Summary and Conclusions

Oral delivery is the easiest and most convenient way for drug delivery. But, the challenges associated with oral route include exposure to extreme pH variations, hepatic first pass effect, intestinal motility, mucus barrier, P-glycoprotein efflux pump and impermeable epithelium. Most of drugs for treatment of cancer and AIDS suffer from abovementioned problems and cannot be given orally. Anticancer drugs are given by intravenous chemotherapy sessions which are very painful to patients. For AIDS, resistance to most of drugs is another barrier in efficient eradication of virus. Oral delivery of anticancer drugs and avoidance of resistance to antiretroviral drugs can be beneficial to patients. Oral chemotherapy can provide a long-time, continuous exposure of the cancer cells to the anticancer drugs of a relatively lower thus safer concentration.

Oral delivery of nanoparticles is extremely researched now days by scientists. Nanoparticle technology has the capability of improving the stability of drugs, minimizing the metabolic degradation and cellular efflux by virtue of colloidal surface properties. Nanoparticles given orally are efficiently taken up by M cells of Peyer's patches of intestine, thereby reaching GALT and systemic circulation directly.

**Gemcitabine HCl**, an anticancer agent, is currently in clinical use for the treatment of several types of cancer. Gemcitabine is a difluoro analogue of deoxycytidine. Unfortunately, the drug is rapidly metabolised with a short plasma half-life and its cytostatic action is strongly exposure-time dependent. It is rapidly and extensively deaminated by cytidine deaminase in blood, liver, kidney and other tissues. In order to achieve the required concentration over sufficient periods of time, repeated application of relatively high doses is required. This, in turn, leads to dose-limiting systemic toxicity. The plasma half life after intravenous infusion is 8-17 min in human plasma. Therefore, it is required in high doses. Furthermore, Gemcitabine is highly hydrophilic molecule with log P value 1.4. Till now, there is no oral formulation of Gemcitabine HCl in the market.

**Lopinavir** is a potent protease inhibitor used as a leading component in combined chemotherapy commonly referred as Highly Active Anti-Retroviral Therapy (HAART). Lopinavir has poor oral bioavailability due to poor drug solubility characteristics as well as extensive first pass metabolism, primarily mediated by cytochrome P450 and P-
glycoprotein efflux which limits intestinal uptake. In marketed preparations, Lopinavir is always co-administered with ritonavir, as ritonavir inhibits the cytochrome P450 enzyme, responsible for extensive first pass metabolism.

The present investigation was aimed to develop PLGA based nanoparticles for Gemcitabine HCl and Lopinavir for bioavailability enhancement after oral delivery. For both the drugs chosen, action at lymphatic site is desirable. Gemcitabine HCl acts on lymphatic tumours and Lopinavir at lymphatic viral reservoirs to combat resistance. PLGA nanoparticles were formulated and optimized by factorial design. It was hypothesized that drug loaded NPs would be taken up intact by M Cells in GALT, thereby will reach the lymphatic circulation and then to systemic circulation. Therefore, the drug will first reach at site of action by bypassing the hepatic first pass metabolism and opsonization in blood. Also, nanoparticles due to smaller size less than 200 nm, directly delivered inside the cells bypassing the p-glycoprotein flux through the cell wall; which is another issue in oral delivery.

**Gemcitabine HCl Loaded NPs**

**Gemcitabine HCl** loaded nanoparticles were formulated by multiple emulsification and solvent evaporation method using sonication. As Gemcitabine HCl is highly hydrophilic drug, its entrapment inside nanoparticles was a challenge. The process and formulation parameters were optimized systematically. After preliminary experiments, pH of internal aqueous phase was identified as critical parameter. By optimizing pH of internal aqueous phase to 3, higher entrapment was able to be achieved. The important parameters such as PLGA concentration ($X_1$), surfactant concentration ($X_2$) and sonication time of secondary emulsion ($X_3$) were optimized by $3^3$ factorial design using Particle size and entrapment efficiency as responses. Optimization by $3^3$ factorial design confirmed that Polymer concentration was the major contributing factor for particle size and entrapment efficiency. Contour and response surface plots showed utility in explaining the effects of various formulation variables on responses. Full model and reduced model equations were generated and ANOVA explained the omission of non significant terms. The formulation was optimized by desirability criteria of maximum entrapment with minimum particle size less than 200 nm.
The optimized nanoparticles were evaluated for particle size, zeta potential, entrapment efficiency, drug loading, surface morphology, DSC, FTIR, *in vitro, ex vivo* drug release studies in rat stomach and intestine. Qualitative and quantitative uptake studies by confocal microscopy, transepithelial permeability studies in Caco 2 cells, cytotoxicity studies in Caco 2 cells as well as K562 leukemic cancer cell lines, *in vivo* single dose bioavailability studies in male Wistar rats and stability studies.

