Introduction

The current state of chemotherapy for leishmaniasis is more promising than it has been for several years due to both new drugs and new formulations of old drugs, either recently approved or in clinical trial (1). In the past decade 4 new potential therapies for visceral leishmaniasis (VL) have been introduced: a parenteral formulation of amonisidine (paromomycin) (2), amphotericin B liposome (3,4), and the orally active drug miltefosine (5,6) and sitamaquine (6). Treatment of cutaneous leishmaniasis (CL) has been improved by various topical formulations of paromomycin (7-9) and oral miltefosine can also be beneficial (10).

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Abstract: In vitro culture systems were used to assess the growth inhibition of kinetoplastids by a variety of aminoglycosides. The parasites were allowed to grow to the stationary phase in the presence of varying concentrations of the drugs. The IC-50 for every drug was calculated by comparison with the control. The sensitivity of various leishmanial strains to these drugs was in the order of: G418 > hygromycin > paromomycin > neomycin; however, under our assay conditions gentamycin and kanamycin were non-leishmanicidal. The effects of the drugs on the intracellular form of the parasite in the macrophage cell line were also tested. Other kinetoplastids, such as Crithidia spp., and Blastocrithidia culicis, were tested and showed resistance to all these drugs. Secondary structures for the 3' region of the SSUrRNA genes for these organisms were constructed, and correlations between drug sensitivity and the secondary structures are presented. In Leishmania it is a T-A pair in the secondary structure instead of a C-G pair at position 1409-1491 (E. coli), as reported in other organisms, which is responsible for paromomycin sensitivity. The residue responsible for hygromycin sensitivity remained G(1494). The 3' loop-stem U-structures are different for organisms in this family, which might be of significance in determining the overall sensitivity to these aminoglycosides. This might provide rational approaches to the development of drugs specific for Leishmania. Because of the sensitivity of mammalian cells to this drug, we suggest that paromomycin may be used for testing against leishmaniasis.

Key Words: Kinetoplastids, aminoglycosides, SSUrRNA, Leishmania
Several other drugs, including itraconazole, ketoconazole, dapsone, and allopurinol, have been tested in limited clinical trials, often with equivocal results. Drug treatment is complicated by the variation in sensitivity of *Leishmania* species, different disease manifestations, lack of controlled clinical trials of new (and old) drugs for CL, and more recently, increasing levels of antimonial resistance. This paper examines the problems that produce variation in drug sensitivity (paromomycin), tries to separate them from acquired drug resistance, and finally discusses methods of monitoring resistance.

Aminoglycoside antibiotics exert their effect primarily by interacting with small subunit ribosomal RNA (SSUrRNA) (11-14). The 3' region of SSUrRNA plays a crucial role in protein biosynthesis (15-19). Its gene, universally present in all organisms, is an extremely useful tool for phylogeny (20). More than 2000 SSUrRNA genes have been sequenced (21), including those of *Crithidia* spp. and *Blastocrithidia culicis*, as recently reported from our laboratory.

The aminoglycoside antibiotic paromomycin is a potent antileishmanial agent (22). The World Health Organization (WHO) is conducting clinical trials of paromomycin ointment in various countries as a topical therapy for cutaneous lesions in humans. A similar kind of study is underway in Pakistan, where cutaneous leishmaniasis is endemic (11-14). A 12% paromomycin ointment supplied by the WHO was tested in human volunteer patients and preliminary data obtained revealed encouraging results.

Current chemotherapy for leishmaniasis also employs heavy metal compounds (antimony and arsenic) and the antibiotic amphotericin B, all of which induce toxic side effects in the host. The aminoglycoside antibiotic aminosidine (paromomycin) has recently shown some promise, although its drawbacks are poor penetration and the inducement of painful inflammation in some cases (23). To the best of our knowledge this is the first report of the use of aminoglycosides as potential antiparasitic drugs, specifically against an intracellular parasite.

Due to the considerable clinical importance of paromomycin, the mode of action of this antibiotic is being studied by various researchers. Interference with protein biosynthesis or direct action on ribosomes is the main target of a large group of antibiotics (21). The SSUrRNA gene, universally present in all organisms, is an extremely useful tool for phylogeny (24). The 3' region of SSUrRNA plays a crucial role in protein biosynthesis and has been characterized as the site of action of several aminoglycoside antibiotics.

In the case of paromomycin's interaction with *E. coli*, it has been established that the base pair at position 1409 (C) and 1491 (G) in the 3' loop-stem U structure of the secondary structure of SSUrRNA is involved. Resistance to this aminoglycoside occurs in mutants in which this particular base pair is disrupted [(A) itself] (25). This was established in cases of other organisms, such as *Giardia Lamblia* [(B) itself] (25) and *Tetrahymena thermophila* [(C) itself] (26). In the case of *Leishmania*, Fong et al. developed paromomycin-resistant clones in which there was no mutation at the 1409-1491 equivalent position of SSUrRNA (27); however, Mearouf et al. (28) recently reported Leishmania resistance to paromomycin.

