CHAPTER-5
EFFECT OF BERBERINE CHLORIDE ON
*L. DONOVANI* INFECTED MACROPHAGES
L. donovani infected macrophages and Berberine chloride

Introduction

Macrophages ubiquitously present in all body tissues carry out fundamental protective functions against invading pathogens through phagocytosis and destruction of microorganisms, thereby serving as an integral component of the body's innate immunity (Johnston, 1988; Alexander and Russel, 1992). Macrophages also play an essential role in acquired immunity to microorganisms by serving as antigen presenting cells and secreting a variety of cytokines during development of the immune response (Gordon and Taylor, 2005). As Leishmania resides within macrophages (Sunderkotter et al., 1993, Muller et al., 2001), their survival within this hostile environment hinges on their ability to modulate various macrophages related immune functions including production of Reactive nitrogen intermediates (RNI) (Naderer et al., 2008).

Nitric oxide (NO) is an important biological signaling molecule in inflammation and immunity and its importance in killing intracellular parasites is well established (Bogdan, 2001). It is synthesized by nitric oxide synthase (NOS) during the conversion of L-Arginine to L-citrulline. There are three isoforms of NOS namely neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3). The nNOS and eNOS isoforms are also known collectively as constitutive NOS (cNOS), because unlike iNOS which is inducible, they usually exist as constitutively expressed cellular proteins and are primarily regulated by Ca\(^{2+}\) fluxes and subsequent binding of calmodulin (Macmicking et al., 1997). The inducible form is produced mainly by macrophages in great quantities during inflammation and other diseases.

Macrophage and Leishmania interaction

In order to develop a successful relationship of the parasite with its host, the Leishmania must evade both innate and adaptive immune responses. Leishmania first enter the human body, as promastigotes which get engulfed by macrophages but being resistant to proteolysis and degradation in the phagosome, can reside inside the macrophages. Promastigotes are transformed into amastigotes and continue to live inside the macrophage. During each of the steps described, the protozoa cleverly evade the human immune system.
Opsonization and Phagocytosis

Both the two developmental forms, promastigotes and amastigotes, exploit different cellular components to facilitate their entry and survival within host macrophages. Promastigotes utilize host complement component C3, whereas the amastigotes use host IgG (Peters et al., 1995; Guy and Belosevic, 1993). The ability of *Leishmania* promastigotes to resist serum lysis is dependent on their expression of gp63.

The complement protein C3b is one of the most potent immune opsonins as it binds to foreign material and promotes parasite uptake, via C3b receptors present on phagocytic cells. Interestingly, *Leishmania* parasites have evolved to resist and circumvent full complement lysis by several mechanisms; firstly, metacyclic promastigotes are more resistant to complement lysis because of membrane alteration during development which prevents the insertion of C5b - C9 complex into the parasites outer membrane. Secondly, the *Leishmania* have a special surface glycoprotein, called gp63 and Lipophosphoglycan (LPG), which converts C3b into iC3b and mediates binding of iC3b to the parasites surface which favors phagocytosis but not subsequent lytic clearance (Dominguez et al., 2003; von Stebut, 2007). Finally, *Leishmania* parasites are able to express protein kinases that phosphorylate C3, C5, C9, which leads to inhibition of complement (von Stebut, 2007). *Leishmania* are very resistant to degradation once phagocytosed, and therefore, this conversion makes sense in terms of *Leishmania*'s survival. Another interesting point is that iC3b is also proposed to promote phagocytosis by B cells. This opsonin-mediated phagocytosis being a non-antigen-specific phenomenon results in a humoral response against irrelevant epitopes which are ineffective against *Leishmania*. In case of amastigotes, it was demonstrated that IgG on the surface of amastigotes was opsonic and allowed amastigotes to specifically interact with the macrophage Fcγ receptors (Peters et al., 1995; Guy and Belosevic, 1993). Importantly this IgG on *Leishmania* amastigotes is a potent inducer of IL-10 in infected cells (Peters et al., 1995; Kane and Mosser, 2001; Nylen and Gautam, 2010), was able completely abrogate production of IL-12 and also dramatically reduce TNF-α production by macrophages, collectively facilitating intracellular survival of parasites.

