DUAL DRUG LOADED LIPOSOMES FOR CONTROLLED DELIVERY OF ANTICANCER AGENTS

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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).

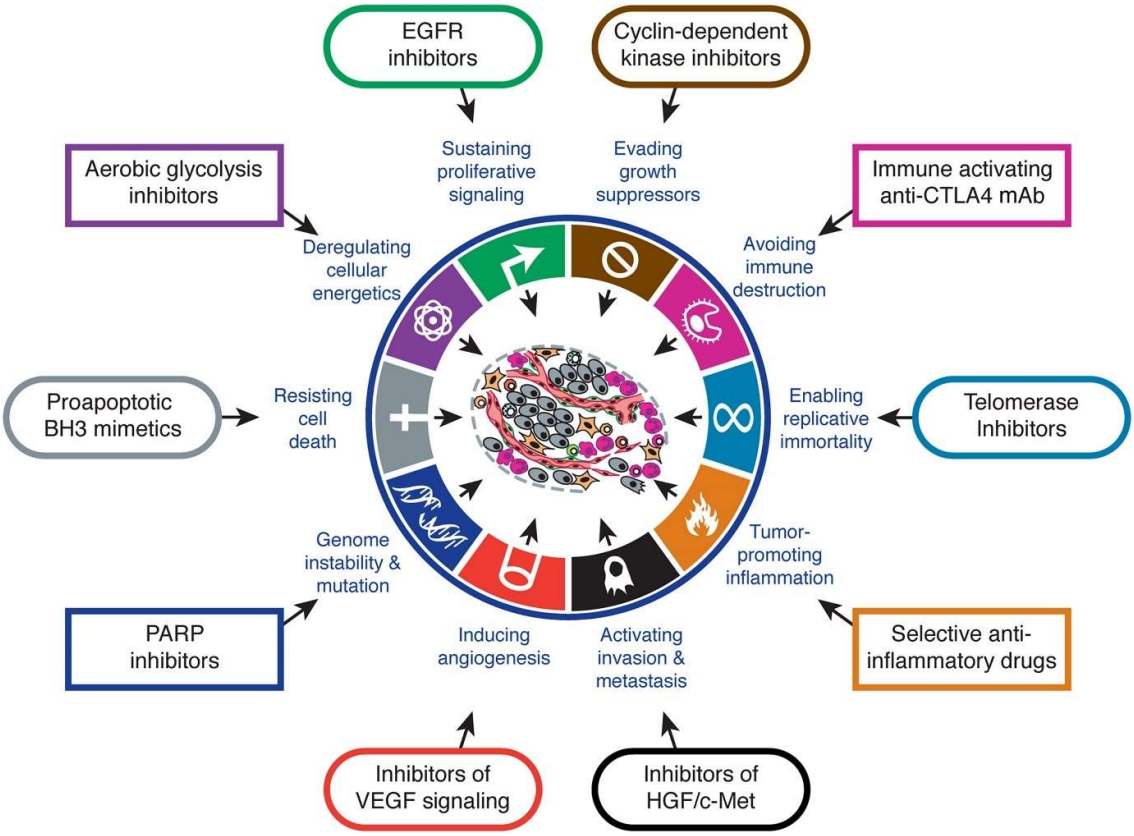

Figure 1: Hallmarks to effective treatment of cancer and possible approaches [adapted with permission from (7)]

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).

