Tuberculosis (TB), a ubiquitous and highly contagious chronic granulomatous bacterial infection, is a leading killer of young adults worldwide. Nanotechnology-related rational drug delivery has improved therapeutic success by minimizing adverse drug effects and frequency of administration, ultimately resulting in patient compliance. Further, researchers can improve the effectiveness of approved drugs and extend their applicability by providing means to overcome technical limitations such as low bioavailability, resistance, cellular and anatomical barriers, etc.

Most of the anti-tubercular drugs presently in use, fail to penetrate macrophages within which bacilli reside and survive. This necessitates pursuing the research with an aim to develop delivery systems and their modified versions in order to optimize therapeutic effects. The project was aimed at delivery of the drug(s) by utilizing target oriented nanocarriers directly to the specific cells (macrophages) simultaneously controlling the release of the therapeutics. Further, the encapsulation of antibiotics modifies their pharmacokinetics by increasing the serum half-life and area under the concentration–time curve and decreasing the apparent volume of distribution so allowing an increase in the maximum tolerated dose.

The encapsulated formulations, therefore show an improved therapeutic index as compared with free antibiotic. Another major advantage of antibiotic delivery by carriers is reduced toxicity including hepatotoxicity and other side effects of antitubercular drugs. By utilizing ligands, i.e., tuftsin which potentiates the natural killer activity of the macrophages and other phagocytosing cells and has been shown to enhance the nonspecific resistance of the host against intracellular infections, thus the drug therapy has been improved. This will help in clearing the bacterial load form the macrophages, thus rendering the therapy clinically effective.
PREFORMULATION

The drug identification studies were conducted which confirmed that drug(s) matches with the standard described for identity and purity as given in IP, 1996. The UV-visible absorption maxima of RIF was 479.0 nm while that of INH was found to be 262.0 nm matching with the values reported in IP, 1996. The peaks in IR spectrum of RIF and INH were in accordance with that of those reported in literature. Solubility study of the drug(s) was conducted in different solvents. RIF was found to be freely soluble in ethanol, methanol and acetone, chloroform, ethylacetate and acetone and slightly soluble in water and phosphate buffer saline (PBS) pH 7.4. INH was soluble in aqueous solvents. Spectrophotometric method was selected for the estimation of drug(s). For both the drug(s) absorbance data obeyed Beer-Lambarts’ law in the concentration range of 2-20 µg/mL. The correlation coefficient of more than 0.99 for both the drug(s) confirmed an excellent linearity between concentration and absorbance. The calibration curve of both RIF and INH was linear between 100-1000 ng/mL ($r^2 \geq 0.99$). It can be concluded that the drug(s) samples of RIF and INH were authentic and results of the study complied with the standards given in official monographs. Through UV/IR analysis the chemical identity of the supplied drugs was confirmed. The data generated regarding the solubility, spectroscopy and standard curves preparation were reproducible and may be used reliably for formulation studies in further work.

PREPARATION AND CHARACTERIZATION

Synthesis of mannosylated PLGA

The exclusive presence of mannose receptors on macrophages has been exploited for developing an efficient macrophage-directed drug carrier. Intracellular delivery of drugs to macrophages by receptor mediated endocytosis using mannose and scavenger receptors for various macrophage-specific diseases has been extensively reviewed. Mn-
PLGA was synthesized and characterized by FTIR. The FTIR results confirmed the synthesis of these novel conjugates.

**Synthesis of tuftsin conjugated PLGA**

Tuftsin is a natural macrophage activator tetrapeptide (Thr-Lys-Pro-Arg), which is a part of the FC portion of the heavy chain of the leukophilic immunoglobulin G (residues 289–292). The tetrapeptide is released physiologically as a free peptide fragment after the enzymatic cleavage. The peptide is known to bind specifically to macrophages, monocytes and polymorphonuclear leukocytes and possesses a broad spectrum of activities related primarily to functional activation of the immune system including potentiation of various cell functions, such as phagocytosis, pinocytosis, motility, immunogenic response, bactericidal and tumoricidal activities.

Structure and consequent activation of mononuclear phagocytic cells is dependent upon rather strict conservation of its molecular structure. Modification of the peptide at its N-terminus, leads to a significant reduction or even loss of biological activity and also an ability to bind to polymorphonuclear leukocytes. However, it has been shown that extending the peptide at its C terminus does not alter or affect the biological activity. In the present study, the C-terminus of tuftsin was conjugated to the terminal amino groups of activated PLGA. Protection of amino groups of tuftsin with Fmoc was carried out as per standard procedure for protection of primary amino groups. The formation of Fmoc-Tu was confirmed by the fact that the final product (Fmoc-Tu) is formed as a precipitate insoluble in water and also by IR spectroscopy. The synthesis was confirmed by FTIR.

**Preparation and optimization of Nanoparticles**

PLGA has been approved by FDA for several therapeutic applications and exhibits biodegradability, biocompatibility and safety in humans. There are a number of techniques available for encapsulation of drugs in nanoparticles (NPs) such as the emulsion solvent evaporation/extraction method, spray drying, phase separation-
coacervation, interfacial deposition, precipitation method, in situ polymerization, etc. Each method has its own advantages and disadvantages. The choice of a particular technique depends on the characteristics of the polymer and physicochemical and pharmacokinetic parameters of the drug(s), molecular biology of the site of the drug action, and the duration of the therapy. NPs were prepared by double emulsion solvent evaporation method because the NPs prepared by this method have been reported to be spherical and exhibit small particle size and low PDI.

The size of the NPs can be affected by selection and quantitative variation of the polymer concentration, temperature, viscosity, the stirring rate, and the amount of emulsifier employed. Considering this fact, the prepared NPs were optimized in respect to polymer concentration, surfactant concentration and sonication time while keeping the stirring speed constant. The purpose of this work is largely focused on exploring the targeting potential of surface modified NPs for selective targeting to the macrophages and further, the role these modified nanosystems might play on the activation status of the macrophages as compared to the optimization of critical attributes. So, keeping this in mind we have optimized the necessary critical variables so that the in process reproducibility can be achieved.

NPs were prepared with various quantities of PLGA and effect of polymer amount on size, PDI and % entrapment efficiency were recorded. The formulations were prepared by taking different PLGA concentrations (0.5-4 %) at constant concentration of PVA (2% w/v).

It was observed that an increase in size of NPs was resulted on increasing the amount of PLGA. It might be attributed to increased viscosity of polymer solution. Increase in polymer concentration results in an increased viscous forces resisting droplets breakdown on sonication. Viscous forces tend to oppose the shear stress in the organic phase and final size and size distribution of particles thus depends on the net shear stress
available for droplet breakdown. The results of PDI in case of formulations of both RIF and INH suggested that as the amount of PLGA deviated from 2% w/v a sharp increase in PDI was recorded. Such sharp change could be due to availability of an excess amount of surfactant for early and immediate droplets stabilization, resulting in to reduction of NPs. On varying polymer amount to higher or lower levels, the surfactant and surface phenomenon predominates over viscosity and hence availability of surfactant affects the size of NPs.

