# *Chapter 1: Introduction*

# 1.0 Introduction

## 1.1 Introduction

### 1.1.1 Barriers to effective treatment of cancer

Cancer has been defined as a refractory and resistant group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. This global epidemic had 19.3 million new cases and 10.0 million deaths worldwide reported worldwide in 2020. Traditionally, it has been treated by surgery, chemotherapy, radiation therapy, hormonal therapy, and targeted therapy (including immunotherapy such as monoclonal antibody therapy) (1).

Effective drug delivery has been a key factor toward better cancer treatment. Inefficient drug delivery has led to poor tumor response, caused severe side effects, and has led to rise of notorious cancer drug resistance. Since anticancer drugs have been typically toxic towards healthy proliferating cells, drug dosage must be restricted to avoid potentially lethal side effects (2). Therapeutic efficacy of such restricted drug dosage can be further diminished by factors such as limited systemic circulation lifetime, undesirable biodistributions, non- specific cellular uptake, and poor tumor vascularity. As a result, each course of chemotherapy has typically induced partial treatment, which has led the surviving cancer cells to a selective pressure that favoured mutations and drug resistance. Drugs that have showed favourable initial response have often been rendered ineffective following repeated administrations with increased cases of the relapsed tumor being more difficult to treat (3).

Several research efforts undertaken to overcome the serious clinical challenge have been aimed at development of potent therapeutics that can be efficiently delivered to various cancers. Clinically, drug-loaded nanoparticulates (NPs) such as liposomes have emerged as a powerful and versatile carrier platform for improving the delivery efficiency and therapeutic efficacy of chemotherapeutics (4). The effective treatment of cancers with minimized drug resistance requires the safer delivery of high doses of potent therapeutics to tumoral sites. Achievement of such outcomes have been difficult for small-molecule anticancer drugs due to numerous barriers encountered during transit from the point of intravenous administration to their

intended diseased destination. These barriers can be classified into three separate levels: the physiological barrier, the cellular barrier, and the molecular barrier (4). On the physiological level, small-molecules drugs are rapidly cleared upon systemic injection from plasma degradation, reticuloendothelial system (RES) uptake, and renal filtration. Because of their poor pharmacokinetics and the short circulation, the majority of administered drugs simply cannot stay in the circulation long enough to reach the tumor (Figure 1) (5).

Therefore, an important requirement for cancer drug delivery has been prolongation of the in-vivo residence time of therapeutic compounds (5). On the cellular level, the cellular membrane of cancer cells has presented a major barrier to transit of molecules. Anticancer drugs have typically relied on facilitators of passive diffusion and membrane translocators to cross the cellular membrane. However, these mechanisms of cellular transit have precluded bulky and polar drugs from effective penetration (5, 6). In addition, the presence of overexpressed membrane bound drug-efflux pumps in drug resistant cancer cells, have actively transported the drug molecules from the intracellular to the extracellular space. Therefore, therapeutically effective drug delivery would require delivering the drugs to the cellular cytoplasm and overcoming these membrane barriers (6).

Further, major barriers have been reported to exist on the molecular level in cancers, which have often survived effects of drug through their mechanistic pathways by activating and strengthening the alternative pathways. Such complexity of cancer biology has been likened to "webs of interconnected routes with multiple redundancies", in which single-drug therapies and their one-dimensional action mechanisms are usually inadequate to treatment of cancer (7). The emergence of chemoresistance has been associated with the emergence of mutations. The identified types of mutations include compromised apoptotic signaling, enhanced damage repair mechanisms, increased drug metabolism, altered drug targets, and upregulation of drug-efflux pumps (8). Therefore, an effective way of improved treatment of cancer cells would encompass activating multiple pathways for negating the possibility of acquiring mutations in tumor cells. Further, most of the cancers have been associated with multiple genetic alterations or abnormalities leading to tumor heterogeneity (9). The use of single chemotherapeutic agents

to treat cancer has led to the development of resistance to use of that drug which has been a major impediment to the success of cancer therapy (9).

