Summary & conclusions

7. Summary and Conclusions

Cancer has become one of the most dreadful diseases to mankind. It has become the leading cause of death worldwide. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030. The cancer patients are poorly served by the present treatment options. While an arsenal of methods, including surgery, chemotherapy and radiation therapy are brought to bear on the disease, success is often marginal and serious side-effects are common. Thus, an important step in improving treatment regimes is to better harness the potency of chemotherapeutic agents by more effectively targeting them to tumor tissues. The effectiveness of cancer therapy in solid tumors depends on adequate delivery of therapeutic agent to tumor cells. Inadequate delivery would result in residual tumor cells, which in turn would lead to regrowth of tumors and possibly development of resistant cells.

The first successful chemotherapy of human cancer led soon to the realization that multidrug resistance (MDR) was going to be a major impediment to cure or long term palliation. It is defined as the resistance of the tumor cell to a broad spectrum of structurally and mechanistically diverse antitumor agents. Tumor cells carrying MDR phenotype are often associated with over expression of some of the drug efflux pumps, known as P-glycoprotein (Pgp) pumps and multidrug resistance associated protein (MRP) pumps. Pgp belongs to the ATP-binding cassette (ABC) family of transport proteins that represents one of the largest families of proteins in living organisms. The members of this family play a central role in cellular physiology. Pgp prevents intracellular accumulation of many anticancer agents (that are its substrates) and hence causes a reduction in their cytotoxic activity mainly by increasing cellular efflux of positively charged amphipathic drugs in an ATP-dependent manner.

The different strategies that can be used to inhibit or circumvent the Pgp pump are as described below:

- Formulation of anticancer agent into Novel Drug Delivery Systems like nanoparticles, liposomes, micelles etc.
- Non substrate strategy

- By interfering with ATP hydrolysis
- By altering integrity of cell membrane lipids
- Use of modulators or inhibitors
- Controlling the expression of MDR proteins.

Paclitaxel (PTX), discovered by the National Cancer Institute in 1967, is a diterpenoid pseudoalkaloid extracted from the bark of the Pacific yew or Western yew tree (*Taxus brevifolia*). It is found to be very effective against a broad spectrum of solid tumors including breast cancer, advanced ovarian carcinoma, lung cancer, head and neck carcinomas and acute leukemias. However, its low therapeutic index and low solubility in water and other acceptable solvents for parenteral administration limits its clinical use. Moreover, PTX is a Pgp substrate and hence there is a reduction in the efficacy of the drug as the intracellular concentrations are not maintained because of the drug efflux by Pgp.

By formulating PTX into Novel Drug Delivery Systems like nanoparticles (NPs) it is possible to avoid/inhibit the drug efflux by Pgp. The rationale behind association of drugs with colloidal carriers for reversal of MDR is the fact that Pgp probably recognizes the drug to be effluxed out of the tumoral cell only when it is present in the plasma membrane and not in case when it is located in the cytoplasm or lysosomes after its endocytosis. . It can be assumed that the Pgp substrates that are not able to access the transporter protein (Pgp) from within the plasma membrane are not recognized by Pgp efficiently. Hence the use of a drug delivery system to transport a Pgp substrate across the plasma membrane would allow to by-pass Pgp and result in intracellular drug accumulation. The other advantage of using these colloidal carriers are firstly the toxic effects of the anticancer agents will be reduced due to passive or active targeting of cancer cells. It has also been reported that drug encapsulated into nanoparticles and coated with stealth polymers like Pluronics® or PEG's are effective in overcoming the Pgp mediated MDR of cancer cells. Also the targeted NPs that target the drug to the cancer cells are endocytosed by receptor mediated endocytosis and are retained within the cell for longer time periods and because of receptor mediated endocytosis these NPs

are not recognised by Pgp present on the cellular membrane. Hence, we can conclude that drug loaded targeted NPs would be more effective in the treatment of MDR of cancer cells.

Poly (d,l-lactic-co-glycolic acid) (PLGA) and poly (n-butyl cyanoacrylate) (PBCA) was selected for the preparation of the polymeric NPs because of their excellent biodegradable and biocompatible attributes. Moreover, PLGA is approved by the US FDA and has a GRAS status. Pluronic®P85 is well known for its Pgp inhibitory properties. Hence, it was used to prepare Pluronic®P85 coated PLGA NPs loaded with PTX. Also it will increase the surface hydrophilicity of the NPs resulting in decreased opsonisation by plasma proteins and hence providing long circulating attributes to the NPs. Targeting of anticancer agents via transferrin (Tf) receptors which are over-expressed by 2- to 10-folds in most of the cancer cells have been demonstrated as an effective approach for treatment of MDR tumors. Hence, an attempt was made to conjugate Tf on the surface of PTX PLGA NPs with a view to actively targeting the MDR tumor cells and enhancing the therapeutic efficiency of PTX. Drug loaded PBCA NPs has been widely used for treatment of MDR tumors. Hence, PTX was entrapped into PBCA NPs for overcoming the Pgp mediated drug efflux.