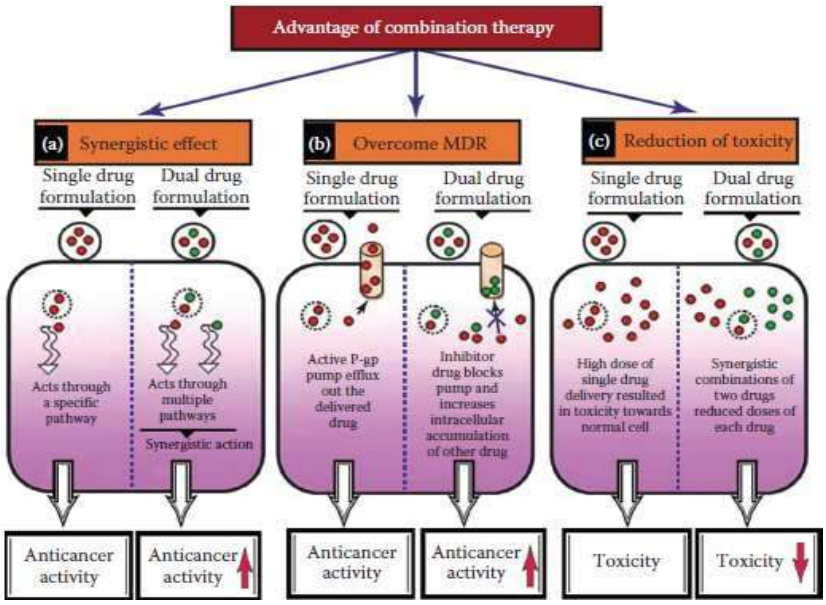

Figure 2: Advantages of combinatorial drug treatment over single drug treatments against cancer [adapted with permission from (19)].

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.

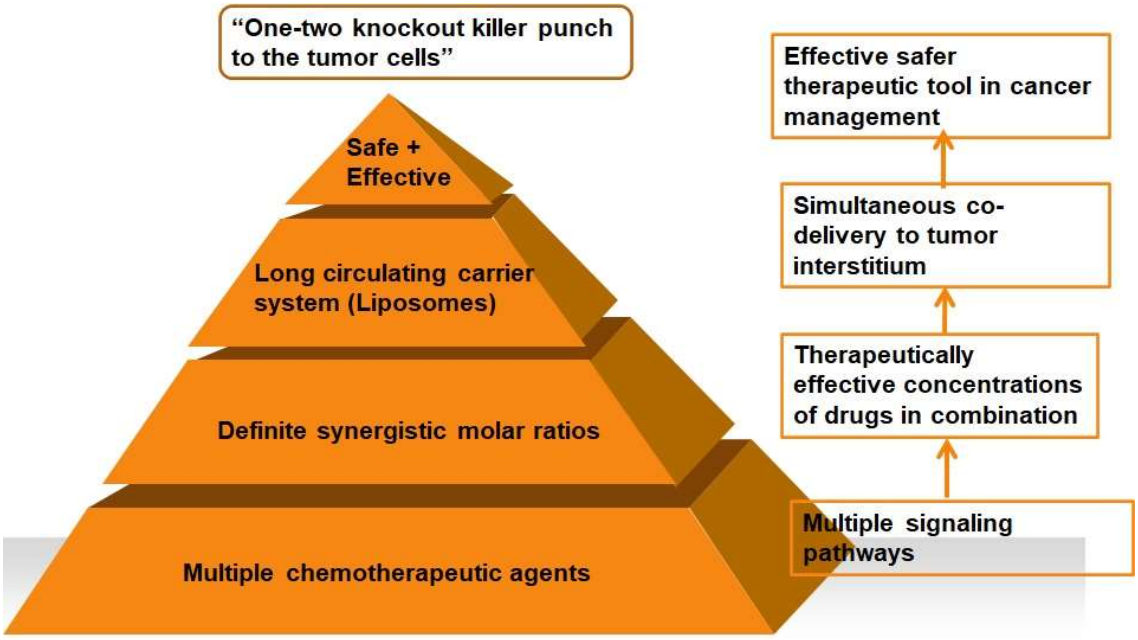

Figure 3: Hypothesis of the present study.

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.

