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EXPERIMENTAL THERAPEUTICS

In Vitro and *In Vivo* Characterization of Potent Antileishmanial Methionine Aminopeptidase 1 Inhibitors

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ABSTRACT *Leishmania major* is the causative agent of cutaneous leishmaniasis (CL). No human vaccine is available for CL, and current drug regimens present several drawbacks, such as emerging resistance, severe toxicity, medium effectiveness, and/or high cost. Thus, the need for better treatment options against CL is a priority. In the present study, we validate the enzyme methionine aminopeptidase 1 of *L. major* ($MetAP1_{Lm}$), a metalloprotease that catalyzes the removal of N-terminal methionine from peptides and proteins, as a chemotherapeutic target against CL infection. The *in vitro* antileishmanial activities of eight novel MetAP1 inhibitors (OJT001 to OJT008) were investigated. Three compounds, OJT006, OJT007, and OJT008, demonstrated potent antiproliferative effects in macrophages infected with *L. major* amastigotes and promastigotes at submicromolar concentrations, with no cytotoxicity against host cells. Importantly, the leishmanicidal effect in transgenic *L. major* promastigotes overexpressing $MetAP1_{Lm}$ was diminished by almost 10-fold in comparison to the effect in wild-type promastigotes. Furthermore, the *in vivo* activities of OJT006, OJT007, and OJT008 were investigated in *L. major*-infected BALB/c mice. In comparison to the footpad parasite load in the control group, OJT008 decreased the footpad parasite load significantly, by 86%, and exhibited no toxicity in treated mice. We propose MetAP1 inhibitor OJT008 as a potential chemotherapeutic candidate against CL infection caused by *L. major* infection.

KEYWORDS *Leishmania major*, antiparasitic agents, cutaneous leishmaniasis, drug discovery, methionine aminopeptidase 1, molecular parasitology, murine model of cutaneous leishmaniasis, parasitology, target validation

The leishmaniases are a complex of infectious diseases caused by more than 20 kinetoplastid protozoan parasites that belong to the Trypanosomatidae family and genus *Leishmania*. Roughly 12 million people are infected, with an increasing incidence of 2 million per year (1). Moreover, approximately 350 million people are at risk of contracting leishmaniasis in 98 countries across five continents, and it is included in the neglected tropical diseases (NTD) group (1). Clinical manifestations range from nodular and ulcerative skin lesions to progressive mucocutaneous and visceral forms. Cutaneous leishmaniasis (CL) is the predominant human clinical manifestation, and it is characterized by particular localized skin ulcers (2, 3). CL is considered a tropical disease. In the Old World, CL is mainly caused by *Leishmania aethiopica*, *Leishmania tropica*, and *Leishmania major*, affecting the Middle East, Mediterranean littoral, Arabian Peninsula, Africa, Near Asia, Indian Subcontinent, and other areas (4, 5). In the New World, CL is caused by several species, such as *Leishmania mexicana*, *Leishmania amazonensis*, *Leishmania venezuelensis*, or members of the subgenus *Vianna*, which includes *Leish-*

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mania Vianna braziliensis, *L.* (*V.*) *guyanensis*, *L. (V.) panamensis*, and *L. (V.) peruviana* (6). Nonetheless, with increases in travel, military activities, and migration, the disease presents a risk for populations that were previously unaffected, including in the United States, where CL is nowadays considered an emerging concern (7–10).

Currently, there are no available vaccines against leishmaniasis (11, 12), and therefore, therapies rely solely upon a reduced number of drugs (13). These drugs, such as the pentavalent antimonials meglumine antimonate and sodium stibogluconate (Glucantime and Pentostam, respectively), miltefosine (Impavido), and liposomal amphotericin B (AmBisome), pose several challenges because of their numerous toxic side effects, high cost, and parenteral administration and the potential emergence of chemoresistant parasites (14). Hence, there is an urgent need for the development of less toxic, more cost-effective, and more therapeutic interventions against leishmaniasis.

Essential enzymes like methionine aminopeptidase (MetAP) have been suggested as promising targets for the development of novel antiparasitic agents. Methionine aminopeptidases are classified into two different types, MetAP1 and MetAP2. The latter contains a 60-amino-acid insertion that distinguishes it from MetAP1 (15, 16). MetAP1 is a dinuclear metalloprotease that catalyzes the removal of N-terminal methionine residues from peptides and proteins (17). MetAP1 proteins bind to metal ions like cobalt or zinc for their activity (18), and disruption of MetAP1 impairs proper protein folding, posttranslational modifications, biologic maturation, and translocation of some newly synthesized peptides and proteins within the cell (19). The functionality and importance of MetAP1 has been shown in several organisms, including *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Mycobacterium tuberculosis*, where the knockdown of the MetAP1 gene leads to lethal effects or reduced viability (20–22). In *Saccharomyces cerevisiae*, the knockdown of MetAP1 leads to slow growth, while the knockdown of MetAP1 and MetAP2 leads to nonviable yeast strains (23). Furthermore, studies have been made of MetAP1b in the protozoan *Plasmodium falciparum* (*Pf*MetAP1b), one of four types of MetAP found in *P. falciparum*. The observation of antiproliferation effects on several *P. falciparum* strains by highly selective inhibitors of *Pf*MetAP1b has led to the discovery of selective MetAP inhibitors (15). Moreover, MetAP inhibitors have shown promising results against tuberculosis, fungal infections, rheumatic disease, various forms of cancer, malaria, leishmaniasis, and other diseases (15, 22–30). Unlike the protozoan *P. falciparum*, only one methionine aminopeptidase has been discovered in *L. major* ($MetAP1_{Lm}$), which has a 50% sequence similarity with human MetAP1 (MetAP1 of *Homo sapiens* [*Hs*MetAP1]) and less than 14% similarity to human MetAP2 (*Hs*MetAP2) (Fig. S1 in the supplemental material). Another report highlighted the potential role of type 2 MetAP in *Leishmania donovani* (31), and a recent study reported the expression, purification, and characterization of MetAP1 in *L. donovani*, giving more evidence of MetAP1 as a drug target for *Leishmania* spp. (32). Therefore, we selected methionine aminopeptidase 1 (MetAP1) as a prospective chemotherapeutic target.

