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Research Article

Nano-Lipoidal Azithromycin: A Strategy to Improve the Antimicrobial Performance of the Drug

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ABSTRACT

Azithromycin (AZT) is prescribed for the treatment of respiratory tract, skin and genital infections caused by *S. pneumonia*, *H. influenza, L. monocytogenes, S. aureus*, *Mycobacterium Avium* Complex (MAC) infections and enteric bacterial pathogens. With a view to improve its oral bio-availability and antimicrobial performance, AZT was encapsulated in Glyceryl Monostearate (GMS) nanoparticles by employing solvent diffusion evaporation method. The optimized AZT-SLN nanoformulation had a size of 380.33 ± 13.31nm, PDI of 0.25±0.06 and % Encapsulation Efficiency (% EE) of 66.73 ± 4.02 %. The *in vitro* drug release study of the AZT-SLN showed an initial fast release of 35% in 12 h followed by slow and sustained release upto 60% in 96h. Pharmacokinetic profile of a single oral administration of AZT-SLN showed the maintenance of therapeutic drug concentrations (> 2µg/mL) in plasma for 4 days and in the tissues (lungs, liver and spleen) for 9 days. The relative bioavailability of AZT from SLN was 2.5 fold higher as compared to administration with AZT suspension. The effect of sustained release and increased bioavailability was reflected in the antimicrobial efficacy of the AZT-SLN against *Staphylococcus aureus,* which was confirmed by zone of inhibition assay and time kill assay. The results were compared with AZT suspension, which also corroborated the sustained release nature of AZT-SLN. The intent of using lipid nanocarriers is primarily to enhance the oral bioavailability of azithromycin, decrease the dose and dosing frequency and thus eventually increase the antimicrobial efficacy against bacterial infections.

INTRODUCTION

Azithromycin, a nitrogen-containing macrolide (azalide-derived from erythromycin)is used for treating respiratory tract infections, skin and genital infections [1,2]. Azithromycinis generally recommended for a plethora of infections ranging from Gram positive (*Staphylococcus aureus*), Gram negative bacteria (*Haemophilus influenzae, Moraxella catarrhalis* and protozoal infections (*Toxoplasma gondii, Plasmodium falciparum*). It is estimated that 20% of the global population are long-term carriers of *S. aureus* causing variety of illnesses ranging from minor skin infections to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, Toxic Shock Syndrome (TSS) causing nosocomial infections [3]. Azithromycin can effectively inhibit the growth of bacteria by interfering with their protein synthesis. It

binds to the 50S subunit of the bacterial ribosome, thus inhibiting translation of mRNA [2]. It has bactericidal activity against strains of *S.pneumonia*, *H.influenza, L.monocytogenes, S. aureus*, *Mycobacterium Avium* Complex (MAC) infections and a variety of enteric bacterial pathogens [4]. Its ability to concentrate inside human cells, particularly neutrophils and phagocytes (200 times higher than extracellular conc.), makes it particularly useful in the treatment of infections caused by pathogens that invade host tissues [5].

However, the therapeutic use of this potent antibiotic is limited due to its low bioavailability (36%), high dose (500 mg, as used in tuberculosis and MAC infections) and dose related adverse effects (diarrhoea, abdominal pain, chest pain, vomiting, dizziness), which leads to poor patient compliance [6]. The low bioavailability of drug indicates that following an oral dose, it is poorly absorbed leading to low concentrations in plasma and insufficient amount of drug reaching the site of action. Hence, the beneficial pharmacologic effects of the new chemical entity may not be realized. For this reason, a high dose of AZT (500 mg) is given, which invariably causes adverse effects. The need to lower the concentration and dosing of the drug and to prevent its untoward adverse effects, necessitates a carrier-mediated delivery of the drug to infected cells. Colloidal carriers such as liposomes, polymeric nanoparticles, lipid nanocarriers have been investigated and developed as targeted strategies especially for the treatment of intracellular infections [7-9]. For example, ciprofloxacin- loaded nanoparticles were more active in human macrophages infected with *Mycobacterium avium* complex than free drug [10]. Amongst the available nanocarriers, Solid Lipid Nanoparticles (SLN) have attracted increasing attention due to their low cost, biocompatibility, low toxicity and ease of large scale production [11-13]. Their ability to easily incorporate lipophilic drugs in biocompatible lipid matrix offers advantages in drug delivery , which could be used for drug targeting in the treatment of complex diseases and disorders, such as cancer, arthritis, etc. [14-16]. In addition, SLNs have also been used to enhance the oral absorption and bioavailability of drugs such as anti-tubercular drugs [17], insulin [18], nitrendipine [19], vinpocetine [20]. The enhancement in oral bioavailability is attributed to reduced exposure to enzymatic degradation during the process of absorption, direct uptake of nanoparticles through GI tract, high permeability to the intestinal membrane and ability to bypass liver first pass metabolism [21]. In our recent work, we have shown the increased bioavailability of rifabutin through its encapsulation into glyceryl monostearate-lipidic nanocarriers [22]. Glyceryl monostearate is a biocompatible and FDA approved lipid for oral use [23].We hypothesize that the encapsulation of the drug inside the lipid nanocarriers will increase the bioavailability of the drug, which will eventually increase the anti-microbial performance of the drug. The present study investigates the sustained- release nature of azithromycin loaded in glyceryl monostearate solid lipid nanoparticles and anti-microbial properties against *Staphylococcus aureus*. This novel strategy has the potential to reduce intracellular persistence of *S. aureus* in the pathogenesis of several disease processes.

