PART II/III: INVESTIGATIONS ON ALTERNATE APPROACH TO TARGET MANNOSE RECEPTORS ON MACROPHAGES USING 4-SULFATED N-ACETYL GALACTOSAMINE MORE EFFICIENTLY AS COMPARED TO MANNOSE DECORATED LIPOSOMES, ESPECIALLY FOR THE CONDITIONS OF LEISHMANIASIS.
PART II/III: INVESTIGATIONS ON ALTERNATE APPROACH TO TARGET MANNOSE RECEPTORS ON MACROPHAGES USING 4-SULFATED N-ACETYL GALACTOSAMINE MORE EFFICIENTLY AS COMPARED TO MANNOSE DECORATED LIPOSOMES, ESPECIALLY FOR THE CONDITIONS OF LEISHMANIASIS.

4.1 INTRODUCTION

In past few decades, macrophage-specific delivery systems gained much attention, as macrophages act as “natural ecological niche” for several microorganisms, which may result to epidemic of so many fatal diseases like tuberculosis, leishmaniasis, filariasis etc. Despite the rapid development in medicinal and pharmaceutical technologies, the targeting of drugs to macrophages for related diseases still remain an open question. In macrophage related diseases, microorganisms inhabit inside resident macrophages, thereby evading host defence mechanisms and their concealed position is responsible for impairing the accession of chemotherapeutic agents. Macrophages articulated with a variety of surface glycoproteins, called mannose receptors (MRs) that are able to bind “pathogen associated molecular patterns” (PAMPs) and several polyanionic molecules. Mannose binding proteins alternatively called lectin (MBL) whose function appears to be pattern recognition in the first line of defense in the pre-immune host and play important role in innate immunity and homeostasis. Mannose receptors (MR) are overly expressed in macrophages especially in infected ones, which can be efficiently targeted by drug delivery systems. Several research groups explored these mannose binding receptors for specifically targeting of drug delivery systems (Yoo, Park et al. 2008; Nahar and Jain 2009; Nimje, Agarwal et al. 2009; Prakash, Beljaars et al. 2010). MR is unusual as it can function to mediate both clathrin-dependent and phagocytic internalisation. Since long time, applicability of macrophage specific liposomal delivery system critically tested against macrophage related diseases using therapeutic agents. Mannosylated liposomes appear to be a promising for delivery of chemotherapeutics and proteins and it has been reported to modestly increase lymph node as compared to control liposomes.

MR is a 180-kD type I transmembrane protein that contains three extracellular regions: a C-type lectin carbohydrate- recognition domains (CRDs), NH2-terminal cysteine rich domain (Cys-MR) and a domain containing fibronectin type II repeats,
(Ezekowitz, Sastry et al. 1990; Taylor, Conary et al. 1990). The extracellular domains are linked to a transmembrane region and a small cytoplasmic domain. The CRDs of the extracellular region mediate calcium-dependent binding to sugars such as mannose, fucose, and N-acetylglucosamine that are commonly found on microorganisms, but rarely seen in sufficient density in terminal positions of mammalian oligosaccharides (Taylor, Bezouska et al. 1992; Taylor and Drickamer 1993). Recent studies demonstrate that the Cys-MR region also has carbohydrate-binding properties. Cys-MR binds to Glycoproteins containing sulfated sugars those terminate in 4-sulfated-N-acetylgalactosamine (4-SO₄GalNAc) such as the sulfated hormones lutropin and thyroid stimulating hormone (Fiete, Beranek et al. 1998). The Cys-MR interaction with cells in the spleen results from the binding of sulfated carbohydrates (Leteux, Chai et al. 2000). Available data suggest that Cys-MR directs MR-bearing cells toward germinal centers during immune responses and that sulfated carbohydrate ligands regulate the trafficking and function of cells bearing MR. Sulfate moiety on 4-SO₄GalNAc makes strong hydrogen bonds with cystein group on mannose receptor similar to mannose interaction with CRD (Liu, Teng et al. 2009).

Working with the above premise, we explored the applicability and targeting potential of 4-SO₄GalNAc for drug delivery purposes in present communication. This novel moiety provides an additional choice for targeting mannose receptors on macrophages, which can be easily coated on cationic liposomes due to strong anionic nature (Colonna, Conti et al. 2008). Thus in this work we prepared and characterized AmB loaded Sulf-Lip and compared with Man-Lip for in vitro targeting ability by studying macrophage uptake, intracellular localization, in vivo pharmacokinetic and organ distribution studies. Enhanced uptake can lead to smaller doses sufficient for optimal therapeutic effect, thereby reducing the toxicity of the medication and developing more efficient drug delivery system (Chen, Wang et al. 2008). Conclusively, sulfated liposomes are the impending candidate for macrophage targeted drug delivery and are anticipated to be promising nanomedicine strategy in the treatment of diseases like leishmaniasis.

4.2 MATERIALS AND METHODS
4.2.1 Materials
Dimyristoyl phosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids (AL, USA). 4-sulfated-N-acetylgalactosamine (4-SO₄GalNAc), stearyl amine (SA),
cholesterol, D-mannose, palmitoyl chloride, dialysis bag (cut off mol. wt. 12 KD) were purchased from Sigma-Aldrich (USA, St. Louis). Dimethylacetamide (DMA), pyridine, diethyl ether, and absolute ethanol were of spectroscopic grade and purchased from Merck (India). All other reagents and chemicals were of analytical grade. For cell culture, Roswell Park Memorial Institute-1640 media (RPMI-1640), fetal bovine serum (FBS) and antibiotic solution (penicillin/streptomycin, 0.1% v/v) were purchased from Sigma. Nile red dye and MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) were also from Sigma. Well plates for cytotoxicity and uptake studies were from Greiner Bio One (Frickenhausen, Germany). All materials were used without further purification. The water used in all experiments was prepared in a three-stage Millipore Milli-Q plus 185 purification system (Bedford, MA, US) and had a resistivity greater than 18.2 mW/cm.