In recent years, increasing attention has been addressed to solid lipid nanoparticles (SLNs), because of their biodegradability and ability to entrap a variety of biologically active compounds. Most of solid lipids have an approved status, such as the GRAS (Generally regarded as safe) status, due to their low toxicity. SLNs were prepared using Dynasan®118 as lipid material which is tri-glyceride derivative of 18 carbon fatty acid stearic acid and glycerol. SLN are stabilized by using poloxamer 188 which also has Pgp inhibitory properties.

7.1 Analytical methods

Drug identification by FTIR showed that the drug supplied by Sun Pharma Advanced Research Company Ltd. matches the reference FTIR spectra. The UV spectra showed a λ_{max} of 230nm in ethanol, acetonitrile, chloroform: methanol mixture (9:1). The UV spectrophotometric method for estimation of PTX in different formulations was

developed at λ_{max} of 230nm in acetonitrile. The interference of the polymers at 230nm was also checked and PBCA was found to interfere with analysis of PTX at 230nm. Hence, a first derivative spectroscopic method was developed for analysis of PTX in PBCA NPs. The zero crossing point (wavelength at which polymer has no absorbance/interference) for determination of PTX in PBCA NPs was 248.5nm. Phosphate buffer (pH 7.4) containing 20% ethanol (to enhance the solubility of PTX) was used as release medium to study the in-vitro release of PTX from NPs and SLNs. HPLC method was developed for determination of PTX in rat plasma and different tissue/organs of rats. Fluorimetric method was developed for determination of 6-coumarin (marker) in formulations and in cell lysates. A colorimetric method was developed for determination of residual PVA in PLGA formulations and also for determination of Tf by BCA protein estimation method. These analytical methods were evaluated for accuracy and precision and subjected to statistical analysis to establish the statistical parameters. The statistical results reveal that the developed methods were highly accurate and precise. The values of correlation co-efficient were found to be high which proves the goodness of fit of the developed methods for analysis of PTX.

7.2 PTX loaded PLGA NPs

PTX loaded PLGA NPs were prepared by nanoprecipitation technique. Nanoprecipitation technique involves a spontaneous gradient-driven diffusion of amphiphilic organic solvents into the continous aqueous phase resulting in precipitation of the polymer and simultaneous entrapment of the drug into the polymer. Ethanol is used in the organic phase at acetone to ethanol ratio of 4:1. Ethanol in the organic phase will reduce the solubility of PLGA in organic solvent which in turn will initiate early precipitation of the polymer upon contact with the aqueous phase and formation of a polymeric wall at a shorter distance from the primary nanodroplet centre, associated with a decrease in the size of NPs. The critical formulation variables (concentration of polymer, concentration of PVA and organic: aqueous phase volume ratio) were identified and optimised by a 3^3 factorial design. A multilinear stepwise regression analysis was performed using Microsoft Excel software and mathematical modelling was carried out to obtain a second-order polynomial equation (Full model equation). Reduced model equations were derived after neglecting the non-significant terms from full model. Results of

analysis of variance (ANOVA) of full model and reduced model indicated that the omitted terms were non-significant. Validation of established relationships confirmed that the differences between predicted and experimental values of responses were non-significant and that nanoprecipitation technique can be used for preparation of PTX loaded PLGA NPs. The optimised values of the critical variables are 150 mg of PLGA at a PVA concentration of 1% and organic to aqueous phase volume ratio of 0.5. The entrapment efficiency (EE) was found to be 76.31 ± 0.88 % with an average particle size (PS) of 169.3 ± 4.16 nm and zeta potential of -8.4 ± 1.6 mV. The optimised formulations were further surface modified with Pluronic®P85 and Tf for passive and active targeting respectively. Coating of Pluronic®P85 on surface of PTX PLGA NPs was done by overnight incubation with 1% solution of Pluronic®P85. The EE, PS and zeta potential of Pluronic®P85 coated PTX PLGA NPs was found to be 75.87 ± 0.95 %, 180 ± 5.32 nm and -3.45 ± 0.58 mV respectively. For the purpose of active targeting the PTX PLGA NPs were conjugated to Tf via the hydroxyl groups of residual PVA present on the PLGA NPs surface through an epoxy linker SR4GL. The EE, PS and zeta potential of Tf conjugated PTX PLGA NPs was found to be 69.47 ± 1.76 %, 182.8 ± 3.78 nm and -11.72 ± 2.27 mV respectively. Trehalose at 2 times with respect to the total solid content was found to be optimum to be added during freeze drying for preserving the particle size of the NPs. The in-vitro drug release profiles indicate a biphasic drug release pattern with an initial burst followed by sustained release effect. About 83% of the drug is released in 18 days in unconjugated NPs whereas in Tf conjugated NPs the drug release after 21 days were found to be 80%. These PTX PLGA NPs can be effectively used to target the resistant tumor cells. It can be concluded that biodegradable polymeric carrier systems made up of PLGA can effectively entrap PTX by nanoprecipitation technique and provide sustained drug release. NPs with suitable size for parenteral administration are formed by this technique.