With increase in amount of PLGA the % entrapment efficiency also tended to increase. The higher concentration levels of PLGA in effect increase the viscosity of organic phase which as a result increases the diffusional resistance to drug(s) molecules from organic to aqueous phase, thereby retaining and entrapping more amount of drug(s) in NPs. The formulations PLGAR3 and PLGAI3, where 2% PLGA concentration was used were optimum in terms of homogenous size distribution and were selected and used in further studies.

To optimize the concentration of PVA; the formulations were prepared by the keeping concentration of PLGA constant (2% w/v) while PVA concentration was varied (0.5% to 2% w/v). It has been known that higher surfactant concentration generally increases the stability of the primary emulsion, resulting in a higher loading efficiency. Moreover, addition of a surfactant to the aqueous phase further improves the stability of the resultant emulsion, as it limits the exchange between the phases. In fact, during solvent evaporation the thin layer of surfactant around the droplets prevents their coalescence. PVA is frequently used as a colloidal polymeric stabilizer that surrounds particles forming a hydrophilic polymer layer. Therefore, the increase of PVA concentration has been associated with a decrease in particles size and polydispersity (Jeffery et al., 1993). The size of NPs were decreased with an increase in PVA concentration but an excess PVA was difficult to remove by washing with water because
of its association with the particles through interconnected networks, hence low concentration of PVA was selected that shows optimum size of NPs (Gupta et al., 2007). On the basis of results, formulation PLGPR2 and PLGPI2 prepared by using 1% w/v PVA and 2% w/v PLGA were selected for further studies and characterization.

Sonication time is very important to be optimized for nanoparticulate formulations since it substantially affects the mean diameter of the particles. Sonication time was optimized by studying its effect on average particle size and % entrapment efficiency. For each formulation the time of sonication was varied from 2 to 5 min during primary emulsion formation. It was observed that upon increasing the sonication time from 2 min to 5 min the average size tended to decrease drastically. This could be due to longer sonication time, which is associated with a release of high energy leading to rapid and effective dispersion of the polymeric organic phase in the form of nanodroplets with monomodal distribution profile.

**Morphology, Particle Size, Polydispersity Index, Zeta potential**

External morphology and the shape of formulations were characterized by SEM. Scanning electron micrographs show the formation of spherical and reasonably monodisperse NPs, all exhibiting a smooth surface morphology.

The particle size, PDI and zeta potential were measured for optimized formulations. The particle size of plain NPs recorded was 464±10.0 nm, of RIF loaded NPs was 470±12.2 nm and INH loaded NPs was 433±12.3 nm, respectively; whereas PDI was 0.16±0.015 for plain NPs, 0.18±0.014 for RIF loaded NPs and 0.13±0.022 for INH loaded NPs. The particle size of mannose conjugated NPs was 485±11.5 nm of blank, 498±13.5 nm for RIF loaded and 483±18.9 nm for INH loaded mannosylated NPs; whereas PDI was measured to be 0.19±0.015 for blank, 0.22±0.018 for RIF loaded and 0.19±0.014 for INH loaded mannosylated NPs.
The particle size of tuftsin conjugated NPs measured was 492±12.4 nm for blank, 505±12.0 nm for RIF loaded and 495±20.2 nm for INH loaded tuftsin conjugated NPs; whereas PDI was 0.21±0.021 for blank, 0.28±0.017 for RIF loaded and 0.24±0.015 for INH loaded tuftsin conjugated NPs. The average particle size of ligand conjugated formulations was higher as compared to plain counterparts. This could be due to an additional bulky molecules layer on the surface of NPs.

The zeta potential of a particle is an overall charge that the particle acquires in a particular medium. The magnitude of the measured zeta potential is an indication of the repulsive force that operates and defines the long-term stability of the product. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there is no tendency for the particles to come together. However, if the particles have low zeta potential values then there is no force to prevent the particles coming together flocculate.

The zeta potential of blank, RIF loaded and INH loaded plain NPs was -19.2±2.7, -17.7±2.1 and -16.3±1.4; and of mannose conjugated blank, RIF loaded and INH loaded NPs it was measured to be -16.3±2.3, -15.3±1.5 and -14.6±1.2. The zeta potential of tuftsin conjugated blank, RIF loaded and INH loaded NPs was -9.4±1.2, -8.0±1.3 and -7.5±1.1. The zeta potential of NPs was negative due to the presence of terminal carboxylic groups in the polymer. The reason behind slight reduction in value of zeta potential in case of mannosylated formulations when compared to plain counterparts may be due to the shielding effect imparted by the mannose present on the surface of NPs. The reduction in zeta potential after mannosylation was owing to masking of free carboxylic groups responsible for negative zeta potential as some of these were utilized for conjugation with mannose. In case of tuftsin conjugated formulations the zeta potential was significantly reduced as compared to plain formulations. The zeta potential was recorded towards more positive side. The presence of amine group in tuftsin
conjugated on the surface of the NPs could be the possible reason behind this significant change in zeta potential.

**MMAD and GSD**

The optimum size-range of particles for inhalation is usually considered to lie within 1–5µm, but the key metric of suitability for lung delivery is a particle’s MMAD. Generally, particles below ~0.5µm are exhaled undeposited as they do not have enough inertia to go through impaction for sedimentation in the lung, while particles larger than ~5 µm get entangled in the oropharyngeal and upper airway regions of the respiratory tree. Particles in the respirable size range deposit in the deep lung and are readily taken up by alveolar macrophages. The MMAD of the developed formulations was found to be in the range of 1.85 to 2.59.

Aerodynamic diameter is a crucial parameter for controlling inertial impaction. A geometrically large particle with a small aerodynamic diameter essentially means that the particle moves as if it were much smaller particle of unit density. A smaller particle carried with it a smaller amount of inertia and so this eventually translates to a lower susceptibility to inertial impaction.

**Percent Entrapment Efficiency**

Encapsulation efficiency is an important index in the evaluation of drug(s)-loaded NPs as it is more economical when high encapsulation efficiency can be obtained. An O/W emulsion technique is mostly used for the encapsulation of drugs. All formulations showed good encapsulation efficiency, ranging from 39% to 67%. The entrapment efficiency of RIF loaded plain NPs was 66.7±3.5 and INH loaded plain NPs was 47.9±2.7. The entrapment efficiency of mannose conjugated RIF loaded NPs was 60.3±4.5 and INH loaded NPs was 40.7±4.3. The entrapment efficiency of tuftsin conjugated RIF loaded and INH loaded NPs was found to be 58.2±6.3 and 39.0±3.8. The results confirmed the better encapsulation efficiency of RIF as compared to INH in both
plain and ligand conjugated NPs. The possible reason may be the lipophilicity of RIF as compared to the hydrophilic nature of INH. Being hydrophobic in nature polymeric matrix provided a safe home to lipophilic RIF. Further the encapsulation efficiency of the ligand conjugated formulations, in case of both the drug(s), was found be less in comparison to that of plain nanoparticulate formulations; this may be due to the increase in heterogeneity of the polymeric matrix because of ligand conjugation.

