Role of Leishmanial Lipid in Hepatocellular Carcinoma
Summary

Lipids and their metabolites from cellular components of micro-organisms show promising effects in different aspects from system inflammation regulation to cancer angiogenesis. Azurin, a protein isolated from *Pseudomonas aeruginosa*, induces apoptosis in hyper proliferating cancer cells toward controlled cell growth. The microbial metabolite myriocin isolated from *Isaria sinclairii*, and 6-MFA obtained from both *Aspergillus ochraceus* and the marine sponge *Hippospongia communis* afford protection against hepatic injury. More interestingly, a lipid of the yeast strain *Wickerhamiella domercqiae* shows significant growth regulating role in human pancreatic cancer cells with anti-inflammatory activity. On the other hand, lipids from *Leishmania major* are reported to inhibit nitric oxide production and exert leishmanicidal activity (Proudfoot et al., 1995).

Earlier, we reported that the lipid from an attenuated strain of *L. donovani* promastigotes (MHO/IN/1978/UR6) suppresses several inflammatory mediators by inducing apoptosis in synovial fluid cells of rheumatoid arthritis patients and also suppresses the inflammatory responses of gram negative bacteria induced septic injury. We found earlier that the lipid from an attenuated strain of *L. donovani* promastigote suppresses several inflammatory mediators in adherent synovial fluid mononuclear cells (SFMCs) isolated from rheumatoid arthritis patients (Majumdar et al., 2008). Additionally, the lipid from pathogenic *L. donovani* (pLLD) acts as an anti-inflammatory agent, protects from endotoxin induced sepsis in hepatic impairment, and shows efficacy in chemical induced hepatic damage towards oxidative stress and inflammation response to regulate apoptosis. These findings encouraged us to evaluate the efficacy of pLLD in hepatocellular carcinoma.
Background

Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide. It is the third leading cause of cancer mortality and is considered to be the outcome of chronic liver inflammation. However, survival rates are suboptimal partially because of tumor recurrence in the remaining liver (Jemal et al., 2011). It is estimated that about 15% of human hepatic cancers are associated with chronic infections and inflammation. Persistent infections and inflammation in these organs lead to continuous cell death and long-lasting local infiltration of inflammatory cells. Even those cancers, whose development is not associated with pre-existing infection or inflammation, are accompanied by massive inflammatory cell recruitment into the tumor, a phenomenon which led Virchow to his original suggestion that inflammation and cancer are linked (Bonacchi et al., 2003). This inflammatory response is likely caused by necrotic cell death in the core of rapidly growing tumor mass due to lack of oxygen and nutrients. Continuous cell death and inflammatory cell infiltration during cancer development are accompanied by the production of a great number of cytokines, chemokines and growth factors, favoring increased cellular proliferation (Dominguez et al., 2009; Guo et al., 2009; Miura et al., 2010; Seki et al., 2009; Seki et al., 2007). In addition, reactive oxygen and nitrogen species generated by both oncogene-expressing cells and inflammatory cells could cause oxidative damage to host DNA, resulting in activation of oncogenes and/or inactivation of tumor suppressor genes, and various epigenetic changes that favor tumor progression. Therefore, etiologies that influence either cell survival or ensuing inflammatory responses are likely to have an impact on the course of cancer development. The promotion of tumor growth is nurtured by angiogenesis which precedes the activation of endothelial cells, supplies the nutrient to hyper proliferating cells, and ignites metastasis by activating several growth factors with transcription of few integral molecules (Folkman, 2002; Veikkola et al., 2000).

Uses of alternative medicine that may even come from microbial sources have gained increasing importance for potential use in intervention against malignant neoplastic diseases including the use of microbial cellular components to improve the therapeutic efficacy of existing drugs. Often therapeutics of anerobic bacteria origin like bacterial LPS(s) and ceramide(s) potentially induce
apoptosis in malignant cells (Hla, 2003). Interestingly, a lipid molecule isolated from a fungus has shown its therapeutic efficacy in multiple sclerosis and neoplasia (Azuma et al., 2003). Previously, we reported that the lipid from the attenuated *L. donovani* can be used to control the inflammatory response (Chatterjee et al., 2014). Very recently, we have also explored the bioactivity of virulent leishmanial lipid (pLLD) in protection against sepsis associated inflammatory injury and hepatic injury (Chatterjee et al., 2015). Here, we would like to divulge the therapeutic implication of the activities of the bioactive lipid isolated from *L. donovani* that induces apoptosis in *in vitro* system and also its application *in vivo* with involvement of several inhibitory effects in neoplastic lesion in hepatocellular carcinoma model.
Materials and Methods

