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The New Pyrazolyltetrazole Derivative MSN20 Is Effective via Oral Delivery against Cutaneous Leishmaniasis

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An orally delivered, safe and effective treatment for leishmaniasis is an unmet medical need. Azoles and the pyrazolylpyrimidine allopurinol present leishmanicidal activity, but their clinical efficacies are variable. Here, we describe the activity of the new pyrazolyltetrazole hybrid, 5-[5-amino-1-(4′-methoxyphenyl)1H-pyrazole-4-yl]1H-tetrazole (MSN20). MSN20 showed a 50% inhibitory concentration (IC_{50}) of 22.3 μM against amastigotes of *Leishmania amazonensis* and reduced significantly the parasite load in infected mice, suggesting its utility as a lead compound for the development of an oral treatment for leishmaniasis.

Leishmaniasis is a zoonosis that is naturally transmitted between vertebrate animals and humans through an invertebrate vector (the sandfly). The parasitic infections are endemic in 98 countries, with a prevalence of 12 million cases worldwide and 2 million new cases occurring each year (1, 2). Since their first use as a trivalent salt formulation in 1912 (3, 4) and their later use as pentavalent complexes since the 1940s (5), antimonials have saved thousands of lives due to their being a first-line treatment for leishmaniasis. Unfortunately, however, antimonials have a very adverse toxicological profile (6, 7, 8) and have not been approved for clinical use by several national health agencies, including the U.S. FDA. Furthermore, antimonials can be delivered only by parenteral routes, and decades of their use have led to the development of parasite resistance in certain regions, especially in India and Sudan (9, 10). Lipid formulations of amphotericin B and miltefosine are alternatives that have emerged in recent years, but there are concerns about the costs of production, teratogenicity, and the development of resistance (11, 12, 13). Cyclic compounds containing nitrogen have been used as antileishmanial drugs in some instances. Azole compounds, such as ketoconazole and miconazole, exhibit antileishmanial activity *in vitro*, but the efficacies of azoles in clinical trials have been variable (7). Allopurinol, a pyrazolylpyrimidine, has been used as an antileishmanial drug since the 1970s, and it is employed as an alternative to antimonials in specific cases. In previous studies, we planned, synthesized, and evaluated the antileishmanial activities of a series of pyrazolyltetrazole hybrids with halogen substitutions in the phenyl ring (14). In the present study, following a rational drug design approach, we replaced the halogens with an electron-donating substituent (OMe), yielding 5-[5-amino-1-(4′-methoxyphenyl) 1H-pyrazole-4-yl]1H-tetrazole (MSN20), which exhibits improved antileishmanial activity and is orally bioavailable in mice, as described here.

For 50% inhibitory concentration (IC_{50}) determination on infective metacyclic promastigotes, *Leishmania amazonensis* cultured for 3 days (1×10^7/ml) was incubated with MSN20 (12.5 to 200 μM) for 24 h at 26°C in Schneider's insect medium. The leishmanicidal activity was assessed by adding 500 μg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich) or by counting in a Neubauer chamber, with both techniques yielding similar results.

The previously evaluated pyrazolyltetrazoles exhibited IC_{50}s against metacyclic promastigotes of *L. amazonensis* that ranged from 75 μM to greater than 800 μM (14). In a new effort to improve the leishmanicidal activity, we introduced a methoxy group in the pyrazolyltetrazole core, generating the compound MSN20 (Fig. 1, inset). This modification enhanced the antipromastigote activity to 37.1 μM.

We further evaluated whether MSN20 was able to reach the amastigotes within the parasitophorous vacuole without affecting the host cell. Resident peritoneal macrophages were plated in RPMI medium (Sigma-Aldrich) at 2×10^6/ml in Lab-Tek eight-chamber slides (Nunc, Roskilde, Denmark) and incubated at 37°C in 5% CO_2 for 1 h. Adherent cells were then incubated with *L. amazonensis* promastigotes at a parasite/macrophage ratio of 3:1 for 4 h. After incubation, MSN20 (0 to 200 μM) was added to the cultures for 72 h. Next, the slides were stained using a hematology staining kit (Instant Prov; Newprov, Curitiba, Brazil). The results were expressed as the infection index (percentage of infected cells multiplied by the number of amastigotes, divided by the total number of macrophages).

