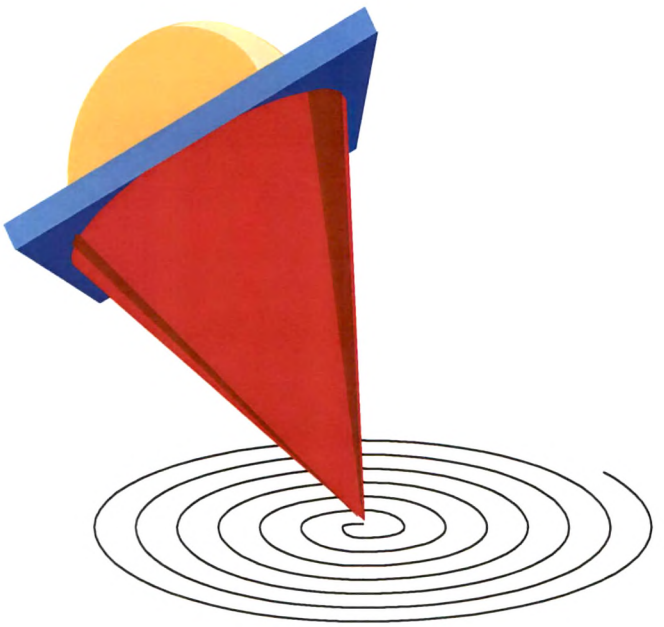

Summary and Conclusions

Every stone is a Master Piece, if it is properly shaped

The development of drug resistance to cancer chemotherapy is one of the biggest obstacles to the successful treatment of cancer disease. Recent advances in biomedical science and combinatorial chemistry have resulted in the design and synthesis of hundreds of new agents with potential activity against a wide range of therapeutic targets in vitro. However, most of these new drugs fail to live up to their potential in the clinic. For instance, although there are numerous anticancer agents that are highly cytotoxic to tumor cells in vitro, the lack of selective antitumor effect in vivo precludes their use in clinic. One of the major limitations of antineoplastic drugs is their low therapeutic index (TI). The low TI of such drugs may be due to: (i) their inability to achieve therapeutic concentrations at the target site (solid tumors); (ii) nonspecific cytotoxicity to critical normal tissues such as bone marrow, renal, GI tract and cardiac tissue; and: or (iii) problems associated with formulation of the drug, for example, low solubility in pharmaceutically suitable vehicles, leading to the use of surfactants or organic co-solvents which have their own undesirable side effects. Thus, there is a need for effective delivery systems that not only act as a formulation aid but also alter the biodistribution of drugs in such a way that a greater fraction of the dose reaches the target site.

Since then, interest in liposomes as devices for drug delivery gradually increased, and they have been one of the main players in the cancer drug delivery area for the last 25 years. Liposomes are micro-particulate or colloidal carriers, usually 0.05–5.0 μm in diameter that form spontaneously when certain lipids are hydrated in aqueous media. Liposomes are composed of relatively biocompatible and biodegradable material, and they consist of an aqueous volume entrapped by one or more bilayers of natural and: or synthetic lipids. Drugs with widely varying lipophilicities can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume or at the bilayer interface. Conventional liposomes are limited in effectiveness because of their rapid uptake by macrophage cells of the immune system, predominantly in the liver and spleen. Short circulation times severely limit the use of liposomes as micro reservoir systems for the slow release of biologically active molecules, which are normally degraded rapidly within the vasculature.

Liposome membranes containing bilayer-compatible species such as poly (ethylene glycol)-linked lipids (PEG-lipid) or the gangliosides are being used to prepare

"Stealth" liposomes. These, so called "Stealth" liposomes have a relatively long plasma half-life approximately 1 day (whereas the conventional liposomes have only few minutes) in blood circulation and show an altered biodistribution *in vivo*. PEG prevents the liposomes from sticking to each other and to blood cells or vascular walls. Disappointments in performance of stealth liposomes include lack of general strategy for tissue targeting, drug concentrations, which are sometimes, lower than desired, difficulty of large scale production and unpredictable shelf life. Moreover, such lipids are very expensive, inherently unstable, very difficult to scale up to commercial production, and difficult to engineer.

Microbubbles consist of a gas (air or perfluorocarbon), which is stabilized by shell (denatured albumin, phospholipids, or surfactants or cyanoacrylates) having diameter of from 1 to 10 μm. The formation of the monolayer microbubbles is solely by molecular self-assembly; no organochemical reactions or derivatization is needed to assemble the microbubble structure, which avoids all the potential problems and complications associated with PEGylation of liposomes.