We used in vitro culture systems to assess the growth inhibition of Kinetoplastids by a variety of aminoglycosides. The effect of paromomycin, in particular, on intracellular Leishmania in the in vitro culture and of its topical ointment in treating cutaneous lesions in human volunteers was studied. The 3' region of the secondary structure of these Kinetoplastids was analyzed and then correlated with the experimental determination of drug susceptibility.

**Materials and Methods**

The aminoglycoside antibiotics (paromomycin 1, neomycin complex 2, kanamycin A 3, gentamycin A 4, genetricin 5) were obtained from Sigma, USA and some of them were synthesized by known procedures, parasite cultures, and macrophage infection; *L. amazonensis* (LV78), *L. major* (MHOM/PK/88/DESTO), *L. tropica*, and *L. infantum* were maintained in the in vitro culture. They were grown at 25 °C in medium 199 at pH 7.4 with 25 μM HEPES (N-2-hydroethylpiperazine-N-2-ethanesulfonic acid) and 20% heat inactivated fetal bovine serum (HIFBS) supplemented with the antibiotics penicillin (100 U ml⁻¹) and streptomycin (100 μg ml⁻¹). *Crithidia* spp. and *Blastocrithidia culicis* were cultured in brain heart infusion medium. To study the effect of aminoglycosides on promastigotes in culture, 4 × 10⁶ promastigotes of the respective *Leishmania* isolates were grown in the above-stated medium in the presence and absence of the drug. After 5 days the promastigotes were counted in both experimental and control groups, and the percentage of growth inhibition was calculated. For studying the effect...
of the drugs on intracellular parasites, the permanent cell
line of mouse macrophage J774G8 was used. The
macrophages were cultured in medium RPMI 1640 with
HEPES (25 μM, pH 7.3), which contained 20% heat
inactivated fetal bovine serum and antibiotics. Macrophages (cell density: 10^6/flask) were infected with
stationary phase promastigotes at a rate of 5 parasites
per macrophage, and after 24 h the infectivity was over
80%. The infected macrophages were washed by
replacing the old medium with fresh culture medium in
the absence (control) or presence of different
concentrations of the drugs. After 4 days the total
number of amastigotes in 100 macrophages was
determined as under, with each reading taken as a mean
of 3 experiments.

The 15% paromomycin simple ointment that
contained 12% benzethonium chloride was prepared
according to B.P. The ointment was applied on the
cutaneous lesion twice daily for 15 days.

Secondary Structure Construction: The Leishmania
SSUrRNA gene sequences came from GenBank. The
different strains had > 90% homology and were identical
in the 3' U-loop region. The SSUrRNA genes for Crithidia
spp., and Blastocrithidia culicis were recently sequenced.
The secondary structures were constructed with the
LoopDLoop program (Don Gilbert, Indiana University,
USA). Numbering is according to that of E. coli.

Cell Culture: Leishmania cells were cultured in M199
plus 10% fetal bovine serum. Four species of Leishmania
were used: L. major, L. amazonensis, L. tropica, and L.
infantum. Crithidia spp. and Blastocrithidia culicis were
cultured in brain heart infusion medium.

Results and Discussion
Activity of the Drug against Promastigotes in
Culture
The antileishmanial activity of various aminoglycosides
against the old world and new world Leishmania isolates
is shown in Figures 1 and 2. The potency of the 4 drugs
was in the order of geneticin > hygromycin >
paromomycin > neomycin sulphate. Their ED_{50} values
ranged between 1.0 μg/ml for geneticin to about 30 μg/ml
for neomycin sulphate. Some of the cells transfected with
NEO were resistant to the drugs. In the present study
gentamycin and kanamycin showed no leishmanicidal
activity up to the concentration of about 200 μg/ml,
which is in agreement with El-On et al. (12,13). The
results presented in Figures 1 and 2 show the response of
the drug varied from strain to strain, signifying the
speciation of the parasite for chemotherapeutic purposes.

Effect of Paromomycin on Intracellular
Amastigotes
Only the effect that produced successful infection of
the murine J 774 G8 macrophage cell line for L.
amazonensis strain was studied. The infected
macrophages were treated with different quantities of
paromomycin; at a concentration of 12 μg/ml of
paromomycin the intracellular amastigotes decreased by
> 50%. Addition of the drugs in fresh medium further
reduced the number of intracellular parasites, bringing
the ED_{50} value to about 3 μg/ml of paromomycin.

Treatment of Cutaneous Lesions in Human
Volunteer Patients with Paromomycin Ointment
To test the efficacy of the paromomycin ointment in
treating cutaneous lesions, 2 patients with
parasitologically confirmed cutaneous lesions were
treated twice daily for 15 days. On the eighth day the
inoculum from the lesion did not give a positive culture.
Between 40 and 50 days the lesions were totally cured.
Figures 1 and 2 show the results of such an experiment
in 1 patient.