Gemcitabine HCl loaded NPs showed particle size of 166.68 ± 3.59 nm (PDI 0.136) and entrapment efficiency of 56.39 ± 2.583%. Drug loading for optimized formulation was found to be 10.39 ± 2.131%. Zeta potential was -20.6 ± 2.321 mV. Particle size below 200 nm, with entrapment efficiency greater than 50% was achieved.

The TEM micrographs of nanoparticles showed discrete round structures below 200 nm sizes. It confirmed that multiple emulsification solvent evaporation method was effective for formulating nanoparticles of hydrophilic drug using PLGA. DSC studies showed change in crystallinity of Gemcitabine HCl compared to plain drug. It confirmed amorphization of drug and entrapment inside nanoparticles. DSC scan of bulk Gemcitabine HCl sample showed a single sharp endothermic peak at 277.49 °C ascribed to the melting of the drug while PLGA showed endothermic peak at 51.06 °C corresponding to its glass transition temperature (Tg). But in NPs, disappearance of melting endothermic peak was observed which indicated substantial crystalline change and amorphization of drug in the polymer matrix.

FTIR spectra of native gemcitabine showed characteristic peaks of amine bands at 1680 cm⁻¹ and characteristic peak of ureido group at 1721 cm⁻¹ with 3393 cm⁻¹ for stretching vibration of (NH₂). The spectrum PLGA showed peaks at (3511.18 cm⁻¹ and 1757.40 cm⁻¹), the intensity of both the peaks N-H of pure drug at 1721.25 cm⁻¹ and at 1680 cm⁻¹ were found to be weak in the spectra of Gemcitabine HCl loaded PLGA NPs. The peak of PLGA at 3511.18 cm⁻¹ was present with a slight shift to 3497 cm⁻¹, while peak for Gemcitabine HCl was absent, indicating that the drug was completely incorporated in the PLGA NPs. The Gemcitabine HCl loaded nanoparticles were found to be stable for the period of 3 months at 2-8 °C conditions.

The drug release from nanoparticles was slow and sustained. The plain drug solution released more than 40% drug in 1h and within 4h all of drug was released. Whereas
from drug loaded NPs, 25.96±3.254 % of drug was released in initial 4h, followed by 56.89±4.259% in 24h and 98.2± 2.371% of drug released at the end of 120h. The release followed Korsmeyer–Peppas model and fickian diffusion. The regression coefficient of the plot of log Mt/M∞ versus log t for NPs was found to be 0.995 with value of release exponent (n) as 0.37 which was less than 0.5.

The ex vivo drug release from plain drug solution showed that nearly complete drug was released in the stomach, whereas from NPs, 10% of drug was released in the stomach in initial hours and most of the drug was released in the intestinal segment. At the end of 6h study, nearly 40 % of drug was released. Because of entrapment of drug inside NPs, release from NPs was sustained. The drug release from NPs in stomach and intestine followed Korsmeyer-Peppas model and fickian diffusion (0.985 and n=0.486).

