

Anticancer effect of atorvastatin loaded bsa nanoparticles biology essay

[Science](#), [Biology](#)



**ASSIGN
BUSTER**

Abstract:

The purpose of the study was to develop a new therapeutic approach for atorvastatin loaded BSA nanoparticles for controlled delivery to cancer cells. Atorvastatin is a drug widely prescribed to treat hypercholesterolemia. This HMG Co-A inhibitor blocks the mevalonate pathway that aids in the synthesis of cholesterol and various membrane proteins. Production of important lipid moieties like geranylgeranyl pyrophosphate and farnesyl pyrophosphate which help in the post translational modifications of the G protein subunits Ras and Rho are blocked by statins thereby exhibiting an anticancer effect. This property was exploited to make this a chemotherapeutic drug for treating various cancers. The disadvantages of the drug like low bioavailability and extensive hepatic first pass metabolism can be overcome by the encapsulation of the drug in a nanocarrier. BSA nanoparticles were preferred due to its biodegradable, non-toxic, non-immunogenic nature and its preferential accumulation in the tumour cells. Atorvastatin loaded BSA nanoparticles were synthesised by desolvation technique and characterisation was done using FE-SEM, Zeta sizer, FTIR and Fluorescence spectrophotometer. The nano drug formulation with the best encapsulation efficiency was taken for the in vitro studies. Cell viability was estimated using MTS assay and the toxicity was established. ROS assay was used to analyse the mechanism of toxicity and the extent of damage caused to the DNA was done through PI staining. Haemolysis assay was also done for the assessment of the toxic haemolysis potential of the nanoparticles. Keywords: Atorvastatin, BSA nanoparticles, anticancer effects, toxicity, MTS assay, ROS assay, PI staining.

1. Introduction:

The unique size-dependent and structural properties of nano scale materials have significantly impacted all spheres of human life making nanotechnology a promising field for biomedical applications. These nanomaterials can be used as an active ingredient in pharmaceutical products which can be used for imaging, diagnosis and therapy. The basic aim behind the nano pharmaceutical industry is to reduce the size of the drug to the nanometric regime and conjugate it with appropriate excipients namely polymer based nanoparticles, liposome and engineered viral nanoparticles. Natural macromolecules such as proteins have proved their efficacy in terms of their non-toxic nature, biocompatibility and possibly less opsonisation by the immune system. These molecules can also be used as charged moieties in which the drugs can be effectively encapsulated. Albumin can accommodate a wide variety of drugs in a non-specific fashion and hence can be used for encapsulating any drug. These colloidal nanoparticles have increased cellular uptake and the undesired toxicity in the body can be reduced. HMG Co-A reductase inhibitors also known as statins are group of eight compounds that are either synthesised artificially or by fungal fermentation. These compounds inhibit the 3-hydroxy-3-methylglutaryl coenzyme A in the rate limiting step of the mevalonate pathway, an important biosynthetic pathway for the synthesis of cholesterol. This makes it a widely prescribed drug for treating atherosclerosis and Coronary heart disease related to hypercholesterolemia. Mevalonate pathway also is the major pathway for the synthesis of membrane proteins and production of lipid moieties like geranylgeranyl phosphate and farnesyl pyrophosphate that aid in the post

translational modifications of G-protein subunits like Ras and Rho.

Atorvastatin calcium, [R-(R*, R*)]-2-(4-fluorophenyl)- β , δ -dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrole-1-heptanoic acid, calcium salt (2: 1) trihydrate, is one of the lipid lowering agent which is shown to exhibit anticancer properties. The drug also has been shown to exhibit toxicity in various cancer cell lines. It was also used in combination with the traditional anticancer drugs like doxorubicin and the growth of cancer cells was completely suppressed. Experiments were done in human neuroblastoma cells and it was unveiled that statins aid in the accumulation of doxorubicin. Multidrug resistance (MDR) has also been overcome when this drug combination was used in the studies in SH-SY5Y cells and the cell viability was greatly reduced. Though the drug is shown to have pleiotropic effects like anti-proliferation and anti-angiogenesis, it has many drawbacks. The drug is shown to have poor solubility, low bioavailability of about 12% and extensive hepatic first pass metabolism which requires frequent dosage of the drug to an individual. To overcome the disadvantages of the drug alone we resort to the usage of nanocarriers. Hence a biodegradable carrier like albumin can be used as an excipient for atorvastatin for the sustained release of drug to cancer cells. Moreover albumin has also shown to preferentially adhere to the tumour tissues making it an ideal carrier for drug delivery to cancer cells. In the present study we have formulated the synthesis and characterisation of drug (atorvastatin calcium) loaded BSA nanoparticles. The various parameters that influence the synthesis and the drug encapsulation efficiency were also studied. The in vitro anti-tumour activity of the atorvastatin loaded BSA

nanoparticles were evaluated using MiaPaCa-2 cell lines as model and various assays like MTS assay, ROS assay, PI staining, Haemolysis assay were done to assess the extent of toxicity of the nanoformulation. 2.

