COMBINATIONAL NANOFORMULATIONS FOR CIRCUMVENTION OF CHEMO-RESISTANCE IN CANCER

By

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Tapan Kumar Dash

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Tapan Kumar Dash

Bhubaneswar

Date:

List of Publications

Journals

1. Selection and optimization of nano-formulation of P-glycoprotein inhibitor for reversal of doxorubicin resistance in COLO205 cells. J of Pharmacy and Pharmacology. **2017**, Tapan K. Dash and V Badireenath Konkimalla. (Article in press)
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3. Formulation and Optimization of Doxorubicin and Biochanin A Combinational Liposomes for Reversal of Chemo-resistance. Tapan K. Dash and V Badireenath Konkimalla. AAPS PharmSciTech. **2016,** 1-9. (Article in press)
4. Nano-formulations for Delivery of Bio-Molecules: Focus on Liposomal Variants for siRNA Delivery. Tapan K Dash and V Badireenath Konkimalla. Critical Reviews™ in Therapeutic Drug Carrier Systems, **2013**, 30 (6), 469-493.
5. Polymeric Modification and its Implication in Drug Delivery: Poly-ε-caprolactone (PCL) as a Model Polymer. Tapan K. Dash and V Badireenath Konkimalla. Molecular Pharmaceutics **2012**, 9 (9), 2365-2379.
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Others

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2. Reaction between lawsone and aminophenol derivatives: Synthesis, characterization, molecular structures and anti-proliferative activity. L Kathawate, PV Joshi, TK Dash, S Pal et al. Journal of Molecular Structure **2014**, 1075, 397-405. *

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Tapan Kumar Dash

Dedicated to my father, mother, family and thesis supervisor

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Table of Contents

Synopsis

1. Introduction

Cancer chemotherapy was started 70 years ago, when sulfur compounds from nitrogen mustard was used for non–Hodgkin's lymphoma during World War II. From then different categories of anti-neoplastic agents were being developed belonging to several categories such as alkylating agents, anti-metabolites, microtubule synthesis modulators, antibiotics, steroids etc. Although the drugs were effective, there were several problems like toxicity, non-specificity, relapse etc. associated with them. Thus combination therapy and adjuvant based therapy was adopted in 1960s. In sequential developments of combination chemotherapy drug resistance evolved as a major problem. (1) Up on mechanistic insight into drug resistance it can be found out that, tumor often exists as heterogeneous mass with improper differentiation hierarchy and divisional plasticity. Chemotherapy treatment results in killing of significant portion of tumor but in many cases leaves behind resistant cells. The further growth of resistant cells leads to relapsing cancer and failure of chemotherapy. (2, 3) From research over past few decades, various resistance mechanisms such as altered drug absorption, uptake, metabolism or efflux were postulated. (4, 5) In several cancers, drug efflux involving ABC transporters (MDR, MRP, MXR etc) were demonstrated to be the major mechanism for conferring reduced efficacy by expelling cyto-toxic drugs out of cell. Out of these several transporters of this family, ABCB1 or P-glycoprotein (P-gp) have indelible prognostic significance in experimental as well as clinical level. (5, 6)

In order to overcome acquired drug resistance, the most commonly adopted approach is to use combinational therapeutics i.e. an anticancer agent with a pharmacokinetic modulator. Here in P-gp inhibitors as a pharmacokinetic modulator has gained focus. Among them, natural sourced P-gp inhibitors belonging to various categories such as flavonoids, isoflavonoids,

terpenoids, alkaloids, steroidal saponins etc. have been trialed widely as pharmacokinetic modulators for reversal of drug resistance. (7-9) Although much emphasis has been given to natural products owing to their abundant availability and non-toxic nature; nevertheless, most natural products are associated with problem of poor solubility and bioavailability. Improvement in the solubility and pharmacokinetic profile of natural p-gp inhibitors by formulating them in to a nano-formulation such as liposomes, nano-particles etc. were reported. (10, 11) Furthermore, studies involving lipid or polymer based nano-formulations over past decades, reported to circumvent drug resistance in several ways. Amongst the possible mechanisms, prime one is cellular internalization through endocytosis which bypasses efflux mediated resistance. (12) Secondly drug incorporation in to nano-formulations improves its pharmacokinetic parameters due to controlled release and improved stability in vivo. Again systemic distribution pattern aided with enhanced permeation and retention (EPR) effect of nano-formulations enhance tumor specific accumulation modulated by their size and functionality. (13, 14) Importantly recent advancement of delivery systems involved modification of polymers or lipids for enriching them with smart delivery or cellular efflux pump inhibitory activity. (15, 16) Studies also suggest reversal of drug resistance by polymers from natural as well as synthetic sources such as alginates, gums, PEG based copolymers, Pluronics etc. by interfering functionality of P-gp. (11, 17-19) Therefore, combination of P-gp inhibitor with a chemotherapeutic agent in nano-formulations will improve physicochemical properties of natural products and have advantages of nano-formulation such as EPR affect, endocytic uptake etc. Among these formulations, nano-particles and liposomes have been experimented widely with significant number of successful outcomes. Thus a formulation containing chemotherapeutic agent with P-gp inhibitor will

possess pharmacokinetic interaction among these agents and distributional as well as retention advantage of nano-formulations.

2. Aim and objective

In the perspective of using combinational nano-formulations for drug resistance, Hu et al recently presented their implication and possibilities.(13) Polymers and lipids used in the preparation of nano-formulation have been modified in different way to render those suitable for various treatment conditions and to enrich them with multimodality.(15, 20) In my PhD work, nano-formulations containing combination of drugs will be evaluated for reversal of doxorubicin (DOX) resistance in different cell lines. For fulfilling above aim following objective were framed.

i. Development of DOX resistance cell line as a model system and analysis of growth pattern.
ii. Validation of reversal of developed resistance by standard P-gp inhibitor.
iii. Screening of P-gp inhibitors from natural sources for P-gp inhibitory activity and reversal of DOX resistance in developed resistance cell lines.
iv. Optimization of nano-formulation of the P-gp inhibitor along with DOX in terms of size, zeta potential and encapsulation efficiency.
v. In vitro evaluation of combinational nano-formulation for reversal of DOX resistance in developed resistance cell lines.

3. Materials and methods

Resistant leukemic and colon cancer models were been developed and used to study reversal of resistance by P-gp inhibitors from natural sources. For my study K562 (K562N) and COLO205 (ColoN) cells were treated with gradually increasing concentration of DOX varying from 10 nM-

500 nM and 10 nM-1 µM to produce resistant counter parts K562R and ColoR respectively over a period of 6-8 months. The resistant cells were then maintained DOX free media with intermittent DOX stress. Resistance developed was analyzed by XTT reduction based cell viability assay. To validate reversal of acquired resistance by standard P-gp inhibitor, verapamil was co-treated along with DOX and XTT assay was performed. Following the successful validation, P-gp inhibitors from natural sources such as biochanin A (BioA), curcumin (Cur), daidzein (Daid), dihydrofisetin (DHF), genistein (Gen), resveratrol (Resv), and silymarin (Sily) were evaluated for their P-gp inhibitory activity and efficacy to reverse drug resistance. Their P-gp inhibitory activity was accessed by calcein-AM uptake measurement and reversal of DOX resistance by co-treatment was evaluated by XTT assay. Accumulation calcein (a fluorescence substrate of P-gp) in resistant cells was measured after pretreatment with P-gp inhibitors. Calcein accumulation was considered as an inverse measure of P-gp activity. From these experiments suitable P-gp inhibitor was selected to combine with DOX, in both K562 and COLO205 cell lines. From the screening BioA and Cur for COLO205 and Cur for K562 cell lines were selected for reversal of DOX resistance.