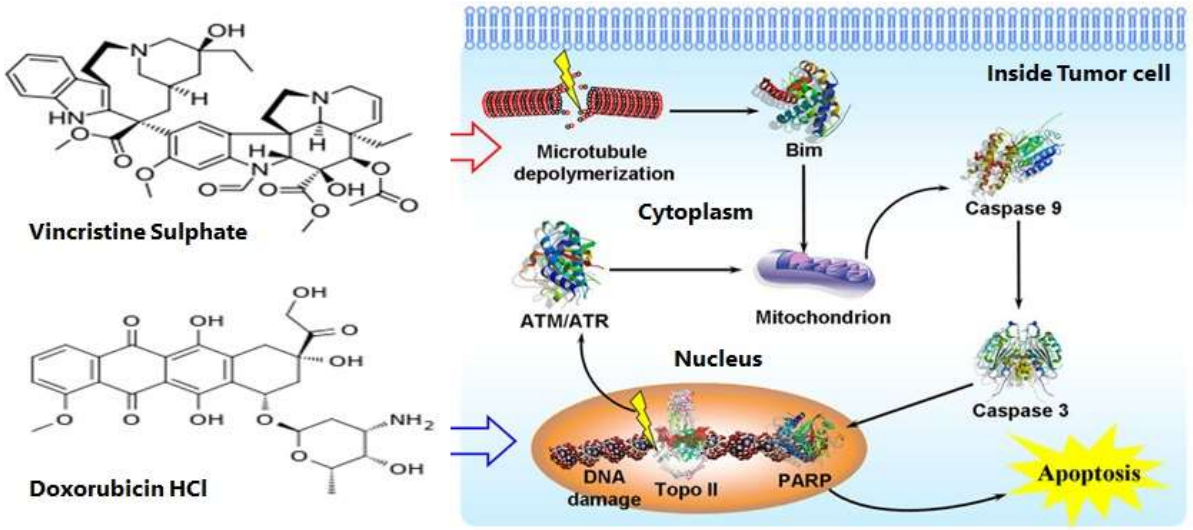

Figure 4: Possible mechanism of simultaneous action of Doxorubicin Vincristine combination on the tumor cells

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

2.0 Research Methodology

2.1 Cell culture and animals

The cell lines MDA MB-231 and A549 were procured from NCCS (National Centre for Cell Science), Pune, India and cultured for cell line studies using methods as previously described (47). Female nude athymic mice (weighing 30±5 g) and Sprague Dawley female rats (weighing 160±20 g) were used for *in-vivo* study. All animals were maintained as per requirements of IAEC (Institutional Animal Ethics Committee) of SPIL (Sun Pharmaceutical Industries Limited) in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines. Polysulfone individually ventilated cages (IVC) were used for housing of the animals with the mice being housed individually and 2-3 rats being housed per cage. The cages were kept under standard light conditions (12hr dark/ 12 hr light cycle) with humidity (50±5%RH) and controlled temperature conditions (20±2°C). The animals were fed Teklad Diet (Envigo) and water ad libitum.

2.2 Materials

VCR and DOX were procured from Minakem (France) and Synbias Pharma (Ukraine) respectively. Fully hydrogenated soy phosphatidylcholine (HSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)2000] (mPEG-DSPE) were obtained from Lipoid GmbH (Switzerland). Cholesterol was procured from Dishman Netherlands BV (Netherlands). All other chemicals used in the study were of analytical grade.

2.3 Combinatorial index and optimal synergistic ratio

The A549 and MDA-MB 231 cells were seeded in sterile 96 well plate (Corning, USA) at the concentration of 5 X 103 cells per well in 200 µl of 10% FBS supplemented DMEM media. These were incubated at 37°C under 5% CO2 atmosphere for 24 hr to facilitate the growth as well as adherence of the cells. Post this period, the medium was removed and the cells were exposed in triplicate to various molar ratios of VCR and DOX (dissolved in DMSO) for 72 hr. The cell viability of the treated cells was then tested as per previously established methods (48)

while being expressed as ratio relative to the untreated control cells (47). The fraction of the affected cells (fa) was determined for each treatment ratio and analyzed for the median-effect analysis for determination of the combinatorial index (CI) using CalcuSyn software (version 2.0, Biosoft, UK) (49). Briefly, based on the combination index theorem (CIT), median effect plot and equations, the CI values for drug combination were calculated using the Chou-Talalay method equation:

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} = \frac{(D)1,2[P/(P+Q)]}{(Dm)1[fa/(1-fa)]1/m1} + \frac{(D)1,2[Q/(P+Q)]}{(Dm)2\,[fa/(1-fa)]1/m2}$$

where, (Dx)1, (Dx)2 were concentrations of drugs when used alone to get x% cellular killing; (D)1, (D)2 were concentrations of the drugs when used in combination to get the same response; m1,m2 are slopes of median effect plot for the drugs; (Dm)1,(Dm)2, (D)1,2 were the median effect dose (IC50) of the drugs when used alone and in combination; P,Q: the ratio of the drugs used (49).

