3. LITERATURE REVIEW
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Technological innovations in drug delivery systems in recent years promise radical change in the field of pharmacotherapy. Many predict that, by the next ten years, drugs will be more specific in pharmacodynamic action, more site selective and will be administered less often and in lower quantities \cite{37, 38}. There are many potentially effective drug delivery systems and vehicles. They differ in their drug capacities, structure, *in vivo* and storage stability, modes of administration and possible applications. Each of them has favorable attributes and limitations. Major technical challenges include drug loading, size, cost and stability. Major biological challenges include immunity, drug denaturation, targeting and non-specific toxicity. Combination of two or more advanced drug delivery systems may be more effective than any one particular system applied on its own.

During the last 25 years intensive investigations have been undertaken on the use of delivery systems to carry pharmaceuticals to target sites. The purpose of using these delivery systems is to convey active materials to target site. In this way the tissue is protected against the side effects of the drug and at the same time the stability of the drug can be maintained. In this regard, many micromolecular carriers such as liposomes, niosomes, pharmacosomes etc. have been designed and studied extensively to modulate biodistribution characteristic of the drug.

### 3.1 CANCER THERAPEUTICS

A tumor is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the adjacent normal tissue and persists in the same excessive manner after cessation of the stimuli which evoke the change initially. This definition brings out three cardinal features of cancer:

- That an abnormal mass of tissue is present and that it is essential component of a malignant tumor made up of actively growing cells and of the supportive stroma or vascular network.

- That the growth is in excess of and uncoordinated with the normal tissue. These attributes distinguish cancer from other proliferative processes such as inflammation, repair and hyperplasia which are orderly and coordinated.
That such excessive growth persists even after cessation of the initial stimuli, is the most distinctive characteristic of cancer and one that makes it such a formidable disease.

The war against cancer has been launched in four segments namely, (i) prevention of cancer, (ii) early detection of cancer, (iii) regional cancer cure, and (iv) systemic cancer control. The most commonly employed modalities for the treatment of established cancer are surgery, radiotherapy and chemotherapy. Immunotherapy and gene therapy are the other two therapeutic tools that are gaining importance in recent years [39, 40].

Chemotherapy of cancer has survived several years of criticism and is now an established means of cancer treatment. It was almost many decades before firm evidence was obtained that chemotherapy could actually cure cancer. At present time, there are dozens of anticancer drugs available for clinical use and the rate of new drug development is steadily increasing. Today, with cures documented in some proportions for a dozen different malignancies, it can be said that chemotherapy has joined surgery and radiotherapy as a significant treatment modality. Cytotoxic drugs can act at the cellular level and leads to a direct kill of tumor cells (cytoidal effect). The cytoidal mechanism involves the blockage of protein synthesis and the cytostatic effect is due to the inhibition of DNA synthesis. Certain drugs are specifically active in tumors with high mitotic activity. They are designated as being “cycle specific agents” which are, mainly the antimetabolites. The resulting tumor cells however, constitute the major population of tumor mass. Such cells are insensitive to cycle specific agents and are affected by alkylating agents and antibodies, termed as “non cycle specific” drugs.

Tumor heterogeneity and development of multi-drug resistance are the two unresolved problems in cancer research and no clear cut understanding in this regard has been achieved till date. Besides the above two, the inherent problem of non-specificity is due to very subtle metabolic differences that exist between a cancerous and a normal cell. So, unlike in a bacterial infection, a cancer chemotherapeutic agent cannot exclusively act on
the metabolic pathways of cancer cells while leaving the rapidly dividing normal cells unaffected.

Antineoplastic agents are therefore, neither specific nor targeted to cancer cells. Improved delivery of anticancer drugs to tumor tissues thus, appears to be a challenging, but achievable effort. Significant efforts have been directed towards the improvement of anticancer drug delivery in the recent years. At present, specificity of antineoplastic agent is achieved by one of the three approaches, namely,

- Chemical conversion of a drug to an inactive pro-drug form. Conversion to an active drug is controlled by intrinsic physiological processes, and thus inappropriate drug activity is theoretically reduced.
- Utilization of simple soluble macromolecules to which a drug is immobilized. The macromolecular carriers exhibit intrinsic targeting properties and delivery of an active drug bound to the macromolecular carriers is controlled by processes intrinsic to the carrier and endogenous ligands.
- Utilization of more complex particulate multicomponent carriers within which the drug is shielded from degradative processes during transit. Delivery of an active drug is controlled by enhanced drug survival and may be targeted by the addition of specific ligands to the carrier.

Ideal cancer chemotherapeutics would involve a relatively constant drug concentration maintained between the minimum therapeutic level and the toxic level of the antineoplastic agent for appreciably prolonged duration of time. This treatment modality would not only avoid/reduce the systemic toxicity commonly associated with the antineoplastic agent but also facilitate effective destruction of tumor cells during their proliferative phase itself wherein, the tumor cells are most sensitive to chemotherapeutic agents.
3.2 LIPOSOMES
Liposomes are among the latest, most widely applicable and exciting of the new drug delivery system. Liposomes are microscopic vesicles (from 0.02 to 6.0 µm in diameter), composed of one or several lipid membranes surrounding discrete aqueous compartments. These vesicles can encapsulate water soluble drugs in their aqueous spaces and lipid soluble drugs within the membrane. Depending on the gel-liquid crystalline transition temperature (Tc) of phospholipids (i.e., the temperature at which acyl chains melt), liposomal membrane can attain varying degree of fluidity at ambient temperature. This, in fact, can be controlled quite accurately to achieve wide range of Tc values by using appropriate mixtures of two or more phospholipids. In addition, liposomal surfaces can be charged negatively or positively by the incorporation of charged amphiphiles, or enriched with reactive groups to which ligands can be covalently linked. It is also possible to adjust the average vesicle size by sonication, detergent dialyses, microfluidization, homogenization and other similar techniques [41]. The nature of the liposome causes it, and therefore its cargo, to be distributed within the body in a pattern dramatically different from that of free drug. It is by taking advantage of this altered biodistribution that superior intravenous therapies can be designed [42].

In the early stages of liposome research, the focus in cancer chemotherapy was on formulations with high affinity for the reticuloendothelial system (RES), using negatively charged phospholipids such as cardiolipin, phosphatidyl-glycerol and phosphatidyl-serine. A gradual shift towards formulation with prolonged circulation took place in the late eighties. This was achieved to a limited extent by using small (< 200 nm diameter) neutral vesicles and to a large extent with surface modified vesicles coated with glycolipids or hydrophilic-polymers such as polyethylene glycol (PEG). In parallel, improved drug loading methods, based on transbilayer gradients resulted in greater efficiency and stability of encapsulation of cationic amphiphiles, such as anthracyclines [43, 44]. Stability problems for liposomes are very severe. Varied changes can occur in liposomes with the passage of time. Liposomal phospholipids can undergo chemical degradation
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such as oxidation and hydrolysis. Either as a result of these changes or otherwise, liposomes maintained in aqueous suspension may aggregate, fuse or leak their contents [45]. Injectable liposomal drug should be stable for at least 18 months at 4°C, but 24 months would be preferable. It does not appear likely that this degree of stability can be achieved for every liposomal drug formulation stored in suspension form and alternative approaches using procedures such as freeze-drying and remote loading have been devised [42].

Freeze drying of liposomes had been precluded in the past because the liposomes were subjected to massive fusion and leakage during the dehydration and rehydration processes. However, the remarkable ability of sugars such as glucose, sucrose, trehexose and maltose to protect liposomes during freeze drying and subsequent rehydration, now make this approach feasible. Sugars are added during the liposome formulation process to create equivocal concentration of sugars on both sides of phospholipid membranes. The preflowing powdered liposomal preparations that result have excellent storage characteristics and retain 90% or more of entrapped agents after rehydration with no change in the size of the liposomes [46]. Another approach to overcome stability problems are to load drugs into preformed liposomes at the time of use [47]. The stability of such preparations can be increased by storing liposome preparations in freeze-dried form, using synthetic saturated lipids and adding antioxidants such as alpha-tocopherol and beta-hydroxytoluene to liposomal preparations [48].

In general, four major factors influence the in vivo behavior and biodistribution of liposomes. If cholesterol is not included in the membrane of the liposome, the liposome leaks substantially when it is introduced intravenously. This phenomenon has been attributed to interactions between the liposome and plasma proteins or lipoproteins [49, 50]. Second, smaller liposomes are cleared more slowly than are equivalent dosed of their larger counterparts. The half life (t½) of a liposomal preparation can be increased from minutes to hours if small unilamellar vesicles are used in place of multilamellar vesicles [51]. Third, the t½ increases as the lipid dose increases. This effect presumably is related to saturation of the reticulo-endothelial system at high lipid doses [52]. Finally,
charged liposomal systems are cleared more rapidly than uncharged systems, an effect that may be related to greater affinity to serum proteins for charged liposomes \[^{[53]}\].

Researchers originally assumed that fixed phagocytic cells in the liver, spleen and to a lesser extent in the lung, lymph nodes and bone marrow could rapidly internalize the liposomes by active process, and thus, if this assumption is valid, the clinical applications of liposomes necessarily would be limited to the treatment of diseases of those cells. Liposomes encapsulated drugs have been used effectively to treat conditions outside the reticuloendothelial system, including extra cellular infections and a diverse array of ascetic and solid tumors \[^{[42]}\].

Although the ultimate fate of liposomes may be well within a phagocyte, this process may occur slowly. That is to say, the mere presence of liposomes in the liver does not indicate uptake by Kupffer cells. Similarly a high concentration of liposomes in the spleen does not imply that the vesicles are located intracellularly. Early accumulation of liposomes in the reticuloendothelial system organs may be more a function of physical filtration than active uptake. This is reasonable when one considers the fact that liposomes are only constrained to the blood vessel lumen when the walls of the vessels are intact. Liposomes can enter the interstitial spaces of organs such as the liver and spleen because the vessels in those organs are lined cells that are not tightly joined (fenestrated). The extended presence of intact extracellular liposomes expands the potential use of liposomes as drug delivery vehicles because it allows for the creation of intra organ depots of drug that can be released slowly into the bloodstream. In the case of antineoplastic agents, such as sustained release could substantially reduce the acute and chronic toxicity of the drugs \[^{[42]}\].

It is unlikely that slow release alone can explain the activity of liposome encapsulated drugs in non reticuloendothelial diseases. Internalization of liposomes into circulating reticuloendothelial cells may also be important. For example, in response to an infection, scavenger cells are recruited to the infected site as part of the normal immune response. If these cells have previously internalized a liposome carrying a drug that is resistant to intracellular inactivation, then the scavenger cells must transport the active drug to the
infected area. In essence, the liposomes will have converted reticuloendothelial cells into a targeted drug delivery system. In addition, areas of infection, inflammation and solid tumors often have a high content of macrophages that could remove liposomes from circulation [42].

Liposomes will circulate in the bloodstream until one of several events occur; they are broken down by exchange of lipids with various serum components or degraded by phospholipases; they are taken up by circulating phagocytic cells; or they are filtered out of the blood through fenestrated capillaries. This latter condition is not limited to the liver and spleen. Fenestrated or incomplete capillary networks may be found at many sites of infection and inflammation and in several types of solid tumors [54, 55]. Liposomes can also leave the circulation at these sites, providing a potential mechanism for concentrating drug at the desired location.

Ogihara et al. studied the accumulation of liposomes containing gallium citrate (⁶⁷G) in subcutaneous tumors in rats and found that small unilamellar vesicles preferentially accumulate in these tumors [56]. Accumulation of small unilamellar vesicle encapsulated indium (¹¹¹In) in a variety of solid tumors in humans was demonstrated by Present et al [57].

As with regions of infection and inflammation, the precise mechanism whereby liposomes accumulate in tumor is not clear. However, it is generally believed that leakage of vesicles from an incomplete or destroyed tumor vasculature is at least partly involved [42]. Liposomes have been used experimentally in virtually every area of medicine and have been administered by every traditional route. Encouraging results with liposomal drugs in the treatment or prevention of a wide spectrum of diseases in experimental animals and in humans indicate that liposome based products for clinical and veterinary applications may be forthcoming [58].

Physical and chemical evaluation of liposomes plays a major role during liposome preparation. Gabriels et al have discussed about the physical and chemical evaluation of liposomes, containing artesunate [59].
3.3 TOXICOKINETIC STUDY

The importance of toxicokinetic (TK) studies in the drug development from discovery phase to NDA has been recognized in the last decade for many reasons other than the requirement of FDA in the course of conducting toxicity studies [60, 61].

As a unique expansion of the science of pharmacokinetics, TK studies provide the information on the systemic exposure of the drug in species used for toxicity studies after single and multiple dose administration and better characterize gender and species differences in exposure relative to the dose [62, 63].

The TK information is not only used to support the selection of species, dosing route and form, dosing regimen, and the study design of the subsequent pre-clinical toxicity studies, it also helps to establish the correlation of systemic exposure with toxicological findings. These parameters are all critical to assessing the relevance of pre-clinical toxicity study results to clinical safety [65-68] and aid in the determination of the margin of safety between nonclinical safety studies and human plasma concentrations achieved in clinical trials.

The TK data can also be used to predict the drug exposure and possible bioaccumulation under multiple dose regimes in humans by interspecies scaling in conjunction with other pharmacokinetic and metabolism data [69-71] These data become an essential parameter for establishing the clinical dose regimen [72, 73].

The awareness of the relationships between the time course of the drug to circulating metabolite(s) and the onset of toxic events are setting an entirely new dimension to the conduct, interpretation, and meaning of toxicology studies. By monitoring the concentrations of major circulating metabolites, the correlation between the metabolite plasma concentrations and the toxicological findings, as well as the species differences, can also be determined. The ADME data derived from toxicology studies will also help to establish the relevance to humans of drug and metabolite exposure in animals, which permit the estimation of a safe starting dose in humans [74].
3.4 IRINOTECAN

The antineoplastic agent Irinotecan hydrochloride (7-ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy-camptothecin (CPT-11) is a semisynthetic derivative of the natural product camptothecin.\textsuperscript{[75, 76]} SN-38, 7-ethyl-10-hydroxycamptothecin is an active metabolite of Irinotecan (CPT-11).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{structure.png}
\caption{Structure of Irinotecan hydrochloride trihydrate}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{structure2.png}
\caption{Structure of 7-ethyl-10-hydroxycamptothecin (SN-38)}
\end{figure}

In the 1970s preliminary clinical trials with the water-insoluble natural alkaloid camptothecin demonstrated promising antitumor activity. Unfortunately, severe side effects were also observed. When it was discovered that the antitumor activity of camptothecin resulted from its ability to inhibit topo-isomerase I inhibitors, its structure was modified to create analogues with better water solubility and less severe side effects.
Camptothecin and its analogues contain a lactone group which is believed to be essential for inhibition of the DNA-enzyme complex. The biological activity of CPT-11 results from its capacity to bind to the transient cleavable complex formed between DNA and topoisomerase I, a nuclear protein involved in DNA replication, preventing dissociation of the DNA-topoisomerase I complex and thereby inhibiting enzyme activity [77–80]. In vivo, prodrug CPT-11 is converted by carboxylesterase into its active metabolite SN-38 (Fig. 8), the cytotoxicity of which is far greater than that of unchanged CPT-11 [81]. SN-38 is further conjugated by UDP-glucuronosyltransferase 1A1 [82] primarily in the liver to yield SN-38 glucuronide (SN-38-G) excreted in urine and bile. Hydrolysis of SN-38-G by the intestinal microflora can occur and allows possible recycling of SN-38 in humans [83]. Several oxidative CPT-11 metabolites have been identified in the human plasma: the major derivative is 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]-carbonyloxy camptothecin (APC) [RPR121056A (figure no.8)], resulting from a double oxidation of the terminal piperidine ring and generated by CYP450 3A4 [84].

Dodds et al. [85] identified in the plasma of patients receiving CPT-11 and in human liver microsomal incubations a new metabolite 7-ethyl-10-[4-(1-piperidino)-1-amino]-carbonyloxy camptothecin (NPC), produced by cleavage of the distal piperidine ring of Irinotecan by cytochrome CYP3A4. Several other oxidation metabolites have been identified in the bile and urine of a female patient (53-yr-old) treated with CPT-11 [86]. Only APC has been evaluated for its biological activity and demonstrated a weaker inhibitory capacity of cell growth in culture in comparison to SN-38 [87]. The cytotoxic capacity of other metabolites remains unknown. Research on toxicity study suggests that the cytotoxicity of SN-38 is due to double-strand DNA damage produced during DNA synthesis when replication enzymes interact with the ternary complex formed by topoisomerase I, DNA and SN-38. Mammalian cells cannot efficiently repair these double-strand breaks [88].
A correlation between the plasma level of SN-38 and the efficacy of CPT-11 has been demonstrated in effective concentration analysis [89]. Plasma levels of SN-38 affect antitumor efficacy and the presence of toxic side effects [89, 90]. Delayed diarrhea is thought to be a dose-limiting toxicity following administration of CPT-11. The relationship among the plasma levels of CPT-11, SN-38 and SN-38G has been studied in terms of CPT-11-induced intestinal toxicities [90-92]. Thus, the relation among those three compounds is essential for efficacy and toxic side effects. However, the \textit{in vivo} behavior of CPT-11, SN-38 and SN-38G have not been performed extensively for the prolonged-release systems of CPT-11.

The conversion of CPT-11(micromolar plasma levels) to SN-38 (nanomolar plasma levels) is highly variable and interpatient variability in the metabolism of SN-38 to form SN-38 glucuronide has been reported [93]. The variability and unpredictability in the

\textbf{Figure No: 8. Metabolism of CPT-11 into SN-38, SN-38-G, APC, and NPC}
CPT-11 to SN-38 metabolic conversion rates pose significant life threatening toxicity risks and complicate clinical management of patients. SN-38, on the other hand, has an advantage over its camptothecin precursors in that it does not require activation by the liver, thereby eliminating the interpatient variability. Additionally, SN-38 is approximately 1000-fold more potent than CPT-11 as a topoisomerase I inhibitor [88]. \textit{In vitro} cytotoxicity studies suggest that SN-38 is up to 1000-fold more potent than CPT-11 against several tumor cell lines [88, 94]. Moreover, the biological half-life of SN-38 is much longer than that of CPT-11, thereby representing a great pharmaceutical advantage over CPT-11 as a potentially highly effective antineoplastic agent. Nonetheless, SN-38 is poorly soluble in aqueous solutions, and is practically insoluble in most physiologically compatible and pharmaceutically acceptable solvents, including ethanol, polysorbate 80 and cremophor. Formulation of SN-38 in concentrated pharmaceutical delivery systems for parenteral administration is thus very difficult. In addition, SN-38 has a low affinity to lipid membranes and tends to precipitate into aqueous phase resulting in a very low drug-to-liposome entrapment [95].

The basic labile characteristic of lactone E ring in Irinotecan is reversible and pH dependent hydrolysis yields the inactive carboxylate species (Figure No. 9) [96]. Only lactone ring can inhibit topoisomerase I but this active molecule is very rarely found under physiological conditions [97]. Consequently finding an effective drug delivery system to reduce the toxicity of the drug is important. Liposomal carriers have the potential to improve the therapeutic activity of anticancer drugs. Improvements manifested by encapsulation of the active agent can include reduced toxicity, increased drug stability, improved drug distribution measures, and most importantly, improved therapeutic effects [98-100].

Literature reports a novel method for the preparation of SN38 liposomes [101]. This SN-38 liposome shows a very high trapped ratio (95%). However, SN-38 is 100 times more expensive than Irinotecan. Nevertheless, very few published reports investigated in depth the preparation and stability of liposomal Irinotecan.
Several HPLC methods have been developed to quantify Irinotecan and its major metabolites in human plasma \[102-111\]. These methods involve the simultaneous quantification of the lactone and carboxylate forms or quantification of the total forms, i.e., lactone plus carboxylate. In most of these methods, both CPT-11 and SN-38 were quantified and the related compound, camptothecin was used as the internal standard. In some of these published methods, chromatography was carried out with fluorescence detection. Most reported approaches involve long run time and tedious sample preparation procedures; Liquid chromatography–mass spectrometry methods have also been described \[112-116\] to support preclinical and clinical studies of CPT-11. Most of these
HPLC and LCMS methods have been reported in human, monkey and mouse plasma and also in urine, tissue homogenates and feces.

The carboxylate form of Irinotecan was completely converted into the lactone form by performing protein precipitation using a mixture of methanol and 5% perchloric acid in water (1:1, v/v). For the compounds included in this assay fluorescence detection appeared to be on an average two times more sensitive than MS detection. Camptothecin analogues may have a higher fluorescent response than ionization efficiency. In addition, assay performance was better for data generated using fluorescence detection than for data generated using MS detection when quality control samples with the same concentration for both methods were compared. However, for the determination of APC, which is expected to be a major product in clinical samples, selective MS detection is preferred as other metabolites may overlap its peak in the LC-fluorescence assay \[^{113}\].

The preclinical studies are the preliminary studies for novel drug delivery systems like liposomes, microspheres and nanoparticles. Although several published methods are available for clinical pharmacokinetic evaluation, a small number of reports are available for Irinotecan in rat models. A sensitive, simple and reliable method with wide calibration range is a prerequisite for pharmacokinetic and toxicokinetic evaluation of Irinotecan novel drug delivery systems.