After being engulfed, *Leishmania* parasites must endure harsh conditions inside the phagosome, which undergo a maturation process involving fusion with endocytic organelles, including endosomes and lysosomes resulting in a mature phagolysosome.
L. donovani infected macrophages and Berberine chloride (Lodge and Descoteauxa, 2005). The mature phagolysosome is the major microbicidal site in macrophages, major contributory factors being lysosomal hydrolyzing enzymes, acidic pH and toxic molecules that include nitric oxide (NO) and other reactive oxygen intermediates. *Leishmania* produces acid phosphatases on its surface which inhibits this burst. In addition to the oxidative burst, macrophages often attempt to degrade parasites with acidic enzymes. This occurs when lysosomes fuse with the phagosome. *Leishmania* can resist this attack because it has a proton pump on its surface which ensures that the intracellular pH remains close to neutral (Sharma and Singh, 2009).

In this regard it is noteworthy that the promastigotes surface molecule LPG appears to be responsible for delaying phagolysosome maturation (Winberg et al., 2009). Delaying the maturation process of the phagolysosome allows sufficient time for differentiation from promastigotes to amastigotes to occur which takes about 48 h following phagocytosis.

**Survival in macrophages through impairment of the acquired immune response**

*Leishmania* species are considered as master manipulators of the host innate and acquired immune mechanisms and succeed by mainly modulating macrophage related immune functions (Naderer et al., 2008). Indeed, their unique mechanism(s) of survival to evade the host immune response is a very critical feature of the disease. The engulfed pathogen by preventing the formation of phagolysosomes suppresses MHC-II mediated presentation of the parasite antigen to CD4+ T cells, therefore, macrophages are unable to kill the phagocytosed pathogen (Engwerda et al., 2004). The resultant inactivation of the macrophage also fails to initiate a respiratory burst or release of NO, thus making the macrophage a safe heaven for the parasite. Therefore, modulation of the macrophage function may be looked upon as a *de novo* chemotherapeutic strategy for Leishmaniasis.

The outcome of leishmanial infections is determined by two functionally distinct T-helper (Th) cell populations, Th1 and Th2. The Th1 cells release IFN-γ, IL-2 whereas Th2 secrete IL-4, IL-10 and IL-5. With regard to cutaneous Leishmaniasis, it is the best documented example of the differential activation Th1 and Th2 subsets (Reiner & Locksley, 1995). Uncontrolled non healing infections i.e. susceptibility to disease is
associated with the proliferation of Th2 cells and production of IL-4, 5 and 10. On the other hand, healing responses i.e. resistance to disease is dependent on the expansion of IFN-γ producing CD4+ Th1 helper cells which controls the primary infection, by activating macrophages (Bhaumik et al., 2009). Indeed, pretreatment of macrophages with IFN-γ and IL-12 has been shown to induce resistance to Leishmania major at the early phase of infection (Ota et al., 2008). Accordingly, key roles have been identified for IFN-γ in mediating expansion of protective, Th1 cells and for IL-4 in mediating progressive infection following expansion of Th2 cells.

With regard to the visceral form, the immunological mechanisms underlying susceptibility or resistance to disseminated visceral parasitism remains less clearly defined. It is generally accepted that VL like all forms of Leishmaniasis is also associated with a marked impairment of macrophage activation. It is accompanied by the inability to generate IL-2 and IFN-γ production thereby allowing for parasite persistence (Thakur et al., 2004). An accompanying increase in IL-4 and IL-10 levels points towards an initial Th2 response (Sacks & Noben-Thrauth, 2002). Recovery from active disease is associated with rise in IFN-γ levels indicating a shift towards a Th1 response (Ghalib et al., 1995). However, measurement of splenic and bone marrow cytokine mRNA levels have shown increased levels of both IL-10 (Th2) and IFN-γ (Th1) at disease presentation. Resolution of infection showed a simultaneous decrease in both IL-10 and IFN-γ indicating that both Th1 and Th2 are increased during active disease and both regress with effective treatment (Khoshdel et al., 2009; Goto and Prianti, 2009).

