Chapter XI

Summary and Conclusions
1. The causative agent of Kala-azar, *Leishmania donovani* is rapidly developing resistance to commonly used antimonial compounds pentostam and glucantime leading to the need for the development of novel chemotherapeutic agents against the parasite.

2. Biochemical characterization of leishmanial strains from the Asian subcontinent was done as strain characterization is an integral part of pathogenesis and treatment of the disease. This involved studying the growth pattern, the polyamine levels, the ODC activity and the effect of various inhibitors of the polyamine biosynthetic pathway and commonly used drugs for treating leishmaniasis. Putrescine uptake in *Leishmania donovani* (strain UR6) was also characterized.

3. The linked thiol and polyamine metabolic pathway that leads to the formation of a compound called trypanothione was used as a target for the chemotherapy of visceral leishmaniasis. Since tubulin is a major cell component of the parasite, an inhibitor of microtubular disassembly was also used as a chemotherapeutic agent against Kala-azar.

4. The inhibitors used for this study were as follows:
   i) DFMO, CGP 40215A, CGP 48664A and MDL 27695, which are inhibitors of the polyamine biosynthetic pathway.
   ii) BSO, an inhibitor of glutathione synthesis and
   iii) Taxol, an inhibitor of microtubular disassembly.

5. The ODC inhibitor DFMO as an antileishmanial agent
   i) DFMO was found to inhibit promastigote growth *in vitro* with an IC$_{50}$ of 125 $\mu$M.
   ii) This compound also had an inhibitory effect on amastigote multiplication within murine macrophages.
   iii) DFMO was found to cause a significant inhibition of ODC activity in the parasite. There was no effect on AdometDC activity. Inhibitor treated parasites were found to have decreased putrescine and spermidine levels.
iv) The inhibitory effect of DFMO was evident at lower concentrations in the case of the promastigotes as compared to the macrophages.

v) DFMO led to increased uptake of putrescine within promastigotes. Thus, there appears to be a need for simultaneous administration of some polyamine transport inhibitor along with DFMO to maximize the effects of this inhibitor on the parasite.

vi) DFMO probably caused parasite killing by causing elicitation of nitric oxide levels within macrophages infected with the parasite.

vii) DFMO was not effective against all types of antimony resistant *Leishmania*.

6. The AdoMetDC inhibitors CGP 40215A and CGP 48664A as antileishmanial agents

i) Both CGP 40215A and CGP 48664A were found to be inhibitory to promastigote growth with CGP 48664A being a more potent inhibitor. The IC₅₀ values of the respective inhibitors were 18 μM and 10 μM respectively.

ii) These compounds also had an inhibitory effect on amastigote multiplication within murine macrophages. *In vivo* studies with CGP 40215A confirmed the above finding.

iii) CGP 40215A inhibited both ODC and AdoMetDC activity significantly causing an accumulation of putrescine and a decrease in spermidine levels within promastigotes. CGP 48664A led to an increase in ODC activity and a decrease in AdoMetDC activity. The polyamines putrescine, spermidine and spermine increased in CGP 48664A treated cells.

v) CGP 40215A had a selective action against *L. donovani* promastigotes as compared to bone marrow macrophages. CGP 48664A was quite toxic to host macrophages as compared to CGP 40215A. Thus CGP 48664A was not used for subsequent studies.

vi) Neither of these two inhibitors had any effect on putrescine transport in leishmanial promastigotes. This could be due to the fact that these inhibitors led to putrescine accumulation within treated promastigotes; any further increase in putrescine may be toxic to the cell.

vii) Nitric oxide elicitation in macrophages could be one of the leishmanicidal mechanisms of the CGP compounds *in vivo*.
viii) CGP 40215A was found to be highly efficacious against antimony resistant *Leishmania donovani* *in vitro*.

7. Antileishmanial effect of the bis (benzyl) analogue of spermine, MDL 27695  
i) MDL 27695 was found to affect leishmanial growth with an IC₅₀ value of 18 μM.  
ii) This inhibitor also inhibited amastigote multiplication within murine macrophages.  
iii) MDL 27695 was found to inhibit both ODC and Adomet activity significantly leading to a decrease in putrescine and an increase in spermidine levels within drug treated cells.  
iv) This inhibitor had an almost identical dose dependent inhibitory effect on both parasites and macrophages. However, promastigotes were affected at earlier time points than macrophages. This was probably due to the differential rates of breakdown of MDL 27695 in the host versus parasite systems as determined by the estimation of polyamine oxidase activity in the parasite and macrophage systems. MDL 27695 may also reduce the capacity of host cells to bind or ingest the parasite. Also inhibition of polyamines in the parasite affected their invasive capacity.  
v) Treatment of the parasite with this inhibitor did not lead to uptake of putrescine by the cells probably due to competitive inhibition between the inhibitor and the polyamine for transport inside the parasite.  
vii) Nitric oxide elicitation in macrophages could be one of the leishmanicidal mechanisms of MDL 27695 *in vivo*.  
viii) MDL 27695 was also found to be highly effective against antimony resistant *Leishmania*.