Consequently, an effective way to increase therapeutic efficacy would include the treatment strategies having agents acting through multiple mechanisms, to reduce the acquirement of drug resistance phenotypes. A promising way to overcome all the aforementioned barriers would include delivery of multiple agents through efficient nanocarrier platforms (10).

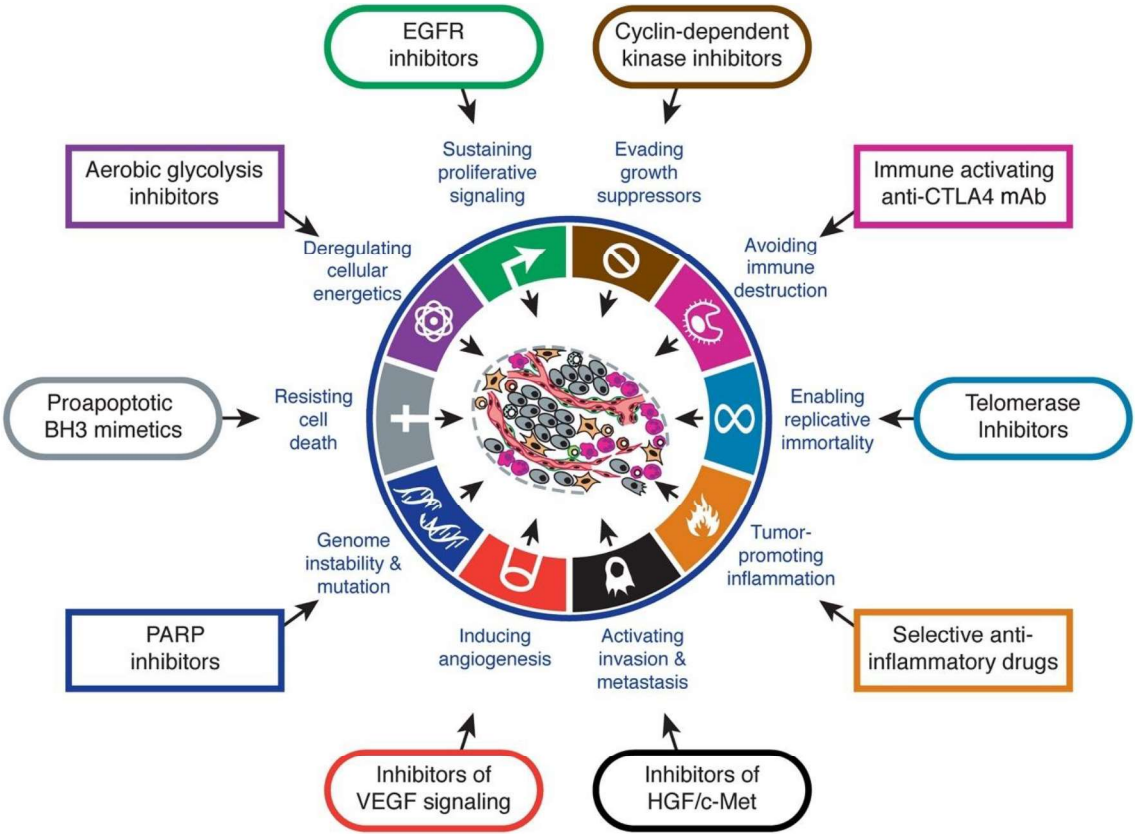


**Figure 1:** Hallmarks to effective treatment of cancer and possible approaches [ adapted with permission from (7)]

### 1.1.2 Nanoscale carriers as effective cancer treatment vehicles

Nanoparticles (NP) have been reported to possess the capability to break down the physiological barriers in cancer drug delivery by extending the circulation time of small-molecule drugs and enabling passive targeting to tumors. Tumor vessels have been reported to

