Summary
Leishmaniasis imposes a substantial burden of mortality and morbidity, affecting 12 million people in more than 88 countries of tropical and subtropical zones of the world. This is a disease complex caused by obligatory intracellular protozoan parasite of the genus *Leishmania* and is manifested by self-healing skin ulcers to visceral infection. Visceral leishmaniasis (VL) is the most severe, fatal and systemic form of the disease which results in absolute fatality if left untreated.

VL is prevalent in more than 80 countries of Asia, Africa, S. Europe and S. America with prevalence of 500,000 and annual death rate of approx. 50,000. Out of these cases nearly 100,000 cases occur in only three countries i.e. Bangladesh, India and Nepal. The problem has been complicated by co-existence of VL with HIV and has recently declared as a major emerging public health problem by WHO.

Extensive efforts have been made for development of vaccine to prevent the disease but till date no vaccine is available therefore, measures are solely dependent upon chemotherapy. Sodium antimony gluconate [SAG, Sb(V)] has been the first line treatment for more than 60 years. Unfortunately, the treatment is now eroded by large-scale increase in clinical resistance (Faraut-Gambbarelli et al., 1997; Lira et al., 1999) and becomes a major obstacle in the treatment. An increasing incidence of development of SAG resistance is a critical issue in India where over 50–65% of the estimated 2,50,000 annual cases are non-responsive to this first-line agent (Croft et al. 2006). The second line drugs include pentamidine and amphotericin B. Pentamidine use has already been abandoned in India because of its ineffectiveness to treat resistant *Leishmania*. Amphotericin is in use to resistant *Leishmania*. However, its wider use in VL endemic regions is limited by its high cost and toxicity. Because of very few drugs in pipeline, resistance to first line drug(s) has a very big impact on the treatment of leishmaniasis. Therefore, understandings of resistance mechanisms operating in *Leishmania* parasite are very important for designing the strategy to deal with parasite by developing tools to recognize resistance early in infection and prevent useless and often toxic chemotherapy, for more rational use of drugs and drug combinations to minimize development of resistance; for pinpointing intracellular drug targets and defence mechanisms allowing the development of drug analogues that evade the most common defences.

Limited studies on field isolates revealed that the mechanism of natural antimony resistance is multi-factorial and may differ from laboratory resistance. Working with clinical isolates has some challenges viz. the relation between clinical non-responsiveness to antimonials and the corresponding status of drug resistance phenotype under laboratory conditions.
Therefore, it is necessary to establish that the isolates are indeed resistant to SAG before conducting any study on the exploration of mechanism.

To determine and explore the drug resistance mechanism in the field isolates, the *Leishmania donovani* clinical isolates were collected from VL patients from Institute of Medical Sciences, B.H.U., Varanasi and its affiliated hospital at Muzaffarpur, Bihar. The isolates were categorized on the basis of their clinical response viz. sensitive (S) and resistant (R) to SAG. The field isolates were cultivated under laboratory conditions and their growth pattern was studied. *Leishmania donovani* Dd8 strain was taken as reference strain for all the studies. The promastigotes of all the strain grew in sigmoid fashion, multiplied exponentially between 2-4 days and reached a maximum density on 5th day. The parasite of resistant strains grew little faster than laboratory strains and maintained in stationary phase for 1-2 days, while in sensitive strains, the stationary phase was very short lived (<1 day), immediately followed by lysis phase. The growth pattern of isolates was almost comparable with reference strain indicating proper adaptation of clinical isolates in laboratory conditions.

Since, present methods for evaluation of drug sensitivity of parasite are either not accurate or difficult to scale, labour intensive, expensive, lengthy or often generate ambiguous data, present study was firstly aimed to develop luciferase reporter gene assay to monitoring drug resistance phenotype of the field isolates.

