CHAPTER 5:

SUMMARY AND CONCLUSION
5.0 SUMMARY AND CONCLUSIONS

Taxanes are the most common anticancer drugs used against a broad range of human malignancies, including ovarian cancer [Green et al., 2009], breast cancer [Overmoyer, 2008], non-small cell lung carcinoma [Schittenhelm et al. 2009] and melanoma [Rowinsky and Donehower 1995]. Taxol® and Taxotere® are commercially available formulations of paclitaxel and docetaxel, respectively, which are administered intravenously at different dosages and infusion schedules [Singla et al., 2002]. Taxane delivery by the oral route would be very valuable with respect to improving patient compliance and ease of administration, as well as the development of chronic treatment schedules, which would in addition decrease the costs of therapy. However, oral treatment with taxanes is not possible until now because of their low oral bioavailability (approximately 1% for Paclitaxel and 8% for Docetaxel) owing to several factors. Taxanes show limited aqueous solubility and dissolution, affinity to the membrane-bound drug efflux pump P-glycoprotein (P-gp) [Malingré et al., 2001a; Sparreboom et al. 1997b], and metabolism by cytochrome P450 3A4 (CYP3A4) [Walle, 1996], as they belong to BCS Class IV group.

The self-emulsification technique provides a unique opportunity for the improvement in the in vitro and in vivo performance of poorly water-soluble drugs and thus acts as a suitable system for the delivery of drugs belonging to BCS classes II and IV. Self micro-emulsifying drug delivery systems (SMEDDS) may be used as a potential alternative to conventional oral formulations for lipophilic compounds. SMEDDS is a pre-concentrate mixture of oil, surfactants, and co-surfactants, which self-emulsifies rapidly in the gastrointestinal tract under gentle agitation to present the drug solubilized in small droplets of a size typically below 100 nm) [Gursoy and Benita, 2004]. Excipients used for the formulation of SMEDDS have the potential to increase the dissolution and permeability of drugs by significantly decreasing droplet size and restraining the secretion by drug efflux transporter P-gp [Shen and Zhong, 2006].

P-glycoprotein (P-gp) efflux mechanism also plays an important role in decreasing the oral bioavailability of taxanes. P-gp is a member of the ATP-binding cassette super family of transport proteins and is expressed in numerous tissues such as the luminal membrane of the
small intestine and blood-brain barrier, and the apical membranes of excretory organs such as liver and kidney [Ayrton and Morgan, 2001]. Co-administration of a potent inhibitor of p-glycoprotein (1st and 2nd generation P-gp inhibitors), results in enhancement in the oral bioavailability for taxanes [Terwogt et al., 1999; Terwogt et al., 1997; Woo et al., 2003]. This therapeutic approach, however, is fraught with various drawbacks, such as toxicity, non-specificity, low potency and unpredictable pharmacokinetic interactions of inhibitors with the drug [Terwogt et al., 1999]. To overcome this problem, 3rd generation P-gp inhibitors came into existence, which had no pharmacological response, high specificity and minimal pharmacokinetic interactions [Thomas and Coley, 2003]. GF120918 is a novel 3rd generation P-gp inhibitor and has been shown to be a potent blocker of P-glycoprotein in tumor cells in vitro and in vivo.

The current research work was performed to define the role of nanometric carrier system for the delivery of anticancer bioactives, i.e. taxanes. In the present study self-emulsifying formulation and a novel P-gp inhibitor, i.e. GF120918 was investigated for the oral delivery of the anticancer drugs, i.e. paclitaxel and docetaxel.

The obtained drug samples were subjected to identification studies using MS/MS scan and were found have similar molecular weight and product ions as reported. The in vitro analytical method was successfully developed and validated for both drugs, i.e. paclitaxel and docetaxel. To carry out plasma studies in animals, a sensitive, rapid and efficient UPLC-MS-MS method was developed for both drugs.

The SMEDDS formulation is a pre-concentrate mixture of one or more surfactants and oil with drug dissolved in it. Solubility studies was performed to determine suitable oils having good solubilizing properties for paclitaxel and docetaxel, respectively. Table 4.2 and 4.17 depicts the results of the solubility study of paclitaxel and docetaxel in various excipients at 37 °C, which was determined by HPLC. Excipients selected for oral SMEDDS formulations of both drugs in this study were of comprehensive regulatory status.

