CHAPTER 3

Mechanism of pentamidine resistance in *Leishmania donovani*
1. Introduction

*Leishmania donovani*, a flagellated protozoan parasite, is the causative agent of visceral leishmaniasis. Sandflies transmit promastigote forms of the parasite to the mammalian host, where they invade macrophages and transform into amastigotes. Pentavalent antimonials are the standard first-line treatment for leishmaniasis (Aggarwal *et al.*, 1999, Berman, 1996), although resistance is a growing problem (Khalil *et al.*, 1998, Lira *et al.*, 1999, Thakur *et al.*, 1993, 2001). The aromatic diamidine pentamidine represents a second line of treatment (Amato *et al.*, 1998, Becker *et al.*, 1999, Sands *et al.*, 1985, Soto *et al.*, 1994). Pentamidine is a dicationic drug that has been used for more than 50 years in the therapy and prophylaxis of African trypanosomiasis and *Pneumocystis carinii* pneumonia in AIDS patients (Sands *et al.*, 1985, Goa *et al.*, 1987) as well as antimony resistant leishmaniasis. However the mode of action of this drug against parasites is still not understood.

It has been reported that in various kinetoplastidae, pentamidine inhibits S-adenosyl-L-methionine decarboxylase (Bitonti *et al.*, 1986), uptake of radioactive putrescine, interferes with polyamine synthesis (Bachrach *et al.*, 1979), mitochondrial topoisomerase II (Shapiro *et al.*, 1990, Shapiro, 1993), mitochondrial membrane potential (Vercesi *et al.*, 1992), calcium transport (Benaim *et al.*, 1993), thymidylate synthetase (Kaplan *et al.*, 1977). Resistance to pentamidine has been described for trypanosomes (Berger *et al.*, 1993, 1995), *L. donovani* (Khalil *et al.*, 1998, Thakur *et al.*, 1993, Wijers *et al.*, 1974) and other *Leishmania* species (Basselin *et al.*, 1997, 1998, Giri *et al.*, 1994, Sereno *et al.*, 1997). Yeast are also susceptible to pentamidine (Ludewig *et al.*, 1994), and a gene called *PNT1*, when overexpressed, confers resistance to the drug in *Saccharomyces cerevisiae* (Ludewig *et al.*, 1994). The protein encoded by this gene localizes to the inner membrane of the mitochondrion and appears to play a role in secretion of proteins from the mitochondrion (He *et al.*, 1999). Disruption of mitochondrial function has been proposed as a likely toxic effect of pentamidine in *S. cerevisiae* (He *et al.*, 1999). The mitochondrion has also been implicated in the action of pentamidine against trypanosomatids. Electron microscopy revealed that treatment of *Leishmania* species with pentamidine leads to disintegration of the kinetoplast and mitochondrion (Croft *et al.*, 1982, Hentzer *et al.*, 1997) and a collapse in mitochondrial potential (Vercesi *et al.*, 1999), is one of the first manifestations of treatment of these parasites with the drug.
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The mechanism of action of pentamidine remains a matter of debate but selective toxicity may relate to high levels of accumulation of the drug in the case of African trypanosomes (Carter et al, 1999, Damper et al, 1976a). Damper and Patton (Damper et al, 1976a, 1976b) first reported that high affinity transporters that are energy dependent and competitively inhibited by other diamidines mediate the uptake of pentamidine by Trypanosoma brucei brucei bloodstream forms. The P2 amino-purine transporter of Trypanosoma brucei (Carter et al, 1995) appears to play a key role in the transport of this drug, although the situation is complex (Carter et al, 1999, Maser et al, 1999) with at least two additional transporters also capable of carrying the drug (De Koning et al, 2001). Loss of P2 nucleoside transporter can mediate resistance to diamidines including diminazene aceturate (berenil) in African trypanosomes (Barrett et al, 1995, 1999, Carter et al, 1993). However, the presence of multiple pentamidine transporters in T. brucei ensures that P2-defective parasites are still sensitive to this drug (De Koning et al, 2001).

In Leishmania sp. uptake of pentamidine has also been shown to be saturable, involving a carrier-mediated, energy dependent process (Basselin et al, 1996, Berman et al, 1987). Pentamidine was found to be a competitive inhibitor of arginine transport (Kandpal et al, 1995, 1996) and a noncompetitive inhibitor of putrescine and spermidine transport in L. infantum (Reguera et al, 1994), L. donovani, and L. mexicana (Basselin et al, 2000). However, experiments using radiolabelled pentamidine in L. mexicana, failed to show reciprocal inhibition with arginine or polyamines. The physiological substrate for the transporter that carries pentamidine has yet to be identified in Leishmania. Exclusion of diamidines from the mitochondrion, in a manner associated with diminished mitochondrial membrane potential, has been proposed to contribute to the resistant phenotype in L. mexicana.

A separate study showed that episomal expression of a P-glycoprotein, Pentamidine Resistant Protein1 (PRP1) belonging to the ABC transporter superfamily, could confer low-level resistance to pentamidine in L. major (Coelho et al, 2003). PRP1 mediated pentamidine resistance could be reversed by verapamil and PRP1 overexpressors were 1.7-3.7 fold resistant to pentamidine and also cross resistant to trivalent antimony but not pentavalent antimony. The subcellular localization of that transporter was not clear.

It has recently been proposed (Mehta et al, 2003) that inhibition of respiratory chain complexes can induce apoptosis in Leishmania donovani. Moreover, inhibitors of
complex II of the respiratory chain are synergistic with pentamidine’s leishmanicidal activity – again pointing to a possible role of the mitochondrion in the action of this drug.

In this study we set out to determine the basis of resistance to pentamidine in \textit{L. donovani}, the most important pathogenic \textit{Leishmania} species that is responsible for visceral leishmaniasis in India.

2. Main Objectives
To study the mode of action of pentamidine and its mechanism of drug resistance in \textit{L. donovani}
A Biochemical and molecular basis of pentamidine resistance in \textit{L. donovani}
B Comparative proteomics to reveal changes in pentamidine resistant \textit{Leishmania donovani}

3. Materials and Methods
3.1 Materials
Cell culture materials like M-199, RPMI-1640, were purchased from Sigma, USA. FBS was purchased from Gibco/BRL, Life Technologies, Scotland, UK and bacto-agar was procured from Difco.
Antibiotics like penicillin-G, streptomycin sulphate, G418, hygromycin-B, ampicillin were purchased from Sigma, USA.
General chemicals like Tris, DTT, glycerol, EDTA, SDS, Proteinase K, MOPS, sodium acetate, bromophenol blue, xylene cyanol FF, HEPES, PLP, ornithine, dansyl chloride, perchloric acid, diaminohexane, proline, putrescine, spermidine, spermine, coomassie brilliant blue G-250, PMSF, leupeptin, aprotinin, protease inhibitor cocktail, MTT, BSA, dimethyl formamide, sodium carbonate, digitonin, mineral oil were procured from Sigma, USA. Ethanol, dibutylphthalate was purchased from Merck.
Analytical grade and molecular biology grade chemicals that were purchased locally are listed below: acetic acid, acetone, ammonium acetate, chloroform, glucose, glycine, glycerol, \( \text{H}_2\text{O}_2 \), HCl, isoamyl alcohol, isopropanol, KCl, \( \text{KH}_2\text{PO}_4 \), \( \text{MgCl}_2 \), methanol, NaCl, \( \text{NaH}_2\text{PO}_4 \), \( \text{Na}_2\text{HPO}_4 \), NaOH, potassium acetate.
Fluorescent dyes like rhodamine 123, nonyl acridine orange, nile red, TMA-DPH were purchased from Sigma, USA.
Chemicals for enzyme activities like DCIP, phenazine methosulphate were purchased from Hi-media, NADH, Potassium ferricyanide, cytochrome c, sodium succinate, fumaric
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Acid disodium salt, ammonium molybdate, ATP, EGTA were purchased from Sigma, USA.

Inhibitors like pentamidine isethionate, berenil, DAPI, verapamil, trifluoperazine dihydrochloride (TFP), prochlorperazine dimaleate (PCP), vinblastine, sodium arsenite, were purchased from Sigma (St. Louis, MO). Pentostam was a gift from The Wellcome Foundation Ltd, UK and glucantime from Rhône-Poulenc, Specia, France. Propamidine and Stilbamidine were kindly donated by Dr Harry De Koning, (University of Glasgow) and DB99 was kindly donated by Dr David Boykin (University of Georgia). CCCP, DCCD, valinomycin, oligomycin, sodium azide, monensin, ouabain, sodium orthovanadate were also procured from Sigma (St. Louis, MO).

Antibodies: Anti TKT antibody was a kind gift from Dr. Richard Burchmore, University of Glasgow, goat anti rabbit IgG HRP conjugated secondary antibody was purchased Santa Cruz Biotechnology.

Kits used were: Qiagen R Plasmid midi kit, Mini Elute™ Gel extraction kit, QIAprep R Miniprep were purchased from Qiagen, Germany. ECL detection kit was used from Santa Cruz Biotechnology, Inc, California, USA.

Radioactive materials like [Ring-3 H] pentamidine (98 Ci/mmol, 5 mCi/ml) was custom synthesized by Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). L-\(^{14}\)C- ornithine was purchased from NEB, England

Molecular biology enzymes: Restriction enzymes, ligases, other modifying enzymes etc. were purchased from MBI Fermentas, Promega, New England Biolabs, USA. RNase free DNase was bought from Qiagen, USA. Pfu DNA polymerase was from MBI fermentas, USA.

Filters, membranes and films: Membrane filters of 0.22 \(\mu\)m pore size was purchased from Millipore, USA. Other filter papers were purchased from Whatman, USA. Hybond™-P, membrane were purchased from Amersham Biosciences, USA. Amersham Hyperfilm™ ECL™ was used for autoradiography and ECL detection.

DNA constructs like psp72\(\alpha\)neo\(\alpha\), was gifted by Prof. Marc Ouellette, Laval University, Canada, PRPI cloned in pSNBR was gifted by Dr. Paulo Cotrim, Brazil.

Chemicals used for DiGE analysis: Urea, thiourea, CHAPS, Tris, iodoacetamide, sypro orange, ammonium bicarbonate, trypsin were purchased from Sigma, USA. CyDyes, IPG strips, IPG buffer, were from GE healthcare. Decyder software to analyse the DiGE gels was purchased from Amersham, Biosciences.
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*E. coli* cells: DH5α was from Gibco BRL, Ltd., USA.

*Leishmania* strains: *L. donovani* (MHOM/IN/1983/AG83) was kindly provided by Prof. A.N. Bhadhuri, Indian Institute of Chemical Biology (IICB), Calcutta.

3.2 **Strains and culture conditions**

*L. donovani* AG83 (MHOM/IN/1983/AG83) promastigotes were grown *in vitro* at 22°C in modified M199 medium supplemented with 10% heat inactivated fetal bovine serum (FBS).

The pentamidine sensitive wild type strain of *L. donovani* was cloned on semisolid M199 medium containing 1% Bacto Agar (Difco) and 10% heat inactivated fetal bovine serum. Colonies were picked and transferred separately into the above-described liquid medium. The cloned wild-type cells were then cultured in the presence of 1 μM pentamidine. The culture was stabilized for 6 subcultures before increasing the drug level at one passage a week. The line growing in 1 μM drug was plated on M199-agar plates and a single clone (R1) was picked. This was subjected to stepwise increasing drug pressure with final concentrations of 2 μM, 4 μM and 8 μM. Cells from cultures growing at each concentration were cloned on semi-solid medium in the presence of drug. The clones were designated as R2, R4 and R8 respectively (Fig. 1).

Axenic amastigotes were obtained after transformation of promastigotes to amastigotes and were grown in RPMI-1640 medium (pH 5.5) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin sulphate and 20% heat inactivated serum in a CO₂ incubator (5% CO₂) at 37°C.

3.3 **Cross resistance studies**

The growth sensitivities of Wild type (WT) and pentamidine resistant strain (R8) to different cytotoxic agents were determined by plating the cells in 96 well microtitre plates. Briefly, 1 × 10⁵ parasites in 200 μl medium were incubated with different concentrations of the drugs. Drugs were added at 20 times the final concentration in a volume of 10 μl in triplicate (with the concentration range depending on the drug). After 72 h of drug addition, cell densities were determined by a Neubauer hemocytometer. The effective concentration of the drugs, which inhibited 50% of cell proliferation of wild type and R8 after 72 h, was denoted as the IC₅₀ value.

3.4 **Viability test of amastigotes**

To estimate 50% inhibitory concentrations (IC₅₀) of pentamidine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micromethod was used in
96 well tissue culture plates (Sereno et al., 1997). 200 µl of culture containing 5 x 10^7 cells/ml was plated in each well. Pentamidine was added at different concentration after 24 h and the plate was incubated at 37°C for 72 h. MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/ml and filter sterilized. At the end of incubation the MTT dye-reduction assay was performed. 20 µl of MTT (5 mg/ml) was added to the wells and further incubated for 4 h. The reaction was stopped by addition of 200 µl of acidic isopropanol (0.4 ml HCl 10 N in 100 ml isopropanol). The absorbance was measured at 570 nm.

