Development of Carboplatin, 5-Fluorouracil and Oxaliplatin Loaded Protein Nanoparticle Formulation for Chemotherapeutic Application

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DOCTOR OF PHILOSOPHY

By

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DECLARATION

The research work presented in this thesis entitled **"**Development of Carboplatin, 5-Fluorouracil and Oxaliplatin Loaded Protein Nanoparticle Formulation for Chemotherapeutic Application**"**, has been carried out by me at Department of Biotechnology and Bioinformatics, School of Lifesciences, University of Hyderabad, Hyderabad, under the guidance of Prof. Anand K. Kondapi.

I hereby, also declare to the best of my knowledge that no part of this dissertation is earlier submitted for the award of any research degree or diploma in full or partial fulfilment in any other university. I hereby agree that my thesis can be deposited in Shodhganga/INFLIBNET.

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CERTIFICATE

This is to certify that **Farhan Ahmed** has carried out the research work embodied in the present thesis under the guidance of **Prof. Anand K. Kondapi**, for a full period prescribed under the Ph.D. ordinance of this university and also adhered to the UGC 2009 Ph.D. regulation. We recommended this thesis entitled **"Development of Carboplatin, 5-Fluorouracil and Oxaliplatin Loaded Protein Nanoparticle Formulation for Chemotherapeutic Application"** to submission for the degree of Doctor of Philosophy in this university. The work is original and has not submitted in part or full for any other degree or diploma of any other university.

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Farhan Ahmed

List of Abbreviations

Apo-nano-carbo:	Carboplatin loaded apotransferrin nanoparticle
Lacto-nano-carbo:	Carboplatin loaded lactoferrin nanoparticle
Sol-carbo:	Soluble or free carboplatin
Lacto-nano-5FU:	5-fluorouracil loaded lactoferrin nanoparticle
Lacto-nano-oxalo:	Oxaliplatin loaded lactoferrin nanoparticles
Sol-5FU:	Soluble or free 5-fluorouracil
Sol-oxalo:	Soluble or free oxaliplatin
5-FU:	5-fluorouracil
IV:	Intra venous
IP:	Intra peritoneal
COLO-205:	Human colon adenocarcinoma cell line
Y79:	Human retinoblastoma cells lines
ACF:	Aberrant crypt foci
AUC:	Area under curve
MRT:	Mean Retention Time
C_{max}:	Maximum concentration achieved after single dose
T1/2:	Elimination half-life
PBS:	Phosphate Buffer saline
Tf :	Transferrin
SDS:	Sodium Dodecyl Sulphate
RES:	Reticuloendothelial System
PEG:	Poly (ethylene Glycol)
BSA:	Bovine Serum Albumin

LF:	Lactoferrin
IL-6:	Interleukine-6
TNF-α:	Tumor Necrosis Factor
MCP-1:	Monocyte Chemoattractant protein-1
DSS:	Dextran Sodium Sulphate
SGOT:	Serum Glutamic Oxaloacetic Transaminase
SGPT:	Serum Glutamic-pyruvic Transaminase
SEM:	Scanning Electron Microscopy
TEM:	Transmission Electron Microscopy
UV:	Ultra Violet
MTT:	3-(4,5-dimethylthiozol- 2-yl)-2,5-diphenyltetrazolium bromide
DLS:	Dynamic Light Scattering
PDI:	Poly Dispersity Index
PTA:	Phospho Tungstic Acid
RP-HPLC:	Reverse Phase High Performance Liquid Chromatography
PLA:	Poly Lactic Acid
PLGA:	Poly(lactic-co-glycolic acid)
ELISA:	Enzyme Linked-Immunosorbent Assay
AOM:	Azoxy Methane
FT-IR:	Fourier Transform Infrared Spectroscopy
EPR:	Enhanced Permeability and Retention

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CHAPTER 1

INTRODUCTION

1.1 Cancer

Cancer is a complex disease state that include unregulated cell division, transformation competence, replicative immortality and resistance to apoptosis. Cancer cells grow and spread into several tissue types including hematopoietic, lymphatic systems and the bone marrow [Stein GS et al 2004]. Cancer is primarily originated by mutation or damage of proto-oncogenes that code for proteins responsible for the induction of cell proliferation and differentiation, and tumor suppressor genes that code for proteins that generate inhibitory signals of cell growth and/or stimulate cell death. Abrasions in both oncogenes and tumor suppressor genes are frequently associated with tumor development and are favoured by mutations in the tumor vulnerable genes, which encode a group of proteins associated in the control of DNA damage. The mutations that induce a tumor are clonally selected to help aberrant and uncontrolled cell division, the absence of cell growth inhibition, evasion of the immune system, blockage of apoptosis and transmission and accumulation of errors in genetic material. [Pollock P.M et al 2002, Hanahan D. 2011, McKinnell RG et al 1998].

Cancer cells are metabolically active and over express specific receptors those are associated with the assimilation of factors vital for growth and transformation. In the initial stages of cancer, cells are restricted within the normal boundaries of the tissues that is called benign. In the later stages of cancer, cells invade boundaries of healthy tissue and move across the body through blood and lymphatic system and colonize at in different organs, that state of cancer is called malignant. The malignant cancer cells secrete proteases that armed them to degrade the extracellular matrix around tissue boundaries that facilitate the invasion of cells. This ability of cancer cells to dysregulate normal control mechanisms is highlighted as the "hallmarks" of cancer: (1) ability to divide in the absence of stimulation mediated by growth factor, (2) ability to divide in the presence of anti-growth signals, (3) the inability to undergo apoptosis, (4) the ability to maintain telomere length despite repeated cell

divisions, (5) stimulation of angiogenesis, (6) the ability to invade surrounding tissues and move to other parts of the body (Hanahan D et al., 2000).

1.2 Therapy

Various treatment options for cancers is based on the type and stage of disease, if cancer cells are confined within particular tissue then it can be removed surgically or destroyed by radiation therapy followed by cycles of chemotherapy regime. Once cancers cells metastasize to different tissue, then cancer drug therapy (chemotherapy, biological therapy and hormone therapy) is only option left to treat cancer, since they are able to reach into every organ thorough systemic circulation [Chabner BA, et al 2005]. Brief account of different therapeutic regimes are given below.

1.2.1 Surgery

Surgery is the process of removal of the tumor and neighboring tissue. It is the preliminary treatment for various types of cancer, and some of them can be completely removed with surgery alone. The side effects of surgery depends on the surgical procedure, complexity of the cancer and the health of patient.

1.2.2 Radiation therapy

Radiation therapy is the process in which high-energy radiations (e.g. gamma rays, X-rays and high charge particle) are used to kill or shrink the cancer cells [Lawrence TS et al 2008]. Source of radiation can be used from outside the body by machine called external-beam radiation therapy, or it can come from radioactive material which is implanted into the body nearby cancer tissue called "internal radio therapy or brachytherapy". More recently systemic radiation therapy are being used in which radioactive iodine administered into blood stream and travel along to kill the cancer cells. Almost half of the cancer patient take radiation therapy in their life time of therapeutic regime.

Radiation therapy is considered as localized treatment regime, since it only affects the specific part of the tissue. The important purpose of radiation therapy include shrinking the volume of tumor

before surgery, deterring the tumor relapse after surgery, eliminating cancer cells in the neighboring tissue. The major aim of radiation therapy is to debilitate as much of the tumor as possible while abating the exposure of healthy tissue. Radiation therapy also inflict some unwanted reversible side effects, which may include fatigue, mild skin reactions, effect on digestive system, and loose bowel movements. Internal radiation therapy may cause bleeding, infection, or irritation after the implant is removed.

1.2.3 Cancer Drug Therapy

This treatment is carried out by biological and non-biological drug entity and this process has been very handy to manage cancer at benign stage and most importantly at metastatic phase. Different types of drug based therapy are briefly explained in under following section.

1.2.3.1 Biological therapy

Biological therapy also known as immunotherapy is designed to activate the humoral and cell-mediated immunological responses to fight against abnormal cancer cells (Snook AE et al 2013). This therapy are mediated by recombinant or synthetic antibodies, interleukins to block cancer growth and strengthen, or restore immune system function. The side effects of immunotherapy generally include flu-like symptoms, such as chills, nausea, and fever.

1.2.3.2 Anti-hormonal therapy

Activation of few specific type of cancers e.g. breast and prostate cancer are mediated by hormones. So some hormones are vital to support the growth of cancer cells. Therefore, anti-hormonal therapy are being used to reduce the hormones level in the body to check the cancer growth (Hinev A et al., 2012; Hatoum et al., 2013). It is employed for treatment of cancers of the prostate, breast, thyroid, and reproductive system. The side effects depend on the drug used and gender.

1.2.3.3 Chemotherapy

Chemotherapy is the use of chemical agents to treat various diseases, but most commonly it refers to use of chemicals for treatment of cancers. Various type of chemotherapeutic drugs are being used to treat different cancers either in combination or alone. These drugs belongs to different category of molecules based on their chemical structure and origin, some of them having broad spectrum activity and some are specific against particular cancer. Efficacy of conventional chemotherapy drugs are reported to be meagre because of following limitation associated with this regimes.

a) **Lack of target specificity-** Because of cytotoxic nature of chemotherapeutic drugs, these are not only kills cancerous cells but also rapidly proliferating normal cells e.g. hair follicle, bone marrow and gastrointestinal tract cells, leads to undesirable side effect in cancer patient [Chabner BA,et al 2005]. The side effects of toxic drug are mainly due to inability of drugs to discriminate between cancerous and healthy cells. This unspecific strategy has been changed with the finding of the cell signaling pathways associated with the control of cell proliferation and differentiation that open avenues to design of drugs that specifically affecting those pathways, laid down the path for targeted therapy [Chabner BA, 2005]. Targeted therapy has been more promising than conventional chemotherapy, still their efficacy became limited due to following reasons which are also valid in the case of conventional chemotherapy drugs.

b) **Poor aqueous solubility**- most of the drugs obtained either from plant sources or from chemical synthesis, are hydrophobic in nature and required excipient to formulate with drugs to enhance their aqueous solubility and eventually biological fluid solubility that enhance the drug half-life in plasma. These excipient often cause greater side effects along with chemotherapy drugs and thus reduces the overall drug efficacy [Kwon GS 2003]. So excipient

related toxicity are still tagged with conventional chemotherapy and that need to be addressed to increase therapeutics index.

c) **Multi drug resistant (MDR) –** cancers are becoming resistant against chemotherapy mainly due to excessive use of drugs. Drug efflux pump like Pgp glycoprotein said to be responsible for chemo resistant cancers. This is the major bottleneck of chemotherapy regime since it reduces the therapeutics index of drugs [Stavrovskaya AA. 2000].

Additional limitation apart from above that includes, drug stability in the biological fluids and short half-life because of rapid clearance and metabolism. Similarly, high protein binding capacity of certain drugs such as protease inhibitors (Sawyer AJ et al., 2006) limits their diffusion across the organs.

Side effect of chemotherapy- The side effects of chemotherapy depend on the individual and the drug dose used, which include mild symptoms e.g. fatigue, risk of infection, nausea and vomiting, loss of appetite, and diarrhoea as well as life threating side effect e.g. bone marrow suppression, renal failure and hepatic abnormality. Side effects are major reason of suspension and/or change in mode of therapy.

Above given limitation of conventional chemotherapy are currently being addressed by different researcher globally cutting across the different discipline, most notably invention of drug delivery platforms for chemotherapy agents, those are being discussed explicitly in following sections.

1.3 Drug delivery

Drug delivery is a fascinating field of research that has catapulted the interest of researchers, since delivering a medicine to its site of action is one of the main limitations of pharmaceutical industries. In modest terms, drug delivery can be defined as the process of delivering a therapeutic agent at a specific rate and on a specific site but in the existing scenario, targeted drug delivery is a

limitation that should be overcome to use thousands of new therapeutics effectively, that are limited due to lack of a safe and effective drug-delivery system [Orive G et al 2003, Langer 1990]. As discussed earlier most of the drugs are limited due to their poor aqueous solubility, high dose related toxicity, aggregation due to poor solubility, non-targeted delivery, systemic degradation and short plasma half-lives. Therefore, the development of new techniques that could specifically deliver drugs to the site of action is presently one of the most important areas of drug delivery research.

A number of effort have been put forth to deal current bottleneck associated with conventional therapy, some of limitation were addressed by conjugation of drugs with biodegradable polymer and antibody to enhance the stability, solubility and plasma half-life of drugs along with specificity [Edgar PH et al 2015]. Nanoparticle mediated drug delivery platform gain more interest than other form of delivery vehicle and it will be explained comprehensively in the following section.

1.4 Nanoparticle based formulation for targeted drug delivery:

Nanotechnology is the formation and application of materials, devices and systems through the control of matter in a nanometer size scale, at atomic, molecular and supramolecular level [Sahoo SK et al 2007, Sahoo SK et al 2003]. Nanoparticle possesses very unique property that set them apart from bulk and single molecule. Such unique property of nanoparticle has been exploited to develop therapeutics and diagnostic platform. Due to their small size and large surface area, nanoparticles could be a very interesting drug delivery platform to deliver drug across different biological barriers. Large surface area of nanoparticle provide enough surface to interact with biological membrane and also provide enough surface to functionalise with different moiety. In nanoparticles drug can be dissolved, encapsulated, entrapped in nanoparticle matrix or attached to surface. Based upon the method of preparation, nanoparticles can be two different type nanospheres or nanocapsules. Nano capsules are vehicular systems in which the drug is loaded and enclosed into a cavity surrounded by polymer membrane, whereas, nanospheres are matrix systems in which the drug is physically and

uniformly distributed [Suphiya P et al 2012]. Nanoparticle alone have some unique advantage over other drug delivery modalities which make them attractive candidate for drug delivery vehicle. Some of the advantages of nanoparticles are listed below

Advantage of nanoparticle as drug delivery vehicle

- Release drugs at predetermined rate over an extended period of time (Sustained drug release).
- Release drugs especially at sites of action with the prospects of controlled release rates (targeted delivery).
- Sustain drug concentrations within therapeutically suitable ranges in circulation and within tissues (Improved bio availability).
- Protect drugs from hepatic inactivation, enzymatic degradation and rapid clearance from the system
- Enhance aqueous solubility of drugs
- Decrease potential side effect of drugs
- Provide appropriate form for different route of administration
- Permit facile-formulation development.

1.5 Strategies for targeted nanoparticles

The efficacy of a cancer therapeutic platform is measured by its ability to subdue and eradicate tumors without harming healthy tissue. Therefore, a distinct approach to target tumors is essential for the success of the therapeutic platform. An Enhanced site specificity and internalization can improve the effectiveness of treatment and reduce the possibility of the critical side effects that cancer patients often encounter. Nanoparticle mediated systems offer major advances in therapeutics by site specificity, ability to escape from multi-drug resistance, and the efficient delivery of drugs [Brannon-

Peppas L et al 2004]. Nanoparticles cargo can be targeted for release of their pay load at therapeutic sites, mediated by two major targeting strategies as explained follow.

Passive targeting of nanoparticles

Nanoparticle exploit the innate characteristics of tumor growth environment for the use of a passive mode of targeting. The tumor mass at a volume of 2 mm^3 or above becomes diffusion limited, diffusion limitation hampers nutrition uptake, waste excretion, and oxygen transportation. The tumor uniquely overcome the diffusion barrier by increasing the adjacent vasculature, called angiogenesis [Jones A et al 1998, Baban DF, et al 1998]. The incomplete tumor vasculature, a characteristics of angiogenesis results into leaky vessels with pore sizes of 100 nm to 2 μm subject upon the tumor type [Hobbs SK et al 1998]. The amalgamation of leaky vasculature and poor lymphatic drainage constitute the Enhanced Permeation and Retention (EPR) effect phenomenon. Size of nanoparticles smaller than endothelial pore size can enter into the interstitium and be entrapped in the tumor [Haley B., et al 2008]. Virtually, every type of nanoparticle loaded with molecule of interest with size less than 200nm can be passively targeted to tumor mass by leaky vasculatures facilitated by EPR effect around them. The EPR effect has been witnessed for a wide range of macromolecules such as proteins; including immunoglobulin G (IgG), micelles, liposomes, drug-polymer conjugates, polymeric NPs and different other types of NPs [Maeda H. et al 2009, Saha RN et al 2010, Torchilin VP 2010]

Active targeting of nanoparticles

Active targeting of nanoparticle involves the use of affinity ligands to facilitate the binding of nanoparticles to antigens, those are differentially overexpressed on the plasma membrane of tumor cells or to the extra-cellular matrix proteins that are differentially expressed in the tumor tissue. Nanoparticle can be actively targeted for intracellular delivery if target antigen present on the surface of tumor cells or extracellular targeting if target antigen present on extra cellular matrix of tumor tissue. Actively targeted nanoparticles for intracellular delivery may be internalized via clathrin-

dependent endocytosis pathways, caveolin-assisted pathway, cell adhesion molecule directed pathway, or lipid raft associated mechanisms, induce endosome formation, which ultimately fuse to lysosomes and deliver the drugs into cytosol [Bareford LM et al 2007].

A number of cell surface receptors that have been over expressed on the cancerous cells including, transferrin receptor [Davis ME et al 2010], folate receptor [Dohmen C. et al 2012], integrin receptor [Han HD et al 2010], LDLR [Jin H. et al 2012], GPCRs [Hild W et al 2010] and receptor tyrosine kinase (EGFR, VEGFR) [Tseng CL et al 2009] are being used to target particular cancer. Various type of targeting moieties have been used to conjugate on the surface of nanoparticle against particular surface receptor overexpressed on tumor cells includes, small molecule(Folate), antibody against receptor, small peptide against receptor, whole ligand protein and aptamers against surface antigen has been successfully demonstrated.

A new class of whole ligand nanoparticle system for active targeting has been developed by our group in which apotransferrin and lactoferrin protein prepared into Nano particulate formulation for doxorubicin and carboplatin delivery into cancerous cells. The advantage of this system are , whole nanoparticle itself act as targeting moiety and reduce the extra effort for conjugation and make entire process easy [Saikrishna ADS et al 2009, Ahmed F et al 2014].

Actively targeted nanoparticle system is more efficacious than passively targeted, because previous one is delivering drug directly into the cells rather than around the tumor that is shown in figure 1.1a&b.

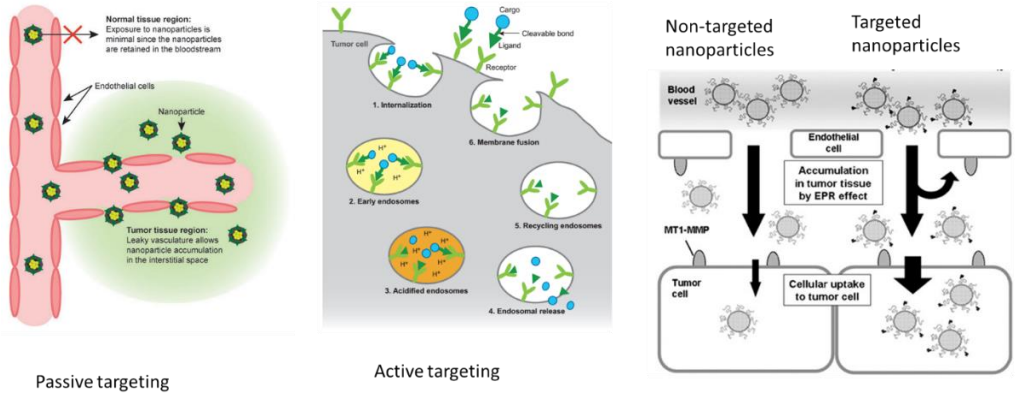


Figure 1-1:a) passive targeting in tumor mass through EPR effect where endothelial space is more compare to normal tissue region. b) Non-targeted nanoparticle accumulate into tumor by EPR effect from where it enter into the cells are less in amount as compare to targeted nanoparticles. Adapted from Byrne JD (2008) et al © Advanced Drug Delivery Reviews, and Nie S (2007) et al . © Annu. Rev. Biomed. Eng.

1.6 Current nanoparticle System for cancer therapy

Various type of nanoparticle systems currently being developed for cancer therapeutics. The properties of material for each nanoparticle system have been developed to boost delivery to the tumor, e.g. hydrophilic surfaces of nanoparticles provide the stealth properties for longer circulation and positively charged surfaces can enhance cellular entry. The types of nanoparticles materials presently used in research for cancer therapeutic applications that includes, liposomes [Bellocq NC et al 2003], polymeric nanoparticles [Betancourt T et al 2007] , protein nanoparticles [Saikrishna ADS et al 2009, Ahmed F et al 2014, Golla K et al 2012,Veronese FM et al 2005], ceramic nanoparticles [Montet X et al 2006], dendrimers [Kukowska-Latallo JF et al 2005], viral nanoparticles [Flenniken ML et al 2005], metallic nanoparticles, [Lowery AR, et al 2006], micelles [Sutton D. et al 2007] and carbon nanotubes [Kam MW et al 2005].

Functionalization of nanoparticles to develop a stealth surface prevent from opsonisation, binding serum proteins to the nanoparticle surface, is vital to increase circulation times, avoiding clearance by the reticuloendothelial systems (RES) [Bhadra D. et al 2003 and Fawaz F. et al 1993].

Short circulatory half-life hamper the efficiency of the delivery of nanoparticle to tumor site. Doping of a hydrophilic polymer on the surface of nanoparticles such as poly (ethylene glycol) (PEG), inhibit the opsonisation eventually reduces Clearance by the RES [Gref R et al 2000]. Various physicochemical properties that effect the in-vitro and in-vivo behavior of nanoparticle are explained in Fig 1.2 [Nazila K et al 2012]. Protein nanoparticles and its advantages will be briefly explained in next section, since my entire work is based on protein (apotransferrin and lactoferrin) nanoparticles.

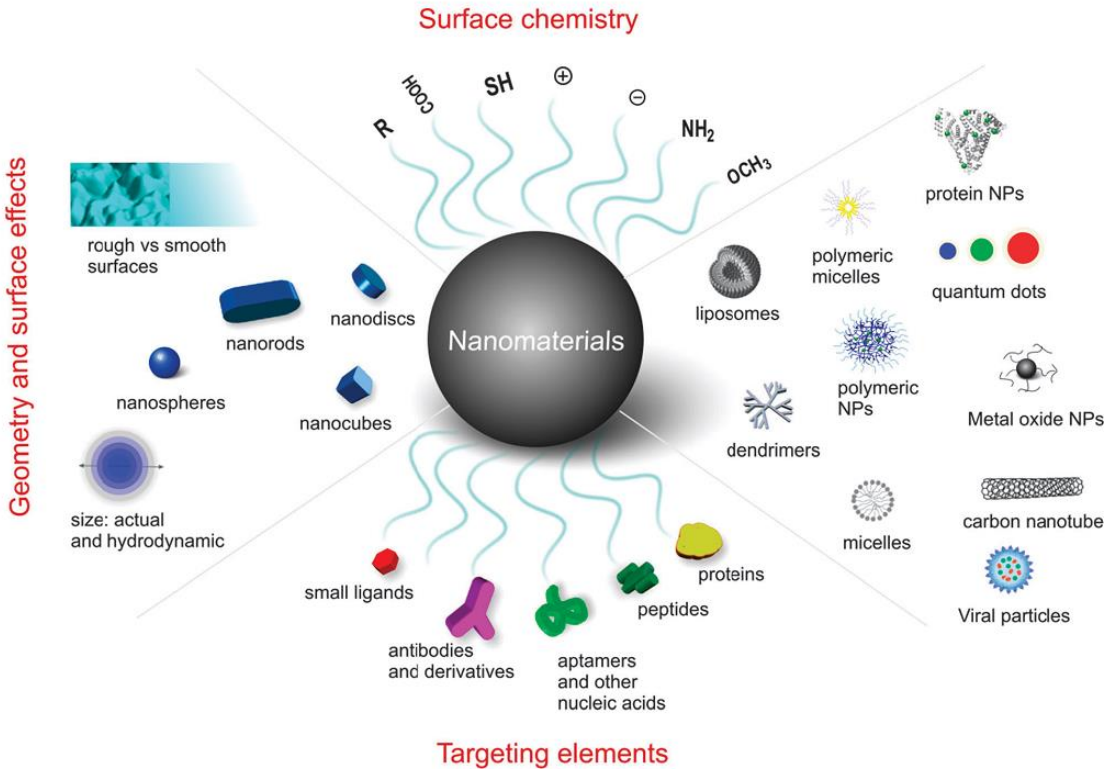


Figure 1-2: Nanoparticles with their physicochemical properties that effects the behaviour in in-vitro and in-vivo situation adapted from Nazila K. et al Chem. Soc. Rev., 2012, 41, 2971–3010 © Chem. Soc. Rev.

1.7 Protein nanoparticles

In the recent time nanoparticle made up of protein polymers draw great attention because of their easy biodegradability, systemic tolerance, and easy surface manipulation. Bovine serum albumin (BSA) nanoparticle is widely used for drug delivery because of its abundance, low cost, ease of

purification, unusual ligand-binding properties, bio degradable and its wide acceptance in the pharmaceutical industry (Hu YJ et al., 2006; Tantra R et al., 2010). BSA nanoparticle alone, lack target specificity until unless functionalise with targeting moiety that itself make entire process complex. Most recently fibrinogen nanoparticle loaded with 5-FU were developed for sustained drug delivery but it also suffer from target non specificity [Rejinold NS et al 2011].

Apotransferrin and lactoferrin nanoparticle have been developed in our laboratory with different drugs that is based on active targeting principle without conjugation with targeting moiety and it possesses several advantage over other nanoparticles. Brief account of apotransferrin and lactoferrin proteins are given below.

1.8 Transferrin

Iron play a central role in DNA replication as one of the major enzyme ribonucleotide reductase, requires iron as a co-factor. Iron is also a co factor for Heme (Li H et al., 2002). Though, free iron can be toxic, stimulating free radical formation via the Fenton and Haber-Weiss reactions, thus effects in oxidative damage to tissues (van Campenhout A et al., 2003). Free iron causes lipid peroxidation by changing hydro peroxides into reactive peroxyl and alkoxyl radicals. Furthermore, Tf promotes anti-oxidation reactions involving carbohydrate aldehyde groups and protein amino groups, which end up in the formation of glycated products. For above reason it is important that iron remains transported in a redox-inactive form. The crucial role of Tf is, therefore to transport iron safely throughout the body to growing cells. The binding and release of iron from Tf regulated by several factors, i.e., pH, temperature, metal chelator and ionic concentrations (He QY et al., 2000).

1.9 Lactoferrin

Lactoferrin (LF) is an iron-binding glycoprotein of the transferrin family with molecular weight of 80 KDa. This protein was secreted in most of the biological fluids and is a major component of the mammalian innate immune system, also present in neutrophil granules in mammals. Lactoferrin share common structural and functional features with transferrin. This is one of the members (Baker EN 1994) of the transferrin family like serum transferrin (sTf), and ovotransferrin (oTf). These proteins are glycoproteins in nature, having molecular weight of about 80 kDa and naturally they exhibit 50–70% pairwise sequence identity. Lactoferrins share 60% sequence identity with serum transferrin. Amongst lactoferrins of different species, the similarity is~70% (Baker EN 1994). Lactoferrin is secreted out in mucosal secretions, including nasal and bronchial secretions, bile, gastrointestinal fluids, urine [Öztas et al., 2005], tears, saliva, vaginal fluids, semen, and most abundantly in milk and colostrum (7 g/L) with sole purpose of providing innate immunity against broad range of microbes (Rodriguez DA et al., 2005), making it the second most abundant protein in milk [Connely OM 2001]. Lactoferrin receptor (LfR) are overexpressed in certain epithelial and endothelial cells, for example endothelial cells of brain capillary [Huang RQ et al 2007], intestinal and colon epithelial cells (Caco2) [Jiang R et al 2011]. That receptor can be exploited for the delivery of drug to the brain as well as to colon and intestine.

1.10 Transferrin based delivery system

Rapidly proliferating malignant cells are high in metabolic activity and to sustain that it needs high amount of iron [Xiu-Lian D et al., 2004], highly metabolic active brain cells express higher amount of transferrin receptors to facilitate iron transport. The use of transferrin receptor antibodies as vehicle of drug for targeted delivery to cells that overexpressed transferrin receptors is extensively studied [Kratz F et al., 2000]. Since, Apo transferrin being an abundant plasma protein so it is an ideal

candidate for purification from plasma [von Bonsdorff L et al., 2001]. In this line, delivery systems can be designed for specific delivery of drugs to rapidly dividing cells.

1.11 Carboplatin and Oxaliplatin

Carboplatin (cis-diammine (1, 1-cyclobutanedicarboxylato)-platinum (II)), a second generation drug synthesised from first generation drug, cisplatin (Fig 1.3). It induces DNA damage by cross linking of double stranded DNA leading cell death though apoptosis [Knox RJ et al 1986]. Mode of action is similar to parent drug cisplatin though rate of reaction is slower than that, thus require higher dose [Elferink F et al 1987]. Toxicity of carboplatin is reported to be less than cisplatin. Carboplatin is recommended for the treatment of ovarian cancer, small or non-small cell lung cancer, retinoblastoma, head and neck cancer, and lung cancer [Vaughn DJ et al 2000,. Zatloukal P et al 2002, Alberts DA 1999]. However, the side effects of carboplatin including dose related myelosupression with severe thrombocytopenia and leucopenia. In older patients renal impairment also observed who have previously received chemotherapy [Wagstsff AJ et al 1989]. Therefore, it is very important to formulate carboplatin with protein nanoparticle for targeted drug delivery to reduce dose related toxicity.

Similarly Oxaliplatin (*trans-l*-diaminocyclohexane oxalatoplatinum), a third-generation platinum antitumor drug (fig1.4), more advance and safe than cisplatin and carboplatin is currently sanctioned in combination therapy with 5-fluorouracil (5-FU)/ leucovorin for ordinary first- and second-line treatment of metastatic or advanced-stage colorectal cancer[Hind D et al 2008, de Gramont A et al 2000]. It's mode of action is similar to their parent compound. It is also associated with some side effect, even though less serious than parental compounds, for example, acute reversible neuropathy, thrombocytopenia. However, oxaliplatin does not show effective antitumor activity *in vivo* when used alone. This lower antitumor activity is mainly because of the high accumulation in

erythrocytes and less delivery in tumor tissues following intravenous administration [Pendyala L, et al 1993, Abu-Lila et al 2009]. Therefore protein nanoparticle can play major role in delivering of drug to tumor tissue and reduce side effect of drugs.

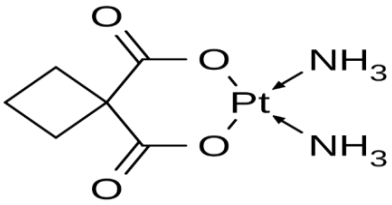


Figure 1-3:Oxaliplatin

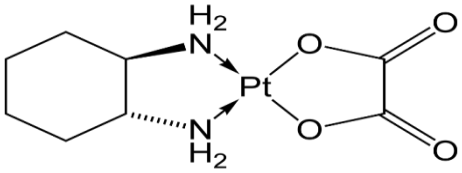


Figure 1-4: Carboplatin

1.12 5-fluorouracil (5FU)

5-FU is structural analogue of pyrimidine (fig 1.5), and it's an antimetabolite inhibits the thymidylate synthase and deplete nucleotide pool ultimate decrease the rate of DNA and RNA synthesis. It can also cause break in the DNA strand by incorporating into DNA during replication. This drug has been approved for the treatment of various cancer including colorectal cancer, head and neck cancer, gastrointestinal cancer, breast cancer hepatomas either in combination of leucovorin or alone. It cause some dose related side effect that includes mild myelosuppression, nephrotoxicity and hepatotoxicity. That side effect can be overcome by loading 5-FU in to nanoparticles based formulation.