As far as diagnosis part is concerned, various technologies exist in which part of animal or human body may be imaged so as to aid in diagnosis or therapy of medical disorders. X-ray is the most well known imaging techniques but it is limited to visualize skeletal parts of animal and humans. The use of X-ray for some of the organs and blood vessels is unsatisfactory and is dangerous if the amount of exposure is excessive. Radionuclide imaging involves the injection of radioactive substances but it requires the use of very expensive and sophisticated machinery. It produces images of only a limited number of views of the heart and those images may not be of exceptional clarity. Ultrasound imaging is safe, cheap, relatively easy to operate and image produced is in real-time. Now the lack of clarity or ultrasound could be solved using ultrasonic contrast agents. The maximum ultrasonic refection obtained depends on the interface between liquid and gaseous media. That is why most accepted contrast agents were liquid containing microbubbles of gas.

The present investigation describes the preparation and comparative evaluation of stealth liposomes and microbubbles as carriers for anti-cancer agents and diagnostic agents. The contents of thesis mainly divided into three parts. Part I consists of introduction, literature review and profiles of the drugs used for the study. Part II

involves method development, preparation and characterization of liposomes and microbubbles containing flutamide (FLT) and 6-Mercaptopurine (6-MP) and their in vivo studies. Part III describes the application of stealth liposomes and microbubbles in ultrasound imaging.

Part I: - Introduction and Literature Review on Cancer, Dosage forms and Drugs

Part I of the thesis divided in to three chapters. Chapter 1 consists of introduction and proposed plan of the work. Chapter 2 shows the literature review of the cancer mainly prostate cancer and blood cancer (leukemia), conventional and stealth liposomes, and microbubbles. Chapter 3 gives the profiles for the drugs (FLT and 6-Mercapto purine) used in the study.

Part II: - Method development, Preparation and Comparative Evaluation of Stealth liposomes and Microbubbles for therapeutic purpose

Part II divided in to eight different chapters (4-11).

Chapter 4: Method Development of FLT and 6-MP

Chapter 4 describes the development of analytical methods for estimation of FLT and 6-MP in different vehicles by different analytical techniques. Spectrophotometric methods for both drugs used for evaluation of drug content in the drug-encapsulated liposomes and microbubbles. High performance liquid chromatographic methods used for estimating the amount of drug present in plasma and different tissue homogenates. A selective, sensitive and reproducible high performance liquid chromatographic method was developed for the separation and determination of FLT in pharmaceuticals from biological samples and the rest of excipients and materials used to prepare the dosage form. The analytical method was subjected to statistical analysis and evaluated for the accuracy and precision. The precision and accuracy of the proposed method are within acceptable limits and the quantitation limit is also low. The method involved less time-consuming sample preparation steps. The method was linear over the concentration range 20-1000 ng/ml and 50-1000 µg/ml in mobile phase and rat blood plasma, respectively. The mobile phase, Methanol: Water

(80:20, v/v) was run at a flow rate of 1 ml/min for 8 min. The retention times for IS and FLT were 3.03 and 4.02 min, respectively. This method permits the determination of FLT in a small amount of rat plasma and tissue homogenates without any complicated extraction procedure. The method was shown to be highly reproducible and it seems to be adequate for routine therapeutic drug monitoring. It could be used without any interference from lipids, endogenous substances from the plasma samples and tissue homogenates. Chapter 4 also involves the method development for the estimation of 6-MP in rat plasma and various tissue homogenates. The retention times were 4.0 min and 6.2 min for drug and internal standard, respectively. A mixture of 0.01M KH_2PO_4 buffer: Acetonitrile (80:20, v/v) was used as a mobile phase at a flow rate of 1 ml/min. The plot of ratio of area of 6-MP and IS vs. concentration of 6-MP in ng/ml was found linear in the range of 50-1000 ng/ml in plasma, and 100-1000 ng/ml in all tissues. High correlation coefficients were observed with plasma (0.998), liver (0.999), lung (0.999), kidney (0.999), heart (0.993) and spleen (0.990). The developed method showed great potential of estimation of 6-MP in plasma and tissues of rat and may substitute other methods, which are complex, time consuming and consumption of high quantities of organic solvents for estimation. Therefore, these methods can be considered to be of real interest for the rapid and reliable clinical, pharmacokinetic and biodistribution studies of FLT and 6-MP.

