Chapter 10
Summary and Conclusion

10.1 Summary

Despite of intensive research and recent advances in drug delivery, the effective and non-toxic delivery of chemotherapeutic agents for the management of cancer remains a challenge for the pharmaceutical industry. Cancer is one of the most disastrous diseases for mankind from centuries even after a lot of research carried out in this arena. Breast cancer is the most frequently diagnosed cancer and now the leading cause of deaths amongst women. It is a malignant tumor that starts in the cells of the breast and grows into surrounding tissues or spread to distant areas of the body. Breast cancer can be treated by surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapies and biologicals. Cancer chemotherapy is generally accompanied by lack of specificity and is often associated with several severe toxicities including bone marrow depression, aplastic anaemia, lymphocytopenia and inhibition of lymphocyte function that results in suppression of host immunity. If an anticancer drug is delivered at the right site of action in the right concentration at the right time, cancer can be cured with very less side effects.

Docetaxel (DTX) and Vinorelbine Tartrate (VBT) are the active chemotherapeutic agents for treatment of metastatic breast cancer but their clinical applications are limited by high toxicity, rapid elimination from the systemic circulation, accumulation in non-targeted organs and tissues, enzymatic and hydrolytic degradation, and/or inefficient cell entry. Additionally polysorbate 80 used in commercial formulation of docetaxel (Taxotere®) have known to exhibit serious adverse effects including hypersensitivity reactions and peripheral neuropathy in human whereas the commercial formulation of vinorelbine tartarate (Navelbine®) is known to have injection-site reactions including transient local pain, swelling, erythema and the local reactions including phlebitis. In order to eliminate these side effects and to maintain the drug solubility of DTX various novel drug delivery systems were developed including liposomes, polymeric nanoparticles, solid lipid nanoparticles, polymeric micelles, polymer-drug conjugates etc. However, these novel drug-delivery systems also suffer from one or more drawbacks; specifically, low encapsulation efficiency and poor storage stability of liposomes; limited drug loading capacity and amenability for only small molecules in case of polymersomes and micelles; and small size of dendrimer causing their diffusion into unwanted regions.

Protein carriers are emerging as good alternative to overcome the challenges resulting from unfavorable properties of cytotoxic drugs and shortcomings of novel drug-delivery system in the development of chemotherapeutics. Albumin (Protein) nanoparticles encapsulating above cancer therapeutics is an appropriate option to overcome the limitations of currently available treatments. Because protein nanoparticulate system offers various advantages such as it protect therapeutic agents from degradation in the biological environment and mediate improved cell entry. Binding/encapsulation of anticancer drug to protein nanoparticles may diminish their toxicity, increase bioavailability, optimize their body distribution and may overcome multidrug resistance. Further surface modification of protein nanoparticulate system using cell surface receptor specific ligand help targeting cytotoxic drug to tumor and reduce the toxicity to the surrounding tissues.

The epidermal growth factor receptor (EGFR) is a cell-surface receptor belonging to ErbB family of tyrosine kinase and it plays a vital role in the regulation of cell proliferation, survival and differentiation. However EGFR is aberrantly activated by various mechanisms like receptor overexpression, mutation, ligand-dependent receptor dimerization, ligand-independent activation and is associated with development of variety of tumors. Therefore, specific EGFR inhibition is one of the key targets for cancer therapy. By combination of EGFR targeting agents and cytotoxics, synergy can be attributed to a great extent on cell division, apoptosis and angiogenesis.

Several anti-EGFR monoclonal antibodies have been used for cancer treatment in patients but they have a relatively large size which limits the number of ligands that can be linked to the surface of a nanoparticle and impedes intratumoral distribution due to interstitial tumor pressure. Generation of single-chain fragment variable (scFv) consists of antibody heavy and light-chain variable domains connected with a flexible peptide linker for EGFR targeting is a good option, as the single chain anti-EGFR antibody (scFv EGFR) provides much smaller ligand for targeting and overcomes the size based limitations of conventional antibodies.

