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Research Article

Development and Characterization of Docetaxel loaded Anti-FGFR-1Modified Solid Lipid Nanoparticles for Breast Cancer Targeting

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ABSTRACT

Surface modification of anticancer drug loaded particulate carriers using growth factor receptor inhibitors is an established approach to avoid dose related toxicity of anticancer drugs by selective distribution. Anti-FGFR 1 is one such approach that offers an excellent drug targeting to breast cancer cells because of it's over expression on the primary tumour as well as on metastatic sites. In our present study, anti-FGFR-1 monoclonal antibody appended docetaxel loaded solid lipid nanoparticles were developed using tristearin and were evaluated. Different formulation conditions were optimized and evaluated for the preparation of anti FGFR-1-conjugated lipid nanoparticles. The integrity of anti-FGFR-1 antibody conjugated on the lipid nanoparticles surface was analysed by SDS-PAGE and was confirmed by comparing it with the native anti-FGFR-1 antibody. Biological activity study conducted on MDA-MB-453 cell lines demonstrated that the anti-FGFR-1 antibody conjugated lipid nanoparticles can effectively target breast cancer cells and exhibited good cytotoxicity.

Keywords: Solid lipid Nanoparticles, Docetaxel, Anti-FGFR-1 Monoclonal antibody, Tristearin.

1. INTRODUCTION

Antibodies are well-established ligands and are extensively researched to target drugs or colloidal carriers to specific cell types particularly cancer cells (Reichert et al., 2005). This principle is based on a defined receptor Ligand interaction which enables the surface binding and even cellular internalisation of drugs or drug carriers conjugated to the antibody (Nahar et al., 2006). Chemical modification of the antibody has become necessary not only to enable their conjugation with a drug carrier (Keegan et al., 2004) but also to perform conjugation reactions maintaining biological activity and full receptor binding of the antibody. Anti-FGFR1 antibody is a humanised IgG1 monoclonal antibody directed against the extracellular domain of the human fibroblast growth factor receptor 1 (FGFR 1) (Eswarakumar et al., 2005). FGFR family contain a cytoplasmic tyrosine kinase domain, a single transmembrane domain, and an extracellular domain that is involved in ligand binding and receptor dimerisation (Jeffers.M et al., 2006). FGFR family consists of four FGFR receptors, FGFR 1, FGFR 2, FGFR 3, and FGFR 4 (Furdui et al., 2006). Over expression of FGFR 1 at the cell surface may lead to a spontaneous formation of homodimers, but more likely the availability of the receptor for ligand-driven hetero-dimerisation increases (Olsen et al., 2004). Moreover, FGFR 1 signalling is less accessible to normal inactivation processes ensuring rapid termination of signals (Smith et al., 2006). Therefore, FGFR 1 over expression translates into signals that potentiate deregulated growth, oncogenesis, metastasis and possibly resistance against apoptosis-inducing therapeutic agents (Beenken et al., 2009).

FGFR 1 is amplified at 20–30% incidence in human breast cancer. Amplification also occurs in ovarian, lung and gastric cancers, but the normal expression in adult tissue is weak (Xian et al., 2009). FGFR 1 amplification leads to a shortened time to relapse and overall survival. Anti-FGFR 1 represents a humanised anti- FGFR 1 antibody for the treatment of patients with metastatic breast cancer (Xu G, 2001). It acts in a cytostatic manner

by blocking and down modulation of the FGFR 1-receptor and by antibody dependent cellular cytotoxicity as major mechanism of antibody action (Jeffers.M et al., 2006). However, the mechanism of anti-FGFR 1 has not yet been defined compleately. It also induces anti-FGFR 1 internalisation and degradation in breast cancer cells (Wong et al., 2002) and the internalisation ability of FGFR 1 allows an efficient uptake of the antibody alone as well as conjugated to drugs or drug carrier systems (Shaul & Seger, 2007).