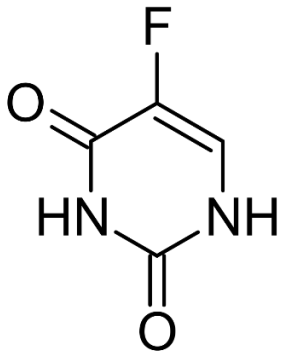


Figure 1-5:5-fluorouracil

1.13 Advantages of Protein nanoparticles

Protein nanoparticles drug delivery system is novel and effective in terms of

1. Vehicle itself act as targeting moiety.
2. No chemical modifications are required for the drug or protein.
3. No conjugation required on the surface of nanoparticle for targeted delivery
4. Any protein ligand specific to particular receptor of a diseased cell can be used for target specific delivery
5. Well tolerated because of high biodegradability.

1.14 Rationale of the study

Current treatment of

- Retinoblastoma using Carboplatin via peri ocular route is reported with reduced efficacy and myelosuppression
- Treatment of Colon adenocarcinoma using Oxaliplatin and 5-fluorouracil reported with nephrotoxicity along with myelosuppression.

Since Lactoferrin nanoparticle based drug delivery illustrated lower localization of drug in heart, bone marrow, kidney, spleen etc. (Golla K et al 2013), it would be interesting to evaluate improvement in bioavailability and safety of

- Carboplatin when loaded in apotransferrin and Lactoferrin nanoparticles and administered through periocular route in eye.
- Oxaliplatin and 5-fluorouracil when loaded in Lactoferrin nanoparticles and administered through *intravenous* route.

1.15 Objectives

1. **Preparation and physicochemical characterisation of carboplatin, Oxaliplatin and 5-fluorouracil loaded protein nanoparticles**
2. **Evaluation of efficacy of Carboplatin loaded Protein nanoparticles in retinoblastoma cells**
3. **Analysis of safety and bioavailability of Carboplatin loaded Protein nanoparticles when administered through periocular route**
4. **Safety, bio distribution and efficacy of Oxaliplatin and 5-fluorouracil loaded lactoferrin nanoparticle against Colon adenocarcinoma: an *in-vtro* and an *in-vivo* study.**

CHAPTER 2

Materials & Methods

2.1 Materials

Drugs: Doxorubicin, Carboplatin, Oxaliplatin and 5-Fluorouracil were purchased from Sigma Aldrich.

Cell lines: COLO-205 and Y79 were obtained from NCCS PUNE, INDIA.

Antibodies: Mouse anti-human transferrin receptor MAb (CD71) (Calbiochem). Rabbit Anti-human Intestinal lactoferrin receptor polyclonal antibody, from Biorbyt.

ELISA kits: IL-6 (Rat) from Peprotech (USA), TNF-α (Rat) and MCP-1 (Rat) from BD Biosciences.

Biochemical assay kits: SGOT analysis kit (span diagnostics), SGPT analysis kit (span diagnostics), Creatinine analysis kit (span diagnostics), blood urea nitrogen analysis kit (span diagnostics),

Solvents: Absolute alcohol (Ethanol), Diethyl ether, acetone, methanol, surgical spirit, HPLC grade ethyl acetate, acetonitrile all from Sigma Aldrich.

Instruments: Centrifuge (REMI C30BL and Hermle Z36HK), Vortexer, ultrasonic homogenizer or sonicator, (300V/T, Biologics Inc., Manassas, Virginia, USA) fluorimeter (Shimadzu RF5000), UV & visible spectrophotometer (Jasco v550), Scanning electron microscope (Philips FEI-XL 30 ESEM, USA), Transmission electron microscope (FEITECNAI G^2S-TWIN 200kv,Germany), Zeta sizer (Malvern instrument), gold in Sputter Coater, Laser Confocal microscope (Carl zeiss), HPLC with UV-vis detector (Waters) Gel electrophoresis apparatus, ELISA reader (Teccan).

Reagents: Phosphotungstic acid, TrisHCl buffer, potassium chloride, potassium dihydrogenorthrophosphate, potassium hydrogen orthrophosphate, sodium hydrogen orthrophosphate, disodium hydrogen orthrophosphate, tween20, , acryl amide, bisacryl amide, TEMED, KOH, SDS, EDTA, Glycene, ammonium persulphate, sodium acetate buffer, Bradford reagent, NaCl, rhodamine^{123} (sigma), phosphate buffered saline (PBS), olive oil, hyderochloric acid (HCl), monobasic potassium phosphate, NaOH, lysotracker dye (Invitrogen), silver nitrate, saline (0.9% NaCl), trichloro acetic acid, acetic acid, Azoxy methane (sigma), Dextran Sodium Sulphate

(DSS M.wt, ~40000 from MP Bio), Agarose, phosphate buffer, Sodium pentabarbitrate, formalin, paraformaldehyde, hematoxylinand eosin (H&E), 3-(4,5-dimethylthiozol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma).

Software's: Kinetica v5.0 software (Thermo Scientific, USA). Sigma Plot v11.0

2.2 Methods

2.2.1 Preparation of apo-nano-carbo, lacto-nano-carbo and rhodamine^{123} loaded nanoparticles

Nanoparticles were prepared using sol-oil chemistry as the procedure described previously [Saikrishna ADS et al 2009]. Briefly 15 mg of Apo transferrin and lactoferrin dissolved in 500 µl of phosphate-buffered saline (pH 7.4) was slowly mixed with (5, 10, 15) mg of carboplatin, (Sigma-Aldrich) separately and the mixture was incubated on ice for 30 min, same amount of oxaliplatin and 5-fluorouracil were added into lactoferrin solution. The mixture of Apo transferrin or lactoferrin with drugs were slowly added to 15 ml of olive oil in two different bottles at 4°C with continuous dispersion by gentle vortexing. The samples were sonicated 15 times at 4°C using a narrow stepped titanium probe of ultrasonic homogenizer (300V/T, Biologics Inc., Manassas, Virginia, USA). The sonication amplitude was 5 mm and the pulses were 30 sec long with an interval of 1 min between successive pulses. The resulting mixture were immediately frozen in liquid nitrogen for 10 min and then transferred to ice and incubated for 4 h. The particles were pelleted by centrifugation at 8000 rpm for 10 minutes and the pellet was extensively washed (3 to 5 times) with diethyl ether and dispersed in 1xPBS by sonication. Apo transferrin and lactoferrin were incubated with 50 µl of 20% Rhodamine ^{123} dye for 30 min at 4^0C then Nanoparticle were prepared by similar procedure as stated above. The content of protein in particles was estimated by the Lowry method and the amounts of protein were used to determine the nanoparticle concentration.

2.2.2 Evaluation of loading efficiency

The prepared nanoparticles were suspended in 1xPBS (pH-5) and kept for 30 min at RT for release of drug from nanoparticle (n=3). Suspended nanoparticles were centrifuge at 15000rpm for 15 min and supernatant were taken for drug estimation. Carboplatin solutions of different concentrations were prepared and estimated by UV spectrophotometer (JASCO) to develop a standard curve for the calculations of encapsulation efficiency of carboplatin loaded into apotransferin (apo-nano-carbo) or lactoferrin (lacto-nano-carbo) nanoparticles. While oxaliplatin (lacto-nano-oxalo) and 5-FU (lacto-nano-5FU) were estimated by RP-HPLC method against their standard curve. RP-HPLC profile of oxaliplatin was (mobile phase-buffer:methanol in ratio of 30:60, flow rate-1ml/min, retention time-8min, detection 229nm) and 5-FU was (mobile phase-buffer:methnol in the ratio of 60:40, flow rate-1min/ml, retention time-6min, detection at 258nm). Supernatant of samples were analysed in triplicate and the drug loading efficiency was calculated by following formula of mass balance.

LE= amount of loaded drug (A)/amount of total drug used (T) X 100

Amount of drug loaded into the nanoparticle can be calculated by following formula.

Input [total drug used in preparation] = output [unloaded drug] + accumulation [loaded drug]

Now, accumulation (A) = input (T) - output (W), then LE=A/T X 100.

2.2.3 In vitro release assay

Pelleted nanoparticles (200μg) were suspended into I ml of phosphate buffer saline (1xPBS), (pH-7.4) and kept under gentle shaking at 200 rpm in shaker incubator. Individual sample were collected by centrifugation at 15000 rpm for 15 min at defined time interval .5hr, 1hr, 2hr, 4hr, 8hr, 12hr, 18hr, 24hr, 48hr, 72hr, 120hr, 240hr, 360hr and 480hr. Supernatant were treated with equal volume of isopropanol and kept for overnight at 4^0C. Entire mixture were centrifuged at 12000rpm for 15min, supernatant were filtered and stored at -20^0C till estimation. Supernatant were used for

estimation of drug that releases by HPLC as described above. Each sample were measured in triplicate (n=3).

2.2.4 pH dependent release assay

Pelleted nanoparticles (200µg equivalent to carboplatin, oxaliplatin and 5-FU) were suspended into I ml of phosphate buffer saline (1xPBS) of different pH ranges (1-9) and kept under gentle shaking at 200 rpm in shaker incubator for 4 hours. Individual sample were collected by centrifugation at 15000 rpm for 15 min at different time interval. Carboplatin was estimated in supernatant by spectrophotometer at 220nm and Oxaliplatin and 5-FU were estimated by HPLC method. Standard curve of different concentrations were prepared with same incubation media for quantification of drugs. Each sample were measured in triplicate (n=3).

2.2.5 Physicochemical Characterisation of nanoparticles:

Structure and morphology of all mentioned nanoparticles were characterised by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Scanning Electron microscopy. Nanoparticle samples were analysed using a PHILIPS FEI-XL ESEM (USA), operated at 100 kv. Nanoparticle sample were spread uniformly at glass Slide in proper dilution and left for some time in dust free environment till slide dried completely. Now glass slide with nanoparticle sample were pasted on metal stubs with the help of double-sided adhesive tape and dried specimen coated with gold in Sputter Coater. Specimens were stored in dry, dust free environment till the analysis. Images are recorded using appropriate resolution.

Transmission Electron Microscopy. Drug loaded protein nanoparticle sample were prepared for electron microscopy by air-drying small drops of a sample solution onto carbon-coated copper electron microscopy grids. Now the grids containing air-dried samples were incubated with a 2% (w/v) aqueous Phosphotungstic acid (PTA) for 10 min at room temperature and washed 2-3 times with distilled water. Protein nanoparticle images were examined using the FEITECNAIG^{2}S-TWIN

200 kv electron microscope. Electron diffraction patterns were recorded from a selected area that is well occupied with protein nanoparticles in order to obtain high diffraction intensities and data were analysed.

Mean size distribution, poly dispersity index (PDI) and zeta potential were analyse by dynamic light scattering (DLS) method (zetasizer from Malvern). FT-IR spectra of all drugs proteins and their nano formulation were recorded in Perkin Elmer FT-IR spectrophotometer employing KBr mode. Mean sedimentation speed and time of nano formulation were calculated by simple centrifugation method. 1 ml of 200μg of nanoparticles were taken in 1.5 ml tubes and rotate at different speed and time to calculate the g force and respective time at which entire nanoparticle got settle down.

2.2.6 Cell culture

Human retinoblastoma cell line Y79 and human colon adenocarcinoma cell line (COLO-205) were purchased from the national cell repository (NCCS Pune, India) were cultured in RPMI 1640 (GIBCO Invitrogen) with 10% foetal bovine serum (biological industries, Israel) and 1% penicillin–streptomycin (Penstrep, GIBCO) at 37^0C in a humidified 95% air and 5% CO_2 atmosphere (SANYO incubator, japan).

2.2.7 Cellular uptake

Cellular uptake assay were carried out in Y79 and COLO-205 cells by following methods.

2.2.7.1 Cellular uptake assay by confocal microscopy

Y79 cells (.2 million) were seeded in serum free media for 60 min in 12 well plate(corning) then incubated with 30μg of apotransferrin or lactoferrin nanoparticles tagged with rhodmaine123 green fluorescent dye and equivalent amount of soluble dye (soluble-rhodamine 123), for the different time points 30minutes, 1, 2 & 4 hours. Similar number of COLO-205 cells at similar concentration were incubated with rhodamine tagged doxorubicin loaded lactoferrin nanoparticle and free doxorubicin (as tracking dye) for similar time points of incubation. Cells were harvested at defined

time points and washed thrice in 1XPBS (phosphate buffer saline, PH 7.4), subsequently cells were mounted on glass slide. Then the amount of intracellular nanoparticle and free rhodamine was assessed through laser confocal microscopy (Carls Zeiss) employing the intrinsic fluorescence of rhodamine123 (green Ex=505 and Em=530), doxorubicin (Red Ex=470 and Em=650).

2.2.7.2 Cellular uptake assay by quantitative measurement of drug

Y79 Cells ($2x10^6$) were suspended in serum-free medium for 60 min in a 12-well plate. Apo-Nano-carbo, Lacto-nano-carbo or soluble-carbo (equivalent to 60µg carboplatin) were added to the each well in triplicate for different time points 30minutes, (1, 2, 4, 8, 12, 16, 24, 36, 48, 60, 72) hours. In different set of experiment 2 million COLO-205 cells in serum free media were incubated with 60µg equivalent Lacto-nano-5FU, lacto-nano-oxalo, sol-5FU and sol-oxalo and collected at specific time points .5, 1, 2, 4, 8, 12 ,16 and 24 hours. After incubation, each type of cells were washed thrice with 1XPBS (phosphate-buffered saline, pH 7.4) and lysed in 1 ml of 1XPBS (pH 7.4) with .1% TritonX-100 under mild sonication for 30 second. The lysate was cleared by centrifugation at 12,000 rpm for 20 min at 4 °C then supernatant were collected in fresh tube. Proteins were precipitated from supernatant by adding equal volume of isopropanol 1:1 ratio and incubate overnight at 4^0C then whole mixture was centrifuged at 12000 rpm for 15 min. Supernatant were collected and each drugs were estimated by RP-HPLC method with specific as give below.

Carboplatin- Column-C-18, Mobile phase- (methanol: potassium phosphate buffer pH-6.5 30:70), Retention time – 6.5min, Flow rate-1ml/min, Detection-229nm.

5-fluorouracil- Column-C-18, Mobile phase- (potassium phosphate buffer pH 6.5: methanol 60:40), Flow rate -1ml/min, Retention time – 6min, Detection-258nm

Oxaliplatin- Column-C-18, Mobile phase – (potassium phosphate buffer pH 6.5: methanol 30:60), Flow rate-1ml/min, Retention time-8min, Detection- 229nm

Concentration of drug from unknown samples were estimated on basis of standard curve of known concentration for each drugs.

2.2.8 Receptor Blocking Assay

Analysis of the role of specific receptor involved in nanoparticle delivery inside the cells was done through receptor blocking assay in the presence of receptor binding antibody (pierce antibody, USA). In this experiment Y79 Cells ($2x10^6$) were suspended in serum-free medium for 60 min in a 12-well plate (Corning). Apo-nano-carbo and lacto-nano-carbo (equivalent to 60 μg carboplatin) was added to the cells in either the presence or absence of 400 ng/ml monoclonal anti-human transferrin receptor and anti-human lactoferrin receptor antibodies and incubated for 1hr and 2hr. After incubation, cells were washed thrice with phosphate-buffered saline (pH 7.4) and carboplatin were extracted as above method given in section. In another set of experiment .2 million Y79 cells were incubated with Rhodmaine 123 tagged apotransferrin and lactoferrin nanoparticle, similar number of COLO-205 cells were incubated with rhodamine123 tagged lactoferrin nanoparticle in receptor blocked medium and unblocked medium. Cells were harvested at similar time point as above and wash thrice with 1X PBS (pH 7.4). Cells were mounted on glass slide and scanned under Confocal microscope (Ex=505nm and Em=530 nm).

2.2.9 Subcellular localization by Lysosome tracking assay

Y79 cells and COLO-205 (0.2 million) were seeded in serum free media in 30 mm culture dishes for 60 minutes under 5% CO_2 injection. Cells were incubated with Rhodamine 123 tagged apo transefrrin or lactoferrin nanoparticles for 2 hours, further lysosome tracking dye (lyso sensor, Invitrogen) yellow were added into the media 30 min prior to harvesting of cells. Cells were harvested and washed thrice with 1XPBS (pH 7.4) and mounted on the glass slides. Slides were scanned under confocal microscopy at (Ex=505nm, Em=530nm, green and Ex=384nm, Em=540nm, yellow) for rhodamine 123 and lysosensor respectively.

2.2.10 Anti-proliferative Assays

Anti-proliferative assay in Y79 cells were carried out by following experiments.

2.2.10.1 MTT Assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay were performed to evaluate the anti-proliferative potential of carboplatin loaded nanoparticle which is a colorimetric assay, based on the cleavage of a yellow tetrazolium salt to insoluble purple formazan crystals by the mitochondrial dehydrogenase of live cells [Al-Joudi F.S. et al 2005]. Y79 cells (50 thousand) were seeded in a 96-well plate and incubated at 37 °C for 4 h in a 5% CO_2 incubator (SANYO japan).These cells were treated with increasing concentrations of carboplatin (5μg, 10μg, 15μg, 20μg) either soluble or equivalent loaded into nanoparticles (apo-carbo-nano and lacto-carbo-nano), and incubated for 36 hours with media changes at every 12 hours. In another set of experiment similar number of COLO-205 cells treated with increasing concentration of 5-fluorouracil and oxaliplatin (1 μg,2 μg, 4 μg, 8 μg) either in free (sol-5FU and Sol-oxalo) or lactoferrin nanoparticle loaded (lacto-nano-5FU and lacto-nano-oxalo), incubated for 36 hours with media changes at every 12 hours. In a different set of experiment 20 μg (fixed concentration) of carboplatin, equivalent amount of nano formulation in Y79 cells and 4 μg 5-FU and oxaliplatin and equivalent amount of nano formulation in COLO-205 cells incubated for different time points. The cells were pelleted at 1200 rpm for 10 min and re suspended in new medium. To this, 20 μl of 5 mg/ml MTT (Sigma-Aldrich) was added and incubated for 4 hr. The cells were then pelleted at 1200 rpm for 20 minutes, the medium was removed, and formazan crystals were dissolved in DMSO and read in an ELISA reader at 595 nm. Percentage viability, percentage inhibition and IC50 valued were calculated in respect to control by following formula.

% Viability = OD of test (treated cells) / OD of control (untreated cells) x 100.

% Inhibition = OD of control−OD of test / OD of test x 100.

$$IC_{50} = \frac{\textbf{Concentration at which close to half inhibition achieved/\%inhibition close to half inhibition}}{2} \times 100$$

2.2.10.2 DNA laddering Assay

Fragmentation of DNA is a peculiar characteristic of apoptosis and the percentage of fragmented DNA can be directly correlated with percentage of apoptotic DNA [Ioannou Y.A. et al 1996]. In this experiment 2 million Y79 cells were seeded in RPMI-1640 media with 10% FBS into the 60 mm culture dishes and cells were incubated with 20 μg of different formulation (apo-nano-carbo, lacto-nano-carbo, apo-nano, lacto-nano, sol-carbo) for 36 hrs. In another set of experiment 2 million COLO-205 cells were incubated with 4 μg of various formulation (Sol-5FU, Sol-oxalo, lacto-nano-5FU and lacto-nano-oxalo) for 36 hours. Media were changed after every 12 hrs without addition of any further drug. Each group of cells were pellet down and wash thrice with PBS (137mM NaCl, 2.7mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) and dissolve into 500 μl lysis buffer contain (0.2M tris-HCl, 0.5M NaCl, 0.01M EDTA, 0.5% SDS). Genomic DNA were extracted by adding phenol:chloroform:isoamyl alcohol at 25:24:1 ratio into the lysate. Solution were separated into two phase, aqueous phase were collected into fresh tube and DNA were precipitated by adding ½ volume 3M sodium acetate and two volume of 90% ethanol. Resulting mixture were centrifuged at 10,000 r.p.m. for 30min, pellet was dissolved into TE (10mM Tris-HCl, 1mM EDTA) and resolved into .4% agarose gel.

2.2.10.3 Caspase-III Assay

Caspase-III is a pro-apoptotic factor involved in promoting apoptosis and its presence indicate a highly activated state during apoptosis [Reidl SJ et al 2004. In this experiment 4 million Y79 cells were seeded with RPMI-1640 media with 10%FBS into the 60mm culture dishes. Cells were incubated with 20 μg of sol-carboplatin and 20 μg carboplatin equivalent to apo-carbo-nano and lacto-carbo-nano for 36 hours. In another set of experiment 4 million COLO-205 were incubated with 4 μg of Sol-5FU, Sol-oxalo, lacto-nano-5FU and lacto-nano-oxalo for 36 hours. After incubation each type of

cells were pelleted down and washed thrice with PBS and total cellular protein were extracted by protein extraction buffer (20mM Tris-HCl pH 7.4, 0.1mM β-Mercaptoethanol, 1.0mM $MgCl_2$,0.1mM EDTA, 5% glycerol, 0.1% Triton X-100, 0.5mM KCl, 1mg/ml pepstatin and leupeptin, 0.5mM PMSF). Proteins were quantified by Bradford reagent and 100 μg of protein were used in each caspase-III reaction. 100 μg of protein were mixed with Caspase buffer containing 5 μg of caspase-III substrate (Ac-DVED-AFC) and incubated at 37^0C for one hour then whole mixture was subjected to fluorimetric estimation.

2.2.11 Animal Study

Wistar rats were used in entire animal study, each and every sets of animal were approved by Institutional Animal ethics committee of university of Hyderabad. Rats were maintained in university animal house as per institute guide lines.

2.2.11.1 Ocular localization of Apotransferrin and Lactoferrin nanoparticle in rats

Rats were injected with 200 μl (100μg) of Doxorubicin loaded rhodamine123 tagged nanoparticle and free Doxorubicin in periocular space (specifically peri bulbar route) by 28 gauge BD™ insulin needles. Animals were sacrificed at specified time points post injection (1^{st}, 3^{rd} and 15^{th} day PI) and eye were enucleated and fixed into 4% paraformaldehyde. After fixation eyes were dehydrated in gradually increasing concentration of ethanol. Dehydrated eyes were fixed into paraffin and cut into thin section followed by H&E staining. Stained tissue sections were mounted and scanned under confocal microscope to detect the tagged nanoparticle as well as free doxorubicin in different section of eyes. In this study doxorubicin is acting as a fluorescence tracking agent.

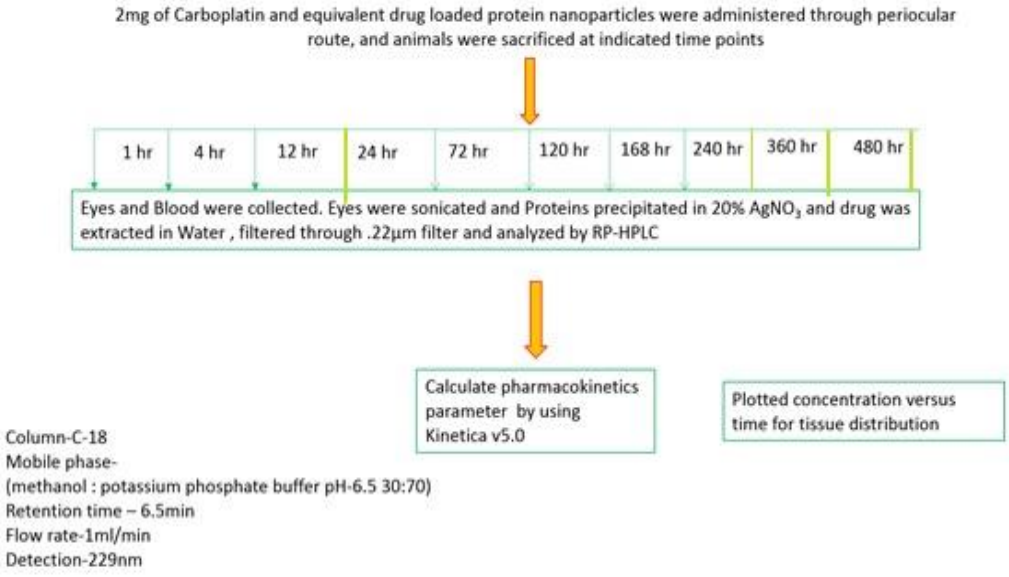
2.2.11.2 Comparative ocular Delivery of carboplatin loaded protein nanoparticles and free carboplatin in Vitreous cavity and blood (Ocular pharmacokinetics)

Single dose of Carboplatin loaded protein nanoparticles and free carboplatin equivalent to 2mg were injected through periocular route in rats by 28 gauge syringe. Animals were euthanized and

eyes enucleated at specified time points post injection (1, 4, 12, 24, 72, 120, 168, 240, 360, 480 hours PI) and blood were also collected directly from hearts at same time points for evaluation of carboplatin concentration with the time.

Collected eyes were sonicated in water for 5 min in order in ensure the complete dispersion of drug into water from tissue. Further, eye extract were treated from 20% Silver nitrate solution for precipitation of protein. Now extract were centrifuged at 12000 rpm for 15 min, supernatant were taken and filtered through .2µm filter and filtrate were stored in -20 C until estimation by HPLC. Similar procedure were adopted to extract carboplatin from blood except sonication step.

Extracted carboplatin were estimated by revers phase HPLC (RP-HPLC) method. In this method C-18 column from Restek were used to estimate the drug. Mobile phase (ethanol and potassium phosphate buffer) in the ratio of 30:70 were used with flow rate of 1ml/min and 6.5min retention time. Carboplatin were detected at 229nm wave length and concentration of carboplatin from test samples were measure against the calibrated curve with linearity range 100ng to 20µg. Pharmacokinetics parameters were calculated by kinetica 5v software and distribution of carboplatin



were represented by plotting the concentration against time. Entire scheme of experiment are summarised in following flow chart.

2.2.11.3 Study of Ocular safety of carboplatin loaded protein nanoparticles

2.2.11.3.1 Ocular Inflammation study

Ocular inflammation is one of the major concern as for as ocular tolerance of different types of nanoparticle delivery platform is concern. So we have evaluated inflammation by the experiment of cellular infiltration assay and whole vitreous protein assay as well as estimation of inflammatory cytokines level.

2.2.11.3.2 Cell infiltration assay and estimation of whole vitreous protein

Carboplatin loaded Protein nanoparticles (Apo-nano-carbo and lacto-nano-carbo) and soluble carboplatin (Sol-carbo) were injected into rat eyes via periocular route with the concentration of 2mg/eye equivalent of carboplatin in triplicate. After defined time point (1^{st}, 3^{rd} and 15^{th} day PI) rats were sacrificed and eyes enucleated. Vitreous humor (VitH) were collected by opening up the sclera, some part of fluid were stained with trypan blue to calculate the absolute cell number by haemocytometer under light microscope and data represented as number of cells/ml of vitH, other part of humor were used to calculate the total protein by BCA estimation method and represent as mg/ml of vitH.

2.2.11.3.3 Estimation of inflammatory cytokines

Remaining vitreous fluid and ocular tissue were suspended in 200µl/eye of RIPA buffer with protein inhibitor cocktail. Samples were homogenised in that buffer incubated on 4^0C for 30min, after incubation homogenate were centrifuged at 12000rpm for 20min. supernatant were collected and protein were estimated by BCA method (kit Thermofisher).

Now equal concentration of protein (100µg) from each sample were used for determination of different cytokines includes IL-6, TNF-α and MCP-1 by ELISA method. ELISA were done by

following standard protocol provided with kit (GE health care). Each and every sample were done in triplicate and concentration of respective cytokines were measured against their standard curve.

2.2.11.3.4 **Histopathology analysis of rat's eye after peri ocular injection**

Another sets of rats were injected with same aforementioned amount of carboplatin and equivalent nano formulation of carboplatin through peri ocular route. Rats were sacrificed by cervical dislocation and eyes enucleated, make a little incision and fix into 4% paraformaldehyde in 1XPBS (pH=7.4). Fixed tissue were kept for ethanol dehydration by gradually increasing the ethanol concentration. Dehydrated tissue were fixed into stab and thin section were cut in microtome. Tissue were stained with haematoxylin and eosin stained and mount and fixed onto glass slide. Tissue sectioned were observed under compound light microscope on 20X-40X magnification. Data were recorded and analyse for structural integrity and infiltratory cells.

2.2.11.3.5 **Study of myelosuppression**

Rat's blood were withdrawn by heart puncturing from same sets of pharmacokinetic experiment after 20^{th} day of injection. Blood were collected in heparinised collection tube and total blood count were done by haemocytometer. Each individual group of cells were counted and plotted against respective treatment groups.

2.2.11.4 Comparative Pharmacokinetic and tissue distribution of free 5-FU/oxaliplatin and loaded in lactoferrin nanoparticle after I.V. route of administration in healthy rats

Healthy rats were injected with 5-FU and oxaliplatin loaded lactoferrin nanoparticles and similar amount of unloaded drugs at the concentration of 40mg/kgBW via I.V. route of administration. After specified time points (5,15,30,60, 120,240,480,720,960) rats were sacrificed by cervical dislocation, blood were collected directly from heart puncturing in .5mM EDTA and liver, kidney and colon were also collected. Whole Tissues were homogenized in 1XPBS (pH-7.4) and

plasma were isolated from blood by centrifugation at 1000 rpm. Tissue homogenates and plasma were incubated with 20% $AgNO_3$ followed by 20%NaCl, mixed well by vortexing and kept at RT for 30min. After 30 min homogenates were centrifuged at 12000rpm for 15 min, supernatant were collected and filtered by .2μm syringe filter (PALL life sciences) , stored in -20^0 C until estimation.

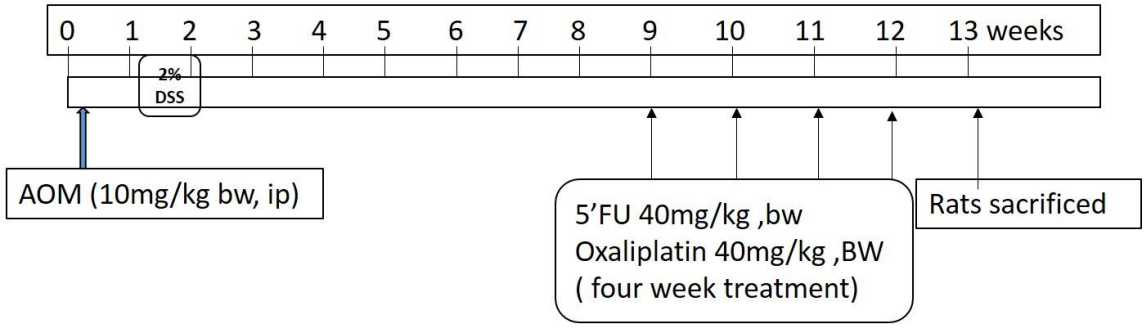
Concentration of 5-FU from plasma and various tissue were estimated by RP-HPLC from filtrates. For HPLC analysis, filtrates were further diluted 10 times and injected in machine with C-18 column (Restek) at the flow rate of 1ml/min under mobile phase of buffer (5mM phosphate buffer pH-6.5) and methanol at the ratio of 60:40 with 6 min of retention time. Drug were estimated by integrated UV detector at the wave length of 258nm. Drug were quantified against the pre maid standard of 5-FU and data were plotted at concentration versus time graph. Pharmacokinetics parameter from quantified drug were calculated by kinetica V.5 with two compartmental model of estimation. Each experimental groups were contains three animals (n=3).

