Efficacy of the Herbicide Trifluralin against Four P-Glycoprotein-Expressing Strains of Leishmania

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Drug resistance has emerged as a major obstacle to chemotherapy for many infectious diseases. Trifluralin, an antimicrotubule herbicide, is a new experimental drug for treatment of leishmaniasis. Here, we found that it was effective against two strains of Leishmania that express the multidrug-resistant genes ldmdr1 and lmpgpA and two strains that express proteins that are immunologically cross-reactive with mammalian P glycoproteins. These results suggest that trifluralin is not subject to counteraction of these multidrug resistance mechanisms of Leishmania species.

Drug resistance has emerged as a major obstacle for chemotherapy for several infectious diseases. Leishmaniasis is a major tropical disease affecting 12 million people in the Third World and a possible cause of Gulf War Syndrome. The causative protozoan parasites, Leishmania species frequently acquire resistance to pentavalent antimonial agents, the recommended treatment since the Second World War (19, 26). In areas where leishmaniasis is endemic, primary resistance to Pentostam occurs in about 5 to 70% of the cases (14). Patients with tropical parasitic infections are often subjected to circumstances that lead to suboptimal treatment, a leading cause for the development of drug resistance. Therefore, it is important to consider the potential of drug resistance when developing new chemotherapy.

Trifluralin is a herbicide whose mechanisms against Leishmania species include binding to tubulins (1, 17, 18, 20). Experimentally, it inhibits promastigote proliferation and amastigote infectivity of all Leishmania species that were tested and, as a topical ointment, reduces the size of cutaneous lesions on BALB/c mice (4–9). Moreover, it is also effective against the in vitro proliferation of Trypanosoma brucei trypomastigotes, the amoeba-flagellate Naegleria fowleri, and the erythrocytic forms of the malaria parasite Plasmodium falciparum (7, 15, 21, 25).

The most frequently reported mechanism for Leishmania drug resistance is gene amplification, although other means, such as decrease in drug uptake and overexpression as a consequence of gene rearrangement, have also been observed (12, 22). The parasite either overproduces enzymes that the drugs normally inhibit or activates membrane proteins that prevent drug accumulation. A group of multidrug-resistant Leishmania major strains have an amplified H region (11). This genomic region contains at least two genes that are highly homologous to the mammalian P glycoprotein genes. In mammalian cells, P glycoproteins confer drug resistance by preventing the intracellular accumulation of hydrophobic drugs. In Leishmania species, their amplification has been associated with simultaneous resistance to many compounds (2, 10, 22, 23).

Since trifluralin is a hydrophobic compound, we investigated whether the Leishmania P glycoprotein may counteract the effects of trifluralin. We have compared the efficacy of the drug against four strains that express multidrug-resistant genes or P-glycoprotein molecules with that for the drug-sensitive parental strains. These strains were an L. major strain transfected with lmpgpA, an L. donovani strain with amplified ldmdr1, and L. mexicana amazonensis and L. bresilienis panamensis strains that had been induced to overexpress P-glycoprotein cross-reactive molecules.

Methodologically, the L. major and L. donovani strains were maintained in RPMI medium with 10% fetal calf serum. For the L. major transfectants, pSNAR and pSNAR-lmpgpA, 8 μg of genetin per ml was added. L. m. amazonensis and L. b. panamensis were maintained in LIT medium (24) containing 1 and 6 mg of Pentostam (sodium stibogluconate with 0.1% chlororesol; Wellcome, London, England), respectively. The parasites were washed to remove these compounds before use. Proliferation inhibition assays were performed as described by Chan et al. (9). Hybridoma medium (Hybri-Max; Sigma), with 1% dimethyl sulfoxide and 1% fetal calf serum, was used for L. major. LIT medium with 0.1% aceton was used for all the other parasites. Trifluralin was obtained from Dow Elanco (9).

For statistical analyses, the 50% effective dose (ED50) values were deduced from each individual experiment by linear regression of each datum point, and then the averages of the ED50 values from the different experimental repeats were determined and compared by the Student t test, using the program MyStat (Macintosh).

Efficacy against Leishmania ldmdr1-amplified parasites. Vinblastine is a microtubule inhibitor that has been widely used in the treatment of cancer; cancer cells may develop resistance by overexpressing P glycoproteins to prevent drug accumulation. Henderson et al. (16) developed a strain of L. donovani, VINB1000, which continues to proliferate in 1.0 mM vinblastine and 100 μM puromycin. This multidrug-resistant strain has an amplified gene, named ldmdr1, that is homologous to those of the mammalian P glycoproteins. Its drug resistance profile is comparable to that for mammalian P glycoproteins. It is resistant to puromycin, vinblastine, anthracyclines, daunomycin, doxorubicin, and ZnCl2. The only discrepancy is that the multidrug resistance phenotype of the Leishmania strains is not reversible by verapamil or desipramine.