MATERIALS AND METHODS

Materials

Azithromycin and Resazurin were purchased from Sigma Aldrich, USA. Imwitor® 900 F (glyceryl monostearate) was supplied as a gift sample by Sasol, Germany. Tween®80 was purchased from Fisher Scientific, USA. All other chemicals and reagents were of analytical grade. The solvents used in HPLC (acetonitrile, water, isopropyl alcohol) were of HPLC grade. *Staphylococcus aureus* strain (SA 22359) was obtained from Microbiology department, Panjab University, Chandigarh, India. Agar for nutrient broth medium was purchased from Himedia, Mumbai, India.

Preparation of azithromycin loaded solid lipid nanoparticles

Azithromycin loaded solid lipid nanoparticles (AZT-SLNs) were prepared by initially reported 'solvent diffusion evaporation' method with slight modification [17]. Briefly, weighed amount of drug (100 mg) and lipid (1:2 ratio) was dissolved in a mixture of acetone: methanol (10 ml) (1:1 v/v) by heating at 60-70^{0}C. This organic phase was added dropwise to 1% Tween®80solution with constant stirring. The above mixture was kept overnight for stirring (700 rpm), at room temperature. The nanoparticulate dispersion formed by precipitation of lipid engulfing drug, was centrifuged at 22,000 g for 45 minutes. The pellet formed was washed

thoroughly with double distilled water (DDW) and re-centrifuged. The process was repeated twice, to remove any unentrapped drug. The pellet formed was then dried in vacuum oven, weighed and used for calculating % EE and % Drug Loading (DL). Empty-SLNs were prepared as above without the drug azithromycin.

Characterization of solid lipid nanoparticles

Size and polydispersity index (PDI): Size and PDI of SLNs were analyzed by using Zetasizer Nano ZS90, Malvern Zetasizer, UK. The SLN suspension was diluted 10 times before the size measurements. All the measurements were done in triplicates.

Entrapment efficiency and drug loading: Drug loading and encapsulation efficiency of nanoparticles were determined by using HPLC with UV detector. The chromatographic analysis was performed on C18 column (Gemini NX, 150 x 4.6, 5µ), at an isocratic elution of mobile phase comprising acetonitrile: 2 mM disodium hydrogen phosphate buffer: isopropyl alcohol (65:25:10v/v/v), with flow rate of 1 ml/min and detected at 215 nm using a UV detector. The % Entrapment Efficiency (EE %) and % Drug Loading (DL %) were calculated by using following equations.

$$EE\ (\%) = \frac{\text{Amount of drug encapsulated in SLN}}{\text{Initial amount of drug}} \times 100$$

$$DL\ (\%) = \frac{\text{Amount of drug encapsulated in SLN} \times 100}{\text{Weight of SLNs}}$$

Surface Morphology: The morphology of AZT-SLN was evaluated using the Transmission Electron Microscopy (TEM) (JEM-2100, JEOL Ltd., Japan) at Advanced Instrumentation Research Facility, J.N.U., New Delhi). Briefly, a drop of diluted colloidal dispersion of nanoparticles was placed on a copper grid and stained with 1% w/v of Phosphotungstic Acid (PTA). After 5- 10 minutes the grid was loaded on microscope for imaging.

***In vitro* drug release study**

The *in vitro* drug release was determined by using the conventional dialysis bag diffusion method. Briefly, a small amount of the AZT-SLN(equivalent to 1 mg of drug) was kept in a dialysis bag (cellulose acetate membrane with molecular weight cut-off value of 10,000) (HiMedia, Mumbai, India) closed at both ends. The dialysis bag was immersed in a beaker containing 30 ml of dissolution medium (PBS pH 7.4) with 0.5% Tween®80 (to maintain sink condition). The beaker was maintained in a shaker water bath at 100 rpm at 37 ±0.5°C. At predetermined time intervals, 1 mL of sample was withdrawn from the dissolution media and analyzed by RP-HPLC method. Equal quantity of fresh media was added to maintain the definite volume.

***In vivo* studies**

Pharmacokinetic study with AZT-suspension and AZT-SLN

Plasma profile: Male Balb C mice (20-25 g) (42 mice) were utilized for pharmacokinetic and tissue distribution studies. The animals were kept in plastic cages in a 12 h dark–light cycle, with a controlled temperature and humidity conditions. Water and food were provided ad libitum throughout the study. The animals were housed in Central Animal Facility (CAF) of National Institute of Pharmaceutical Education and Research (NIPER). All protocols were approved by Institutional Animal Ethics Committee (IAEC) and experiments were performed in accordance with CPCSEA. All the in vivo experiments were performed according to the previously published article [22]. A single dose of AZT-suspension as well as AZT-SLN (encapsulated with an equivalent amount of drug, (3.2mg/mL) was administered orally to mice. AZT suspension was prepared by making homogenous suspension of AZT in water by trituration, using sodium carboxymethyl cellulose as suspending agent. At various time points the animals were bled by the retro-orbital vein and blood samples were obtained at 0.5h, 1h, 2h, 4h, 8h, 12h, 24h, 36h, 48h in case of AZT suspension and 0.5h, 1h, 2h, 4h, 8h, 12h, 24h, 36h, 48h, 3rdto5th day in case of AZT-SLN. Plasma was separated from blood samples and stored at -20°C. For analysis, 100 µl of plasma was deproteinized with 400 µL of Acetonitrile (ACN) and centrifuged at 10,000g for 5 min; the supernatant was analyzed on a dedicated HPLC system (as discussed in section 2.3.2)

Tissue distribution study: For tissue drug distribution study, the animals administered orally with a single dose AZT-SLN were sacrificed on 5th, 7th and 9th day and liver, lungs and spleen were excised. A 20% tissue homogenates of liver, lungs and spleen were prepared in normal saline and stored at -20°C for analysis. For analysis, 100 µl of tissue homogenate

was deproteinized with ACN and centrifuged at 10,000 rpm for 5 minutes. The supernatant was used for the estimation of azithromycin in free form. The pellet obtained on centrifugation was lysed with chloroform (1 mL) and used for the estimation of azithromycin in encapsulated form. Data interpretation was done by using Kinetica™ (Kinetica DB Inc., USA) and pharmacokinetic parameters such as Cmax(maximum concentration), tmax (time to reach maximum conc.), t1/2 (half life), AUC (area under curve) , MRT (mean residence time) were calculated [22,24].