Both DTX-HSA-NPs and VBT-HSA-NPs were prepared by different techniques. DTX-HSA-NPs were prepared by high-pressure homogenization technique (Nab Technology), whereas VBT-HSA-NPs prepared by desolvation method. Both nanoparticulate formulations were optimized using suitable optimization designs. Box-Behnken design was used to optimize DTX-HSA-NPs whereas Central Composite Design was used to optimize VBT-HSA-NPs. The results of optimization suggest that

optimized formulation of DTX-HSA-NPs consisted of DTX (0.83 mg), Albumin (4.92 mg) and Homogenization Pressure (15078.87 PSI) while VBT-HSA-NPs consisted of Albumin (17 mg/ml), Drug (1 mg/ml) pH (8.5) and Glutaraldehyde Conc. (125%). These optimized formulations were further characterized for several parameters including various physicochemical properties like particle size, zeta potential, morphology etc.; in-vitro drug release study; various in-vivo parameters like pharmacokinetics, acute toxicity, VBT induced phlebitis study and stability study.

The principle of the DTX-HSA-NPs formation by high pressure homogenization technique was crosslinking of protein (HSA) as result of exposure to high shear condition in a high pressure homogenizer. High pressure homogenization produces cavitation in the liquid that causes tremendous local heating due to high shear condition that result in the formation of superoxide ions which is capable of crosslinking the protein. A new crosslinking disulfide bond could be formed by disrupting existing disulfide bond or by oxidizing the sulfhydryl residues. The particle sizes, zeta potential and % EE of optimized DTX-HSA-NPs was found 150 ± 3.52 nm, -23.60 ± 1.34 mV and 70.65 ± 2.27 % respectively. DTX as such present in needle shape crystals and the presence of crystals in a drug formulation for intravenous injection is obviously detrimental due to potential blockage of capillaries. It is also known that with the increase of drug loading in a formulation, the tendency of crystallization also increases. X-ray powder diffraction and DSC analysis were performed to investigate the physical states of the drug in nanoparticles and it's very important aspect that could influence the *in vitro* and *in vivo* release of the drug from systems. From the XRD results of different samples it was concluded DTX in albumin nanoparticles was present in an amorphous form. In DSC analysis the DTX-HSA-NPs showed an identical result to blank NPs indicating no crystalline characteristic of DTX exists in the NPs. TEM imaging of optimized DTX-HSA-NPs exhibit a spherical shape without aggregation. The diameters of NPs were in the range of 140-200 nm similar to particle size results obtained by Malvern Zetasizer. *In vitro* drug release studies were carried out for both plain DTX solution and for DTX-HSA-NPs by dialysis method and results were compared by different kinetic models. The results of *in-vitro* drug release studies have confirmed that at the end of 8 h, 96.84 ± 1.132 % of the drug was released from plain drug dispersion compared to 43.78 ± 1.532 % from DTX-HSA-NPs while at the end of 24 h, 67.86 ± 1.54 % of the drug was released from the DTX-HSA-NPs indicating prolonged release of drug from DTX-HSA-NPs. After application of

different drug release kinetic models, it was observed that the DTX-HSA-NPs followed the Korsmeyer Peppas model because R^2 value (0.9885) was nearer to 1 and having n value of 0.5721 indicating that drug transport mechanism is non-Fickian transport. Stability study results of DTX-HSA-NPs for drug content and particle size were evaluated and it was observed that DTX-HSA-NPs were stable over the period of 6 months at 2-8°C. The IC_{50} of DTX-Solution and DTX-HSA-NPs determined in breast cancer cells and it was observed that DTX-HSA-NPs showed better cytotoxicity compared to Taxotere in cell-based *in vitro* cytotoxicity assays.