Material

MTT and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO, USA), p-NBT-BCIP systems from Amresco (Solon, OH, USA), RPMI 1640, FBS (Fetal Bovine Serum), and penicillin–streptomycin–neomycin from Gibco-BRL (Grand Island, NY, USA), tissue-culture plasticware from Nunc (Roskilde, Denmark), Bradford protein assay reagent from Fermentas (Pittsburgh, PA, USA), DAPI (4',6-diamidino-2-phenylindole dihydrochloride), acridine orange (AO), and ethidium bromide (EtBr) from Invitrogen (Carlsbad, CA, USA), and rabbit and goat anti-BAX, BAD, BCL-2, p38, p-P38, ERK, p-ERK, JNK polyclonal and secondary antibodies in alkaline phosphatase, and FITC and PE-conjugated from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

*L. donovani* cell culture and isolation of lipid

*L. donovani* strain AG83 (MHOM/IN/1983/AG83) used for the present experiments was obtained originally from Indian kala-azar patients and maintained in golden hamsters (Mukhopadhyay and Madhubala, 1994). Promastigotes obtained after transforming amastigotes from infected hamster spleen were maintained at 22 °C in M199 (Invitrogen), supplemented with antibiotics and 10% FBS. The Bligh and Dyer method (Bligh and Dyer, 1959) of lipid extraction was used to isolate the total lipid from *Leishmania* cells (1×10<sup>10</sup>). The total lipid, obtained from the lower organic phase after evaporation to dryness at 40 °C, was then stored at 4 °C in vacuum desiccators until used.

Thin layer chromatography

pLLD was dissolved in 2:1 chloroform:methanol. TLC was performed in chloroform:methanol:water (30:60:10) and lipid spots were visualized using iodine spray (Das et al., 2014).
Cell culture

Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (PSN) at 37 °C in a humidified atmosphere under 5% CO\textsubscript{2}. After confluency, cells were harvested with 0.025% trypsin and re-equilibrated a day before the start of experiment. Isolated LSPL was added to 10% FBS containing media to achieve the desired final concentration and the mixture was sonicated in a water bath sonicator for use in subsequent experiments.

Cell viability assay

MTT assay was done to evaluate cell viability (Mosmann, 1983). The cells were seeded in 96-well plates and treated with or without different concentrations of pLLD for 0-36 h. Four hours after the addition of MTT, cells were lysed, formazan was solubilized with acidic isopropanol, and the absorbance of the solution was measured at 595 nm using an ELISA reader as also by flow cytometric analysis using propidium iodide.

Assessment of cell morphology

Cells (3×10\textsuperscript{4}/well) were grown in 30 mm culture plates after treatment with pLLD and morphological changes were observed with an inverted phase contrast microscope. To detect nuclear damage or chromatin condensation, cells were treated with 10 μg/ml of DAPI. The conventional acridine orange/ethidium bromide (AO/EtBr) staining procedure followed by observation under fluorescence microscope (OLYMPUS IX70, Olympus Optical Co. Ltd) was used to differentiate the live, apoptotic and necrotic cells and images were acquired with excitation and emission wavelengths of 488 and 550 nm respectively (Das et al., 2012).

Apoptosis assay using Annexin-V

Apoptosis was assayed by using an Annexin-V FITC apoptosis detection kit (Calbiochem, CA, USA). Briefly, cells were treated with pLLD, and then washed and stained with PI and Annexin-
V-FITC in accordance with the manufacturer's instructions. The percentages of live, apoptotic and necrotic cells were determined by flow cytometric method using the equipment LSR Fortessa™ of Beckton Dickinson, San Jose, CA, USA (Pramanik et al., 2013). Data from 10^6 cells were analyzed for each sample.