7.3 PTX loaded poly(n-butyl cyanoacrylate) (PBCA) NPs

The poly (alkyl cyanoacrylate) NPs are reported as a novel tool to deal with resistant cancer because of its unique drug delivery mechanism. The PBCA nanoparticles are prepared by anionic polymerization reaction in aqueous acidic medium. The different factors that were found to affect the final PS and EE are monomer concentration,

surfactant concentration, pH, amount of drug, stirring speed and temperature. It was found that with increase in monomer concentration the PS and EE increased whereas with increase in the concentration of surfactant there was a decrease in PS and an increase in entrapment efficiency due to effective coverage of the porous particles which resulted in a decreased drug loss from NPs. The PS was found to increase significantly at 60°C temperature and also a decrease in entrapment efficiency was found. The optimum temperature was found to be 25°C. The particle size increases slightly with increasing stirring speed. This increase is caused by the higher kinetic energy of the system at higher agitation forces. It was observed that increase in concentration of PTX added during polymerization would increase the entrapment efficiency up to a concentration of 5mg in case of EP and 7.5mg for DP technique. The PS was found to increase with increase in PTX concentration. A pH of 2.5 was found to be optimum for obtaining sufficient drug loading. The optimised batch was prepared with 1% surfactant, 1% monomer, 5mg drug at a speed of 500rpm and pH of the polymerization medium was 2.5. The PS and EE were found to be 103.63 ± 6.45 nm and 75.93 ± 0.15% for emulsion polymerization and 194 ± 2.67 nm 78.13 ± 2.35% for dispersion polymerization respectively. Trehalose at 3 times the total solid content was used as a cryoprotectant during freeze drying to preserve the PS of the NPs. The in-vitro drug release study of lyophilized drug-loaded NPs conducted in phosphate buffer (pH 7.4) containing 20% ethanol showed biphasic release profile (initial burst release followed by sustained release) for emulsion as well as dispersion polymerization. We can conclude that PTX loaded PBCA NPs with high drug entrapment efficiency and smaller particle size can be formulated. This carrier system may be used for controlled release as well as for targeting PTX to the resistant tumor cells.

7.4 PTX loaded glyceryl tristearate (GTS) SLNs

PTX loaded GTS SLN were prepared by solvent diffusion technique at 10°C above the melting point of the lipid. The critical formulation variables (Drug: lipid ratio, concentration of lecithin and concentration of surfactant) were identified and optimised by a 3^3 factorial design. A multi linear stepwise regression analysis was performed using Microsoft Excel software and mathematical modelling was carried out to obtain a second-order polynomial equation (Full model equation). Reduced model equations

were derived after neglecting the non-significant terms from full model. Results of analysis of variance (ANOVA) of full model and reduced model indicated that the omitted terms were non-significant. Validation of established relationships confirmed that the differences between predicted and experimental values of responses were non-significant and that solvent diffusion technique can be used for preparation of PTX loaded GTS SLN. The optimised values of the critical variables are drug to lipid ratio of 1: 15 at 30mg lecithin and 1.5% surfactant (poloxamer 188) concentration. The EE was found to be 82.46 ± 3.49 % with an average particle size PS of 210.7 ± 1.92 nm and zeta potential of -28.7 ± 3.17mV. Trehalose at 3 times the total solid content was used as a cryoprotectant during freeze drying to preserve the PS of the NPs. PTX loaded GTS SLN exhibited a biphasic release profile consisting of an initial burst effect followed by a sustained release of PTX from the lipid matrix. The initial burst release is probably caused by the drug adsorbed on the nanoparticle surface or precipitated from the superficial lipid matrix. The sustained release following the initial burst release is probably due to the diffusion of drug from the lipid matrix. The XRD patterns of PTX, GTS and PTX GTS SLN reveal that the PTX in SLN is in amorphous state and the crystalline structure of the lipid has been modified by incorporation of PTX between the crystal lattice of the lipid. The DSC studies also revealed the amorphous nature of the PTX GTS SLN. The in-vitro steric stability test suggests that the PTX GTS SLN is sterically stable and hence there will be reduced opsonization of plasma proteins.