The optimal quantitative degree of synergism for treatment using both the drug combination in both the cell lines was chosen as the synergistic ratio to be encapsulated in the liposomal nano-carrier.

2.4 Preparation of Liposomes

Combinatorial liposomes of DOX and VCR were prepared by active loading of the drugs into optimised liposomal placebo. The optimal synergistic ratio of DOX and VCR was determined using Chou-Talalay method and weight ratio of 2:1 was used for active loading against modified ammonium ion gradient of small unilamellar vesicles (SUV) of size 100±20 nm as per previously described method (10, 32, 49).

2.5 Characterization of nanoliposomes

2.5.1 Quantification of drugs and lipids

The quantification of the drugs in the liposome was evaluated simultaneously using reverse phase high performance liquid chromatography (HPLC, Agilent, Santa Clara, California, USA) as per previously described methods (32). The entrapment efficiency and loading efficiency was calculated using following equations (50) :-

$$\text{Entrapment Efficiency} = \frac{\text{Amount of drug loaded in liposomes}}{\text{Amount of drug taken for liposome preparation}} \text{ X } 100$$

$$\text{Loading Efficiency} = \frac{\text{Amount of drug loaded in liposomes}}{\text{Amount of lipids used in liposomes}} \text{ X } 100$$

2.5.2 Particle size and Zeta Potential

The hydrodynamic diameter, polydispersity index and surface potential were measured using quasi-elastic light scattering (QELS) using Zetasizer Nano ZS (Malvern Instruments, Malvern, Worcester, UK) equipped with DTS software 7.11 as described earlier (50).

2.5.3 Microcalorimetry

The Differential scanning calorimetry measurements for liposomal formulations were done using the high-sensitivity Nano-DSC (TA Instruments, Newcastle, Delaware, USA) using previously described methods (51).

2.5.4 Attenuated Total reflection -Fourier transform infrared spectroscopy (ATR-FTIR)

The DFL, VCR-L, DOX-L and DDL prepared using the optimized composition were evaluated for the ATR-FTIR using previously described methods (51). The experiments were carried out

for liposomal suspensions using FTIR Microscope & Imaging System (Microscope-Cary 620 FTIR, Spectrometer-Cary 670 FTIR, Agilent Technology Limited, USA) with a total of 120 scans each having a spectral resolution of 4 cm^{-1} being collected for each sample. The obtained data was analyzed using Resolutions Pro FTIR Software. The reported spectra were obtained post the baseline correction using blank crystal (51).

2.5.5 Fixed aqueous layer thickness study (FALT)

The thickness of the aqueous layer around the PEGylated liposomes were evaluated using the previously established methods (52). Briefly the effect of addition of varying concentrations of electrolyte sodium chloride (0,10,20,50 mM) to liposomal suspension in sucrose was evaluated by measurement of the zeta potential. The thickness of the fixed aqueous layer was calculated as the slope of the plot of zeta potential and Debye-Huckel parameter. All measurements were performed in triplicate (52).

2.5.6 Electrolyte-induced aggregation (EIA)

The uniformity of PEGylation on surface of the optimized liposomes was evaluated using the EIA method (32). Briefly, the liposomal suspensions were diluted (1:1) with 10% (w/v) sodium chloride solution and 10% (w/v) sucrose solution and incubated at room temperature for 8 hr. The aliquots of diluted liposomal suspensions were withdrawn at 0 hr, 4 hr and 8 hr time points and evaluated for particle size. All measurements were performed in triplicate.