IL-12, a proinflammatory heterodimeric cytokine secreted by macrophages, plays a critical role in the initiation and regulation of Th1 immune responses as it stimulates the production of IFN-γ producing CD4+ cells. The neutralization of IL-12 has been shown to exacerbate Leishmania infection (Bhaumik et al., 2009). As IL-10 is known to promote intracellular infection by disabling Th1 mediated cellular responses, blockade of IL-10 has been proposed as a possible therapeutic option. Carvalho et al., 1985 have demonstrated that in normal mice with established Leishmaniasis, administration of anti IL-10 induced near cure by itself. It has been shown that anti-IL-10 mAb can protect against experimental Visceral Leishmaniasis via induction of Th1 cytokines and nitric oxide (Bhattacharjee et al., 2009).
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The intracellular signaling encompassing IL-10 mediated attenuation of the host response has implicated a role for mitogen activated protein kinases (MAPKs), a group of serine/threonine kinases responsible for phosphorylation of cellular proteins which in turn triggers signals required for cell proliferation, differentiation and survival (Karin M, 1992). With regard to Leishmania infection, CD40 mediated MAPKs have been reported to promote parasite survival by modulating the expression of IL-10 and IL-12 in macrophages (Mathur et al., 2004). It has been shown that CD40 signalling exerts dual counteractive effects on immune system, but the differential signaling depends on the composition of the signalosomes assembled on the membrane. When CD40 binds TRAF 2, 3, 5, its signals primarily through p38MAPK, whereas binding to TRAF6 signals primarily through ERK1/2 (Bhardwaj et al., 2010). This has been proposed to be achieved via Leishmania lipophosphoglycans that stimulate ERK pathway, which in turn, inhibits production of IL-12 in macrophages (Feng et al., 1999). It has also been demonstrated that parasites modulated the TLR2-stimulated mitogen-activated protein kinase (MAPK) pathway by suppressing p38 MAPK phosphorylation and activating extracellular regulated kinase ERK1/2 phosphorylation (Chandra and Naik, 2008). Nitric oxide, a crucial mediator for leishmanicidal activity, was found to be dependent on iNOS expressions and was linked to the mitogen-activated protein kinases (MAPK) signaling pathway (Gupta et al., 2009). Ben-Othman et al., 2008, shows that Leishmania initially activates but subsequently down-regulates intracellular mitogen-activated protein kinases and nuclear factor-kappaB signaling in macrophages. Thus, as ERK and p38 MAP kinases differentially regulate induction of macrophage effector molecules which dictate the course of infection, one is tempted to propose that these kinases be considered as potential targets for development of novel strategies to combat Leishmaniasis.
Materials and methods

Parasite culture
Promastigotes from an Indian *Leishmania donovani* isolate, NS2 were routinely passaged in BALB/c mice and after transformation, cultured at 24°C in Medium 199 supplemented as described in Materials and methods. For infection of macrophages, 24 h prior to the experiment, stationary phase promastigotes i.e parasites kept in culture for 5 days were centrifuged and the pellet resuspended in Schneiders medium (pH 5.5, da Luz et al., 2009).

Ex vivo anti amastigote activity in macrophages
Peritoneal macrophages lavaged from BALB/c mice were seeded in 16 chambered slides and infected with stationary phase *L. donovani* promastigotes as described in Materials and methods. At the end of the experiment, cells were fixed, Giemsa stained and examined microscopically for intracellular amastigotes. At least 100 macrophages per well were counted for calculating the percentage of infected macrophages (Sen et al., 2010).

Safety index of Berberine chloride
The safety index of Berberine chloride was evaluated in murine macrophages and the MTS-PMS assay was performed as described in Materials and methods (Ganguly et al., 2006).

Determination of intracellular NO in *L. donovani* infected macrophages
Intracellular generation of NO was measured in *L. donovani* infected macrophages following treatment with Berberine chloride, using a NO specific probe 4,5 diaminofluorescein -2 diacetate (DAF-2DA) as described in Materials and methods (Chatton and Broillet, 2002, Rathel et al., 2003).

Measurement of extracellular NO in *L. donovani* infected macrophages
Extra cellular production of NO was measured in *L. donovani* infected macrophages using Griess reagent as described in Materials and methods (Sarkar et al., 2008).

