Chapter 6

General Discussion
This chapter synthesizes the novel findings of this study, presented in different chapters, to interpret possible functions of heat shock proteins in *Leishmania donovani*. The results of our study indicate that in response to heat shock at 37°C, a cascade of events is initiated in *Leishmania* that can ultimately result in either cell death or survival. While death occurs primarily by apoptosis, survival is characterised by differentiation into amastigotes, forms that are adapted to survival at 37°C.

In accordance with the duality of their fate at 37°C, parasites exposed to heat shock *in vitro* display the phenotype typical of apoptosis in mammals and other systems – characterised by shrinkage of cells, loss of motility, increased asymmetry of the cell membrane, and increased mitochondrial membrane permeabilisation – all of which are typical of the process of differentiation of the parasite into its amastigote forms. In addition to these changes, it is significant that heat shock *in vitro* in a host-free system induces increased DNA fragmentation in *Leishmania* promastigotes, a change typical of the execution phase of apoptosis in most organisms (Kerr *et al.*, 1972; Arends *et al.*, 1990; Nagata 2000; Nagata *et al.*, 2003). That this change is unique to the death pathway is reflected in the dramatic decrease that we observe in the *in vitro* infectivity of promastigotes exposed to chronic heat shock at 37°C prior to infections; and confirmed by the observation that the *in vitro* infectivity of promastigotes at 26°C and the differentiated or amastigote forms of the parasite are comparable. All of which clearly indicate that in spite of its physiological relevance, heat shock at 37°C is primarily perceived as a cellular stress signal by the parasite.

Cell fate is determined not just by the length of exposure to heat shock, but also upon certain environmental cues that the parasite is exposed during heat shock. For example, our studies show that promastigotes exposed to short periods of heat shock for periods ranging between 1 – 6 hours undergo certain cellular changes like the loss of physiological activity, mitochondrial membrane potential, promastigote phenotype, viability (Raina and Kaur, 2006) and cell number – all of which are rapid and involve a significant percentage of the original promastigote population. However, promastigotes that survive this period show changes in their protein profile and survival parameters – which are gradual and involve only a relatively small percentage of the original promastigote population. It seems likely, thus, that the parasite responds to chronic exposures to the physiologically relevant temperature of 37°C as a cue signalling its transmission from the sand fly vectors to mammalian hosts. Perception of this cue
is strengthened by an additional change in the growth conditions of the parasite – their exposure to pH shock at 5.5. During its life cycle in vivo, Leishmania promastigotes encounter such a change only subsequent to their phagocytic uptake by macrophages and transport to phagolysosomes (Clos and Krobitsch, 1999). Since this environment is ideal for the survival of the amastigote forms of the parasite, it triggers differentiation of promastigotes into these forms.

Since their identification in Leishmania sp. (Lee et al., 1988), several studies have indicated that the parasite HSP70 gene homologues exist in two forms (Lee et al., 1988; Wallace et al., 1992) called Hsp70 I and II. These initial studies hypothesised that these two forms were identical in the sequence of their coding regions, but differed in the sequence of their 3'-untranslated regions. Subsequent to the discovery that these 3'-untranslated regions were regulatory in function, variations in their sequence were observed to result in differences in the relative stabilities and translational efficiencies of the transcripts of the two types of Hsp70 genes, at 26°C and 37°C – the two temperatures that were relevant in the life cycle of the parasite (Lee et al., 1988; Wallace et al., 1992). A recent study (Brochu et al., 2004) established the fact that the two Hsp70 genes, tandemly linked in the parasite genome, were not just different in their regulatory sequences but also had small but significant differences in their coding regions. Based on these differences, this study identified the two genes to be homologues of their mammalian cHSC73 and iHSP72 counter-parts. However, ours is the first study to show that the proteins encoded by these homologues of the mammalian cHSC73 and iHSP72 genes in Leishmania donovani are differentially expressed at 26°C and 37°C. It is also the first study to show the effects of chronic heat stress at 37°C on the transcriptional levels of the two HSP70 genes, confirming previous findings that indicate that their regulation is primarily at the post-transcriptional level. In addition, this study correlates these differences in expression levels of the two genes with survival parameters of the parasite and their commitment to death or differentiation pathways under conditions important in the in vivo physiology of Leishmania donovani.

