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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 starins 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 ssurRNA 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.

KEYWORDS

Kinetoplasides;
Aminoglycosides
ssrRNA,
Leishmania.

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 visceral 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 antimonial 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 (ssRNA)[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 fo phylogeny[12]. More than 2000 ssrRNA genes have been sequenced [13], including that of the Crithidia spp. And Blastocrithidia culicis, reported recently from our laboratory.

The aminoglycoside antibiotic paromomycin is a potent antileishmanial agent[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 underway in our group, where cutaneous leishmaniasis is highly endemic[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[15]. To our knowledge, this is the first report of aminoglycosides as a potential antiparasitic drug, especially against an intracellular 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[13]. The small subunit of ribosomal RNA (ssur RNA) gene, universally present in all organisms, is extremely useful tool for phylogeny[16]. The 3'-end of ssur 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 *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][17]. This is being established in cases of other organisms such as *Giardia Lamblia*[17] [(B) itself] and *tetrahymena thermophila* [(C) itself][18]. In case of *Leishmania* Fong et al.[19] developed paromomycin resistant clones of this parasite and has evidenced that there was no mutation at the 1409-1491 equivalent position of ssurRNA in the paromomycin resistant clone. However Mearouf et al.[20], has recently reported that resistance to paromomycin involves.

We have used *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 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 μg/ml for geneticin to about 30μg/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 200μg/ml. However EI-On et al.[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.

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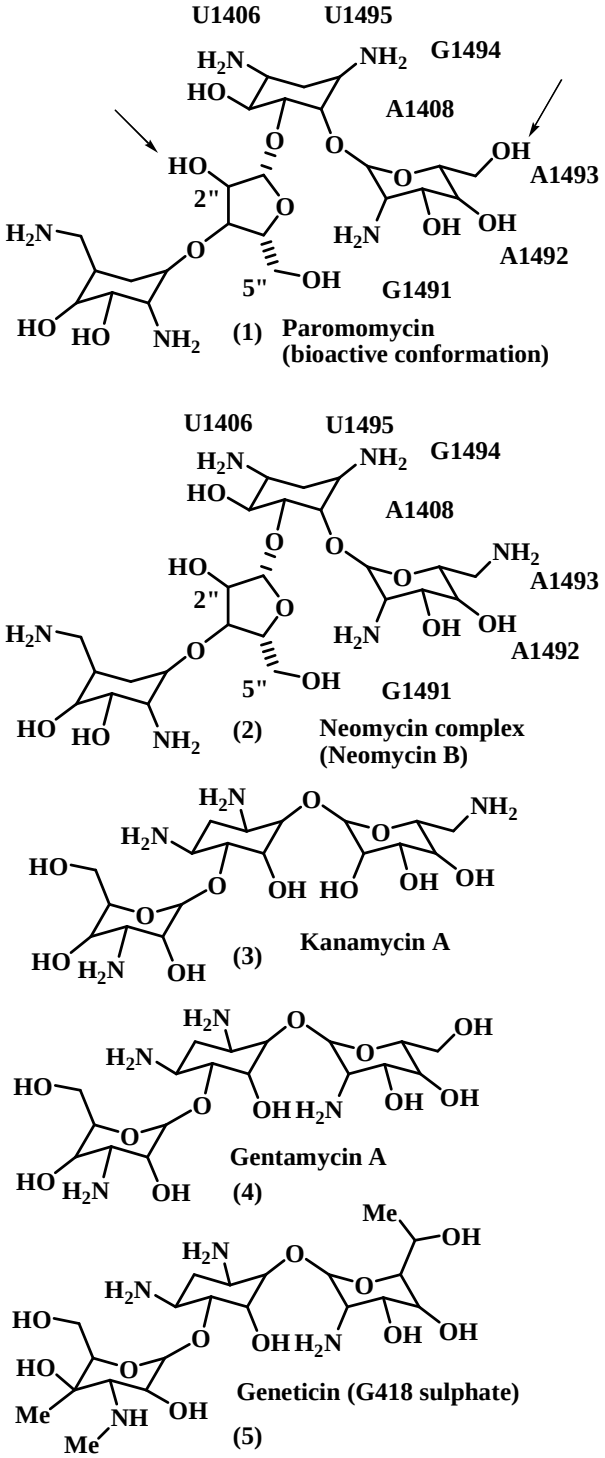

Figure 1: The structures of paromomycin 1, its bioactive conformation with relevant nucleotides in the A-site binding domain, and proposed site for diversification (solid arrow), neomycin complex 2, Kanamycin A 3, Gentamycin A 4, Geneticin 5, the dashed arrow indicates the replacement of -OH by -NH_2 for the paromomycin 1 into Neomycin 2

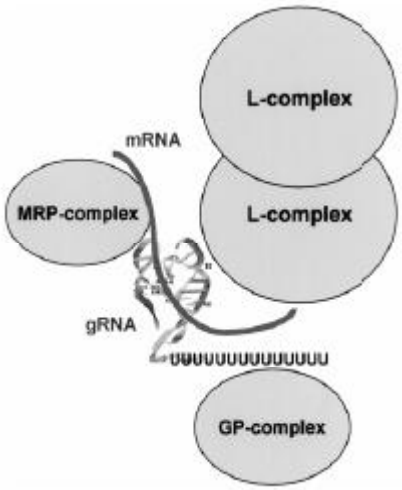

Figure 2: Diagrammatic model for higher-order organization of editing complexes. The L-complex is shown as a dimer, for which the evidence is suggestive, and the three complexes are shown as interacting via RNA. The gRNA and mRNA indicated are the most likely candidates, but the existence of other RNA species has not been ruled out. The gRNA structure shown is taken from the model of [Hermann et al. 1997][21].

