INHIBITION OF INTRACELLULAR PROLIFERATION OF Leishmania PARASITES IN VITRO AND SUPPRESSION OF SKIN LESION DEVELOPMENT IN BALB/C MICE BY A NOVEL LIPID A ANALOG (ONO-4007)

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Abstract. A synthetic lipid A analog (ONO-4007) exhibits antileishmanial activity by activating Leishmania-infected macrophages in experimental leishmaniasis. In the present in vitro study, ONO-4007 at concentrations between 0.01 and 1.00 mg/mL markedly inhibited the proliferation of Leishmania major and L. amazonensis promastigotes. Ultrastructurally, L. major-infected macrophages showed degenerated intracellular amastigotes after exposure to ONO-4007. Leishmania-infected macrophages treated with ONO-4007 showed poorly developed parasitophorous vacuoles. High levels of tumor necrosis factor-alpha were induced by ONO-4007 in Leishmania-infected macrophages. In this in vivo study, L. amazonensis-infected BALB/c mice were treated with a dose of 30 mg/kg of ONO-4007 by perilesional and peritoneal injections. The skin lesion size was assessed before treatment with ONO-4007 and at eight weeks after injection. The lesion size was significantly suppressed in mice perilesionally injected with ONO-4007 (P < 0.01) compared with the controls. The data from our present in vitro and in vivo studies indicate that ONO-4007 has an antileishmanial effect.

INTRODUCTION

Leishmaniasis is a widespread, infectious parasitic disease caused by Leishmania spp. The World Health Organization estimates that 350 million people are at risk of infection with leishmaniasis in endemic areas and that approximately 12 million are currently infected, with 400,000 new cases each year. It is now a worldwide problem and a great challenge exists in the fight against this disease. At the present time, commercially available, antimonial compounds remain the first-line drugs of choice in spite of reported severe toxicity affecting the heart, liver, and kidneys. However, instead of using metallic antimonial agents in the treatment of leishmaniasis, investigators have been introducing different chemotherapeutic agents with different methods of administration. Studies in endemic areas have reported strains of Leishmania that are resistant to antimonial compounds. Safe, effective, and sensitive chemotherapeutic agents that kill Leishmania parasites have been the subjects of intense research to discover new drugs for solving this worldwide health problem.

ONO-4007, a novel synthetic lipid A analog, has been reported to exhibit strong antitumor activity in various experimental animal models via intratumor production of the tumor necrosis factor-alpha (TNF-α) with lower toxicity than has been exhibited with bacterial lipopolysaccharide (LPS). Lipid A is an active part of LPS, a component of the cell wall of gram-negative bacteria that has a variety of biologic activities, including both beneficial immunopotentiating activity and toxicity. The chemical structure of ONO-4007 (sodium-2-deoxy-2-[3S-(9-phenylnonanoyloxy)tetradecanoyl]-amino-3-0-(9-phenylnonanoyl)-D-glucopyranose-4-sulfate) is shown in Figure 1. This analog activates macrophages, which then release large amounts of TNF-α in a dose-dependant manner. Moreover, in vitro studies have shown that pretreatment of human monocytes with granulocyte-macrophage colony-stimulating factor enhances the production of TNF-α in response to ONO-4007. It has been reported that ONO-4007 contributes to the induction of nitric oxide (NO) synthesis in macrophage cell lines J774.2, resulting in the formation of cytotoxic NO, which may show antitumor activity in vitro.

Macrophages are activated when exposed to cytokines to eliminate intracellular infections by microorganisms. Infections with Leishmania, cytokines such as interferon-gamma (IFN-γ), migration inhibitory factor, and TNF-α are capable of inducing leishmaniacidal activity in vitro. The leishmaniacidal activity of macrophages is also mediated by NO after activation by cytokines and LPS. These data indicate that ONO-4007 may contribute to the elimination of Leishmania parasites from infected subjects.

In this study, we examined the antileishmanial efficacy of ONO-4007 in experimental leishmaniasis in vitro and in vivo, with the aim of introducing a new drug strategy for treatment of leishmaniasis. The inhibitory effects of ONO-4007 on Leishmania proliferation were investigated in promastigotes and amastigotes, and in L. amazonensis-infected BALB/c mice.

