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Sensitivity of Kinetoplastids to Aminoglycoside: Correlation with the 3' Region of the Small Subunit rRNA Gene

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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

Kinetoplastidlerin Aminoglikozide Duyarlılıkları: rRNA Geninin Küçük Alt Biriminin 3' Bölgesi ile İlgisi

Özet: Kinetoplastidlerin bir seri aminoglikozidlerce büyümesinin engellenmesi için in-vitro kültür sistemleri kullanılmıştır. İlacın çeşitli derişimlerinin varlığında parazitler durgun faza kadar geliştirilmişlerdir. Her ilacın IC-50 değeri kontrolle karşılaştırılarak hesaplanmıştır. Çeşitli leishmanial suşların ilaca olan duyarlılığı sırasıyla G418> Hygromycin> Paromomycin> Neomycin şeklindedir. Buna rağmen bizim koşularımızda Gentamycin ve Kanamycin nonleishmanicidal bulunmuşlardır. İlacın parazitin makrofaj hücre hattındaki hücre içi formuna olan etkisi de denenmiştir. Crithidia spp., ve Blastocrithida culicis gibi kinetoplastidler de denenmiş ve tüm bu ilaçlara dayanıklı bulunmuştur. Bu organizmalar için SSUr-RNA genlerinin 3' bölgeleri için ikincil yapılar oluşturulmuştur. Leishmania' da ikincil yapıda diğer organizmalarda Paromomycin duyarlılığından sorumlu olduğu bildirilen 1409-1491 (*E. coli*) pozisyonunda C-6 yerine T-A baz eşleşmesi bulunur. Higromisin duyarlılığı için görevli kök G(1494) olarak kalmıştır. Bu ailenin organizmaları için 3' loop-stem U-yapıları farklıdır ve bu aminoglikozidlere tüm duyarlılığı belirlemede önemli olabilirler. Bu ise leishmania' ya özgü ilaçların geliştirilmesinde gerçekçi yaklaşımlar sağlayabilir. Memeli hücrelerinin bu ilaca olan duyarlılıkları yüzünden leishmaniasisin test edilmesinde paramomisinin kullanılmasını önerebiliriz.

Anahtar Sözcükler: Kinetoplastid, aminoglikozide, ssurRNA, Leishmania

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 aminosidine (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).

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, geneticin 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 10^6 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. amazonesis*, *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 staudy 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

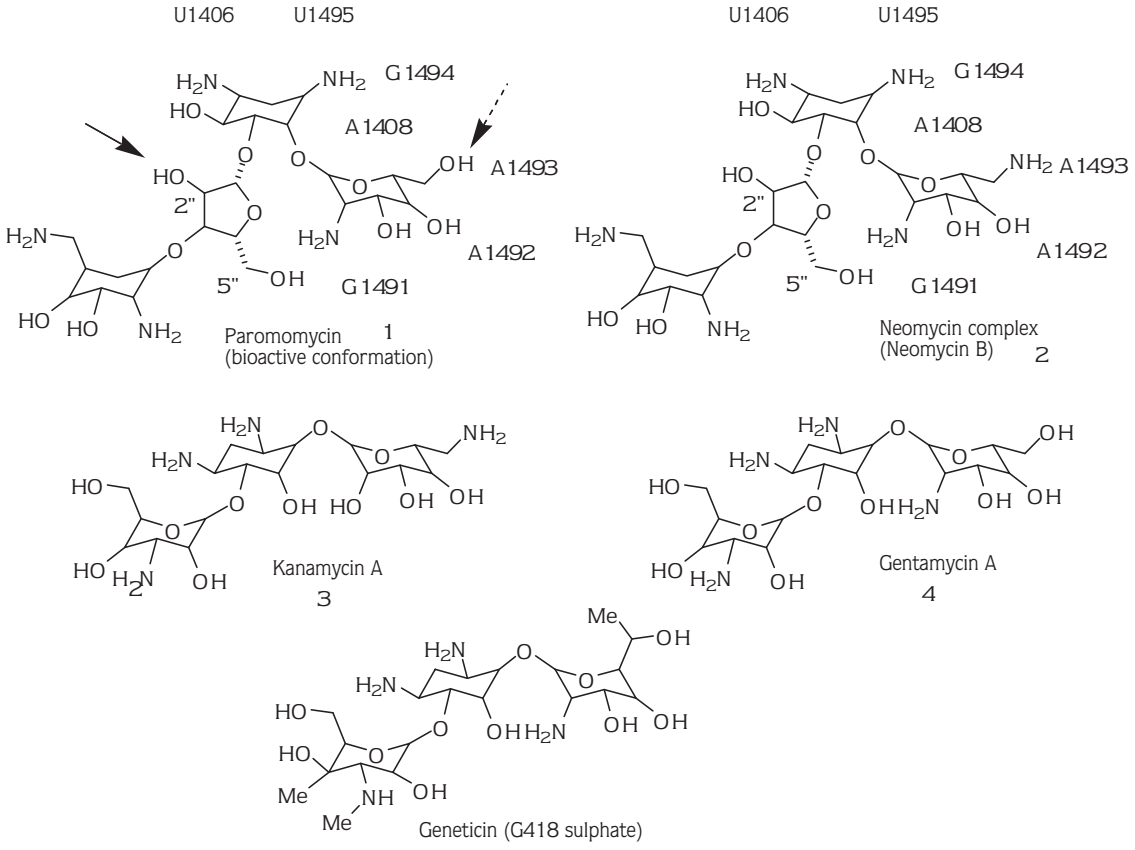

Figure 1. The structure of paromomycin 1, its bioactive conformation with relevant nucleotides in the A-site binding domain and the proposed site for diversification (solid arrow), neomycin complex 2, kanamycin A 3, gentamycin A 4, geneticin 5,. The dashed arrow indicates the replacement of -OH by -NH_2 for paromomycin 1 into neomycin 2.