When treated with trifluralin, the ED50 for L. donovani VINB1000, 3.8 ± 0.4 μM, was similar to that for its parental strain D1700, 4.0 ± 0.9 μM (Table 1). Thus, trifluralin was not susceptible to the action of Leishmania ldmdr1-encoded P-
TABLE 1. Leishmania P-glycoprotein homologs do not protect against trifluralin

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Strain</th>
<th>Gene or protein expressed</th>
<th>ED_{50} (μM)</th>
<th>SD</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. donovani</td>
<td>D1700</td>
<td>lmdr1</td>
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<tr>
<td></td>
<td>VNB1000</td>
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<td>3.8</td>
<td>0.4</td>
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<td>CC-1/pSNAR</td>
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<td></td>
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<tr>
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<tr>
<td></td>
<td>WR746R</td>
<td>P glycoprotein</td>
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<td>0.5</td>
<td>0.072</td>
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</table>

glycoprotein-like proteins. The parasites were checked for resistance to puromycin at the end of the series of trifluralin testing. Comparable to the titer originally reported by Henderson et al. (16), VNB1000 was resistant to 16 μM puromycin (Sigma) whereas proliferation of D1700 was completely inhibited, indicating that the Leishmania lmdr1 gene remained stably amplified, even though in our experiments the parasites were grown in the absence of puromycin.

**Efficacy against Leishmania lmpgpA-expressing parasites.** Callahan and Beverley (3) identified lmpgpA by hybridization to lppgs, which confers resistance to arsenite, in L. tarentolae. Then they generated a multidrug-resistant strain by transfecting L. major CC-1, an arsenite-susceptible strain, with the Leishmania expression vector, pSNAR, inserted with a 7-kb HindIII fragment which contained the lmpgpA gene. This transfectant is resistant to antimonal agents (including anti-monal tartrate, stibophen, SbO_3, and SbCl_3) and metals (such as sodium arsenate). However, it is not resistant to the lmdr1 substrates puromycin, vinblastine, CdCl_2, and ZnCl_2. Although it confers a different drug resistance phenotype, the sequence of lmpgpA, like that of lmdr1, is highly similar to those of the mammalian P-glycoprotein genes.

When treated with trifluralin, the ED_{50} values for the strain with the gene insert (L. major CC-1/pSNAR-lmpgpA), the strain with the vector alone (L. major CC-1/pSNAR), and the parental CC-1 strain were 7.6 ± 1.7, 5.8 ± 2.4, and 8.2 ± 2.0 μM, respectively. The Student t test indicates that lmpgpA did not confer resistance to trifluralin when compared with the parental strain or the strain which is transfected with vector alone, at P < 0.5.

**Efficacy against strains that express P-glycoprotein cross-reactive proteins.** L. b. panamensis (MHOM/CR/87/WR746 CL-1) and L. m. amazonensis (MHOM/BR/73/M2269/WR669 CL-4) are drug-resistant strains developed by sequential exposure to higher concentrations of Pentostam preparations which contained 0.1% chlorocresol. These strains of Leishmania have a stable 30-fold resistance. They express two proteins of 96 to 106 and 23 to 25 kDa. Although smaller in size, these proteins are cross-reactive with C219, a monoclonal antibody specific for a conservative domain of P-glycoprotein homolog in multidrug-resistant human and Chinese hamster ovary cells (13).

The trifluralin ED_{50} value for L. m. amazonensis WR669R (resistant) was 3.2 ± 0.9 μM, and that for the parental strain (WR669S [suscetible]) was 4.0 ± 0.6 μM. For L. b. panamensis, the ED_{50} for the resistant strain (WR746R) and the susceptible strain (WR746S) were 2.8 ± 0.5 and 5.3 ± 0.9 μM, respectively. According to the Student t test, at P < 0.5, the pairs were equally susceptible.

In summary, results from studying four Leishmania strains with amplified production of P-glycoproteins indicate that trifluralin is not subject to the action of these proteins, although there are numerous other strains, developed in laboratories and isolated in the field, that have not been tested. Simultaneously, these results suggested that trifluralin is unlikely to activate amplification of P-glycoprotein homologs in Leishmania. This information strengthens the possibility of and enhances the impetus for developing trifluralin as a therapeutic agent for the treatment of leishmaniasis.

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