8. BSO, the glutathione biosynthesis inhibitor as an antileishmanial agent  
i) BSO was found to inhibit promastigote growth with an IC₅₀ of 10 mM.  
ii) It was also found to significantly inhibit amastigote multiplication within macrophages.
iii) Macrophage inhibitory studies showed that at the concentrations used to inhibit promastigote growth, BSO was not very effective against host macrophages.
iv) BSO was found to deplete GSH levels in *Leishmania donovani* promastigotes within 12 hr of treatment.
v) This inhibitor was also found to cause a significant inhibition in the SOD levels in the parasite, indicating that this inhibitor might lead to increased parasite killing by increasing reactive oxygen species within the parasite.
vii) BSO also led to an increase in reactive nitrogen species within macrophages. This was probably related to the inhibition of GSH by this inhibitor; thereby leading to parasite killing.
viii) A combination of BSO with other inhibitors of the polyamine biosynthetic pathway showed an increased inhibitory effect on parasite growth than either of the compounds used alone. This suggested that a combined chemotherapeutic schedule involving the use of polyamine inhibitors along with BSO might be a more effective strategy for treating visceral leishmaniasis.

9. Signal transduction in macrophages by *Leishmania donovani* lipophosphoglycan
i) Signal transduction events mediated by the surface component of the parasite, LPG, were studied in J774A.1 murine macrophages, using ODC as a marker of macrophage activation.
ii) LPG was found to stimulate ODC activity in macrophages in a dose and time dependent manner, confirming the possible role of ODC as an early marker of macrophage activation. LPG stimulated ODC activity within 30 min of macrophage treatment, suggesting that interaction of LPG with its receptor stimulated a specific signal transduction pathway within the macrophages. This induction of ODC was a transient event since the ODC levels reached basal levels at 3 hr after exposure to LPG.
iii) The parasite *Leishmania donovani* also stimulated ODC activity in macrophages. The effect on ODC activity was dependent on the parasite to macrophage ratio and the time of macrophage infection. However, the effect on ODC activity with the parasite was less than that observed with purified LPG.
iv) LPG stimulated ODC through protein kinase signal transduction as judged by the use of a broad spectrum inhibitor of protein kinase, staurosporine.

v) The use of okadaic acid along with staurosporine showed that a protein serine-threonine kinase may not be the relevant target for staurosporine within macrophages. However, it may also be possible that the treatment time with OKA may not have been sufficient to cause changes in the degree of protein phosphorylation. Specific inhibitors of individual protein kinases may help to sort out which of the many protein kinases present in the macrophage could be the relevant target for staurosporine.

vi) ODC activation appeared to be coupled to the activation of PKC in macrophages as activators of PKC stimulated a rapid increase in ODC.

vii) LPG inhibited induction of ODC activity in macrophages by activators of PKC. This was contrary to the result that ODC induced ODC activity in macrophages within 30 min of treatment but could be explained on the basis that the induction of ODC by LPG was a transient event; with the ODC activity reaching basal levels at 3 hr after exposure.

viii) LPG did not inhibit the induction of ODC activity by dibutyryl cyclic AMP ruling out the role of PKA in LPG stimulation of ODC activity.

10. The antileishmanial effect of the microtubule stabilizing agent, taxol
i) Taxol was found to affect promastigotes growth significantly with an IC₅₀ of 35 nM.

ii) It was also found to inhibit amastigote multiplication within host macrophages.

iii) Macrophage inhibitory studies with this compound showed that the concentrations used to inhibit the promastigotes were ineffective against host macrophages.

iv) Taxol led to a significant blockage of cells in G₂ - M phase of the cell cycle and the percentage of cells in S phase got decreased significantly.

v) This inhibitor had a dose dependent effect on tubulin assembly in leishmanial promastigotes in vitro.

11. The mechanism of resistance to taxol in Leishmania donovani promastigotes.
i) A mutant strain of *Leishmania donovani* that was resistant to 500 nM taxol was raised by exposing wild type cells to gradually increasing concentrations of taxol.

ii) This strain was approximately 30 fold more resistant to taxol, 2 fold more resistant to Adriamycin and 5 fold more resistant to Vinblastine as compared to the wild type strain. T-500 was also found to be 2 fold and 5 fold more resistant respectively to Pentostam and Glucantime, the drugs commonly used to treat leishmaniasis. The resistance exhibited by T-500 to unrelated drugs suggested that this cell line had a multidrug phenotype.

iii) This multidrug resistance phenotype was reversed by the calcium channel blocker Verapamil.

iv) DNA amplification was observed in the T-500 strain as compared to the wild type strain. However, DNA hybridization studies with the human mdr-1 probe ruled out the possibility that there was amplification of the mdr gene in the resistant strain as compared to the wild type strain.

v) *In vitro* tubulin polymerization studies with wild type and T-500 strain showed that the taxol resistant strain required much higher concentrations of taxol for its polymerization as compared to the wild type strain. The higher resistance of polymerization to taxol in the T-500 strain may be due to alterations in the tubulin of the parasite.

From the existing results, it may be concluded that the linked glutathione-polyamine biosynthetic pathway leading to the formation of a novel compound trypanothione, which is present only in the parasite and not in the host, could be used as a target for treating leishmaniasis. A combination of inhibitors of glutathione and the polyamine biosynthetic pathway appear to have a better scope for treating leishmaniasis than either of these inhibitors used individually.

Also the role of taxol, a microtubule stabilizing agent, as a possible chemotherapeutic agent in leishmaniasis and the mechanisms of drug resistance to taxol opens up an entirely new vista in this field.