Chapter-1

Review of Literature
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1.1 The parasite *Leishmania*

*Leishmania* are protozoan parasites and members of the family Trypanosomatidae. These unicellular eukaryotic organisms are characterized by the presence of a flagellum and of a DNA-rich, mitochondria-like organelle, the kinetoplast. Multiple species of *Leishmania* organisms are known to cause human diseases involving the skin and mucosal surfaces, or the visceral reticulo-endothelial organs. Three distinct forms of leishmaniases are known from the symptoms and clinical manifestations caused by *Leishmania* species:\(^1\); (a) cutaneous leishmaniasis, an infection that causes ulcerative lesions of the skin, is caused by *L. major*, *L. tropica* and *L. mexicana*, (b) a variant form of cutaneous disease, mucocutaneous leishmaniasis, caused by *L. braziliensis braziliensis*, and (c) visceral leishmaniasis (kala azar) caused by *L. donovani*. The visceral form of infection is chronic and may be fatal in untreated cases. Currently no vaccine is available to deal with this widespread infectious disease and the chemotherapeutic treatment involves antimonials and diamidines that are toxic.\(^2\)

During its life cycle, *Leishmania* alternates between two distinct developmental stages\(^3\) spanning human host and sandfly vector. In the mammalian host, the parasite proliferates intracellularly as nonmotile amastigote forms, within the acidic and hydrolase-rich phagolysosomal compartment of host macrophages\(^4\) - \(^6\). Transmission of the parasite is mediated by the blood-sucking sandfly which while feeding on the infected mammal, takes up amastigote-containing macrophages/monocytes. The amastigotes differentiate into motile promastigotes, which attach to the sandfly midgut epithelium to avoid being excreted together with the digested blood meal. Virulence is acquired during metacyclogenesis, a process by which dividing, noninfective promastigotes (procyclic) transform into a nondividing infective form. These metacyclic promastigotes detach from the gut epithelial cells and migrate towards the anterior end of the digestive tract. Upon the next blood meal of the infected sandfly, metacyclic promastigotes are introduced into the mammalian host, the promastigotes get entry inside macrophages.

Throughout its digenetic life cycle, *Leishmania* deals with various environments where it must avoid destruction\(^3\): (i) the sandfly midgut, where the parasite is
exposed to a variety of digestive enzymes, (ii) the bloodstream of the host, where the parasite transits and must deal with the lytic complement pathway, and (iii) the phagolysosome of host macrophages, where the parasite is exposed to a number of hydrolytic enzymes, acidic pH, and the microbicidal oxidative burst. To deal with such hostile surroundings, the parasite has evolved mechanisms and molecules which are beginning to be understood in some detail. One such protective mechanism involves the expression of unique cell surface glycoconjugates\textsuperscript{7,8} which play a major role in the ability of \textit{Leishmania} to adapt and persevere in the hostile environments encountered in both the invertebrate and the mammalian host. \textit{Leishmania} parasites express three major and functionally important types of molecules on their cell-surface, all of which are anchored to plasma-membrane through a Glycosylphosphatidylinositol (GPI) anchor. These include a major surface glycoprotein (gp63), lipophosphoglycan (LPG) and protein-free glycosyl inositol phospholipids (GIPLs)\textsuperscript{9,10}. 
1.2 Glycosylphosphatidylinositol anchors in protozoan parasites

GPIs are a class of eukaryotic glycolipids containing the structural motif Manα(1,2)Manα(1,6)Manα(1,4)GlcNα(1,6)myo-inositol-1-phospholipid as shown in Figure-1.

![Figure-1: Structure of a typical GPI anchor](image)

The GPI-anchors were first reported during identification of the variant surface glycoprotein (VSG) of trypanosomatigote forms of *T. brucei*\(^{19,20}\) and later on a number of GPI-linked proteins were identified from higher eukaryotic cells\(^9\). Mammalian cells typically express in the order of 100 thousands copies of GPI-anchor per cell, whereas parasitic protozoa express upto 10-20 million copies of GPI-anchor (and related structures) per cell. The most fundamental function of the GPI-anchor is to afford the stable association of proteins with the surface.
membrane. GPI membrane-anchored proteins have attracted considerable attention as markers of detergent-insoluble cholesterol/sphingolipid-rich "lipid rafts". Although these glycolipids were originally discovered covalently linked to eukaryotic cell-surface glycoproteins, many cells contain large pools of non-protein linked or "free" GPIs. Many of these GPIs represent end products of biosynthetic pathways or by products not consumed in protein or glycoconjugate anchoring reactions.

The major surface macromolecule on the surface of the promastigote stage of *Leishmania* spp. is the hyperglycosylated GPI glycolipid, termed lipophosphoglycan (LPG) and has been shown to be essential for parasite survival and virulence. This molecule contains a highly conserved GPI anchor and a long phosphoglycan backbone that can be elaborated with species- and stage-specific glycan side chains. In all pathogenic *Leishmania* spp., LPG is expressed in high levels (~5 x 10^6 molecules/cell) and forms a distinct glycocalyx and polymorphisms in LPG has been observed in different *Leishmania* species. The surface coat of *Leishmania* promastigotes also contains a number of GPI proteins and proteophosphoglycans (PPGs). *Leishmania* promastigotes also synthesize free GPIs (GIPLs) that are 10-fold more abundant (~5 x 10^7 copies/cell) than LPG. While most of these glycolipids are found in the exoplasmic leaflet of the plasma membrane, a significant pool may be located in the inner leaflet of the plasma membrane.