Antimicrobial efficacy

Zone of inhibition (ZOI) assay of AZT-suspension and AZT-SLN: The antimicrobial efficacy of the AZT-SLN against *Staphylococcus aureus* strain (SA 22359) was determined by the Zone of Inhibition Test and compared with AZT suspension. Briefly, an aseptically prepared overnight culture (10^8 CFU/mL) in Luria broth was prepared and 100µL were spread on Luria agar plates. Wells (d = 5 mm) were aseptically bored using a 5 mm sterile tip and 100 µL each of AZT suspension and AZT-SLN (at different concentrations) were inoculated into each well. Wells containing empty-SLN served as a negative control. The plates were incubated at 37°C for 24 h and zone of inhibition around each well was measured [25].

Minimal inhibitory concentration (MIC) determination of AZT-suspension and AZT-SLN by microtiter broth dilution method: Resazurin-based 96-well plate microdilution method, was used for the determination of minimum inhibitory concentration [26]. In a 96 well microtiter plate, first two rows i.e. Rows A and B correspond to AZT- suspension while the 4th and 5th rows (D and E) correspond to AZT-SLN. In all these wells, nutrient agar broth (100 µl) was dispensed. 50 µL of AZT- suspension/ AZT-SLN was added to column 1 of the respective wells.

The serial dilution of this added concentration was done from column 1 to 8 using multichannel pipette. Thus, the concentrations for AZT suspension/AZT-SLN achieved through serial dilutions from columns 1 to 8 were 20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.106 µg/ml. 50 µl of the bacterial suspension (conc. 1 x106 CFU/mL) was then added to all wells containing AZT- suspension or AZT-SLN, except negative control wells, which contains only nutrient agar broth. The well contents were thoroughly mixed to obtain uniform suspension and the 96 well microtiter plate was incubated for 24 h at 37°C. Resazurin dye (0.015 %w/v) was added to all wells (30 µL per well) and the plate was further incubated for 2 to 4 h. On completion of the incubation, the plate was read on an ELSA reader at a wavelength of 595 nm. A change in colour of the dye from blue to pink due to reduction of dye indicates the growth of bacteria. The lowest concentration that showed inhibition of bacterial growth, as determined by no change in colour, was taken as Minimum Inhibitory Concentration (MIC) of that formulation [26].

Time kill assay: To investigate the sustained release nature of AZT-SLN, the time kill assay was performed. Mid log-phase cells of microbial strain *S. aureus* (1 x 10 6CFU/mL) were inoculated in flasks containing AZT- suspension (10 µg/mL), and AZT-SLN (10 µg/mL). The flask containing S. Aureus without any active agent served as control. Aliquots were removed at 12, 24, 48, and 72 h and serial dilutions were plated on nutrient agar broth. After the predetermined time intervals, total bacterial CFU/mL was determined [27,28].

Statistical analysis

The statistical analysis was run using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, California) by applying one-way analysis of variance (ANOVA). The statistical significant difference was considered if ($p < 0.05$).

RESULTS AND DISCUSSION

Preparation of azithromycin loaded SLNs (AZT-SLN)

In this study, azithromycin was encapsulated into solid lipid nanoparticles (AZT-SLN) to enhance its oral bioavailability. For making SLNs, glyceryl monostearate [Imwitor®] was selected as solid lipid and Tween®80 as a surfactant. Several batches of AZT-SLN were prepared by varying the drug : lipid ratio from (1:1 to 1:5) and concentrations of surfactant (Tween®80). The formulations with high drug : lipid ratio showed less entrapment of drug (<45%), while those with low ratio (1:3 to 1:5) showed higher particle size (>500 nm).

The formulation with 1:2 drug : lipid ratio and 1% surfactant (Tween®80) was selected as the optimal formulation condition, as it yielded higher entrapment of the drug inside the nanoparticles and was of optimum particle size. The nanoparticles produced were further characterized for their

characteristics, loading capacity and evaluated for their *in vitro*/ *in vivo* performance.

Characterization of azithromycin loaded SLNs

The average particle size of the nanoparticles with optimized drug: lipid ratio of 1:2 was found to be 380.33 ± 13.31nm with a PDI of 0.25 ± 0.06. The AZT-SLN had an EE of 66.73 ± 4.02 % w/w and a DL of 46.56 ± 0.65 % w/w. Table 1 depicts the characteristics of the optimized formulation AZT-SLN.

Table 1: Characteristics of AZT-SLN w.r.t. Size, PDI, % EE and % DL.

Particle size (nm)	PDI	% (w/w) EE	% (w/w) DL
380.33 ± 13.3	0.25±0.06	66.73 ± 4.02	46.56 ± 0.65

The surface morphology of the prepared SLNas evaluated by TEM depicted the nanometric range and spherical-shaped particles with smooth and round edges (Figure 1).