4.2.2 Synthesis, characterization of O-palmitoyl mannose (PM)

O-palmitoylmannose (PM) was synthesized by esterification of mannose by the reaction of palmitoyl chloride in dimethylformamide (DMF) under anhydrous catalytic conditions by the process reported elsewhere in the literature (Hammerling and Westphal 1967) and adopted with minor modification in our laboratory. Briefly, 2.0 g mannose was dissolved in 100 mL dry DMF at 70°C. To the resulting solution, 2mL dry pyridine and 0.2 g palmitoyl chloride dissolved in 0.5 mL dry DMF were added. The mixture was stirred using a magnetic stirrer (Remi India Ltd, Mumbai, India) for 3 h at 60°C followed by 2 h at room temperature. This mixture was then slowly poured into 100 mL absolute ethanol with stirring. The precipitate so formed was collected and washed thrice with 120 mL absolute ethanol and 80 mL dry diethyl ether. The white solid material obtained was dried in vacuum at 50°C for 2h. The FTIR spectrum was recorded on FTIR multiscope spectrophotometer (Perkin Elmer, Buckinghamshire, UK) equipped with spectrum v3.02 software. Hydrophobised mannose was characterized by infra red (IR) spectroscopy to identify carbonyl groups, and thus, to ascertain that mannose is covalently bound to palmitoyl anchor. The IR spectrum of PM and mannose (1%), incorporated into a KBr disc, was run on a FT-IR single beam spectrometer (Figure 4.2).

4.2.3 Preparation and development of liposomal formulation

Ethanol injection method based on proliposome has been employed for preparation of liposomes due to its simplicity and reproducibility. This method was modified to enable development of AmB liposomes for in situ application. DMPC (250mg),
cholesterol (50 mg) and stearylamine (7.5) [molar ratio: 7:3:0.5] were dissolved in 1.5 ml of ethanol to prepare lipid ethanol solution (heating at 60-70°C for 5 minutes in well closed vial to reduce ethanol evaporation using water bath). Separately 50 mg of AmB was dissolved in 1.5 ml of acidified DMA (acidified with 0.1% HCl). Ethanolic lipid solution and AmB solution in acidified DMA were mixed together to form proliposome concentrate containing lipids and drug in ethanol and acidified DMA. This 3 ml of proliposome was rapidly injected into 50 ml of phosphate buffer saline (PBS; pH 7.4; where appropriate) with the help of 5 ml dispovan syringe (26G needle) to get the final AmB concentration of 1 mg/ml. Immediately after injecting, the characteristic opalescence of colloidal dispersions appeared in all preparations. This results in formation of small and uniform liposomes. For in vitro cell uptake study, 0.1 mol% of lipid soluble Nile red dye was added to the lipid mixture.

4.2.4 Coating of liposomes with PM and 4-SO₄GalNAc

For coating, 2 mL of uncoated liposomal formulation (Lip) was incubated with PM and 4-SO₄GalNAc solution in PBS, pH 7.4 and stirred gently at room temperature. After completion of coating, the excessive unbound PM/SO₄GalNAc was removed by passing the resultant suspension through a Sephadex G-50 column at 2000 rev min⁻¹ for 10 min. The process variables i.e. PC to 4-SO₄GalNAc weight ratio was optimized by measuring the change in zeta-potential of the dispersion in PBS at 25°C (Zetasizer 3000 HS; Malvern Instruments Co., UK).

4.2.5 Characterization of liposomes

Developed formulations were characterized prior to and after surface ligand anchoring. The different liposomal formulations were evaluated for their shape and vesicle type by transmission electron microscopy (TEM). Vesicle characterization for size and shape was performed using TEM. Phosphotungstic acid (1%) was used as a negative stain (JEM 1200, EX 11, JEOL, Japan). Carbon coated samples were placed over a copper grid and subjected to TEM analysis. Average particle size and polydispersity index (PDI) of plain and surface decorated liposomes were determined by Zetasizer (Malvern Instrument, Malvern, UK). Similarly the zeta potential was determined by laser Doppler anemometry using a Malvern Zetasizer. The instrument is a laser-based multiple angle particle electrophoresis analyzer. Both formulations were dispersed in PBS (pH 7.4) and the zeta potential was determined.