Direct linking of several drug molecules to an immunoglobulin is possible but may lead to a decreased biological activity and receptor specificity (Byron et al., 2008). Colloidal systems such as nanoparticles (Nahar et al., 2006) or liposome, on the other hand, provide higher drug carrier capacities than antibodies and, therefore, are especially suited for conjugation to antibodies (Hans M., 2002). Solid Lipid based nanoparticles represent a colloidal drug carrier system with high drug loading capacity. These particles are biodegradable, non-antigenic, non-irritative for tissues and non-toxic (Chen et al, 2001). It also provide protection against degradation and controlled release of the loaded drug (Bargoni et al, 1998). The aim of the present study was the direct covalent coupling of the anti- FGFR 1 antibody to the surface of Docetaxel loaded solid lipid nanoparticles in order to achieve a cell type specific drug carrier system.

2. MATERIALS AND METODS

2.1 MATERIALS

Drug, Docetaxel was a kind gift from Sun Pharma Advance Research Centre, Vadodara. DSPE (Di-sterayl Phosphatidyl Ethanolamine) and Hydrogenated Soya Phosphatidylcholine (HSPC) was a kind gift from Lipoid, Germany. Cholesterol (Chol), and Sephadex G-50 were purchased from sigma chemical Co., USA, and used as supplied. Glutaraldehyde was purchased from Rankem Laboratory reagents, New Delhi. Leibovitz's L-15 medium, Foetal bovine serum and Trypsin-EDTA solution were purchased from Hi-Media (Mumbai, India). The breast cancer cell lines MDA-MB-453 were obtained from the National Centre for Cell Science (Pune, India). It was maintained in Leibovitz's L-15 medium supplemented with 10% FBS, 2 to 3 ml of Trypsin-EDTA solution. All other chemicals used were of analytical grade and procured from local suppliers unless mentioned. Double distilled water was used throughout the study.

2.2. METHODS

2.2.1. PREPARATION OF DOCETAXEL LOADED LIPID NANOPARTICLES

The solid lipid nanoparticles were prepared by ethanol injection method (Stevens et al., 2004) with little modifications. Briefly, lipid mix containing HSPC, Tristearin, cholesterol and DSPE in 4:3.4:2.5:1.3 molar ratio was dissolved in ethanol at a concentration of 10 mg/ml and injected into stirred solution (1200 rpm) of phosphate buffer saline (pH 7.4) containing Docetaxel (Xt mgs) and Tween 80 (0.5%). Both the aqueous medium and the lipid solution were pre-warmed to and kept at 70–75ºC during the mixing. The preformed lipid suspension was then sonicated using a probe sonicator to form solid lipid nanoparticles. The unentrapped drug from SLNs suspension was removed by passing the suspension through Sephadex G-50 minicolumn. Elutes of free drug fractions and nanoparticle fractions collected quantitatively were pooled separately and estimated for the drug content (Xe) by UV spectrophotometry at 231 nm. The pooled fraction of lipid nanoparticle elutes were Lyophilized and stored in the refrigerator at 4°C. The percentage drug entrapment was calculated as under:

$$\% \text{ DEE} = [Xe / Xt] * 100$$

2.2.2. PREPARATION OF SOLID LIPID IMMUNONANOPARTICLES

The immunonanoparticles were prepared by the method reported by Hun et al (2009) with slight modifications. Briefly, 10 mg of the SLNs was dispersed in the PBS buffer (pH 7.4) containing 0.25% glutaraldehyde (100 fold molar excess) for about 2 h. The excess of Glutaraldehyde was removed from the nanoparticle suspension by dialysis using dialysis membrane (12 kDa) until the nanoparticle suspension yields negative result for free glutaraldehyde when tested using Schiff reagent. The nanoparticles were then incubated with anti-FGFR1 antibody (0.1µg/ml) for 12 h at 4^{o}C with shaking. The anti-FGFR1 antibody conjugated SLN's produced were recovered by centrifugation at 22000 rpm at 4^{o}C for 20 min and were stored at 4^{o}C. Percentage drug entrapment was determined by the method given above.

2.2.3. PARTICLE SIZE AND ZETA POTENTIAL ANALYSIS

The nanoparticles size was determined by dynamic light scattering (DLS) (Zetasizer 2000, Beckman Coulter Ltd. USA) at 25 °C at an angle of 90°. The particle dispersion was diluted with water to get the highest possible signal to noise ratio, yet small enough to prevent multiple scattering to occur. Zeta-potential measurements by laser Doppler electrophoresis were performed on particles redispersed in 10 mM NaCl at the same concentration as used for particle analysis (Zetasizer 2000,Beckman Coulter Ltd. USA). The average of three measurements were recorded.