Similarly, concentration of oxaliplatin from plasma and tissues were calculated by same method but with different HPLC running para meter. Filtrates were loaded into C-18 column with mobile phase of buffer (5mM phosphate buffer pH-6.5) and methanol at the ratio of P30:70 at flow rate of 1ml/min with the retention time 8min. Pharmacokinetic parameter were calculated as above method.

2.2.11.5 Induction of aberrant crypt foci (AFC) by azoxy methane (AOM) and treatment of the same by drugs loaded lactoferrin nanoparticle and free drugs

Aberrant crypt foci in colon of rats were induced by Azoxy methane (AOM) carcinogen "gold standard". Two dose of AOM in consecutive week were injected through intra peritoneal (i.p.) route in one month old rats at the concentration of 10mg/kg BW. Dermatan Sulphate Sodium (DSS) salt were also given by drinking water up to 1^{st} week at the concentration of 2% to accelerate the process of ACF induction [Kazuto Yoshimi et al 2009].At the end of the two dose, twenty five rats were

randomly divided into five groups (saline control, sol-5FU, sol-oxalo, lacto-nano-5FU and lacto-nano-oxalo) and started treatment from 9th week onwards till 12th week (four dose in four consecutive week) through i.v. route of injection at the concentration of 40mg/kg BW of both free drugs (Sol-5FU and Sol-oxalo) and equivalent amount of lactoferrin loaded drugs (lacto-nano-5FU and lacto-nano-oxalo) as clearly explain in following flow chart. At the end of 13th week rats were sacrificed by cervical dislocation, colon were excised and fixed into 4% paraformaldehyde for histopathological assay. Blood were also collected by heart puncturing in two separate tube one for total blood count and another for safety analysis from serum. Body weight were also recorded for each treatment group from the 9th week to 13th week (total treatment period) and plotted as treatment days versus body weight.



2.2.11.5.1 **Quantification of aberrant crypt foci in colon**

Paraformaldehyde (4%) fixed Colon were cut and opened, stained with methylene blue for 10 min. To evaluate the number of aberrant crypt foci (ACF), stained colon were observed under bright field microscope (Olympus) to find out the number ACFs. Aberrant crypt were differentiated from normal colon crypt on the basis of enlarges size and protrusion form normal crypt surface. Total number of ACF were counted on the basis of above characteristics for each group and plotted over ACF number versus treatment groups.

2.2.11.5.2 **Histopathological analysis of Colon**

Colon stored in 4% paraformaldehyde were dehydrated by increasing percentage of ethanol and fixed into paraffin block. Paraffin block were subjected into microtome (Leica) to generate thin sections and mount on glass slide, mounted section were stained with haematoxylin and eosin then fixed by plastic cover slip. H&E stained sections were observed under bright field microscope (Olympus) at 20X magnification and data were recorded for further analysis.

2.2.11.5.3 **Hepatic and renal safety assays**

Hepatic safety assays were carried out by scoring the enzymatic activity level of serum SGPT and serum SGOT. Kit based colorimetric assay were used to evaluate their activity by following the manufacturer guidelines for experiment. Renal safety assay were carried out by the estimation of amount of serum creatinine and blood urea nitrogen (BUN). Colorimetric assay were done to estimate the amount of serum creatinine and BUN according to manufacturer guidelines of experiment.

2.2.11.5.4 **Study of Myelosuppression**

At the end of treatment period blood were collected from each treatment group in heparinized collection tube. Total blood count were done by hemocytometer to find out cells number of different populations. Each cells population were plotted separately as number of cells versus treatment groups.

2.2.11.5.5 **Statistical analysis**

All experiment was repeated three times and statistical analysis was performed via student t test and one way ANOVA. A value of $P< 0.05$ was considered to be significant.

CHAPTER 3

Preparation and physicochemical characterisation of carboplatin, Oxaliplatin and 5-fluorouracil loaded protein nanoparticles.

3.1 Introduction

Chemotherapy is an indispensable method of cancer management, but full potential is limited due to 1) non-specificity of drug, 2) serious systemic toxicity, 3) reduced effective drug concentration due to poor solubility and stability of drugs. These limitations especially for carboplatin, Oxaliplatin and 5-fluorouracil were overcome by development of wide variety of targeted and non-targeted nanoparticle delivery systems.

Various type of carboplatin Nano formulation mostly involve in the loading of drug to different biomaterials such as biodegradable polymers has been reported e.g. carboplatin loaded in PLGA [Tanmoy S. et al 2014] , Methamethylcrylate nanoparticle for delivery to posterior eye [Kalita D et al 2014], PCL for the intranasal delivery [Alex AT et al 2014] and functionalised albumin nanoparticle for the treatment of breast cancer [Conlin AK et al 2010]. Similarly effort has been made to developed nano formulation of another platinum containing drug oxaliplatin for the treatment of various cancers. Oxaliplatin has been loaded in range of biodegradable material derived nanoparticle in order to improve pharmacological efficacy [Lila AS et al 2014] e.g. poly (ethylene glycol)-b-poly(glutamic acid) [PEG-b-P(Glu)] block copolymer loaded for the treatment of colon adenocarcinoma [Cabral H et al 2007], hydroxypropylcellulose–poly(acrylic acid) (HPC–PAA) loaded nanogel against gastric cancer [Chen Y et al 2008], PEG-liposome and transferrin conjugated PEG-liposome[Suzuki R et al 2008]. Non polymeric nanoparticle has been also developed with oxaliplatin for the cancer management e.g. Mesoporus silicia nanoparticle for the control of liver cancer and multi-walled carbon nanotubes (MWCNTs) in order to overcome the limitation that is associated with free oxaliplatin [He H et al 2014, Wu L et al 2013].

A number of 5-fluorouracil loaded nanoparticle has been investigated to overcome aforementioned shortcoming associated with this drug. It has been loaded in a variety of materials, but most notably polymeric material such as: poly(ε-caprolactone) [Guerra GD et al 2001] and

chitosan [Chang SJ et al 2007,Tıglı RS et al 2012], Gelatin [Sun W et al 2013], Poly(alkylcyanoacrylates)[Arias JL et al 2008] Polymers-based on methacrylic acid [Ashwanikumar N et al 2012], Polyacrylamide [Hadjikirova M et al 2005], Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) [Chen AZ et al 2006, Li XM et al 2008, Nair KL et al 2011], Hydrogels [Wang Y et al 2010], fibrinogen nanoparticle and also liposomal formulation for the treatment of Colo-rectal cancer.

Though these materials are biocompatible, still some of them are lacking the target-specificity and require high energy for biodegradation, thus posing extra burden to diseased tissue. In order to enhance target specificity, many drug-loaded materials are conjugated or coated to iron binding protein transferrin/apotransferrin for recognition and binding to the cancer cells (Thorstensen et al., 1993; Qian ZM et al., 2002; Li H et al., 2002; Vincent et al., 2009). Transferrin receptors are abundantly expressed in active and rapidly proliferating cells and thus targeting such proteins enables preferential localization of drugs in those cells. Another advantage is that the drug can be localized in all transferrin receptor abundantly expressed cancer cells and tissues (Gatter KC et al., 1983), thus widening the scope of drug action on various types of cancers. Similarly lactoferrin is also iron binding and storage protein, it is multifunctional protein found in exocrine secretion sucv as breast milk and mucosal secretion. Various type of lactoferrin peptide conjugated nanoparticle have been developed for targeted drug delivery. Our group have developed protein-based nano formulation composed apotransferrin and lactoferrin itself, which act as a nano carrier as well as targeting-specific recognition moiety. So in this technology we do not need separate steps for conjugation of targeting ligand to nanoparticle thus makes entire process easy and facile.

The objectives are

(1) Preparation and physicochemical characterisation of carboplatin loaded apotransferrin and lactoferrin nanoparticle;

(2) Preparation and characterization of combination of drugs 5-fluorouracil and Oxaliplatin loaded lactoferrin nanoparticles.

3.2 Result

3.2.1 Preparation of Nanoparticles

Nanoparticles were prepared as per procedure explained in Chapter II. Stoichiometry of encapsulation efficiency of apo-nano-carbo and lacto-nano-carbo were analysed at three different concentration of carboplatin, while protein concentration was kept constant. Maximum encapsulation efficiency of carboplatin into apo-transferrin nanoparticle was found to be in formulation II (50%-±2.3). While lactoferrin nanoparticle exhibited highest loading efficiency under conditions of preparation of formulation II (52%-± 3.9). The loading efficiency of both nanoparticles with various concentration of carboplatin was illustrated in (Table3.1, Table 3.2). Formulation II of both nanoparticles Apo-carbo-nano and lacto-carbo-nano were used in entire cellular assay throughout the manuscript until otherwise stated.

In another set of lactoferrin nanoparticle preparation loaded with 5-fluorouracil (5-FU) and oxaliplatin evaluated for loading efficiency. Similar experimental design were used, results showed that maximum loading efficiency of 5-FU into lactoferrin nanoparticle found to be in formulation III (48.6±3.5) and in oxaliplatin loading efficiency found to be in formulation III (51.3±4.9) that is illustrated in table 3.3 and 3.4. Formulation III were used in entire in-vtro and in-vivo experiment. We used only lactoferrin protein for preparation of nanoparticle with 5-FU and oxaliplatin, because both the protein behave in similar fashion and moreover it is readily available and quite economical, subsequently it will help in scaling up the process.

Table 3.1: Encapsulation efficiency of carboplatin loaded apotransferin nanoparticle

Formulations	Conc. Of Apo transferrin(mg)	Conc. Of carboplatin (mg)	% Encapsulation efficiency
I	15	5	40±3.2
II	15	10	50±2.3
III	15	15	41±1.5

Table 3.2: Encapsulation efficiency of carboplatin loaded lactoferrin nanoparticle

Formulations	Conc. Of Apo transferrin(mg)	Conc. Of arboplatin (mg)	%Encapsulation efficiency
I	15	5	47±4.1
II	15	10	52±3.9
III	15	15	50±2.7

Table 3.3: Encapsulation efficiency of 5-fluorouracil (5-FU) loaded lactoferrin nanoparticle

Formulations	Conc. Of Apo transferrin(mg)	Conc. Of carboplatin (mg)	%Encapsulation efficiency
I	15	5	38±3.8
II	15	10	44±4.7
III	15	15	48±3.6

Table 3.4: Encapsulation efficiency of Oxaliplatin loaded lactoferrin nanoparticle

Formulations	Conc.OfApo transferrin(mg)	Conc. Of carboplatin (mg)	% Encapsulation efficiency
I	15	5	42±4.5
II	15	10	49±3.1
III	15	15	51±4.9

3.2.2 Characterization of Apo-nano-carbo, Lacto-nano-carbo, Lacto-nano-5Fu and Lacto-nano-oxalo

Formulation II containing 15mg of apotransferrin or lactoferrin and 10mg of carboplatin was used in entire physicochemical characterisation of nano formulation. Size, zeta potential and hydrodynamic size of Carboplatin loaded apotransferrin and lactoferrin nanoparticles were measured by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and DLS. The size of carboplatin loaded apotransferrin nanoparticle measured through SEM analysis, ranges 82 to 92 nm (Fig. 3.1A), while size of carboplatin loaded lactoferrin nanoparticles ranges from 68 to 81nm (Fig.3.1B), almost similar size were observed in TEM analysis with spherical in shape and serrated surface (Fig. 3.1C & D). The hydrodynamic size of carboplatin loaded apotransferrin and lactoferrin nanoparticles are found to be 142±15nm and 263±20nm respectively (Fig. 3.2C & D). Poly despersity index (PdI) of Apo-carbo-nano and lacto-carbo-nano were 0.155±.05 and 0.340±.04 observed respectively. Zeta potential of Apo-carbo-nano and lacto-carbo-nano were -23±2mV and -10±3mV observed respectively that explain in (table 3.5). Whereas 5-FU and Oxaliplatin loaded lactoferrin nanoparticles were characterised by SEM and TEM, size of these nanoparticles ranges from 60nm-110nm, spherical in shape with serrated surface fig 3.3 A, C and 3.3 C, D respectively. The hydrodynamic size of lacto-nano-5FU and lacto-nano-oxalo were 161±13 and 158±9 (fig 3.3 E & F), Poly dispersity index (PDI) were .170±.07 and .219±.09, Zeta potential were -3.2±1 and -2.3± .9 (table 3.5) respectively.

In the FT-IR spectrum of bulk proteins (apotransferrin and lactoferrin) a characteristics peak of amide I (C=O stretch), amide II (C-N coupled with N-H bonding mode) and peptide N-H stretching frequency were observed at 1650-1660 cm^{-1} , 1450 cm^{-1} and 3300 cm^{-1} respectively, while in nanoparticle formulations (apo-carbo-nano & lacto-carbo-nano) a slight shift in stretching frequency were observed (Fig 3.4A & B). In FT-IR spectrum of carboplatin, a featured peak of different bonds

e.g. (Pt-N) at 545 cm^{-1} , (Pt-O) at 575 cm^{-1}, strong peak of C-O and C-C stretching vibrations of the co-ordinated dicarboxylated ring at 1347 cm^{-1}, a very strong peak at 1375 cm^{-1} of C_6-O_4 and C_7-O_5 , in the region of 1550-1700 cm^{-1} C=O and NH_3 stretching vibration, methylene stretching vibrations at 2900-2995 cm^{-1} and very strong peak at 3260 cm^{-1} to N-H stretching vibration of two ammonia group of carboplatin were observed (Fig 3.4E). Though there was some slight but not significant shift in stretching vibration of either carboplatin or proteins in nano formulation were observed (Fig 3.4C & D).

Almost similar pattern of stretching frequencies were observed in free 5-FU/ Oxaliplatin and loaded in lactoferrin nanoparticle. There were no major shift occurred in stretching frequencies of any major group of 5-FU and Oxaliplatin after loading into lactoferrin nanoparticle as observed in Fig 3.5 A,B,C,D and E.

Though slight change in stretching vibration was observed after loading into nanoparticle, it may be due to dipole moment of bonds as a result of electrostatic interaction between drugs (carboplatin, Oxaliplatin and 5-fluorouracil) and proteins molecules in the nano formulations. These FT-IR data suggest that all above drugs physically entrapped/ adsorbed with the protein nanoparticles rather than through any sort of covalent interaction.

Table 3.5: Physicochemical characterization of protein nanoparticles (mean±SD n=3)

Nanoparticle formulations	**Size distribution [a] (nm)**	**Zeta potential [b] (mV)**	**PdI [c]**	**Sedimentation force and time**
Apo-nano-carbo	140±15	-23±2	.155±.05	3100(g)/30min
Lacto-nano-carbo	263±20	-10±3	.340±.04	1800(g) /30min
Lacto-nano-5FU	161±13	-3.2±1	.170±.07	2300(g)/30min
Lacto-nano-Oxalo	158±9	-2.3± .9	.219±.09	2100(g) /30min

a- Size distribution measured by Dynamic light scattering method.

b- Zeta potential measured by zetasizer of Malvern instrument.

c- Poly dispersity index

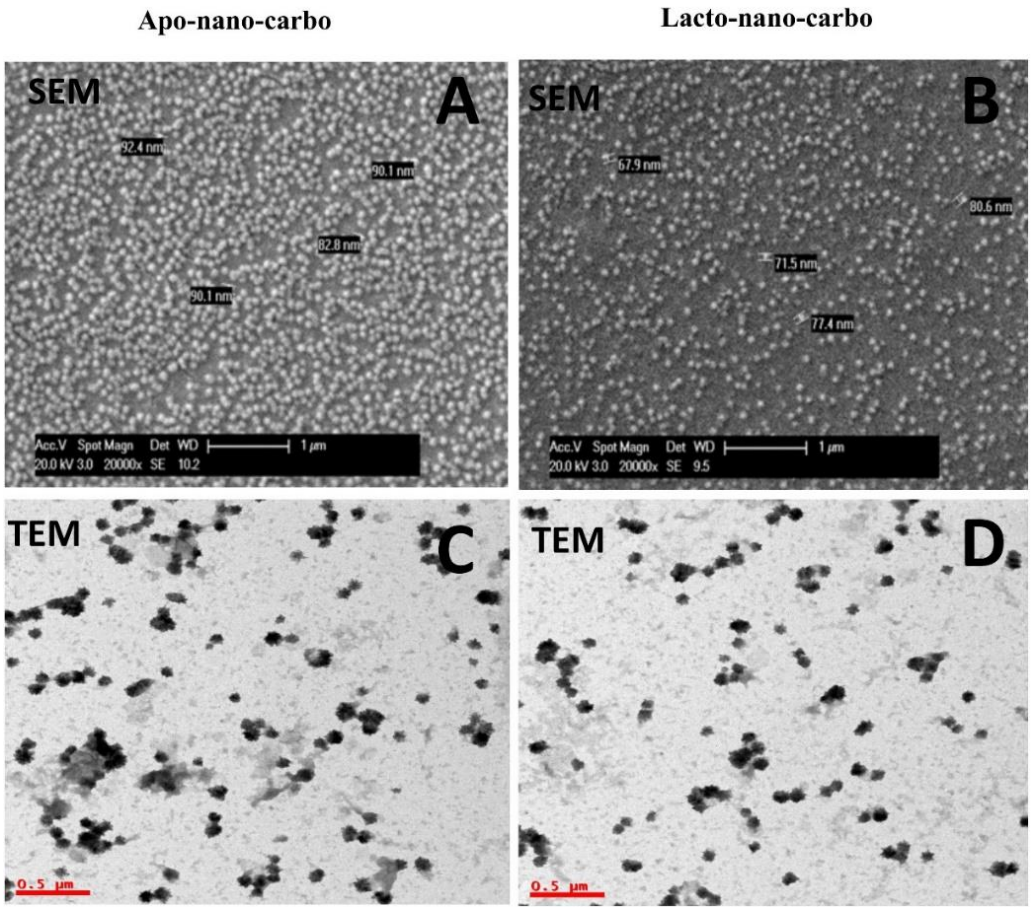


Figure 3-1: SEM analysis of nanoparticles A) carboplatin loaded apotransferrin nanoparticle (Apo-nano-carbo), B) Carboplatin loaded lactoferrin nanoparticle (Lacto-nano-carbo) and TEM analysis of nanoparticle C) Apo-nano-carbo, D) Lacto-nano-carbo.

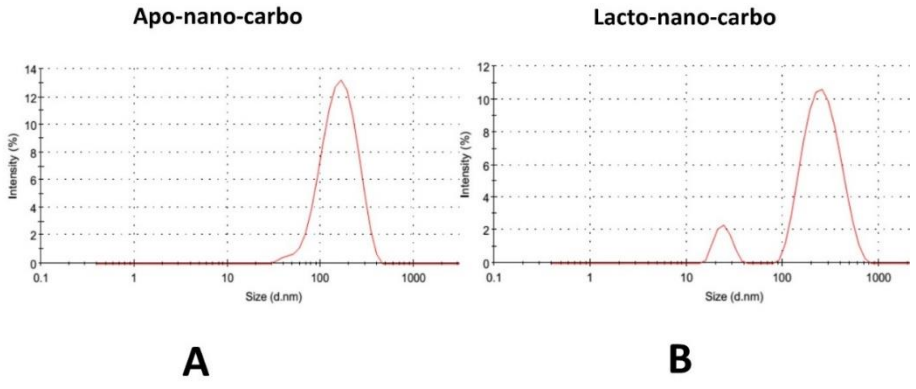


Figure 3-2: Analysis of size distribution of carboplatin loaded Apotransferrin (A) and Lactoferrin nanoparticle (B) by Dynamic light scattering method (DLS).

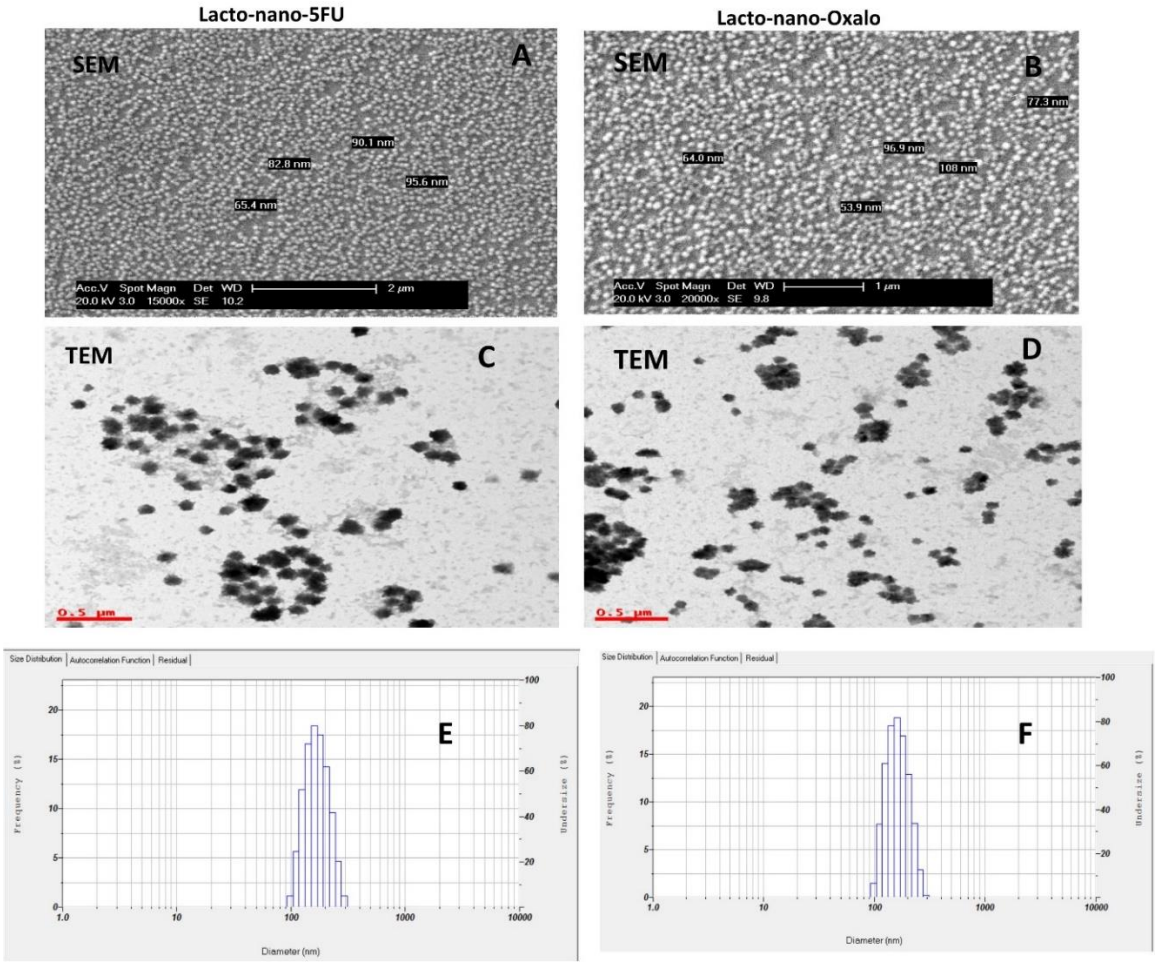


Figure 3-3: SEM analysis of lactoferrin nanoparticles A) 5-FU loaded lactoferrin nanoparticle, B) Oxaliplatin loaded lactoferrin nanoparticles and TEM analysis of C) lacto-nan-5FU, D) lacto-nano-oxalo. Size distribution by DLS analysis E) lacto-nano-5FU and F) lact

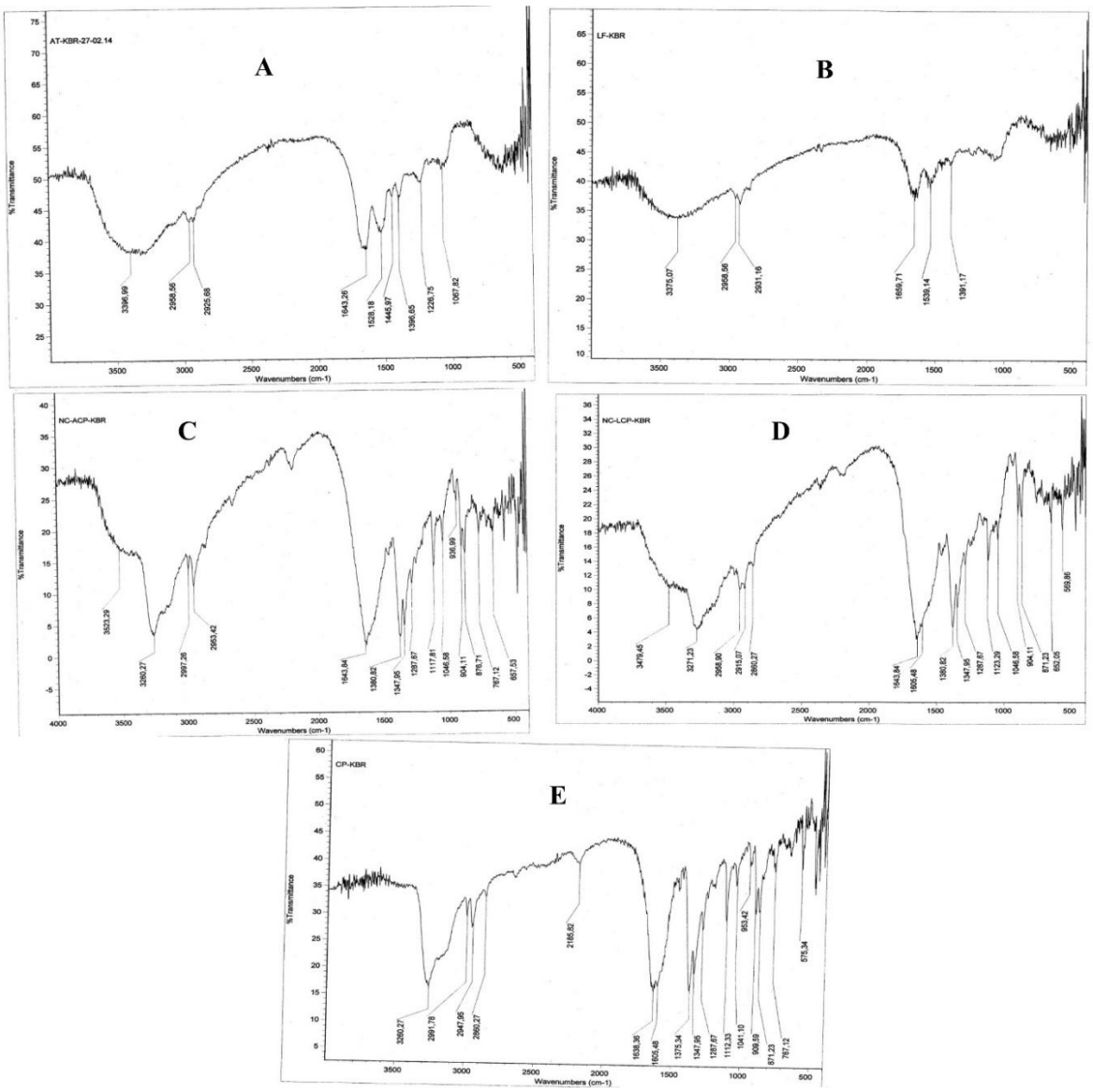


Figure 3-4: FT-IR spectral analysis of A) apotransferrin proteins, B) Lactoferrin proteins, C) carboplatin loaded apotransferrin nanoparticle (Apo-nano-carbo), D) carboplatin laoded lactoferrin nanoparticle (lacto-nano-nano-carbo) and E) carboplatin molecule.

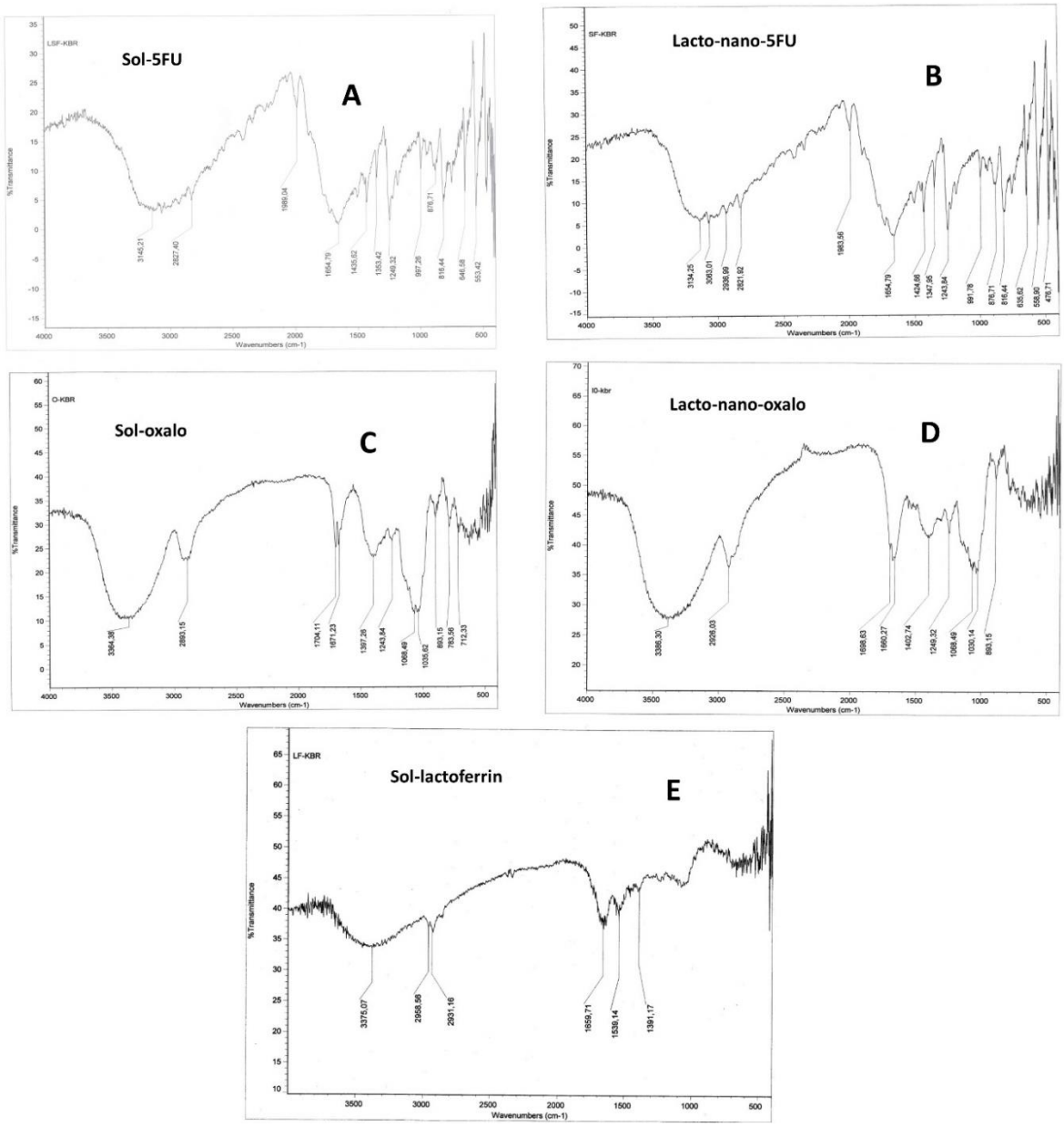


Figure 3-5: FT-IR spectral analysis of A) 5-fluorouracil (5-FU), B) 5-FU loaded lactoferrin nanoparticle (lacto-nano-5FU, C) Oxaliplatin molecule , D) Oxaliplatin loaded lactoferrin nanoparticle (lacto-nano-Oxalo) and D) lactoferrin protein.