The choice of excipients (especially oil) to prepare SMEDDS depends on their drug dissolving capacities. Basically, fatty acid esters of glycerol, i.e. long chain triglycerides (LCT) and medium
chain triglycerides (MCT), are commonly used in the formulation of SMEDDS [Constantinides, 1995]. Because of higher ester content per gram in medium chain triglycerides, most of the drugs show higher solubility and enhanced bioavailability in MCT as compared to LCT [Cao et al., 2004; Khoo et al., 1998]. The explanation for higher bioavailability shown by MCT is found in passive diffusion phenomena from the GI tract to the portal system, while longer fatty acids are absorbed into the lymphatic system, which requires modification in the structure of long chain fatty acids. Additionally, MCT do not require bile salts for digestion [Martena e al., 2006].

In the present study the efficiency of LCT and MCT for the preparation of taxane-loaded SMEDDS was evaluated. The solubility study data obtained shows paclitaxel having high solubility in commercially available modified medium chain triglycerides, Capryol PGMC (Propylene Glycol Caprylate) and Castor oil (long chain triglycerides). Docetaxel shows high solubility in commercially available modified medium chain triglycerides, Capryol 90 and Corn Oil (long chain triglycerides), respectively.

The important parameter for the selection of the surfactant is its self micro-emulsification efficiency, which is dependent on its hydrophilic–lipophilic balance (HLB) value. Commercially available surfactants having sufficient regulatory compliance for oral administration including Cremophore EL (HLB=13.5), Cremophor RH 40 (HLB=14–16) and Labrasol (HLB=14) were evaluated in this study [Raymond et al., 2003]. For surfactants, paclitaxel shows good solubilization in Cremophor EL, Cremophor RH 40 and Transcutol HP. Accordingly, docetaxel shows good solubilization in Transcutol HP, Labrasol and Cremophor EL.

A co-surfactant possesses a unique property of enhancing the solubility of compounds by increasing the interfacial fluidity, which subsequently develops a disordered film due to the void space among surfactant molecules. Co-surfactants are more likely to be retained by the oil phase upon dilution with aqueous media, thus avoiding precipitation of compounds [Pouton, 2000]. For paclitaxel, co-surfactants showing good solubilization are PEG 300 and Lauroglycol 90. Docetaxel showed good solubilization in PEG 300, Lauroglycol 90, and Lauroglycol FCC.

On the basis of the solubility study, miscibility and self-emulsification efficiency of all surfactants and co-surfactants, Cremophore EL and Cremophor RH 40 were selected as
surfactants and Lauroglycol 90 and PEG 300 were selected as co-surfactants for the preparation of ternary phase diagrams for paclitaxel SMEDDS. Cremophore EL and Transcutol HP were selected as surfactants and Lauroglycol 90, Lauroglycol FCC and PEG 300 were selected as co-surfactants for the preparation of ternary phase diagrams for docetaxel SMEDDS.

Introduction of SMEDDS preconcentrate into the aqueous media under gentle agitation results in the formation of fine oil in water microemulsion. Since the free energy required to form an emulsion is very low, the formation is thermodynamically spontaneous [Craig et al., 1995; Kim et al., 2000]. Surfactants form a layer around the emulsion droplets and reduce the interfacial energy as well as providing a mechanical barrier to coalescence. Phase diagram were constructed with different combination of surfactants, co-surfactants and oils [Pouton, 1985; Khoo et al., 1998; Kang et al., 2004]. These combinations were presented in table 4.3 (paclitaxel SMEDDS) and table 4.18 (docetaxel SMEDDS). The visual test was conducted to observe the apparent spontaneity of microemulsion formation (Table 3.3).

Pseudo-ternary phase diagrams were constructed to identify the self-emulsifying regions and to optimize the concentration of oil, surfactant (S) and co-surfactant (CoS). Paclitaxel pseudo-ternary phase diagrams are presented in (Fig. 4.6 A-D). Self-emulsifying region of the pseudo-ternary phase diagrams were used to select the best composition of oils, surfactants and co-surfactants). In our study extensive microemulsion region was observed with combination 3, which is comprised of Capryol PGMC, Cremophore RH 40 and PEG 300. This combination offers the advantage of good clarity at high oil concentration (up to 55% w/w). Emulsification was good when the surfactant (Cremophor RH 40) concentration was more than 30% of formulation. These results are in accordance with other studies, where the use of high concentration of surfactants was found to be necessary to achieve fast and efficient self-emulsification [Shah et al., 1994; Khoo et al., 1998; Kommuru et al., 2001; Kang et al., 2004]. A definite ratio of oil, surfactant and co-surfactant from each combination comprising maximum amount of oil was selected for further evaluation.