possess abnormal vasculature owing to lack of adequate pericyte coverage and presence of large fenestrations (11). The presence of abnormal porosity has led to "enhanced permeability and retention" (EPR) effect which enabled therapeutic NPs having particle size of 50-150 nm to escape from tumor capillaries and accumulate passively in the extracellular tumor matrix. Additionally, the accumulated NP have presented increased retention times due to lack of well-developed lymphatic drainage in tumors (12). However, the EPR effect has shown negligible benefit for systemically administered free anticancer drugs because of their short circulation lifetime and high rates of clearance. These small molecule drugs have been rapidly removed from the blood by nonspecific cellular uptake, immune opsonization, plasma degradation, glomerular filtration and hepatic clearance. In general, small molecule anticancer drugs delivered without nanocarrier systems have been cleared from the blood within hours post administration (13). NPs have presented excellent pharmacokinetic profiles that allowed them to take advantage of these leaky vasculatures near the tumoral environment (14).

Clinical studies have revealed a striking difference in circulation halflife between free drugs and their NP-encapsulated formualtions. In a detailed review, Gabizon et al. have compared the pharmacokinetics between free doxorubicin (Dox) and PEGylated liposomal Dox (Doxil). Doxil showed improved pharmacokinetic profiles in both human and animal studies with dose of 50 $mg/m^2$ in humans, resulted in 300-fold increased plasma drug concentrations as compared to free Doxorubicin (15). In the last few decades, with the advancement of nanotechnology, the feasibility of synthesis of nanoscale, biocompatible and biodegradable drug delivery vehicles have improved. Many types of nanocarriers including liposomes, solid lipid NPs and polymeric NPs have been developed to deliver a variety of drugs. These nanocarriers have demonstrated desirable drug delivery characteristics such as prolonged systemic circulation lifetime, reduced non-specific cellular uptake, targeting abilities, controllable drug release, and multidrug encapsulation for combinatorial treatment. Recently, NPs with a size range of 50-150 nm have emerged as a promising drug delivery platform for cancer treatment with increased number of NP-based cancer drugs being tested in clinical trials and translated to effective therapies (16).

### 1.1.3 Tumor heterogeneity and use of multiple therapeutic agents in combination

The molecular barrier in cancer drug delivery has been manifested in the emergence of cancer drug resistance. Cancer cells over treatment schedules acquire defence mechanisms against the presence of therapeutic compounds. Importantly, emergence of heterogenous populations of cells within the tumor (Tumor heterogeneity) has resulted in increased rates of failure of drug treatments. The use of multiple therapeutic agents in combination has become one of the strategies to combat this molecular barrier in cancers (Figure 2) (17). It has been reported that usage of proper drug combinations has promoted synergistic actions, improved target selectivity, and deterred the development of cancer drug resistance. However, administration of combinatorial regimens has been limited by the varying pharmacokinetics of different drugs, which resulted in inconsistent drug uptake and suboptimal drug combination at the tumor sites (18).

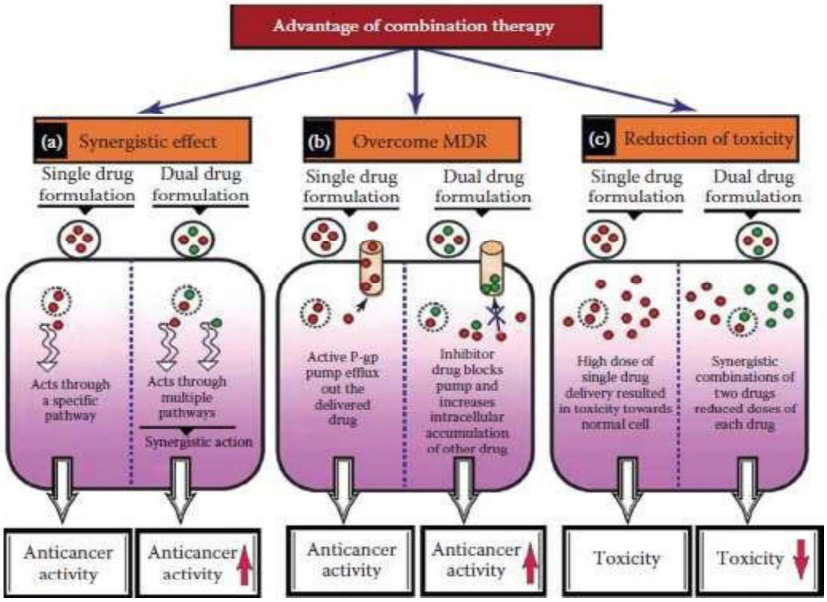


**Figure 2:** Advantages of combinatorial drug treatment over single drug treatments against cancer [ adapted with permission from (19)].