2.2.4. MORPHOLOGY

To examine the shape and morphology of the nanoparticles, samples were analyzed using Scanning Electron Microscopy (SEM) after coating the nanoparticles with gold-palladium alloy (150-250Å) using a sputter coater.

2.2.5. IN-VITRO DRUG RELEASE STUDY

An amount equivalent to 10 mg of drug loaded nanoparticles were suspended in 1.5 ml of PBS (pH 7.4) and were incubated in 50 ml PBS in shaking incubator (LabTech, LSI- 2005RL) maintained at 37°C. At defined time intervals, Samples were withdrawn (4 ml) and was replaced with an equal volume of fresh buffer solution. The samples were centrifuged and the amount of drug (docetaxel) in the supernatant was estimated using UV spectrophotometer (λmax = 231 nm). Percentage drug release was calculated.

2.2.6. SDS-PAGE (SDS Polyacrylamide Gel Electrophoresis)

SDS gel electrophoresis was performed in order to authenticate the anti-FGFR1 antibody procured and to find out the integrity of the antibody in the immuno-nanoparticles prepared. Hence SDS-PAGE was performed, before and after the preparation of the final formulation. The integrity of anti-FGFR1 antibody in the immuno-nanoparticles was confirmed by comparing the SDS-PAGE result with that of the native anti-FGFR1 antibody.

2.2.7. CYTOTOXICITY STUDIES

Cell viability was tested using MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay on MDA-MB-453 cell lines. The assay is based on the cleavage of yellow tetrazolium salt MTT by metabolically active cells to form an orange formazan dye which was quantified using ELISA reader. Cells were seeded in 96 well microtitre plates (2×10^4 cells /200 µl growth medium/well) followed by overnight incubation. Supernatants from the wells were aspirated out and fresh aliquots of growth medium were added. After 24 h, supernatants were aspirated out and the cell monolayers in the wells were washed with 200 µl PBS (0.1M, pH 7.4). Subsequently, MTT reagent (150 µl, 0.8 mg/ml) was added in each well, incubated for 5 h. DMSO (dimethyl sulfoxide, 100 µl) was added in each well after aspirating out the supernatant and incubated at 37ºC for 1 hour. Absorbance at two wavelengths (570 nm for soluble dye and 630 nm for cells) were recorded using ELISA reader. Concentrations of samples showing 50% reduction in cell viability (i.e. IC_{50} values) were then calculated. An OD value of control cells (Unexposed cells) was taken as 100% viability (0% cytotoxicity).

2.3. Statistical analyses

All the experiments were carried out three times, independently. The data obtained were expressed in terms of 'mean ± standard deviation' values. Wherever appropriate, the data were also subjected to unpaired two tailed Student's t-test. A value of $p<0.05$ was considered as significant. Asterisk (*) in Figure denotes a statistically significant difference compared to control ($p < 0.05$).

3 RESULTS AND DISCUSSIONS

3.1 Preparation of Nanoparticles

The Ethanol injection method (Stevens et al., 2004) was used for encapsulating hydrophobic drug, docetaxel. An important step in the preparation of nanoparticles is the formation of the emulsion, as the droplet size determines the final nanoparticles size. Emulsion droplets smaller than 0.5 µm were obtained by sonication which eventually leads to the formation of particles of required size range. However, other parameters like emulsion volume and viscosity played an important role in controlling particle size of the SLN prepared. Furthermore, the drug payload during emulsification process affected the morphology and physio-chemical properties of the resultant nanoparticles. Hence major influencing parameters like drug to lipid ratio and sonication time were optimized keeping the other parameters like the composition of the lipid mix (10 mg HSPC, 3 mg DSPE, 3 mg Cholesterol) and sonication frequency (80W) constant.