Docetaxel pseudo-ternary phase diagrams [Fig. 4.21 A-D] demonstrate that a large microemulsion region was formed with combination 1, which comprised of Capryol 90,
Cremophore ELP and Transcutol HP. Phase diagrams showed that a bigger microemulsion area was achieved when using MCT (Capryol 90) instead of LCT (Corn oil). This is due to the differences in polarity between the lipids, as more hydrophobic LCT is more difficult to emulsify. This is parallel to the findings of Deckelbaum et al., 1990 showing that MCT is more soluble and has a higher mobility in the lipid/water interfaces than LCT associated with a more rapid hydrolysis of MCT. In general, when using LCT, a higher concentration of Cremophore ELP was required to form microemulsions compared with MCT. Various other investigators have also documented that the use of high concentration of surfactants is necessary in these combinations to achieve fast and efficient self-emulsification [Shah et al., 1994; Khoo et al., 1998; Kommuru et al., 2001; kang et al., 2004]. A definite ratio of oil, surfactant and co-surfactant from each combination comprising maximum amount of oil was selected for further evaluation.

On loading of both drugs in their respective combinations, no significant difference was observed with regard to self-emulsifying performance, when compared with the corresponding placebo formulation. The four different SMEDDS formulations loaded with paclitaxel were coded as PS1, PS2, PS3 and PS4 (Table 4.8), and four different SMEDDS formulations loaded with docetaxel were coded as DS1, DS2, DS3 and DS4 (Table 4.23). The effect of dilution (i.e. 1:20 and 1:1000) was investigated for various diluents, including simulated gastric fluids (i.e. de-ionized water, 0.1 N HCl and phosphate buffer, pH 6.8). All PCT SMEDDS formulations do not show any effect on dilution study except PS2 formulation which turned hazy after 8 h of dilution (Table 4.9). While in case of DCT SMEDDS DS2 and DS3 formulations turned hazy after 8 h of dilution (Table 4.24).

The mean droplet size of the diluted SMEDDS pre-concentrate mixture for both drugs was found to be very low and in the nanometric range (<100 nm). PS3 and DS1 were found to have the smallest mean droplet size of 22.1 ± 2.4 nm and 28.4 ± 2.3 nm, respectively (Table 4.10 and 4.25). Droplet size of the PS3 and DS1 was significantly lower than of other formulations. This is because chain length of the oil plays a role in the ease of emulsification, stabilization of the emulsions, as well as the emulsion droplet size. PS3 and DS1 formulations have a relatively shorter triglycerides chain, which is the reason behind the smaller mean droplet size observed in
PS3 and DS1. These findings are akin to those reported by previous workers [Shah et al., 1994; Belmonte and Atef, 2008]. In general, the mean droplet size of the DS3 was smaller than the PS3 formulation probably because capability of Transcutol HP in reducing the interfacial tension is greater than PEG 300 and also it has more hydrophilic character than the PEG 300. The zeta potential and percentage transmittance of all the selected SMEDDS formulations for the both drugs do not show any significant change.

Thermodynamic stability study was done to identify and avoid unstable formulations. Formulations selected were subjected to stress tests such as centrifugation and freeze–thaw test. In case of PCT SMEDDS, PS2 and PS4 showed phase separation in the centrifugation study, while PS1 and PS3 were found to be stable and hence selected for the freeze-thaw study. In case of DCT SMEDDS, DS2 and DS3 showed phase separation in the centrifugation study, while DS1 and DS4 were found to be stable and hence selected for further freeze-thaw study.

PS3 and DS1 was found to have the smallest mean droplet size (34.1 nm and 28.4 nm), highest absolute zeta potential (-23.3 mV, -19.4 mV) and transmittance in close proximity to 100%. No significant changes observed after dilution with various diluents. Hence, formulations PS3 and DS1 were selected for further investigation, in ensuing in vitro release study, stability study, permeability, uptake study and pharmacokinetic study. The morphology of the micro-emulsions formed from selected SMEDDS formulations (PS3 and DS1) were examined with a transmission electron microscope. The microemulsion droplet appears dark with the bright surroundings. TEM photographs (Fig. 4.7 and 4.22) further conformed that the globules were spherical in shape.