Effect of paromomycin on intracellular amastigotes

The effect was studied for L. amazonensis strain as that only gives successful infection of murine J 774 G8 macrphage 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 parasitologocally 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 r RNA sequence

In leishmania we have tried to correlate the antibiotic susceptibility pattern with the secondary structure

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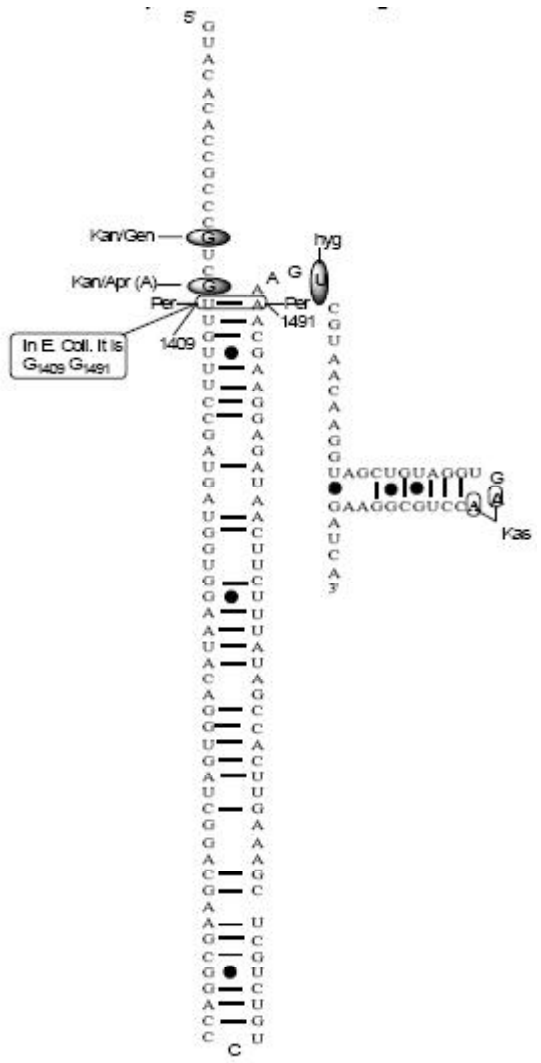

Figure 3: Leishmania ssur RNA shape

prediction. The nucleotide sequence corresponding to the 3' end of the ssrRNA of leishmania crithida and Blastro crithida are shown in figure 2.

Paromomycin resistant rRNA mutations have been isolated in *E.coli* [M-1986][22,11a], Giardia[17], tetrahymena[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 tetra hymena 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 1494 is present which may account for the low susceptipility 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 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 experiment results on Leishmania. The resistance of crithida and blastocrithida 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 ssur 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 theramophila*[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 *Hygromyci*n susceptibility of these organisms. The residue responsible for *Kanamycin*

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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 methrotrexate 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 Leithmainiasis. Hygomycin 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 antileishmania 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 phosphortransfeerse covers a board 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. To study the effect of aminoglycosides on promastigotes in culture, 4×10^6 promastigotes of the respective *Leishmania* isolate were grown in above stated medium in the presence and absence of drug. After 5 days the promastogotes were counted both in experimental and controls and the percentage (%) growth inhibition calculated. For studying the effect of drug on intracellular parasites, the permanent cell line of mouse macrophage J774G8 was used. The macrophages were cultured in medium RPMI 1640 with HEPES (25μM, PH 7.3) containing 20% heat inactivated fetal bovine serum and antibiotics. Macrophages (cell density 10^6 per flask) were infected with stationary phase promastigotes at a ratio of 5 parasites per macrophages, after 24 hrs the infectivity was over 80 percent. The infected macrophages were washed by replacing the old medium with fresh culture medium, in the absence (control) or presence of different concentrations of drug. After 4 days the total numbers of amastigotes in 100 macrophages were determined as under with each reading taken as a mean of three experiments.

Total no. amastigotes = Total no. macrophages × % infection × total amastigotes per macrophage

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

Secondary structure construction

The Leishmania ssurRNA gene sequences are from GenBank. The different strains have over 90% homology and identical in the 3'U-loop region. The ssrRNA genes for Crithidia spp., and Blastocrithidia culicis were recently sequenced. The secondary structures were constructed by LoopDloop program (Don Gilbert, Indiana University, USA). The numbering is according to that of *E.coli*.

Cell culture; Leishmania cells are cultured in M199 plus 10% fetal bovine serum. Four species of leishmania were used, L.major, L.amazonesis, L.tropica and L.infantum. Crithidia spp. And Blastocrithidia culicis are 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 Scientist shared this concern with us.

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