Materials and methods: 2. 1 **Chemicals** The following chemicals were used in the synthesis as received. Bovine serum albumin (BSA), ethanol (99% absolute alcohol) and glutaraldehyde (25%) (Desolvating agent and cross linking agent respectively), mannitol (cryo-protectant), MTS ((3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)), were obtained from Sigma Aldrich, India. Atorvastatin calcium powder used for the experiments was of pharmaceutical grade. Dulbecco's modified Eagle's medium (DMEM) was supplied from Invitrogen. Propidium Iodide (PI) was purchased from Hi-Media chemicals; India. Double distilled Millipore water was used for all the experiments.

2. 2 Cell line:

Human pancreatic cancer cell line (MiaPaCa-2) were obtained frozen in liquid nitrogen (-180°C) from National Centre for Cell Sciences (NCCS), Pune, India.

2. 2 Synthesis of BSA Nanoparticles

The desolvation process was first reported by Marty et al. Briefly 0.1 g of the protein was taken and mixed with 2 ml of water. The pH was made to 8 followed by addition of 8 ml of ethanol drop wise at the rate of 0.8 ml/min under constant stirring. An opalescent solution was observed indicating the formation of the nanoparticles. Then 100 µl of the 8% glutaraldehyde solution was added for cross linking for increasing the stability. The solution was kept overnight stirring to ensure the cross linking of all the amino acid

moieties. The stirred solution was then centrifuged at 15000 rpm for the nanoparticles to settle down. The supernatant was removed and hence the pellets of protein were freeze dried for about 24 hours with mannitol which acts as a cryo-protectant and then lyophilized (Lyophilizer- Christ Alpha 2-4 Lo plus). The lyophilised particles were then suspended in the phosphate buffer saline at pH 7. 4 which resembles the body environment so that the protein do not denature.

2. 3 Synthesis of Drug Entrapped BSA Nanoparticles

The drug was added to the protein solution and incubated overnight before the synthesis of the nanoparticles. Irache et al accounted on the sustained release of drug when it was incubated with the protein prior to the formation of nanoparticles. Incubation was followed by the production of the nanoparticles using desolvation process as mentioned above. The supernatant was collected after centrifugation at 14500 rpm. The coacervates obtained after centrifugation were lyophilised to obtain fine powder of the nanoformulation. Drug encapsulation efficiency was calculated by
$$\text{Encapsulation efficiency} = \frac{\text{Weight of the drug in nanoparticle}}{\text{Weight of the feeding drug}} \times 100$$

2. 4 Optimization of the process parameters:

Various parameters influence the synthesis of the BSA nanoparticles using desolvation technique. The parameters include pH, concentration of glutaraldehyde, and rate of addition of ethanol. These parameters were studied and then optimized for the synthesis of the nanoparticles to obtain minimal particle size and monodispersity.

2. 5 Characterisation of the prepared BSA and drug loaded BSA nanoparticles:

2. 5. 1 Hydrodynamic size and Zeta potential:

The mean particle size, zeta potential and the polydispersity index of the sample was determined using Photon Correlation Spectroscopy (PCS) using a Malvern Zeta sizer 3000 HS. He-Ne laser (633 nm) at a scattering angle of 90°C was used in the analysis of the drug entrapped and BSA nanoparticles.

2. 5. 2 Field emission Scanning Electron Microscopy (FE-SEM):

The morphological features of BSA nanoparticles and the drug entrapped particles were characterized by Scanning Electron Microscopy (JEOL JSM 670F-6701) at a voltage of around 3 keV.

2. 5. 3 FT-IR spectrometry:

FT-IR spectra were recorded on a Perkin Elmer FT-IR spectrometer (Perkin Elmer, India) in potassium bromide discs. Samples were ground with dry potassium bromide powder and compressed using a hydrostatic press. Scanning was done in a range of about 400-4000 cm⁻¹.

2. 5. 4 Fluorescence spectrometry:

Fluorescence scanning was performed using Perkin Elmer fluorescence spectrometer (Perkin Elmer, India). The excitation wavelengths used were 280 nm and 293 nm and the emission spectra were recorded to determine the residues that are involved in the drug-carrier interaction.

2. 6 Drug release kinetics:

The release of atorvastatin was determined by loading the sample in a dialysis bag immersed in Phosphate Buffered Saline (PBS) at pH 7. 4.

Samples were withdrawn at predefined intervals of time and the amount of drug released was calculated by the OD acquired using a UV visible spectrophotometer (Perkin Elmer Lambda 25, Perkin Elmer; India).