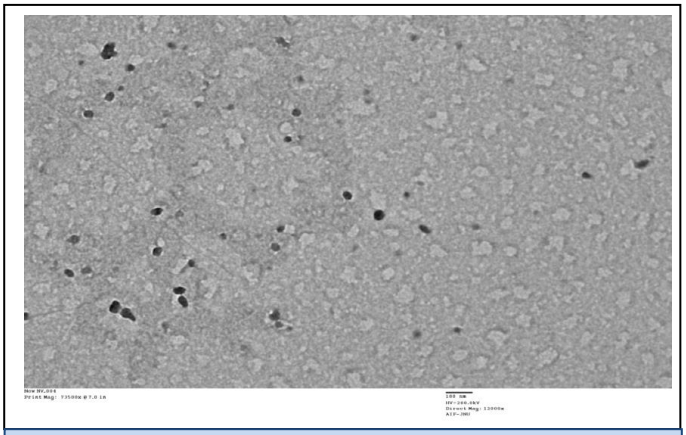

Figure 1: TEM image of AZT-SLN.

***In vitro* drug release study**

The *in vitro* drug release profile of AZT-SLN with PBS (pH7.4) showed a biphasic drug release pattern (Figure 2). There was an initial fast release of AZT upto 12 h with nearly 35% of the drug released. After 12 h, a slower and sustained drug release profile continued upto 96 h with a cumulative drug release of about 60%. The observed low drug release was probably due to the hindrance in drug diffusion by the GMS matrix, which is composed of compact structure wherein its meshes do not allow flow of liquid phase and hence a significant presence of the drug remained within the SLNs [29]. The sustained release nature of the formulation could be advantageous to improve the oral bioavailability of the drug.

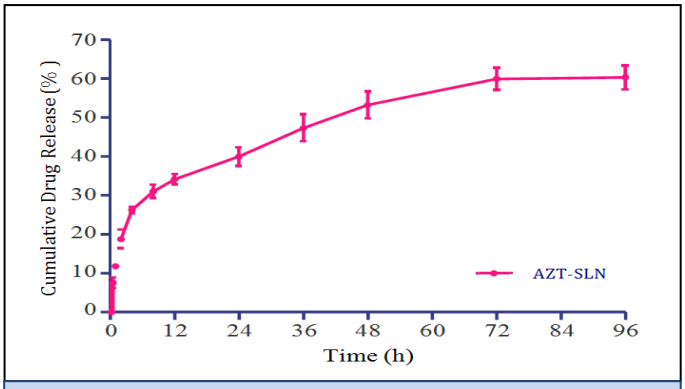

Figure 2: *In vitro* drug release profile of AZT-SLN.

Table 2: Pharmacokinetic parameters of Azithromycin-suspension and AZT-SLN.

Formulation	Cmax (µg/ml)	Tmax (h)	MRT (h)	t1/2 (h)	AUC	Relative Bioavailability (%)
AZT-Suspension	13.64	4	49.89	33.52	596.3	100
AZT-SLN	15.65	8	170.30	117.04	1496.05	251

***In vivo* studies**

In vivo studies were carried out in mice to assess the pharmacokinetic behavior of the formulation. The plasma profiles of AZT-suspension and AZT-SLN are shown in Figure 3 and the pharmacokinetic parameters are shown in Table 2. Following a single oral dose of AZT-SLN (3.2 mg equivalent dose of AZT), a sustained drug release behavior was observed, with drug levels being maintained above the MIC (2 µg/mL) upto 96 h. The initial spike in the release between 1-8 h could be due to the release of some adsorbed drug. After 24 h, the drug concentration again increased in plasma (7.5 to 9.5 µg/mL) and remained in systemic circulation with a slight decline over a period of 96 h, (9.5-3.5 µg/mL). This was due to the slow diffusion of drug from the interior of the nanoparticles. The lipophilic drug gets released slowly from the lipid matrix over a period of time and gets into systemic circulation. The plasma levels peaked at 8h and at 36h. In contrast, following a single oral dose of AZT- suspension, the plasma drug concentration peaked at 2h (13 µg/ml), with slight increase in drug level at 12 h and attained its lowest level at 48 hrs (5.5 µg/ml). After 48 h, the drug was not detected in plasma and hence was not shown any further in the graph.

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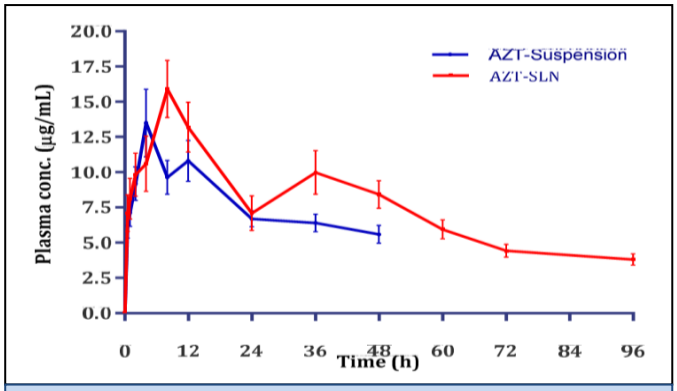

Figure 3: Pharmacokinetic profile of Azithromycin - suspension (AZT-suspension) and SLNs (AZT-SLN).

