

## DEVELOPMENT AND CHARACTERIZATION OF PAROMOMYCIN-RESISTANT *LEISHMANIA DONOVANI* PROMASTIGOTES

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### Summary :

Paromomycin is an antileishmanial chemotherapeutic agent. *Leishmania donovani* promastigotes resistant to 800  $\mu$ M of paromomycin were selected by increasing drug pressure and cloned. These promastigotes did not acquire multidrug resistance. Paromomycin resistance was stable in the absence of the drug in the culture. It remained stable also in amastigotes isolated after a passage in mice. Furthermore the resistant parasites were still infective to macrophages *in vitro* and for mice *in vivo*. A sensitive method to detect and to quantify intracellular paromomycin by HPLC was developed and allowed to show that the main mechanism of resistance seems to be due to decreased drug uptake probably as a consequence of altered membrane composition.

**KEY WORDS :** paromomycin, *Leishmania donovani*, drug resistance.

### Résumé : DÉVELOPPEMENT ET CARACTÉRISATION DE PROMASTIGOTES DE *LEISHMANIA DONOVANI* RÉSISTANT À LA PAROMOMYCINE

La paromomycine est un médicament ayant aussi une activité antileishmanienne. Des promastigotes de *Leishmania donovani* résistants à 800  $\mu$ M de paromomycine ont été obtenus par pression médicamenteuse et clonage. Ces promastigotes n'ont pas acquis de polychimiorésistance. La résistance à la paromomycine était stable en l'absence de cette molécule dans le milieu de culture. Elle est restée stable également dans les amastigotes isolés après passage chez la souris. De plus, les parasites résistants sont restés infectieux pour les macrophages *in vitro* et pour la souris. Une méthode sensible pour détecter et quantifier la paromomycine intracellulaire par HPLC a été développée et a permis de montrer que le principal mécanisme de résistance semble être dû à une diminution de l'absorption de la paromomycine comme une conséquence de l'altération de la composition membranaire.

**MOTS CLÉS :** paromomycine, *Leishmania donovani*, résistance médicamenteuse.

## INTRODUCTION

Paromomycin (PR) is an aminoglycoside antibiotic used for treatment of bacterial, Katsu *et al* (1968) parasitic infections, such as amoebiasis, giardiasis, Gillin & Diamond (1981), Cryptosporidiasis, Armitage *et al* (1992) and cutaneous and visceral leishmaniasis, Scott *et al* (1992).

In prokariotic and eukariotic cells (*E. coli*, *S. cerevisiae*) resistance to PR is generally related to mutation in the small subunit of rRNA (De Stario, 1989) or to enzymatic inactivation of the antibiotic like in *Pseudomonas aeruginosa* (Maeda *et al.* 1968).

We selected PR-resistant *Leishmania donovani* promastigotes by increasing drug pressure and studied biochemical alterations in comparison to the parental wild type cells. A new and sensitive method to detect

and to quantify intracellular paromomycin was developed and allowed to show that the resistance of these parasites to PR is related rather to decreased drug uptake than to a mutation in the small subunit rRNA.

## MATERIALS AND METHODS

Paromomycin sulfate, o-phthalaldehyde (OPA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (France). The ion-pairing agent sodium pentanesulfonate was supplied by Janssen (France) and sodium sulfate from SDS (France). Culture medium components were purchased from Gibco (France) and the fetal calf serum from FLOBIO (France). Coomassie Blue reagent for protein assays was from Bio-Rad Laboratories (France). Water was purified with Milli-Q water purification system of Millipore (France). All HPLC buffers and reagents were of analytical or HPLC grade.

### PARASITES

*Leishmania donovani* strain MHOM/ IN/ 80/ DD8, originating from the WHO reference collection at the London School of Hygiene and Tropical Medicine

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(United Kingdom) was provided by Dr. D. Evans. Promastigotes were routinely grown in a semi-defined RPMI 1640 medium containing 2 mM glutamine, 25 mM HEPES (pH 7.4) and 10% heat inactivated fetal calf serum. Paromomycin (Sigma) was dissolved in sterilized water prior to its addition to the cultures.