**Cell cycle analysis**

Upon treatment, cells were collected and fixed in 70% ethanol for 24 h at 4 °C. These were centrifuged (1500 g) and the cell pellet was resuspended in PBS (400 µl) containing RNaseA (10 mg/ml, 50 µl) and PI (2 mg/ml, 10 µl). The mixture was incubated in the dark at 37 °C for 30 min and analysed by flow cytometry (Das et al., 2012).

**Reactive oxygen species (ROS) assay**

ROS were detected using the cell-permeable fluorescence probe 2,7-dichlorofluorescein diacetate or H2DCFDA (Sigma-Aldrich, USA), a non-fluorescent compound, which is converted into the highly fluorescent dichlorofluorescein (DCF) by cellular peroxides. Briefly, cells were exposed to various agents for the indicated times and then loaded with H2DCFDA (20 mM). Following incubation at 37 °C for 30 min, cells were washed with PBS and the fluorescence was assayed by flow cytometric method at excitation wavelength of 488 nm and emission wavelength of 530 nm (Das et al., 2012).

**Mitochondrial membrane potential (MMP/Δψm) measurement**

Cells were treated with the voltage-sensitive lipophilic cationic fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) to measure MMP. JC-1 monomers fluoresce red in stable mitochondria but combine to form green fluorescent dimers upon exclusion. Briefly, cells were collected, washed with cold PBS, incubated with JC-1 (5 mg/ml) for 15 min, and analyzed by flow cytometric method (Das et al., 2015).
Confocal microscopy

During apoptosis induction in U937 cells, released cytochrome C and AP-1 were measured by immunocytochemical analysis. Cells cultured on chambered plastic slides were fixed with ethanol for 30 min at 4 °C and the detergent was extracted with 3% Triton X-100 for 10 min at room temperature. After washing with PBS and blocking with 3% bovine serum albumin (BSA) for 30 min, samples were incubated overnight with a primary antibody at 4 °C. Excess primary antibody was removed by washing with PBS, samples were incubated with FITC-conjugated secondary antibody for 2 h at room temperature, and mitochondria were stained with Mito Red. After washing with PBS, slides were mounted using DAPI to visualize the nuclei. Specimens were covered with cover slips and evaluated under an Andor spinning Disc laser scanning confocal microscope (Chatterjee et al., 2014).

Animal treatment and measurement of serum ALT, AST and ALP

With proper husbandry, mice were treated multiple doses of DEN (35mg/kg) and co-treated with p-LLD up 180 days. Blood samples were withdrawn by retro-orbital puncture and centrifuged (3500 r/min, 10 min, 4 °C) to obtain the serum. Activities of both alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and levels of alkaline phosphatase (ALP) and bilirubin in serum were measured according to the instructions supplied with the commercial assay kits.

Histological evaluation

The livers were removed from sacrificed mice and stored in fixative. The fixed tissues were embedded in paraffin, sectioned, deparaffinized, and rehydrated using the standard techniques. The evaluation for several pathological changes was assessed by using H&E, PAS, Masson’s trichrome and Van Gieson stains. The standard histopathological gradations were followed - Grade 0: Normal histology; Grade 1: Presence of degenerated hepatocytes with only rare foci of necrosis; Grade 2: Mild centrilobular necrosis around the central vein, occupying only a part of Rappaport’s zone; Grade 3: Established necrosis limited to zone; and Grade 4: Extensive,
confluent centrilobular necrosis and the fibrotic injury was measured by the reported method (Fujii et al., 2010).

**Immunohistochemistry**

Paraffin-embedded blocks of liver tissues were cut into 5 μm sections and mounted onto slides. The sections were deparaffinized with xylene and dehydrated over several series of alcohol. Antigen retrieval was performed by trypsin (0.05% trypsin, 0.1% CaCl$_2$) and blocking was performed using 5% BSA in TBS (20 mM Tris HCl, pH 7.4, containing 150 mM NaCl) for 4 h at room temperature. Finally the sections were incubated with primary antibody in dilution 1:300 at 4 °C overnight in a humidified chamber. The tissue sections were washed with TBST and incubated with FITC and PE conjugated secondary antibody (Santa Cruz Biotechnology, USA) solution (1:500) for 2 h at room temperature, and the nucleus was visualised by DAPI (Invitrogen). The images were observed in confocal microscope (Andor Revolution XD Spinning Disk Microscope with Andor ixon 897 EMCCD camera) (Das et al., 2014).