3.2 Influence of Drug to lipid ratio

The effect of drug concentration on the particle size and entrapment efficiency of drug is shown in Fig.1. An inverse relationship between the drug concentration and the particle size was observed without affecting the Polydispersity index. This indicate the possibility of preparing fairly monodisperse particles over a size range of 100-150 nm by using fairly higher drug to lipid ratio (D/P ratio). However, with the increasing D/P ratio between 0.3 to 0.7, drug entrapment efficacy decreased gradually, but a sharp decrease in DEE was observed at D/P ratio of 0.7. The docetaxel recovery results indicated that the drug could be incorporated into SLNs up to approximately 56% in case of the batch with 0.7 D/P ratio. Increase in the sonication time (30-90 sec) decreased in particle size till 60 sec. as a result of smaller droplet formation and thus formation of smaller particle size (Fig 1). However, further increase in the sonication time (60 sec. to 90 sec.) resulted considerable increase in particle size probably due to aggregation of globules prior to the formation of particle as a result of high kinetic energy driven collision of globules.

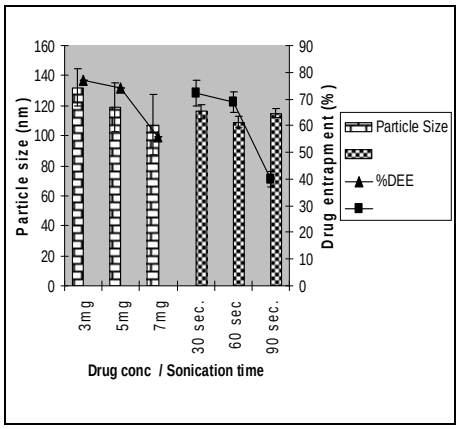

Fig. 1: Effect of drug concentration and sonication time on size of nanoparticles and % Drug Entrapment Efficiency

3.3 Morphology and Zeta potential

Morphological studies conducted using scanning electron microscopy showed the particles to be spherical, smooth and almost uniform in shape and size (Figure 2). The SLN samples exhibited zeta potential of -30.8 mV. The value is practically acceptable for electrostatic stabilization of non flocculated systems like SLN suspension even though some theoretical values suggested are ideally in the range of -30 to -60 mV. (Muller, & Heinemann, 1992)

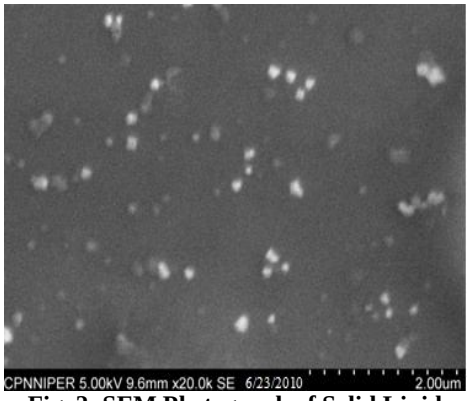

Fig. 2: SEM Photograph of Solid Lipid Nanoparticles

3.5 In-vitro Release Profile

In vitro drug release profile of lipid nanoparticles is shown in figure 3. The studies carried out in PBS (pH 7.4) at 37°C exhibited low but uniform drug release of around 30% in 48 hours characterizing very slow release probably due to low partitioning of the drug between the dissolution medium and the lipid mix in the SLN matrix. Low burst release (<10% of the loaded drug) observed here is an indicative of high encapsulation of the drug in the polymer matrix with insignificant adsorption of the drug on the particle surface. Kinetics of the drug release calculated from the profile was found to follow zero-order (r^2= 0.9874) confirming the release mechanism to be predominantly by lipid degradation rather than diffusion through the matrix (Higuchi's Kinetic model).