Conventional drug combination strategies aimed to maximize therapeutic efficacy based on maximum tolerated dose has not accounted for the therapeutic synergism as such phenomenon

are sensitive to both dosing and scheduling of multiple drugs. The issue associated with such combination therapies have been highlighted when the USFDA approved Gemcitabine and nab-paclitaxel as first-line treatment for advanced pancreatic cancer. In a phase III randomized, open-label, multicenter trial (MPACT) mean overall survival, progression free survival and tumor response rates were seen to have significantly improved in the gemcitabine plus nab-paclitaxel group compared with gemcitabine alone (8.5 versus 6.7 months, $p < 0.001$; 5.5 versus 3.7 months, $p < 0.001$; 23% versus 7%, $p < 0.001$, respectively). However, higher incidence of myelosuppression, peripheral neuropathy, neutropenia, febrile neutropenia, thrombocytopenia and sensory neuropathy was observed in the patients. These side effects were found to similar to the approved clinical regimen of FOLFIRINOX (a combination of 5-fluorouracil, irinotecan, oxaliplatin and folinic acid) (20).

### 1.1.4 Co-delivery of multiple therapeutic agents in combination

Advances in nanotechnology have opened up unprecedented opportunities in novel combination strategies. Recently, nanocarriers have been fomulated for their ability to co-encapsulate multiple therapeutic agents and to synchronize their delivery to the diseased cells. One distinctive advantage of NP-based combination therapy over traditional cocktail combinations has been their ability to maintain the synergistic drug-to-drug ratio in vivo post at tumor site. Drug-to-drug ratio has been found to govern the efficacy of combination treatments. Multiple studies suggested that the degree of synergism and antagonism of a combination therapy were highly dependent on the relative concentrations between the combined drugs (21).

By unifying the pharmacokinetics of different drug cargoes, combinatorial nanoparticles have opened the avenues to co-delivering multiple drugs at a predetermined ratio that has maximized the combination efficacy (22). Dual drug loaded liposome with precise molar ratio of cytotoxic drugs and different mechanisms of action has presented a promising alternative. The effectiveness of such strategies has been highlighted by the USFDA approval of the combinatorial liposomal nanocarrier Vyxeos® (Celator Pharmaceuticals) for treatment of acute myeloid leukemia (AML) (23). Enhanced benefits of dual-drug liposomes with precise molar ratios over traditional combination therapy were highlighted by the clinical trials of CPX-351

for AML (Phase III) and CPX-1(1:1 irinotecan and floxuridine for colorectal cancer, Phase I) (24). These liposomal formulations have demonstrated the ability to maintain the synergistic drug ratios in vivo while being reported to be more effective than the cocktail administration of the free drugs (25).

Thus, therapeutic NPs such as liposomes have emerged as a safer and more effective drug delivery option as compared to their small molecule chemotherapy counterparts. They have shown numerous favorable features including long systemic circulation lifetime, targeting ability, cellular internalization through endocytosis, and co-delivering multiple therapeutic agents. These desirable features make multi-drug multi-target therapeutic liposomes highly promising in treating cancer.

**1.2 Hypothesis of study**

There has been a paradigm shift in cancer therapy from drug monotherapy to use of multiple chemotherapeutic agents. However, this strategy of using multiple agents administered individually suffers from various drawbacks: -

1) Lack of beneficial therapeutic effectiveness when considering theoretically nonoverlapping mechanisms of action of each anticancer agent.
2) Treatments in cancer are far from being perfect with moderate enhanced efficacy but additive toxicity.
3) Without pharmacokinetic modification free drugs get distributed and eliminated independently of each other.
4) Combining molecularly targeted agents provides an improved strategy, but added complications of use of such agents including patient compliance is an issue.
5) Further it is virtually impossible to achieve uniform temporal and spatial co-delivery at tumor site.

