# STRATEGIES TO OVERCOME PHYSICOCHEMICAL AND BIOLOGICAL BARRIERS IN CHEMOTHERAPY BY FORMULATION AND DRUG DELIVERY DEVICE COMBINATION

**By**

**MILINKUMAR N. SHAH**

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And approved by

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## ABSTRACT OF THE DISSERTATION

## STRATEGIES TO OVERCOME PHYSICOCHEMICAL AND BIOLOGICAL BARRIERS IN CHEMOTHERAPY BY FORMULATION AND DRUG DELIVERY DEVICE COMBINATION

**By MILINKUMAR N. SHAH**

**Dissertation Director:**

**Professor Tamara Minko, PhD**

The success of chemotherapy depends on efficacy of drug to enter the cancer cell to exert cytotoxicity. This feat is extremely challenging for drugs with low solubility and permeability which leads to inadequate concentrations in cancer cells resulting in therapeutic failure. For potent drugs like Paclitaxel (PTX), this drawback can be overcome by reformulation approach. The objective of the research is to improve PTX aqueous solubility and permeability by using suitable approach such as formulating inclusion complexes of PTX with various β-cyclodextrin (CD) derivatives. It was hypothesized that complexation will improve aqueous solubility of PTX and expand cellular internalization. This concept was verified by testing prepared complexes and understanding their physicochemical interactions. Later, the complexes were further evaluated with respect to excipient safety and efficacy of reformulated PTX under *in vitro* conditions. Achieving desired therapeutic levels within the brain for the treatment of neurological conditions and brain cancers is a formidable task due to blood brain barrier's (BBB) ability to restrict different drugs. A non-invasive approach of nose to brain delivery to bypass BBB is proven but has many

limitations such as small dose volume and precise delivery. Drug distribution to brain can be improved by using a device that can precisely deliver the formulation in the olfactory region of nasal cavity and provide rapid and direct access to brain. The objective was to evaluate the feasibility of using intranasal liposomal formulation to carry cargo of variety of drugs and oligonucleotides to brain. The hypothesis was verified by applying liposomal formulation in combination with a drug delivery device and comparing brain accumulation with intravenous route of administration.

**DEDICATION**

**To my parents who taught me the value of hard work and discipline**

**To Mital, my wife who has been with me throughout**

**To my daughter Maahi who has been an inspiration for me for now and ever**

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## 1. INTRODUCTION

In the last fifteen years of drug discovery research, many of the new entities came through combinatorial chemistry and high throughput screening. The resultant new chemical entities have higher molecular weight, increasing lipophilicity and lower aqueous solubility. This has resulted in about 40% of the currently marketed drugs to have an aqueous solubility of less than 100µg/ml or defined as practically insoluble.[1] The aqueous solubility of a drug substance is a critical physicochemical property for its physical and biological processes. To achieve pharmacological activity, the molecules have to be in dissolved state in physiological fluids so as to reach the site of action. During the early drug discovery and screening phase, molecules with the best receptor binding are selected for further pre-clinical studies.[2] From this very first step drug solubility is one of the most important physicochemical property because it serves as initial estimates of pharmacological and toxicological profiling. The solubility profile of compounds needs to be carefully and thoroughly evaluated during the drug development phase and necessary steps should be taken to produce dosage forms that consistently deliver the desired quantities of drug at the site of absorption. Before the early phases of clinical trials in humans, sufficient and well characterized solubility becomes even more critical. At this juncture, a comprehensive solubility profile of the new entity is created and reported to the regulatory agencies so assure reproducible performance of the formulated drug substance and will be expected remain unchanged for the final development and eventually marketing.[3] It is well accepted throughout the scientific community that aqueous drug solubility is an issue for the drug discovery as well as the early and late stage pharmaceutical development. Thus, careful and comprehensive efforts in the area of formulation development are needed to achieve reproducible bioavailability and pharmacokinetic profile in humans for the new drug substances. Solubility is an intrinsic

material property that cannot be altered unless chemically modified by salt, complex or prodrug formation. Two main approaches can be applied to alter the solubility of the drug substance, through material engineering of the drug substance or through formulation approaches. Care must be is taken to assure that such enhancement or modification approach should be scalable to a commercially viable process later in the development phase.[4] It may be suitable to go for chemical modification in the early phase of the drug development. However, chemical modification during the late phases of clinical studies or post-marketing poses huge regulatory hurdles as well as increased time and cost involved in clinical studies since these modified entities are treated as completely new entities.[5] Hence, formulation approach is more suitable during the later phase or post marketing era.

In addition to choosing a formulation approach, it is very important to choose safe and inert excipients that may enhance the solubility without affecting the pharmacological activities of the drug in the early phase of formulation development for any drug candidate.[6] There have been several ways reported to improve solubility and thereby the bioavailability of the drug. The most classical example is to increase the surface area of the drug particles by milling or micronization. Although the method is easy and rapid, the thermal stress associated with the process is a concern for thermolabile drugs. In addition, it improves the rate of solubilization but doesn't alter the equilibrium solubility of the drug. Therefore this method is not useful for drugs with solubility of 100µg/ml or less.[7] Another approach is using solid dispersion method wherein the hydrophobic drug is within the hydrophilic matrix of several excipients.[8] Although solid dispersions can improve the solubility of the drug within the matrix, many of the methods employed use high amount of heat and shear and cannot be applied to many drug candidates which may have low thermal stability. A more suitable approach to improve solubility is to prepare

inclusion complexes with β-cyclodextrin (CD). Using functional excipient like CD which is inert and biocompatible in nature to enhance solubility has been widely used in recent years in pharmaceutical, food and consumer industries. Macrocyclic hosts like CD can be used to make supramolecular delivery systems by forming non-covalent inclusion complexes with a wide variety of drug molecules. These inclusion complexes exhibit higher aqueous solubility and thermodynamic stability for the complexed drug molecule.[9] Several derivatives of CDs are reported as non-immunogenic, biocompatible and suitable for a human use by oral as well as parenteral routes.[10] Thus CD provide a suitable alternative for solubility enhancement for poorly soluble drugs for parenteral and oral drug delivery.

Besides the aqueous solubility permeability is another important criteria bioavailability. Permeability across biological membranes is a key factor in the absorption and distribution of drugs. Poor permeability can arise due to a number of structural features and membrane-based efflux mechanisms. It can lead to poor absorption across the gastrointestinal mucosa or poor distribution throughout the body. This may result in large inter- and intra-subject variability during clinical trials in terms of absorption and distribution which may eventually challenge the success of the new drug candidate as a viable product.[11] Although the two key physicochemical parameters the solubility and the permeability have been comprehensively studied separately, little work has been done to understand the relationship and interplay between them. Therefore, in order to aid drug product development, Biopharmaceutical Classification System (BCS) was introduced in the mid-1990s to classify the drug substances with respect to their aqueous solubility and membrane permeability.[12] The BCS classification system provides indication of difficulty during the development phases. According to BCS, drugs belonging to class IV are the most difficult to formulate into a successful clinical formulation due to challenging

molecular properties of low solubility and permeability. There have been several approaches reported to enhance solubility of the drug in order to improve absorption or distribution. This approach alone cannot help BCS Class IV drugs which have very low solubility as well as impaired permeability. Hence, a combined approach of simultaneous enhancement of solubility and permeability can improve drug performance, reduce the dosage and thereby possibly associated side effects.

Therefore, one of the aims of this proposal is to evaluate the reformulation strategy of a marketed drug which has poor solubility and permeability. By employing novel formulation approaches to enhance solubility and permeability of the drug, the reformulated product can improve the clinical performance of the drug and provide added advantage to the patients and thereby improve patients' compliance.

Another significant barrier to drug delivery is delivering drugs to brain and central nervous system (CNS). Brain tumors, stroke, Parkinson's disease and Alzheimer's disease are some of the most difficult to treat. The underlying reason is the occlusive Blood Brain Barrier (BBB) which hampers passage of most of the drugs to brain and cerebrospinal fluid (CSF).[13] BBB is consists of tightly knit set of cells that protects brain from infections or exposure to harmful molecules present in systemic blood stream. Unfortunately, the blood-brain barrier also blocks most drugs from entering the brain, making it difficult to treat neurological disorders. Effectiveness of the drug treatment of brain pathologies is often hindered by insufficient drug delivery across the BBB.[14] It spurs researchers to search for methods for effective delivery of drugs and diagnostics across the blood-brain barrier with non-invasive approaches. Many of the new developments in the treatment of neurological disorders will be biological therapies consists of large molecules such as recombinant proteins, peptides, antisense oligonucleotides, monoclonal antibodies and gene therapies. Essentially, none of these molecules can cross the BBB owing to their

large molecular weights.[15] Thus novel drug discovery for neurological disorders will need innovative ways to deliver drugs across BBB. Successful and potentially non-invasive drug delivery systems to deliver drugs across BBB will allow not only reaching better therapeutic outcomes but also having impact on drug's commercial potential. Comprehensive efforts in the area of innovative drug delivery to brain can not only be applied to newer molecules but also to the existing drugs so as to improve their therapeutic outcomes and possibly extend their patent life.

There have been several approaches reported to deliver the drug across the BBB. One of the approaches is the most invasive approach commonly referred as trans-cranial drug delivery wherein an intra-cerebroventricular injection or intracerebral implantation method is used to deliver the drug directly in the desired area of the brain. Both of the above approaches are not only extremely painful and inconvenient to the patient but also need surgical intervention. The method also exhibited significant adverse events related to trans-cranial implantations.[16] In addition, such systems usually exposes the brain to very high levels of drug which results in irreversible damage to the brain tissues.[17] Another approach is to use of chemical or physical stimuli to disrupt the BBB. The major problem with this approach is that the resultant leaky BBB let blood albumin to enter the brain which is toxic to the astrocytes resulting in chronic neuropathologic changes in brain.[18] Prodrug based approaches are also applied to many drugs so as to take the advantage of the fact that lipid soluble molecules can easily cross the BBB. However, the molecular weight threshold for BBB crossing is only 400 Da beyond which the permeability does not increase irrespective of its lipid solubility. Hence, despite of many efforts in this area, there is no drug available in the market till date.[19] The prodrug based approach was slightly modified to use a transporter based system to deliver the drugs across BBB such as L-DOPA. Dopamine cannot cross the BBB while L-DOPA can cross the BBB via

endogenous carrier mediated transporters.[20] Although this approach was found to be successful, it may not be possible to use the same approach for larger biologics. For such large molecular drugs, colloidal carriers encapsulating the drug in the matrix can be used to deliver the cargo across BBB. Liposomes and polymeric and lipid nanoparticles have been used with several biologics for brain delivery.[21] Some of the major advantages of colloidal carriers are controlled delivery, enhanced therapeutic efficacy by improving bioavailability and distribution. For successful endocytosis across the BBB, colloidal carriers need surface modification with ligands specific to transporters available on the BBB apical site.[22]

In order to deliver the drugs and more specifically biologics to brain, an ideal system should in principle circumvent the BBB at all. The unique relationship between nasal cavity and cranial tissues makes intranasal (IN) delivery as a promising shortcut for drugs to circumvent the BBB. Some of the major advantages of nose to brain delivery are rapid onset, ease of self-administration, avoidance of systemic and hepatic clearance and non-invasive nature of the delivery.[23] Not only small molecules but also polar molecules, peptides, proteins, oligonucleotides and stem cells can be delivered to CNS via nasal route.[24] Due to quicker onset, there are currently several treatments under trial to treat emergency situations such as breakthrough pain in cancer using nose to brain drug delivery pathways.[25] Despite of such advantages, IN route bears some limitations. In humans, the total volume of administration is limited to 200 µL. Compared to rats, humans olfactory regions comprises of approximately 10 % of the total surface area which limit the access to brain directly through nasal cavity. Hence, IN can be a route of choice for brain delivery of drugs which are potent and required at very low brain concentration in order to exert their pharmacodynamics effects.[23] Experiments in animal models have shown that colloidal carriers can also be used to deliver the drug to brain via IN route. These

animal data are very encouraging since it opens the gate of numerous options to efficiently deliver drugs to brain which cannot be delivered otherwise due to their physicochemical properties or stability. Colloidal carriers can protect the encapsulated drug from enzymatic degradation as well as from efflux transporters such as P-glycoprotein.[26] It was shown that such colloidal carriers enter the brain via transcellular pathway using receptor mediated endocytosis. Another important parameter to consider is the particle size. Ideally particles of 50-100 nm in size are rapidly internalized by the cells than larger particles.[27] In addition, cationic charge also helps the particles to circumvent the lysosomal pathway.[28]

Therefore, to gain the maximum benefit of nose to brain pathway of delivery, we propose several novel ways combined together to achieve synergistic results. Firstly, the IN delivery will circumvent the BBB which is considered a formidable hurdle in brain delivery. Also, using IN will improve a penetration of more drug in the brain without causing any damage to BBB thereby preserve to function its physiological role of protection of brain. The proposed delivery system is capable of self-administration and thereby reduces number of clinic visits for patient. In order to overcome some of the shortfalls of the IN route such as low volume and high mucociliary clearance, we devise a novel strategy to improve cargo in a smallest possible volume and enhance localization in the olfactory region to achieve higher amount of drug delivered to the brain. Combining all, the proposed formulation will bring higher patient compliance compared to current regimen.

# 2. BACKGROUND AND SIGNIFICANCE

## 2.1. Cancer

Cancer is a group of disease where malignant growth or tumor results due to abnormal cell growth and failure to regulate such growth that can invade and spread to other parts of the body. It initiates from an uncontrolled cellular growth to form heterogeneous primary tumor with leaky and premature vasculature followed by spread or metastasis of the cancer cells to other parts of body to form secondary tumors. The pathogenesis of cancer is very dynamic as it involves constantly altered cellular and molecular interactions in different pathways. The complexity of pathogenesis of cancer have been further reduced to six common traits that are believed to govern the transformation of normal cells to cancer cells (Figure 2.1).[29] These characteristics are described as:

1. It begins with acquired cellular and genetic mechanism that allows the growth and division of cells in the absence an external growth factor or stimuli.
2. Mutation of tumor suppressor genes or altered proteins responsible to halt such uncontrolled growth. Thus cells become insensitive to internal or external signals that can stop or suppress the growth.
3. Damaged cells which have such abnormalities can result in self-destruction by cascade of events known as “programmed cell death” or apoptosis. However, during the transformation cancer cells have acquired capability to evade this check-point.
4. The next acquired characteristics of cancer cells is the ability of limitless replication by suppressing tumor suppression genes and upregulation of telomerase, an enzyme that controls doubling frequency of the cells creating immortal cancer cells.

5. In order to sustain such uncontrolled growth and to fulfill the nutrients and oxygen requirements, small clusters of cancer cells will start producing various growth factors that will promote blood vessels growth or “angiogenesis”.
6. One of the most important acquired trait of the cancer cells is their ability to disseminate to invade neighboring or distant tissues, adapt to the new environment and start uncontrolled growth.

In addition to the above, recent research added that such cancer cells have abnormal metabolic pathways to generate energy as well as ability to evade body’s immune system and several server chromosomal abnormalities that can worsen as the disease progress. Such complexity exhibits that cancer is a disruption or failure of several cellular and genetic control mechanisms and may differ from one patient to the other based on stage of cancer and the organ or tissue involved. This adds further complexity to the treatment of the disease.

The treatment can be divided into three broad classes of surgery, chemotherapy and radiation. Isolated cancers which haven’t spread to other organs can be removed by surgery. Recent advances in diagnostic procedures and identifying correct stage of the tumor has significantly helped improving the surgical interventions by prolonging patient survivals. However, not all cancers are easily accessible or diagnosed at a stage where surgery can be an option. In such circumstances, one or more anti-cancer drugs given to a patient as standardized regimen. Ionizing radiation as cytotoxic agent to cure or improve cancer symptoms have been commonly used, mostly in combination with either surgery and/or chemotherapy. The focus and purpose of the research data presented here is mainly on improving chemotherapy.

Although tremendous amount of resources around the world are heavily invested in finding prevention and cure for the disease, the overall epidemiological and statistical data provides a very disturbing outlook for the patient survival and quality of life. According to one of the estimates recently presented by the National Cancer Institute, there are approximately 1.6 million new cancer patients will be diagnosed in the United states and sadly more than half a million people will die fighting the disease in 2016.[30] According to 2014 data, there are more than 14.5 million patients living in the United States who have been diagnosed with cancer earlier. Still, cancer is the second leading cause of death in the US and other developed nations. In 2010, US alone have spent a staggering $ 125 billion towards national expenditure for cancer care. There have been some positive outlook for the treatment of some cancer as death rate has declined, it has remained either stagnant or increased for the most of the cancers.[30] There are several challenges in the treatment of cancer such as ineffective therapeutic drug concentrations at the tumor site, toxic and life-threatening side effects of some of the drugs due to non-specific distribution to healthy tissues and acquired resistance to the treatment by the cancer cells that renders the regiment ineffective. Traditional chemotherapy treatment involves use of cytotoxic drugs that will damage the cell's ability to multiply and thus kill cancer cells. Unfortunately, some mechanisms in cancer cells will evolve over the period of the chemotherapy that will increase the resistance to the drug's ability to kill the cell and thus resistance to the treatment will emerge. To make the matter worse, this evolved resistance is not against only single drug but several other drugs which are either from the same chemical class or somehow utilize same mechanism of action. This phenomenon is known as multidrug resistance which is frequently correlated with amplification of certain genes involved in resistance by either preventing drug accumulation within the cell or by increasing intracellular metabolism of the drug.[31] Thus, it warrants a path forward in the

area of drug discovery and development to design drugs which targets specific underlying mechanism of the pathology to make the drug more effective and safer. Alternatively, a faster route is to design drug delivery systems that can effectively target cytotoxic drug to tumors to improve efficacy, reduce dosing and severe side effects.

### 2.2. Anticancer drugs discovery and development

The shortcomings of chemotherapy and associated severe side effects have provided great momentum to investigate and evaluate newer areas of drug discovery and developments. The recent paradigm shift in the drug discovery as well as involvement of proteomics and genomics have resulted in better understanding of cancer pathogenesis and its underlying mechanism to identify potential molecular targets for chemotherapy.[32] The renewed focus of current chemotherapy is not just to apply high doses of cytotoxic drugs that has severe systemic side effects but to apply newer therapies that specifically target cancer cells, has lower systemic side effects and either kill the cancer cells completely or slow their progression. Increased knowledge of the disease and tumor progression has led us to design better drugs which will targeting specific mechanisms within the cancer cells. In addition, new and precise diagnostic technics provide tool to characterize each and every tumor at their molecular levels. Such diagnosis helps to better understand the tumor and possibly precise defects in the genes that led to uncontrolled growth and thereby select specific treatment or drugs that can target them and kill them and slow the progression. Following the identification of potential target and discovery or synthesis phase, the drug enters to the screening phase where appropriate *in vitro* screening is performed to evaluate pharmacodynamic properties of the compound. The National Cancer Institute (NCI) has developed 60 different human tumor cell lines of varying histology derived from different tumors is a widely used model to perform early

screening. The high throughput screening data collected on several agents using NCI's 60 cancer cell lines has resulted in a large database of compounds based on similar mechanisms and sensitivity to target specific tumor cell lines to develop treatment focused on specific types of tumor. Although NCI's cell line screening is a great tool in the early discovery phase to provide high throughput screening on large libraries of data, the *in vitro* screening largely ignores the complexity of tumor histology, heterogeneity, micro environment and role of immune system. Hence despite of being a rapid in vitro discriminatory tool, drug's sensitivity to above mentioned tumor factors can sometimes leading to false-positives or negatives that can lead to failures in early clinical studies. One of the biggest advantage of in vitro screening is it has helped identify or predict drug resistance with high accuracy thereby exclude such compounds from further screening. Following *in vitro* screening, selected compounds are further evaluated in preclinical animal models to gather more information about the therapeutic index, dose limiting toxicities, metabolism and pharmacokinetics. Xenograft and orthotropic animal models were developed using well characterized human cancer cell lines to further evaluate the effects of anticancer drugs with respect to their therapeutic outcomes as well as toxicities. The information gathered during the early preclinical studies are pivotal with respect to human toxicities, dosage and safety. Though these models mimic morphology and growth characteristics to clinical disease, animal models do not completely represent clinically relevant models for cancer especially in case of metastasis. Combining with well characterized *in vitro* data, preclinical studies can provide significant amount of information that can accurately predict clinical response and impetus to evaluate such agents in early clinical trials. Many newer anticancer agents with well-defined molecular targets and safety profiles were discovered using the above approach. In many cases, the translation of such target into a feasible therapy has been disappointing due to incomplete

understanding of the role of the target in pathogenesis of malignancy and the overall success of early preclinical models. However, this ideal scenario is not always true with respect to anticancer drug discovery. There are still several potential anticancer agents developed which lack of specificity and selectivity results in severe systemic side effects to healthy organs and tissues.[33] Poor drug solubility, permeability, systemic metabolism before reaching the tumor, rapid clearance from body and drug efflux are some of the major challenges in order to prepare an effective and safe formulation. Since these drugs have already proven their efficacy in clinics, there is an opportunity utilize them in a safer way by developing formulations for them so as to increase drug localization in the tumor and reduce systemic concentration. Such approach will not only improve the therapeutic outcomes but also minimize side effects as well as possibly reduce dosage and incidence of drug resistance.

### 2.3. Anticancer Drug Formulations

Sharp increase in cost of development of newer drugs and increased scrutiny from the regulatory agencies with regards to patient safety has resulted in decline in number of new drugs approvals. According to one estimate the attrition during the various preclinical and clinical phase is so high that for every 10,000 molecules in the preclinical agents only one may reach the clinic and possibly receive marketing approvals (Figure 2.2).[34] To mitigate the high attrition rate and high anticancer drug discovery and development costs, approaches like drug delivery and formulation design have become integral and essential component. Designing newer formulations for already established drugs help to bring to the market a safer alternative to already established regimen that can not only improve therapeutic outcomes but also improves patient compliance. Since the drug is already being used in the clinic, the regulatory safety requirements are significantly lower

compared to a new drug thus can be brought to the market significantly faster and at relatively lower cost.

With more than 40 % of the agents are withdrawn at various stages mainly due to pharmacokinetic reasons anticancer drugs are the most suitable candidates that can possibly be revived by applying recent formulation technologies and approaches. In addition, newer biopharmaceutical agents such as proteins, peptides, antibodies and polynucleotides such as siRNA are prone to proteolytic degradations as well as immunogenic reactions which can be minimized by using appropriate drug delivery technologies. Using appropriate formulation approach suitable to anticancer drugs, the pharmacokinetic parameters such as solubility can be improved to enhance amount of drug available within the tumor to improve the efficacy or minimize distribution to other bystander tissues can be reduce toxicities. An ideal anticancer drug formulation should provide high distribution to tumor with minimal or no distribution to non-tumor sites, possibly at controlled or sustained release to minimize dosing and using safe and biodegradable or biocompatible excipients. In addition, formulation design can also target some other problems with tumor delivery such as avoid or bypass efflux mechanism, overcome poor vasculature within the tumor to localize more drug and prevent drug from the destabilizing microenvironment within the tumor. Hence, formulation approaches and drug delivery technologies are universal tools that can be applied to newer small molecules and biotechnology based drugs to overcome associated problems and overcome attrition and failure as well as established drugs to improve their efficacy and safety. The data presented here in this report mainly focus on how to employ newer approaches on already established drugs to improve their cytotoxicity and minimize distribution to healthy tissues to reduce toxicities and adverse drug reactions.

## 2.4. Drug Solubility

Drug solubility is one of the most important physicochemical properties during drug discovery and development. The thermodynamic solubility of a compound in a solvent is the maximum amount of the most stable crystalline form of the compound that can remain in solution in a given volume of the solvent at a given temperature and pressure under equilibrium conditions. In order to exert pharmacological action, sufficient and reproducible amount of drug should reach the target site that largely depends on the solubility of the drug in plasma and other physiological fluids.[3] For drug to be absorbed it first has to undergo dissolution in the physiological fluids.[35] In the early discovery phase, solubility information serves a wide range of needs for the preformulation scientist that provide a rationale for formulation design with reproducible bioavailability and pharmacokinetic profile for early clinical use. Parenteral products development may face significant hurdles due to low aqueous solubility and it may also limit the absorption and bioavailability of oral formulations.[36] The issue of drug solubility became more pertinent in the modern era of drug discovery emphasizing heavily on combinatorial chemistry and high throughput screening resulting in new entities with higher lipophilicity and low aqueous solubility.[4] The process of drug solubilization can be essentially divided into three main steps: In the first step involves the breaking of the bonds between drug molecules followed by void creation in the bulk of the solvent sufficient to accommodate the drug molecule. The final step is the insertion of drug molecule in the solvent void when essentially a drug molecule is dissolved in a solvent (Figure 2.3). The energy of solubilization depends on the molecule and its crystal lattice. A more thermodynamically stable crystal structure needs higher energy to overcome and thus have lower solubility. Since drug solubility is being an equilibrium measure, a more dynamic parameter of rate of dissolution is more critical. In order to achieve complete or nearly complete absorption

from the oral formulation, the rate of dissolution must exceed significantly to the rate of transit.[37] Since solubility is an intrinsic characteristic of a drug molecule that largely depends on the strength of the crystal lattice and affinity of the drug molecule to the aqueous environment, the United States Pharmacopoeia has classified them on the basis of their equilibrium solubility into seven classes (Table 2.1).[38] Low solubility for injectable drugs are more challenging since there is a heightened need to design a simple dosage form. Upon administration of injectable delivery system the components of the formulations are in immediate contact with blood and vasculature of the circulatory system, heart, liver, spleen, lungs and kidney upon administration. Drug precipitation at the site of injection, pain and irritation at the site of injection, hemolysis, embolism and acute immune response due to drug and/or excipients present in the dosage form are of critical concerns from the product development and safety aspects. Hence, choices of safe and inert excipients for parenteral formulations are of critical importance. While developing formulations for poorly water soluble drugs, the route of administration is an important consideration. For oral formulations the aim is to enhance the dissolution rate under the physiological conditions, while for parenteral formulations the entire dose should be completely dissolved prior to administration. One of the approaches reported in literature employ structural modification by medicinal chemistry to design more water soluble compounds. In many instances during the early discovery phase, it is apt to apply medicinal chemistry approach in order to avoid manufacturing related issues and design a conventional dosage form. Although a very promising strategy to significantly alter the physicochemical properties of a compound, such approaches are challenging to apply in the later phases of drug discovery and development. In addition, altering the molecular structure to enhance solubility may also change the bioactivity of lead compound.[39] Hence, a formulation based approach is more suitable during the later phase of drug

development and also for marketed product due to associated time, cost and regulatory considerations.

### 2.4.1. Solubility Enhancement Approaches

The three fundamental approaches that can be used to enhance dissolution and apparent solubility are: a. reducing the intermolecular forces in the solid state of solute b. increasing the strength of solute-solvent interactions and c. increasing the surface area for solute-solvent interactions. Increasing the surface area by micronization is one of the earliest approach applied in the for solubility enhancement for oral solid formulations since by simply increasing the surface area the rate of dissolution can be significantly increased without adding any excipients to aid solubilization. Spray drying, micronization by milling and grinding are some of the common methods use mechanical stress to deaggregate the drug particles to the size of 2-3 μm thereby provide an efficient and reproducible means of solubility enhancement.[7] Drugs such as griseofulvin, progesterone, spironolactone and fenofibrate are some of the examples where significant difference in dissolution rate and bioavailability was observed for micronized drug particles compared to the unprocessed.[40] Although a very simple and efficient method, it cannot be applied universally as higher surface area also increases the surface free energy and affect the shelf-life of the formulation. In addition, the method can only be applied to very potent drug compound with low dose since the method essentially doesn't alter the apparent solubility but merely enhance the dissolution rate.

Converting crystalline drug into amorphous solid has been widely used approach to enhance the apparent solubility and dissolution rate. Use of crystal engineering to deliberately design and control molecular packing within the crystal to exhibit desired solid-state properties is common by the preformulation scientists.[7] Polymorphs and cocrystals are some of the examples of crystal engineering used for the purpose of solubility

enhancement. The ability of a drug molecule to form more than one type of crystal structure that differ by their molecular arrangement in the crystal lattice is termed as polymorphism while materials lacking any type of crystal lattice structure are considered as amorphous. Polymorphs and amorphous solids exhibit different surface free energy which is why they differ in some of the physicochemical properties such as solubility. Thus, by simply designing a process that will end up in polymorph (or amorphous structure at all) exhibiting higher solubility, the drug can potentially improve its bioavailability.[41] Since the polymorphs are thermodynamically unstable, they may undergo transformation to a more stable polymorph with less surface free energy and hence low solubility. This may occur very rapidly or during storage over a long period of time resulting in aberrant dissolution and bioavailability profiles. A very well-known example of such transformation is Abbot's Ritonavir. The identification of low solubility polymorph after the product launch forced product recall and reformulation.[42] Following the example of Ritonavir, it has become a routine for a formulation scientist not only to provide a comprehensive information about different drug polymorphs and their transition to the regulatory agencies but also justify use of an excipient that stabilizes the drug in a high energy form in a formulation over a long periods of shelf-life.

A very similar approach is a formation of cocrystals for solubility enhancement. The Food and Drug Administration (FDA) has recently issued a guideline on pharmaceutical co-crystals that defines co-crystals as ''solids that are crystalline materials composed of two or more molecules in the same crystal lattice''.[43] Although this approach is very similar to salt formation, a weak non-covalent bond formation takes place between crystal former and a drug. Hence, this method is not limited to ionizable drugs. Unlike polymorphs, cocrystals are thermodynamically stable and thus issues related with shelf-life can be circumvented.[44] One important consideration while choosing a coformer is to assure

high solubility (at least 10 fold to the drug) of the coformer to generate cocrystals with higher aqueous solubility for the drug. Cocrystals of several drugs such as Carbamazepine, Theophylline and Caffeine were prepared and found to have significantly higher solubility than the drug alone.