3.2.3 In-vitro release profiling of carboplatin from apotransferrin/ lactoferrin nanoparticle and 5-FU/Oxaliplatin from lactoferrin nanoparticles

In-vitro release assay of Carboplatin loaded apotransferrin (Apo-nano-carbo) and lactoferrin (Lacto-nano-carbo) nanoparticles were carried out from 30 minutes to 480 hours. Results showed that the release of carboplatin from nanoparticle follow a biphasic release pattern, initial burst followed by sustained release. In the first 24 hrs of experiment ~55-60% of drug was released from the nanoparticles. Further controlled release of drug was observed till 480 hrs, ~80-85% of drug was released during this period (Fig. 3.6). Almost Similar pattern of in-vitro drug released were observed in Oxaliplatin (lacto-nano-oxalo) and 5-FU (lacto-nano-5FU) loaded lactoferrin nanoparticles. In case of lacto-nano-5FU ~50-60% of drug released in the first 8hrs of experiment and followed by sustained release ~80-85% of drug up to 480 hrs of experiment were observed. Whereas, lacto-nano-oxalo in the first 24hrs ~50-55% of drug released from the nanoparticle followed by sustained release up to 480 hrs post incubation were observed Fig.3.7.The observed pattern of sustained drug release after initial burst is significant because sustain drug delivery system having huge pharmacological implication in cancer treatment.

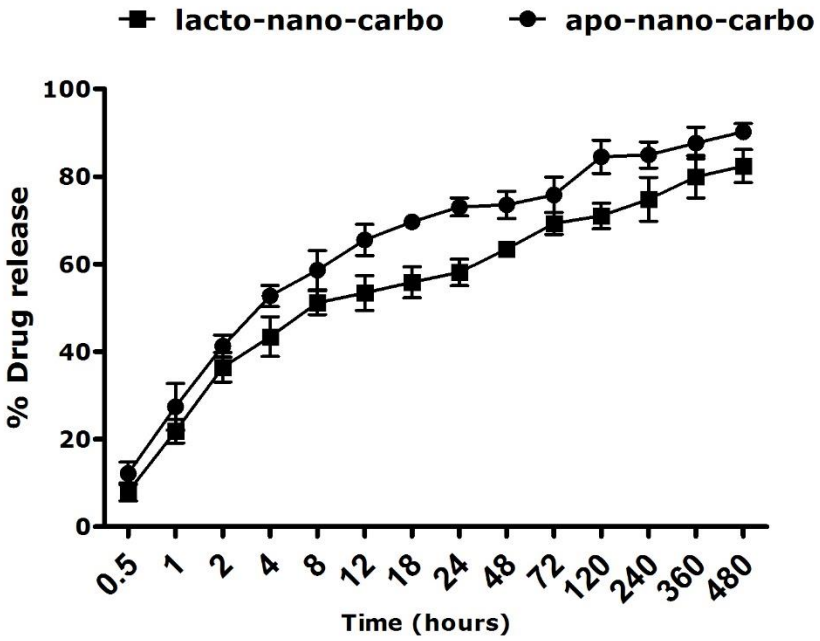


Figure 3-6: In-vitro Drug release assay in 1XPBS of carboplatin loaded Apotransferrin (Apo-nano-carbo) and lactoferrin (lacto-nano-carbo) nanoparticles. All data are represented in triplicate with SEM.

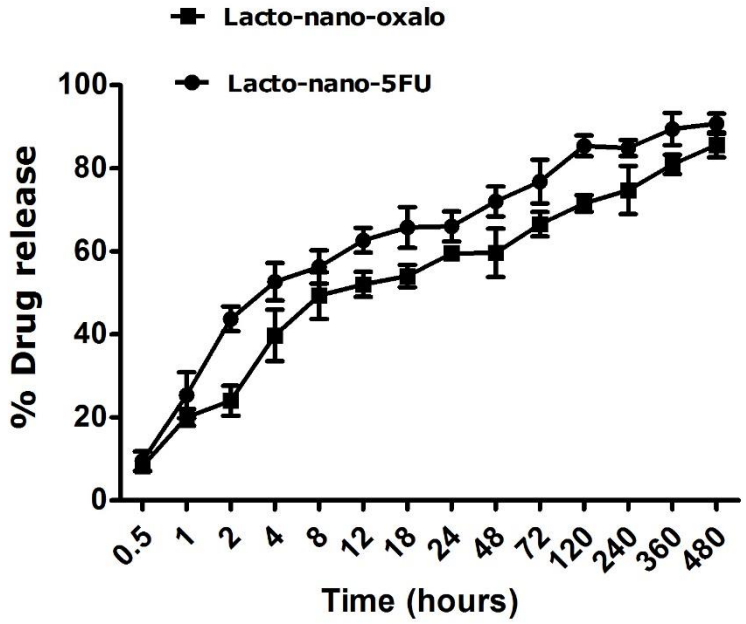


Figure 3-7: In-vitro drug release assay of 5-fluorouracil and oxaliplatin loaded lactoferrin nanoparticle in 1 XPBS at RT. Data are represented in triplicate with SEM.

3.2.4 pH-dependent release of carboplatin, Oxaliplatin and 5-FU from protein nanoparticles

The pH-dependent release assay of carboplatin loaded apo transferrin (apo-carbo-nano) and lactoferrin (lacto-carbo-nano) nanoparticles were carried out at various pH range (1-9). The carboplatin were released to maximum ~ 80% at pH 5 to 6 from both the nanoparticles (Fig 3.8) and similar release pattern was observed in 5-Fu and Oxaliplatin loaded lactoferrin nanoparticles (Fig.3.9).

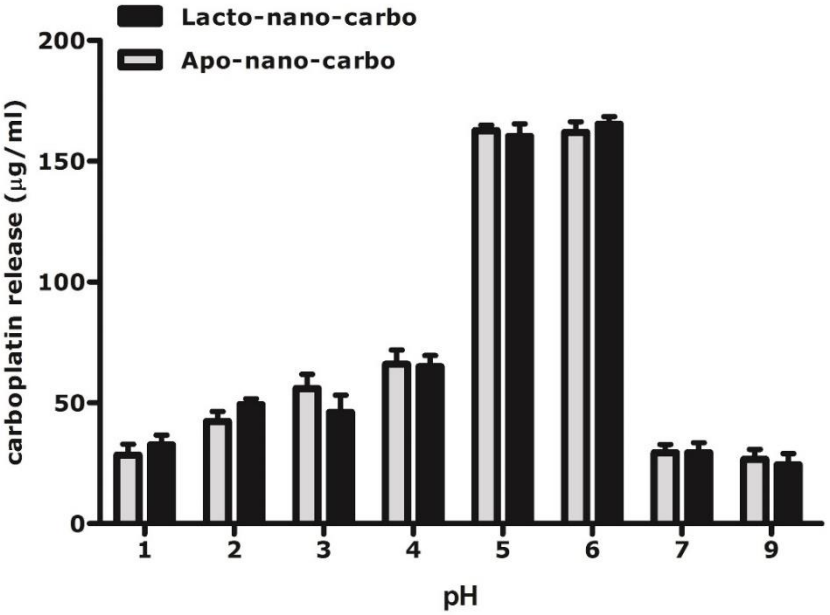


Figure 3-8: pH dependent release profile of carboplatin from Apo transferrin (apo-nano-carbo) and lactoferrin (lacto-nano-carbo) nanoparticle. Each data points were taken in triplicate (n=3) and presented in Mean ± SEM.

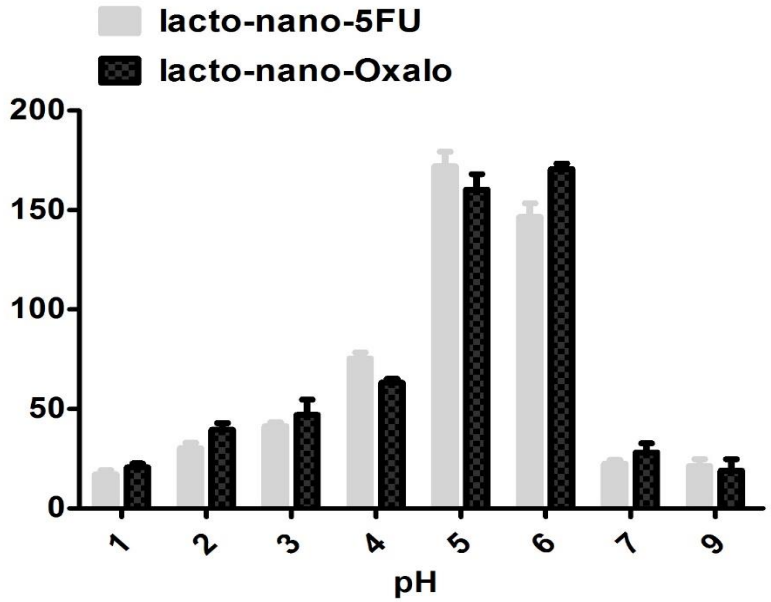


Figure 3-9: pH dependent release profile of 5-fluorouracil (lacto-nano-5FU) and oxaliplatin (lacto-nano-oxalo) from lactoferrin nanoparticle. Each data points were taken in triplicate (n=3) and presented in Mean ± SEM.

3.3 Discussion

Carboplatin loaded apotransferrin/lactoferrin nanoparticle and 5-fluorouracil/loaded lactoferrin nanoparticle were prepared as described earlier [ADS Kishna et al 2009]. It is very simple and fast procedure without any chemical modifications in protein. In contrast to our procedure, protein co-acervation method takes longer time and involves crosslinking of proteins [Shome D et al 2009], which could alter the structural and conformational property of the proteins. Their Size was found in appropriate range, 82-92nm and 68-81nm respectively (Fig.3.1A&B) by SEM analysis, and by TEM (Fig 3.1C & D), but in DLS analysis size were found to be significantly higher than SEM. In both the formulation, DLS sizes of nanoparticles were higher than their SEM sizes. This is quite rational because in light-scattering analysis, the average hydrodynamic radii of nanoparticles are often affected by the interactions with the water shell and particle's surface charge. Conjointly these factors contribute to an overall increase in particle size that gives different particle size measurements using SEM and DLS [Regev O et al 2004, Chaleawlert-umpon S et al 2009]. Zeta potential of Apo-nano-carbo is coming under colloidal stable range -23±2mV, while zeta potential of lacto-nano-carbo is coming under moderate colloidal stable range -10±3mV. Lesser colloidal stability of lacto-nano-carbo facilitate the aggregation that may attribute to bigger particle size in aqueous medium (263±20nm) than highly stable Apo-nano-carbo of size 142±15nm (Fig 3.2 A &B). Poly dispersity index of Apo-nano-carbo were observed 0.155±.5 and that seems to be more homogenous than Lacto-nano-carbo which is having higher poly dispersity index 0.340±.4 (table 3.5). Despite the bigger hydrodynamic size of lacto-nano-carbo, it shows the same antiproliferative activity as compare to apo-nano-carbo formulation. Similarly size and shape of 5-FU/oxaliplatin loaded lactoferrin nanoparticles are found to be similar as above nanoparticles characterised by SEM and TEM (Fig 3.3 A, B, C and D). This smaller size, stable surface charge and optimum poly dispersity index of nanoparticle may be able to be attributed better pharmacological efficacy of these drugs into different system of application.

Maximum Encapsulation efficiency of apo transferrin and lactoferrin of formulation II were found to be 50% and 52% respectively (Table 3.1 and Table 3.2), which is relatively good as compared to other reported Nano formulation with carboplatin such as ~3.9% in PLGA nanoparticle [Tanmoy S. et al 2012]. While in case of 5-fluorouracil and oxaliplatin, maximum encapsulation efficacy were found to be high in formulation III 48% and 51% respectively (table 3.3 & 3.4). Our group has reported that physical entrapment may be the likely mechanism by which doxorubicin is loaded into the apo transferrin nanoparticles (Saikrishna A.D.S. et al 2009)). In this study FT-IR spectra of above nanoparticle shows that there is no significant shift in stretching frequencies from both the drugs and bulk protein as described in (Fig 3.4 and 3.5). Though there was minute shift observed in nano formulation, it may be due to electrostatic interaction of drugs and protein. Carboplatin, oxaliplatin and 5-FU being a positively charged drug like doxorubicin; it is thought that the same mechanism would be involved in drug loading into protein nanoparticles. High loading efficiency as shown in Table 3.1 to 3.4 indicates the prominent encapsulation of drug and protein nanoparticles. pH dependent drug release assay apparently shows that most of the drugs releases from protein nanoparticles at pH 5&6 (Fig.3.8 and 3.9) Acidic pH is the property of endocytotic vesicles which involves in receptor mediated endocytosis.

CHAPTER 4

Development of Carboplatin loaded Protein nanoparticles with the aim of treatment of retinoblastoma

4.1 Introduction

Retinoblastoma (RB) is the most common intraocular tumor in children, affecting 1:15,000 live births per annum [Sanders BM et al 1988, Shields JA, et al 1994]. The recovery rate of retinoblastoma in developing countries is decreased substantially compared to the developed countries. One of the main reasons which account for this poor survival rate is delayed diagnosis because of lower socio-economic status, meagre education and inadequate healthcare system [Rodriguez-Galindo C et al 2008].

Retinoblastoma is caused by mutation in both alleles of the retinoblastoma tumor suppressor gene (*rb1*). As a consequence of the mutation in this gene, it leads to induction of the proliferative pathway, which causes a plethora of malignancies [Van Quill KR et al 2005]. Treatment of retinoblastoma had been limited to enucleation [Ellsworth RM 1977] but most recently, procedures such as external radiation beam therapy (ERBT), episcleral plaque radiotherapy (EPR) and focal therapy are being routinely used to treat RB [Amendola BE et al 1989]. However, these procedures are often accompanied with side effects such as cataract, radiation retinopathy, incidence of secondary malignant neoplasms and facial deformities [Murray TG et al 1998]. Recent therapy of RB management involves systemic platinum based chemotherapy in conjunction with focal therapy [Doz F, et al 1994]. Platinum based anticancer therapy involves the use of Carboplatin. Carboplatin is considered to be clinically safer than its parent molecule cisplatin, as it induces mild severe side effects [Ho YP et al 2003, Chen D. et al 1994]. In spite of its efficacy for the systemic treatment for wide variety of malignant diseases, there were efforts to utilise carboplatin for the treatment of localized tumors most notably retinoblastoma (RB), both way by direct local administration and by systemic treatment [Shields CL et al 1996, Friedman DL et al 2000, Kiratli H. et al 1998]. The poor targeting and penetration of this drug into eye ball through systemic administration confines the efficacy of treatment. High doses of localised administration of this drug has been effectively tried by injecting

via peri ocular route or into vitreous cavity to minimise the systemic toxicity and maximise the local concentration [Hayden BH et al 2000, Gallie BL et al 1996, Harbour JW et al 1996]. However, this therapy too is associated with numbers of life threating side effects such as neutropenia, thrombocytopenia, renal toxicity and hepatotoxicity [Abramson DH 1999]. In addition to this the clinical scope of this therapy is limited due to systemic toxicity, rapid blood clearance and resistance to cancerous cells [Chan HS et al 1989].

Drug delivery in eye remains a challenge for the physicians because it is protected from various defensive barriers. Intense research efforts are being carried out to develop and improve method for drug delivery into the eye. Amongst the methods are being developed, nanoparticle is one of them. The method of drug delivery by nanoparticles formulation has also been found to be promising to overcome the above limitations. Nanoparticles are submicron particle that can encapsulate the therapeutic molecule in order to reduce the problem associated with drug delivery, like penetration across the blood-retina barrier and retention time in blood circulation. They are polymeric colloidal particles with diameters oscillating from 10 to 1000nm, in which the therapeutic molecule of interest can be loaded within the polymeric matrix or adsorbed or conjugated on the surface [Sahoo SK et al 2007].

Biodegradable polymeric nanoparticles, a form of NP, have gained considerable research interest in recent years because of their compatibility with the various tissues of the body, including eye. A range of polymeric nanoparticle has been developed specifically for ocular drug delivery such as PLGA, PLA, chitosan-alignate, poly-e-caprolactone [Tanmoy S. et al 2014, Ashwin N. 2008]. Although these nanoparticles are biocompatible, however, they lack target specificity and have high energy demand for degradation, thus limiting their scope. So, natural macromolecules, such as proteins have gained interest as biomaterials due to their innate property of biodegradability and lack of toxicity [Moghimi SM et al 2001]. The presence of charged groups on protein nanoparticles makes them a

promising matrix in which drug can be physically entrapped [Merodio M et al 2002]. In order to improve the target specificity of nanoparticle many polymeric materials are being coated or covalently conjugated to transferrin/apotransferrin and lactoferrin protein to facilitate their entry into the target cells [Qian ZM et al 2002, Subramani, K.et al 2009]. So keeping this in view drug loaded apotransferin (protein) nanoparticle were developed as a sole drug carrier to enhance the target specificity against the cancerous cells [Saikrishna A.D.S. et al 2009].Apo-transferrin and lactoferrin are iron transporting protein belonging to the transferrin group of protein of molecular weight (~ 80kd). Transferrin receptors are known to be highly expressed on the surface of cancerous cells because these cells require high amount of iron to sustain their enhanced rate of metabolism [Gatter KC et al 1983].

We hypothesize that various side effects of carboplatin can be remarkably reduced and its efficacy can be greatly enhanced by encapsulating into the apotransferrin and lactoferrin nanoparticles. This study is focused on the use of the nanoparticles for sustained delivery of carboplatin for the treatment of retinoblastoma. In Chapter III, nanoparticles were prepared and characterised, the carboplatin loaded apotransferrin and lactoferrin nanoparticle and studied their loading efficiency and *in-vitro* drug release profile. In this chapter, we present results on the mechanism of nanoparticles entry into the cells and comparative anti-proliferative activity of nanoparticles compared to soluble drug. Furthermore, In-vivo study has been carried out to investigate the ocular and plasma pharmacokinetics, ocular and systemic toxicity of free and loaded carboplatin on healthy wistar rats.

Objectives of this chapter: This chapter contains two major objectives (II & III) of thesis as given below

2. Evaluation of efficacy of carboplatin loaded protein nanoparticle against retinoblastoma cells, an *in-vitro* study

3. Analysis of safety and bioavailability of Carboplatin loaded Protein nanoparticles when administered through periocular route

4.2 Results

Objective 2. Evaluation of efficacy of carboplatin loaded protein nanoparticle against retinoblastoma cells, an *in-vitro* study

4.2.1 Cellular uptake of nanoparticles

The result of comparative cellular uptake assay shows that soluble rhodamine[123] taken up quickly by the cells, cresting up in 30 minutes then rapidly started eliminating in 1 hr, 2 hr and completely eliminated in 4hr (Fig.4.1). In contrast, apotransferrin and lactoferrin nanoparticle loaded with rhodamine [123] slowly localised into the cells and remains high for up to 4 hr. The results confirmed that these nanoparticles slowly deliver into the retinoblastoma cells and remain for longer time compare to soluble dye, thus provides more exposure time to anticancer drug to act against retinoblastoma cells.

In another set of experiment comparative drug localization of soluble carboplatin, apo-nano-carbo and lacto-nano-carbo nanoparticles loaded with carboplatin were demonstrated by spectrophotometric estimation of drug into the retinoblastoma cells. Result of this experiment indicates that delivery of carboplatin through protein nanoparticles are significantly increases from 30 min to 4hr in a linear manner and remain constant for up to 24 hr, followed by steep reduction in carboplatin concentration for up to 72 hr is observed (Fig 4.2). Whereas in case of soluble carboplatin, the concentration of delivered drug inside the cells was high at 30 min after incubation and then a gradual reduction observe in the carboplatin concentration from 1 hr to 72 hrs. Further this result is corroborated (for up to 4hr) with the result of confocal study in previous sub section. When compared to soluble drug, these results confirms that drug loaded protein nanoparticles are delivered high amount of drug as well as remains for longer time inside the retinoblastoma cells.

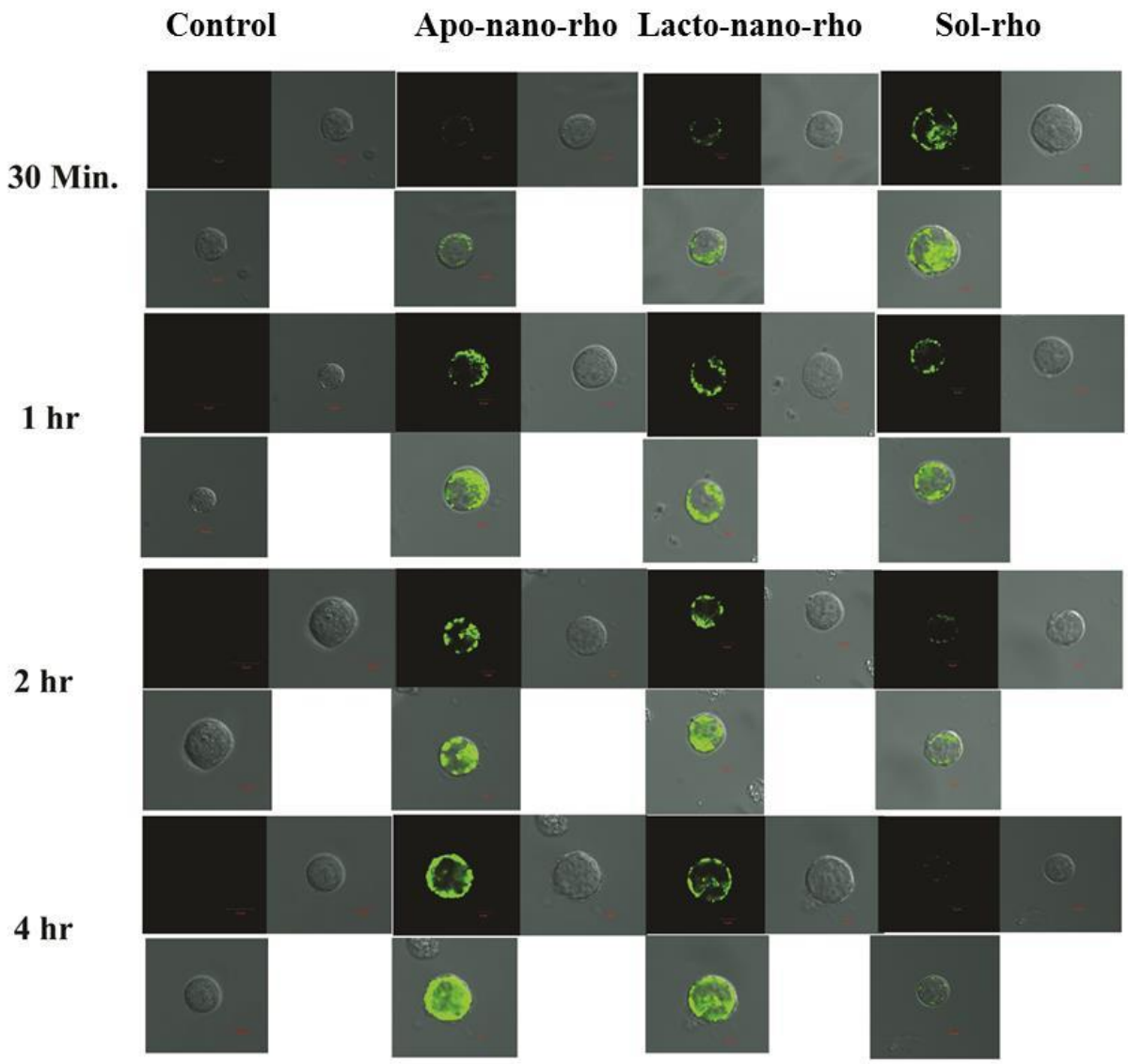


Figure 4-1: Cellular uptake of Apotranferrin (Apo-nano-rho) and Lactoferrin (Lacto-nano-rho) nanoparticles loaded with rhodamine123 and soluble rhodamine (sol-rho) in Y79 cells. Time course experiment shows that intracellular retention of free dye is decrease with time.

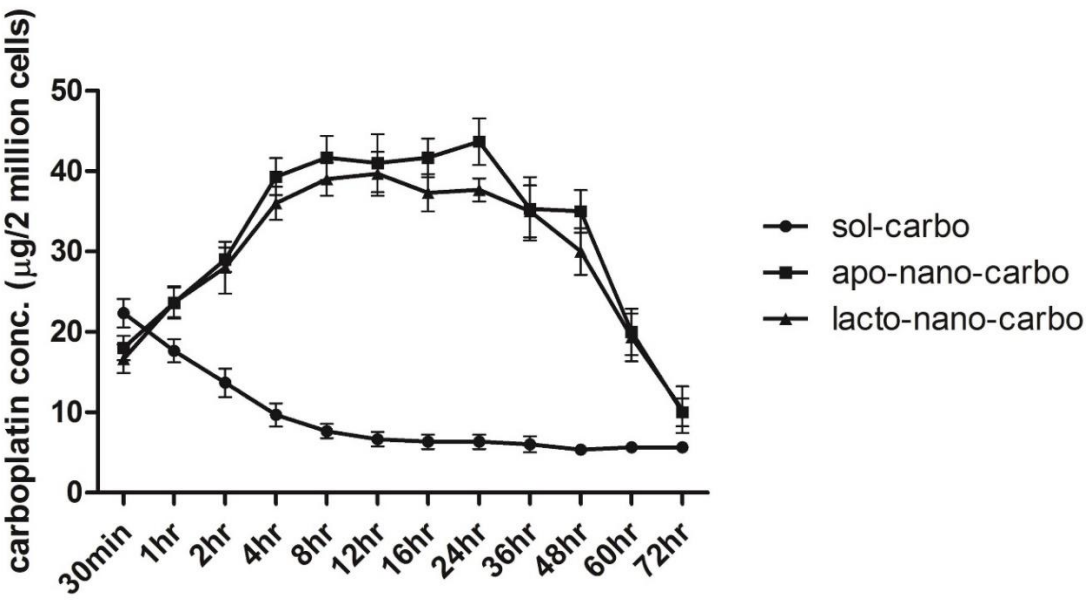


Figure 4-2: Quantitative measurement of comparative cellular uptake of carboplatin with and without loading into the Apotransferrin (Apo-nano-carbo) & Lactoferrin (Lacto-nano-carbo) nanoparticles across the various time points. Carboplatin were estimated by spectrophotometric method at (220nm) and quantified by using the standard curve. Data of each points were taken in triplicate (n=3) and presented in Mean ± SEM.

4.2.2 Receptor-mediated entry of nanoparticles

We have carried out this assay to prove the possible role of transferrin and lactoferrin receptor in the delivery of apotransferrin and lactoferrin nanoparticle into the retinoblastoma cells. In this experiment it is observed that nanoparticle localisation into the cells is dramatically decreased in the presence of transferrin and lactoferrin receptor-specific antibody at different time points (1hr,2hr) compared to absence of antibody that shown in confocal images Fig 4.3A. In carboplatin estimation assay similar type of result was being replicated. Carboplatin localisation got significantly reduced with receptor blocking antibody at different time points (1hr and 2hr) compared to absence of receptor blocking antibody (Fig. 4.3B&C.). Above result indicate that blocking of receptors of apotransferrin and lactoferrin on the cell surface could inhibit cellular entry of nanoparticles, thus suggesting nanoparticles entering into the Y79 cell through their specific receptors.

Further, we have studied sub cellular localisation of protein nanoparticle in Y79 cells. This confocal microscopy based experiment carried out in the presence of lysosome marker (yellow fluorescence) to demonstrate that protein nanoparticle (green fluorescence) co-localised with lysotracker suggesting nanoparticles are localized into lysosome. Lysosomal localisation of nanoparticles further ascertains that it follows the endocytotic route for entering into retinoblastoma cells as shown in (Fig.4.4).

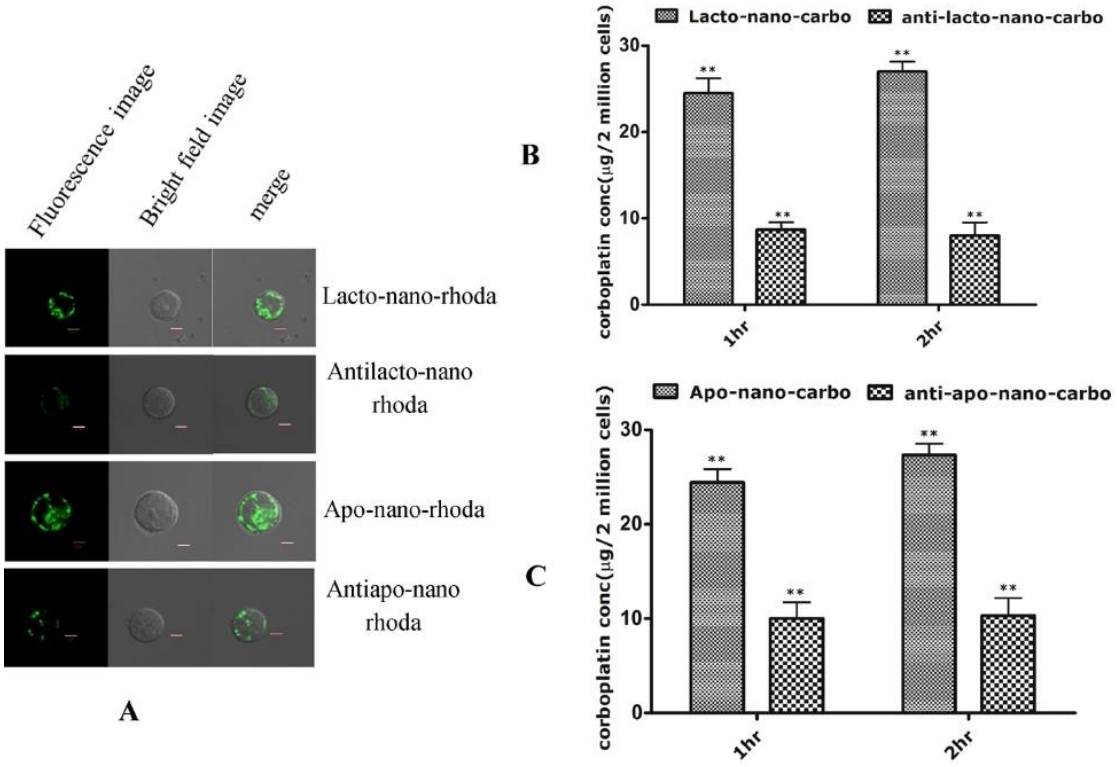


Figure 4-3: Receptor blocking assay as a proof of possible role of Apo transferrin and lactoferrin receptor in nanoparticle transportation inside the cells. (A) confocal analysis shows reduction of nanoparticle(green) entry into the cells, bar length is equivalent to 20 µm. (B&C) quantitative measurement of carboplatin delivery inside the cells after blocking the receptor in comparison to unblocked receptor .Data are present in mean ± SEM, n=3, and value of significance **P<.0080.