Reverse transcriptase polymerase chain reaction (RT-PCR) of iNOS, IL-12p40 and IL-10
Total RNA was isolated from normal and *Leishmania* infected BALB/c murine peritoneal macrophages that had been treated with Berberine chloride (0-10 μM) for 18 h, using the RNAqueous® Kit and RT-PCR was carried out as described in Materials and methods (Ganguly et al., 2007).
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**Measurement of IL-12 by sandwich ELISA**
Levels of mouse IL-12p40 present in supernatants of uninfected and *L. donovani* infected macrophages that had been treated with Berberine chloride (24 h) were measured using an ELISA kit as per the manufacturer's instructions as described in Materials and methods.

**Western blotting in pp38 MAPK and pERK1/2**
Peritoneal macrophages isolated from BALB/c mice were infected with stationary phase promastigotes and after being treated with Berberine chloride were processed for western blotting as described in Materials and methods.

**Statistical Analysis**
Results were expressed as mean ± SD/SEM as indicated. Statistical analysis was evaluated by one way ANOVA (Tukey's Multiple Comparison Test) using Graph Pad Prism software (version 4), p<0.05 was considered as statistically significant.

**Results**

**Anti-amastigote activity of Berberine chloride**
During *Leishmania* infection, promastigotes transform into amastigotes within phagolysosomal vacuoles of macrophages. Accordingly, the anti-leishmanial activity of Berberine chloride (0 - 25 μM, 72 h) was evaluated in intracellular amastigotes wherein the infection rate of *Leishmania* infected macrophages was normalized to 100%; with the addition of Berberine chloride, a dose dependent reduction in parasitic load was evident, the IC₅₀ being 2.54 μM (Figure 1).

To evaluate the safety index of Berberine chloride, its effect on the viability of murine macrophages was evaluated by the MTS-PMS assay. Macrophage viability remained unaffected up to 25 μM and at the highest concentration (100 μM), cell viability decreased to 55.5 % (Figure 1, inset); therefore, the IC₅₀ of Berberine chloride in macrophages was at least 39 fold higher than the IC₅₀ obtained in amastigotes.
Figure 1: Anti amastigote activity and safety index of Berberine chloride

The anti-leishmanial activity of Berberine chloride (0 - 25 µM, 72 h) was tested in intracellular amastigotes as described in Materials and methods. Each point corresponds to the mean ± SD of at least three experiments in duplicate.

Inset: The safety index of Berberine chloride in murine macrophages was evaluated by the MTS-PMS assay as described in Materials and methods. Each point corresponds to the mean ± SD of at least three experiments in duplicate.

**Effect of Berberine chloride on production of NO in parasitized macrophages**

In uninfected macrophages, Berberine chloride caused no morphological changes as evidenced by an unchanged forward (FSC) and side scatter (SSC), with cells remaining predominantly in the R1 gate (Figure 2A). However, infection with *L. donovani* parasites effected morphological changes by enhancing the internal granularity in macrophages resulting in cells moving out of the R1 gate into R2, which remained unchanged with the addition of Berberine chloride (Figure 2A).

As NO is an important biological signaling and effector molecule necessary for killing intracellular parasites, *Leishmania* to survive and propagate within host macrophages inhibits several macrophage functions, including production of RNI. Therefore, it is anticipated that an anti-leishmanial compound will influence production of NO. To measure intracellular NO, a dye 4, 5 diamino fluorescein (DAF-2DA) was used which has the propensity to enter the cell, release the diacetate groups of DAF-2DA following hydrolysis by cytosolic esterases, the resultant DAF-2 in the presence of NO gets converted into an impermeable and importantly, highly fluorescent triazolofluorescein...
In uninfected macrophages, Berberine chloride (2.5 and 10.0 μM, 24 h) increased the baseline GMFC by a 1.24 and 1.52 fold increase in production of NO, the baseline GMFC increasing from 54.57 ± 3.17 to 68.06 ± 1.3 and 82.88 ± 1.66 respectively (Figure 2B). Infection with *Leishmania* translated into a significant decrease in production of NO as compared with uninfected macrophages, GMFC being 32.84 ± 4.76 (p< 0.001) (Figure 2B), which was reversed by Berberine chloride (2.5 and 10.0 μM), as it caused a significant 1.7 and 2.0 fold increase in generation of NO to 56.22 ± 2.22 (p< 0.001) and 65.78 ± 3.22 (p< 0.001) respectively (Figure 2B).