Solid dispersion has been another popular method for solubility enhancement applied to many drugs in the last decade.[8] Since pure amorphous drugs are thermodynamically unstable, they have limited application for commercial development. However, combining such amorphous solids in a matrix of excipients that can assure the amorphous state of the drug throughout the shelf-life of the drug can be a viable and attractive option. These systems are better known as solid dispersions. Essentially, in solid dispersions a hydrophobic drug is dispersed in a matrix of hydrophilic polymer to prepare single phase amorphous mixture. The choice of polymer plays an important role since it not only enhance the solubility and dissolution of the drug but also stabilizes the amorphous drug by preventing nucleation and crystal growth. Due to vast choice of polymers available to prepare solid dispersions and varying methods of preparation available, solid dispersion method has gained significant attention from formulations scientists and has resulted in several marketed products such as Griseofulvin-PEG dispersion (Gris-PEG®, Novartis), Nabilone-PVP (Cesamet®, Eli Lilly) and Lopinavir and Ritonavir solid dispersion (Kaletra®, Abbott). However, the polymer screening process is very comprehensive since many of the product attributes are determined by the choice of the polymer and its compatibility with the drug. The associated cost with development and scale-up is higher than other conventional dosage forms. Since the drug is in supersaturated form in the dispersion, there have been several instances of phase separation reported.[45]

### 2.4.2. Solubility Enhancement for Parenteral Formulations

Unlike oral delivery, solubilization techniques for injectable delivery has more limitations owing to safety and toxicity of the excipients used for the purpose and stricter standards for quality, purity, sterility and product consistency. A thorough understanding of physicochemical properties of the drug has enabled a step by step process to design an appropriate injectable dosage form for poor solubility drugs (Figure 2.4).[46] One of the simplest approach used for polar compounds with pH dependent solubility. Buffered systems of weak acid and conjugate base are used in parenteral drug development for a long time. Some of the notable examples of such drugs are Warfarin (Coumadin®) using phosphate buffer system (pH range 6-8), Ertapenam (Invanz®) using bicarbonate buffer system (pH range 4-9) and Mitoxantrone (Novantrone®) using acetate buffer system (pH range 4-6). Although such pH adjustment are done in a narrow range around the physiological pH (7.4), there are few products marketed with extreme pH difference such as Thiopental sodium injection Many of such pH adjustment are made with weak buffer capacity so as to avoid irritation at the site of injection, it may affect the formulation since there is a high probability of drug being precipitated upon rapid injection at the injection site. Such precipitation upon dilution can end up in therapeutic failure as the drug is unable to reach the target site. The application of cosolvent and surfactants to aid solubilization has limited application for the injectable drug delivery since cytotoxicity and genotoxicity are major impediment. High osmotic pressure associated with cosolvent also limits the applicability of their use in injectable formulations. Common water miscible cosolvents used in practice are Ethanol, Propylene glycol and glycerin. Melphalan Hydrochloride for Injection (Alkeran®) exemplifies the application of cosolvency. The drug is supplied as a lyophilized powder with a sterile diluent for reconstitution that is comprised of sodium citrate (0.2 g), propylene glycol (6.0 mL), ethanol (96% grade, 0.52 mL), and Water for Injection, diluted to a total volume of 10 mL. Non-polar drugs without any ionizable groups

and high water-octanol partition coefficient (Log P) values are suitable examples for such approach. Similar to cosolvents, surfactants are also excellent solubilizer but have limited application due to toxicities. Ionic surfactants can cause hemolysis and damage to lymphocytes can result in histamine release leading to anaphylactic reactions that may necessitate use of anti-histamine drug treatment prior to treatment.[46] In many instances, a combined approach of using a surfactant and a cosolvent is also applied in order to solubilize drug especially with large hydrophobic groups. Paclitaxel is one of such drugs which have extremely low solubility that limited its clinical applications after early discovery period due to lack of suitable vehicle. A mixture of polyethoxylated castor oil and Ethanol known as Cremophor was used as a solubilizer.[47] Although not an inert excipient, Cremophor has variety of adverse effects reported which are sometimes fatal.[48] Despite of that, Cremophor has been still used in some clinical formulations till a suitable alternative is made available for drugs like Paclitaxel.

### 2.5. Drug Permeability

Once the drug has been solubilized, it has to pass through a biological barrier in order to reach to the receptors or site of action. Thus drug permeability is a key factor in the absorption and distribution and thereby influences the overall pharmacokinetic behavior of the drug. More importantly macromolecules such as recombinant proteins, monoclonal antibodies, oligonucleotides and vaccines differ significantly from small organic compounds with respect to their permeation across the membrane. Similar to solubility, permeability is an intrinsic property that is largely dependent on the molecular structure of the drug. However, there are external biological factors such as membrane transporters, efflux mechanisms and metabolizing enzymes can adversely affect the drug permeability. Permeability limitations can hinder significant developments to design formulations not only for oral but also for transdermal, pulmonary and other routes as well. Although lot of

emphasis has been placed on enhancing solubility for a drug there has been little work done in order to enhance intestinal permeability of the drug. The advanced understanding and availability of information about drug's physicochemical properties, metabolism mechanisms and transporters involved in transport and efflux of the drug can help a formulation scientist to come up with a suitable approach to achieve reproducible pharmacokinetic parameters of the drug.[49] The fundamental of permeability enhancement mechanism is to shield the drug while in transit across the membrane. Due to lipophilic nature of the membranes, many of the approaches for permeability enhancement vastly use lipid based or amphiphilic excipients for the purpose. Bile acids, nanoemulsion, liposomes, lipid nanoparticles and self-emulsifying systems are some of the most popular approaches applied for the permeability enhancement of oral delivery systems.[50] The key factors that control the bioavailability and pharmacokinetic profile of any drug are solubility and permeability. Combining these two factors and studying their interplay can help understand and predict drug's pharmacokinetic behavior but also help designing suitable formulations to overcome its limitation. In 1995 a Biopharmaceutical Classification System (BCS) was developed to classify the drugs based on its solubility and permeability and has been widely used not only by the scientists but also by regulatory bodies worldwide.[12]

### 2.6. Biopharmaceutical Classification System (BCS)

The Biopharmaceutical Classification System (BCS) covers the fundamental key physicochemical parameters that govern the overall rate and extent of drug absorption are drug solubility and permeability.[12] According to the BCS, class I molecules are those having both high solubility and high permeability (and therefore likely few problems with

oral absorption); class II compounds are those that have low solubility and high permeability (where solubility is the primary limitation to absorption); class III compounds have high solubility but low permeability (where absorption is limited by membrane permeation and not solubility); and class IV compounds are those in which both poor solubility and poor permeability limit drug absorption (Figure 2.5). According to BCS, a drug is considered highly soluble when highest dose strength is soluble in 250 mL of aqueous media over the pH range of 1-7.5 at 37 $^{o}$C. Similarly, a drug is highly permeable when more than 90% of the administered dose is absorbed in the systemic circulation.[51] Over the period of time, BCS has emerged as an essential tool in modern preformulation and biopharmaceutics due to the core reason that the system describes the role of solubility and permeability combined. Although solubility and permeability were separately studied comprehensively and several approaches were reported to enhance either permeability or solubility of the drug, a combined approach or interplay of solubility and permeability has been largely overlooked. For example, drugs that belong to BCS Class IV have low solubility and permeability. Hence, enhancing one of the parameter irrespective of the other may not help the drug improve its bioavailability. Thus solubility enhancement may improve the dissolution test *in vitro*, but may be misleading with regards to predicting its absorption and bioavailability owing to low permeability of the drug. Thus, BCS and the solubility-permeability interplay must be taken into account prior to formulation development to strike the optimal balance between these two parameters and maximize drug absorption and bioavailability.[11] In the modern drug discovery era where solubility enabling formulations are widely developed and applied that significantly enhance the apparent solubility, the fraction of dose absorbed is erratic due to the fact that permeability has been ignored resulting in failure of such formulations. Hence, after understanding the effect of permeability on bioavailability and with the help of BCS,

formulation approaches for solubility enhancement can be customized based on the requirements. For example, solubility enhancement for BCS class II drug will significantly improve its bioavailability whereas the same approach may not work for BCS class IV drug due to its low permeability. For drugs that fall under BCS class I have no rate limiting steps in their dissolution or absorption, making them some of the easiest candidates to formulate in a simple formulation. For BCS class II drugs, the low solubility is a rate limiting step. By improving the dissolution rate of BCS class II drugs, a significant bioavailability improvement can be achieved. Methods earlier discussed such as particle size reduction, increase in saturation solubility or preparing amorphous solids are typically used for the formulation development of drugs belonging to BCS class II. In comparison to BCS class II, drugs from BCS class III suffer from poor permeability which requires significantly different approach as dissolution rate is not a rate-limiting step but permeability. Fatty acid, bile salts, surfactants, and polysaccharides that may aid drug transport across the membrane by enhancing the permeability of drugs are used for BCS class III candidates. However, acute or chronic damage to the intestinal membrane is a major safety concern. Due to lack of approaches available and less is known about the efficacious and safe dosage options for BCS class III drugs, generally an oral controlled release formulation design is avoided for clinical use. Low solubility and low permeability of BCS class IV drugs are challenging physicochemical properties to transform them in a dosage form with reproducible pharmacokinetic profile so as to minimize inter and intra-subject variability. Since some of the options applied to BCS class II drugs can also be used for class IV drugs, the safety of permeability enhancement still put class IV drugs as most challenging ones. In recent years, surfactants, emulsification, cyclodextrin complexation and several other approaches have been used for class IV drugs. Out of many approaches, cyclodextrin based approaches have resulted in several products approved by the FDA.

The newer safe and biocompatible chemically modified variants of cyclodextrins have been approved by regulatory agencies for use not only in oral but also in injectable drug delivery systems.

### 2.7. Cyclodextrins

Cyclodextrins (CDs) are class of cyclic oligosaccharides produced by enzymatic degradation of starch. The monomeric units or CDs are α-D-glucopyranose are linked by α-1-4 link to each other creating a cyclic structure. The chair confirmation of the monomeric units gives molecule a truncated cone or toroid shape to the molecule than a perfect cylinder. In a three dimensional space, the carbons of monomers in CDs are aligned inside the cavity and thus creates a lipophilic core while the primary and secondary hydroxyl groups of glucose moieties are on the periphery of the molecule are reasons for its hydrophilicity. Cyclodextrins are classified based on the number of glucopyranose units in them. The most common and naturally available CDs are α-, β- and γ-cyclodextrin consist of 6, 7 and 8 units respectively (Figure 2.6).[52] The natural derivatives have been previously applied in some pharmaceutical applications and found to have hemolytic and nephrotoxic properties. While small internal hydrophobic cavity limited applications of α-cyclodextrin, γ-cyclodextrin was found to be unstable in physiological fluids to bond cleavage. Low solubility and hemolytic potential of β-cyclodextrin renders it not suitable as a safe excipient. Also, being a natural derivative, it is difficult to maintain consistent quality for large scale applications. Due to internal hydrogen bond formations highest aqueous solubility at room temperature for unsubstituted β-cyclodextrin can be only up to 2%. Low solubility of unsubstituted β-cyclodextrin makes it unsuitable especially for parenteral applications since it can form microcrystalline precipitates in kidney. Modifying and substituting hydroxyl groups of the glucopyranose units of β-cyclodextrin has helped

to minimize internal hydrogen bond interactions and resulted several high aqueous solubility β-cyclodextrin derivatives. In this work, we have employed several synthetic β-cyclodextrin derivatives and are further discussed in the subsequent section.

### 2.8. β-cyclodextrin Derivatives

Such highly purified and substituted β-cyclodextrin derivatives are biocompatible, have significant higher aqueous solubility and are for safe applications as excipients for oral and parenteral formulations (Figure 2.7).[9] In aqueous media, interior lipophilic cavity of CD creates hydrophobic micro-environment that can host hydrophobic molecules within the cavity to form a molecular inclusion complexes which exhibit higher aqueous solubility for the host drug molecule. Such increase in solubility results in higher dissolution rate and thereby improve bioavailability of the drug. More importantly, inclusions complex formation involves only weak hydrophobic interactions by simply displacing water molecule from the cavity and no covalent bonds are formed or broken. Intravenous injections of β-cyclodextrin derivatives excreted unchanged in urine and oral doses are not absorbed from the gastrointestinal tract. Due to non-toxic and biocompatible properties, several β-cyclodextrin derivatives are now have regulatory approvals to use in pharmaceutical applications and are gaining more popularity as suitable functional excipients. Some of the main applications in pharmaceuticals are to improve solubility and bioavailability for poorly soluble drugs. CDs have also been found to improve drug stability as well as other applications such as reduce gastric irritation, reduce eye irritation and taste or odor masking.[53-55] There are more than 30 drug products approved by regulatory agencies are being marketed that contains β-cyclodextrin inclusion complexes with an active drug molecule for various applications (Table 2.2). There are several reported applications which are currently either at pre-clinical or clinical levels applying β-

cyclodextrin derivatives as functional excipients. Due to their hydrophobic cores, CDs can stabilize protein confirmation and behave as artificial chaperons to refold aggregated or denatured proteins.[56] A quaternary amine group based has been developed to be used in ophthalmic formulations that can serve as a solubilizing agent as well as an antibacterial agent that exhibit bacteriostatic properties with less eye irritation compared to other quaternary amine based preservatives.[57] In case of cytotoxic drugs where high lipophilicity and poor aqueous solubility make cytotoxic drugs difficult to formulate. Most of the cytotoxic agents owing to their low solubility are either classified in BCS class II or IV are suitable for such CD based inclusion complexation. CDs provide suitable and biocompatible way to enhance solubility and bioavailability for such drugs. In the last few years, CDs and their derivatives have found increased attention and efforts to apply them in various pharmaceutical formulations. Their biocompatibility and safety are some of the attractive attributes that led the efforts to develop more than 30 approved products currently in the market. However, there are still unmet need in the area of developing such inclusion complexes for cytotoxic drugs to improve solubility, stability and bioavailability. Applying modified CDs to cytotoxic drugs can improve the solubility of cytotoxic drugs and thereby potentially help to reduce dosage, serious adverse drug reactions and enhance cytotoxic response and patient survival.

### 2.9. Barriers to drug delivery to brain

When Paul Ehrlich and Edmond Goldman failed to stain the brain upon systemic administration of a dye, they put forward a concept of a barrier that shelters the brain and the majority of the central nervous system. Today we know that permeating a therapeutic agent across the BBB is one of the most challenging aspect of drug delivery to brain (Figure 2.8).[58] By applying passive and active mechanisms to regulate access to brain,

BBB prevents early diagnosis and effective treatment of brain tumor that results in poor prognosis for the patients of primary and metastatic brain tumors. With more than 10,000 patients diagnosed with glioblastoma and more than 100,000 patients with metastatic brain tumors in 2015 who have median survival after diagnosis is between 4 and 15 months.[59] Anticancer therapeutic agents which are effective in the treatment of peripheral tumors are unable to access the tumor cells due to intact BBB. Systemic delivery of anticancer drugs by oral or intravenous route for adequate concentrations in brain largely depends on BBB permeability of the drug or its carrier. Offsite toxicities of such agents and sub therapeutic brain levels are some the reasons for the failures of such treatments. Pre-systemic metabolism and presence of efflux transporters on BBB limits brain bioavailability of drugs to less than 10 %. Upon reaching BBB via systemic circulation, the probability delivering drug across the BBB is still a challenge due to their extremely high trans- endothelial electrical resistance of 1500-2000 $\Omega*cm^2$ compared to 3-30 $\Omega*cm^2$ across most micro vessels.[24] Recent advancement where transient damage to BBB is caused by either hyperthermia or ultrasound showed promising results where larger quantities of the active drug could cross the damaged BBB. However, patient safety of such damage on long term is yet to be tested on a large scale clinical trials.[60]

### 2.10. Intranasal delivery to brain

On the other side IN route of administration provide a suitable non-invasive path to bypass BBB and deliver drugs to brain. The unique relationship between nasal cavity and cranial tissues makes intranasal delivery as a promising short cut for drugs to circumvent the blood brain barrier.[23] Some of the main advantages of IN delivery to brain include rapid onset, ease of self-administration, avoidance of systemic circulation and hepatic first pass metabolism. Using IN route, several challenging drugs such as small polar molecules,

peptides, proteins, oligonucleotides, viral vectors and stem cells can be effectively delivered to brain.[24] There are several drugs used in clinic developed for IN delivery to various parts of the brain. Since IN delivery provide quicker onset, several formulations are under trial to treat emergency situations such as breakthrough pain in cancer.[25] Despite of such advantages, IN route bears some limitations. In humans, the total volume of administration is limited to 200 µL. Compared to rats, humans olfactory regions comprises of approximately 10 % of the total surface area which limit the access to brain directly through nasal cavity. Formulations that are not mucoadhesive are cleared within 15-20 minutes of application.[61] Hence, IN can be a route of choice for brain delivery of drugs which are potent and required at very low brain concentration in order to exert their pharmacodynamic effects.[23]

The nasal cavity can be divided in three parts: Olfactory region (10 % of total surface area), respiratory region (80-90 % of total surface area) and vestibule region. Out of these, the olfactory region plays the most important role in direct drug transport to brain. Responsible for the sense of smell and located on the most superior aspect of the cavity, the olfactory neurons form epithelium of the olfactory region are directly connected to olfactory bulb (Figure 2.9).[62] There are two major pathways involved in delivery of drugs to brain via nasal cavity: (a) olfactory epithelium region absorption and trigeminal nerve pathway. The olfactory region is about 10 $cm^2$ in area. Unlike BBB, the nasal epithelium has higher permeability due to low electrical resistance of only 40 $\Omega*cm^2$ measured in rabbits' nasal epithelium. Therefore, drug can either take paracellular pathway or transcellular pathway to reach the brain areas via olfactory region (Figure 2.10).

Another pathway for nose to brain delivery is trigeminal nerve pathway. A portion of trigeminal nerve cells endings are located in the respiratory region. However, transfer

through trigeminal nerve can be much slower as compared to olfactory region. The choice of pathway depends upon physicochemical properties of the drug being delivered such as lipophilicity and molecular weight to name a few.[23] Despite of such promises, rapid mucociliary clearance is a major obstacle for efficient delivery. Formulation approaches are being employed to increase the residence time for the drug at the site of absorption. Using mucoadhesive polymer based formulation is one of them. Chitosan is polymer of choice among several candidates as it is biodegradable, bio-adhesive and inert on nasal membranes. In addition, chitosan has been shown to increase the paracellular transport of polar drugs by transiently opening the tight junctions between the epithelial cells.[61] Thus to achieve maximum benefit of the nose to brain pathway and overcome the shortcomings of IN route we anticipate that by incorporating chitosan based thermosensitive gel, we can significantly increase the residence time of the delivery system and increase absorption across the membrane. Also, we expect that in addition to transcellular pathway, paracellular pathway will be active to transfer the drug due to transient opening of tight junctions by chitosan. The selective presence of lectin receptors in the olfactory region will help liposomes to bind selectively and thereby cross the olfactory region and enter the brain.

**2.11. Liposomes**

In order to understand and establish the model phospholipid bilayer membrane, Alec Bangham and colleagues published their work describing phospholipid bilayer membrane vesicles as swollen phospholipid systems which were termed as liposomes at a later stage. It was Gregory Gregoriadis was the first one to use liposomes to entrap the drug and use them as drug carriers and change the *in vivo* distribution of the drug.[63] Liposomes are nano-sized vesicles consists of at least one phospholipid bilayer

surrounding an aqueous solution core. The amphiphilic nature of liposomes allow them to encapsulate both hydrophilic drugs in aqueous core as well as hydrophobic drugs in the lipid bilayers. The hydrophobic bilayer and aqueous core in a single vesicle also enable to use a combinatorial system where a hydrophobic and hydrophilic drugs can be simultaneously encapsulated for drug delivery.[64] Advantages of liposomes to carry variety of cargo, improve drug stability, limit distribution to target organs and achieve controlled drug release encouraged pharmaceutical companies to apply them for several anticancer drug delivery which has successfully resulted in formulations currently being used in clinics. Liposomes are a great example of how a formulation based approach can improve application of a classical and potent anticancer drug like Doxorubicin. Doxorubicin has narrow therapeutic index and severe cardio and renal toxicities if administered as such. However, liposomal Doxorubicin has altered the distribution of the drug to not only improve concentrations in the tumor but significantly reduced cardio and renal toxicities than the parent compound. In the last 15 years, there have been several liposomal products were approved by the USFDA and other regulatory agencies as well as several clinical trials are underway using liposomal as drug delivery for anticancer drugs, nucleic acids such as siRNA, vaccines and diagnostic agents. Some of the reasons for high popularity of liposomes as delivery systems are their versatility to carry cargo of hydrophobic or hydrophilic (or both in some cases) drugs, biocompatibility and safety of the excipients used for the preparation of liposomes and suitability for controlled or site specific delivery. With regards to anticancer drug delivery, it has been seen that the liposomal drug is less toxic than the parent compound, more active within the tumor due to higher accumulation which may help improve the therapeutic index and broaden the clinical application of the drug and act as a carrier. To gain the advantage of liposomes,

we applied these carriers to encapsulate and deliver a hydrophobic drug and siRNA to brain using intranasal route.

### 2.12. Liposomes for IN delivery to brain

It is now clear that direct nose to brain pathways exist which facilitate brain delivery by obviating a need to pass the formidable BBB. Experiments in animal models have shown that colloidal carriers can also be used to deliver the drug to brain via IN route. These animal data are very encouraging since it opens the gate of numerous options to efficiently deliver drugs to brain which cannot be delivered otherwise due to their physicochemical properties or stability. Also, relatively larger cargo of the drug can be specifically delivered to the desired region by using targeting moieties. Colloidal carriers can protect the encapsulated drug from enzymatic degradation as well as from efflux transporters such as P-glycoprotein.[26] It was shown that such colloidal carriers enter the brain via transcellular pathway using receptor mediated endocytosis. Another important parameter to consider is the particle size. Ideally particles of 50-100 nm in size are rapidly internalized by the cells than larger particles.[27] In addition, cationic charge also helps the particles to circumvent the lysosomal pathway.[28]

### 2.13. Nose to brain delivery device

In order to achieve maximum efficiency of nose to brain delivery of IN formulation, localization of larger quantity of formulation in the olfactory region will significantly promote internalization.[65] Conventional devices can only load up to 5 % of the total delivered formulation in the olfactory region. Using a high pressure based device will significantly improve olfactory localization due to deep delivery inside the nasal cavity where olfactory epithelium is located. Thus enhanced delivery per dose will reduce the loss and frequency

of dosing. One such device was studied and it was found that high pressure indeed help to localize the formulation in the olfactory region (Figure 2.11).

**2.14. RNA interference in Cancer**

Andrew Fire and Craig Mellow discovered key gene expression regulatory mechanisms in the living cells known as RNA interference (RNAi). RNAi is used as a powerful tool to test function of almost any gene in a cell. Functionally active siRNA can bind in a sequence specific manner to target mRNA causing gene silencing either by mRNA degradation or translational inhibition. Hence, using synthetic exogenous siRNA to bind to a very specific site and provide gene silencing is considered to be very potent and promising approach in therapeutics. siRNA is a double stranded molecule usually 21-24 nucleotides base pairs in length. After introduction in a cell, it might be further processed by enzymatic complex known as Dicer and incorporated in a RNA interference silencing complex (RISC). The RISC will bind to target mRNA and either degrade the target mRNA or stop the process of translation.[66] Despite of being such promising therapeutic moieties, a limiting factor for effective *in vivo* siRNA efficacy is delivery systems. Using siRNA without delivery approach may result in either siRNA degradation or transient gene knockdown owing to very short half-life of siRNA.[67] The use of RNAi in cancer is based on a compelling rationale: many cancers are at least in part associated with toxic gain of function mutations.[68] Due to this, several RNAi based therapeutics are reported in literature to treat several diseases including cancer.[69-72] However, one of the major challenges in treatment of cancer by RNAi is a development of non-invasive delivery system which can be used in clinical settings and can deliver siRNA in a sufficient quantity in the tumor to produce therapeutic gene silencing. Stability of siRNA is a major challenge which limits its efficacy due to rapid degradation *in vivo*. A suitable carrier based targeted

system is essential for effective RNAi.[73] We intend to use liposomes as carrier for siRNA along with a cytotoxic drug. The combinatorial approach of using drug and siRNA simultaneously has shown promising results as siRNA targeted to down regulate proteins within the cancer cells responsible for resistance to cytotoxic drug.

### 2.15. Paclitaxel

Paclitaxel (PTX) belongs to taxane class of anticancer drugs which is used as a first-line therapy alone or in combination for many cancers. It is also used as a subsequent therapy as well as for several advanced and metastatic cancers. The drug has been listed on the World Health Organization's list of essential medicines that shows the importance of the drug in today's global healthcare system.[74] Discovered in the early 70s by Wall and Wani, who first reported the successful isolation of the drug from the natural source. Although the drug indeed showed promising results as well as unique mechanisms in the early studies, it took twenty years to come up with semi-synthetic method of synthesis to produce large quantities needed for Phase II trials.[75] To meet the demand of the drug on a large scale and reduce the ecological impact, pharmaceutical companies designed a novel fermentation process using plant cell line propagated in an aqueous medium and drug synthesized during the fermentation is extracted, purified and crystallized. In addition, the fermentation based method also eliminates use of hazardous chemicals and more efficient. Chemically, PTX is a diterpenoid contains 8 member taxane ring as it nucleus. (Figure 2.12). PTX is a strong hydrophobic drug with very low aqueous solubility and therefore administered with a delivery vehicle of 50 % Ethanol and 50 % polyethoxylated castor oil (Cremophor EL®). PTX has a unique mechanism of action with its ability to promote microtubule formation by specifically binding to β-tubulin subunit of microtubule to antagonize disassembly of microtubules resulting in cell cycle arrest during the

mitosis.[76] Arrest of cell cycle in G2/M phase induces cytotoxicity of the drug leading to apoptosis in time and concentration dependent manner. In clinics, the drug is administered as 3-hour infusion with a half-life of 10 to 14 hours. PTX undergoes extensive hepatic metabolism via Cytochrome P450 enzymes and goes through biliary clearance. One of the major problem with PTX is the vehicle used for infusion Cremophor EL®. The vehicle has been reported for severe hypersensitivity reactions, anaphylaxis and neurotoxicity necessitates pre-treatment with corticosteroids and antihistamine agents prior to the treatment. Cremophor EL® was also found to leach out plasticizers from the PVC infusion sets that require special attention during compounding and infusion to use specifically either glass of polyethylene-lined infusion sets. Alternative formulation of PTX to avoid Cremophor EL® has been developed using albumin-bound paclitaxel for infusion (Abraxane®). However, unlike PTX infusion approved to be used in several clinical applications, the albumin-bound PTX is only indicated for advanced stages of cancer when other treatment options have been exhausted. Hence, there is still a gap to come up with suitable PTX formulation that can effectively replace PTX infusion without using Cremophor EL®. Another problem is the therapeutic efficacy of PTX has been found to induce multi drug resistance through various cellular mechanisms which are not fully understood. The drug has been also identified as a key substrate for efflux transporter p-glycoprotein responsible for drug efflux resulting in low cellular internalization and rapid clearance. These two distinct resistance are also termed as non-pump and pump mechanisms resulting in therapeutic failures.[77] The pump mechanism can be overcome by shielding the drug in a suitable carrier such as nanoparticles or liposomes to improve intracellular drug concentrations. This report describes two such formulations of PTX wherein one of the formulations uses a simple non-ionic complexing excipients to replace Cremophor EL® while the second approach uses liposomes as a carrier for PTX.

| Descriptive Forms (solubility class) | Parts of solvent required for one part of solute | Solubility Range (mg/mL) |
|---|---|---|
| Very Soluble | <1 | >1000 |
| Freely Soluble | From 1 to 10 | 100-1000 |
| Soluble | From 10 to 30 | 33-100 |
| Sparingly Soluble | From 30 to 100 | 10-33 |
| Slightly Soluble | From 100 to 1000 | 1-10 |
| Very slightly soluble | From 1000 to 10,000 | 0.1-1 |
| Practically insoluble | >10,000 | <0.1 |

Table 2.1: Classification of drugs by solubility according to USP 34. Reproduced from ref. [38]

| Cyclodextrin | Drug | Route of Administration |
|---|---|---|
| **α-cyclodextrin** | Alprostadil | Intravenous |
| **β-cyclodextrin** | Dexamethasone | Dermal |
| | Iodine | Topical |
| | Nicotine | Sublingual |
| | Nitroglycerin | Sublingual |
| | Omeprazole | Oral |
| **2-hydroxypropyl β-cyclodextrin** | Hydrocortisone | Buccal |
| | Indomethacin | Eye drops |
| | Itraconazole | Oral, intravenous |
| | Mitomycin | Intravenous |
| **Randomly methylated β-cyclodextrin** | 17β-Oestradiol | Nasal spray |
| | Chloramphenicol | Eye drops |
| **Sulphobutylether β-cyclodextrin** | Voriconazole | Intravenous |
| | Ziprasidone maleate | Intramuscular |

Table 2.2: List of drug products and their intended route of administration approved by regulatory agencies which are formulated with various cyclodextrins. Reproduced from Ref [9]

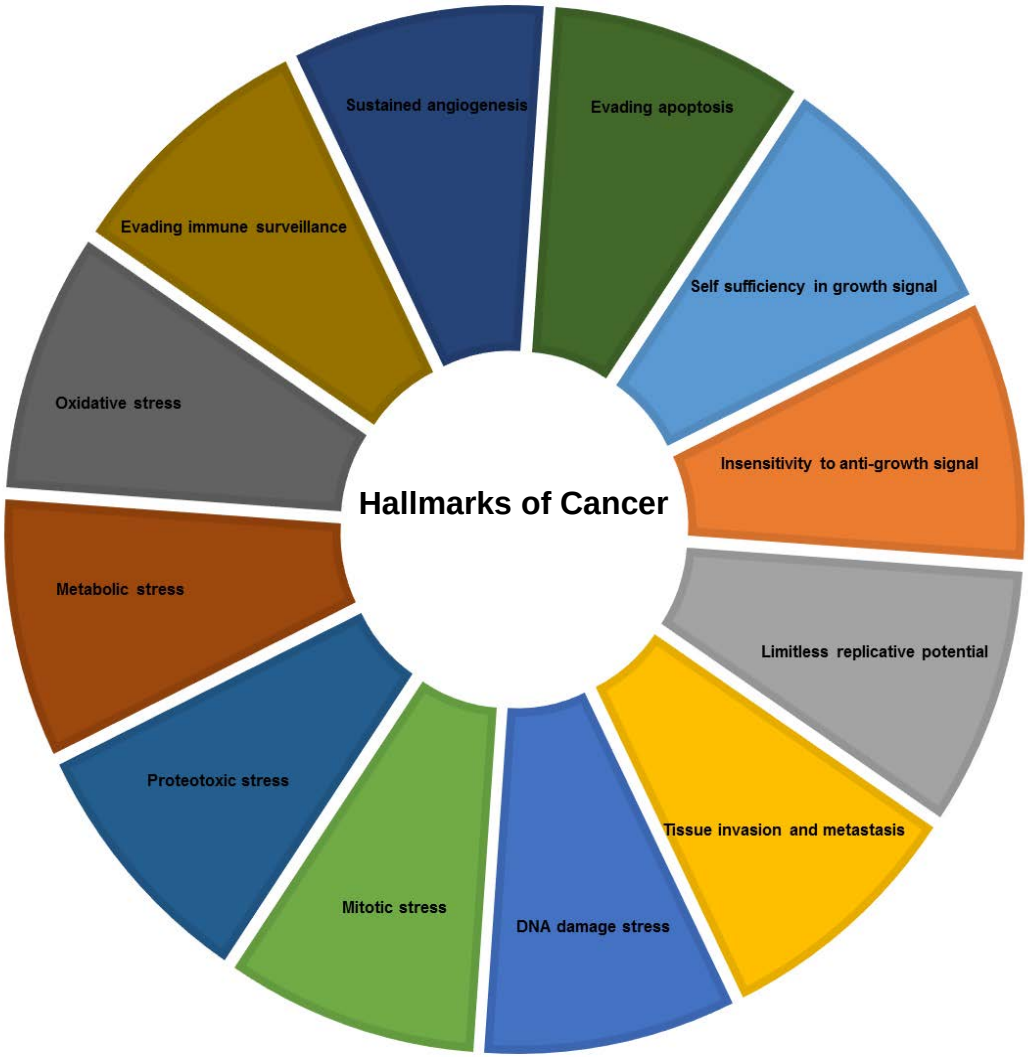



Figure 2.1: Common traits of pathogenesis of cancer. The modified representation above displays common pathways that leads to development of cancer. Modified from Ref. [29]