**In vitro ligand agglutination**

The development of novel multivalent carbohydrate ligands for the modulation of biological processes continues to be an attractive target for research. Con A, a lectin from Jack Bean (*Canavalia ensiformis*) well investigated lectin, is a tetrameric protein with four binding sites that binds specifically with saccharides, such as mannose, fructose and glucose residues.

Turbidity of mannosylated formulations increased on addition of Con A in various concentrations to mannosylated formulations. The rate of agglutination depended on lectin concentration. The extent of aggregation increased as Con A concentration was increased from 25 µg/L to 200 µg/L and incubation time from 5 min to 60 min. Beyond 200 µg/L lectin concentration, aggregation decreased indicating saturation of the binding sites. Similarly, on increasing the incubation time beyond 60 min, no further change in turbidity was recorded. This suggests the presence of mannose on the surface of the nanoparticulate formulations. Further, the results clearly indicate that mannose in nanoparticulate formulations is capable of interacting with the lectin receptors followed by its subsequent internalization in the cells.

**Surface Analysis**

To further prove the surface modification, the surface elemental composition of plain and ligand conjugated PLGA NPs was determined by using X-ray photoelectron spectroscopy (XPS). Typical XPS survey scans were obtained. The peaks at binding energies of 280-
305, 396-416, and 528-548 eV were ascribed to the elements of carbon, oxygen, and nitrogen, respectively. The chemical composition percents of carbon, oxygen, and nitrogen on the surface of plain PLGA NPs were 75.0%, 25.0%, and 0%, respectively; those on the surface of mannosylated PLGA NPs they were 56.08%, 36.32%, and 8.60%, respectively and those on the surface of tuftsin conjugated PLGA NPs were 59.62%, 16.42%, and 23.95%, respectively.

There was a significant change in chemical composition after the conjugation of tuftsin. The nitrogen atom was not detected on the surface of plain PLGA NPs, however it was detected on the surface of tuftsin conjugated PLGA NPs. This nitrogen content is due to the free amine groups of the tuftsin. The detection of 8.60% nitrogen in the case of mannose conjugated PLGA NPs is surprising but this might be due to deeper penetration (6 nm) of X-ray into the PLGA bulk phase. When attaching mannose on the surface of PLGA, ethylene diamine is used as a linker. The deeper penetration of X-ray may have caused the detection of these nitrogen atoms of the linker.

**In vitro drug release**

*In vitro* drug release data reveal fundamental information on the structure (e.g., porosity) and behavior of a formulation at the molecular level, as well as possible interactions between drug and polymer, and their influence on the rate and mechanism of drug release and model release data.

Release studies were carried out by using two different release medium, phosphate buffer at pH 7.4 and at pH 5.0. pH 5.0 buffer was selected in order to have the same pH values present within phagosome and lysosome and to evaluate the effect of pH on RIF and INH release from PLGA NPs. *In vitro* release profile of RIF and INH loaded plain and ligand conjugated NPs are presented in Fig. 4.22 and Fig. 4.23 at pH 7.4 and Fig. 4.24 and Fig. 4.25 at pH 5.0, respectively.
The release profiles of the drug(s) were different in the respective release media. Normally, the release medium pH is able to affect the drug release pattern from PLGA based NPs. The solubility of the drug(s) and the characteristic property of the polymer play a central role in determining the drug(s) release in the different release mediums.

The release of RIF was found to be pH dependent during the 120 hours experiments. At pH 7.4, RIF loaded plain, mannose conjugated and tuftsin conjugated formulations showed percent cumulative release of 83.42±6.48%, 75.74±4.76% and 72.64±5.82% at the end of 120 h. However at pH 5.0 the percent cumulative RIF release was 65.51±5.61% for RIF loaded plain NPs, 62.39±5.73% for mannose conjugated NPs and 61.46±4.92% for tuftsin conjugated NPs, at the end of 120 h. The swelling of polymeric matrix takes place at higher rate at acidic pH. Also, under the acidic conditions, the degradation rate of PLGA is known to increase more than that in the solution of neutral pH. So, it is expected that the release of encapsulated drug might take place at faster rate in acidic media. But this was not found in case of RIF as the solubility of RIF is low at acidic pH. Further the swelling and erosion of PLGA an acidic microenvironment is created at the vicinity which further decrease the solubility and hence release of RIF from matrix. So the release rate of RIF will be slower. Makino et al., 2004 has further suggested that the although due to the swelling of the matrix the diffusion of RIF will be faster from PLGA matrix but the diffused RIF will be precipitated as solid particles and dissolute at slower rate.

Due to the aqueous solubility, the % cumulative release of INH from PLGA NPs is higher as compared to RIF. At pH 7.4, INH loaded plain, mannose conjugated and tuftsin conjugated formulations the percent cumulative release recorded was 96.4±5.1%, 92.2±4.9% and 89.3±5.2% respectively at the end of 120 h, which was found to be relatively higher as compared to RIF formulations. The % cumulative release of INH was 84.7±5.0% for RIF loaded plain NPs, 80.3±5.3% for mannose conjugated NPs and 78.4±5.2% for tuftsin conjugated formulations NPs at the end of 120 h. At acidic pH the
swelling of PLGA matrix takes place which could enhance the drug(s) mobility and diffusion. It can be concluded that the developed nanoparticulate formulations were able to release the drug in sustained manner in the phagolysosomal bio milieu.

The release pattern of RIF and INH from the nanoparticulate formulation followed a biphasic pattern, characterized by an initial burst, followed by a slower sustained release. The initial burst release may be due to the release of the drug(s) present at or just beneath the surface of NPs. Thereafter the release occurred due to the polymer erosion and diffusion of the drug(s) molecules. The diffusion of drug(s) is through small pores and channels in the polymer matrix. O’Hara and Hickey (2000) studied the release profiles of RIF from PLGA microspheres prepared by the spray-drying method and reported that the release pattern displayed two phases of drug release. They described that the initial rapid release was attributed to release from superficial areas of microspheres and could involve both dissolution and diffusion, and that the second phase could be contributed to diffusion of drug through small pores or channels in the polymer matrix.

The main reason that can explain this behaviour is correlated to the transition temperature (Tg) of the polymer. In fact it is well know that PLGA polymer Tg is commonly above the physiological temperature of 37°C, which gives it enough mechanical strength to be fabricated into delivery devices. Tg increases with increase of lactic acid content in copolymer, because the extra methyl group on the lactic acid moiety increases the rigidity of the polymer chain because of the steric hindrance. Moreover, as polymer molecular weight increases reduced polymer chain mobility is obtained. In fact, increase in the polymer chain length enhances the intra- and interpolymer chain interactions such as chain entanglement and packing, which decrease the polymer chain mobility and consequently increase Tg. On the other hand, it has been found that the release of RIF or other drugs from PLGA particles is influenced by PLGA monomer composition (lactid acid/glycolic acid).
STABILITY STUDIES

The stability studies were undertaken in order to contemplate and predict the integrity and stability of the formulations after intratracheal administration as well as to validate their optimal storage conditions. Long term storage stability of drug delivery systems is one of the foremost requirements in the development of clinically acceptable commercial formulation.