#### ISOLATION OF PR RESISTANT PROMASTIGOTES

PR resistance was induced by increasing drug pressure. The wild type strain *L. donovani* DD8 was cloned on semi-solid RPMI 1640 medium containing 1% Bacto Agar (Difco) and 10% fetal calf serum as described by Iovannisci & Ullman (1983). Colonies were picked up and transferred separately into liquid RPMI 1640 medium. The cloned wild type was then cultured in the presence of increasing PR concentrations starting with 150  $\mu\text{M}$ . The cultures were transferred by seeding 10<sup>6</sup> promastigotes ml<sup>-1</sup> with the same concentration until the growth rate became stable, approximately three passages with one passage a week. PR concentration was then increased until the final 800  $\mu\text{M}$  concentration was reached. The resistant culture was then re-cloned. Using the same medium and 96-wells Nunclon plates, different dilutions were prepared from a culture to obtain one well with one parasite. PR resistant clones appeared after 2-3 weeks. Each clone was then transferred to 800  $\mu\text{M}$  PR-containing medium and the drug pressure maintained by weekly passage of 10<sup>6</sup> promastigotes ml<sup>-1</sup> in 5 ml medium in 50 ml Nunclon Flasks.

#### DETERMINATION OF THE PHENOTYPE

The susceptibility of both wild-type and PR-resistant clones to PR and other drugs was measured in 24-well Nunclon plates. Each well was inoculated with 10<sup>6</sup> parasites in 500  $\mu\text{l}$  medium. The drugs to be studied were added 5-6 h later in 25  $\mu\text{l}$  using two wells for each concentration. In each plate, two wells with untreated parasites were maintained as controls. After three days of incubation, viability was estimated by the colorimetric MTT dye reduction assay of Mossman (1983).

#### STABILITY OF RESISTANCE

PR was removed from the PR<sup>R800</sup> promastigote culture by centrifugation and washed twice with PBS to remove adherent drug. The cells were then resuspended in fresh RPMI 1640 medium lacking the inhibitor. The culture was separated into two parts. PR 800  $\mu\text{M}$  was added (PR<sup>R8001+</sup>) to one flask while the second was cultured without inhibitor (PR<sup>R8001-</sup>). When the cultures reached the end of the exponential growth phase, two new cultures were prepared from the later culture at a cell density of  $2 \times 10^6$  promastigote ml<sup>-1</sup>,

one with 800  $\mu\text{M}$  PR (PR<sup>R8001+</sup>) and one without inhibitor (PR<sup>R8002-</sup>). PR<sup>R8002-</sup>, at the end of the log phase, was again divided into PR<sup>R8003-</sup> and PR<sup>R8002-1+</sup>. This was repeated through 8 passages without PR (PR<sup>R8000-</sup> and PR<sup>R8008-1+</sup>). The growth curves were recorded, and the doubling times and the cell densities at the stationary phase were measured and compared. Thus, the PR resistance remained stable at least for two months in the absence of drug pressure.

#### INFECTIVITY OF PR-RESISTANT CLONES

##### In vitro

Peritoneal macrophages were isolated from CD1 mice (Charles River Ltd, Margate, UK) and were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum in Labtek 8 chamber tissue culture slide (Nunc, Inc, Naperville, IL, USA). Wild-type or PR-resistant promastigotes and PR were added at the same time to macrophages and maintained in a CO<sub>2</sub> incubator at 37°C for 48 hours. At the end of the experiments, cultures were fixed with methanol and stained by Giemsa. The percentage of infected macrophages was determined for each drug concentration using a light microscope.

##### In vivo

Infectivity and resistance to PR were also analysed after a passage in mice. Five female Balb/C mice were infected by wild-type promastigotes and another group of five mice by PR-resistant promastigotes by retro orbitary "sinus" injection of a 0.2 ml inoculum ( $4 \times 10^7$  cells). Three weeks later, mice of each group were killed, their livers were removed, and weighed. Impression smears were made, fixed in methanol and stained by Giemsa. Isolated amastigotes from the liver were resuspended in culture medium and their susceptibility to PR determined.

