CHAPTER 1

INTRODUCTION AND PLAN OF WORK
1.1. Introduction to neoplastic disorders

Cancer is a condition characterized by abnormal and uncontrolled growth of cells, leading to the formation of either benign or malignant tumors. It is a mutagenic, multistage process involving series of sub-cellular alterations, determining the effect of tumor progression on the host.

Neoplastic cells display two prime features viz. unlimited proliferation and metastasis. Cancer cells divide at a higher rate than the normal somatic cells, compete with them and place a strain on the nutrient supply and excretion of metabolic waste materials. Ultimately, all the normal cells are replaced by tumor cells and the tumor continues to grow until maximum size is attained. Normally, the balance between process of proliferation and cell death in human body is thought to be disrupted by DNA mutations that lead to cancer (Si-Shen and Chien, 2003). Metastasis on the other hand involves the invasion of host tissue by multicellular clumps of cells from the primary neoplastic site, followed by transport via circulation to distant organs (Liotta and Stevenson-Stetler, 1991).

![Fig. 1.1: Difference between normal and cancer cell division](image)

Cancer results from a series of molecular events that significantly alter the customary cellular properties of somatic cells. Hence, these cells differ from normal cells in many aspects. Most importantly, the restriction of cell division in normal cells is absent in cancerous cells (Fig. 1.1). In cancer cells, the nucleus completely occupies the cell volume, whereas in normal cells, nucleus fills only one fifth of the cell. Cancer cells do not produce and release mucus unlike normal cells. Mitosis is a continuous event in cancer cells, whereas this process is rarely seen in normal, somatic cells (Pardal et al, 2003).
1.2. Liver cancer and its current treatment options

1.2.1. Anatomy of the Liver

Liver is one of the largest, wedge shaped, glandular organ in the human body (Fig. 1.2). It lies on the right hand side of the abdomen, below the diaphragm. Liver comprises of three lobes viz. right, left and caudate lobe. The blood supply to the liver is via two sources i.e. hepatic artery and hepatic portal vein. These vessels are further divided into capillaries which carry blood to large number of hepatic cells. Liver performs many critical functions in humans like secretion of bile, synthesis of proteins, storage of vitamins, metabolism of food, drugs, toxic materials and regulation of blood glucose (Ramadori et al, 2008).

![Fig. 1.2: Anatomical divisions of the liver](image)

1.2.2. Liver Cancer

Liver cancer is one of the most widespread forms of cancer and the major reason for tumor related mortality in Asia (Tsukuma et al, 2005). It ranks fifth with respect to incidences of cancer in the male population and seventh in females. Globally, more than 6,00,000 cases of death per year are associated with hepatic cancer. Although, liver cancer is observed more commonly in the developing countries, its instances are increasing at an alarming rate throughout the world (El Serag, 2011).

The prognosis of liver cancer is based on the stage of tumor growth at the time of treatment and concurrent hepatic function. Median survival time for hepatic cancer patients ranges from 4 to 21 months for patients not receiving any treatment and around 3 to 6 months after the beginning of symptoms (Veenok, 2000).
1.2.3. Types of Liver Cancer

1.2.3.a. Primary liver cancers - Numerous subtypes like hemangiomas (cluster of abnormal blood vessels forming a swelling), adenomas (clumps of hepatic tissues), cholangiocarcinoma (cancer of liver bile ducts), hepatocellular carcinoma (cancer of hepatic parenchymal cells), carcinoids (tumor of hormone producing cells in liver) and lymphomas (tumors of immune cells in the liver) of primary liver cancer have been reported (Stagg, 1992). Hepatocellular carcinoma comprises of around 95% of the primary cancers (Fig. 1.3) followed by cholangiocarcinoma (3.4%) and other cancers (1%). Hence, most of the antineoplastic treatments in patients are directed towards hepatocellular carcinoma (Imawari, 2002).

1.2.3.b. Secondary liver cancers - Liver is found to be the second site for metastasis of tumors after regional lymph nodes. Metastatic tumors are seen in those patients who have primary cancer originating in organs drained by the portal vein like tumors of stomach, small intestine, pancreas, colon and gall bladder.

Fig. 1.3: Hepatocellular carcinoma and its histology

1.2.4. Liver cancer statistics

Each year, the American cancer society (ACS) and National cancer institute (NCI) publish data on new cases of cancer along with the number of cancer related mortalities. Cancer is the second most common disease worldwide, after cardiovascular disorders. In the year 2014, approximately 33,190 cases of liver cancer are expected to occur in the U.S. with more than 80% of these cases constituting of hepatocellular carcinoma (HCC). About 23,000 patients are expected to die from HCC with the mortality rates increasing by 3.7% in men to 1.4% in women (Cancer Facts and Figures, 2014).
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In India, around 4 million new cases of cancer are reported each year with the incidence expected to grow five folds by the year 2025. Lung, liver, colon and breast cancers have lead to maximum mortality in Indian patients. Incidences of hepatic cancer are also growing in Asian American population due to the higher prevalence of Hepatitis B and C infections, in addition to alcohol consumption (Siegel et al, 2012). The cancer trouble in India is thought to increase still further in the future.

1.2.5. Etiology of liver cancer

Multiple factors increase the risk of liver cancer in patients. In general, males are at a higher risk of developing liver cancer than females with a ratio of 3:1. This gender difference is found to be prominent in areas of high cancer occurrence than low incidence areas (Chuang et al, 2009).

1.2.5.a. Alcohol consumption- The prime cause of hepatic cancer is due to excess drinking of alcohol (Boffetta and Hashibe, 2006). A dose response relationship has been observed between alcohol consumption and hepatic tumors with increasing relative risks in correlation with higher alcohol intake per day (Corrao et al, 2004). Alcohol is known to cause cancer by inflammation and scarring of tissues, liver cirrhosis and consequent liver damage.

1.2.5.b. Hepatitis B and C infections- Patients who are suffering from chronic hepatitis B (HBV) and hepatitis C (HCV) infections are likely to develop liver cancer at a later stage. HBV infections have contributed to more than 80% of hepatocellular carcinoma cases worldwide (Yu et al, 2000). HBV and HCV occur via blood transfusions and use of contaminated intravenous needles and blood products.

1.2.5.c. Alfatoxin- Alfatoxin is a carcinogen from the fungus Aspergillus flavus in the tropical region. Contamination of food products viz. peanuts, vegetable and cereals with this chemical can occur upon storage in hot and humid environment.

1.2.5.d. Other carcinogenic chemicals- Thorium dioxide, used for examination of X-rays and Vinyl chloride, employed in plastic manufacturing are the major causes of angiosarcoma (London and McGlynn, 1996). Mestranol, a component of oral contraceptives and anabolic steroids are the prime reasons for liver cancers in women.

1.2.5.e. Diabetes and Obesity- Obesity is the cause of majority of cancers, apart from hepatic tumors. Obesity and diabetes can cause liver tumors due to fatty liver or non-alcoholic
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steatohepatitis. However, the cause of liver cancer due to these factors is difficult to establish unless cirrhotic liver is seen in the patients.

1.2.6. Symptoms of liver cancer

The most frequent symptoms of liver cancer are pain and inflammation in the abdominal region, weight loss, swelling of stomach, loss of appetite, cirrhosis in the liver, intestinal bleeding, fatigue due to ammonia buildup in the body, jaundice, fever, nausea and vomiting.

1.2.7. Diagnosis of liver cancer

1.2.7.a. Physical examination- Liver, abdomen, spleen and adjacent organs are inspected for changes in size, shape, formation of lumps and skin of the patient is examined for jaundice.

1.2.7.b. Blood tests- Blood sample from the patients is checked for levels of alkaline phosphatase, AST and ALT, since they are expected to rise due to underlying liver cancer. Alpha fetoprotein is a useful marker for diagnosis of liver cancer and is found to be elevated in approximately 60% of the cancer patients (Colleoni et al, 1998).

1.2.7.c. Imaging of the liver- Number, mass and location of tumors are identified by the use of magnetic resonance imaging, computed tomography scan, ultrasound and percutaneous transhepatic cholangiography.