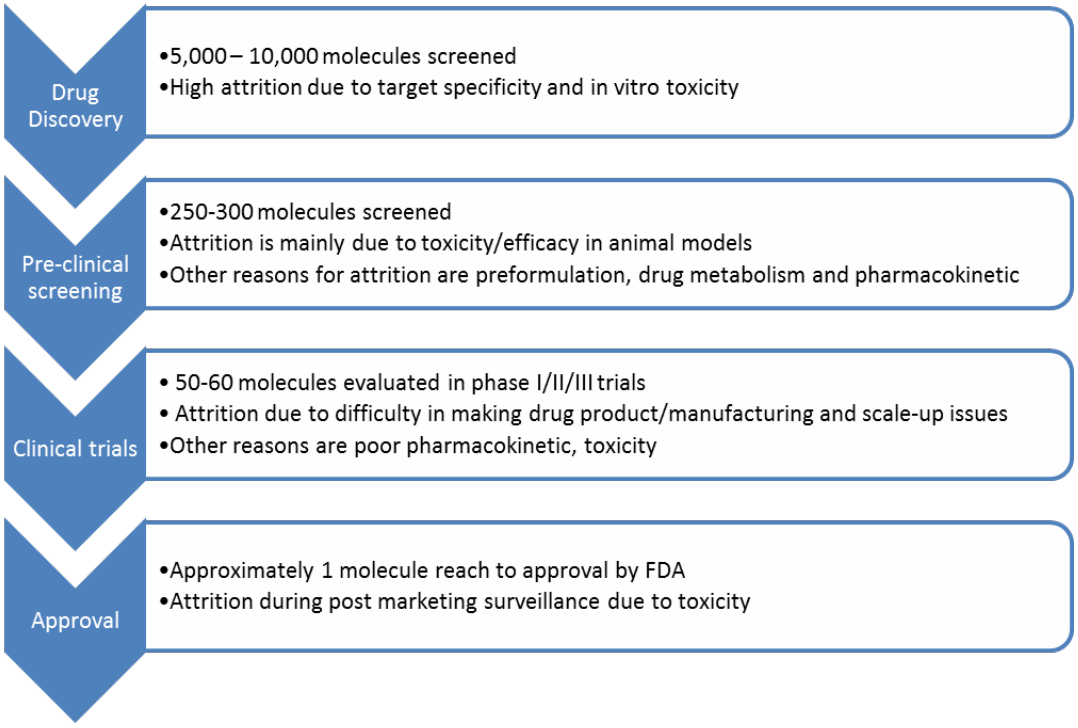
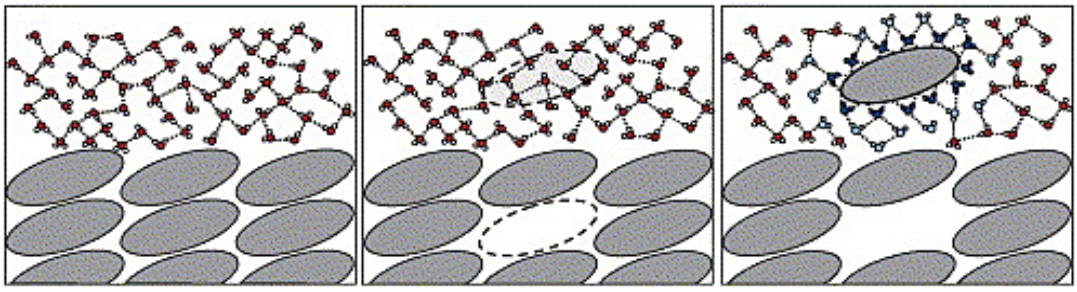



Figure 2.2: Typical phases of anticancer drug discovery and development in pharmaceutical industry. Each phase shows number of molecules screened at each phase followed by common possible reasons for discontinuation of further research for that molecule. Modified from ref. [34]



(a) (b) (c)

Figure 2.3: The three essential steps of solubilization of crystalline solids (a) Solvent and solutes are mixed but do not interact with each other. (b) The crystal lattice energy is overcome that removes the solute molecule from the lattice to make it available for interaction with solvent for solubilization (c) The solute molecule is surrounded by solvent creating new stabilizing interactions between the two to form solution releasing the energy. Reproduced from ref. [3]

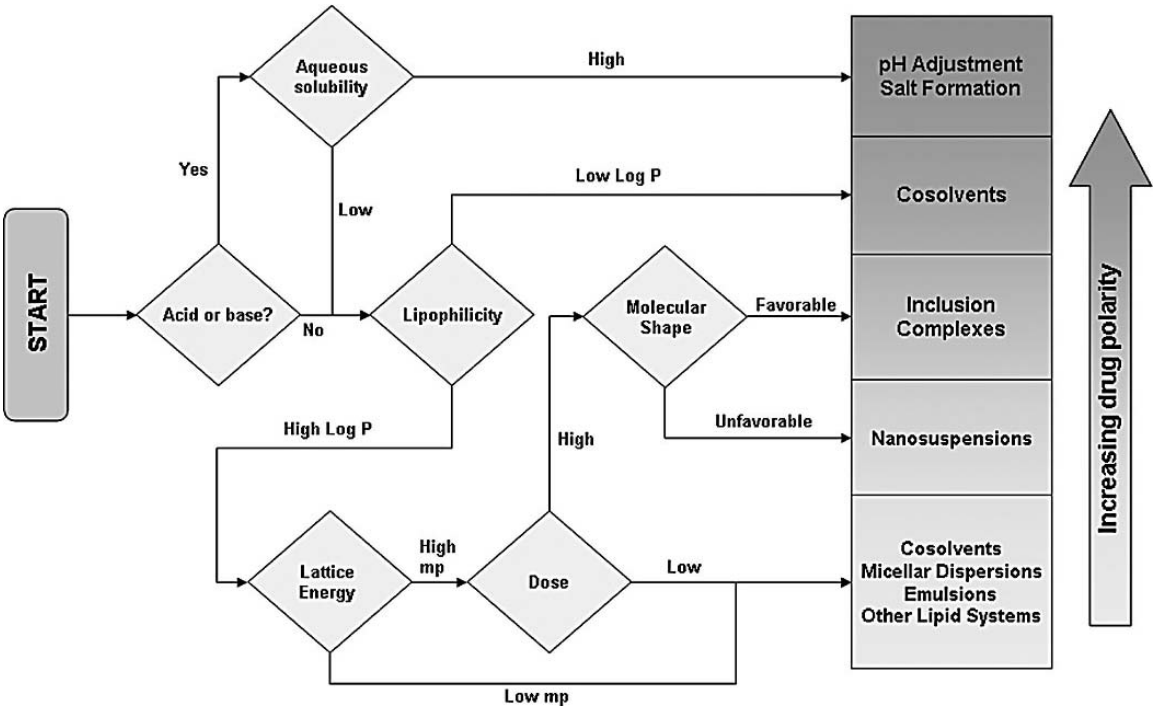



Figure 2.4: A step by step flow chart to choose appropriate solubility enhancement approach for injectable drugs with poor solubility. Reproduced from ref. [46]

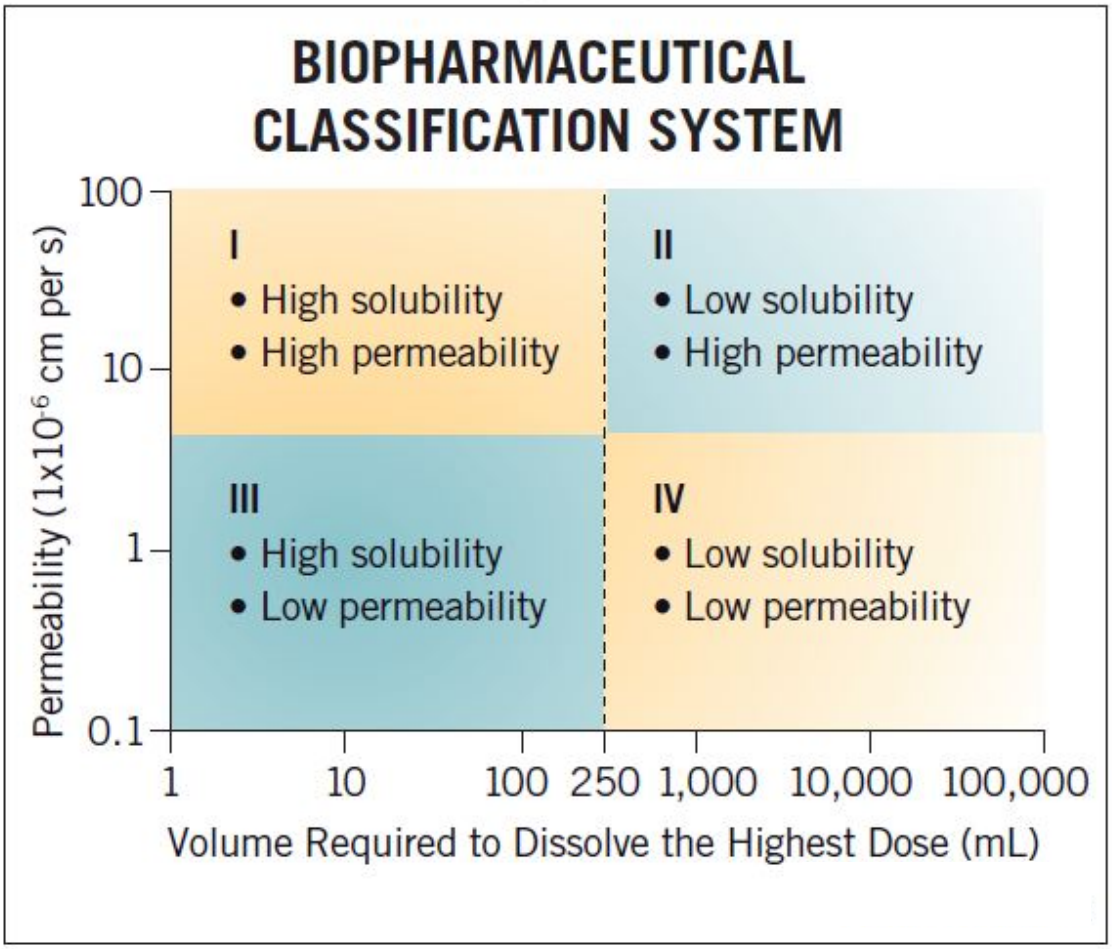



Figure 2.5: Biopharmaceutical Classification System (BCS) of drugs. Reproduced from ref. [78]

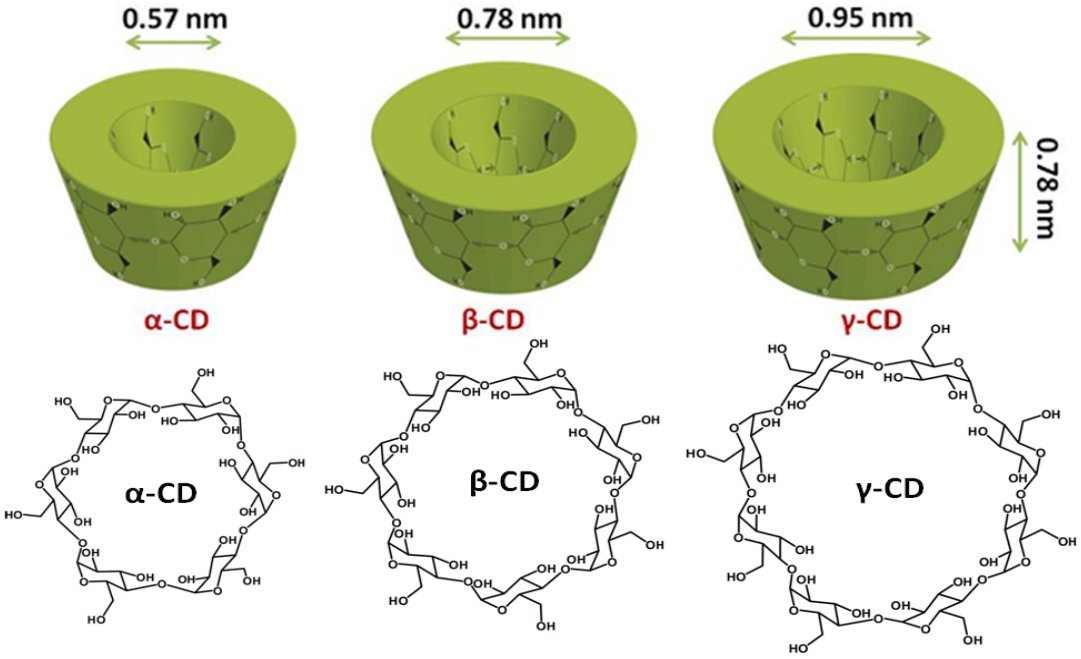
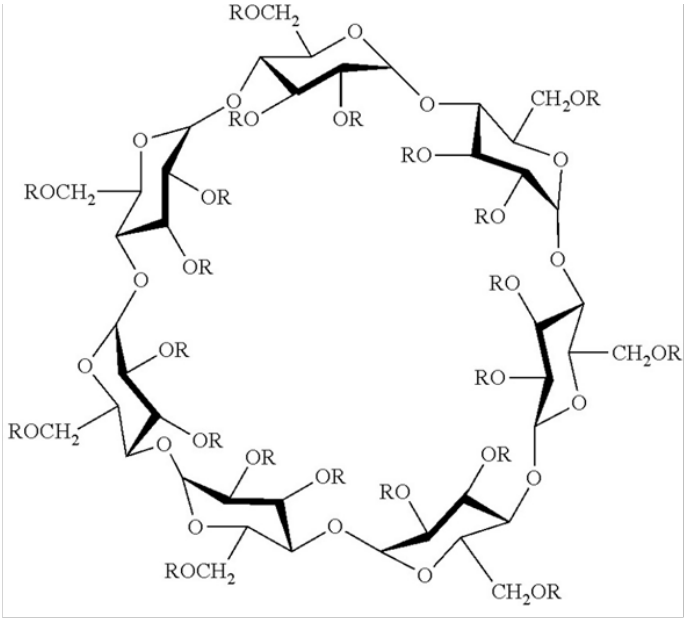



Figure 2.6: Three dimensional and chemical structure of different types of cyclodextrins. Naturally available α-Cyclodextrin, β-Cyclodextrin and γ-Cyclodextrins contains 6, 7 and 8 glucopyranose units respectively. Modified from ref. [52]




| β-cyclodextrin derivative | R- group modification | Water solubility at 25°C |
|---|---|---|
| β-cyclodextrin | -H | 18.5 mg/mL |
| Dimethyl β-cyclodextrin | $-CH_3$ | >300 mg/mL |
| Hydroxyethyl β-cyclodextrin | $-CH_2CH_2OH$ | >300 mg/mL |
| 2-Hydroxypropyl β-cyclodextrin | $-CH_2CHOHCH_3$ | >600 mg/mL |
| Sulfobutylether β-cyclodextrin | $-(CH_2)_4SO_3Na$ | >500 mg/mL |

Figure 2.7: Chemical structure of β-cyclodextrin and its derivatives. The table lists various derivatives and chemical group modifications of the structure and impact of chemical structure modification on water solubility. Reproduced from ref. [9]

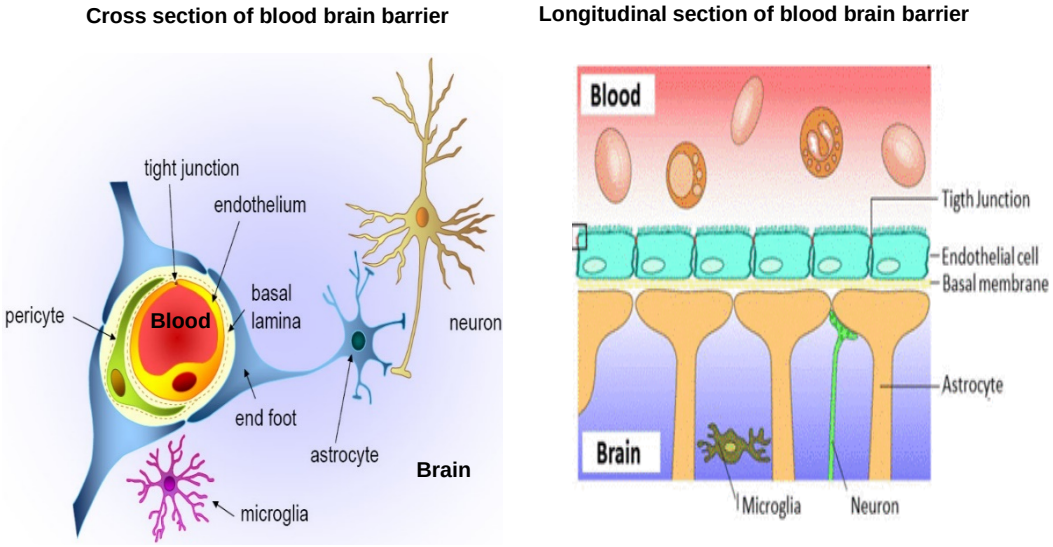



Figure 2.8: Schematics of blood-brain barrier displaying tight endothelial cell arrangements and tight junctions that restrict entry of several molecules from blood across to the brain. Modified from ref. [58]

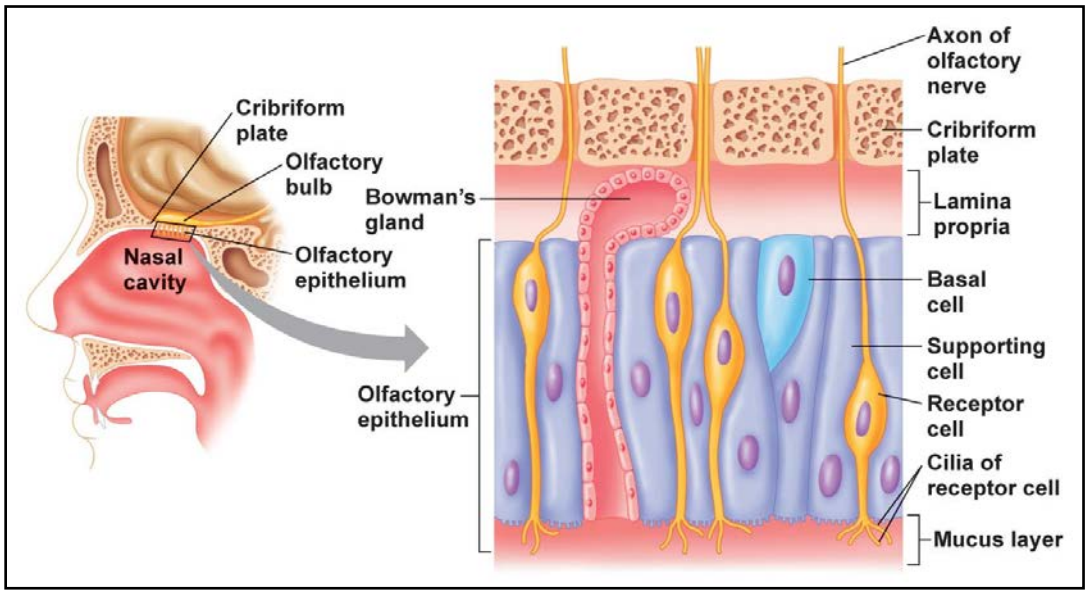



Figure 2.9: Anatomy of nasal cavity. The expanded olfactory epithelium shows direct access from the nasal cavity to brain [Reproduced from Ref. 62]

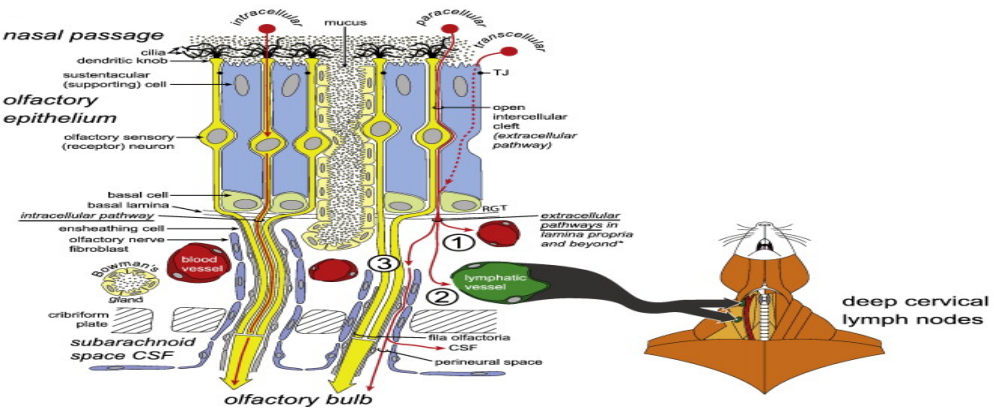



Figure 2.10: Pathways of drug transport from nasal cavity. 1. Systemic circulation 2. Paracellular transport and 3. Transcellular transport. Reproduced from ref. [24]

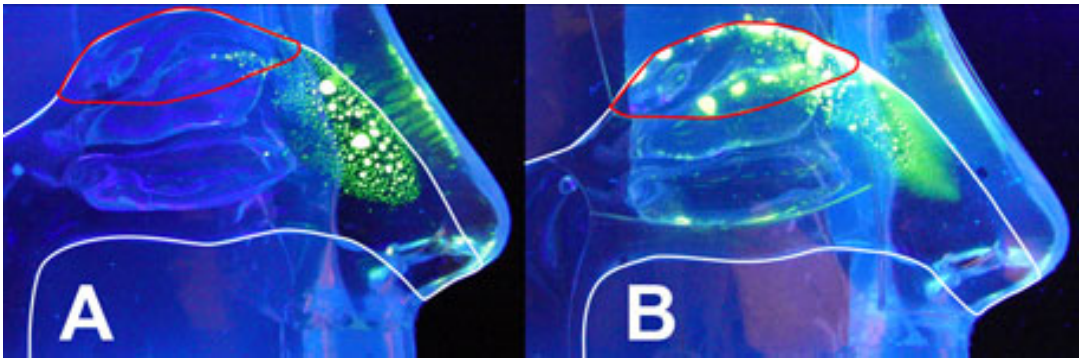



Figure 2.11: (A) Deposition of nasal spray following conventinal spray (B) Deposition after using high pressure nasal spray device localize more drug in te olfactory region (red outline). Reproduced from ref. [79]

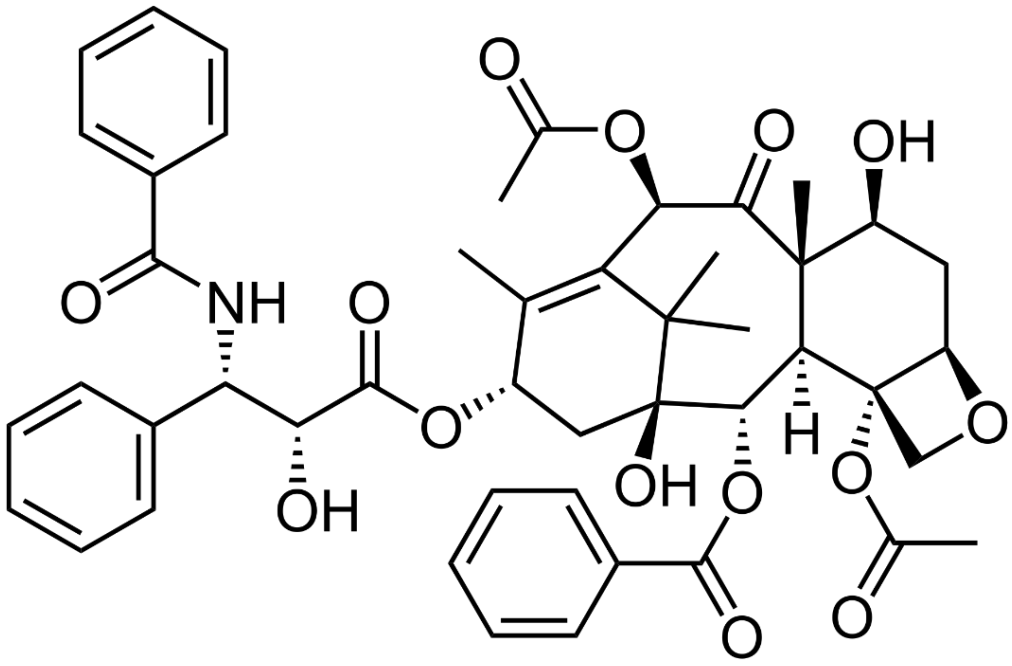



Figure 2.12: Chemical structure of Paclitaxel. Reproduced from ref. [75]

## 3. SPECIFIC AIMS

### Specific Aim 1: Design and develop molecular inclusion complexes of Paclitaxel with β-cyclodextrin derivatives at low molar ratios to improve aqueous solubility of Paclitaxel and evaluate their physicochemical interactions

Despite the anticancer effects of the Paclitaxel was known since 1970, the clinical formulation Taxol® was approved by the USFDA in 1992 for first-line treatment for breast and ovarian carcinoma, non-small lung cancer and AIDS related Kaposi sarcoma. One of the reasons for the long gap is to come up with a suitable formulation since PTX being a BCS class IV drug that exhibits low solubility and permeability. This makes it difficult to deliver sufficient therapeutic dosage and achieve effective plasma drug levels using traditional formulation approaches. Taxol® is a nonaqueous solution uses mixture of Cremophor EL (polyethoxylated castor oil) and Ethanol (1:1) as a vehicle to solubilize PTX. This vehicle is further diluted in saline or 5 % dextrose solution to 0.3 – 1.2 mg/mL PTX concentration and administered as infusion over the period of 1-24 hours to the patients. Although slow and longer duration infusions have been found beneficial in breast cancer patients, the stability of PTX in the infusion solution limited the application for extended time. Cremophor EL has been found to be responsible for some of the severe hypersensitive reactions such as anaphylaxis, neurotoxicity, nephrotoxicity as well as incompatibility with PVC infusion sets and leaching of plasticizers from the infusion bags. Due to the shortcomings of Cremophor EL, prophylactic pre-treatment with corticosteroids and anti-histamine is necessary for patients which increase hospital stay for the patients and requires polypropylene or polyolefin infusion sets. In order to overcome the problems of low solubility of PTX and hypersensitivity reactions to Cremophor EL, there is an unmet need to design PTX formulation that can improve the aqueous solubility of the drug and

obviate Cremophor EL as vehicle. The ideal formulation should be designed using inert and safe excipients that can enhance PTX solubility to deliver the drug using the same infusion dosage used in clinics today. Such approach can improve the usage of the potent but highly hydrophobic drugs like PTX in the clinics but also reduces the prophylactic pre-treatment and save associated cost and hospital stay for the patients. Recently, several synthetic derivatives of β-cyclodextrin were developed and used for BCS class IV drugs to improve aqueous solubility and dissolution rates. These derivatives have significantly higher solubility than native β-cyclodextrin present an alternative approach for solubility enhancement for potent anticancer drugs like PTX and other belonging to BCS class IV by forming molecular inclusion complexes. The purpose of this study is to evaluate the feasibility of approach by preparing molecular inclusion complexes of PTX with various CD derivatives so as to improve aqueous solubility of PTX at relatively lower molar ratios. Once such complexes are formed, it is important to understand the physicochemical interactions between the PTX and complexing CD derivatives. Quantitative estimations of PTX complexation with CD derivatives and understanding the physicochemical interactions within the complex will help deduce the encapsulation mechanism as well as any incompatibilities between PTX and CD derivatives. Once the method is optimized and physicochemical interactions are understood, group of selected formulations can be further evaluated to understand the PTX activity within the complex.

**Specific Aim 2: To evaluate the safety of β-cyclodextrin derivatives as excipients and assess cytotoxicity of Paclitaxel inclusion complexes mechanism of cellular internalization**

The overall aim is to design an alternative formulation of PTX for the clinic that can replace use of Cremophor EL and Ethanol as vehicle and thereby avoid associated severe

hypersensitivity reactions and prophylactic pre-treatment. An important aspect is to choose safe excipients for the study such that possess no biologic or pharmacological implications. Multiple dosages over the course of chemotherapy expose cancer patients to excipients that are used to formulate the anticancer drugs. Chronic administration can result in accumulation of excipients that can lead to variety of acute to chronic adverse effects at cellular and genetic levels. Special attention is required when such excipients over the long term accumulation or chronic usage can alter the genetic information and lead to mutation that can end up in cell death, loss of physiological functions or tumor. Hence, evaluating the safety profile and estimating the genotoxicity potential of the CD derivatives used to prepare PTX complexes is as important as designing a successful formulations with them. The objective is to evaluate genotoxic potential of CD derivatives using *in vitro* micronucleus test and ascertain that replacing Cremophor EL with CD derivatives provides a safer alternative for PTX. Once, the safety is established, the following objective is to determine the anticancer activity of complexed PTX. By assessing *in vitro* cytotoxic potential of the PTX and CD complexes and compare those with PTX can reveal the effect of CD complexation on cytotoxicity potential. It is also important to understand the underlying mechanism involved in complexation that affect or alter cytotoxic potential of PTX after complexation. The altered cytotoxic potential could be attributed to cellular internalization of PTX and hence measuring the effect of CD complexation on cellular internalization should be compared with free PTX. Combining quantitative cellular internalization data with *in vitro* cytotoxicity studies can help reveal underlying mechanism to explain effect of complexation on PTX activity.

**Specific Aim 3: Design and develop liposomes containing paclitaxel and siRNA for nose to brain targeting and evaluate their *in vitro* efficacy, cellular internalization and *in vivo* distribution**

Brain tumors are one of the most challenging to treat due to the presence of an occlusive blood brain barrier (BBB) that restrict passage of most of the anticancer drugs and results in either slow onset of action or incomplete therapeutic levels in the brain leading to therapeutic failure or emergence of drug resistance. Another possible route to reach brain via intranasal route has been known for years. However, the limitations to achieve full benefit of this route have slowed the progress. Since many anticancer drugs are very potent, low dose and volume can be successfully administered using IN route. To combine the effectiveness of the IN route, a nanocarrier based approach is proposed where a drug delivery carrier encapsulating an anticancer drug with oligonucleotides such as siRNA. Such nanomedicine platform with a combinatorial delivery of anticancer drug and siRNA can improve drug cytotoxicity by suppressing underlying cellular resistance mechanism. Although PTX has shown very high activity against brain tumors, its failure to reach at sufficient concentration in the brain using traditional approaches has limited its therapeutic applications. To gain the advantage of this gap, the proposed nanocarrier system should encapsulate PTX simultaneously with siRNA. Liposomes are foreseen to be ideal carriers due to their versatility to carry hydrophobic and hydrophilic drugs together. The first step is to design and develop suitable liposomes that can effectively encapsulate PTX and siRNA. In order to design effective safe and inert delivery system that lacks any biological or pharmacological implications, assessing the safety profile of excipients used for the preparation of liposomes is crucial. An *in vitro* micronucleus test to measure the genotoxic potential of liposomes. Suitable *in vitro* studies to be employed to further evaluate their ability to carry the cargo across the cell membrane followed by evaluation of cytotoxic

potential of such delivery system using suitable *in vitro* methods. The success of the delivery system prepared largely depends on its ability to be used via IN route for brain delivery. Hence, a suitable animal model to be employed to gain more understanding and evaluate the feasibility wherein such delivery system is applied via IN route to estimate its brain localization and compare it with free PTX.