Another unique aspect of this study is that it is the first to show differences in the expression levels of the parasite homologue of the mammalian Hsp90 protein under chronic heat stress at 37°C. Based on reports that identify an important role for the parasite Hsp90 protein in the promastigote stage of differentiation of the parasite (Wiesgigl and Clos, 2001;
Figure 6.1. Model summarising the physiology of heat shock in *Leishmania donovani*.
Graefe et al., 2002), this study is the first to show that changes in expression of this protein are similar to those of the cHsc73 protein of the parasite – an observation that forms the basis of our hypothesis that the cHsc73 protein may have a role similar to that of the parasite Hsp90 protein.

The coupling of the stress signal induced death pathway to its differentiation pathway seems to confer an important advantage to Leishmania - the conserved increase in expression of cyto-protective heat shock proteins like iHsp72 allows the parasite to survive in a dramatically changed environment for a considerably longer period of time. Considering the fact that the initial parasite load in vivo is much lower than that in vitro, it seems plausible that this increased period of survival significantly improves the chances of the parasite in being capable of establishing a successful infection in their mammalian hosts. Three observations seem significant in this context. One, we observed that even after a chronic exposure to heat shock at 37°C that results in death of almost the entire infecting population of promastigotes, a small percentage of parasites remain capable of establishing an infection. Two, our results confirm that when promastigotes exposed to this chronic period of heat shock are given a pH shock at 5.5, a certain sub-population of promastigotes is capable of in vitro differentiation into amastigotes (Saar et al. 1998; Gupta et al. 2001; Somanna et al., 2002; Debrabant et al., 2004; Barak et al., 2005). Three, it is a well established fact that promastigotes differ significantly in their physiology from amastigotes (Mukkada et al., 1985; Mazareb et al., 1999). Thus, heat induced changes that simulate apoptosis may prepare the parasite for this stage-related difference. For example, the physiological activity and motility of the promastigote forms of the parasite may be best suited to its commensal mode of survival within sand flies, but may confer no advantage to the amastigote forms of the parasite in their role as obligate intracellular parasites in macrophages of their mammalian hosts. Similarly, increased membrane PS reversal in amastigote forms of the parasite has been shown to increase their chances of uptake by macrophages (Wanderley et al., 2005; Wanderley et al., 2006). In contrast, this phenotype is observed in promastigotes only on exposure to cytotoxic signals like hydrogen peroxide or potassium antimony tartrate. The fact that our studies strongly indicate that even after exposure to chronic heat shock, a change in pH is capable of triggering differentiation into amastigotes is immensely important in the context of Leishmania physiology.
In addition to an increase in their period of survival at 37°C prior to infection, enhanced expression of heat shock proteins might also increase chances of survival of the parasite during the process of contact and uptake by macrophages. For example, it is well established that at the time of their entry into macrophages, promastigotes elicit a respiratory burst that kills all but a small percentage of promastigotes. Phagocytosis of amastigotes, on the other hand, is known to elicit either a lesser respiratory burst or none at all (Wilson et al., 1994). Considering that increased expression of iHsp72 is known to protect a variety of organisms from this form of stress and heat induced changes in physiology are common to differentiation of the parasite into amastigotes, it seems likely that the heat induced decrease in promastigote number is matched by a corresponding increase in survival of the viable population of parasites once they come into contact with their macrophage hosts. In this context, it is significant that a recent study has shown that the presence of dead promastigotes in the infecting inoculum significantly enhances the survival and infectivity of Leishmania (van Zandbergen et al., 2006). The cyto-protective role of the inducible Hsp70 protein (iHSP72) of the parasite during exposure to a signal perceived as stress, in addition to the high degree of homology that it shares with its counterparts in a variety of organisms, underlines the conserved nature of this protein across evolution. It is significant that our study is the first to show that the overexpression of just the C-terminal domain of iHsp72 along with its EEVD motif can protect parasites against stress. Not just does this indicate that some, if not all, differentiation related events are independent of the ability of these proteins to function as molecular chaperones; but, also, as signalling modulators, the fact that the increased expression of iHsp72 protein has been linked to a wide range of extremely diverse roles in a variety of organisms (Morimoto, 1993) seems more plausible. Thus, it is significant that inhibition of iHSP72 transcription as well as over expression of the C-terminal domain of this protein, are both capable of significantly modulating the in vitro infectivity of the parasite.