#### DETERMINATION OF PR UPTAKE BY HPLC

No analytical method for detection and quantification of PR using HPLC has yet been described. The only known procedure to detect PR is by gas column chromatography (Tsuji & Robertson 1970), but this method requires derivatization of PR. As the derivatization reactions are frequently incomplete, the procedure is not reproducible. Our protocol to measure the intracellular concentration of PR in promastigotes by HPLC and reversed phase column C18 is sensitive. Concentrations as low as 0.25 nmol of PR could be detected within 40 min.

Log-phase promastigote cultures, treated or not with 150  $\mu\text{M}$  PR for various times, were harvested and washed twice with cold BPS to remove adherent drug for the wild type and with 800  $\mu\text{M}$  for the resistant

clone. The pellets were suspended in 5% perchloric acid (PCA). Following 20 min of incubation at 4°, the acid soluble components were separated from promastigote proteins by centrifugation for five min at 5,000 g. The supernatant was removed, filtered through at 0.45 µm Nalgene filter and 20 µl (equivalent to 0.8 mg of protein) was injected by an injector model 715 ULTRA WISP directly for chromatography.

#### CHROMATOGRAPHY SYSTEM AND DETECTION CONDITIONS

The HPLC system consisted of Waters Assoc. (France) model 600E system controller. Analyses were carried out at 20 ± 2°C, on a reversed phase Kromasil C18 column 250 mm × 4.6 mm (5 µm particle size) purchased from Eka-Nobel (France). The mobile phase used for the analysis of *Leishmania* extracts was made up of 200 mM sodium sulfate and 20 mM pentane-sulfonate sodium salt adjusted to pH 6.8 or pH 8.5 with concentrated NaOH. Using a flow-rate of 1.5 ml/min, runs were initiated at pH 6.8 for eight min, the pH was then increased to 8.5 over 30 s. The later pH was then held for 15.5 min before being dropped to 6.8 over 30 s and kept for 16.5 min before the injection of another sample.

#### POST-COLUMN DETECTION

The reagent was made up of 20 ml of a solution of 4% OPA in methanol, 250 ml of 100 mM borate buffer pH 11 and 1 ml of β-mercaptoethanol. The post-column detection system consisted of an isocratic pump which assured a flow-rate of 0.5 ml/min of the reagent. The mixture reagent-eluate was driven through a water-bath at 45°C and then to a detector of fluorescence (Waters, model 420E) which was coupled with a chromato-integrator Merck D-2500 (France). Exciting and emission wavelengths were 340 nm and 455 nm respectively.

#### STANDARD CURVE

From a 1 mM PR solution, several dilutions were made with sterilised water. 2° µl of every concentration was injected for the analysis.

#### PROTEIN DETERMINATION

Protein concentration was measured by the dye-binding method of Bradford (1976) using bovine serum albumin as the standard.

#### VERIFICATION OF AN EVENTUAL INACTIVATION OF PR BY THE RESISTANT CLONES

Cultures grown with or without PR were arrested after different times of incubation and the supernatant fil-

tered after centrifugation of the cells. The activity of the supernatant was tested as described for the determination of the phenotype, in comparison to authentic PR after adequate dilutions.

#### DNA ISOLATION

Promastigotes were washed with PBS and homogenized in 1 ml of extraction buffer containing 100 mM Tris-HCl pH 8.5, 10 mM EDTA, 100 mM NaCl, SDS 0.1% and dithiothreitol 50 mM. The lysate was incubated with proteinase K for two hrs at 50°C. After centrifugation for five min at 10,000 g, the supernatant was extracted first by phenol and then by phenol-chloroform-isoamyl alcohol (50/49/1). DNA was precipitated by 3 M sodium acetate pH 5.3 and absolute ethanol. After centrifugation for 15 min at 10 000 g at 4°C, the DNA was washed with 70% ethanol, dried and then resuspended in 100 µl of sterile water.