Chapter 5: Preparation and Optimization of Liposomes and Microbubbles

Chapter 5 describes the preparation and optimization of conventional liposomes (CL) containing FLT and 6-MP by thin film hydration technique using 3^3 factorial 26-term logit model and 4^2 factorial design, respectively. A detailed study of effect of process parameters such as vacuum, rotation speed, hydration temperature on film formation and formulation parameters such as drug/lipid molar ratio, PC/Chol molar ratio, volume of organic phase and aqueous phase on entrapment efficiency (% EE) of drug in liposomes were studied. In case of FLT liposomes, the data of the study indicated that % EE is more dependent on the drug/PC/Chol molar ratio and the volume of organic phase than the volume of aqueous phase. From the results of contour plots, it was concluded that the range of volume of aqueous phase and the drug/PC/Chol molar ratio in conventional thin film hydration technique was from 1.175 ml to 1.8 ml and from 1:14:2 to 1:15:2 molar ratio, respectively at volume of organic phase 5

ml (0 level) to achieve maximum % EE. While in case of 6-MP liposomes, 4^2 factorial design offered the possibility of investigating four independent variables at two levels after performing only sixteen experiments. The drug/lipid molar ratio and PC/Chol molar ratio had a statistically significant influence on the % EE of 6-MP liposomes. Although the volume of aqueous phase and organic phase had a slight influence on entrapment efficiency, they had a significant influence on the stability of liposomes. The optimum setting for preparing 6-MP liposomes was 1:20 drug/lipid molar ratio, 7:3 PC/Chol molar ratio, 5 ml of organic phase and 2 ml of aqueous phase. The optimized batches were selected further for the preparation of stealth liposomes (SL) using synthesized $mPEG_{2000}$-PE (methoxy polyethylene glycol 2000-phosphotidylethanolamine). The aggregation of liposomes or steric stabilization was studied by electrolyte induced flocculation test. It was found that 5-mole% of $mPEG_{2000}$-PE gives maximum steric stabilization to liposomes and showed the highest % EE.

Chapter 5 also describes preparation of microbubbles containing both drugs by various methods such as vortexing with freeze and thaw cycles (Microbubbles (MBs)), double emulsion solvent evaporation technique (Gas filled Microspheres (GFMs)) and mixing cum sonication technique (Acoustically active lipospheres (AALs)). The effect of drug: lipid mass ratio, oil concentration and the gas volume on particle size of microbubbles were determined. All methods are appropriate to form microbubbles with good stability.

Chapter 6: Characterization of Liposomes and Microbubbles

Chapter 6 describes physical and chemical characterizations of liposomes, which are very important for a meaningful comparison of different liposome preparations. The prepared liposomes both conventional (CL) and sterically stabilized (SL) of FLT and 6-MP were characterized for drug entrapment, particle size and size distribution by light diffraction technique using Malvern particle size analyzer, morphological behavior using photomicrography and transmission electron microscopy (TEM), lamellarity by ^{31}P-NMR studies, particle charge (zeta potential) by Malvern Zeta Sizer and differential scanning calorimetric studies. In case of FLT liposomes, FLT could be entrapped into CL and SL by thin film hydration technique with the % EE of 99.32% and 98.56%, respectively. The sonication time of 20min was found to be

adequate to reduce the size of the CL of FLT to around 1 μm. The relatively narrow particle size distributions were achieved with an average particle size of 136±5.8 nm and 158±6.3 nm of CL and SL formulation after three cycles of homogenization. The particle size of the SL was smaller as compared to that of the corresponding CL. The majority of the prepared liposomes were spherical and multilamellar before homogenization. The TEM studies showed that the surface of the CL and SL did not show any visual difference. In ^{31}P-NMR studies, upon addition of $MnCl_2$, The percentage of the relative loss of signal (RLOS) was found to be 15% indicated that FLT liposomes are oligolamellar systems having more than 2 lamellae per vesicle. The zeta potential of FLT-CL and SL were -9.42 mV and -11.03 mV, respectively indicating insignificant difference after steric stabilization of liposomes by $mPEG_{2000}$-PE. In DSC studies, all the thermograms show a single-phase transition peak indicates that FLT was completely miscible with the components of the bilayer of liposomes. The phase transition temperature of the liposomes lies between 30° to 35°C in CL and SL, which indicated no change in bilayer rigidity was found after addition of $mPEG_{2000}$-PE in liposomes. While in case of 6-MP liposomes, the formulation composed of the drug: lipid molar ratio of 1:20 and HSPC/Chol molar ratio of 7:3 was found to have good % EE (97.26±2.6 %) and particle size of 120.24 ± 5.6 nm. Stealth liposomes with the highest % EE (96.38±4.5) were obtained at 5-mole% of $mPEG_{2000}$-PE of total lipids with particle size of 132.26 ± 4.8 nm. Here, the prepared liposomes were also spherical in shape and multilamellar before homogenization and bilamellar after homogenization. The TEM studies showed the same results as the FLT liposomes. The RLOS was found to be 10%, after addition of Mn^{+2} indicated that the hydration of the lipid films, followed by annealing, led to the formation of multilamellar systems with 5 lamellae per vesicle; after the homogenization, the number of lamellae per vesicle was reduced to 2 (25% RLOS). Thus, a conversion of multilamellar to bilamellar systems was achieved which proves that the homogenization process also determined a significant reduction of the number of lamellae per vesicle. The zeta potential of 6-MP-CL and SL were -7.28 mV and -8.67 mV, respectively. After addition of DSPG in liposomes, the zeta potential was reduced to –22.4 mV. In DSC studies, the thermogram of 6-MP showed endothermic peak at 319°C. The thermogram of 6-MP liposome dispersion showed no melting endothermic peak of cholesterol at 150°C, which was found in thermogram of cholesterol. It indicated that all the lipid components interacted with each other to a great extent while forming the lipid bilayer.