As depicted in Table 2, the MRT and $t1/2$ of AZT-SLN showed a ~3.5 fold and ~ 2 fold increase as compared to AZT-suspension. Thus, these results show an enhanced AUC and increased relative bioavailability of SLNs (2.5 fold) as compared to free drug. The enhanced bioavailability of SLN formulation could be attributed to direct uptake of nanoparticles through GI tract. Lipid particles have a high permeability to the intestinal membrane. The drug embedded into a solid lipid matrix, not only reduces the drug's exposure to enzymatic degradation during the process of absorption, but also offers a long contact time *in vivo* [30]. As the average particle size of SLNs were < 500 nm, they bypassed liver first pass metabolism and were taken up by M cells of Peyer's patches (lymphatic transport) directly into the systemic circulation [31,32]. Thus, an increase in the bioavailability of the drug encapsulated into SLNs was a result of combined mechanisms including a) adherence of the SLN to the mucosal wall, which allows penetration through the intestinal membrane, resulting in direct entry into systemic circulation; b) enhanced lymphatic transport, via the M cells of Peyer's patches, which can absorb particles up to 500nm in size [33].

In tissue distribution study (Figure 4), individual groups of mice were administered a single oral dose of AZT- SLN and sacrificed on 5th, 7th and 9th day. The liver, lungs and spleen were excised and 20% tissue homogenate was analyzed for drug content. The drug was detected in lungs, liver and spleen upto day 9 following oral administration of AZT-SLN. The amount of drug found in any of tissues at any point was above the MIC level (2 µg/ml). Thus, bactericidal concentration was maintained in tissues up to 9 days. This finding is also relevant to the fact that azithromycin gets deposited in tissues 30-50 times higher in concentration than in plasma [34].

In this case, both drug as well as SLNs are lipophilic. Hence, their affinity for the tissues is higher, as compared to blood. Thus, their tissue distribution is higher than in plasma. SLNs tend to deposit in the tissues for a longer period of time, causing a sustained release of the drug into the systemic circulation [34,35]. Thus SLNs can be used as an effective means of improving the biopharmaceutical performance i.e. MRT and bioavailability of the drug.

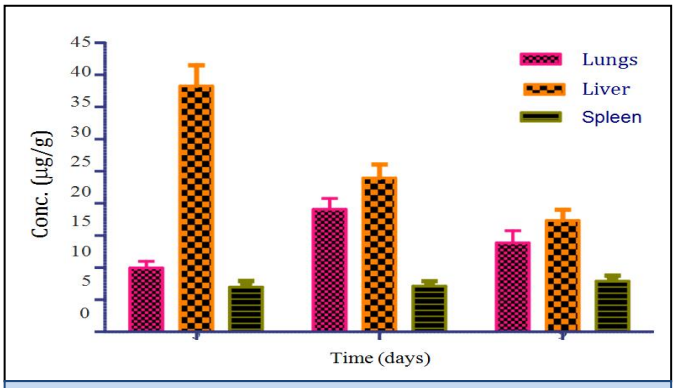

Figure 4: Tissue distribution profile of AZT-SLN.

Zone of inhibition: antimicrobial efficacy of AZT-SLN versus AZT suspension

To evaluate the influence of the increased bioavailability of the AZT-SLN formulation on the antimicrobial performance, the developed formulation was tested against *Staphylococcus aureus* bacteria and compared with AZT suspension. It was earlier reported that the encapsulation of the drug in SLN not only maintains, but also improves the antimicrobial activity against pathogenic microorganisms [36]. The antimicrobial effect was evaluated for AZT-suspension (10 µg/mL) and for AZT-SLN (10 µg/mL; 20 µg/ml). Wells containing empty- SLN served as negative controls (Figure 5). There was no significant difference in the ZOI between AZT-SLN (27mm) and AZT-suspension (28mm) at a conc. of 10 µg/ml. However with 20µg/ml, the zone of inhibition around wells containing AZT-SLN was more significant (38%), than the one containing 10µg/ml. The efficacy of AZT-SLN to inhibit *Staphylococcus aureus* increased with a higher concentration of encapsulated AZT in nanoparticles, as more amount of drug diffused out of SLNs. This showed that lipid matrix released the drug in a similar manner and that the release was proportional to different concentration tested No zone of inhibition was observed surrounding the empty SLN. This confirmed that lipid

has no antibacterial action and all bacterial inhibition was due to encapsulated AZT.

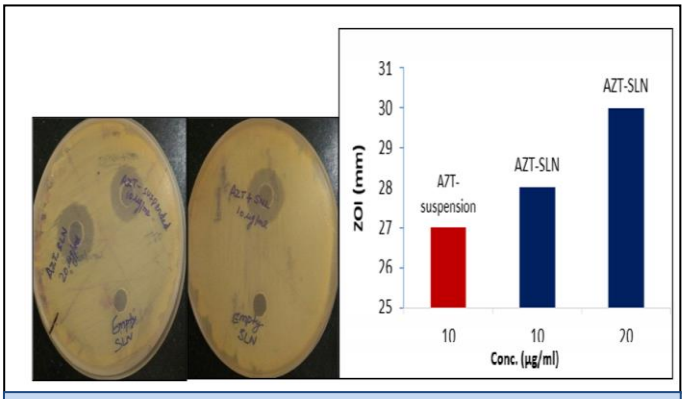

Figure 5: Zone of Inhibition assay of AZT-suspension and AZT-SLN against bacterial colonies of *Staphylococcus aureus*.

MIC determination by microtiter broth dilution method

For broth dilution, often determined in 96-well microtiter plate format, *S.aureus* bacteria were inoculated into a liquid growth medium in the presence of various concentrations of AZT-suspension and AZT-SLN (20 µg/ml to 0.105 µg/ml). Growth was assessed after incubation for 24h and the MIC value was read. Pink color denoted growth of bacteria, while blue color denoted inhibition of growth (Figure 6B). With AZT-suspension there was an inhibition in growth from 5 µg/mL to 20 µg/mL. On the other hand with AZT-SLN, the inhibition in growth was from to 2.5 µg/mL to 20 µg/mL. Our studies and results are in agreement with the earlier published report on the antibacterial activity of tilmicosin- SLN, which could inhibit the microbial growth at smaller concentration than pure drug itself [37].