Prior to formulate them in to a nano-formulation along with DOX, the physicochemical properties of both the compounds must be considered. For improvement of lower aqueous solubility of Cur, HP-β-CD encapsulated Cur (CurN), liposomal Cur (CurL), micellar Cur (CurM) and DMSO assisted nano-dispersed Cur (CurD) were prepared and evaluated for their P-gp inhibitory and reversal of drug resistance effects. For CurN, the reported processes yielded very low encapsulation efficiency ranging from 5-20%. After considering possible reasons, we proposed presence of stabilizer would increase the encapsulation by restricting the inter-molecular interaction of Cur molecules. For the above purpose we optimized different stabilizer

and organic solvents. In other case, micelles of methoxy-polyethyleneglycol-polycaprolactone (MPEG-PCL) encapsulating Cur was prepared by emulsification solvent diffusion method and liposomal Cur by thin film rehydration technique. In another approach for BioA, effect of pH on solubility was analyzed. Further HPLC method for detection and quantification of BioA and DOX was developed and validated using acetonitrile -0.1% orthophosphoric acid as mobile phase. Method where in solvent B (acetonitrile) concentration: 0.1-2.6 min; 80%, 2.6-3.0 min; 80-70%, 3.0-6 min; 70%,6.0-9.0 min; 70-80%, 9.0-10.0 min; 80%, was used. Quantification of BioA and DOX was done from correlation function of 5-point calibration curve.

We prepared liposomal DOX co-encapsulated with either BioA or Cur. Liposomes were prepared by thin film rehydration technique where in, all the lipids along with BioA or Cur was deposited as a layer of round bottom flask by using rotary evaporator. The film formed was rehydrated by addition of 250 mM ammonium sulfate and dialyzed in 150 mM sodium chloride in a dialysis bag of MWCO 14 KD. Later up on creation of sulfate gradient DOX was actively loaded by ammonium sulfate gradient produced in the above process. These prepared formulations were evaluated for their efficacy to reverse drug resistance in both the model systems developed as presented below.

K562		COLO205	
Curcumin and DOX combinational liposomes	DMSO nano-dispersed/ nano/micellar/liposomal curcumin and DOX	Biochanin A and DOX combinational liposomes	Nano-curcumin and liposomal DOX

4. Results and discussion

a) Studies performed with K562 cells

Resistance development was validated by XTT assay. From the growth pattern studies it was observed that resistance cells grow slowly in comparison to non-resistant cells. When P-gp activity was analyzed by calcein uptake assay, it was noted that in comparison to non-resistant cells, resistant counterpart accumulated only 40% of calecin in K562R cells. Standard P-gp inhibitor verapamil reversed DOX resistance dose dependently. Then above enlisted P-gp inhibitors were screened for their P-gp inhibitory activity as well as efficacy to reverse drug resistance. From the results it was inferred that among screened P-gp inhibitors, BioA and Cur were able to inhibit P-gp activity comparable to that of verapamil in K562R cell and had corresponding DOX resistance reversal effect. However, calcein accumulation by Cur was similar to that of standard P-gp inhibitor verapamil at comparatively lower dose (20 µM) to that of BioA (40 µM). Thus Cur was considered for K562R to develop combinational nano-formulation along with DOX.

Co-loaded liposomes were planned to prepare where both the agent remain in the liposomal core. For this, process of Cur solubilization by formulating it into different nano-formulations was adopted. The solubilized Cur then planned for incorporation into the liposomal core by rehydration of lipid layer with solubilized Cur containing 250 mM ammonium sulfate. Here DOX could be actively loaded in to core by sulfate gradient. Thus different nano-formulation of Cur such as DMSO assisted Cur nano-dispersion (CurD), nano-Cur (CurN), liposomal Cur (CurL), micellar Cur (CurM), were prepared and evaluated for cyto-toxic, P-gp inhibitory and DOX resistance reversal effects. To solubilize Cur by encapsulation in to HP- β-CD by emulsification solvent evaporation process was adopted and where in lower loading ration

(1-5%) of Cur to the carrier observed. This observation was supported by various literatures. We proposed presence of stabilizer during process of emulsification, and selective evaporation of organic solvent will increase the loading. For this purpose, we encapsulated Cur using different stabilizers and organic solvents. Here in acetone dispersed emulsion with polyvinyl alcohol stabilized system produced most optimal nano-Cur with size ranging about 40 nm that had loading efficiency of about 60%. Other nano-formulations prepared were having encapsulation efficiencies of 85% and 100% for CurL and CurM respectively. When cyto-toxicity activity was studied long with other formulations, CurL and CurM had lower cyto-toxicity attributed to their lower cellular uptake. Irrespective of uptake, CurM had highly compromised P-gp inhibition and reversal of DOX resistance. Among CurL and CurN, CurN had superior internalization as well as biologic activity but may need another carrier for in vivo delivery. For CurL, although it decreased the P-gp inhibitory activity moderately, but can provide prolonged release and in vivo stability. Thus for reversal of drug resistance and a carrier of Cur, CurL and CurN were found to be more applicable.

However the encapsulation of CurN in to liposomal core was very low after development of sulfate gradient by dialysis. Thus to study reversal of DOX resistance in K562 cells by combinational nano-formulation, Cur was loaded into the liposomal bilayer and DOX was actively loaded in to the core of liposome by sulfate gradient. This co-loaded liposomes prepared internalized in to K562R cells and were able to deliver almost comparable amount of Cur to that of nano-dispersed Cur. Liposomes released Cur gradually for a longer duration period of time and retained the P-gp inhibitory as well as re-sensitization potential to DOX in K562R cells.

b) Studies performed with COLO205 cells

Resistance cell lines (ColoR) developed showed significant sensitivity difference in XTT assay and growth pattern was observed to be slower. P-gp activity was analyzed by calcein uptake assay indicated only 20% of calcein accumulation in ColoR cells in comparison to ColoN cells. ColoR cells were able to be re-sensitized to DOX by verapamil in a dose dependent manner. From the screening studies it was inferred that non-of the selected agents led to increased calcein accumulation up on pre-treatment in ColoR cells. However, BioA and Cur were able to reverse DOX resistance exerting an additive effect at lowest dose of about 40 µM. Thus BioA and Cur was considered for ColoR to develop combinational nano-formulation along with DOX.

When formulating BioA in to nano-formulation was being considered, so far there is no report on liposomal co-formulation of BioA and DOX. Thus we performed solubility studies and it was inferred that BioA is aqueous soluble at basic pH. Furthermore, BioA and DOX have closer ƛmax of 262 nm and 232 nm respectively. Thus, to avoid any interference a binary gradient system in HPLC was developed to separate detect and quantify BioA and DOX simultaneously. For liposomal preparation, the lipid composition was optimized to find optimal cholesterol concentration and surface charge for BioA encapsulated liposomes. The cholesterol: egg phosphatidyl glycerol: Soy phosphatidylcholine ration 40:40:20 was fixed to be optimal. The liposomal BioA was loaded with DOX by sulfate mediated active loading. After DOX loading, prepared DOX and BioA containing liposomes were of size 125 nm and having zeta potential -19.5 mV with BioA EE about 70%. The co-loaded liposomes were able to reverse drug resistance additively in a similar manner to that of dissolved BioA, and thus maintained its pharmacological activity. Similarly, CurN and liposomal DOX in combination were studied for

reversal of DOX resistance in ColoR cells. Here in, activity of curcumin was as par to that of nano dispersed curcumin.

