Sensitivity of Kinetoplastids to aminoglycoside: Correlation with 3’ small subunit rRNA gene

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ABSTRACT

In-vitro culture systems were used to assess the growth inhibition of Kinetopasids by a variety of aminoglycosides. The parasites were allowed to grow to the stationary phase in the presence of varying concentrations of the drug. The IC-50 for every drug was calculated by its comparison with the control. The sensitivity of various leishmanial strains to these drugs is in the order G418 > Hygromycin > Paromomycin > Neomycin. However under our assay conditions Gentamycin and Kanamycin were nonleishmanicidal. The effect of the drug on the intracellular form of the parasite in the macrophage cell line was also tested. Other Kinetoplastids, such as crithidia spp. And Blastocrithida culicis were tested which showed resistance to all these drugs. Secondary structures for the 3’ region of the ssrRNA genes for these organisms were constructed, and the correlation between the drug sensitivity and the secondary structures is presented. In leishmania it is a T-A pair in the secondary structure instead of C-G at position 1409-1491 (E.coli) as reported in the other organisms which is responsible for paromomycin sensitivity. The residue responsible for hygromycin sensitivity remained as 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 used for testing leishmaniasis.

INTRODUCTION

The current situation for the chemotherapy of leishmaniasis is more promising than it has been for several years with both new drugs and new formulations of old drugs either recently approved or on clinical trial[1]. In the past decade four new potential therapies for vis-ceral leishmaniasis (VL) have been introduced: a parenteral formulation of aminosidine (paromomycin)[2], amphotericin B liposomes[3,4], and the orally active drugs miltefosine[5] and sitamaquine (WR6026)[6]. Treatment of cutaneous leishmaniasis (CL) has been improved by various topical formulations of paromomycin[7,8,9] and could also benefit from oral miltefosine[10].
Several other drugs including itraconazole, ketoconazole, dapsone and allopurinol have been on limited clinical trials, often with equivocal results. Drug treatment is complicated by the variation in sensitivity of Leishmania species, the different disease manifestations, the lack of controlled clinical trials of new (and old) drugs for CL and recently, the increasing levels of antimonal resistance. This paper examines the problems that produce variation in drug sensitivity, tries to separate these from acquired drug resistance and finally discusses methods to monitor resistance.

Aminoglycoside antibiotics mostly exert their effect by interacting with the small subunit ribosomal RNA (ssrRNA)\textsuperscript{[11a]}. The 3’ end of the ssrRNA plays a crucial role in protein biosynthesis. Its gene universally present in all organisms is extremely useful tool for phylogeny\textsuperscript{[12]}. More than 2000 ssrRNA genes have been sequenced\textsuperscript{[13]}, including that of the Crithidia spp. And Blastocritidium culicis, reported recently from our laboratory.

The aminoglycoside antibiotic paromomycin is a potent antileishmanial agent\textsuperscript{[14]}. World health Organization is conducting clinical trials of paromomycin ointment in various countries for the tropical therapy of cutaneous lesions in humans. Similar kind of study is under way in our group, where cutaneous leishmaniasis is highly endemic\textsuperscript{[11]}. The 12% paromomycin ointment supplied by WHO was tried in human volunteer patients. Preliminary data obtained reveal 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 for the host as well. An aminoglycoside antibiotic aminosidine (paromomycin) has recently shown some promise, although it has the drawback of poor penetration and induces painful inflammation in some cases\textsuperscript{[15]}. To our knowledge, this is the first report of aminoglycosides as a potential antiparasitic drug, especially against an intracellulair parasite.

Due to the considerable clinical importance of paromomycin, the mode of action of this antibiotic is being studied by various groups. Interference with protein biosynthesis or direct action on ribosomes is the main target of a large group of antibiotics\textsuperscript{[13]}. The small subunit of ribosomal RNA (ssrRNA) gene, universally present in all organisms, is extremely useful tool for phylogeny\textsuperscript{[16]}. The 3’-end of ssr RNA play a crucial role in protein biosynthesis and has been characterized as the site of action of several aminoglycoside antibiotics.

In case of interaction of paromomycin with \textit{E.coli} it is established that the base pair at position 1409(C) and 1491(G) in the 3’ loop-stem U structure of the secondary structure of ssrRNA is involved. Resistance to this aminoglycoside occurs in mutants in which this particular base pair is disrupted [(A) itself]\textsuperscript{[17]} This is being established in cases of other organisms such as \textit{Giardia Lamblia}\textsuperscript{[17]} [(B) itself] and \textit{tetrahymena thermophila} [(C) itself]\textsuperscript{[18]}. In case of \textit{Leishmania} Fong et al.\textsuperscript{[19]} developed paromomycin resistant clones of this parasite and has evidenced that there was no mutation at the 1409-1491 equivalent position of ssrRNA in the paromomycin resistant clone. However Mearouf et al.\textsuperscript{[20]}, has recently reported that resistance to paromomycin involves.

We have used \textit{in vitro} culture systems to assess the growth inhibition of Kinetoplastids by a variety of aminoglycosides. The effect of paromomycin in particular on the intracellular \textit{Leishmania} in the in-vitro culture and of its topical ointment in treating cutaneous lesions in human volunteers is studied. The 3’-end of the secondary structure of these Kinetoplastides is analyzed and then correlated with the experimental determination of drug susceptibility.