Chapter 6 also describes the characterization of microbubbles prepared by various techniques. The prepared microbubbles were characterized for particle size and particle number by Malvern particle size analyzer and hemocytometer, morphology by photomicrography, survival rate measurement, gas content by densitometry, effect of sonication and centrifugation on particle size, in-vitro drug partitioning and effect of different ultrasound transducers on microbubble number. Data of particle size, number and survival rate showed that the microbubble number per ml of dispersion and the size increased as the amount of lipid increased in microbubbles prepared by vortexing method. Freeze and thaw cycle played an important role in particle size reduction. After 7 cycles of freeze and thaw treatments, microbubbles showed the least particle size. AALs showed less particle size, more particle number and the highest survival rate as compared to GFMs and MBs. Photomicrograph of microbubbles prepared by all three methods indicated that all the microbubbles were of spherical in shape and less particle size distribution after extrusion. The sonication time of 5 min was found to be adequate to reduce the size of the microbubbles to 1-10 μm. There was no effect of sonication on particle size observed after 5 min. The centrifugation up to 500 RPM for 5 min showed the least particle size in all type of microbubbles. In vitro drug partitioning data indicated that as the concentration of sucrose increased from 30 to 90% w/v, percentage drug leakage increased from 0.58% to 6.5% in case of FLT and 0.75% to 8.5% in case of 6-MP microbubbles, respectively. Microbubble number reduced as the frequency of transducer decreased from 2 MHz to 0.5 MHz in case of all microbubbles. Ultrasound exposure per unit time (T) is more in case of 0.5 MHz (0.1067) than that of 2 MHz (0.0266), which is responsible for more destruction of microbubbles with less frequency of transducer.

Chapter 7: In vitro drug release studies of drugs from Liposomes and Microbubbles

Chapter 7 describes the drug release of FLT and 6-MP from liposomes. These studies are an essential part of the product development process in case of liposomes. The release of drug from liposomes depends on the nature of drug and lipid used in liposomes. The drug release studies were conducted using cellulose dialysis bag having cut off of 12, 000 Dalton It was found from the drug release profiles of various FLT dosage forms that 40 % of the drug was released in just 30 min and it

took 4 hr to release 96% of drug from pure FLT. FLT CL showed 42% of drug release initially in 1 hr because of the large surface area and the enrichment of drug present in the outermost bilayer of the liposomes, which did not traverse any membrane before encountering the external medium and acted similar to free drug. CL showed release of drug for 36 hr while SL showed the release of drug for more than 48 hr. It indicates that the drug release from FLT SL is slower than the that of CL. Incase of 6-MP, 30 % or the initial amount of the drug was released in 30 min. Initial release of 6-MP (38 % in 1 hr) from liposomes was due to the large surface area and amount of drug present in outermost layer. This portion of the drug was associated with the bilayer due to its intrinsic hydrophobicity and the presence of phosphatidylcholine in the bilayer, which enhanced the solubility of 6-MP in the lipidic region. The drug release from the 6-MP CL exhibited for 48 hr and for SL 54 hr, respectively. It indicated that liposomes could successfully control 6-MP release for prolong time.

Chapter 7 also describes the drug release from different types of microbubbles of both drugs. In case of FLT and 6-MP, GFMs showed sustained release of drug for 48 hr while 90% of drug released after 4 hr and 24 hr from microbubbles prepared by MBs and AALs, respectively. The initial burst effect in drug release form all microbubbles may be due to drug present on the upper surface of microbubbles. The slower release rate of the drug from GFMs could be due to hydrophobic interaction between the drug and the polymer as it has higher lactide content.