#### POLYMERASE CHAIN REACTION

Genes of cytoplasmic and mitochondrial small subunit (SSU) rRNA were amplified by polymerase chain reaction (PCR) using the following primers: ATC-CAGCTGCAGGTTACCTAC, (162 pb; ATCCA is located between sequences 4012 and 4038) and GTCCCTGCATTTGTACACACCG, (GTCCC is located between sequences 3781 and 3804) for nuclear genes, Looker *et al* (1988) and TTTAGAAGGTATTGTTGCC and TATATTAATTACTGCACGTT for the mitochondrial gene (61 pb), De La Cruz *et al* (1985). TTTAG is located between the sequences 3786 and 3807 and TATATT is located between the sequences 3988 and 4013. *Taq* polymerases purchased from [Promega] or from [Appligène] were used to amplify nuclear and mitochondrial genes respectively. The PCR conditions for nuclear gene amplification were as follows: heat denaturation at 95°C for five min, and 30 cycles: denaturation at 95° for 45 s, annealing at 45°C for 45 s and elongation at 72°C for 45 s. The last elongation was lengthened to five min. Reaction products were electrophoresed on 1.4% agarose gels.

#### MOLECULAR CLONING

Both wild-type and paromomycin-resistant *L. donovani* SSU rRNA genes were amplified and then cloned into the vector T (2887bp, lac Z<sup>+</sup>, Ap<sup>R</sup>) using the pMOS Blue T-vector kit (Amersham). The experimental conditions of the manufacturer were applied. Positive white bacterial clones were selected and the presence of the insert was detected by a rapid method using the conditions of PCR mentioned above. A miniprep was made for each positive clone and the presence of the insert was checked.

SEQUENCING OF PCR PRODUCTS AND CLONES

Double stranded PCR products were directly sequenced using DNA sequencing kit (USB), Amersham after purification by exonuclease I and alkaline phosphatase. Inserted DNA was sequenced using the T7 polymerase (Pharmacia kit).

RESULTS

CHARACTERIZATION OF THE PR-RESISTANT CLONES

We developed by increasing drug pressure promastigotes resistant to 800 µM PR, this concentration is six-seven times higher than the IC 50 measured for the wild type. The acquisition of resistance was associated with decreased growth rate. The generation time of the resistant promastigotes was 24 hrs which is about three times longer than that of the wild-type. The cellular yield of the resistant strain at the end of the stationary phase was 27 % lower in comparison to that of the wild-type promastigotes.

STABILITY OF THE RESISTANCE

In the absence of drug pressure, resistance to PR was maintained up to eight passages (two months) in the cultures. Promastigotes grown in the absence of the drug for two months could multiply in the presence of 800 µM PR without a lag phase.

INFECTIVITY OF THE RESISTANT CLONES *IN VITRO* AND *IN VIVO*

Wild-type and PR-resistant promastigotes had the same degree of infectivity for the macrophages *in vitro*. The wild-type infected macrophages responded to PR treatment, as the extent of the infection decreased with the increase of PR concentration. On the contrary, the macrophages infected with the PR-R cells remained resistant to PR treatment up to 1250 µM, indicating that

Treatment [µM] PR	% of infested macrophages after 48 hrs	
	Wild type	Resistant
	70 %	70 %
10	62 %	70 %
125	36 %	69 %
250	18 %	70 %
1250	n. d.	70 %

Macrophages were isolated, infected by promastigotes and treated with PR as described in Materials and Methods. (n. d): not determined.

Table I. – Response to PR-treatment of macrophages infected with wild type and PR-resistant promastigotes.

they conserved the resistant phenotype upon transformation to amastigotes (Table I). BALB/c mice injected with wild-type and resistant promastigotes, became infested after three weeks. The parasites isolated from the liver and the spleen of the mice infected with the resistant clone conserved their resistance, as they could grow in a PR-containing medium.

PHENOTYPE

Growth inhibition of wild-type and resistant clones by several drugs was measured (Table II). Susceptibility of the resistant clone to amphotericin B and sinefungin did not change relative to that of the wild type strain, but it showed somewhat lower susceptibility to pentamidine and allopurinol. Verapamil, a calcium channel inhibitor did not reverse PR-resistance, suggesting that the drug resistance of this clone does not involve the MDR-phenotype.

Molecules	Wild type	Resistant
Pentamidine	7.50	15
Amphotericin B	0.33	0.30
Paromomycin	120	> 1,250
Sinefungin	0.024	0.035
Allopurinol riboside	2.85	> 10

Values representing the IC<sub>50</sub> (µM) were measured after an exposure for 72 hrs to the different drugs described in Materials and Methods.

Table II. – Phenotype of wild-type and resistant clones.