VBT-HSA-NPs were prepared using desolvation technique in which ethanol was used as desolvating agent and glutaraldehyde was added as cross-linker. After formation both formulations were lyophilized to improve their storage stability. In lyophilization, albumin itself act as cryoprotectant. The particle sizes and zeta potential of optimized VBT-HSA-NPs were measured in triplicates and the results were 154.3 ± 4.72 nm and -15.6 ± 2.074 mV respectively. The % EE of VBT-HSA-NPs was found 75.24 ± 2.23 %. The TEM images of the nanoparticles revealed their regular spherical shape, as well as a range of diameters. This is much closer to the ideal particle size for parenteral purpose, which is between 100 and 200 nm. For particles larger than 200 nm, the phagocytic uptake is faster because of enhanced opsonization. Particles smaller than 100 nm are able to cross the fenestration of the hepatic sinusoidal endothelium, accumulate in the liver and have the tendency of nonspecific uptake in all tissues. In DSC Thermogram, VBT-HSA-NPs no melting process was observed which indicates that nanoparticles were nanostructured and non-crystalline.

The in vitro drug release studies of VBT-HSA-NPs were carried out in trypsin phosphate buffered saline (pH 7.4) by dialysis method for 60 hr and the results were compared by different kinetic models. An initial burst of more than 60% of the plain VBT in first 6 hr was observed then a slow release up to 24 hr. A cumulative release reached 96% for plain VBT, while VBT-HSA-NPs showed a slow release of drug up to 60 hr, releasing approximately 86% of VBT. It is evident that the sustained release of drug from VBT-HSA-NPs will provide a better therapeutic efficacy than plain VBT. After application of different drug release kinetics models, it was observed that the VBT-HSA-NPs followed the Korsmeyer Peppas model because R^2 value (0.9379) which was nearer to 1 and having diffusional release exponent (n) value of 0.3798 indicating that drug transport mechanism is Fickian diffusion even though, the value lies below 0.5. Stability study results of VBT-HSA-NPs for drug content and particle

size are evaluated and it was observed that VBT-HSA-NPs were stable over the period of 6 months at 2-8°C. The IC_{50} of Navelbine and VBT-HSA-NPs determined in tumor cells and it was observed that VBT-HSA-NPs shown better cytotoxicity compared to Navelbine in cell-based in vitro cytotoxicity assays. After IV administration VBT-HSA-NPs have not shown any sign of phlebitis indicate that VBT-HSA-NPs are safer than Navelbine.

In order to target EGFR, immunonanoparticles (INPs) were formulated. For targeting single chain anti-EGFR antibody was isolated and conjugated to optimized DTX and VBT NPs. Initially transformation of pDNA was successfully carried out in E.coli DH5α (for determination of pDNA) and BL 21 (DE3) (for protein expression). When transformed cells in E.coli DH5α and BL 21(DE3) were subjected to single colony purification, easily identifiable single colonies of pDNA transformed cells were observed on antibiotic containing LB-Agar medium plates. pDNA was successfully transformed and carefully isolated from the transformed cells using alkaline lysis method and purified pDNA was further confirmed by the restriction enzyme digestion studies. After single digestion studies, a single band of around 4.0 kilo base pairs was observed on the agarose gel indicating that the DNA was cut using Xho I and digested the pCYN2 B10 scFv leading to linear fragment of the said pDNA. The relative distance travelled by linearized plasmid DNA after digestion studies was compared with standard DNA ladder and results of the agarose gel electrophoresis confirmed that the size of linearized plasmid DNA under study was around 4.0 kilo base pair.

Protein expression study was successfully carried out in E.coli BL21 (DE3) strain. Expression of scFv was clearly observed after induction (Carried out at 30°C) of scFv using IPTG. The culture was then pelleted down and processed further for SDS-PAGE analysis. Results of the SDS PAGE analysis clearly indicated the induction of scFv in the cell lysate under the optimized conditions. Highly expressed band of scFv around 25 kDa (when compared to protein marker which is observed between band of 20 and 29 kDa). However, no induction of expression was observed in uninduced scFv. Results indicated that IPTG is helpful to induce the expression of 25 kDa scFv protein. Purification of histidine tagged protein was done by using HIS-Select Spin Columns and purified form of scFv protein clearly showed single band around 25 kDa. Purified scFv was quantified by using BCA protein assay kit and the yield of the protein was found to be 1311.5 µg/mL isolated from 1000 mL bacterial culture calculated from the standard curve.