# 4. DESIGN AND DEVELOP MOLECULAR INCLUSION COMPLEXES OF PACLITAXEL WITH β-CYCLODEXTRIN DERIVATIVES AT LOW MOLAR RATIOS TO IMPROVE AQUEOUS SOLUBILITY OF PACLITAXEL AND EVALUATE THEIR PHYSICOCHEMICAL INTERACTIONS

## 4.1. Introduction

Anticancer drugs like PTX are found to be very potent and so essential in the clinics for the treatment of several types of solid tumors. The drug however is extremely hydrophobic, has poor permeability and aqueous solubility that makes it extremely challenging to achieve desired pharmacokinetic parameters with a simple formulation approach. Taxol®, the first formulation approved by the USFDA to be used in clinics in 1992 employed non aqueous mixture of Cremophor EL and anhydrous Ethanol (1:1) as a vehicle. For administration, Taxol is diluted in either saline or 5 % dextrose solution at a low concentration of 0.3-1.2 mg/mL. The solubilizing vehicle of Cremophor EL and anhydrous Ethanol have been found to be responsible for many adverse reactions that requires pre-treatment and also reported to have interactions with polyvinyl chloride infusion sets. The resultant infusion solution was reported to be unstable with time that does not allow using slow infusion which has been found beneficial in some types of cancer chemotherapy. PTX is also susceptible to degradation by hydrolysis under mild alkaline conditions by taxane ring cleavage which results in formation of various active as well as inactive metabolites. Although there have been other formulations of PTX have been approved by the USFDA, there hasn't been any formulation developed or in the clinical trials that can replace Cremophor EL based PTX formulations in the clinics. Due to its high potency and wide application, it is very important to reduce the clinical attrition of PTX and salvage it by applying re-formulation approach. This can be fulfilled by

designing a formulation that can enhance PTX solubility without using Cremophor EL and anhydrous Ethanol and can be diluted in a large volume infusion bag to be slowly infused over a period of time. Such formulation should improve solubility and thereby efficacy as well as prevent hydrolytic degradation of PTX to enable longer infusion duration. Thus reformulation seems a suitable, cost effective and rapid approach to replace Cremophor EL and anhydrous Ethanol and avoid associated side effects, stability issues and possibly prophylactic pre-treatment. There have been several solubility enhancement approaches used till date for various drugs and can be applied to PTX to achieve this goal. However, the number of approaches available to screen can lead to exhaustive study. A wiser way is to look at the chemical structure and physicochemical properties of PTX and decide appropriate solubility enhancement approach to screen. Reviewing the structure and physicochemical properties reveals that lack of ionizable functional groups in PTX indicates that pH dependent solubility, salt formation or charge based complexation will not be feasible to enhance solubility. A prodrug and conjugation based approach can be applied but since a prodrug is treated as a new entity by the regulatory agencies with respect to safety and efficacy of the drug, this venture will ultimately lead to lengthy and costly clinical trials makes this approach non-suitable. A co-solvent based approach is another simple way to achieve desired solubility by using water miscible solvents. Although solution of PTX at high concentration resulted from using co-solvent method, drug precipitation upon dilution in the infusion bag or in circulation is a significant impediment to co-solvent approach. Similar results were also observed when PTX was emulsified but upon dilution in 5 % dextrose solution, the drug slowly precipitated. One of the suitable approach that surfaced with similar drugs is to use macrocyclic hosts such as CD to form non-covalent inclusion complexes. These complexes have exhibited higher aqueous solubility and thermodynamic stability for the complexed drug. CD are cyclic

heptamers of glucose α-1,4 D-glucopyranose that creates a toroid structure with a hollow hydrophobic core capable of forming weak hydrogen bonds with hydrophobic molecules. Earlier unmodified CD investigated for such approach was found to have hemolytic and renal toxicities. But modified CD derivatives have not only been found to be safer but also exhibit significantly higher aqueous solubility and better solubilizing abilities than the native CD. Such Several derivatives of CD are reported as non-immunogenic, biocompatible and suitable for human use by various regulatory agencies including the United States Food and Drug Administration (USFDA). The results indicate that complexation with CD derivatives is a suitable alternative to Cremophor EL based formulations for PTX and can effectively substitute the current formulation in the clinics. Preliminary studies to complex PTX with various CD derivatives have shown that such complexes have shown higher aqueous solubility as well as reported better stability. These investigations have encouraged to evaluate it further by preparing series of complexes of PTX with several new CD derivatives and assess the effect of complexation on solubility enhancement. The first objective is to evaluate methods to complex PTX with CD derivatives such that relatively higher quantities of PTX can be efficiently complexed at low molar ratios and exhibit higher aqueous solubility. Out of the methods evaluated, a selected method to be further used to evaluate other studies. The next step is to decipher the mechanism of complex formation. It is very important that to understand that the complexes formed should retain the drug without having any significant ionic or chemical interactions that can alter the stability and thereby activity of PTX. One of the rapid methods to evaluate and detect any possible interactions in the solid complex of PTX and CD is to apply differential scanning calorimetry (DSC). DSC measures the temperature and heat flow changes associated with transitions occurring in the material with temperature in a controlled atmosphere. Since any physical or chemical transition of a substance is associated with

either heat gain or loss, studying the heating and cooling thermograms obtained by normal and modular DSC studies can reveal sufficient information about such physical and chemical changes in the complex. Another method to study complexation and physical state of PTX within the complex is to employ spectroscopic methods. By measuring or observing discrete signals characteristics arising in a spectrogram due to specific chemical groups or side chains of PTX, one can qualitatively and quantitatively determine the PTX within the complex. Lastly, if such complexes exhibit higher aqueous solubility, one has to understand the effect of complexation on the crystal structure of PTX. Powdered X-ray diffraction is rapid and simple method to characterize the complexes with regards to crystalline nature of PTX within the complex and PTX compatibility with CD.

## 4.2. Materials and Methods

### 4.2.1. Materials

Paclitaxel was purchased from Sigma Aldrich (St. Louis, MO). Hydroxypropyl β-cyclodextrin (HPCD, Kleptose®) and Methyl β-cyclodextrin (MeCD, Crysmeb) were purchased from Roquette Pharma (Lestrem, France). Sulfobutyl ether β-cyclodextrin (SBCD) was purchased from Cydex Pharmceuticals (Lenexa, KS). Tert-butyl alcohol, Ethanol and Acetonitrile were purchased from Alfa Aesar (Ward Hill, MA). Deionized water from Millipore water system was used for all the experiments.

### 4.2.2. Preparation of PTX-CD inclusion Complexes

The inclusion complexes of PTX and CD derivatives were prepared using two different methods. In order to match the stoichiometry, molar ratios were chosen over weight ratio to create comparative results. Molar ratios of 1:1, 1:2 and 1:5 of PTX and CD derivatives respectively were prepared using two different methods. In the first method, 8.55 mg of PTX was dissolved in 1 mL of absolute Ethanol. CD derivatives were dissolved in 1 mL

of deionized water. The PTX solution was added drop wise to CD derivative solution followed by vortex mixing for approximately 1 minute. After vortex mixing the solution was kept under stirring for 1 hour at room temperature.[80] After 1 hour, the resultant mixture was filtered using 0.2µ syringe filter (VWR International, Radnor, PA) to remove any undissolved PTX. The solution lyophilized using bulk lyophilization (Labconco, Kansas City, MO) overnight to remove water. Next day, the lyophilized complex powder was transferred in a glass vials and stored at 2-8 $^{o}$C until further use. Similarly in another method, a modified co-solvent method was employed to prepare complexes at a same molar ratios.[81] To prepare complexes using this method, in one vial required quantity of CD derivative was dissolved in 5 mL deionized water. In another vial, 8.5 mg of Paclitaxel was added followed by 100 µL of Acetonitrile and 400 µL of Tert butyl alcohol. Both phases were vortexed thoroughly to ensure clear solution. Finally, Paclitaxel solution was added to aqueous solution containing cyclodextrin in a drop-wise manner while stirring. The resultant mixture was kept for six hours at room temperature under stirring. After six hours the mixture was filtered using 0.2µm syringe filter and lyophilized overnight. The resultant lyophilized formulation was stored in glass vials at 2-8 $^{o}$C till further use.

### 4.2.3. Assessment of Complexation Efficiency and Solubility Enhancement

In order to assess the amount of Paclitaxel in the inclusion complex, a modified chromatographic technique was used.[82] The chromatographic equipment was consists of an auto sampler (Waters 717plus Auto sampler), a pump (Waters 1525 binary pump) and a detector (Waters 2487 dual λ detector). The samples were run using mobile phase of water and methanol at a ratio of 35:65 and flow rate of 1 mL/min with detection wavelength was set to 227 nm using C18 column (3.9 mm x 150 mm, Waters) at 25 $^{o}$C. All lyophilized formulations prepared using both methods were dissolved in water and

centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and subjected to measurements. Complexation efficiency was measured using the following formula:

$$Complexation\ efficiency\ (\%) = \frac{PTX\ complexed}{PTX\ total} \times 100$$

Standards of PTX were prepared by dissolving PTX in 95% Ethanol. PTX calibration curve was made ranging from 1-50 µg/mL. The aqueous solubility of PTX was based on the assay from the HPLC measurements. Solubility enhancement of PTX was calculated as a ratio of solubility in the complex and inherent solubility of PTX. The aqueous solubility of pure Paclitaxel was considered 0.34 µg/mL at 25 $^{o}$C.[82]

### 4.2.4. Differential Scanning Calorimetry (DSC) of PTX - CD Complexes

To evaluate drug-excipient interactions and solid state stability of the inclusion complexes, DSC studies were carried out. In order to reduce the number of samples, PTX-CD complexes with highest solubility enhancement were selected. To carry out DSC studies, each sample was accurately weighed (3-10 mg per sample) in an aluminum pan to be used for the study. Once weighed the sample pans were carefully picked up using tweezers and placed on a press to seal them with an aluminum top. A small pinhole was created on the top of the pan using a sharp needle. The pan was then picked up using metal tweezers and kept in a sample pan stage inside the instrument. Similarly an empty pan was placed on a reference pan. Once the sample and reference pans were placed, the instrument lid was closed and study was started using heating rate of 2 oC/min for all the samples (Mettler Toledo, Columbus, OH). In addition to PTX-CD complexes, individual samples of CD derivatives and PTX were also run as controls. The thermograms obtained from the DSC studies were compared against respective CD and PTX thermograms to understand the physicochemical interactions and polymorphic state of PTX within the complex.

### 4.2.5. $^{1}$H-NMR Studies of PTX-CD Complexes

Similar to DSC studies, $^{1}$H-NMR studies of PTX-CD Complexes were carried out using similar sampling plan to better understand the physicochemical interactions between PTX and CD derivatives in the complexes as well as confirm the complexation. To prepare samples, each complex was accurately weighed (1-5 mg) and transferred to an NMR tube (Sigma Aldrich, St. Louis, MO). To this tube, Dimethyl sulfoxide –$d_6$ was added to dissolve the solid and yield clear solution. The solution height was adjusted to 4 to 5 cm in the tube to gain maximum resolution. The tube was covered with a Teflon tape and capped. The samples were then subjected to $^{1}$H-NMR studies using Bruker 700 MHz advanced spectrometer (Bruker Corporation, Billerica, MA). PTX and three CD derivatives were used as a controls to compare them with complexes and understand physicochemical interactions.

### 4.2.6. Powdered X-ray Diffraction Studies of PTX-CD complexes

Powder X-ray diffraction analysis was performed on Bruker D8 Advance, controlled by diffrac plus X-ray diffraction (XRD) commander software (Bruker AXS Inc., Madison, WI). Samples were prepared by spreading powder sample on a polymethyl methacrylate (PMMA) specimen holder rings from Bruker (AXS Inc., Madison, WI) and were scanned from 2 to 40 degrees at the rate of 2 degrees/min with 0.02 degrees step size and 0.6 s/step at 40 KV and 40 mA. The divergence and anti-scattering slits were set to 1 degree and the stage rotated at 30 rpm. Data analysis was performed using EVA Part 11 version 14.0.0.0 software. PTX was used as a control to compare the crystal structure of pure drug to the complexed PTX.

### 4.2.7. Statistical Analysis of Data

All quantitative data presented as mean ± standard deviation from five to ten independent measurements. Data analysis was performed using descriptive analysis and single factor ANOVA. Student's t-test was applied to data sets and P value of less than 0.05 was considered as statistically significant.

## 4.3. Results

### 4.3.1. Solubility Enhancement and Entrapment Efficiency of PTX-CD Complexes

All lyophilized complexes were reconstituted at 25 $^{o}$C in deionized water into clear colorless solution. 9 formulations were prepared using the co-solvent method at three different molar ratios of 1:1, 1:2 and 1:5 for PTX and CD respectively while three formulations were prepared using Ethanol method. Solubility enhancement and data for all formulations are summarized in Table 4.1. Compared to inherent solubility of 0.34 µg/mL, CD complexes exhibited substantial increase in aqueous solubility of PTX. Also, it was clear that the complexes prepared using modified co-solvent method resulted in significantly higher aqueous solubility compared to Ethanol solution method. The solutions of complexes prepared from modified co-solvent method were easily reconstituted to aqueous PTX concentration of more than 0.1 mg/mL. All solutions were visually observed to be clear colorless solutions and free of any visible particulates. A general trend was observed that as molar fraction of CD was increased, the aqueous solubility of PTX improved as well. Comparing different formulations, it can be seen that PTX complexes with HPCD exhibited higher aqueous solubility than MeCD and SBCD complexes prepared at the same molar ratios. Similarly, complexes of MeCD with PTX exhibited lowest aqueous solubility when compared with other two CD derivatives at the same molar ratios.

### 4.3.2. Differential Scanning Calorimetry Studies of PTX-CD Complexes

The DSC studies were performed on inclusion complexes of the highest molar ratio studied of PTX to CD (1:5). A very broad heating temperature range of 20 $^{o}$C to 240 $^{o}$C was employed for all DSC samples to understand the physicochemical interactions between PTX and CD in the complexes and compared with thermograms of PTX and CD alone. All pans were weighed before and after DSC studies and showed no or little weight difference (less than 0.5 mg). The data suggests that all samples had negligible amount of water and/or any other volatile substances. Residual amount of water suggests that all samples were lyophilized for sufficient time to yield nearly dry powder. Representative thermograms for PTX alone (Fig 4.1), CD derivatives alone and their respective complexes are displayed (Fig 4.2-4.4). Sharp endothermic peak at around 220 $^{o}$C was observed representing the melting point of PTX (Fig. 4.1). However, in all three complexes prepared with different CD derivatives, this sharp peak was visibly absent. Comparing the thermograms of complexes with their respective CD, the overall trend was observed to be similar with respect to the peaks and no indications of any physicochemical interactions between PTX and CD were observed. All CD derivatives and their respective complexes showed an endothermic trough between 65 -100 $^{o}$C which could be attributed to loss of volatile substances in the CD and their complexes. Overall, the thermograms of the complexes suggest absence of any physicochemical interactions between PTX and CD that could have any negative effect on PTX stability within the complex.

### 4.3.3. $^{1}$H-NMR Studies of PTX-CD Complexes

Representative images of spectrograms of CD derivatives and their respective complexes are compared with PTX alone (Fig. 4.5-4.8). Dimethyl sulfoxide –$d_6$ was used instead of $D_2O$ due to low aqueous solubility of PTX. Due to complete solubility of the complex,

complete feature of the spectra representing PTX and CD were clearly seen. Comparing the spectra to PTX to the complexes, distinctive changes in the aromatic protons of PTX were seen to have significantly less resolved peaks and lack of well-defined resonance (Fig 4.6-4.8, magnified sections). The shielding of aromatic protons of PTX are due to their transfer within the CD cavity resulting in weak hydrophobic interactions between the protons of PTX and CD. The lower resonance supports the hypothesis of core-shell structure wherein the PTX molecule is within the hydrophobic cavity of CD. These weak hydrogen bonds illustrate the underlying mechanism of complexation between a hydrophobic drug like PTX and water soluble molecules like CDs. The $^{1}$H-NMR studies revealed that PTX was complexed within the CD cavity and held by weak hydrogen bonds within the hydrophobic cavity of CDs. In addition, no other interactions have been observed negates possibility of any other interactions that may affect the stability of PTX within the complex.

### 4.3.4. X-ray Diffraction Studies of PTX-CD Complexes

Powdered X-ray diffraction patterns of PTX-CD complexes were compared with PTX to understand the scattering pattern of PTX within the complex that can provide key information about the atomic arrangement and crystal structure within the complex. An overlay of spectra of representative samples of PTX and PTX-CD complexes was prepare to study the differences between three complexes and PTX crystal structure (Fig. 4.9). PTX in its free form represents a discrete crystalline structure reflected in various peaks on the spectra. These distinct peaks of PTX were absent in all three complexes indicating that the crystal structure of PTX within the complex is not the same with the free PTX. Upon further investigation, there are no other distinct peaks of PTX were observed within the complex. Given the facts that CDs are amorphous and do not represent any specific

crystalline nature, absent of distinct peaks indicated that PTX also lost its crystalline structure within the complex and is in the amorphous form.

### 4.4. Discussions

Inclusion complex involves formation of non-covalent bond based host-guest relationship between PTX and CD derivatives. The classical mechanism of complex formation is also explained as compensation where a water molecule inside the hydrophobic cavity is replaced by hydrophobic drug that favors weak hydrophobic interactions resulting in molecular inclusion complex formation. These complexes exhibit physicochemical properties that are different from free PTX or CD. The spatial arrangements of each glucopyranose units of CD create chair confirmation resulting in a truncated cone type structure with a hollow hydrophobic cavity. The cavity serves as a host for PTX molecules allowing them to encapsulate by forming non-covalent interactions resulting in molecular inclusion complexes. The complexes have either entire or some parts of PTX molecule enter the hydrophobic cavity of CD derivatives and shielded resulting in higher apparent solubility for PTX. Another important property of the PTX-CD complex is that there is a dynamic equilibrium in the aqueous solution between the PTX and CD. This has very important benefit as PTX does not precipitate over dilution and minimize the risk of precipitation in the infusion bag, at the injection site or under physiological conditions. By preventing strong intramolecular hydrogen bonding within the CD by various chemical substitution of the hydroxyl group improve aqueous solubility as well as complexation efficiency of the CD derivatives compared to native CD. In addition, CD derivatives are reported to improve the stability as well as bioavailability of low solubility drugs.[83] These properties make CD derivatives more suitable macrocyclic hosts for drugs like PTX.[9] The unique solubility enhancement mechanism makes a strong argument to

evaluate CD derivatives as suitable excipients to reformulate highly lipophilic BCS class IV drugs such as PTX. Previous data has suggested that while evaluating various hydrophobic drugs for CD complexation, a linear relationship was found hydrophobicity of drug and complexation with CD that clearly favored PTX significantly compared to other anticancer drugs. Especially for PTX, the new formulation not only enhances the solubility, it also replaces toxic Cremophor EL in the formulation. The effect of solvent system on solubility enhancement and complexation efficiency is summarized in Table 4.1. Higher PTX solubility was observed when a co-solvent method was used. In order to form successful complexes, water is essential since water occupies the cavity and later released upon PTX entering the cavity and forming complex.[10] During complexation, there is a dynamic equilibrium between complex, PTX and CD. The rate of complexation is directly dependent on the PTX concentration. Hence, in case of Ethanol based system, the solubility of PTX will be a rate-limiting step and govern the complexation and solubility enhancement. To overcome, one has to employ higher cyclodextrin concentrations and larger aqueous volumes. The co-solvent system is designed in such a way that it overcomes the rate-limiting PTX solubility and favored higher complexation efficiency that reflected in higher solubility enhancement. In presence of organic solvents, PTX is less globular in confirmation that favored high inclusion as the solvent polarity was reduced and higher complexation was achieved at relatively lower CD concentration and molar ratios.[83] Also, presence of organic solvents reduces the inherent tendency of PTX to form stable hydrophobic clusters with other PTX molecules.[82] Understanding the impact of hydrophobicity and effect of PTX concentration helped understand the mechanism of complexation to prepare complexes with a co-solvent method that showed significantly higher solubility and complexation at relatively lower molar ratios than previously reported.[82] It can be seen that compared to HPCD and SBCD, MeCD had relatively

lower complexation and solubility enhancement. Low complexation efficiency with MeCD could be attributed to the methyl substitution on CD ring. Previous studies have shown that direct substitution of CD hydroxyl groups with methyl group increased the steric hindrance and thus prevents PTX inclusion in the cavity.[81] The effect of molar ratio on complexation and solubility is explained by plotting drug solubility versus CD concentration known as phase solubility diagram. A linear solubility increase with increase in CD concentration is defined as $A_L$-type curve. With co-solvent method, enhancement of PTX solubility with increased molecular ratio indicated that all CDs showed $A_L$-type solubility isotherms.[84] This relationship also indicates that the complexes exhibit first-order relationship with respect to CD and PTX concentration. From stoichiometric point of view, if all cyclodextrins molecules formed 1:1 complexes, there is no need to use higher amount of cyclodextrins. However, with an equilibrium method used, it is not possible to take the full advantage of every single cavity in all the CD molecules. This is the reason why we have to employ higher amount of cyclodextrins in order to complex more Paclitaxel. Recent phase solubility studies of several drugs have revealed that some drugs employ more than 1 molecule of cyclodextrin per molecule of a drug which may result in either 1:2, 1:3 or sometimes a mixture of these complexes.[85] Although worth understanding, it is difficult to deduce the actual molecular ratio of complexes in the formulations prepared. It is worth mentioning that the phase solubility curves do not verify complex formation but describe impact of CD concentration on PTX solubility. The main objective of the solid state physicochemical study was to demonstrate presence of PTX in the CD cavity. The second aim was to show absence of any potential chemical interactions that may affect the stability and/or activity of complexed PTX. The DSC studies revealed that disappearance of endothermic melting point peak of PTX that suggested that PTX was complexed within the CD cavity. CD prevented thermal transition of PTX near its melting

point suggested that upon complexation CDs prevented crystalline phase formation of PTX which further confirms inclusion complex formation at molecular level. Lack of melting point peak also indicated that in PTX may be present in amorphous form. Since all complexes were lyophilized and if PTX was effectively complexed within the CD cavity, it is very likely that PTX is in amorphous form. This can be further investigated by performing other tests such as powdered X-ray diffraction studies. The thermograms also displayed no signs of any interactions between CD and PTX. Though based on current data and previous work one can conclude the formation of inclusion complex, absence of PTX melting behavior posted reasonable doubts for any artifacts. This led to more confirmative studies using 1H-NMR studies. In case of 1HNMR studies, characteristic resonance peaks of PTX in the region of 7.5-8.5 ppm were not seen in the spectra of CD alone. Unlike PTX alone, the complexes showed very high resonance for the PTX peaks in the 7.5-8.5 ppm region. These peaks mainly originate due to the aromatic ring attached to the taxane ring at C-13 position. The data indicate weak interactions between the protons of CD and PTX led to high resonance in the $^{1}$H-NMR spectra of the complexes. The results of $^{1}$H-NMR studies thus indicated that PTX was effectively complexed within the cavity of CD. To further support the claim that complexed PTX is not in crystalline form, we analyzed the powdered X-ray diffraction patterns of the complexes. None of the three complexes showed any crystalline character of PTX complexed within the CD cavity. XRD studies of all 3 samples appeared to show that samples were amorphous. However, in case of SBCD complexes, faint noise near the drug substance peaks was observed, which may suggest that there may be a small quantity of PTX in a crystalline form in SBCD. Though it cannot be confirmed, one or more of the factors may have played role such as degree of substitution or nature of substitute (Sulfobutyl ether is slightly negative in charge compared to other two which are neutral). Combining DSC, 1H-NMR and X-ray

diffraction studies we can infer that CD efficiently complexed PTX within the cavity and lack of interactions suggested that the complex was mainly formed due to weak hydrophobic interactions between the CD and PTX. The DSC results suggested that the drug has lost its crystalline structure. Although concluding the amorphous nature of the API only on the basis of DSC data may not provide sufficient evidence. The most important conclusions made based on DSC data were: 1. the absence of any type of physicochemical interactions between the API and any of our CD and 2. All API was in complexed form. The second conclusion hinted that due to the lyophilization of complexes and high aqueous solubility, PTX may be present in amorphous phase. The data of DSC to claim the amorphous nature of the API was not sufficient. To further investigate the crystalline nature of PTX, X-ray diffraction studies were performed that confirmed the amorphous state of PTX in all three batches. Hence, the results of the DSC data, the X-ray diffraction provided confirmative evidence to claim the amorphous nature of PTX within the complexes. In addition, the data suggested that in complexes the amorphous nature of PTX could be attributed to CDs, wherein CDs either prevented formation of crystalline PTX or formed molecular inclusion complexes with PTX.

### 4.5. Conclusions

Results demonstrated the efficiency of β-CDs to form complexes with PTX at lower molar ratios to increase the solubility of PTX. The complex formation and solubility enhancement was found to be dependent on PTX solubility in the aqueous medium and was rate-limiting. Upon changing it to co-solvent system, marked increase in solubility enhancement was observed. The combined results of DSC, $^{1}$H-NMR and powdered X-ray diffraction concluded that the drug was encapsulated in the CD cavity. Powdered X-ray diffraction confirmed the amorphous nature. Complete absence of melting point peak in DSC and

resonance peaks in the aromatic region in $^{1}$H-NMR led to conclusion that the all CD derivatives formed complexes with PTX. CD played an important role in masking either complete or some part of PTX and thereby increased aqueous solubility. Thus, such formulations provide a feasible substitute to the current formulations used in clinic. However, further studies are required to assess the cytotoxic potential of complexed PTX.

| Sr. No. | Method | Batch | PTX:CD (molar) | Complexation Efficiency | PTX Solubility µg/mL | Solubility Enhancement |
|---|---|---|---|---|---|---|
| **1.** | Ethanol | HPCD | 1:5 | 92.35 ± 0.44 | 13.56 | 33.9 ± 1.15 |
| **2.** | Ethanol | MCD | 1:5 | 81.15 ± 0.17 | 8.91 | 24.49 ± 3.52 |
| **3.** | Ethanol | SBECD | 1:5 | 99.34 ± 0.23 | 10.68 | 26.7 ± 2.44 |
| **4.** | Co-solvent | HPCD1 | 1:1 | 98.62 ± 0.43 | 154.43 | 454.2 ± 1.44 |
| **5.** | Co-solvent | HPCD2 | 1:2 | 92.09 ± 0.19 | 190.98 | 561.7 ± 1.11* |
| **6.** | Co-solvent | HPCD5 | 1:5 | 90.90 ± 0.34 | 204.37 | 601.1 ± 1.88* |
| **7.** | Co-solvent | MeCD1 | 1:1 | 79.99 ± 0.27 | 155.72 | 458.0 ± 0.6 |
| **8.** | Co-solvent | MeCD | 1:2 | 87.12 ± 0.69 | 138.21 | 406.5 ± 0.58 |
| **9.** | Co-solvent | MeCD | 1:5 | 79.94 ± 0.19 | 178.98 | 526.4 ± 0.69# |
| **10.** | Co-solvent | SBCD1 | 1:1 | 97.13 ± 0.25 | 156.57 | 460.5 ± 1.75 |
| **11.** | Co-solvent | SBCD2 | 1:2 | 102.28 ± 0.18 | 162.08 | 476.7 ± 0.82§ |
| **12.** | Co-solvent | SBCD5 | 1:5 | 98.49 ± 0.47 | 160.58 | 472.3 ± 1.31§ |

Table 4.1: Solubility enhancement of PTX-CD inclusion complexes prepared by different methods. *, P<0.05 when compared to HPCD. #, P<0.05 when compared with MCD. §, P<0.05 when compared with SBECD

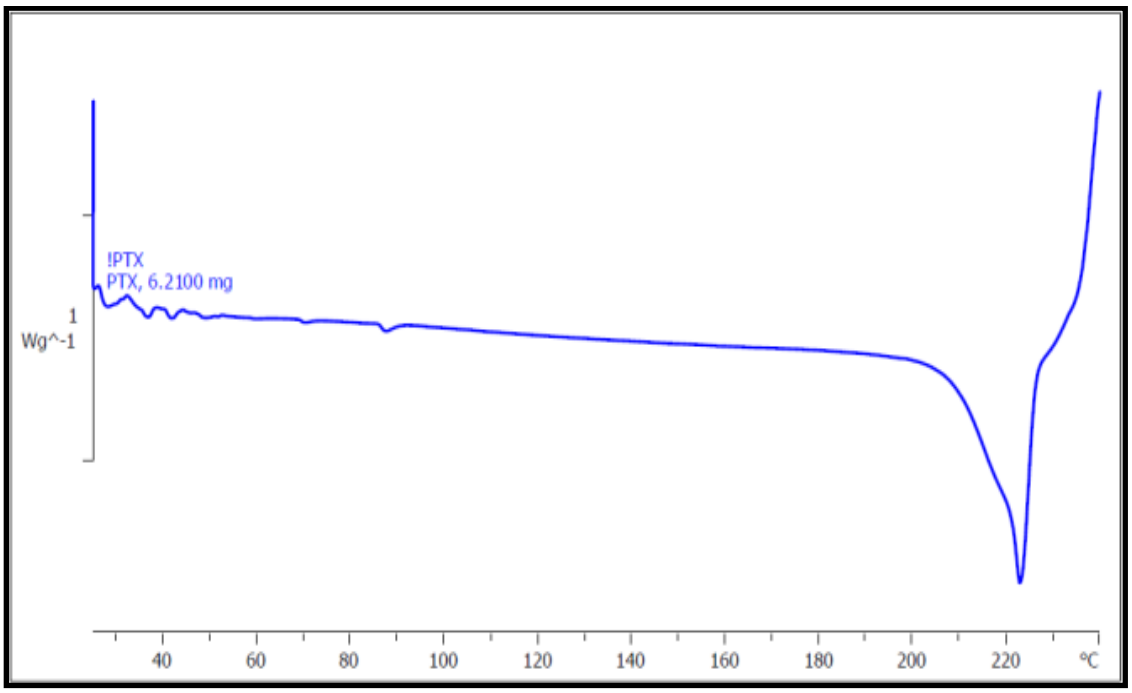



Figure 4.1: DSC thermogram of Paclitaxel

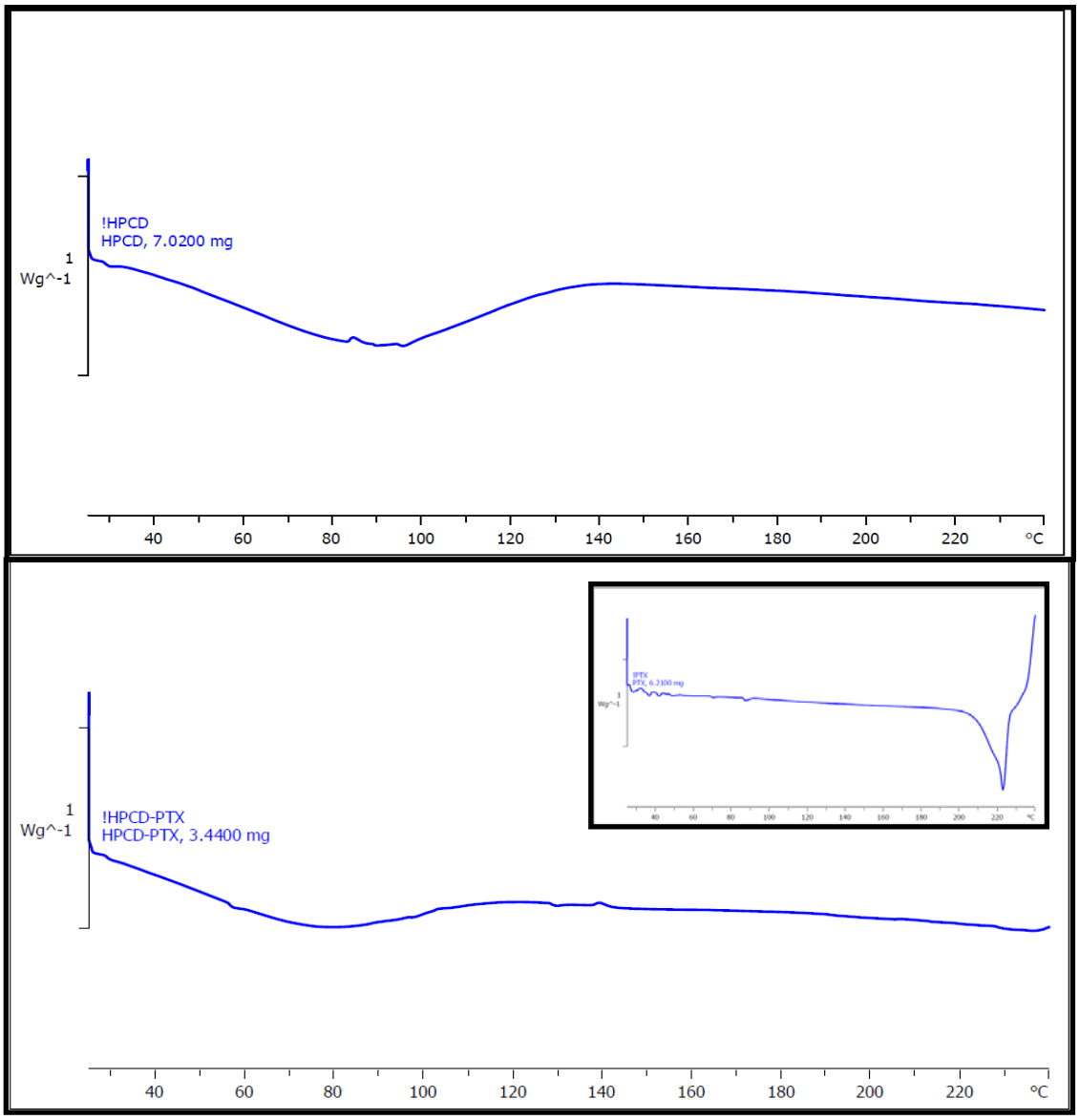



Figure 4.2: Comparative DSC studies of HPCD (above) and HPCD-PTX complex (below). The inset image of below thermogram shows a typical thermogram of PTX.