1.2.7.d. Biopsy- A small tissue sample is removed and examined microscopically for the presence of abnormal, cancerous cells using fine needle aspiration method or laproscopy.

1.2.8. Staging of Liver cancer

Therapy of liver cancer is dependent on the stage of development of cancer in the body, size of the tumor, invasion to adjacent organs and its spread to lymph nodes. Four stages for hepatic cancer (Stage I to Stage IV) have been stated by American Joint Commission on Cancer (AJCC) (Table 1.1).

1.2.9. Treatment options for liver cancer

Liver cancer is difficult to treat with current therapies and the efficacy of these treatments is unconvincing in liver cancer (Varela et al, 2003; Adam, 2004).

1.2.9.a. Surgery- Surgery remains the prime therapy for initial stages of liver cancer. The main aim of surgical resection is to remove the neoplastic segments with preservation of liver
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parenchyma to the maximum, to avoid liver failure. Overall only 20% of the hepatic cancers are suitable for surgical treatment (Kooby and Jarnagin, 2004).

Table 1.1: AJCC staging for liver cancer

<table>
<thead>
<tr>
<th>Stage</th>
<th>Particulars like size and number of tumors, lymph node involvement and metastasis</th>
<th>Treatment options</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1, N0, M0, Single, &lt; 2 cm, no VI</td>
<td>Localized and resectable</td>
</tr>
<tr>
<td>II</td>
<td>T2, N0, M0, &lt; 2 cm with VI, &gt; 2 cm with no VI, &lt; 2 cm with unilobar/multiple with no VI</td>
<td>Localized and resectable</td>
</tr>
<tr>
<td>III</td>
<td>T1-T3, N+, M0, &gt; 2 cm with VI, &gt; 2 cm unilobar/multiple</td>
<td>No benefit by surgery, multiple treatment modalities required for maximum benefit</td>
</tr>
<tr>
<td>IV</td>
<td>T4 or M+, Bilobar/multiple or major VI or adjacent organ invasion</td>
<td>No benefit by surgery, multiple treatment modalities required, chemotherapy is the best option</td>
</tr>
</tbody>
</table>

T- Number of tumors
N- Involvement of lymph nodes  N0- Absence of lymph node involvement  N+- Lymph node is involved
M- Metastasis  M0- Absence of metastasis  M+- Presence of metastasis  VI- Vascular invasion

1.2.9.b. Liver transplantation- Liver transplantation is utilized mainly for cancers restricted to the liver when partial hepatectomy cannot be employed due to rapid spread of tumor or altered liver function in cirrhotic subjects (Venook et al, 1995). Liver transplantation has shown to prove effective post chemotherapy in patients due to removal of microscopic metastatic areas in the liver.

1.2.9.c. Percutaneous ethanol injection- Percutaneous ethanol injection is a localized therapy which has been applied to tumors less than 3 cm in diameter with volumes ranging from 5 ml to 99 ml. Injection of alcohol in the tumor region is known to cause degeneration of proteins combined along with thrombosis, leading to drying of tumor cells and cell death (Shina et al, 1987), with overall survival rate of 60% in patients.
1.2.9.d. **Radiofrequency ablation (RFA)**- In this procedure, radiofrequency energy is supplied via a metal probe which leads to generation of heat up to 100°C, resulting in vibration and friction in the tumor cells, ultimately leading to their destruction. A diagrammatic representation of RFA is as shown in Fig. 1.4.

![Fig. 1.4: Schematic representation of radiofrequency ablation](image)

1.2.9.e. **Cryoablation**- Cryoablation is a technique where extremely low temperatures are used for destruction of tumors with the help of a cryogen like liquid nitrogen (Fig. 1.5). This helps in destruction of cells by affecting their vascular structure and alteration in physicochemical properties.

![Fig. 1.5: Schematic representation of cryoablation](image)

1.2.9.f. **Radiation therapy**- Radiation therapy is of minor importance in liver cancer treatment due to destruction of normal cells and thus the non-specificity to cancer cells (Chamberlain et al, 1983). In this method, radioactive radium, cesium, iridium, yttrium, and palladium are implanted into or adjacent to the cancerous mass. Clinical trials for yttrium-90 were a success due to its pure β emission, short half-life and greater penetration (Yu et al, 2003).

1.2.9.g. **Systemic chemotherapy**- Hepatic cancer is quite resistant to conventional chemotherapeutic agents. Neither single agent nor combination chemotherapy have resulted in
significant effect on median survival of patients and have exhibited less than 25% survival rates. Doxorubicin has exhibited a median survival of four months and an overall response rate of 15% in liver cancer (Ikai et al, 2004). The best response in hepatic cancer was observed with a combination of Doxorubicin, Cisplatin, Alpha-interferon and 5-Fluorouracil (Leung et al, 1999).

1.2.9.h. Intra-arterial chemotherapy- Intra arterial therapy is popular due to the direct delivery of chemotherapeutic drugs to liver parenchyma via the hepatic artery, which minimizes the systemic side effects. Response rates of upto 40% have been seen with Fluorouracil and anthracyclines after intra arterial administration (Doci et al, 1988). Similarly, combination therapy of Doxorubicin, Floxuridine, Cisplatin and Leucovorin has lead to 64% response rate (Patt et al, 1994; Rajakumari, 2007).

1.2.9.i. Chemoembolization- Chemoembolization is a variant of the intra arterial therapy wherein an embolizing agent is inserted into the hepatic artery to block the blood supply to tumor after treatment with anticancer drugs. Thus a synergistic response could be obtained in chemoembolization by employing both chemotherapy and embolization (Chen et al, 2004).

1.2.9.j. Hepatic artery ligation- Surgical ligation of the hepatic artery has been carried out in number of patients with benign liver tumors in order to produce tumor necrosis and cell death. However, the median survival in patients was not improved with this treatment.

1.2.9.k. Hormonal therapy- Primary liver cancer is known to express receptors for hormones like estrogen, androgen and somatostatin. In a particular randomized clinical trial, an analogue of somatostatin was found to extend the survival of hepatic cancer patients (Kouroumalis et al, 1998).

1.2.10. Need for newer treatment options for liver cancer

Liver resection is the best suitable treatment for non-cirrhotic liver cancer subjects. The chances of resection and post-operative survival are better in non-cirrhotic patients than those suffering from cirrhosis by this treatment. However, this therapy holds limitations like suitability for small tumors (Qian et al, 2003). Liver transplantation proves to be a boon for patients with small tumor size (<5 cm), but suffers from restricted amount of donors and delay due to the donor non-availability (Franco and Usatoff, 2001).
Chemotherapy although widely used to treat tumors, results in more side effects and toxicity due to the destruction of normal, non-malignant cells. Drug delivery to the tumors occurs through endothelial junctions, fenestrations and vesicular organelles. Factors like P-glycoprotein mediated drug efflux and changes in enzyme activity add to failure via chemotherapy. Pharmacokinetics is an important parameter to be considered during chemotherapy. Ideally, cancerous cells should be exposed to suitable concentrations of drug over an extended time period. Current chemotherapy treatments are given over a short period of time with time intervals of 2-3 weeks. This results in speedy growth of tumors and thus diminishes the therapeutic effects of drugs with untoward side effects. Also, it was seen that prolonged drug exposure to tumors produced better results rather than short term exposure to elevated drug concentrations.

Percutaneous ethanol injection is an excellent option for small tumors; however it cannot be used in case of patients with ascites. Radiofrequency ablation has a curative effect on liver cancer because of lesser sessions required for therapy, but possesses more complications than percutaneous ethanol injection. Therapeutic effects with other treatments prove to be inadequate due to metastasis of tumors, lack of suitable embolizing materials, non-specificity to tumor cells and greater number of complications (Durand and Belghiti, 2002; Achenbach et al, 2002; Sturm et al, 2001). Inspite of various therapies available for hepatocellular carcinoma, none have sufficed in complete and side effect free treatment of hepatic cancer. In this respect, many other alternative treatments have been explored for treatment of liver cancer.

1.3. Introduction to Nanoparticles

1.3.1. Definition of Nanoparticles

Nanoparticles are minute, solid particulates in the size range of 10-1000 nm where, an anticancer agent is either entrapped in the core, adsorbed on the surface or both (Mohanraj and Chen, 2006). Nanospheres or nanocapsules could be prepared by varying the methodology for synthesis of these colloidal carriers.