Using an integrated whole-cell-based screening and chemogenetic approach, we systematically identified and characterized three novel $MetAP1_{Lm}$ inhibitors. Previously, a high-throughput screen consisting of a library of 175,000 structurally diverse small molecules was conducted by Olaleye et al. (22). Their study successfully identified lead MetAP1 inhibitors against *M. tuberculosis* (22). As part of the drive to find new antileishmanial treatments, we screened and characterized the antiparasitic activity of these novel MetAP1 inhibitors against CL infection caused by *L. major in vitro* and in an *in vivo* model. $MetAP1_{Lm}$ inhibitors OJT006, OJT007, and OJT008 showed potent leishmanicidal activity and remarkable selectivity indexes *in vitro*. More importantly, OJT008 significantly reduced the parasitic load with no evident toxicity in a preclinical *in vivo* model. These findings suggest $MetAP1_{Lm}$ as a potential therapeutic target for the development of efficient and nontoxic drugs against CL. MetAP1 can serve as a potential target for the development of novel anti-infective agents to combat the emergence of drug-resistant pathogens.

TABLE 1 Antiparasitic activities of OJT compounds at 72 h in *L. major* promastigotes and cytotoxicities of the compounds to intraperitoneal mouse macrophages

Compound	Value ± estimated interval for:		
	EC50 (μM)[a]	CC50 (μM)[b]	SI[c]
OJT001	10.9 ± 1.3	NA[d]	NA
OJT002	11.96 ± 0.84	NA	NA
OJT003	14.36 ± 0.45	NA	NA
OJT004	3.36 ± 0.13	NA	NA
OJT005	6.8 ± 0.57	NA	NA
OJT006	~0.6	79 ± 2.34	131.6
OJT007	0.38 ± 0.006	40.68 ± 2.18	107.05
OJT008	~0.24	~148.1	617.08

[a]EC_{50}, median effective concentration. Measure of antiparasitic activity against *L. major* promastigotes.
[b]CC_{50}, median cytotoxic concentration. Measure of cytotoxicity in mammalian cells (intraperitoneal mouse macrophages [IPΦ]).
[c]SI, selectivity index (CC_{50}/EC_{50}).
[d]NA, not applicable.

RESULTS

MetAP1$_{Lm}$ inhibitors have potent antileishmanial activities and nontoxic effects in intraperitoneal murine macrophages. The efficacy of theMetAP1 inhibitors tested in this study has been previously demonstrated against the two MetAP1 proteins from *M. tuberculosis* through a high-throughput screening assay (22, 33). Thus, to identify new MetAP inhibitors for the potential treatment of CL, we tested eight MetAP1 inhibitors (OJT001, OJT002, OJT003, OJT004, OJT005, OJT006, OJT007, and OJT008) (Table 1 and Fig. S2A) to determine their effectiveness against the promastigote form of *L. major*. First, parasites were incubated with each of the eight inhibitors (OJT001 to OJT008) for 24 or 48 h. The most potent antileishmanial agents found were OJT006, OJT007, and OJT008, exhibiting low 50% effective concentrations (EC_{50}) of 780 nM, 500 nM, and 500 nM, respectively, after only 24 h of incubation (Fig. S2C). Interestingly, after 48 and 72 h of incubation, their antiparasitic effects increased slightly (Table 1 and Fig. S2B and D). Next, the cytotoxic effects of MetAP1$_{Lm}$ inhibitors (OJT006, OJT007, and OJT008) were determined by the addition of alamarBlue to intraperitoneal macrophages (IPΦ) after 24 or 48 h of treatment. None of the three inhibitors displayed cytotoxicity against IPΦ at concentrations up to 20 μM (Table 1 and Fig. S2E and F). Importantly, complete inhibition of extracellular promastigotes of an *L. major* strain expressing firefly luciferase (*L. major-luc*) was detected at a low concentration of 3.12 μM. Therefore, a wide window of selectivity (the selectivity indices [SI] were 131.6, 107.05, and 617.08 for OJT006, OJT007, and OJT008, respectively) between parasite and mammalian cell was observed (Table 1).