**Statistical analysis**

Results were expressed as mean ± SEM. Statistical analyses were performed with ANOVA, followed by Dunnett’s test. P < 0.05 was considered significant.
Results

Effect of leishmanial lipid on the growth of cancer cell lines

The TLC profile of pLLD used in our study showed six to seven spots of lipids in the TLC plate upon iodine staining; this product was used for further studies. To determine the cytotoxicity and the effect on cell proliferation, a panel of cancer cell lines including HepG2, U937, Mia-PaCa, MCF7, B16F10 and HCT 116 were treated with different concentrations (0-150 µg/ml) of pLLD (Figure 1). It was found that pLLD exhibited potent cytotoxicity in HepG2 cells at 150 µg/ml, a better value compared to the other cancer cells.

Figure 1: Growth inhibitory effect of pLLD on a panel of cancer cells in concentration (0 - 150 µg/ml). The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

Further, we have studied the cytotoxicity effect of pLLD on a panel of ten cancer cell lines including HepG2, U937, Mia-PaCa, MCF7, B16F10, HCT 116, A549, A498, PC3 and Sarcoma 180 using different time periods to note that it showed most significant cytotoxicity with 150 µg/ml at 24 h in HepG2 cells.
Figure 2: Growth inhibitory effect of pLLD on a panel of cancer cells at concentration 150 µg/ml in time dependent manner. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

Moreover, we did not find any cytotoxic effect in normal blood, i.e. PBMC in time or concentration dependent manner as depicted in Figure 3.

Figure 3: Growth inhibitory effect of pLLD on peripheral blood mono nuclear cells in concentration dependent manner (0 - 150 µg/ml) at 24 h. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).
For the morphological analysis, treatment with pLLD (150 µg/ml) followed by observation under light and fluorescence microscope at 24 h showed characteristic apoptotic changes like cell rounding, cell shrinkage, and blebbing. Figure 4 reveals a number of condensed bright fragmented nuclei (employing DAPI) in pLLD 150 µg/ml treated cells unlike control. Upon staining with A.O./Et.Br, yellowish, orange and red colored nuclei or a combination of these were observed in treated cells while greenish intact nuclei were found in control cells.

![Image of morphology changes](image.png)

**Figure 4.** Morphological and nuclear changes observed under light and fluorescence microscopy with DAPI & A.O./Et.Br respectively (20×).

Treatment with pLLD significantly and time dependently reduced the viability of HepG2 cell lines as compared with cells present at 0 h. PI positive cells reveal the percentage of death cells. Thus, at 24 h pLLD (150 µg/ml) showed highest potency of cell death of HepG2 cells that may be via apoptotic pathway as shown in Figure 5.
Figure 5. Flow cytometric analysis of cell viability measured by PI staining with the treatment of pLLD (150 µg/ml) in HepG2 cells for 0, 12, 24, and 36 h.

**Induction of apoptosis with pLLD in HepG2 cells**

At the initial phase of apoptosis, phosphatidyl serine is exposed from the inner membrane to the outer membrane of the cells. This externalized phosphatidyl serine can be detected through flow cytometry using annexin V with FITC. The percentage of annexin V positive cells was significantly increased (in Q2 & Q4) in HepG2 cells compared to the control following treatment of the cells with 150 µg/ml of pLLD for 0, 12, 24, and 36 h as found in Figure 6.
Figure 6. Flow cytometric analysis of apoptosis with Annexin-V and PI by pLLD (150 μg/ml) in HepG2 cells for 0, 12, 24, and 36 h. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

The reflection of apoptosis process is found in cell cycle progression, an integrated part of cell growth. The cell cycle analysis study was performed in HepG2 cells with 150 μg/ml concentration by flowcytometry analysis. Treatment with pLLD for 0, 12, 24, and 36 h showed higher number of cells at G0/G1 phase as compared with the control as found in Figure. 7.
Figure 7: Analysis of cell cycle arrest in HepG2 cells by flow cytometry. Study of cell cycle arrest in HepG2 cells was carried out by propidium iodide staining. Percentage of G0/G1 cell population increases after treatment of 150 μg/ml of pLLD in time dependent manner. The data are represented as mean ± SEM from triplicate independent experiments (*P<0.05; ** P<0.01).

