Conclusions

Epidemics of fatal Visceral Leishmaniasis (VL) caused by the intracellular protozoan Leishmania are a public health problem in tropical and subtropical regions of the world. A major drawback in the treatment of Leishmaniasis is the emergence of resistance to current chemotherapeutics. Since, to date, vaccination approaches have failed to enter clinical trials, chemotherapy is currently the main option (Polonio and Efferth, 2008) highlighting the need for developing novel drugs with improved features to the pool of current chemotherapeutics.

Leishmania parasites possess an impaired antioxidant system and triggering of oxidative stress seems a valid chemotherapeutic modality. In fact, SAG, the first line of treatment for Leishmaniasis (Sundar & Chatterjee, 2006) generates reactive oxygen species (ROS) both within the parasite (Mehta & Shaha, 2006; Mandal et al., 2007) and macrophage phagolysosomes (Sudhandiran & Shaha, 2003) and triggers apoptosis (Das et al., 2001; Mukherjee et al., 2002) by mitochondrial dysfunction in promastigotes.

Several plant-derived anti leishmanial compounds including Aloe vera (Dutta et al., 2007a,c), Artemisinin (Sen et al., 2007), Piper betle Linn (Sarkar et al., 2008), Luteolin (Mitra et al., 2000) and Curcumin (Das et al., 2008) amongst others have also been shown to induce apoptosis in Leishmania parasites by inflicting an enhanced oxidative insult upon susceptible Leishmania parasites (Sen et al., 2007; Das et al., 2008; Mittra et al., 2000).

In the present study, we have characterized the apoptotic pathway induced by Berberine chloride in L. donovani promastigotes, the causative species of VL. Berberine chloride, a benzodioxoloquinolizine (Vennerstrom & Klayman, 1988) displays a broad spectrum of anti-microbial activity (Schmeller et al., 1997) and its effectiveness in leishmaniasis has been amply demonstrated (Ghosh et al., 1983; Ghosh et al.,1985; Vennerstrom et al., 1990). With regard to the IC_{50} in promastigotes, our data corroborated with previous studies (Ghosh et al., 1983) but in amastigotes, we demonstrated a >3 fold decrease in the IC_{50} as compared to promastigotes (7.1 μM vs 2.54 μM) which was not in concordance with previous studies possibly due to variation displayed by different strains (Ghosh et al., 1983). Importantly, Berberine chloride showed a high safety index (> 39 fold), and therefore could be considered as a potent antileishmanial drug. This leishmanicidal activity of Berberine chloride was initiated by its pro-oxidant effect, evidenced by enhanced generation of reactive oxygen intermediates that was accompanied by depletion of non protein thiol. Furthermore, pre incubation with N-acetyl cysteine, anti-oxidant, enhanced cell viability, corroborating that generation of free radicals triggered the parasiticidal activity of Berberine chloride. It induced externalization of phosphatidyl serine; elevation of intracellular calcium in promastigotes,
Conclusions

depolarization of the mitochondrial membrane potential, which translated into an increase in the sub G_0/ G_1 population, and was accompanied by DNA laddering, hallmarks of apoptosis. The failure of Berberine chloride to induce caspase activity and the unchanged antileishmanial activity in the presence of a pan caspase inhibitor, Z-Val-Ala-DL-Asp (methoxy)-fluoromethylketone, indicated that the apoptosis observed was caspase independent. Collectively, our data indicates that Berberine chloride, a potent anti leishmanial compound triggers an apoptosis-like death following enhanced generation of reactive oxygen species, thus meriting further pharmacological investigations.

Infection with the obligate intracellular protozoan, Leishmania is thought to be initiated by direct parasitization of macrophages, but the early events following transmission to the skin by vector sand flies are difficult to examine directly. Using dynamic intravital microscopy and flow cytometry, it has been reported that a rapid and sustained neutrophilic infiltrate occurs at localized sand fly bite sites and phagocyted L. major remained viable within infected neutrophils (Peters et al., 2008). Since parasites entering macrophages via the uptake of infected apoptotic PMN may survive and multiply within macrophages, apoptotic neutrophils are proposed to serve as "Trojan horses" (Laskay et al., 2008). Neutrophils are naturally short-lived and spontaneously undergo apoptosis (Laskay et al., 2008), while the presence of Leishmania parasites delays their apoptosis, via interference in production of reactive oxygen intermediates (Laufs et al., 2002), thus increasing the life span of neutrophils, which facilitate parasite survival (Aga et al., 2002). To trigger apoptosis, neutrophils utilize specific signalling pathway, MAPK pathways (Aoshiba et al., 1999). As Berberine chloride, is an effective anti-leishmanial agent that mediates its leishmanicidal activity by generation of reactive oxygen species culminating in apoptosis, we evaluated its effect in Leishmania infected neutrophils. We demonstrated that Berberine chloride induced apoptosis in Leishmania infected neutrophils via generation of oxidative burst leading to a reduction in the parasite burden. Berberine chloride in Leishmania infected neutrophils upregulated both ERK ½ and p38 MAPK pathways, suggesting that Berberine chloride in neutrophils exerts an immunomodulatory action via the mitogen activated protein kinase (MAPK) pathway, highlighting its importance as an potential antiparasitic target.

Since amastigotes, the causative form of Leishmaniasis, is responsible for cell to cell spread of Leishmania and resides within macrophages, amastigote-macrophage interactions represent an important target for anti-leishmanial drugs. Macrophages respond to infectious pathogens through regulation of T helper 1 (Th1) and T helper 2 (Th2) cells, wherein Th1 cells secrete IFN-γ that enhances macrophage microbicidal activity, protecting the host from intracellular Leishmania pathogens (Robert MT, 2006).
Conclusions

The parasite cleverly augments the Th2 response, which is followed by increased secretion of IL-4 and IL-10. The generation of host protective Th1 cells hinges on IL-12 secreted by macrophages and dendritic cells. Nitric oxide (NO) is an important biological signaling and effector molecule in inflammation and immunity, and is known to be vital for killing intracellular parasites. The inducible form of NOS (iNOS) is also induced by Th1 type cytokines and suppressed via Th2 cytokines. In Leishmania infected macrophages, Berberine chloride enhanced the production of NO, mRNA expression of iNOS and IL-12p40 along with down regulation of IL-10 expression.

The intracellular signaling encompassing IL-10 regulation has implicated a role for mitogen activated protein kinases (MAPKs), a group of serine/threonine kinases responsible for phosphorylation of cellular proteins (Karin M, 1992). In Leishmania infection, MAPKs promote parasite survival by modulating expression of IL-10 and IL-12 in macrophages (Mathur et al, 2004) which is achieved via Leishmania lipophosphoglycans that stimulate phosphorylation of ERK pathways which in turn, inhibits production of IL-12 (Feng et al, 1999). Thus, as ERK and p38 MAP kinases differentially regulate induction of macrophage effector molecules, one is tempted to propose that these kinases be considered as potential targets. As Berberine chloride has been shown to induce IL-12 following activation of p38 MAPK, (Kang et al, 2002), it prompted us to study whether this accounted for its anti leishmanial activity. Berberine chloride caused a time dependent increase in phosphorylation of p38 MAPK, concomitant with reduction in phosphorylation of extracellular signal related kinase (ERK½); with a p38 MAPK inhibitor, SB203580 the involvement of p38 MAPK pathways was confirmed. Taken together, our results indicate that Berberine chloride exerts its immunomodulatory action via the mitogen activated protein kinase (MAPK) pathway in macrophages, highlighting the importance of MAPKs as a target for future drug development.