5. Conclusion

Acquired drug resistance is one of the prime causes of relapse of cancer and failure of chemotherapy. Out of several postulated mechanisms of drug resistance, over-expression of efflux pump (P-glycoprotein) considered to be the prime one with significant prognostic relevance. Thus P-gp inhibitors, especially from natural sources have been studied widely for reversal of acquired resistance. This pharmacological approach along with pharmaceutical approach for reversal of drug resistance by nano-formulations has been combined here. Nano-formulations combining P-gp inhibitor along with chemotherapeutic agent (DOX) was studied for circumvention of acquired DOX resistance in COLO205 and K562 cell lines. From a comparative study of different nano-formulation of Cur, it was inferred that CurN (nano-Cur) as most suitable nano-formulation for reversal of DOX resistance in K562 cells. BioA and Cur were combined with DOX in a combinational liposome those were reversed DOX resistance in both the cell lines.

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List of abbreviations

µ	Micro
µl	Microlitre
µM	Micromolar
ABC	ATP binding cassette
CAN	Acetonitrile
hTR	Human telomerase RNA
AXL	A tyrosine-protein kinase receptor
BCL-2	B-cell lymphoma 2
BCRP	Breast cancer resistant protein
BioA	Biochanin A
Calcein-AM	Calcein acetoxymethylester
Chol	Cholesterol
CMC	Carboxy methylcellulose
Conc	Concentration
COLO205	Colorectal adenocarcinoma cell line
ColoN	COLO205 non-resistant
ColoR	COLO205 resistant
Cur	Curcumin
Daid	Daidzein
DAPI	4',6-diamidino-2-phenylindole
DCM	Dichloromethane
DHF	Dihydrofisetin
DMSO	Dimethyl sulphoxide
DLS	Dynamic light scattering
DN-hTERT	Dominant negative-hTERT
DOPE	1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine
DOX	Doxorubicin
DSPE-PEG	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(PEG))]

EE	Encapsulation efficiency
EMT	Epithelial mesenchymal transition
EPG	Egg phosphatidyl glycerol
FACS	Fluorescence- assisted cell sorting
FBS	Fetal bovine serum
FL-1	Fluorescence channel-1
Gen	Genistein
GST	Glutathione-S-transferase
h	Hour
HP-β-CD	Hydroxypropyl-β-cyclodextrin
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
HSPC	Hydrogenated soy phosphatidyl choline
hTERT	Human telomerase reverse transcriptase
IC_{50}	50% Inhibitory concentration
K562	Chronic myelogenous leukemia cell line
K562N	K562 non-resistance
K562R	K562 resistance
L	Litre
MET	Mesenchymal-epithelial transition
MFI	Mean fluorescence intensity
min	Minute
mL	Millilitre
mM	Millimolar
MPEG-b-PCL	Poly(ethylene glycol)-block-poly(ε-caprolactone) methyl ether
mV	Milivolts
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCCS	National centre for cell sciences
NSCLC	Non-small cell lung cancer

PACA	Polyalkylcyanoacrylate
PCL	Polycaprolactone
PD-1	Programmed cell death protein-1
PDA	Photodiode array
PDI	Poly dispersity index
PD-L1	programmed death ligand-1
PDGFR-α	Platelet-derived growth factor receptor alpha
PEG	Poly ethylene glycol
PFA	Para formaldehyde
P-gp	P-glycoprotein
PLGA	Polylactic-glycolic acid
PMEA	6-mercaptopurine and 9-(2-phosphonyl-methoxyethyl) adenine
PMS	Phenazine metho-sulfate
PVA	Polyvinyl alcohol
Resv	Resveratrol
ROS1	Proto-oncogene tyrosine protein kinase
RPMI 1640	Rosewell Park memorial institute medium
RT	Room temperature
Sec	Seconds
Sily	Silymarin
SPC	Soy phosphatidyl choline
TIE-3	Tyrosine kinase with immunoglobulin-like and EGF-like domains-3
Verp	Verapamil
VGFR	Vascular endothelial growth factor receptor
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

List of figures

List of tables

Chapter 1. Introduction and review of literature

1.1. Cancer and chemotherapy

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. (1) These cells are often dedifferentiated and have modified inherent features those results in loss of functionality. When their growth is not controlled, the rapidly dividing cells reposition to different location of body causing multiple organ damage and death. Irrespective of tumor site, the mass of cells are highly heterogeneous with improper differentiation hierarchy and divisional plasticity. These features have been marked essential for tumor malignancy and metastasis. (2, 3) The different treatment modalities adopted for cancer therapy, so far are surgical excision, radiation and chemotherapy. Where a tumor is associated with non-visceral organ, the tumor burden is initially reduced by surgical excision or destruction of tumor tissue by radiation in situ followed by chemotherapy if needed. On the other hand for cancers in visceral organ are often treated with combination of chemotherapeutic agents. (4) In addition to these treatment options such as immune-therapy, hormonal therapy etc. are also adopted in various studies. (5)

Cancer chemotherapy in modern medicine has been in practice for several decades since the end of World War II where nitrogen mustard was used for treating lymphomas. Since then different categories of anti-cancer agents such as anti-metabolites (anti-folates, thiopurines and fluorouracils), microtubule modulators, adjuvant chemotherapy and immuno-therapy are sequentially developed for treatment of cancer therapy (figure 1.1). (6) These anticancer agents target different cellular process such as folate synthesis, nucleotide synthesis, DNA replication, microtubule polymerization, proteosomal degradation, cell division signals etc. Figure 1.2 shows different agents acting on such targets. Other than these intracellular targets, extracellular target based therapies such as inhibition of tumor vasculature (ADH-1, genistein, minocycline, coxibs),

immune therapy (pembrolizumab, nivolumab, atezolizumab), anti-inflammatory agents (NSAIDs, corticosteroids, coxibs), extra-cellular vesicles, extra cellular matrix (simtuzumab), matrix metallo proteinase (marimastat, prinomastat) etc. has been identified recently and studied for cancer therapy. (7-12)

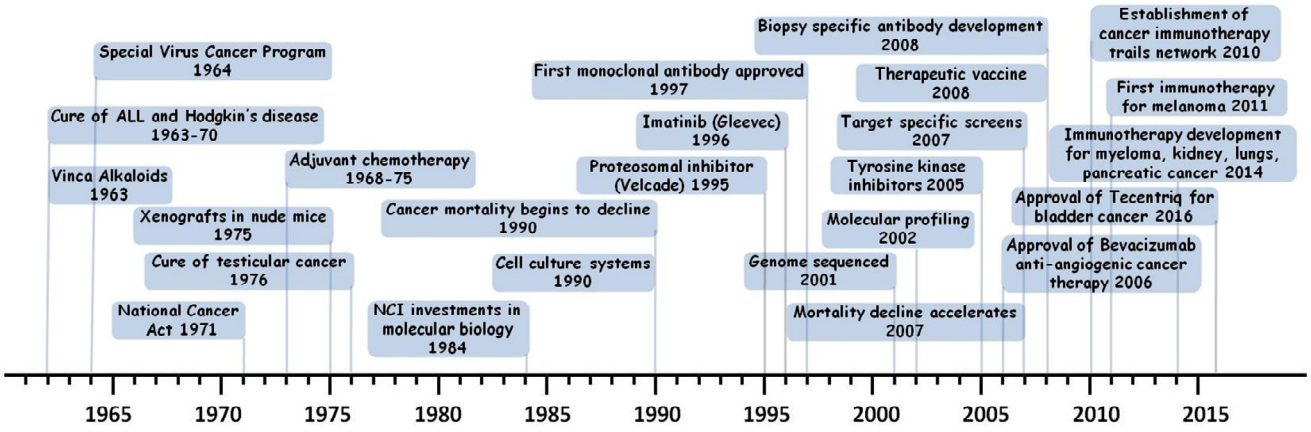


Figure 1.1. Timeline of development of cancer chemotherapy (6).