MetAP1$_{Lm}$ inhibitors reduce the proliferation of *L. major* intracellular amastigotes. The most potent MetAP1$_{Lm}$ inhibitors (OJT006, OJT007, and OJT008) were chosen to further study their effects against intracellular amastigotes proliferated inside IPΦ. Since we are interested in the potential antiproliferative properties of these inhibitors, we first incubated *L. major-luc*-infected BALB/c IPΦ for 48 h with OJT006, OJT007, or OJT008 treatment. We observed that at a concentration of 0.312 μM, OJT006, OJT007, and OJT008 were able to inhibit the proliferation of intracellular amastigotes by approximately 80%, 90%, and 85%, respectively (Fig. 1). Taken together, these results indicated that OJT006, OJT007, and OJT008 have high antileishmanial effects in both the extracellular and intracellular forms of the parasite with no cytotoxicity to mammalian cells. Thus, OJT006, OJT007, and OJT008 were further selected for evaluation in a preclinical *in vivo* model of CL. The assay Z factor was 0.5, indicating this is a satisfactory assay.

MetAP1$_{Lm}$ inhibitors act on target. To determine whether our three lead candidates were specific against MetAP1$_{Lm}$, we first treated 1×10^6 transgenic promastigotes/ml (*LucMetAP1$_{Lm}$/p1RlHYG*, a transgenic parasite that simultaneously expresses luciferase and overexpresses MetAP1$_{Lm}$) or wild-type parasites for 96 h with OJT006,

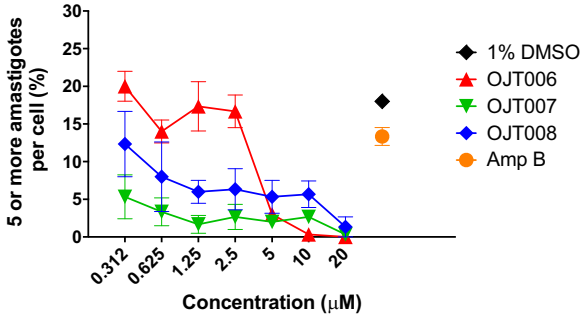


FIG 1 MetAP1$_{Lm}$ inhibitors OJT006, OJT007, and OJT008 reduced the intracellular proliferation of *L. major* amastigotes. Data from high-content imaging assay (HCIA) analysis of intraperitoneal mouse macrophages (IPΦ) infected with *L. major-luc* metacyclic promastigotes and treated with OJT006, OJT007, or OJT008 inhibitor from 0.312 μM to 20 μM for 48 h are shown. Controls were treated with 1% DMSO (drug diluent control) or amphotericin B (Amp B) at 5 μM (reference drug; positive control). Data are represented as the percentages (%) of infected IPΦ with 5 or more amastigotes per cell. Error bars indicate standard errors of the means (SEM).

OJT007, or OJT008. As expected, the antileishmanial activity of amphotericin B (reference drug; control) was not altered in the transfected *LucMetAP1$_{Lm}$/p1RlHYG* parasites (Fig. 2B). In contrast, increases of more than 10-fold were observed in the EC$_{50}$ values of OJT006, OJT007, and OJT008 when tested against transfected LucMetAP1$_{Lm}$ promastigotes compared to the values for treatment of wild-type *L. major-luc* (Fig. 2A and B). These data strongly suggest that OJT006, OJT007, and OJT008 successfully inhibited MetAP1$_{Lm}$, acting on target.

Potent *in vivo* activity of inhibitor OJT008 against *L. major* infection. The *in vivo* activities of MetAP1$_{Lm}$ inhibitors OJT006, OJT007, and OJT008 were characterized in *L. major-luc*-infected BALB/c mice. First, we evaluated the oral drug administration of different formulations by assessing their antiparasitic activities and potential toxicity in mice. Mice treated with a formulation in 70% deionized (DI) water–30% polyethylene glycol 400 (PEG 400) showed it to be well tolerated, with no weight loss observed, maintaining the antiparasitic activity of OJT006, OJT007, or OJT008 (Fig. S3A and B). Therefore, this formulation was selected for subsequent experiments. Next, BALB/c mice ($n = 5$) were infected, and after 18 days postinfection (dpi), mice were orally treated at 20 mg/kg of body weight/day with OJT006, OJT007, or OJT008. After 13 consecutive days of treatment, inhibitors OJT006 and OJT007 were shown to have lower efficacies than OJT008. However, all showed decreases in the lesion sizes in treated mice compared to the effect of the placebo control (Fig. S4). Nevertheless, small lesion sizes were observed through the course of the infection in OJT008-treated mice (Fig. S4). To further study and corroborate the efficacy of OJT008 in the preclinical