*Leishmania* promastigotes and amastigotes also secrete copious amounts of soluble proteophosphoglycans (PPGs). The secreted (and surface-bound) PPGs characteristically contain very long polypeptide backbones, which are modified with phosphoglycans similar to those added to LPG. Individually or after self-association, the PPGs characteristically contain very large macromolecular filamentous structures that may facilitate promastigote aggregation and the transmission of large parasite clusters within the sand fly bite. These secreted PPGs may also deplete the level of lytic complement components in the mammalian lesion via nonproductive activation of the complement pathway and modulate processes in the macrophage, such as the number and size of parasitophorous vacuoles and cytokine production.
1.3 Structure of LPG and GIPLs of *Leishmania* parasite

1.3.1 Structure of LPG

The molecular basis of these adaptations has been investigated largely in the context of the structure and function of the surface lipophosphoglycan, the dominant surface glycoconjugate on promastigotes of all *Leishmania* species studied to date. LPG is organized as a densely packed glycocalyx that covers the entire surface of the cell, including the flagellum. Each parasite cell contains several million molecules of LPG. The structure of LPG consists of four domains\textsuperscript{10,29} as shown in Figure-2. (a) a phosphatidylinositol lipid anchor, (b) a conserved glycan core, (c) a repeating phosphorylated saccharide region, and (d) a small oligosaccharide cap structure.

![Figure-2: Structure of Lipophosphoglycan of *Leishmania donovani*](image_url)
The lipid anchor of LPG is an unusual phospholipid derivative 1-O-alkyl-2-lyso-phosphatidyl (myo) inositol. Probably in all the species of *Leishmania*, the aliphatic chain consists of either a C\textsubscript{24} or a C\textsubscript{26} (mostly in 60-40 mol\%) saturated, unbranched hydrocarbon\textsuperscript{10} e.g. quantitative analysis of lipid moiety in *Leishmania donovani*\textsuperscript{36} indicated that 78\% of LPG possessed a C\textsubscript{24} hydrocarbon and the remaining 22\% possessed a C\textsubscript{26} species. Similar analysis showed a 50-50 ratio in *L. mexicana*\textsuperscript{37}. The lipid anchor is conserved amongst all *Leishmania* species studied till date.

Attached to the inositol of the lipid anchor of LPG is the hexasaccharide glycan core region. This core is conserved\textsuperscript{10} across the *Leishmania* species. An unacetylated glucosamine is attached to the inositol of the lipid anchor by an \(\alpha 1,6\) linkage. On the non-reducing end, glucosamine is attached to two mannoses. The distal mannose is then attached to a unique galactofuranose residue. The presence of this galactofuranose is unique especially because the furanose is internal in the carbohydrate chain. The galactofuranose is followed by two galactoses linked to each other in an \(\alpha (1,6)\) configuration; completing the glycan core backbone of Gal \(\alpha (1,6)\) Gal \(\alpha (1,3)\) Galf \(\beta (1,3)\) Man \(\alpha (1,3)\) Man \(\alpha 1\). A substantial percentage of *L. major* LPG also contains a glucosyl \(\alpha 1\)-phosphate substitution at the distal mannose\textsuperscript{38}.

Whereas the glycan core and lipid anchor are highly conserved between species, the repeating oligosaccharide phosphate units display extensive interspecific polymorphisms\textsuperscript{10,25,29}. The phosphoglycan moieties of all LPG’s studied to date share a common backbone consisting of repeating disaccharide units of Gal \(\beta (1,4)\) Man \(\alpha 1\)-PO\textsubscript{4}-6 where the 3-position of Gal residue can either be unsubstituted (as in *L. donovani*) or almost completely substituted with a variety of saccharide side chains (as in *L. major*). On the basis of the nature of the side chain substitutions on the repeat unit, some have classified the leishmanial LPGs into three classes\textsuperscript{29}. The type-1 LPGs contain no side chain substitution as exemplified by the LPGs from the East African *L. donovani* strains S-1 and LV9 and *L. peruviana*. Type-2 LPGs contain a variety of saccharide side chains, all of which are linked to the C-3 position of the Gal residue in the disaccharide repeat unit. These side chains vary from a single Glc residue, as in *L. mexicana* LPG, to the more complex side chains
of *L. tropica* and *L. major* LPG. The *L. tropica* LPG contains the most complex side-chain profile of any of the LPGs examined to date\textsuperscript{29}. The type-3 LPGs, exemplified by the *L. aethiopica* LPG, contain side chains on both the C-2 position of the mannose residue, as well as on the C-3 position of the galactose residue, in the disaccharide repeats. In the *L. aethiopica* LPG, approx. 35% of the disaccharide repeat units were substituted with single $\alpha$ Man residues\textsuperscript{29}. This type of side chain may have a profound effect on the three-dimensional conformation of the phosphoglycan chain. In this regard, studies using NMR\textsuperscript{38,39} and molecular dynamics modeling suggest that a chain of unsubstituted phosphorylated disaccharide repeat units assumes an extended helical conformation in which the C-3 position of the Gal residues is exposed on the outer face of the helix, while the C-2 position of the Man residues are directed towards the inside of the helix. These studies predict that addition of side chains to the Gal could occur without major change in the conformation of the backbone, while the opposite would be true for additions to the Man residue. As several stable conformers can exist about the phosphodiester linkages, the molecule is allowed to expand or contract somewhat like a slinky spring.

The non-reducing terminus of the phosphoglycan is capped\textsuperscript{10} with a mannose-containing, neutral oligosaccharide, which for *L. major* is primarily the sequence Man$\alpha$(1,2)Man$\alpha$1. The capping oligosaccharide *L. donovani* LPG display considerable heterogeneity, with the most abundant structure made up of the branched trisaccharide Gal $\beta$(1,4)[Man$\alpha$(1,2)]Man$\alpha$1. The cap structure of *L. tropica* has been characterized\textsuperscript{29} as Gal $\beta$(1,4)Man, [Man$\alpha$(1,2)]$\alpha$1,2Man$\alpha$1 and Man$\alpha$(1,2)Gal $\beta$(1,4) Man.