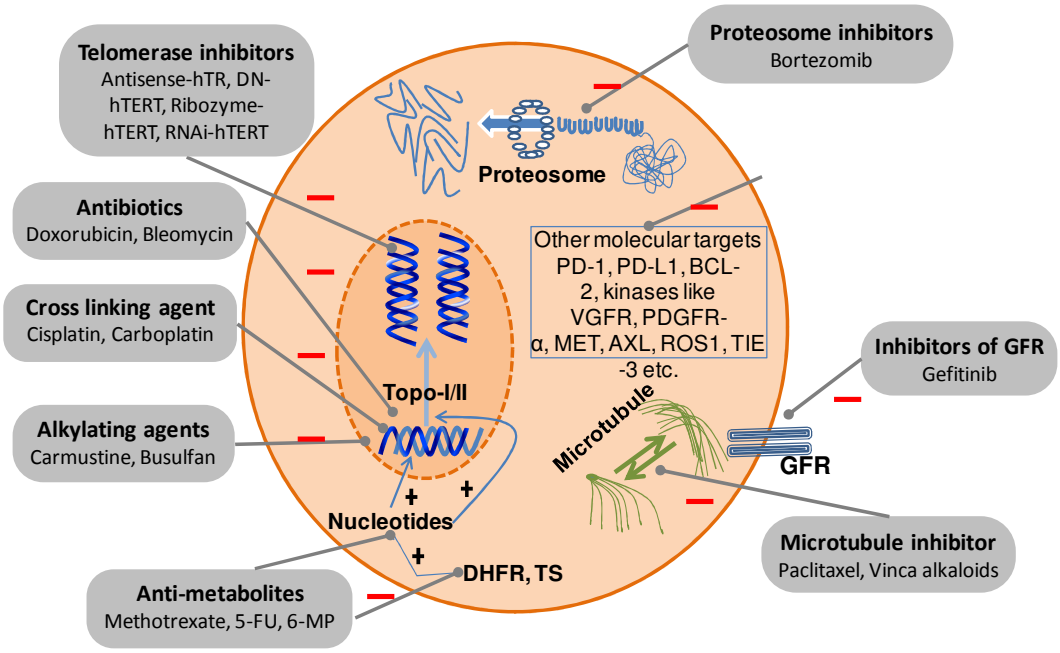


Figure 1.2. Molecular targets of cancer chemotherapy.

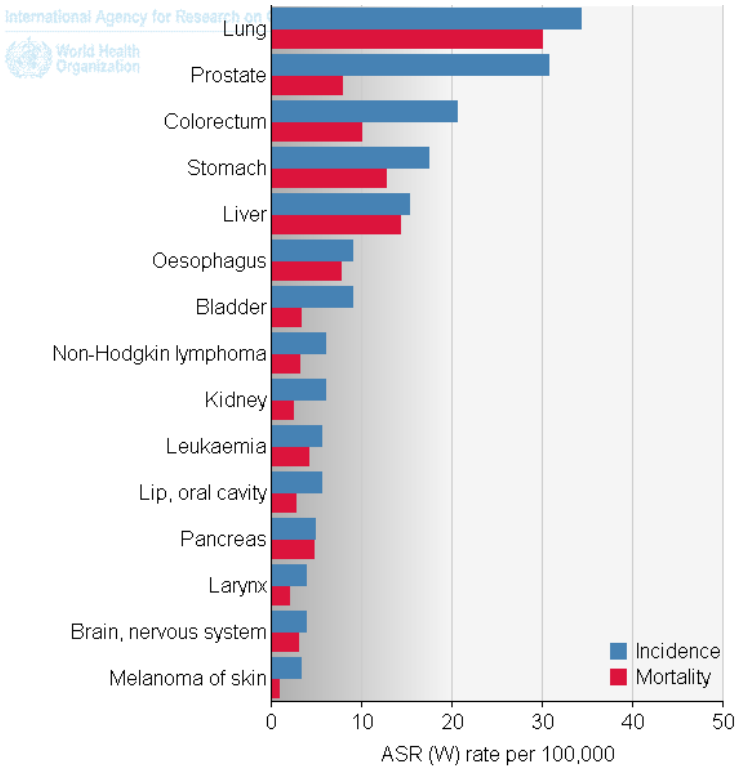


Figure 1.3. Comparative presentation of incidence Vs mortality for various cancers. (13)

Despite these developments, mortality rate is reported to be very high especially in cancers associated with visceral organs such as liver, lungs, esophagus, pancreas etc. (figure 1.3). From studies on high mortality rates, it was observed that cancer develop acquired resistance to chemotherapeutic agents rendering them ineffective. The resistant cells later grow back and results in a therapeutic failure and relapse of resistant cancer. (14-16)

1.2. Acquired drug resistance and mechanisms

Drug resistance is a process by which cancer cells develop mechanisms to survive during chemotherapeutic stress. Drug resistance affects patients with variety of cancers associated with blood, breast, liver, lungs, gastrointestinal tract etc. Initial stage of treatment with chemotherapy results in killing of significant portion of heterogeneous tumor but leaves behind resistant clonal cells. These resistant cells grow again causing a cancer relapse that is mostly insensitive to

chemotherapy. (16, 17) Molecular mechanisms of acquired drug resistance have been studied and several mechanisms are postulated that may act singly or in combination leading to resistance. Figure 1.4a represents different cellular adaptations developed by cancer a cell to confer resistance. Figure 1.4b represents number of publications that report on different mechanism of resistance since 1990 as indexed in PubMed. (18, 19)