To mimic the acidic and basic condition of the gastrointestinal fluid the release study of selected SMEDDS formulations of both drugs (PS3 and DS1) were carried out in different media (a) 1.0% Tween 80 in PBS solution pH 7.4; (b) 1% Tween 80 (w/v) in pH 4.5 acetate buffer; (c) 1% Tween 80 (w/v) in simulated gastric fluid without enzymes (pH 1.2). The release study shows the complete dissolution of both the drug in 60 min in all the media (Table 4.11 and 4.26; Fig 4.8 and 4.23).
Chapter 5

Summary and Conclusion

Taxanes show poor bioavailability because of restricted aqueous solubility and affinity to membrane-bound P-glycoprotein (P-gp) mediated drug efflux system. Inhibition of P-gp may, therefore, be an attractive strategy for increasing the permeation of taxanes across the intestinal epithelium. For this purpose a number of P-gp inhibitors has been identified so far [Teodori et al., 2002]. GF120918 is a recently developed 3rd generation P-gp-inhibitor [Hyafil et al., 1993]. In comparison to the early generation of P-gp inhibitors (e.g. cyclosporin-A, verapamil), GF120918 has a higher affinity for P-gp and a lower cellular toxicity [Wallstab et al., 1999]. The present study was focused on the evaluation of P-gp inhibition activity of GF120918 when administered along with taxanes loaded SMEDDS formulations (PS3 and DS1) across Caco-2 cell monolayers.

Cell cytotoxicity study was performed to identify the dose of paclitaxel, docetaxel and GF120918 at the caco-2 cell can maintain their integrity during the course of study (Fig. 4.9 and 4.24). From the study paclitaxel and docetaxel at a concentration of 5µM and GF120918 at a concentration 10µM were selected for the permeability assay.

The quality of tight junction status was monitored by measuring the transepithelial electrical resistance (TEER) across Caco-2 cell monolayers. Taxane concentrations were determined in the apical and basolateral compartments in order to calculate the apparent permeability (P_app) by previously developed HPLC method. The results after 3 h of incubation showed that microemulsion formed from paclitaxel loaded SMEDDS (PSME) along with GF120918 (16.17 x 10^{-6} cm/sec) mediated a four-fold increase in permeability as compared to the plain paclitaxel solution (4.29 x 10^{-6} cm/sec) (Table 4.12; Fig. 4.10). Microemulsion formed from docetaxel loaded SMEDDS (DSME) in the presence of GF120918 (18.25 x 10^{-6} cm/sec) showed a nintimes increase in permeability as compared to plain docetaxel solution (2.12 x 10^{-6} cm/sec) (Table 4.27; Fig. 4.25).

The results clearly demonstrate the potential of GF120918 included in SMEDDS formulations, to at least partially block the P-gp efflux pump, which therefore results in a significant increase in permeability of paclitaxel and docetaxel, respectively. Apart from this, SMEDDS alone also demonstrated their contribution in the enhancement of permeability of these drugs. The reason
behind this may be the excipients used for the preparation of SMEDDS, as Cremophore, PEG, Transcutol HP, etc., have the ability to modify the activity of P-gp efflux pump system, as reported previously [Gursoy et al., 2003].

The cellular uptake of both SMEDDS formulations along with GF120918 were qualitatively and quantitatively evaluated by using Rhodamine 123 (Rh123) incorporated in SMEDDS and employing fluorescence microscopy and the measurement of fluorescence intensity by spectrofluorimetry in A549 cells. The drugs were replaced with Rh123 in their respective formulations. Quantitative analysis of intracellular fluorescence revealed that microemulsions formed from rhodamine loaded SMEDDS I and SMEDDS II along with GF120918 exhibited 3- and 2.5-times higher uptake than plain rhodamine solution in A549 cells (Fig. 4.12 and 4.26).