SEQUENCES OF THE SMALL SUBUNIT rRNA GENES

The region sequenced for both nuclear and mitochondrial SSU rRNA genes encompasses the region supposed to be involved in PR-resistance. The two critical paired nucleotide sites are located at the root of the stemloop 47 in the variable domain V9 in the secondary structure of the 16S rRNA of *Escherichia coli* (nucleotides 1409 and 1491). Their homologous counterparts are nucleotides 2067 (T) and 2185 (A) for the nuclear gene of *L. donovani* (Looker *et al.*, 1988) and nucleotides 558 (T) and 572 (A) for the mitochondrial gene of *L. tarentolae* (De la Cruz *et al.*, 1985). The sequences obtained for sensitive promastigotes were identical to these published sequences. For the resistant strain we have directly sequenced the PCR products but, in spite of some non specific arrests on the gels, no difference was noted. However, in order to detect a possible microheterogeneity of the amplified DNA, we have sequenced 20 clones of the nuclear gene and 17 clones of the mitochondrial gene. Before determining the sequences of the cloned PCR products, we amplified the inserts of bacterial clones and obtained PCR products of the expected lengths. All the sequences obtained were identical to those of the

sensitive strain. A singular nucleotide was however observed in one clone of the nuclear gene in position 2111 where a T was replaced by C.

#### COMPARISON OF PR UPTAKE BY WILD TYPE AND RESISTANT CLONES

We developed a sensitive method which could detect concentration as low as 0.25 nmol PR and to quantify

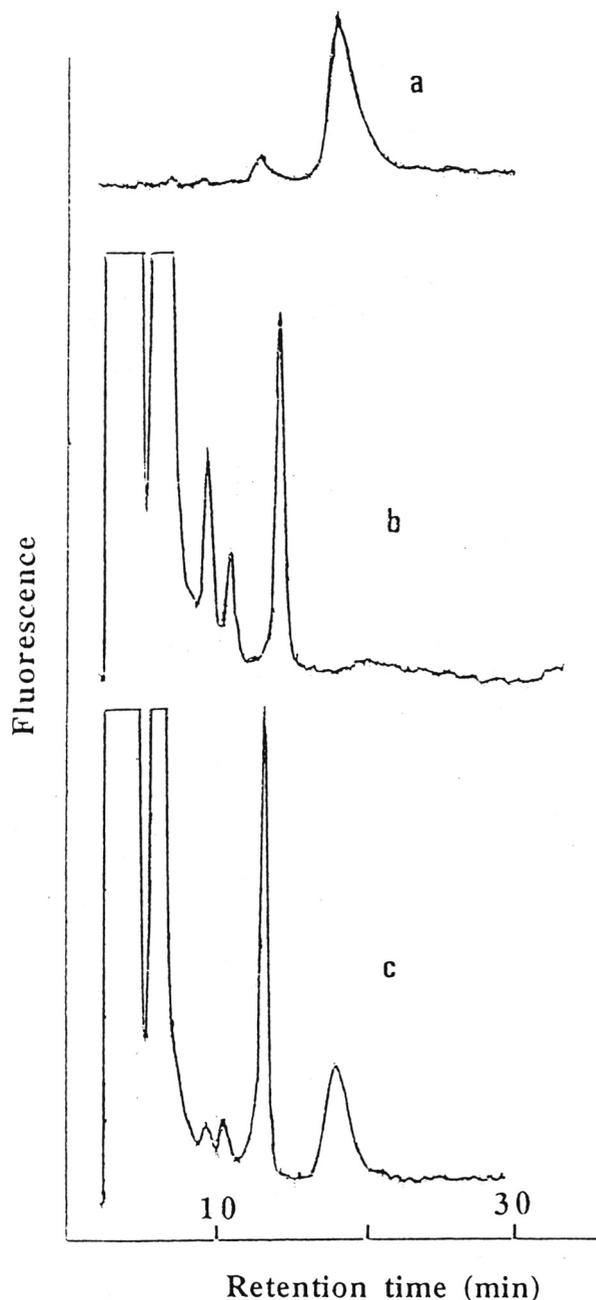


Fig. 1. – Profiles of the PR standard and the extract of untreated and PR-treated *L. donovani* promastigotes. *a*: standard PR, *b*: extract of untreated promastigotes prepared as mentioned in the experimental section, *c*: extract of PR-treated promastigotes, cells were treated by 150  $\mu\text{M}$  PR as mentioned in the experimental section.