The *in vitro* stability in SLF of the selected formulations was evaluated by incubating each formulation with SLF for a period of 24 h and the quantity of drug leached out from the NPs was analyzed at different time intervals. The study revealed that after 24 h of incubation 18.6±1.1%, 14.9±1.3% and 13.3±1.7% of RIF was leached out from formulations R-NPs, R-Mn-NPs and R-Tu-NPs, respectively. INH leached out from I-NPs was 20.8±2.0%, I-Mn-NPs was 16.7±1.5% and I-Tu-NPs was 17.3±1.8%. The results suggest that the conjugation of the ligand significantly reduced the leaching of drug(s) from the formulations may be due to the presence of a layer of bulky group on the surface of NPs which could have inhibited the leakage of drug and offering protection against drug leakage. The protective effect of bulky group anchored on the surface is well documented in literature.

To study the effect of storage temperature and duration on particle size and drug content, NPs were stored at refrigerated (5±3°C) and at room temperature (25±2°C) for 6 months. The formulations were then evaluated periodically for particles size and percent residual drug content.

No significant change in particle size was observed for RIF and INH containing NPs after 180 days of storage at 5±3°C whereas a marginal increase in particle size was observed at room temperature. It is evident that increase in average particle size was more pronounced when stored at room temperature in comparison to refrigerated conditions. This may be attributed to the fusion and aggregation of NPs at higher temperature.
The MMAD determined was 2.21 ±0.07, 1.89 ±0.05 and 1.73 ±0.05 for R-NPs, R-Mn-NPs and R-Tu-NPs, respectively and 2.42 ± 0.04, 2.01 ±0.07 and 1.91 ±0.06 for I-NPs, I-Mn-NPs and I-Tu-NPs, respectively at refrigerated conditions. While the MMAD of these formulations at room temperature was found to be 2.11 ±0.04, 1.62 ±0.05 and 1.75 ±0.06 for R-NPs, R-Mn-NPs and R-Tu-NPs, respectively and 2.32 ± 0.05, 1.98 ±0.06 and 1.89 ±0.07 for I-NPs, I-Mn-NPs and I-Tu-NPs, respectively. It is clearly evident from the results that there is no significant change in MMAD when the formulations were stored at refrigerated conditions. The MMAD was slightly changed when the formulations were stored at room temperature however it was still within the limit of respirable particles which can be taken up efficiently by the alveolar macrophages.

The elicitation of the therapeutic effect of the formulation depends on the amount of drug that has been incorporated in the formulation; hence the residual drug content was monitored and compared with that obtained before stability testing period. The percent residual drug content of the NPs was determined periodically after storing the formulations at refrigerated and room temperature. The data clearly suggested that refrigerated conditions are suitable for the storage of nanoparticulate formulations as after three months the residual drug content estimated was 95.8±1.8%, 96.5±2.1% and 95.8±2.2% for RIF and 94.3±2.4%, 95.2±2.1% and 94.7±2.5% for INH containing plain, mannosylated and tuftsin conjugated NPs. Whereas the residual drug content at room temperature was estimated to be 82.5±2.3%, 83.9±1.3% and 83.4±1.4% for RIF and 81.2±1.1%, 82.6±1.8% and 82.5±1.6% for INH containing plain, mannosylated and tuftsin conjugated NPs.

Conclusively, the formulations should be stored at low temperature in the refrigerator for better stability. Physical instability in systems was recorded when systems were stored at room temperature.
**EX VIVO STUDIES**

**Cytotoxicity studies**

MTT assay is a simple non-radioactive colorimetric assay used to measure cell cytotoxicity, cell proliferation or viability. The colourless MTT is cleaved to formazan (highly coloured end product) by the succinate-tetrazolium reductase system which belongs to the mitochondrial respiratory chain and is active only in viable cells. Almost no cytotoxicity was observed in case of NPs, Mn-NPs and Tu-NPs at concentrations as high as 1.0 mg/mL. The percent of cell viability was 65.44±3.71%, 87.66±4.38%, 86.76±4.08% and 81.45±3.98%, respectively for RIF, R-NPs, R-Mn-NPs and R-Tu-NPs treated cells, at 1.0 mg/mL concentration after 72 h of treatment. While INH, I-NPs, I-Mn-NPs and I-Tu-NPs treated cells showed 61.38±3.57%, 88.33±4.29%, 87.85±4.07% and 82.21±3.74% percent viability, respectively after 72 h incubation. These results clearly indicated that PLGA-NPs though with a varied degree of modification were biocompatible and non-toxic to normal J774 cells and significantly lowered the cytotoxicity of the free drug(s). The percent cell viability of mannosylated system was found to be more as compared to tuftsin conjugated systems. This might be due to number of free amino groups at the surface of Tu-NPs.

**Macrophage uptake**

In the present study, J774 macrophages were incubated with rhodamine loaded nanoparticulate formulations and analyzed at different time intervals. The percent uptake by cells depicted greater uptake of ligand conjugated formulations as compared to plain NPs. In case of plain NPs, Mn-NPs and Tu-NPs, initially a steady increase in percent uptake was recorded but did not increase considerably after a definite period. The cell associated fluorescence at 4 h was 16.39±2.6% for NPs, 42.43±3.4% for Mn-NPs and 49.91±4.6% for Tu-NPs. After 4 h, the increase in percent uptake was found to be marginal. This may be due to the fact that the mannose and tuftsin receptors on the cell surface may have become saturated. Hasegawa et al., 2008 reported that phagocytosis is
very active for the first hour and then becomes gradually saturated. Our data are consistent with this idea.

The results indicated that the uptake of ligand conjugated formulations were significantly higher as compared to the plain counterpart. This clearly suggests that the conjugation of specific ligands on the surface improved the rate and extent of uptake of the particles by the macrophages. Hence, it can be concluded that surface decoration of NPs with ligands make them endocytosable than plain NPs.

Besides quantitative determination by FACS analysis, the qualitative uptake study of rhodamine loaded NPs (NPs, Mn-NPs and Tu-NPs) by J774 macrophages after 1 h and 4 h incubation was also studied using fluorescence microscopy. The study revealed that the intensity of fluorescence was substantially higher in case of Mn-NPs and Tu-NPs as compared to NPs formulations. This could be attributed to the affinity of the ligands (mannose and tuftsin) to the macrophage receptors. The results clearly demonstrated the efficacy of Mn-NPs and Tu-NPs for intracellular delivery to macrophages.

Confocal microscopy was also performed to further evaluate the intracellular localization of plain and ligand conjugated nanoparticulate formulations. In the control culture only nucleus stained with DAPI were visible. Cells incubated with NPs however showed small green spots, in the cytoplasm that could be attributed to the FITC in the internalized NPs. The ligand conjugated NPs were endocytosed by the macrophages affluent as compared to the plain counterparts. This study further confirmed the data obtained by the FACS analysis.