7.5 Stability studies

All PTX loaded nanoparticulate formulations stored at 5°C ± 3°C were in the form of white porous cake and were stable with no significant change ($p>0.05$) in PS, drug content, zeta potential and in-vitro drug release at the end of 6 months. All PTX loaded nanoparticulate formulations stored at 25 ± 2°C/60 ± 5% RH were in the form of white porous cake and were stable with no significant change ($p>0.05$) in PS, drug content, zeta potential and in-vitro drug release at the end of 3 months. But a long term stability study is required to establish the storage conditions. In the present study it can be concluded that the PTX loaded NPs formulations must be stored at 5°C ± 3°C for optimum stability of the formulations.

7.6 In-vitro cell line studies

The different formulations were subjected to in-vitro cell line studies including intracellular uptake (qualitative and quantitative) and cell cytotoxicity studies. C6 rat glioma cell line was used for the purpose as it is known to express Pgp that are responsible for multidrug resistance of tumor cells. PTX is a Pgp substrate hence it gets effluxed out of the cell resulting in a decreased efficacy of the antitumor agent. By formulation of PTX into nanoparticles the Pgp efflux can be bypassed. NPs labeled with 6-coumarin, a fluorescent lipophilic dye were used to study the intracellular uptake by fluorescent microscopy. From the fluorescent images it was evident that the green fluorescence in the cell cytoplasm was attributed to the endocytosis of the dye loaded NPs within the cell cytoplasm and not to the free dye. The fluorescence intensity was more pronounced in case of Tf-conjugated PLGA NPs (attributed to receptor mediated endocytosis) and Pluronic®P85 coated PLGA NPs in comparison with unconjugated PLGA NPs. The fluorescence was also pronounced in case of PBCA and GTS SLN.

From the cell cytotoxicity studies it was evident that blank NPs exhibited negligible cytotoxicity on the cells suggesting that NPs are suitable carriers for drug without any significant cytotoxic effects. PTX encapsulated into different nanoparticulate carriers showed a significant increase ($p<0.05$) in cytotoxicity as compared to the free drug as greater amount of PTX could be delivered intracellularly by endocytosis in the nanoparticulate form and also due to the sustained drug release feature of the nanoparticulate form. Also it can be concluded that PTX can be effectively delivered to resistant cells in the form of NPs without the drug getting effluxed out as in the case with free drug because C6 glioma cells express Pgp. In case of Tf conjugated PLGA NPs, receptor mediated endocytosis occurs which results in sustained drug delivery intracellularly. Since the action target of PTX is microtubules in the cytoplasm, the NPs are suitable carriers to deliver PTX into the cells.

7.7 In-vivo pharmacokinetic and biodistribution studies

Male SD rats weighing 250-300gms implanted with a subcutaneous C6 rat glioma were used for the in-vivo pharmacokinetic and biodistribution studies. Visible tumors were developed on the back of the rats around 14-15 days after inoculation of the C6 rat

glioma cells in the subcutaneous tissue of the rats. All the rats under study developed visible tumors indicating a 100% success rate for tumor development in SD rats. It serves as a useful model for studying new therapeutic modalities. PTX was administered by intravenous routed via the tail vein at a dose of 20mg/kg for the pharmacokinetic and biodistribution studies. The concentration-time profiles were fitted into non-compartmental model and the mean pharmacokinetic parameters were calculated. From the results it is evident that PTX solution is rapidly cleared from the blood compartment. In case of PTX encapsulated into different nanoparticulate formulations PTX was retained in the blood for more than 24 hrs indicating the long circulating properties of the NPs. This increase in the residence time may be attributed to decreased opsonisation from blood due to smaller size of nanoparticle (≤200nm) and hydrophilicity of the surface of NPs which imparts stealthiness. . The plasma $AUC_{(0\rightarrow\infty)}$, MRT and $t_{1/2}$ of PTX NPs (for all NP formulations) were found to be significantly higher than PTX solution. The MRT of PTX-PLGA NPs, Pluronic®P85 coated PTX-PLGA NPs, Tf conjugated PTX-PLGA NPs, PTX-PBCA NPs and PTX-GTS SLN was found to be 2.68, 3.0, 3.76, 3.11 and 3.13 folds higher than PTX solution respectively which further supports the long circulating properties of the drug loaded NPs. The $AUC_{(0\rightarrow\infty)}$ of PTX in PTX-PLGA NPs, Pluronic®P85 coated PTX-PLGA NPs, Tf conjugated PTX-PLGA NPs, PTX-PBCA NPs and PTX-GTS SLN was found to be 3.34, 3.61, 5.09, 4.06 and 4.2 times higher than PTX in solution