3.5 Determination of mitochondrial transmembrane potential, mitochondrial biomass and in vivo dehydrogenase activities

Wild-type and resistant cells, treated or not treated with pentamidine were stained with 0.05 µM rhodamine 123 for 30 min to determine the mitochondrial membrane potential. The relative "biomass" was estimated with 10 µM of the cardiolipin dependent marker nonyl acridine orange (NAO). Mitochondrial membrane potential and biomass were assessed by flow cytometry. The excitation from an argon laser was set at 488 nm, 400 mW, emission at 525 nm using HBO100 lamp. (Maarouf et al., 1997a). Data were gathered with a Becton Dickinson LSR, flow cytometer. Rhodamine 123 and NAO fluorescence was collected in a photomultiplier tube designated as FL1. Data analysis was carried out with Win MDI software.

The MTT test also reflects mitochondrial dehydrogenase activities (Mossman et al., 1983). Activities were measured in 96 well plates by the method previously described in section 3.4 except for the fact that promastigotes were plated at a density of 1 x 10^6 cells/ml in a volume of 200 µl/well (Maarouf et al., 1997b). Activities of mitochondrial dehydrogenases were estimated as a function of cell density.

3.6 Isolation of mitochondria from promastigotes

Promastigotes of wild type L. donovani and R8 were collected by centrifugation and washed twice in ice cold phosphate buffered saline with 2 mM glucose, pH 7.4 (PBSG). Subsequent steps were performed at 4°C. Cells were resuspended at 1.2 x 10^9 cells/ml in 1 mM Tris, pH 8.0, 1 mM EDTA, briefly homogenized in an all glass homogenizer and lysed by passage through a 26-gauge needle. 60% sucrose was immediately added to the above lysates so that the final concentration of sucrose was 250 mM. The lysate was centrifuged for 10 minutes at 25,800 x g (Sorvall SS-34 rotor, 11,500 rpm). The pelleted material was resuspended at one-fifth of the original lysate
volume in 250 mM sucrose, 20 mM Tris, pH 8.0, 2 mM MgCl₂ and treated for 30 min with 9 μg/ml Rnase free Dnase (Qiagen). This pellet was further diluted 5-fold with 250 mM sucrose, 20 mM Tris, pH 8.0, 2 mM EDTA and centrifuged at 15,600 × g as described above. The pelleted material was resolved on a 20-35% Percoll gradient (45 min, Beckman SW rotor, 24,000 rpm), and a mitochondrial vesicle fraction was collected. Mitochondria were washed 4 times with an excess of 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA to remove percoll. Mitochondria could be stored for up to a month in 50% glycerol, 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA (Harris et al, 1990).

3.7 Biochemical assays of mitochondrial enzymes

All enzymatic assays from mitochondrial fractions except for the ATPase assay were assessed by a modified method originally described by Denicola-Seoane et al, 1992. The assays were performed using a final protein concentration of 0.04 mg/ml in 1 ml cuvettes using 50 mM phosphate buffer pH 7.5 with 5 mM azide to inhibit cytochrome oxidase and divert electrons to the artificial acceptor. Pentamidine was injected directly into the cuvette during the time of measurement.

(I) Succinate Dehydrogenase (SDH) activity was measured spectrophotometrically at 600 nm (ε = 20.5 mM⁻¹ cm⁻¹) using 3 mM succinate, 0.5 mM 2,6- dichlorophenolindophenol, 0.1 mM phenazine methosulphate. Succinate in the presence of PMS gets oxidised to fumarate and PMS gets reduced to PMSH₂. PMSH₂ reduces DCIP to DCIPH₂. The bleaching of DCIP to DCIPH₂ was measured at 600 nm.

(II) NADH dehydrogenase (NDH) activity was determined spectrophotometrically at 420 nm by measuring the rate of potassium ferricyanide reduction in the presence of NADH (ε = 1 mM⁻¹ cm⁻¹). The reduction of ferricyanide to ferrocyanide in the presence of NADH was measured at 420 nm.

(III) Succinate and NADH cytochrome c reductase (SCC and NCC) activities were measured spectrophotometrically at 550 nm (ε = 18.9 mM⁻¹ cm⁻¹) in the presence of 20 μM cytochrome c and either 5 mM succinate or 0.2 mM NADH. The reduction of ferricytochrome to ferrocytochrome by succinate or NADH was measured at 550 nm.

(IV) Mitochondrial ATPase activity was measured by an end point inorganic phosphate (Pi) release colorimetry assay. The amount of inorganic phosphate
(Pi) released over 10 min at 25°C was measured. Briefly, a standard 100 μl reaction mixture containing mitochondrial extract (0.04 mg) was incubated with ATPase buffer (150 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 0.5 mM EGTA), 100 μM Na-orthovanadate (inhibitor of P-type ATPases), supplemented with 3 mM ATP (Das et al, 1993). The transfer of the mixture from ice to room temperature initiated the reaction. The reaction was terminated by addition of 1 ml stop solution (0.5% SDS, 2% sulphuric acid, and 0.5% ammonium molybdate), followed by addition of 10 μl of freshly prepared coloring reagent (10% ascorbic acid) and absorbance was taken at 750 nm using UV-Vis 160A Shimadzu spectrophotometer. ATPase activity was plotted using a standard curve for 0-100 nmol of inorganic phosphate.

3.8 Transport studies

Parasites were harvested during the mid logarithmic phase of growth by centrifugation at 2,100 × g, for 10 min at 4°C. Cells were then washed twice with phosphate buffered saline supplemented with 1% D-glucose (PBSG) at pH 7.4. Cells were resuspended in this buffer at densities appropriate for the procedure to be used, as indicated in the text and figure legends. Parasite suspensions (100 μl, containing 2 × 10⁷ cells) were warmed to 25°C and mixed with 100 μl of assay buffer containing labeled molecule plus or minus other test compounds at the concentration indicated in the figure legend. Transport was terminated after times indicated in the figure legends by the rapid separation of parasites from the buffer components by centrifugation through a 9:1 mixture of dibutylphthalate (specific gravity, 1.04) and mineral oil (specific gravity, 0.875 to 0.885). The sample tubes were immediately flash frozen in liquid nitrogen, and the tubes were cut to separate the pellet from the transport medium. The pellet was dissolved in 2% sodium dodecyl sulfate (200 μl) and 3 ml of scintillation fluid (Ecoscint A, National Diagnostics). These were left overnight to remove the effects of chemiluminescence, and then incorporated radioactivity was counted in a Scintillation counter.

3.9 Inhibition studies

The effect of various metabolic inhibitors, ionophores and uncouplers on pentamidine accumulation in wild-type and resistant promastigotes was studied by resuspending cells in PBSG containing inhibitors at concentrations indicated in the figure
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legends for 15 min at 25°C. After this treatment, 5 μM [3H] pentamidine was added to the mixture and uptake was measured for 2 min. Accumulated label was counted as above.

3.10  Fluorescence microscopy

Cells from a mid-log phase culture were pelleted by centrifugation, washed (in PBSG) and incubated with 10 μM 2,5-bis (4-amidophenyl) 3,4 dimethyl furan, DB99, (a fluorescence analog of pentamidine) for 5 min and then transferred to a microscope slide and viewed by fluorescence microscopy (excitation at 330 nm, emission at 400 nm) with a Zeiss Axioscope and images were captured using the Improvision OpenLab Software.

3.11  Measurement of polyamine levels by High Pressure Liquid Chromatography (HPLC) and ODC activity

Mid log phase L. donovani promastigotes were harvested by centrifugation (2,000 x g, 15 min at 4°C). The resultant cell pellet was washed with phosphate buffered saline (pH 7.4, PBS) and resuspended in 50 mM Tris-HCl, pH 7.5, 10 μM EDTA and 2.5 mM DTT. The cells were then lysed by repeated freezing/thawing alternatively in liquid nitrogen and water. The lysates were centrifuged at 15,000 x g (20 min, 4°C) and the supernatant was used for determination of ODC activity, polyamines and protein content.

Assay of Ornithine decarboxylase (ODC): Ornithine decarboxylase activity was assayed by following the release of 14CO2 from L- [-14C] ornithine (Seely et al. 1982). The standard assay mixture was prepared. To the vials containing cell lysates of promastigotes, equal volume of reaction mixture (200 μM PLP, 12.5 mM DTT, 250 mM Tris pH-7.5, 2 mM ornithine, 3 μCi of the radiolabelled ornithine) was added and incubated at 37°C for 1 hour. Released 14CO2 was trapped in benzethonium hydroxide. The reaction was terminated by injecting 5N H2SO4 to the reaction solution and benzethonium hydroxide was put in scintillation vials containing equal volume of liquid scintillation fluid (COCKTAIL-O). "Total Count" vials were also taken containing scintillation fluid, as before, and reaction mixture. Counts were taken in β- scintillation counter. Activity is expressed in enzyme units, in which 1 unit is nmol of CO2 / mg protein/ h.

Polyamine estimation: Quantitative determination of polyamines in crude lysates of L. donovani was obtained by C18 reversed-phase high performance liquid chromatography (HPLC) after derivatization with dansyl chloride (Seiler et al, 1978). Briefly, to 200 μl of supernatant of cell lysate, 6 μl of 60% perchloric acid was added in the eppendorf. To this 10 μM internal standard, 1, 6-diamino hexane (DAH) and 100 μl saturated sodium
carbonate was added. 100 µl of 10mg/ml dansyl chloride solution was then added to the above reaction mixture (stock solution of dansyl chloride was in acetone to which NaOH was added to dissolve the powder). The mixture was incubated at 60°C for 1h. Following incubation, 50 µl of 100 mg/ml proline was added, vortexed and incubated at 60°C for 30 min. Acetone was removed from the mixture by vacuum drying. 400 µl toluene was added and vortexed for 1 min. It was centrifuged at 2000 x g for 10 min. 200 µl of toluene containing dansylated polyamines were aliquoted and evaporated to dryness. It was dissolved in 100 µl HPLC grade methanol. Standards of 10 µM putrescine, spermidine, spermine and DAH were prepared and processed in the same manner.

The column of HPLC (Shimadzu) was first equilibrated with methanol–water (57.5:42.5). 20 µl of the sample was applied to the column. Gradient elution was started with the same methanol concentration, which was then increased linearly by an increment of 0.5% per min. From 20 to 30 min the methanol increment was maintained at 1.5% per minute and it was further increased to 3% per min after 30 min. Elution with pure methanol was continued for 4 min to elute impurities. At 40 min the gradient was switched to the initial methanol - water mixture.

**Protein concentration determination:** Bradford method using bovine serum albumin as standard (Bradford, 1976) was used. Bradford reagent was prepared by mixing 100 mg of Coomassie brilliant blue G-250 in 50 ml of 95% ethanol, to which 100 ml of 85% phosphoric acid was mixed and the volume was made up to one liter with double distilled water.

The Bradford method is based on the detection of a blue colour that results after mixing a protein sample with Coomassie brilliant blue G-250. The blue colour is proportional to the amount of protein in the sample. By determining the optical density (OD) at a wavelength of 595 nm and by extrapolation of this OD₅₉₅ on a standard linear graph, the concentration of the protein can be determined. The standard linear graph is obtained by plotting known concentrations of BSA against their corresponding OD₅₉₅ after treatment with the Coomassie reagent.

### 3.12 Determination of lipids by flow cytometry

A stock solution of the phenoxazine dye Nile red was prepared at 100 µg/ml in acetone and stored in darkness at 4°C. Promastigotes (wild type, pentamidine treated, R8) were diluted with PBS to a density of 10⁷ promastigotes/ml and then stained with 100 ng/ml Nile red for 5 min and then individually analyzed by flow cytometry (BD-LSR,
Becton Dickinson, USA). The excitation wavelength of the argon laser was set at 488 nm and 400 mW. The fluorescent emission was filtered through a 515 nm long-pass filter (interference and absorbance) and split with a 590 nm dichroic mirror, sending the red component through a 610 nm long-pass filter to one photomultiplier and the yellow component through a 560 nm short-pass filter to another photomultiplier. Cellular fluorescence in the absence of dye was negligible. (Maarouf et al 1997b). In each analysis 10,000 promastigotes were studied. The intensity of red (IRF) and yellow fluorescence (IYF) was measured.

3.13 Determination of plasma membrane fluidity

The fluorescent probe 1-[4-(trimethylammonio)-1,6-diphenylhexal]-1,3,5-triene (TMA-DPH) was prepared as a 2 mM stock in dimethylformamide and stored in darkness at 4°C. Promastigotes (wild type, pentamidine treated, R8) were diluted with PBS to a density of $10^7$ promastigotes/ml and then stained with 2 μM TMA-DPH for 5 min and then analyzed in a spectrofluorimeter (Varian) with excitation at 357 nm and emission at 430 nm. Membrane fluidity was assessed by measurement of the fluorescence anisotropy (r) of TMA-DPH following its insertion into plasma membrane.

3.14 Western blot analysis

Late log phase promastigotes ($1 \times 10^8$) were harvested and the resultant cell pellet was resuspended in lysis buffer (20 mM MOPS, pH 7.2; 1 mM DTT; 2 mM phenylmethylsulfonyl fluoride (PMSF); 5 μl protease inhibitor cocktail). The cell pellet was lysed by sonication (6 cycles 10 sec each with 50 sec break) and cell supernatants were prepared by centrifugation at 20,000 g, 15 min at 4°C. Protein (50 μg) of promastigotes was fractionated by SDS-Polyacrylamide gel electrophoresis, blotted on to PVDF membrane using electrophoretic transfer cell (Bio-Rad) and probed with anti leishmania transketolase (TKT) antibody raised in rabbit (1/5000 dilution), and then with 1/10000 dilution of HRP-conjugated mouse anti-rabbit secondary antibody. Western blot analysis was done using Chemiluminescence kit (Amersham) and the film was Hyperfilm ECL from Amersham Biosciences. The results shown are from a single experiment typical of at least three giving identical results.