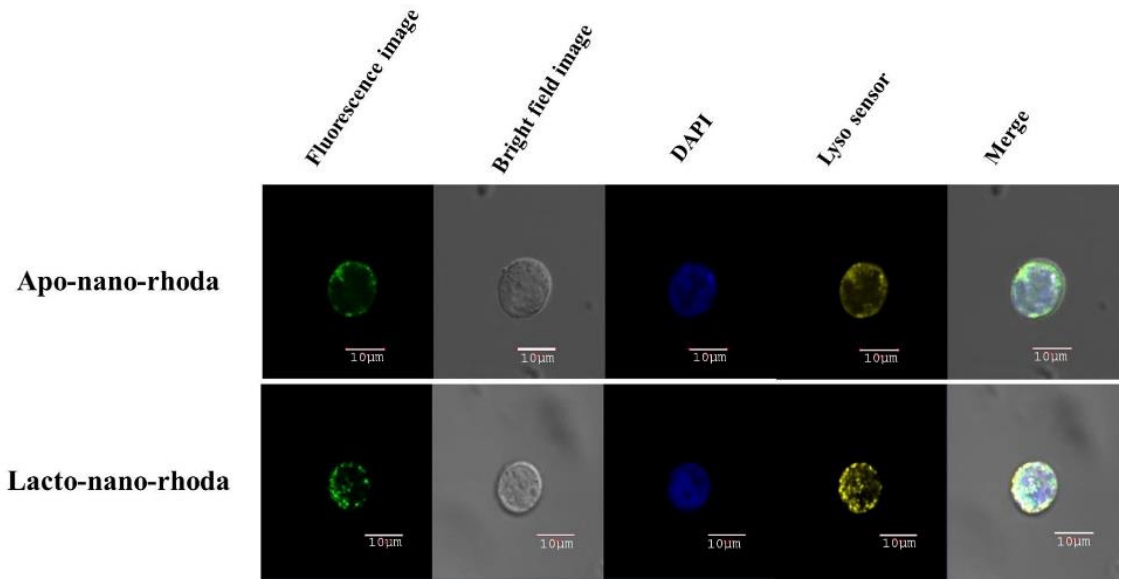


Figure 4-4: The Confocal microscopy assay to the study of Co-localization of Apo transferrin & lactoferrin (green color) nanoparticles inside the lysosome (Yellow color) of Y79 cells. Nanoparticles are tagged with rhodamine-123 (green), lysosomes are stained with lysotracker (yellow color) and nucleus is stained with DAPI (blue color).

4.2.3 Antiproliferative activity of carboplatin when loaded in nanoparticle formulation

The results of cell proliferation assay shows that Apo-nano-carbo and lacto-nano-carbo exhibit significantly high antiproliferative activity at all concentrations tested in comparison to soluble carboplatin (Fig.4.5 and B). Carboplatin loaded Apo-nano-carbo and lacto-nano-carbo shows IC_{50} value as low as (4.31µgml^{-1}) and (4.16µgml^{-1}) respectively in Y79 cells, whereas IC_{50} of soluble carboplatin was as high as (13.498µgml^{-1}). In another set of experiments, viability of cells was measured at different time points in a fixed carboplatin or equivalent nanoparticles concentration (30ug). Cell viability at 4 hr incubation of free carboplatin is less than nanoparticle counterpart, but 4hrs onwards nanoparticle loaded carboplatin shows lesser cellular viability than free carboplatin (Fig.4.5C). This result is corroborated with cellular uptake of free and loaded drugs. Encapsulation of carboplatin into

Apo transferrin and Lactoferrin increases growth inhibition may be as a result of higher uptake of loaded drug than soluble drug into the Y79 cells.

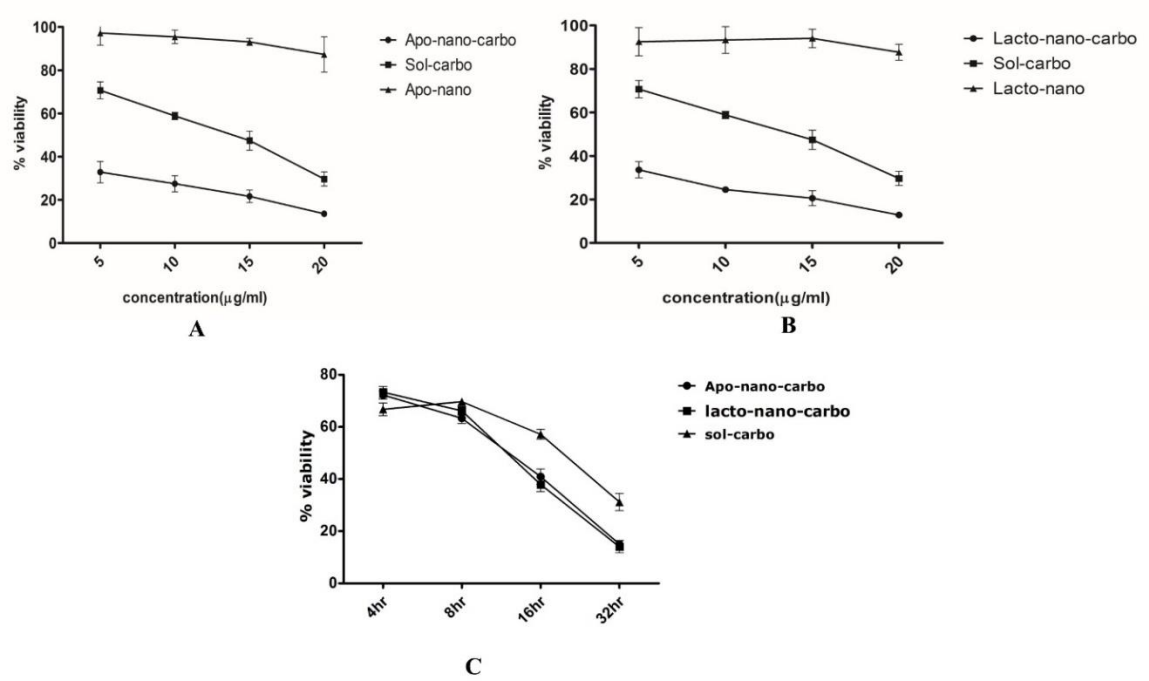


Figure 4-5: Dose dependent anti proliferative potential of carboplatin loaded Apo transferrin (A) & lactoferrin (B) nanoparticles and time dependent activity (C) in comparison to soluble carboplatin. Apo-nano-carbo, Lacto-nano-carbo and Sol-carbo represents, carboplatin loaded Apo transferrin nanoparticle, carboplatin loaded Lactoferrin nanoparticle and soluble nanoparticle respectively. Data are represent in Mean ± SEM, n=3 and value of significance ***P <.001.

4.2.4 Nanoparticles induces higher cell death in Y79 cells

Carboplatin causes cell death in retinoblastoma cell line by mechanism of induction of apoptosis [Di Felice V et al 1998]. DNA laddering assay were carried out into Y79 cells as DNA fragmentation is one of the important characteristic of apoptotic cells. The result of DNA fragmentation assay clearly shows that carboplatin loaded nanoparticles induces higher DNA

fragmentation in comparison to soluble carboplatin in human retinoblastoma cell line (Fig.4.6A). This result corroborated with result of anti-protleferation assay.

Further, we have carried out Caspase-III assay to show that carboplatin inducing apoptosis in Y79 cells. Caspase-III is proapototic enzyme, which is the final executioner of cellular apoptotic pathway as their enzymatic activity increases with apoptosis, so increase in Caspase-III activity is the directly proportional to the increase in apoptosis [Vento R et al 2000]. As result clearly shows that Caspase-III activity increases by two folds when cells treated with Apo-nano-carbo and Lacto-nano-carbo as compared to soluble carboplatin in Y79 cells (Fig.4.6B). At similar concentration, carboplatin loaded Apo transferrin and lactoferrin nanoparticle induces greater apoptosis into the human retinoblastoma cells as compare to their soluble counterpart. Thus nanoparticle increase the anti-proliferative activity of carboplatin in Y79 cells as it further corroborated with the result of MTT assay.

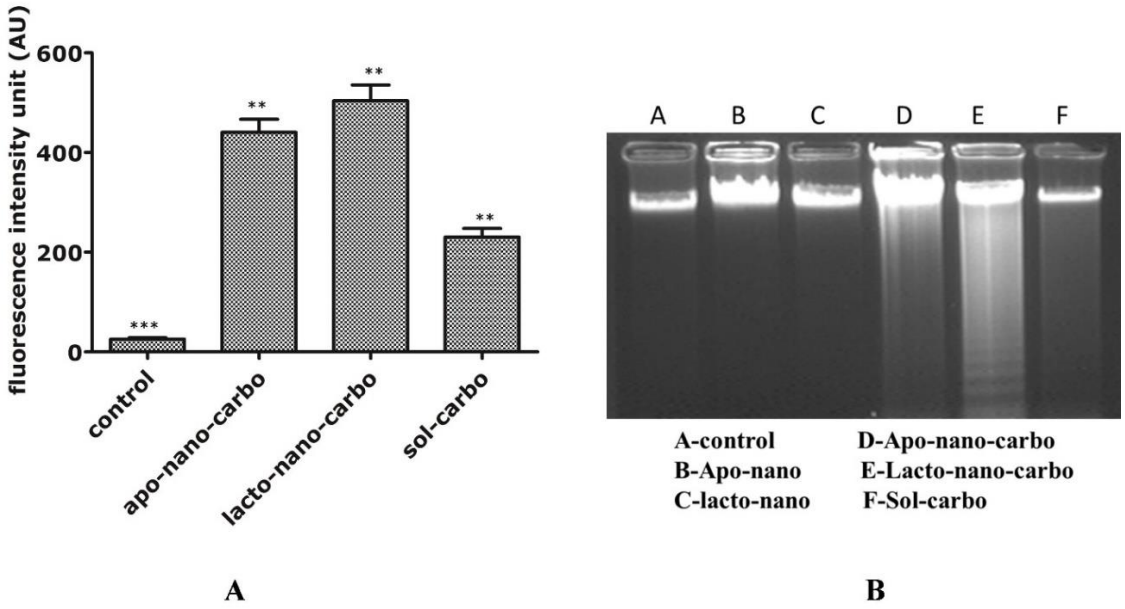


Figure 4-6: Carboplatin loaded Apo transferrin & lactoferrin nanoparticle in comparison to soluble carboplatin at 20µg each, induces Caspase-III activation in Y79 cells. Data are shown in Mean ± SEM, n=3 and value of significance, **P<.0026, ***P<.0001. (B) Carboplatin loaded Apo transferrin and lactoferrin nanoparticle causes significant DNA fragmentation while soluble carboplatin causes mild DNA fragmentation at similar concentration (20µg each) in Y79 cells. Apo-nano-carbo, Lacto-nano-carbo, Apo-nano, Lacto-nano and sol-carbo represents carboplatin loaded Apo transferrin nanoparticle, carboplatin Lactoferrin nanoparticle , blank Apo transferrin nanoparticle, blank Lactoferrin nanoparticle and soluble carboplatin respectively.

Objective 3. Analysis of safety and bioavailability of Carboplatin loaded Protein nanoparticles when administered through periocular route.

4.2.5 Ocular localization of protein nanoparticle in rat eyes post peri ocular injection

Rats were injected periocularly with nanoparticle tagged with rhodamine[123] (green fluorescent) and loaded with doxorubicin (red fluorescent as tracking agent). Eyes were collected at defined time points and sectioned to scan under confocal microscope. Confocal microscopic analysis shows that apotransferrin & lactoferrin nanoparticle (green colour) localised and deliver relatively more drug (doxorubicin red in colour) in every subsection of eye (retina, choroid and lens) of rat eye as compared to free doxorubicin at each time point (fig 4.7).

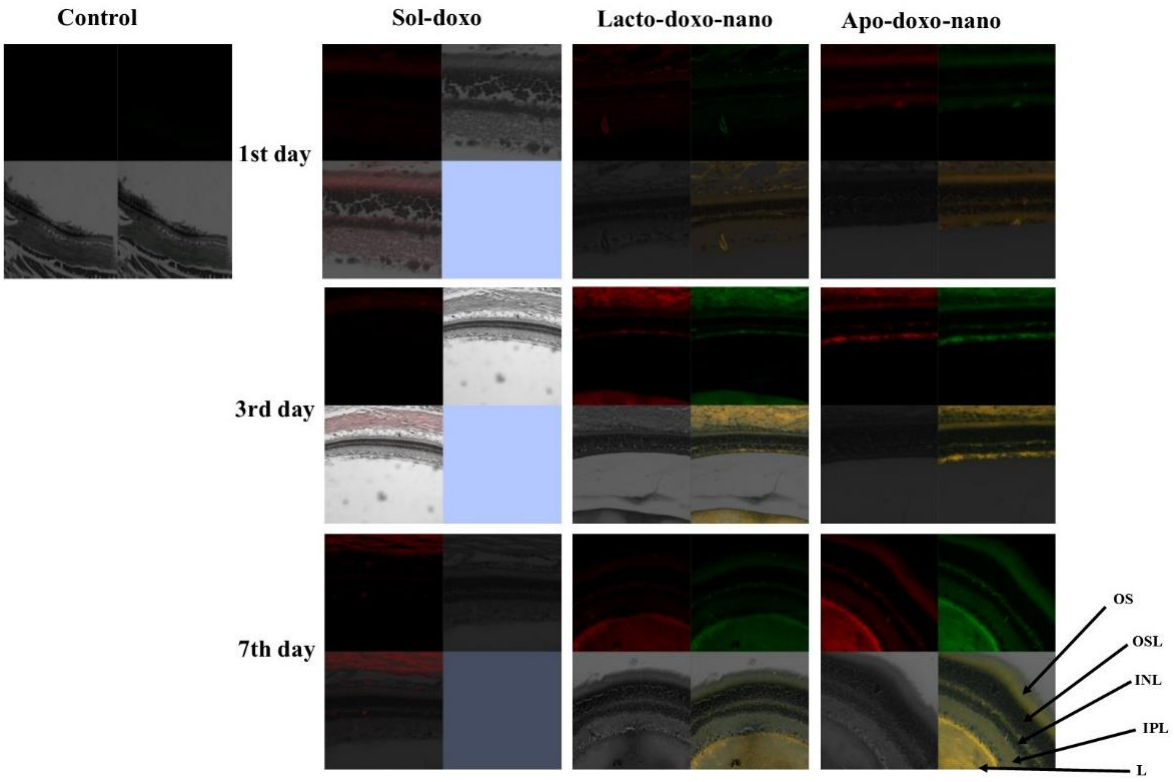


Figure 4-7: Comparative Ocular localization of doxorubicin loaded apotransferrin & Lactoferrin nanoparticle and free doxorubicin. Green colour indicates the nanoparticle and red colour indicates the doxorubicin. OS-outer segment, ONL-outer nucelus layer, INL-inner nucelus layer, IPL-inner plexiform layer, L-lense.

4.2.6 Comparative Pharmacokinetics of free Carboplatin and Carboplatin loaded Protein nanoparticle after periocular injection around the rat's eyes

Ocular and plasma pharmacokinetics of free carboplatin (Sol-carbo) and loaded carboplatin into protein nanoparticles (Apo-nano-carbo and lacto-nano-carbo) were carried out upon injection of single dose via peri ocular route. After single dose, drug concentrations in vitreous cavity as well as blood was measured by HPLC method and data were plotted on concentration against time curve. Apo-nano-carbo and lacto-nano-carbo exhibit better distribution profile in vitreous cavity having relatively improved area under curve (AUC). AUC of apo-nano-carbo and lacto-nano-carbo are

(87666±5635 and 92928±2385 µg/dL/hr) respectively, while area under curve of free carboplatin (Sol-carbo) found to be 17806±1908 µg/dL/hr (Fig.4.8 and table 4.1). Maximum concentration after single dose of (C_{max}) of carboplatin loaded Nano formulations are far higher than free carboplatin (C_{max} µg/ml of Apo-nano-carbo= 238.43±13, lacto-nano-carbo= 275.10±5 and Sol-carbo= 62.50±46.8). Half-life of drug into vitreous cavity ($T_{1/2}$) in case of free drug is less than loaded drug (table 4.1). Whereas plasma pharmacokinetics parameter shows that large amount carboplatin enter into systemic circulation by sol-carbo formulation after first hour of injection while very less amount of carboplatin enter into blood by protein Nano formulations. Complete pharmacokinetics parameter into plasma are given in table 4.1.

Table 4.1: Pharmacokinetic profile of protein nano formulation in vitreous fluid and blood/plasma of wistar rats post peri ocular injection.

Formulations	**AUC (µg/dL.hr)**	**C_{max} (µg/ml)**	**$T_{1/2}$ (hrs)**
Vitreous			
Apo-nano-carbo	87666±5635	238±13	116±31
Lacto-nano-carbo	92928±2385	275±5	118±12
Sol-carbo	17806±1908	62±46	37±8.9
Plasma			
Apo-nano-carbo	625±111	2.04±.43	101±19
Lacto-nano-carbo	554±38	1.75±.29	118±43
Sol-carbo	1144±80	7.5±.5	222±7.6

AUC-Area under curve
C_{max}- maximum concentration achieved after single dose
$T_{1/2}$- time at which half of the drug eliminated from the system

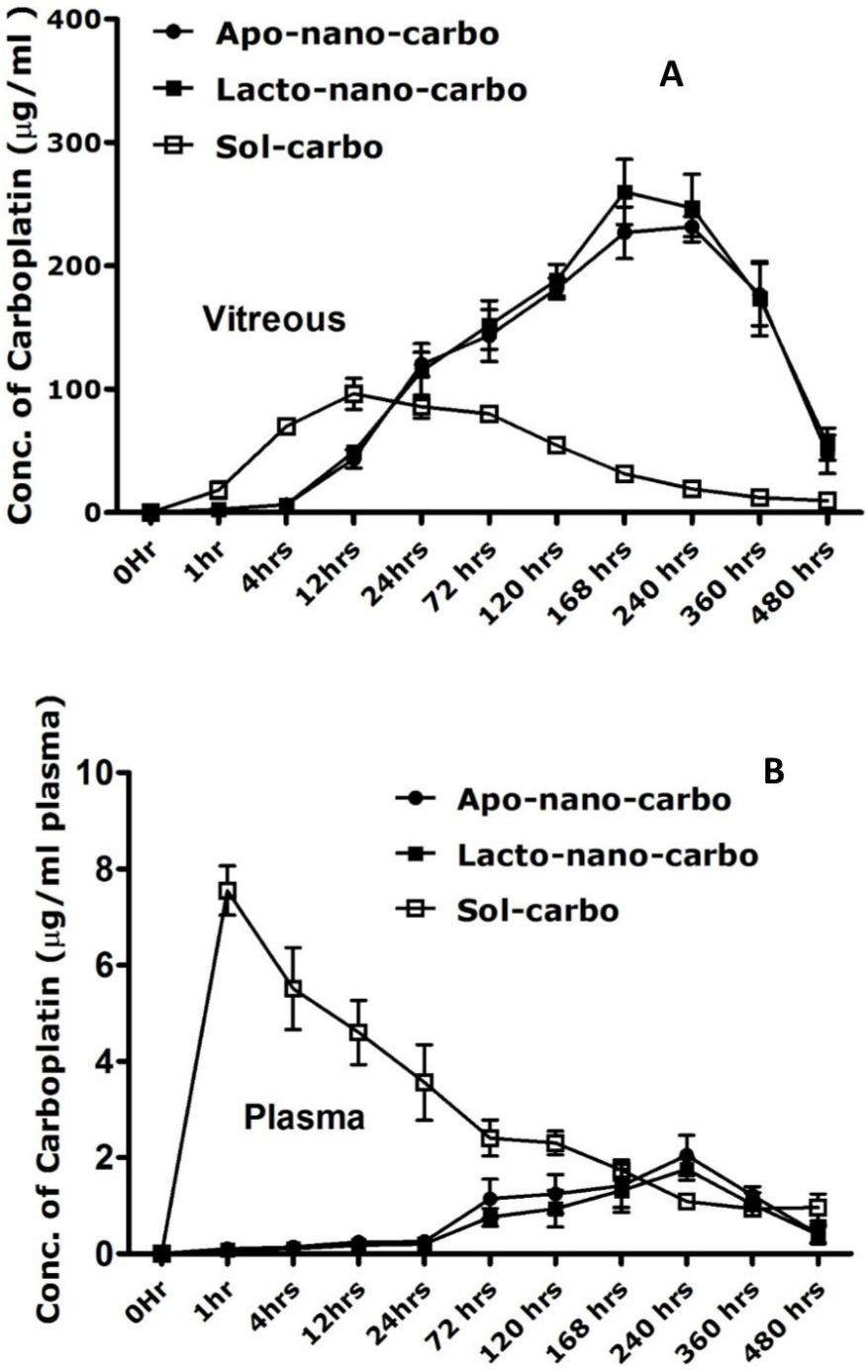


Figure 4-8: Comparative ocular delivery of apo-nano-carbo, lacto-nano-carbo and sol-carbo into the vitreous (A) and (B) in blood after Single periocular injection at different time point. Data are plotting at X-axis (time points) and at Y-axis (carboplatin concentration in μg), each data have been taken in triplicate and data are represented in SDM.

4.2.7 Ocular safety analysis of rat after injection of carboplatin loaded protein nanoparticle and free carboplatin

4.2.7.1 Cell infiltration and total protein assay from Vitreous humor of rat eye

Carboplatin (2mg equivalent) loaded in protein nanoparticle and free carboplatin were injected periocularly in the rat. Since total protein and cells in vitreous humor is an indicator of ocular inflammation, we have collected the eyes at defined time point then ocular humor were collected to estimate the protein and total cells count. There were no significant change noticed in amount of humor protein and cells count post injection of carboplatin loaded nanoparticles as compared to control rat, while significant change noticed in free carboplatin treated rats. Above result suggest that free carboplatin induces inflammatory response in 1st and 3rd day post injection, but came back to normal at 15th day post injection. In contrast to free carboplatin there were no significant elevation of either protein level or cells count in vitreous humor at any given time point after injection of nano formulations, thus nano formulation doesn't induce any inflammatory response in eyes. LPS has been used as a positive control for inflammation study (Fig 4.9A &4.9B).

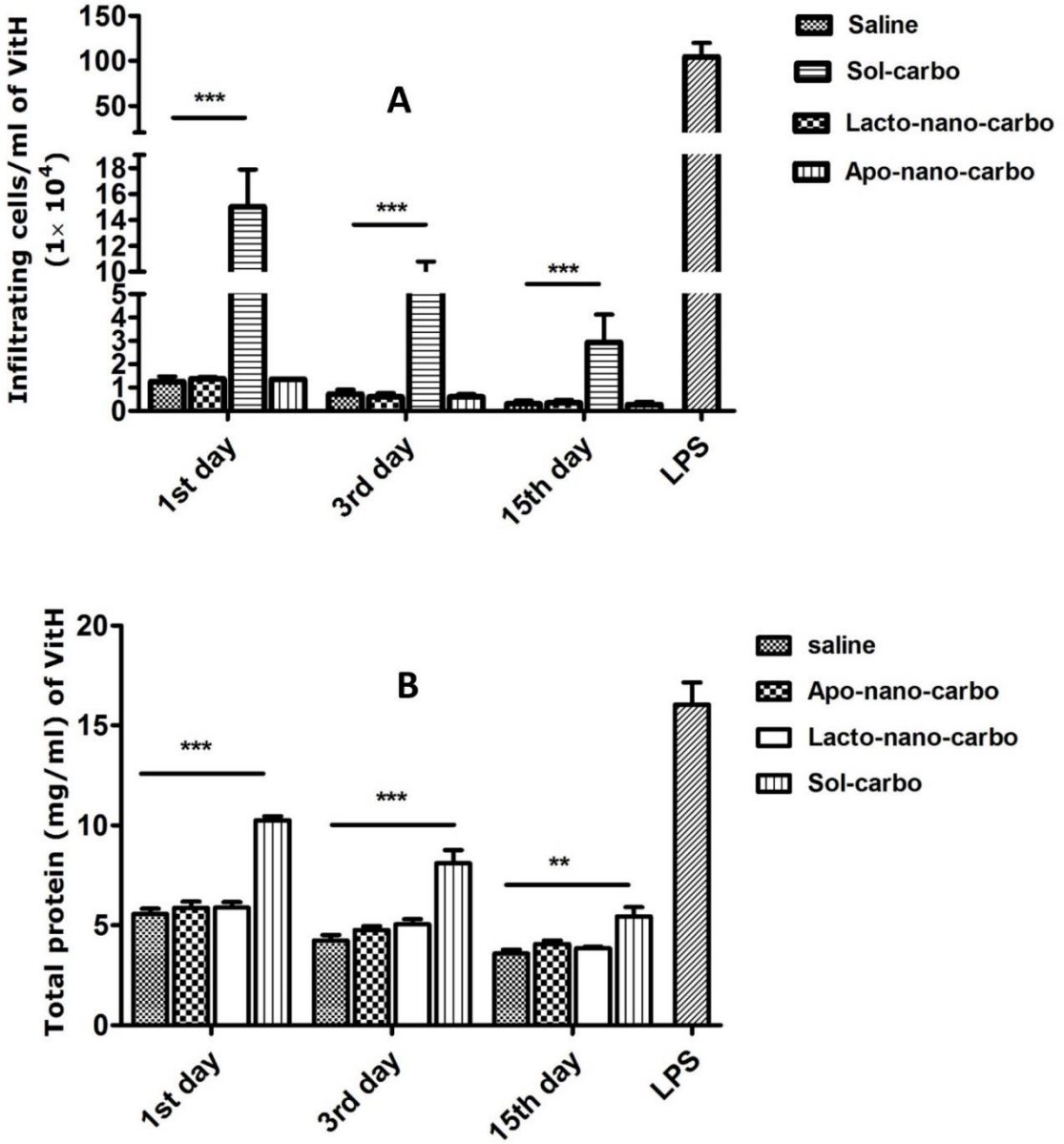


Figure 4-9: Time course Study of ocular inflammation after periocular injection of free and protein nanoparticle loaded carboplatin carried out by calculation of infiltratory cells into vitreous cavity (A) and estimation of total humour protein from aqueous humour (B). Saline and LPS were used as control and positive control respectively. Data are presented with mean±SEM, value of significance P*<.005 are considered to be significant.

4.2.7.2 Estimation of inflammatory cytokines from whole eye extract

Cytokines are potent pro-inflammatory mediator since its hold centre stage in any kind of ocular inflammation. Mostly IL-6, TNF-α and MCP-1 cytokines level found to be elevated during Ocular inflammation [Jo N et al 2003, Yoshida S et al 2003]. So, we have taken whole eye extract to estimate these cytokines by ELISA method. The result shows that level all above cytokines got elevated after treatment with free carboplatin as compare to control eye at initial time point (1st and 3rd day PI) but came back to normal after 15th day PI. While there were no significant change observed in cytokines level in carboplatin loaded nanoparticle treated groups (Fig 4.10A, 4.10B, and 4.10C). This observation shows that Protein nano formulation doesn't induce ocular inflammation. LPS treated group used as positive control for ocular inflammation.

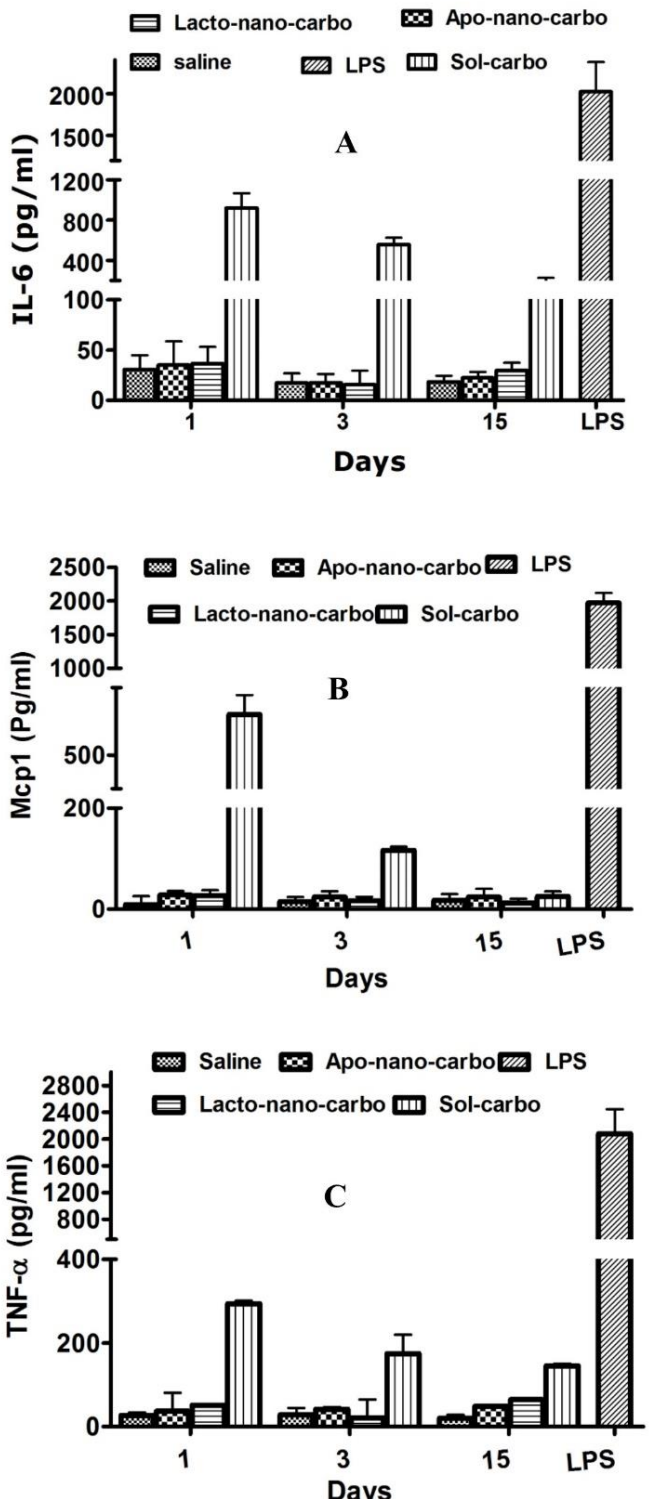


Figure 4-10: Estimation of ocular pro-inflammatory cytokines (IL_6, TNF-alpha and Mcp1) after periocular injection at different time points of nanoparticle loaded and free carboplatin are carried out by ELISA method. Saline and LPS groups are represents control and positive control of inflammation respectively. Data are demonstrated as mean ± SEM and value of significance P*<.005 considered to be significance

4.2.7.3 Histopathology analysis of rat eyes after peri ocular injection

To visualise the infiltratory cells into vitreous cavity and structural deformity into retinal sub layer was evaluated by histopathology analysis of rat's eye section. Microscopic observation of H&E section of rats eye shows that no significant level of infiltratory cells found into vitreous cavity as well retinal sub layer and there is no structural deformity were observed in carboplatin loaded protein nanoparticles treated groups at all-time points. Whereas in carboplatin treated groups shows that few infiltratory cells were found in retinal sub layer after first day post injection as compare to saline treated group but no cells were observed after first day onwards (fig 4.11). LPS treated group were used as a positive control for inflammation.