**Ex-vivo antitubercular activity**

The antimycobacterial effectiveness of RIF and INH loaded NPs in the J774 macrophages cell lines was examined both after individual and combination drug treatment. Following the infection of cell lines with H37Rv, cells were treated with RIF and INH; added either directly to culture medium or as drug(s) loaded different nanoparticulate formulations as individual or combination treatment. Infected cells were
treated with RIF and INH concentrations equal to their $C_{\text{max}}$ values in human plasma (i.e., 10 µg of RIF/ml and 3 µg of INH/ml) (Sharma et al., 2007). After individual drug treatment in infected J774, RIF delivered in plain nanoparticulate formulations resulted in significant reduction in log CFU (2.07±0.05 for R-NPs, 1.41±0.05 for R-Mn-NPs and 1.12±0.04 for R-Tu-NPs, respectively). The higher reduction in the numbers of CFU was observed with ligand conjugated systems (R-Mn-NPs and R-Tu-NPs) as compared to R-NPs. The similar result was observed in case of INH individual therapy. The log CFU obtained was 2.95±0.16 for free INH, 2.25±0.08 for I-NPs, 1.66±0.06 for I-Mn-NPs and 1.17±0.06 for I-Tu-NPs, respectively. The data suggested that ligand conjugated formulations have better efficacy in combating the infection as compared to plain counterparts and further superior than treatment with free drug(s). Further the mannose present beneath the surface may have acted as a tag to target the phagosomes containing $Mtb$ through the mannose 6 phosphate receptors within the cells. In contrast, tuftsin plays its role by activating the macrophages which further could themselves clear up the bacterial load and aid in drug(s) therapy.

The combination therapy is generally preferred due to different modes of action of drugs which cause rapid elimination of the mycobacteria and ultimately complete eradication of the infection without development of resistance. Keeping in view aforesaid fact, the effect of drug combination either in free forms or in nanoparticulate formulations for treatment of TB was attempted. The nanoparticulate formulations loaded with RIF and INH were mixed in such proportions in order to produce recommended dose in the combination.

The combination drug treatment exhibited a more reduction in numbers of CFU as compared to individual drug treatment. The free drug combination resulted in significant reduction in CFU count (log CFU- 2.34±0.11) as compared to individual drug therapy. Moreover, the nanoparticulate formulations administered in combination resulted into significant killing of mycobacteria, i.e., log CFU was 1.59±0.04 for R-NPs + I-NPs,
1.14±0.05 for R-Mn-NPs + I-Mn-NPs and 0.85±0.03 for R-Tu-NPs + I-Tu-NPs, respectively. The combination therapy was found to be more effective in controlling the infection. This may be due to the use of two drugs simultaneously having different modes of action which resulted in greater killing of the bacterial colonies.

Delivery of the drug(s) in NPs resulted in intracellular accumulation and subsequent release which is capable of producing significant reduction in numbers of *Mtb* actively dividing in host macrophages. In each experiment, doses of RIF and INH either individually or in combination, when used as nanoparticulate formulations exhibited significant reduction in log CFU of *Mtb* as compared to the control. More importantly, doses of drugs delivered by mannosylated and tuftsin conjugated NPs were able to significantly reduce CFU as compared to drug loaded plain NPs. The results clearly reflected the utility of these site specific formulations for delivery of drugs, particularly to host macrophages.

Targeting to macrophages could be achieved by chemically attaching target specific ligands on the surface of NPs. In addition delivery of drugs by means of nanoparticulate formulations might be able to reduce toxicity of RIF and INH because of changed disposition characteristics of drug(s) administered in NPs.

**Macrophage activation for bactericidal activity**

Particle’s phagocytosis is sufficient to induce a ‘classical’ activation response in the macrophages, which mobilizes intracellular and extracellular calcium, undergoes a respiratory burst, generates free radicals and initiates mechanisms to acidify the phagosome, induces phagosome-lysosome fusion or autophagy, and thereby destroys and digests the foreign particle. These responses have collectively been referred to as the “activation” response (Gordon and Martinez, 2010 and Cohn, 1978). Various surface receptors present on macrophages—Toll-like receptors (TLRs), mannose receptor, complement receptors, Fc receptor, etc. when stimulated induce high levels of proinflammatory mediators like Th1 cytokines, generation of reactive nitrogen
intermediate (RNI) and reactive oxygen species (ROS) (Mosser, 2003). Classically, activated macrophages with high levels of inflammatory mediators possess improved capacity of killing intracellular bacteria, such as Mtb (Day et al., 2009).

ROS and RNI generated as a consequence of phagocytosis are important components of the antimicrobial defense mechanism within macrophages or other phagocytic cells (Hansen and Mossman, 1987 and Dostert et al., 2008). These are involved in direct killing of invading pathogens. Phagocytosis and internalization of Mtb into phagosomes induces macrophages to sharply increase their oxygen consumption. This phenomenon is called a respiratory burst, and is important in killing intracellular Mtb (Nau et al., 2002).

Infected macrophages, and lymphocytes that interact with them, initially produce inflammatory cytokines that signal the presence of an intracellular pathogen. Th1 cells amplify phagocyte mediated defense mechanisms against infection by secreting macrophage activating cytokines such as IFN-γ, TNF, IL-2, IL-12, and chemokines like IL-18 (Raveh et al., 1998). These cytokines promote the ability of macrophages to phagocytose and destroy intracellular microbes. IFN-γ and TNF are considered to play a central role in the activation of macrophages. TNF also plays a role as modulator of macrophage activation and contributes to the elimination of mycobacteria (Engele et al., 2002). Another Th1 cytokine, IL-12 primarily produced by macrophages and dendritic cells, induces production of IFN-γ and TNF which further enhance microbial killing (Feng et al., 2005). Recent evidence showed that stimulation of macrophages by cytokines like IFN-γ and TNF induces activation of inflammasomes, autophagy and apoptosis of the infected macrophage.

Macrophages are known to generate reactive oxygen species (ROS) and nitric oxide (NO) during inflammatory responses. High production of ROS and NO causes tissue injury and apoptosis of these cells. NO was not detectable in the control group. The results suggested that uptake of NPs is accompanied by significantly higher production of
RNI. NO concentration (nM/10^6 cells) were 38.36±6.51 for untreated control, 2.25±0.32 for RIF+INH, 55.34±5.23 for R-NPs + I-NPs, 69.76±4.56 for R-Mn-NPs + I-Mn-NPs and 73.25±6.11 for R-Tu-NPs + I-Tu-NPs respectively. Nitrite concentrations peaked at 9 h in all the groups except untreated control, but fell when measured at 24 h. At 9 h the NO concentration (nM/10^6 cells) were 45.6±5.81 for untreated control, 55.98±4.87 for RIF+INH, 105.92±7.21 for R-NPs + I-NPs, 145.21±10.46 for R-Mn-NPs + I-Mn-NPs and 172.58±15.30 for R-Tu-NPs + I-Tu-NPs respectively. It is probable that downstream reactions of NO_2 ensued in the interim, as may be expected from the concurrent elevation of ROS. It is also interesting to note that infection alone did not result in similar kinetics, and that drug treatment actually had the effect of lowering RNI levels during the first 3 h.