Chapter 8: In vitro cell cytotoxicity studies of Liposomes and Microbubbles

Chapter 8 describes the comparison of the in-vitro cell cytotoxicity studies of pure drugs with their respective conventional and stealth liposomes. The in vitro cytotoxicity of anti-cancer drugs and their liposomal preparations were evaluated by using MTT assay. In case of FLT, the pure drug, CL and SL of various concentrations were exposed to fix number of cells of PC-3 cell lines. The cytotoxic effect of liposomes was found after 24 hr of incubation, but appreciable amount of cytotoxicity was found after 48 hr. IC_{50} (concentration of agent required to kill 50 % of cancer cells) was found 0.32, 0.38, and 0.35 mmoles for pure FLT, CL and SL and were not significantly different from each other. While in case of 6-MP, the pure drug, CL and SL of various concentrations were exposed to cells of HL-60 cancer

cell lines. There was gradual decrease in the % cell viability was found, as the concentration of 6-MP increased. After 48 hr of incubation, 0.015, 0.018 and 0.016 mmoles of IC_{50} was found for pure 6-MP, CL and SL and 20% of cells remained at concentration of 2 mmoles after 48 hr of incubation. The addition of polymers in the bilayer to produce steric stability did not cause significant change in the cytotoxicity of the drug.

Chapter 8 also describes the in-vitro cell cytotoxicity studies of microbubbles containing FLT and 6-MP. Effect of 0.5 MHz and 2 MHz frequency ultrasound transducers and their exposure time on % cell viability was also determined. In case of FLT, the all three types of microbubbles in various concentrations were exposed to fix number of cells of PC-3 cell lines for 48 hr of incubation. If the exposure time of 2 MHz transducer was less than 45 sec, the more was the % cell viability. The % cell viability was found 12-15% after the exposure for 45 sec of 2 MHz transducer and for 30 sec of 0.5 MHz in all types of microbubbles. In case of 6-MP, microbubbles were exposed to HL-60 cancer cell lines for 48 hr of incubation. The % cell viability was found 34-36% after the exposure for 45 sec of 2 MHz transducer and for 30 sec of 0.5 MHz transducer in all types of microbubbles. It was found that 0.5 MHz ultrasound transducer is enabled to burst all microbubbles within 30 sec of exposure time, which may help during in-vivo studies. AALs showed the cytotoxic effect similar to that of pure drugs. The in-vivo experiments were conducted using AALs and 0.5 MHz ultrasound transducer.

Chapter 9: Stability studies of Liposomes and Microbubbles

Chapter 9 describes the stability of the lyophilized product of CL and SL of FLT and 6-MP at various conditions and in presence of human plasma at 37°C. The evaluation of % EE, particle size and steric stability of the liposomes were considered as evaluating parameters. The prepared liposomal dispersions were subjected to stability studies, in triplicate, at conditions according to ICH guidelines i.e. 2-8°C with ambient humidity and 30 ± 2°C/60 ± 5% RH. The stability data of FLT liposomes showed that the liposomal preparations were physically stable for more than 3 months in lyophilized form at 2-8°C and retained 90% of their initial content over that period. Leakage of FLT from CL was faster than from SL in human blood plasma at 37°C. The % drug retained in CL (48.12 %) was less than that of SL

(66.38%) after 24 hr and no more drugs was retained after 2 days. Maximum instability was observed at 37°C in presence of plasma of CL and SL. Thus, from these studies, it was observed that liposomes remained more stable at refrigeration temperature. The lyophilized products of 6-MP liposomes were also physically stable for more than 3 months at 2-8°C. The % drug leakage from conventional liposomes (64.78 %) was more than that of all stealth liposomes formulations after 24 hr in human plasma at 37°C. The SL with 5-mole % of $mPEG_{2000}$-PE may effectively prevent adsorption of aggregation promoting components such as proteins in plasma as well as leak less amount of drug. The particle size of reconstituted lyophilized liposomes was not altered much after storing at different temperatures up to 3 months. The presence of steric stabilizing agents in the lipid bilayer in the SL prevented them to aggregate with each other and change in particle size. The steric stability of all liposomes was maintained for 1 month at 2-8°C and less than two weeks at 30 ± 2°C/60 ± 5% RH.