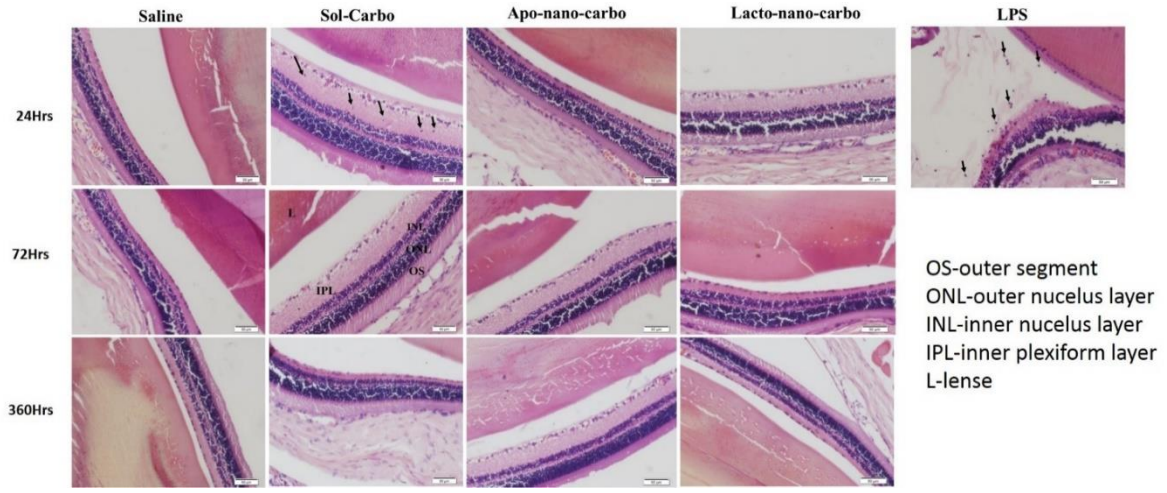


Figure 4-11: Study of structural integrity of retina and visualisation of infiltrating cells into retina and vitreous cavity is demonstrated by H&E staining of rat's eyes section post periocular injection of free (Sol-carbo) and loaded (Apo-nano-carbo&Lacto-nano-carbo) carboplatin. Black arrows indicates the infiltrating cells. LPS is used as positive control of ocular inflammation.

4.2.7.4 Evaluation of systemic toxicity of free and loaded carboplatin

As carboplatin cause systemic side effect especially bone marrow suppression and that is well documented. We wanted to check whether carboplatin loaded protein nanoparticle induced bone marrow suppression after 20 days post injection so, we carried out complete blood count for each group to find out the number of different population of blood cells. Carboplatin loaded protein nanoparticle doesn't induce bone marrow suppression as number of each type of blood cell remains in normal range while, free carboplatin induces suppression into the number of each population of blood cells e.g. RBCs, lymphocytes , neutrophils and platelets (fig.4.12A, B, C, and D). As we have seen in pharmacokinetics parameter, significant amount of free carboplatin enters into blood stream compare to loaded carboplatin that may be the cause of bone marrow suppression.

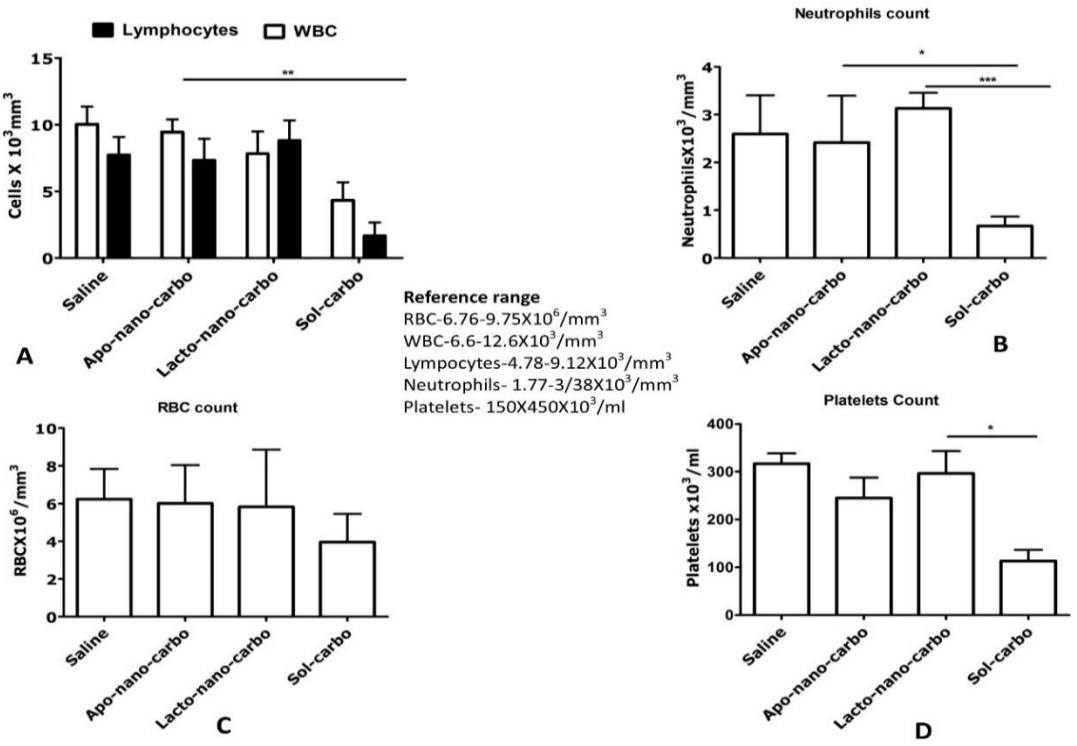


Figure 4-12: Extent of myelosuppression is calculated by total blood count of rats at 480 hrs post periocular injection of both free as well as nanoparticle loaded carboplatin. Number of different population of cells are plotted at y axis versus formulation at x axis (A-count of WBC and Lymphocytes, B-Neutrophils count, C-RBC and D-Platelets count). Data are presented in Mean ± SEM n=3.

4.3 Discussion

In Chapter III, we have demonstrated the preparation, characterisation, loading efficiency and pH dependent release of carboplatin loaded apo transferrin (apo-nano-carbo) and lactoferrin (lacto-nano-carbo) nanoparticles as cancer therapeutic delivery vehicle. In this chapter, we have validated their cellular uptake efficiency and possible cellular uptake mechanism in retinoblastoma cell line (Y79). In addition to this we have also demonstrated their antiproliferative potential in retinoblastoma cells compared to soluble carboplatin by different methods. In-vivo experiments in Wistar rats were carried out to evaluate the Ocular localisation, pharmacokinetics and safety profile of above formulations.

In retinoblastoma, sustain ocular drug delivery without affecting the intraocular structure, as well as combats the various harmful effects of systemic drug exposure is remains a challenge. Presence of the various types of barriers around the retina impedes the drug delivery into the posterior part of the eye for the treatment of retinoblastoma. In order to overcome above limitations, a number of efforts have been put forth in recent years to develop advance biomaterials such as Nano biomaterial and hydrogel. So, anticancer agents can be encapsulated in Nano materials with the idea of improving their pharmacokinetic properties and therapeutic index by minimising dose limiting side-effects.

Intra cellular uptake of nanoparticle was investigated by tagging of rhodamine^{123} to the nanoparticle with comparison to soluble rhodamine ^{123} employing confocal microscopy. Rhodamine ^{123} was chosen to study the intracellular delivery of protein nanoparticles loaded with non-fluorescence anticancer drug is based on modified protocol as described earlier [Rejinold NS et al 2011]. Apo transferrin and lactoferrin nanoparticles were localised slowly over a period of time and remains for a longer time in the cells as compared to free rhodamine 123 this is illustrated in (Fig.4.1). Similar result obtained in another set of experiments which was based on quantitative measurement of carboplatin delivered into the retinoblastoma cells by nanoparticle loaded with carboplatin and soluble

carboplatin. Soluble carboplatin enter rapidly into the cell and eliminated quickly too as compared to nanoparticle form, which deliver the carboplatin slowly and remains for longer time. It may be due to the fact that nanoparticle enters into the cells by receptor mediated endocytosis, while soluble carboplatin enters by passive diffusion. It has been shown that different type of nanoparticles enters into cells by different processes are well documented by various groups [Rejinold NS et al 2011, del Pozo-Rodríguez et al 2008, and Mo, Y et al 2007]. This type of delivery is valued in treatment of cancer because drug accumulates for longer period of time and provides enough time to act on cancerous cells, such studies has been reported earlier [Tanmoy S. et al 2014, Yang H et al 2009]. Additional benefit of this system is that, it may also decrease the dose related toxicity into the healthy tissue.

Cellular uptakes of Apo transferrin nanoparticles are mediated by transferrin receptors that are known to be highly expressed on cancerous cells [Saikrishna A.D.S. et al 2009]. We have established the same pattern in retinoblastoma cell line. Using the receptor blocking experiment we have shown that apotransferin and lactoferrin nanoparticle enters into the cells by their native receptor as shown qualitatively and quantitatively (Fig 4.3A, 4.3B and 4.3C). Transferrin receptor has been utilised for targeted drug delivery into cancerous cells by many researchers [Gatter KC 1983]. Therefore, a number of polymeric nanoparticles coupled with transferrin and lactoferrin peptides have been developed with the idea of targeted drug delivery against particular target [Gan CW, Feng SS 2010, Yu Zheng et al 2010, and Kaili Hu et al 2009]. Furthermore, as we have believed that nanoparticle enters into the cells by receptor mediated endocytosis then it should go through the lysosome. In order to prove the same, we carried out experiments to show that proteins nanoparticles are co-localised with lysosome as shown in Fig.4.4 and it further strengthen the proposed mechanism of nanoparticle uptake into the cells. Even though we have proved that nanoparticle enter into cells through receptor mediated endocytosis but some other delivery mechanism might also involve because of minute size of nanoparticles. A minor fraction may enter through this pathway.

Carboplatin is an important component of chemotherapeutic regimens of the retinoblastoma treatment, as clinical data have recognised the efficacy of carboplatin in the treatment of the RB [Shields CL et al 1996]. However, it is accompanied by serious side effects [Griesinger F et al 2004], so the need for a drug delivery system that could successfully overcome such side-effects as well as improving its therapeutic efficacy would be very useful in the treatment of retinoblastoma. We then performed studies to demonstrate the anti-proliferative activity of carboplatin loaded Apo transferrin and lactoferrin nanoparticles with comparison to soluble carboplatin in concentration dependent manner. The IC_{50} of apo-nano-carbo, lacto-nano-carbo is ($4.31\mu gml^{-1}$, $4.16\mu gml^{-1}$) respectively and soluble carboplatin is ($13.498\mu gml^{-1}$) as shown in (fig. 4.5A &B). Cell viability with different time point incubation of nanoformulation and free carboplatin shows that in first four hour of incubation free drug exhibit higher efficacy than nano formulation but at latter time points nano formulation shows better anti proliferative activity than free drug. This observation also indicate that nano formulation shows sustained release and antiproliferative activity for longer time, thus it may be advantageous than free carboplatin. In addition, resultls ofDNA laddering and caspase-III assay (fig 4.6A &B) further supporting the above observations. It may be due to the drug loaded nanoparticle localised into the cells better than soluble counterpart that have demonstrated in our study thus, bypassing the multiple drug barriers [Jabr-Milane LS et al 2008]. Furthermore, polymeric capsule protecting the drug and shield it from being degraded in harsh in-vivo environment [Panyam J et al 2003].

Promising in-vitro data prompt us to evaluate the ocular localisation, pharmacokinetics and safety of protein nanoparticle via peri ocular route of injection most specifically (peri bulbar) to avoid the systemic clearance and toxicity. As ocular localisation data shows that both the protein nanoparticles (green in color) enter into retinal sub layer by crossing the outer scleral layer by unknown mechanism. Not only nanoparticles but it also carrying along the doxorubicin (as a tracking agent) into the eyes as compare to free doxorubicin. It's clearly indicate that when doxorubicin loaded into

these nanoparticles then localised more into retinal sublayer as compare to free doxorubicin. Protein nano formulations and route of delivery jointly enhance the localisation into the posterior eye without breaching the ocular barrier unlike other research report where different nano formulation were injected into the vitreous cavity that may cause retinal detachment [Kawakami S et al 2004, Merodio M et al 2002, Bourges JL et al 2003]. Various nano formulation has been developed with biodegradable nanomaterial and injected through peri ocular route in order to minimise the material toxicity as well as retinal injury. However, these nanoparticle are well tolerated but their scope are limited as for as delivery of drug into posterior segment of eye is concern. Whereas this protein nanoparticles deliver more drug compare to other biodegradable nanoparticles for ocular delivery application [Shome D. et al 2009,]

Carboplatin loaded protein nanoparticles exhibit better vitreous pharmacokinetics profile as compared to free carboplatin. Since compare to free carboplatin nanoparticle loaded carboplatin shows large area under curve (AUC), nanopartciles exhibits improved distribution pattern into vitreous cavity, better C_{max} value to indicate elevated vitreous concentration of carboplatin(Fig 4.8A, table 4.1). As plasma pharmacokinetics data shows that AUC plasma and Cmax plasma of free carboplatin is higher than that of protein nano formulated carboplatin. Free carboplatin enter into the blood 1 hour post peri ocular injection at significantly higher concentration from where it cleared rapidly from the system while minute amount of nano formulation of carboplatin enter into the blood. Through peri ocular route anything less than 20nm can easily enter into the circulation probably by both lymphatic and circulatory system thus it make less available around the sclera to enter into retina or vitreous cavity [Liu H et al 2005, Aniruddha CA et al 2008].Subsequently free carboplatin can freely move into systemic circulation as a result of this vitreous concentration of free carboplatin decreases as compare to carboplatin loaded protein nanoparticles because majority of nanoparticles are bigger

than 20nm, which will not easily enter into circulation and it will remain deposited around the sclera and constitute a drug depot that keep continue to deliver drug across the sclera.

To determine whether nanoparticle induce any sort of ocular inflammatory response followed by peri ocular injection. We have carried out different assay to find out the cellular infiltration and inflammatory cytokines level. Total protein estimation from vitreous humour and total cells count from vitreous humour could give indication of ocular inflammation. During ocular inflammation infiltrating cells most importantly polymorphonuclear leukocytes (PMN) are rushed into eye against injury or toxic stimuli [Ramadan RT et al 2006, Giese MJ et al 1998] which in turn further recruit other immune cells at inflammatory sites causing increased in total number of cells in vitreous cavity. We also performed the hematoxylin and eosin staining of cross section of treated eye to find out any infiltratory cells or structural aberration followed by peri ocular injection of free carboplatin and carboplatin loaded protein nanoparticles. From all these above assay it can be clearly demonstrated that free carboplatin induce mild ocular inflammation 1st day post injection and came back to normal after 1st day onwards. While carboplatin loaded nanoparticles doesn't induce ocular inflammation at all. Lipopolysaccharide has been used as positive control for ocular inflammation as it is well established mode for generation of ocular inflammation model [Justine RS et al 1998].

If infiltratory cells are rushing at inflammatory site then responsible cytokines level should elevate. Since eye is immune-active and very sensitive toward any environmental change including ischemic injury [Jo N et al 2003], oxygen induced retinopathy [Powers MR et al 2005], infection [Chintakuntlawar AV et al 2007] etc. MCP-1, Interlukine-6 (IL-6) and TNF-α are known to be responsible for ocular inflammation. So we have checked the level of these prominent pro-inflammatory cytokines by ELISA method. MCP-1 is product of gene family called chemokines, produced by various immune cells in response to infection, or ocular injury. Its recruit the monocytes at the site of action to induce inflammation. MCP-1 has been shown to be upregulated in retina has

been observed in the rat model of endotoxin induced uveitis (EIU) [De Vos AF et al 1994]. Free carboplatin induced the level MCP-1 in rat eyes after 1st day of peri ocular injection but slowly it came to normal up to 15th days PI, whereas nanoparticle loaded carboplatin doesn't change the level. IL-6 and TNF-α are cytokines release from different types of immune cells and mediate the ocular inflammation. Both the cytokines are well observed in different model of EIU and ocular injury [Hoekzema R et al 1992, Planck SR et al 1994]. Nanoformulation doesn't induce these cytokine and get the same pattern as MCP-1. These protein nanoparticle doesn't induce inflammation, hence well tolerated by eyes. Ocular tolerance is very important parameter to be looked into especially if one want to move their product for further study. It is become very important to evaluate the ocular inflammation since some recent report suggest that few nano formulation are reported to be cytotoxic [Hantzschel N,et al 2007, Jan E et al 2008, Lee J et al 2009].

Our present study, clearly validate the significance of the carboplatin loaded apotransferin and lactoferrin nanoparticles as a more promising system, compared to the free carboplatin due to the greater drug retention and intracellular uptake attributed better therapeutic efficiency against retinoblastoma cells. Consequently, a drug delivery approach that is focused not only on enhancing the cellular drug uptake but also on prolonging its intracellular retention might be more useful for retinoblastoma therapy. However, these protein nanoparticle carriers have great potential as a drug delivery system for the ocular tissues since it is well tolerated by ocular tissue, still further studies are needed on retinoblastoma model to evaluate the pharmacological efficacy of protein nano-formulation.

4.4 Conclusion

These carboplatin loaded protein nanoparticles exhibited significantly greater cellular uptake, sustained intracellular drug retention in retinoblastoma cells. Data suggest that major entry mechanism of nanoparticle into the cells might be receptor mediated endocytosis, however other mechanism cannot be rule out. Because of greater cellular uptake and sustained intracellular retention, causes high anti proliferative activity of carboplatin loaded protein nanoparticle than their soluble counterpart, as demonstrated by MTT, DNA laddering assay and caspase-III assays. In-vivo experiment conclude that protein nanoparticles are localising into retinal sublayer and remain their up to 15^{th} day of after peri ocular injection. Pharmacokinetics profile also indicating that after loading carboplatin shows better vitreous distribution and less in blood circulation as contrary to soluble carboplatin. These particle are well tolerated by ocular tissue and doesn't induce mylo suppression. These carboplatin loaded protein nanoparticle might be advantageous for targeted drug delivery to treat the ocular malignancies because of their biocompatible properties.

CHAPTER 5

Safety, bio distribution and efficacy of Oxaliplatin and 5-Fluorouracil loaded lactoferrin nanoparticle against Colon adenocarcinoma: an *in-vitro* and an *in-vivo* study

5.1 Introduction

Colon adenocarcinoma has appeared as a major public health problem, with incidence of more than 1 million new cases and acclaim over half a million mortality worldwide each year [Sharma M et al 2010 , Twelves C et al 2005]. It is second most common cause of cancer related death and most common type of gastrointestinal tract cancer [Terzic J et al 2010]. It is developed due to series of genetic mutations, both in somatic tissues and germ line, and it is widely believed that the cancer phenotype fallouts occur due to an accumulation of genetic changes in the cell clone. However, there is no common mutation pattern exist in all colon cancers, and the chronology of these genetic events does not follow a stern order, indicates more than one pathway leading to the disease (Draznin J et al 2006, Ponz de Leon M et al 2001, Ponz de Leon et al 2000).

Currently therapeutic strategies including surgery, chemotherapy and radiotherapy are being used separately or in combination to manage colon adenocarcinoma. Selection of these modalities is mainly based on the stage of colon cancer [Haller DG et al 2011]. At the early stage of disease surgical resection in combination with chemo and radiation therapy is efficient, but subsequent reversion and metastasis often left with only chemotherapy option, hence chemotherapy is indispensable treatment regime. Hydrophilic drugs including 5-fluorouracil and oxaliplatin are frequently used in chemotherapy module for treatment of colon cancers. These drugs are administered through IV route in combination of each other or with other drugs, because individually these drugs are inefficient. Therapeutic efficacy of these drugs are limited due to short plasma half-life, rapid metabolism, low bioavailability, systemic toxicity and development of resistance by malignant cells [Peters GJ et al 2002, Guichard SM et al 2005, Song M et al 2012, and Amr Selim ALa et al 2014]. Therefore, a novel therapeutic strategies are desired to overcome above limitations and to improve current colon cancer treatment.

Nanoparticle mediated drug delivery system can provide substantial advantages over the use of free drugs in cancer treatment. Moreover, nanoparticle systems can potentially affect drug bio distribution, hence increasing drug concentration and specificity toward cancerous tissue. Furthermore, nanoparticles can extend the plasma half-life of chemotherapeutic drugs while keeping to a minimal level of dose associated drug toxicity (Wang AZ et al 2012). Remarkably, these nano particulate system have been further reported to overcome drug resistance by significantly increasing the drug accumulation within these cells (Patel NR et al 2013). As for as colon adenocarcinoma treatment is concern, various type of biodegradable polymeric nanoparticle systems have been studied to optimize chemotherapy (Prados J et al 2013), especially with 5-FU and oxaliplatin. Amongst them, poly(ε-caprolactone) [Guerra GD et al 2001] and chitosan [Jain A et al 2010 , Chang SJ et al 2007, Tıglı RS et al , 2012] are widely studied due to its biocompatibility and mucoadhesion properties, apart from these polymers few others were investigated such as Gelatin [Sun W et al 2013], Polymers-based on methacrylic acid [Ashwanikumar N et al 2012], Poly(alkylcyanoacrylates) [Arias JL et al 2008], Polyacrylamide [Hadjikirova M et al 2005], Hydrogels [Wang Y et al 2010], Poly(D,L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA) [Chen AZ et al 2006, Li XM et al 2008, Nair KL et al 2011] and Vesicular systems (liposomes) [Yang C et al 2011 , Alvi IA et al 2011]. In addition to these carriers, a number of effort have been put forth to increase tumor specificity of 5-FU and oxaliplatin carriers, though these systems often failed to perform efficiently in vivo [Wang Y et al 2015].

Despite being improvement in conventional chemotherapy of colon cancer by biodegradable nano particulate systems, it suffers the limitation of effective tumor targeting and high energy demand for degradation exerts tremendous burden on diseased individual. So, taking this into consideration new class of whole ligand protein nanoparticle based on iron transporting protein (Apo-tranferrin and lactoferrin), has been developed [Saikrishna A.D.S. et al 2009]. These protein nanoparticles are based on the active targeting principle against cancerous tissue, since the receptor of these iron transporting

proteins are reported to be highly expressed on the surface of cancerous cells [Ahmed F. et al 2014]. Therefore, whole ligand protein nanoparticles based drug delivery module especially lactoferrin nanoparticle seems have potential to target colon adenocarcinoma and pose minimal side effect to healthy tissue [Golla K et al 2012].

In this current study we hypothesized that various side effects of current chemotherapy regime by 5-fu and oxaliplatin for colon cancer can be reduced and therapeutic index can be enhanced by employing lactoferrin based targeted nanoparticle system. This study undertake the evaluation of in-vitro efficacy of lactoferrin nanoparticle loaded 5-FU and oxalipatin drug as compare with free drug in COLO-205 cells. The mechanism of nanoparticle delivery into cells has been also reported. In addition to this in-vivo study of these formulation such as plasma pharmacokinetics, tissue distribution and safety analysis in wistar rats have been carried out. Moreover, the study of efficacy of these formulation against colon aberrant crypt foci in azoxy methane treated wistar rats has also been performed to establish the potential of this particular drug delivery platform.

Objectives of this chapter: This chapter contains major objective-4 of thesis and further divided into two sub objective for ease of comprehending the study.

I. Localisation and efficacy of oxaliplatin and 5-fluorouracil loaded lactoferrin nanoparticle against Colon adenocarcinoma cells, an *in-vitro* study.

II. Study of pharmacokinetics, Tissue distribution, Efficacy and Safety of Oxaliplatin and 5-fluorouracil loaded lactoferrin nanoparticles administered through intra venous route in wistar rats.

5.2 Results

Objective I. Localisation and efficacy of oxaliplatin and 5-fluorouracil loaded lactoferrin nanoparticle against Colon adenocarcinoma cells, an *in-vitro* study

5.2.1 Cellular uptake of nanoparticles

Comparative cellular uptake of soluble Dox and Dox loaded rhodamine^{123} tagged lactoferrin nanoparticles were carried out at different time point (30 min, 1 hr, 2 hr, and 4 hr) in COLO-205 cells, visualising the green and red fluorescence of rhodamine^{123} and doxorubicin respectively by confocal microscopy. This study is showing that soluble Dox taken up quickly by the cells, cresting up in 30 minutes then rapidly started eliminating in 1 hr, 2 hr and completely eliminated in 4hr (Fig.5.1). In contrary, Dox loaded rhodamine ^{123} tagged lactoferrin nanoparticle slowly localised into the cells and remains high for up to 4 hr. Further, results confirmed that these nanoparticles slowly deliver into the colon adenocarcinoma cells and remain for longer time compared to soluble drugs, thus provides longer time to act against cancerous cells. In above experiment Doxorubicin were used solely as a tracking dye.

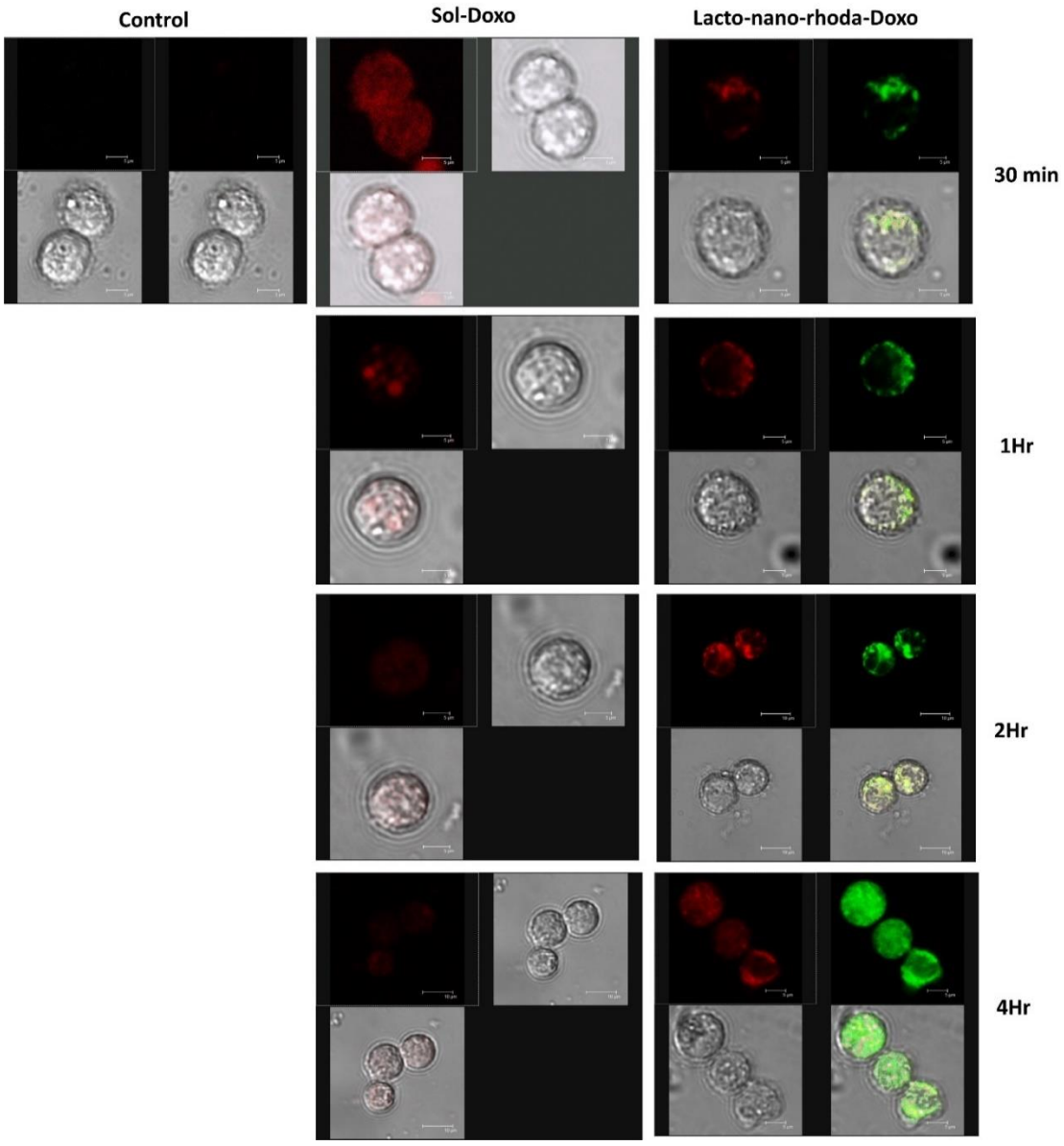


Figure 5-1: Scanning laser confocal images of COLO-205 cells with localised nanoparticle (lacto-nano-rhoda-doxo) and free dye (Sol-dox). Green colour represent the lactoferrin nanoparticle tagged with rhodamine 123, red colour represent the doxorubicin that used as a tracking agents. Left panel represent the control cells (unstained),middle panel represents the localisation of free doxorubicin with different time point and right panel represent the localisation lactoferrin nanoparticle as well as loaded doxorubicin at different time points. Rhodamine123 scanned at Ex=505 and Em=530 and doxorubicin at Ex=470 and Em=650.

In another set of experiments, comparative cellular uptake assay with the time (In-vitro pharmacokinetics), an important observation to demonstrate the potential of anti-cancerous agent with respect to resident time into the cells and subsequent anti-proliferative activity against cancer cells. This experiment indicates that delivery of 5-FU through lactoferrin nanoparticles (Lacto-nano-5FU) are significantly increases from 30 min to 8hr in a linear manner and followed by steep reduction in 5-FU concentration for up to 24 hr is observed (Fig5.2a). Oxaliplatin delivery via lacto-nano-oxali shows an increase from 30min to 4hr and linear up to 8hr followed by steep decline up to 24 hr is observed (Fig 5.2b). Whereas in case of soluble 5-FU and Oxaliplatin, the concentration of delivered drug inside the cells was high at 30 min after incubation and then a gradual reduction observed in concentration from 1 hr to 24 hrs. Moreover this result is corroborated (for up to 4hr) with the result of above confocal study. As compared to soluble drug, these results confirms that drug loaded lactoferrin nanoparticles are delivered high amount of drug as well as remains for longer time inside the Colon adenocarcinoma cells.