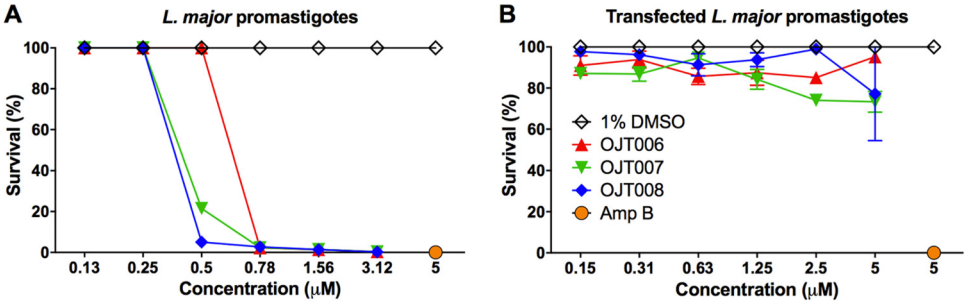


FIG 2 Action of MetAP1$_{Lm}$ inhibitors OJT006, OJT007, and OJT008 is on target. (A) Data from viability assay of *L. major-luc* promastigotes (wild type) treated with inhibitor OJT006, OJT007, or OJT008 for 96 h in a concentration range of 0.13 μM to 3.12 μM are shown. (B) Data from viability assay of transfected (*LucMetAP1$_{Lm}$/p1RlHYG*) *L. major* promastigotes treated with the OJT006, OJT007, or OJT008 inhibitors in a concentration range of 0.13 μM to 3.12 μM for 96 h are shown. Controls were treated with 1% DMSO (diluent drug control) or amphotericin B (Amp B) at 5 μM (reference drug). Error bars indicate SEM.

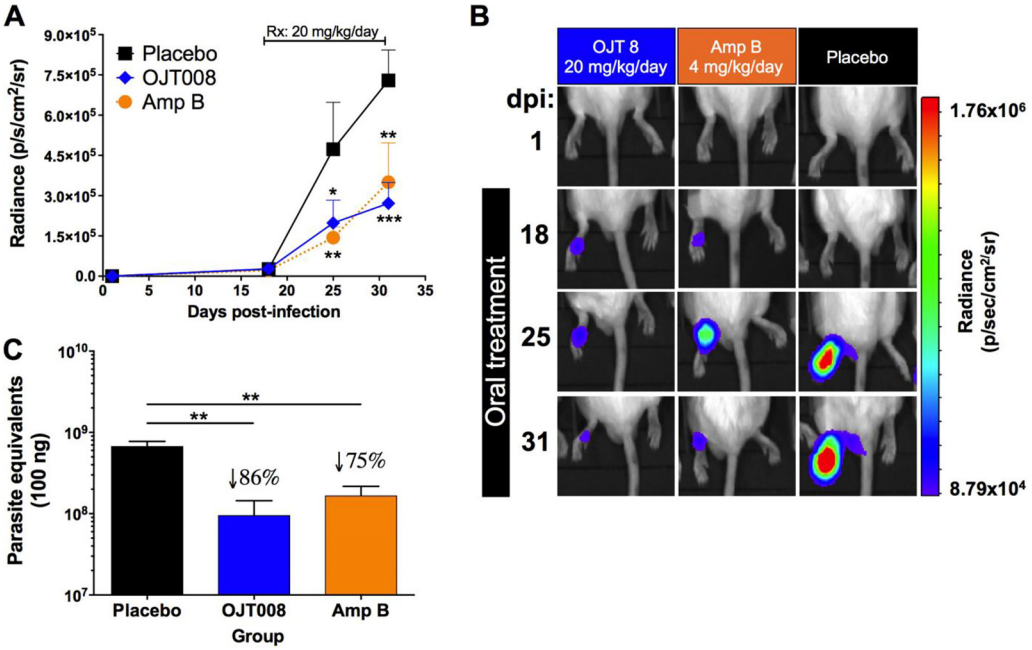


FIG 3 Oral treatment with OJT008 significantly reduced the parasitic burden caused by *L. major* infection. (A) Quantification of parasite bioluminescence emitted in BALB/c mouse footpads infected with *L. major-luc* metacyclic promastigotes and treated with 20 mg/kg/day of OJT008, 4 mg/kg/day of amphotericin B (Amp B; reference drug group), or placebo (PBS; control group). Two-way ANOVA with Dunnett's multiple-comparison test (compared to placebo group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$. Error bars indicate SEM. (B) Representative images of *in vivo* bioluminescence acquired at 1, 18, 25, and 31 dpi from *L. major-luc*-infected BALB/c mice treated with OJT008, Amp B, or placebo. (C) Quantification of parasitic load (parasite equivalents/100 ng) by qPCR at experimental endpoint (31 dpi). One-way ANOVA (compared to placebo; control group). **, $P < 0.01$. Error bars indicate SEM.

model, we decided to follow the infection during the course of treatment, using *in vivo* bioluminescence imaging. Thus, BALB/c mice ($n = 5$) were infected and treated using the same conditions as before, and images were acquired at 18, 25, and 31 dpi (Fig. 3A and B). Similarly to the results for amphotericin B (reference drug), OJT008 significantly ($P < 0.0001$) decreased the parasite's bioluminescence signal (Fig. 3A and B). Furthermore, quantitative PCR (qPCR) was performed to analyze the parasite burden of mice treated with OJT008. As expected, compared to the parasite loads in the placebo group, OJT008-treated mice had a significant ($P < 0.01$) reduction in parasite load, by 86% (Fig. 3B). Taken together, these findings suggest that OJT008 successfully reduced and controlled *L. major* infection in a preclinical murine model of CL, representing the therapeutic potential of the inhibitor.