In fact, chemotherapies activating multiple signalling pathways can lead to different cell death outcomes. Thus, there is a need to investigate novel platform approaches by incorporating nanotechnology with combination anticancer treatment. Such nanotechnology platforms can lead to synchronized and controlled pharmacokinetics of each drug and with enhanced bioavailability providing aggressive therapy.

We hypothesize that use of multiple chemotherapeutic agents acting through various cancer treatment pathways in definite synergistic molar ratios when co-delivered together through a single long circulating carrier system (liposomes) can be a more effective safer therapeutic tool in cancer management than either the individual drugs used in combination or carrier encapsulated individual drugs (Figure 3). Such carrier mediated co-delivery shall not only ensure simultaneous delivery of therapeutically effective concentrations of drugs to the tumor interstitium providing a "one-two knockout killer punch to the tumor cells" but also provide a safer less toxic therapeutic regimen.

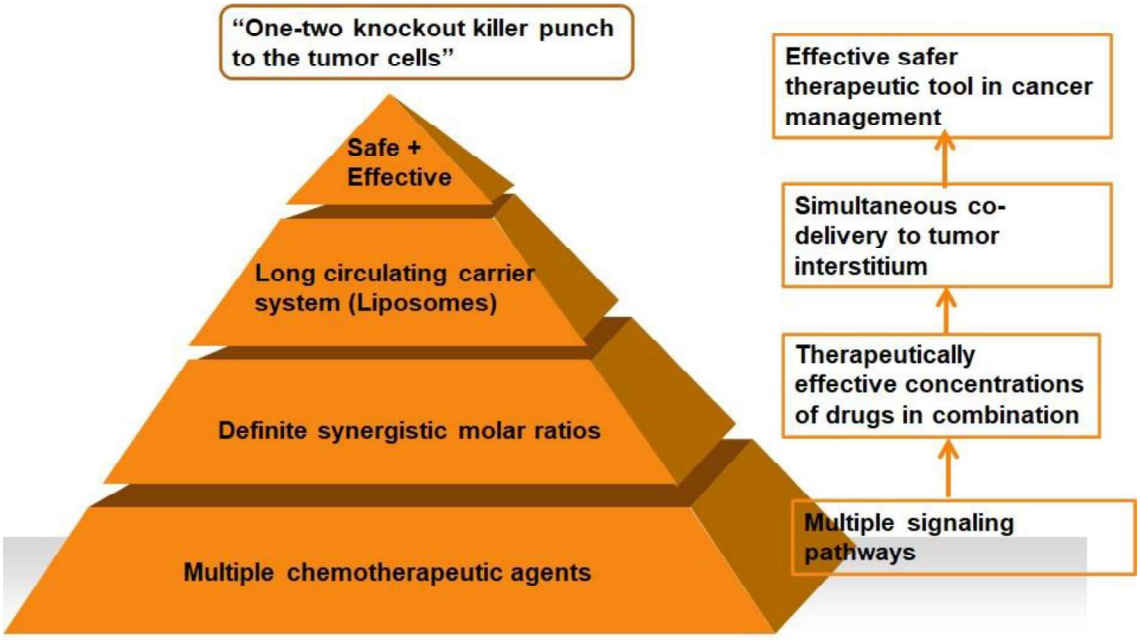


**Figure 3:** Hypothesis of the present study.

### 1.3 Research Statement

#### 1.3.1 Role of Liposomes in cancer combination therapy

Cancer as a broad cluster of disorders may be defined as the abnormal uncontrolled growth of the cells with inherent ability to spread to other tissues of the human body facilitated majorly