2.5.7 Morphology evaluation

The morphological evaluation of the dual drug liposome was evaluated using the atomic force microscopy (AFM), Field emission scanning electron microscopy (FESEM) and cryogenic Transmission electron microscopy (cryo-TEM) using previously described methods (50, 53, 54).

2.5.8 Interaction with serum proteins, protein adsorption and plasma stability

The interaction potential of liposomal formulations with the serum proteins was evaluated using the blood from female SD rats (55). The albumin adsorption potential on in-vivo administration of optimized nanoliposomes were evaluated by measurement of the *in-vitro* changes in particle size and zeta potential post interaction with albumin protein (56). *In-vitro* plasma stability of dual drug liposome was evaluated over 24 hours after dilution with 50% plasma with measurement of entrapped drug and particle size using previously described methods (57, 58).

2.5.9 Hemolysis Study

Hemolysis potential of the optimized formulations was evaluated by performing the toxicity study on erythrocytes as reported earlier (59). Briefly, blood from the anesthetized SD rats (20 ml from 10 rats) were collected through retro-orbital plexus puncture in EDTA containing centrifuge tubes, centrifuged at 3000 rpm for 5 minutes at 5°C and the resultant pellet erythrocytes) was resuspended to achieve 2% suspension in 0.9% saline. The positive control (considered as 100% lysed) and negative control values were determined by measurement of the absorbance of the hemolyzed RBC cells (using 0.5% Triton X solution) and non-lysed RBC in saline respectively at 540 nm. The drug solutions and liposomes were diluted to various concentrations (5000, 500, 50 and 5 µg/ml) with normal saline and incubation with the RBC suspension for 1 hr at 37±2˚C in an incubator. Post incubation, the samples were analyzed for the absorbance of the centrifuged supernatant. These samples were then observed under the inverted optical microscope (Nikon Eclipse TS100 with NIS elements imaging software) for the morphological evaluation after1 hr of incubation.

2.5.10 Small Angle X-ray scattering (SAXS)

The SAXS intensity profiles of the optimized formulations (DFL,DOX-L,VCR-L, DDL) were obtained using SAXSpace (AntonPaar, Austria) according to previously described methods (60). The instrument was run using 2.2 kW Sealed tube (line collimation) of X-ray source

operating at 40 kV and 50 mA with wavelength of 1.5418 Å. The intensity profiles were collected at 10°C under vacuum for 5 minutes in quartz capillary in triplicate. The collected SAXS data was processed and analyzed using PRIMUST software of ATSAS 2.7.2 suite of program available at EMBL-Hamburg website (61). The analyzed plots included: Double log plot; Kratky plot; Guinier approximation; pair distance distribution and rigid body modelling.

2.5.11 In-vitro drug release and kinetics

The release of the drugs from optimized liposomes were evaluated at 37°±0.5 °C in comparison with lipid free drug solutions. Briefly, liposomal suspensions and drug solutions (equivalent to 2 mg/ml DOX and 1 mg/ml VCR) were placed in 5 ml Float-A-Lyzer G2 (MWCO: 10kD, Spectrum USA) while being immersed in 250 ml of the release medium phosphate buffered saline pH 7.4, pH 6.4 and acetate buffer pH 5.5 (47).

Additionally, the in-vitro stability in presence of biological fluids was assessed by drug release measurement in presence of bovine serum albumin and 50% human plasma as previously described (58). All the release study experiments were conducted in triplicate and the results were reported as cumulative amount of the drug released at the individual time points. The drug release kinetics was evaluated using model fitting with zero-order kinetics, first-order kinetics, Higuchi model, Korsmeyer–Peppas model, and Hixson–Crowell model being analyzed while regression coefficients (R^2) being determined for model suitability (54).

2.5.12 Liposome Membrane Integrity

The intactness of the liposomal membrane to retain the drugs on storage was evaluated using highly hydrophilic dye 5,6-carboxyfluorescein (CF) in presence of fetal bovine serum (FBS) as previously described (47, 62).