The OJT008 inhibitor is nontoxic in a murine model of CL. Elevated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes are recognized as markers for cardiac and hepatic damage, respectively (34). As observed in the experiments whose results are shown in Fig. 4A and B, serum AST and ALT levels of mice treated with OJT008 were not elevated and were similar to those in the placebo group, indicative of drug safety. These results were further supported by the observation that the mouse weights in the OJT008-treated group were not statistically different from the weights in the placebo-treated control group (Fig. 4C). Additionally, OJT008 caused no changes in the behavior, appetite, waste elimination, appearance, or survival of treated mice compared to these parameters in placebo- and amphotericin B-treated animals (Fig. 4C). These results demonstrate the oral safety of the $MetAP1_{Lm}$ inhibitor OJT008 in a preclinical murine model of CL.

DISCUSSION

Despite the advances in understanding the protozoan parasite *L. major*, CL continues to cause significant morbidity. The drugs available to treat this disease (i.e.,

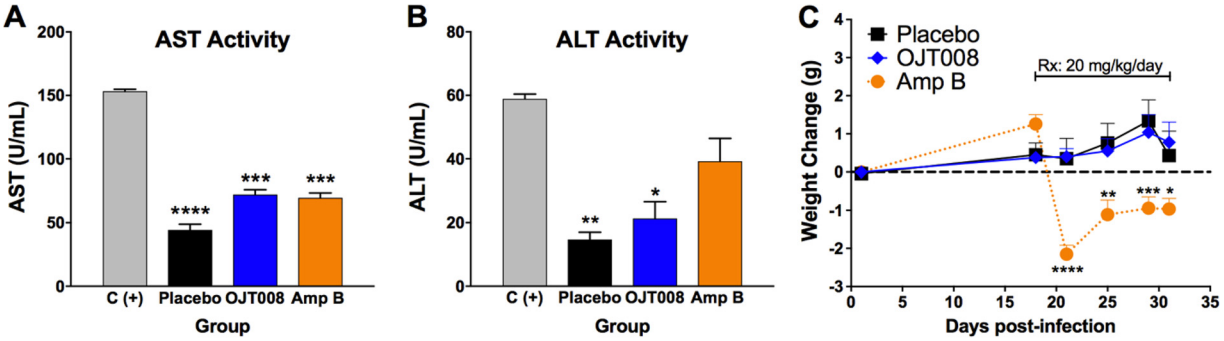


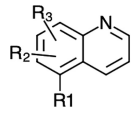
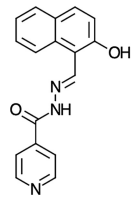
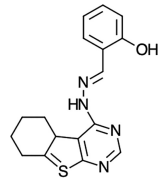
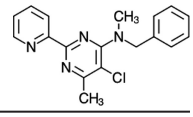
FIG 4 OJT008 is nontoxic *in vivo*. (A and B) Evaluation of systemic toxicity by serum levels of alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) in *L. major-luc*-infected BALB/c mice dosed with 20 mg/kg/day of OJT008, 4 mg/kg/day of Amp B, or placebo (PBS). Pooled serum samples were collected at 31 dpi (endpoint). Positive control [C (+)] was provided by the kit's manufacturer (Sigma-Aldrich). Data are represented as units/ml (U/ml). Ordinary one-way ANOVA with Dunnett's multiple-comparison test (compared to positive control). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (C) Assessment of treatment toxicity by weight change (grams) in *L. major-luc*-infected BALB/c mice treated with 20 mg/kg/day of OJT008, Amp B, or placebo (PBS). Two-way ANOVA with Dunnett's multiple-comparison test (compared to PBS group). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Error bars indicate SEM.

pentavalent antimonials and amphotericin B) are aged, limited in efficacy, and present severe side effects, and drug resistance continues to be reported. Consequently, there is an urgent need for new chemotherapeutic approaches to treat CL (35). Herein, we present data that demonstrate the potential success of novel MetAP1 inhibitors as chemotherapeutic agents against *L. major* infection. MetAP1 is a metalloprotease that removes the N-terminal methionine from proteins and peptides, a process involved in the highly conserved N-terminal methionine excision (NME) pathway (17). Since NME is an essential process in both prokaryotes and eukaryotes (19, 36), inhibitors of MetAP have been suggested as novel chemotherapeutic agents against different forms of cancer and bacterial, fungal, and parasitic infections (22, 24–29, 33, 37). Moreover, it has been reported that deletion of MetAP1 in yeast and other eukaryotic cells is detrimental and leads to cell death (32, 38, 39). Nonetheless, despite the obvious importance of this metalloprotease in *L. major*, insufficient effort has been taken in exploiting MetAP1 as a drug target for CL.