**pLLD induced apoptosis in HepG2 cells via regulating mitochondrial death pathway**

Initiation of apoptosis proceeds with significant involvement of pro- and anti-apoptotic markers to regulate cancer cell proliferation. Down-regulation of the anti-apoptotic Bcl-2 protein and binding of pro-apoptotic protein Bax to the mitochondrial membrane trigger the release of cytochrome C from mitochondria to cytosol and generation of ROS (87.2% at 24 h). This ROS generation is a significant marker of mitochondrial death pathway. We have performed ROS generation study by DCFDA staining in HepG2 cells with pLLD (150 μg/ml) in time dependent manner as found in Figure 8.
Figure 8: Effect of pLLD (150 µg/ml) in apoptosis induction in HepG2 cells through measurement of ROS by DCFDA at time dependent manner. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

Flowcytometric analysis for mitochondrial membrane potential in HepG2 cells revealed that 9.21 % of the cell population exhibited fluorescence at the FITC channel indicating a higher level of cells having a healthy ΔΨm, whereas of the cell population receiving 150 µg/ml pLLD, 17 % did so at 12 h and 20.6 % at 36 h, hinting at a loss of ΔΨm in 24 h (19.6 %) as evident from Figure 9. This alteration of mitochondrial membrane potential indicates cell death in apoptotic pathway by increasing the ROS generation.

Figure 9: Effect of pLLD (150 µg/ml) on apoptosis induction in HepG2 cells through alteration of MMP measurement JC-1 positive cells at time dependent manner analyzed flow cytometric study. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).
By image analysis in confocal microscopic study, we found that the intensity of FITC positive cells identified using JC-1 dye was higher in pLLD (150 μg/ml) treated cells as shown in figure 10 and in time dependent manner.

**Figure 10:** Effect of pLLD (150 μg/ml) on apoptosis induction in HepG2 cells through alteration of MMP measurement JC-1 positive cells at time dependent manner analyzed by confocal microscopic analysis.

Thus, generation of ROS is a key factor in apoptotic cell death and maximum ROS generation was exhibited with pLLD treatment with concentration 150 μg/ml. The study demonstrates that pLLD induced apoptosis in HepG2 cells may proceed via the ROS mediated pathway.

pLLD also enhanced the expressions of caspase-3 and caspase-9 in a time dependent manner as described in Figure 11. It also suppressed the elevated expression of Bcl-2, activated the expression of Bax, and promoted PARP cleavage in HepG2 cells in time dependent manner as found from Figure 11. These results indicate that pLLD may induce apoptosis via the mitochondrial pathway.
Figure 11: Expression of pro- and anti-apoptotic molecules was assessed by western blot. pLLD caused time-dependent decrease in the expression of Bcl-2; in addition, the expression levels of Bax, cleaved PARP, caspase 9 and 3 were increased.

Gradually elevated levels of cytosolic cytochrome C were also observed after pLLD treatment (150 μg/ml) at 0, 12, 24, 36 and 48 h intervals Figure 11.

Clear evidence from microscopic study revealed in Figure 12 demonstrated that AIF release from mitochondria is able to induce apoptosis upon treatment of pLLD (150 μg/ml) as compared to control HepG2 cells.
pLLD triggers apoptosis in HepG2 cells with involvement of different transcriptional factors

Apoptosis of cancer cells is profoundly dependent on the tumor suppressor protein, p53. The interaction of different pro- and anti-apoptotic proteins effectively represses p21 induction by regulation of p53. Treatment of HepG2 cells with pLLD (150 μg/ml) resulted in increased expression of p53 in the nucleus at 24 h along with increased expression of p21 as compared to the untreated cells as found in Figure 13.
Figure 13: Effect of pLLD (150 µg/ml) in tumour suppressor protein activation, i.e. up-regulation of p53 and p21. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

Thus the expression of p53 is regulated by the transcription of protein NF-kBp65, a ubiquitous transcriptional factor in cancerous state. A contrasting result was found in case of p65. Treatment of pLLD (150 µg/ml) resulted in reduced expression of p65 at 24 h as compared with untreated control as shown in Figure 14. The result was analyzed by confocal microscopic study.
**Figure 14:** Effect of pLLD (150 µg/ml) in NF-κb p65 and p50 regulation in HepG2 cells. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