MSN20 was more potent, with an IC_{50} of 22.3 μM, against intracellular amastigotes, promoting a concentration-dependent reduction of parasite load (Fig. 1). No morphological alterations were observed in the infected macrophages that had been treated for 72 h with MSN20 at concentrations up to 200 μM (data not shown). To specifically assess the cytotoxicity of the drug, resident peritoneal macrophages were plated (2×10^6/ml) and incubated with different concentrations of MSN20 at 37°C under 5% CO_2. After 72 h of incubation, cell viability was estimated by measuring

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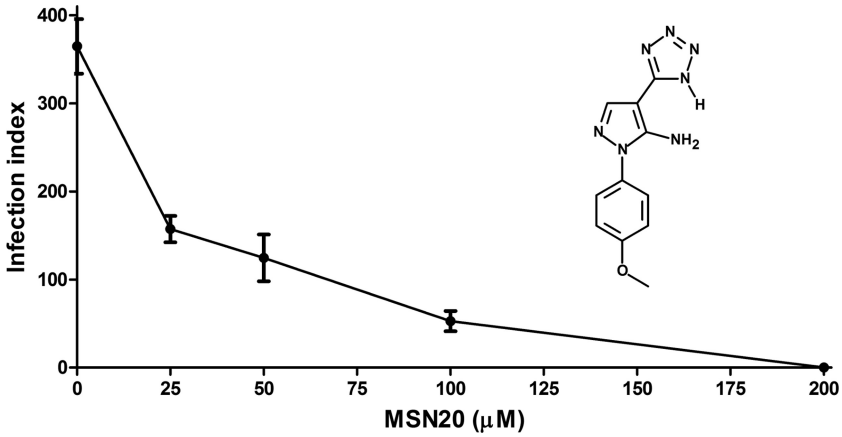

FIG 1 Structure and activity of MSN20 against intracellular amastigote infection. Murine peritoneal macrophages were infected with *L. amazonensis* and incubated with MSN20 concentrations that ranged from 0 to 200 μM.

the reduction of MTT. MSN20 presented a 50% lethal dose (LD_{50}) of 210.6 μM and a selectivity index (SI) of 9.4 (SI = LD_{50}/IC_{50} for intracellular amastigote), which means that the drug causes host cell cytotoxicity at a concentration that is 9 times higher than that required to elicit a therapeutic effect.

In accordance with laboratory animal welfare policies, we analyzed the theoretical biological effects of MSN20 before proceeding to *in vivo* tests. The absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties of MSN20 were evaluated using the admetSAR tool (15), and Lipinski's rule of five was calculated using Advanced Chemistry Development (ACD/Labs) software version 11.02. MSN20 satisfied Lipinski's rule of five and presented a probability greater than 99% of human intestinal absorption, suggesting a good chance of druggability and oral bioavailability (Table 1). The Ames test predicted a medium mutagenic potential probability of 63%, but the simulation for carcinogenesis showed an 88% probability that MSN20 was noncarcinogenic. We also noted that MSN20 was not likely to act as an inhibitor of CYP3A4, unlike other azoles, such as ketoconazole.