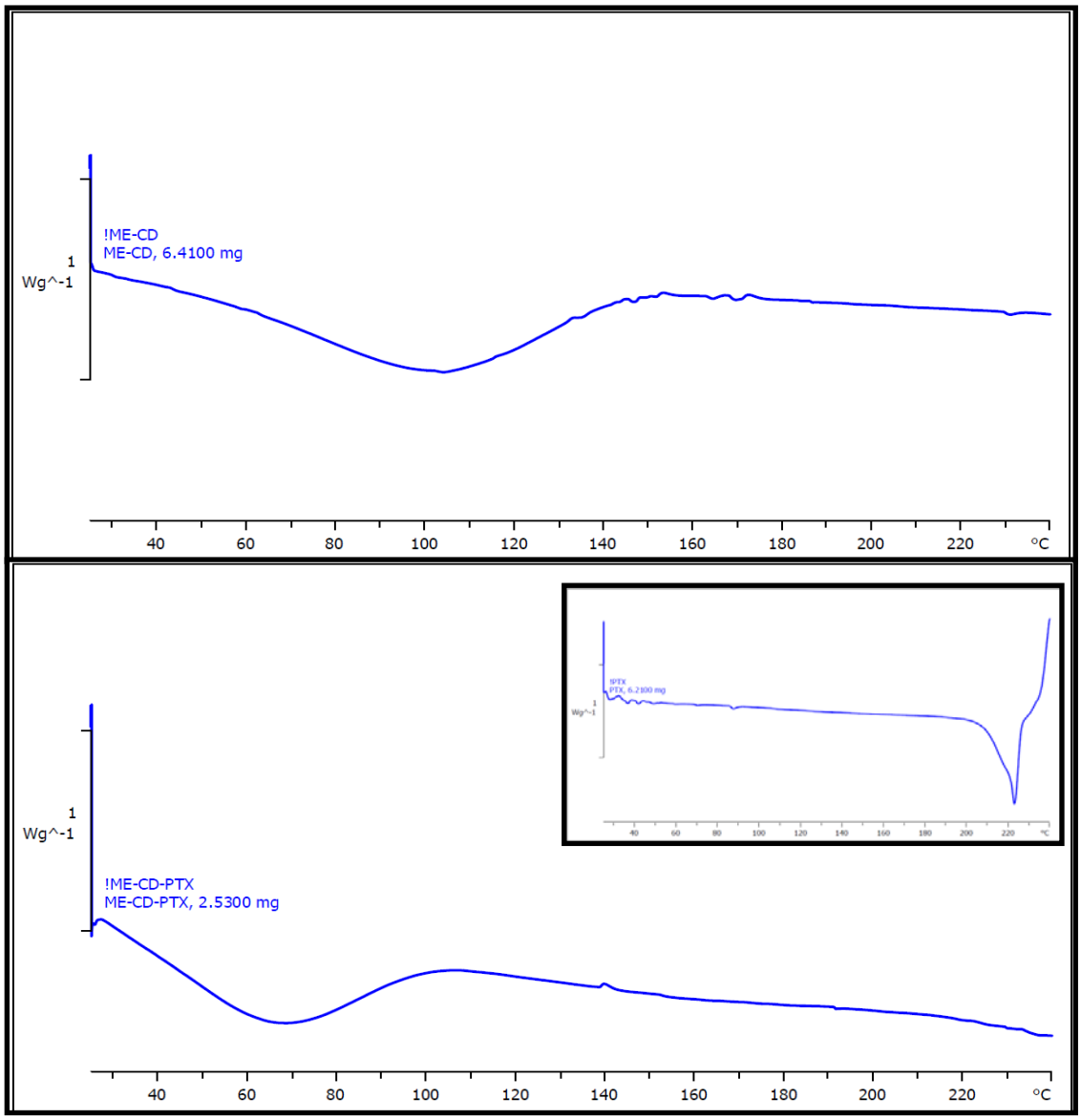



Figure 4.3: Comparative DSC studies of MeCD (above) and MeCD-PTX complex (below). The inset image of below thermogram shows a typical thermogram of PTX.

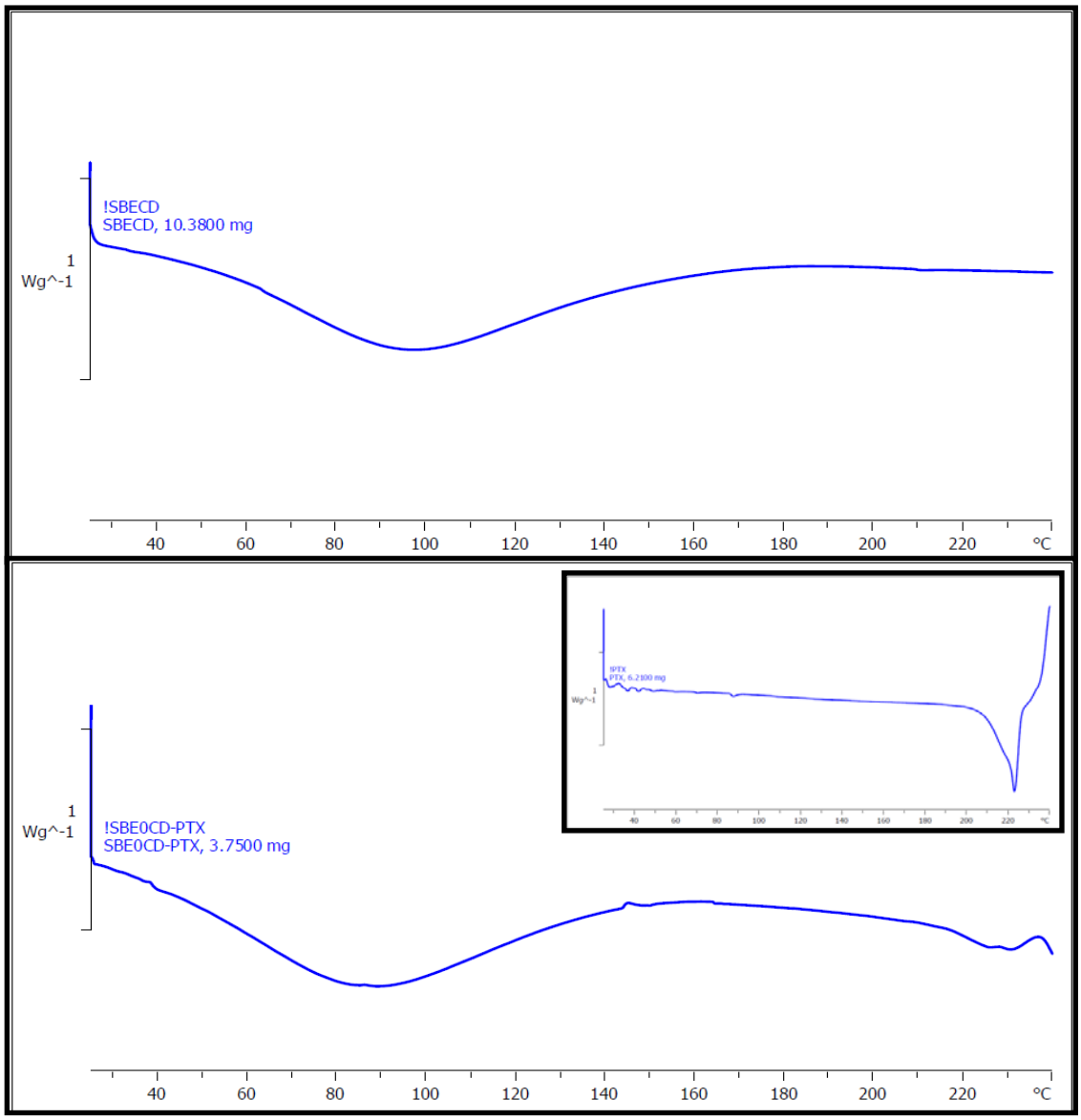



Figure 4.4: Comparative DSC studies of SBCD (above) and SBCD-PTX complex (below).

The inset image of below thermogram shows a typical thermogram of PTX.

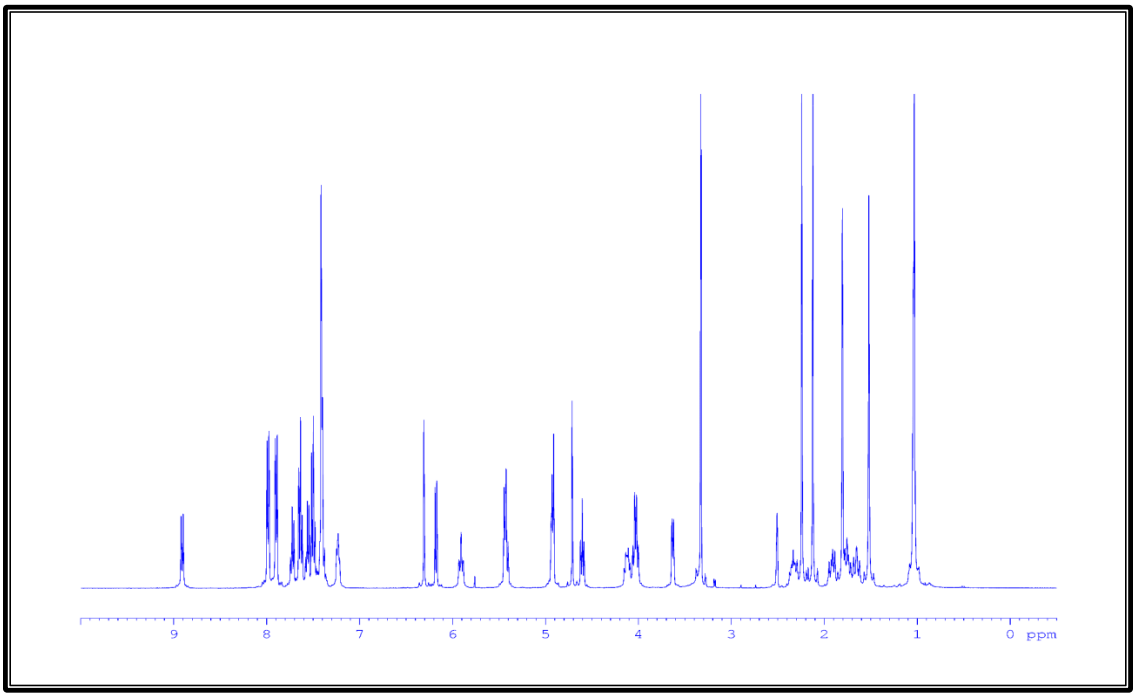



Figure 4.5: Typical $^{1}$H-NMR spectra of Paclitaxel

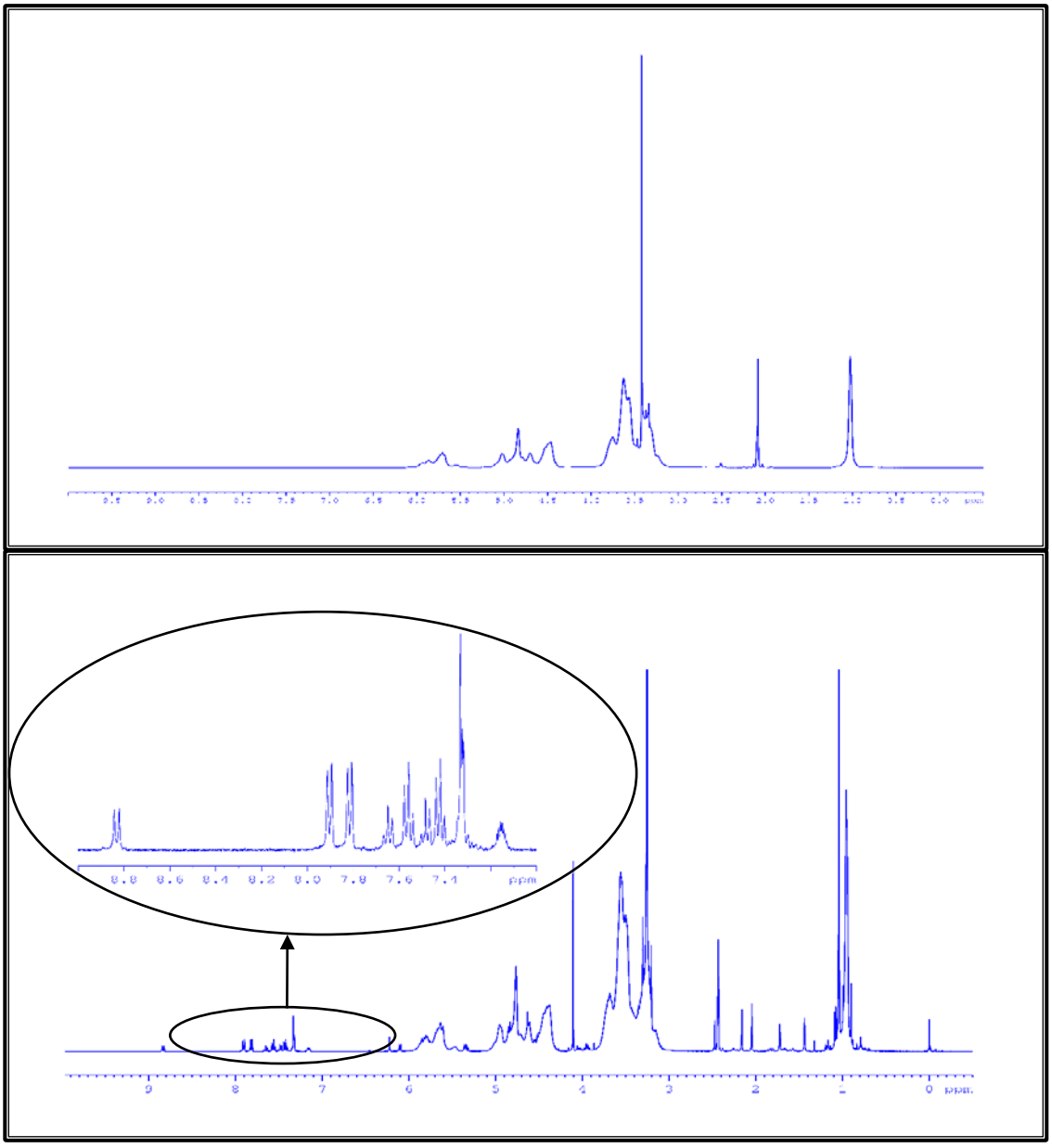


Figure 4.6: Comparative $^{1}$H-NMR studies of HPCD (above) and HPCD-PTX complex (below). The magnified inset image of below spectrogram shows typical peaks due to PTX.

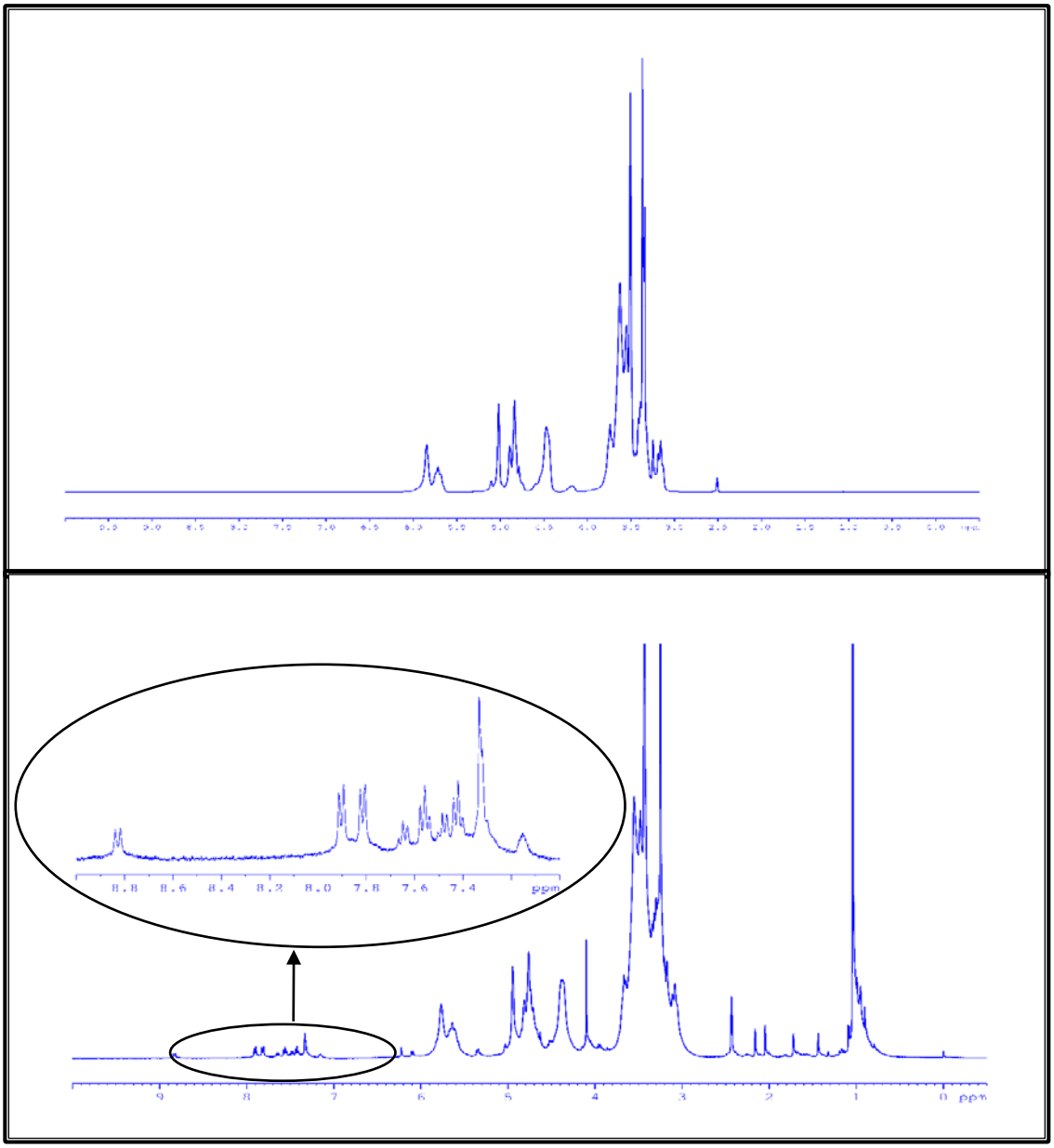



Figure 4.7: Comparative $^{1}$H-NMR studies of MeCD (above) and MeCD-PTX complex (below). The magnified inset image of below spectrogram shows typical peaks due to PTX.

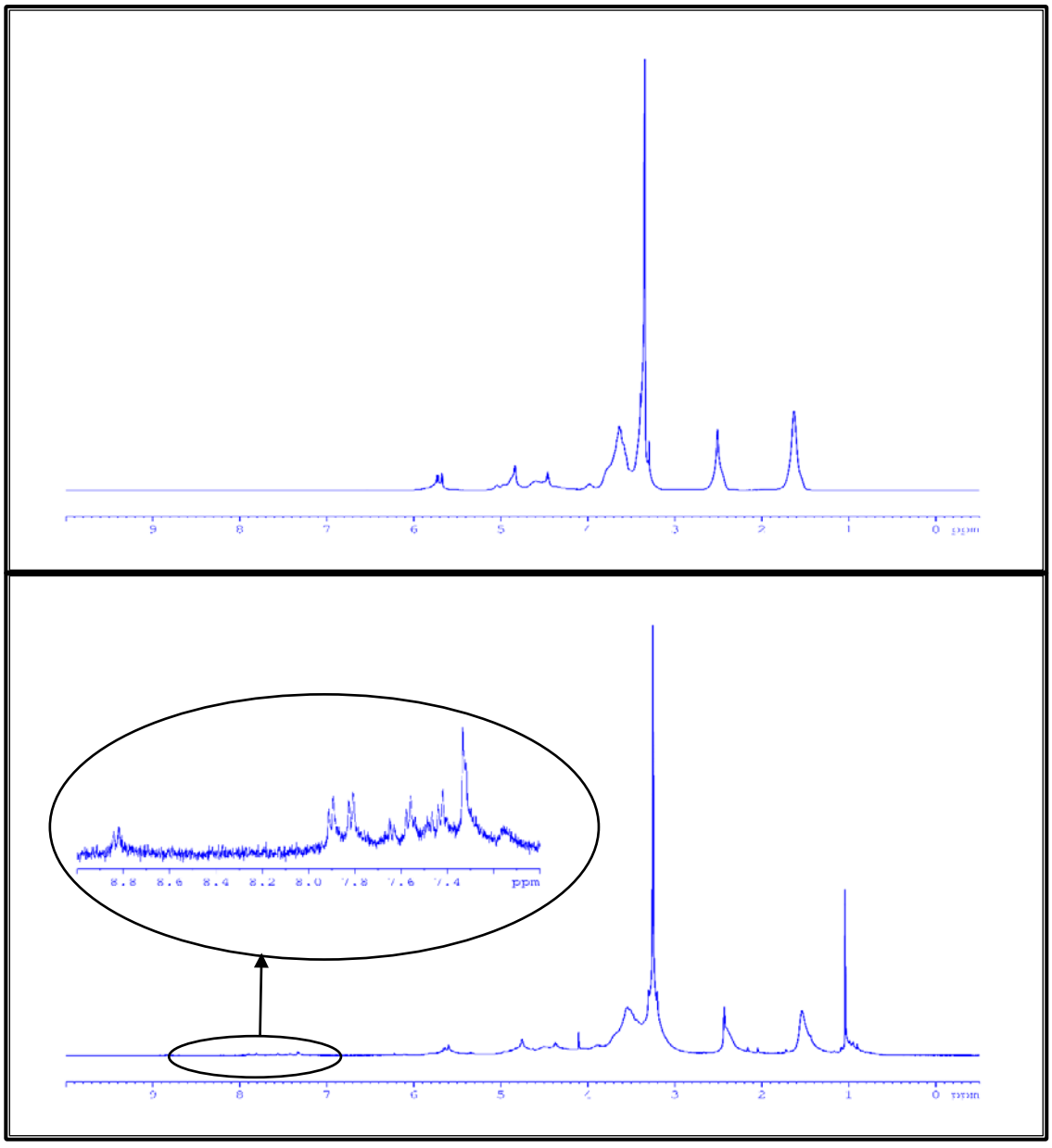



Figure 4.8: Comparative $^{1}$H-NMR studies of SBCD (above) and SBCD-PTX complex (below). The magnified inset image of below spectrogram shows typical peaks due to PTX.

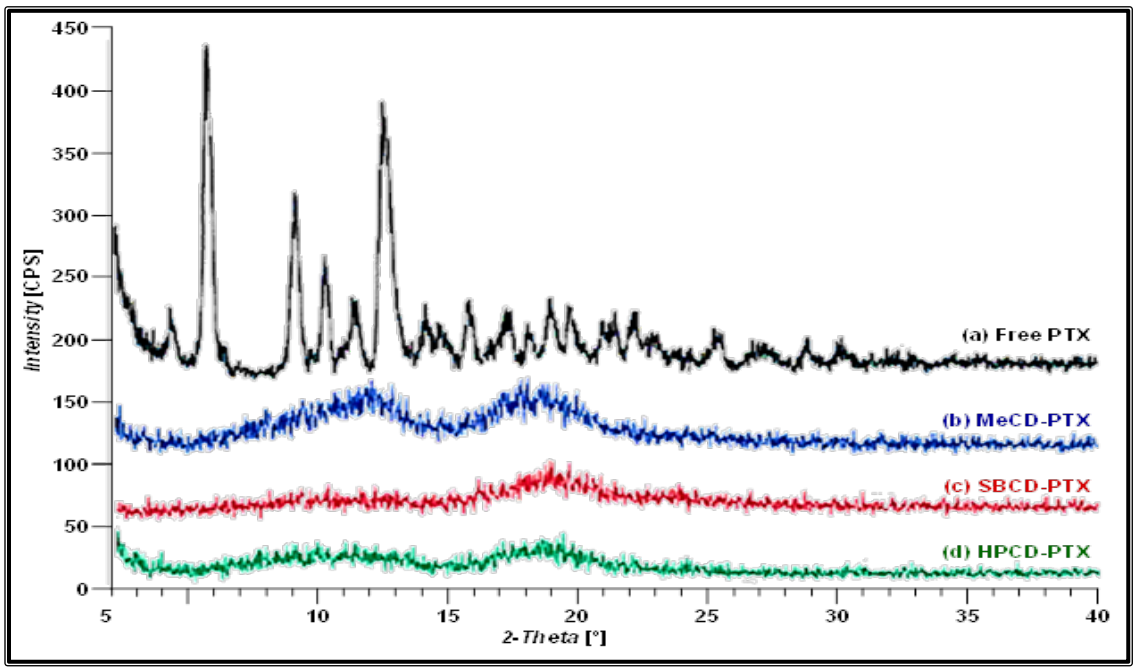



Figure 4.9: Powdered X-ray diffraction patterns of (a) Free PTX, (b) MeCD-PTX, (c) SBCD-PTX and (d) HPCD-PTX

# 5. TO EVALUATE THE SAFETY OF β-CYCLODEXTRIN DERIVATIVES AS EXCIPIENTS AND ASSESS CYTOTOXICITY OF PACLITAXEL INCLUSION COMPLEXES AND MECHANISM OF CELLULAR INTERNALIZATION

## 5.1. Introduction

Water soluble complexes of PTX with CD offer very attractive solution and lead to the overcoming of the drawbacks of the current regimen of Taxol®. These complexes exhibited higher aqueous solubility for PTX and showed high complexation at relatively lower molar ratio offers significant advantages to overcome the shortcomings of Taxol®. Although PTX is very potent anticancer drug with established mechanism of action, the efficacy of PTX-CD complexes are yet to be determined. In addition, it is equally important to assess the safety of the excipients like CD so as to avoid prophylactic treatments as seen with formulations prepared with toxic excipients like Cremophor EL. The molecular mechanism of PTX necessitate cellular internalization exert cytotoxicity. PTX binds to the β-tubulin subunit within the microtubule and stabilizes the microtubule assembly.[86] The prerequisite of cellular internalization for PTX cytotoxicity has two major stumbling blocks due to PTX itself are solubility and permeability. Overcoming these obstacles by improving aqueous solubility of PTX the cellular internalization can be improved that can be reflected in higher cellular accumulation and thus improved cytotoxicity. Similar to early drug discovery studies, selecting appropriate quantitative test methodologies can provide sufficient information to evaluate safety and efficacy of the PTX-CD complexes. Screening of these complexes can be performed using biochemical assays as used in early drug discovery to provide rapid results. However, the physiological parameters and effect on organisms are largely overlooked during these assays and may not reveal helpful information. Since cytotoxicity of PTX is well established, the aim of the evaluations

should be to distinguish the activity of native PTX compared to PTX-CD complexes to discern meaningful conclusions. Cell based studies can be used as a suitable, rapid and economic alternatives that are more biologically relevant to predict response of the organism as compared to the biochemical assays. Technology advancement to detect signals better as well as specific assay chemistry has improved the cell based assays significantly recently. Cultured human tumor cell lines are suitable representative cell lines that have been used to understand the pharmacological properties of cytotoxic drugs for a long time.[87] There are several cancer cell lines and large number of cell based assays to choose from requires understanding of what information is required from the assay and determining the end points of the cell based assays. This report illustrates use of three different cell based assays to understand the cytotoxicity of the complexes, assessment of PTX internalization and quantitative estimation of safety profile of CD derivatives used. Cell viability assays are suitable tool to demonstrate the cytotoxicity of the PTX-CD complexes and compare them with native PTX. A simple colorimetric assay to measure the cellular metabolic activity directly proportional to the number of viable cells present. Reduction in number of viable cells in presence of cytotoxic drugs such as PTX reflects its cytotoxic potential. Comparing the dose response curve of standard (e.g. PTX alone) with samples (e.g. PTX-CD complexes) can help to assess cytotoxic potential of the complexes in comparison to PTX as well as help to understand impact of improved aqueous solubility and thereby cellular internalization. Though the cell viability assay data illustrate impact of cellular internalization, the test itself do not quantify amount of drug internalized. Since PTX-CD complexes have demonstrated significantly higher aqueous solubility than PTX, it is hypothesized that the complexes may exhibit higher intracellular concentrations. Thus, a suitable test to be used to quantify the effect of complexation on cellular internalization. To establish the evidence of better cellular internalization,

qualitative and quantitative tests can be designed to show the impact of complexation. Commercially available molecular markers such as fluorescent Paclitaxel conjugates have been used for such studies previously for variety of evaluations.[86] Comparing the fluorescent microscopic images of adherent cancer cell lines treated with such conjugates and complexed conjugates can establish evidence of cellular internalization and measuring the fluorescence intensity by flow cytometry can establish quantitative effect of CD complexation on cellular internalization. Cell based assays can also be used to evaluate safety of excipients such as CD derivatives used to complex PTX in the earlier studies. One of the main objective of the study is to establish safety of CD derivatives in a quantitative manner and display absence of acute as well as serious DNA damage upon chronic exposure and accumulation. Despite of inherent DNA-repair mechanisms excipients and other chemicals may pose risk that primary damages exerted by such chemicals are transformed to mutations due to lack of repair and may lead to inflammation or carcinogenesis.[88] Both bacterial and mammalian cell culture based assays are available to evaluate genotoxic potential of a substance. Mammalian cell culture based assays are preferred since the prokaryotic cells differ with respect to uptake, metabolism, chromosome structure and repair mechanisms. For the ease and better predictability micronuclei test was selected to assess the genotoxic potential of CD derivatives. *In vitro* micronuclei test is a multiple target genotoxic end-point quantitative assay of chromosome aberration which is simple to execute and provide easy to score micronuclei as quantitative output. A micronucleus is a cytoplasmic erratic bodies formed during cell division when a chromosome or a fragment of chromosome is not incorporated into one of the daughter nuclei.[89] Formation of micronuclei results in lack of part or all of the chromosomes in the daughter cell. To assess the genotoxic potentials of the CD derivatives, mammalian cell culture is exposed to CD derivatives with and without

metabolic activation systems. The metabolic systems are employed if any enzymatic transformation will render these substances genotoxic potential. At a defined interval, the cells are treated with test substances and incubated to reach metaphase. The cells are then harvested and stained to observe formation of micronuclei under microscope and perform quantification. Under this specific aim, the PTX-CD complexes formed earlier were subjected to cytotoxicity studies using cell viability assay using different cancer cell lines. Further to understand the impact of solubility enhancement and complexation, quantitative and qualitative estimations of cellular internalization were carried out. Lastly, genotoxicity study using *in vitro* micronuclei test were performed to establish the safety of CD derivatives.