Percent drug entrapment was determined and expressed as the ratio of experimentally measured amount of drug in dispersion and initial amount used for
entrapment. Gel Exclusion Chromatography was employed to determine entrapment efficiency. The formulation was passed through Sephadex G-100 column to separate unentrapped drug. The vesicular formulations were lysed with 0.1% v/v Triton X-100 and AmB content was estimated by RP-HPLC method.

\[
\% \text{ Entrapment} = \frac{\text{Total amount of drug} - \text{Free drug}}{\text{Total amount of drug}} \times 100
\]

4.3 **IN-VITRO STUDIES**

4.3.1 **Macrophage uptake studies**

The adherent mouse macrophage cell line J774A.1 and macrophage murine cell line RAW 267.4 were used for the present study using fluorescence activated cell sorter (FACS) instrument (BD Biosciences, FACS Aria, Germany). Aliquots (100 μL) containing J774A.1 and RAW 267.4 cell lines (1 × 10^5) were suspended in 0.9 mL of fresh RPMI-1640 medium supplemented with penicillin 10 U/mL, 10% fetal bovine serum, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES medium (Duverger, Jackson et al. 2006). These were transferred into 24-well plates containing fresh medium and suspended in a 37°C humidified incubator with 5% CO₂ atmosphere. After 24 h the culture medium was replaced with fresh culture medium containing mannose and 4-SO₄GalNAc decorated liposomal formulations incorporating Nile red as fluorescent marker (0.1 mol%). For separation of the internalized and surface bound liposomes, the cells were washed three times with acetate buffer (pH 4.0). The cell-associated fluorescence was measured by FACS at an excitation wavelength of 485 nm and an emission wavelength of 628 nm (Seelagy, Schwartz et al. 1994; Nimje, Agarwal et al. 2009).

4.3.2 **Measurement of haemolysis**

Various targeted liposomal formulations (mannose and 4-SO₄GalNAc decorated liposomes) containing equivalent concentration of AmB (50 μg/ml) along with corresponding blank formulations having similar excipient composition were incubated with 1.0 ml of blood and processed as described above to determine (section 3.2.4.7) percentage haemolysis by haemoglobin released into the supernatant.

4.3.3 **Cytotoxicity studies**

Biocompatibility studies were carried out using MTT assay (Mosmann, 1983) on J774A.1 cell lines. MTT assay is a laboratory test and a standard colorimetric assay (an assay which measures changes in color) for measuring cellular proliferation (cell growth). It can also be used to determine cytotoxicity of potential
medicinal agents and other toxic materials. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced.

Figure 4.1: Conversion of MTT to Formzan crystal

The in-vitro cytotoxicity of blank formulations (composition of excipients similar to 1mg/ml AmB liposomes) was measured by the MTT proliferation assay (Seymour, Sheldon et al. 1994). The experiments were carried out on cells in the exponential growth phase. J774A.1 and RAW 267.4 macrophage cells were seeded into 24-well plates at 5 × 10⁴ cells/well and were allowed to adhere overnight. The growth medium was replaced with a fresh medium and then cells were incubated for 24 h with different formulations (Man-lip and Sulf-lip). Cells were then washed twice with 1 ml PBS. Cells were then incubated in a growth medium containing 1 mg/ml MTT for 4h at 37°C, and 500 µl DMSO was added to each well to ensure solubilization of the formazan crystals. The optical density was measured using a multiwell scanning spectrophotometer (MRX Microplate Reader, Dynatech Laboratories Inc., Chantilly, VA, US) at a wavelength of 570 nm.

4.4 IN VIVO STUDIES
4.4.1 Intracellular localization and organ biodistribution studies in wistar rats

The studies were carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Experiments were conducted after
ethical clearance by the Institutional Animal Ethics Committee of the institute (IAEC approval no. 116/08/Pharmaceutics/IAEC/130/09).

4.4.1.1 Intracellular localization with the help of fluorescent marker.

The intracellular localization of the developed formulations was studied by fluorescence microscopy. The Nile red loaded formulations (Lip, Man-Lip and Sulf-Lip) were administered to wistar rats (n=3, which is the number of animals taken in one experiment for each formulation) through intravenous route. After 30 min, the rats were sacrificed and livers and spleens were isolated, washed with physiological saline, fixed, embedded in wax and sections were made using microtome (LEICA RM 2155, Germany) and photographs were taken using fluorescent microscope (Leica, DMRBE, Bensheim, Germany).

4.4.1.2 Organ distribution studies

Wistar rats weighing about 150–200 g were divided in three groups of 3 rats each. Plain-Lip, Man-Lip and Sulf-Lip containing equivalent doses of AmB (1mg/kg body weight) were administered intravenously to different groups. Animals from each group were sacrificed at 0.5, 1, 2, 4, 6 and 24 h after administration of the formulations. Blood samples were collected by cardiac puncture. Liver and spleen of the dissected rats were excised, isolated, washed with distilled water and were blot dried using tissue papers (van Etten et al. 1995). Drug content in the blood and organs was determined by HPLC method (van Etten et al. 1993). The serum was harvested from collected blood samples, deproteinized and analyzed using the similar protocol as described.

4.4.2 Activity studies in infected Syrian golden hamsters

Laboratory bred male Syrian golden hamsters (Mesocricetus auratus, 45–50 g) were used for the experiments. All animals were housed in individual cages under constant temperature (22⁰ C) and humidity with a 12-h light/dark cycle, and had access to chow and water ad libitum throughout the study.