Considering the *in vitro* and *in silico* results, we evaluated the activity of MSN20 in a murine model of cutaneous leishmaniasis using oral administration. BALB/c mice ($n = 5$ per group) were infected in the ear with 2×10^6 *L. amazonensis* promastigotes. After 72 h of infection, MSN20 was administered orally at 30 mg/kg body weight/day for 10 days without interruption and then five times per week, until the 48th day postinfection. Control animals were intraperitoneally injected with meglumine antimoniate (60 mg Sb^{5+}/kg/day) without interruption for 46 days. Negative controls were treated orally with vehicle alone (phosphate-buffered saline [PBS]-5% ethanol). On day 49, the animals were euthanized, and blood was collected for toxicological analysis. The parasite burden in the ears was evaluated by limiting dilution analysis. This study was approved by the Animal Ethics Committee of the Oswaldo Cruz Foundation (license number LW07/2010). Statistical analysis was performed by analysis of variance with a Bonferroni posttest. The effect of oral administration of MSN20 to BALB/c mice infected with *L. amazonensis* is shown in Fig. 2. MSN20 was effective in reducing both the development of lesions (Fig. 2A) and the parasite burden (Fig. 2A, inset) compared with those in the vehicle-treated control group. At the end of the treatment, serological markers of toxicity were evaluated, and no significant differences in serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, creatinine, uric acid, and urea were observed between the treated animals and the control group (Fig. 2B). All levels of the toxicity markers were within the reference values, suggesting the absence of liver and kidney damage.

Imidazoles and triazoles, such as ketoconazole and posaconazole, are potent inhibitors of the sterol C_{14}-demethylase (CYP51) of fungi and trypanosomatids. This inhibition is mediated by the

TABLE 1 Oral bioavailability, molecular properties, and predicted ADMET properties of MSN20[a]

Property	Result	Probability (%)
Absorption		
BBB	+	96.55
HIA	+	99.56
Caco-2	+	57.78
Metabolism		
CYP450 2C9 substrate	NS	77.29
CYP450 2D6 substrate	NS	85.11
CYP450 3A4 substrate	NS	59.47
CYP450 1A2 inhibitor	I	78.90
CYP450 2C9 inhibitor	NI	56.76
CYP450 2D6 inhibitor	NI	87.79
CYP450 2C19 inhibitor	I	54.48
CYP450 3A4 inhibitor	NI	89.02
Toxicity		
Ames toxicity	Ames toxic	63.56
Carcinogens	Noncarcinogenic	88.24
Lipinski molecular descriptor		
NHBA (≤10)	8	
NHBD (≤5)	3	
clogP (≤5)	2.30 ± 0.99	
MW (≤500)	257.251	

[a] BBB, blood-brain barrier; HIA, human intestinal absorption; Caco-2, human epithelial colorectal adenocarcinoma cells; I, inhibitor; NI, noninhibitor; NS, nonsubstrate; NHBA, number of hydrogen bond acceptors; NHBD, number of hydrogen bond donors; clogP, logarithm of the compound partition coefficient between *n*-octanol and water; MW, molecular weight; +, positive.