Upon stimulation or during phagocytosis, monocytes produce reactive oxygen metabolites and other oxygen independent products (Robinson and Badwey, 1994). Enhancement of oxidative radical production clearly indicates classical activation of macrophages as a consequence of treatment with NPs. Such a conclusion is derived on the basis of an emphasis on observation that infection left untreated did not induce ROS. Similarly, Prior et al., 2002 and Sharma et al., 2007 have demonstrated generation of oxidative radicals in response to biodegradable microparticles. The oxidative radical response was higher when infected cells were given mannose and tuftsin conjugated NPs. It is reported that liposomes containing palmitoyl tuftsin (I) could specifically recognize macrophages and PMN leukocytes (Singhal et al., 1984). Treatment of macrophages with these liposomes considerably increased their respiratory burst activity as reported (Singh et al., 1992). Nevertheless, it is encouraging to observe that the NPs were apparently regarded as biocompatible by uninfected cells, but they turned to be stimulatory when _Mtb_ infection was present in the cell population.

Intracellular _Mtb_ in macrophages downregulates proinflammatory modulators such as IL-12, TNF, IL-6, etc. and evokes antinflammatory cytokines like IL-10, IL-13
and IL-4 and IL-10 (Friedland et al., 1995). The kinetics of cytokines induction were determined using J774 cells infected *Mtb* and treated with drug(s), plain NPs and ligand conjugated NPs. The levels of cytokines recorded in the case of the uninfected and untreated control group were considered as a ‘normal’ or baseline picture, and up-regulations/down-regulations were inferred with reference to these ‘normal’ levels. Of the four cytokines assayed, TNF-α and IL-12 were observed to be significantly induced on treatment with drug-containing ligand conjugated NPs, especially tuftsin conjugated formulations. Drugs in solution and plain NPs however did not have the same effect.

While there is consensus on the protective roles of IL-12, IFN-γ and TNF-α in the immunology of TB (Flynn and Chan, 2001), the relative contributions of each of these remain to be ascertained in the early stages of infection when the key cytokine producers are infected macrophages. In studies reported here, treatment with tuftsin and mannose conjugated NPs most significantly affected the secretion of TNF-α. Kornfeld and colleagues have extensively demonstrated the role of TNF-α as a mediator of caspase-induced apoptosis of macrophages infected with *Mtb* H37Ra (Kornfeld et al., 1999), especially at levels of infection which we have used in our study. It is possible that ligand conjugated NPs evoke the same strategy that the host macrophage employs against *Mtb*. If this were so, this system could represent a mechanism of anti-TB therapy akin to that of treatment with exogenous TNF-α (Cho et al., 2005).

As in the case of pro-inflammatory cytokines, enhanced production and release of ROS and RNI by macrophages upon exposure to various particulates may have both positive and negative effects. It may cause lung tissue damage, or destroy the pathogen, or both. Indirectly elevated ROS and RNI activate signal transduction pathways via kinases and transcription factors, processes that result in a complex cascade of events that may contribute to the eradication of pulmonary infection.

Host-defense responses have traditionally been of little concern when microbicidal drugs are administered. The underlying assumption is that chemotherapy
contributes the major part of antimicrobial action, and that host responses are secondary. Concurrent with our study and also as reported by other researchers, drug therapy with particulate systems and further surface decoration with specific ligands, a remarkable enhancement in efficacy of drugs has been achieved. Further results reported here indicate that treatment of macrophages with tuftsin (a natural macrophage activator) conjugated nanoparticulate systems but not the drugs themselves in solution or encapsulated in plain NPs, leads to several cellular and biochemical events that are in conformity with the profile of a classically ‘activated’ macrophage.

**IN VIVO STUDIES**

The objective of *in vivo* study was to evaluate the optimized nanoparticulate formulations containing RIF and INH for their selective and preferential delivery and hence accumulation at the target site and assessing their antitubercular activity. The experiments were primarily focused on providing information on the availability of the therapeutic concentration of the drug(s) in a sustained manner at the target sites, i.e., macrophages. The free drug(s), plain, mannosylated and tuftsin conjugated drug(s) loaded NPs were administered individually as well as in combination.

**Biodistribution study**

*In vivo* biodistribution study of the optimized formulations was carried out in Balb/c mice after intratracheal administration of a single dose of various formulations. The formulations were administered via intratracheal route individually as well as in combination, since single-drug therapy of TB is disfavored in clinical practices for fear of drug resistance. Also, combination therapy was expected to improve the efficacy by maintaining bactericidal concentrations at the target site and synergizing the drug action thereby enhancing the bactericidal efficacy. At the selected time intervals, animals were sacrificed and blood was collected by cardiac puncture. The major organs, i.e., lungs, liver, spleen and kidney were isolated, washed and homogenized. RIF and INH in the extracts and plasma was determined at specified time intervals by HPLC.
Clinical use of antitubercular drug(s) is limited by a number of factors (Dube et al., 2012). Amongst these one major factor is lower serum concentrations of the drugs. At standard doses of INH and RIF, serum drug concentrations are comparatively low and are cleared within 24 h. Our observations were consistent with these findings indicating that drugs have to be administered daily in order to maintain effective therapeutic levels throughout the period of treatment or antitubercular therapy.

Following the administration of various nanoparticulate formulations either individually or in combination, the therapeutic concentration was maintained in the plasma for up to 96 h. On the other hand free drug(s) were not detectable in the plasma beyond 12 h after the administration in case of INH and 24 h after the administration in case of RIF. This suggests that the nanoparticulate systems were able to contain the release of both the drug(s). It was previously shown by Pandey et al., 2003 that the encapsulation of different first-line anti-TB drugs (RIF, INH and PZA) within biodegradable PLGA NPs leads to therapeutic plasma concentrations up to 9 days in contrast to free drugs which were cleared within 24 h. It should be noted that the % dose recovered means the sum of drug(s) which is released from the formulation as well as the drug(s) encapsulated within the nanoparticulate formulations.

In the case of individual administration, after 48 h, the % recovered dose in plasma following administration of R-NPs was 15.32±1.4, of R-Mn-NPs was 5.35±1.2 and of R-Tu-NPs was 4.27±1.2. After 96 h % recovered dose was 7.21±0.75 from R-NPs, 3.12±0.25 from R-Mn-NPs and 3.07±0.73 for R-Tu-NPs, respectively. When administered in combination with INH, the % recovered dose in plasma was 12.17±1.4 for R-NPs, 5.36±0.7 for R-Mn-NPs and 4.83±0.5 for R-Tu-NPs, respectively, after 48 h. After 96 h % dose recovered in plasma was 7.15±0.2 for R-NPs, 3.26±0.3 for R-Mn-NPs and 2.07±0.2 for R-Tu-NPs, respectively.