1.3.2. Nanoparticles: new era in the treatment of cancer

The aim of any cancer treatment is to ensure optimum drug delivery to the desired cancerous site. In this context, nanotechnology offers immense scope to deliver the anticancer agent specifically to the cancerous site, with higher concentrations being achieved in the tumor region.
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The potential of using nanoparticles for delivery of anticancer drugs is massive and can be applied to various areas of medicine.

Nanoparticles due to their colloidal nature, offer numerous advantages over conventional treatment strategies. Small sized particles possess large surface area and hence increase the dissolution properties of antineoplastic agents with poor solubility. Nanoparticles can be tailored to reach specific tumor sites by modulation of their size, surface characteristics, and particle charge (Keyser et al, 2005). Delivery of anticancer agents in the form of nanoparticles also ensures reduction in their therapeutic dosage and frequency of administration due to the controlled release properties of the nanosystems. Additionally, nanoparticulate drug delivery systems reduce the untoward toxicities associated with antitumor agents and prevent their degradation. Site specific targeting is possible with nanoparticles after attachment of certain ligands, antibodies or carriers on the surface by physical adsorption or covalent attachment (Bamrungsap et al, 2012).

Higher drug loading can be achieved in case of nanocarriers, which is prime factor for any particulate drug delivery system. Nanoparticles possess the ability to increase the absorption of anticancer agents from the body upon administration, thus causing a concomitant rise in their bioavailability. These particles can act at cellular levels and can be endocytosed / phagocytosed by cells, with resulting cell internalization of the encapsulated drug. Also, the small size of colloidal carriers makes them suitable for parenteral delivery of drugs. Irritation potential at the site of injection can be minimized with nanocarriers as compared to conventional therapy (Nesalin and Smith, 2011). Nanoparticles are biocompatible and non-immunogenic and can entrap both hydrophilic and hydrophobic drugs. These particles can be delivered by various routes of administration like nasal, oral, dermal and ocular, apart from parenteral route.

There are numerous barriers encountered by anticancer drugs after administration into the body. Delivery of the drug moiety to the interstitium is dependent on a number of physiological factors like physicochemical properties of the drug (size, shape, surface properties and hydrophobicity) and properties of the interstitium itself (structure, charge and composition). Hence, in order to ensure drug delivery to the desired site of action, following problems have to be solved:

(i) Overcome the drug resistance at the tumor level due to physiological barriers,

(ii) Prevent the resistance to the drug moiety at the cellular level,
(iii) Prevent distribution, metabolism and elimination of drug from the body.

One of the strategies to overcome these barriers is to tag the drug with colloidal nanoparticles in order to evade the cellular resistance mechanisms and deliver the drug at tumor location (Nie, 2010). In this perspective, nanoparticles are being increasingly explored for drug therapy.

1.3.3. Drug targeting

Drug targeting is associated with the transport of drugs to receptors, organs or any other specific parts of the body with minimum distribution to non-target tissues. Desired differential distribution of the drug would spare rest of the body and significantly reduce the overall toxicity; while retaining its therapeutic effect. Targeted drug delivery is one of the most potential ways to improve therapeutic index of drugs. Targeting of nanocarriers to tumor occurs majorly via two processes viz. passive and active targeting.

1.3.3.a. Reasons for requirement of targeted drug delivery systems

Target oriented drug delivery systems can provide maximum therapeutic benefit through controlled and predetermined release rate kinetics, prevent drug degradation or inactivation during transit to target sites and prevent the body from adverse reactions. For drugs that possess low therapeutic index, these systems can provide effective treatment at low concentrations (Sachdeva, 1998).

1.3.3.b. Passive targeting of nanoparticles to tumors

Passive targeting is associated with deposition of the drug at the tumor site due to certain anatomical and physiological factors (Garnett, 2001). Nanoparticles move through the leaky capillaries of tumor cells by passive diffusion or convective transport (Haley and Frenkel, 2008). It has been observed that convection process is weak in cancerous cells and as a result diffusion constitutes the major path for transport of drugs in tumor (Danier et al, 2010). Nanoparticles and other molecules gather at tumor site due to the distinct pathophysiology of tumor vessels. Growing cancerous cells are in continuous demand of nutrients and oxygen resulting in formation of new vessels or redirection of the existing vessels to the tumor region (Carmeliet and Jain, 2000). However, the resultant instability in growth factors and matrix metalloproteinases causes development of multiple gaps and pores in the tumor vessels and improper lymphatic drainage. This phenomenon of ‘Enhanced permeability and retention effect’
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i.e. EPR effect, causes retention of nanoparticles within tumor interstitium. Nanoparticles with
drug carriers once inside the tumor are not removed with ease and thus remain within cancerous
tissues. Endothelial junctions within tumors allow passage of nanoparticles up to 400 nm in size
(Maeda, 2001).

Several other factors which are associated with the drug accumulation by EPR process include
surface properties of the nanoparticles and degree of tumor vascularization. Particles taken up via
the EPR mechanism are degraded within the lysosomes and are ultimately eliminated from the
body. Later, the drug released from nanoparticles acts either at the local sites or after diffusion
outside the cellular organelles (Grislain et al, 1983).

Drug loaded nanoparticles are transported primarily to three organs of mononuclear phagocytic
system viz. liver, spleen and bone marrow. Hence, the ability of nanoparticles to increase the
efficacy of anticancer agents is restricted to tumors at these organs. For example, Kupffer cells of
the liver are thought to be involved in the phenomenon of passive targeting of macromolecules.
This strategy can be employed in some situations for treatment of diseases like listeria,
candidiasis and leishmaniasis (Daemen et al, 1995). This strategy has also been employed for
various anti-infective agents like antivirals and antibiotics.

However, nanoparticles could be cleared by the phagocytes and this strategy of delivering
anticancer agents could be of limited application in case of hepatocellular carcinoma which
originates in the parenchymal cells of the liver. Generally, Kupffer cells of the liver form an
unwanted location for the nanoparticulate transport. Additionally, passive delivery of
nanoparticles could lead them being accumulated in all the RES organs. Hence, alternative
techniques have been adapted for delivery of active agents, particularly to hepatocellular
carcinoma.

1.3.4.c. Active tumor targeting by nanoparticles

Active targeting involves coupling of ligands or other specific molecules/components to
nanoparticles resulting in selective attachment of these carriers to the desired receptor at the site
of action (Vasir et al, 2005). This type of targeting reduces the side effects of drugs and
maximizes the therapeutic outcome. An optimum targeted nanoparticulate system would reduce
the toxicities related to the antineoplastic agent, thus allowing the utilization of lower doses of
the same for treatment. The final beneficial effects could be achieved by selective recognition
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techniques like antigen-antibody identification and ligand-receptor attachment. Additionally, the pharmacokinetic properties of the free drug should be same irrespective of employed carrier system. A drug may follow definite anatomical and physiological pathways to reach its target site. Various methods have been utilized for active targeting including non-covalent and covalent bonding on the surface of nanoparticles (Nobs et al, 2004). A comparison of passive and active targeting is presented in Fig. 1.6.

Since hepatocellular carcinoma occurs on parenchymal cells of the liver, nanoparticles would be apparently targeted to receptors present on these cells. It is seen that the liver cells possess gaps/channels though which the nanoparticles may reach the hepatocytes. Also, the nanoparticle surface needs to be modified in order to evade the Kupffer cells and deliver the nanocarriers to the desired location at parenchyma.

![Fig. 1.6: Passive targeting verses active nanoparticle targeting](image)

1.3.4.d. Asialoglycoprotein receptors (ASGPR) for hepatoma targeting

Asialoglycoprotein receptors, located on the surface of liver parenchymal cells have been widely explored for active targeting of drugs to hepatic tumors (Li et al, 2008). It is a glycoprotein oligomer with molecular mass of 41 KDa and comprised of two subunits $H_1$ and $H_2$. The unique advantage of this receptor includes internalization of molecules/drugs into the intracellular compartments by receptor mediated endocytosis (Fig. 1.7), thus providing an opportunity for specific delivery to cancer region (Stockert, 1995). This receptor is involved in the elimination of lipoproteins and glycoproteins with sugar residues. These receptor sites have the ability of
identifying several carbohydrates including glucose, galactose, mannose, pullulan and other N-acetyl-galactosamine moieties. The density of sugars or sugar linked moieties at the receptor surface influences the affinity of these moieties for ASGPR receptor (Spiess, 1990).