4.4.2.1 Organisms and Reagents

The WHO reference strain, L. donovani (M HO-M/IN/ 80/Dd8) promastigotes were cultured in RPMI-1640 medium (Sigma, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA), penicillin (100 U/ml) and streptomycin (100 µg/ml). Parasites were also maintained through in vivo serial passage (amastigote to amastigote) in hamsters (Dube et al. 1998, 1999).
4.4.2.1 Experiment

Laboratory bred male Syrian golden hamsters (Mesocricetus auratus, 45–50 g) from animal house facility of Central Drug Research Institute (CDRI) were used as the experimental host after approval from Institutional Animals Ethical Committee (IAEC) of CDRI. They were housed in plastic cages in climatically controlled rooms and fed with standard rodent food pellet (Lipton India Ltd, Bombay) and water ad libitum. The study was carried out with the guidelines of Council for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The parasites were isolated from spleen of infected hamster and injected to naïve hamsters as described earlier (Gupta, Dube et al. 2007). Infected hamsters having 37-40 amastigotes/100 macrophages nuclei were selected for the treatment with various formulations in the following manner (i) infected controls (no therapy was given), (ii) Sulf-Lip, (iii) Man-Lip, (iv) Plain-Lip (v) Blank Sulf-Lip (vi) Blank Man-Lip (without AmB), (v) Blank Plain-Lip (without AmB). Infected hamsters were treated with 5 mg/kg of AmB in all formulations as single dose therapy (three in each group). After eight days post treatment splenic biopsies were performed and parasite burden was determined by counting number of amastigotes (Guru, Agrawal et al. 1989).

4.5 STATISTICAL ANALYSIS

All results are given as means±SD (n = 3). Differences between formulations were compared using one-way analysis of variance (ANOVA) followed by the Turkey-Kramer multiple comparison test, using Graph pad Instat software (Graph Pad Software Inc. CA, USA). P < 0.05 denotes significance in all cases.

4.6 RESULTS

4.6.1 Formulation development and characterization

4.6.1.1 Characterization of O-palmitoyl mannose (PM)

Comparison of the infra red spectra of PM with mannose revealed the presence of extra peaks due to C-C band deformation (2859 cm\(^{-1}\)) and C-H deformations (1490 cm\(^{-1}\)) arising from alkyl group in the product. Strong peaks at 1666 cm\(^{-1}\) confirmed the carbonyl group (C=O) (Pavia 1979; Vyas, Katare et al. 2000). Lower frequency stretching vibrations of the O-H band (at about 3379 cm\(^{-1}\)) and enhanced intensity and higher bandwidth confirmed the presence of hydrogen bonding. All these
characteristic peaks appeared in the infra red spectra indicates the formation of an ester bond between mannose and O-palmitoyl anchor.

![IR spectra of palmitoyl mannose](image)

**Figure 4.2: IR spectra of palmitoyl mannose**

### 4.6.1.2 Preparation of liposomes

Modified ethanol injection method was used for the preparation of AmB liposomal formulations. Entrapment efficiency (%EE) in liposomes was found to be 97.8±2.1%. Liposomes decoration with targeting moiety has not significantly affected the %EE. The liposomes had an almost spherical shape and were well dispersed without any aggregation (Figure 4.3). Table 4.1 shows the particle size and size distributions of different formulations measured by DLS. The results indicate that the mean diameter of uncoated liposome was 114.4 ± 7.8 nm.
**Figure 4.3**: Visualization of (A) plain uncoated liposomes, (B) Man-Lip and (C) Sulf-Lip by Transmission Electron Microscopy (TEM). Outer dark layer continuous covering over liposomes in Mannose and 4-sulfated galactosamine decorated liposomes is clearly visible. Scale bar = 200nm. Magnification, ×15,000

**4.6.1.3 Liposomes coated with mannose and 4-SO_4GalNAc terminating ligands:**

After characterizing the uncoated liposomes (Lip), they were coated with PM and 4-SO_4GalNAc by incubation to form Man-Lip and Sulf-Lip respectively. PM to PC w/w ratio of 0.15:1 was used to successfully decorated liposomes with mannose, which was previously reported and optimized by Vyas et al. 2000 (Vyas, Katare et al. 2000). 4-SO_4GalNAc to PC w/w ratio was optimized by measuring the change in zeta potential of dispersions (Figure 4.4). Change of zeta potential was constant above 4-SO_4GalNAc to PC w/w ratio of 0.20:1, therefore this ratio was selected for liposome decoration. Table 4.1 shows the particle size and size distributions of Sulf-Lip and Man-Lip measured by DLS. The results indicate that the mean diameters of Sulf-Lip
...and Man-Lip were 139.4 ± 7.4 nm and 147.4 ± 8.6 nm both with narrow size distributions. Figure 4.3B and C shows the morphological characteristics of Man-Lip and Sulf-Lip by TEM study. Coating is signified by the continuous opaque layer of mannose (Figure 4.3B) and 4-SO₄GalNAc (Figure 4.3C) over plain liposomes.

![Graph](image.png)

**Figure 4.4:** Formulations with different ratios of lipid and 4-sulfated N-acetyl galactosamine (4-SO₄GalNAc) were prepared. Optimization of total lipid-to- 4-SO₄GalNAc weight ratio (zeta-potential values after time period of 24 h at 25°C). Values are expressed as mean ± s.d., n = 6.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Zeta potential ±S.D. (mV)</th>
<th>Size (nm) ±S.D.</th>
<th>Polydispersity Index (PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearyalmine containing liposome's (Lip)</td>
<td>(+)48.2 ± 3.7</td>
<td>114.4 ± 7.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Sulfated Liposomes (Sulf-Lip)</td>
<td>(+)12.2 ± 1.3</td>
<td>139.4 ± 7.4</td>
<td>0.12</td>
</tr>
<tr>
<td>Mannose decorated liposomes (Man-Lip)</td>
<td>(+)17.3 ± 2.6</td>
<td>147.4 ± 8.6</td>
<td>0.23</td>
</tr>
</tbody>
</table>

**Table 4.1:** Characterization of coated and uncoated liposomes.

### 4.6.2 In vitro studies

#### 4.6.2.1 In-vitro uptake studies

Figure 4.5[A] and [B] shows the macrophagic uptake of various liposomal formulations in J774A.1 and RAW 267.4 respectively by flow cytometry study.
Figure 4.5: Flow cytometric diagram for uptake studies in macrophagic J77.4 and RAW 267.4 cells exposed to Nile red-labelled liposomes. X-axis represents fluorescence inside the cells. [A] uptake studies in J774A.1: [B] uptake studies in RAW 267.4 cells. Figure shows fluorescence in (a) control cells, (b) Lip, (c) Man-Lip and (d) Sulf-Lip.