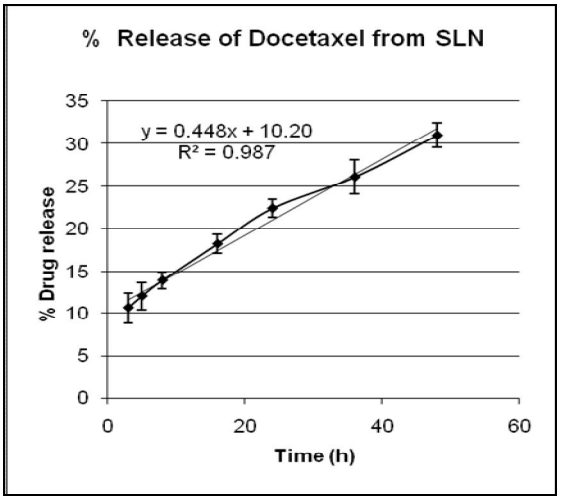

Fig. 3: In- vitro release profile of docetaxel loaded nanoparticles in PBS (pH 7.4) at 37°C

3.6 Conjugation of Anti-FGFR 1 Mab to Solid Lipid Nanoparticles

The major objective of this study was to prepare immune nanoparticles derived from solid lipid mix (HSPC, Tristearin, cholesterol and DSPE) containing amino groups at the ends, allowing nanoparticles surface modification for targeting purposes. Amine groups were chosen to couple targeting units by applying Glutaraldehyde chemistry in aqueous media. The coupling of anti FGFR 1 mAb (as an antibody for breast cell for targeting units) to Glutaraldehyde activated nanoparticles at the surface was studied. Figure 4 shows the scheme of the coupling mechanism of the Anti-FGFR 1 Mab at the surface of solid lipid nanoparticles.

The particle size and size distribution along with drug entrapment efficiency of docetaxel loaded immune nanoparticles is shown in Table 1. The particle size of solid lipid immune nanoparticles was slightly higher than plain lipid nanoparticles and the polydispersity index was also higher. The reason could be the possibility of bridging of amine groups of two different particles through glutaraldehyde leading to increase in size and PDI of the particles. The anti-FGFR 1 antibody attachment slightly increases the nanoparticles size due to increased molecular dimension. However, this had little effect on drug entrapment efficiency.

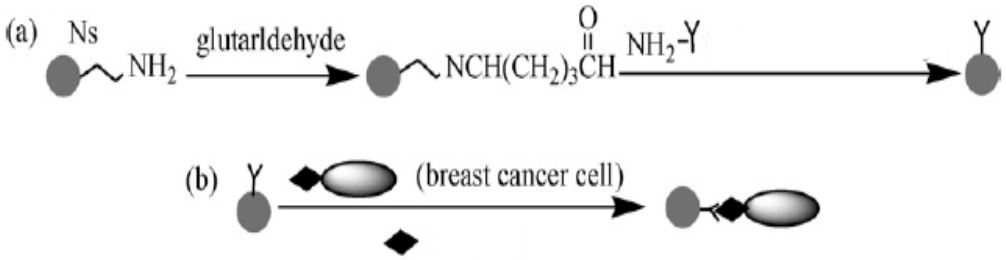

Fig. 4: (a) Shows the Glutaraldehyde mediated cross linking scheme of Docetaxel loaded Solid Lipid Nanoparticles to Anti-FGFR1 Antibody. (b) Shows the Anti-FGFR1 mediated uptake of Docetaxel loaded Nanoparticles to the Breast cancer cells expressing FGFR1 receptors.

Table 1: Represents Size, Polydispersity and Drug Entrapment Efficiency of Solid lipid Nanoparticles and Immunonanoparticles

S. No.	Parameters	Solid Lipid Nanoparticles	Immunoparticles
1	Particle size (nm)	109.5±13.5	128.4
2	Polydispersity index	0.286±0.03	0.321
3	Zeta Potential	-30.8±7.8	-22.96
4	% Drug entrapment efficiency	69.16±1.14%	57.12%

3.7 SDS-PAGE (SDS Polyacrylamide Gel Electrophoresis)

Anti-FGFR-1 antibody may become inactive due to irreversible denaturation and aggregation during the immunonanoparticle preparation process. The integrity of anti-FGFR-1 antibody after conjugation with the nanoparticle surface was analysed by SDS-PAGE (figure 5) in comparison with the native anti-FGFR-1 antibody. From the SDS-PAGE photos it can be concluded that antibody conjugated on nanoparticle surface was almost same in structure as the native anti-FGFR-1 antibody.