MATERIALS AND METHODS

In vitro study. Parasites. Two strains of Leishmania promastigotes, L. major (MHOM/SU/73/5ASKH) and L. amazonensis (MHOM/BR/73/M2269), were cultured in RPMI 1640 medium (GIBCO-BRL, Tokyo, Japan) medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL of penicillin, and 50 μg/mL of streptomycin. Macrophase culture. Macrophage cell line J774 was obtained from the Dai Nippon Pharmaceutical Co. (Osaka, Japan), and was cultured in Dulbecco’s minimal essential medium (GIBCO-BRL) supplemented with 10% FBS, 50 units/mL of penicillin, and 50 μg/mL of streptomycin. Laboratory-Tek® tissue culture chamber slides (Nalge Nunc International Corp., Naperville, IL) were used for cell culture and kept in an incubator at 37°C in an atmosphere of 5% CO₂ and a humidity of 95%.

Drug. ONO-4007 was generously provided by the Ono...
Pharmaceutical Co. (Osaka, Japan). The drug was prepared in 5% ethanol in a 4.5% glucose solution.

**Drug administration.** Promastigotes of *L. major* and *L. amazonensis* were exposed to ONO-4007 at concentrations of 0 mg/mL, 0.01 mg/mL, 0.10 mg/mL and 1.00 mg/mL for 48 hours. Similarly, macrophages infected with *L. major* were incubated with ONO-4007 at the same dosages for 48 hours.

**Parasite count.** Promastigotes in the medium were counted with a hemocytometer.

**Enzyme-linked immunosorbent assay (ELISA) for TNF-α.** The expression of TNF-α in culture supernatants was determined by a sandwich ELISA using a commercially available TNF-α kit (Endogen, Woburn, MA) according to the protocol provided by the manufacturer. The level of TNF-α was expressed as micrograms per milliliter/2.08 × 10⁶ macrophage cells. All measurements were carried out in triplicate.

**Electron microscopy.** Macrophages were harvested and washed twice with phosphate-buffered saline. They were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, and then treated with phosphate-buffered 1% osmium tetroxide for one hour. The samples were dehydrated with a graded ethanol series and propylene oxide. The samples were then embedded in Epon 812 resin and ultrathin sections were cut. The sections were stained with uranyl acetate, counterstained with lead citrate, and then observed with a JEOL 2000EX electron microscope (JEOL, Tokyo, Japan).

**In vivo study. Infection of mice with parasites.** Female BALB/c mice (6–8 weeks old) were used in these experiments. They were strictly maintained under the rules and regulations of the Animal Welfare Center (University of the Ryukyus, Okinawa, Japan) in specific pathogen-free conditions with laminar air flow. All mice were inoculated intradermally on the shaved skin of their backs with 2 × 10⁷ *L. amazonensis* promastigotes.

**Drug administration.** After the skin lesion was confirmed (five weeks after inoculation), a 30 mg/kg dose of ONO-4007 was perilesionally or peritoneally injected into the mice twice a week for eight consecutive weeks. Mice were divided into four groups: group A (vehicle only, perilesional injection, n = 7), group B (vehicle only, peritoneal injection, n = 8), group C (ONO-4007, perilesional injection, n = 6), and group D (ONO-4007 peritoneal injection, n = 6).

**Assessment of the lesion size.** Lesion size was measured with a dial caliper before and after eight weeks of treatment with ONO-4007 and expressed as the area of lesion (long axis × short axis in mm²).

**Statistical analysis.** Statistical differences were examined by the unpaired *t*-test. The level of significance was determined at *P* < 0.05.

**FIGURE 1.** Chemical structure of ONO-4007.

**FIGURE 2.** Inhibition of growth of *Leishmania* promastigotes in culture medium incubated with ONO-4007. Promastigotes of *L. major* (A) and *L. amazonensis* (B) showed a gradual reduction in numbers when exposed to 0.01–1.00 mg/mL of ONO-4007 for 48 hours. Both strains of promastigotes proliferated in the absence of ONO-4007. A significant reduction in number of promastigotes was observed following incubation with the drug for 24–48 hours. The proliferation of *L. major* and *L. amazonensis* was inhibited by ONO-4007. Bars show the mean ± SD of four experiments.
RESULTS