Chapter 9 also describes the stability of microbubbles at different conditions as per ICH guideline i.e. 2-8°C with ambient humidity, 30 ± 2°C/60 ± 5% RH and in presence of human plasma at 37°C. The particle size and number were considered as evaluating parameters. Stability data showed that AALs for both drugs showed stability less than 4 weeks at 30 ± 2°C/60 ± 5% RH and of three months at 2-8°C. AALs were stable for 24 hr and the change in particle size and number was observed in presence of human plasma at 37°C.

Chapter 10: In vivo studies of drug encapsulated Liposomes and Microbubbles

Chapter 10 describes the comparison of pharmacokinetic behavior and biodistribution of pure drug with their respective liposomal preparations. In case of FLT, the different formulations were injected through i.v. at the FLT dose of 25 mg/kg in albino wistar rats of either sex having weight of 200-300 gm for pharmacokinetic and biodistribution studies. The amount of drug present in plasma and various organs were estimated after extracting from respective parts by HPLC method as described in chapter 4. The pharmacokinetic parameters of free drug, CL and SL indicated that there was a rapid elimination of the free drug was found from the blood circulation and only 3.6 % of initial drug level per ml in blood was remaining after 4 hr. A marked increase in Cmax in blood following the

administration of SL was found as compared to CL. The half-life of SL (17.4 hr) resulted from the slower release of FLT from liposomes, as it remained circulated in the body. The mean clearance value of FLT was 6 and 16 folds greater than CL and SL, respectively. The biodistribution studies showed that stealth liposomes were shown lower uptakes by reticulo endothelial system (liver and spleen) and more concentrated in prostate as compared to conventional liposomes and free drug and remained for more than 24 hr. In contrast, CL was accumulated intensely in liver and in spleen, though the size of liposomes was only 150 nm. It indicated that SL could remain in blood for prolonged time, reduce the uptake of liposomes to RES, more concentrated in prostate and thereby reduce the possibility of the risk of toxicity to RES (hepatotoxicity) generally seen with free FLT.

Chapter 10 also describes the pharmacokinetic studies of 6-MP on albino wistar rats of either sex having weight of 200-300gm. Each animal was treated with CL or SL intravenously via tail vein and compared with the free drug solution of 6-MP at dose of 5 mg/kg. There was a rapid elimination of the free drug from the blood circulation and only 16 % of initial drug level was remaining after 8 hr of administration of free drug. Stealth liposomes showed much longer circulation time with half-life of about 4.15 hr after i.v administration. Comparatively, CL were distributed to the tissue in few short times and cleared from circulation with in 24 hr. Stealth liposomes exhibited high Cmax, have long circulating capacity in blood and biological half-life, which may help to increase therapeutic efficacy of 6-MP and to reduce amount as well as frequency of the dose. Biodistribution studies showed that free 6-MP was more concentrated in each organ specifically in liver. It also showed that there was considerable decrease in drug uptake in the reticulo endothelial system (RES)-containing organs (liver and spleen) after 1 hr of i.v injection of stealth liposomes. It indicated that stealth liposomes could remain in blood for prolonged time, reduce the uptake of liposomes to RES and thereby reduce the possibility of the risk of toxicity to RES generally seen with free 6-MP.

It further describes the in-vivo studies of microbubbles of both drugs in rats. Pharmacokinetic studies showed that 30 sec of exposure of ultrasound from 0.5 MHz transducer on tail vein was enabled to burst microbubbles and drug started to release from microbubbles. In case of FLT microbubbles, it showed Cmax of 8.89 μg/ml after 30 min. Pharmacokinetic parameters AALs indicated that they could

successfully remain in blood for prolong time and release drug slowly up to 36 hr with total clearance rate of 0.298 l h^{-1} kg^{-1}. In case of 6-MP microbubbles, it showed Cmax of 1016.21 ng/ml after 30 min and 40.87 ng/ml of drug remained after 36 hr in blood. There was no significant difference found between pharmacokinetic parameters of drug-loaded liposomes and microbubbles. Biodistribution studies showed that FLT was more concentrated in each organ specifically in liver, spleen and prostate after released from AALs. Rapid uptake of both the drugs by RES (liver and spleen) after 4 hr indicated that drug started to metabolize after released from AALs. The prostate targeting studies of FLT showed that microbubbles could enter in to prostate within 30 min after the exposure of ultrasound for 30 sec on prostate to burst microbubbles. FLT remained present in prostate after 24 hr, which indicated that microbubbles could be used successfully as a novel sustained and targeting delivery of FLT to prostate.