At 48 h, the basal levels of NO increased in uninfected macrophages, GMFC being 79.65 ± 3.08, but remained unaltered with Berberine chloride (Figure 2C). However, *Leishmania* infected macrophages showed a further decrease in production of NO as compared with their uninfected counterparts, GMFC being 24.42 ± 1.98 (p< 0.001) (Figure 2C). Once again, Berberine chloride (2.5 and 10 μM) increased generation of NO by 3.23 and 3.50 fold, GMFC being 79.11 ± 6.9 (p< 0.001) and 85.57 ± 8.33 (p< 0.001) respectively (Figure 2C). Methanol, the vehicle control, showed no effect thereby confirming its immunological inertness (data not shown).

Additionally, measurement of extracellular NO in uninfected macrophages at 24 h showed generation of NO to be 4.08 ± 0.52 μM (mean ± SEM), which with the addition of Berberine chloride (2.5 and 10 μM) increased marginally to 5.37 ± 0.33 and 5.69 ± 0.16 respectively (Figure 2D); infection with *L. donovani*, caused minimal changes in generation of NO, and remained unchanged in the presence of Berberine chloride (2.5 and 10 μM) (Figure 2D).

At 48 h, extracellular NO in uninfected macrophages increased 2.1 fold as compared to levels measured at 24 h (8.68 ± 1.16 μM) which was slightly decreased by Berberine chloride (2.5 and 10 μM) to 6.45 ± 1.50 μM and 6.15 ± 1.29 μM respectively (Figure 2E). Infection with *Leishmania* caused a 1.68 fold decrease as compared with uninfected macrophages, mean ± SEM being 5.14 ± 0.77 μM, p<0.01 and with the addition of Berberine chloride, 2.5 and 10 μM, production of NO significantly increased by 1.67 and 1.4 fold to 8.61 ± 2.60 (p<0.01) and 7.18 ± 2.25 respectively, reaching levels comparable to uninfected macrophages (Figure 2E).
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Figure 2A

Figure 2B

Figure 2C
**Figure 2D**

**Figure 2E**

**Figure 2: Effect of Berberine chloride on generation of NO and expression of iNOS**

A: A representative dot plot of uninfected (a) and *Leishmania* infected (b) murine peritoneal macrophages, that were treated with Berberine chloride (10 µM, 48 h, c, d). Cells were gated on the basis of characteristic linear forward and side scatter features of macrophages and subsequently DAF-2T fluorescence was measured on a logarithmic scale in the FL1 channel. A representative histogram of uninfected macrophages (e, —) and *L. donovani* infected macrophages (f, —) for DAF-2T that were treated with Berberine chloride (...) macrophages as described in Materials and methods.

B: Uninfected macrophages (1 X 10^6/mL, □, a) or *L. donovani* infected macrophages (■, a) were treated for 24 h with Berberine chloride 2.5 µM (b) and 10 µM (c), and processed for measurement of DAF-2T fluorescence as described in Materials and methods. Data are expressed as the mean GMFC ± SEM of at least 3 experiments in duplicate.

C: Uninfected macrophages (1 X 10^6/mL, □, a) or *L. donovani* infected macrophages (■, a) were treated for 48 h with Berberine chloride 2.5 µM (b) and 10 µM (c) and processed for measurement of DAF-2T fluorescence as described in Materials and methods. Data are expressed as the mean GMFC ± SEM of at least 3 experiments in duplicate.

D: Uninfected macrophages (1 X 10^6/mL, □, a) or *L. donovani* infected macrophages (■, a) were treated for 24 h with Berberine chloride 2.5 µM (b) and 10 µM (c) and assayed for levels of extracellular NO as described in Materials and methods. Each point represents the mean ± SEM of NO_2^- (µM) of at least 3 experiments in duplicate.