Considering that the inducible component of heat shock proteins in mammals is intimately associated with a variety of cellular kinases – like, JNK, Akt and ERK (Gabai and Sherman, 2002) – the relatively recent discovery of MAP kinase like proteins in many Leishmania sp. and their stage specific expression patterns (Wiese, 1998; Wiese and Görcke, 2001; Wiese et al. 2003; Kuhn and Wiese, 2005; Naula et al., 2005; Olivier et al., 2005; Wang et al., 2005; Erdmann et al., 2006) have important implications in our understanding of host –
parasite interactions. It is likely that in its capacity as a modulator of cellular signalling pathways under the physiologically relevant stress factor of heat shock at 37°C, the parasite iHSP72 protein interacts with one or more of these kinases.

The high levels of expression of parasite iHSP72 protein that we observed in promastigotes exposed to heat stress is also significant in view of their identification as the immuno-dominant antigens in infected mammals. That host immune responses are mainly targeted against the C-terminal domain of this protein underlines its utility as a parasite specific target for the design of vaccines and modulation of parasite-related pathology. This species specificity is explained by the fact that the C-terminal domain, also called the peptide binding domain, of the iHsp72 protein has a sequence that allows it to interact with the peptide pool unique to *Leishmania*. In this context, it is promising that *Mycobacterium* hsp65 DNA entrapped in trehalose dymicolate (TDM) - loaded biodegradable PLGA microspheres protects mice against *Leishmania major* infections, indicated by a significant reduction in the edema and parasite loads of infected tissues (Coelho et al., 2006).

In this context, it is also notable that parasite counterparts of mammalian heat shock proteins like cHsc73, Hsp90, and Hsp100 are constitutively and abundantly expressed only in any one stage of the parasite life cycle. For example, our study shows that expression of the parasite Hsp90 and cHsc73 protein homologues is limited to the promastigote stage of development of the parasite. Other studies show that Hsp100 has a key role in the amastigote stage of parasite development (Hübel et al., 1995; Hübel et al., 1997; Krobitsch et al., 1998).

Thus, our study establishes chronic heat shock at 37°C as a cellular stress signal, with the ultimate fate of the parasite exposed to this signal being determined by its ability to infect macrophages in its mammalian hosts. Till such an infection is established, the signalling cascade initiated by heat shock is skewed towards cell death by apoptosis. We also show that the parasite homologue of mammalian iHsp72 protein, *via* its ability to act as signalling modulator, has an important role in determining this balance by modulating the survival and infectivity of *Leishmania donovani* under *in vitro* conditions simulating their mammalian hosts. Finally, it provides evidence to suggest that the relative expression levels of heat shock proteins, like cHsc73, iHsp72 and Hsp90, act as molecular sensors of the extra-cellular
environment of *Leishmania donovani* and modulate its response to temperature related changes in this environment.

With respect to its contribution to possible advances in studies on *Leishmania* biology, this study provides a rationale for research on the role of kinases and their interactions with parasite iHsp72 proteins in modulating parasite biology. In addition, it provides a platform that makes the study of roles of the parasite cHsc73, iHsp72 and Hsp90 proteins in the biology of cutaneous infections caused by *Leishmania major* both *in vitro* and *in vivo* animal models seem feasible. In conclusion, this body of work identifies the parasite iHsp72 protein as an important and specific target in research on the identification of drugs and vaccines to combat Leishmaniasis.

**Bibliography**