A screening of 175,000 diverse small molecules conducted by Olaleye et al. (22) led to the discovery of eight potent MetAP1 inhibitors (OJT001 to OJT008). The eight MetAP inhibitors tested belong to four structurally diverse classes of small-molecule compounds affiliated with four structurally distinct chemical classes. Compounds OJT001 to OJT005 are five analogues belonging to the 8-hydroxyquinoline chemical class and are structurally related analogues with the same pharmacophore (26), while compounds OJT006, OJT007, and OJT008 are all structurally different, with diverse pharmacophore classes. OJT006 is a pyridoxal isonicotinoyl compound, OJT007 has the hydrazine-1-ylidene-containing pharmacophore, and OJT008 has the pyrimidin-4-amine pharmacore (Table 2). Treatment of *L. major* promastigotes and intracellular amastigotes with inhibitors OJT001 to OJT008 revealed three potent $MetAP1_{Lm}$ inhibitors, OJT006, OJT007, and OJT008, with EC_{50}s in the low range of 0.243 μM to 0.640 μM. Interestingly, although the first five hydroxyquinoline compounds (OJT001 to OJT005), with similar pharmacophores, have been reported to have potent activity against *M. tuberculosis* MetAP1 and/or antimycobacterial activity (26), they were not potent against *L. major* promastigotes, while compounds OJT006, OJT007, and OJT008, with three different novel pharmacophores, showed potent activity against *L. major* promastigotes. These observations suggest the enzyme specificity and selective toxicity of the MetAP inhibitors.

In addition, we have demonstrated that the antiparasitic activity observed for the inhibitors is due to a specific on-target effect by overexpressing MetAP1. We observed a 10-fold increase in resistance to the antiparasitic activity of the compounds compared to the drug resistance of wild-type *L. major-luc*. Therefore, we can conclude that since

TABLE 2 Structural classes of methionine aminopeptidase inhibitors

MetAP inhibitor class	Core structure
I. 8-Hydroxyquinolines	
II. Pyridoxal isonicotinoyls	
III. Hydrazin-1-ylidenes	
IV. Pyrimidin-4-amine	

there is an excess of MetAP1 enzyme on the transgenic parasites, a higher concentration of the drugs is required to achieve a similar antiparasitic effect. Similarly, these effects were previously described in an *M. tuberculosis* model by Olaleye et al. (22). These data provide evidence that the OJT006, OJT007, and OJT008 compounds specifically inhibit MetAP1 from *L. major*.

Drug accessibility and parenteral administration are two of the main reasons for treatment interruption for leishmaniasis (13, 14). Several reports show that patients with conditions ranging from cancer to autoimmune and infectious diseases have an inclination toward oral chemotherapy administration rather than intravenous administration (40–42). Here, we present evidence of potent oral efficacy of MetAP1 inhibitor OJT008 in a preclinical mouse model of CL. OJT008 significantly decreased the parasite load, by 86%, as shown by bioluminescence assay and qPCR. More importantly, OJT008 did not generate adverse or toxic effects in treated infected BALB/c mice, as observed by the low systemic levels of AST and ALT that were measured. Furthermore, these data correlated with no significant weight loss and no behavior changes during the course of treatment. Given these findings, we propose the $MetAP1_{Lm}$ inhibitor OJT008 for further preclinical studies as a novel chemotherapy agent, representing an excellent candidate for the oral treatment of CL.

To summarize, in the present study, we identified and characterized $MetAP1_{Lm}$ as a target for the development of novel antileishmanial drugs. We have discovered three (OJT006, OJT007, and OJT008) novel small-molecule inhibitors of $MetAP1_{Lm}$ with diverse pharmacophores for potential development of agents for CL treatment. This is the first report of a new pharmacophore targeting *L. major*-specific MetAP1 ($MetAP1_{Lm}$), in inhibitor OJT008, with significant antileishmanial activity *in vitro* and *in vivo*. Further delivery experiments are planned, seeking to improve the antileishmanial activity of OJT008. Our discovery of three new pharmacophores as potent $MetAP1_{Lm}$ inhibitors makes these pharmacophores and the $MetAP1_{Lm}$ target an attractive combination for further optimization. In addition, structure-activity relationships and X-ray

crystallography structure studies will accelerate the rational design and synthesis of more potent MetAP1$_{Lm}$ inhibitors. Furthermore, these inhibitors could be used as chemical probes or tools in the future to better understand the physiologic relevance of MetAP1$_{Lm}$ in N-terminal methionine excision, as well as the essentiality and substrate specificity of this class of enzymes in *L. major*.

MATERIALS AND METHODS

Animals and ethics statement. BALB/c mice aged 6 to 8 weeks were bred and maintained in a pathogen-free animal biosafety level 2 (ABSL-2) facility at the Laboratory Animal Resources Center (LARC) at The University of Texas at El Paso (UTEP). All animal studies and procedures were performed so as to minimize the distress and pain for the animals in accordance with the NIH guidance and animal protocol A-201107-1, approved by UTEP's Institutional Animal Care and Use Committee (IACUC).