3.15 Cloning, transfection and overexpression of Transketolase (TKT) and Pentamidine Resistance Protein 1 (PRP1) construct in Wild type L. donovani

Primers were designed (Oligo program) for amplifying transketolase (TKT) from L. donovani genome. This was done based on the known sequence of TKT of L. major
and L. infantum from GeneDB. A sense primer with a flanking Xba I site, 5'-TGCTCTAGAATGGCCTCTATTGAGAAAGATTG-3' and the antisense primer with a flanking HindIII site, 5'-CCCAAGCTTTTCACATCTTGCTGAATGAGGAG-3' were designed. PCR was performed in 50 µl reaction volumes containing 100 ng of genomic DNA, 25 pmol each of the gene-specific forward and reverse primers, 200 µM of each dNTP, 2 mM MgCl₂ and 5 units of Pfu DNA polymerase. The conditions for PCR were as follows: 94°C for 10 min, then 35 cycles of 94°C for 45 sec, 51°C for 45 sec and 72°C for 4 min. Final extension was carried out for 10 min at 72°C. A single 2 kb PCR product was obtained and subcloned into psp-72αeoα Leishmania shuttle vector, containing neomycin phosphotransferase as the selection marker (obtained as a gift from Prof. Marc Ouellette, CHUL, Laval University, Quebec, Canada) and subjected to automated sequencing. Comparisons with other sequences of the database were performed using the search algorithm BLAST.

20 µg of TKT construct (TKT cloned as 2 kb XbaI-HindIII fragment in Leishmania shuttle vector psp-72αeoα) was transfected into L. donovani promastigotes by electroporation. For transfection 4 x 10⁷ log phase wild type L. donovani promastigotes were harvested. The cells were centrifuged at 3,500 rpm for 12 min. The pellet was washed with PBSG (10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 145 mM NaCl, 2% glucose, filter sterilized) and then with EP buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH₂PO₄, 6 mM glucose, filter sterilized) and centrifuged at 3,500 rpm for 12 min. To the pellet 400 µl EP buffer and 20 µg of the construct was added and then transferred to 2 mm gap electroporation cuvette. It was incubated on ice for 10 min. Electroporation was done with a single pulse with the following parameters 450 V, 500 µF, (Bio-RAD). After the pulse, 1 ml of M-199 medium was immediately added to the cuvette and incubated on ice for 10 min. The transfectants were transferred in a flask with 9 ml of medium. They were grown for 24 h without selection. After 24 h, G418, the selection marker was added at a concentration of 40 µg/ml.

Pentamidine resistance Protein 1 (PRP1) gene from L. major, cloned as 8 kb SmaI fragment in Leishmania shuttle vector, pSNBR, was obtained as a gift from Dr. Paulo Cotrim, Brazil. Wild type L. donovani was harvested from late log phase and transfected with 20 µg of PRP1 construct by electroporation as described above.
3.16 Effect of pentamidine on TKT and PRP1 overexpressors

Effect of pentamidine in *L. donovani* wild type, TKT overexpressors and PRP1 overexpressors was determined by calculating the IC₃₀ in these promastigotes. Cells were plated at a density of 1 x 10⁶ cells in 200 μl M-199 medium. After 24 h pentamidine was added at different concentrations. After 72 h of drug addition viable cells were counted using a neubaur hemocytometer.

3.17 Preparation of cellular lysate from total cellular proteome and preparation of mitochondrial fraction for Differential Gel Electrophoresis (DiGE)

For preparation of total cellular lysate 4 replicate promastigote cultures for each condition were grown to similar density in 50 ml culture medium. R8 promastigotes were grown in the presence of pentamidine (R8+) and both R8 and pentamidine sensitive (AG83) promastigotes were grown in the absence of pentamidine (R8- and AG83-). Promastigotes were harvested and washed 2X with PBS.

For preparation of mitochondrial subproteome, a mitochondrial fraction was prepared from wild type and R8 lines, grown in the absence of pentamidine (Harris *et al.*, 1990) as described in the section 3.6.

The washed pellets were lysed in 1 mL DiGE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris base) by 3 x 10 sec cycles of probe sonication (MSE soniprep) in an ice water bath, followed by 10 minutes centrifugation at 13,000 x g to remove insoluble material. Soluble proteins were precipitated and washed with 80% acetone, and resuspended in a small volume of lysis buffer. Protein content was assayed (Biorad) and adjusted to 5 mg/ml. A pooled standard sample was prepared by combining equal quantities of every sample. 10 μl aliquots (0.05 mg protein) were labelled with 100 pmoles DiGE CyDye (GE Healthcare), according to manufacturers’ protocol. Labelled aliquots were combined in groups of 3, such that each mixture comprised 2 protein samples from different culture conditions (labelled with Cy3 and Cy5) and an aliquot of the pooled standard (labelled with Cy2). An additional sample was prepared that comprised 500 μg of unlabelled pooled standard (sample for preparative gel). All samples were adjusted to a final volume of 460 μl by addition of DiGE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% DTT, 0.5 % non-linear pH 3-10 JPG buffer, 0.002% bromophenol blue), incubated at room temperature for 60 min and centrifuged 3 min at 13,000 rpm.
3.18 2D gel electrophoresis

The supernatant from each sample mix (450 μl) was loaded onto a dehydrated non-linear pH 3-10 IPG strip, overlayed with mineral oil and placed in an IPGphor (GE Healthcare). A small voltage was applied for 12 h to permit complete rehydration of the IPG strip, then voltage was ramped to achieve isoelectric focussing (300 V, 2 h; gradient to 600 V, 2 h; gradient to 1000 V, 2 h; gradient to 8000 V, 3 h; 8000 V, 8 h). Immediately upon completion of this protocol, strips were transferred to equilibration buffer I (2% SDS, 50 mM TrisCl, pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, 1% DTT) for 15 min and then to equilibration buffer II (2% SDS, 50 mM Tris-Cl, pH 8.8, 6 M urea, 30% glycerol, 0.002% bromophenol blue, 2.5% iodoacetamide) for 15 min. Equilibrated IPG strips were transferred to the top of homogeneous 12% acrylamide gels and placed in a DALT II electrophoresis apparatus. Electrophoresis was performed under standard conditions at 180 W until the bromophenol blue dye front reached the base of the gels.

3.19 Gel scanning and analysis.

DiGE gels were scanned immediately after electrophoresis, using a Typhoon 9400 instrument and default laser and filter settings for each CyDye. PMT voltage was optimised for each channel, to maximise sensitivity without permitting image saturation. Scans were performed at 100 μm resolution. Preparative gels were fixed for 2 h in 10% methanol, 7% acetic acid, stained for 2 h with Sypro Orange (Sigma), diluted 1:10,000 in 7% acetic acid. The gel was rinsed briefly in 7% acetic acid and scanned on a Typhoon 9400 instrument, using the green laser and an emission filter at 580 nm. All images were cropped to exclude the edges of the gels.

Gel analysis was performed using Decyder software (version 5, Amersham). Each set of gel images was processed via the Decyder DIA module, to detect spots and generate a spot map. Spot maps were loaded in Decyder BVA module and gel-to-gel matching inter-gel spot matching was initiated manually by selecting a number of Cy2 labelled (pooled internal standard) spots that were identical on all gels. These “landmark” spots facilitated automated spot matching in the Decyder BVA module, although spot matching was subsequently assessed by manual inspection of all Cy2 images. Cy3 and Cy5 spot intensities were expressed relative to Cy2 spot intensity, generating spot volume ratio and the average spot volume ratios for matched spots were generated. Statistical
analysis of variation in spot volume ratio between gels was performed using Student’s t-test.

Protein spots selected for identification were marked in the Decyder BVA module and matched to the image of the Sypro Orange-stained preparative gel. The coordinates of the selected spots (centred on the maximum volume of each spot) were assigned relative to internal reference markers applied to preparative gel. This “pick list” was exported from Decyder as a text file.

3.20 Protein processing and peptide analysis by mass spectrometry

Spots were cut and processed using an Ettan Spot handling workstation using a 1.4 mm diameter pick head. Gel plugs were washed twice with 50 mM ammonium bicarbonate, 50% methanol and dried. 0.2 μg trypsin in 10 μl of 20 mM ammonium bicarbonate was added to each gel plug and digestions were performed for 4 h at 37°C. Peptides were extracted from gel plugs by washing twice with 50% acetonitrile, 0.1% trifluoroacetic acid. These 2 washes were pooled, dried and stored at −20°C.

Dried peptides were dissolved in 0.5% formic acid and fractionated by nanoflow HPLC on a C18 Pepmap reverse phase column eluting with a continuous gradient to 40% acetonitrile over 20. Eluate was analysed by online electrospray mass spectrometry using a Qstar Pulsar (Applied Biosystems). MS analysis was performed in IDA mode (Analyst QS software, Applied Biosystems), selecting the 4 most intense peaks from each 3 sec MS survey scan for MSMS analysis. 5 sec MSMS scans from 50-2000 Da were performed using standard rolling collision energy settings. Peaks were extracted using the mascot script (Bioanalyst, Applied Biosystems) and exported to the Mascot search engine (Matrix Science), maintained on a local server, to search the L. major and L. infantum genome sequence databases.

In the few cases where more peptides were matched to a L. infantum contig than to a L. major predicted open reading frame, the region of the contig from where coverage was obtained was BLASTed against the L. major database. In all these cases, a highly significant match was obtained to a L. major predicted protein.

4. Results

4.1 Raising and characterization of pentamidine resistant parasites

Pentamidine resistant promastigotes were raised by subjecting wild type (WT) Leishmania donovani to increasing drug concentration. WT AG83 strain of L. donovani was first subjected to a pressure of 1 μM pentamidine and passaged in that particular drug
Pentamidine resistance in Leishmania

concentration for 5-6 times till the cultures stabilized in that drug pressure. These resistant promastigotes were plated on semisolid M199-agar plate and a single colony was picked up. This was further subjected to 2 μM drug pressure and the above steps were repeated till clones resistant to 8 μM pentamidine were obtained (Fig 1). The 4 cloned lines, designated R1, R2, R4 and R8, displayed 4, 18, 24 and 40 fold resistance to pentamidine respectively (Fig 2). Doubling time for the exponentially grown wild type L. donovani and R8 was 14 h and 22 h respectively.

R8 expressed cross-resistance to other diamidines; propamidine (9 fold), stilbamidine (12 fold), berenil (4 fold) and fluorescent analogs including DAPI (19 fold) and DB99 (11 fold) (Table 1).

These aromatic diamidines inhibited pentamidine uptake when the initial uptake of 1μM [3H] pentamidine was measured in the presence of 100 μM of these inhibitors thereby suggesting that they share the common route of uptake (Table 2).

The R8 line did not show any cross-resistance to antimonials (pentostam, glucantime), arsenite and to multidrug resistance pump substrates including vinblastine, adriamycin or the Ca^{2+} antagonist and P-glycoprotein inhibitor verapamil (Bitonti et al, 1988, Safa et al, 1987) (Table 3).

Promastigotes of the resistant line (R8) were transformed in vitro to amastigotes (without drug pressure because pentamidine inhibits the transformation of promastigotes to amastigotes) (Sereno et al, 1997). It has previously been shown that while low-level resistance to pentamidine in L. mexicana is rapidly lost once drug selection was removed, high-level pentamidine resistance is stable (Sereno et al, 1997). In L. donovani high-level pentamidine resistance was also maintained for at least 8 generations, without drug pressure. The IC_{50} of pentamidine for amastigote derived from R8 was greater than 50 μM in comparison to 1 μM for sensitive amastigotes (Table 4).

The resistant phenotype was stable in drug free medium for up to 2 months, although the IC_{50} of R8 had declined to 18 μM after a period of 5 months without drug pressure in promastigotes (Table 4).