2.5.13 Stability Study

The stability studies of DDL suspension was evaluated on exposure to storage conditions of 2-8°C for 12 months and at 25°C/60% RH for 1 month. The encapsulated drug content, particle

size and the zeta potential were evaluated on stability. All measurements were performed in triplicate.

2.6 In-vitro cell line studies

2.6.1 Cellular uptake study by confocal microscopy and flow cytometry

Liposomal formulations were analysed for the cellular uptake in A549 and MDA-MB 231 cells by confocal microscopy (seeding concentration of 5 X 10^4 cells/well) and by flow cytometer (4 X 10^5cells/well seeding) using previously reported methods (63, 64).

2.6.2 Cell viability assay

Cellular viability of MDA-MB 231 and A549 (cell seeding at 5 X 10^3 cells/well) in presence of various treatment conditions was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay upto 72 hours as previously described (47). The IC_{50} (inhibitory concentration) were obtained from the cellular viability vs concentration plots with the optical density of untreated cells taken as 100%.

2.6.3 Wound Scratch Study

Potential of cellular migration of MDA-MB 231 and A549 cells in presence of the drug treatments were evaluated using the wound healing study as described earlier (47).

2.6.4 Cell cycle analysis

The MDA-MB 231 and A549 cells were seeded at a concentration of 0.8 million/ well and incubated with formulations to assess their effect on cell cycle over 24 hours as described earlier (65).

2.6.5 Annexin V assay

The free drug solution and liposomal formulations were evaluated for the apoptosis induction in MDA-MB 231 and A549 cells (0.8 million cells/well) using Annexin V cellular binding assays as previously described (63).

2.7 In-vivo studies

2.7.1 Acute toxicity study

The maximum tolerated dose (MTD) of the liposomal formulations was analysed by conducting acute toxicity study in healthy female nude athymic mice (n= 6/group) using previously described method (66). Liposomal DOX (clinically approved formulation) has been previously reported to have MTD of 6 mg/Kg and 9 mg/Kg (67, 68).

2.7.2 Tumor regression

The comparative efficacy of formulations was evaluated using tumor regression studies in xenograft model of A549 and MDA-MB 231 in female nude athymic mice (n= 6/group) following intravenously administered q7d*4 dosage regimen. The disease progression as monitored over 35 days with tumor volume, body weight and mortality being evaluated (66). The comparative efficiency of the various treatments were evaluated statistically using analysis of variance (ANOVA) and percentile ratio of test with control (%T/C) (69).

2.7.3 Pharmacokinetics

Comparative pharmacokinetic profile of the formulations (drug solutions, single drug and dual drug liposomes) were evaluated in female Sprague-Dawley rats (n= 6/group) at dose equivalent to 4 mg/Kg of liposomal DOX and 2 mg/Kg of liposomal VCR (considering the synergistic ratio) as per method described earlier (68).

2.7.4 Tissue Distribution studies

The comparative tissue distribution studies (liver, spleen, kidney, heart, lung, tumor, plasma) of formulations were carried out in female athymic nude mice (n= 6/group) bearing xenograft

MDA-MB 231 model with dose equivalent to 6 mg/kg liposomal DOX as previously described (68, 69).

2.8 Statistics

All experiments were performed in triplicate with results being presented as values in mean ± SD (standard deviation) unless mentioned otherwise. Statistical significance of the data ($p<0.05$) was determined using ANOVA and Student's t-test using GraphPad Prism 6.0 (San Diego, California, USA).

3.0 Key findings

The development of a stable liposomal formulation co-encapsulated with synergistic ratio of doxorubicin and vincristine for ensuring their temporospatial presence at the tumour site of the two solid tumors (triple negative breast cancer and non-small cell lung cancer) was intended (70-72). The synergistic ratio against the two cancers was established using in-vitro cytotoxicity studies by treatments of drug combinations (10:1 to 1: 10 weight ratios of DOX:VCR) in both the cell lines A549 and MDA MB231 using Chou-Talalay method. The best effective synergistic ratio against both carcinomas having the lowest cellular viability was determined. The most optimum drug ratio exhibiting highest degree of synergism in both the cell lines was found to be 1:2 w/w Vincristine sulphate: Doxorubicin hydrochloride (with Combinatorial Index = 0.26 for A549 cell line and 0.42 for MDA-MB-231 cell line).