Culture of *Leishmania major*. *L. major-luc* Friedlin clone V1 promastigotes expressing firefly luciferase Lmj-FV1-LUC-TK (*L. major* strain Friedlin [MHOM/JL/80/Friedlin]) were cultured at 28°C in M199 medium (Sigma-Aldrich) supplemented with hemin, 10% heat-inactivated fetal bovine serum (iFBS; Gibco), 1% 10,000 U/ml penicillin, 10 mg/ml streptomycin (Gibco) and treated with 50 ng/ml of streptothricin neosulfate (GoldBio) for maintenance of the luciferase (*luc*) gene.

Culture of mammalian cells. Starch-induced intraperitoneal BALB/c mouse macrophages (IPΦ) were obtained as described previously (43) and cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% iFBS (Gibco), 1% 10,000 U/ml penicillin, and 10 mg/ml streptomycin (Gibco).

MetAP1$_{Lm}$ inhibitor formulations. The MetAP1$_{Lm}$ inhibitors were synthesized and three oral formulations were developed by the Olaleye group at Texas Southern University, Houston, TX. However, two of the oral formulations were toxic for the *in vivo* experiments: formulation one, which consisted of 23% PEG-400, 75% glycerin, 0.05% cremophor EL (all from Sigma-Aldrich), and 0.5% Labrasol (Gattefosse), and formulation two, which consisted of 33.3% capryol 90 (Sigma-Aldrich), 33.3% cremophor EL, and 33.3% Labrasol. Therefore, for *in vivo* experiments, inhibitors OJT006, OJT007, and OJT008 were dissolved in a nontoxic oral formulation of 70% deionized (DI) water and 30% PEG-400. Stock solutions were dissolved in pure dimethyl sulfoxide (DMSO) at a concentration of 1 mM for *in vitro* studies.

Luciferase viability assay. MetAP1$_{Lm}$ inhibitors OJT001, OJT002, OJT003, OJT004, OJT005, OJT006, OJT007, and OJT008 were screened against *L. major-luc* promastigotes. First, parasites at 1×10^6/ml were added to 96-well, white, flat-bottom Nunc plates (Thermo Fisher Scientific) together with the inhibitors in a final concentration range from 0.78 μM to 100 μM, in triplicates, followed by 96 h of incubation at 28°C. Amphotericin B (Sigma-Aldrich) was used at 5 μM as the drug of reference. The efficacies of OJT006, OJT007, and OJT008 were further evaluated. The efficacies of the compounds were assessed by monitoring parasite survival by luciferase activity. The substrate 5′-fluoroluciferin (ONE-Glo luciferase assay system; Promega) was added according to the manufacturer's protocol, and the signal read in a luminometer (Luminoskan; Thermo Fisher Scientific).

alamarBlue assay of mammalian cell cytotoxicity. The cytotoxicity of OJT006, OJT007, and OJT008 was evaluated using BALB/c mouse IPΦ. First, IPΦ were harvested and seeded at a density of 1×10^6/ml, followed by 8 h of incubation. Next, cells were washed, compounds added at an initial concentration of 1 mM, and cells serially diluted and incubated for an additional 24 or 48 h at 37°C, 5% CO_2. The cytotoxicity of the compounds was determined by the addition of alamarBlue (Invitrogen) following the manufacturer's recommendations. Plates were read using a fluorometer (Flouroskan; Thermo Fisher Scientific). The drugs were tested in triplicates, and three independent experiments were performed.

***In vitro* evaluation of MetAP1$_{Lm}$ inhibitors by high-content imaging assay.** Intraperitoneal mouse macrophages were seeded in a BD Falcon 96-well, clear-bottom, black imaging plate and infected with *L. major-luc* metacyclic promastigotes (44) in a ratio of 10 parasites per macrophage, followed by 24 h of incubation at 37°C, 5% CO_2. The cells were then washed twice and treated for 48 h with MetAP1$_{Lm}$ inhibitors (OJT006, OJT007, and OJT008). Each drug was tested in triplicate. To determine the quality of the assay, 10 replicates of each control, 1% DMSO and amphotericin B, were carried out to calculate the Z factor. Three independent experiments were performed. The procedure was performed as previously described (45). BD Pathway Bioimager 855 was used to determine the percentage of infected cells containing at least 5 intracellular parasites.

Homologous overexpression of MetAP1$_{Lm}$. The MetAP1$_{Lm}$ gene was amplified from *L. major* genomic DNA using the oligonucleotides MetAP1$_{Lm}$-XbaI sense (5′-TCTAGAGGATCCATGCCCTGCGAAG GCTGCGGC-3′) and MetAP1$_{Lm}$-XbaI antisense (5′-TCTAGAGAATTCTCAGATTTTGATTTCGCTGGGGTCTTCG G-3′). PCR was performed using PCR master mix (Promega), 420 ng of *L. major* genomic DNA, and MetAP1$_{Lm}$ sense and antisense primers under conditions of denaturation of 5 min at 95°C, followed by 40 cycles of 60 s at 95°C, 60 s at 68°C, and 90 s at 72°C, and a final 5-min elongation period at 72°C. The PCR product was purified using the Wizard SV gel and PCR clean-up system (Promega). The amplified MetAP1$_{Lm}$ gene was then cloned into the XbaI restriction site of the *Leishmania* expression vector *p1RlHYG*. The *plR1HYG* expression vector was kindly provided by Stephen M. Beverley at Washington University, St. Louis, MO. The identification of the clone *MetAP1$_{Lm}$/p1RlHYG* was confirmed by DNA sequencing (DNA Analysis Core Facility, Border Biomedical Research Center, El Paso, TX). *L. major-luc* promastigotes were transfected with 25 μg of *MetAP1$_{Lm}$/p1RlHYG*. The transfected (*LucMetAP1$_{Lm}$/p1RlHYG*) parasites were plated in M199 medium, 0.0005% hemin, 10% iFBS (Gibco), 50 ng/ml streptothricin (GoldBio), 1% agarose, and incubated at 28°C. After 10 days, parasite colonies were observed, and an individual colony (clone of parasites) was grown in liquid medium supplemented