4.2 Effect of pentamidine on mitochondrial transmembrane potential and mitochondrial biomass

The ability of wild type parasites, and the R8 clone to accumulate the hydrophobic cation rhodamine123 (Rh123), which is widely used as an indicator of mitochondrial membrane potential (Chen, 1988) was studied. Cationic drugs like
Wild Type AG83 strain of *L. donovani*

\[ \downarrow \]

Subjected to pressure of 1µM pentamidine

\[ \downarrow \]

Passaged in medium with 1 µM drug for 5-6 times

\[ \downarrow \]

Plated on M199 agar plate

\[ \downarrow \]

Single clone was picked up and subjected to higher drug pressure (2 µM pentamidine)

\[ \downarrow \]

Above steps repeated till we got a clone resistant to 8 µM pentamidine (R8)

---

**Fig 1:** Flowchart represents the strategy employed to raise pentamidine resistant *L. donovani* by stepwise increase in drug pressure. AG83 strain of wild type *L. donovani* was subjected to slow increase in pentamidine concentration till clones resistant to 8 µM pentamidine were obtained which was used for all studies.
Fig 2: IC$_{50}$ of pentamidine in all the cloned resistant lines of *L. donovani* to pentamidine

To determine the IC$_{50}$, the cells were plated at a density of 1 x $10^6$ cells/ml (200 µl/well) in 96 well tissue culture plates. Pentamidine was added 24 h after plating. The cells were incubated at 22°C for 72 h after drug addition and then the viable cells were counted with a hemocytometer. Resistance of each of the cloned line to pentamidine was determined by measuring the 50% inhibitory concentration (IC$_{50}$) of the drug when compared to the control. The numbers on top of the bars represent respective IC$_{50}$ for each of the resistant strain isolated. The values are the mean ± S.D. of triplicate measurements of 3 independent experiments.
Table 1: Cross resistance profile of wild type and pentamidine resistant (R8) L. donovani promastigotes to aromatic diamidines (structural analogs of pentamidine).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean IC$_{50}$ ± S.D (µM)</th>
<th>Wild type</th>
<th>R8</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine</td>
<td>0.81 ± 0.01</td>
<td>40 ± 6.36</td>
<td>49.4</td>
<td></td>
</tr>
<tr>
<td>Propamidine</td>
<td>10.4 ± 1.5</td>
<td>95 ± 12.8</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Stilbamidine</td>
<td>8.5 ± 0.4</td>
<td>104 ± 7.5</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td>Berenil</td>
<td>24.0 ± 6.3</td>
<td>99 ± 1.4</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>DAPI</td>
<td>1.03 ± 0.03</td>
<td>20 ± 2.5</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>DB99</td>
<td>1.25 ± 0.06</td>
<td>14 ± 1.6</td>
<td>11.2</td>
<td></td>
</tr>
</tbody>
</table>

Effects of different diamidine drugs on in vitro growth of pentamidine sensitive and resistant promastigotes after a 72 h incubation period at 25°C. R8 was cross resistant to other aromatic diamidines. IC$_{50}$ (µM) values are the mean values ± S.D. of 3 different experiments.
Table 2: Effect of different aromatic diamidines on pentamidine uptake by wild type *L. donovani* promastigotes.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean uptake (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propamidine</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>Stilbamidine</td>
<td>27 ± 4</td>
</tr>
<tr>
<td>Berenil</td>
<td>33 ± 3</td>
</tr>
<tr>
<td>DAPI</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>Isometamidium</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>83 ± 10</td>
</tr>
</tbody>
</table>

Initial rate of 1 μM pentamidine uptake by wild type *L. donovani* was determined with different drugs added at 100 μM. Results were normalized to the control value. All values are given as a percentage of the wild type control. Results are mean ± S.D. of 2 independent experiments done in triplicates.
Table 3: Cross resistance profile of wild type and pentamidine resistant *L. donovani* (R8) promastigotes to antileishmniaial drugs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean IC$_{50}$ ± S.D (µM)</th>
<th>Wild type</th>
<th>R8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentamidine</td>
<td>0.81 ± 0.01 µM</td>
<td></td>
<td>40 ± 6.36 µM</td>
</tr>
<tr>
<td>Pentostam</td>
<td>0.06 ± 0.01 mg/ml</td>
<td></td>
<td>0.06 ± 0.06 mg/ml</td>
</tr>
<tr>
<td>Glucantime</td>
<td>0.52 ± 0.02 mg/ml</td>
<td></td>
<td>0.46 ± 0.03 mg/ml</td>
</tr>
<tr>
<td>Na$_2$AsO$_3$</td>
<td>14.63 ± 0.88 µM</td>
<td></td>
<td>15.75 ± 0.88 µM</td>
</tr>
<tr>
<td>Verapamil</td>
<td>100 µM ± 9.5 µM</td>
<td></td>
<td>92.50 ± 10.6 µM</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>75.66 ± 6.65 µM</td>
<td></td>
<td>52.33 ± 4.50 µM</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>1.73 ± 0.08 µM</td>
<td></td>
<td>1.35 ± 0.14 µM</td>
</tr>
</tbody>
</table>

Effects of different drugs on *in vitro* growth of pentamidine sensitive and resistant promastigotes after a 72 h incubation period at 22°C. Cross resistance was not evident for compounds like pentostam, glucantime, arsenite, verapamil, vinblastine and adriamycin. IC$_{50}$ (µM) values are the mean values ± S.D. of 2 different experiments.
Table 4: IC\textsubscript{50} of promastigotes and axenic amastigotes of wild type, R8 and revertants of *L. donovani* to pentamidine

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mean IC\textsubscript{50} ± S.D (\textmu M)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Promastigotes</strong></td>
<td><strong>Amastigotes</strong></td>
</tr>
<tr>
<td>Wild type</td>
<td>0.81 ± 0.01</td>
<td>1.0 ± 0.02</td>
</tr>
<tr>
<td>R8</td>
<td>40 ± 6.6</td>
<td>54 ± 5.2</td>
</tr>
<tr>
<td>Revertant (5 months)</td>
<td>18 ± 0.4</td>
<td>N.D</td>
</tr>
</tbody>
</table>

Effect of pentamidine on *in vitro* growth of pentamidine sensitive and resistant promastigotes after a 72 h incubation period at 22°C. R8 was grown without the drug for 5 months and was designated as Revertant. The values are mean ± S.D. of duplicate independent experiments done in triplicates.
rhodamine123 are known to be concentrated into mitochondria across the membrane potential. Because of their positive charge they move into mitochondria through a mechanism driven electrophoretically by the inside negative membrane potential. Wild-type cells, wild type treated with pentamidine and R8 were used for this study. After 30 min of staining, Rh123 showed decreased fluorescence in treated parasites and also in R8, indicating probably a decrease in the membrane potential (Fig 3) compared to untreated wild-type cells.

The relative mitochondrial biomass was estimated by the fluorescence of Nonyl acridine orange (NAO). It is known that NAO binds to cardiolipin present exclusively in the mitochondrial membrane (Petit et al, 1992). Pentamidine did not modify the biomass, and there was no significant difference between the fluorescence intensity between wild type and resistant parasites indicating the mitochondrial biomass is unchanged (Table 5).

4.3  Pentamidine uptake by wild type and resistant parasites

Uptake of 1 μM pentamidine was measured over 3 h at 25°C in pentamidine resistant L. donovani and compared with that in wild type cells. The resistant promastigotes accumulated significantly less drug in comparison to the sensitive strain. After 3 h of incubation, the intracellular pentamidine concentration was 0.77 μM in R8 whereas in the sensitive promastigotes the intracellular pentamidine concentration reached 3.8 μM, representing a 5.4-fold enhanced accumulation of the drug within these cells (Fig 4).

Apparent kinetic constants for pentamidine uptake into resistant and wild type promastigotes were determined with a range of substrate concentrations between 1 μM and 40 μM for 2 min. The apparent K_m value and the V_max value did not differ significantly in the wild type and resistant lines (Fig 5). The K_m value indicates the presence of a relatively high affinity transporter for pentamidine. In addition to this saturable high affinity transporter, pentamidine uptake in wild-type L. donovani promastigotes also appears to occur through a secondary, nonsaturable route. The presence of this second route was indicated by a failure of up to 1 mM cold pentamidine to completely inhibit uptake of 1 μM [^3]H] pentamidine (Fig 6). In another experiment, increasing concentration of labelled pentamidine to 1mM also showed the presence of a substantial non-saturable component in wild-type but not in resistant cells (Fig 7).
Fig 3: Rhodamine 123 staining of pentamidine resistant and sensitive promastigotes of *L. donovani* and determination of mitochondrial membrane potential.

Promastigotes of wild type and resistant parasites were incubated with rhodamine 123 as described. Cell suspensions were subjected to flow cytometry and the fluorescence distribution was plotted as frequency histograms. The figure shows a set of histograms, representative of two duplicate determinations, for both the populations: (a) Wild type untreated, (b) Wild type treated with 2 μM pentamidine for 24 h, (c) R8. Differences in the degree of probe accumulation are seen.
Table 5: Estimation of mitochondrial biomass as estimated by fluorescence of Nonyl-Acridine Orange in wild type, treated and pentamidine resistant promastigotes

<table>
<thead>
<tr>
<th>Strains and treatment</th>
<th>Mean fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>2893 ± 69.5</td>
</tr>
<tr>
<td>Wild type +2 µM pentamidine (24 h)</td>
<td>2320 ± 6.5</td>
</tr>
<tr>
<td>R8</td>
<td>2291 ± 22.5</td>
</tr>
</tbody>
</table>

Mitochondrial biomass, that is the abundance of internal membranes per cell, was estimated by fluorescence of nonyl acridine orange which stains their specific lipid, cardiolipin. The wild type cultures were treated or not with pentamidine for 24 h and the fluorescence in WT, treated and R8 was measured in a flow cytometer. The values are mean ± S.D of triplicates of a single experiment.
Fig 4: Pentamidine uptake by promastigotes as a function of treatment time.
Promastigotes in the exponential phase of growth were incubated for various times with 1 μM pentamidine at 22°C and uptake was measured between 1 min and 3 h. Values are mean ± S.D. of 3 independent experiments.
Fig 5: Kinetic parameters of pentamidine transporter in WT and R8 promastigotes

Apparent kinetic constants for pentamidine uptake into resistant and wild type promastigotes were determined with a range of substrate concentrations between 1 µM and 40 µM for 2 min. Kinetic constants were calculated using Michaelis-Menten equation to a non-linear fitted curve using Enzyme Kinetics Pro (version: 2.36) software. The results show mean values of an experiment performed in triplicates.
Fig 6: Second nonsaturable route of uptake of pentamidine in wild versus resistant strain
In the presence of high extracellular cold pentamidine concentration, the inhibition of uptake of $[^3]$H pentamidine was measured in WT (●) and R8 (■). The results are mean values of triplicates of 2 independent experiments.
Fig 7: Effect of increasing concentration of labeled pentamidine on pentamidine uptake by wild-type and pentamidine resistant *Leishmania donovani* promastigotes. The graph shows a substantial amount of nonsaturable uptake that is less pronounced in the resistant line. The results are average of triplicates from 2 independent experiments. WT (●) and R8 (□).
4.4 Second route of entry of pentamidine into the sensitive strain

Both the wild type and the resistant promastigotes have a high affinity transporter with similar $K_m$ and $V_{max}$ values. When pentamidine accumulation was measured in both the cell lines in the presence of a high range of extra-cellular pentamidine concentration (up to 1 mM), the accumulated pentamidine in the wild-type did not reach saturation (Fig 8). This could indicate the presence of a secondary route with very low affinity into cells for pentamidine. This secondary route was not as pronounced in the R8 cell line. Treatment of wild-type cells with CCCP for 10 min led to loss of measurable uptake via the secondary route. Uptake into CCCP treated wild-type cells was very similar to uptake into the resistant line (Fig 8). It is possible that the secondary route is an independent low affinity carrier. Alternatively, the unsaturable component could relate to a limitation in the kinetic experiments and at higher concentrations uptake into the mitochondrion of wild-type, but not resistant cells, could alter transmembrane equilibria and influence measurable uptake.

4.5 Relative accumulation of pentamidine in wild type and pentamidine resistant $L$. donovani promastigotes in the absence and presence of different metabolic inhibitors

Pentamidine uptake was also investigated in the presence and absence of different inhibitors to investigate the nature of the force-driving uptake (Table 6). Inhibitors of mitochondrial metabolism including KCN and sodium azide, led to significant reductions in net pentamidine uptake in wild type parasites. Ouabain, an inhibitor of plasma membrane Na$^+$/K$^+$ ATPase, and monensin, a Na$^+$ ionophore did not decrease pentamidine uptake in the promastigotes. These data indicate that pentamidine uptake is independent of Na$^+$ ions. The K$^+$ ionophore valinomycin and the protonophore carbonylcyanide (CCCP) reduced pentamidine uptake. The nonspecific H$^+$ATPase inhibitor, N,N'-dicyclohexylcarbodiimide (DCCD), which reduced the plasma membrane pH gradient and the membrane potential in $Leishmania$ sp. (Glaser et al, 1988, 1992), oligomycin A, a mitochondrial ATPase inhibitor all had inhibitory effects on pentamidine uptake. These results suggest that energy is required for the entry of drug into the promastigotes and that an actively metabolizing mitochondrion plays an important role in the net accumulation of pentamidine in $L$. donovani.

In $L$. mexicana promastigotes, the energy dissipating activity of mitochondrial inhibitors stimulated drug uptake, indicating that energy was expended in excluding the drug from the cell (Basselin et al, 2002). However, pentamidine uptake in resistant $L$. donovani has not been studied yet.
Fig 8: Effect of the mitochondrial uncoupler, CCCP, on pentamidine uptake in wild type, in comparison to resistant promastigotes.

Increasing the concentration of the labelled drug, uptake of pentamidine in wild-type *L. donovani* promastigotes is higher than resistant parasites, indicating the nonsaturable route of transport of pentamidine. However, pretreatment of sensitive promastigotes with CCCP at 22°C for 10 min, resulted in similar accumulation of pentamidine in sensitive parasites like that of resistant ones, suggesting that high mitochondrial potential is responsible for the increased secondary uptake in wild-type parasites. Results are mean ± S.D. of triplicates from 2 independent experiments.
Table 6: Relative accumulation of pentamidine in wild-type and pentamidine resistant *L. donovani* promastigotes in the absence and presence of different inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor (conc)</th>
<th>Mean % of control accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>KCN (1mM)</td>
<td>22.1 ± 5.2</td>
</tr>
<tr>
<td>CCCP (10µM)</td>
<td>45.3 ± 6.5</td>
</tr>
<tr>
<td>DCCD (50µM)</td>
<td>46.6 ± 6.1</td>
</tr>
<tr>
<td>Verapamil (50µM)</td>
<td>57.3 ± 21.4</td>
</tr>
<tr>
<td>Valinomycin (10µM)</td>
<td>52.6 ± 8.6</td>
</tr>
<tr>
<td>Oligomycin A (12µg/ml)</td>
<td>27.0 ± 3.6</td>
</tr>
<tr>
<td>Azide (5mM)</td>
<td>21.0 ± 8.5</td>
</tr>
<tr>
<td>Monensin (50 µM)</td>
<td>136.0 ± 58.3</td>
</tr>
<tr>
<td>Ouabain (1 mM)</td>
<td>101.3 ± 15.9</td>
</tr>
<tr>
<td>Orthovanadate (10 µM)</td>
<td>36.0 ± 2.6</td>
</tr>
</tbody>
</table>

Cells were pretreated with inhibitors at 22°C for 5 min and then incubated with 5 µM pentamidine for 1 min in PBSG. All values are given as a percentage of wild type and resistant control (without inhibitor pretreatment) and are means ± S.D. of 3 independent experiments.
*Pentamidine resistance in Leishmania*

*donovani* also decreased in the presence of mitochondrial inhibitors, although the extent of inhibition was less pronounced than for wild-type cells. The protonophore CCCP did not alter the uptake of the drug significantly in comparison to control. Resistant parasites already have a lowered mitochondrial membrane potential which may contribute to the exclusion of drug from the mitochondrion.