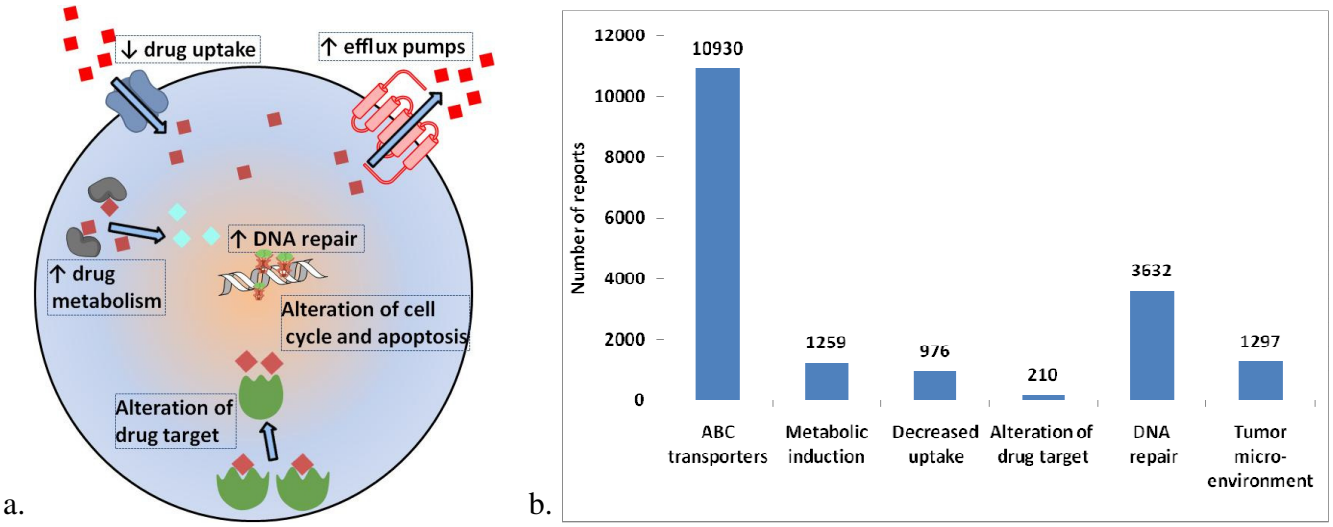


Figure 1.4. Mechanisms of acquired drug resistance in cancer (a) and their reports since 1990 (b).

1.2.1. Over-expression of efflux pumps

Efflux pumps or ABC transporters are present in normal cells for cellular detoxification process. In physiological conditions, these pumps are present in cell membrane of almost all organs (especially intestine, liver, pancreas, kidney, adrenal, brain etc) by which cellular toxic metabolites expelled out of the cell by efflux pumps in an energy dependent process.(20, 21) However, these transporters are constitutively over-expressed in tumor tissue to efflux the chemotherapeutic agent out of cell. Thus, cells become resistant due to unavailability of chemotherapeutic agent at the site of action.

Table 1.1. ABC transporters, their tissue distribution and cyto-toxic substrates.

Name/ Common Name	Tissue	Exogenous cyto-toxic compounds
ABCA2	Brain, monocytes	Estramustine
ABCA3	Lungs	Daunorubicin
ABCB1/ MDR1, P-gp	Intestine, liver, kidney, placenta, blood–brain barrier	Anthracyclines, actinomycin D, colchicine, podophyllotoxin, mitomycin C, mitoxantrone, taxanes, vinca alkaloids, S-38, topotecan
ABCB4	Liver	Paclitaxel, vinblastine
ABCB5	Melanocytes, retinal pigment epithelial cells	Doxorubicin
ABCB11	Liver	Paclitaxel
ABCC1/ MRP1	All tissues	Anthracyclines, colchicine, etoposide, heavy metals, vincristine, vinblastine, paclitaxel, methotrexate
ABCC2/ MRP2	Liver, kidney, intestine	Cisplatin, CPT-11, DOX, etoposide, methotrexate, SN-38, vincristine, vinblastine
ABCC3 / MRP3	Pancreas, kidney, intestine, liver, adrenal glands	Cisplatin, doxorubicin, etoposide, methotrexate, teniposide, vincristine,
ABCC4 / MRP4	Prostate, testis, ovary, intestine, pancreas,	Methotrexate, nucleotide analogs, PMEA
ABCC5 / MRP5	Most tissues	Doxorubicin, methotrexate, nucleotide analogs, topotecan,
ABCC6 / MRP6	Liver, kidney	Doxorubicin, etoposide, teniposide
ABCC10	Pancreas	Paclitaxel, vinca alkaloids
ABCC11/ MRP8	Breast, lungs, liver, kidney, placenta, prostate, testes	5'-Fluorouracil, 5'-fluoro-2'-deoxyuridine, 5'-fluoro-5'-deoxyuridine, PMEA
ABCG2/ BCRP	Placenta, intestine, breast, liver	Anthracyclines, bisantrene, camptothecin, epirubicin, flavopiridol, mitoxantrone, topotecan, imatinib,

Modified from ((16, 19, 20)

ABC transporters are a super family comprising of highly conserved 48 transporters. Of which, several are involved in conferring resistance to anticancer drugs. The most commonly involved are ABCB1 or P-glycoprotein (P-gp), ABCC1, ABCG2, ABCC2 etc. ABC transporters are able to transport a wide range of chemical entities including hydrophobic, neutral or positively charged agents (table 1.1). Over-expression of these transporters, confer resistance to

several anti-cancer drugs such as doxorubicin, daunorubicin, vincristine, paclitaxel, etoposides etc. (21, 22) In addition over-expression of specific exporter proteins is reported to have role in resistance of some anti-cancer agents. For example, copper transporting ATPase such as ATP7A, ATP7B etc. are over over-expressed in cisplatin resistant cancers.(23)

1.2.2. Decreased uptake of drug

Several of chemotherapeutic agents enter in to the cell by cellular transport system. Copper and folate transporter are used as transport machinery by anticancer agents. (24) Anticancer agents act by malfunctioning or inhibiting folate pathway such as the dihydrofolate reductase inhibitor (methotrexate) and thymidylate synthase inhibitor (tomudex) enter the cell by folate carrier. (25) Further, platinum based anti cancer agents such as cisplatin, carboplatin and oxaloplatin enter in to cells by solute carrier transporter family of copper transporters. (24) Following initial exposure, cells often down regulate these carriers (folate transporters, copper transporters; Ctr1) by which cellular accumulation of these anti-cancer agents are lowered. In several experimentations with acute lymphoblastic leukemia, chronic myeloid leukemia, ovarian cancer etc reduced accumulation of chemotherapeutic agents conferred resistance to methotrexate, cisplatin etc. (23, 26) In certain cell lines, cisplatin accumulation was reported to be lower in resistant cells and depletion uptake mediated by receptor or endocytosis was postulated as the major cause. (19) Recently developed immune-toxins enter the cell by endocytic uptake. Cancer cells with reduced, defective or mutated endocytosis machinery acquire resistant to immuno-therapy as well as anticancer agents. (19, 27)

1.2.3. Inactivation or increased metabolism of drugs

Metabolism by cellular enzymes is a physiologic process by which chemical entities get oxidized, reduced or conjugated to facilitate its excretion. Cancer cells up-regulate enzymes that modify the drug to produce an inactive metabolite. In some instances cancer cells down-regulate enzyme responsible for drug activation.(28, 29) Metabolisms of drugs are mainly mediated by cytochromes. They oxidize the xenobiotic drugs to introduce polar groups that inactivate it. Over expression of these enzymes leads to rapid metabolism of anti-cancer agents to inactive metabolites, thereby resulting in resistance.(30) In addition, thiol containing molecules such as glutathione-S-transferase and metallothioneins are detoxifying enzymes expressed in the cell to protect the cell from oxidizing radicals and considered as second step of drug metabolism.(21, 25) Over-expression of GST correlates with drug resistance as demonstrated in cancers of ovary, neck, stomach, esophagus, lungs, colon, urinary bladder by inactivating anticancer agents such as cisplatin, chlorambucil, melphalan, nitrogen mustard, phosphoramide mustard etc. (21, 30-32) In other case, 5-fluorouracil is metabolized by liver enzyme; dihydropyrimidine dehydrogenase, and its over-expression directly correlated with its resistance in colon cancer. (33)

Other than cellular detoxification, some of enzymes are also responsible for activation of prodrugs.(28) For instance GST is involved in activation of 6-mercaptopurine, TER286, S-CPHC-ethylsulfoxide etc.(34, 35) In another case, cytarabine that is used for treatment of acute myelogenous leukemia is activated after multiple phosphorylation events to convert it to Ara-C triphosphate. (21) In these cases cancer cells often down regulate these enzymes responsible for activation of these prodrugs.