Anti-promastigote activity of ONO-4007. Promastigotes of *L. major* and *L. amazonensis* were cultured with 0, 0.01, 0.10, and 1.00 mg/mL of ONO-4007 for 48 hours. In the absence of ONO-4007, both strains of *Leishmania* promastigotes proliferated over time. In contrast, both types of promastigotes treated with ONO-4007 gradually decreased in number in a time-dependent fashion. After 24 and 48 hours of incubation with three different drug concentrations, *L. major* promastigotes showed a significant reduction in number when compared with the controls. No significant differences in the number of *L. major* promastigotes were observed for the three different concentrations of drug after 48 hours of incubation (Figure 2A). However, the number of *L. amazonensis* promastigotes was significantly reduced at the three different drug concentrations when compared with the controls following incubation for 24 and 48 hours (Figure 2B). Statistically significant differences were observed at a concentration of 1.00 mg/mL compared with 0.01 mg/mL (*P* < 0.01) and at a concentration of 1.00 mg/mL compared with 0.10 mg/mL following 48 hours of incubation (*P* < 0.01). These results demonstrate the inhibition of the growth of *L. major* and *L. amazonensis* promastigotes by ONO-4007.

Promastigotes cocultured with macrophages. This study was performed to estimate the time required for *Leishmania* promastigotes to enter murine macrophages. Cocultivation showed that approximately 80% of the promastigotes of *L. major* and *L. amazonensis* in the medium entered macrophages after 24 hours (Figure 3). Thus, ONO-4007 should be given after 24 hours of cocultivation with the macrophages.

Expression of TNF-α by *L. major*-infected macrophages. Production of TNF-α by macrophages infected with *L. major* following incubation for 24 hours with three concentrations (0.01 mg/mL, 0.10 mg/mL, and 1.00 mg/mL) was examined by sandwich ELISA. The culture supernatant was collected and analyzed after incubation for 6, 12, and 24 hours with ONO-4007. Production of TNF-α was significantly induced by ONO-4007 at all three concentrations. The highest level of TNF-α was observed after incubation for six hours with all concentrations of drug, and gradually decreased in a time-dependent manner (Figure 4). After incubation for six hours, the level of TNF-α was significantly higher at drug concentrations of 0.01 mg/mL, 0.10 mg/mL, and 1.00 mg/mL when compared with the controls (*P* < 0.01, *P* < 0.001, and *P* < 0.01, respectively). Induction of TNF-α after incubation for six hours at ONO-4007 concentrations of 0.01 mg/mL and 0.10 mg/mL was higher than at the concentration of 1.00 mg/mL.

Electron microscopy of *L. major*-infected macrophages. Macrophages in the absence of ONO-4007 showed many intracellular amastigotes within markedly large parasitophorous vacuoles (Figure 5A). At a higher magnification, oval amastigotes showed well-developed cell organelles (Figure 5C). Conversely, macrophages treated with ONO-4007 showed only a few amastigotes and poorly formed parasitophorous vacuoles in the macrophages (Figure 5B). At a higher magnification, intracellular amastigotes showed degenerative changes and poorly formed parasitophorous vacuoles (Figure 5D).

Lesion development in *L. amazonensis*-infected mice. BALB/c mice infected on their backs with *L. amazonensis* promastigotes showed skin lesions as a single nodule with or without ulceration five weeks after inoculation. The lesions developed to full size in most of the infected mice. However,
FIGURE 5. Ultrastructure of *Leishmania major*–infected macrophages. **A**, *L. major*-infected macrophages used as a control show large parasitophorous vacuoles (P) containing many *Leishmania* parasites (arrows). **B**, an *L. major*-infected macrophage treated with ONO-4007 contains a single *Leishmania* parasite (arrows), but parasitophorous vacuoles are too small to identify. **C**, Higher magnification view of control macrophages, showing formation of a parasitophorous vacuole with a well-developed membrane (arrowhead). **D**, Higher magnification of macrophages treated with ONO-4007, showing poor formation of parasitophorous vacuoles compared with the control (arrowhead) and an amastigote (Am) inside the parasitophorous vacuole with degenerative changes.
four mice in group C (ONO-4007 perilesionally injected mice) showed healed lesions eight weeks after treatment (Figure 6C). The mean (SD) size of the skin lesions in each group is shown in Figure 7. Lesion development was significantly suppressed in mice perilesionally injected with ONO-4007 ($P < 0.01$) compared with mice injected with the vehicle (group A). No statistically significant difference in lesions was observed between mice peritoneally injected with ONO-4007 (group D) and mice injected with the vehicle (group B).