This study represents comparative uptake study between plain liposomes, Man-Lip and Sulf-lip. Figure 4.5[A] shows almost two times (>1.75) times enhanced uptake of Sulf-Lip over Man-Lip in J774A.1 cell lines. Similarly Sulf-Lip showed almost two and half times (>2.46) enhanced uptake in RAW 267.4 compared to Man-Lip.

4.6.2.2 Determination of haemolysis by targeted liposomes (Man-Lip, Sulf-Lip)

To evaluate the safety profile of the developed formulation, the haemolytic activity was measured with different liposomal formulations.
The plain liposomes showed haemolytic activity of 15.6 ± 2.5%, suggesting that stearylamine was relatively toxic to erythrocytes. Surface modification of liposomes with palmitoyl-mannose and 4-SO₄GalNAc had notable effect on haemolytic activity, which was 7.2 ± 3.1% and 6.2 ± 2.8% respectively. Moreover, because most of the surface-modified liposomes will be cleared from the circulation within 2 hours, observed haemolysis would be insignificant from a safety point of view. The safety of sulfated decorated after 2 h can be understood because similar sulfated sugar that terminate in SO₄-4GalNAc are present in sulfated hormones lutropin and thyroid stimulating hormone (Fiete and Baenziger 1997; Fiete, Beranek et al. 1997; Fiete, Beranek et al. 1998).

4.6.2.3 Cytotoxicity studies

The cytotoxicity of Sulf-Lip and Man-Lip was found in acceptable limit when tested against J774A.1 and RAW 267.4 macrophage cell lines by MTT assay. The cell viability remained >90% for all the liposomal formulations against J774A.1 and RAW 267.4 macrophage cell lines indicating safety of excipients used. The cell viability of Man-Lip was found to be 95 and 93% while Sulf-Lip showed 94 and 92% cell viability after 24 h of incubation in J774A.1 and RAW 267.4 macrophagic cell lines respectively.
4.6.3 *In vivo studies (Intracellular localization and organ biodistribution studies in Wistar rats)*

4.6.3.1 **Intracellular localization with the help of fluorescent marker**

Intracellular localization of formulations is revealed by intensity of fluorescence in the fluorescence photomicrographs of the liver and spleen (Figure 4.7 & 4.8). The fluorescence observed in the case of plain uncoated liposomes in both liver and spleen sections (Lip, Figure 4.7B, Figure 4.8B) was relatively lower than the fluorescence intensity of the coated formulation [Man-Lip (Figure 4C, 5C) and Sulf-Lip (Figure 4.7D, 4.8D)]. Between Sulf-Lip and Man-Lip, Sulf-Lip showed higher fluorescence intensity in both liver and spleen.

![Figure 4.7: Intracellular localization of fluorescent marker (Nile Red) in the spleen cells of Wistar rats after 30 minutes intravenous administration of plain and ligand appended liposomes. (A) Control spleen cells without any treatment. (B) Spleen cells after plain liposomes (Lip) treatment (C) Spleen cells after Man-Lip treatment and (D) Spleen cells after Sulf-Lip treatment.](image-url)
Figure 4.8: Intracellular localization of fluorescent marker (Nile Red) in the liver cells of Wistar rats after 30 minutes intravenous administration of plain and ligand appended liposomes. (A) Control liver cells without any treatment. (B) Liver cells after plain liposomes (Lip) treatment (C) Liver cells after Man-Lip treatment and (D) Liver cells after Sulf-Lip treatment.

4.6.3.2 Pharmacokinetics of AmB formulations in rats

The concentration of AmB in case of Lip ($C_{\text{max}}=22.38\ \mu g/ml$) was significantly higher (P<0.05) than Man-Lip ($C_{\text{max}}=16.62\ \mu g/ml$) and Sulf-Lip ($C_{\text{max}}=14.11\ \mu g/ml$) at all time points (Figure 4.9). Higher Volume of distribution ($V_{ss}=113.42\ \text{ml/kg}$) and lower clearance ($\text{CL}=11.21\ \text{ml/h/kg}$) was observed in Lip compared to Man-Lip ($V_{ss}=78.39\ \text{ml/kg}$, $\text{CL}=25.32\ \text{ml/h/kg}$), and Sulf-Lip ($V_{ss}=67.3\ \text{ml/kg}$, $\text{CL}=35.17\ \text{ml/h/kg}$). Man-Lip showed higher plasma conc., higher Vss and lower clearance compared to Sulf-Lip (P<0.05). The pharmacokinetic parameter of Lip, Man-Lip and Sulf-Lip has been given in Table 4.2.
Figure 4.9: Represents the effect of ligand anchoring liposomes on serum conc. of AmB. Total percent drug recovered from serum has been recorded as function of time.

Table 4.2: Pharmacokinetic parameters following intravenous administration of various formulations of AmB in rats.