After administration of the nanoparticles, the carbohydrate recognition domain (CRD) on ASGPR receptors binds to the specific carbohydrate residues in nanocarriers. Endocytosis leads to release of the ligands into endosomes and after segregation of ligand from the receptor, receptor molecules recycle back to the plasma membrane.

In the past, numerous strategies have been attempted for liver tumor delivery by formulating either covalent drug-sugar conjugates or physical adsorption techniques which have resulted in improvement in targeting efficacy to the liver cancer cells (Hashida et al, 1997). Other popular ligands for liver selectivity include sweetening agents like glycerrhetinic acid and glycyrrhizin, obtained from liquorice root. Studies have shown that these molecules are uptaken by receptors present on the surface of hepatocytes in rats (Negishi et al, 1991).

![Image of nanoparticle uptake by ASGPR receptors]

**Fig. 1.7: Uptake of nanoparticles by asialoglycoprotein receptors**

1.3.5. Types of nanosystems used in anticancer drug delivery

Different types of polymeric, lipid and inorganic nanoparticle systems and strategies have been developed for the delivery of drug moieties to tumors, based on the fact that they are able to modify the systemic distribution of antineoplastic drugs.

1.3.5.a. Liposomes

Liposomes are constituted of vesicles fabricated from non-toxic, amphiphilic phospholipids and cholesterol, which have been widely utilized for encapsulation of lipophilic drugs in lipid bilayer and hydrophilic drug molecules in inner compartment (Jiang et al, 2007). They are versatile,
efficient and most extensively studied class of carrier systems. Primarily, lipid materials used in the preparation of liposomes are FDA approved natural or synthetic phospholipids like lecithin, phosphatidylserine, cephalin etc. After intravenous administration, liposomes are rapidly removed from the blood stream by RES and Kupffer cells of the liver and hence have been utilized in the treatment of liver and RES organ diseases. They have been also employed for delivery of immunomodulating agents to macrophages, targeting to primary tumors and sites of infection.

Liposomal formulations have also shown promising results in preclinical developmental stages. These include liposomes of 5-Fluorouracil analogue (Doi et al, 1994), Bleomycin, Vincristine, Paclitaxel and Mitoxantrone. Combination of Cisplatin with Vanlomycin liposomes has also exhibited enhanced efficacy after animal testing (Daoud, 1994). Numbers of anticancer liposomal products are available in the market and many more are expected to reach clinical developmental stages (Allen and Cullis, 2013). Doxil®, Myocet®, Daunoxome® are the most successful examples of parenteral liposomes for Kaposi’s sarcoma and Breast cancer. Other promising formulations in clinical development include Thermodox (hepatocellular carcinoma), SPI-077 (solid tumors) and Lipoplatin (non-small cell lung cancer).

1.3.5.b. Niosomes

Niosomes are made using non-ionic surfactants and are more rigid and stable than the former due to the materials used in their fabrication process. It has been reported that the encapsulation of Methotrexate and doxorubicin (Rogerson et al, 1988) into niosomes increased the tumor accumulation with greater tumoricidal activity. Similarly, doxorubicin-polymer conjugates entrapped in niosomes have provided a depot delivery to the liver. The free drug was gradually cleaved off and the drug concentration in the liver was found to increase over a period of 24 hours, thus proving effective in hepatic neoplasm therapy (Uchegbu et al, 1995). Although niosomes are advantageous over liposomes, they suffer from particle aggregation, leaking of encapsulated drug and hydrolysis of drug moiety leading to limited shelf life of the dispersions (Pola Chandu et al, 2012).

1.3.5.c. Solid lipid nanoparticles

Solid lipid nanoparticles constitute colloidal drug delivery carriers which are fabricated from lipid materials and generally designed with an aim to improve safety and bioavailability of
poorly water soluble drugs (Muller et al, 1995). These systems exhibit numerous advantages like controlled release of active moieties, excellent biocompatibility, enhancement in tissue distribution and site specific delivery.

Chemotherapeutic agents have been encapsulated into SLN’s and examined for changes in their in-vitro and in-vivo efficacy. Solid lipid nanoparticles resulted in improvement of therapeutic index of 5-Fluorouracil by directing the drug moiety to desired site of action (Jenning et al, 2000). Similarly, liposomes fabricated for Cucurbitacin B using galactosylated lipid N-hexadecyl lactobionamide have shown improved liver targetability and cytotoxicity (Wang et al, 2010). Other drugs for which solid lipid nanocarriers have been formulated include Tamoxifen, Methotrexate, Camptothecin and Mitoxantrone. However, none of these solid lipid nanocarrier preparations are successful till date in reaching the market.

1.3.5.d. Nanosuspensions and nanocrystals

Nanosuspension technology has been primarily employed for solubility enhancement of poorly water soluble drugs. Nanosizing is achieved using various techniques like homogenization, spray drying, wet milling or formation of nanocrystals from supersaturated solutions (Sarkari et al, 2002). Formulation of a nanosuspension of anticancer agent Tacrolimus enhanced its bioavailability. Nanosuspensions can also improve other problems encountered with poorly soluble drugs like erratic absorption pattern leading to faster onset of action and better dose proportionality. Nanosuspensions are suitable for regional drug delivery due to their sustained dissolution profile and uptake by the mononuclear phagocytic system. Commercially, nanosuspensions like Rapamune®, Emend® and Tircor® based on pearl milling have been marketed by Elan Nanosystems (Muller et al, 2001).

1.3.5.e. Nanoemulsions

Nanoemulsions are transparent, kinetically stable systems with a particle size in the range of 20-200 nm, requiring high energy for production as compared to microemulsions (Abolmaali et al, 2011). Nanoemulsions are advantageous in improving the solubility and bioavailability of hydrophobic drugs. However, due to their small size, they are prone to damage by Ostwald ripening, leading to their instability. Inspite of stability issues with nanoemulsions, some local anaesthetic and parenteral nutrition products are present in the market.
1.3.5.f. Inorganic nanoparticles

Inorganic nanoparticles include class of nanocarriers derived from metals like gold and silver, iron oxide, silica and alumina which hold important thermal, electrical and magnetic characteristics and are used to an advantage in anti-tumor treatment. Lately, these nanocarriers are being investigated for commercial purposes; however the growth in this area is slow due to delay in designing these particles with lower toxicity. Inorganic nanoparticles are required to be designed in a manner so as to elude the RES based uptake upon the changes in composition and particle size (Faragi and Wipf, 2009).

1.3.5.g. Dendrimers

Dendrimers are branched molecules with three dimensional arrangements, created from monomeric or oligomeric components. They possess unique properties like definite molecular mass and presence of large surface area for entrapment of actives (Tang et al, 2010). Large variety of molecules could be included in these nanosystems by modification in the functional groups. However, dendrimers are not explored much in the cancer area because of several issues like toxicity, stability and bulky structures which are difficult to handle in terms of synthesis and purification (Madaan et al, 2014).

1.3.5.h. Polymeric nanoparticles as drug delivery systems in cancer

Polymeric nanoparticles are categorized as nanocapsules, nanospheres or nanoparticles wherein, the drug is either inside the particle cavity or forms a part of the polymer matrix. Polymeric nanocarriers lead to release of drug by virtue of their swelling characteristics, hydration properties and polymer breakdown due to enzymatic action (Ghosh, 2000). Several modifications can be made in these systems without any change in physical, chemical and biological characteristics. In the area of chemotherapeutics, polymeric drug delivery systems would prove to be beneficial by selective delivery of drugs along with negligible damage to the adjoining healthy cells (Brewer et al, 2011). Both hydrophilic and hydrophobic moieties could be entrapped in the polymeric nanosystems by physical entrapment or chemical reaction. Due to these advantages, polymeric nanoparticles are being extensively utilized in the field of anticancer research.