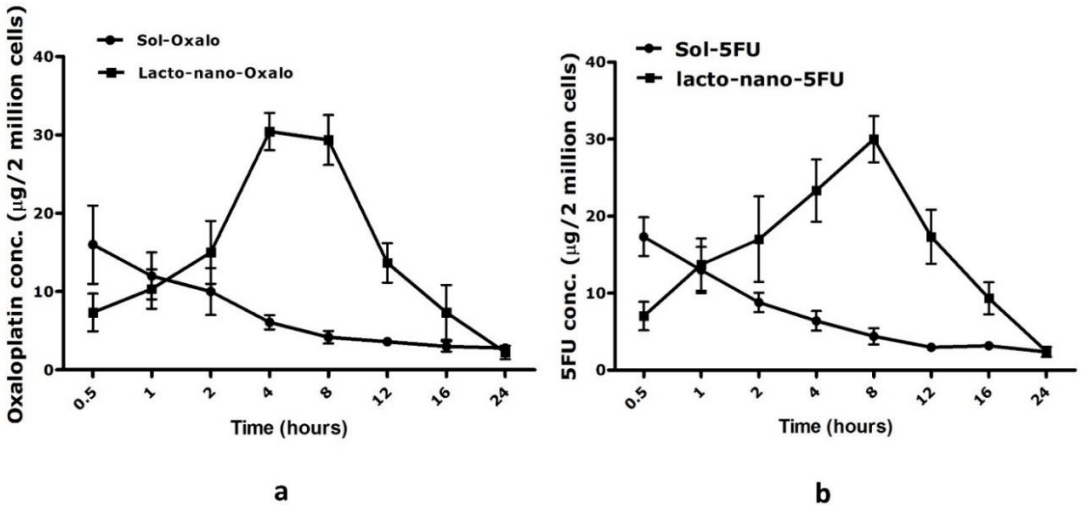


Figure 5-2: Quantitative estimation of comparative drugs localisation in COLO-205 cells a) oxaliplatin loaded lactoferrin nanoparticle (lacto-nano-oxalo) versus free oxaliplatin (sol-oxalo), b) 5-FU loaded nanoparticles (lacto-nano-5FU) versus free 5-FU (sol-5FU). Oxaliplatin and 5-FU were estimated by RP-HPLC and detected by UV detector at absorption maxima λ 220nm and λ 254nm respectively. Triplicate data were taken for each points (n=3) and represents as Mean±SDM.

5.2.2 Mechanism of Nanoparticle Delivery into COLO-205 cells

5.2.2.1 Receptor blocking and lysosome tracking assay

We have carried out this assay to prove the possible role of lactoferrin receptor in the delivery of lactoferrin nanoparticle into the colon adenocarcinoma cells. In this assay COLO-205 cells were incubated with lactoferrin nanoparticle tagged with rhodamine[123] in the presence and absence of lactoferrin receptor blocking antibody. Localisation of nanoparticles (green colour) into the cells is dramatically decreased with lactoferrin receptor blocking antibody post 2hr incubation compared to absence of antibody that shown in confocal images Fig 5.3a. Above result critically shows that lactoferrin nanoparticles enter into the COLO-205 cells through specific receptor.

Furthermore, we have studied sub cellular localisation of nanoparticle in COLO-205 cells. The Confocal microscopy based experiment carried out in the presence of lysosome marker (yellow fluorescence) to demonstrate that protein nanoparticle (green fluorescence) co-localised with lysotracker suggesting nanoparticles are localized into lysosome. Lysosomal localisation of nanoparticles further ascertains that it follows the endocytotic route for entering into colon adenocarcinoma cells as shown in (Fig. 5.3b).

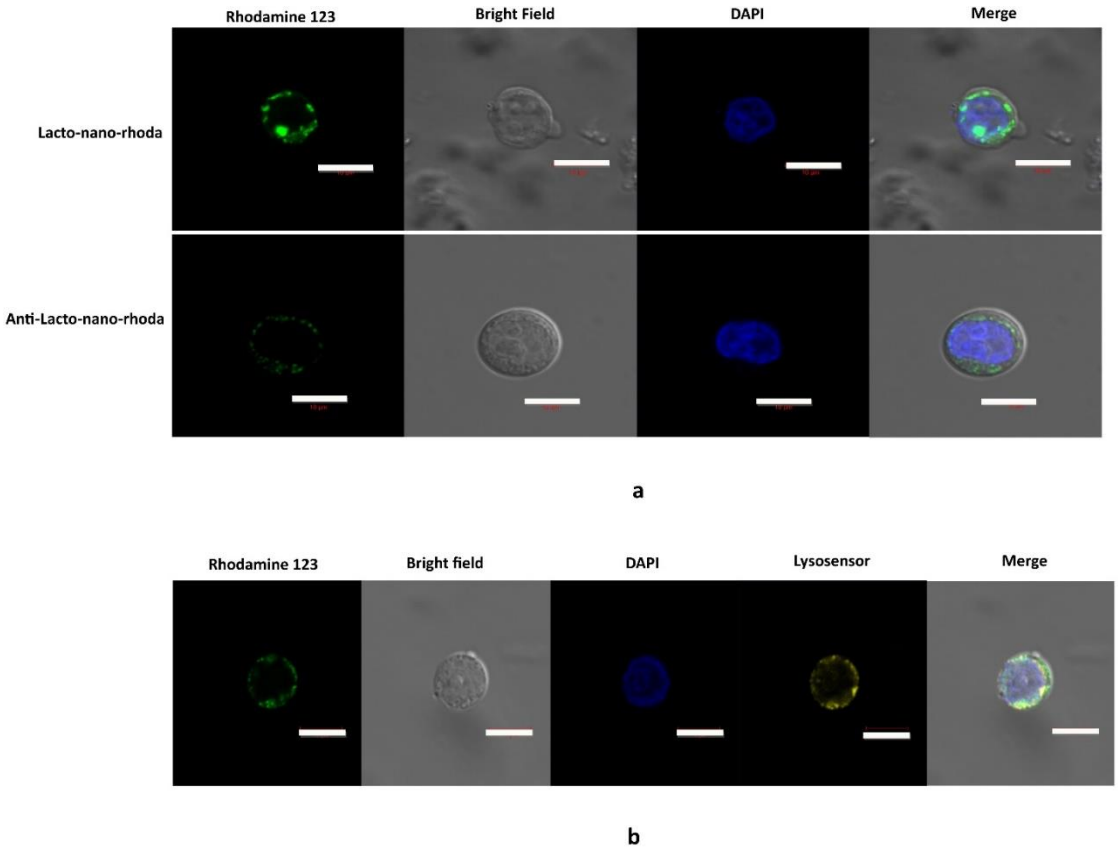


Figure 5-3: a) Receptor blocking assay observed by confocal microscopy upper panel of fig 5.3a represent lactoferrin nanoparticle tagged with rhodamine 123 in absence of receptor blocking antibody and lower panel represents lactoferrin nanoparticles in presence of receptor blocking antibody in COLO-205 cells. Green colour indicates the nanoparticle and blue colour of DAPI indicated the cellular nucleus b) Co-localization of lactoferrin nanoparticles (green colours) with lysosome in COLO -205 cells, lysosome stained with lysotracker (yellow colour), nucleus stained with blue colour of DAPI and each white bar represents 10μm of length.

5.2.3 Antiproliferative activity of oxaliplatin and 5-FU nanoparticle formulation

To evaluate the comparative anti-proliferative activity of soluble 5-FU and oxaliplatin with lacto-nano-5-FU and Lacto-nano-oxalo in Colo-205 cells, were incubated with increasing concentrations (1, 2, 4, 8, μg) of each of the formulations and cell death was assessed by MTT assay. Lacto-nano-5-FU and lacto-nano-oxalo exhibit significantly high antiproliferative activity at each concentrations in comparison to soluble 5-FU and Oxaliplatin (Fig.5.4a and Fig.5.4b) respectively. In comparison with sol-5FU, nanoparticle loaded 5-FU shows low IC_{50} value (sol-5FU-2.27 $\mu g ml^{-1}$ and

Lacto-nan-5FU- 1.08 μgml^{-1}), whereas IC50 value of Sol-oxalo and lacto-nano-oxalo are 2.22 μgml^{-1} and .89 μgml^{-1} respectively in colon adenocarcinoma cells as given in table 5.1. Encapsulation of 5-FU and oxaliplatin into lactoferrin increases growth inhibition may be as a result of higher uptake of loaded drug than soluble drug into the colon adenocarcinoma cells.

Table 5.1: IC50 value of different formulation against COLO-205 cells

Formulations	**IC_{50}-μgml^{-1}**
Sol-5FU	2.27
Lacto-nano-5FU	1.08
Sol-oxalo	2.22
Lacto-nano-oxalo	0.98

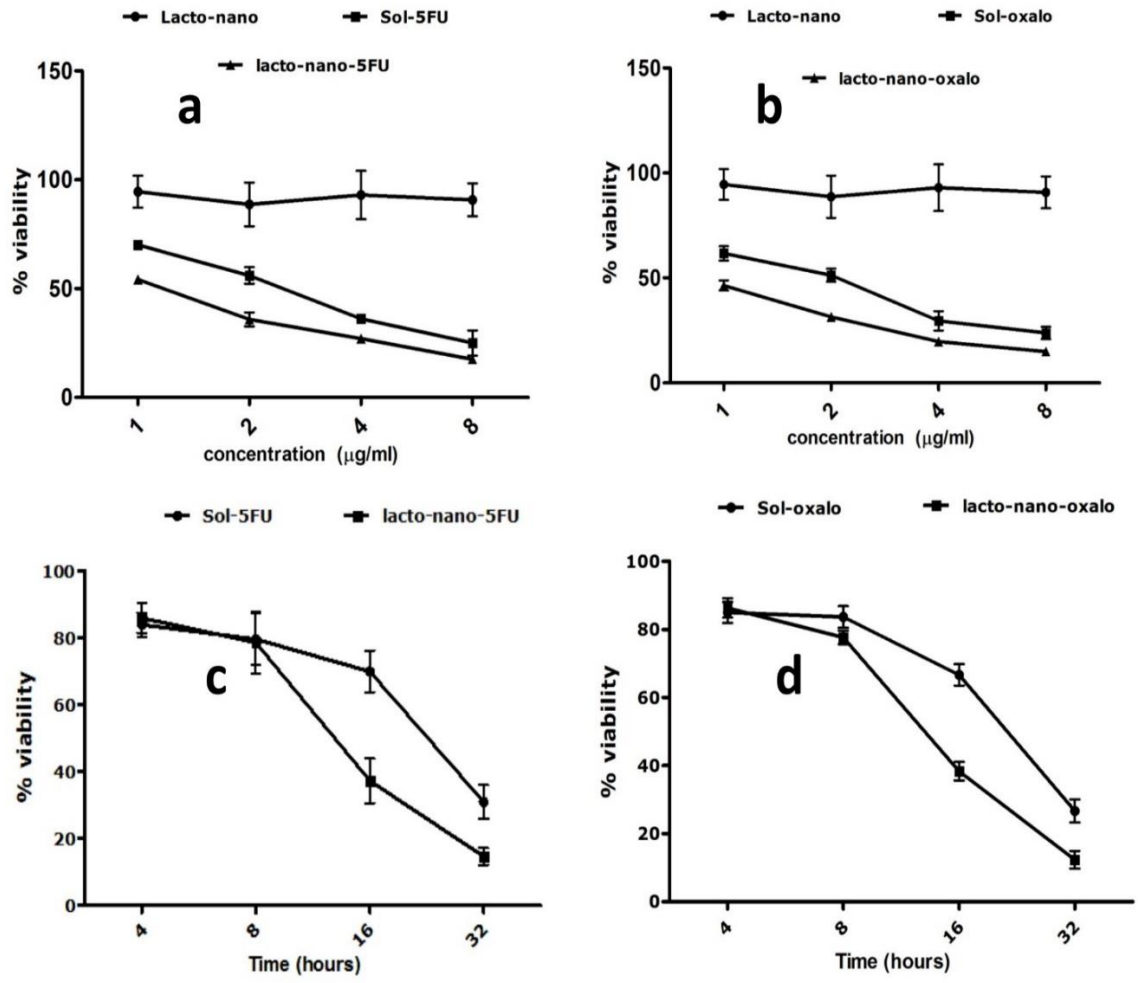


Figure 5-4: Concentration dependent antiproliferative assay (a & b) and time dependent assay by MTT method (c & d). Lacto-nano-oxalo, lacto-nano-5FU, lacto-nano, Sol-oxalo and sol-5FU represents oxaliplatin loaded lactoferrin nanoparticle, 5-FU loaded lactoferrin nanoparticle, blank lactoferrin nanoparticles, free oxaliplatin and free 5-fluorouracil respectively. Each time point have multiple values (n=5) and data were presented in Mean± SDM.

5.2.3.1 DNA laddering and Caspase-III assay

Oxaliplatin and 5-fluorouracil causes cell death in colon adenocarcinoma cell line by mechanism of induction of apoptosis [Nita M. E. et al 1998]. DNA laddering assay were carried out into Colo-205 cells as DNA fragmentation is one of the important characteristic of apoptotic cells [Ioannou YA et al 1996]. The result of DNA fragmentation assay clearly shows that 5-FU and

oxaliplatin loaded nanoparticles induces higher DNA fragmentation in comparison to soluble counterpart in human colon adenocarcinoma cell line (Fig.5.5b).

Further, we have carried out Caspase-III assay to demonstrate that 5-FU and oxaliplatin inducing apoptosis in Colon adenocarcinoma cells. It is well documented that Caspase-III is the final executioner of cellular apoptotic pathway as their enzymatic activity increases with apoptosis, so increase in Caspase-III activity is the directly proportional to the increase in apoptosis [Su CC et al 2006]. The results shows that Caspase-III activity is increases by two folds when cells were treated with lacto-nano-5FU and Lacto-nano-oxalo as compared to soluble counterpart in Colo-205 cells (Fig 5.5a). At similar concentration, 5-FU and oxaliplatin loaded lactoferrin nanoparticle induces enhanced apoptosis into the human colon adenocarcinoma cells line as compared to their soluble counterpart. Thus nanoparticle increase the anti-proliferative activity of 5-FU and oxaliplatin in Colo-205 cells as it further corroborated with the results of MTT assay.

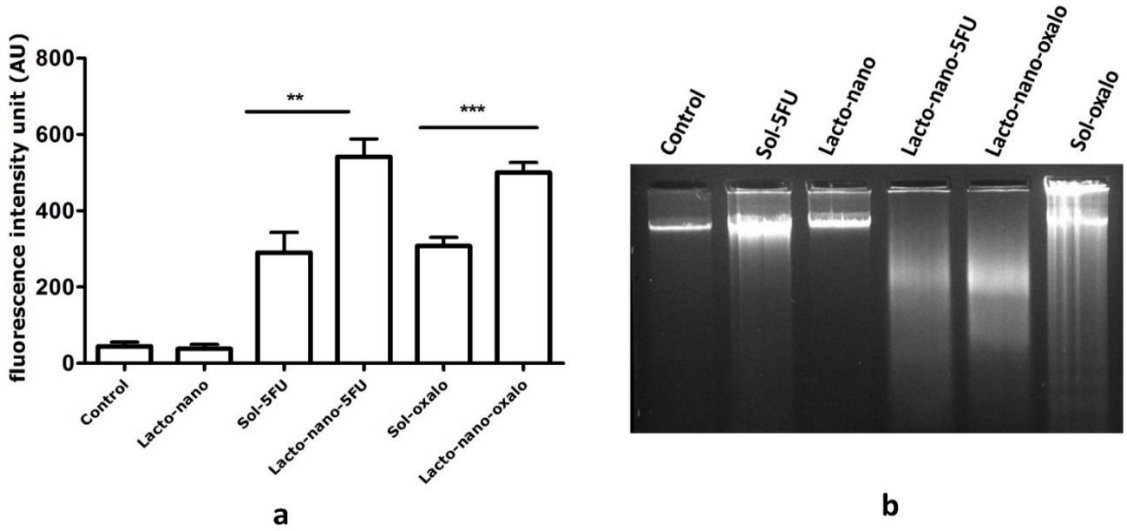


Figure 5-5: a) Caspase-III activation assay based on degradation of Caspase-III substrate upon activation, furnished products with fluorescence intensity that measured by fluorometry and data represented in fluorescence intensity unit (AU). Oxaliplatin and 5-FU loaded lactoferrin nanoparticles (lacto-nan-5FU and lacto-nano-oxalo) shows increased Caspase-III activation than free drugs (Sol-5FU and Sol-oxalo) data are represented as mean± SEM b) DNA fragmentation assay by simple agarose gel electrophoresis shows nano formulation causes serious DNA fragmentation, hence induced stronger apoptosis than free drugs .

Objective II. Study of pharmacokinetics, Tissue distribution, Efficacy and Safety of Oxaliplatin and 5-fluorouracil loaded lactoferrin nanoparticles administered via intra venous route in wistar rats

5.2.4 Comparative pharmacokinetics and tissue distribution of 5-FU and oxaliplatin loaded lactoferrin nanoparticle and free 5-FU or oxaliplatin via I.V. route of administration

Pharmacokinetics study of free 5-Fluouracil (Sol-5FU) and free oxaliplatin (Sol-oxalo) in comparison with 5-FU and Oxaliplatin loaded lactoferrin nanoparticle (lacto-nano-5FU and lacto-nano-oxalo) were carried out by administering single dose of mentioned formulation into Wistar rat through intra venous (IV bolus) route of administration. Results of this experiment shows that 5-FU and oxaliplatin loaded lactoferrin nanoparticles exhibit improved pharmacokinetics profile than their soluble counterpart. Concentration of 5-FU in rat plasma after single dose of free 5-FU (sol-5FU) shows maximum at first five minute of injection and later comes down and eliminate at 960 minute PI. Almost similar kind of drug distribution pattern shown in 5FU loaded nanoparticle (lacto-nano-5FU), even though, distribution area found to be quite large in comparison with free drug as shown in fig (5.6b). Other pharmacokinetic parameters of sol-5FU are found to be ($Cmax_{\mu g/ml}$=82±1.6, $AUC_{\mu g/dL/hr}$=4635±954 and $t1/2_{min}$=115±27.9) and of lacto-nano-5FU are ($Cmax_{\mu g/ml}$=66±7.9, $AUC_{\mu g/dL/hr}$=12892±1938 and $t1/2_{min}$=312±101) as given in table 5.2.

Almost similar pattern of distribution in plasma of free oxaliplatin (sol-oxalo) and loaded oxaliplatin (lacto-nano-oxalo) has been shown in figure (5.6a). Pharmacokinetic parameters as given in (table 5.2) of sol-oxalo ($Cmax_{\mu g/ml}$=95±4.5, $AUC_{\mu g/dL/hr}$=4386±833 and $t1/2_{min}$=100.4±19) and of lacto-nan-oxalo ($Cmax_{\mu g/ml}$=80±4, $AUC_{\mu g/dL/hr}$=12529±311 and $t1/2_{min}$=367±72) are shows that nanoparticle loaded drug have better distribution pattern as compare with free drug.

Distribution of drugs in different tissue were also calculated after single dose of IV injection. The concentration of Sol-5FU after 60 min of injection in liver found to be 19.7µg/gram of liver and

sol-oxalo after 60 min of injection found to be 21 μg/gram of liver (Fig. 5.7a & 5.8a) whereas, concentration of 5-FU and Oxaliplatin in nano formulation group shows higher localisation in liver with the value of 24.7 μg/gram in liver at 60 minute and 41 μg /gram in liver at 30min respectively (Fig 5.7a and 5.8a). The maximum concentration of free 5-FU and oxaliplatin in kidney are found to be 215 μg /gram of kidney and 185.3 μg /gram of kidney at 60 minute post injection (fig 5.7b and 5.8b) respectively, whereas, amount of same drugs with lactoferrin nanoparticle (lacto-nano-5FU and lacto-nano-oxalo) are found to be 102 μg /gram of kidney and 70 μg /gram of kidney (fig. 5.5.7b and 5.8b) respectively. The amount of drug estimated from the kidney in case of free drugs is way higher than their nano formulation counterpart. The amount of drugs localised in the colon after single dose via i.v. route of free 5-FU and oxaliplatin are found to be 6.1μg/gram of colon and 7μg/gram of colon (fig 5.7c and 5.8c) respectively whereas, in case of lactoferrin nanoparticle loaded with 5-FU and Oxaliplatin found to be 8.1 μg/gram of colon and 11.5 μg/gram of colon (fig 5.7c and 5.8c) respectively. Significantly higher amount of drugs shown to be localised in colon through nanoparticle loaded formulation as compare to free drugs, is very important to enhance the bioavailability of these drugs around the target organ.

Table 5.2: Plasma pharmacokinetic parameters of lactoferrin Nano formulated drugs and free drugs (Mean±SDM).

Formulations	AUC(µg/dL/hr)	C_{max} (µg/ml)	$T_{1/2}$ (minutes)	MRT (minutes)
Sol-oxalo	4386±833	95±4.5	100±19	97±17.4
Lacto-nano-oxalo	12529±311	80±4.1	367±72	363±17.6
Sol-5FU	4635±954	82±1.6	115±27	105±5.1
Lacto-nano-5FU	12892±1938	66±7.9	312±101	377±58

AUC-area under curve

C_{max}- maximum drug concentration attained in blood after single dose

$T_{1/2}$- elimination half-life of drug

MRT- mean retention time of drug in the blood

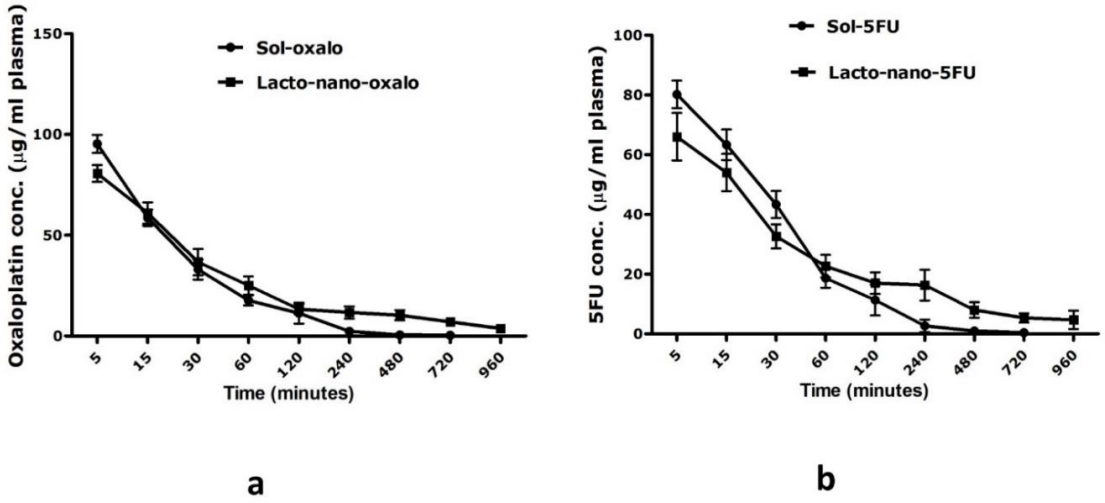


Figure 5-6: Distribution of drugs in Rat's plasma represented by concentration versus time graph a) concentration of oxaliplatin with Nano formulated (lacto-nano-oxalo) and free drug (Sol-oxalo) b) concentration of 5-fluorouracil with Nano formulated (lacto-nano-5FU) and free drug (Sol-5FU). Each data set represented as Mean±SEM.

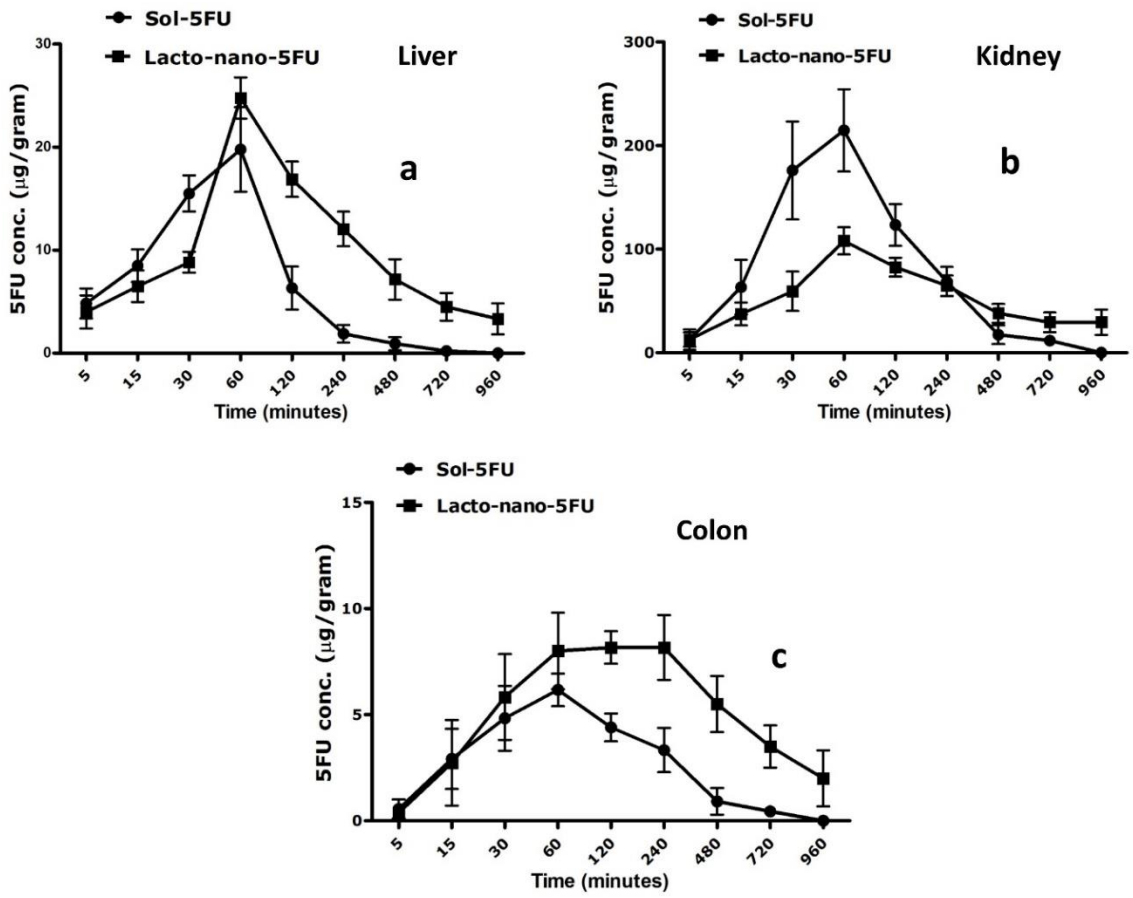


Figure 5-7: Tissue distribution analysis of free drug (sol-5FU) and loaded drug (lacto-nano-5FU) in different organs a) Livers, b) Kidney and c) Colon estimated by RP-HPLC method. All data points are in triplicate (n=3) and presented as Mean±SD).

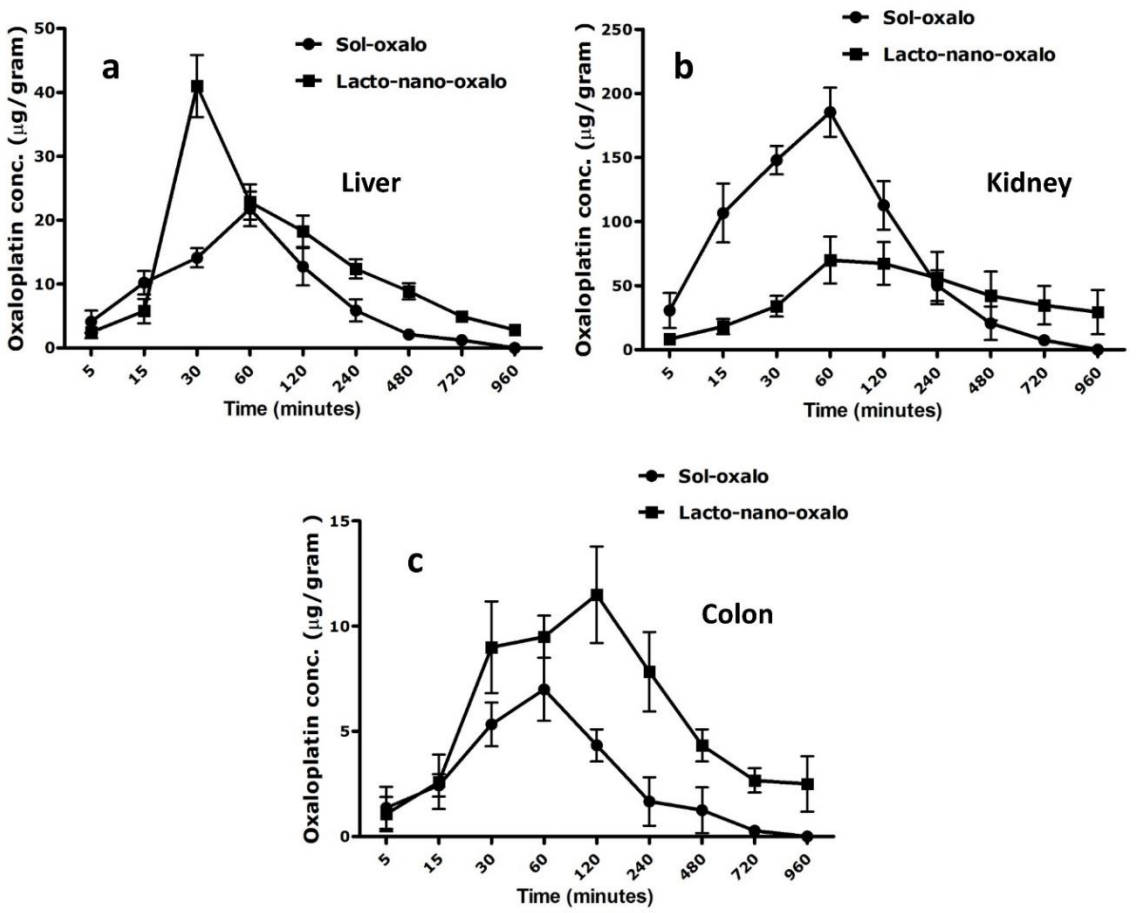


Figure 5-8: Tissue distribution analysis of free drug (sol-oxalo) and loaded drug (lacto-nano-oxalo) in different organs a) Livers, b) Kidney and c) Colon estimated by RP-HPLC method. All data points are in triplicate (n=3) and presented as Mean±SD).

5.2.5 Treatment of azoxy methane induced aberrant crypt foci (AFC) in Wistar rats by free 5-FU/Oxaliplatin and loaded into lactoferrin nanoparticle

Aberrant crypt foci (AFC) are known to be initial structural deformity, later leads to colon adenocarcinoma. So we have checked the comparative efficacy of lactoferrin nanoparticle formulation of 5-FU and oxaliplatin with free counterpart. This was performed by induction of AFC in Wistar rat through azoxy methane (AOM) as described in chapter II. Rats were given IV injection of mentioned formulation and efficacy of each formulation were scored in the terms of number of AFC per colon

and histopathological analysis. Body weight of rats were significantly reduced form starting point after one month treatment regime when treated with free 5-FU and oxaliplatin , while body weight were remain same or slightly increase from starting point when treated with nano formulation of same drugs as shown in (fig 5.9a). Reduction in the body weight is the initial evidence of toxicity of given formulations. After treatment with lactoferrin nano formulation number of ACF in colon found to be significantly reduced as ~7 fold as compare to positive control, whereas, free formulation of these drugs were able to reduce the number of ACF were found to be ~3 fold as given in (fig 5.9b). Colon histopathology result shows that number of abnormal crypts get reduced or becoming normal after the treatment by nano formulation as compare to positive control. Free formulation of 5-FU and oxaliplatin also reduced the number of abnormal crypt but not as efficient as nano formulation as given in fig 5.10. Abnormal crypts have some peculiar structural feature compared to normal crypt that can be easily scored by H&E section of colon.