Further the optimized NPs were conjugated with scFv EGFR for site specific targeting by carbodiimide reaction. These targeted nanoparticles were characterized for particle size, zeta potential, in-vitro drug release, stability etc. to determine whether conjugation had any effect on such parameters. Additionally SDS-PAGE was performed to determine whether scFv EGFR has conjugated to the nanoparticulate surface or not. The targeted nanoparticles were also checked for their immunoreactivity by FACS. The mean particle size prepared DTX-HSA-INPs and VBT-HSA-INPs were found 160.7 ± 5.5 nm and 164.5 ± 5.2 nm respectively and TEM imaging of optimized DTX-HSA-INPs and VBT-HSA-INPs shown spherical shape without aggregation. Slight decrease in drug release of DTX-HSA-INPs and VBT-HSA-INPs was observed compared to DTX-HSA-NPs and VBT-HSA-NPs respectively. DTX-HSA-INPs followed the Korsmeyer Peppas model because R^2 value (0.9873) which was nearer to 1 and having n value of 0.5746 indicating that drug transport mechanism is non-Fickian transport. VBT-HSA-INPs followed the Korsmeyer Peppas model because R^2 value (0.9401) which was nearer to 1 and having n value of 0.3931 indicating that drug transport mechanism is Fickian diffusion.

MTT assay is a widespread method to assess cell cytotoxicity. In vitro cytotoxicity studies were performed on EGFR positive (MDA-MB-468, MDA-MB-231) and EGFR negative (MCF-7) cell line and it is demonstrated that scFv EGFR antibody conjugated NPs showed higher cytotoxicity compared to non-targeted NPs at all concentrations and at all-time points in MDA-MB-468 and MDA-MB-231 cells than MCF-7 cells. Enhanced cytotoxic activity of DTX-HSA-INPs and VBT-HSA-INPs for MDA-MB-468 and MDA-MB-231 cells as compared with MCF-7 cells clearly demonstrates the high affinity of INPs towards EGFR positive cancer cells. Qualitative uptake study results of coumarin HSA-INPs (For DTX) and rhodamine HSA-INPs (For VBT) by confocal microscopy showed augmented fluorescence activity in MDA-MB-468 and MDA-MB-231 cells treated with INPs as compared to MCF-7 cells. The enhanced cellular uptake INPs in MDA-MB-468 and MDA-MB-231 cells explained on the basis of EGFR expression in cells and specificity of EGFR receptor mediated binding of INPs. Moreover, the enhanced uptake level of INPs can be attributed to the lesser binding and uptake of INPs by non-EGFR expressing MCF-7 cell lines.

Quantitative intracellular uptake of NPs in MDA-MB-468 cell line was performed by flow cytometry and it was observed that the intracellular uptake of NPs was incubation time dependent. MFI of coumarin HSA-INPs (1142.5 ± 19.3) is more

than thrice than that of coumarin control (380.9 ± 21.1) and twice than that of coumarin HSA-NPs (575.8 ± 24.7) after 180 min whereas the MFI of rhodamine HSA-INPs (1245.6 ± 17.8) is about thrice than that of rhodamine control (420.9 ± 20.7) and 2.5 times than that of rhodamine HSA-NPs (508.7 ± 23.6) after 180 min due to receptor (EGFR) mediated endocytosis. The fluorescence intensity increased gradually with the incubation time and so also the uptake of NPs indicating time dependent uptake. Results clearly indicate the enhanced cellular uptake of INPs than non-conjugated and control system. The greater uptake of INPs can be explained on the basis of EGFR expression in cells and specificity of EGFR receptor mediated binding of INPs. Moreover, the enhanced uptake level of INPs can be attributed to the lesser exocytosis of the conjugated system than non-conjugated NPs. Higher cellular uptake observed with INPs is attributed to their greater intracellular delivery by receptor mediated endocytosis.