DISCUSSION
This study provides evidence that ONO-4007 inhibits promastigote proliferation. However, in the elimination of *Leishmania* parasites *in vivo*, activation of macrophages may be the most critical factor because the disease course is influenced by several cytokines. Activation of macrophages can be estimated by endogenous production of TNF-α and confirmed by ultrastructural findings, such as well-developed mitochondria, endoplasmic reticulum, and other cell organelles. In previous reports, ONO-4007 induced higher levels of endogenous production of TNF-α and cytoidal activities against MM46 mammary/KDH-8 hepatoma cells than LPS *in vitro* and *in vivo*.6,11

In the present study, ONO-4007 induced production of TNF-α in *Leishmania*-infected macrophages at concentrations of 0.01–1.00 mg/mL, especially at 0.10 mg/mL. Tumor necrosis factor-alpha is also a pleiotropic cytokine that may stimulate a variety of potent antimicrobial mechanisms and have a protective role against infection with *L. major*.18,19 Our ultrastructural observations of *Leishmania*-infected macrophages treated with ONO-4007 revealed degenerative change in amastigotes, dilated rough endoplasmic reticulum, and large mitochondria. These results supported our theory that ONO-4007 promotes antileishmanial activity by activation of *Leishmania*-infected macrophages. Ultrastructural study showed that in the ONO-4007-treated macrophages, the number of amastigotes was much less than in the controls.

![Figure 6](image-url)

**Figure 6.** Area (long axis × short axis in mm²) of skin lesions of *Leishmania amazonensis*-infected BALB/c mice before and after eight weeks treatment with ONO-4007. **A.** Mice in group A (n = 7) were perilesionally injected with vehicle. **B.** Mice in group B (n = 8) were peritoneally injected with vehicle. **C.** Mice in group C (n = 6) were perilesionally injected with ONO-4007 (note that the lesions healed in four mice). **D.** Mice in group D (n = 6) were peritoneally injected with ONO-4007.
The most striking feature of the study was the formation of parasitophorous vacuoles in the infected macrophages. The parasitophorous vacuoles were very large in cells not treated with ONO-4007, and they contained several amastigotes. In contrast, cells treated with ONO-4007 cells did not show extensive formation of these vacuoles. Parasitophorous vacuoles are a sign of intracellular parasite survival. Poor formation of these vacuoles in cells treated with ONO-4007 cells suggests that amastigote activity was inhibited by the treatment.

In our **in vitro** study, we used ONO-4007 in a murine model of leishmaniasis. After checking the development of lesions, we injected this drug perilesionally and peritoneally. In mice injected perilesionally with ONO-4007 (group C), lesion development was significantly suppressed compared with the controls (group A). It is noteworthy that four mice in group C showed healed lesions, although lesion development in these mice was variable. In the peritoneally injected group, there was no significant difference between the drug and control groups. These results suggest that ONO-4007 might have an antileishmanial effect in the **in vivo** model, as well as in the **in vitro** model. The toxic effect of ONO-4007 was very negligible, a 1,000-fold less than that of *Escherichia coli* LPS. This has been shown in previous reports. In human monocytes/macrophages, ONO-4007 stimulates higher levels of TNF-α production with lower toxicity than bacterial LPS. Lipopolysaccharide has never been used in humans due to its strong toxicity. Based on these observations, ONO-4007 may be a practical therapeutic agent for treatment of leishmaniasis. We used only one dose (30 mg/kg) of the drug in the **in vivo** study. Several dosage levels should be tested in mice in future studies.

Killing of intracellular parasites by macrophages has been shown to be mediated by NO both **in vitro** and **in vivo**. Recent studies have shown that NO is a potent antimicrobial agent derived from L-arginine by the enzyme NO synthase. The cytokines TNF-α and IFN-γ are the important immunologic mediators in the induction of NO synthase in macrophages. The conversion of L-arginine to NO is catalyzed by the enzyme NO synthase, which is induced in macrophages after activation of cytokines by LPS. The Th1 cytokine response and the production of IFN-γ are required for the induction of inducible NO synthase in infected macrophages. ONO-4007 induced NO synthase resulting in the formation of cytotoxic NO, which may contribute to the antitumor activity that has been shown in both **in vivo** and **in vitro** studies. It has been reported that intracellular killing of *Leishmania* parasites within macrophages was enhanced in the presence of TNF-α together with IFN-γ or LPS. Neither IFN-γ nor LPS alone induced leishmaniacidal activities **in vitro**, indicating that LPS was necessary as a secondary stimulus. Although our experiment was not specifically designed to determine the exact mechanism(s) by which ONO-4007 exerted an antileishmanial agent, it is suggested that the role of NO and Th1 cytokine induction should be the subject of intense research in further **in vivo** studies using experimental leishmanial mice.

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