with 16 μg/ml hygromycin. *L. major LucMetAP1$_{Lm}$/p1RlHYG* transgenic parasites were used to confirm that the activity of MetAP1$_{Lm}$ inhibitors (OJT006, OJT007, and OJT008) was on target by a luciferase viability assay.

Luciferase assay of *Leishmania major* overexpressing MetAP1$_{Lm}$. The inhibitors OJT006, OJT007, and OJT008 were screened in parallel with 1×10^6/ml *L. major LucMetAP1$_{Lm}$/p1RlHYG* or wild-type *L. major-luc* promastigotes for 96 h at 28°C. The assay was performed using the same conditions described above for the luciferase viability assay.

***In vivo* antiparasitic activity of MetAP1$_{Lm}$ inhibitors.** Male BALB/c mice (6 to 8 weeks old) were injected in the left hind footpad with 50 μl of *L. major-luc* metacyclic promastigotes in DMEM (1×10^6/ml) after purification by Ficoll step gradient as previously described (44). After 18 days postinfection (dpi), animals were treated orally with 20 mg/kg/day (100 μl/day) of OJT006, OJT007, or OJT008 or 4 mg/kg/day intraperitoneally of reference drug amphotericin B (Sigma-Aldrich) for a total of 13 days ($n = 5$ mice per group). Infection was monitored by footpad lesion measurements using a digital caliper or by bioluminescence imaging in an IVIS Lumina III *in vivo* imaging system (Perkin Elmer). Bioluminescence images were acquired at 18, 25, and 31 dpi after administration of 200 μl of 150 mg kg^{-1} D-luciferin in phosphate-buffered saline (PBS; Gold Biotechnology) as previously described (46). After D-luciferin injection, mice were kept conscious for 10 min to allow luciferin to be metabolized and circulate and then anesthetized with 2.5% gaseous isoflurane and imaged after 5 additional minutes. Luminescence data were analyzed using Living Image software (Perkin Elmer). Quantification of bioluminescence per footpad is represented as radiance (photons per second per square centimeter per steradian [p/s/cm^2/sr]).

Parasite load by quantitative PCR. At the experimental endpoint, mice were euthanized by CO_2 overdose and the infected footpads were harvested from all groups. Genomic DNA was extracted from 20 to 30 mg of tissue using the high pure PCR template preparation kit (Roche), following the manufacture's protocol. Parasite footpad burden was determined by absolute quantification based on a standard DNA curve ranging from 0.5 to 10^5 *L. major* parasite equivalents/ml. A standard curve was produced by extracting DNA from a 20- to 30-mg tissue fragment spiked with 10^5 *L. major* promastigotes. Amplification of a 120-bp fragment from kinetoplastic DNA was performed using 100 nM forward primer (5′-CTTTTCTGGTCCTCCGGGTAGG-3), 100 nM reverse primer (5′-CCACCCGGCCCTATTTTACACCAA-3′), and *Taq*Man probe (5′-FAM-TTTCGCAGAACGCCCCTACCCGC-TAMRA-3′) (47). As an internal control, a linearized pUC57 plasmid containing a sequence from *Arabidopsis thaliana* was spiked before all DNA extractions as previously described (48). *Taq*Man chemistry allowed a 2-step temperature cycle. PCR conditions were set at 50°C for 2 min, 94°C for 10 min, followed by 45 cycles at 94°C for 15 s and 55°C for 1 min (47). Samples were run in triplicate in the StepOnePlus real-time PCR System (Applied Biosystems), and parasite equivalents per 100 ng were plotted. All the conditions were followed as previously described (49).

Toxicity monitoring and assessment. Treatment toxicity was evaluated by monitoring mouse weight changes periodically. Weight changes (grams) were normalized by subtracting from the mouse's initial weight. Moreover, blood was collected by cardiac puncture at the endpoint and serum obtained by centrifugation at 2,000 rpm for 10 min. The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes in OJT008-treated mice were measured according to the manufacturer's recommendations (ALT or AST activity kit; Sigma-Aldrich).

Statistical analysis. All data were analyzed and plotted using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA). The median lethal dosage (LD_{50}), half-maximal cytotoxic concentration (CC_{50}), and half-maximal effective concentration (EC_{50}) were calculated. Ordinary one-way analysis of variance (ANOVA) or two-way ANOVA was employed in the statistical analysis. Values were considered significant when P was <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

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