Apoptosis studies were carried out using annexin V procedure to determine whether drug loaded NPs caused apoptosis or necrosis of MDA-MB-468 cells. After 24 h and 48 hrs of exposure with Taxotere solution only 6.2 and 5.4 % cells were in apoptotic phase (early and late apoptosis) whereas exposure with DTX-HSA-NPs and DTX-HSA-INPs showed 34.2 % and 60.9 % cells in apoptotic phase which increased to 38.2% and 56.2%, respectively. After 60 and 90 hrs of exposure Navelbine, VBT HSA NPs and VBT HSA INPs showed 5.9 %, 9.2 % and 24.5 % cells in apoptotic phase which increased to 6.4 %, 19.1 % and 54.7 %, respectively. With increase in exposure time the percentage of cells in early apoptotic phase decreased, at the same time percentage of cells in late apoptotic phase were found to be increased. This is possibly due to conversion of early apoptotic phase into late apoptotic phase. Similarly, percentage of cells undergone necrosis also increased with time but in less proportion than apoptosis indicating apoptosis as the possible mode of cell death. Targeted drug delivery showed enhanced apoptosis due to receptor mediated endocytosis which resulted in higher concentration of drug available for action at target site.

Cell cycle analysis demonstrated that DTX treated cells showed stronger arrest at G0/G1 phase as compared to nanoparticulate formulations. INPs showed lesser percentage of cells at G0/G1 phase (36.57 %) as compared to 63.24 % and 55.57 % cells in PBS (control) and Taxotere treated cells. However, exposure to INPs showed stronger arrest at G2/M phase (27.64 %) which was almost 1.67 times higher than Taxotere. Whereas in cell cycle analysis VBT treated cells showed stronger arrest at

G0/G1 phase as compared to nanoparticulate formulations. INPs showed lesser percentage of cells at G0/G1 phase (40.85 %) as compared to 63.34 % and 58.74 % cells in PBS (control) and Navelbine treated cells. However, exposure to INPs showed stronger arrest at G2/M phase (24.31 %) which was almost 2.05 times higher than Navelbine. Greater efficiency of INPs in arresting more number of cells depends on the intracellular drug level in the target cells due to enhanced cellular uptake following receptor mediated endocytosis and sustained drug release as compared to drug solution and non- targeted NPs.

DTX-HSA-NPs showed significantly higher AUC, MRT and $t_{1/2}$ (16.89±1.54 µg. h/mL, 6.65±0.65 h and 7.45±1.24 h) compared to Taxotere® (8.79±1.12 µg. h/mL, 2.49±0.46 h and 2.69±1.65 h) whereas The DTX-HSA-NPs also showed significantly decreased in the clearance (592.2±15.1 mL/h) compared to Taxotere® (1136.9±23.5 mL/h). VBT-HSA-NPs showed significantly higher AUC, MRT and $t_{1/2}$ (40.03 ± 1.28 µg. h/mL, 33.25 ± 1.67 h and 33.34 ± 1.54 h) compared to Navelbine® (7.314 ± 2.13 µg. h/mL, 3.407 ± 0.85 h and 3.926 ± 1.35 h) whereas The VBT-HSA-NPs also showed significantly decreased in the clearance (124.90±11.62 mL/h) compared to Navelbine® (683.59±23.41 mL/h). The results of *In vivo* pharmacokinetic studies clearly shown that better pharmacokinetic properties of DTX-HSA-NPs and VBT-HSA-NPs compared to that of marketed formulations (Taxotere and Navelbine) respectively.

LD_{50} was determined by observing the signs of morbidity and mortality in the mice after dosage administration of all three formulations. LD_{50} of Taxotere was at 155 mg/kg which was concordant to previously estimated LD_{50} (>154 mg/kg) in mice via i.v. route (Taxotere Label Information). The LD_{50} of both DTX-HSA-NPs and DTX-HSA-INPs was about 200 mg/kg which is better than that of marketed formulation. LD_{50} of Navelbine was roughly 37 mg/kg which was concordant to previously estimated LD_{50} (36.1 mg/kg) in mice via i.v. route. The LD_{50} of both VBT-HSA-NPs and VBT-HSA-INPs was found 60 mg/kg which is better than that of marketed formulation.

