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Ex-Vivo Efficacy of Rifabutin Loaded Solid Lipid Nanoparticles

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ABSTRACT

Tuberculosis is a ubiquitous, highly contagious chronic granulamatous communicable bacterial infectious disease caused by Mycobacterium tuberculosis and other species of same genera. "Rifabutin" which is useful in the management of tuberculosis. Formulated Rifabutin in the form of solid lipid nanoparticle evaluated for their efficacy *ex-vivo* by checking for various interactions. Thus, physiological parameters like cellular uptake, MTT cytotoxicity assay and hemolytic toxicity of rifabutin loaded mannosylated solid lipid nanoparticles are determined and compared**.** The present work establishes the suitability of rifabutin loaded mannosylated solid lipid nanoparticles as a delivery system.

Keywords: Rifabutin, Tuberculosis, solid lipid nanoparticle, preformulation studies.

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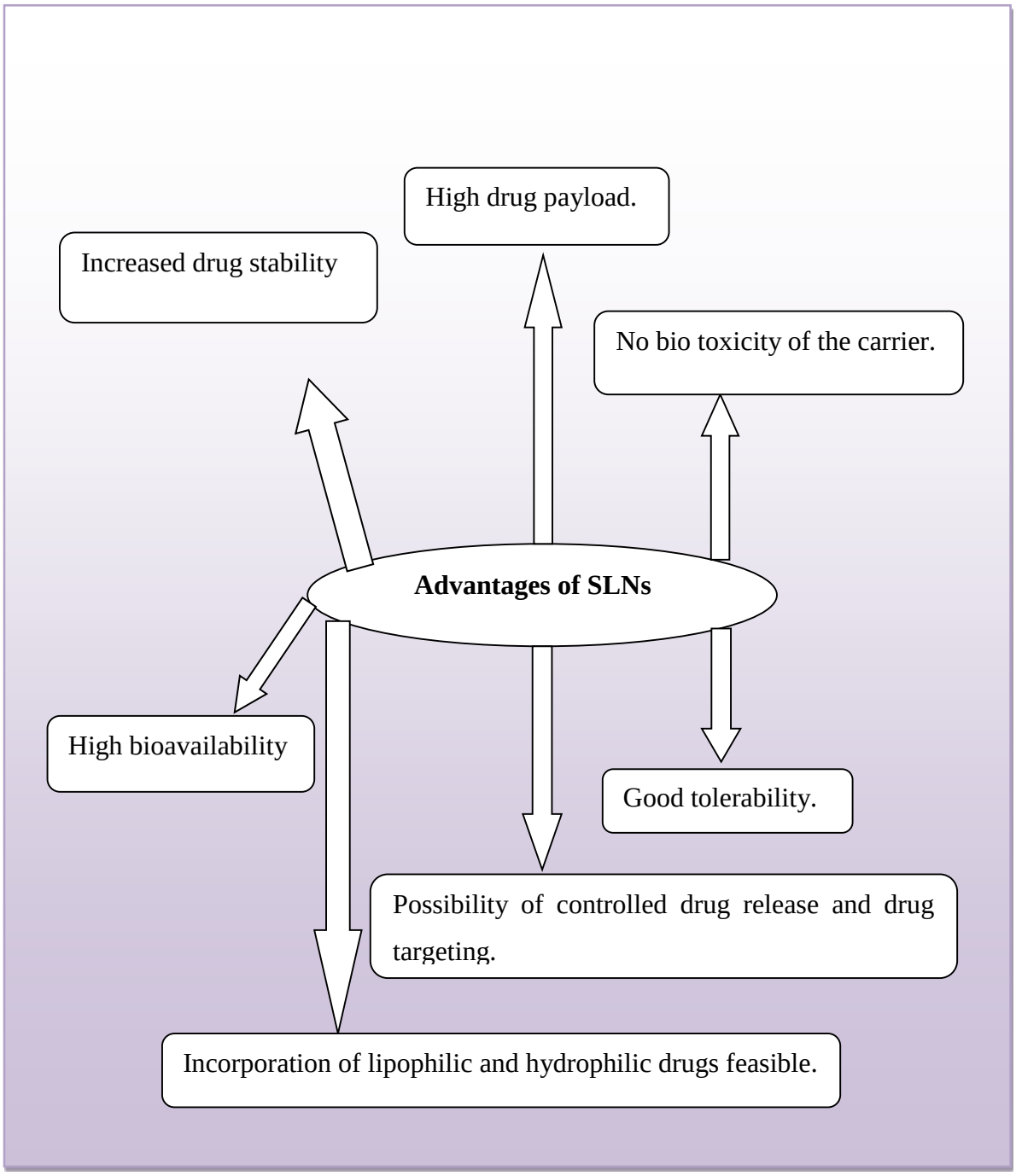
INTRODUCTION