By controlling the extent of binding to lectins or lectin-like receptors present in the midgut of different sandfly vectors, the interspecies variations in LPG structure have been implicated in the species specificity of vectorial competence observed in nature\textsuperscript{40,41}. For example, binding of parasites to midgut of *Phlebotomus papatasi*, which is a natural vector of *L. major*, but nonpermissive for all other species of *Leishmania*, requires expression of LPG containing Gal$\beta$(1,3)-terminating side chains. Furthermore, developmentally regulated polymorphisms\textsuperscript{21,41} in LPG
structure appear to control the stage specificity of midgut adhesion. *Leishmania* displays regulated variations in LPG throughout the different stages of the parasite life cycle\(^4\). During metacyclogenesis of promastigotes, the LPG undergoes extensive modifications. These include an elongation of the molecule due to an approximate doubling in the number of oligosaccharide-phosphate units expressed. As a result of which, the cap becomes cryptic, enabling detachment of the metacyclic promastigotes from the fly midgut\(^4\). Structural changes of LPG repeat units also are important for intraspecies polymorphisms. LPGs of an Indian isolate of procyclic *L. donovani* and the New World *L. chagasi*\(^4\) have a Glc\(\beta(1,3)\) residues that branch off the Gal\(\beta(1,4)\)Man\(\alpha(1)\)-PO\(_4\) backbone repeat units.

Such changes in LPG structure may have implications on function and suggest that there are important points of regulation of the glycosyltransferases involved in LPG biosynthesis.

### 1.3.2 Glycosylinositolphospholipids (GIPLs)

The GIPLs are the major glycolipids synthesized by *Leishmania* parasites. Three distinct lineages of GIPLs have been identified, which are expressed in markedly different levels in different species or developmental stages\(^9\). The type-1 and type-2 GIPLs have glycan headgroups, which are structurally related to the GPI protein anchors and the LPG anchor, respectively. The hybrid-type GIPLs have branched glycan headgroups which share features common with both types of GPI anchor, as shown in Figure-3. The lipid moieties of *Leishmania* GIPLs are either alkylacyl-PI or lysalkyl-PI. The lipid composition in hybrid and type-1 GIPLs are predominantly C\(_{18:0}\) alkyl chains and, in contrast, type-2 GIPLs contain a more heterogenous alkyl chain composition, which includes C\(_{24:0}\) and C\(_{26:0}\) found in the LPG anchor. The GIPLs coat a significant proportion of the plasma membrane and, in the case of the type-2 GIPLs, are highly immunogenic. Although the function of these glycolipids is unknown, it is possible that, together with LPG, they are involved in protecting the parasite in the insect midgut and macrophage phagolysosome. *L. donovani* promastigotes and amastigotes, which both express mannose-terminating GIPLs, are able to utilize the mannose receptor on the macrophage surface. These functions are crucial to the intracellular amastigote
stage, which dramatically downregulates the surface expression of the major macromolecules, LPG and gp63, leaving the GIPLs as the major components in the surface glycocalyx. In *L. major* promastigotes the GIPLs belong to the type-2 series\(^9,45\) which are structurally related to the core sequence of LPG ie Galβ(1,3)Manα(1,3)Manα(1,4)GlcNα(1,6)myo-inositol (termed GIPL-1). In GIPL-1, Galβ is present as a terminal residue at the non-reducing end. Galβ can also be present as a non-terminal residue as in GIPL-2 which is [Galpα(1,3)GIPL-1] and GIPL-3 which is [Galpα(1,6)GIPL-2].

The role of GIPL in the induction of iNOS in murine macrophage cell line, J794 has been studied.\(^45\) GIPL markedly inhibited the production of NO by macrophages and enhanced survival of *L. major* in activated macrophages. The terminal Galβ in GIPL-1 has been shown\(^46\) to play a central role in the survival of the parasites by blocking action of the host’s glycosidase against glycoconjugates of the parasite. The structure of some of the GIPL molecules found on the *Leishmania* parasite are shown in Figure-3.
Figure-3: Structure of GIPLs of *Leishmania donovani*
1.4 Biosynthesis of *Leishmania* LPG

1.4.1 Biosynthesis of *Leishmania* LPG

Since LPGs are the major surface macromolecules of most human-pathogenic *Leishmania* and are essential for the pathogenicity and survival of the parasite, a detailed knowledge of the biosynthesis of LPG will help in designing efficacious drugs.

![Figure-4: Proposed biosynthetic pathway for the glycan core of Lipophosphoglycan](image)
Although not much is known about the enzymes involved in the biosynthetic pathway, the possible pathway that leads to the assembly of PI-anchor of LPG has been postulated (as shown in Figure-4), based on the information from the other eukaryotic systems. Synthesis of ether phospholipid of dihydroxyacetone phosphate has been proposed as the first step, a reaction catalyzed by dihydroxyacetone phosphate acyl transferase. The acyl group must then be replaced by a fatty alcohol. The alkyl dihydroxyacetone phosphate is then reduced with NADPH and acylated at the sn-2 position forming 1-O-alkyl-2-O-acyl phosphatidic acid. The CDP-derivative of this alcohol is then coupled to myo-inositol to form 1-O-alkyl-2-O-acylPI. The first committed step of GPI-anchor biosynthesis is mediated by GPI-N-acetylglucosaminyl transferase (GPI-GnT), where an N-acetylglucosamine is transferred from UDP-GlcNAc to alkylacylPI. This GPI-GnT is a complex glycosyltransferase and it consists of at least four proteins. The next steps include a deacetylation step mediated by N-acetylglucosaminyl phosphatidylinositol de-N-acetylase, followed by O-deacetylation at the sn-2 position of glycerol to give GlcN-alkyl-lysoPI intermediate.