In *L. mexicana* it was possible to load pentamidine to equilibrium levels across the plasma membrane by exposing the cells to drug for 15 min on ice (Basselin *et al.*, 2002). In *L. donovani*, however, we were unable to stimulate significant uptake of drug on ice, making simple efflux experiments difficult to conduct in this species.

### 4.6 Intracellular compartmentalization of diamidines

2,5-bis (4-amidophenyl) 3,4, dimethyl furan (DB99), is a fluorescent analogue of pentamidine. The compound is toxic to *L. donovani* promastigotes with an IC₅₀ close to that of pentamidine, and cells resistant to pentamidine are cross-resistant to DB99. Moreover, the compound inhibits pentamidine uptake into *L. donovani*, indicating that the two compounds share a common route of internalization. Distribution of 10 µM DB99 was analyzed by adding it to the cells and viewing with fluorescence microscopy. Within 2 min of staining bright blue fluorescence was found to be associated with the kinetoplast DNA of wild type but not with the R8 resistant line (Fig 9a). Thus diamidines selectively accumulate in the mitochondria of WT but not of resistant parasites. Treating both WT and R8 cells with 1µM digitonin, however led to clear fluorescence associated with the kinetoplast in both types of parasites within 3-5 min (Fig 9b). This indicates that the drug is bound to the kinetoplast DNA regardless of the resistance phenotype. However, the compound is apparently selectively accumulated across the mitochondrial membrane of WT but not pentamidine resistant parasites.

### 4.7 Effect of pentamidine on mitochondrial dehydrogenase activity in vivo by MTT assay

Using the MTT method (Mossman, 1983) the mitochondrial dehydrogenase activities were estimated in relation to growth, expressed as an increase in cell number. The optical density, OD at 570 nm is due to the reduction of thiazolyl blue, MTT, into formazan as a consequence of dehydrogenase activities. R8 promastigotes showed lower overall mitochondrial dehydrogenase activity than the wild type parasites (Fig 10).
(a) Exponentially growing promastigotes were pelleted, washed and were incubated with 10 μM DB99 at 22°C. Bright fluorescence was associated with the kinetoplast of sensitive cells but not with resistant parasites within 5 min of staining. A and C represent phase contrast and fluorescence images of sensitive promastigotes. B and D represent that of resistant promastigotes.

(b) Wild type and R8 were treated with 1μM digitonin and then incubated with DB99 at 22°C. Within 3-5 min of staining the kinetoplast of both wid type and R8 showed blue fluorescence under the microscope.

Fig 9: Fluorescent images of DB99 stained promastigotes of wild-type and pentamidine resistant *L. donovani*.
Fig 10: Mitochondrial dehydrogenase activities detected in wild type and pentamidine resistant *L. donovani* promastigotes

MTT test detects the mitochondrial dehydrogenase activity. The O.D at 570 nm is due to the reduction of thiazolyl blue, MTT, into formazan as a consequence of dehydrogenase activities. The abscissa was the cellular density at which the absorbance was measured in the promastigotes. The result is mean of triplicate of an independent experiment.
4.8 Effect of pentamidine on mitochondrial dehydrogenases and ATPases in vitro

To test if pentamidine directly affects mitochondrial dehydrogenases, mitochondrial extracts were prepared as mentioned in materials and methods section, and different enzymatic activities were measured in the presence of pentamidine. Table 7 shows that in vitro addition of pentamidine did not alter any of the enzyme activities. However, in the resistant line, the activity of NADH dehydrogenase (NDH), NADH cytochrome c reductase (NCC), succinate dehydrogenase (SDH) and mitochondrial ATPase were less in comparison to the wild type strain. Thus, the activity of most of the enzymes responsible for maintaining the mitochondrial potential decreased in a coordinated fashion in the resistant parasites. No difference in the activity of succinate cytochrome c reductase (SCC) was observed in wild-type compared with the resistant strain.

4.9 Role of multidrug pumps and Pentamidine Resistance Protein (PRP1), a member of ABC transporter superfamily to confer pentamidine resistance in L. donovani

In L. mexicana, PCP and TFP which are known to inhibit P-glycoprotein mediated efflux of compounds from Leishmania (Essodaigui et al, 1999) increased the rate at which pentamidine could accumulate into the resistant cells (Basselin et al, 2002). This was taken to indicate that drug efflux was operative in these cells. Pentamidine accumulation over a 3 h period was also investigated in wild-type and R8 L. donovani in the presence of 10 μM PCP and 10 μM TFP (wild type cells) and 20 μM PCP and 20 μM TFP (resistant cells). In the presence of these inhibitors the resistant cells accumulated drug to similar levels as in control cells (without drug), (Fig 11), indicating that P-glycoprotein pumps do not contribute to the net accumulation of pentamidine in L. donovani to the same extent as they do in L. mexicana (Basselin et al, 2002).

It has been reported earlier that overexpression of a P-glycoprotein gene PRP1, which is a member of the ABC transporter superfamily results in pentamidine resistance in L. major (Coelho et al, 2003). In order to check the role of PRP1 in pentamidine resistance in L. donovani we transfected wild type L. donovani promastigotes with PRP1 construct (8 kb Smal fragment of PRP1 cloned in shuttle vector, pSNBR). To check the effect of pentamidine on the overexpressors of PRP1, we determined the IC50 of these overexpressors to pentamidine. The IC50 of wild type L. donovani control to pentamidine was 1 ± 0.1 μM and that of PRP1 overexpressors was 1.25 ± 0.15 μM (Fig 12). This
Table 7: Spectrophotometric analysis of *in vitro* mitochondrial dehydrogenase activities of a mitochondrial fraction from untreated wild type (WT) and pentamidine resistant (R8) *Leishmania donovani* promastigotes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity nmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>NADH Dehydrogenase</td>
<td>211±10.4</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>29 ±3.2</td>
</tr>
<tr>
<td>NADH cytochrome c reductase</td>
<td>35 ±5.1</td>
</tr>
<tr>
<td>Succinate cytochrome c reductase</td>
<td>49.05 ±19</td>
</tr>
<tr>
<td>Mitochondrial ATPase</td>
<td>158.3 ±34.6</td>
</tr>
</tbody>
</table>

The results are mean values of triplicates of 2 independent experiments. To see the effect of pentamidine on the mitochondrial dehydrogenase activities, the drug was injected at a concentration of 2μM and 10μM into the cuvette at the time of measurement.
Fig 11: Effect of P-glycoprotein inhibitors on the time course of pentamidine accumulation in wild-type and pentamidine resistant promastigotes.

The effect of 10 μM PCP and 10 μM TFP in sensitive wild-type and 20 μM PCP and 20 μM TFP in resistant cells on pentamidine influx was studied. Results are mean ± S.D. of triplicates from 3 independent experiments.
**Fig 12: IC$_{50}$ of Wild type and PRP1 overexpressors to pentamidine.**

To determine the IC$_{50}$, the cells were plated at a density of $1 \times 10^6$ cells/ml (200 µl/ well) in 96 well tissue culture plates. Pentamidine was added 24 h after plating. The cells were incubated at 22°C for 72 h after drug addition and then the viable cells were counted with a hemocytometer. Resistance of each of the line to pentamidine was determined by measuring the 50% inhibitory concentration (IC$_{50}$) of the drug when compared to the control. The values are the mean ± S.D. of triplicate measurements of 3 independent experiments. There is no significant difference in IC$_{50}$ of wild type and PRP1 overexpressors to pentamidine.
further excludes the possibility of involvement of multidrug pumps/P-glycoproteins in pumping out the drug from *L. donovani*.

4.10 Examination of alteration of other putative targets in pentamidine resistant *L. donovani*

4.10.1 Effect of pentamidine on polyamine level and biosynthesis in wild type, pentamidine treated and pentamidine resistant *L. donovani*

Earlier reports indicate that pentamidine affects the polyamine biosynthetic pathway being a diamidine. Analyses of polyamines by HPLC method showed that the level of putrescine in resistant and pentamidine treated promastigotes were 3 fold less than that compared to the control parasites (*p*=0.03) as shown in Table 8. The low level of putrescine in the resistant promastigotes could be responsible for their slower growth rate though the viability is not altered. The intracellular spermidine level was also measured but there was no significant difference between treated, resistant and control parasites.

To examine if the decreased putrescine pool was a direct consequence of diminution in the enzymatic conversion of ornithine to putrescine, the level of ODC was assessed at the protein level. Western blot analysis with ODC antibody revealed lower expression of the protein in R8 and WT treated with pentamidine when compared to the control promastigotes (Fig 13A). The activity of ODC was around 140 nmol/mg protein and when WT cells were pretreated with 2 μM pentamidine for 24 h, the activity was less by 2 folds (*p* value= 0.02). Resistant promastigotes also have a lower ODC activity and was decreased by 1.8 fold (*p* value= 0.028) (Fig 13B). It appears that the establishment of pentamidine resistance requires adaptation to this low putrescine concentration.

4.10.2 Effect of pentamidine on membrane fluidity and lipid metabolism

Changes in anisotropy of TMA-DPH in treated WT and R8 were seen using a spectrofluorimeter using excitation wavelength of 357 nm and emission wavelength of 430 nm. Pentamidine did not affect the membrane fluidity after 24 h of treatment. Also, in R8 there was no change of emission anisotropy of TMA-DPH indicating that resistance is not due to fluidification of the membrane (Table 9).

Nile red is a vital dye that emits a predominantly red fluorescence in polar hydrophobic domains (phospholipids) and a yellow fluorescence in neutral hydrophobic domains (cytoplasmic neutral lipid droplets). Intensity of red fluorescence (IRF) and intensity of yellow fluorescence (YF) was not changed significantly in pentamidine
Table 8: Comparison of polyamine levels in wild type and pentamidine resistant *L. donovani*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Putrescine (nmol/mg protein)</th>
<th>Spermidine (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>32.8 ± 5.2</td>
<td>30.8 ± 6.7</td>
</tr>
<tr>
<td>R8</td>
<td>10.5 ± 2.3(*)</td>
<td>18.4 ± 3.8</td>
</tr>
</tbody>
</table>

Quantitative determination of polyamines in crude lysates of *L. donovani* was obtained by C18 reversed-phase high performance liquid chromatography (HPLC) after derivatization with dansyl chloride. The results are mean ± S.D. of triplicates of a single experiment. The mean level of putrescine in R8 was significantly less than wild type (* indicates p value of 0.03). The level of spermidine was not significantly different (p > 0.05).
Fig 13: Analysis of ODC protein expression and ODC activity in Wild type, pentamidine treated and pentamidine resistant *L. donovani*

(A) Western blot analysis to determine the amount of ODC protein expressed in log phase promastigotes of wild type (lane 1), wild type treated with 2 µM pentamidine for 24 h (lane 2) and pentamidine resistant (lane 3) promastigotes. 50 µg of whole cell lysate was fractionated by SDS-Polyacrylamide gel electrophoresis, transferred on to PVDF membrane using electrophoretic transfer cell (Bio-Rad) and probed with ODC antibody against Leishmania. Molecular weights were determined using Bangalore Genei protein molecular weight marker. Fold decrease in expression of ODC in treated and R8 promastigotes are was determined by densitometry scanning in Fuji film FLA-5000, Japan. The same blot was reacted with antibody against GAPDH protein to normalize the loading on to each lane of the gel.

(B) ODC enzymatic assay to determine the activity of ODC enzyme in WT *L. donovani*, pentamidine treated *Leishmania* and R8. Ornithine decarboxylase activity was assayed by following the release of $^{14}$CO$_2$ from L- $[^{14}$C] ornithine. Activity is expressed in enzyme units in which 1 unit is nmol of CO$_2$ /mg protein/h and is represented by mean ± S.D. of triplicates from 2 independent experiments.* denotes a p value of <0.03.
Table 9: Membrane fluidity of Wild type (WT), treated (WT +2 μM pent) and pentamidine resistant (R8) promastigotes as assessed by emission anisotropy of TMA-DPH.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Polarization</th>
<th>Emission anisotropy</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.41± 0.002</td>
<td>0.31 ± 0.004</td>
</tr>
<tr>
<td>WT + 2 μM pent (24 h)</td>
<td>0.41 ±0.003</td>
<td>0.31 ± 0.003</td>
</tr>
<tr>
<td>R8</td>
<td>0.42 ± 0.005</td>
<td>0.32 ± 0.001</td>
</tr>
</tbody>
</table>

Promastigotes were diluted in PBS at a cellular density of 1x 10⁷ cells/ml and then stained with 2 μM TMA-DPH for 2 min before being analyzed by flow cytometry. Membrane fluidity was assessed by measurement of the fluorescence anisotropy of TMA-DPH following its insertion into the plasma membrane of promastigotes. Values are mean ± S.D. of 2 independent experiments done in triplicates.