In order to develop reporter gene assay, the complete ORF of the luciferase gene was amplified and cloned into the pKS-Neo *Leishmania* shuttle expression vector which was than transfected into *L. donovani* Dd8 reference strain and field isolates. The luciferase expressing cell lines exhibited very high level of luciferase activity which was reached to maximum level after three subcultures in G418 (20µg/ml)-containing medium and then stabilized. Interestingly, the luciferase expression was stable even in the absence of G418 for up to six subcultures (30 days) suggesting the copy number of the episomal vector per parasite was maintained during the period. Expression of luciferase gene in parasite was maintained under as low drug pressure (G418) as 10µg/ml which was 2-100 fold less as compared to the reported concentrations. There was a linear correlation of cell number and luminescence with the detection limit of 10 cells.

The luciferase transfectants were infective to macrophages and drug pressure did not affect their infectivity. Further, the expression of luciferase was not affected after transformation of the promastigote stage to the amastigote stage and a good correlation was also observed between the numbers of intracellular amastigotes per 100 macrophage cell nuclei.
We established that the resistance is an inherited property of parasite as in vitro drug response was correlated well with clinical outcomes. SAG did not affect promastigotes multiplication even at very high dose while Sb(III), the active form of drug significantly inhibit promastigote growth. The sensitivity of field isolates towards Sb(III) varied according to their clinical response to antimony. Resistant isolates (R1 & R2) exhibited significantly higher IC\(_{50}\) values of Sb(III) than the sensitive ones (S1 & S2) and were almost 3-4 fold resistant as compared to the sensitive isolates and reference Dd8 strain. The data clearly suggests that \(L.\) donovani parasites isolated from non-responders patients maintain the resistance phenotype under laboratory conditions also. It was also established that the promastigote/Sb(III) assay can be used for characterization of drug response profile of field isolates rapidly without going for tedious intramacrophagic amastigote/Sb(V) assay.

The SAG resistant field isolates were not found cross-resistant to other antileishmanials drugs viz. pentamidine and amphotericin B rather, amphotericin B was found more active on clinical resistant isolates and therefore, may be a drug of choice in SAG unresponsive cases. Miltefosine was also found active on all isolates as well as reference strain.

The LUC assay was validated for the evaluation of drug toxicity in HTS and compared with more conventional MTT assay. Except Sb(III), the IC\(_{50}\) values of the other three drugs determined by LUC assay were almost comparable to the values obtained by the MTT assay. Interestingly, the luciferase assay could be used to determine the IC\(_{50}\) value of SAG, which otherwise could only be determined by either parasite counting method or the hazardous \([3H]\)thymidine incorporation assay.

Therefore, a simple, rapid, reliable, quantitative screening method for antileishmanial compounds using luciferase-expressing clinical isolates of \(L.\) donovani was established and evaluated. The assay is straightforward and basically requires nothing but the Steady-Glo reagent and a luminometer. The method has been automated in a 96-well plate format. These cell lines also helped in present study to explore the mechanism of drug resistance.

Multiplicity of the mechanism of antimony resistance in field was explored by transcriptome analysis using genomic DNA microarray of \(L.\) donovani containing 4224 genomic fragments. Twenty two differentially expressed genes were identified. Sequencing of these differentially expressed clones and annotation by searching their homology to the genomic and protein data base led to identification of three clones namely, G13H6, G36D10 and G51G7 that were consistantly down-regulated in resistant isolate(s) and exhibited significant homology to 3' UTR and ORF of mitogen-activated protein (MAP) kinases homologue of \(L.\) mexicana (LmxMPK1) and \(L.\) major. Since role of MAPKs in chemo-resistance is well documented in
cancer cells, it would be interesting to explore the role of MAPK1 in drug resistance in *Leishmania*.