Significant portion of the injected dose was found to be distributed into the reticuloendothelial system organs. PTX concentration in liver was found to be less in case of NPs in comparison with the drug solution. The overall uptake of PTX NPs in comparison to PTX solution increased in the spleen after intravenous administration attributed to the retention of nanoparticles in the reticular fibre meshwork and the macrophages in red pulp of spleen resulting in high accumulation of NPs. The distribution pattern of PTX NPs and PTX solution in kidneys indicate higher values for solution than the NPs initially due to rapid clearance of PTX in solution whereas PTX concentration at 24 hrs post injection was higher in case of NPs than in drug solution. The concentration of PTX in heart in case of NPs was significantly lower than that in

solution indicating a potential reduction in cardiotoxicity in comparison to the drug solution.

Most important of all the tissues under study is the subcutaneous tumor. The tumor accumulation of free PTX was significantly ($p<0.05$) low at all time points in comparison to PTX NPs attributed to the Pgp present on the cell membrane that efflux free PTX out of the C6 glioma cells. When PTX in incorporated into the NPs, the Pgp cannot recognise PTX hence it does not get effluxed out. Hence, a greater concentration of PTX in tumor is achieved in case of NPs. Also, via active targeting with Tf it is possible to achieve higher PTX concentrations in tumor due to sustained intracellular drug delivery. The tumor $AUC_{(0\rightarrow\infty)}$ of PTX-PLGA NPs, Pluronic®P85 coated PTX-PLGA NPs, Tf conjugated PTX-PLGA NPs, PTX-PBCA NPs and PTX-GTS SLN was found to be 6.77, 7.71, 15.79, 8.14 and 8.9 folds higher than PTX solution respectively. The MRT of PTX in tumors in case of PTX-PLGA NPs, Pluronic®P85 coated PTX-PLGA NPs, Tf conjugated PTX-PLGA NPs, PTX-PBCA NPs and PTX-GTS SLN was found to be 6.8, 4.85, 9.03, 5.95 and 7 folds higher than PTX solution respectively. This can be attributed to inhibition of the drug efflux by membrane Pgp. Pluronic®P85 coated NPs show a greater accumulation of PTX in tumour in comparison to uncoated PLGA NPs because of the Pgp inhibitory activity of the Pluronic®P85. Also in case of PTX PBCA NPs and PTX GTS SLN due to the surface hydrophilicity because of Poloxamer 188 and due to its Pgp inhibitory activity. The effectiveness of drug loaded NPs as drug delivery systems can be attributed to their small size, reduced drug toxicity to other organs, controlled drug release and modification of drug pharmacokinetics and biodistribution. Tumor vasculature has been described as "leaky" due to the presence of interendothelial junctions and transendothelial channels, which for several tumor models have been reported to have sizes ranging between 0.2 and 1.2 μm. Therefore, it has been demonstrated that the use of colloidal systems improves tumor therapy due to enhanced permeability and retention effect (EPR) within the tumor site.

7.8 Concluding remarks

The problem of drug resistance in cancer appears to be inextricably linked to the larger question of finding new agents with more specific modes of action that are directed

against the underlying processes that drive the neoplastic state itself. There is an urgent need to develop drug delivery systems that are consistent with the science and the great clinical need and not simply with established bureaucratic procedures. The present study demonstrates some new findings, which may be exploited in improving the therapeutic efficacy of anticancer agents that are substrates to Pgp by using nanoparticles as drug delivery systems for active and passive targeting. However, extensive clinical trials need to be performed to establish the efficacy and safety of these novel drug delivery systems in clinical practice. We believe that these systems have the potential to improve the chemotherapy of multidrug resistant tumors. Their effective use will not only increase patient non compliance, it will possibly reduce the dose of the antitumor agent for a given degree of therapeutic response and also reduce the overall side-effects associated with the drug. Hence, these novel drug delivery systems can play an imperative role in annihilating the present shortcomings associated with the cancer chemotherapy and lead to successful chemotherapy.