Table 10: Fluorescence of promastigotes of wild type (WT), treated with pentamidine (WT+2 μM pent) and resistant (R8) labeled with Nile red.

<table>
<thead>
<tr>
<th>Strains and treatment</th>
<th>IRF</th>
<th>IYF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1525 ± 116.9</td>
<td>2020 ± 118.9</td>
</tr>
<tr>
<td>WT+2 μM pent (24 h)</td>
<td>1843 ± 133</td>
<td>1985 ± 39</td>
</tr>
<tr>
<td>R8</td>
<td>1143 ± 127.5</td>
<td>1551 ± 138</td>
</tr>
</tbody>
</table>

Promastigotes were diluted in PBS at a cellular density of 1x 10⁷ cells/ml and then stained with Nile red (100 ng/ml) for 5 min and then analyzed by flow cytometry. The parameters, intensity of red fluorescence (IRF) and intensity of yellow fluorescence (IYF) were measured. Values are mean ± S.D. of triplicates of a single experiment. The means of IRF and IYF in treated and R8 parasites were statistically insignificant (p> 0.05) when compared to wild type.
treated and resistant promastigotes indicating pentamidine did not alter the lipid metabolism in *L. donovani* (Table 10).

### 4.11 2-D Difference Gel Electrophoresis (DiGE) analysis of total cellular proteome

2-D Difference gel electrophoresis (DiGE) (Alban *et al*, 2003) is a novel approach to comparative proteomics that overcomes the problems of conventional 2-D electrophoresis by enabling analysis of different samples for comparison on the same gel by labelling the samples differentially, and running them on the same gel along with a pooled internal standard. A number of studies (Alban *et al*, 2003, Yan *et al*, 2002, Zhou *et al*, 2002) have shown that DiGE can highlight small but reproducible differences in contrast to conventional 2-D- electrophoresis. The detection of CyDye labelled proteins is comparable to fluorescent or silver stains, and we have developed a method to enable recovery of sufficient protein for mass spectrometric analysis of even faint spots.

Comparison of protein expression in drug sensitive and drug resistant cells has the potential to reveal proteins that are involved in mediating drug resistance. Stable drug resistance can arise by modulation of protein expression, so it is critical that comparative approaches be quantitative in order to highlight important differences that might be quite small.

The experimental plan of DiGE is depicted in Figs 14 and 15. Protein extracts were prepared from late log phase promastigotes of wild type and pentamidine resistant cells. The 2 different samples were labelled differentially with the dyes, Cy5 and Cy3. Same amount of both the samples (i.e. same amount of protein from both the samples were mixed (pooled internal standard) and labelled with the third dye, Cy2. The extracts with labelled proteins with 3 dyes were mixed and separated by 2-D electrophoresis on the same gel. The gel was imaged and excited at 3 different wavelengths depending on the emission of the dyes. The overlaid image showed the proteins differentially regulated as volumes expressed a ratios relative to pooled internal standard. Software expresses spot intensity relative to the pooled standard, enabling pairwise comparison between gels.

DiGE gels were labelled and loaded as depicted in the Fig 15 and 16. Approximately 2,000 protein spots were detected on each gel image (Fig 17). After spot matching, approximately 650 spots were determined to be reproducibly expressed in replicate samples. No spots were reproducibly modulated (p=0.05) by more than 5-fold in a pairwise comparison of R8 grown in the presence of pentamidine and R8 grown in the absence of pentamidine. However, 81 spots were reproducibly modulated (p=0.05) by
**Difference Gel Electrophoresis (DiGE)**

1. **Pooled internal standard**
   - Label with Cy2
2. **Protein extract 1**
   - Label with Cy3
3. **Protein extract 2**
   - Label with Cy5

**Mix labelled samples**

**Separate by 2-D PAGE**

- Cy2 excitation wavelength
- Cy3 excitation wavelength
- Cy5 excitation wavelength

**Volumes expressed as ratios relative to pooled internal standard**

**Fig 14: Diagram showing the principle of Difference Gel Electrophoresis.**

Protein extracts from 2 different samples are labelled differentially with Cy5 and Cy3. Same amount of both the samples (i.e. same amount of protein from both the samples were mixed (pooled internal standard) and labelled with the third dye, Cy2. The extracts with labelled proteins with 3 dyes are mixed and separated by 2-D electrophoresis on the same gel. The gel was imaged and excited at 3 different wavelengths depending on the emission of the dyes. The overlaid image showed the proteins differentially regulated as volumes expressed a ratios relative to pooled internal standard. Software expresses spot intensity relative to the pooled standard, enabling pairwise comparison between gels.
Cy5  |  Cy3  |  Cy2
---|---|---
WT (1) | R8 (-pent) 1 | PS
WT (2) | R8 (-pent) 2 | PS
R8 (+ pent) 1 | WT (3) | PS
R8 (+ pent) 2 | WT (4) | PS
R8 (- pent) 3 | R8 (+ pent) 3 | PS
R8 (- pent) 4 | R8 (+ pent) 4 | PS

Fig 15: Experimental plan of the DiGE experiments.
First experiment was done in duplicates where WT was labelled with Cy5 and R8 growing in the absence of pentamidine was labelled with Cy3. In the next experiment R8 growing in the presence of pentamidine was labelled with Cy5 and WT with Cy3. In the third experiment R8 growing in the absence of pentamidine and presence of pentamidine was labelled with Cy5 and Cy3 respectively. Pooled standard was included in each experiment where the pooled standard was labelled with Cy2. All the experiments were done in duplicates.
Fig 16: Diagrammatic representation of each DiGE experiment

WT (CTRL) was labelled with Cy5, R8 (Test) was labelled with Cy3 and the pooled standard (pool) with Cy2. (3, 5 and 2 represent Cy3, Cy5 and Cy2 respectively). The pooled standard sample was prepared by combining equal quantities of every sample. An additional sample was prepared that comprised 500 µg of unlabelled pooled standard (sample for preparative gel) for the preparative gel. The experiment was repeated 4 times before analysis.
Fig 17: One of the 2-D gels of promastigote lysate of *L. donovani*

The proteins were separated in a pH gradient of pH 3 and 10 and then subjected to SDS-PAGE. Proteins of mol wt in the range of 10 kDa and 250 kDa were finally separated.
more than 5-fold in a pairwise comparison of AG83 and R8 grown in the absence of pentamidine. Of these, 53 spots were increased in abundance in R8 and 28 were reduced in abundance in R8.

In parallel to the analytical DiGE gels, a preparative gel was prepared. This gel was loaded with 500 µg of the pooled standard sample that was represented on each gel as 50 µg of Cy2 labelled internal standard. The preparative gel was visualised by staining with Sypro Orange and manual matching was performed between the total protein stain image of the preparative gel and the assigned master gel from the DiGE analysis. 53 spots of the 81 selected spots were confidently matched. These were picked for tryptic digest and LC-MSMS analysis. 39 protein spots produced statistically significant database hits (p<=0.05). The results of this analysis are presented in Table II. There was a general bias toward identification of the more intense gel spots in a total protein stain of the preparative 2D gels that was submitted for spot picking. In the few cases where more peptides were matched to a *L. infantum* contig than to a *L. major* predicted open reading frame, the region of the contig from where coverage was obtained was BLASTed against the *L. major* database. In all these cases, a highly significant match was obtained to a *L. major* predicted protein.

The proteins, which were more than 5 fold upregulated in R8, are shown in Fig 18. They include NADP dependent alcohol dehydrogenase (27 fold upregulation, p= 5.0 x 10^{-7}), carboxypeptidase was 10 fold upregulated in R8 (p = 7.8 x 10^{-6}). Enzymatic regeneration of the thiol pool during oxidative stress in trypanosomatids is dependent on the enzyme, Trypanothione reductase (TR). In pentamidine resistant *L. donovani*, there is upregulation of TR by 5 fold, p = 9.5 x 10^{-5}. Transketolase, one of the important enzymes of the nonoxidative pathway of pentose phosphate pathway was also overexpressed in R8 by 9 fold, p = 0.0053. Transketolase is important in production of NADPH which in turn functions mainly in keeping the thiols in the reduced state, which are important in combating oxidative stress.

4.12 Western blot analysis to check level of transketolase (TKT) expression in Wild type and R8 promastigotes

50 ml of Wild type and R8 cells were harvested at mid log phase for western blot analysis in WT and R8 with anti transketolase antibody at 1/5000 dilution. Western blot revealed increased expression of TKT in R8 (Fig 19) though the increase was not by 9
Table 11: Proteins identified by MALDI after DiGE which gave statistically significant database hits (p=<0.05)

<table>
<thead>
<tr>
<th>Master No.</th>
<th>Protein ID</th>
<th>Protein AC</th>
<th>Av. Ratio</th>
<th>T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>2350</td>
<td>NADP dep alcohol dehydrogenase</td>
<td>LmjF23.0360</td>
<td>27.04</td>
<td>5.00E-07</td>
</tr>
<tr>
<td>990</td>
<td>methionine synthase</td>
<td>LmjF31.0010</td>
<td>13.58</td>
<td>1.00E-05</td>
</tr>
<tr>
<td>1795</td>
<td>carboxy peptidase</td>
<td>LmjF33.2540</td>
<td>9.58</td>
<td>7.80E-06</td>
</tr>
<tr>
<td>1450</td>
<td>Transketolase</td>
<td>LmjF15.1550</td>
<td>8.19</td>
<td>0.0053</td>
</tr>
<tr>
<td>1870</td>
<td>enolase</td>
<td>LmjF14.1160</td>
<td>6.67</td>
<td>7.00E-06</td>
</tr>
<tr>
<td>2844</td>
<td>P28 of cruzihipo</td>
<td>LmjF25.2010</td>
<td>6.57</td>
<td>3.60E-06</td>
</tr>
<tr>
<td>1517</td>
<td>amino peptidase</td>
<td>LmjF11.0630</td>
<td>5.95</td>
<td>0.00018</td>
</tr>
<tr>
<td>2962</td>
<td>proteosome beta 1 subunit</td>
<td>LmjF12.0030</td>
<td>5.73</td>
<td>0.0032</td>
</tr>
<tr>
<td>1729</td>
<td>trypanothione di sulphide reductase</td>
<td>LmjF14.1160</td>
<td>5.13</td>
<td>9.50E-05</td>
</tr>
<tr>
<td>2228</td>
<td>ATP dep dead box helicase</td>
<td>LmjF35.0370</td>
<td>4.44</td>
<td>0.0003</td>
</tr>
<tr>
<td>1786</td>
<td>carboxypeptidase</td>
<td>LmjF33.2540</td>
<td>4.38</td>
<td>0.00031</td>
</tr>
<tr>
<td>1826</td>
<td>aspartyl t RNA synthatase</td>
<td>LmjF30.0460</td>
<td>4.26</td>
<td>0.00062</td>
</tr>
<tr>
<td>2348</td>
<td>NADP dep alcohol hydrogenase</td>
<td>LmjF23.0360</td>
<td>3.65</td>
<td>0.029</td>
</tr>
<tr>
<td>1548</td>
<td>Glucose-6-phosphate isomerase</td>
<td>LmjF12.0530</td>
<td>3.55</td>
<td>0.00048</td>
</tr>
<tr>
<td>1605</td>
<td>t complex prt</td>
<td>LmjF23.1220</td>
<td>3.42</td>
<td>0.0079</td>
</tr>
<tr>
<td>2003</td>
<td>thiol dependent reductase</td>
<td>LmjF33.0240</td>
<td>3.17</td>
<td>0.0014</td>
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<tr>
<td>1742</td>
<td>proteosome reg non ATPase subunit 5</td>
<td>LmjF21.0760</td>
<td>3.06</td>
<td>0.0057</td>
</tr>
<tr>
<td>1510</td>
<td>fumarate hydratase</td>
<td>LmjF29.1960</td>
<td>3.04</td>
<td>0.00024</td>
</tr>
<tr>
<td>2318</td>
<td>Cytochrome c oxidase subunit iv</td>
<td>LmjF12.0670</td>
<td>2.85</td>
<td>0.00027</td>
</tr>
<tr>
<td>2150</td>
<td>cystathione gamma lyase</td>
<td>LmjF35.3230</td>
<td>2.75</td>
<td>0.0012</td>
</tr>
<tr>
<td>2304</td>
<td>beta tubulin</td>
<td>LmjF21.1860</td>
<td>2.4</td>
<td>0.0034</td>
</tr>
<tr>
<td>2155</td>
<td>hypothetical</td>
<td>LmjF30.0280</td>
<td>2.4</td>
<td>0.047</td>
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<tr>
<td>3080</td>
<td>tagatose 16 bis phos aldolase</td>
<td>LmjF25.0960</td>
<td>2.12</td>
<td>8.60E-05</td>
</tr>
<tr>
<td>1882</td>
<td>gamma tubulin</td>
<td>LmjF36.3910</td>
<td>1.98</td>
<td>0.0039</td>
</tr>
<tr>
<td>1739</td>
<td>s adenosyl homocysteine hydrolase</td>
<td>LmjF36.3910</td>
<td>1.93</td>
<td>0.015</td>
</tr>
<tr>
<td>2090</td>
<td>glutamate dehydrogenase</td>
<td>LmjF28.2910</td>
<td>1.91</td>
<td>0.041</td>
</tr>
<tr>
<td>2972</td>
<td>Hsp 70</td>
<td>LmjF28.2770</td>
<td>1.68</td>
<td>0.0011</td>
</tr>
<tr>
<td>2151</td>
<td>ATP dep Dead Box RNA helicase</td>
<td>LmjF35.0370</td>
<td>-1.36</td>
<td>0.0063</td>
</tr>
<tr>
<td>1799</td>
<td>mito processing peptidase beta sub</td>
<td>LmjF35.1380</td>
<td>-1.52</td>
<td>0.038</td>
</tr>
<tr>
<td>1806</td>
<td>mito processing peptidase beta sub</td>
<td>LmjF35.1380</td>
<td>-1.62</td>
<td>0.00088</td>
</tr>
<tr>
<td>1713</td>
<td>HMG coA synth ( Hypo )</td>
<td>LmjF24.2110</td>
<td>-2.71</td>
<td>0.00034</td>
</tr>
<tr>
<td>2347</td>
<td>pyruvate dehydrogenase E1 beta subunit</td>
<td>LmjF25.1710</td>
<td>-2.96</td>
<td>0.0013</td>
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<tr>
<td>1886</td>
<td>proteosome reg subunit 5</td>
<td>LmjF22.0620</td>
<td>-3.02</td>
<td>3.30E-05</td>
</tr>
<tr>
<td>2106</td>
<td>aspartate aminotransferase</td>
<td>LmjF35.0820</td>
<td>-3.19</td>
<td>0.0023</td>
</tr>
<tr>
<td>1895</td>
<td>beta tubulin</td>
<td>LmjF33.0798</td>
<td>-4.83</td>
<td>0.0083</td>
</tr>
</tbody>
</table>
A  
NADP dependent alcohol dehydrogenase ~ 27 fold