1.2.4. Alteration of drug targets and DNA repair mechanisms

Intact cellular pathways are essential components for several anti-cancer drug activities. Any mutation of target or alteration of expression results in insensitivity of chemotherapeutic agent. (21) For instance, a point mutation in topoisomerase-II resulted in resistant to amsacrine in a human leukemic cell line. (36) Tyrosine kinases from EGFR family are over-expressed in many of cancers. The EGFR inhibitors rapidly become resistant with in periods of one year of use due to a mutation in the gate keeper residue, EGFR T790M. (37) Similarly, reports suggest point mutation in *ABL* gene and amplification of *BCR-ABL* fusion gene as a major cause of imatinib resistance in chronic myeloid leukemia.(38) Another cellular target, β-tubulin is mutated or expressed at different levels in cancers to acquire resistance to taxols and vinca alkaloids. (25, 39) Resistance to 5-flurouracil and leuprolide was rendered by over-expression of thymidylate synthase and androgen receptor respectively that causes insufficient therapeutic agents to act on all the molecular targets.(16, 37) Collectively, reduction of expression of topoisomerase-I and its mutation bring out resistance to SN-38 (active metabolite of CPT-11) in different studies.(21, 25, 36, 40) Similarly reduction of topoisomerase-II enzyme level results in gaining of resistance to etoposide and doxorubicin. (25)

Improved DNA repair mechanisms can reverse the DNA damage induced by chemotherapeutic agents. (25) Nucleotide excision repair and homologous repair mechanisms are evidenced to be upregulated in cisplatin resistant cancer. Importantly only few proteins involved in nucleotide excision repair system such as excision repair cross-complementing protein are up regulated and their expression status correlates well with cisplatin sensitivity in ovarian, gastric and non-small scale lungs carcinoma. (41-43) Here the efficacy of anticancer

drug acting by damaging DNA (alkylating agents and cross-linking agents) is directly related to DNA damage response by cancer cells.(21, 44)

1.2.5. Impaired apoptotic pathway and alteration of cell cycle

Many chemotherapeutic agents impair different cellular pathways and induce cell death *via* apoptosis. Cell death through apoptosis is induced either by an intrinsic pathway (mediated by the mitochondria involving BCL-2 family proteins, caspase-9 and Akt), or an extrinsic pathway (involves cell surface receptors). In different cancers, cells acquire resistant phenotype by directly modifying apoptotic pathway or indirectly inhibiting signaling molecules at downstream and consequently they acquire resistance to different chemotherapeutic agents. (21) For instance upon chemotherapeutic treatment, apoptosis is promoted by the tumor suppressor protein p53 which is mutated or deleted in 50% of cancers resulting resistance to chemotherapy. Wild type P53 was observed to be determinant for the sensitivity for several of anticancer drugs including 5-fluoro uracil, cisplatin, doxorubicin, etc. However, there are several contradictory reports and high case to case variation to draw any consensus of P53 status and sensitivity to anticancer drugs.(25) In addition inactivation of P53 regulators, such as caspase-9 and Apaf-1 can also lead to drug resistance. (45-47)

In other cases, increased GST level in cell indirectly suppress induction of apoptosis due to its ROS scavenging activity or via MAP kinase inhibition.(30) Although apoptosis and cell cycle are closely related, they mediate drug resistance differently. Cell cycle based resistance is the relative insensitivity of chemotherapeutic agent due to its position in cell cycle. This mode of resistance is highly significant for cell cycle specific chemotherapeutic agents like taxols, camptothecins, fluorouracils etc. For paclitaxel, cyto-toxicity is maximal at G2-M and minimal

at G1-S phase of cell cycle. For these drugs, cell cycle arrest by modulation of regulators of cell cycle results in insensitivity to respective agents.(48)

1.2.6. Other mechanisms involved in development of resistance to anti-cancer agents

Other than above well studied mechanism of acquired drug resistance, there are several other mechanisms postulated on a case to case basis. Mechanisms involving cancer stem cells, tumor micro-environment and autophagy are reported in this context. It is observed that, out of heterogeneous population of cancer mass, about 0.1-30% possesses stem cell like features and possess tumorigenic potency. Genetic flexibility, ability to form multi-lamellar spheroids, and EMT are some of the properties of stem cells which enhance their resistance towards chemotherapeutic agent. (49) Tumor microenvironment forms a niche which facilitates survival and growth of cancer stem cells. Niche is a dynamic supportive system enriched with specific anatomic and functional features that contains a variety of cell types, cytokines and signaling pathways. In addition, hypoxic microenvironment and neo-angiogenesis are two important parameters for development of resistant to chemotherapy. (50, 51) Role of EMT in drug resistance is considered to be indispensable since the cells undergoing EMT possess stem cell line features in Wnt, Hedgehog and Notch signaling pathways. However the proper mechanism by which EMT leads to drug resistance is not known yet. (52, 53)

1.3. P-glycoprotein (P-gp) expression and its role in conferring chemo-resistance

Among ABC transporters involved in anti-cancer drug resistance, over expression of ABCB1 or P-glycoprotein is reported to be one of major mechanism of acquired drug resistance in various cancers. (54) Although several ABC transporters can act together to confer resistance to a chemotherapeutic agent, ABCB1 or P-glycoprotein is contributing resistance in almost

categories of chemotherapeutic agents. (54) A brief structural and functional outline of P-gp is presented below.

P-gp is a membrane bound glycoprotein of 1280 amino acids consisting two homologous half transporters arranged to have both C-and-N terminus domain in the cytoplasmic side (figure 1.5 (a)).(24) Each homologous half contains six trans-membrane segments and a cytoplasmic nucleotide binding domain. The two half are connected through a linker region that is crucial for proper interaction between two subunits for their ATPase activity and drug transportation.(24) The trans-membrane domain provide site for substrate binding where hydrophobic drug substrates that are either neutral or positively charged. Binding triggers hydrolysis of first ATP that cause conformational change (figure 1.5 (b)) for expulsion of substrate through P-gp. Hydrolysis of second ATP is postulated for transforming P-gp to its actual conformation. (55)

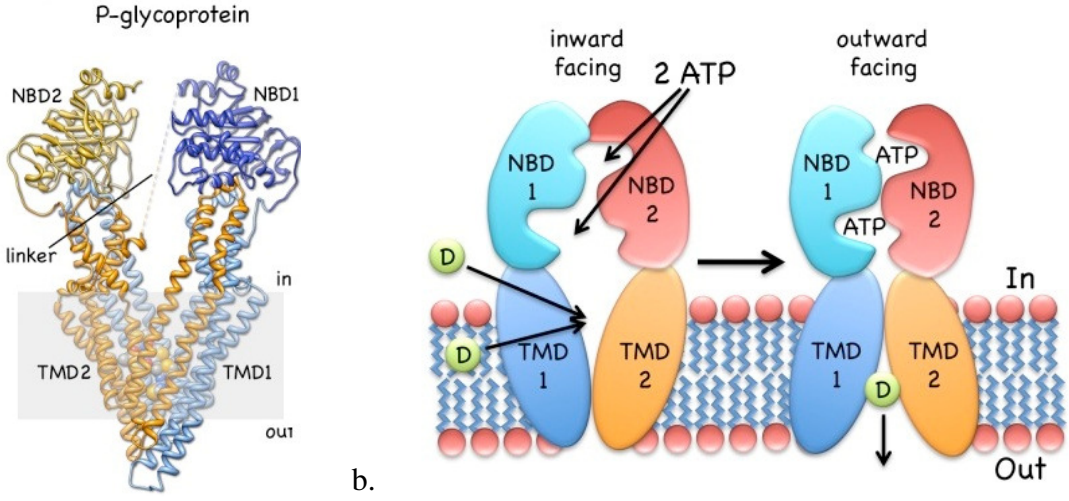


Figure 1.5. Structure of P-gp (a) and its mechanism of drug efflux (b). (56)