The uptake studies in Caco 2 cell lines confirmed the internalization of nanoparticles in the Caco-2 cells. The NPs were observed as red fluorescence spots in the perinuclear regions. The uptake of NPs in Caco-2 cells was time dependent, increased with incubation time upto 2h. Whereas, the plain dye solution did not show the uptake due to hydrophilic nature of the dye. We could find that, the uptake efficiency of a hydrophilic drug can be improved by encapsulating the drug in nanocarriers, as colloidal and surface properties of nanoparticles are different from native drug. Further, transport studies were performed to assess the increased permeation through intestine barrier. The permeability coefficient for Gemcitabine HCl solution was found to be 0.72×10^{-5}. The permeability coefficient for Gemcitabine HCl loaded NPs was found to be 4.6×10^{-5} which is 6.38 times more than the plain drug solution. The higher permeability coefficient for nanoparticles corresponds to the lipophilicity of PLGA NPs in which the drug is entrapped, whereas lower permeability coefficient for plain drug solution was because of high hydrophilicity of drug as well as poor permeation (log P 1.4). As the drug is substrate for p glycoprotein pump, the low permeability can also be attributed to that. The uptake studies and transport studies revealed that permeability of Gemcitabine HCl after loading it in nanoparticles was greatly improved. It would lead to enhancement of absorption via The nanoparticle formulation was found to be safe on Caco 2 cells with lesser cytotoxicity as compared to free drug.

Cytotoxicity studies by MTT assay confirmed that Gemcitabine HCl loaded nanoparticles were found to be 2.4 fold, 3.4 fold and 8.59 fold more cytotoxic on K 562 cancer cells
after 6, 24h and 48h incubation respectively, which could be attributed by higher uptake via endocytosis and more internalization of PLGA NPs with time duration. 

*In vivo* uptake studies by confocal microscopy in rat intestinal tissue after oral delivery of Rhodamine loaded NPs confirmed the penetration and absorption of NPs in intestinal tissue after oral administration. 

*In vivo* single dose bioavailability studies results showed Cmax for PLGA nanoparticles was found to be 1586.23±122.5 ng/ml, which was significantly higher than the plain drug solution. A higher Cmax for NPs could be achieved as drug loaded in PLGA NPs was capable to bypass hepatic first pass metabolism and able to reach directly to systemic circulation by virtue of size and surface properties of nanocarrier system. Cmax for plain drug solution was only 489.43±60.06 ng/ml, due to very low bioavailability of Gemcitabine HCl by oral route as Gemcitabine (dFdC) is extensively deaminated by cytidine deaminase into 2, 2-difluorodeoxyuridine (dFdU), which has been reported to be present mainly in liver of humans and kidney of rats. Tmax for NPs was found to be (4h) while for plain drug solution was found to be 1 h. Delayed Tmax would be justified by the fact that, after intestinal transit, major drug would be released at lymphatic site and then reach systemic circulation. Also, T1/2 was increased upto 18.99 h for NPs, which is 3 times higher as compared to plain drug solution; t1/2 of 6.42h. AUClast for Gemcitabine HCl loaded NPs was found to be 54,444.7± 3200 ng.h/ml, which is significantly higher than AUC last for Plain drug solution; 2534.72±686.5 ng.h/ml (P<0.01, student's unpaired t test). Plasma concentration from plain drug solution were undetectable after 4h and AUC last was very less as due to extensive metabolism to inactive metabolite, while NPs could achieve higher AUC due to slow and sustained release from NPs, which would protect the drug from metabolism . 

Gemcitabine loaded PLGA NPs showed 21.47 fold increase in relative bioavailability in comparison to plain drug solution after oral delivery. Improvement in bioavailability could be attributed to ability of nanoparticles to reach the oral lymphatic region after absorption through M cells of Peyer’s patches and reaching to systemic circulation through mesenteric lymph duct. Thus, PLGA nanoparticles could play important role in improving entrapment, uptake, permeability, Cmax and t1/2 Gemcitabine HCl which will ultimately lead to enhancement in its bioavailability. Oral chemotherapy would be possible in coming years for a drug like Gemcitabine HCl having hydrophillicity and
given by continuous IV infusion due to shorter half life in minutes, leading to too higher concentration and undesirable toxicity to tissues.