To confirm microarray data, mRNA abundance of MAPK1 was compared in resistant and sensitive isolates by real-time PCR. There was significant down-regulation of the *L. donovani* MAP kinase homologue (LDPK) in resistant isolates as compared to reference sensitive strain Dd8. To clone and characterize MAPK1 of *L. donovani*, complete ORF of LDPK was PCR amplified, cloned and sequenced. Sequence analysis, multiple sequence alignment with other MAPK and phylogenetic analysis revealed that the gene was highly conserved within spp. of *Leishmania* and exhibited significant homology (~96%). It also exhibited significant homology to protein kinases and MAP kinases of other organisms particularly in conserved domains. The protein has all characteristic feature of MAPK e.g. consensus 11 subdomains (I-XI), amino acid residues characteristic of proline-directed serine/threonine kinases in its catalytic domain, phosphate anchor ribbon, the P+1 specificity pocket, catalytic loop, dual phosphorylation motif, DFG motif, invariant Lys and presence of ser150 Interestingly, LDPK has conserved motif of tyrosine-auto-phosphorylating protein kinases which indicates that it may autophosphorylate at tyrosine residue to become active. The recognition sequence for activators, substrate and regulators (common docking (CD)-domain) is also present in this protein. The number of amino acids between subdomain VII and VIII of LDPK is 14 amino acids which is similar to ERK. Further, the X residue in the activation site (TXY) of LDPK was TDY (Thr-Asp-Tyr) again similar to ERKs suggests that LDPK may be a member of ERK subfamily. The alignment of LDPK to other MAP kinases identified in *L. mexicana* (LmxMPK1-15) revealed that it has very less homology to LmxMPK3-15 (22-32%), moderate with LmxMPK2 (44%) and high with LmxMPK1 (96%). The southern blot analysis revealed that LDPK gene was present in one copy per haploid genome of *L. donovani*.

The gene was over expressed in *E. coli* as a His-tagged fusion protein. As LDPK was expressed in aggregate form, protein was purified from inclusion bodies after denaturation with urea using Ni-NTA affinity chromatography and electroelution. The polyclonal anti-LDPK antibody was raised in rabbit and affinity purified on nylon membrane. The anti-LDPK antibodies hybridized with approx 40kDa protein band of all strains of *L. donovani* lysate on membrane. However western analysis did not reveal any significant difference in the expression levels of LDPK between resistant and sensitive isolates.

To establish the role of LDPK in antimony resistance, the gene was overexpressed in laboratory sensitive strain of *L. donovani* Dd8 using leishmania shuttle vector. Transfection of gene in wrong orientation did not exhibit any change in sensitivity towards Sb(III) while
transfection of LDPK in right orientation conferred increased sensitivity to the parasite for antimony. It was observed that the IC$_{50}$ of transfectants (17µg/ml) was significantly (48%) less than WT Dd8 (33.5µg/ml). The increase in sensitivity towards antimony was due to over expression of LDPK, as there was no change in morphology or the growth pattern of transfectants. This is first ever report demonstrating the association of MAPK with antimony resistance in *Leishmania*.

It is evident that antimony augment the generation of reactive oxygen species (ROS) i.e., oxidative stress and apoptosis like death in *Leishmania* and mammalian cells. Recently, level of expression of ERK and other MAPKs have been found to be decreased in arsenic/antimony treated or drug resistant cancer cell lines. In accordance, LDPK was found down regulated in antimony resistant parasites indicating its role in antimony resistance. Most importantly, increase in sensitivity of LDPK over expressing parasites towards antimony indicates that this protein may play a role in cell death pathway induced by antimony. However, it has to be confirmed further by other methods.

As a whole the results of the present study provides the first ever indication of role of MAP kinase in antimony resistance. By exploring the regulation of the LDPK expression, finding regulatory kinases and by targeting them, the antimony resistant parasite can made sensitive again.

In addition the LUC-expressing recombinant *L. donovani* cell lines developed in this study are now in routine use at the Central Drug Research Institute for the evaluation of large numbers of compounds for their antileishmanial activities. The infectivity of a transgenic cell line made possible to study the effects of compounds on intracellular amastigotes using a semi-*in vivo* amastigote-macrophage method in the HTS mode. Further, LUC-expressing resistant *L. donovani* cell lines provide a useful tool for the screening of compounds against resistant *Leishmania*. These cell lines can also be used to study the mechanism of drug resistance and to screen for clones in functional complementation studies.