E: Uninfected macrophages (1 X 10^6/mL, □, a) or *L. donovani* infected macrophages (■, a) were treated for 48 h with Berberine chloride 2.5 µM (b) and 10 µM (c) and assayed for levels of extracellular NO as described in Materials and methods. Each point represents the mean ± SEM of NO_2^- (µM) of at least 3 experiments in duplicate.
**Berberine chloride enhanced mRNA expression of iNOS**

As increased generation of NO is an established event necessary for elimination of *Leishmania* parasites, its production which is dependent upon activation of iNOS has been shown to be upregulated by anti-leishmanial compounds (Ukil et al., 2005; Bhattacharjee et al., 2009). In uninfected macrophages, Berberine chloride (2.5 and 10 μM) induced a 10 and 17 fold increase in the mRNA expression of iNOS (Figure 2F). Following parasitization, a 3.7 fold down regulation of mRNA expression of iNOS ensued which with the addition of Berberine chloride (2.5 and 10 μM), dramatically increased (Figure 2F).

![Figure 2F](image-url)

**Figure 2F: Effect of Berberine chloride on iNOS in macrophages**

Uninfected macrophages (a) and *L. donovani* infected macrophages (d) were treated for 24 h with Berberine chloride, 2.5 μM (b, e) or 10 μM (c, f). RWA was isolated and subjected to RT-PCR and the products of β-actin and iNOS mRNA were resolved on an agarose gel (1.5%) and quantified densitometrically using Total lab software as described in Materials and methods.

**Berberine chloride enhanced mRNA expression of IL-12p40**

Macrophages upon stimulation by Th1 cells secrete several pro-inflammatory cytokines including IL-1β, IL-6 and IL-12 (Trinchieri et al., 1998). Amongst these, IL-12 a heterodimeric cytokine is critical for development of Th1 cells, which in turn ensures
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macrophage activation (Murphy et al., 1995; Ma et al., 1997). In uninfected macrophages (Figure 3A), Berberine chloride (2.5 and 10 μM) effectively induced a 1.5 and 1.6 fold increase in mRNA expression of IL-12p40 (Figure 3A). Following infection with Leishmania parasites (as confirmed by Giemsa staining), a down regulation in mRNA expression of IL-12p40 was observed, which increased 1.9 and 2.3 fold with the addition of Berberine chloride (Figure 3A).

This transcriptional upregulation by Berberine chloride of IL-12p40 was corroborated by quantifying its levels in culture supernatants. In uninfected macrophages, the mean ± SEM was 474.00 ± 26.94 pg/ml which with the addition of Berberine chloride (2.5 and 10 μM) significantly increased by 1.88 and 1.93 fold to 895.00 ± 28.87 (p<0.001) and 915.00 ± 25.98 pg/ml (p<0.001) respectively (Figure 3B). Following successful intracellular Leishmania infection, the levels of IL-12 reduced significantly by 1.6 fold, mean ± SEM being 293.4 ± 22.12 pg/ml (p<0.05). Importantly, their treatment with Berberine chloride (2.5 and 10 μM) significantly increased IL-12p40 production by 1.72 and 1.87 fold, mean ± SEM being 505.6 ± 62.88 (p<0.05) and 551.2 ± 30.86 pg/ml (p<0.01) respectively (Figure 3B).

Figure 3A
IL-10, a class II α-helical cytokine, has been well documented to have potent immune inhibitory capacity (Yang et al., 2007) and importantly contributes to disease progression in Leishmaniasis by disabling Th1 driven responses, deactivating macrophages and eventually enhancing disease progression (Bhattacharyya et al., 2001; Tripathi et al., 2007). In uninfected macrophages, Berberine chloride (2.5 and 10 μM) caused minimal changes in mRNA expression of IL-10 (Figure 4) whereas following infection with Leishmania parasites, the observed 1.4 fold up regulation was effectively decreased by Berberine chloride (2.5 and 10 μM) 2.4 and 1.8 fold respectively (Figure 4).
Figure 4: Effect of Berberine chloride on expression of IL-10
Uninfected (a) and L. donovani infected (d) macrophages were treated for 24 h with Berberine chloride, 2.5 μM (b, e) or 10 μM (c, f). RNA was isolated, subjected to RT-PCR and the products of β-actin and IL-10 mRNA were resolved on an agarose gel (1.5%) and quantified densitometrically using Total lab software as described in Materials and methods.