through the components of haematic systems. The current treatment options for these diseases involve the usage of a cocktail of chemotherapeutic drugs with the approach of having multiple agents acting through multiple mechanistic pathways on the tumor cells (26). Although this conventional approach has provided some benefits in the therapy, it is saddled with the un-coordinated pharmacodynamic (PD) and pharmacokinetic (PK) profiles of the individual drugs being used. Additionally, such combination approach has being clinically mired with the issues of presentation of non-synergistic drug ratios and lack of simultaneous presence at the site of action resulting in limited therapeutic efficacy, increased drug resistance and increased toxicity profile (27). However, the establishment of synergistic drug combination to be presented as combination therapy may not be sufficient to elicit the desired response. The effective translation of the combination drug therapy necessitates the controlled delivery of the agents through carrier systems ensuring spatial and temporal presence (23). Traditionally, nanoliposomal carrier systems have been evaluated for the delivery of chemotherapeutic agents due to their ability to provide altered PK-PD profiles resulting in clinically effective EPR (enhanced permeation and retention) mediated specific controlled drug delivery to the desired loci of action with reduced toxicity profiles (28). While various liposomal formulations (Lipodox™, Onivyde™, Marqibo™ among others) have been clinically used as a component of established combination chemotherapies in place of the conventional naïve agents, the problem of lack of desired efficacy and their co-presence along with the other chemotherapeutic agents in the desired synergistic ratio cannot be ascertained using the conventional combination therapy (29). Encapsulation of the chemotherapeutic agents in the pre-determined effective molar ratios into the liposomal carriers-single or multiple may be an approach to solve the issue. However, combination of single chemotherapeutic drug loaded into similar composite liposomes to ferry the drugs to the tumor site may not guarantee the co-spatial presence of the agents in tumor while presenting disadvantages of higher lipid load to the human system and subsequent immune response (10). Consequently, another alternative for effective delivery of synergistic combination therapy may be the encapsulation of chosen drugs in the same nanocarrier as illustrated in previously tested combinations of cytarabine/daunorubicin (Vyxeos™), irintotecan/floxuridine and topotecan/vincristine (22, 25). Such manifestation may ensure the simultaneous drug release and presence at the tumor

site due to tumor microenvironment induced membrane rupture as well as destabilization of the drug loading ion gradient (30). Importantly, a rationalistic approach considering the clinical risk-to-benefit ratio needs to be adopted for the choice of the drugs in combination which may be encapsulated in such combinatorial nanocarriers. Determination of synergism and encapsulation of chemotherapeutic agents which have been clinically used in the traditional cancer treatment regimens may serve as good basis for such nano-constructs.

### 1.3.2 Conventional doxorubicin liposome (PEGylated liposomal doxorubicin)

Doxorubicin HCl Liposome®, the first USFDA approved nano-drug (1995), was based on three unrelated principles: (i) prolonged drug circulation time and avoidance of the RES due to the use of PEGylated nano-liposomes; (ii) high and stable remote loading of doxorubicin driven by a transmembrane ammonium sulfate gradient, which also allows for drug release at the tumor; and (iii) having the liposome lipid bilayer of phosphatidylcholine, and cholesterol. Due to the EPR effect, Doxorubicin HCl Liposome is "passively targeted" to tumors and drug is released while becoming available to tumor cells by as yet unknown means (31). A generic liposomal doxorubicin injection (Doxorubicin HCl Liposome) was approved by USFDA in 2013 (32). Doxorubicin HCl Liposome/ Doxil® is a sterile stealth liposome encapsulated doxorubicin for intravenous use clinically indicated in ovarian cancer, Kaposi sarcoma and multiple myeloma besides being used in various solid and haematological cancers. The advantage it offered over Adriamycin (Doxorubicin) injection was reduced cardiotoxicity and myelosuppression (30). However, this did not translate into increased efficacy of doxorubicin which may be attributed to various reasons such as longer half-life in blood circulation, impaired release at the tumor site due to stability of doxorubicin sulphate complex and development of drug resistance. Further, Doxorubicin HCl Liposome is associated with another potential toxicity, Hand-Foot Syndrome (31). Different approaches have been used to improve the liposomal doxorubicin like pH sensitive liposomes, Thermodox, dual drug liposomes etc (21, 33).