Time kill assay

Studies conducted to investigate the role of intracellular active antibiotics in the elimination of intracellular *S. aureus* revealed that withdrawal of antibiotics over time leads to killing of host cells due to recurring infection caused by the surviving population of *S. aureus* inside the phagocytic cells [38]. This situation demands some alternative strategy which may help to eliminate intracellular pathogen load and also concurrently aid in survival of the phagocytic cells. The AZT-SLN formulation was developed in order to increase the drug availability inside the cells. AZT-SLN was found to exhibit sustained release properties and have increased residence time in the tissues as well as in plasma. To further test the effect of this sustained release formulation on the microbial population and growth, the time kill assay was carried out against *S. aureus* strain and results are summarized in (Figure 7).

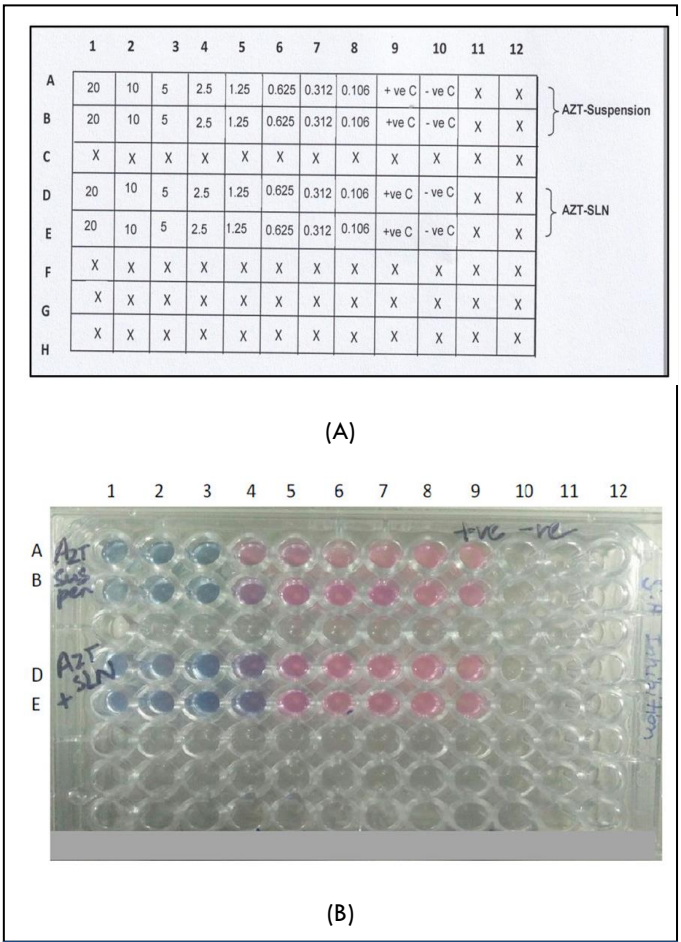

Figure 6: MIC assay by Microtiter Broth Dilution Method using 96 well plate (A) Setup of microtiter plate : Concentrations: Rows A & B (AZT-Suspension)- Well 1 : 20 µg/ml, Well 2: 10 µg/ml, Well 3: 5 µg/ml; Well 4: 2.5 µg/ml; Well 5: 1.25 µg/ml; Well 6: 0.625 µg/ml; Well 7: 0.312 µg/ml; Well 8: 0.106 µg/ml; Well 9 : +ve Control (+ve C); Well 10 : -ve Control (-ve C) Concentrations: Rows D & E (AZT-SLN)- Well 1 : 20 µg/ml, Well 2: 10 µg/ml, Well 3: 5 µg/ml; Well 4: 2.5 µg/ml; Well 5: 1.25 µg/ml; Well 6: 0.625 µg/ml; Well 7: 0.312 µg/ml; Well 8: 0.106 µg/ml Well 9 : +ve Control (+ve C); Well 10 : -ve Control (-ve C) X : Blank wells ; Positive control (+ve C) well contains nutrient agar broth and bacterial inoculum Negative control (- ve C) well contains nutrient agar broth only. (B) Result of MIC : Blue Color : Inhibition of growth; Pink color : Growth of bacteria.

All three groups, control group, AZT-suspension and AZT-SLN were inoculated with 6 log CFU/mL. The control group showed growth of more than 6 log CFU/mL after 12 h. The bacterial

colonies steadily increased at every time point and at the end of 72 h, the bacterial growth was > 9 log CFU/mL. On the other hand, the AZT-suspension showed some inhibitory action on bacterial growth upto 24 h. wherein the bacterial growth decreased from 6 log CFU/mL to < 4 log CFU/mL. Subsequently no significant effect was observed on bacterial growth and at the end of 72 h, the bacterial growth was around 4.5 CFU/mL. In case of AZT-SLN, the bacterial growth decreased to 4.5 log CFU/mL at 12 h, with a continuous decrease to < 2 log CFU/mL at the end of 72 h. The significant decrease in bacterial growth with AZT-SLN was due to the accumulation of the released drug over a period of time, resulting in inhibition in the growth of bacteria and a reduction in the number of bacterial colonies. The results are a confirmation of the entrapment of drug within the SLNs and the sustained release of drug from SLNs.