The first mannose residue from Dol-P-Man is then added to GlcN-PI yielding Manα(1,4)GlcN-PI, a common precursor to LPG and GPI-anchored proteins. The addition of the second mannose is at a branch point in the biosynthetic pathway of leishmanial GPI anchors, which is added to give Manα(1,3)Manα(1,4)GlcN-PI. This is followed by the addition of a unique galactofuranose residue which must require a UDP-galactopyranose mutase to convert the UDP-galactopyranose (UDP-Galp) to UDP-galactofuranose (UDP-Galf), the activated sugar donor of galactofuranose. A galactofuranosyl transferase would then transfer the Galf from UDP-Galf to the growing glycan chain. Though there is no direct evidence for the presence of UDP-Galp mutase and UDP-Galf transferase in Leishmania, a Leishmania mutant R2D2 was found to synthesize LPG that was truncated at the Galf residue within the core region. Complementation by a single gene LPG1 restored full length LPG synthesis, leading to the proposal that LPG1 encoded the Galf transferase. The two galactoses are then subsequently added from UDP-Gal to give the glycan core Galα(1,6)Galα(1,3)Galβ(1,3)Manα(1,3)Manα(1,4)GlcN-PI.
After the glycan core has been formed, a unique initiating mannosyl phosphoryl transferase (iMPT) transfers a Man-α-1-PO₄ from GDP-Man to the 6-OH of galactose. This is followed by β1,4-galactosyl transferase mediated transfer of galactose from UDP-Gal to form the first unit of Galβ(1,4)Man-α-1-PO₄. Depending on the stage in the life-cycle of the parasite, the Gal-Man unit is reiteratively synthesized by the action of elongating mannosyl phosphoryl transferases (eMPTs) (which transfers Man-α-1-PO₄ from GDP-Man) and β1,4-galactosyl transferase⁴⁹,⁵⁰

All leishmanial LPG cap contain a Manα(1,2)Man structure. Therefore a Manα(1,2)mannosyl transferase is speculated. This is an important activity as it acts as a stop signal for LPG elongation. These chain termination mannosyl transferases may prove to be an important target for chemotherapeutic intervention¹⁰.

1.4.2 Inhibition of LPG biosynthesis
The proposed roles of LPG during mammalian infections have been established by inhibiting its biosynthesis. This was done by generating LPG-deficient mutants by chemical mutagenesis. These mutants synthesized truncated forms of LPG and have been shown to be avirulent. One such Leishmania donovani mutant, JEDI, generated by antibody selection against cells expressing repeat unit epitope of LPG⁵¹, was shown to accumulate a truncated form of LPG, where the LPG synthesis had terminated after the addition of the first repeat unit onto the glycan core. Though the parasite had the iMPT to initiate the PG synthesis, it did not have the eMPTs to elongate the chain.

L. donovani mutant, R2D2, was found¹⁷,¹⁸ to synthesize LPG that was truncated at the Galβ residue. Complementation with gene LPGI restored full-length LPG synthesis, proposing that LPGI encoded the Galβ transferase. Though this mutant was generated by chemical mutagenesis, studies led to the generation and investigation of targeted LPGI knockout mutants in L. mexicana and L. major. These mutants were defective in LPG synthesis only but retained normal levels of all other important PG containing glycoconjugates.

Another L. donovani mutant, OB1, accumulates a truncated LPG containing only the Manα1-PO₄ residue of the first repeat unit. Functional complementation and
gene knockout approach identified a \textit{LPG3} gene\textsuperscript{52} that was responsible for this defect.

The \textit{LPG2} gene of \textit{L. donovani} was identified by functional complementation of the \textit{lpg2}" mutant that was deficient in biosynthesis of LPG\textsuperscript{53,54}. The altered LPG made by \textit{lpg2}" mutants lacked the repeat units of mannose phosphate but these mutant cells contained functional Golgi localized enzymes for the addition of these units to the LPG backbone. \textit{lpg2}" parasites failed to transport radiolabeled GDP-Man, but restoration of the \textit{LPG2} gene restored this ability, confirming that the \textit{lpg2} gene is responsible for GDP-Man transporter, a protein absent in mammalian cells.

Not much work has been done on designing synthetic inhibitors that inhibit the LPG biosynthesis. A paper\textsuperscript{55} reports the synthesis of Gal\beta(1,4)Man\alpha1-P-decenyl and its analogues. This is a synthetic acceptor for the characterization of eMPT activity especially in terms of substrate specificity using these analogues.

Anallogues of Gal\textsuperscript{56,57}, UDP-Gal\textsuperscript{58-61} and UDP-Galp\textsuperscript{62} have been synthesized and thus they have been used to inhibit UDP-Galp mutase activity in other organisms such as \textit{Mycobacterium tuberculosis}.

1.4.3 Topology of GPI and LPG biosynthesis

The GPI anchors are assembled on the same pool of alkylacyl-PI as the protein anchor precursors. The initial steps (upto the formation of GlcN-PI) occur in the cytoplasmic side of the ER membrane\textsuperscript{63}. The later steps are catalyzed by luminally oriented enzymes\textsuperscript{64}. The first mannose residue is transferred from Dol-P-Man to form Man\alpha(1,4)GlcN-PI. The LPG biosynthesis pathway diverges from the protein anchor pathway with the addition of second mannose residue in \alpha1-3 rather than \alpha1-6 linkage. The \alpha1,3-linked mannose is probably added on the cytoplasmic face of the ER by a GDP-Man-dependant mannosyltransferase\textsuperscript{51}. In contrast, all other GPI mannosylation reactions are thought to occur in the ER lumen\textsuperscript{23}. The LPG anchor precursor is subsequently elongated with a galactofuranose. The early LPG intermediates must be flipped from the cytosolic face of the ER to the luminal face of the ER or the Golgi for this step to occur. The remaining steps of the core synthesis include the addition of the two galactoses and deacylation of the lipid moiety. However, the ordering and enzymology of these reactions have not been
carried out as yet. The phosphoglycan moiety of LPG is subsequently assembled by
the multiple rounds of Manα1-PO₄ and Gal additions to the mature glycan core. Mutants lacking the GDP-Man transporter (the product of LPG2 gene) were unable to synthesize these phosphoglycan chains, but this mutant contained functional Golgi localized enzymes for the addition of mannose phosphate units to the LPG backbone, supporting the notion that GDP-Man is the donor for all mannose additions, that lpg2 mutant has an impaired transport activity and that phosphoglycan synthesis occurs in the Golgi apparatus. In L. major, families of galactosyl- and arabinosyltransferases are required for addition of a unique set of glycan side chains to the LPG phosphoglycan backbone. The arabinose (Arap) residues are transferred from GDP-Arap, which is synthesized in the cytosol and possibly transported into the Golgi by the LPG2p GDP-Man transporter. How the developmentally regulated changes in the average length of the LPG phosphoglycan chains and the degree of Ara capping are regulated during promastigote growth remain intriguing questions. Figure-5 on the next page shows a representation of the topology of synthesis of GPI molecules in Leishmania mexicana.
Figure-5: GPI biosynthetic pathway in *Leishmania mexicana* promastigotes. All reactions, except for LPG anchor and phosphoglycan chains, occur in the ER.
1.5 Functions of LPG