Abraxane® is a prime example of the success of polymeric albumin nanoparticles for the treatment of multiple cancers. Most of the trials favour such organic nanocarriers due to their
excellent biocompatibility, biodegradability and low toxic potential. An example includes camptothecin, a topoisomerase-I inhibitor, for which two polymer-drug conjugates are being tested for clinical trials. CRLX-101 containing camptothecin conjugated to a cyclodextrin-based polymer which self-assembles into nanoparticles was also in Phase-I clinical trials. XMT-1001, a conjugate of camptothecin with a biodegradable polyacetal polymer poly (1-hydroxymethylene-hydroxymethylformal), was in Phase 1b studies for non-small-cell lung and gastric cancers (Davis, 2009; Yurkovetskiy and Fram, 2009). Examples of other commercial polymeric nanoformulations currently in different phases of clinical trials and in pipeline are as presented in Table 1.2.

Table 1.2: Examples of polymeric nanoformulations in clinical trials (Wang et al, 2013)

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Nanoplatform</th>
<th>Product name</th>
<th>Company/Institution</th>
<th>Phase of clinical trial</th>
<th>Route</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doxorubicin N-(2 hydroxypropyl)methacrylamide galactosamine nanoformulation</td>
<td>PK2</td>
<td>UK Institute for cancer studies</td>
<td>Phase-I</td>
<td>I.V</td>
<td>Primary and metastatic liver cancer</td>
</tr>
<tr>
<td>2</td>
<td>PEG-polyaspartate formulation of Cisplatin</td>
<td>NC-6004</td>
<td>Nanocarrier Inc.</td>
<td>Phase-I</td>
<td>I.V</td>
<td>Various Cancers</td>
</tr>
<tr>
<td>3</td>
<td>PEG-polyaspartate with Doxorubicin</td>
<td>NK-911</td>
<td>Nippon Kayaku Co. Ltd</td>
<td>Phase-II</td>
<td>I.V</td>
<td>Various Cancers</td>
</tr>
<tr>
<td>4</td>
<td>PLGA or PLA nanoparticles of Docetaxel</td>
<td>BIND-014</td>
<td>BIND Biosciences</td>
<td>Phase-I</td>
<td>I.V</td>
<td>Prostate cancer and others</td>
</tr>
<tr>
<td>5</td>
<td>Paclitaxel nanoparticle Injection</td>
<td>Nanoxel</td>
<td>Dabur Pharma</td>
<td>Approved in 2006</td>
<td>I.V</td>
<td>Breast cancer and others</td>
</tr>
<tr>
<td>6</td>
<td>Cyclodextrin based PEGylated nanoparticles containing siRNA (TfR targeted)</td>
<td>Cyclosert</td>
<td>Insert therapeutics</td>
<td>Phase-I</td>
<td>I.V</td>
<td>Solid tumors</td>
</tr>
<tr>
<td>7</td>
<td>Doxorubicin polymeric nanoparticles</td>
<td>Transdrug®</td>
<td>Bioalliance Pharma</td>
<td>Approved</td>
<td>I.V</td>
<td>Advanced Hepatic carcinoma</td>
</tr>
</tbody>
</table>
1.3.6. Methods of preparation of polymeric nanoparticles

The method selected for preparation of polymeric nanoparticles should be simple, reproducible, independent of the solubility of the drug moiety and polymer and easy to scale up. Nanoparticles can be prepared by polymerization of monomers or from prefomed polymers.

1.3.6.a. Solvent evaporation technique

The polymer and drug are dissolved in an organic solvent such as dichloromethane, chloroform or ethyl acetate and this mixture is then emulsified in an aqueous solution containing a surfactant or emulsifying agent to form an o/w emulsion. Later, the organic solvent is evaporated under reduced pressure or removed by stirring. In order to produce small particles, a high-speed homogenizer or ultrasonicator may be employed (Hood et al, 2002).

1.3.6.b. Solvent diffusion method

In this method, both water miscible and immiscible solvents are used in a combined form as oil phase. Due to the spontaneous diffusion of solvents, an interfacial turbulence is created between two phases leading to the formation of small sized particles. With increase in the concentration of water miscible solvent, a decrease in the particle size can be achieved. In the case of hydrophilic drugs, it is necessary to form a multiple w/o/w emulsion, with the drug dissolved in the internal phase (Niwa et al, 1993).

1.3.6.c. Polymerization technique

Here, monomers are polymerized to form nanoparticles in an aqueous solution. Drug is incorporated either by dissolving in the polymerization medium or by adsorption onto the nanoparticles after polymerization is completed. The nanoparticle suspension is then purified to remove various stabilizers employed for polymerization by ultracentrifugation and re-suspending the particles in an isotonic surfactant-free medium. This technique has been reported for making polybutylecyanacrylate or poly-(alkylcyanacrylate) nanoparticles (Puglisi et al, 1995).

1.3.6.d. Ionic gelation method

Normally, this method has been employed for preparation of nanoparticles using polymers like chitosan, sodium alginate and gelatin. The process involves mixture of two aqueous phases, of which one is the polymer, a di-block co-polymer ethylene oxide or propylene oxide (PEO-PPO) and the other is a polyanion like sodium tripolyphosphate. In this process, positively charged
amino group of the polymer interacts with negatively charged tripolyphosphate to form coacervates within nanometer size range due to electrostatic interaction (Calvo et al, 1997).

1.3.6.e. Antisolvent precipitation

Drug crystals with narrow size distribution could be prepared by direct controlled crystallization. This process involves antisolvent precipitation of drug solution in a water-miscible organic solvent, followed by addition of a bridging solvent, which is immiscible or partially miscible with water. Growth-retarding stabilizing additives, such as hydroxylpropylmethylcellulose are usually added in the medium to yield particles with small size. The precipitated drug crystals exhibit high fine particle fraction and low amorphous content (Lonare and Patel, 2013).

1.3.6.f. Double emulsion/Solvent evaporation technique

This methodology involves preparation of o/w emulsions with subsequent removal of the oil phase (i.e. volatile organic solvent) through evaporation. The emulsions are usually prepared by emulsifying the organic phase containing the drug, polymer and organic solvent in an aqueous solution containing emulsifier. The organic solvent diffuses out of the polymer phase and into the aqueous phase and is then evaporated, forming drug-loaded polymeric nanoparticles. This process has been utilized for fabrication of poly (l-lactic acid), poly-glycolic acid and PLGA nanoparticles (Jean C. Sung et al, 2007).

1.3.6.g. Nanoparticles produced in external oily emulsion

This process has been employed primarily for albumin nanoparticles and is characterized by the method of stabilization viz. thermal treatment at elevated temperatures (95–170°C) or chemical treatment in vegetable oil, iso-octane emulsions, or aqueous medium. Other techniques involve slight modification of either of these two methods. Nanospheres are formed by homogenizing the oil phase containing the albumin droplets and later thermally stabilized by heating at 175 to 180°C. This mixture is cooled and diluted with ethyl ether to reduce viscosity of the oil phase to permit separation by centrifugation. This technique is applicable for drugs that are not sensitive to heat. For this reason, nanoparticles have also been produced by emulsifying polymer solution in cottonseed oil, followed by its denaturation by resuspending the particles in ether containing cross-linking agents (Widder et al, 1979).
1.3.6.h. Solvent displacement technique

This method is based on the spontaneous o/w emulsion formation where the polymer is dissolved in the organic phase (intermediate polarity solvent) and is precipitated from the organic solution when injected in aqueous medium. The polymer is deposited on the interface of water and organic solvent, leading to formation of nanoparticles. If a small amount of oil is incorporated in the organic phase, nanocapsules are formed by this procedure. This procedure is specifically applicable to hydrophobic drugs (Quintanar-Guerrero et al, 1998).

1.3.6.i. Nanoparticles produced by desolvation of molecules

Another technology applicable to a wide range of polymers is based on desolvation by charge and pH changes, or by addition of a desolvating agent (ethanol, acetone or concentrated inorganic salt solutions). The main advantage is that this process does not require an increase in temperature and hence is best suited for heat sensitive drugs. Here, the nanoparticles are prepared using the process of reversible swelling of macromolecules using gelatin, human serum albumin, bovine serum albumin, and casein as the macromolecular materials. This process offers the advantage of producing nanoparticles directly in aqueous suspension. Absence of surfactant for formation of nanoparticles leads to formulations with reduced toxicity.