### 1.3.3 Conventional vincristine liposome

The cell cycle specific drug vincristine has been widely used in the treatment of haematological cancers and solid tumors. The drug has been widely known for biexponential kinetics of elimination, large $V_d$ (volume of distribution), its rapid elimination profiles with high organ bio-distribution. These PK-PD (pharmacokinetic pharmacodynamic) properties of the drugs limits the usage of the agent as the intravenous delivery of the naïve agents presents reduced exposure of the intended payload to the target site (34). The mitotic drug has been associated with neutropenia, myelosuppression and peripheral neuropathy among others as their side effects (35). As a proposed formulation strategy, the drug was loaded into conventional liposomes but the formulation presented poor drug carrying capabilities owing to the high permeability and leakage potential from the lipid bilayer. Long circulating PEGylated liposomes with ammonium sulphate gradient were formulated to present high circulation time and improved neoplastic accumulation to the drug molecule (36). However, such approach presented with higher leakage of the drug (due to interactions with DSPE) during storage and during transit to tumor cells post intravenous injection resulting in the increase toxicity with reduced efficacy (37). Further, to reduce the leakage of the drug, active loading of the drug was done using sulphobutylether cyclodextrin gradient into PEGylated liposomes. The approach resulted in reduced leakage and delayed release of the agent from the liposome resulting in reduced efficacy (38). The regulatory agency USFDA approved sphingomyelin and cholesterol based nanoliposomal formulation of Vincristine sulphate (Marqibo®) in 2012 for adults' patients with acute lympholastic leukamia (ALL) and Non-hodgkin's lymphoma (NHL). The formulation presented improved dosing, pharmacokinetic limitations, increased circulation time and targeted delivery presented by VCR formulations. The Marqibo kit presented by Talon Therapeutics consisting three vials of buffer, liposomal and vincristine sulphate USP injection while requiring 32-step tedious process for in-situ preparation of liposomes and intended application by the healthcare professionals (39).

### 1.3.4 Doxorubicin - Vincristine drug combination and it's benefits

The drug combination of Doxorubicin hydrochloride (DOX) and Vincristine sulphate (VCR) was selected on the basis that it has been indicated together in low doses for treatment of breast

cancer (40), small cell lung cancer (41), multiple myeloma (42) and non-Hodgkin's lymphoma (43). These drugs act through different cellular pathways acting on multiple targets during different cell cycle phases ensuring a more efficient reduction in tumor cell survival. The anthracycline antibiotic DOX arrests the cell cycle at various stages but majorly at $G_2$/M phase through its activity as DNA intercalator, inhibitor of topoisomerase-II mediated DNA repair and generation of free radicles (44). The vinca alkaloid vincristine has been reported as mitotic inhibitor of the cell cycle due its microtubule depolymerization properties (34). Since both drugs are amphipathic bases, it presented a suitable opportunity for their active co-encapsulation using the modifications of ammonium ion transmembrane gradient (45). Interestingly, although both the drugs have been encapsulated into individual liposomes which have been used clinically in the treatment of various cancers, both the liposomal formulations have never been used together in any treatment regimens. Consequently, in addition to the determination of ideal drug combinations and their synergistic molar ratios, the development of stable co-encapsulated liposomal formulation is absolutely necessary for the achievement of effective simultaneous delivery of the chemotherapeutic nanocarrier based combination therapy (46). The possible mechanism of simultaneous action on the tumor cells is presented in Figure 4.

