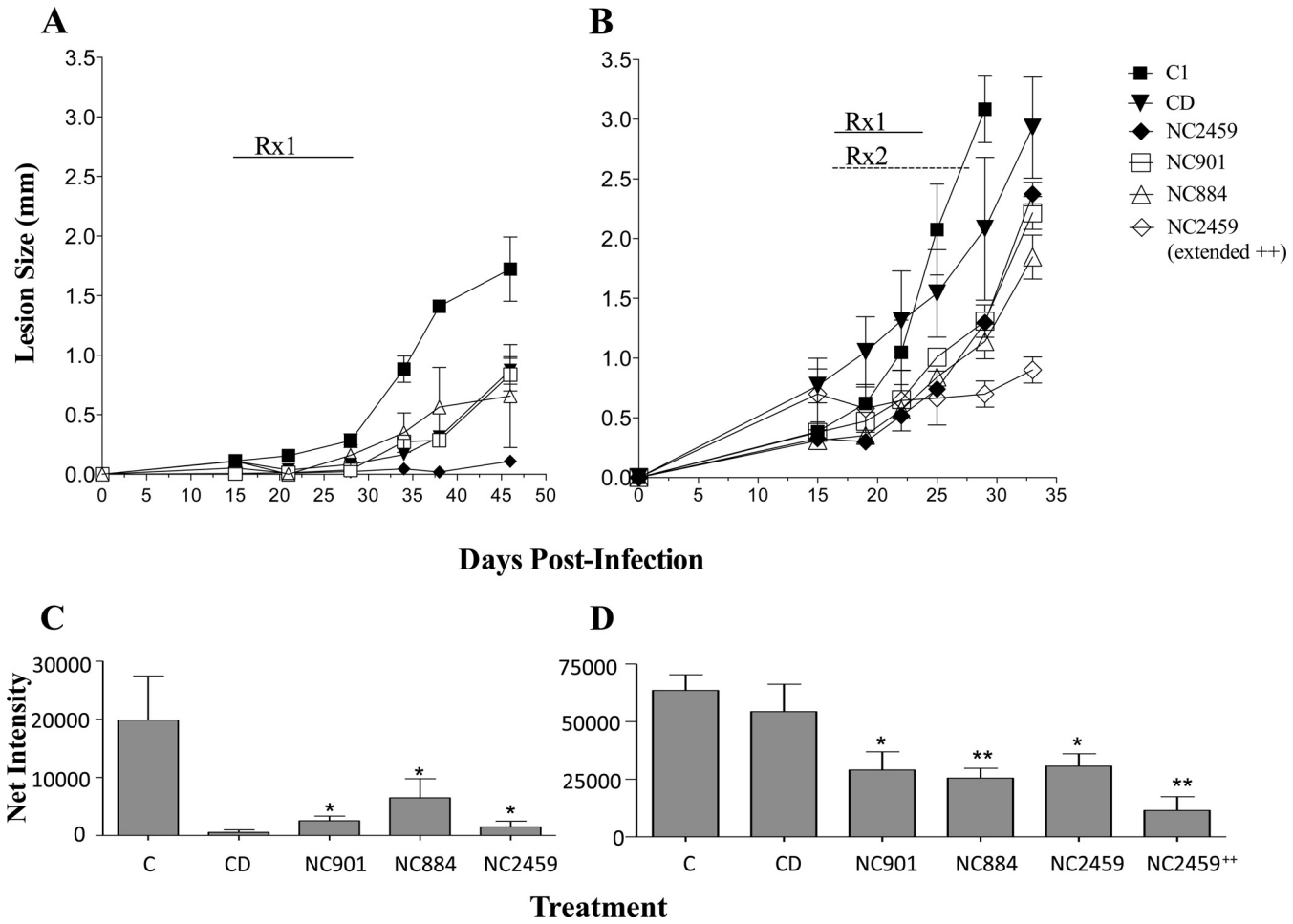


FIG 1 *In vivo* activity of compounds NC901, NC884, and NC2459 in the murine model of CL. (A and B) Footpad size of BALB/c mice infected with 10^5 and 10^6 *L. major* metacyclic promastigotes, respectively. The Rx1 line refers to the time period in which treatment was administered daily, and the Rx2 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 10^5 metacyclic promastigotes. *, $P < 0.05$. (D) Luminescence of BALB/c mice footpads at 29 days postinfection with 10^6 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 10^5 and 10^6 experiments was carried out using the two-sided unpaired *t* test. *, $P \leq 0.05$; **, $P \leq 0.01$.

TABLE 2 Treatment regimens of the *in vivo* experiments

Compound	Treatment (mg/kg/day) after infection with:	
	10^5 promastigotes[a]	10^6 promastigotes[b]
DMSO (100 μl)		
AmpB[c]	8	8
NC901	2	4
NC884	4	8
NC2459	1	4 (3 mice/14 days)/ 8 (3 mice/days 8–17)

[a] The mice infected with 10^5 *L. major* metacyclic promastigotes were treated for 14 consecutive days at the stated dosages.
[b] The mice infected with 10^6 *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).
[c] AmpB, amphotericin B.

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