<table>
<thead>
<tr>
<th>Formulations</th>
<th>$V_{ss}$ (ml/kg)</th>
<th>$t_{1/2}$ (h)</th>
<th>MRT (h)</th>
<th>$C_{max}$ (µg/ml)</th>
<th>Clearance (CL) ml/h/kg</th>
<th>AUC (µg/ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip</td>
<td>113.42±10.31</td>
<td>7.30±1.2</td>
<td>9.82±3.60</td>
<td>22.38±3.60</td>
<td>11.21±2.42</td>
<td>89.20±6.54</td>
</tr>
<tr>
<td>Man-Lip</td>
<td>78.39±4.61</td>
<td>2.84±0.2</td>
<td>4.03±0.3</td>
<td>16.82±0.82</td>
<td>25.32±4.21</td>
<td>39.49±2.98</td>
</tr>
<tr>
<td>Sulf-Lip</td>
<td>67.31±3.42</td>
<td>2.12±0.2</td>
<td>3.01±0.2</td>
<td>14.11±0.71</td>
<td>35.17±2.91</td>
<td>28.43±2.14</td>
</tr>
</tbody>
</table>

Sulf-Lip, sulfated galactosamine decorated liposomes; Man-Lip, Mannose coated liposomes; Lip, Uncoated liposomes; $C_{max}$, maximum conc. of drug in serum; $t_{1/2}$, half-life; MRT, mean residence time; $V_{ss}$, volume of distribution at steady state; CL, clearance of drug; AUC, area under the curve.

4.6.3.3 Liver and spleen distribution of different AmB formulation

AmB conc. in liver and spleen after i.v. injection of different AmB formulations are shown in Figure 4.10 and 4.11. Sulf-Lip and Man-Lip showed significantly higher localization of AmB at all time points compared to Lip (P<0.05). Similarly Sulf-Lip showed enhanced drug distribution compared to Man-Lip in liver and spleen.
Figure 4.10: Represents the effect of ligand anchoring liposomes on hepatic uptake of AmB. Total percent drug recovered from liver has been recorded as function of time. 4-sulfated galactosamine decorated liposomes (Sulf-Lip) significantly (P<0.05) enhanced uptake in comparison to Man-Lip.

Figure 4.11: Represents the effect of ligand anchoring liposomes on splenic uptake of AmB. Total percent drug recovered from spleen has been recorded as function of time. 4-sulfated galactosamine decorated liposomes (Sulf-Lip) significantly (P<0.05) enhanced uptake in comparison to Man-Lip.

4.6.3.4 In vivo antileishmanial activity testing

Highly significant activity (P<0.01 as compared to Plain Lip) was observed using formulation Sulf-Lip causing 83.8±2.9% inhibition of splenic parasitic burden. Whereas, formulation Man-Lip and Plain Lip caused only 75.5±3.1 and 67.2±3.4%
parasite inhibition respectively (P<0.05 for Sulf-Lip vs. Man-Lip and Man-Lip vs. Plain Lip) in leishmania infected hamsters. Blank Sulf-Lip as well as Blank Man-Lip formulation also showed 17.8 ± 2.9 and 14.2±3.1% parasite inhibitions respectively. Blank Sulf-Lip shows significantly higher (P<0.05) parasite inhibition in comparison to Plain-Lip (10.2±2.8%) (Figure 4.12).

**Figure 4.12:** Antileishmanial activity of AmB formulations against established infection of Leishmania donovani in hamsters. Drug formulations (equivalent to 5 mg/kg) and formulations without drug were injected intracardially into each hamster on day 31st post infection. Parasite burden was estimated by splenic biopsy on day 8 post treatment and percent parasite inhibition was calculated in comparison to the parasite burden of untreated animals (mean ± SD) (n = 3). *P<0.05 (*Depicts comparison of Sulf-Lip vs. Man-Lip and Plain-Lip vs. Man-Lip and Blank Sulf-Lip vs. Blank Plain-Lip), ** P<0.01 (**Depicts comparison of Sulf-Lip vs. Plain-Lip)

4.7 DISCUSSION

Rapid ethanol injection method is suitable for preparation of liposomes because of simplicity, reproducibility and to obtain the spontaneous formation of liposomes having a small average radius (Stano, Bufali et al. 2004; Wagner, Platzgummer et al. 2006; Shende and Gaud 2009). This method was suitably modified for preparation of AmB liposomes. Use of biocompatible acidified DMA for solubilization of AmB enables us to prepare AmB liposomes by modified ethanol injection method.

Liposomes initially had positive zeta-potential due to the presence of stearylamine. This positive zeta-potential decrease with adsorption of 4-SO₃GalNAc (anionic) and it approaches a minimum value at a 0.20:1 ligand-to-lipid weight ratio (Figure 4.4). The positive zeta-potential value is changed to a less positive value on
adsorption of 4-SO₄GalNAc, which indicates the masking of positive charge by 4-SO₄GalNAc. On further addition of 4-SO₄GalNAc (beyond a 0.20:1 ligand-to-lipid weight ratio) there is no change in zeta-potential. This is an indication of complete intrinsic charge quenching on 4-SO₄GalNAc coating of liposomes. No significant change in zeta-potential can be attributed to the fact that the lipid bilayer gets saturated with 4-SO₄GalNAc at this concentration. The increase in the average diameter of liposomes and continuous outer coating over liposomes as revealed by TEM might confirm the coating of ligand.