Tuberculosis is a ubiquitous, highly contagious chronic granulamatous communicable bacterial infectious disease caused by Mycobacterium tuberculosis and other species of same genera. Tuberculosis, which is easily transmitted through the air, already infects 1.9 billion people, and takes the lives of about two million people each year. The situation has been excerbated because of the presence of numerous other complicating factors like multi drug resistant tuberculosis and HIV-coinfection. Tuberculosis is a leading cause of death amongst infectious diseases. Furthermore, this re-emerging disease has become one of the most important infections affecting human immunodeficiency virus (HIV)-positive patients worldwide. TB also is becoming increasingly resistant to existing drugs. It is estimated by the World Health Organization (WHO) that more than 2 billion people in the world are infected with Mycobacterium tuberculosis[1]. Mycobacterial infection is a challenging health problem that requires particular attention worldwide. The characters of Mycobacterium tuberculosis are distinctly contrast to many other common bacteria[2]. The mycobacterium cell wall, lipid (e.g. mycolic acid) is linked to the underlying arabinogalactan and peptidoglycan. The structure is responsible for very low permeability of cell wall and thus for ineffectiveness of most of antibiotic against the organism. Clinical management of tuberculosis possess serious problem because the efficacy of chemotherapy has been reduced which may be attributed to the degradation of drugs before reaching the target, the low level of cell permeability to drugs, or primary drug resistance. Other reason for the failure of chemotherapy may be the difficulty in achieving adequately high concentration at the infection site, inadequate penetration of drug into macrophages and low level in cells. These problem, which arises with conventional dosage forms of antitubercular drugs, may be overcome by designing and developing a site specific delivery of antitubercular drug using surface modified solid lipid nanoparticles[3]. Drug selected for present study was “Rifabutin” which is useful in the management of tuberculosis. Rifabutin is a first line drug for the treatment of tuberculosis and it is a derivative of Rifamycin S. Rifabutin inhibits the essential rpoB gene product b-subunit of DNA dependent RNA polymerase activity, acting early in transcription. It is thought to bind to the b subunit, close to the RNA/DNA channel, and physically blocks the transit of the growing RNA chain after nucleotides have been added. In Escherichia coli bactericidal action may come from the triggering of apoptosis via activation of the “suicide gene module” mazEF, and the same system has been identified in Mycobacterium tuberculosis. Rifabutin does not inhibit the mammalian enzyme.

Advantages of SLNs[4-6]



MATERIALS AND METHOD

Cellular Uptake of Fluorescein Isotiocyanate (FITC) Loaded Solid Lipid Nanoparticles By Macrophages

The study was performed at Jalma Institute of Leprosy and Other Mycobacterium Diseases, Agra. Macrophage cell lines J774[7] was used for cellular uptake study. The uptake study was performed

at 1,2 and 6 hours time interval using fluorescence activated cell sorters (FACS) instrument ("BD" Biosciences FACS Aria, Germany).

One hundred µl aliquots containing macrophage cell lines i.e. J 774 (1×10^3) with more than 95% viability were suspended in 0.9 ml of fresh supplemented RPMI – 1640 culture medium (penicillin 10 U/ml; 10% fetal bovine serum; 100 µg/ml streptomycin; 1mM sodium pyruvate and 10 mM HEPES medium) [8] and transferred into a dish with 24 wells and incubated for 12 hours at 37°C under 5% carbon dioxide atmosphere.