## 5.2. Materials and Methods

### 5.2.1. Materials

Paclitaxel was purchased from Sigma Aldrich (St. Louis, MO). Hydroxypropyl β-cyclodextrin (HPCD, Kleptose®) and Methyl β-cyclodextrin (MeCD, Crysmeb) were purchased from Roquette Pharma (Lestrem, France). Sulfobutyl ether β-cyclodextrin (SBCD) was purchased from Cydex Pharmceuticals (Lenexa, KS). Cyclophosphamide (CP), Ethylmethyl sulfone (EMS), Glucose 6-phosphate, Methanol Dimethyl Sulfoxide (DMSO), Tween 20, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), Thiazolyl blue tetrazolium bromide, (MTT), Sodium dodecyl sulfate (SDS) and Dimethyl formamide (DMF) were acquired from Sigma Aldrich (St. Louis, MO). Nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Fisher Chemicals (Suwannee, GA). Sodium phosphate buffer and Aroclor-1254 induced rat liver S9 fraction was obtained from Molecular Toxicology Inc. (Boone, NC). Fluorescent conjugate of PTX Flutax-2 was obtained from Molecular Probes (Carlsbad, CA).

### 5.2.2. Cell lines

PC-3 human prostate adenocarcinoma, A549 human lung adenocarcinoma, HT-29 human colorectal adenocarcinoma, MCF-7 human breast adenocarcinoma and Chinese hamster ovary - K1 (CHO-K1) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). A2780 human ovarian carcinoma cell line was obtained from Dr. T.C. Hamilton (Fox Chase Cancer Center). CHO-K1 cells were cultured in F-12K medium (ATCC, Manassas, VA) with 10 % Fetal bovine serum (Life Technologies, Grand Island, NY) and penicillin-streptomycin solution (100 UI/mL-100µg/mL) obtained from Life Technologies. All other cells were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA) with 10 % Fetal bovine serum and penicillin-streptomycin solution (100 UI/mL-100µg/mL). Cells were grown at 37 $^{o}$C in humidified conditions with 5 % CO2 (v/v) in air. All cell-based experiments were performed on cells in the exponential growth phase.

### 5.2.3. Cytotoxicity of PTX-CD complexes

Cytotoxicity screening of PTX-CD inclusion complexes were performed using modified MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as previously described.[90] All cell lines (PC-3, A2780, MCF-7, HT-29 and A549) were subjected to similar treatment for the MTT assay. For the assay, 10,000 cells were seeded per well in 96-well plates and incubated for 24 h. Next day, the media was replaced with fresh media; different concentrations of each PTX-CD complexes were added in the media and incubated for 24 hours. After 24 hours, the solution was again replaced by fresh media and MTT reagent (25 µL of 5 mg/mL). Plates were further incubated in cell culture conditions for 3 h. Viable cells would convert MTT reagent into insoluble formazan crystals which were dissolved overnight by adding 50 % (v/v) dimethylformamide in water with 20 % (w/v) sodium dodecyl sulfate. The absorbance was measured at 570 nm with

background correction at 630 nm. Based on these measurements, $ID_{50}$ (concentration of active ingredient necessary to inhibit cell growth by 50 %) of free PTX and PTX-CD complexes were calculated and compared.

### 5.2.4. Cellular Internalization of PTX-CD complexes

To analyze the intracellular localization of PTX-CD complexes, complexes were prepared using PTX labeled with Oregon green® 488 (Flutax-2, Molecular Probes®, Life Technologies, Carlsbad, CA) using co-solvent method at PTX-CD molar ratio of 1:5. For cell internalization studies, 10,000 cells (PC-3) were seeded per well in a six well plate and incubated overnight. Next day, the media was replaced and inclusion complexes were added to wells (equivalent to 1 μM of Flutax-2). Flutax-2 alone was used as a control. The cells were incubated for 3 hours and washed 3 times with DPBS. After the wash, 1 mL of media was added to each well and visualized using fluorescence microscopy (Olympus, Waltham, MA) using filters 470/40 nm and 525/50 nm for excitation and emission respectively. Quantitative estimation of cellular internalization of the complexes were performed using flow cytometry studies. In this study the cells (PC-3) were cultured in a reduced serum media at 500,000 cells per well in a six well plate. Next day, the cells were washed with DPBS and media was replaced with samples inclusion complexes (equivalent to 10 μM Flutax-2). The cells were incubated for 3 h. After incubation, the cells were harvested and washed using DPBS and re-suspended in 1 mL of reduced serum media for fluorescence assisted cell sorting (FACS) analysis using a Gallios flow cytometer (Beckman Coulter, Brea, CA). Cells treated with Flutax-2 alone were used as a control and measured fluorescence intensity of each complex was compared with control.

### 5.2.5. Genotoxicity of CD derivatives

Modified *in vitro* micronuclei test was used to evaluate genotoxic potential of cyclodextrins. The test was adopted from Organization for Economic Cooperation and Development (OECD) protocol and performed as previously described.[89] Briefly, in a 25 $cm^2$ flask, about 300,000 CHO-K1 cells were cultured for 24 hours before treatment. After 24 hours, the cells were treated with cyclodextrins (50 mg/mL) for three hours followed by replacement of fresh media and incubation for another 24 h. Then, the cells were stained to detect the presence of micronuclei. The set of experiments included following series of samples: (1) Negative control – media only; (2) DMSO (100 µL) –negative control for the solvent; (3) Cyclophosphamide 10 mg/mL with metabolic activator (S9 mix, 0.3 mL) – positive control; (4) Ethyl methanesulfone (EMS) 400 µg/mL – positive control that does not require a metabolic activation; (5) CD samples 50 mg/mL and (6) CD (50 mg/mL) with S9 mix (0.3 mL). The S9 mixture is a metabolic activation system that enables the detection of mutagenic activity for the samples that require metabolic transformation (e.g. cyclophosphamide). In order to prepare the S9 mix, following chemicals were added to a rat liver S9 fraction (0.2 mL) after Arclor-1254 induction: sterile water for injection (560 µL), sodium phosphate buffer (0.1 M, pH 7.4, 1 mL), 4mM NADP (150 µL), glucose-6-phosphate (120 mM, 14 µL) and potassium-magnesium salt solution (8 mM – 33mM, 60 µL). After incubation, the cells were fixed in a cold solution of 100 % methanol. Methanol was removed, cells were washed with phosphate buffer, and nuclei staining was performed using 600 nM 4, 6 diamidino-2-phenylindole (DAPI) for 8 minutes. After 8 minutes the solution was removed and cells were washed using PBS with 0.02 % v/v Tween 20. The micronuclei were observed under fluorescent microscope (Olympus, Waltham, MA) and the number of micronuclei observed per 1000 cells was calculated.

### 5.2.6. Statistical Analysis of Data

All quantitative data presented as mean ± standard deviation from five to ten independent measurements. Data analysis was performed using descriptive analysis and single factor ANOVA. Student's t-test was applied to data sets and P value of less than 0.05 was considered as statistically significant.

## 5.3. Results

### 5.3.1. Cytotoxicity of PTX-CD complexes

The cytotoxicity studies of complexes were performed using inclusion complex batches of highest molar ratio of CD to PTX (5:1). The study showed lower $ID_{50}$ values (the concentration that kills 50% of cells) for all five human cancer cell lines used and three tested CD-PTX complexes were statistically significantly lower when compared with free non-complexed PTX indicating substantially higher cytotoxicity of PTX complexes (Figure 5.1). In the absence of PTX, all CDs did not show any signs of cytotoxicity in concentrations up to 50 mg/mL. It was observed that PTX complexes with HPCD showed significant reduction in $ID_{50}$ concentrations as compared to PTX in four of the five cell lines studied. All three showed significant reduction in $ID_{50}$ concentration for MCF-7, A2780 and HT-29 cells. Although all complexes exhibited lower $ID_{50}$ values for A549 cells, it was not statistically significant.

### 5.3.2. Cellular internalization of PTX-CD complexes

Fluorescent tagged conjugate of PTX was complexed with three CD derivatives at a molar ratio of 5:1 and used for the qualitative and quantitative estimations of cellular internalizations. Representative images of fluorescent microscopy shows visibly higher fluorescence for the cells treated with PTX-CD complexes as compared to PTX alone (Figure 5.2). The evidence of higher internalization observed fluorescence microscopy was quantitatively measured by fluorescence assisted cell sorting (FACS) analysis using

flow cytometry. In FACS, fluorescent intensity of each group of cells were measured after treatment and compared with control. The FACS analysis showed 1.5 times higher fluorescence intensities with complexes as compared to samples without CDs reflecting higher cellular internalization in presence of CDs (Figure 5.3).

### 5.3.3. Genotoxicity of CD derivatives

*In vitro* formation of micronuclei, a DNA fragment nuclei in the cytoplasm in presence of a test substance, is an indication of mutagenic potential of the tested substance. This test is widely used for quantitative analyze a genotoxic potential of various substances. Representative images of cells (with stained nuclei) incubated in the present study with different testing substances are shown in the figure (Figure 5.4). Analysis of fluorescence images as well as quantitative data revealed that the positive controls (Cyclophosphamide and Ethyl methyl sulfonate) induced substantial DNA damage leading to high number of micronuclei formation while none of CD showed any sign of genotoxicity with or without metabolic activation system (Figure 5.5).

## 5.4. Discussions

The cytotoxicity studies of all CDs without a complexed drug revealed that they did not exert any cellular toxicity. This, in conjunction with prior studies, showed that CD lack any cytotoxic potential and can be considered safe for such delivery systems. Cremophor EL which is used as a solubilizer for PTX in the current clinical formulation has been earlier reported to be cytotoxic during *in vitro* studies and exert pharmacodynamic effects *in vivo*.[91] In addition, the cytotoxicity data confirmed that the activity of PTX was preserved and was not altered after complexation. Lower $ID_{50}$ values (higher cytotoxicity) of complexed PTX can be explained by enhanced solubility of PTX which makes more soluble drug available at the cell surface for internalization and exert pharmacological

effects at lower concentrations. According to the Biopharmaceutical classification system (BCS), PTX belongs to a class IV drug that exhibits poor solubility and permeability. Consequently, lower $ID_{50}$ values compared to PTX alone cannot only be attributed to the enhancement in solubility. As PTX exhibiting poor solubility and permeability, some factor should play an important role to facilitate its permeation through cell membrane once it is solubilized. This led us to hypothesize that CD is playing an important role not only in solubilization of PTX but also on facilitating internalization of PTX. To assess the hypothesis, two studies were performed, namely fluorescent microscopy and flow cytometry studies. Both the studies showed that higher PTX internalization could be achieved in presence of CD. Thus, we could conclude that the higher internalization of PTX from the CD complex formulation was due to two most possible mechanisms of enhancements: (1) Solubility enhancement and (2) Enhancement in cellular internalization. To support this statement, one could propose the following hypothesis. CDs interact with cell membrane cholesterol and form inclusion complexes with them. This will result in altered fluidity of the membrane and create transient channels for internalization of PTX across the membrane.[92] Thus, a higher amount of PTX available in the extracellular environment will be internalized. While in absence of such mechanism, there will be larger quantity of insoluble PTX waiting in the extracellular environment to be first solubilized and further internalized. Low solubilization and internalization of PTX will pose a significant challenge for the drug to be effective at lower concentrations as there may be insufficient amount of PTX solubilized and internalized. CDs on the other side do not alter PTX chemically, but by simply forming molecular inclusion complex through hydrophobic interaction within their cavity, significantly increase the aqueous solubility of the drug. When in the vicinity of the cell membrane, such complex should interact with membrane cholesterol and release a molecule of PTX simultaneously altering the fluidity

of the membrane. The released molecule of PTX will have to face less resistance from the membrane and will be easily internalized (Figure 5.6).

The aforementioned results showing interaction of CD and cell membrane cholesterol inspired us to carry out a further study involving assessment of safety of CD as an excipient. At this level of formulation development, it is very important to evaluate the safety and toxic potential of CD prior to any pre-clinical or clinical investigations. Hence, the genotoxic studies of CDs were performed using the micronuclei test as per the OECD guidelines. OECD has two studies described under the genotoxicity study: 1. Structural aberration study which is the most common (Test # 473 In vitro mammalian cell chromosomal aberration test) and 2. Micronuclei assay (Test # 487, In vitro mammalian cell micronucleus test). Though chromosomal aberration test has been used most commonly, we evaluated CDs using the micronucleus test since it is a better indicator of genotoxicity potential. In the former, the chromosome aberration observed during metaphase which may not transmit to daughter cells while a micronucleus represents a damage that has been transmitted to daughter cells. Hence, micronucleus formation is a better indicator of genotoxicity. In addition, the aberration assay is more qualitative since it needs observational assessment of nucleus morphology to differentiate an arrested cell in the process of cell division. This needs an experienced individual who can differentiate such cells from the normal cells. On the other side, micronucleus test is rapid, easy and allows quantification of data to arrive at discrete conclusion. The cytotoxicity results described above revealed that CDs showed practically no sign of cytotoxicity (nearly all cells were viable after incubation with CDs alone). Genotoxicity studies also concluded that none of CD found to be genotoxic. The concentrations of CDs used in the present investigation are safe and showed no cytotoxic or genotoxic effects. Lack of cytotoxicity

and genotoxicity reflects that higher cytotoxicity observed with CD complexes is only associated with PTX owing to its higher solubility and internalization in presence of CD.

### 5.5. Conclusion

Despite of being a highly potent cytotoxic drug, PTX solubility and permeability are challenging to achieve desired levels within the tumor with the current clinical formulation. Formation of inclusion complexes with CD helped improve solubility significantly. The cytotoxicity of PTX was intact and not affected due to complexation. Increase in cytotoxicity of PTX-CD complexes was thought to be the effect of improved solubility. However, the cellular internalization studies showed the CD play important role in the cellular internalization. Thus, a simultaneous improvement in solubility and cellular internalization due to complexation led to higher cellular concentrations and enhanced cytotoxicity. The genotoxicity data confirmed that CD displayed no indication of genotoxicity and found to be safe. The data suggests that these formulations can be promising candidates to evaluate further as a suitable replacement of Taxol®.

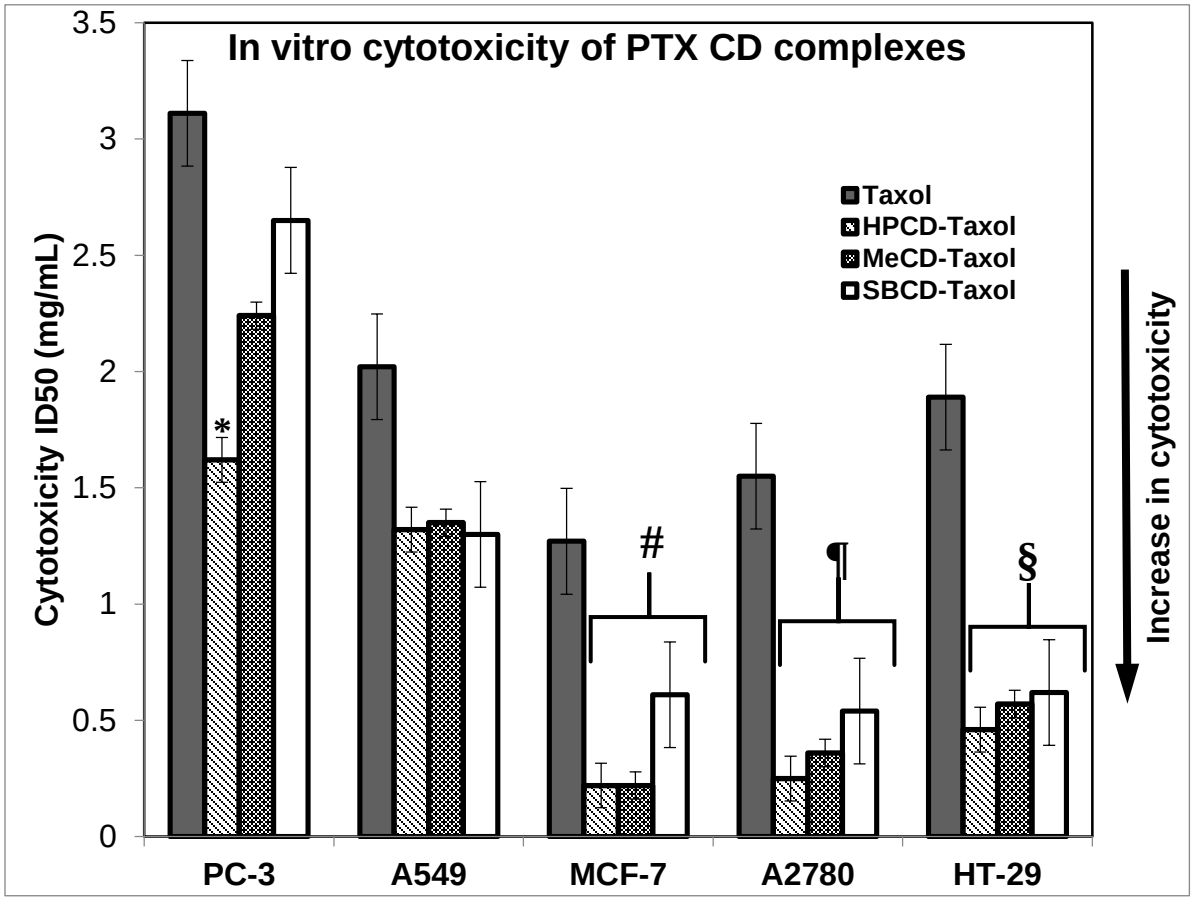



Figure 5.1: Comparison of cytotoxicity of PTX CD complexes on various cancer cell lines. *, P<0.05 when compared with PTX treatment for PC-3. #, P<0.05 when compared with PTX treatment for MCF-7. ¶, P<0.05 when compared with PTX treatment for A2780. §, P<0.05 when compared with PTX treatment HT-29.

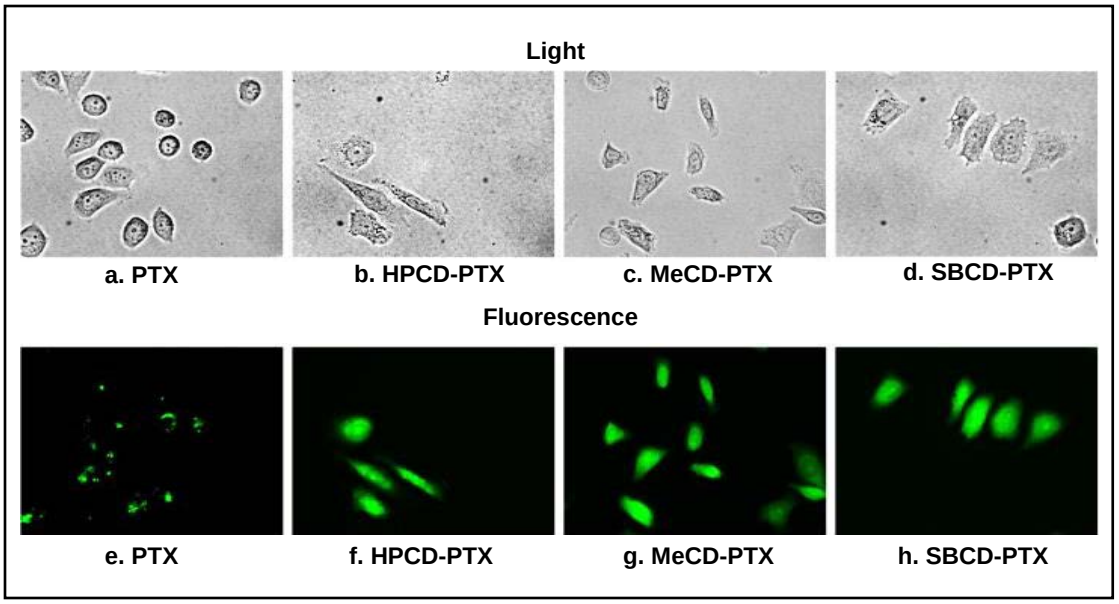
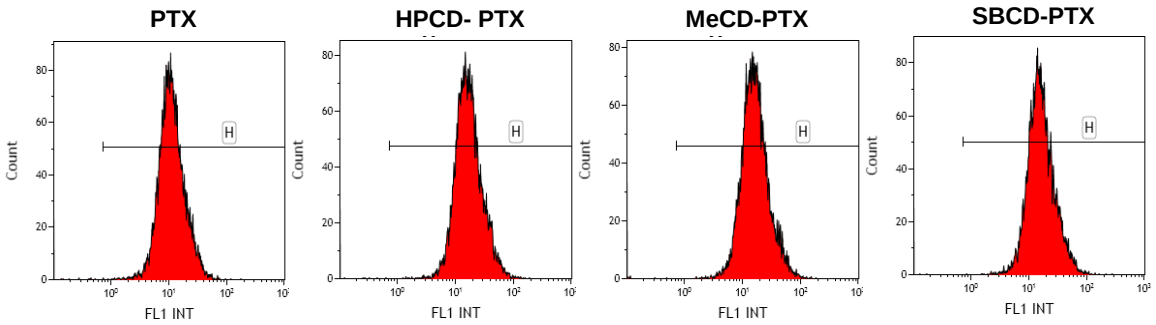



Figure 5.2: Light (a-d) and fluorescent (e-h) microscopic images of PC-3 cells upon FITC-PTX internalization. Optical microscopic images: (a) PTX, (b) HPCD-PTX, (c) MeCD-PTX and (d) SBCD-PTX. Fluorescent microscopic images (e) PTX, (f) HPCD-PTX, (g) MeCD-PTX and (h) SBCD-PTX.




| H ratio | | | |
|---|---|---|---|
| **PTX** | **HPCD-PTX** | **MeCD-PTX** | **SBCD-PTX** |
| 1.0 | 1.54* | 1.51* | 1.46* |

Figure 5.3: Flow cytometry fluorescence intensity plots to study the effect of CDs on cell internalization. The table below the chart represents H-ratio of the respective CD compared to PTX. * P<0.05 when compared with FITC-PTX treatment.

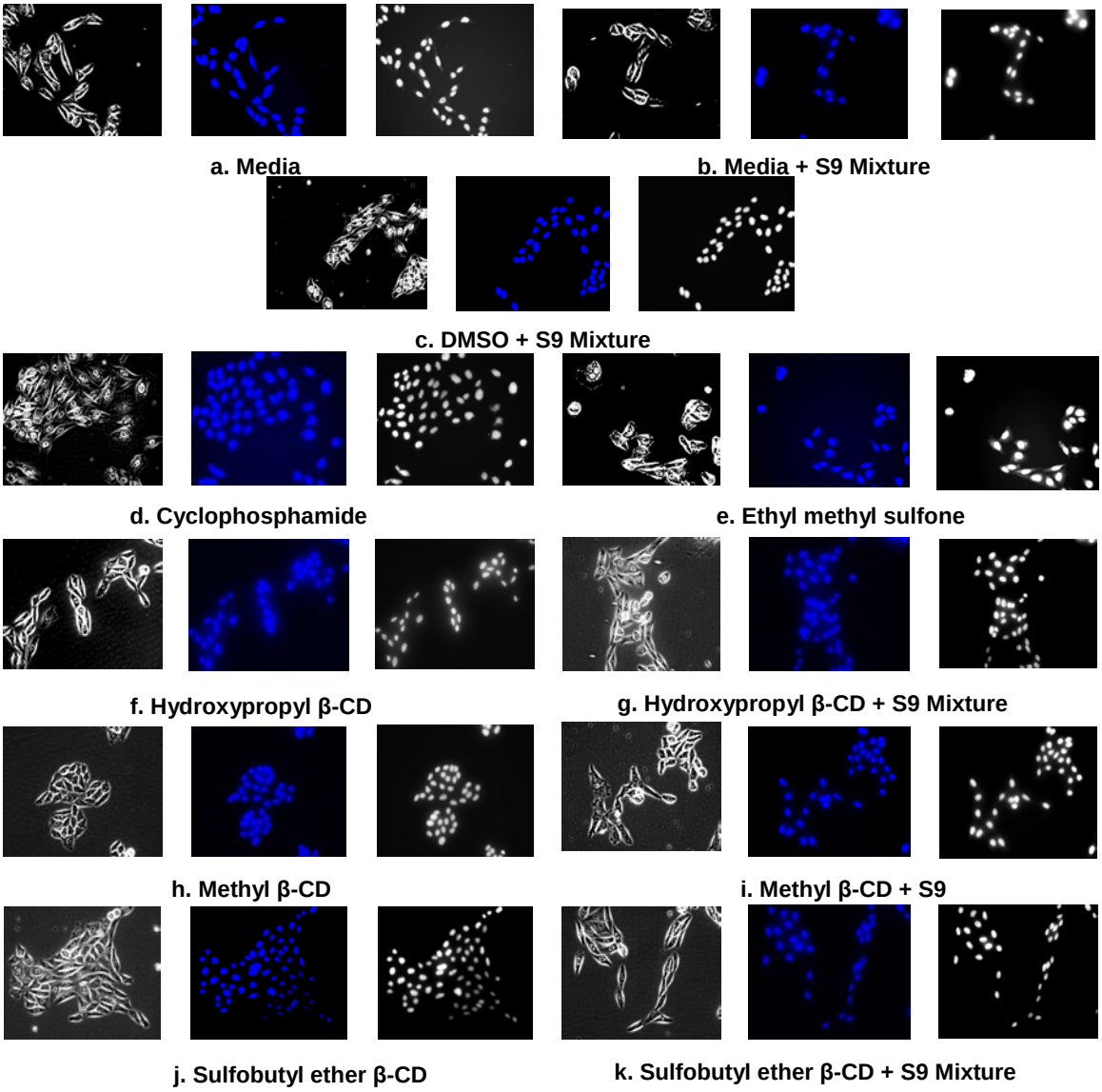



Figure 5.4: Optical and Fluorescent microscopic images of Chinese Hamster Ovary Cells after treatment with various CD derivatives to evaluate the genotoxic potential of the CD.

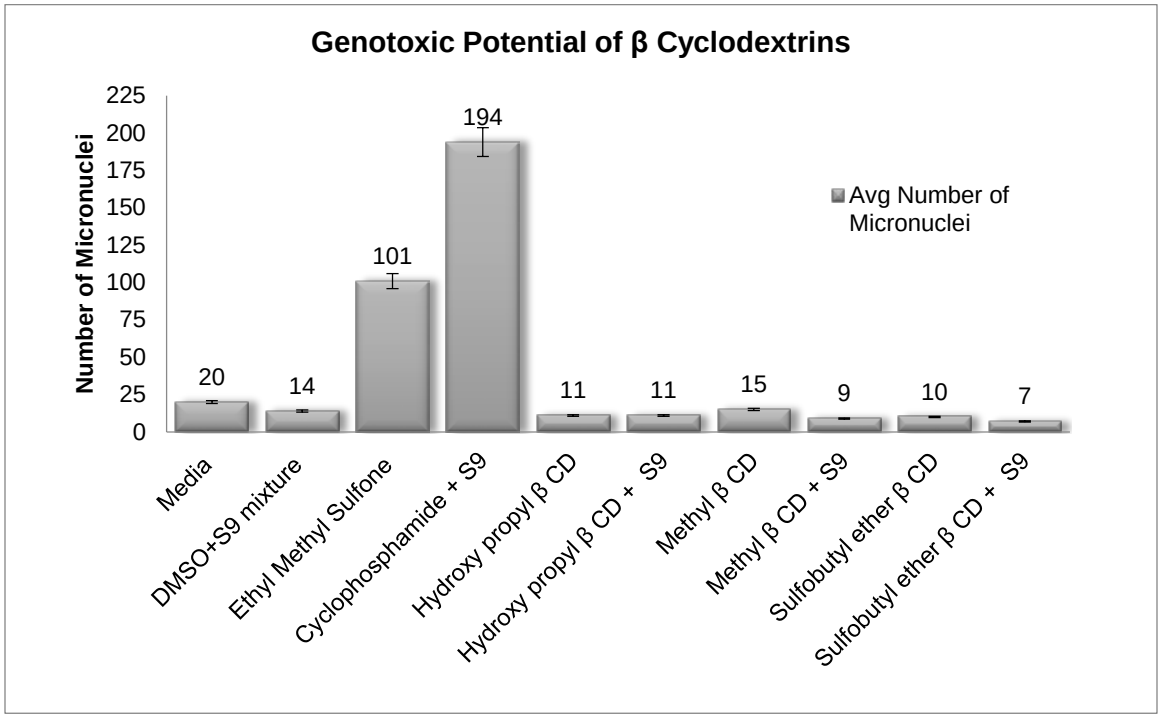



Figure 5.5: Genotoxicity of CD. The bar represents number of micronuclei per 1000 cells

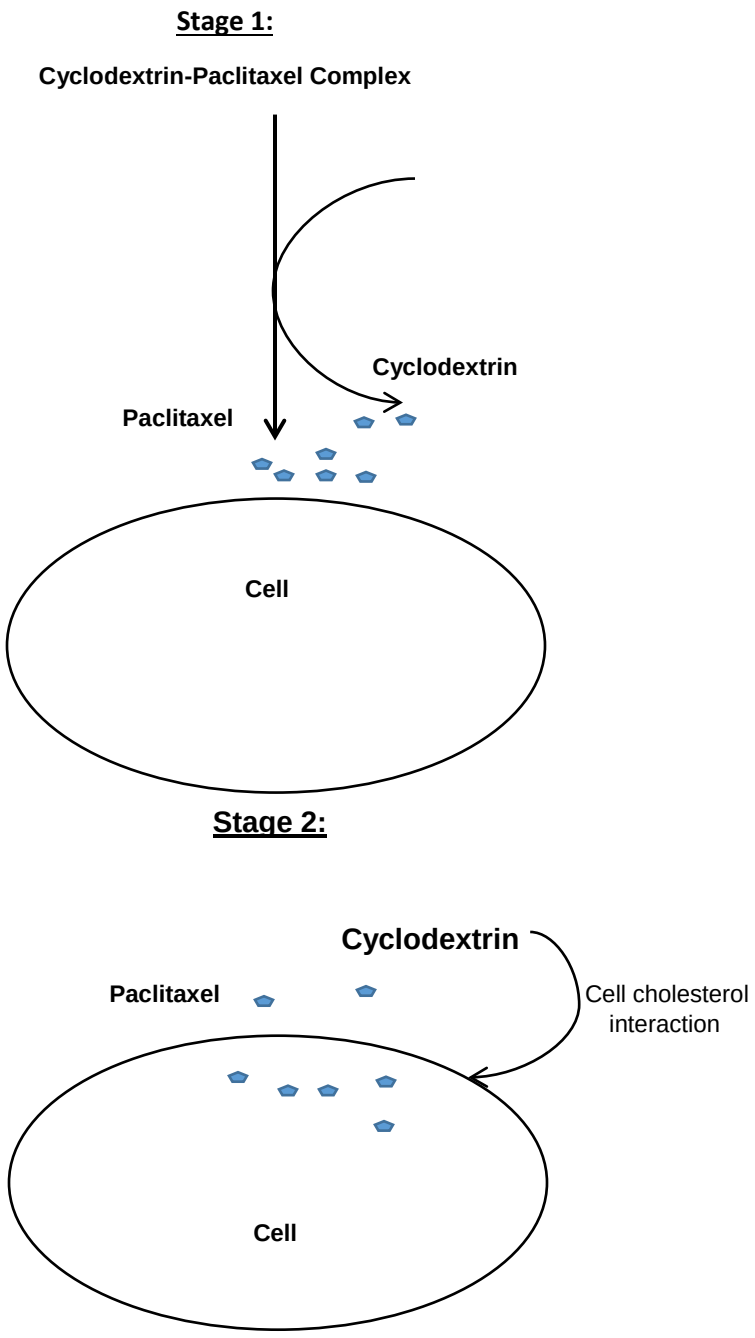



Figure 5.6: Schematics explaining role of CD in combined solubility and internalization enhancement