VBT induced phlebitis study was performed in rats. Inflammation/edema of the vein was observed in Plain VBT solution whereas sign of inflammation/edema of the vein was not observed in other formulations containing VBT [Albumin + VBT Physical mixture, VBT-HSA-NPs and VBT-HSA-INPs] which confirmed that the entrapment VBT inside HSA reduces exposure to free VBT and thereby prevents development of inflammation/edema of the vein in rats.

10.2 Conclusion

To conclude DTX-HSA-NPs and VBT-HSA-NPs were successfully prepared and optimized by using different optimization designs. Further both the formulations were successfully conjugated with scFv-EGFR (DTX-HSA-INPs and VBT-HSA-INPs). Result of this study clearly showed that the specific binding (MDA-MB-468) and intracellular accumulation of biodegradable HSA-INPs in EGFR expressing breast cancer cells (MDA-MB-468 and MDA-MB-231). From the result of this study it can be concluded that the DTX-HSA-INPs and VBT-HSA-INPs are a promising tool for the specific transport of DTX and VBT to EGFR-expressing tumor cells. As the NPs provide the possibility of efficient drug transport, preventing adverse effects and injection site reactions. Due to the biocompatibility and specificity of the system and decreased toxicity profile of both the drugs (DTX and VBT), such a targeted formulation will provide better treatment option for the patients suffering from breast cancer.

Awards

1. Awarded with Prestigious National "**Ranbaxy Science Scholar Award-2013**" By Ranbaxy Science Foundation.
2. Selected as "**Maharashtra State Youth Icon**" by Sakal Media and Saam TV for Research on Breast Cancer in the field of Science and Technology.

Publications

Paper Published

1. **Yewale C**, Baradia D, Imran V, Patil S and Misra A. Epidermal Growth Factor Receptor Targeting in Cancer: A Review of Trends and Strategies. Biomaterials. 2013; 34(34):8690-707 **(Impact Factor (IF): 7.6)**
2. **Yewale C**, Baradia D, Imran V and Misra AN. Protein: Emerging carriers for Delivery of Cancer Therapeutics. In Expert Opinion on Drug Delivery. 2013; 10(10): 1429-48. **(IF: 4.87)**
3. Butani D. **Yewale C,** Misra AN. Amphotericin B Topical Microemulsion: Formulation, Characterization and Evaluation. Colloid and Surface B: Biointerfaces. 2014 Apr 1;116:351-8. **(I.F: 3.55)**
4. Patel H, **Yewale C**, Rathi M and Misra AN. Mucosal Immunization: A Review of Strategies and Challenges. Critical Reviews™ in Therapeutic Drug Carrier Systems. **(I.F: 3.02)**

Papers under Communication

5. **Yewale C**, Misra AN. Protein Nanoparticles of Docetaxel for Safe and Effective Management of Metastatic Breast Cancer.
6. **Yewale C,** Misra AN. Formulation and Optimization of Vinorelbine Tartrate Loaded Human Serum Albumin Nanoparticles Using Response Surface Methodology by Application of Box Wilson Central Composite Design.
7. **Yewale C**, Misra AN. Multifunctionality of Single Chain Antibodies: A Boon in Cancer Diagnostics and Therapeutics.
8. **Yewale C,** Patil S, Kolate A, Kore G, Misra AN. Oral Absorption Promoters: Opportunities and Challenges.

Book Chapters

9. Chapter Title: Application of Polymers in Transdermal Drug delivery System.
Book: Application of Polymers in Drug Delivery
Authors: **Chetan P. Yewale**, Hemal Tandel, Ambikanandan Misra
Publisher: Smithers Rapra, North America

10. Chapter Title: Conjugation of Monoclonal Antibodies to Increase Drug Delivery Efficiency and Its Specificity.
Book: Monoclonal antibodies – development, delivery and applications.
Authors: **Chetan P. Yewale**, Manisha Lalan, Ambikanandan Misra
Publisher: Future science group, Unitec House, 2 Albert Place, London, UK