1.3.7. Characterization of nanoparticles

Nanoparticles are key constituents in the development of advanced delivery systems. The characterization of nanocarriers is important for understanding the parameters involved in controlling the nanoparticle properties and synthesis. Nanoparticles differ from other drug delivery carriers in terms of their small particle size, large surface area and brownian motion in solution form. Hence, nanoparticles require different methods of characterization from other pharmaceutical systems (Hall et al, 2007).

Direct and indirect methods available for nanoparticle characterization are as listed in Table 1.3. Direct techniques include imaging methods like S.E.M and T.E.M which can immediately measure and infer the nanoparticle size. Disadvantages involved with these techniques are measurement of small number of particles at a particular time and tedious sample preparation. Indirect techniques are used to characterize nanoparticles using neutrons or X-rays, where the results are analyzed using a mathematical equation for eg. small angle neutron scattering (SANS).
technique. Indirect methods like SANS are relatively simple and need no sample processing before actual analysis.

### Table 1.3: Different methods of nanoparticle characterization (Nagvekar et al, 2009)

<table>
<thead>
<tr>
<th>Type of characterization</th>
<th>Available methods</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size determination</td>
<td>Dynamic light scattering</td>
<td>Most widely employed, fast and accurate</td>
<td>Not very useful for dilute solutions</td>
</tr>
<tr>
<td></td>
<td>Sedimentation method</td>
<td>Useful tool for microparticles</td>
<td>Particles in nano range settle slowly, hence tedious</td>
</tr>
<tr>
<td>Physical state determination</td>
<td>T.E.M</td>
<td>Liquid particles can be analyzed, two dimensional imaging</td>
<td>Instrument requires large amount of space, costly technique</td>
</tr>
<tr>
<td></td>
<td>S.E.M</td>
<td>Particles analyzed in powder form, surface morphology also determined</td>
<td>Instrument requires large amount of space</td>
</tr>
<tr>
<td></td>
<td>A.F.M</td>
<td>3D imaging possible, surface texture seen</td>
<td>Costly technique</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>Zetasizer</td>
<td>Accurate and fast method</td>
<td>Limitation application in case of dense particles</td>
</tr>
<tr>
<td>Physical state determination</td>
<td>D.S.C</td>
<td>Accurate and reliable</td>
<td>Preferably used for solid samples &amp; not liquids</td>
</tr>
<tr>
<td></td>
<td>X.R.D</td>
<td>Accurate and reliable</td>
<td>Instrument requires large amount of space</td>
</tr>
<tr>
<td></td>
<td>FT-I.R</td>
<td>Accurate and reliable</td>
<td>Time consuming sample preparation</td>
</tr>
<tr>
<td></td>
<td>N.M.R</td>
<td>Accurate and reliable</td>
<td>Instrument requires large space, costly technique</td>
</tr>
<tr>
<td>In-vitro dissolution</td>
<td>Diffusion chamber</td>
<td>Easy to perform</td>
<td>Difficult to maintain sink conditions</td>
</tr>
<tr>
<td></td>
<td>Dialysis technique</td>
<td>Easy to perform</td>
<td>Accurate only with low sample volume</td>
</tr>
<tr>
<td>Entrapment efficiency</td>
<td>Centrifugation/Ultracentrifugation</td>
<td>Easy and fast to perform</td>
<td>Accurate only if all nanoparticles settle well</td>
</tr>
<tr>
<td></td>
<td>Column chromatography</td>
<td>Determination of entrapment in addition to particle purification</td>
<td>Standardization of procedure is required</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Brookfield viscometer</td>
<td>Easy to perform, accurate estimation possible</td>
<td>Accurate only if the nanoparticles are homogenous</td>
</tr>
</tbody>
</table>
1.3.7.a. Lyophilization of nanoparticles

Lyophilization or freeze drying involves removal of water molecules from sensitive pharmaceutical products like nanoparticles for improvement in their physical and chemical stability (Franks, 1998). Freeze drying is aimed at removal of water from a frozen product by sublimation and desorption under vacuum, for conversion into a solid form. This process comprises of three steps viz. freezing (solidification), primary drying (ice sublimation) and secondary drying (desorption of unfrozen water).

If a formulation is intended to be lyophilized, it is important to take the physical and thermal properties of the nanoparticle suspensions into consideration. The crystallization stress generated during this process could destabilize the nanoparticle preparation, hence special excipients like cryoprotectants are added to polymeric nanocarrier formulations. These include bulking agents like mannitol, lactose, trehalose, sucrose; stabilizers like glucose, sorbitol, glycine, alanine; various buffers and tonicity adjusters (Niresha et al, 2013).

The choice of appropriate type and concentration of the above can be made by optimization of freeze-thaw cycles. For polymeric nanoparticles, the formulations are frozen along with cryoprotectants at temperatures from -40°C to -60°C in liquid nitrogen and freeze dried under vacuum for time period ranging from 24 hours to 90 hours. Desired nanoparticle size can be achieved by right choice of lyophilization parameters like freezing temperature, pressure and velocity (Anhorn et al, 2008).

1.3.7.b. Sterilization of nanoparticles

Sterility is a prerequisite for intravenously administered nanoparticles and can be achieved by aseptic membrane filtration, autoclaving or gamma sterilization. Generally, sterilization by γ-irradiation is the preferred technique for nanoparticles because of drawbacks of earlier methods like high heat and pressure imparted by autoclaving and ineffectiveness of filtration process. However, exposure of nanoparticles to radiations may lead to irreversible changes in the nanoformulations (Zheng et al, 2011). Hence it is necessary to critically select the radiation dose and time and evaluate the final product for changes in physicochemical parameters like nanoparticle size, shape drug content and drug release profile to arrive at optimum sterilization conditions.
1.3.8. Polymers used in preparation of nanoparticles

Nanoparticles based on synthetic polymers like PLA, PLGA and PGA represent the most extensively investigated polymers in drug delivery. In addition to these polymers, natural macromolecules such as chitosan, sodium alginate, agarose, albumin, zein, collagen and gelatin represent a second fundamental class of materials for nanoparticle synthesis. Among these, nanoparticles of proteinaceous origin i.e. albumin, collagen and gelatin have raised specific interest. Due to their intrinsic protein structure with high number of different accessible functional groups, they present multiple opportunities for coupling of targeting ligands, crosslinkers, and shielding substances (Kompella et al, 2004).

Normally, synthetic polymers like polycaprolactone, polyacrylamide, poly-(methylmethacrylate) etc. used for encapsulation of various drugs require organic solvents for drug solubilization. The use of organic solvents is mainly associated with safety issues, high cost, toxicity and they are difficult to remove from the final nanoformulation. The use of natural polymers like albumin, gelatin, starch, chitosan offers an added advantage of preventing the use of toxic chlorinated solvents, ease of availability in large quantities and low cost in the context of commercial development (Pinto Reis et al, 2006).

1.3.8.a. Albumin as a carrier for nanoparticles

Albumin is the most important soluble protein in human body, which is synthesized in the liver. This protein accounts for around 60% of the total proteins present in blood with a molecular mass of 66.5 kDa. It is the primary protein in human body controlling the osmotic pressure of serum. Other functions imparted by albumin include binding of toxic products and their transport to the liver, distribution and transport of metal ions and other exogenous components (Peters, 1996). Albumin is a GRAS listed excipient and has been approved by the Food and Drug administration, USA for administration via the injectable route.