2. 7 In vitro assays for estimation of cell viability:

2. 7. 1 ROS assay:

2', 7'-dichlorofluorescein diacetate (DCFH-DA) was used in the estimation of the amount of ROS production in the cells. Dichlorofluorescein, a highly fluorescent compound is obtained when DCFH-DA passively enters the cell and reacts with the ROS. Briefly MiaPaCa-2 cells were seeded in the 96 well plates and incubated for 24 hours. The cells were then incubated with the different concentrations of the drug, drug loaded nanocarriers and nanocarriers for about 48 hours. The media was removed and washed with PBS thrice. Approximately 150µL of the reagent was added to the cells and then kept at 37°C for 1 hour. After the removal of the reagent, fluorescence was determined at 485-nm excitation and 520-nm emission using a micro plate reader. The ROS level is expressed as the ratio of $(F_{\text{test}} - F_{\text{blank}}) / (F_{\text{control}} - F_{\text{blank}})$. F_{test} is the fluorescence intensity of the cells exposed to the nano particles. F_{control} is the fluorescence intensity of the control cells. F_{blank} is the fluorescence intensity of the wells without MiaPaCa-2 cells.

2. 7. 2 MTS assay:

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) dye-reduction assay measuring mitochondrial respiratory function. Pancreatic cancer cell lines were seeded in a 96 wells and then treated with test samples at various concentrations. MTS dye was then added to the cells and incubated at room temperature for 4 hours. Absorbance was determined in a Tecan plate reader at 490 nm. The absorbance is directly related to viable cell number.

2. 7. 3 Haemolysis assay:

Blood was collected from healthy adult volunteers and loaded into test tubes containing EDTA. Dilution was done with 0. 9% saline . After that, 0. 2 mL of diluted blood was added into fresh test tubes containing test samples and incubated for 60 minutes at room temperature. Positive and negative controls were prepared accordingly and the haemolysis percentage (HP) was calculated by using the formula $HP (\%) = \frac{D_t - D_{nc}}{D_{pc} - D_{nc}} * 100$ Where D_t is the absorbance of the test sample and D_{pc} and D_{nc} are the absorbances of the positive and negative test samples respectively.

2. 7. 4 DNA fragmentation assay:

DNA fragmentation is one of the major characteristics of apoptosis of cells. DNA fragmentation can be determined by using agarose gel electrophoresis. Briefly 0. 52 g of agarose is mixed in TAE buffer for the casting of gel. Proteinase K and RNAase was added to the cell extract that is free from media and washed twice with PBS, for the complete removal of the proteins

and RNA. EtBr was used as an intercalating agent for DNA and the gel was run for about 1-2 hours depending on the voltage set. The gel with the DNA bands was then viewed with a UV-illuminator.

2. 7. 5 PI staining and FITC staining:

Fluorescence microscopy was used in the analysis of the cell structure and cell morphology after the treatment of the cells with the desired formulations. Propidium Iodide has a unique characteristic of binding to the nucleic acids. They cannot penetrate through the cell membranes that are intact. In other words, they permeate through the membrane of the apoptotic cells and intercalate with the DNA and RNA staining it red. For qualitative uptake studies, the cells were seeded with FITC labelled nanoparticles for four hours at 37°C and then washed three times with cold phosphate-buffered saline. Fluoroscopic images were then taken using.....

3. Results and Discussion:

3. 1 Influence of the process parameters:

Glutaraldehyde is used for cross linking of the amino acid residues on the BSA particles. The addition of glutaraldehyde was studied by varying the concentration from 4% to 25% to ensure effective cross linking. On 4% glutaraldehyde addition the cross linking of the amino acid residues of the protein nanoparticles was minimal and hence the nanoparticle yield was less. When 25% glutaraldehyde was added the particle size increased drastically and fell in the micro regime. During 8% addition of glutaraldehyde the cross linking was found to be optimum. So 100µl of 8% glutaraldehyde proved to

be sufficient for the linkage of all the amino moieties in the lysine group of the BSA particles. Another criterion that decides the formation of the nanoparticles is pH. Hence synthesis was performed at varying pH conditions (acidic and basic). The isoelectric point of the protein was found to be at 4.9. When the pH is far away from the isoelectric point the protein solvent interactions are higher and hence coagulation decreases by decreasing the particle size. The small negative charge that is present in BSA enhances the coacervation of the small particles at basic condition while at acidic conditions the electrostatic interactions gets altered thus enhancing the coagulation increasing the particle size. It was found that at pH 8 the particle had a more uniform distribution in size and was spherical. The rate of addition of ethanol has great influence in the particle size distribution. Puff structured nanoparticles were obtained when the rate of addition of solvents were particularly high. The rapid desolvation process leads to the formation of the large size particles and this rate has to be optimized. Uniform distribution was seen when the rate of addition of ethanol was about 0.8 ml/min and drug encapsulation within the nanoparticles was ascertained.