**Effect of pLLD in metastasis growth and different enzyme parameters of hepatocellular carcinoma**

Tumorigenesis and its sequential progression constitute a multi-step process that comprises four overlapping series of events: initiation, proliferation, infiltration, and metastasis. To derive further confirmation on the effect of pLLD on regulation of cancer cell growth, we have examined its efficacy in DEN induced hepatocellular carcinoma system. No significant changes in toxic sign and symptoms was noted with intraperitoneal administration of pLLD in BALB/c mice as described in chapter II (toxicity study).

The survival rate was significantly improved in mice bearing carcinoma induced by DEN, when receiving pLLD (Dose-1 and Dose-2) and observed up to 180 days. There was also reduction in liver weight, as compared with only tumour bearing mice as found from Figure 14 (A-C).
**Figure 14:** Effect of pLLD Dose -1 (25 mg/kg) & Dose 2 (50 mg/kg) to regulate the DEN induced hepatic nodules. Photograph of mice liver of control, DEN, DEN + Dose 1, and DEN + Dose 2 treated animals. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).
The liver enzyme parameters including ALT, ALP and AST also improved in DEN induced hepatocellular carcinoma model after the treatment of pLLD compared to untreated mice, as found in Figure 15.

![Figure 15: Effect of pLLD on serum biomarker in CCl4 induced hepatic injury: ALT (A), AST (B), ALP (C). The data are reported as the mean ± SEM of triplicate experiments. (*P<0.05, **P<0.01).](image)

The histological analysis with H&E, M&T and PAS found in Figure 16 describes that the fibrotic nature of DEN induced liver improved with treatment of pLLD at the end of experiment.
Figure 16: Histopathological examination of eosin–haematoxylin, Masson’s trichome and PAS stained liver section in DEN induced carcinoma bearing, pLLD (Dose 1= 25 mg/kg and Dose 2 = 50 mg/kg) treated mice.

**pLLD improves growth factor level to regulate metastatic vasculature formation**

Angiogenesis is the major concern to regulate the spread of cancerous cells in various parts of the body via neovascularization aided by inflammatory stimuli in HCC. The cancerous microenvironment encompasses numerous signaling cascades that influence the angiogenic response in a way limiting its metastatic progression. We therefore investigated whether pLLD plays a role in angiogenesis in *in vivo* systems. High levels of expression of vascular growth factor VEGF along with CD34 were observed in liver sections of mice with DEN induced carcinoma, with consequent higher expression of angiogenic receptor Ang2 and hypoxic factor HIF-1α. But upon treatment with pLLD, the expression levels of VEGF and CD34 were reduced, while simultaneous reduction of HIF-1α and Ang-2 levels were observed in pLLD treated DEN induced liver sections as found in Figures 17 and 18.
Figure 17. Effect of pLLD (50 mg/kg) in regulation of angiogenesis in DEN induced carcinoma bearing mice. Immune fluorescence localization of VEGF and CD34 in liver tissue section. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).
Figure 18. Effect of pLLD (50 mg/kg) in regulation of angiogenesis in DEN induced mice. Immune fluorescence localization of VEGF and CD34 in liver tissue section. The data are reported as the mean ± SEM of triplicate experiments (*P<0.05; ** P<0.01).

Thus, we may conclude that pLLD significantly reduced the metastatic severity of hepatocellular carcinoma by regulating the signaling pathway both in vitro and in vivo systems.
Discussion