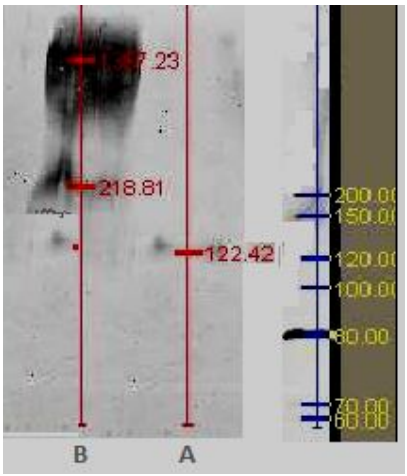

Fig. 5: SDS-PAGE of anti- FGFR1 antibody: (A) Native anti-FGFR-1 antibody and (B) FGFR-1 antibody after conjugation with the nanoparticle

3.8 CYTOTOXICITY STUDIES (MTT assay)

In cytotoxicity study, Docetaxel concentrations in all the formulations were adjusted to be the same. Percentage cell viability of all formulations at different concentrations in MDA-MB-453 cell lines was evaluated and documented in figure 6. Solid lipid immunonanoparticles showed significant cytotoxic effect at lesser drug concentration than solid lipid nanoparticles after 24 h incubation period in MDA-MB-453 cell lines.

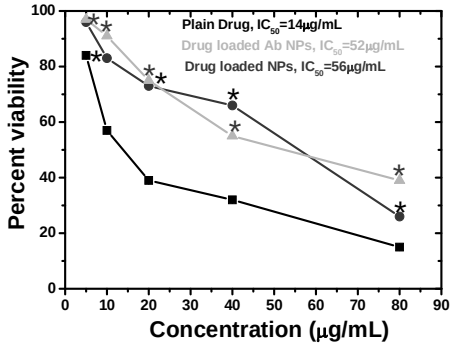

Fig. 6: Percentage cell viability of Docetaxel loaded AbNP (▲), Docetaxel loaded NPs (●) compared with the plain drug (■) in MDA-MB-453 cell lines

The IC_{50} of Solid lipid immunonanoparticles was 52 µg /ml in comparison to the simple drug loaded solid lipid nanoparticles (IC_{50} 56 µg/ml) after 24 h incubation period in MDA-MB-453 cell lines. However, the IC_{50} observed for the pure drug was

comparatively very low (14 µg/ml) on the cell lines obviously due to immediate availability of the drug for internalization. When solid lipid immuno nanoparticles when compared with simple SLN, cell uptake was more in case of Anti FGFR-1 antibody conjugated nanoparticles (Immunonanoparticles) than unconjugated lipid nanoparticles, indicating increased internalization of nanoparticles by antibody attachment. This proves the targeting potential of anti-FGFR-1 monoclonal antibody tagged on nanoparticle surface.

The blank solid lipid nanoparticles and immunonanoparticles when tested exhibited low cytotoxicity in MDA-MB-453 cell lines in comparison to pure drug. It implied that these nanoparticles could be useful as drug carriers without any cytotoxic effects of it's own. Such a nanoparticle system for drug delivery is multifunctional, which provides a way to formulate anticancer drugs with increased effectiveness and reduced side effects.

CONCLUSIONS

A solid lipid nanoparticle offers specific advantage of simple and rapid preparation method which is easy to scale up. Potential of this carrier system for receptor mediated target delivery is investigated in this study. Anti- FGFR-1 appended solid lipid immuno-nanoparticles loaded with anticancer drug docetaxel were prepared by direct covalent coupling of antibodies to Glutaraldehyde activated nanoparticles and evaluated for in-vitro cytotoxicity on MDA-MB-453 breast cancer cell lines. Immuno nanoparticles were of the size range suitable for IV administration and showed sustained released of the loaded drug. SDS-Page results confirmed the intactness of the antibody after conjugation to nanoparticles also. The results of ex-vivo cytotoxicity experiment on MDA-MB-453 cells proved the targeting capability of anti-FGFR-1 when appended on the lipid nanoparticles. Moreover this also concludes that proposed nanoparticle system was able to achieve a specific uptake in FGFR-1-positive breast cancer cells. This provides the basis for an efficient targeted delivery system design for docetaxel in SLN based system for breast cancer with possible reduction of side effects of the pure drug.

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