*Leishmania* species have developed unique adaptive mechanisms to ensure their survival in the harsh environment faced throughout their life cycle. LPG is the most abundant cell surface molecule expressed by the infectious promastigote stage of the parasite. There is now substantial evidence that phosphoglycans i.e. the LPG and PPG and the other glycoconjugate molecules covering the surface of the promastigote are antigenic and are multifunctional virulence factors\(^{10,24-28}\), essential for the infectivity and survival of the parasite. LPG plays a critical role in subverting the host immune system, binding of the parasite to the sandfly-midgut epithelial cells and human macrophages\(^{3-6}\). It is also known to inhibit Protein Kinase C (PKC) dependant signaling and thus allows *Leishmania* to survive and proliferate. LPG deficient mutants of *Leishmania* cannot survive in the sandfly vector or infect mammalian macrophages\(^{25,26,51-54}\). Both the functions can be restored on insertion of exogenous LPG into the plasma membrane of deficient strains. The uniqueness of the overall structure of LPG and its highly unusual domains are responsible for its multifunctionality\(^{10,66,67}\).

Some of the well known functions of LPG are:

1.5.1 LPG and its interaction with host serum components: LPG and other glycoconjugates play an important role in *Leishmania*-complement interaction. LPG prevents the access of the C5b-9 membrane attack complex to the promastigote membrane. The mannose-binding protein (MBP) binds to the oligosaccharide cap of LPG. This leads to the formation of C3 and hence C3b, which in turn participates in attachment to macrophages. Phagocytosis of *L. donovani* promastigotes is also enhanced following opsonization by C-reactive protein (a serum protein), which binds to Galβ(1,4)Manα1-PO₄ of LPG. Although promastigotes bind to macrophages in absence of serum also, presumably through the mannose receptor, attachment involves complement receptor CR1, CR3 etc. CR1 and CR3 favor survival of *Leishmania* promastigotes, since these receptors promote phagocytosis without triggering the oxidative burst. CR3 ligation also inhibits IL-12 production.
1.5.2 Inhibition of Protein Kinase C: *Leishmania* infected macrophages display an impaired responsiveness to IFN-γ, lipopolysaccharide (LPS) and activators of PKC. In vitro studies have revealed that LPG is a potent inhibitor of PKC activity ($K_i<1 \mu M$)\(^{68}\). GIPLs, which represent the most abundant glycoconjugate of the amastigote stage, also display an inhibitory activity towards PKC, which modulates a wide variety of cellular functions. Treatment of macrophages with purified LPG inhibited several PKC-dependant events, including induction of oxidative burst, LPS- and diacylglycerol- induced c-fos gene expression. It has been shown\(^{69}\) that the glycerolipid domain of LPG may be responsible for inhibition of PKC mediated c-fos expression. Despite the positions of LPG and PKC on opposite sides of the membrane, LPG is capable of trans-bilayer inhibition of PKC activity. A full length LPG molecule is necessary for maximal inhibition of PKC, which may be the consequence of alterations in the physical properties of the membrane, when LPG inserts into membrane and makes the rearrangement of proteins in membranes more difficult.

1.5.3 Inhibition of phagosome maturation: Whereas amastigotes reside inside acidic, hydrolase-rich phagolysosomes, *L. donovani* promastigotes inhibit phagosome-endosome fusion during the early phase of infection. Further analyses revealed that promastigotes containing wild-type *L. donovani* promastigotes fail to acquire the late endocytic and lysosomal markers rab7 and LAMP1, whereas, LPG repeating unit-defective mutant containing phagosomes, mature rapidly into phagolysosomes by acquiring rab7 and LAMP1.

LPG gives rise to an effective steric repulsion between phagosomal and endosomal membranes or reduces the negative curvature strain in bilayers, increasing the energy barrier for forming highly curved fusion intermediates, thereby preventing fusion.

Alternatively, it is also proposed that LPG may inhibit this fusion by inhibition of phagosome associated PKC. PKC phosphorylates MARCKS\(^{70}\) (a phagosome membrane protein, associated with actin-based motility and membrane trafficking), which results in the displacement of LAMP-1 positive lysosomes and thus participates in fusion. Inhibition of PKC may block all these processes. In presence
of LPG, an accumulation of periphagosomal F-actin is seen, which correlates with an impaired recruitment of PKC-α.

1.5.4 LPG and lipid rafts: Lipid rafts are defined as specialized membrane microdomains, enriched in sphingomyelin, glycosphingolipids and cholesterol. Rafts are predominantly found at the plasma membrane and at the early endocytic and late exocytic compartments. Rafts are enriched in several proteins with lipid modifications. These include GPI-anchored proteins, cytoplasmic proteins with dual acylation (palmitoylation and myristoylation) like Src-family etc. Lipid rafts present on phagosomes are devoid of LAMP-1 and are rich in flotillin-1. Phagosomes containing *L. donovani* promastigotes display a marked inhibition of flotillin-1 enriched lipid rafts. Though the mechanism is not yet known, but LPG-deficient mutants do not show such inhibition. Since LPG is transferred to host cell membranes, it is possible that its unusual GPI anchor inserts itself preferentially into lipid rafts.