Furthermore, P-gp can transport numerous chemical entities with diverse physicochemical properties. In the initial phase different compounds interact at different points of P-gp, but during expulsion, single transport channel is involved irrespective of their initial point of interaction.(57) However, lipophilicity of compounds is the determinant factor for

interaction with P-gp. Hydrophobic or amphipathic drugs those are either neutral or positively charged may interact with P-gp. Diverse group of compounds interacting with P-gp are classified as per their modulation after interaction. Some agents interact and get transported are called substrates where as other agents those block the interaction or transport of other compound after interaction are called antagonists. (58) Chemotherapeutic agents such as vinca alkaloids (vincristine, vinblastine), anthracyclines (doxorubicin, daunorubicin, and epirubicin), epipodophyllotoxins (etoposide, teniposide), taxol (paclitaxel), actinomycin-D, topotecan, mithramycin, mitomycin C etc are also substrates of P-gp. (22, 58) Thus, over-expression of P-gp results depletion of intracellular concentration of these chemotherapeutic agents and highly compromise therapeutic activity. In different cancers associated with blood, bone marrow, breast, lungs, ovary, urinary bladder, cervix etc. expression of P-gp was increased post treatment and corresponded to decreased clinical response to chemotherapy. (19, 58)

1.4. Approaches to circumvent drug resistance

1.4.1. Development of new therapeutic entities

Drug discovery tools have been adopted for finding suitable agents those target novel cellular pathways and possess unaltered cyto-toxic activity in by acquired resistance mechanisms. (54) Several of new cellular targets have been identified for development of chemotherapeutic agents. As detailed in a review by Ma et al, “the new chemotherapeutic targets can be conceptualized based on membrane-bound receptor kinases (HGF/c-Met, human epidermal growth factor receptor and insulin growth factor receptor pathways), intracellular signaling kinases (Src, PI3k/Akt/mTOR, and mitogen-activated protein kinase pathways), epigenetic abnormalities (DNA methyltransferase and histone deacetylase), protein dynamics (heat shock protein 90,

ubiquitin-proteasome system), and tumor vasculature and microenvironment (angiogenesis, HIF, endothelium, integrins). (59) Table 1.2 represents few novel chemotherapeutic agents targeting different molecular pathways and their implication in different cancers.

Table 1.2. Novel anti-cancer agents, their molecular target and implication in different cancer.

Drug name	Molecular target	Cancer	Reference
Temsirolimus, Everolimus, OSI-027	mTOR	Renal cell carcinoma, mantle cell lymphoma, solid tumors	(59, 60)
Dinitroazetidines	Cellular redox pathways	Cancerous cell lines of different tissue origin	(61)
Ethacraplatin	DNA damage	Breast cancer	(62)
Vorinostat, ITF2357,	Histone deacetylase inhibitors	T-cell lymphoma, Hodgkin lymphoma	(63)
N-alkylated isatins	Microtubule	Lymphoma, uterine sarcoma cell line	(64)
DT-010	Glycolytic pathway and inhibition of GRP78	Breast	(65)
Baicalein	Mismatched DNA	Colon	(66)
Tigatuzumab	TRAIL agonist	Pancreatic	(67)
Dalotuzumab	IGFR1	Lungs, breast	(68)
Abiraterone acetate, Enzalutamide	Androgen receptor or pathway	Prostate	(69, 70)
Patupilone,	Microtubule	Hepatic	(71, 72)
Sagopilone, Ixabepilone	Microtubule	Lungs, breast, colon	(73-75)
ST1481	Topoisomerase-I	Colon	(76)

Other than finding new targets, several agents developed to target the existing cellular pathways but designed to minimize interaction with efflux pumps. Microtubule targeting epothilones class of drug are been developed which are poor substrate of efflux pumps. Different epothilones demonstrate varying susceptibility to efflux by P-glycoprotein.(73) For example, sagopilone (ZK-EPO) is not a substrate of P-gp hence highly active in cell lines expressing P-glycoprotein. (74) Ixabepilone (BMS-247550) is a substrate of P-gp (but not ABCG2) but appears less affected than paclitaxel. In P-gp expressing cell line SNU-449, it was reported to be

108 to 529 times more potent than taxanes or doxorubicin.(71, 72, 77) In another modification, camptothecin modified to ST1481 that was not a substrate of ABCG2 and accumulated well in resistant HT29 cells.(76)

1.4.2. Combination of P-gp inhibitors and chemotherapeutic agent

Over expression of P-glycoprotein are major mechanisms of acquired drug resistance in cancer. Thus, P-gp inhibitors from various sources such as natural products, marketed drugs, novel synthetic agents and natural as well as synthetic polymers are been discovered and studied for their efficacy to reverse drug resistance in different model systems.(78, 79) P-gp inhibition could result from the blockage of substrate recognition, ATP binding or hydrolysis as well as ATP coupling hydrolysis to translocation of the substrate. (22) Inhibitors act either competitively or non-competitively after binding to substrate (cyclosporine A) or modulator binding site respectively. However there are inhibitors binding at surface (flavonoids) or vicinity (steroids) of nucleotide binding domain as well as cytoplasmic domain (kaempferide) and block either binding or hydrolysis of ATP (cyclosporine A, Vanadate).(20, 22)

Natural p-gp inhibitors belonging to various categories such as flavonoids, isoflavonoids, terpenoids, alkaloids, steroidal saponins etc have been trialed widely as pharmacokinetic modulators for reversal of drug resistance. (18, 80, 81) Among these, flavonoids have gained significant attention for reversal drug resistance and increased cyto-toxicity of chemotherapeutic agents. In studies on various cancers models, flavonoids in combination improved effect of vinblastine, doxorubicin, irinotecan, daunomycin, paclitaxel etc. (78, 82) Additionally, flavonoids are believed to have high therapeutic index and thus being studied widely as chemo-sensitizer. (20) These promote much emphasis on natural products supported by abundant

availability and non-toxic nature; but most natural products have poor solubility and bioavailability. (83) For the above problem, pharmaceutical nano-formulations containing these agents have provided significant improvement. Many of natural products have been formulated in to different nano-formulations for solubility and bioavailability improvement, controlled release and targeted delivery.(84, 85) In addition, reversal of drug resistance by polymers from natural as well as synthetic sources such as alginates, gums, PEG based copolymers, Pluronics etc. has been reported interfering functionality of P-gp. (86-88)

In addition several available synthetic compounds are also studied for their P-gp inhibitory activity. Verapamil, cyclosporine-A, quinidine etc. are among the first P-gp inhibitors used. These are non-specific and have side effects or toxicity with varying binding affinity for transporter. (20) Thus P-gp inhibitors are designed based on available structural details and QSAR studies to improve specificity and binding affinity. For example, cyclosporin A had promising P-gp inhibitory activity, but it was associated with immune suppression, hepatic and renal toxicity. Thus its non immune suppressant analogue; valspodar was developed with higher chemo-sensitizing efficacy. (20) Subsequently, several chemically synthesized compounds were developed and studied for their P-gp inhibitory activity and reversal of chemo-resistance. (79) Based on improvements obtained they are classified in to several generations as shown in table 1.3.