WT  R8 (+pent)

B  
Transketolase ~ 9 fold regulation

WT  R8 (+pent)
Carboxypeptidase ~9 fold regulation

Fig 18: Proteins strongly upregulated in R8 as determined by DiGE.
A, B, C, D depict the strongly upregulated spots in R8(+ pent) are selected in Decyder software. The 3-D view of each of those spots is also shown. The abundance of these proteins in R8(+pent), R8 (-pent) and WT are plotted in the log scale.
Fig 19: Western Blot Analysis with anti TKT antibody in cell lysate of WT and R8 promastigotes.
Cells were harvested in late log phase and 50 μg of whole cell lysate was loaded on SDS-PAGE gel. The gel was transferred to a PVDF hybond membrane and blotted with Anti TKT antibody at a dilution of 1/5000. The blot was probed with 1/10000 dilution of HRP-conjugated mouse anti-rabbit secondary antibody and then developed using ECL kit. The same blot was reacted with antibody against GAPDH protein to normalize the loading on to each lane of the gel.
fold as seen in DiGE analysis may be due to saturation which is common for highly overexpressed genes (Guimond et al., 2003).

4.13 Overexpression of transketolase in Wild type L. donovani

We next tried to check whether overexpression of TKT in WT L. donovani could confer pentamidine resistance. In order to clone the gene encoding TKT, PCR was performed using specific oligonucleotides whose sequence was based on the Leishmania Genome Sequencing Project of Leishmania infantum (http://www.ebi.ac.uk/parasites/LGN/). Genomic DNA from L. donovani AG83 (MHOM/IN/1983/AG83) promastigotes was used as a template. A single 2 kb PCR product was obtained, cloned in a Xba I- Hind III site of a shuttle vector, psp72aneoα (Fig 20), and was confirmed by sequencing.

TKT construct was transfected in WT L. donovani by electroporation. The transfectants were grown in the presence of G418. To check the effect of pentamidine on the overexpressors of TKT, we determined the IC₅₀ of these overexpressors to pentamidine. The IC₅₀ of wild type L. donovani control to pentamidine was 1 ± 0.1 μM and that of TKT overexpressors was 2.5 ± 0.1 μM (Fig 21). TKT overexpressors were 2.5 fold (p=0.01) more resistant to pentamidine than wild type (Fig 21).

Further work is going on in the laboratory to overexpress other upregulated proteins in wild type and check their response to pentamidine.

4.14 Effect of oxidative stress inducing agents and inhibitors of glucose metabolism on WT L. donovani and R8

DiGE analysis showed that proteins involved in handling oxidative stress were upregulated in R8. Therefore we tried to test if R8 is more tolerant to oxidative stress. The effect of oxidative stress inducing agents was determined by calculating the IC₅₀ of these parasites to these agents. The IC₅₀ of hydrogen peroxide in these parasites were not different. However R8 was more resistant to cumene hydroperoxide (1.5 fold, with p=0.0006) and T- butyryl hydroperoxide (1.46 fold, p=0.0008) than wild type promastigotes (Table 12).

From the biochemical data and proteomics results we also hypothesized that the R8 line probably requires a higher glycolytic flux because it is more dependent on substrate level phosphorylation. To determine that, we checked the effect of 2-deoxy-glucose (2-DOG) on WT and R8 promastigotes. 2-DOG is a synthetic glucose analog and an antimetabolite. This compound has been shown to inhibit glucose metabolism. The
Fig. 20: Cloning of transketolase (TKT) from Leishmania donovani

A. Strategy followed for cloning of Transketolase (TKT) in Leishmania specific shuttle vector, pSP72αneoα from Leishmania donovani.

B: TKT PCR. PCR was performed using L. donovani genomic DNA as template.
Lane 1: HindIII digested λDNA. Lane 2: PCR amplified TKT from L. donovani genomic DNA.
Fig 21: *IC*$_{50}$ of Wild type and TKT overexpressors to pentamidine.

To determine the *IC*$_{50}$, the cells were plated at a density of 1 x 10$^6$ cells/ml (200µl/well) in 96 well tissue culture plates. Pentamidine was added 24 h after plating. The cells were incubated at 22°C for 72 h after drug addition and then the viable cells were counted with a hemocytometer. Resistance of each of the line to pentamidine was determined by measuring the 50% inhibitory concentration (IC$_{50}$) of the drug when compared to the control. The values are the mean ± S.D. of triplicate measurements of 3 independent experiments. There is a significant difference (* p=0.01) in IC$_{50}$ of wild type and TKT overexpressors to pentamidine.
Table 12: Effects of different oxidative stress inducing agents and 2-deoxy glucose on wild type and R8 promastigotes

<table>
<thead>
<tr>
<th>Oxidative stress inducing agent</th>
<th>IC_{50} (μM)</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>R8</td>
<td></td>
</tr>
<tr>
<td>Cumene Hydroperoxide</td>
<td>44 ± 3.5</td>
<td>67 ± 2**</td>
<td></td>
</tr>
<tr>
<td>T Butryl Hydroperoxide</td>
<td>35.7 ± 0.8</td>
<td>52 ± 3**</td>
<td></td>
</tr>
<tr>
<td>2-deoxy-glucose</td>
<td>2.0 ± 0.2</td>
<td>0.5 ± 0.01*</td>
<td></td>
</tr>
</tbody>
</table>

Effects of different oxidative stress inducing agents on *in vitro* growth of pentamidine sensitive and resistant promastigotes after a 72 h incubation period at 22°C. R8 was more tolerant to hydroperoxides than wild type cells. IC_{50} (μM) values are the mean values ± S.D. of 3 different experiments. ** denotes that p value is less than 0.0008. * denotes a p value of 0.008
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Effect of 2-DOG was determined by calculating the IC50 of these parasites to this antimetabolite. The IC50 of WT to 2-DOG is 2.0 ± 0.2 mM whereas that of R8 is 0.5 ± 0.01 mM, showing that WT is 4 fold (p=0.08) more tolerant to 2-DOG than R8 parasites (Table 12), thereby suggesting that the resistant parasites are more dependent on substrate level phosphorylation when the mitochondria is dysfunctional.

4.15 DiGE analysis of the mitochondrial subproteome

A mitochondrial fraction was prepared from wild type and R8 lines, grown in the absence of pentamidine (Harris et al, 1990). 50 µg of each preparation was analysed on a single DiGE gel that was additionally loaded with 200 µg of unlabelled material from each of the two-mitochondrial preparations. Approximately 2000 protein spots were detected and the relative volume of each spot from Cy3 (AG83) and Cy5 (R8) images was compared (Fig 22). No replicates were available in this analysis, due to the difficulty in obtaining sufficient mitochondrial material. Therefore, a modulation of 10 fold or more was selected as the cut off for further analysis. 18 spots showed modulation of more than 10 fold in spot volume. 15 of these were stronger in the R8 mitochondrial preparation and 3 were stronger in the AG83 mitochondrial preparation. Where these could be matched with corresponding protein spots in a Sypro Orange protein stain, spots were excised for tryptic digest and LC-MSMS analysis. This resulted in identification of 8 protein spots that were strongly modulated in pentamidine resistant Leishmania, the 3 protein spots which were downregulated in R8 could not be identified. Proteins, which could be identified as upregulated in R8, were protein with signal sequence and single TM domain (~ 140 fold). This was the most dramatically upregulated protein. The others were protein with some homology to nucleoside diphosphate kinase (~30 fold), 3 isoforms of mitochondrial processing peptidase (10-20 fold), Rieske iron-sulphur protein (8 fold).

5. Discussion

Pentamidine, a cationic aromatic diamidine, is a drug, which has been widely used in the treatment of early stage sleeping sickness caused by Trypanosoma brucei, pneumonia caused by Pneumocystis carinii, fungal infections and antimony-refractory leishmaniasis. Resistance to antimonials is widespread in India; hence the use of non-antimonial drugs is of pressing urgency in this region. Understanding the mechanisms by which resistance might develop is of clear importance if efforts might be put in place to delay this process. Previous studies have given insight into mechanisms of resistance in L. mexicana (Basselin et al, 2002), but less is known of L. donovani. It is tempting to
Fig 22: 2-D DiGE gel showing the mitochondrial subproteome.

200 μg protein was loaded on the gel. A 3-10 nonlinear IPG strip was used for 1st dimension and then the proteins were separated in the second dimension using 12% DALT SDS-PAGE. The gel was stained with Sypro Orange stain.
Table 13: Mitochondrial proteins up-regulated in pentamidine resistant *Leishmania*

<table>
<thead>
<tr>
<th>Mitochondrial proteins</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein with signal sequence and single TM domain</td>
<td>~140 fold</td>
</tr>
<tr>
<td>Protein with some homology to nucleoside diphosphate kinase</td>
<td>~30 fold</td>
</tr>
<tr>
<td>3 isoforms of mitochondrial processing peptidase</td>
<td>10-20 fold</td>
</tr>
<tr>
<td>Rieske iron-sulphur protein</td>
<td>8 fold</td>
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</table>
draw conclusions from one *Leishmania* species across the range of species. However, it is important to learn about what actually happens in each individual species as clear biological differences can be ascertained across the spectrum of *Leishmania* species. We therefore focused on *L. donovani*, the causative agent of Kala-azar in India.

The pentamidine resistance developed by the clone of *L. donovani* in vitro, studied here, is specific to pentamidine and other diamidines and does not involve the multidrug-resistance phenotype. These diamidines competitively inhibit pentamidine uptake, indicating a common transport system. Diamidine transport has been studied in *T. brucei* where the situation regarding pentamidine uptake into *T. brucei* is complex (Damper *et al.*, 1976) with the drug entering via at least three transporters: The P2 amino-purine transporter, a high affinity pentamidine transporter (HAPT1) and a low affinity pentamidine transporter (LAPT1) (De Koning, 2001). *Leishmania* parasites are related to trypanosomes; however it was previously shown in *L. mexicana* (Basselin *et al.*, 2002) that pentamidine does not enter the parasites by a nucleoside transporter. Moreover, *Leishmania* sp. do not have a functional equivalent of the *T. brucei* P2 amino-purine transporter (Iovannisci *et al.*, 1984, Landfear *et al.*, 2004).

Biochemical evidence shows that pentamidine resistance in *L. donovani* is associated with reduced accumulation of the drug in the resistant promastigotes. Wild-type *L. donovani* accumulated 5.4 fold more pentamidine than resistant cells. The uptake of pentamidine into the cells is carrier dependent. Metabolic inhibitors including KCN, azide and oligomycin all decreased pentamidine uptake. A relatively high affinity (with an apparent *K_m* of around 6 μM) transporter could be measured in both wild-type and resistant parasites.

The affinity for pentamidine shown by its transporter in *L. donovani* is unaltered in resistant parasites. A similar situation has been noted in resistance to pentamidine in *L. mexicana* (Basselin *et al.*, 2002) and resistance to isometamidium in *T. congolense* (Wilkes *et al.*, 1997). Resistance in the latter case was concluded not to associate with alterations at the level of a plasma membrane transporter, but with changes in the mitochondrial membrane potential. Isometamidium rapidly enters the *T. congolense* mitochondrion, where it binds tightly to kinetoplast DNA. The decrease in apparent *V_max* in resistant cells correlated to diminished capacity to accumulate drug intracellularly (Wilkes *et al.*, 1997).
Diamidines are organic cations and like isometamidium they have a high affinity for DNA. Ultrastructural changes to kinetoplast DNA and the mitochondrion are observed in parasites exposed to these drugs (Hentzer et al, 1997, Johnson et al, 1998, Vercesi et al, 1992). DB99, a fluorescent analogue of pentamidine accumulates in the kinetoplast of sensitive strain of *L. donovani* but not in the resistant promastigotes, suggesting that the mitochondrion is a site of accumulation of pentamidine, as reported in previous study done with *L. mexicana* where DAPI, another fluorescent diamidine, was used. When cells were treated with digitonin to make their membranes permeable, the kinetoplasts of both the wild type and resistant strains fluoresced indicating that it is access to the kinetoplast, rather than their binding abilities to kinetoplast DNA, that differentiates between sensitive and pentamidine resistant strains.