**Lopinavir Loaded NPs**

*Lopinavir* loaded nanoparticles were formulated by nanoprecipitation method using water miscible solvent acetone. The process and formulation parameters were optimized systematically. After preliminary experiments, the important parameters such as drug concentration ($X_1$), PLGA concentration ($X_2$) and surfactant concentration ($X_3$) were optimized by $3^3$ factorial design using particle size and entrapment efficiency as responses. Optimization by $3^3$ factorial design confirmed that increase in drug and polymer concentration lead to increase in entrapment and particle size. Contour and response surface plots showed utility in explaining the effects of various formulation variables on responses. Full model and reduced model equations were generated and ANOVA explained the omission of non significant terms. The formulation was optimized by desirability criteria of maximum entrapment with minimum particle size less than 200 nm.

The optimized nanoparticles were evaluated for particle size, zeta potential, entrapment efficiency, drug loading, surface morphology, DSC, FTIR, *in vitro* drug release, *ex vivo* drug release studies in rat stomach and intestine, qualitative cell uptake studies by confocal microscopys, transepithelial permeability studies in Caco 2 cells, cytotoxicity studies to confirm tolerability in Caco 2 cells, *in vivo* single dose bioavailability studies in male Wistar rats and stability studies.

Optimized formulation of Lopinavir loaded NPs had EE of 93.03±1.27% and particle size of 142.16±2.13 nm at +1, -1, and -1 levels of $X_1$, $X_2$ and $X_3$ respectively. Drug loading for optimized formulation was found to be 25.11±3.141%. Zeta potential was -27.2±2.423 mV. Thus, this confirmed that nanoprecipitation method was successful for formulating lipophilic drug loaded PLGA NPs.

The TEM micrographs of nanoparticles showed discrete round structures below 200nm sizes. DSC studies confirmed amorphization of drug and entrapment inside nanoparticles. Disappearance of melting endothermic peak was observed which indicated crystallinity change and amorphization of drug in the polymer matrix.
FTIR studies of Lopinavir loaded NPs showed absence of peak at 3376 cm⁻¹ corresponding to Lopinavir but peak corresponding to PLGA was present, indicating the incorporation of drug inside the NPs. Lopinavir loaded nanoparticles was found to be stable for the period of 3 months at refrigerated conditions.

In vitro drug release data showed that plain drug suspension released nearly 50 % drug in 3h, whereas drug release from NPs was near to 10 % in 3 hour reaching to 50 % in 24 h and near to 100 % in 120 hours. The drug release was sustained from the NPs. Lopinavir loaded PLGA NPs followed the Korsmeyer peppas model and non fickian diffusion with r² value of .9969 and n =0.647.

Ex vivo drug release studies in stomach and intestine showed that from plain drug suspension, more than 60% of drug was released in stomach, whereas drug release from NPs was slow and sustained; nearly 13% of drug was released in stomach in initial hours. Only small fraction of drug was released before the NPS could reach to the Peyer’s patches in intestine, indicating the stability of NPs in stomach. Low aqueous solubility of drug entrapped in polymeric system was the reason for slow release. When data of drug release from NPs were fitted to various kinetic equations, the drug release from NPs was found to be diffusion controlled as it follows Korsmeyer peppas model with r² value of 0.9652 and mechanism of drug release was non fickian diffusion (n=0.879 ).

The uptake studies in Caco 2 cell lines confirmed the internalization of nanoparticles in the Caco-2 cells. The NPs were observed as green fluorescence spots in the perinuclear regions. The uptake of NPs in Caco-2 cells was time dependent, increased with incubation time. Plain dye solution showed limited uptake. The uptake efficiency of a lipophilic drug could also be enhanced by encapsulating the drug in nanocarriers, which are uptaken by endocytosis and bypass p-glycoprotein efflux. Further, transport studies were performed to assess the increased permeation through intestine barrier.

Quantitative uptake studies using FACS showed that mean fluorescent intensity of 6-Coumarin loaded nanoparticles in Caco 2 cells was doubled from 1h to 4h. 6-Coumarin loaded NPs had 2.1 fold higher MFI at 1h, which increased to 3.6 fold to 6.4 fold higher MFI than plain dye solution at the end of 4h.