Correlation of Drug Sensitivity Data with the
SSUrRNA Sequence
In Leishmania we attempted to correlate the antibiotic
susceptibility pattern with the secondary structure
prediction. The nucleotide sequence corresponding to the
3' region of the SSUrRNA in Leishmania, Crithidia, and
Blastocrithidia are shown in Figure 3.

Paromomycin-resistant rRNA mutations have been
isolated in E. coli (11,30), Giardia (25), tetrahymena
(26), and yeast mitochondria (31), as outlined in Figure
2. These mutations are at the sites C-1409 and G-1491,
pairing at the circular location in the secondary structure.
Comparing the secondary structure of our tested
organisms with that of E. coli and Giardia, we found that
T-1409 and A-1491 exists in these organisms, strongly
suggesting that it may not be the individual bases at these
positions, but rather that the disruption of the pair may
account for paromomycin resistance. In Leishmania,
therefore, this T-A (1409-1491) confers susceptibility to
paromomycin. Hygromycin-resistant mutants of
tetrahymena, in which U-1495 is altered, have been
isolated (26). This is also present in the kinetoplastids studied; therefore, susceptibility can be predicted as evidenced by the in vitro studies of the effects of this drug. Chemical probing experiments with E. coli have shown that A-1408 and G-1495 are protected by neomycin antibiotics (11-14). At position 1408 in Leishmania A is replaced with base G, while base G at position 1494 is present, which may account for the low susceptibility of kinetoplastids to neomycin, as evidenced by the in vitro experiment results on Leishmania. Kanamycin was observed to bind G at position 1408. In our kinetoplastid the A at position 1408 was replaced with G, except in Blastocrithidia; therefore, on the basis of the gene structure the resistance to this drug can be predicted and this is supported by the in vitro Leishmania experiment results. The resistance of Crithidia and Blastocrithidia to these drugs can be presumably explained as such: they either do not take in these drugs or their MDR efflux pump rapidly pumps out the drugs before any damage is done to the cells. We have constructed the secondary structure for other organisms (Endotrypanum, Bodo caudatus, Leptomonas, Trypanosoma cruzi, and Trypanosoma brucei) in this group as well. All the essential residues of this family are the same.

To further substantiate the drug sensitivity test at the molecular level, we constructed the secondary structure of the 3' region of the SSUrRNA gene, as outlined in Figure 2, which is responsible for the sensitivity to these drugs in all these organisms. After comparing the secondary structures with that of E. coli (11-14) and Giardia, we determined that at position 1409 and 1491, a new T-A pair was formed instead of C-G, as in Giardia. Previously, disruption of this pair also resulted in resistance.

Figure 1. The structure of paromomycin 1, its bioactive conformation with relevant nucleotides in the A-site binding domain and the proposed site for diversification (solid arrow), neomycin complex 2, kanamycin A 3, gentamycin A 4, geneticin 5., The dashed arrow indicates the replacement of -OH by -NH₂ for paromomycin 1 into neomycin 2.
Figure 2. The shape of *Leishmania* SSuRNA.
Paromomycin and *Tetrahymena thermophila*

In humans, G at position 1491 is substituted by A and disrupts the paring (23) so that the base pairing rather than G or C at those particular residues is responsible for the paromomycin binding (22). For hygromycin, we observed that G is at position 1494 in these organisms, the same as in *E. coli* and *Giardia*, which is supposed to bind to the drug (11). It is T that followed, which was also shown to be the residue responsible for the drug effect (21,22). These accounted for the hygromycin susceptibility of these organisms. The residue responsible for kanamycin sensitivity is G at position 1408 in these organisms, except in *B. culicis*, so they are as resistant to drug as A. Actually, this *Crithidia* sp. failed to be inhibited by all the drugs tested, i.e. arsenates and tunicamycin, and they developed methotrexate resistance after a short period of treatment. Their multi-drug resistance feature requires further investigation.

In *Leishmania* the sensitivity pattern correlated well with the secondary structure prediction. This gave us the molecular basis for using these aminoglycosides in chemotherapy for leishmaniasis. Hygromycin has no useful practical application in this case, as it does not exhibit selective toxicity. The spectrum of its activity may extend to all organisms because its target nucleosides are universally conserved. Paromomycin is a potentially useful anti-*Leishmania* agent. Understanding the mechanism of this drug action is of great clinical significance, as resistance may appear in patients during treatment. The possibility of the existence of more than 1 mechanism cannot be ignored. We observed that transfection of *Leishmania* with the NEO gene renders them resistant to all these drugs, as the substrate to the enzyme. Neomycin phosphotransferase covers a broad range, including neomycins, paromomycin, kanamycin, and gentamycin. It is possible that this kind of mechanism may exist in *Leishmania*.

**Conclusion**

This manuscript described the susceptibility of several kinetoplastid species to a small panel of aminoglycosides, and correlated the results with rRNA sequence and secondary structure analysis. While the data are sound, they are sufficiently novel, even though similar research previously demonstrated the efficacy of paromomycin for the treatment of cutaneous leishmaniasis.

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