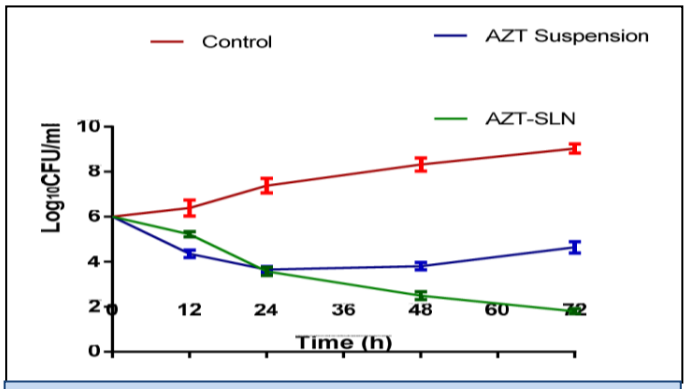

Figure 7: Colony forming Unit Assay (CFU) for AZT-suspension and AZT-SLN.

CONCLUSION

Azithromycin loaded solid lipid nanoparticles were prepared successfully by solvent diffusion evaporation technique. The particles were nanometric in size (< 400 nm), with uniform size distribution PDI < 0.5 and > 65% of drug entrapment efficiency. The *in vitro* studies showed that the formulation exhibited sustained release of the drug, which could be helpful in achieving sustained level of drug in systemic circulation. Pharmacokinetic and tissue distribution study also confirmed that AZT-SLNs could effectively deliver the drug in systemic circulation in a sustained manner and also get deposited in tissues. At all the time points, drug level was maintained above MIC in plasma upto 96 h and in tissues upto 9 days. The efficacy of the formulation against *S. aureus* due to the sustained release nature of the drug from AZT-SLN was corroborated by Time kill assay study which demonstrated reduction in the bacterial colonies for 3 days. The current results suggest that the AZT-SLN formulations can achieve sustained concentrations above MIC in systemic circulation, enabling inhibition of microbial growth. This sustained release nature of formulation can be helpful in reducing dosing frequency as well as reducing dose related adverse effects, which would eventually increase the patient compliance. Conclusively, AZT-SLN worked as a biocompatible carrier for sustained release of the drug for effective management of bacterial infections.

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REFERENCES

1. Lai PC, Walters JD. (2016). Relative effectiveness of azithromycin in killing intracellular Porphyromonas gingivalis. Clin Exp Dent Res. 2: 35-43.
2. Solleti VS, Alhariri M, Halwani M, Omri A. (2015). Antimicrobial properties of liposomal azithromycin for Pseudomonas infections in cystic fibrosis patients. J Antimicrob Chemother. 70: 784-796.
3. Das MC, Biswas A, Chowdhury M, Saha J. (2014). Screening Antimicrobial Susceptibility of Gentamicin, Vancomycin, Azithromycin,Chloramphenicol and Cefotaxime Against Selected Gram Positive and Gram Negative Bacteria. 2: 324-331.
4. Rakita RM, Jacques-Palaz K, Murray BE. (1994). Intracellular activity of azithromycin against bacterial enteric pathogens. Antimicrob Agents Chemother. 38: 1915-1921.
5. Stepanić V, KoŠstrun S, Malnar I, Hlevnjak M, Butković K, et al. (2011). Modeling cellular
6. pharmacokinetics of 14- and 15-membered macrolides with physicochemical properties. J Med Chem. 54: 719-733.
7. Pouretedal HR. (2014). Preparation and characterization of azithromycin nanodrug using solvent/antisolvent method. Int Nano Lett. 4: 103.
8. Aparna V, Shiva M, Biswas R, Jayakumar R. (2018).

Biological macromolecules based targeted nanodrug delivery systems for the treatment of intracellular infections. Int J Biol Macromol. 110: 2-6.