Previous reports have pointed to a decrease in mitochondrial transmembrane potential in pentamidine resistant sp. (Basselin et al, 1998, 2002). In this study we have also shown that there is a decrease in accumulation of rhodamine 123 (Rh 123) in the resistant promastigotes and in pentamidine treated sensitive parasites. However, less Rh 123 accumulation could also be due to decrease in the mitochondrial biomass, but this is not the case in pentamidine resistance in *L. donovani* as NAO fluorescence revealed that the mitochondrial biomass is unaffected, therefore indicating that it is possibly reflecting a decrease in the mitochondrial membrane potential. Rhodamine 123 is also a substrate for P-glycoprotein efflux (Gueiros-Filho et al, 1995). However, we noted no overexpression of efflux pumps in the resistant line suggesting that enhanced efflux of this marker does not explain its reduced intracellular accumulation. A decrease in mitochondrial membrane potential is thus the most likely reason for decrease rhodamine 123 accumulation in resistant parasites.

The studies of drug localization implicated the mitochondrion and subsequently the kinetoplast of *L. donovani* in accumulation and mode of action of pentamidine. We further analyzed the role of mitochondrial membrane potential on the uptake of pentamidine in wild type and resistant parasites. Studies on the accumulation of pentamidine reveal that in addition to the transporter that appears to be saturable over a 2 minute uptake period in resistant cells, an additional non-saturable component can be measured in wild-type cells. This secondary component can be abolished by interfering with the mitochondrial membrane potential with agents such as CCCP. Two minutes was required to give significant counts of accumulated pentamidine, and this appeared to fall
within the linear range of uptake. However, it cannot be ruled out that this time period does not actually represent initial uptake rates involving simple accumulation at the plasma membrane. It is clear from studies using the fluorescent analogue, DB99 that within this time period drug also commences its accumulation into the mitochondrion. In this event, we can surmise that a single plasma membrane transporter is operative and unaltered in resistant parasites. However, in wild-type cells, but not in resistant cells, accumulation of drug into the mitochondrion removes drug from the cytosol and maintains a concentration gradient across the plasma membrane, allowing continuous uptake in wild-type cells. In this case, the second non-saturable component represents uptake into the mitochondrion. In resistant cells, loss of uptake into the mitochondrion, ensures that equilibrium is reached and thus over the 2 min time frame uptake appears saturable. Net uptake into *L. donovani* is significantly lower over these time frames than into *L. mexicana* (Basselin et al, 2002). The concentration within the *L. donovani* cell would be equivalent to the 1 μM external concentration assuming the intracellular volume of *Leishmania* was around 1.5 μl per 10^6 cells, close to that previously measured (Basselin et al, 1996) indicating that in the resistant line it could be approaching a transmembrane equilibrium between cytosol and extracellular fluid. The drug is clearly accumulated in wild-type cells, probably within the mitochondrion.

In *L. mexicana*, a minor, but significant efflux activity for pentamidine at the plasma membrane was noted (Basselin et al, 1996). Inhibitors of P-glycoprotein pumps, including PCP, TFP and verapamil, all enhanced net pentamidine uptake into resistant *L. mexicana* and also partially reversed the drug resistance phenotype. In the case of *L. donovani*, none of these agents had any impact on either net drug uptake or drug resistance. This indicates that P-glycoprotein mediated efflux of pentamidine is not operative in *L. donovani* as it is in *L. mexicana*. In *L. major*, the basis of pentamidine resistance is overexpression of a P-glycoprotein, PRP1. We tried to see if overexpression of PRP1 in WT *L. donovani* confers pentamidine resistance. But unlike *L. major* and *L. mexicana* P-glycoproteins had no role in pentamidine resistance in *L. donovani*. This result is important as it clearly distinguishes the three *Leishmania* species, with *L. mexicana*, and *L. major* but not *L. donovani* apparently having an efflux system for pentamidine. Inspite of this difference in apparent P-glycoprotein mediated efflux, part of the mechanism of drug resistance in both *L. mexicana* and *L. donovani* appears to be the same. Namely that uptake of drug at the mitochondrial membrane is diminished in
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resistant cell lines and this leads to diminished net uptake of drug in both species. Resistance to isometamidium in *Trypanosoma congolense* (Wilkes et al, 1997) and to pentamidine in both *L. mexicana* and *L. donovani* thus appears to relate to diminished mitochondrial membrane potential and this mechanism of drug resistance with respect to cationic drugs appears to be widespread in this group of organisms. Further work is required to elucidate the genetic basis of the alteration of mitochondrial membrane potential. The decrease in mitochondrial transmembrane potential correlates with the decrease in the activities of numerous mitochondrial dehydrogenases and also the F$_1$F$_0$ ATPase. The basis of this coordinated downregulation in expression of several enzymes in relation to the selection of resistance is not known, but it would be of great interest if all of these enzymes are under coordinated regulation activities in both treated and resistant promastigotes.

The effect of pentamidine on other putative targets was checked in *L. donovani*. Membrane fluidity was assessed by measurement of the fluorescence anisotropy of TMA-DPH following its insertion into the plasma membrane of promastigotes. Pentamidine does not alter the membrane fluidity of *L. donovani*. The resistant parasites and pentamidine treated parasites also doesn't have any significant alteration in the level of membrane polar lipids and cytosolic neutral lipids. The fluidification is classically correlated with two membrane processes: a decrease in the cholesterol/ phospholipid ratio and an increase in the proportion of unsaturated fatty acids in membrane phospholipids. Pentamidine does not alter the lipidic metabolism in *L. donovani* promastigotes. The results are in contrary to what was proposed by Basselin et al in *Leishmania amazonensis* and *L. donovani* (Basselin et al, 1998).

Analysis of polyamine levels in wild type and R8 shows that amount of putrescine is 3 fold lower in R8. The intracellular level of spermidine is not significantly different. Maintainance of the intracellular spermidine concentration seems to be an important requirement for cell viability. This correlates well with the slow growth rate of pentamidine resistant promastigotes, though the viability is not affected as the polyamines are necessary for growth and differentiation. To examine if the decreased putrescine pool is a direct consequence of diminution in the enzymatic conversion of ornithine to putrescine, the expression level of ODC was assessed, which again decreased in both pentamidine treated and resistant promastigotes. Therefore, it seems that the establishment of pentamidine resistance requires adaptation to this low putrescine
Pentamidine resistance in Leishmania concentration. Though it is not sure whether the diminution is a direct effect of pentamidine or a secondary effect.

Comparison of protein expression in drug sensitive and drug resistant cells can reveal proteins that are involved in mediating drug resistance. Stable drug resistance can arise by modulation of protein expression, so it is critical that comparative approaches be quantitative in order to highlight important differences that might be quite small. Gel-based protein separation is readily quantitative, and 2-dimensional gel electrophoresis has the power to resolve substantial numbers of proteins. However, a limitation of comparative 2-D analysis is the high degree of gel-to-gel variation in spot patterns that makes it difficult to distinguish any true biological variation from experimental variation.

2-D Difference gel electrophoresis (DiGE) (Alban et al, 2003) is a novel approach to comparative proteomics that overcomes these problems by enabling analysis of samples for comparison on the same gel. A number of studies (Alban et al, 2003, Yan et al, 2002, Zhou et al, 2002) have shown that DiGE can highlight small but reproducible differences. The detection of CyDye labeled proteins is comparable to fluorescent or silver stains, and we have developed methods to enable recovery of sufficient protein for mass spectometric analysis of even faint spots.

Acquisition of pentamidine resistance is accompanied by a reduction in mitochondrial membrane potential (MMP). Since MMP is required for efficient oxidative phosphorylation and mitochondrial biogenesis/maintenance, mitochondria from pentamidine resistant cells must be dysfunctional. Disregulation of mitochondrial function can lead to decreased ATP production by electron transport chain. As the mitochondrion is dysfunctional, the cells will now depend on substrate level phosphorylation as the oxidative phosphorylation pathway is compromised. In support of this hypothesis, several key enzymes of glycolysis are upregulated, as would be anticipated where efficiency of oxidative phosphorylation is compromised. That the resistant promastigotes are more dependent on glycolytic flux than WT promastigotes was also proved by seeing the effect of 2-deoxy glucose on them. WT cells were 4 times more tolerant to 2-DOG (an inhibitor of glucose metabolism) than the resistant parasites. Disregulation of mitochondrion can also lead to increased production of reactive oxygen species, due to the incomplete oxidation. These will attack and damage proteins and lipids, and must be scavenged efficiently. Therefore proteins involved in managing oxidative stress, protein turnover and protein processing should be overexpressed in the
drug resistant cells. Consistent with this hypothesis, many of the proteins that are found to be upregulated in pentamidine resistant *Leishmania* are involved in response to increased oxidative stress, in the protein turnover and processing that will be required to degrade damaged proteins and to replace functional mitochondrial proteins. Trypanothione reductase (TR), the key enzyme involved in combating oxidative stress is upregulated by 5 fold in R8. Trypanothione reductase (TR) is an unique enzyme in parasites that maintains the reduced environment inside cells (as they lack glutathione reductases), important for the reduction of disulfides, the detoxification of peroxides and the synthesis of DNA precursors (Fairlamb *et al.*, 1985, Schirmer and Schulz, 1987). TRs are members of the NADPH dependent flavoprotein oxidoreductase family. TR and its subordinate thiols are proposed to play a vital role in maintaining an intracellular reducing environment and in protecting these parasites from oxidative damage (Dumas *et al.*, 1997). In the presence of NADPH, they reduce trypanothione disulphides to T(SH)$_2$. This hypothesis is confirmed by checking the effect of peroxides on R8. Pentamidine resistant promastigotes have significant higher IC$_{50}$ to hydroperoxides than wild type cells confirming that they are more tolerant to oxidative stress.

These two conditions i.e energy production and managing oxidative stress can be fulfilled by the resistant parasites by fluxing glucose into the pentose phosphate pathway which produces glycolytic intermediates (fructose 1,6 bisphosphate and glyceraldehyde 3-phosphate) and NADPH. This NADPH is required for combating oxidative damage and the glycolytic intermediates may form ATP by substrate level phosphorylation. Transketolase (TKT) is an important enzyme of the non oxidative step of pentose phosphate pathway and converts 5- carbon sugar phosphates into glycolytic intermediates like fructose-6-phosphate and glyceraldehyde- 3- phosphate. Supporting this hypothesis, DiGE analysis revealed that TKT is overexpressed by 9 fold in the resistant promastigotes. Overexpression of TKT is also confirmed by western blot analysis. We next overexpressed TKT in wild type *L. donovani* to see if TKT conferred pentamidine resistance. Cells overexpressing TKT are 2.5 fold resistant to pentamidine in comparison to wild type cells. Although there is a modest level of pentamidine resistance observed in transfected cells, TKT gene clearly mediates pentamidine resistance as the resistance level is statistically significant when calculated from 3 independent experiments.

Based on the above molecular and biochemical studies involving transport of pentamidine, the following model for pentamidine susceptibility versus resistance can be
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proposed (Fig 23). In WT and pentamidine resistant *L. donovani* there is a high affinity pentamidine transporter, which is energy dependent and saturable and is unaltered in resistant parasites. However, in WT but not in pentamidine promastigotes accumulation of pentamidine (PMD) into the mitochondria occurs because of high mitochondrial membrane potential ($\psi$). This removes drug from the cytosol and maintains a concentration gradient across plasma membrane allowing continuous uptake in WT cells. In this case the second nonsaturable component represents uptake into mitochondria. In resistant cells, loss of uptake into mitochondria, ensures that equilibrium is reached and thus over the 2 min time frame, uptake appears saturable.

The proteome studies further show that dysfunctioning of mitochondrion makes the resistant parasites more dependent on glycolytic flux and substrate level phosphorylation. Overexpression of transketolase leads to higher formation of glycolytic intermediates. Also, a compromise in oxidative phosphorylation leads to the formation of reactive oxygen species. Overexpression of trypanothione reductase combats the oxidative damage in these resistant cells and makes them more tolerant to oxidative stress.

Thus, the great majority of proteins that are observed to be strongly modulated have functions, which fit with phenotypic changes that could compensate for mitochondrial dysfunction. As such, they may be secondary to the changes that confer pentamidine resistance. Alternatively, a complex remodelling of the *Leishmania* proteome may give rise to cells that can tolerate the cytotoxic effects of pentamidine.
In WT and pentamidine resistant *L. donovani* there is a high affinity pentamidine transporter which is energy dependent and saturable and is unaltered in resistant parasites. However, in WT but not in pentamidine promastigotes accumulation of the drug into the mitochondria occurs because of high mitochondrial membrane potential (Ψ). This removes drug from the cytosol and maintains a concentration gradient across plasma membrane allowing continuous uptake in WT cells. In this case the second nonsaturable component represents uptake into mitochondria. In resistant cells, loss of uptake into mitochondria, ensures that equilibrium is reached and thus over the 2 min time frame uptake appears saturable.

**Fig 23:** Proposed model of pentamidine resistance in *L. donovani.*