3.2 Surface morphology, Hydrodynamic diameter and Zeta potential:

ATV-BSANPs were prepared by one step desolvation process with ethanol and glutaraldehyde as desolvating and cross linking agents respectively. The particles prepared by this method were found to be highly stable in both water and cell media. The particle size was found to be in the range of about 97-125 nm and polydispersity index in the range of 0.29-0.40. Particles with uniform size were obtained by controlling the rate of addition of ethanol and

rotation speed of the magnetic stirrer. Hydrodynamic diameter of ATV-BSANPs was comparatively higher than the BSANPs alone thus enumerating the entrapment of the drug inside the nanoparticle. The stability of the nanoparticles can be attributed to the significantly higher zeta potential. Negatively charged particles contribute to the high stability of the colloidal solution. Columbic repulsive forces between the particles prevent them from agglomerating in the colloid state thus maintaining the stability of the system. The surface morphology of the particles was determined by the Scanning Electron Microscopy (SEM). The images of the BSANPs and ATV-BSANPs reveal a spherical morphology of the particles. Majority of the particles show uniform size distribution and no crystal precipitation of the particles were seen. Figure shows BSANPs and ATV-BSANPs freeze dried with mannitol. The aforementioned characterisation studies confirm that the particles were round and well dispersed without an aggregation.

3. 2 FT-IR spectroscopy:

Proteins are shown to exhibit characteristic amide I and amide II bonds in the FT-IR spectra. Amide I bonds can be directly related to the C=O stretching vibrations at 1650 cm^{-1} . Amide II bonds correspond to the C-N stretching vibrations and N-H deformational vibrations at 1520 cm^{-1} . At 1390 cm^{-1} the peak corresponding to the carboxylic anion can be seen. Amide III bonds ascribe to the 1220-1330 cm^{-1} peak range (C-N stretching vibrations and N-H rotating vibrations). The following table represents the characteristic peaks of the FT-IR spectra. On interaction we look for the amide I and amide II

bands frequency shifts. The shifts detect the secondary structural changes caused by the interaction of atorvastatin and BSA.

Groups

Peak Energy(cm-1)

-OH

3365. 5

-NH

3228. 3

-CH(aromatic)

2903. 5

-C= C-(aromatic)

1595. 1 There are no significant band shifts seen in the amide I and amide II bonds. Amide II band shifts (1500-1560 cm⁻¹) can be ascribed to the changes in the secondary structure of the proteins. The movement of the vibration frequency peak of the N-H and disappearance of 1543 cm⁻¹ and 1535 cm⁻¹ suggests indicated that the end residues are involved in the ATV binding to BSA. The sulphur groups in cysteine residues of BSA help in the interaction with ATV and also aids in the bond formation.

3. 3 Fluorescence studies:

The structure and the dynamics of the binding of protein molecules in a solution can be determined using fluorescence spectroscopic studies. They also give a clear picture on the internal environment around the vicinity of

the fluorophore. When HSA is excited at 293 nm a strong decrease in the intensity is observed in ATV-BSANPs on comparison with BSANPs alone. This quenching of the intensity can be attributed to the changes in the microenvironment of the tryptophan residues which suggests interaction of ATV and BSA. Tertiary structural changes can also be indicated through this significant decrease of intensity. The hydrophobic binding pocket in the sub domain IIA has undergone conformational changes which can be either substantiated with the possible interaction of BSA and ATV or overexposure of the residue to water. The same quenching effects can be seen in figure when the ATV-BSANPs are excited at 280 nm. This excitation wavelength corresponds to the tryptophan and tyrosine residues of the test sample. The microenvironment of both these residues can be well explained by the interaction of ATV and BSA which corresponds to the decrease in intensity seen.

3. 4 Drug release profile:

The release of the drug in the Phosphate Buffered Saline was monitored for about 72 hours continuously. Readings were taken at predefined time intervals and OD was estimated at an absorbance wavelength of 265 nm. Following the acquisition of the OD, concentration of drug released at each time interval was calculated from the standard graph containing known concentrations and OD. As we can see from figure, an initial burst release of the drug occurred during the first few hours. The plateau region seen after few hours corresponds to the sustained release of the drug making it an effective carrier for the delivery of anticancer drugs to the cells. The cross

linking of the BSA nanocarriers using glutaraldehyde is the major reason for the stability in PBS. The degradation of the nanoparticles is comparatively slow leading to the slow release of the drug in the media which makes it a novel system for the drug delivery.

3. 5 Cellular toxicity:

3. 5. 1 Estimation of ROS: