INTRODUCTION
1. Introduction

Cancer can be broadly defined as a disease involving heritable defects in cellular control mechanisms resulting in the formation of malignant and usually invasive tumors. Next to heart disease, cancer is the major cause of death in the world. In simple words, cancer is the result of uncontrolled replication of a ‘rebel’ cell, which does not obey the rules of nature. Cancer can occur anywhere in the body (except hair and nail). The cancerous cells (cells that have unlimited proliferating capability) can spread to other areas in the body (metastases). Cancer carries high morbidity and mortality.

The development of cancer in a tissue depends upon a combination of the following factors:

(a) Exposure to cancer-causing agents (carcinogens)
(b) Hereditary factors (Oncogenes)
(c) Tissue susceptibility.

The exposure to carcinogens causes the highly reactive carcinogenic species to alter the growth characteristics of the cell thus converting it into a cancerous cell. The conversion of a normal cell into a cancer cell – “Carcinogenesis” – mediated by a carcinogen is a multi-step process and is schematically illustrated in Fig. 1.1.
Fig 1.1: Schema of multi-step carcinogenesis
Exposure to potent carcinogens can occur in any of the following ways.

a) Pollutants in the air (CO\(_x\), SO\(_x\), NO\(_x\), tobacco smoke, automobile exhaust, industrial fumes etc.)

b) Dietary factors (high animal fat content, low fiber content 'junk foods', excessive alcohol consumption, high salt, spicy and deep fried food, consumption of adulterated food and vegetables containing persistent pesticides)

c) Occupational exposure (exposure to hazardous chemicals in the industries like Dyeing, Mining, Plastic, Insecticide, Rubber, Cement, Metal finishing, Chemicals etc.)

d) Ultraviolet radiation exposure (Mainly from the solar ultraviolet rays that reach the earth due to ozone layer depletion)

e) Ionizing radiation (Cosmic rays from space and from man-made sources in earth)

f) Infectious agents (Epstein Barr virus, Hepatitis B virus, Human Papilloma virus, Human Immunodeficiency Syndrome virus etc.)

g) Drugs

h) Electromagnetic radiation (from power lines, cellular phones etc.)

1.1 Cancer therapy

Different methods of therapy are being used and are under investigation to cure cancer. Still, a complete cure for cancer is yet to be achieved. The nature and basic approaches to cancer treatment are constantly changing. Some of the major therapeutic measures used widely to combat cancer are:
(1) Surgery (Remove the cancerous portion surgically)

(2) Radiotherapy (Kill the cancer cells using high energy radiation)

(3) Brachytherapy (Kill the cancer cells by implanting a radioactive source within the tumor)

(4) Hypothermia (Arrest the growth of cancer cells by freezing them)

(5) Ultrasonic therapy (Kill the cancer cells by using ultrasound)

(6) Gene therapy (Use programmed genes to reverse carcinogenesis — Under development)

(7) Chemotherapy (Use of cytotoxic chemicals to kill cancer cells)

Among the various methods listed above, a single method is not sufficient to eradicate cancer. A combination of various methods is essential to control the spread of cancer. An early detection of malignancy gives a better chance to combat cancer. Drug research (chemotherapy) might provide the ultimate cure for cancer, though it seems far away. Many new compounds with potential anti-cancer activity are being discovered and synthesized regularly. A thorough understanding of their pharmacology, drug interactions and clinical pharmacokinetics is essential for their safe and effective use in human beings. It is unlikely that new therapies will totally replace existing drugs, as these drugs have become increasingly effective over the years. Drugs from both natural sources and those prepared synthetically have been used successfully in the treatment of cancer.

Anti-cancer drugs are usually classified according to their site and mode of action on the cancer cell. The important classes of anti-cancer drugs are:

(a) Alkylating agents (cyclophosphamide, chlorambucil, busulphan, thiopeta)

(b) Antimetabolites (methotrexate, fluorouracil, mercaptopurine, thioguanine)
Natural products

1) Plant products (vincristine, vinblastine, paclitaxel, teniposide, camptothecin)
2) Antibiotics (doxorubicin, bleomycin, daunorubicin, mitomycin)
3) Enzymes (L-asparaginase)
4) Biological response modifiers (interferon-alfa)

Hormones (prednisone, tamoxifen, testosterone propionate, estrogens)

Miscellaneous agents

1) Platinum coordination complexes (cisplatin, oxaliplatin)
2) Adrenocortical suppressant (mitotane, aminogluthethimide)
3) Substituted urea (hydroxy urea)
4) Methyl hydrazine derivative (procarbazine)
5) Anthracenedione (mitoxantrone)

These drugs are used either individually or in combination with each other for cancer therapy. Drugs are generally more effective in combination and may be synergistic through biochemical interactions. These interactions are useful in designing new multi-drug regimens. Many successful trials have been conducted with multi-drug regimens and as a result combination chemotherapy is now almost a standard approach to combat cancer.

An ideal anticancer drug should eradicate cancer cells without harming normal tissues. Unfortunately, no currently available agents meet this criterion and the clinical use of these drugs involves a weighing of benefits against toxicity in a search for a favorable therapeutic index. A drug is any small molecule that, when introduced into the
body, alters the affected organ's function by interactions at the molecular level. The therapeutic and toxic effects of drugs are a result of their interactions with different molecules and ions present within the cells in the body.

The usefulness of drugs in cancer chemotherapy has been limited by its numerous side effects. These side effects occur because the anti-cancer drugs are unable to differentiate between cancer cells and rapidly dividing normal cells. Some of the side effects might lead to severe deterioration in the health condition of the patient and might result even in death. Therefore, a complete knowledge of the molecular mechanisms by which a drug acts is essential in determining its efficacy, toxicity and optimal dosage.

The primary site a drug encounters before entering into a cell is the cell membrane. Therefore, a detailed study of the interaction of these drugs with the cell membranes might provide an insight into the molecular mechanisms underlying the toxic side effects of drugs.

1.2 The cell membrane

The functions of the various cells within the body and the sub-cellular organelles vary widely. However, it was observed that the intracellular membranes, which are the structural elements of the organelles, had a common structure consisting of lipids and proteins. Cell membranes form the boundary between the internal aqueous compartment of the cell and the external environment. Carl Negeli first discovered the cell membrane in 1855. These observations were confirmed by the experiments of Wilhem Pfeffer in 1897. Charles Overton studied the properties of the cell membrane in 1899 and suggested that the cell membrane is selectively permeable to the passage of ions and molecules through it and therefore presents a water-resistant barrier. The composite
arrangement and functions of the membrane components have led to the development of
the specialized field called ‘membranology’.

The measurements of the physical properties of the cell membranes such as
electrical capacitance, resistance, surface tension, osmotic properties and surface area
resembled those of isolated lipids, which led to the conclusion that the cell membrane
consists mainly of lipids\textsuperscript{5,6}. The structure of the plasma membrane was not resolved until
1925 when Gorter and Grendel suggested that the plasma membrane contains lipids in the
form of a bilayer\textsuperscript{5-9}. This conclusion was based upon experiments conducted by
Langmuir on surface films of lipids. Davson and Danielli confirmed these suggestions in
1935 and proposed their ‘sandwich model’ for the arrangement of the lipids. According
to them, the membrane is a bimolecular lipid layer; with the polar ends of the lipids
facing outward and hydrophilic proteins coat these polar ends\textsuperscript{5-9}. J.D.Robertson in 1955
altered this concept and proposed the now famous ‘unit membrane concept’ to explain
the structure of the cell membrane. According to this model, all membranes are based on
a “unit” with a lipid bilayer sandwiched between different proteins on the exterior and
interior surfaces\textsuperscript{5-9}. Advances in electron microscopy and biophysical techniques during
the 1960s led to a revision of the unit membrane model. After a thorough analysis of the
membrane structure, J.Singer and G.Nicolson proposed in 1972, the widely accepted
‘fluid-mosaic theory’ of membrane structure. The fluid-mosaic structure of the biological
membrane is given in Fig.1.2.
Fig. 1.2: Fluid-mosaic structure of the biological membrane
The fluid mosaic model builds on the asymmetric bilayer proposed by the unit membrane theory. The proteins are however disposed more variably in the structure. Some of them span the bilayer completely and have part of their structures facing both the inside and outside compartments. A few may be inserted into the bilayer from one side or the other without passing completely through. These classes of proteins are known as the 'integral' or 'intrinsic' membrane proteins. They interact with the lipids primarily by hydrophobic forces and cannot be removed unless the bilayer is disrupted. They also interact with the hydrophilic heads of the lipids. The 'peripheral' or 'extrinsic' proteins comprise the other class of proteins that are attached to the head groups of lipids, or to the superficial portions of the integral proteins. They can be easily dislodged by mild treatments. The relative independence of the proteins from one another gives rise to the 'mosaic' concept. The idea of 'fluidity' depends upon the mobility of the lipids comprising the bilayer. Any change brought about in the fluidity of the membrane lipids would bring about a corresponding change in the conformation of proteins and the lipid-lipid, lipid-protein and protein-protein interactions. This forms the crux in many drug-membrane studies. The cell membrane is an important part of the cell and performs many functions. Some of the important functions of the cell membrane are listed below5-8.

(1) The cell membrane exhibits 'selective permeability', i.e., it can select among different molecular species, slowing down the permeation of some materials while allowing others to pass almost unimpeded.

(2) It can bring about 'active transport', i.e., transport of material inward (accumulation) or outward (excretion, secretion) against both concentration and electrical potential gradients.
(3) Membranes provide a structural framework for the cell and some membrane-associated enzymes.

(4) Membranes contain 'receptors' for transmission of information from and into the cell. These receptors are sensitive to extremely low concentrations of substances in the environment.

(5) Membranes sometimes contain cellular extensions (cilia), extracellular structures (flagella), and intracellular structures (cytoskeleton) apart from the proteins, receptors and enzymes.

The protein and lipid compositions of the cell membrane depend upon its specialized function. For instance, the mitochondrial membrane is rich in proteins and enzymes, as it has to generate energy from various oxidation and reduction reactions. The nerve membrane, on the other hand, has fewer proteins as it is mainly involved in the electrical signal conduction. Though the structure, conformation and number of proteins in the membrane vary, it is widely accepted that the bimolecular layer of lipids is a basic structural feature of membranes.

1.3 Membrane lipids

Phospholipids and sterols make up about half the mass of biological membranes. The most abundant membrane lipids are the glycerophospholipids or phosphoglycerides, in which the hydrophobic regions are composed of two fatty acids joined to glycerol. This fact is presented in Table 1.1. Other membrane lipids include cholesterol, sphingomyelin and cardiolipin.
Table 1.1: Lipid composition of membranes from various sources\(^6\).
* The values denote the percentage composition of the total lipid dry weight.

<table>
<thead>
<tr>
<th>Source</th>
<th>Phospholipids* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma membrane</td>
<td>70</td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>85</td>
</tr>
<tr>
<td>Endoplasmic reticulum (rough)</td>
<td>81</td>
</tr>
<tr>
<td>Endoplasmic reticulum (smooth)</td>
<td>76</td>
</tr>
<tr>
<td>Mitochondria (outer)</td>
<td>83</td>
</tr>
<tr>
<td>Mitochondria (inner)</td>
<td>79</td>
</tr>
<tr>
<td>Human erythrocyte</td>
<td>70</td>
</tr>
</tbody>
</table>

Phospholipids contain a glycerol chain where two carbon atoms are acylated with bulky long chain fatty acids that are non-polar and the third carbon atom has a smaller, polar, phosphate-containing moiety. The polarity of this group is derived from the negatively charged phosphate group and sometimes, a positively charged amine group. Thus, all phospholipids exhibit an amphipathic character with distinct hydrophilic (polar) and hydrophobic (non-polar) regions. Since each of these lipids can contain many combinations of fatty acyl groups, the general name refers to the family of compounds, and not to a single compound. The most common phospholipid is phosphatidylcholine (PC). Other phospholipids include phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). The structures of the two common membrane phospholipids PC and PE are given in Fig. 1.3.
Fig. 1.3: Structure of phosphatidylcholine (left) and phosphatidyl ethanolamine (right).
The fatty acyl chains $R_1$ and $R_2$ in the phospholipids might be the same or different. More than 100 different fatty acids have been identified in microorganisms, plants and animals. The fatty acids differ from one another in the length of their hydrocarbon tails, the degree of unsaturation and the positions of the double bonds in the chains. These factors affect the nature of packing of the lipids in the bilayer of biological membranes. Some fatty acids commonly found in biological membranes are shown in Table 1.2.

<table>
<thead>
<tr>
<th>No. of Carbon atoms</th>
<th>No. of double bonds</th>
<th>Common name</th>
<th>IUPAC name</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>0</td>
<td>Laurate</td>
<td>Dodecanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{10}\text{COO}^-$</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>Myristate</td>
<td>Tetradecanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{12}\text{COO}^-$</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>Palmitate</td>
<td>Hexadecanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{14}\text{COO}^-$</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>Stearate</td>
<td>Octadeconoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{16}\text{COO}^-$</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>Arachidate</td>
<td>Eicosanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{18}\text{COO}^-$</td>
</tr>
<tr>
<td>22</td>
<td>0</td>
<td>Behenate</td>
<td>Docosanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{20}\text{COO}^-$</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>Lignocerate</td>
<td>Tetracosanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{22}\text{COO}^-$</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>Palmitoleate</td>
<td>$\text{Cis} , \Delta^9$ - Hexadecanoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{7}\text{CH}=\text{CH}(\text{CH}<em>2)</em>{3}\text{COO}^-$</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>Oleate</td>
<td>$\text{Cis} , \Delta^9$ - Octadeconoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{7}\text{CH}=\text{CH}(\text{CH}<em>2)</em>{3}\text{COO}^-$</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>Linoleate</td>
<td>$\text{Cis}, \text{cis} , \Delta^9,12$ - Octadecadienoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{14}(\text{CH}=\text{CH}\text{CH}<em>2)</em>{2}\text{COO}^-$</td>
</tr>
<tr>
<td>18</td>
<td>3</td>
<td>Linolenate</td>
<td>All $\text{cis} , \Delta^9,12,15$ - Octadecatrienoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{16}(\text{CH}=\text{CH}\text{CH}<em>2)</em>{3}\text{COO}^-$</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>Arachidonate</td>
<td>All $\text{cis} , \Delta^5,8,11,14$ - Eicosatetraenoate</td>
<td>$\text{CH}_3(\text{CH}<em>2)</em>{18}(\text{CH}=\text{CH}\text{CH}<em>2)</em>{4}\text{COO}^-$</td>
</tr>
</tbody>
</table>

Table 1.2: Common fatty acids present in biological membranes.
Depending on the precise conditions and nature of the lipids used, three types of lipid aggregates can be formed with amphipathic lipids.

(a) **Micelles**, which are relatively small, spherical structures involving a few dozen to a few thousand lipid molecules arranged such that their hydrophobic hydrocarbon chains aggregate in the interior, excluding water, and their hydrophilic head groups are at the surface, in contact with water. Micelle formation is favored when the cross-sectional area of the head groups is greater than that of the acyl side chains, as in free fatty acids and lysophospholipids.

(b) **Lipid bilayers**, which are formed when two lipid monolayers combine to form a two-dimensional sheet. Bilayer formation occurs readily when the cross-sectional areas of the head groups and acyl chains are similar as in glycerophospholipids. The hydrophobic portions in each monolayer interact, excluding water. The hydrophilic head groups interact with water at the two surfaces of the bilayer. This is the arrangement of lipids in biological membranes.

(c) **Liposomes** are formed when a lipid bilayer folds back on itself to form a hollow sphere. These structures can enclose water or other molecules in an aqueous compartment. By forming liposomes, the bilayer sheets lose their hydrophobic edges. The formation of liposomes is promoted by external sources such as ultrasound.

Fig. 1.4 gives the structures of these three aggregates.
Fig. 1.4: Structures of some lipid aggregates

a) Micelle

b) Planar Lipid Bilayer

c) Liposome
1.4 The lipid bilayer of biological membranes

Only amphipathic lipids can form the bilayer structure of membranes while hydrophobic lipids such as triacyl glycerols do not form bilayers. The lipid bilayer is typically about 50-60 Å thick and consists of two leaflets or layers. In each leaflet, the polar head groups of the amphipathic lipids are in contact with the aqueous environment and the non-polar hydrocarbon tails point towards the interior of the bilayer.

The cell membrane is analogous to the protective wall around a house with the proteins and receptors acting as the doors and windows. The cell organelles like DNA, mitochondria etc., are analogous to the valuables in the house. In order to interact with the subcellular organelles, the drug has to first get inside the cell. Permeation of drugs through the membrane can occur by:

(a) Aqueous diffusion
(b) Lipid diffusion
(c) Facilitated diffusion (via special carriers) and
(d) Pinocytosis (receptor-mediated endocytosis)

The permeation of drugs via (a), (c) and (d) requires the presence of specific protein receptors. Each drug or molecule has a unique receptor, which aids its transport across the cell membrane. The receptor for a particular drug might not serve as a receptor for another. The receptor for a particular drug may not be available in all cell membranes and might be restricted to the membranes in some specialized areas. Therefore, when a drug reaches the cell membrane, it can get inside the cell via specialized carriers or receptors, if available, or else its entry into the cell will depend upon its lipid solubility.
and interactions with the lipid bilayer. This characteristic gives rise to two kinds of interaction:

(a) The interaction of drugs with the unique proteins and receptors comprising its specialized transport systems (Specific interaction) and

(b) Non-specific interactions with the lipid bilayer, which is present in all membranes.

In a layman’s words, a person (drug) can enter a house (cell) either through the windows and gates (proteins) or can break through the wall (lipid bilayer) like an intruder. The breaking of the wall could take place as a quick ‘demolition’ or could be a gradual ‘infiltration’ with a steady rise in tempo. As the specific transport into the cell is programmed and controlled by the cell, it is the non-specific entry of the drug molecules into the cell through the lipid bilayer that arouses interest, as the cell does not control it. These non-specific interactions could take place even in non-target cells, as it requires only the lipid bilayer construct. Since the lipid bilayer matrix is the common structural construct in all membranes, such type of drug-lipid interactions could well be responsible for the undesired side effects caused by the drugs.

The transport of drugs into the cells through specific receptors and carriers has been well studied using both in vitro and in vivo models. But in order to study the interactions of the drugs with the lipid bilayers, model systems simulating the lipid bilayer architecture have been found extremely helpful. This is because of the complex nature of the biological membrane. It becomes difficult to separate the interactions between lipids and the drugs in a complex system due to interference from the proteins and other receptors. Hence, lipid-drug interactions are investigated using
artificially constituted bilayer model systems\textsuperscript{10}. The rationale behind this investigation is that an elucidation of these simpler model systems will provide an understanding of similar and comparable phenomenon in biological membranes\textsuperscript{10-13}. After having understood the simple model system, further complexity can be introduced, different models may be combined, \textit{in vitro} and \textit{in vivo} studies can be performed to understand the complete mode of interaction of drug with the biological membrane\textsuperscript{13}.

1.5 Model systems for lipid bilayers

The function of a biological membrane is determined to a great extent by the structural and dynamic properties of its two main components namely, the lipids and proteins, as well as by their reciprocal interactions. Because of the complicated nature of the biological membrane, it is a sound strategy to study the properties of these components using experimental models. Some of the widely used models to simulate the lipid bilayer architecture of biological membranes include lipid monolayers at the air-water interface, planar Bilayer Lipid Membranes (BLM), micellar liposomes or in general, organized systems of lipids\textsuperscript{10-14}. Among the different models, the most important are the molecular lipid bilayers, which are very useful in studying the transport properties of membranes. One prime objective in using phospholipid models is to reduce the chemical complexities of biological membranes. Apart from their simplicity, electrical stress can be easily applied to either side of the model membrane. Ion transport studies can be easily followed since both sides of the membrane are amenable to variations in experimental parameters such as ionic concentration, ionic nature, pH and hydrostatic pressure. Moreover, the investigation of a possible analogous mechanism in biological membranes can be developed based on the results using model membranes\textsuperscript{14}. Liposomes
or phospholipid vesicles are widely used as drug delivery systems. The charge and magnetic properties of these liposomes can be manipulated and they are used to hoodwink the defense barriers presented by the biological membrane, and deliver the drug into the target cell. Such liposomes known as the ‘stealth liposomes’ have been developed to deliver drugs that are otherwise unable to partition into the target cell\textsuperscript{15,16}.

1.6 Planar Bilayer Lipid Membranes (BLM)

Hanke and Schlue\textsuperscript{13} have defined planar lipid bilayers as lipid double layers in a hydrophilic environment. Donald Rudin, William Wescott, Paul Mueller and H.Ti Tien developed the technique for the formation of planar bilayer lipid membrane (BLM) in 1962. The BLM can be easily prepared as a diffusion barrier separating two aqueous compartments, each of which is open and accessible for sampling and medium control\textsuperscript{11,13}. Wobschall has described BLMs as excellent models of the lipid component of biological membranes\textsuperscript{17}. Similarly White et al., describe BLM as an important model system for studying molecular interactions that occur in biological membranes\textsuperscript{18}. Fig.1.5 gives the schematic diagram of a lipid bilayer and Fig. 1.5.a shows the magnified picture of a stabilized BLM given by White et al\textsuperscript{18}. 
Fig. 1.5: Structure of planar lipid bilayer membrane (BLM)
Fig. 1.5.a: Photograph of a planar lipid bilayer membrane. The magnification factor is 33. The broad dark circle is the annulus whose outer edge corresponds to the aperture diameter. The inner narrow white circle is the beginning of the transition between the annulus and bilayer film.

The formation and stabilization of the BLM is spontaneous as it involves the formation of a structure with the least free energy. Tien et al., have suggested that the thinning and spontaneous phase transitions of BLM occur due to the Plateau-Gibbs border suction, gravitational flow and diffusion\textsuperscript{11}. The formation of BLM, once initiated, proceeds until the whole film reaches a configuration having the lowest possible free energy. The overall thinning process is suggested to be a combination of simple mobile, regular mobile and rigid thinning\textsuperscript{11,12}. The final thinning step has been attributed to chance contacts between the two interfaces of the lipid, caused by thermal motion, mechanical vibration, trace impurities such as dust particles or local variations in the interfacial tension. As the membrane gradually thins by diffusion of lipid solution into the aqueous phase and border suction, the probability of chance contact increases. Once the two interfaces come close enough together, van der Waals’ attractions between the opposing hydrocarbon chains are strong enough to produce a bilayer. In forming this bilayer, adjacent molecules are drawn sufficiently close to be attracted to the opposite interface. This effect known as the “Zipper effect” is responsible for the growth of the BLM\textsuperscript{11,12}. Fig. 1.6 depicts the various stages observed during the thinning of the BLM.
Fig. 1.6: Various stages during the thinning of BLM
1.7 Characterization of drug-membrane interactions

The experimental parameters to study these model systems include variations in temperature, pH, bath medium, measurement of electroosmosis and permeability of water, ions and other solutes and physical characterization by spectroscopic, thermal and electrical measurements involving capacitance, resistance and diffusion potentials\(^{11}\). Many sophisticated techniques such as spectrofluorimetric analysis\(^{20-23}\), phase transition studies\(^{24-26}\), NMR spectroscopy\(^{27}\), FTIR spectroscopy\(^{18}\), Differential Scanning Calorimetry (DSC)\(^{28-30}\) etc., have been used to probe the drug-membrane interactions. The conclusions derived from these studies have been useful in correlating membrane function to the macroscopic structure and additive-induced changes. Electrical measurements also have been employed extensively to characterize the BLM properties as they provide simple means to monitor the interactions between the BLM and the drug\(^{31-36}\). They are also reliable and economical. A.C measurements provide an insight into the mechanism of drug-membrane interactions, emphasizing the surface interactions\(^{37}\) while D.C studies provide an insight about the changes produced in the permeability characteristics of the BLM\(^{11}\). The present study employs both A.C and D.C electrical measurements to characterize the drug-membrane interactions.

1.8 Importance of drug-lipid interactions

Many investigative studies have been carried out using model systems to ascertain the importance of lipid-drug interactions\(^{14,38-39}\). The binding to phospholipid membranes has been related to the biological effects of drugs, for instance, sodium channel blockade and drug-induced lipidosis\(^{24}\). Various functional and structural changes in biomembrane
interfaces have been reported to be directly or indirectly associated with changes in interfacial polarity that affects the extent and stability of the electrostatic interactions at the charged membrane surface\textsuperscript{40-41}. Ricardo Diaz et al., have reported that the myelin (associated with the nerves) lipids are the primary targets for heavy metal neurotoxicity\textsuperscript{42}. Neurotoxic organotin derivatives such as triethyltin produce a specific damage in the myelin membrane as observed in experimental animals\textsuperscript{43}.

Drug-membrane interactions play a crucial role in anti-cancer drug cytotoxicity. For instance, the alterations observed in the membrane-mediated cellular responses of tumor cells have been attributed to the differences in the ability of the anticancer drug daunomycin to partition within the lipid bilayer\textsuperscript{44}. Dietmar Porschke et al., have attributed the lipid phase transitions to be responsible for the effective transmission of electric signals to molecular processes as these transitions were capable of inducing large change in conformations of membrane associated proteins and lipids by relatively small changes in the electric field strength\textsuperscript{45}.

Perhaps, the most significant findings emphasizing the importance of lipid-drug interactions come from the extensive studies done on anesthetics by earlier researchers. It has been now firmly established that the general anesthetics act by disordering the lipid region of membranes\textsuperscript{46}. The anesthetic effects of n-alkanes have been postulated to be due to their interactions with the lipid bilayer\textsuperscript{47}. The n-alkane chains intercalate between the lipid hydrocarbon chains and this hypothesis was supported by both x-ray and diffraction data\textsuperscript{47}. General and local anesthetics have been established to intercalate readily into the hydrophobic bilayer core of membranes thereby altering the packing of lipid molecules and also the proteins\textsuperscript{46}. The interactions of charged anesthetic molecules
with the surface of the lipid bilayer could constitute an additional mechanism by which
the electrophysiological properties of the membrane-bound proteins could be regulated\textsuperscript{46}.
Ueda et al., and Deamer et al., have confirmed the non-specific action of anesthetics on
the lipid bilayer of membranes through various model systems\textsuperscript{40,48,49} such as liposomes
and planar BLMs. The correlation between the responses of animals (\textit{in vivo} studies),
synaptic vesicles and artificial bilayers to anesthetics\textsuperscript{48} has demonstrated the validity of
using model membranes to study anesthesia.

The pharmacological activity of neuroactive drugs and their relative potency were
assessed using investigations on lipid bilayer model systems by Jayaraman et al\textsuperscript{50}. According to Beigi et al., there exists a strong interaction between the drugs and
phospholipid bilayers, which influences the permeability of the drug into the
membrane\textsuperscript{51}. Qiang Hao et al., have suggested that the bilayer aggregation might
participate in the transport of proteins across membranes\textsuperscript{52}. Wolf et al., have reported that
the anti-fungal drug amphotericin B inserts disruptively within the lipid bilayer when the
lipid packing density is altered by other osmomechanical stresses\textsuperscript{53}. Lundbaek et al., have
suggested that changes in the lipid composition and fluidity alter the mechanical
properties of the lipid bilayer and the function of the associated integral proteins\textsuperscript{54}.

\textit{Escherichia coli} hemolysin has been shown to open ion conducting channels in
lipid bilayer model systems by Menestrina et al\textsuperscript{55}. Agawa et al.,\textsuperscript{56} have reported similar
observations for amphipathic peptides. Madeira et al., have established through
investigations on lipid-insecticide interactions that the perturbation of the bilayer
organization by the insecticides parathion, DDE, DDT, malathion and lindane is the
important step in the molecular mechanism of their insecticide action\textsuperscript{57-59}. Vidal et al.,
suggest that though the pyrethroid insecticide allethrin affects the cellular responses by direct action on the ionic channels and pumps, an indirect action on other membrane systems by inducing modifications of the physical properties of the membrane bilayer is also responsible for its toxic action. Videira et al., have suggested that both the functional and neurotoxic effects of the anti-arrhythmic drug perhexiline are due to the structural perturbations introduced by it on the lipid bilayer of membranes. Similarly, a change in the membrane bilayer organization by another anti-arrhythmic drug amiodarone has been implicated in its biochemical and physiological effects.

1.9 Drugs for the study

The anti-cancer drugs chosen for the present study are cisplatin, methotrexate and vincristine. These three drugs have different modes of action on the cancer cell. While cisplatin acts on the DNA, vincristine acts on the microtubules and methotrexate disrupts DNA replication. The three drugs differ in their structure and also in their activity against different types of cancer cell lines. However, the three drugs have a common feature in that they exhibit neurotoxic side effects. The molecular basis of these side effects is still the subject of active research. The possibility of their non-specific action on lipid bilayers leading to the neurotoxicity has not yet been explored and therefore, this forms the major objective of this study.

1.9.1 Cisplatin – Discovery and Synthesis

Alfred Werner first postulated the existence of cisplatin or cis-diamminedichloroplatinum (II) and its diastereomer transplatin or trans-diamminedichloroplatinum (II) in 1893. Chernyaev in 1926 confirmed the square planar nature of Pt (II) complexes. Grinberg provided proof for the spatial arrangement of
ligands in these diastereomers (cisplatin and transplatin) in 1931 based on their reactions with oxalic acid\textsuperscript{63}.

The structures of cisplatin and transplatin are given below in Fig. 1.7.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{structures_cis_trans_platin.png}
\caption{Structures of cisplatin (a) and transplatin (b)}
\end{figure}

Fig. 1.7: Structures of cisplatin (a) and transplatin (b)
The Pt-Cl bond length is 2.33 Å and the Pt-N bond length is 2.01 Å. Cisplatin is prepared by the direct reaction of potassium tetrachloroplatinate with aqueous ammonia. The *trans* isomer is synthesized in two steps. First potassium tetrachloroplatinate reacted with excess ammonia to form platinum tetra ammine chloride, which was then heated in the presence of excess chloride (ammonium chloride) to produce transplatin. In both synthetic methods, the *trans* effect of ammine and chloride ligands plays an important part in determining the spatial orientation of the product\(^{63-66}\).

Cisplatin is a yellow coloured odourless crystalline substance with the molecular formula \((\text{NH}_3)_2\text{PtCl}_2\). Its molecular weight is 300 and has a melting point of 207°C\(^{67}\). Its solubility in water is limited. The Cl-Pt-Cl bond angle is 90°. The electron micrograph of cisplatin is given in Fig 1.8. Fig. 1.9.a represents the molecular model of cisplatin.

The *trans* isomer – transplatin – is a yellowish green powder with an offensive odour. It exhibits moderate solubility in water. Its molecular model is illustrated in Fig.1.9.b. The bond angle between Cl-Pt-Cl is 130°.
Fig. 1.8: Photomicrograph* (Photograph taken under microscope) of Cisplatin

** The magnification factor is 600

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* Photograph downloaded from Internet, Courtesy: National High Magnetic Field Laboratory, Florida State University.
Fig. 1.9.a: Molecular model of cisplatin.

Fig. 1.9.b: Molecular model of transplatin.
1.9.1.a Pharmacological importance

Cisplatin belongs to the class of pharmaceuticals that are coordination complexes. Though its chemical, physical and structural properties had been thoroughly investigated in co-ordination chemistry, its anti-cancer effects were unknown until Barnett Rosenberg accidentally discovered them in 1965 while investigating the effect of electric field on the growth of bacteria in ammonium chloride medium using platinum electrodes\textsuperscript{4,68,69}. Cisplatin has been used in single as well as multi-drug regimens to treat testicular and ovarian cancers\textsuperscript{4,68,70}. It is also used in the treatment of head and neck, lung, cervical and bladder cancers. The trans isomer – transplatin, has a lesser cytotoxic activity\textsuperscript{68,69}. Nevertheless, it is more reactive kinetically\textsuperscript{63-66}. Recent reports have suggested that certain amine substituents in the trans configuration may render platinum more reactive and some trans analogues have been developed by Farrell et.al\textsuperscript{68}.

1.9.1.b Mechanism of anti-cancer activity

Three factors are reported to be necessary for a platinum compound to be active\textsuperscript{4,63-66,68,69}.

(a) The compound must be neutral.

(b) It must have two groups (like the chloride ligands in cisplatin) that will bind at two sites in a cis (not trans) configuration.

(c) The molecule must have two groups (like the NH\textsubscript{3} in cisplatin) that will not bind.

Cisplatin belongs to the \textbf{pseudo-alkylating} class of chemotherapeutic drugs. The intracellular milieu with low chloride concentration induces the loss of chloride ligands from cisplatin thus generating a reactive electrophile. These electrophiles interact with
the nitrogen atoms (N-7) of the guanine bases in the DNA (Deoxy Ribonucleic Acid)* and to a lesser extent with the nitrogen atoms of the adenine bases of DNA, to produce either inter- or 1,2-intrastrand cis-Pt(NH₃)₂ cross-links. The cisplatin-DNA adducts have been separated using various chromatographic techniques and quantitated by atomic absorption spectroscopy. The binding of cisplatin to DNA is the critical step in producing cytotoxicity. Apparently, once cisplatin binds to DNA, it blocks DNA's replication, hampering the spread of cancer. The trans isomer is unable to form such type of intrastrand bridges due to steric reasons and hence exhibits lesser cytotoxicity.

1.9.1.c Toxicity

The adverse effects of cisplatin are well documented. The general side effects associated with cisplatin are nausea and vomiting, nephrotoxicity (affecting the kidneys), irreversible ototoxicity (affecting hearing), neuropathy (affecting the nerves) and myelosuppression (affecting the blood platelets). Rare effects include visual impairment, seizures, arrhythmias, acute ischemic vascular events, glucose intolerance and pancreatitis. The peripheral neuropathy caused by cisplatin is cumulative. Though the effects of cisplatin neuropathy are reversible, the recovery is slow. The neurotoxicity of cisplatin generally affects the limbs (peripheral sensory neuropathy). Fig. 1.10 illustrates the pathological lesions and various clinical manifestations of cisplatin neurotoxicity.

* DNA contains the genetic code for replication of the cell. Blocking DNA synthesis or its function results in disruption of cell division. Chemotherapeutic agents take advantage of this fact to control the spread of cancer cells.
Fig. 1.10: Neurotoxic effects of cisplatin
Cisplatin-induced neurotoxicity is primarily sensory, with the loss of proprioception (L, Propio: One’s own, Captive: to take, Proprioception is the ability to receive stimuli from muscles, tendons and other organs) and vibratory sense. Though, motor function is not severely affected, the severe proprioceptive loss can lead to ataxia (gait abnormalities)\(^4,67,68\). Both CMTI (Charcot-Marie-Tooth International) and CMTA (Charcot-Marie-Tooth Association) have classified cisplatin as a potent nerve-damaging agent. Reports of neurotoxic manifestations as a consequence of cisplatin therapy have been reported by A.M. Smith, C.M. Bagley et al., R.F.Ozols et al., and Petrioli et al\(^67\).

The side effects caused by cisplatin are thought to be related to the presence of Pt (II) in the complex. This has lead the researchers at Leiden University, Netherlands to investigate the possibility of replacing platinum in cisplatin with ruthenium, which has similar properties but lesser toxicity towards kidneys and nerves\(^73\).

Cisplatin neuropathy also impairs the auditory nerves causing ototoxicity. Tinnitus (noises in the ear which may take the form of buzzing, clicking or thudding) and high frequency hearing loss has been observed in 31% of patients receiving cisplatin therapy. Usually, the hearing loss occurs in the frequency range greater than 2000 Hz, but may sometimes affect the normal hearing range between 250-2000 Hz also. The dose dependent ototoxicity is found to affect children severely. Hearing loss can be unilateral or bilateral and tends to become more frequent and severe with repeated doses and may not be reversible\(^74\).

1.9.2 Methotrexate

Methotrexate belongs to the antimetabolite class of chemotherapeutic drugs, which have been successfully used in the treatment of cancers since 1948\(^4\). The structure
Fig. 1.11: Photomicrograph* (Photograph taken under microscope) of methotrexate
** The magnification factor is 600

* Photograph downloaded from the Internet, Courtesy: National High Magnetic Field Laboratory, Florida State University.
Fig. 1.12: Structure of methotrexate.
1.9.2. a Mechanism of anti-cancer activity

Methotrexate is a tight-binding inhibitor of the enzyme dihydrofolate reductase, which is responsible for maintaining intracellular folic acid as tetrahydrofolate. Methotrexate binds to the active catalytic site of dihydrofolate reductase, thus interfering with the synthesis of tetrahydrofolate. Lack of this cofactor interrupts the synthesis of thymidylate, purine nucleotides and the amino acids serine and methionine, thereby interfering with the formation of DNA, RNA and proteins. These chains of events also lead to the inhibition of the cellular capacity to repair DNA, resulting in breaking of DNA strands. The cytotoxicity of methotrexate is dose dependent.

1.9.2. b Toxicity

The primary toxic effects of methotrexate therapy are myelosuppression (affecting bone marrow and blood platelets) and gastrointestinal mucositis (affecting the intestines). It is also associated with nephrotoxicity (affecting the kidneys) and hepatotoxicity (affecting the liver). Methotrexate causes a poorly defined, self-limited pneumonitis (affecting the lungs) characterized by fever, cough and interstitial pulmonary infiltrates. Methotrexate is extremely toxic to developing embryos. Hence, it is nowadays widely used as an agent to induce abortion. Additional toxicities of methotrexate include alopecia, dermatitis, defective oogenesis and spermatogenesis, and teratogenesis. Methotrexate also possesses neurotoxic side effects, which are being investigated. These effects are pictorially illustrated in Fig.1.13.
NEUROTOXIC MANIFESTATIONS OF METHOTREXATE
(CLINICAL & PATHOLOGICAL)

ORDINARY DOSE THERAPY

ACUTE (IMMEDIATE)
(ACUTE CHEMICAL ARACHNODITIS)

@ Severe Headaches
@ Nuchal Rigidity
@ Vomiting
@ Fever
@ Inflammatory Cell Infiltrate in the CSF (Cerebro Spinal Fluid)

SUB-ACUTE (AFTER 3-4 COURSES)
(USUALLY SEEN IN ACTIVE MENINGEAL LEUKEMIA)

@ Motor paralysis
@ Cranial Nerve Palsies
@ Seizures
@ Coma
@ Death

CHRONIC (AFTER MANY MONTHS/YEARS)
(DEMYELINATING ENCEPHALOPATHY)

@ Dementia
@ Motor Paresis
@ Acute Transient Cerebral Dysfunction
@ Paresis
@ Aphasia
@ Behavioral Abnormalities
@ Seizures

HIGH DOSE SYSTEMIC THERAPY

(ENCEPHALOPATHY)

@ Dementia
@ Motor Paresis

Fig.1.13 : Neurotoxic manifestations of methotrexate
Three distinct neurotoxic syndromes are associated with methotrexate therapy\textsuperscript{4,75}.

(a) Acute chemical arachnoiditis characterized by severe headaches, nuchal rigidity, vomiting, fever and an inflammatory cell infiltrate in the Cerebro Spinal Fluid (CSF). These symptoms can be diminished by decreasing the methotrexate dose or by discontinuation of methotrexate therapy.

(b) A sub-acute form of neurotoxicity, which is common in adults with active meningeal leukemia. Its manifestations include motor paralysis, cranial nerve palsies, seizures and coma. Continued methotrexate therapy might result in death.

(c) A chronic, demyelinating encephalopathy occurs in children after several months of therapy. The symptoms include dementia, limb spasticity and coma. CT (computerized tomography) scan reveals ventricular enlargement, cortical thinning and diffuse intracerebral calcifications in the brain.

Apart from these symptoms that are generally associated with ordinary dose methotrexate therapy, high dose systemic therapy leads to encephalopathy. High dose therapy is usually employed in cases with advanced lymphoblastic leukemia\textsuperscript{75}. The accompanying neurotoxic symptoms consist of dementia and motor paresis. Acute, transient cerebral dysfunction along with paresis, aphasia, behavioral abnormalities and seizures has also been reported\textsuperscript{75}. Intrathecal administration of methotrexate often causes meningismus and an inflammatory response in the CSF\textsuperscript{75}. In most cases, methotrexate neurotoxicity has been reported to be irreversible\textsuperscript{78}. 
The mechanism of central nervous system toxicity due to methotrexate is still unknown. The neurotoxicity of methotrexate is well documented. Mahoney Donald et al., have concluded after many phase trials in children that high dose methotrexate therapy invariably results in neurotoxic side effects. Similarly, O'Marcaigh et al., have described the neurotoxic effects caused in a patient inadvertently administered with an overdose of intrathecal methotrexate. Garcia-Tena et al., after conducting imaging studies on cancer patients undergoing methotrexate therapy have suggested that the neurotoxicity of methotrexate is irreversible. Confirmations of methotrexate-induced neurotoxicity are also found from the results of Winick et al., Millot et al., Balthasar et al., and Freilich et al.

1.9.3 Vincristine

Vincristine is a plant alkaloid and belongs to the antimicrotubule class of chemotherapeutic drugs. Its cytotoxicity was discovered accidentally. The extracts of the periwinkle plant, a species of myrtle (Vinca rosea Linn; Catharanthus roseus G. Don) belonging to the Apocynaceae family were being investigated for their possible beneficial effects in diabetes mellitus by Noble et al., in 1958, when their activity against cancer was first detected. These findings were confirmed by the experiments of Johnson et al., during the same period. Fractionation of these extracts led to the identification of four active dimeric vinca alkaloids: vinblastine, vincristine, vinleurosine and vinrosideine. Among these, vincristine and vinblastine were found to be effective anti-cancer agents. Desacetyl vinblastine or vindesine, a metabolite of vinblastine was introduced in the 1970s, but its efficiency against neoplasms is still uncertain. Recently, another vinca alkaloid, vinorelbine has also been identified as an
effective anti-cancer agent\textsuperscript{85}. The vinca alkaloids are extracted from natural sources and are also synthesized.

The vinca alkaloids are dimeric molecules having two multiringed units – an indole nucleus (catharanthine) and a dihydroindole nucleus (vindoline) Vincristine and vinblastine are structurally identical except for a single substitution on the vindoline nucleus, where vincristine possesses a formyl group and vinblastine a methyl group.

Vincristine is available as 22-Oxovincaleukoblastine sulphate\textsuperscript{67}. Its molecular formula is $\text{C}_{66}\text{H}_{56}\text{N}_{4}\text{O}_{16}\text{H}_{2}\text{SO}_{4}$ and it has a molecular weight of 923. It is a white, odorless, hygroscopic powder that is soluble in water, chloroform, and methyl alcohol, slightly soluble in alcohol and practically insoluble in ether\textsuperscript{67}. The structure of vincristine is given in Fig. 1.14.
Fig. 1.14: Structure of vincristine
Vincristine is effective in the treatment of both Hodgkin’s and non-Hodgkin’s lymphoma, acute lymphoblastic leukemia, Wilm’s tumor, Ewing’s sarcoma, neuroblastoma and rhabdomyosarcoma in children, and multiple myeloma, breast cancer and small cell lung cancer in adults. It is used both individually as well as in many multi-drug regimens. Many trials are being conducted with different combinations of vincristine with other chemotherapeutic agents such as bleomycin, idarubicin, cyclophosphamide, doxorubicin, ifosfamide, etoposide, teniposide, picarubicin, cepharanthin etc.

1.9.3. a Mechanism of anti-cancer activity

The principal site of action of vincristine is on the microtubules, which are integral components of the cell during cell division. Vincristine binds specifically to tubulin, a protein that polymerizes to form the microtubules. As a result, the microtubules become depolymerized and disorganized. This disrupts cell division and consequently arrests the uncontrollable spread of cancer cells.

1.9.3. b Toxicity

The utility of vincristine in chemotherapy has been sidelined mainly because of its numerous undesirable side effects. Vincristine along with cisplatin has been contraindicated for its adverse side effects. The principal toxic effect of vincristine is peripheral neurotoxicity. Initially, symmetric sensory impairment and paresthesias are encountered particularly at the fingers. Some of the earliest signs include numbness, tingling of the extremities (fingers and toes) and weakness of the distal limb musculature. Neuritic pain, loss of deep tendon reflexes followed by foot drop, motor dysfunction, ataxia and paralysis are also reported. Back, bone and limb pains have also
been occasionally reported. Cranial nerves may also be affected by vincristine resulting in hoarseness (due to laryngeal nerve paralysis), diplopia, jaw pain and facial palsies. Rare occurrences of CNS (Central Nervous System) disturbances including confusion, mental status changes, depression, hallucinations, agitation, insomnia, seizures, coma, inappropriate secretion of antidiuretic hormone (SIADH) and visual disturbances have also been experienced. Acute, severe autonomic neurotoxicity may arise as a consequence of high-dose therapy\textsuperscript{85}. The neurotoxic effects of vincristine have been well documented\textsuperscript{90}. Igarashi et al., have reported severe central neurotoxicity due to vincristine administration\textsuperscript{91}. Michelagnoli et al. have presented a similar case report, which highlights the significant after effects of vincristine overdose inspite of immediate salvage treatment\textsuperscript{92}. The neurotoxic side effects due to vincristine are also observed in combination chemotherapy involving vincristine\textsuperscript{93-94}.

Other toxic manifestations of vincristine include constipation, abdominal cramps, paralytic ileus, urinary retention, orthostatic hypotension and hypertension\textsuperscript{4,85}. The neurotoxic manifestations of vincristine therapy in humans are illustrated in Fig.1.15.
NEUROTOXIC EFFECTS OF VINCIRISTINE

CONFUSION, MENTAL STATE CHANGES, DEPRESSION, HALLUCINATION, AGITATION, INSOMNIA, SEIZURES, COMA

DIPOPIA

JAW PAIN

FACIAL PALSY

HOARSENESS

BONE PAIN

REPORTED PATHOLOGICAL LESION

1. NEURITIS
2. AXONAL DEGENERATION

BACK PAIN

MOTOR DYSFUNCTION

Limb PAINS

WRIST DROP

PARASTHESIAS

LOSS OF DEEP TENDON REFLEXES

ATAXIA

SYMMETRIC SENSORY IMPAIRMENT

FOOT DROP