# 6. DESIGN AND DEVELOP LIPOSOMES CONTAINING PACLITAXEL AND siRNA FOR NOSE TO BRAIN TARGETING AND EVALUATE THEIR *IN VITRO* EFFICACY, CELLULAR INTERNALIZATION AND *IN VIVO* DISTRIBUTION

## 6.1. Introduction

Glioblastomas are the type to intracranial tumors are one of the most lethal and aggressive forms of cancers that originate within the brain. About 15 % of the brain cancers diagnosed are glioblastomas with the median survival rates for less than one year and less than 5 % of five year survival rates. Every year, more than 10,000 patients are diagnosed with glioblastomas in United States.[93] Typical treatment includes cytoreductive surgery as a first line of treatment that removes more than 98 % of the tumor. Significant reduction in the tumor mass alleviate symptoms due to tumor mass as well as reduce secondary resistance to chemotherapy and radiation. Although it looks very high, a 99 % reduction therapy typically will reduce a glioblastoma tumor of $10^{11}$ cells to $10^{9}$ cells. A nearly complete resection is not possible due to high infiltrative nature of glioblastoma cells to the other parts of the brain, most patients will develop recurrent tumor at more distant locations within the brain. The surgery is followed by chemotherapy and radiation with the goals to keep maximum resection to improve quality of life and longer survival period. The frequency of recurrence are very high despite of aggressive treatment protocols and there are no second line treatments available. High mortality and low rates of success are mainly attributed to the facts that most of the surgeries cannot eliminate tumors entirely while success of chemotherapy is limited due to inability of cytotoxic drugs to reach the affected tissues as their localization is limited by occlusive blood brain barrier (BBB). In addition, the tumor cells are also found to be very resistant to the chemotherapy. Increasing the dose of chemotherapy not only induce serious systemic side effects but

also inflict irreversible damage to the healthy brain tissues that has limited capacity to repair which may affect other vital physiological functions. Although many clinical trials have shown that chemotherapy alone is not beneficial. Concomitant therapy of standard radiation with Temozolamide have shown slightly better median survival of 14.6 months compared to 12.1 months who received the radiation alone.[94] Glioblastoma tumors are known to have zones within the tumor that exhibit hypoxia that are highly resistant to radiotherapy.[95] There is an unmet need that requires urgent action to develop therapies to enable anticancer drugs to cross the BBB and access the affected tissues in order to improve the therapeutic outcomes and patient survival. Malignant brain tumors not only affect the local functions but also exert systemic and physiological symptoms that affect many other parts and organs such as motor and cognitive dysfunctions. A better treatment with the application of modern anticancer drugs and a combination of them with either surgery or radiation to be explored to reduce the loss of life and debilitating disabilities due to the disease and current treatment. Due to low permeability of BBB, it is always difficult to achieve desired pharmacological concentrations of the drug within the brain tumors. This problem can be overcome by using local drug administration. Although an invasive method, use of intracerebral injections and infusions as well as placement of drug releasing polymer based matrix within the resection site have found some clinical applications.[96] Although these treatments have found some success, leakage of cytotoxic drugs is almost always detected in nearly all patients that expose the healthy neighboring tissues to the serious adverse side effects of the anticancer drugs. A convection enhanced delivery is a continuous infusion by convective flow to deliver Paclitaxel directly to the brain tumor has been used in the clinical trials have shown promising results.[97] However more work is needed to reduce the treatment associated complications and optimize therapeutic outcomes to enhance the efficacy and reduce

toxicities. Gliadel® wafer was approved in 2003 by the FDA for the treatment of glioblastoma as an adjunct to the surgery and radiation.[98] The wafers are implanted into the tumor bed upon resection surgery. Following the implantation the cytotoxic drug is released from the biodegradable matrix for the period of 2-3 weeks provides high concentrations of anticancer drug within the localized tumor region. Although the localized treatment yields high concentrations of anticancer drug within the tumor and prevents systemic side effects of the drug, more than half of the patients receiving Gliadel wafers have reported new or worsened seizures within the five days post-surgery. Other reported side effects are intracranial hypertension, meningitis, impaired wound healing and wafer migration. The serious side effects requires pre-operative anti-seizure therapy and postoperative patient monitoring. An alternative non-invasive way proposed to deliver anticancer drugs to the brain tumor tissue is via intranasal route. Direct localization of drugs through intranasal route has been known for many years and been used for many neurological treatments to efficiently circumvent the occlusive BBB. This phenomenon can be utilized to deliver drugs which are difficult via systemic circulation where it has to permeate through the BBB. Large molecules, water soluble drugs as well as biologics such as antibodies, recombinant proteins, viral vectors and nucleotides can be efficiently delivered directly to the brain via intranasal route. Rapid onset of action and avoidance of systemic circulation it improves the safety profile of the drug by eliminating systemic side effects as well as minimizing the dosing significantly. The non-invasive nature of intranasal drug delivery offers more advantages as it allows self-administration and thereby improves patient compliance.[99]

About 10 % of the total surface area is known as olfactory region through which fast access to the brain can be attained via intranasal route where nerve fibers from the olfactory bulb are exposed to the nasal cavity. The leaky nature of olfactory mucosa help enhance nose-

to-brain drug delivery by olfactory pathway. Anatomically, olfactory neurons are extends their exons into the cribriform plate to different regions of the brain. Thus, high localization of drugs within the olfactory region can lead to rapid delivery to various parts of the brain through the direct olfactory pathway. The trigeminal nerve pathway is another route by which a drug can access the brain regions. About 80-90 % of the total nasal cavity is innervated by trigeminal nerves similar to olfactory region that facilitate drug transport to the brain via intraneuronal or perineuronal pathways. *In vivo* studies using intranasal administration of chemotherapeutic agents like Methotrexate and 5-Fluorouracil for nose to brain delivery in animals have shown significant reduction of implanted tumor size.[100] More importantly, the nose to brain delivery showed less side effects as compared to systemic administration.[101] Recently, antisense oligonucleotides have also been reported to be delivered to brain using nose to brain pathway. Significant improvement in the median survival rates were observed for the rats bearing intracerebral human tumor xenografts treated with antisense oligonucleotides via nose to brain pathway.[102] With the proven pathway and success in animal models, clinical studies were conducted in Brazil using Perillyl alcohol for the treatment of recurrent glioblastoma using nose to brain pathway. Compared to oral treatment that was halted due to severe side effects, the intranasal route patients was well tolerated and showed better survival from 2.3 months to 5.9 months.[103] Despite of all the positive outcomes from the pre-clinical studies and promising clinical study data, the intranasal route of nose to brain delivery has its own shortcomings. Limited volume of application per dose restricts nose to brain pathway only for potent drugs. Another pitfall is to restrict the delivery in the area of olfactory region so as to maximize brain delivery in a relatively short period of time. Mucociliary clearance is another major factor to be considered and the applied drug or formulation should be rapidly internalized to avoid clearance from the site of application. All of the above

shortcomings can be overcome by designing a suitable formulation that can deliver anticancer drugs to brain. In addition, some other benefits of designing a formulation are increasing the payload to brain, protect drug in the nasal cavity and release drug in a controlled manner. A suitable drug delivery platform can help deliver novel anticancer drugs in combination so as to improve the efficacy and overcome drug resistance. An efficient and safe delivery system that can deliver the cargo of combinations of different drugs via nose to brain pathway can be a new therapeutic paradigm shift in the chemotherapy of glioblastoma and other types of brain cancers as well as to prevent or hold brain metastasis. This is especially true in case of potent antisense oligonucleotides since they hold considerable promise in the treatment of cancer in combination with chemotherapeutic agents but needs a delivery system to protect their destruction in the physiological milieu. Although the physicochemical nature of the chemotherapeutic drugs and oligonucleotides are quite different from their aqueous solubility stand point, it is a challenge to encapsulate both in a single cargo. Fortunately, liposomal system renders the capability to load both hydrophobic and hydrophilic drugs together. The hydrophobic lipid bilayer encapsulate the hydrophobic drug while water soluble drugs can be incorporated in the aqueous core. The presented work was perform to evaluate the feasibility of using liposomes as drug carrier for combined anticancer drug and siRNA to brain via nose to brain pathway. Although there are few reports in the literature that have cited use of liposomes for drug delivery to brain, the availability of reports where such delivery system is used for a combination of drugs to treat cancer are scarce.[104] By encapsulating anticancer drug and siRNA, liposomes can carry cargo across via nose to brain pathway that can be administered intranasal in a relatively small volume. Liposomes not only shields the drug and siRNA from the physiological environment until released, it also can facilitate diffusion through olfactory and trigeminal pathways to brain owing to its

lipid composition. Thus, liposomes provides ideal choice for drug delivery system for nose to brain delivery. Although, liposomes can overcome significant obstacles of intranasal delivery, a formulation alone is not successful if it is not applied or delivered in the area of interest. It is well understood that the olfactory region is key in order to achieve efficient and rapid drug delivery to brain, the delivery and localization of liposomes in the area of olfactory region is critical. Since in humans, the olfactory region covers about 10 % of the total nasal cavity, localizing liposomes to this area can improve brain delivery, reduce drug clearance, dose and frequency of dosing. All of these factors can contribute significantly to improve safety of the dosage form, prevent or minimize systemic side effects and better patient compliance. With significant amount of understanding of brain delivery vial intranasal pathways and to maximize the benefit by achieving high localization within the olfactory region, various devices have been designed and currently are under clinical trials. The function of the device is to localize the powder or liquid droplets in the nasal cavity where maximum absorption can be achieved in a relatively short period of time. Combining such device with a drug delivery system can help to overcome the limitations of intranasal route such as low volume, precise localization and rapid absorption. Under this specific aim, we evaluated the feasibility of nose to brain delivery using such device. First, the liposomal delivery system was formulated to encapsulate anticancer drug Paclitaxel and siRNA together. Characterizations of liposomes were performed with respect to size, surface charge and drug encapsulation. The safety and genotoxic potential of excipients used in preparation of liposomes were evaluated using micronucleus assay. Efficacy of liposomal system was evaluated *in vitro* on brain cancer cell line using cytotoxicity assay. Cellular internalization by cancer cells was assessed using confocal microscopy. At last, the proof of concept was evaluated in mice using nose to brain delivery device and compared with systemic administration.

## 6.2. Materials and Methods

### 6.2.1. Materials

Paclitaxel was purchased from Sigma Aldrich (St. Louis, MO). Egg phophatidyl choline (Egg PC), 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP) and Cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Cyclophosphamide (CP), Ethylmethyl sulfone (EMS), Glucose 6-phosphate, Methanol Dimethyl Sulfoxide (DMSO), Ethanol Tween 20, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), Thiazolyl blue tetrazolium bromide, (MTT), Sodium dodecyl sulfate (SDS) and Dimethyl formamide (DMF) were acquired from Sigma Aldrich (St. Louis, MO). Citrate buffer and Nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Fisher Chemicals (Suwannee, GA). Sodium phosphate buffer and Aroclor-1254 induced rat liver S9 fraction was obtained from Molecular Toxicology Inc. (Boone, NC). Fluorescent conjugate of PTX Flutax-2 and near IR fluorescent lipophilic carbocyanine (DiR) were obtained from Molecular Probes (Carlsbad, CA). Transfection indicator siGlo Red was obtained from GE life sciences (Lafayette, CO). Intranasal catheter delivery device for nose to brain delivery was obtained from Impel Neuropharma (Seattle, WA).

### 6.2.2. Cell Lines

Human glioblastoma cell line (LN-229) and Chinese hamster ovary - K1 (CHO-K1) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). CHO-K1 cells were cultured in F-12K medium (ATCC, Manassas, VA) with 10 % Fetal bovine serum (Life Technologies, Grand Island, NY) and penicillin-streptomycin solution (100 UI/mL-100µg/mL) obtained from Life Technologies. LN-229 cells were cultured in DMEM medium (Life Technologies, Carlsbad, CA) with 5 % Fetal bovine serum and penicillin-streptomycin solution (100 UI/mL-100µg/mL). Cells were grown at 37 $^{o}$C in humidified

conditions with 5 % CO2 (v/v) in air. All cell-based experiments were performed on cells in the exponential growth phase.

### 6.2.3. Preparation of Liposomes

The liposomes were prepared using modified thin film hydration method. Egg PC (50 % w/w), Cholesterol (22 % w/w) and DOTAP (28 % w/w) were dissolved in 5 mL Ethanol. To this lipid solution PTX was added at 10 % w/w of total lipids. The solution was transferred to a round bottom flask and Ethanol was evaporated using rotary evaporator (Buchi, New Castle, DE) attached to vacuum pump maintained at 200 mbar. The temperature of water bath was maintained at 40 $^{o}$C. At the end of evaporation, a thin uniform film was obtained on the bottom of the flask. The film was rehydrated using Citrate buffer (pH 4.5) and nuclease free deionized water at a ratio of 3:2. The siRNA was added in rehydration buffer. The resultant suspension was further mixed on rotary evaporator at 40 $^{o}$C for 15 minutes followed by sonication at 25 % amplitude for 30 seconds. After sonication, the suspension was subjected to extrusion at room temperature using 200 nm filters for the first 4 cycles followed by 10 cycles through 100 nm filters. After preparation, the liposomes were purified by dialysis (Mol. Wt. cut off 10,000 Da) and stored at 2-8 $^{o}$C until use.

### 6.2.4. Particle size and Zeta potential measurements

The particle size and surface charge or Zeta potential of the liposomes were measured using dynamic light scattering by Malvern ZetaSizer (Malvern Instruments, UK) using method as recommended by the manufacturer. All measurements were performed at room temperature and multiple measurements of particle size and zeta potential were performed. Results of multiple measurements were recorded as average values and standard deviation.

### 6.2.5. Encapsulation efficiency

Encapsulation of PTX in the liposomes was assess using a modified chromatographic technique.[82] The chromatographic equipment was consists of an auto sampler (Waters 717plus Auto sampler), a pump (Waters 1525 binary pump) and a detector (Waters 2487 dual λ detector). The samples were run using mobile phase of water and methanol at a ratio of 35:65 and flow rate of 1 mL/min with detection wavelength was set to 227 nm using C18 column (3.9 mm x 150 mm, Waters) at 25 $^{o}$C. The liposomes were dissolved in Ethanol centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected, filtered using 0.2 µm syringe filter and subjected to measurements. Encapsulation efficiency was measured using the following formula:

$$Encapsulation\ efficiency\ (\%) = \frac{PTX\ in\ liposomes}{PTX\ total} \times 100$$

### 6.2.6. Cellular internalization

Cellular internalization of liposomes containing PTX and siRNA was carried out using confocal microscopy. Liposomes were prepared using the method as described above. Near IR fluorescent lipophilic carbocyanine (DiR) for blue fluorescence of liposomes was added to the formula at 2 % w/w. Fluorescent conjugate of PTX Flutax-2 (green fluorescent PTX conjugate) replacing PTX and transfection indicator siGlo Red (red fluorescent siRNA) for siRNA were added at 3 µM and 0.40 µM respectively. Human glioblastoma (LN-229) cells were seeded in a 6 well plate overnight at 2000 cells per well. Next day, the cell culture media was replaced with 400 µL of fresh media and 100 µL of liposomal suspension containing 1 mg of liposomes per well and incubated for 24 hours. The day after the treatment, cells were washed three times with DPBS and 1 mL of fresh media was added to each well. Fluorescent images of the cells to display individual substances were taken followed by intracellular distribution of each substance was

evaluated by taking sequential photographs along the vertical axis of the cell from top to bottom using confocal microscope capable of capturing single and multicolor fluorescence simultaneously (G-STED SP8, Leica Microsystems, Buffalo Grove, IL).

### 6.2.7. Cytotoxicity study of liposomes using human glioblastoma cell line

Cytotoxicity screening of liposomes containing PTX and siRNA were performed using modified MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay as previously described.[90] Human glioblastoma cell line (LN-229) was used to evaluate cytotoxicity study using MTT assay. For the assay, 10,000 cells were seeded per well in 96-well plates and incubated for 24 h. Next day, the media was replaced with fresh media; different concentrations of liposomes containing PTX and BCL-2 siRNA were added in the media and incubated for 24 hours. A series of formulations were also tested as control or positive standards as follows: (1) Control (Fresh media); (2) PTX alone; (3) BCL-2 siRNA alone; (4) PTX liposomes; (5) BCL-2 siRNA liposomes; (6) blank liposomes; and (7) PTX and BCL-2 siRNA. After 24 hours, the solution in each well was replaced by fresh media and MTT reagent (25 µL of 5 mg/mL). Plates were further incubated in cell culture conditions for 3 h. Viable cells would convert MTT reagent into insoluble formazan crystals which were dissolved overnight by adding 50 % (v/v) dimethylformamide in water with 20 % (w/v) sodium dodecyl sulfate. The absorbance was measured at 570 nm with background correction at 630 nm. Based on these measurements, $ID_{50}$ (concentration of active ingredient necessary to inhibit cell growth by 50 %) of all formulations were calculated.

### 6.2.8. Genotoxicity of cationic liposomes

A modified *in vitro* micronuclei test was used as previously described to evaluate genotoxic potential of cationic liposomes. The test was adopted from Organization for Economic

Cooperation and Development (OECD) protocol and performed as previously described.[89] Briefly, in a 25 $cm^2$ flask, about 300,000 LN-229 cells were cultured for 24 hours before treatment. After 24 hours, the cells were treated with cyclodextrins (50 mg/mL) for three hours followed by replacement of fresh media and incubation for another 24 h. Then, the cells were stained to detect the presence of micronuclei. The set of experiments included following series of samples: (1) Negative control – media only; (2) DMSO (100 µL) –negative control for the solvent; (3) Cyclophosphamide 10 mg/mL with metabolic activator (S9 mix, 0.3 mL) – positive control; (4) Ethyl methanesulfone (EMS) 400 µg/mL – positive control that does not require a metabolic activation; (5) Cationic liposomes samples 25 mg/mL and (6) Cationic liposomes 25 mg/mL with S9 mix (0.3 mL). The S9 mixture is a metabolic activation system that enables the detection of mutagenic activity for the samples that require metabolic transformation (e.g. cyclophosphamide). In order to prepare the S9 mix, following chemicals were added to a rat liver S9 fraction (0.2 mL) after Arclor-1254 induction: sterile water for injection (560 µL), sodium phosphate buffer (0.1 M, pH 7.4, 1 mL), 4mM NADP (150 µL), glucose-6-phosphate (120 mM, 14 µL) and potassium-magnesium salt solution (8 mM – 33mM, 60 µL). After incubation, the cells were fixed in a cold solution of 100 % methanol. Methanol was removed, cells were washed with phosphate buffer, and nuclei staining was performed using 600 nM 4, 6 diamidino-2-phenylindole (DAPI) for 8 minutes. After 8 minutes the solution was removed and cells were washed using PBS with 0.02 % v/v Tween 20. The micronuclei were observed under fluorescent microscope (Olympus, Waltham, MA) and the number of micronuclei observed per 1000 cells was calculated.

### 6.2.9. Nose to brain delivery by intranasal catheter device in mice

A nasal catheter (Figure 6.1) designed to specifically deliver drugs in the upper olfactory region of nasal cavity for nose to brain delivery of the drugs. The device was purchased

from Impel Neuropharma (Seattle, Washington). The components of the device are: (1) Catheter guide – To introduce catheter tube in the nasal cavity; (2) Base – Ensure catheter guide stability; (3) Catheter tube – Plastic tubing that delivers the drug in the nasal cavity; and (4) Hamilton syringe – For small volume liquid dosing (25 µL). To administer dose to the animal liquid drug formulation was drawn using the 25 µL Hamilton syringe ensuring no air bubbles in the syringe. The catheter tube was attached onto the needle of the Hamilton syringe. Slowly liquid from the syringe was primed to fill up the catheter tube and ensure that air is completely expelled from the tube. The function of the catheter guide is to introduce and locate the catheter tube precisely in the olfactory region. To do so in the next step, the catheter tube was carefully threaded through the catheter guide from the back end of the catheter guide until it reaches the front end of the catheter guide. The catheter guide was then placed and attached to the base to hold in place. To dose the animals, animals were anesthetized with 5% isoflurane for 3 minutes. The animal was kept on its back. The catheter guide was inserted in the naris while holding the base and catheter guide together as a unit (Figure 6.2). Once the catheter guide reached the opening of the naris, the guide was gently pushed forward until it reached the bend. The bent tip of the catheter guide was placed so that it was pointing towards the roof of the nasal cavity while the rest of the catheter guide was horizontal. At this point, the catheter tube was slowly fed into the nasal cavity. Approximately 2 cm of the tube could be inserted until it reached the olfactory region (Figure 6.2).

**6.2.10. Nose to brain delivery of liposomes in mice using intranasal catheter device**

The animal studies were performed on athymic nu/nu mice according to the protocol and institutional guidelines (Approved protocol # 14-077). The study was performed to evaluate capability of the liposomal delivery system along with nose to brain catheter device to reach brain as compared to intravenous system. For the study, the liposomal

formulations were prepared similar to the cellular internalization method as described above. Fluorescent conjugate of PTX Flutax-2 (green fluorescent PTX conjugate) replacing PTX and transfection indicator siGlo Red (red fluorescent siRNA) for siRNA were added at 3 µM and 0.40 µM respectively. For the intranasal dosing, the mice were anesthetized using inhalation anesthesia 5 % isoflurane. While under anesthesia, the animals were placed in supine position (Figure 6.2) and catheter was inserted. Once the catheter inserted in the nasal cavity, the tube was glided inside approximately 1 cm to reach the olfactory region while holding the catheter at the base. The animal was dosed 10 µL of the liposomal formulation in the nostril and kept aside for 30 seconds followed by dosing in other nostril. The animals were kept under maintenance anesthesia in between the dosing. All animals were dosed 100 µL of liposomal formulation in each group. After one hour of treatment, all animals were anesthetized and sacrificed. Brain tissues from each animal were isolated and frozen. Confocal microscopy of 5 mm sections of brain tissues were carried out to evaluated accumulation of liposomes in the brain tissue after intranasal and intravenous administration.

#### 6.2.11. Statistical analysis of data

All quantitative data presented as mean ± standard deviation from five to ten independent measurements. Data analysis was performed using descriptive analysis and single factor ANOVA. Student's t-test was applied to data sets and P value of less than 0.05 was considered as statistically significant.

## 6.3. Results

### 6.3.1. Characterization of liposomal delivery system

The liposomes were successfully prepared encapsulating PTX and BCL-2 siRNA simultaneously. Once the liposomes were formed using thin film hydration method, further size reduction was obtained using extrusion under pressure. Liposomes obtained after extrusion were evaluated for particle size and zeta potential measurements. Particle size distribution was measured using dynamic light scattering revealed that all batches consistently produced monodispersed liposomes of 110 ± 10 nm size. The zeta potential measurements were performed showed that all batches showed positive charge of approximately + 15 ± 5 mV. The objective to add siRNA to the hydration medium was to encapsulate it in the hydrophilic core of the liposomes and removing excess by substantial dialysis. The liposomes prepared with or without siRNA did not show significant difference in the zeta potential measurements. Encapsulation of PTX was measured using HPLC. The data revealed that more than 95 ± 2 % of the PTX was successfully encapsulated within the liposomes.

### 6.3.2. Cellular internalization of liposomal delivery system

Human glioblastoma cell line LN-229 was used to evaluate the in vitro cellular internalization of the liposomal delivery system. Confocal and fluorescence microscopy images were captured to establish the evidence of co-localization of liposomes containing PTX and siRNA within the cell. After the treatment cells were observed under confocal microscope that showed substantial fluorescence within the cytoplasm. High fluorescence intensity of siRNA (red fluorescence), PTX (green fluorescence) and liposomes (blue fluorescence) were observed within the cytoplasm of LN-229 cells (Figure 6.3). In addition, the instrument allowed to superimpose these individual components that resulted in images displaying co-localization. Thus, superimposition of liposomes and siRNA (pink), liposomes and PTX (cyan), PTX and siRNA (yellow) as well as PTX, siRNA and liposomes combined (white) showed that all three components of the delivery system were

distributed throughout the cytoplasm. To further evaluate the intracellular localization of the delivery system, confocal microscopy in the vertical z-sections was performed. Consistent with the earlier results, the z-sections revealed uniform distribution of liposomes, PTX and siRNA throughout the cytoplasm as seen in images captured from the top of the cell surface to the bottom (Figure 6.4).

### 6.3.3. Cytotoxicity study of liposomes using human glioblastoma cell line

Cytotoxic potential of prepared series of formulations were assessed *in vitro* on LN-229 human glioblastoma cell line. Inhibitory concentrations of Paclitaxel were evaluated in four different scenarios. Liposomal Paclitaxel as well as combination of Paclitaxel and BCL-2 siRNA showed significantly lower dose compared to Paclitaxel. A slight reduction in inhibitory concentration was observed in Paclitaxel and BCL-2 siRNA without liposomal delivery system (Figure 6.5). The dose response curve of all formulations showed that the cytotoxicity was only attributed to Paclitaxel as formulations without it did not show any signs of cytotoxicity. Similarly, carrier mediated Paclitaxel showed higher cytotoxicity indicating better internalization via liposomal delivery system resulting in lower inhibitory concentrations. There was a marked difference seen when liposomal delivery system contained both Paclitaxel and BCL-2 siRNA indicating suppression of resistance mechanism.

### 6.3.4. Genotoxicity of cationic liposomes

To understand the toxicity potential of cationic liposomes, genotoxicity studies were conducted on LN-229 human glioblastoma cells. Assessing micronuclei formation in LN-229 cells while incubated with liposomes was compared with positive standards such as cyclophosphamide and ethyl methane sulfone (EMS). As seen in images, cyclophosphamide in presence of metabolic activation system was significantly toxic to

the cells (Figure 6.7 and 6.8). Similarly, EMS also showed high number of micronuclei formation in comparison to blank samples. Assessment of micronuclei was performed on liposomes along with metabolic activation system as well. Both liposomal system in presence or absence of metabolic activation system showed any alarming levels of micronuclei formation.

### 6.3.5. Accumulation of liposomes in mice brain after intranasal delivery

Confocal microscopic slides of various sections of brain were analyzed after intranasal delivery using the catheter device and compared with intravenous delivery system. The animals were sacrificed after 30 minutes of dosing. As can be seen in the images (Figure 6.9), brain sections of animals treated with intranasal delivery system displayed very high fluorescence intensity. The intensity for both Paclitaxel (green fluorescence) and siRNA (red fluorescence) was observed to be significantly higher compared to intravenous route. Both group of animals were treated with same dose and drug delivery system. High accumulation and localization within the brain via intranasal delivery system established the evidence of internalization of liposomal delivery system via olfactory region. A possible mechanism of higher localization compared to intravenous system is direct and rapid pathway through the olfactory region. While the intravenous system had to go through occlusive BBB resulted in lower accumulation. In addition to provide higher accumulation within the brain region, rapid rise in concentration via intranasal delivery system can also help overcome some of the acute conditions and possibly prevent occurrence of drug resistance due to sub therapeutic concentrations. The data confirmed that high brain localization can be achieved by using intranasal delivery systems in a relatively short period of time.

## 6.4. Discussions

Noninvasive drug delivery to brain for neurological diseases and brain cancers has been always challenging due to the presence of insurmountable biochemical barriers such as BBB and brain-cerebrospinal fluid barrier. Especially for brain cancers and other types of cancers that have metastasized in the brain are extremely challenging to treat that leads to poor prognosis and low survival rates. Nasal route to reach brain has been known and several drug formulations are available in clinic for the treatment of various neurological diseases. A direct pathway through the olfactory region to brain helps reduce dose, avoid first-pass metabolism and systemic side effects to improve safety as well as allows self-administration that leads to improved patient compliance. Large intranasal surface area constitutes the olfactory region that helps passage of the drug straight to the brain leads to rapid onset of action that allows treatment of several acute conditions such as migraine and cancer breakthrough pain.[105] Despite of several advantages, nose to brain delivery has been seen limited applications in the clinics owing to some setbacks. The delivery system prepared and data presented here are to address each of these setbacks so to maximize nose to brain delivery in a safe and effective manner. As mentioned above, the selection of anticancer drug for chemotherapy of brain cancers and brain metastasis of other types of cancers is largely governed by the occlusive BBB. A potent drug like Paclitaxel has extremely limited application in brain cancers due its large molecular structure and physicochemical properties. However, recent clinical trial studies have showed that by co-valent attachment of a peptide to facilitate drug penetration into the brain compartment via receptors located on the intact BBB. Not only the drug was well tolerated but it also established evidence of anticancer activity in patients.[106] This data indicates that potent drugs like Paclitaxel have a place in the treatment of brain cancers if a suitable delivery system is designed that can help drug reach the brain compartments beyond BBB. From the nose to brain delivery perspective, highly potent drugs are the

prerequisite due to low dosing volume of 0.4 mL per administration. Paclitaxel is an ideal choice due to its high potency that may help reduce the overall dose. The physicochemical properties of Paclitaxel and low permeability of nasal epithelium to high molecular drugs pose another challenge. Hence, a simple drug solution delivered into the nasal cavity may not help improve drug permeation. In addition, applying paclitaxel solution may lead to local toxicities and mucosal damage. The shielding of drug was achieved by successfully encapsulating drug in a slightly positive colloidal carriers such as liposomes. These colloidal carriers are known to protect the drug from the external environment and load the therapeutic cargo inside the cells via endocytosis or other different molecular mechanisms. Especially in nose to brain delivery, these carriers are reported to take transcellular transport route to reach various brain compartments.[107] The olfactory epithelium internalization of colloidal carriers is largely affected by its size, surface characteristics which could be used to improve uptake in the olfactory epithelial cells. This led to design and formulation of slightly positive liposomes to encapsulate Paclitaxel. All liposomes prepared were of less than 120 nm with a slight positive charge of 15 mV. The cellular internalization and nose to brain delivery in mice revealed that internalization can be achieved with such delivery system in a relatively short amount of time. Net positive charge and particle size are thought to play a pivotal role in rapid internalization of the liposomes since it has been shown that size of less than 200 nm helped rapid internalization while positive charge on the liposomes not only helped to establish electrostatic attachment to mucus layer that improved localization and residence in the olfactory region resisting mucociliary clearance.[107] High localization resulted in better internalization in various brain regions in short period of time for the same dose as compared to the intravenous administration. The efficacy of chemotherapy to brain cancer is limited by rapid development of drug resistance. Resistance to cytotoxic drugs is

developed in presence of BCL-2 proteins that increase the apoptosis threshold and develop apoptotic resistance. Inhibiting BCL-2 proteins in glioblastoma cells have resulted in overcoming resistance and enhance anticancer activity *in vitro* and *in vivo.[108]* siRNA has shown great promise to suppress these proteins and overcome drug resistance in cancer.[109] The liposomal system afforded to design a multifunctional nano delivery system to incorporate drug and siRNA to deliver via nose to brain pathway. The siRNA was encapsulated rather than surface attached to keep the net positive charge that is thought to help facilitate internalization. Degradation of siRNA in the nasal cavity was also prevented by encapsulating it within the aqueous core of the liposome. Thus, the resulting liposomal system provided a multifunctional colloidal cargo to carry drug and siRNA across the olfactory epithelium. The delivery system also helped to concentrate more drug in small volume of dose. Another setback of the nose to brain delivery is the local application of the formulation at high precision within the olfactory region. Lack of precision results in systemic absorption through highly vascularized nasal epithelium that results in low brain localization and systemic exposure to the drug. The olfactory region is located in the upper nasal cavity and encompass large surface area in that region. Local targeting of drug in that region can help prevent systemic exposure and enhance brain targeting. By applying drug formulation via a novel intranasal catheter device enabled local application of liposomal formulation in the olfactory region at high precision resulting in improved brain delivery in a short period of time at relatively low dose. Hence adding to the rationale of choosing a multifunctional liposomal system, using appropriate method such as use of intranasal catheter device to localize formulation specifically in the olfactory region helped improve brain localization compared to application of liposomes via intravenous administration. The approach can be divided in multiple components to overcome the limitations of nose to brain delivery and achieve high brain localization. The

dose volume of administration is very low which was efficiently overcome by using highly potent drug such as Paclitaxel. High encapsulation efficiency and loading of drug in the liposomes further helped concentrate more drug in relatively less volume. Further, in order to reduce dose further and enhance anticancer activity, siRNA targeting to BCL-2 protein was also encapsulated. The objective of encapsulation was not to alter the surface morphology and charge of the liposomes that was thought to improve internalization. Zeta potential measurements before and after encapsulation of siRNA showed no difference that suggested encapsulation. Additionally, encapsulation of both Paclitaxel and siRNA was further confirmed by cellular internalization. Uniform high fluorescence intensity throughout the cytoplasm corresponding to Paclitaxel and siRNA indicated high internalization via liposomal carrier. Simultaneous delivery of Paclitaxel and siRNA significantly enhanced anticancer activity of Paclitaxel as seen in MTT assay which helped to reduce the dose further. Net positive charge on the particles have been found previously to be cytotoxicity as well as genotoxicity.[109] Unlike other genotoxicity studies, human glioblastoma cells were used to understand the genotoxic effect of the delivery system directly on the cells it was applied. This was extremely important since the delivery system could reached various regions of the brain. By reducing the charge of the liposomal system to +15 mV, genotoxicity and cytotoxicity was efficiently reduced. Lower zeta potential was sufficient to facilitate the internalization and high residence time in the olfactory region without inducing any genotoxicities. The MTT assay further confirmed safety of empty liposomes. Finally, combining drug delivery with a device helped to reduce the dose by improving localization specifically within the olfactory region. Once the catheter is inserted in the nasal region, the catheter tube was guided through to reach the olfactory region. The unique but mechanism of gliding tube through the catheter helped to reduce animal to animal variability. This dynamic mode helped to reach the olfactory

region every time in all animals at very high precision. By combining right components of the delivery system the dose of Paclitaxel was significantly reduced and the efficiency of delivery system was further enhanced by precise local delivery in the olfactory region using a catheter device. The results showed that maximum benefits of intranasal drug delivery to brain can be achieved by using suitable drug delivery system encapsulating a potent drug and delivering it in olfactory region for rapid and high internalization.