Significantly higher uptake of Sulf-Lip over Man-Lip in macrophagic cell lines (J774A.1 and RAW 267.4) generate interest that still there is scope of enhanced localization of drug in liver and spleen macrophages. Sulf-Lip was able to localize higher amount of fluorescence inside the cells compared to Man-Lip because of relatively better interaction with mannose receptors on macrophages. Pharmacokinetic parameters showed higher blood level and significantly lower clearance of uncoated liposomes compared to targeted liposomes which indicates rapid clearance of targeted liposomes from the plasma. Similarly significantly lower plasma conc. and higher clearance with Sulf-Lip indicates efficient macrophage targeting and quick removal of drug from the circulation into the liver and spleen compared to Man-Lip. Higher drug concentration of Sulf-Lip and Man-Lip compared to Lip in both liver and spleen authenticate that most of the drug is rapidly localized in liver and spleen due to enhanced uptake in macrophages (Figure 4.10 and 4.11).

Similarly MR binding of hormones is thought to regulate their bioavailability (Baenziger, Kumar et al. 1992) and facilitate rapid clearance of lutropin from the serum through binding to MR on hepatic endothelial cells (Smith, Bousfield et al. 1993). This tendency of higher uptake of targeted liposomes compared to plain uncoated liposomes was further reflected by enhanced fluorescence in both liver and spleen tissues (Figure 4.7 and 4.8). In comparison to Man-Lip and Sulf-Lip, Sulf-Lip showed significant enhanced fluorescence which further supports our hypothesis of enhanced uptake of sulfated galactosamine coated liposomes through mannose receptors.

Macrophagic uptake of 4-SO₄GalNAc decorated liposomes similar to mannose targeted liposomes opens new area of targeting drug delivery systems. The antileishmanial activity of the AmB (5mg/kg) was investigated in vivo against VL by the inhibition of parasitic load in the spleen of L. donovani infected hamsters after
intracardiac injections of Plain Lip, Man-Lip and Sulf-Lip. Highly significant activity (P<0.01 as compared to Plain Lip) was observed using formulation Sulf-Lip causing 83.8±2.9% inhibition of splenic parasitic burden. Whereas, formulation Man-Lip and Plain Lip caused only 75.5±3.1 and 67.2 ± 3.4% parasite inhibition respectively (P<0.05 for Sulf-Lip vs. Man-Lip and Man-Lip vs. Plain Lip) in leishmania infected hamsters.

The primary determinant of mannose specificity in CRD on mannose receptor is the presence of hydroxyl groups in positions equivalent to equatorial 3 and 4-OH groups of D-mannose. The key feature of the interaction is the direct ligation of the calcium ion by the 3 and 4-hydroxyl groups of a terminal mannose to form a coordinate calcium ion complex (East and Isacke 2002; East, Rushton et al. 2002). In functional CRD, interaction with sugars occurs in the hydrophobic fold which brings into contact the residues required for the coordination of Ca\(^{2+}\) ions and sugar residues. Examination of the MR CRD sequences reveals that all eight CRDs contain residues necessary for the formation of the hydrophobic fold but that only ‘CRD 4’ contain the residues required for Ca\(^{2+}\) dependent sugar ligation (Taylor, Bezouska et al. 1992; Taylor and Drickamer 1993). In MR ‘CRD 4’ the side chains of the two asparagine residues each donate a lone pair of electrons from their NH groups to form hydrogen bonds to the 3- and 4-OH groups of the sugar. The carbonyl oxygen forms a coordination bond with the Ca\(^{2+}\) ion that is also ligated to the sugar. (Weis, Drickamer et al. 1992; Dinges, Kribbs et al. 1994; Weis and Drickamer 1996).
Figure 4.14: Schematic diagram presents mannose moiety attached with C-type carbohydrate recognition domain (CRD), while 4-sulfated galactosamine targets cysteine rich domain on mannose receptor. 3 and 4- hydroxyl group on mannose form hydrogen bond with C-type CRD while sulfate moiety on 4-sulfated galactosamine form hydrogen bond with cysteine rich domain.

Schematic diagram (Figure 4.14) reveals the various binding sites; principal site other than CRDs is Cys-MR site for targeting macrophages. The other domain i.e. cysteine-rich region in the MR is able to bind to glycoproteins terminating in 4-sulfated N-acetylgalactosamine such as those found on the pituitary hormones lutropin (luteinizing hormone) and thyrotropin (thyroid stimulating hormone) (Fiete, Beranek et al. 1997; Fiete, Beranek et al. 1998). In the MR cysteine-rich domain, the sulfated 4-SO4-GalNAc ligand binds in a pocket formed by the loop among various strands. The sulfate group points into the pocket and interacts with the cysteine-rich domain via eight hydrogen bonds. Six of the eight hydrogen bonds occur through the free oxygens of the sulfate group and the protein, the first free oxygen accepts two hydrogen bonds, one from the side group of Asn99 and the other from the Asn102 side chain. The second free oxygen accepts hydrogen bonds from the NH groups of Ser114 and Gly115, while the third free oxygen accepts hydrogen bonds from the NH groups of Leu116 and Trp117. Two hydrogen bonds also occur between the 3- and 6-OH groups of the galactose ring and Asn102 and Leu116. Finally, a stacking interaction occurs between the non-polar face of the galactose ring and Trp117 which allows numerous vander waal’s contacts to form with Asn102, Leu116 and Trp117.
The specificity of Cys-MR for sulfated carbohydrates is explained at the structural level by the multitude of interactions between the protein and the sulfate group of 4-SO4-GalNAc.