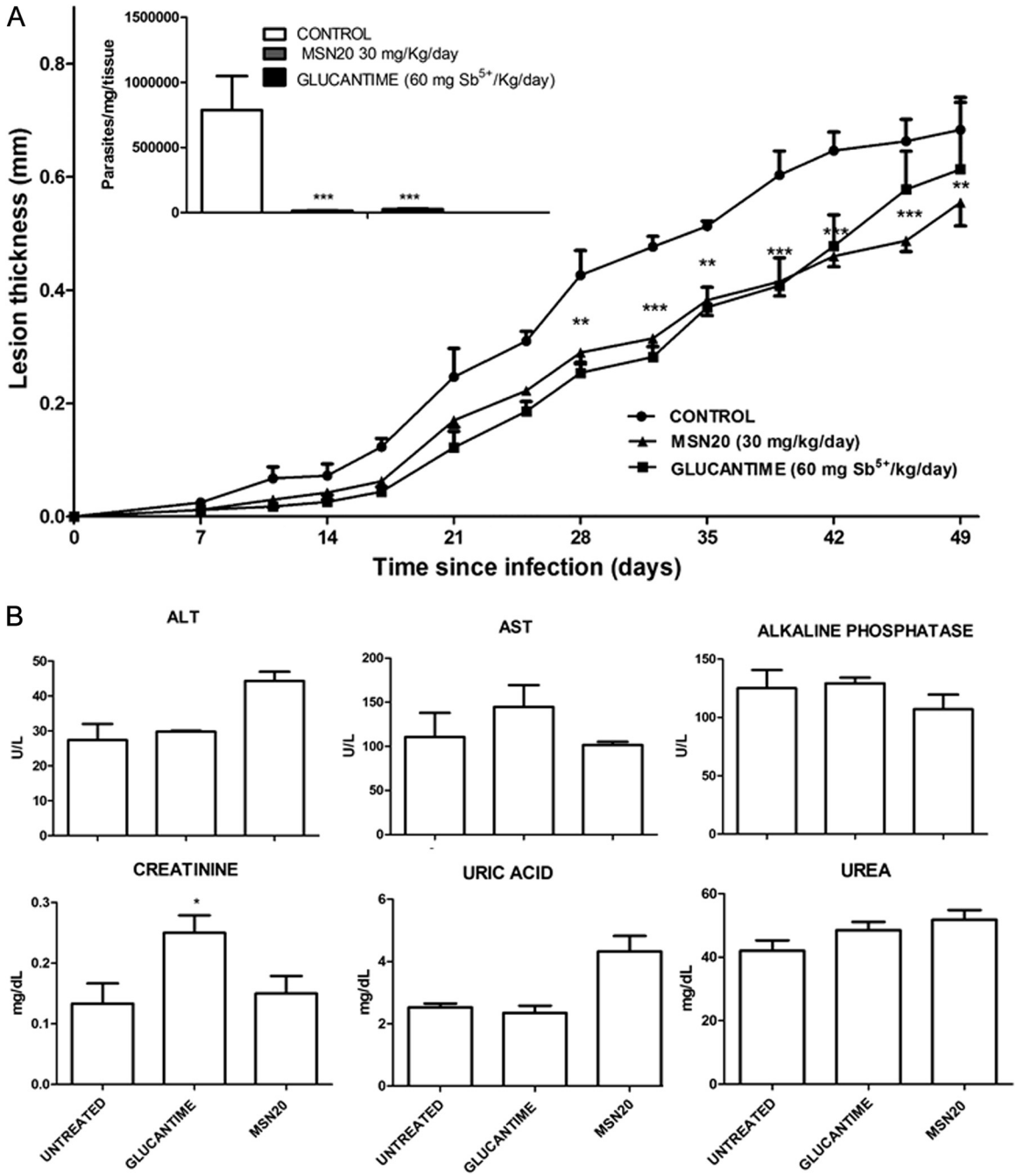

FIG 2 MSN20 is effective *in vivo*. (A) BALB/c mice were subcutaneously infected with 2×10^6 *L. amazonensis* promastigotes in the ear. The animals were treated orally with MSN20 (30 mg/kg/day) or intraperitoneally with meglumine antimoniate (60 mg Sb^{5+}/kg/day). Lesion development was measured with a dial caliper twice a week. At the end of the experiment, the mice were euthanized and the parasite burden was estimated by dilution analysis (inset). (B) After the treatment period, serum samples were collected for colorimetric determination of ALT, AST, alkaline phosphatase, creatinine, uric acid, and urea. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

azole basic nitrogen, which coordinates to the heme iron of the CYP51, interfering with the catalytic center (16). The IC_{50} of posaconazole for intracellular amastigotes of *L. amazonensis* has been demonstrated to be 1.63 μM (17), and we have established the IC_{50} for itraconazole to be ~0.05 μM (data not shown), in agreement with de Macedo-Silva et al. (17). Further studies are necessary to prove whether the mechanism of action of MSN20 is similar to that of the azole antifungals; in which case, modifications to the MSN20 scaffold could be made to improve the binding affinity for the parasite CYP51 enzyme.

The selective activity *in vitro*, the theoretical predictions of druggability, and the *in vivo* activity by oral administration suggested MSN20 as a good prototype to be considered a drug candidate for treating leishmaniasis.

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