1.5.5. Modulation of nitric oxide production: Macrophages express inducible nitric oxide synthase (iNOS) in response to various extracellular signals, including IFN-γ and bacterial LPS. iNOS is required for resistance to *Leishmania* infection in mice. While the intact LPG molecule had no effect on NO production, preincubation of macrophages with the phosphoglycan moiety of LPG potentially inhibited iNOS expression. Simultaneous addition of phosphoglycan and IFN-γ to macrophages however induced leishmanicidal activity and NO secretion. Thus, the production of NO, a key host defense molecule, can be modulated by distinct LPG domains.

1.5.6. Suppression of IL-1β and IL-12 expression: It has been reported that agonist-induced production of IL-1, a key mediator of immunity and inflammation, is impaired. LPG is a potent inhibitor of LPS-induced IL-1 production. LPG inhibits IL-1β gene expression by suppressing transcriptional activity, and involves a unique sequence within the IL-1β promoter that acts a gene silencer. Interestingly the whole of the LPG molecule is required for this inhibitory activity.
parasites have been shown to actively suppress IL-12, an important cytokine involved in the differentiation and expansion of Th1 cells. This action has been attributed to the PG repeat units. Synthetic PGs inhibited IL-12 release in a dose-dependant manner.

1.5.7. Scavenging of toxic oxygen metabolites: It has been shown that LPG scavenges toxic oxygen metabolites like O$_2^{-}$ and H$_2$O$_2$ by the NADPH oxidase system, thus increasing survival of *Leishmania* promastigote.
1.6 Galactofuranose metabolism: a potential target for chemotherapy

The search for new molecules unique to pathogenic microorganisms has led to the identification of a potential chemotherapeutic target; the biosynthesis of sugar galactofuranose (Galf). In the pyranosidic configuration, galactose is abundant in mammalian glycoconjugates, and a conformational alteration in Galp yields Galf (Figure-6). This Galf conformation is present only in microorganisms such as bacteria, protozoa and fungi.\(^{11,12}\)

![Figure-6](image)

Importantly, Galf has been shown to be present in numerous structures considered to be essential for virulence in many pathogenic organisms. These include LPS O-antigen of an increasing number of Gram-negative bacteria; T1-antigen of *Salmonella* spp. etc. Galf is a critical and abundant component of the arabinogalactan of *Mycobacterium* spp. In protozoan parasites, Galf is present in lipopeptidophosphoglycan (LPPG) and GIPLs of *Trypanosoma cruzi* and in LPG and GIPL-1 of *Leishmania* spp.\(^{46,11,12}\). Galf containing epitopes have been shown to be highly antigenic in mammals. Thus the metabolism of Galf has become an attractive drug target.

The pathway of Galf metabolism has been difficult to study due to the instability of Galf itself. UDP-Galf derived from UDP-Galp by the action of UDP-Galp mutase, has been shown to be the source of Galf. This enzyme has been isolated and its activity demonstrated in several microorganisms that synthesize Galf-containing glycoconjugates, including *Escherichia coli* K-12\(^{13,14}\), *Mycobacteria*\(^{15}\) and *Klebsiella*\(^{16}\). Galf is then transferred from UDP-Galf to the respective glycoconjugate molecules by specific galactofuranosyl transferases (Figure-7).
The gene encoding UDP-Galp mutase, Glf (EC 5.4.99.9) was identified and cloned from the *E. coli* K-12 *rfb* region, and purified protein was shown to demonstrate the mutase activity *in vitro*. Biochemical characterization demonstrated that Glf is a flavoprotein to which FAD is noncovalently bound\textsuperscript{14,78,79}. The crystal structure of *E. coli* mutase has been solved\textsuperscript{79} and site-directed mutagenesis\textsuperscript{80} showed that enzyme-bound FAD is located within a cleft containing the active site of the mutase. Reduction of FAD has been shown to be an absolute requirement for Glf activity. No requirement for either NADP\textsuperscript{+} or NADPH has been shown.

In *K. pneumoniae*\textsuperscript{16}, an analogous gene *gifKPOI* (formerly *rfbDKPOI*) was cloned and its protein purified. Investigation of its activity in vitro showed that, as for *E. coli* *glf*, *gifKPOI* encodes *K. pneumoniae* UDP-Galp mutase (*GlfKPOI*). In contrast to *E. coli* Glf, however *GlfKPOI* was shown to absolutely require NADH or NADPH for activity. *wbbO* (formerly *rfbFKPOI*) was recently cloned, expressed in *E. coli* K-12 strains, and shown to encode a product exhibiting both galactopyranosyl and galactofuranosyl transferase activities\textsuperscript{81}. These two genes *wbbO* and *gifKPOI* are a part of the gene cluster *rfbA*-F involved in directing the synthesis of D-galactan I, which comprises the O-antigen backbone structure of *K. pneumoniae* LPS.

It has been shown that UDP-Galp mutase activity is present in *Mycobacterium smegmatis*, and that UDP-Galf derived from this activity is the Galf donor in mycobacteria. This led to the identification, cloning and expression of *M. smegmatis*
glf gene (Rv3809c), encoding the UDP-Galp mutase (Glf), the activity of which has been shown using in vitro assays. The amino acid sequence of *M. smegmatis* Glf is quite similar to *E. coli* and *K. pneumoniae* Glf proteins, with 42.5 and 40.9% identity at the amino acid level, respectively.

A study\(^8\) described the synthesis of arabinogalactan via the addition of Galf residues, using UDP-Galp in presence of Glf and obtaining a Galf transfer to the linker unit intermediate polyprenyl-P-P-GlcNAc-Rha. This identified a specific galactofuranosyl transferase, identified as the product of *M. tuberculosis* gene Rv3808c locus. This transferase was designated as gifT, a novel galactofuranosyl transferase\(^83,84\).

A glf knockout mutant could only be rescued by the presence of plasmids expressing glf and rv3808c, showing both Glf and GifT are essential for the growth of *M. smegmatis*.