Ten µl of (FITC) loaded solid lipid nanoparticles was suspended in the wells containing J 774 macrophage cell lines. After incubation for 1 hour, J774 were detached by pipetting and collected by centrifugation at 5000 rpm for 2 min. To remove solid lipid nanoparticles adhering to cell surface, the cells were washed five times with PBS (pH7.4). The solid lipid nanoparticles incorporated into the cells were extracted by dichloro methane (DCM) and stored in mini FACS tube at 5°C. The first tube was taken as control. Same procedure was followed for studying the uptake after 2 and 6 hours. The cell-associated fluorescence was measured by FACS ("BD" Biosciences FACS Aria, Germany)[9] (Fig. 1 and Table 1). Because of the limitation of facility, cellular uptake of only mannosylated SLNs was performed.

MTT Cytotoxicity Assay

J774 cells were resuspended to a concentration of 1×10^3 cells/ml in fresh culture medium. The cells growth inhibition activity of the samples was evaluated by 3- (4-5 dimethylthiazol-2H)-2,5-diphenyl tetrazolioum bromide (MTT) (Sigma Aldrich) colorimetric assay. The cells were seeded evenly into 24 wells of 96 wells flat-bottomed tissue culture plate (Sigma, Germany) at 1000 cells/well concentration and incubated for 24 hours in a humidified atmosphere of 5% carbon dioxide at 37°C [10]. From stock SLNs containing 1mg/ml of rifabutin, 5µl , 10µl , 20µl of samples solution i.e. rifabutin loaded mannosylated solid lipid nanoparticles were added to each well and plates were incubated. After 72 hours, 50µl of 5 mg/ml MTT solution in PBS (pH 7.4) was added to each well and the plate was incubated for 3 hours in dark at 37°C, allowing viable cells to reduce the MTT into purple formazan crystal. The formazan crystal was lysed by adding 50µl DMSO: Ethyl alcohol (1:1). The absorbance of tissue culture plate was measured at 450 nm in an ELISA plate reader at 25°C using cells only as control (Table 2 and Fig. 2).

Hemolytic Toxicity

The study was carried out on whole human blood, which was collected in Hi-Anticlot vials (Himedia, India). It was centrifuged at 3000 rpm for 15 min and RBCs were separated and washed

with normal saline (0.9% w/v) until a clear, colorless supernatant was obtained above cell mass. The cells were resuspended in normal saline to obtain a hematocrit volume of 5%. To 1 ml of RBC suspension taken in centrifuge tube, 5 ml distilled water was added, which was considered as producing 100% hemolysis. Similarly, 5.0 ml of normal saline was added to 1 ml of RBC suspension in another tube producing no hemolysis, hence acting as blank. To 1 ml of RBC suspension, 0.5 ml of different formulations added and the volume was made up to 5 ml with normal saline[11].

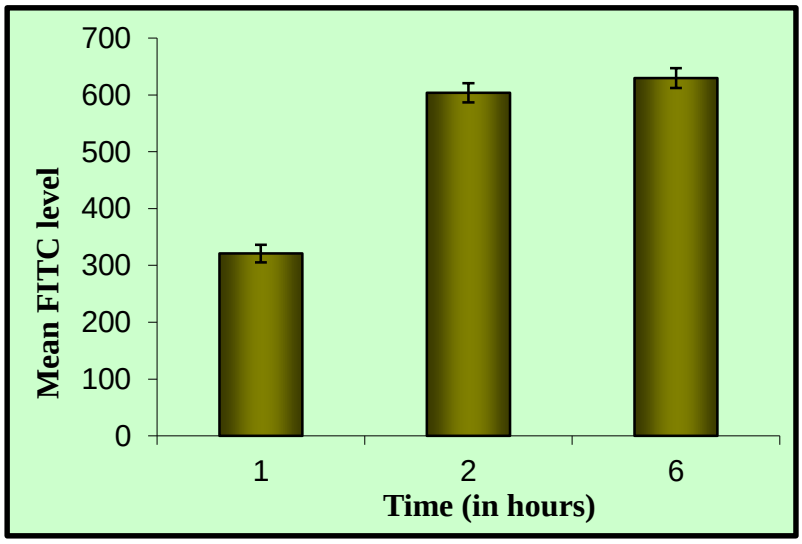
The tubes were allowed to stand for 1 hr at 37°C with continuous shaking. All the tubes were then centrifuged at 3000 rpm for 15 min. The supernatant was taken and diluted with equal volume of normal saline and absorbance was taken at 550 nm. The degree of hemolysis was determined by the following equation:

$$\text{Hemolysis (\%)} = \frac{\text{Abs} - \text{Abs}_o}{\text{Abs}_{100} - \text{Abs}_o} \times 100$$

where Abs, Abs_{100}, and Abs_o are the absorbances of sample, solution of 100% hemolysis, and solution of 0% hemolysis; respectively (Table 3 and Fig. 3).

Table 1: Cellular uptake of FITC loaded mannosylated solid lipid nanoparticles.

S.No.	Time (hrs)	Mean FITC level
1.	1	321
2.	2	604
3.	6	630



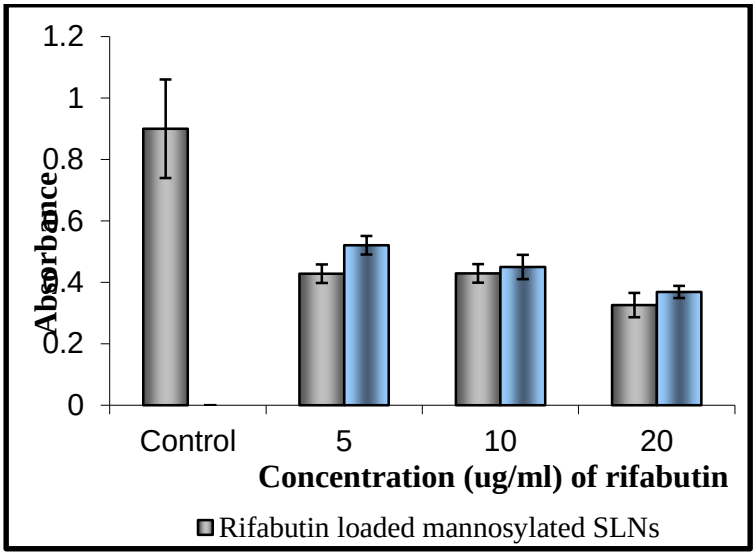
S.D. ± Mean (n=3)

Figure 1: Cellular uptake of FITC loaded mannosylated solid lipid nanoparticles

Table 2: MTT cytotoxicity assay of rifabutin loaded mannosylated solid lipid nanoparticles

Concentration (ug/ml)	Absorbance	
	Rifabutin loaded mannosylated SLNs	Rifabutin loaded SLNs
5	0.428	0.521
10	0.429	0.450
20	0.326	0.369

Absorbance of Control i.e. cells only is 0.9009



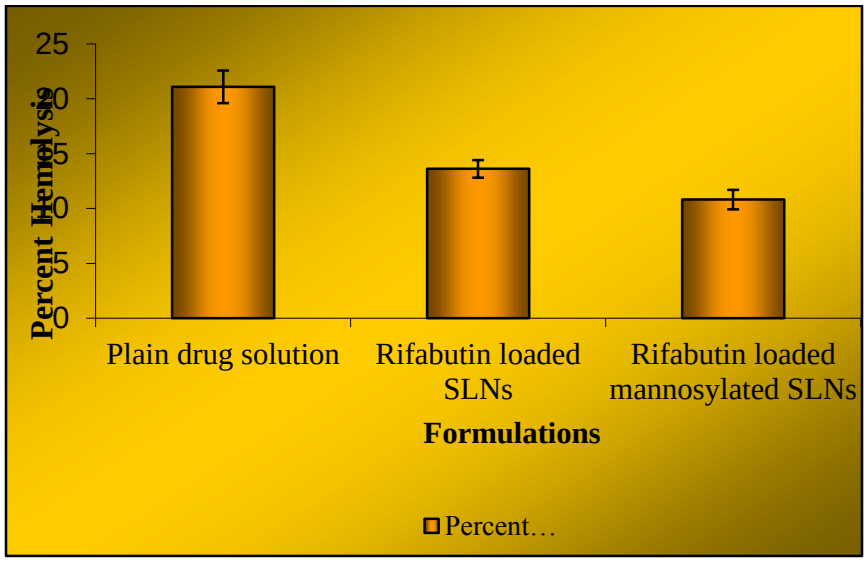
S.D. ± Mean (n=3)

Figure 2:MTT cytotoxicity assay of rifabutin loaded mannosylated solid lipid nanoparticles.