9. Hsu CY, Sung CT, Aljuffali IA, Chen CH, Hu KY, et al. (2018). Intravenous anti-MRSA phosphatiosomes mediate enhanced affinity to pulmonary surfactants for effective treatment of infectious pneumonia. Nanomedicine Nanotechnology, Biol Med. 14: 215-225.
10. Page-Clisson ME, Pinto-Alphandary H, Ourevitch M, Andremont A, Couvreur P. (1998). Development of ciprofloxacin-loaded nanoparticles: Physicochemical study of the drug carrier. J Control Release. 56: 23-32.
11. Combe B, Cantagrel A, Goupille P, Bozonnat M-C, Sibilia J, et al. (2003). Predictive factors of 5-year health assessment questionnaire disability in early rheumatoid arthritis. J Rheumatol. Canada. 30: 2344-9.
12. Müller RH, Maaßen S, Weyhers H, Specht F, Lucks JS. (1996). Cytotoxicity of magnetite-loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles. Int J Pharm. 138: 85-94.
13. Wissing SA, Kayser O, Muller RH. (2004). Solid lipid nanoparticles for parenteral drug delivery. Adv Drug Deliv Rev. 56: 1257-1272.
14. Pandey R, Sharma S, Khuller GK. (2005). Oral solid lipid nanoparticle-based antitubercular chemotherapy. Tuberculosis. 85: 415-420.
15. Garg NK, Singh B, Jain A, Nirbhavane P, Sharma R, et al. (2016). Fucose decorated solid-lipid nanocarriers mediate efficient delivery of methotrexate in breast cancer therapeutics. Colloids Surfaces B Biointerfaces. 146: 114-126.
16. Jain A, Sharma G, Kushwah V, Thakur K, Ghoshal G, et al. (2017). Fabrication and functional attributes of lipidic nanoconstructs of lycopene: An innovative endeavour for enhanced cytotoxicity in MCF-7 breast cancer cells. Colloids Surfaces B Biointerfaces. 152: 482-491.
17. Jain A, Kesharwani P, Garg NK, Jain A, Jain SA, et al. (2015). Galactose engineered solid lipid nanoparticles for targeted delivery of doxorubicin. Colloids Surfaces B Biointerfaces. 134: 47-58.
18. Pandey R, Khuller GK. (2005). Solid lipid particle-based inhalable sustained drug delivery system against experimental tuberculosis. Tuberculosis. 85: 227-234.
19. Sarmento B, Martins S, Ferreira D, Souto EB. (2007). Oral insulin delivery by means of solid lipid nanoparticles. Int J Nanomedicine. 2: 743-749.
20. Kumar VV, Chandrasekar D, Ramakrishna S, Kishan V, Rao YM, et al. (2007). Development and evaluation of nitrendipine loaded solid lipid nanoparticles: Influence of wax and glyceride lipids on plasma pharmacokinetics. Int J Pharm. 335: 167-175.
21. Luo YF, Chen DW, Ren LX, Zhao XL, Qin J. (2006). Solid lipid nanoparticles for enhancing vinpocetine's oral bioavailability. J Control Release. 114: 53-59.
22. Salah E, Abouelfetouh MM, Pan Y, Chen D, Xie S. (2020). Solid lipid nanoparticles for enhanced oral absorption: A review. Colloids Surfaces B Biointerfaces. 196: 1-18.
23. Nirbhavane P, Vemuri N, Kumar N, Khuller GK. (2017). Lipid Nanocarrier-Mediated Drug Delivery System to Enhance the Oral Bioavailability of Rifabutin. AAPS Pharm Sci Tech. 18: 829-837.
24. Zhao B, Gu S, Du Y, Shen M, Liu X, et al. (2018). Solid lipid nanoparticles as carriers for oral delivery of hydroxysafflor yellow A. Int J Pharm. 535: 164-171.
25. Sharma A, Pandey R, Sharma S, Khuller GK. (2004). Chemotherapeutic efficacy of poly (DL-lactide-co-glycolide) nanoparticle encapsulated antitubercular drugs at sub-therapeutic dose against experimental tuberculosis. Int J Antimicrob Agents. 24: 599-604.
26. Azhdarzadeh M, Lotfipour F, Zakeri-Milani P, Mohammadi G, Valizadeh H. (2012). Anti-bacterial performance of azithromycin nanoparticles as colloidal drug delivery system against different gram-negative and gram-positive bacteria. Adv

Pharm Bull. 2: 17-24.

27. Elshikh M, Ahmed S, Funston S, Dunlop P, McGaw M, et al. (2016). Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. Biotechnol Lett. 38: 1015-1019.
28. Loo YY, Rukayadi Y, Nor-Khaizura MAR, Kuan CH, Chieng BW, et al. (2018). *In Vitro* antimicrobial activity of green synthesized silver nanoparticles against selected Gram-negative foodborne pathogens. Front Microbiol. 9: 1555.
29. Zainin NS, Lau KY, Zakaria M, Son R, Abdull Razis AF, et al. (2013). Antibacterial activity of Boesenbergia rotunda (L.) Mansf. A. extract against Escherichia coli. Int Food Res J. 20: 3319-23.
30. Hüttenrauch R, Fricke S. (1979). Dependence of the release of active principles from ointment bases upon the degree of order of the solid phase. Pharmazie. 34: 437-438.
31. Muller RH, Keck CM. (2004). Challenges and solutions for the delivery of biotech drugs - A review of drug nanocrystal technology and lipid nanoparticles. J Biotechnol. 113: 151-170.
32. Trevaskis NL, Porter CJH, Charman WN. (2006). The lymph lipid precursor pool is a key determinant of intestinal lymphatic drug transport. J Pharmacol Exp Ther. 316: 881-891.
33. Kulkarni SA, Feng SS. (2013). Effects of particle size and surface modification on cellular uptake and biodistribution of polymeric nanoparticles for drug delivery. Pharm Res. 30: 2512-22.
34. Partridge GJ, Rao S, Woolley LD, Pilmer L, Lymbery AJ, et al. (2019). Bioavailability and palatability of praziquantel incorporated into solid-lipid nanoparticles fed to yellowtail kingfish Seriola lalandi. Comp Biochem Physiol Part C Toxicol Pharmacol. 218: 14-20.
35. Foulds G, Shepard RM, Johnson RB. (1990). The pharmacokinetics of azithromycin in human serum and tissues. J Antimicrob Chemother. 25: 73-82.
36. Li X, Chang S, Du G, Li Y, Gong J, et al. (2012). Encapsulation of azithromycin into polymeric microspheres by reduced pressure-solvent evaporation method. Int J Pharm. 433: 79-88.
37. Fazly Bazzaz BS, Khameneh B, Namazi N, Iranshahi M, Davoodi D, et al. (2018). Solid lipid nanoparticles carrying Eugenia caryophyllata essential oil: the novel nanoparticulate systems with broad-spectrum antimicrobial activity. Lett Appl Microbiol. 66: 506-513.
38. Wang XF, Zhang SL, Zhu LY, Xie SY, Dong Z, et al. (2012). Enhancement of antibacterial activity of tilmicosin against Staphylococcus aureus by solid lipid nanoparticles *in vitro* and *in vivo*. Vet J. 191: 115-120.
39. Dey S, Majhi A, Mahanti S, Dey I, Bishayi B. (2015). *In Vitro* Anti-inflammatory and Immunomodulatory Effects of Ciprofloxacin or Azithromycin in Staphylococcus aureus-Stimulated Murine Macrophages are Beneficial in the Presence of Cytochalasin D. Inflammation. 38: 1050-1069.