**Mechanism of action of UDP-Galp mutase:** The enzyme's absolute requirement for FAD\(^78\) as a cofactor and its occasional requirement for NADH (as in *K. pneumoniae*), proposed that the substrate is transiently reduced during the reaction. Further studies on *E. coli* Glf, showed that the reaction is not initiated by the oxidation of either the C2- or C3-OH of the sugar substrate, as synthetic C2- and C3- fluorinated UDP-Galp analogues can function as substrates\(^59\) for reduced mutase. Similar results were observed using C2- and C3- fluorinated UDP-Galp analogues as substrates for *K. pneumoniae* UDP-Galp mutase\(^62\). Further characterization using positional isotope exchange (PIX) experimentation revealed that mechanism by which this reaction works involves an anomeric C-O bond cleavage of the substrate\(^85\), a result that was similarly confirmed for *E. coli* Glf. In a recent study\(^86\), however, a radical mechanism has been proposed. The key intermediate in this proposed mechanism is the anomeric radical, which is formed by single-electron transfer from the anionic hydroquinone FADH\(^+\) to the oxocarbonium ion. Till date, this radical based mechanism rationalizes all the previous data. This mechanism has been referred to as the single-electron cryptor-redox process.
In order to inhibit the galactofuranose assembly, either the mutase or the transferase has to be targeted and analogues of Galf\textsuperscript{56,57} and UDP-Galf\textsuperscript{58-61} have been synthesized and their potential as inhibitors tested. The first analogue of UDP-Galf synthesized was UDP-C-Galf\textsuperscript{60}. Though its inhibitory activity on the mutase was not tested then, the group synthesized the same compound by a new and efficient route\textsuperscript{61} and tested it as an inhibitor showing\textsuperscript{61} 2-91% inhibition under various conditions. The paper also reports the synthesis of another inhibitor, UDP-1,4-anhydro-Galp which has been designed as a transition state analogue showing 32-53% inhibition under various conditions. 2-deoxy-2-fluoro and 3-deoxy-3-fluoro analogues of UDP-Galf have also been synthesized\textsuperscript{59} and they inhibit (61% and 10-98.6% respectively, under various conditions) UDP-Galp mutase. Synthesis of a peptidomimetic of UDP-Galf has also been reported\textsuperscript{58} with potential inhibitory activity. Sulphur\textsuperscript{56} and pyrrolidone\textsuperscript{57} analogues of galactofuranose have been synthesized as potential inhibitors of the mutase but the inhibitory results have not been published. A uridine-based library has been developed\textsuperscript{87} and screened as inhibitors for the mutase.

In *Leishmania*, the major Galf expressing glycoconjugate LPG is expressed in a stage-specific manner. Studies were performed in which *Leishmania* mutants were generated using chemical mutagenesis as described earlier in this review. *L. donovani* mutant, R2D2, was found\textsuperscript{11,12} to synthesize LPG that was truncated at the Galf residue. Complementation with gene designated *LPGI* restored full-length LPG synthesis, leading to the proposal that *LPGI* encoded the Galf transferase. Though this mutant was generated by chemical mutagenesis, studies led to the generation and investigation of targeted *LPGI* knockout mutants in *L. mexicana* and *L. major*. These mutants were defective in LPG synthesis only but retained normal levels of all other important PG containing glycoconjugates.

The proposed roles of LPG during mammalian infection had only been shown through in vitro assays. Recently, however, two independent investigations led to the role of LPG during mammalian infection using targeted *LPGI* mutants. *L. mexicana* *LPGI* knockout was examined for its ability to infect macrophages and mice. It was shown that there were minimal differences between the LPG-deficient mutant and the wild-type parasites in their attachment to, uptake by and replication within
macrophages. The mutant parasites were as virulent as the wild type, resulting in disseminated disease and death in both BALB/c (susceptible) and C57/BL6 (resistant) mice. On the other hand, *L. major* *LPG1* knockout highly attenuated in BALB/c mice, with the restoration of virulence upon introduction of *LPG1*. This mutant was also significantly impaired in its ability to survive within macrophages. The difference in reported results may reflect species-specific variations, but it can be certainly warranted that the roles of LPG and Galf metabolism are very critical.

Although homologous Galf metabolism genes have been identified and examined in various pathogenic organisms, whether functions assigned to one enzyme is common to all other pathogenic microorganisms known to possess Galf in their surface glycocojugates is still an unanswered question. As investigation of Galf metabolism still continues, what is evident is a new target for chemotherapeutic intervention.
1.7 Organic synthesis of LPG

It has now been substantially proved that *Leishmania* cell surface molecules like LPG, GIPLs, gp63 and other related glycoconjugates are key mediators of interaction between the host cell and the parasite. These molecules have thus become attractive targets for development of inhibitors and as vaccines. Organic synthesis of these molecules have become attractive, not only because of their importance in biological activity but also because of their challenging structural complexity to develop new synthetic methodologies. In our ongoing work on the organic synthesis \(^{88-92}\), biosynthesis \(^{93-95}\) and immunology \(^{69}\) of *Leishmania* cell surface glycoconjugates, a complete synthesis of LPG was required. Synthetic efforts towards this molecule have been minimal till date and no total synthesis of LPG has been accomplished so far. Different groups have worked on the synthesis of the various domains of LPG.

1.7.1. Synthetic effort towards the cap structure of LPG: The first synthesis of the neutral cap of LPG of *L. donovani* was reported by Fraser-Reid et al.\(^6\). The lower Galβ(1,4)Man unit of the tetrasaccharide was made from the monosaccharides suitably protected galactose and mannose and efficiently coupled using pentenyl chemistry (Figure-8). The coupling of this Gal-Man unit with suitably protected mannobiose donor provided the fully protected cap domain of LPG.