Similarly in case of INH no drug was detected in plasma after 12 h in case of plain drug solution where as the % recovered dose was 9.99±1.2, 3.47±0.6 and 2.76±0.5
for I-NPs, I-Mn-NPs and I-Tu-NPs, respectively, after 48 h and 3.83±0.96, 0.31±0.1 and 0.12±0.1 for I-NPs, I-Mn-NPs and I-Tu-NPs, respectively, after 96 h. When administered in combination with RIF the % recovered dose in plasma after 48 h was 8.91±1.3, 2.95±0.8 and 2.65±1.0 for I-NPs, I-Mn-NPs and I-Tu-NPs, respectively. The % recovered dose of INH in plasma after 96 h was 3.28±0.3, 0.42±0.2 and 0.23±0.1 for I-NPs, I-Mn-NPs and I-Tu-NPs, respectively. These data clearly suggested that the entrapment of the drug in NPs could sustain the release the drugs after individual as well as combination drug(s) administration. The above data justified the efficacy of the developed formulations as sustained release system. Drugs such as INH with short half life (2.29 and 2.67 h) can be successfully made available for longer duration when entrapped in appropriate sustained release systems such as PLGA NPs.

An interesting observation was constant concentration of both the drug(s) in the plasma upto 96 h when administered contained in nanoparticulate formulations administered via intratracheal route. This can be attributed to so called “leakage” of the drug(s) from the target site or due to the migration of the alveolar macrophages (wondering macrophages) from the lungs to blood and secondary lymphoid locuses.

A number of studies support the fact that passively targeted systems as well as actively targeted systems alter the biodistribution pattern of encapsulated drug moieties in a significant manner. Biodistribution of drug(s) from various formulations were evaluated in major target organs in Balb/c mice for 96 h after single intratracheal administration. The results suggested that the free drug(s) have free access to all the organs despite it was given through intratracheal route. In the case of free RIF solution, only 20.12±3.7% of the administered dose was found in the lungs at 1 h post-administration which is less than the amount recovered in serum (29.84±2.3%) at the same time. Similarly, only 26.42±3.1% of the administered dose of INH was found in the lungs at 1 h post-administration.
All the developed nanoparticulate formulations (plain as well as ligand conjugated) showed greater accumulation in the lungs when compared with the controls. Inhalation resulted in immediate peak concentrations, since the NPs were deposited in the lung lumen itself. Direct deposition and initial burst release of the drug(s) could have resulted in immediate peak concentrations. R-NPs and I-NPs showed an initial higher lung accumulation (53.86±5.4% and 45.69±3.9% after 1h administration) but the overall distribution pattern with time was not satisfactory. Whereas in the case of mannosylated system, the % dose recovered was 67.49±6.3 at 1 h and in case of INH the % recovered dose was 69.02±4.5 at 1 h. Similar pattern was also obtained in the case of tufts in conjugated systems suggesting a consistent drug accumulation in the lung over a prolonged period of time. The order of accumulation was RIF/INH Tu-NPs ~ RIF/INH Mn-NPs > RIF/INH nanoparticle > RIF/INH. The faster and higher degree of localization and accumulation of Tu-NPs and Mn-NPs in comparison to plains NPs could be attributed to the presence of specific tufts in and mannose receptors on the cell surface of macrophages.

Khuller and group had extensively investigated the steady-state pharmacokinetics of various anti-TB drugs in different formulations administered to animals by nebulization. Their results generally showed a lag time of 3 to 6 h between administration and appearance of drugs in the blood, first-order kinetics of absorption and elimination from the blood and sustained blood and tissue concentrations over several days. These results arise from the administration of nebulized formulations, often containing particles or vesicles in the nanometer size range. In contrast to this, our data suggested faster appearance of drug at the site of action. This may be due to initial burst release of the drug(s) from the formulations which could have maintained the concentration. Ligand conjugated NPs appear to increase the time frame of action of the drugs incorporated in them by sustained release and drug targeting.
It has also been shown here that receptor mediated endocytosis results in higher intracellular drug concentrations, confirming drug targeting to the site of infection. Targeting drugs to alveolar macrophages appears to have additional merits apart from the obvious. Alveolar macrophages tend to migrate to secondary lymphoid organs especially after taking up particulate material in the lungs. As mycobacteria disseminate not only through the bloodstream, but also to secondary sites particularly where macrophages traffic and accumulate. Loading resident alveolar macrophages with drug-containing particles might conceivably support the transport of drugs to those sites where migratory macrophages accumulate mimicking the course of spread of mycobacteria.

Apart from the main target site drug was also assessed at other macrophage rich organs, liver and spleen and also in peripheral blood mononuclear cell (PBMC). A constant concentration in PBMC was recorded in the case of ligand conjugated systems when compared with free drugs and plain NPs, irrespective of the drug encapsulated and individual or combination administration. In case of free drug(s) very less amount was detected in PBMC, the % dose recovered in PBMC was undetectable at 12 h post administration. The higher concentration of drug(s) in case of nanoparticulate formulations in PBMC may be attributed to receptor mediated uptake of the ligand conjugated systems due to the presence of specific receptors on the surface of the cells.

The results of the above experiments were taken to signify that ligand conjugated NPs containing RIF and INH could establish higher drug concentrations in macrophages as compared to equivalent amounts of drugs in solution; within a concentration ‘window’ imposed by the relative rates of: (i) receptor mediated phagocytosis, (ii) drug release from NPs, and (iii) intracellular diffusion of soluble drugs.

Beside quantitative determination by biodistribution studies, the qualitative uptake study of FITC loaded NPs (NPs, Mn-NPs and Tu-NPs) was also performed by intratracheal administration of FITC solution and these formulations in Balbc mice, after 1 h of administration using fluorescence microscopy. Fig 7.21 - Fig. 7.24 revealed that the
intensity of fluorescence was substantially higher in case of Mn-NPs and Tu-NPs as compared to NPs formulations. This could be attributed to the affinity of mannose and tuftsin ligands to the macrophage receptors. These results clearly demonstrated the efficacy of Mn-NPs and Tu-NPs for intracellular delivery to macrophages.

**Antitubercular activity**

The chemotherapeutic study of the selected formulations was performed on the TB induced Balb/c mice both after individual and combination drug treatment. After 24 h post infection, the animals were divided in various groups and drug therapy was started. Drug treatment was given for 28 days by administering RIF equivalent to 10 mg/Kg body weight and INH equivalent to 5 mg/Kg body weight. Free drug(s) were administered daily for 4 weeks; whereas plain, mannosylated and tuftsin conjugated NPs were given after an interval of 3 days for the same time period.

When drug(s) were used in individual treatment regimen, treatment of all the groups resulted in significant killing of microorganisms as compared to control. The free drug administration resulted in reduction of bacilli count in lungs to $4.68 \pm 0.16$ and $5.83 \pm 0.26$ log CFUs for RIF and INH, respectively. For plain NPs of RIF and INH, more significant reduction in log CFU ($3.44 \pm 0.25$ for RIF and $3.75 \pm 0.28$ for INH) was observed in lungs. The mannosylated and tuftsin conjugated NPs resulted in significant reduction of the number of viable *Mtb* as compared to free drug. The mannosylated NPs resulted in log CFU $1.84 \pm 0.07$ and $2.23 \pm 0.15$ for RIF and INH, respectively. With tuftsin conjugated NPs the log CFU was found to be $1.42 \pm 0.04$ and $2.05 \pm 0.12$ for RIF and INH, respectively.