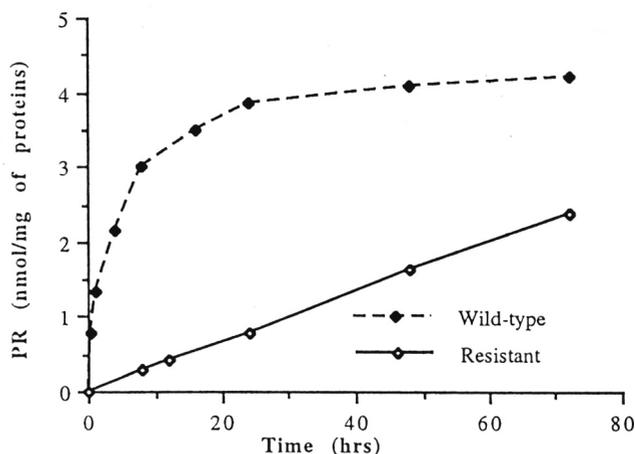


Fig. 2. – Uptake of PR in wild-type and PR-resistant promastigotes. Wild type promastigotes were treated with 150  $\mu\text{M}$  PR for different times. Resistant cells were cultivated without PR and then treated with 800  $\mu\text{M}$  PR for different times. Cellular extracts were prepared as described in materials and methods. Results represent the intracellular amounts of PR, measured as nmol/mg of proteins, as function of the time of treatment.

intracellular paromomycin by HPLC (Fig. 1) (see Materials and Methods). Wild type promastigotes were treated with 150  $\mu\text{M}$  PR corresponding to the IC<sub>50</sub> value, for various times from 30 min. to 72 h. As the calculated cellular volume of a promastigote is 22  $\mu\text{M}^3$  and 1 mg of protein is equivalent to  $2.72 \times 10^8$  promastigotes (Phelouzat *et al.*, 1992), the intracellular concentration of PR could be determined, as a function of incubation times (Fig. 2). The intracellular concentration of PR was 126  $\mu\text{M}$  and 628  $\mu\text{M}$  after treatment of promastigotes for one and 24 h respectively. This indicates that PR accumulated in the cells, its intracellular concentration after 24 h being four times higher with respect to the extracellular concentration. In resistant promastigotes treated with 800  $\mu\text{M}$  PR, the antibiotic enters slowly. Its intracellular concentration is 128  $\mu\text{M}$  after 24 h and 426  $\mu\text{M}$  after 72 h. This concentration is higher than the IC<sub>50</sub> value but lower than the extracellular drug concentration, indicating that in resistant cells PR does not accumulate.

#### CONTROL OF AN EVENTUAL INACTIVATION OF PR BY THE RESISTANT CELLS

PR-resistance in bacteria was shown to be due to enzymatic modification (Maeda *et al.*, 1968). To verify that PR-resistance in *Leishmania* is due to inactivation of the antibiotic, the effect of the supernatants of the resistant cells grown in the presence of PR were tested on wild type promastigotes. According to our results these supernatants even after seven days incubation had the same activity on the wild type than authentic PR indicating that PR-resistance was not due to its inactivation by the resistant cells.

## DISCUSSION

The main molecular mechanisms involved in drug resistance are changes in the uptake qualitative or quantitative modifications of the targets or enzymatic inactivation of the drug. We report here that step-by-step drug pressure induces specific and stable resistance to PR in *L.donovani*. The PR-resistant promastigotes keep their infectivity for macrophages *in vitro* and for mice *in vivo*. No analytical method for detection and the quantification of PR using HPLC has yet been described. The only known procedure to detect PR is by gas column chromatography Tsuji & Robertson (1970), but this method requires the derivatization of PR, which is frequently incomplete, and not reproducible. Reversed-phase kromasil columns are used generally for the analysis of amino acids. As PR is a basic compound and has five amino functions, this column could easily be adapted. We established a protocol to measure the intracellular concentration of PR in *L.donovani* promastigotes by HPLC and a reversed phase column C18. This method is sensitive and reproducible and enabled us to detect low PR concentrations within 40 minutes. The resolution of PR was dependent on the temperature. Temperatures higher than 22°C did not permit good separation and at temperatures lower than 17°C the elution buffer precipitated. For the sake of reproducibility, the analysis should be performed between 18 and 22°C. PR does not absorb U.V., so fluorescence detection was used. A post-column system had been optimized with OPA as chromophore reagent.