1. The interactions are mediated primarily through hydrogen bonds rather than salt bridges or counter ions;
2. Most of the hydrogen bonds involve peptide NH groups, and
3. The peptide NH groups associated with the sulfate group are coupled to a hydrogen bond array within the protein.

These were very encouraging results as till now only mannose as targeting moiety has been widely explored for enhanced drug delivery in macrophages. Various researchers have already proved the enhanced macrophagic uptake of mannose decorated liposomes (Datta, Mukherjee et al. 2003; Medda, Jaisankar et al. 2003; Mitra, Mandal et al. 2005; Nahar and Jain 2009). At the best of our knowledge, there are no reports of using 4-SO4-GalNAc as targeting moiety in novel drug delivery systems. As described above, it can be easily coated over liposomal surface due to strong electrostatic attraction. Schematic diagram (Figure 4.14) shows the different binding sites for mannose and 4-SO4-GalNAc moieties on mannose receptor present preferably on macrophages. Surprisingly enhanced uptake can be attributed to strong hydrogen bonding with cysteine group present on macrophage. It forms eight hydrogen bond (six bond with sulfate group and two with N-acetyl galactosamine) in comparison to four hydrogen bond of mannose with C-type lectins (Table 4.3).

<table>
<thead>
<tr>
<th>Bond type</th>
<th>Ligand atom</th>
<th>Protein group on receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>H bond</td>
<td>OS1</td>
<td>Asn102</td>
</tr>
<tr>
<td>H bond</td>
<td>OS1</td>
<td>Asn99</td>
</tr>
<tr>
<td>H bond</td>
<td>OS2</td>
<td>Ser114</td>
</tr>
<tr>
<td>H bond</td>
<td>OS2</td>
<td>Gly115</td>
</tr>
<tr>
<td>H bond</td>
<td>OS3</td>
<td>Leu116</td>
</tr>
</tbody>
</table>

Table 4.3: 4-SO4-GalNAc and mannose interactions with Cys-MR domain and carbohydrate recognition domain (CRD) respectively.
### Table of H-bonds

<table>
<thead>
<tr>
<th>H bond</th>
<th>OS3</th>
<th>Trp117</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between GalNAC and Cys-MR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H bond</td>
<td>4-OH</td>
<td>Asn102</td>
</tr>
<tr>
<td>H bond</td>
<td>4-OH</td>
<td>Wat-Tyr111</td>
</tr>
<tr>
<td>Between mannose and C-type CRD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H bond</td>
<td>3-OH</td>
<td>Glu185</td>
</tr>
<tr>
<td>H bond</td>
<td>4-OH</td>
<td>Glu193</td>
</tr>
<tr>
<td>H bond</td>
<td>3-OH</td>
<td>Asn187</td>
</tr>
<tr>
<td>H bond</td>
<td>4-OH</td>
<td>Asn205</td>
</tr>
</tbody>
</table>

Also for proper targeting on mannose receptors, there is essential need of calcium ions along with mannose on liposomal or any drug delivery surface. While in case of targeting mannose receptor through Cys-MR, only 4-SO₄GalNAc moiety is required without the need of calcium ion. Alongwith strong anionic charge due to sulfate group suggests strong electrostatic attraction with Cys-MR. The mannose receptor cysteine-rich domain can also mediate association with transmembrane glycoproteins bearing sulfated oligosaccharides, including sialoadhesin (Martinez-Pomares, Crocker et al. 1999).

The extent of drug uptake from liposomal formulations is an important parameter for estimating the dosage of the formulation. It is highly desirable that drug uptake from liposomal formulations be maximal so that a smaller dosage of the formulation would suffice to achieve an optimal therapeutic effect. Moreover, small doses are convenient to the patient in terms of parenteral administration and reduce side effects. Therefore future plan is related to test the activity of liposome encapsulated drugs targeted with the help of 4-SO₄GalNAc against intacellular protozoan parasitic diseases.

To evaluate the safety profile of formulations on cells, a cell viability study was carried in J774A.1 and RAW 267.4 macrophage cells. Negligible cytotoxicity of both Man-Lip and Sulf-Lip in J774A.1 and RAW 267.4 macrophagic cells confirms those formulations components are safe to use. These data indicates that novel 4-SO₄GalNAc moiety attached on liposomes does not increase any cytotoxic effect and is biocompatible similar to mannose coated liposomes.

Similarly, to evaluate the safety profile of the developed formulation, the haemolytic activity was measured with different liposomal formulations. Relatively less haemolysis (<7%) with surface decorated liposome after 2 hour can be understood because similar sulfated sugar that terminate in SO₄-4GalNAc are present.
in sulfated hormones lutropin and thyroid stimulating hormone (Fiete and Baenziger 1997; Fiete, Beranek et al. 1997; Fiete, Beranek et al. 1998). We have also shown that most of the surface decorated liposomes cleared from the circulation within 2 h therefore, observed haemolysis would be insignificant from safety point of view.

**Conclusion**

4-\(\text{SO}_4\)GalNAc can be used as targeted moiety for significantly enhanced drug delivery of AmB compared to mannose decorated liposomes in macrophages for efficient treatment against leishmaniasis. 4-\(\text{SO}_4\)GalNAc binds to extracellular domain i.e. Cys-MR through eight hydrogen bond and cause enhanced internalization in cells compared to mannose targeting. Enhanced uptake directly reduces dosage of formulation which is highly desirable for optimal therapeutic effects with reduced side effects for AmB.