Chapter 11: Histopathology studies and Biochemical analysis

Chapter 11 describes the histopathological and biochemical analysis of free drug and liposomal preparations on albino rats for the assessment of hepatotoxicity. In case of FLT, each animal was treated with 25 mg/kg/day intravenously with control, free drug and encapsulated drug in CL and SL for 7 days and microscopical examination of rat liver was done. It is evident that free drug showed patchy marked necrosis, fatty degeneration changes and eccentrically situated nuclei with bile duct proliferation. CL showed cloudy degeneration and patchy necrosis, while SL showed no changes in hepatocytes and any other liver structure the same as control. After three days of injection of all formulations, 1 ml of blood from each rat was collected and serum Alanine transferase level (ALT) was measured using standard diagnostic kit. ALT level in free drug was also higher than those in CL. ALT level in serum following administration of SL was insignificantly different from control. In case of 6-MP, each animal was treated with 5-mg/kg/day i.v with control, free drug and encapsulated drug in CL and SL for 7 days and microscopical examination of rat liver was done. The histopathological studies of 6-MP indicated that free drug and CL showed injured bile ducts (bile duct proliferation) or canaliculi causing cholestasis without marked damage of hepatocytes as well as fatty degeneration changes, while SL showed no changes or injury to bile ducts or canaliculi hepatocytes and any other liver structure the same as control. Biochemical analysis

of 6-MP showed that there was no significant change in serum ALT level was observed between all above mentioned groups but serum glutamate-oxalate transferase (SGOT) and serum glutamate pyruvate transaminase (SGPT) level were higher in case of 6-MP and CL than SL. Furthermore, whereas no significant difference was observed, when compared with control. It indicated that stealth liposomes could reduce hepatotoxicity associated with 6-MP.

Chapter 11 also describes the histopathology and biochemical analysis of both drugs encapsulated microbubbles. Because of FLT and 6-MP was more concentrated in liver, histopathological studies were carried out particularly on these organs. Microscopical examination of sections indicated that no significant change was found in structure of the liver at cellular and tissue level. Biochemical analysis showed that ALT level after injection of AALs for 3 days was increased but the change was not significantly different from control. The same results were found when serum GOT and GPT determined after injecting AALs for 3 days. The study indicated that microbubbles could be used in treatment of prostate cancer and leukemia without any side effects associated with liver.

Part III: - Comparative Evaluation of Stealth liposomes and Microbubbles in Diagnosis

It consists of chapter 12. It describes the application of stealth liposomes and microbubbles in ultrasound based diagnosis techniques. The main disadvantage of such techniques is lack of clarity and misinterpretation of results. Nowadays, ultrasound contrast agents are used to increase the clarity of images. The developed stealth liposomes and microbubbles were tested as ultrasound contrast agents in sonography and Doppler studies. The experiment was carried out on albino rats of either sex and weight of 200-300 gm. Each animal was treated with dye solution, CL, SL and AALs intravenously and the images of rat heart were taken using sonographic and dopper imaging instruments. The study indicated that the images of rat heart obtained were clear after microbubbles injection and the chambers of heart were visualized clearly. This was not observed after injecting dye solution or liposomes. It indicated that microbubbles could be more suited for visualization of heart for diagnosis.

Conclusion

Liposomes have had a rocky history of a single product approval after its 30th birthday in United States. Disappointments in performance include difficulty in large-scale production, lack of a general strategy for tissue-targeting and unpredictable half-life. The development of microbubbles has increased the possibilities for diagnostic imaging. Ultrasound causes bubble destruction, resulting in micro streaming and increased permeability of cell membrane. Interestingly, this mechanism of microbubbles serves as the platform for a portfolio of products with applications including contrast agents for ultrasound imaging and could also be used as vehicles for therapeutic drug delivery.

The present study focuses on the feasibility of using liposomes (both conventional (CL) and stealth (SL)) and microbubbles (in combination with ultrasound) as drug delivery systems for anticancer drugs. FLT (FLT) and 6- Mercaptopurine (6-MP) that are the drugs of choice for the treatment of prostatic cancer and leukemia respectively are selected for the study. Diagnostic potential of stealth liposomes and microbubbles were assessed and compared each other.

The study demonstrates some new findings, which may help to improve the therapeutic efficacy of anti-cancer agents using liposomal and microbubble in combination with ultrasound based drug delivery systems.

The liposomes of FLT and 6-MP were prepared by conventional thin film hydration technique. The 3^3 factorial 26-term logit model and 4^2 factorial design were tested and found suitable experimental designs to optimize formulation parameters of FLT and 6-MP liposomes, respectively. The microbubbles were prepared by three methods: vortexing with freeze and thaw cycles (Microbubbles (MBs)), double emulsion solvent evaporation technique (Gas filled microspheres (GFMs)) and mixing cum sonication technique (Acoustically active lipospheres (AALs)).