The extraction conditions are important for the recovery of PR in treated promastigotes. Three different methods for the extraction of PR from the cells have been used and compared: (a) PCA, (b) trichloroacetic acid or (c) a mixture of 10 % butanol in 1M formic acid. The best results were obtained when the extract, prepared with PCA, was used directly or after a short period of freezing.

The mechanism of uptake of PR by the promastigotes of *Leishmania* was studied by this method. As shown, paromomycin enters rapidly in these cells where it accumulates, suggesting active transport.

Analysis of PR uptake by resistant clones shows that this antibiotic enters slowly into the cells and suggests a passive diffusion. However its intracellular concentration after 72 hrs of incubation was four times higher in comparison to the IC50 of wild-type suggesting the implication also of biochemical changes other than the membrane.

Several articles have reported that the mechanism responsible for PR-resistance is a mutation at one of the two-paired bases C 1491 which are located at the root of domain 49 of *E.coli* [De Stasio (1985)], several

eukariotes resistant to PR carry a mutation occurring at a homologous site: C → at nucleotide 1514 in the mitochondrial 15S rRNA gene for *Saccharomyces cerevisiae* [Li *et al.* (1982)], G → A at nucleotide 1707 in the nuclear 17S rRNA gene for *Tetrahymena thermophila* Brums *et al.* (1985). According to Edlind (1991), the presence of paired bases at homologous positions in bacterial and eukariotic rRNA predicts sensitivity to PR whereas a disruption is indicative of resistance to this antibiotic. However this relation was overruled by Fong *et al.* (1994) in *L. tropica* where no disruption was detected at the nuclear DNA nor in the RNA product. However, the possibility of sequence heterogeneity could not be entirely ruled out by the authors. In addition, the possibility of a mutation in the mitochondrial gene still remains as we have previously shown that mitochondrial ribosomes are a target of PR in *Leishmania* parasites *in vitro* Maarouf *et al.* (1995).

Resistance can be conferred to a cell by mutation occurring in few copies of repeated genes. As a matter of fact, the number of rRNA operons is only 7 in *E. coli*, a single nuclear gene exists in *Tetrahymena* and in spite of multiple copies of mtDNA molecules in *S. cerevisiae*, the resolution of heteroplasmy is generally achieved in few generations. On the other hand, nuclear genes of rRNA are in multiple copies in the nucleus of *Leishmania*, about 160 according to Leon *et al.* (1978). Similarly, few dozens of maxicircles exist in the kinetoplast, and the resolution of a possible heteroplasmy is probably impeded by their catenation Borst (1991). Our observations involve 20 sequences of amplified rDNA of the nucleus and 17 for its mitochondrial counterpart, all without mutation at the critical nucleotides. If mutations in the rRNA genes are responsible for resistance to PR in *Leishmania*, it seems reasonable to conclude that the critical nucleotides are not the only targets, both in the nucleus and in the kinetoplast. The singular transition observed in one clone cannot be conclusive, even if this mutation is a region involved in translation Dalhberg (1991), because one substitution in 160 sequences is in the range of *Taq* mistakes during amplifications. Thus, the main mechanism of PR-resistance seems to be due to decreased drug uptake.