Post determination of the optimum combinatorial index, the most effective ratio of the drugs was encapsulated in a single nanoliposome. The dual drug loaded nanoliposome was optimised using an array of OFAT studies to determine the factors responsible optimal CQA for encapsulation efficiency of both drugs, particle size, zeta potential and drug release. The independent variables evaluated for the active co-loading of the drugs included transmembrane salt gradient, concentration of cholesterol (lipid molar ratio), drug loading temperature, phosphatidylcholine chain length, sequence of addition of drugs, pH of drug loading, external medium, drug to lipid molar ratio and concentration of Ammonium sulphate. Three dependent variables- concentration of ammonium sulphate, pH of drug loading and lipid molar ratio was found to have significant effect on the tested parameters. These parameters were then evaluated using 2^3 full factorial design to understand the effect of the variation of the factors individually and together on the tested CQAs. The significance of combination of three individual causal factors and corresponding interactions on the dependent outcomes were explored using regression analysis and ANOVA. The results of the DOE studies and regression equation indices indicate that 350 mM ammonium sulphate, drug loading at pH 5.5 and lipid ratio of

56.55:38.19:5.26 (HSPC: Cholesterol: mPEG-2k-DSPE) were found optimal for suitable particle size, zeta potential and entrapment efficiency of both drugs.

The DOE based optimised dual drug liposomes was then physico-chemically characterized for the assessment of the stability of the carrier while drug release kinetics was evaluated simulating the blood and tumour conditions in comparison with the single drug liposomes (63, 73, 74). The optimized co-loaded liposomal formulation exhibited more than 95% encapsulation of both drugs with particle size of 95.74±2.65 nm and zeta potential of -9.17±3.1 mV. The morphological evaluation using cryo-TEM showed the formation of unilamellar, spherical structures with presence of characteristic gel strands inside the liposomes. The co-loaded liposome presented no significant difference in the average size, bilayer thickness and strand size as compared to the liposomal doxorubicin indicating the absence of the morphological changes in response to co-loading of vincristine. Morphological evaluation of the liposomal formulation done using AFM and FESEM indicated the presence of spherical external surface with liposomes having a hydrodynamic diameter of 100 nm.

The ATR-FTIR and microcalorimetric characterization of the formulation showed the lack of any physicochemical interactions between the active agents and excipients. Further, these studies indicated the presence of the drugs within the aqueous core of the liposomes while exhibiting the characteristic thermal melting of the formed doxorubicin sulphate crystals present in the aqueous compartment at 68.75±1.07°C in doxorubicin containing formulations. The in-vitro characteristics and stability of the formulation was further evaluated using studies of fixed aqueous layer thickness (FALT), electrolyte-induced flocculation/aggregation, interaction with Serum proteins and protein adsorption, plasma stability and liposome membrane integrity. These tested parameters indicated the presence of sufficient in-vitro stability at the storage conditions, stability and lack of interaction potential with blood components. These results of morphology, interaction potential, localization of the drugs, the absence of aggregation and other characteristic properties of the dual drug liposomes were confirmed by small angle X-ray analysis (SAXS) evaluation of the formulation.

The drug release from the combinatorial carrier was tested at various pH (7.4, 6.4, 5.5) and biological fluids (in presence of plasma and human serum albumin) representing the physiological conditions the drug may encounter during the transit from the injection site to tumor. The cumulative drug release profiles at these tested conditions showed controlled release of the individual agents in a manner similar to that of the single liposomes but significantly different from the naked drugs. The doxorubicin release from the co-loaded liposomes indicated time-dependent fickinian diffusion-controlled (Higuchi model) along with erosion and diffusion controlled (Korsmeyer-Peppas) release profiles. The vincristine release from the liposomal formulations was found to be biphasic in nature with initial burst release (first order kinetics) followed by slow release of the drug through the lipid bilayer (Higuchi and the Korsmeyer-Peppas models).