As such, bovine serum albumin and human serum albumin possess domains in their structure which are topographically similar however; they have different binding affinities for different molecules. These proteins differ in the number of peptide residues i.e. BSA has 583 peptides while HSA has 585 peptides in the structure (Table 1.4).
Table 1.4: Amino acid residues in BSA and HSA (Mine and Shahidi, 2006)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Human</th>
<th>Bovine</th>
<th>Amino Acid</th>
<th>Human</th>
<th>Bovine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>36</td>
<td>40</td>
<td>Cysteine/2</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Asparagine</td>
<td>17</td>
<td>14</td>
<td>Methionine</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Threonine</td>
<td>28</td>
<td>34</td>
<td>Isoleucine</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Serine</td>
<td>24</td>
<td>28</td>
<td>Leucine</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>62</td>
<td>59</td>
<td>Tyrosine</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Glutamine</td>
<td>20</td>
<td>20</td>
<td>Phenylalanine</td>
<td>31</td>
<td>27</td>
</tr>
<tr>
<td>Proline</td>
<td>24</td>
<td>28</td>
<td>Lysine</td>
<td>59</td>
<td>59</td>
</tr>
<tr>
<td>Glycine</td>
<td>12</td>
<td>16</td>
<td>Histidine</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Alanine</td>
<td>62</td>
<td>46</td>
<td>Tryptophan</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Valine</td>
<td>41</td>
<td>36</td>
<td>Arginine</td>
<td>24</td>
<td>23</td>
</tr>
</tbody>
</table>

Albumin is an acidic protein with an isoelectric point of 4.7. This protein is stable in the pH range of 4-9 and exhibits solubility in water and other aqueous solutions. Albumin can be heated at a temperature of around 60°C, without any change in the protein structure. All these properties along with the preferential tumor uptake, biodegradability, non-toxicity and property to accumulate at inflamed tissues, makes it an ideal polymer for cancer drug delivery.

1.3.8.b. Gelatin as a carrier for fabrication of nanoparticles

Among all the polymers used to produce nanoparticles, gelatin, a natural macromolecule is commonly employed as a pharmaceutical adjuvant and an encapsulating drug material. It is a molecule of great interest due to its biocompatibility and biodegradability. It has been reported that gelatin nanoparticles, prepared by different methodologies and hardened by a suitable cross-linking agent as glutaraldehyde, lead to enhanced tumoral cell phagocytosis. Hence, these systems have been widely studied in parenteral formulations as carriers of cytostatic drugs such as Interferon, 5-Fluorouracil, Methotrexate, and Doxorubicin (Eliana et al, 1997).

Gelatin is obtained mainly by acidic or alkaline process or thermal or enzymatic degradation of the structural protein collagen. According to origin and pretreatment of the utilized collagen, two major types of gelatin are commercially produced. Gelatin Type A (acidic) is obtained from porcine skin with acidic pre-treatment prior to the extraction process. The second gelatin type is
Gelatin Type B (basic) which is extracted from ossein and split from bovine origin (Raja Mohd Hafidz et al, 2011).

Advantages of gelatin for drug delivery include its inexpensiveness, ability to be sterilized, free from contamination with pyrogens and low antigenicity. It has been used for decades in parenteral formulations and is an approved plasma expander. It is documented as a GRAS excipient by the U.S Food and Drug Administration. Gelatin derivatives also form constituents of intravenously administered preparations like plasma expanders (e.g. Gelafundin™, Gelafusal™) and used as sealants for vascular prostheses (Weber et al, 2000).

1.3.9. In-vivo fate of administered nanoparticles

Nanoparticles upon administration via injectable route are removed from the bloodstream by reticuloendothelial system (RES) after binding to opsonin proteins. These proteins facilitate the adherence of phagocytes to the nanoparticle surface, resulting in ingestion of nanocarriers by phagocytes.

Typically, biodegradable polymeric nanoparticles with high molecular weight are not cleared by the renal system but are transferred to mononuclear phagocytic organs (MPS) viz. liver, spleen and bone marrow (Frank and Fries, 1991). Hence, this mechanism is of advantage in targeting anticancer drugs to hepatocellular carcinoma. The delivery of nanocarriers to MPS organs is also dependent on the nanoparticle size, shape, particle charge and surface properties. The pharmacokinetics and biodistribution of nanoparticulate drug delivery systems can be studied using numerous in-vitro and in-vivo studies.

1.4. Cisplatin for treatment of Hepatocellular carcinoma

As per the literature survey, various drugs available for treatment of hepatic cancer are Cisplatin, Doxorubicin, Bevacizumab, Floxuridine, Sorafenib and Erlotinib (Marin et al, 2008). These antineoplastic drugs along with their properties are listed in Table 1.5.

Out of these drugs, Cisplatin was selected by us for the present study due to its reported use as a first line drug in the treatment of liver cancer, small molecular size and adequate aqueous solubility for incorporation in polymeric nanoparticles synthesized with natural polymers.
### Table 1.5: Drugs used for Hepatic cancer

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the drug</th>
<th>Brand name</th>
<th>Mol. wt Daltons</th>
<th>Log P</th>
<th>Mechanism of action</th>
<th>Aqueous solubility</th>
<th>Route of admin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cisplatin</td>
<td>Platinol®</td>
<td>300.1</td>
<td>-2.19</td>
<td>Binds to DNA molecules at guanine bases and inhibits DNA synthesis</td>
<td>1 mg/ml</td>
<td>I.V Injection and Infusion</td>
</tr>
<tr>
<td>2</td>
<td>Doxorubicin</td>
<td>Adriamycin PFS®</td>
<td>543.51</td>
<td>1.27</td>
<td>Intercalates between DNA and stops replication</td>
<td>0.5 mg/ml</td>
<td>I.V Injection</td>
</tr>
<tr>
<td>3</td>
<td>Sorafenib tosylate</td>
<td>Nexavar®</td>
<td>637.02</td>
<td>3.8</td>
<td>Inhibits intracellular and cell surface kinases and decreases tumor cell proliferation</td>
<td>1.71e-03 g/lit</td>
<td>Oral (Tablets)</td>
</tr>
<tr>
<td>4</td>
<td>Floxuridine (Metastatic cancer)</td>
<td>Floxuridine</td>
<td>246.19</td>
<td>-1.16</td>
<td>Inhibits thymidylate synthesis and results in disruption of DNA</td>
<td>4.08e+01 g/lit</td>
<td>Intra-arterial injection</td>
</tr>
<tr>
<td>5</td>
<td>Bevacizumab (Metastatic cancer)</td>
<td>Avastin®</td>
<td>149</td>
<td>----</td>
<td>Blocks vascular endothelial growth factor (VEGF)</td>
<td>2.5 mg/ml</td>
<td>I.V Infusion</td>
</tr>
<tr>
<td>6</td>
<td>Erlotinib</td>
<td>Tarceva®</td>
<td>429.82</td>
<td>2.7</td>
<td>Inhibits intracellular phosphorylation of tyrosine kinase</td>
<td>8.91e-03 g/lit</td>
<td>Oral tablets</td>
</tr>
</tbody>
</table>


1.4.1. Drug profile: Cisplatin

Cisplatin (cis-diaminedichloroplatinum) is an inorganic compound that is widely used for a variety of tumors like bone cancer, lung cancer, head and neck cancer, sarcomas and ovarian tumors (Fig. 1.8).

![Fig. 1.8: Structure of Cisplatin](image)

Molecular formula: $\text{Cl}_2\text{H}_6\text{N}_2\text{Pt}$
Molecular weight: 300.1 Daltons

1.4.1.a. Mechanism of action of Cisplatin

Cisplatin exerts its antineoplastic activity when it possesses cis-configuration and when the molecule is uncharged. The trans-configuration of this moiety is inactive. Cisplatin moves through the cell membranes in unionized form due to the high concentration of chloride ions in the plasma. Intracellularly, the concentration of chloride ions is low as compared to that in plasma. The chloride ions on the Cisplatin molecules are exchanged for water, thus resulting in the formation of positively charged aquated cisplatin complex (Fig. 1.9), which is toxic to the cancer cells (Pil and Lippard, 1997).

![Fig. 1.9: Mechanism of action of Cisplatin](image)
Chapter 1: Introduction and Plan of work

1.4.1.b. Route of administration

Cisplatin is currently available in the market only as an injectable preparation. Cisplatin is not absorbed after oral administration and hence parenteral routes like intravenous, intra-arterial and intra-peritoneal have been explored for treatment with this agent.

1.4.1.c. Dosage and administration

The recommended dose of Cisplatin in children and adults is 50-100 mg/m² as a single i.v dose for 3-4 weeks or 15-20 mg/m² daily for 5 days by intravenous route.