The permeability coefficient of Lopinavir loaded NPs in Caco 2 cells was found to be 8.84×10⁻⁵ whereas for Lopinavir solution was found to be 2.9×10⁻⁵. Lopinavir has a log P
value of 4.67 (Drug Bank) and it is lipophilic drug, therefore it is permeable to Caco 2 cells, but Lopinavir loaded NPs showed a 3.04 times increase in permeability, which could be attributed to the higher uptake of NPs by endocytosis in Caco 2 cells. There was significant enhancement in permeability of Lopinavir by loading the drug in NPs, as nanoparticles also bypass p-glycoprotein pump. The uptake studies and transport studies revealed that permeability of Lopinavir after loading it in nanoparticles was greatly improved. It would lead to enhancement of absorption via The nanoparticle formulation was found to be safe on Caco 2 cells with lesser cytotoxicity as compared to free drug. Confocal microscopy of rat intestinal section after oral delivery 6-Coumarin loaded NPs confirmed penetration and uptake of NPs.

After oral administration, Lopinavir loaded NPs exhibited higher plasma level concentration compared to plain drug solution. The $AUC_{\text{last}}$ for NPs was found to be $22335.3 \pm 2310$ ng.h/ml, which was significantly higher than PD which showed $AUC_{\text{last}}$ of $1596.5 \pm 216.5$ ng.h/ml. Significant improvement in $C_{\text{max}}$ in case of NPs compared to plain drug solution was observed. The $C_{\text{max}}$ for Lopinavir loaded nanoparticles was found to be $834.62 \pm 86.8$, whereas for plain drug suspension, it was $143.42 \pm 22.43$. The $C_{\text{max}}$ for plain drug solution was very less due to extensive metabolism of Lopinavir by liver microsomal CYP3A4 as well as p-glycoprotein efflux pump which secret it back in intestinal lumen. This improvement in $AUC$ and $C_{\text{max}}$ could be explained by the combination of the following effects: firstly, the drug molecules were absorbed from M cells of intestine due to particle size less than 200nm bypassing the efflux pump. Secondly, a decrease in first pass metabolism by liver microsomal enzymes, as NPs directly reaches systemic circulation through GALT. When $T_{\text{max}}$ of the NPs was compared with plain drug suspension, an increase in $T_{\text{max}}$ was observed in case of NPs. When $t_{1/2}$ of the NPs was compared with plain drug suspension, it was observed that there was significant difference in $t_{1/2}$ (17.93) as compared to PD (8.45). Similarly, when the mean residence time (MRT) of the formulations was compared with plain drug suspension, there was marked increase observed which indicated that elimination was extended for nanoparticles. There was about 13.9 times increase in bioavailability of Lopinavir loaded NPs.
This work could be a contribution towards the enhancement of bioavailability of Gemcitabine HCl and Lopinavir which are used in treatment of cancer and AIDS respectively. By encapsulating the drug in PLGA nanoparticles, adopting different methods for formulation depending upon the solubility of drug, a significant enhancement in absorption, uptake and permeation through intestinal barrier could be achieved leading to enhanced bioavailability. Therefore, Gemcitabine HCl, an anticancer agent which is currently administered as continuous iv infusion could be used as an oral chemotherapeutic agent, benefitting the patients by avoiding complications of parenteral administration. Thus, the orally delivered PLGA nanoparticles of Gemcitabine HCl could deliver the drug to the M cells of Peyer’s patches from where it directly reach to systemic circulation, thus protecting the drug from first pass metabolism as well as from gastrointestinal environment and avoiding the serious side effects associated with infusions. Also, Oral NPs for Gemcitabine HCl would be beneficial for lymphatic tumours, as higher concentration to tumour could be easy to achieve, which would be otherwise difficult to reach after iv injection, due to opsonization.

Also, Lopinavir which is always co administered with ritonavir for bioavailability enhancement, could be given alone as 13.9 times increase in bioavailability was achieved as compared to plain drug. Thus orally delivered NPs of Lopinavir has tremendous potential for improving the bioavailability of Lopinavir without co administration of Ritonavir and directly delivering the drug at lymphatic viral reservoir sites.

However, we need to conduct elaborate toxicological studies and preclinical studies and further investigations in human beings under clinical conditions before they can be commercially exploited.