The number of steps involved in the preparation of microbubbles was quite less and simple as compared to liposomes. The prepared liposomes and microbubbles were

subjected to freeze drying using various cryoprotectants to increase the stability and to ensure redispersivity.

The prepared liposomes were characterized for drug entrapment, particle size and size distribution by light diffraction technique using Malvern particle size analyzer, morphological behavior using photomicrography and transmission electron microscopy (TEM), lamellarity by ^{31}P-NMR studies, particle charge (zeta potential) by Malvern Zeta Sizer and differential scanning calorimetric studies. The microbubbles were characterized for particle size and particle number by hemocytometer and Malvern particle size analyzer, morphology by photomicrography, survival rate measurement, gas content by densitometry, effect of sonication and centrifugation on particle size, in-vitro drug partitioning and effect of different ultrasound transducers on microbubble number. The electrolyte induced flocculation test was conducted to assess steric stabilization of these products. The test showed that 5-mole % of the total lipids of $mPEG_{2000}$-PE was found to be adequate for steric stabilization of the liposomes. The prepared liposomes and microbubbles were found stable and had particle size in the range of 0.2-10 μm.

The reversed-phase high-performance liquid chromatography (HPLC) methods for estimation of drugs were developed which required less time-consumption in the sample preparation steps. The methods were shown to be highly reproducible and it seems to be adequate for routine therapeutic drug monitoring. It could be used without any interference from lipids, tablet excipients and endogenous substances from the plasma samples and tissue homogenates.

The in vitro drug release studies from these formulations were conducted using dialysis bag (cut off of 12, 000 Dalton) at 37°C. It was found from in vitro drug release studies that CL, SL and AALs have potential for sustained delivery of drugs.

The in vitro cell cytotoxicity studies were conducted on PC 3 and HL 60 cell lines to evaluate the cytotoxicity potential of the FLT and 6-MP formulations, respectively. The study showed that IC_{50} of all liposomes and AALs were similar to that found for pure drugs. The effect of ultrasound transducers of various frequencies required for bursting bubbles and % cell viability was also determined. It was found that 0.5 MHz

ultrasound transducer was enabled to burst microbubbles within 30 sec of exposure time and so the in-vivo studies designed accordingly.

The stability studies of formulations of both drugs were conducted at conditions according to ICH guidelines i.e. 2-8°C with ambient humidity and 30 ± 2°C/60 ± 5% RH. The stability of formulations was also checked in presence of human plasma at 37°C. The data showed that the liposomal preparations were physically stable for more than 3 months in lyophilized form at 2-8°C and retained 90% of their initial content over that period. AALs were also found stable up to 3 months at 2-8°C. The % drug leakage from CL was faster than that from SL and AALs in human plasma at 37°C. The change in particle size and number per ml of AALs was also found in presence of human plasma at 37°C.

Pharmacokinetic and biodistribution studies were performed in Albino Wistar rats of either sex having weight of 200-300 gm at the dose of 25 mg/kg and 5-mg/kg i.v for FLT and 6-MP, respectively. The animals were treated with CL, SL or AALs and compared with the free drug. The tail vein was exposed to ultrasound for 30 sec using 0.5 MHz ultrasound transducer to burst microbubbles (AALs) in blood vessels. The data indicated that SL and AALs could remain in blood for prolonged time, reduce the uptake to RES, more concentrated in prostate and thereby reduce the possibility of the risk of toxicity to RES (hepatotoxicity) generally seen with pure drugs. There was no significant difference found between pharmacokinetic parameters of drug-loaded liposomes and microbubbles.

Histopathological studies were conducted on Albino Wistar rats of either sex having weight of 200-300 gm for the assessment of hepatotoxicity. Each animal was treated at dose of 25 mg/kg/day and 5-mg/kg/day i.v of FLT and 6-MP formulations, respectively for 7 days and was sacrificed by cervical dislocation and dissected to collect liver after one week of last injection. The liver was fixed in 10% neutral buffered formalin. Sections of 3-5 mm thickness were stained with hematoxylin and eosin (H&E) for microscopical examination. The slides showed that patchy marked necrosis in hepatocytes, fatty degeneration changes and eccentrically situated nuclei with bile duct proliferation was found, when pure FLT and CL were administered i.v. The injured bile ducts (bile duct proliferation) or canaliculi causing cholestasis without marked damage of hepatocytes as well as fatty degeneration changes were