Berberine chloride enhanced phosphorylation of p38MAPK along with decreased phosphorylation of ERK 1/2 in macrophages

To examine the role of Berberine chloride on the MAPK pathway in leishmaniasis, the kinetics of p38 MAPK and ERK 1/2 phosphorylation was initially studied in uninfected macrophages (1/2 - 6 h). Berberine chloride did not alter the status of ERK 1/2 and p38 MAPK (Figure 5A). However, parasitization by Leishmania, as confirmed by Giemsa staining (data not shown) translated into an increased phosphorylation of ERK 1/2 (Figure 5B), which with the addition of Berberine chloride progressively decreased, maximally at 2 h and was sustained up to 6 h (Figure 5B). With regard to p38 MAPK, Leishmania infection resulted in a pronounced decrease in its phosphorylation (Figure
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5B). Which with, the addition of Berberine chloride was enhanced phosphorylation of p38MAPK, and was evident from 1h onwards (Figure 5B).

To confirm whether the enhanced generation of NO by Berberine chloride (Figure 2) was dependent on p38 MAPK pathways, L. donovani infected macrophages were treated with SB203580, a p38 MAPK inhibitor, (10 μM, 1 h), along with Berberine chloride (10 μM). At 24 and 48 h, the presence of SB203580 reduced Berberine chloride induced increase in intracellular NO by 45.27% and 35.64% respectively (data not shown). Furthermore, we confirmed that the Berberine chloride induced IL-12p40 production (Figure 3B) was mediated through the p38 MAPK pathway as treatment of L. donovani infected macrophages with Berberine chloride (2.5 and 10 μM) for 24 h in the presence of a p38 MAPK inhibitor SB203580 (10 μM, 1 h) caused a 31.95 % and 44.37 % decrease in IL-12 secretion thereby confirming the role of the p38 MAPK pathway in mediating its anti-parasitic effect.

Figure 5A

[Graph and images]
**Discussion**

The remarkable propensity of *Leishmania* to survive within macrophages hinges on their ability to devise strategies to evade or impair host defense mechanisms (Naderer and McConville, 2008, Buchmüller-Rouiller and Mauel, 1987). It is known that the major antileishmanial effector molecule produced by activated macrophages is NO, essential to kill established intracellular amastigotes (Gnatt et al., 2001). Berberine chloride demonstrated potent antileishmanial activity in promastigotes (Saha et al., 2009) and as its IC₅₀ decreased 3 fold in amastigotes (Figure 1), it suggests that Berberine chloride acts both directly upon parasites and also exerts an immunomodulatory effect upon...
Leishmania infected macrophages. Its high safety index (>39 fold, Figure 1 inset) is an important consideration for future studies.

Several plant derived compounds with proven immunomodulatory capability in visceral leishmaniasis, have a consistent feature of being able to enhance production of NO (Ukil et al., 2005 and Bhattacharjee et al., 2009). To establish whether Berberine chloride demonstrated a similar pro-oxidant activity, its effect on production of NO was studied in Leishmania infected macrophages. Infection translated into increased granularity and these morphological alterations were evident by flow cytometry (Figure 2A). Parasitization also caused a pronounced decrease in both intracellular and extra cellular production of NO (Figures 2 B, C and E) that was effectively reversed by Berberine chloride. At 24 h, absence of changes in extracellular NO (Figure 2D) are possibly a feature of time as in previous studies we have observed change in extracellular NO are evident after 48 h (Sen et al., 2010). What is worthy of note is that following parasite clearance, Berberine chloride simply restored levels of NO (Figures 2A, B, C, D and E). A similar scenario was also demonstrated by Artemis nin which restored production in L. donovani-infected macrophages (Sen et al., 2010). This is pertinent, as excessive activation of macrophages may have long term deleterious effects.

Th1 cytokines induce iNOS leading to oxidation of L-arginine and subsequent production of citrulline and NO. As synthesis of NO correlates with killing of Leishmania parasites (Kropf et al., 2004), the effect of Berberine chloride on mRNA expression of iNOS was evaluated. In both uninfected and parasitized macrophages, 18 h treatment with Berberine chloride increased mRNA expression of iNOS (Figure 2F) which accounted for the increased production of NO.