Table 3 Percent hemolysis by different SLNs formulations on addition to human RBC.

Formulations	Percent Hemolysis after 1hr
Plain rifabutin solution	21.09±1.5
Rifabutin loaded solid lipid nanoparticles	13.62±0.8
Rifabutin loaded mannosylated solid lipid nanoparticles	10.81±0.9

S.D. ± Mean (n=3)



S.D. ± Mean (n=3)

Figure 3: Percent hemolysis by different SLNs formulations on addition to human RBC suspension.

RESULTS AND DISCUSSION

Cellular Uptake Of Fluorescein Isotiocyanate (Fitc) Loaded Solid Lipid Nanoparticles By Macrophages

Figure 1 shows that with the increase in time, mean FITC level increases in macrophage cell lines (J774) which may be due to cellular uptake of FITC loaded mannosylated solid lipid nanoparticles. This result shows that with rifabutin loaded mannosylated solid lipid nanoparticles, high drug concentration can be achieved in alveolar macrophages as it can bypass the metabolism by kupffer cells of liver.

MTT Cytotoxicity Assay

Limited clinical experience using solid lipid nanoparticles as carrier makes it impossible to designate any particular chemistry intrinsically ‘safe’ or ‘toxic’. The cytotoxicity of solid lipid nanoparticles is dependent on the core, but is most strongly influenced by the nature of solid lipid nanoparticles surface. MTT cytotoxicity assay ensured that solid lipid nanoparticles could serve as biocompatible delivery system for biomedical and pharmaceutical use[10]. The mannosylated solid lipid nanoparticles at a drug concentration of 1mg/ml showed negligible cytotoxicity in J774 cells (Figure 2) possibly due to shielding of the internal cationic charges by surface hydroxyl groups of mannose. It was observed that rifabutin loaded mannosylated solid lipid nanoparticles exhibited significantly lower cytotoxicity in comparison to rifabutin loaded solid lipid nanoparticles cells were low at all concentration.

Hemolytic Toxicity

Loading on to solid lipid nanoparticles can reduce the hemolytic toxicity of rifabutin. The hemolytic toxicity of rifabutin and rifabutin loaded solid lipid nanoparticles was evaluated and the percentage hemolysis on addition of SLNs formulations is shown in fig. 3 with the data given in table 6.2. Rifabutin loaded mannosylated solid lipid nanoparticles showed the lowest hemolytic toxicity as compared to rifabutin loaded solid lipid nanoparticles, which might be due to the increased hydrophilicity of rifabutin loaded mannosylated solid lipid nanoparticles. The hemolytic activity of plain rifabutin was reduced to a considerable extent when loaded in solid lipid nanoparticles. But results for decline in hemolytic activity of rifabutin were obtained when it was present in mannosylated solid lipid nanoparticles. This was supposed to be due to the combined effect of slow release of rifabutin and increased hydrophilicity of mannosylated solid lipid nanoparticles.

CONCLUSION

The present work establishes the suitability of rifabutin loaded mannosylated solid lipid nanoparticles as a delivery system. The MTT assay revealed reduction in *in vitro* cytotoxicity of drug loaded mannosylated solid lipid nanoparticles in J774 cells, justifying biocompatibility of this delivery system. Hemolytic toxicity was also found to decrease with drug loaded mannosylated solid lipid nanoparticles. The present work opens the opportunity for exploring the detailed *in vivo* aspects related to such delivery system. Thus, physiological parameters like cellular uptake, MTT cytotoxicity assay and hemolytic toxicity of rifabutin loaded mannosylated solid lipid nanoparticles are determined and compared**.**

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