### 6.5. Conclusion

The promising route of nose to brain delivery is proven but has several setbacks. The presented data displayed that setbacks can be overcome by using a suitable drug delivery and device combination. A novel catheter device helped improve localization within the olfactory region and cationic liposomal carriers were rapidly internalized at low dose in a short period of time. Multifunctional liposomal system facilitated simultaneous delivery of drug and siRNA which improved the anticancer activity of Paclitaxel and helped reduce the doe further. Lower charge on the liposomal system was found to be sufficient for internalization via olfactory region. The early feasibility work showed that this approach can be further explored in pre-clinical settings to evaluate proposed combination of drug delivery and device for cancer and other neurological diseases.

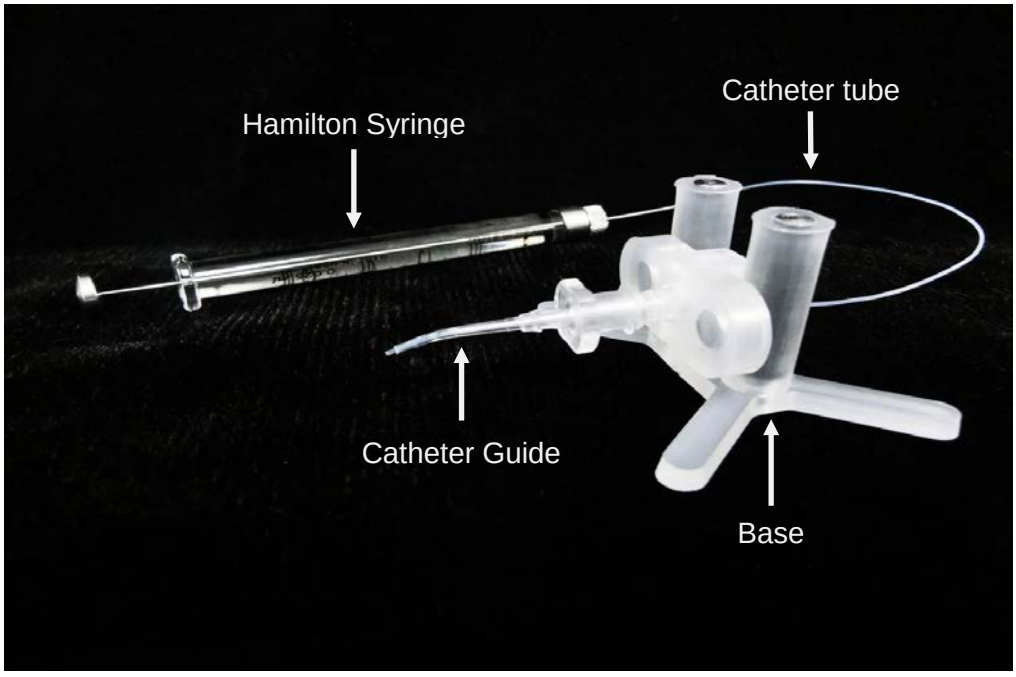



Figure 6.1: Representation of assembled intranasal catheter device for nose to brain delivery with all components. All the components received individually and assembled before dosing as shown in the image above.

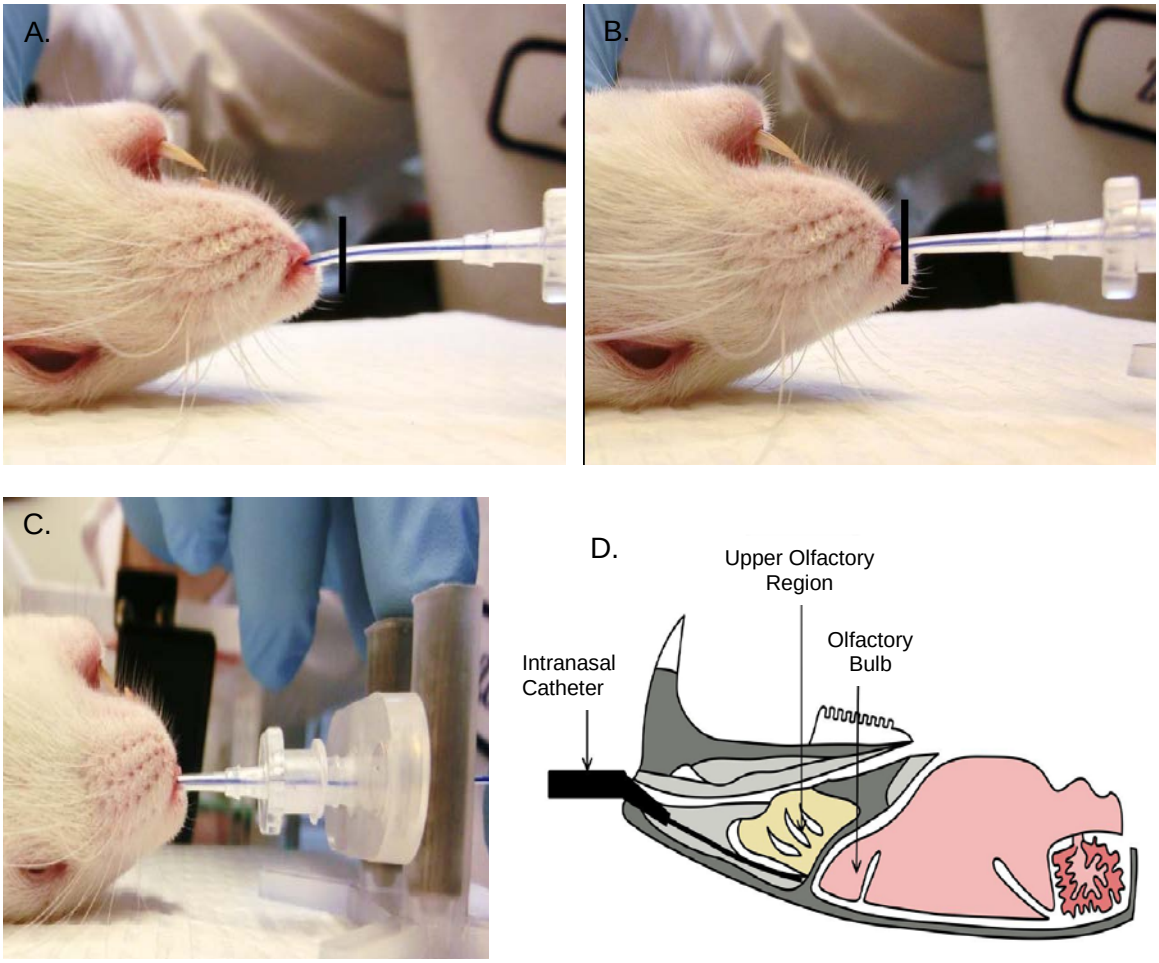



Figure 6.2: Images showing steps to perform intranasal dosing in animals using intranasal catheter device. (A.) Locating naris: The anesthetized animal is placed in supine place and bent tip of the catheter is inserted in the naris; (B.) Insertion: After locating the naris, the tip is gently inserted inside the nasal cavity. The black vertical line indicates approximate length of insertion (C.) Insertion of catheter tube: Once the catheter is inserted, the catheter tube is glided into the nasal cavity until it reaches olfactory region. (D.) A schematic diagram shows the location of inserted catheter and catheter tube before dosing.

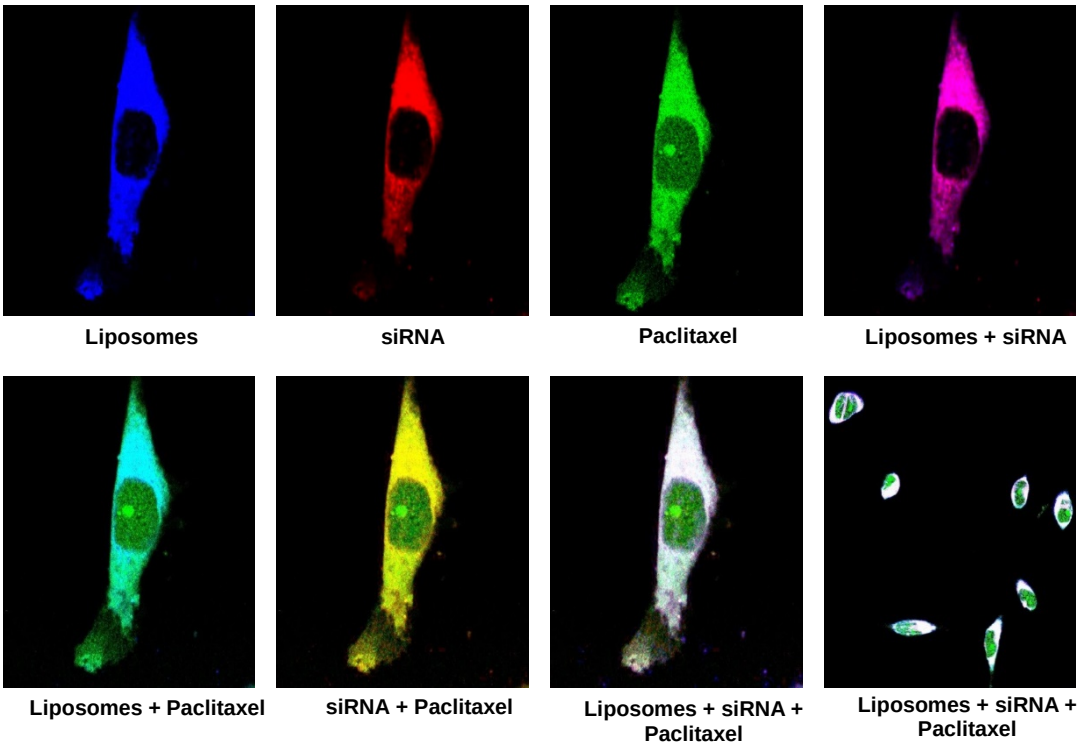



Figure 6.3: Representative confocal microscopic images of human glioblastoma cells (LN-229) after cellular internalization of Liposomes (Blue Fluorescence) Containing siRNA (Red Fluorescence) and Paclitaxel (Green Fluorescence). Superimposition of red and blue colors gives pink color (Liposomes + siRNA); superimposition of blue and green gives cyan color (Liposomes + Paclitaxel); superimposition of red and green colors gives yellow color (siRNA + Paclitaxel); superimposition of red, green and blue colors gives white color (Liposomes + siRNA + Paclitaxel).

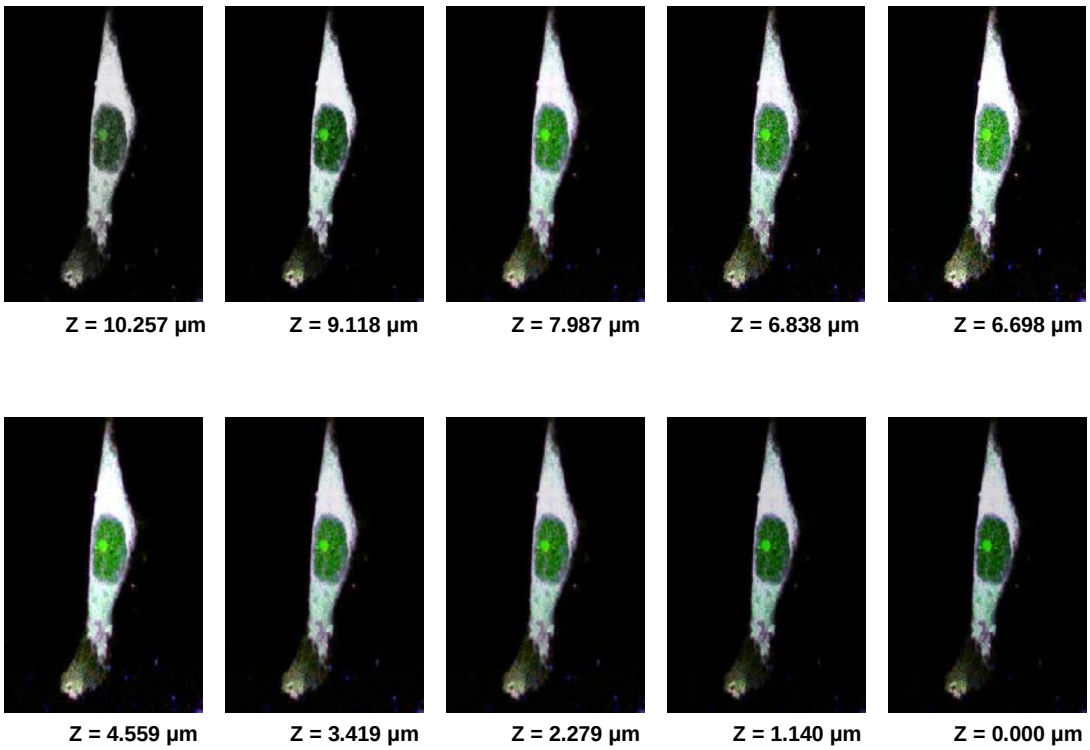
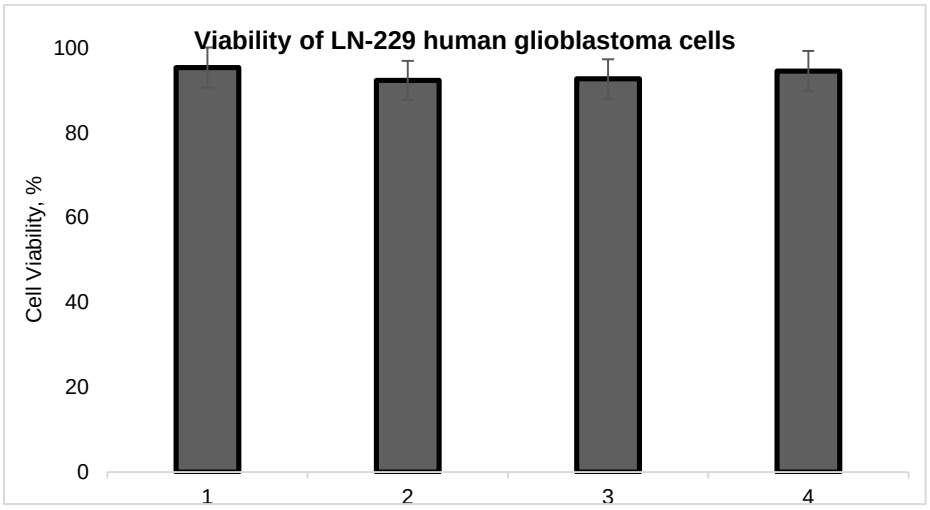



Figure 6.4: Representative z-series confocal microscopic images displaying Co-localization of Paclitaxel (green fluorescence) and siRNA (red fluorescence) delivered by liposomes (blue fluorescence) in human glioblastoma cell line (LN-229) from top of the cell to bottom. Superimposition of red, blue and green gives white color.

A.




B.

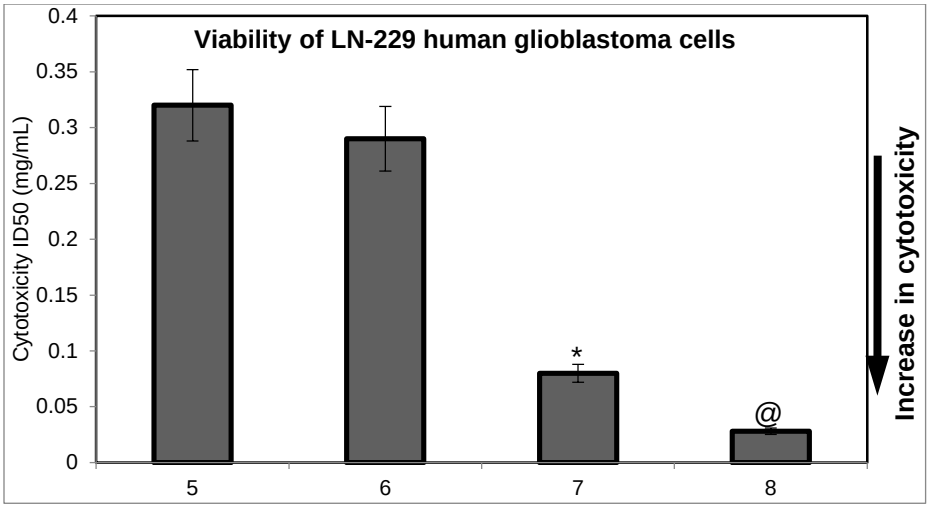



Figure 6.5: Viability of LN-229 human glioblastoma cells incubated with indicated formulations. A. Cytotoxicity of formulations that do not contain Paclitaxel; B. cytotoxicity of formulations that contain Paclitaxel. Data shown as mean ± SD. 1. Blank (Fresh media); 2. Empty liposomes; 3. BCL-2 siRNA; 4. Liposomal BCL-2 siRNA; 5. Paclitaxel; 6. Paclitaxel + BCL-2 siRNA; 7. Liposomal Paclitaxel; 8. Liposomal Paclitaxel + BCL-2 siRNA. * $P < 0.05$ when compared to Paclitaxel; @ $P < 0.05$ when compared to liposomal Paclitaxel

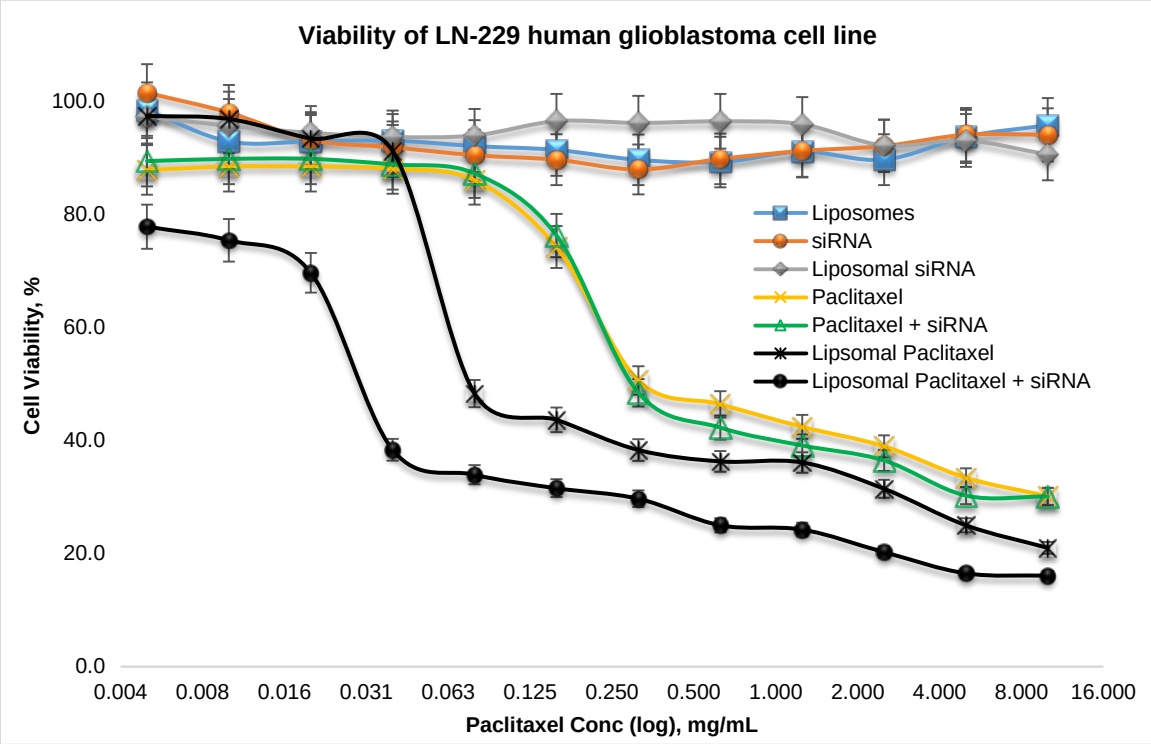



Figure 6.6: Dose response curve of LN-229 human glioblastoma cells incubated for 24 hours with formulations indicated in chart legend. The composition and concentrations of all formulations were same. Viability of cells against Paclitaxel concentration data shown as Mean ± SD.

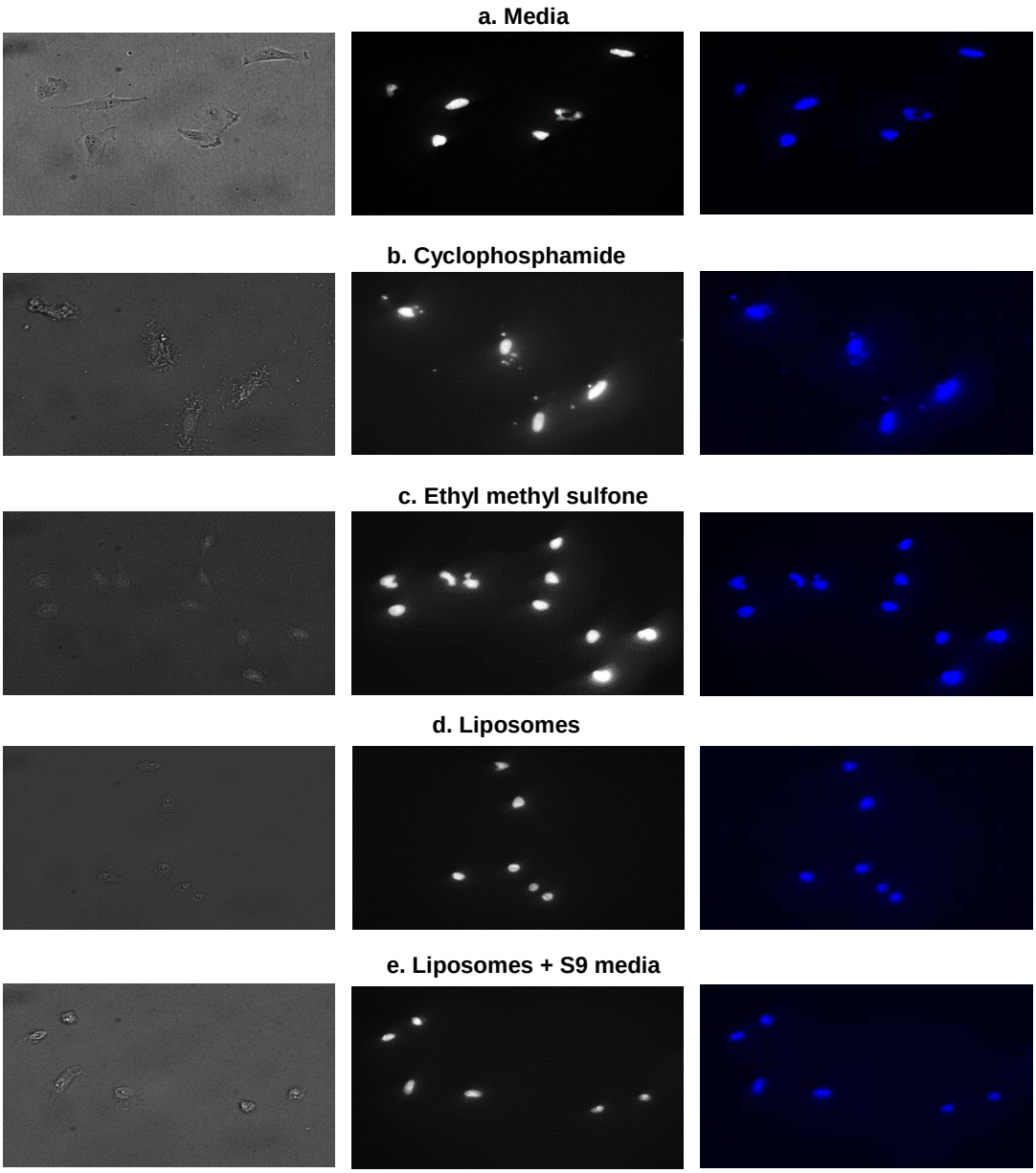



Figure 6.7: Optical and Fluorescent microscopic images of human glioblastoma cells LN-229 after treatment with liposomes to evaluate their genotoxic potential

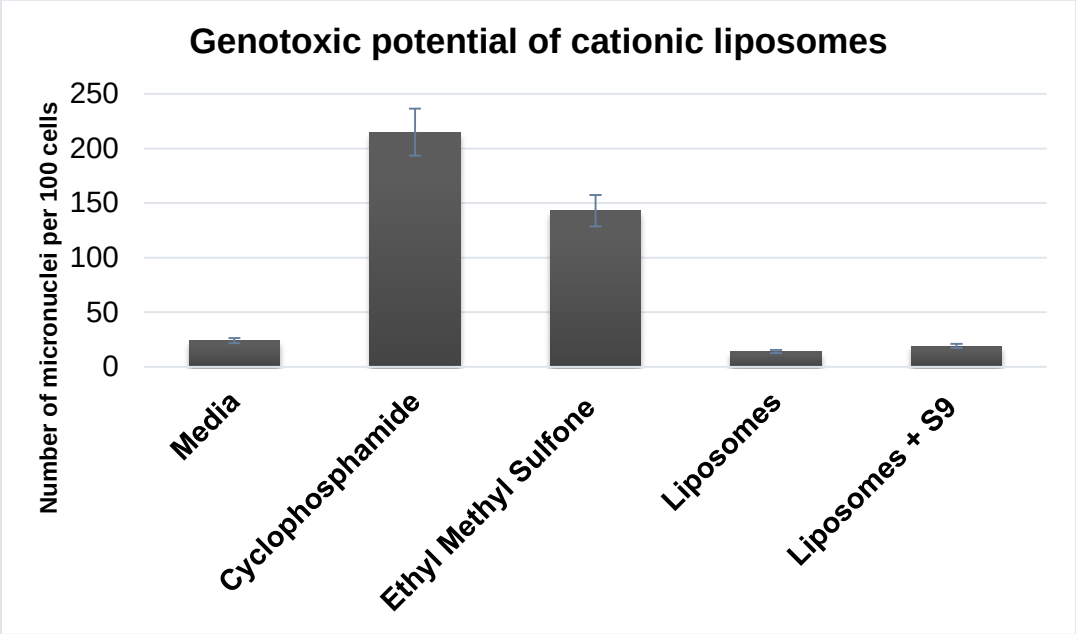



Figure 6.8: Genotoxic potential of cationic liposomes. The bars represent number of micronuclei observed per 1000 cells. Data presented as Mean ± SD.

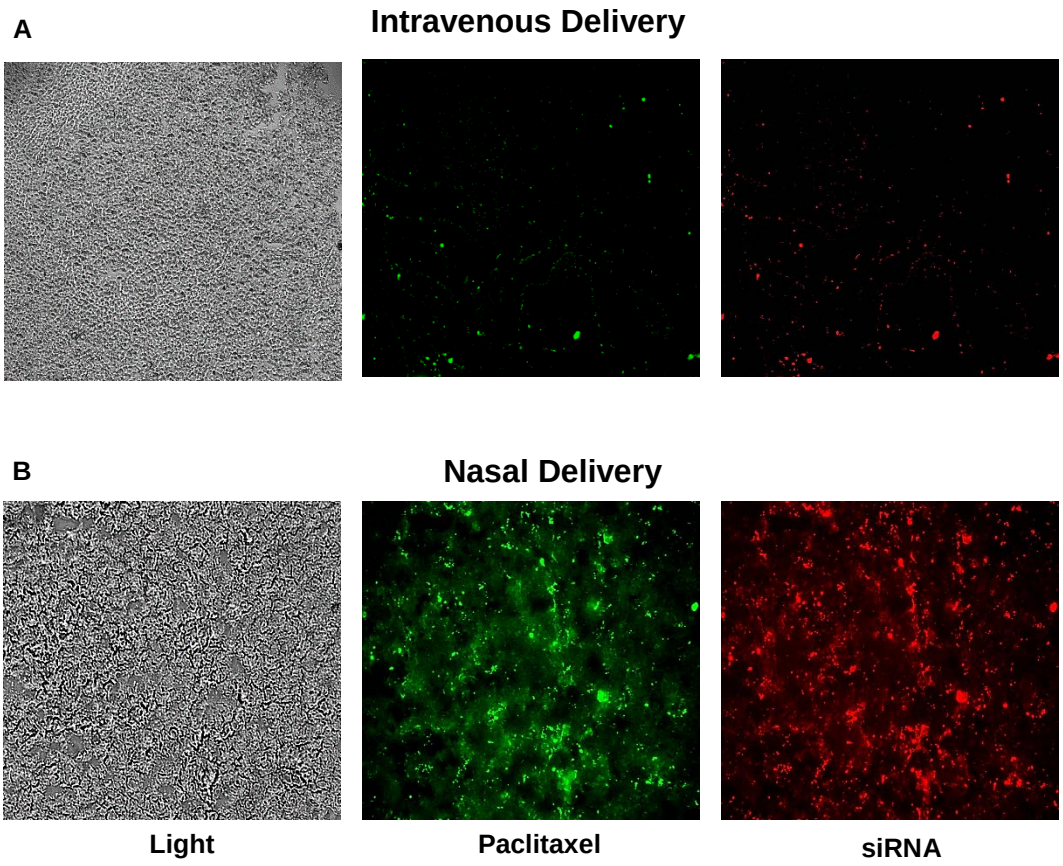



Figure 6.9: Representative confocal fluorescence microscope images of frozen 5 mm brain tissue sections displaying brain accumulation of liposomal Paclitaxel and siRNA after intravenous (Top, A) and intranasal (Bottom, B) delivery. Accumulation of Paclitaxel (green) and siRNA (red) in brain tissue after Intravenous (A) and intranasal (B) Delivery.

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