A new therapeutic development to regulate the cancer cell growth is the modern approach for alternative medicine. Our studies mainly emphasize on the study and development of new agents from sources other than those of common synthetic and semi synthetic origin, that is microbe mediated therapy for restriction of cancer cell growth (Minton, 2003). Microbial cellular components such as endotoxin, protein or cellular components are very popular in tumour cell destruction and also in vaccine development for cancer treatment (Carswell et al., 1975). Now a days, bacteria or their cellular components are preferred for use as carriers for delivering the therapies in cancer treatment (Saltzman et al., 1996). The popular member of this group is FTY720 which is being used in multiple sclerosis and has also been established as an anti-proliferative agent in regulating the growth of several cancer cells via the involvement of apoptotic response (Liu et al., 2008; Wallington-Beddoe et al., 2011). Sophorolipid, a yeast component, is able to induce apoptosis in human liver cancer cells and show beneficial effects in sepsis (Fu et al., 2008; Hardin et al., 2007). We are also developing a new entity, i.e. leishmanial lipid, which has already been reported to have apoptosis inducing effect in synovial cells isolated from rheumatoid arthritis patients (Majumdar et al., 2008). Recently, we have reported the protective role of this lipid against endotoxin induced bacterial sepsis (Chatterjee et al., 2014) and hepatic injury. Thus, the previous findings encouraged us to find out its role in hepatocellular carcinoma in regulating cancer growth.

This is the first report to show that leishmanial lipid causes apoptosis in HepG2 cells while playing an inhibition of cancer cell growth regulating role in murine model. Here, we have described the in vitro study which indicated the inhibition of cancer cell proliferation along with apoptosis induction in time and dose dependent manner. Preliminary observation from morphological evaluation of HepG2 cells treated with pLLD shows the consequent apoptotic series including phosphatidyl serine exposure and cell cycle regulation. ROS are major contributors of apoptosis upon external stimuli and mitochondria are the major source for internal cellular ROS generation via activation of several signaling cascades. Alteration of mitochondrial potentiality releases cytochrome C which is to induce cell death. Our study
revealed that pLLD induced apoptosis may alter mitochondrial membrane potential with generation of ROS by increasing the level of cytochrome C. It was found that pLLD eventually activates caspases 9 and 3 during apoptosis in time dependent manner. Transcriptional factors like p53 trigger the apoptotic series with the involvement of different pro and anti apoptotic molecules in presence of pLLD. It is very interesting that the induction of p53 causes an alteration in the expression of the ubiquitous transcription factor NF-kB. Any activation of NF-kBp65 signaling abrogates p53-induced apoptosis that is essential in p53-mediated cell death and sequential inflammatory phenomenon (Hoesel and Schmid., 2013).

Remarkable changes were found in in vivo system which is relevant to in vitro system. Thus a dose dependent significant reduction was found in liver weight, liver necrotic percentage, and liver fibrotic percentage in DEN induced hepatocellular carcinoma model without any detrimental effect on normal murine subject (Heindryckx et al., 2009). The liver enzyme parameters and peroxidation level also improved in presence of pLLD in carcinoma induced mice. Cancer proliferation associated cells secrete a wide array of pro-inflammatory molecules that trigger the activation of vascular endothelium cells to recruit leukocytes which release angiogenic factors, mitogens, proteolytic enzymes and chemotactic factors, recruiting more inflammatory cells and stimulating angiogenesis to sustain and provoke metastasis (Benelli et al., 2006). VEGF acts as the master regulator of angiogenesis, regulating the proliferation, migration, and differentiation of endothelial cells to determine the consequence of "immune surveillance" against cancer. Furthermore, VEGF levels increase in response to cellular stress such as hypoxia and changes in the tumor microenvironment during cancer development. Higher HIF-1α and VEGF expressions correlate with increased cancer cell invasiveness and metastatic potential (Ferrara, 2002). Thus, angiogenic progress may be quantified by measuring vascular density of endothelial cell marker CD34 and angiogenic binding factor Ang 2 (Maschio et al., ??). Figure 5 shows the reduced expression of HIF-1α, VEGF and CD34 in cancer cells on treatment with LSPL-1. Interestingly, in the hypoxic milieu of hepatic micro environment, p53 tumour suppressor down regulates VEGF in HIF-1α dependent manner that justifies our inference from in vitro data (Kaklamanis et al., 1998).

Our data convincingly showed that pLLD initiated the induction of apoptosis in HepG2 cells via alteration of mitochondrial homeostasis with activation of caspases in vitro. Also the in vivo
study indicated reduction of hepatic nodule formation and restriction of angiogenesis with regulation of liver enzyme parameters.

In conclusion, we suggest that leishmanial lipid is a potential agent for regulation of hepatic cancer cell growth with involvement of several apoptotic molecules and angiogenic factors without having any adverse effect on the system.
References


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