**RESULTS AND DISCUSSION**

**Activity of drug against promastigotes in culture**

The antileishmanial activity of various aminoglycosides against the old world and new world leishmania isolates is shown in figure 1. The potency of the four drugs is in the order of Geneticin>hygromycin>paromomycin>neomycin sulphate. Their ED50 value ranges from 1.0 \(\mu\)g/ml for Geneticin to about 30\(\mu\)g/ml for neomycin sulphate. All these cells transfected with NEO were resistant to these drugs. In our hands Gentamycin and Kanamycin showed no leishmanicidal activity up to the concentration range of 200\(\mu\)g/ml. However EI-On et al.\textsuperscript{[11b-d]} has shown that. The results shown in figure 1 shows that the response of the drug varies from strain to strain. This signifies the speciation of parasite for chemotherapeutic purposes.
Effect of paromomycin on intracellular amastigotes

The effect was studied for L. amazonensis strain that only gives successful infection of murine J 774 G8 macrophage cell line. The infected macrophages were treated with different quantities of the paromomycin. As shown in figure 3, at a concentration of 12μg/ml paromomycin the intracellular amastigotes decreased by less than 50%. Addition of drugs in fresh medium further reduced the number of intracellular parasites bringing the ED-50 value to about 3μg/ml paromomycin.

Treatment of cutaneous lesions in human volunteer patients with paromomycin ointment

To test the efficacy of the paromomycin ointment in treating cutaneous lesions, two patients with parasitologically confirmed cutaneous lesions were treated twice daily for 15 days. On 8th day the inoculum from the lesion did not give +ve culture. In between 40-50 days the lesions were totally cured. figure 3 shows the results of such an experiment in one patient.

Correlation of drug sensitivity data with the ssu rRNA sequence

In leishmania we have tried to correlate the antibiotic susceptibility pattern with the secondary structure
Paromomycin resistant rRNA mutations have been isolated in *E. coli* [M-1986][22,11a], *Giardia*[17], *Tetrahyymena*[18] and yeast mitochondria[23]. 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 find that a T-1409 and A-1491 exists in these organisms, strongly suggesting that it may be the individual bases at these positions, but rather 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 *Tetrahyymena* in which U-1495 is altered have been isolated[18]. This is also present in kinetoplastids studied; therefore susceptibility can be predicted as evidenced by the in vitro studies on effects of this drug. Chemical probing experiments with *E. coli* has shown that A-1408 and G-1495 are protected by Neomycin antibiotics protect[11]. In Leishmania at position 1408 A is replaced with base G, while the base G at position 1495 is present which may account for the low susceptibility of neomycin to kinetoplastids as evidenced by the in-vitro experiment results on Leishmania. Kanamycin is found to bind G at position 1408. In Kinetoplastids studies here the A at position 1408 is replaced with G, except in Blastocithidia. Therefore, on the basis of the gene structure the resistance to this drug can be predicted and this is supported by the in-vitro experiment results on Leishmania. The resistance of crithidia and Blastocithidia to these drugs can be presumably explained, that they either do not take in these drugs or their mdr efflux pump rapidly pumps out these drugs before any damage is done to the cells. We have constructed the secondary structure for other organisms (Endotrypanum, Bodo Caudata, Leptomonas, Trypanosoma cruzi and 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 secondary structure of the 3’ region of the ssr RNA gene, which is responsible for the sensitivity to these drugs, in all these organisms. After comparing the secondary structures with that of *E. coli*[11] and *Giardia*, we found that at position 1409 and 1491, a new T-A pair was formed instead of C-G as found in *Giardia*. Previously, disruption of this pair resulted in the resistance to paromomycin *Tetrahymena thermophila*[14].

In humans that G at position 1491 is substituted by A and disrupts the paring[15]. So that, it is the base pairing rather than G or C at those particular residues which is responsible for the paromomycin binding. For Hygromycin, we found that at position 1494 it is G in these organisms, the same as in *E. coli* and *Giardia*, which is supposed to bind to the drug[11a].

It is T that followed which was also shown to be the residue responsible for the drug effect[13,14]. 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 resistant to drug as A. Actually, this crithidia spp. failed to be inhibited by all the drugs tested, i.e. arsenates, tunicamycin, and they developed methotrexate resistance after short period of treatment. (Yubin Du and K.P.Chang, unpublished results). Their multi-drug resistance feature needs further investigation.

In Leishmania the sensitivity pattern correlates well with the secondary structure prediction. This gives 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 since its target nucleosides are universally conserved. Paromomycin is a potentially useful antileishmaniasis agent. Understanding the mechanism of this drug action is of great clinical significance as resistance may appear in patients during treatment. The possibility of more than one mechanism cannot be ignored. We have 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 neomycin’s paromomycin, kanamycin and gentamicins. It is possible that this kind of mechanism may exist in leishmania.

MATERIALS AND METHODS

The aminoglycoside antibiotics were obtained from Sigma, USA, parasites culture and macrophage infection; L.M. 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 with 25μM HEPES (N-2-hydroethyl piperazine-N-2-ethanesulfonic acid), PH 7.4 and 20% heat inactivated fetal bovine serum (HIFBS), supplemented with antibiotics, penicillin (100Uml⁻¹) and streptomycin (100μg ml⁻¹). Crithidia spp. And Blastocrithidia culicis were cultured in brain heart infusion medium.

CONCLUSION

This manuscript above describes the susceptibility of several kinetoplastid species to a small panel of aminoglycosides, and correlates the results with rRNA sequence and secondary structure analysis. While the data are sound, they are sufficiently novel, even similar analyses were completed, which the paromomycin was
already well established as treatment for cutaneous leishmaniasis. Some other scientists shared this concern with us.

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