1.4.1.d. Solubility

Cisplatin is insoluble in aqueous solutions at a concentration of 1 mg/ml. It is also soluble in dimethylformamide and dimethylsulfoxide. The solubility of Cisplatin in aqueous solutions increases upto 8 mg/ml upon heating at 50-60°C.

1.4.1.e. Marketed formulation

Cisplatin is available as a sterile injection for intravenous administration in 0.9% saline (Cytoplatin, Cipla Ltd). This injection contains Cisplatin at a concentration of 1 mg/ml and is available in the form of 10 ml, 25 ml or 50 ml vials.

1.4.1.f. Therapeutic uses

Cisplatin is indicated in metastatic germ cell carcinoma, head and neck carcinoma, refractory ovarian and bladder carcinoma. Cisplatin is also indicated as a regimen of combination therapy along with other antineoplastic agents for treatment of metastatic testicular tumors. For example, combination therapy of Cisplatin, Bleomycin and Vinblastine has been reported to be highly effective (Rosenberg et al, 1969).

Other uses of this drug include:-

(1) Treatment of locally advanced or metastatic transitional cell carcinoma involving the renal pelvis, ureter, bladder and/or urethra.
(2) In combination with radiation treatment to treat bladder cancer and together with Doxorubicin and Cyclophosphamide to treat locally advanced bladder cancer.
1.4.1.g. Pharmacokinetic pattern

1.4.1.g.a. Absorption

Absorption and peak plasma concentrations of this drug depend on the route of administration.

**Intravenous:** Rapid intravenous injection of Cisplatin over 1-5 minutes or rapid intravenous infusion over 15 minutes results in immediate peak plasma concentration of this drug. If the intravenous infusion is given slowly over a period of 24 hours, the concentration of Cisplatin rises gradually and reaches peak plasma.

**Intra-arterial:** Cisplatin is completely absorbed from this route due to the high local concentration of the drug in the tumor.

**Intra-peritoneal:** Cisplatin is well absorbed systemically after intra-peritoneal administration. Intra-peritoneal fluid concentration of the drug is increased greatly as compared with intravenous administration.

1.4.1.g.b. Distribution

Cisplatin rapidly distributes into different tissues after intravenous administration like kidneys, liver and intestine. Small concentrations of Cisplatin are also found in muscles, bladder, testes, spleen, prostate, heart, lungs and pancreas.

Cisplatin is known to bind extensively to plasma proteins. Protein binding of this moiety in plasma increases with time with about 90% of protein binding.

1.4.1.g.c. Metabolism

The metabolic fate of Cisplatin has not been completely understood till today. The chloride ions on this molecule are known to be displaced by water, leading to the formation of aquated Cisplatin. The rate and extent of metabolism depends on the strength, concentration and accessibility of the nucleophiles.

1.4.1.g.d. Elimination

Intact Cisplatin and its metabolites are principally excreted from urine. This excretion occurs via glomerular filtration but some reabsorption of Cisplatin and its metabolites also occurs. Initially, renal clearance of total platinum equals creatinine clearance and represents elimination of non-protein bound platinum molecules including intact Cisplatin. Due to extensive protein binding,
the clearance rate declines rapidly, resulting in a prolonged excretory phase. A small amount of this drug is excreted via the bile and saliva.

1.5. Rationale and Plan of research work

Globally, liver cancer represents one of the most common malignant tumors. It is categorized into primary cancers, originating from epithelial liver cells and secondary cancers which proliferate in other areas of the body after their transfer via the lymphatic system (Srivatanakul et al, 2004). Secondary cancers merely embrace primary cancer cells and hence pose hurdles in discrimination and treatment of liver cancer. Consequently, most of the current treatment strategies are directed towards primary liver tumors like hepatocellular carcinoma.

Currently, a lot of new drugs are being developed and made available for clinical use in the field of cancer. However, most of these drugs suffer from narrow therapeutic index with an array of side effects. Hence, the major challenge in anticancer drug therapy is to effectively increase the drug concentration at the desired site of action with minimum toxic effects.

Cisplatin is used in the treatment of variety of cancers and is one of the well known antineoplastic agents for hepatic cancer. However, its therapeutic effects are limited by several side effects occurring during the course of therapy like nephrotoxicity, ototoxicity and gastrointestinal toxicity (Cvitkovic, 1998). Additionally, short half life of the drug provides only short term remission to the patients, leading to frequent drug administration. These difficulties have urged the need for development of targeted drug delivery systems which would lead to selective drug transport, without affecting normal cell physiology.

In recent years, nanoparticles are being increasingly used for the treatment of neoplasms. Nanoparticulate pharmaceutical carriers are employed in the cancer arena to enhance the *in-vivo* efficacy of anticancer drugs. Small particle size of nanocarriers makes them suitable for intravenous drug delivery along with easy uptake by the tumor cells (Sivasankar and Kumar, 2010; Torchilin, 2007). Formulation of Cisplatin in the form of targeted delivery to the liver would increase its therapeutic efficacy along with reduction in side effects. Hence, in the present study, targeted delivery was attempted for Cisplatin in the form of polymeric nanoparticles.

As such, conventional nanocarriers are able to reach hepatic metastasis via enhanced permeability and retention mechanism and this phenomenon is of advantage for treatment of
hepatocellular carcinoma. However, the EPR effect not only leads to accumulation of nanoparticles in the liver, but also in other organs like spleen and bone marrow. Hence to ensure selective delivery of the nanocarriers, it is essential to tag the nanoparticles with various molecules like ligands or antibodies.

Nanoparticles can be prepared using both biodegradable and non-biodegradable polymers. However, the latter are preferred due to their biocompatibility, biodegradability, low cost and safety. These nanoparticles can incorporate hydrophilic and hydrophobic moieties, proteins, vaccines and biological macromolecules.

1.5.1. Aims and Objectives of the project

1. To prepare stable, biodegradable nanoparticulate formulations of Cisplatin for hepatocellular carcinoma treatment using BSA, HSA and Gelatin.

2. Optimization and characterization of the developed nanoformulations.

3. *In-vitro* and *in-vivo* studies on developed polymeric nanoparticle formulations to establish the biodistribution and pharmacokinetic pattern, benefits and safety.

1.5.2. Plan of work

1. Literature survey on anticancer drugs for hepatic cancer and evaluation of various biodegradable polymers, excipients and methods of preparation of polymeric nanocarriers for the research work.

2. Selection, procurement and standardization of Cisplatin, excipients, selected polymers and drug excipient compatibility studies.

3. Analytical Method Development for estimation of Cisplatin in formulations, plasma and hepatic tissues with the help of U.V, HPLC and ICP-AES.


5. Selection of optimized batches of the above polymeric nanoparticles.

7. *In-vitro* release of Cisplatin from the developed formulations using *in-vitro* dissolution apparatus to determine the drug release pattern and release kinetics of drug moiety from the developed nanoparticles.

8. Lyophilization of optimized batches using suitable cryoprotectants for long term stability.

9. Stability studies on the optimized formulations as per ICH guidelines.

10. Sterilization of the finalized nanocarrier preparations by $\gamma$-radiation and sterility testing of these batches.

11. *In-vitro* hemolytic assay for optimized polymeric nanoparticles.

12. *In-vitro* cytotoxicity studies against human liver cancer cell line HepG2 using MTT and SRB assays to measure the cell viability of cancerous cells after exposure to chemotherapeutic agents.

13. Other *in-vitro* assays like colony formation assay, wound scratch assay, lighten tube assay to determine the migration potential and inhibitory activity of the formulations on hepatoma.

14. Confocal microscopical analysis and flow cytometry studies to determine the liver cell uptake of nanoparticles in a qualitative and quantitative fashion.

15. *In-vivo* pharmacokinetic and biodistribution studies to determine the *in-vivo* fate of the developed polymeric nanoformulations.

16. Acute and repeated dose toxicity studies to prove the safety of developed formulations.

**1.6. Conclusion**

Based on the literature survey, Cisplatin and polymers BSA, HSA and Gelatin were finalized for fabrication of biodegradable polymeric nanocarriers in the present work.