Additionally, the newly formulated liposomal suspension was predicted to present 18M stability similar to approved product besides presenting with ease in scalability for manufacturing. Thus, the optimized liposomal formulation presented non-significant difference in physicochemical and biochemical characteristics and stability to the clinically used standard, pegylated liposomal doxorubicin.

Next, the optimized formulation was tested for determination of the in-vitro and in-vivo biological characteristics of ratio-mimetic VCR co-loading into the clinically used pegylated liposomal DOX. In-vitro cell line studies (cellular uptake studies using confocal microscopy and flowcytometry, cell viability using MTT assay, cell cycle analysis using FACS, Apoptosis study by Annexin V assay and wound scratch study) were done in MDA-MB 231 as well as A549 cell lines. The cellular uptake studies exhibited significantly increased uptake of dual drug formulation when compared to the liposomal doxorubicin (as well as all other formulations). The in-vitro cell viability studies of the co-loaded formulation showed significantly improved cytotoxicity potential of the drugs when co-encapsulated in a single carrier as compared to neat drugs, individual liposomal carriers and combination of individual liposomal components. The enhanced cytotoxicity potential of the optimized formulation was explained by the increased cellular uptake which resulted in significantly increased cell cycle

arrest in G_2/M phase. Further, higher presentation of the cells in the G_0 phase resulted in the significantly improved apoptotic potential which were in good correlation with the reduced cell viability in both tumor cell lines when presented with co-loaded formulation than with the single drug liposome. The dual liposomes exhibited the highest inhibition of the cellular recovery (in wound scratch study) which indicated significant reduction in cellular viability as well as presenting improved chances of in-vivo anti-angiogenic properties as compared to liposomal DOX.

The optimised liposomal formulation was further tested in-vivo for their acute toxicity, efficacy, pharmacokinetic and biodistribution profiles. While the dose response studies were performed in the tumor free nude athymic mice, the efficacy studies were done in tumor induced xenograft models of MDA-MB 231 as well as A549 in nude athymic mice. The comparative pharmacokinetic (in disease free animals) and biodistribution (in disease induced animals) profiles were established using Sprague Dawley rats. The pharmacokinetic profile analysis was done using the model-independent non-compartmental method (NCA). The bio-distribution studies were done by collecting samples at pre-determined time points from tissues (Plasma, Spleen, Liver, Kidney, lungs, heart, tumor). The new liposomal carrier exhibited similar acute toxicity, pharmacokinetic and tissue distribution profiles with significant increase in tumor regression as compared to currently used liposomal doxorubicin.

4.0 Conclusion

Cancer as a diversified group of diseases is characterized by abnormal proliferation of cells leading to heterogeneity among the affected cells. Current chemotherapeutic treatment options entailing the usage of conventional free drug cocktail therapy targeting multiple signalling pathways have proved to be less effective due to lack of spatial and temporal simultaneous presence at the tumour site. Nanoparticulate ratio-mimetic combinatorial delivery of such agents may offer an efficient cancer cell load reduction.

The present study was aimed to investigate improvement in efficacy of clinically used PEGylated liposomal doxorubicin in NSCLC and TNBC by co-encapsulation of synergistic ratio of two drugs (doxorubicin and vincristine) in an optimized liposomal formulation. The study was divided into two parts: development of optimized dual loaded liposomal formulation; in-vitro as well as in-vivo evaluation of the biological characteristics of the co-loaded formulation.

The results indicated significantly improved efficacy in the in-vitro and in-vivo therapeutic efficacy upon VCR incorporation into currently available therapeutic standard against NSCLC and TNBC. These studies indicate towards extension of therapeutic potential in NSCLC and TNBC of clinically used standard post VCR incorporation and rationale for continued investigation of therapeutic potential of such combinatorial formulation. Thus, ratio-mimetic co-encapsulation of the drugs in combinatorial dual drug loaded liposomal formulation may help in improving their spatial co-presence at the site of action in tumours as compared to the single liposomes of the agents and neat drugs leading to better therapeutic outcomes in both these solid tumors.

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