The second synthesis of the cap was reported by our group\(^8\). The key intermediate hexa-\(\Omega\)-benzyl lactal \(2\) was prepared from lactose (Figure-9). This was followed by a stereoselective \(\alpha\)-epoxidation using 2,2-dimethyldioxirane solution giving the \(\alpha\)-epoxide \(3\), the methanolysis of which gave the corresponding \(\beta\)-glucoside \(4\). The gluco-manno transformation was carried out using Danishefsky approach to get a \(\beta\)-mannoside \(5\) and an acceptor for mannobiose trichloroacetimidate (synthesis not shown in the figure). TMSOTf mediated coupling of compounds \(5\) and \(6\) yielded the fully protected cap. The paper also reports synthesis of the radiolabeled form of the cap, using Swern oxidation-NaB\(^3\)H\(_4\) reduction couple to get the tritiated cap derivative.
Figure-8
Hewitt and Seeberger have also reported a solution and solid phase synthesis of this neutral cap\textsuperscript{97,98}. The hexa-\textit{O}-benzyl lactal was used as the key intermediate for the synthesis of Gal-Man and mannoses were added to get the protected cap. The unique feature of their synthesis was the conjugation of the cap to the immunostimulator Pam\textsubscript{3}Cys to create a fully synthetic vaccine and to the carrier protein KLH to form a semisynthetic vaccine. The paper described a solid-phase synthesis which relied on
the assembly from monosaccharide units and used both glycosyl phosphates and glycosyl trichloroacetimidates as the coupling donors\textsuperscript{98}.

1.7.2. Synthesis of the phosphoglycan repeat units of LPG: Extensive synthetic work on the phosphoglycan repeat units has been done by Nikolaev and Ferguson\textsuperscript{99} who has described the synthesis of oligosaccharide fragments in LPG of \textit{L. donovani}, \textit{L. major} and \textit{L. mexicana}. In all these synthesis, the starting materials were monosaccharides galactose and mannose and they underwent many protection and deprotection steps to give the final product. The phosphate groups in between the repeat units were introduced using standard phosphonate chemistry. Extending this strategy, these authors then developed a polycondensation approach to make a heterogeneous mixture of phosphoglycans\textsuperscript{100}.

A new synthesis of the PG repeat unit has been reported by our group\textsuperscript{90,91}. The key feature of this approach (Figure-10) is that the chemically labile, anomeric phosphodiester-linked repeats can be assembled in an iterative manner without involving any glycosylation steps. This approach was extended to solid-phase and polycondensation method.

Recently, our group has reported\textsuperscript{92} a synthesis of PG repeat unit of \textit{L. major}, using the same strategy of synthesizing the Gal-Man repeat unit from lactose. The only glycosylation reaction involved was the coupling of 2,3,4,6-tetra-\textit{O}-acetyl-\textalpha-D-galactopyranosyl trichloroacetimidate to the Gal-Man in a \textbeta1,3 configuration.
Figure-10
1.7.3. Synthesis of the galactofuranosyl containing glycan core of LPG: The first synthesis of the core has been recently reported\(^{101}\). Reterosynthetic analysis of the core divided it into 4 Building Blocks as shown in Figure-11.

![Figure-11](image)

The synthesis of the Building Block A was reported\(^{102}\) previously by this group. For Building Block B\(^{103}\) (Figure-12), ethyl-3-\(O\)-tertbutyldimethylsilyl-4,6-\(O\)-benzylidene-1-thio-mannopyranoside was converted to the acceptor (3) and the
bromide donor (4). AgOTf mediated coupling gave the mannose disaccharide 5a. The TBDMS group was removed using BF$_3$.Et$_2$O to give 5b, which was ready to couple to Building Block C.

![Chemical structure](image)

**Reagents:** a. BnBr, NaH, DMF b. (i) Me$_3$N.BH$_3$, AlCl$_3$ (ii) BnBr, NaH (iii) BF$_3$.Et$_2$O, CHCl$_3$ c. (i) Me$_3$N.BH$_3$, AlCl$_3$ (ii) ClAcCl, DCM, Py d. AgOTf, collidine, DCM e. BF$_3$.Et$_2$O

**Figure-12**

For Building Block C (Figure-13), ethyl-1-thio-β-galactopyranoside was converted into ethyl-2,3,4,6-tetra-O-benzyl-1-thio-β-galactopyranoside (8) by perbenzylation and into ethyl-2,3,4-tri-O-benzyl-1-thio-β-galactopyranoside (7) by sequential tritylation, benzylation and detritylation. Compounds 7 and 8 were then coupled to give the Galα(1,6)Gal disaccharide (12). The galactofuranose intermediate was prepared from 1,2-O-isopropylidene-3-O-benzyl galactofuranose (9), which was prepared from galactose using a reported procedure$^{104,105}$. The free OH groups were
then acetylated. The 1,2-\textit{O}-isopropylidene group was then cleaved using a 10% solution of trifluoroacetic acid in chloroform.

\textit{Reagents}: a. (i) \textit{TrCl}, Py (ii) \textit{BnBr}, NaH, DMF (iii) \textit{pTSA} in MeOH: CHCl$_3$ b. \textit{BnBr}, NaH, DMF c. (i) \textit{Br$_2$}, CH$_2$Cl$_2$ (ii) \textit{Et$_4$NBr}, CH$_2$Cl$_2$/DMF d. DMTST, 4A$^0$ MS Diethyl ether e. \textit{5b}, TMSOTf, DCM

\textbf{Figure-13}
Acetylation followed by debenzylation gave the galactofuranose acceptor (11) which was then coupled with compound 12 to give the Galα(1,6)Galα(1,3)Galβ unit (13). This paper used compound 13, with a furanoidic anomeric ester because such compounds were shown to be good glycosyl donors. Compound 13 was coupled to the Manα(1,3)Man disaccharide (5b) in presence of TMSOTf and the pentasaccharide (14) thus obtained was coupled to the Building Block A (Figure-14) to get the heptasaccharide 16. The chloroacetyl group in compound 16 was deprotected and 2,3,4,6-tetra-O-benzyl-1-O-α-glucopyranose phosphate was attached using H-phosphonate chemistry, and after global deprotection, the glycan core (17) was obtained.
Reagents: a. 14, DMTST, Diethyl ether, b. (i) NH₃(sat) MeOH/DCM (ii) D, Piv-Cl, CH₃CN/Py (iii) I₂, Py/Water (iv) NaOMe, DCM/MeOH (v) Na, NH₃(l) (vi) 0.1M HCl

Figure-14