IL-12, a heterodimeric cytokine secreted by macrophages and other antigen presenting cells (APCs) are essential for development of Th1 cells (Gee et al., 2009) which in turns by producing IFN-γ, activate macrophages. Berberine chloride has been reported to induce IL-12 production though activation of p38 MAPK and with LPS in increasing IL-12 (Kang et al., 2002). Furthermore, Kim et al., 2003 have shown that Berberine chloride mediated induction of IL-12 skews the CD4+ T cells from a Th2 towards a Th1 response which is potentially favorable for elimination of Leishmania parasites. In uninfected macrophages, Berberine chloride as expected, up regulated expression of IL-12 both at
the mRNA and protein level (Figures 3A & B). What was of greater interest to us was its effect on *Leishmania* infected macrophages, where it sharply increased mRNA expression and secretion of IL-12 (Figures 3A and 3B). From these observations, we can conclude that Berberine chloride up regulated mRNA expression of IL-12 and thereby plays an important role in host protection (Figures 3A and 3B).

The severity of VL is strongly associated with increased levels of IL-10 as it counter regulated secretion of pro inflammatory cytokines and aided parasite survival (Kaye et al., 1991; Sacks and Noben-Trauth, 2002). Although Berberine chloride showed minimal changes in mRNA expression of IL-10 in uninfected macrophages, in *Leishmania* infected macrophages, a notable increase in mRNA expression of IL-10 occurs, that was sharply decreased with addition of Berberine chloride (Figure 4).

Deactivation of macrophage functions by *Leishmania* parasites has been linked to its ability to induce differential signaling of the mitogen activated protein kinase (MAPK) cascade, which consists of three subtypes, ERK, JNK and p38 MAPK (Seger and Krebs, 1995). The MAPK pathways have been identified as the upstream kinases that induce NF-κB activation through phosphorylation of its inhibitor IκBα (Yang et al., 2001) which then rapidly translocates to the nucleus and activates transcription of multiple κB dependent genes including iNOS and Th1 cytokines (May and Ghosh, 1998).

In leishmaniasis, the CD40-CD40L signaling has been proposed to regulate secretion of two counter regulatory cytokines, IL-12 and IL-10 via the p38MAPK and ERK pathways as it skews the CD40 signaling towards ERK 1/2, which then induces IL-10. In turn the increased IL-10 now prevents CD40 induced p38 MAPK activation, translating into a reduction in IL-12 (Mathur et al., 2004; Bhardwaj et al., 2010). Collectively, Berberine chloride promotes the deactivation of ERK along with activation of p38MAPK. Therefore one can extrapolate that a drug with the propensity to up regulate p38MAPK (and therefore up regulates IL-12) along with down regulation of ERK (and thereby down regulates IL-10) would demonstrate anti parasitic activity.

Accordingly, the effect of Berberine chloride upon ERK 1/2 and p38 MAPK was examined. Berberine chloride caused minimal changes in uninfected macrophages with regard to both ERK 1/2 and p38 MAPK (Figure 5A). In *Leishmania* infected
L. donovani infected macrophages and Berberine chloride

macrophages, Berberine chloride caused a pronounced decrease in ERK 1/2 phosphorylation (Figure 5B) which corroborated with its ability to decrease expression of IL-10 (Figure 4). Berberine chloride enhanced amplification of p38 MAPK phosphorylation in Leishmania infected macrophages (Figure 5B) which again correlated with its propensity to increase IL-12 (Figures 3A & B). To confirm that the Berberine chloride induced production of NO and IL-12 in Leishmania infected macrophages was mediated by p38MAPK pathways, we measured production of NO and IL-12 with SB203580, a selective inhibitor of p38MAPK. Our studies showed that p38 MAPK play an important role in Berberine chloride mediated generation of NO and IL-12p40.

Taken together, our data has established that Berberine chloride exerts its leishmanicidal activity both directly, by inducing an oxidative burst in parasites (Saha et al., 2009) and indirectly, via an increase in IL-12 through enhanced phosphorylation of p38 MAPK, accompanied with a down regulation of ERK1/2 and IL-10, thus highlighting the importance of modulation of the MAPK pathways as a potential target for future drug development (Figure 6).

Figure 6: Proposed immunomodulatory action of Berberine chloride as mediated in L. donovani infected macrophages. Upregulation = ↑ Down regulation = ↓