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.

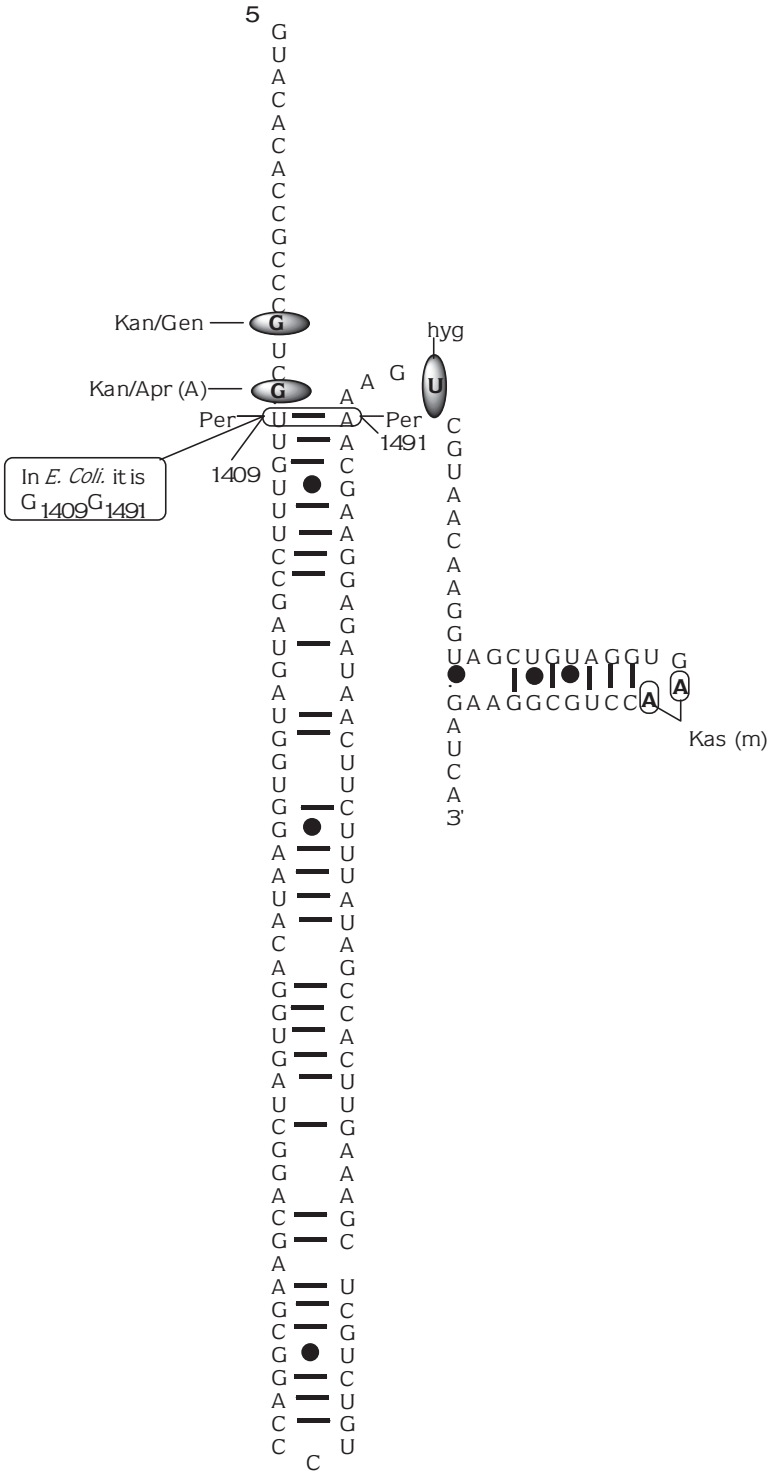

Figure 2. The shape of *Leishmania* SSUrRNA.

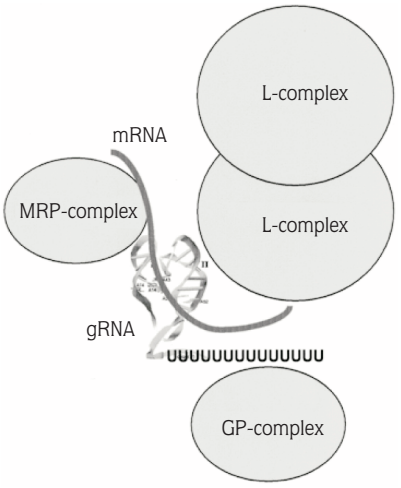

Figure 3. Diagrammatic model for higher-order organization of editing complexes. The L-complex is shown as a dimer for which the evidence is suggestive, and the 3 complexes are shown to interact via RNA. The gRNA and mRNA indicated are the most likely candidates, but the existence of other RNA species has not been ruled out. The gRNA structure shown is taken from the model given by Hermann et al. (29).

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 board 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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