Fluorescence of Rh123 loaded SMEDDS were observed in the cytoplasm of all cells irrespective of particle size, suggesting that microemulsions formed from SMEDDS aids the internalization of dye by the A549 cells at significant rates (Fig. 4.13 and 4.27). Fluorescence intensity was found to be higher in RSME1 (Fig. 4.13A) and RSME2 (Fig. 4.27A), which clearly indicated the significant uptake of dye loaded in SMEDDS into the tumor cells when applied with GF120918.

The oral absorption of taxanes were inhibited by the P-gp efflux pump in the intestine mucosa and was also metabolized by CYP3A in both the liver and epithelial cells of small intestine. Enhancement of oral bioavailability of taxanes may be achieved via an increase in intestinal epithelial permeability, P-gp inhibition, or reduced intestinal metabolism. In the present study the developed taxanes loaded SMEDDS formulation (PS3 and DS1) was administered orally in Sprague-Dawley (S.D.) rats along with novel P-gp inhibitor, i.e. GF120918 and the pharmacokinetic profile was evaluated. Pharmacokinetic parameters in plasma were obtained from the pooled concentration-time data with statistical moment algorithm using WinNonlin program package.

The pharmacokinetic parameters of orally administered paclitaxel and docetaxel in different groups are shown in Table 4.13 and 4.28. The pharmacokinetic data of group, which received oral taxanes SMEDDS with GF120918 (PSME and DSME), revealed that co-administration of GF120918 resulted in a pronounced increase in the mean AUC value of orally administered
paclitaxel and docetaxel (Fig 4.14 and 4.28). AUC is important parameters in evaluating bioavailability of drug from dosage form as it represents the total integrated area under the blood concentration time profile and represents the total amount of drug reaching the systemic circulation after oral administration. The AUC$_{0-24}$ of PSME and DSME group was about 7- and 10-fold higher than PS and DS group, respectively, thus bioavailability enhancing capacity of developed SMEDDS formulation of taxanes along with GF120918 could be successfully proven. Higher amount of drug concentration in blood indicated better systemic absorption of paclitaxel and docetaxel from SMEDDS formulation as compared to the suspension formulation.

The increase in oral bioavailability of taxanes is mainly due to non-competitive and specific blockage of P-gp by novel third generation P-gp inhibitor, i.e. GF120918 (fig. 4.15 and 4.29). Apart from this the developed SMEDDS formulation also shares its contribution in enhancing bioavailability of both the taxanes. Surfactant used for the purpose, i.e. Cremophore RH40, Cremophore ELP, PEG 300 and Transcutol HP are responsible to cause dissolution enhancement, augmentation of tight junction intestinal membrane and effective P-gp blockade which in turn lead to oral bioavailability enhancement of taxanes.

The short term stability study was performed at real time and accelerated condition to evaluate the stability of developed SMEDDS formulations (PS3 and DS1). Both the formulation does not show any degradation at both the stability conditions and found to be stable (table 4.29 and 4.30).

SMEDDS act as an ideal carrier for the delivery of drugs belonging to BCS classes II and IV. The current study aimed to develop and characterize self-emulsifying formulations of paclitaxel and docetaxel (BCS Class IV). The optimized formulation of paclitaxel SMEDDS consisted of Capryol PGMC, Cremophore RH 40, PEG 300 whereas the optimized formulation of docetaxel SMEDDS comprised of Capryol 90, Cremophore EL and Transcutol HP. Both of these formulations showed sufficient drug loading, rapid self-emulsification in aqueous media, and could produce small mean droplet sizes. The average droplet size of the optimal formulation is within 100 nm and shows a Gaussian distribution. The potential of the 3rd generation novel P-gp inhibitor (GF120918) was also investigated for the effective delivery of taxanes. The resulting
SMEDDS formulations were investigated for enhanced intestinal permeability and cell uptake when administered along with GF120918. The oral pharmacokinetic study further confirms the potential of this dual approach for the effective delivery of taxanes by oral route. The results of these studies revealed the strong potential of GF120918 for effective oral delivery. GF120918 is intensively research drug molecule as a P-gp inhibitor because of its obvious advantages. Currently, GF120918 is under clinical investigation for the evaluation of its ability to act as a potent P-gp modulator. Hence, the outcome of this research work will definitely help in strengthening its suitability for the delivery of anticancer drugs which has problem of bioavailability on account of P-gp efflux mechanism. This type of study will definitely helpful in establishing the rationale of SMEDDS and P-gp inhibitor, i.e. GF120918, for the effective oral delivery of anticancer drugs.