Similarly, the reduction in *Mtb* was also recorded in the spleen. All treatment groups showed a significant reduction in the bacilli count when compared to the control. For plain nanoparticulate system, more significant reduction in log CFU ($2.36 \pm 0.15$ for RIF and $1.95 \pm 0.08$ for INH) was observed in spleen when compared to the free drugs. The mannosylated NPs resulted in reduction of log CFU to $1.76 \pm 0.05$ for R-Mn-NPs and
1.09±0.06 for I-Mn-NPs. The tuftsin conjugated NPs resulted in most significant reduction number of log CFU (0.82±0.05 for RIF and 1.0±0.04 for INH).

When drugs were used in combination, they were mixed so that required doses of RIF and INH are incorporated after administration. All the groups resulted in significant killing of microorganisms as compared to control group. After intratracheal administration of plain nanoparticulate formulations (R-NPs + I-NPs), a significant reduction in the number of CFU in lungs (log CFU- 1.97±0.18) and spleen (log CFU-1.02±0.03) was observed when compared to the control groups. Both mannosylated and tuftsin conjugated formulations resulted in almost 10-folds reduction in the bacterial colonies in lungs while no colonies were found in spleen when compared to free drug(s) combination. Deol et al., 1997 obtained similar results after combination therapy by using liposomal RIF and INH. The data suggested that ligand conjugated formulations have greater efficacy in combating the infection as compared to plain counterparts and further they were found to be superior than treatment with free drugs. Further, the combination drug treatment exhibited a more reduction in numbers of CFU as compared to individual drug treatment.

Delivery of the drugs resulted in intracellular drug(s) release which is capable of producing significant reduction in *Mtb* in host macrophages. In each experiment, doses of RIF and INH either individually or in combination, when used as of nanoparticulate formulations have significantly reduced the log CFU of *Mtb* as compared to the control. More importantly, doses of drugs delivered by mannosylated and tuftsin conjugated NPs were able to significantly reduce CFU as compared to drug loaded plain NPs. The results clearly reflected the utility of these targeted formulations for delivery of drugs to host macrophages.

This study revealed that although both free drug(s) (individual and combination) and nanoparticulate formulations were efficacious, yet therapeutically beneficial as against 28 doses of conventional treatment only 10doses of NPs cold produce many fold
better results. These results suggest the NPs based combination therapy is most effective; may result in dose and frequency of administration, thus may ensure of patient compliance and maximum therapeutic effects.

**Hepatotoxicity study**

Estimation of the serum enzymes is a useful quantitative marker of the extent and type of hepatocellular damage. Alanine aminotransferase (ALT, SGPT) is a cytosolic enzyme primarily present in the liver. The levels of this enzyme in serum increase because of leakage of this cellular enzyme into plasma if there is any hepatic injury. Alkaline phosphatase (ALP, SGOT) is a mitochondrial enzyme released from heart, liver, skeletal muscle, and kidney. Increased serum level of SGOT is also associated with liver damage.

*In vivo* hepatotoxicity by the anti-TB drug(s) given in free form or entrapped in NPs was evaluated on day 2 after the last therapeutic dose, i.e., 30 days from the initiation of the treatment.

Hepatotoxicity studies were performed for individual drug(s) based treatments. The entrapment of INH and RIF in NPs reduced the total bilirubin levels compared to those observed for free drugs. Free drugs (RIF and INH) induced toxicity in infected mice as a result of increased levels of ALT, ALP and total bilirubin for RIF and INH was observed.

The differences in the levels induced by free and nanoparticulate drug(s) after individual therapy were significant. The ALT and APL levels were significantly low in mice treated with RIF and INH containing mannosylated and tuftsin conjugated NPs as compared to free drug(s) and control groups. RIF and INH entrapped in plain NPs showed a small increase in level of ALT, ALP, total bilirubin, for RIF and INH. The drug(s) encapsulated in mannosylated and tuftsin conjugated NPs did not show any apparent change in activity of ALT compared to normal mice. Similar results were also observed in combination therapy.
The increased levels of SGOT and SGPT in serum are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman, 1978). Serum analysis ALT, ALP and total bilirubin illustrated that there was no evidence of biochemical toxicity in mice treated with plain and ligand conjugated NPs after individual and combination administration.

From in vivo studies, it could be inferred that there was a significant increase in drug accumulation in the desired cellular tropics when they were administered in nanoparticulated form (plain, mannosylated and tuftsin conjugated) formulations. As expected, the plasma and organ distribution studies had shown higher amount of drug(s) recovery from lungs in the case of both the ligand conjugated formulations as compared to plain counterparts and free drug(s), indicating the efficacy of developed carrier for lung targeting of antitubercular drug(s).

The experiments were primarily focused on providing information on the time course of sustained drug concentration at the target sites, i.e., lung macrophages. Drug concentrations at other macrophage rich sites, including the livers and spleen, was found to be beneficial as the formulations which escaped were majorly got concentrated in macrophage rich organs such as PBMC, liver and spleen. The findings are in agreement with work carried out previously in the lab where liposomes encapsulating RIF were delivered as aerosolized formulations (Vyas et al., 2004). Verma et al., 2008 also reported that inhaled microparticles containing INH and rifabutin reduced the dosing frequency and improved the pharmacological index of the drug combination. The ligand conjugated particles led to transport of drug(s) to those sites where \textit{Mtb} traffic. This will help in clearing the bacterial load in the case of extrapulmonary TB.

Experimental observations also suggested that ligand conjugated NPs, both after individual or combination administration, were more effective against \textit{Mtb} than free drugs or plain NPs. Obviously the combination therapy was more effective in combating the infection as compared to individual therapy. The combination therapy was expected
to improve the efficacy in two ways. First, maintenance of bactericidal concentrations at the target site resulting in enhanced bactericidal efficacy. Second, as the infection comprises strains with various sensitivities, INH might have acted in synergy with maintained RIF concentrations. The second reason could be beneficial with the perspective of a clinical scenario. The hepatotoxicity due to free drug(s) (RIF and INH) after individual as well as combination treatment was minimized.

Therefore, the results confirmed that the developed delivery systems possessed an enhanced antitubercular activity in mice and were also capable of reducing the hepatotoxicity associated with the drug(s) when administered for long term treatment of TB in conventional formulations. Thus, it also inaddition concludes the axiomatic principle of drug targeting.

(Devyani Dube)
Candidate

(Prof. S. P. Vyas) (Prof. G. P. Agrawal)
Supervisor Co-supervisor

Head
Department of Pharmaceutical Sciences
Dr. Hari Singh Gour University
Sagar (M.P.) 470 003