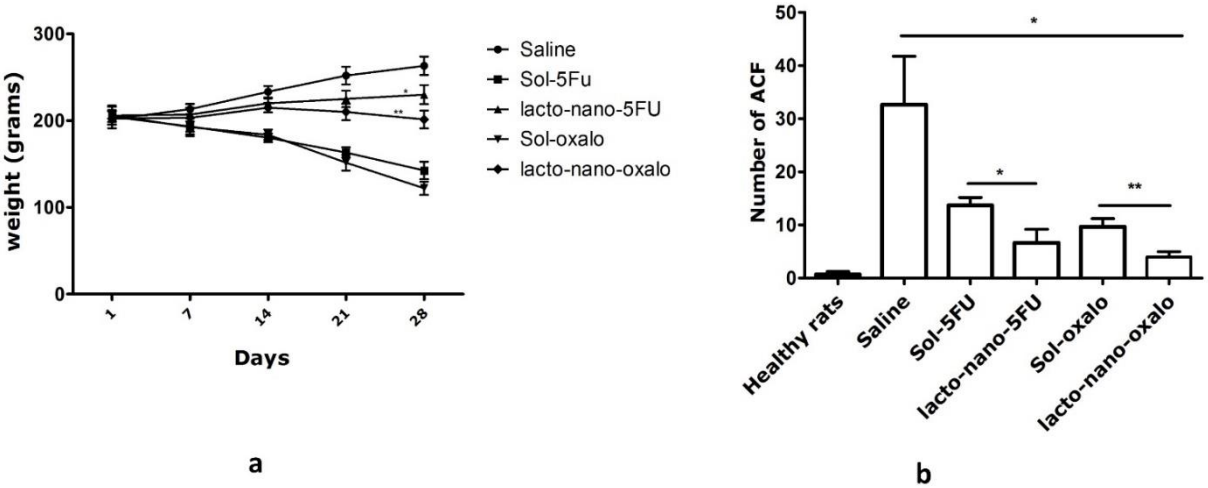


Figure 5-9: a) Weight of rats after one month treatment by lactoferrin nanoparticle formulation of drugs (lacto-nano-oxalo and lacto-nano-5FU) and free drugs (Sol-oxalo and sol-5FU) b) Number of aberrant crypt foci counted by methylene blue staining ,one month post treatment by above formulations. Each data time points are in triplicates and data represents in Mean±SE , value of significance *P <.05 and **p <.005 considered to be significant.

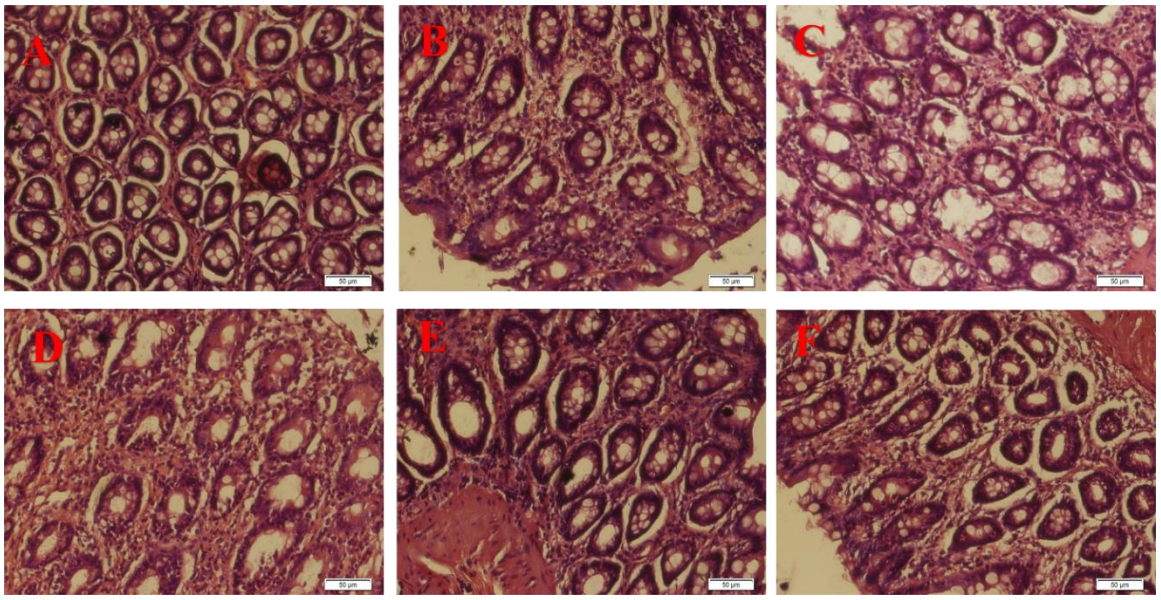


Figure 5-10: Colon histopathology analysis by H&E staining of colon section captured under bright field microscope with 20X magnification and bar size represent 20µm scanned area. Different treatment groups' shows various type of crypt in rat's colon started from A- Healthy rats, B- Sol-5FU treated group, C- lacto-nano-5FU treated group, D- saline treated groups(untreated), E- Sol-oxalo treated group and F- lacto-nano-oxalo treated groups.

5.2.6 Safety analysis

A major bottleneck of conventional 5-FU and Oxaliplatin therapy for colon adenocarcinoma are toxicity of liver, kidney and bone marrow that greatly hamper the efficacy of these drugs. So we have carried out enzymatic assay to check the hepatic/ renal toxicity and total blood cell count to evaluate the bone marrow suppression by these formulations. To observe the hepatic toxicity of drug formulation we have performed the enzymatic activity assay of SGOT and SGPT from serum. As data clearly indicates that enzymatic activity of SGOT were greatly enhanced up to 2-3 fold as compare to saline control in the free 5-FU and oxaliplatin treatment groups, whereas, lactoferrin nanoparticle formulation treated group shows similar level of enzymatic activity as saline group (fig 5.11a). Almost similar pattern of enzymatic activity in SGPT enzyme were shown (fig 5.11b). To evaluate renal toxicity we have estimated the level of creatinine and blood urea nitrogen (BUN) from serum of drug treated rats. As data clearly indicates that level of creatinine and BUN in serum enhance by 2-6 fold and 2 fold as compare to saline control respectively (fig 5.11c &5.11d) after treatment by free 5-FU and oxaliplatin, while there is no significant difference noticed in level of serum creatinine and BUN compared to saline control after the treatment of lactoferrin nanoparticle formulation of same drugs.

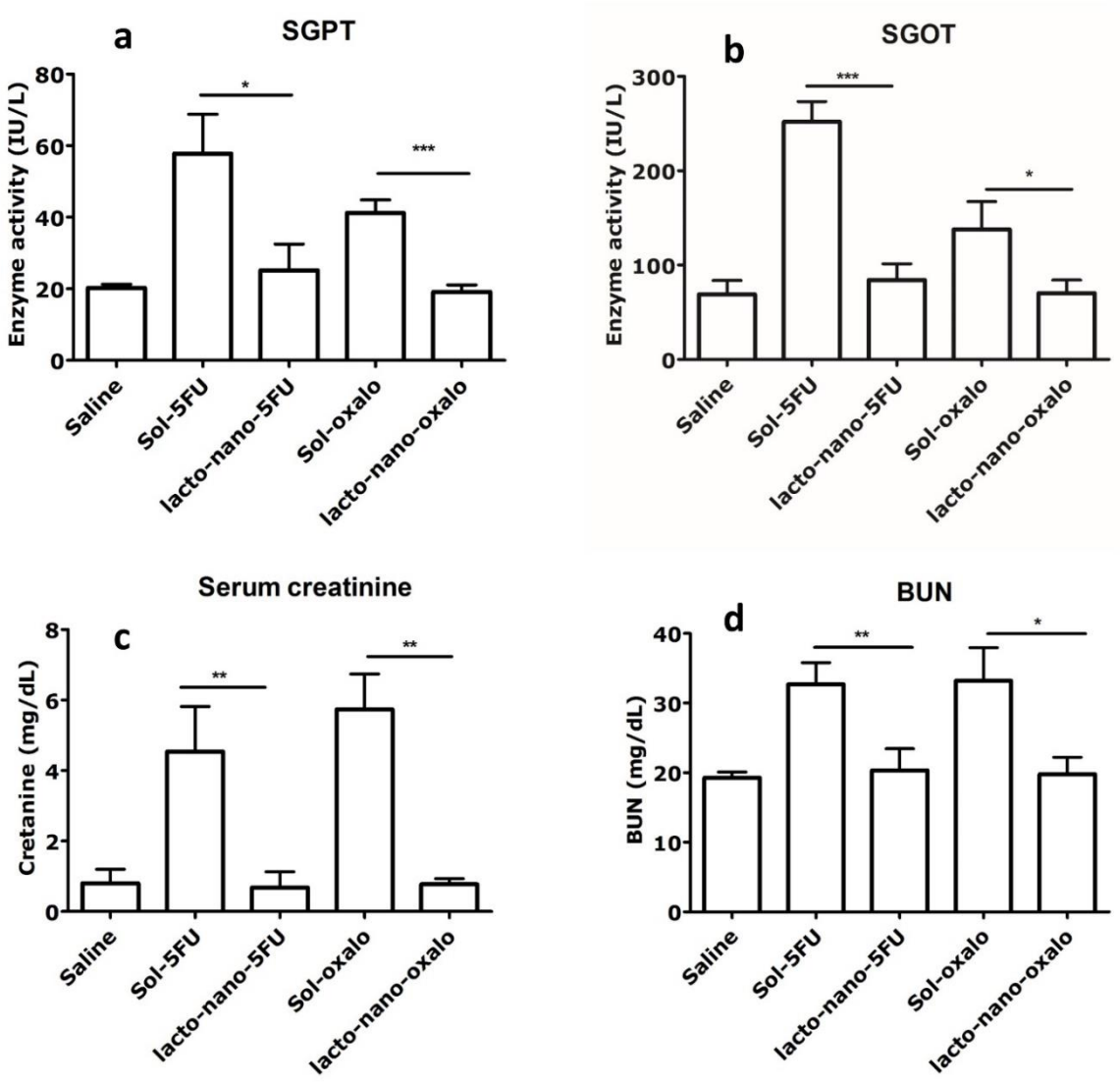


Figure 5-11: Hepatic and renal safety profile of rats after on month treatment by nano formulation and free drugs, a, b) hepatic function test by colorimetric assay of enzyme activity level of SGPT and SGOT shows that nanoformulation (lacto-nano-5FU and lacto-nano-oxalo) induces lesser activation of these hepatic enzyme as compare to free drugs (Sol-5FU and Sol-oxalo), c, d) Renal function test were carried out by of serum creatinine and blood urea nitrogen by colorimetric assay exhibited that nano formulation causes less accumulation of blood urea nitrogen and serum creatinine than free drugs. Data were analysed in triplicate and represented as Mean± SEM, value of significance *p <.05, **p <.005 and ***p < .0005 are considered to be significant.

Hematopoietic toxicity (bone marrow suppression) is the major concern associated with conventional therapy of cancer by these drugs, most importantly with oxaliplatin. To evaluate the extent of bone marrow suppression by these formulations, we have carried out total blood cells count, any reduction in sub population of blood cells gives the indication of possible bone marrow suppression. Out of different treatment groups, free oxaliplatin and 5-FU causes the suppression of almost every cells type in the blood started from WBCs, sub population of WBCs (neutrophils, lymphocytes), platelets, and RBCs as give in (fig 5.12 a, b, c, d and e). Free oxaliplatin shows potent bone marrow suppression activity than free 5-FU. Whereas, nanoparticle formulation does not reduce the number of different types of blood cells significantly as compare with saline control.

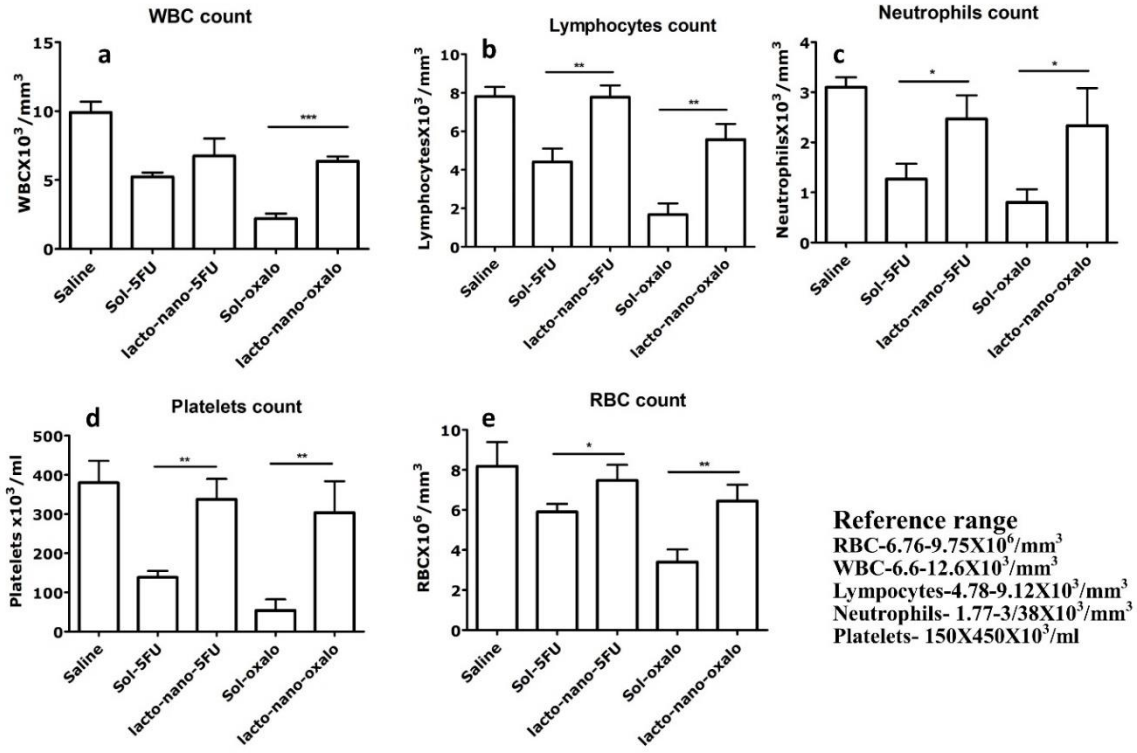


Figure 5-12: Hematopoietic toxicity of free drugs (Sol-5FU and Sol-oxalo) and lactoferrin Nano formulation of same drugs (lacto-nano-5FU and lacto-nano-oxalo) were examined by complete blood counts by haemocytometer and count of different population a) WBCs, b) lymphocytes, c) Neutrophils, d) Platelets and e) RBCs were plotted on treatment groups versus cells count. Each data point are in triplicate and presented in Mean±SEM, value of significance *p <.05, **p<.005 and ***p <.0005 considered significant.

5.3 Discussion

We have investigated comparative cellular uptake efficiency of 5-FU and oxaliplatin loaded lactoferrin nanoparticles versus free drugs in colon adenocarcinoma cells (COLO-205). Furthermore, we have also demonstrated the possible cellular uptake mechanism in same cells. In addition to this, their antiproliferative potential in colon adenocarcinoma cells as compared to free counterpart by different experiments have been also investigated. Based on in-vitro data, In-vivo experiments has been undertaken to find out tissue distribution, plasma pharmacokinetics and safety profile of free as well as loaded 5-FU and oxaliplatin in Wistar rats upon i.v. route of administration. To establish the efficacy of these formulation against the colon abnormality, aberrant crypt foci were induced by azoxy methane in Wistar rats followed by treatment using same formulation (free drugs versus loaded drugs).

Existing chemotherapeutic regime against colon adenocarcinoma is less effective [Meyerhardt JA et al 2005]. Antiproliferative drugs, including oxaliplatin and 5-FU, are often ineffective in separate doses or less effective in combinational therapy and impinge severe side effects due to rapid metabolism, short plasma half-life, resistant to tumor, poor biodistribution and target non-specificity, eventually leads poor patient compliance[Peters GJ et al 2002, Guichard SM et al 2005, Song M et al 2012]. To consider above inherent limitation of conventional chemotherapy, a number of effort has been taken to overcome these limitation. Amongst them nanoparticles are seems to be promising alternative for targeted drug delivery, to improving the cytotoxicity and specificity of chemotherapeutic agents, while keeping drug hostility minimum toward healthy tissue [Prados J et al 2013].

Cellular localisation of nanoparticle in COLO-205 cells was investigated by doxorubicin loaded rhodamine^{123}-tagged lactoferrin nanoparticle with comparison to soluble doxorubicin employing confocal microscopy. Rhodamine^{123}-tagged protein was used to study the intracellular delivery of protein nanoparticles loaded with fluorescence tracking agent (doxorubicin) is based on

modified protocol as described earlier [Rejinold N S et al 2011]. Doxorubicin loaded in lactoferrin nanoparticles localised gradually over a period of time and stay remains for a longer time in the cells as compared to free doxorubicin as illustrated in Fig.5.1. Similar pattern of results were observed in another set of experiments which rely on quantitative measurement of 5-FU (Fig. 5.2a) and oxaliplatin (Fig 5.2b) delivered into the COLO-205 cells by nanoparticle loaded with these drugs and free drugs. Free drugs (sol-5FU & sol-oxaliplatin) enter rapidly into the cells and eliminated shortly too as compare to nanoparticle which transport drugs slowly and stay for longer time. This type of phenomenon may be attributed with the fact that nanoparticle enters into the cells by receptor mediated endocytosis, whereas soluble drugs enters by passive diffusion. Various type of nanoparticles enters into cells by diverse processes are well documented by various groups [Rejinold NS et al 2011, del Pozo-Rodríguez et al 2008, and Mo, Y et al 2007, Ahmed F, et al 2014]. This type of delivery behaviour of nanoparticles are promising in treatment of cancer because drug resides for longer period of time and offer enough time to act on cancerous cells, such studies has been reported earlier [Tanmoy S. et al 2014, Yang H et al 2009]. As a result of these delivery behaviour, it may also mitigate the dose related toxicity against the healthy tissue.

Mechanism of cellular entry for different type of nanoparticles has been well reported in literature most specifically protein nanoparticles enters into cells through their respective receptors, over expressed in diseased situation [Saikrishna A.D.S. et al 2009, Ahmed F et al 2014, Golla K et al 2012, Golla K et al 2013]. Similar pattern of lactoferrin nanoparticles entry into COLO-205 cells were obtained by the blocking of lactoferrin receptor experiment. It is clearly indicates that majority of nanoparticles enter in COLO-205 cells through their native receptors (Fig 5.3a), and alternative pathway of nanoparticle entry cannot be rule out since size of nanoparticles are very small [Ahmed F et al 2014]. Lactoferrin receptors have been exploited for targeting of various cancers by different groups earlier [Huang R et al 2009, Kanwar JR et al 2012]. Therefore, range of liposome and polymeric

nanoparticles coupled with lactoferrin peptides have been developed for targeted drug delivery against specific target [Kaili Hu et al 2009, Feng-Yun J et al 2013 and Hui-le Gao et al 2010]. To further ascertain the delivery of nanoparticle into cells guided by endocytosis process, we have shown that nanoparticles are co-localised with lysosome (Fig. 5.3b) in COLO-205 cells, which is integral part of endocytosis. Since lactoferrin receptors are known to be over expressed in colon adenocarcinoma cells [Jiang R et al 2011], so whole protein nanoparticles of lactoferrin may prove potential for targeted drug delivery vehicle against colon cancers.

Oxaliplatin and 5-fluorouracil are indispensable element of chemotherapeutic modules for the treatment of colon adenocarcinoma, as clinical data are witness of efficacy of these drugs [Calvo E et al 2002]. Nevertheless, associated with serious side effects [Kazuhiko Hanada et al 2010, Thomas Ludwig et al 2004 and Wigmore PM et al 2010], so there are dire requirement for a drug delivery platform that can effectively overcome such side-effects as well as improves its therapeutic efficacy would be advantageous in colon adenocarcinoma treatment. So, we have investigated the comparative antiproliferative potential of nanoparticle loaded and free drugs at different concentration on COLO-205 cells. There were 2 fold improvement in IC_{50} value noticed in lacto-nano-5FU as compare to Sol-5FU (Fig 5.4a) and 2.5 fold improvement in lacto-nano-oxalo as compare to Sol-oxalo (Fig.5.4b). Time course experiments of these nanofromulations indicates that drug loaded nanoparticles release the content in sustained manner and thus increases the activity for longer time as compared to free counterpart (Fig.5.4c &d). Additionally, caspase-III and DNA laddering assay further corroborated the fact that oxaliplatin and 5-FU loaded lactoferrin nanoparticle shows improved antiproliferative activity as compare to free drugs (Fig 5.5 a & b). The phenomenon of improved antiproliferative activity of drug loaded nanoparticle as compared to free drugs may be endorsed by the fact that nanoparticles enhances the delivery and sustenance into the cells as well as overcomes the multi drug resistant of cancerous cells [Jabr-Milane L. S. et al 2008, Nazila K et al 2012].

Encouraging observation from in-vitro analysis of lactoferrin loaded 5-FU and oxaliplatin on colon adenocarcinoma cells guided us to evaluate the in-vivo observation of these formulations that includes pharmacokinetics, tissue distribution in order to establish their efficacy and safety. Data obtained from pharmacokinetics experiments shows that lactoferrin nanoparticles loaded 5-FU and oxaliplatin improved plasma pharmacokinetics profiles as compare to their free counterpart. Oxaliplatin plasma exposure were measured by area under curve (AUC) of the concentration-time curve is 3 fold higher in case of nanoformulation treated than free oxaliplatin (table 5.2), and similar result observed in case of nanoformulation of 5-FU and free 5-FU (table 5.2). Improved AUC value by nanoformulation of these drugs are indication of better and sustained distribution pattern in plasma and this data is in agreement with previous published report of nanoformulation of mentioned drugs [Zhao Y et al 2012, Udofot OC et al 2015].

As compared with sol-oxalo, lacto-nano-oxalo have an extended elimination half-life ($t_{1/2}$), greater mean retention time (MRT), and slightly lower peak concentration (C_{max}) (Table 5.2). Almost similar pharmacokinetics pattern reported in lacto-nano-5FU as compare to sol-5FU (table 5.2). Above observations clearly indicates that 5-FU and oxaliplatin loaded lactoferrin nanoparticles attributed sustained release, improved distribution and prolong half-life as compare to their soluble counterpart when injected via i.v. route of administration. Pervious research by various groups have been also demonstrated the slightly improved pharmacokinetics profile of nanoformulation of these drugs [Yong JIN et al 2005, Haitian Wei et al 2015, Su Li, et al 2008]. Tissue distribution analysis of lacto-nano-oxalo and lacto-nano-5FU as compare to sol-5FU and sol-oxalo shows that, concentration of these drugs are significantly high in liver and colon and less in kidney of rats as given in figure (Fig 5.7 &8 a, b, c ,& d). Concentration of these drugs in kidneys are comparatively lesser in Nano formulation groups, may attributed the slower renal clearance and subsequently enhances the elimination half-life and mean retention time (Yong JIN et al 2005). Furthermore, high concentration

of these drugs in colon by nano formulation gives us indication that it might be a potential drug delivery vehicle against colon diseases. Thus, lactoferrin nanoparticle enhances the pharmacokinetics parameters of 5-FU and oxaliplatin up to great extent through the inherent behaviour of nanoparticles to modulate the solubility and diffusivity of drugs, shielding drugs from premature inactivation during it coarse, reduce the rate of renal clearance and liver degradation of drugs, conjointly all above phenomenon may results into extended blood circulation and enriched tissue accumulation and therapeutic effects (Klaus D. Sattler ,Handbook of Nanophysics: Nanomedicine and Nanorobotics).

Owning to improved pharmacokinetics of these drugs by lactoferrin nanoformulation prompted us to evaluate the comparative efficacy of this formulation versus free drugs against aberrant crypt foci (ACF) induced by Azoxy methane (AOM) in rats. Aberrant crypt foci (ACF) an initial stage of colon adenocarcinoma can be identified from normal crypt by observing the following morphological characteristics: 1) the inflated and elevated crypts than normal mucosa and 2) enlarged pericryptal space and irregular lumens [Hang X. et al 2008]. Result after one month treatment regime clearly indicates that lactoferrin nano formulation of these drugs reduces significant amount of colon ACFs as compare to free drugs (Fig 5.9b), this property might be attributed by improved pharmacokinetic profile of nano formulation. Histopathology analysis of colon also established the same fact that lactoferrin nanoformulations are efficacious in treating the ACF as compare to soluble counterpart (Fig 5.10). Conjointly all above observation prompt us to believe that lactoferrin nanoparticle formulation shows promising efficacy to debilitate the initial stage of colon adenocarcinoma proliferation as compare to free counterpart of same drugs.

Chemotherapy of colon adenocarcinoma by these drugs are associated with severe dose limited side effect including hepatotoxicity, nephrotoxicity and haematopoietic toxicity that greatly limits the therapeutic potential of 5-FU and oxaliplatin [Kazuhiko Hanada et al 2010, Thomas Ludwig et al 2004 and Wigmore PM et al 2010]. In the present study, we have found promising results that

lactoferrin nanoformulation with 5-FU and Oxaliplatin are effectively assuaging the potential side effect of these drugs that observed after one month treatment regime. All parameter of hepatic and renal safety are reported to be in normal range in nanoformulation treated groups while free drug does show sign of toxicity (Fig 5.11 a,b,c,& d). From this result it is evident that nano formulation of these drugs does not induce any hepatic and renal toxicity while free drug as usual shows marked toxicity. Bone marrow suppression was also observed in free oxaliplatin and 5-FU treated groups whereas, nano formulations does not show any sign of bone marrow suppression (Fig 5.12 a,b,c,d,& e). Safety parameters of lactoferrin nanoparticle formulation comes under normal ranges that may prove to be effective to enhance the therapeutic index of 5-FU and oxaliplatin against colon adenocarcinoma.

5.4 Conclusion

In this current study, lactoferrin nanoparticles have shown improved and sustained localisation in colon adenocarcinoma cells (COLO-205) as compared to free dye (doxorubicin). Oxaliplatin and 5-FU loaded lactoferrin nanoparticle demonstrated improved cellular delivery and antiproliferative activity in COLO-205 cells as compared to free counterpart. Though, entry mechanism of lactoferrin nanoparticles in COLO-205 cells found to be receptor mediated endocytosis through their native receptors, however other mechanism cannot be ruled out since particles size are lesser than 100nm. In-vivo study in Wistar rats revealed that lactoferrin nanoparticle loaded with these drugs exhibited improved pharmacokinetics parameters, enhanced therapeutic efficacy against ACF and mitigated the systemic toxicity as compared to free form of counter part of same drugs. So, the current study might add extra weapon into chemotherapy arsenal to manage the colon adenocarcinoma by lactoferrin nanoparticle mediated specific delivery of oxaliplatin and 5-fluorouracil.

CHAPTER 6

Summary

6.1 Summary

Chemotherapy is indispensable regimen to manage various type of cancers, however associated with dose related systemic toxicity. Systemic toxicity greatly limits their therapeutic index that eventually lead to poor patient compliance. This limitation of chemotherapeutic module is basically outcome of poor aqueous solubility, lack of target specificity, short plasma half-life and rapid clearance of drugs. A number of effort has been put forth to overcome these limitation, amongst them nanoparticle based formulation that is rapidly evolving as the most potential drug delivery platform. Nanoparticles for drug delivery application are the containers with dimensions of nanometer, developed from range of material i.e. metal, lipids and different types of biodegradable polymers. Nano particulate system possess unique properties such as targeted drug delivery, sustained drug release, enhance aqueous solubility, protection from hepatic inactivation, improved bioavailability and decreases the systemic clearance conjointly improve the therapeutic index and reduce dose limiting toxicity.

Amongst biodegradable polymeric materials, proteins are the most important material because of their biodegradability and facile method of preparation. Our group has been developed a novel nano particulate platform from iron transporting protein (Apo transferrin and lactoferrin) that successfully demonstrated the efficacy of these module with different drugs in *in-vitro* and *in-vivo* system. These protein nanoparticles are based on the active targeting principal since receptors of these proteins are reported to be overexpressed on cancerous cells. So these receptor can be targeted for specific delivery of drugs through their whole ligand nanoparticles. The very uniqueness of this nanoparticle is that entire ligand nanoparticle acts as a targeting moiety without any surface modification, makes entire preparatory process facile.

Keeping above points in the view we have developed carboplatin loaded apotransferrin and lactoferrin nano particulate system to demonstrate the efficacy against the retinoblastoma cell line

(in-vitro study) and evaluated the ocular pharmacokinetics and safety in Wistar rats (an in-vivo study). Since retinoblastoma is the major health hazard in children and chemotherapy with carboplatin has meagre success rate in management, so we hypothesized that protein nanoparticles may enhance the therapeutic index of carboplatin when administered through peri ocular route. In parallel, we have also developed the lactoferrin nano formulation with oxaliplatin and 5-fluorouracil to debilitate the colon cancer. An in-vitro and an in-vivo study have been performed to evaluate the efficacy, pharmacokinetics, tissue distribution and safety parameters of these nano formulations. These drugs are often use to manage the colon adenocarcinoma, despite being their potential systemic side effects. So we hypothesised that efficacy and safety of these drugs can be greatly enhanced using targeted drug delivery module as lactoferrin nanoparticles.

Two major observations of this entire research are given below

- Carboplatin loaded apotranferrin and lactoferrin nanoparticles are found to be less than 100nm in size, with spherical shape and serrated surface. Drug loaded into the nanoparticle solely by physical interaction and it shows sustained drug release and inductive release between pH 5 to 6. Loaded carboplatin shows improved antiproliferative activity against retinoblastoma cells (Y79) as compare to soluble carboplatin and nanoparticle enter into cells through their native receptors. These nanoparticles are successfully localised into retinal sublayers as compared to free dye by crossing the scleral layer after peri ocular route of injection. Carboplatin loaded protein nano formulation further enhances the ocular pharmacokinetics profile when injected via peri ocular route as compare to free drugs. When compared to free drug, very less amount of carboplatin detected into the blood circulation reported in nano formulation group which is very important observation to enhance the bio availability and reduce the systemic toxicity. Nano formulation doesn't show any sign of ocular inflammation while free drug does induce inflammation by destroying healthy tissue.

Additionally free carboplatin induce myelosuppression, while nano formulation does not exhibit any sign of myelosuppression.

- Oxaliplatin and 5-fluorouracil loaded lactoferrin nanoparticles shows improved antiproliferative activity in colon adenocarcinoma cells (COLO-205) when compared to free counterpart. These nano formulations shows enhanced plasma pharmacokinetics of as compare to free drugs, this is attributed due to fact that protein nanoparticles release drug in sustained manner. Tissue distribution analysis revealed that nano formulated drugs found to be high in liver and colon as compare to free drugs while localisation in kidney is significantly lesser than free drugs hence nanoformulation may decrease the pace of renal clearance eventually enhance the circulation half-life. As compare to free drug, nano formulation found to be more efficacious against the mitigation of aberrant crypt foci in colon which in initial point of cancer development. After one month treatment regime nano formulation found to be safe against hepatic, renal toxicity and myelosuppression.

Conjointly nano formulation of these drugs enhances the pharmacokinetics, therapeutic efficacy and safety profile as compare to free counterpart of same drugs. Current study might act as a proof of principal for development of improved therapeutic module based on protein nanoparticles to manage the menace of retinoblastoma and colon adenocarcinoma.

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