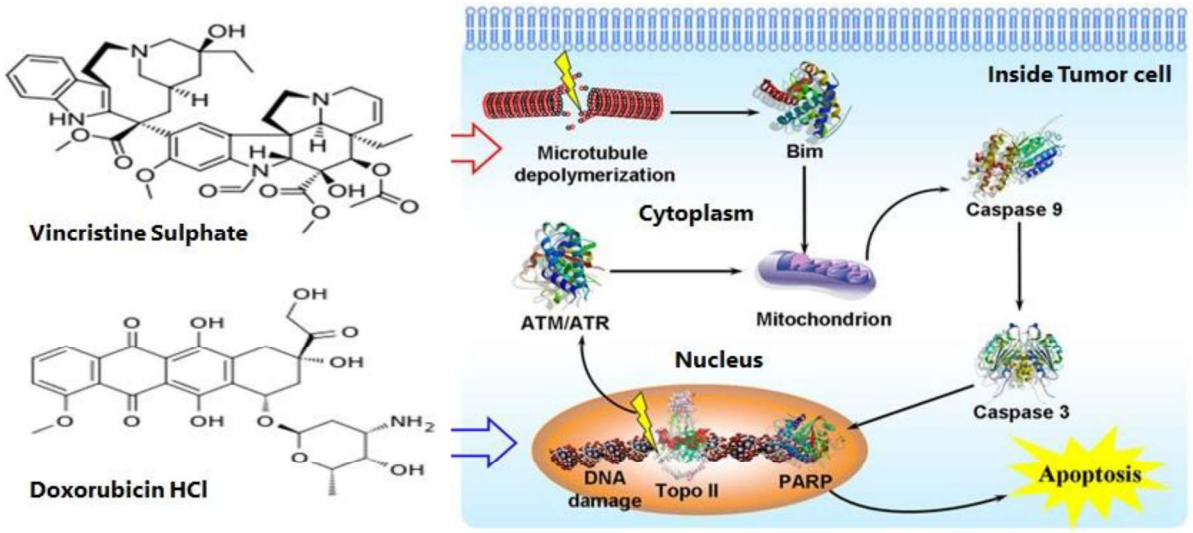


**Figure 4:** Possible mechanism of simultaneous action of Doxorubicin Vincristine combination on the tumor cells

## 1.4 Aims and objectives of study

### 1.4.1 Aims of the study

The proposed study intends to evaluate the efficacy of liposomal co-delivery of vincristine (VCR) and doxorubicin (DOX) through the use of dual drug nanotechnology approach as against use of combination of individual drugs in mixture. Such a combination not only acts on different targets of tumor cells during solid tumor [Triple negative breast cancer (TNBC) and Non-small lung cancer (NSCLC)] treatments but also their combination would alleviate drug resistance to individual drugs besides reducing cardiotoxicity of DOX. Thus, development of doxorubicin- vincristine dual drug liposome may provide a new attractive treatment option with following advantages: -

- Enhanced efficacy
- Improved target selectivity
- Reduced toxicity

### 1.4.2 Objectives of the study

The objectives of the present project to develop stable dual drug loaded liposomes of doxorubicin and vincristine in solid tumor (TNBC and NSCLC) treatment were: -

- In-vitro cytotoxicity and synergy determination of DOX-VCR combination for effective combinatorial strategy in TNBC (MDA-MB 231 cell line) and NSCLC (A549 cell line)
- Design, development, characterization and optimization of stable dual drug liposomes comprising DOX and VCR with optimal synergistic ratio.
- *In-vitro* and *in-vivo* efficacy assessment of doxorubicin-based dual drug liposomes against neat drug and single drug-loaded liposomes against xenograft tumor models of TNBC (MDA-MB 231 tumor model) and NSCLC (A549 tumor model)

### 1.5 Plan of work

The intended work had been subdivided into various targets and the plan of work has been detailed in Table 1.

| SL. No. | Activity |
|---|---|
| 1 | Literature review |
| 2 | Selection and procurement of lipids/excipients & Procurement of drugs |
| 3 | Procurement of cell lines: Determination of synergism between the drugs |
| 4 | Pre-formulation studies and method development of analytical methods |
| 5 | Prototype formulation development and optimization |
| 6 | Characterization of optimized formulation |
| 7 | In-vitro cell line studies |
| 8 | Procurement of animals & In-vivo animal studies |
| 9 | Submission of synopsis to thesis & Submission of thesis |

**Table 1:** Plan of work in present study

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