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## REFERENCES

- ARMITRAGE K., FLANIGAN T., CAREY Y., FRANK I., MACGREGOR R.R., ROSS P., GOODGAME R., & TURNER J. Treatment of cryptosporidiosis with paromomycin: a report of five cases. *Archives of Internal Medicine*, 1992, 152, 2497-2499.
- BORST P. Why kinetoplast DNA network. *Trends in Genetics*, 1991, 7, 139-141.
- BRADFORD M.M. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. *Analytical Biochemistry*, 1976, 72, 248-254.
- BRUMS P.J., KATZEN A.L., MARTIN L. & BLACKBORN E.H. A drug-resistant mutation in the ribosomal DNA of *Tetrahymena thermophila*. *Proceedings of the National Academy of Sciences USA*, 1985, 82, 2844-2846.
- DAHLBERG A.E. The function role of ribosomal RNA in protein synthesis. *Cell*, 1991, 57, 525-529.
- DE LA CRUZ V.F., LAKE J.A., SIMPSON A.M. & SIMPSON L. A minimal ribosomal RNA: Sequence and secondary structure of the 9S kinetoplast ribosomal RNA from *Leishmania tarentolae*. *Proceedings of the National Academy of Sciences USA*. 1985, 82, 1401-1405.
- DE STASIO, E.A. Mutations in 16S ribosomal RNA disrupt antibiotic-RNA interaction. *EMBO Journal*, 1989, 8, 1213-1216.
- EDLIND T.D. Protein synthesis as a target for antiprotozoal drugs. In: *Biochemical Parasitology*, 1991, pp. 578-594, Taylor and Francis, London.
- FONG D., CHAN M.T., RODRIGUEZ R., GATELY L.Y., BERMAN Y.D. & GROGL M. Paromomycin resistance in *Leishmania tropica*: lack of correlation with mutation in the small subunit ribosomal RNA gene. *American Journal of Tropical Medicine and Hygiene*, 1994, 51, 758-766.
- GILLIN F.D. & DIAMOND L.S. Inhibition of clonal growth of *Giardia lamblia* and *Entamoeba Histolytica* by metronidazole quinacrine and other antimicrobial agents. *J. Antimicrobial Agents & Chemotherapy*, 1981, 8, 305-316.
- IOVANNISCI D.M. & ULLMAN B. High efficiency plating method for *Leishmania* promastigotes in semi-defined or completely-defined medium. *Journal of Parasitology*, 1983, 69, 633-636.
- KATSU M., FUJIMORI I., OGAWA J., ITOS, L. & SHIMADA S. The effect of aminosidine on infections in the domain of internal medicine. *Chemotherapy*, 1968, 16, 171-173.
- LEON W., FOUTS D.L. & MANNING Y. Sequence arrangement of the 16S and 26S rRNA gene in the pathogenic haemoflagellate *Leishmania donovani*. *Nucleic Acid Research*, 1978, 5, 491-501.
- LI M., TZAGOLOFF A., UNDERBRINK-LYON K. & MARTIN N.C. Identification of the paromomycin-resistance mutation in the 15S rRNA in the yeast mitochondria. *Journal of Biological Chemistry*, 1982, 257, 5521-5528.
- LOOKER D., MILLER L.A., ELWOOD H.J., STICKEL S. & SOGIN M.L. Primary structure of the *Leishmania donovani* small subunit ribosomal RNA coding region. *Nucleic Acid Research*, 1988, 6, 7198.
- MAAROUF M., LAWRENCE F., CROFT, S.I. & ROBERT-GERO M. Ribosomes of *Leishmania* are a target for the aminoglycosides. *Parasitology Research*, 1995, 81, 421-425.
- MAEDA K., KONDO S., OKANISHI M., UTAHARA R., UMEZAWA M. Isolation of paromamine inactivated by *Pseudomonas aeruginosa*. *Journal of Antibiotics*, 1968, 21, 458-459.
- MOSSMAN T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 1983, 65, 55-63.
- PHELOUZAT M.A., LAWRENCE F., MOULAY L., BORO, C., SCHAEVERBEKE J., SCHAEVERBEKE M. & ROBERT-GERO M. *Leishmania donovani*: antagonistic effect of S-adenosyl methionine on ultrastructural changes and growth inhibition induced by methionine sinefungin. *Experimental Parasitology*, 1992, 72, 177-187.
- SCOTT J.A.M., DAVIDSON R.N., MOODY A.H., GRANT H.R., FELMINGHAM D., SCOTT G., OLLIARO P. & BRYCESON A.D.M. Aminosidine in the treatment of leishmaniasis imported into the United Kingdom. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1992, 86, 617-619.
- TSUJI K. & ROBERTSON J.M. Gas-liquid chromatographic determination of amino-glycoside antibiotics: kanamycin and paromomycin. *Analytical Chemistry*, 1970, 42, 1661-1663.

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