Table 1.3. Classification of P-gp inhibitors.

First generation	**Second generation**	**Third generation**
Verapamil, cyclosporine A, tamoxifen, amiodarone, quinidine, quinine, nifedipine	XR9576, valspodar, biricodar dexniguldipine, PSC833	Zosuquidar, tariquidar, elacridar, ontogeny, elacridar, laniquidar, disulfiram, CBT-1
High toxicity and low efficacy at tolerable doses, non-specific	Improved efficacy and safety but low specificity	High potency, high specificity and low toxicity

Modified from (54, 58, 89, 90)

1.4.3. Delivery of therapeutic agent in pharmaceutical nano-formulations

In the initial phase of development, polymeric novel formulations containing therapeutic agent were developed for solubility improvement and controlled release. In comparison to conventional formulations, novel formulations improved the stability of encapsulated drug in systemic circulation and increased the bioavailability. These formulations mainly composed of biodegradable polymers either from natural or synthetic source as shown in table 1.4. (15, 91-93)

Table 1.4. Biodegradable polymers from natural and synthetic sources.

Natural sourced polymers	Synthetic polymers
Proteins (albumin, gelatin, collagen) polysaccharides (starch, dextran, chitosan, inulin, hyaluronic acid) cellulose (CMC, HPMC)	PLGA, poly-anhydrides PCL, poly-phosphazenes pseudo poly-amino acids, poly-carbonates poly-urethanes, poly-amides

Out of these polymers, PCL is one of widely studied that is formulated in almost all types of novel formulation by virtue of its amiable modification. Figure 1.6 represents different novel formulations employed in pharmaceutical technology.

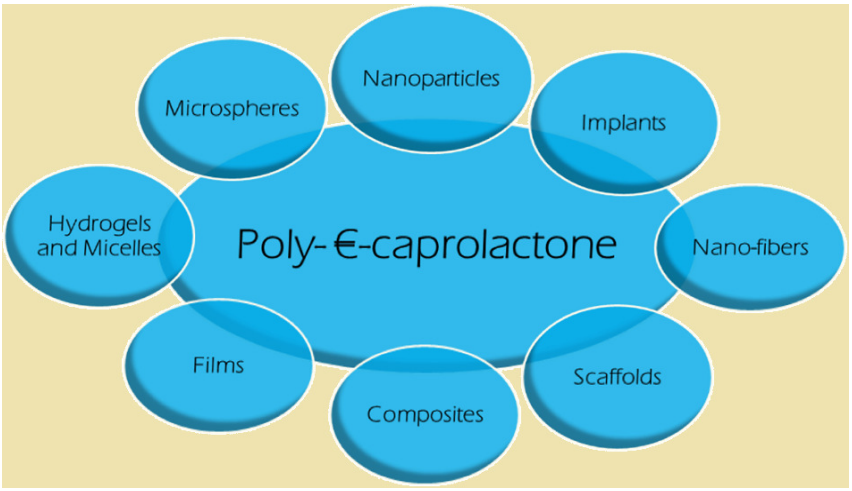


Figure 1.6. PCL based novel formulations adopted in pharmaceutical technology. (94)

From the past few decades, design of functional polymers for targeted or environment sensitive drug delivery or render then biologically active has been demonstrated in various studies. For example, PCL has been modified in different ways to enrich it with different functionalities as shown in figure 1.7.

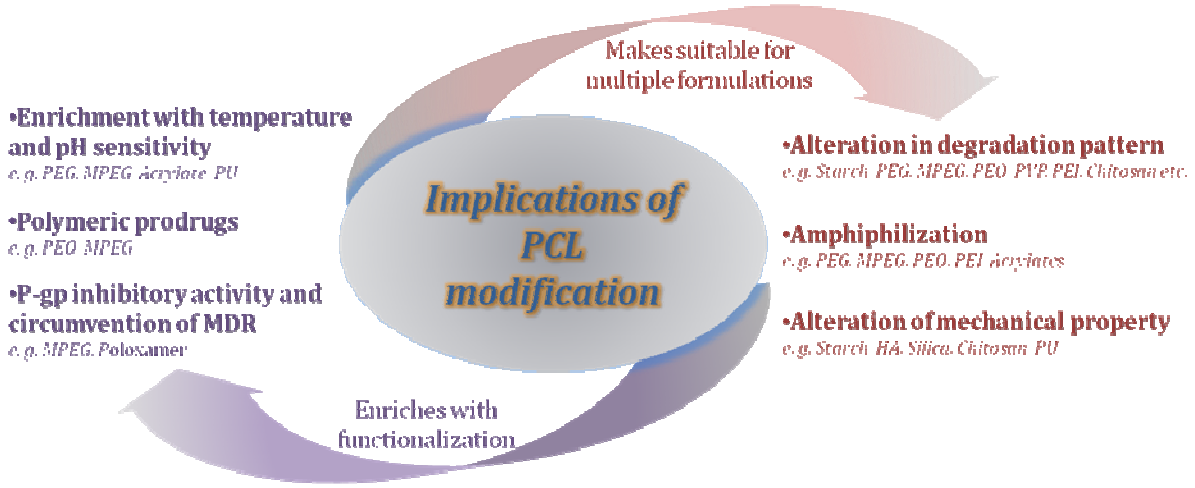


Figure 1.7. Modification of PCL for different formulations and to have functionality. (95)

In addition, nano-formulations provide many opportunities for circumvention of chemo-resistance. Firstly their endocytic uptake in to the cells bypasses transport related resistance mechanisms. (96) Secondly drug incorporation in to nano-formulations stabilizes the encapsulated agent and releases it in more sustained manner. Furthermore, nanoformulations possess EPR effect and tumor targeting features based on their size and functional properties.(97, 98) Importantly recent advancement of multimodal delivery through modification of polymers or lipids for enriching them with smart delivery or functional properties established their application for reversal of drug resistance. (95, 97, 99) Reports also suggest reversal of drug resistance by polymers from natural as well as synthetic sources such as alginates, gums, PEG based copolymers, Pluronics etc. by interfering functionality of P-gp. (86-88)

1.5. Combinational nano-formulations

This approach combines pharmacological and pharmaceutical approach for circumvention of drug resistance. Pharmacologically, multiple drugs or drug in combination with a chemo-sensitizer is been treated together to treat resistant cancer effectively. In other ways pharmaceutical nano-formulation has shown to circumvent drug resistance by enhanced solubility, bioavailability, uptake in to the cell and EPR effect. Here, a chemotherapeutic agent was combined along with another chemotherapeutic agent or other agents such as efflux pump inhibitors, pro-apoptotic compounds or siRNA targeting MDR in different formulations such as liposomes, nano-particles, and polymer drug conjugates etc. (100-102)

1.5.1. Advantages and challenges of combinational nano-formulation

Although the combinational nano-formulations are advantageous, there are certain challenges need to be addressed for each combination. These are mainly associated with physicochemical properties and pharmacokinetic parameters of each entity. Some advantages and challenges of combinational nano-formulation are as follows.

Advantages of combinational nano-formulation

- Pharmacokinetic or pharmacodynamic interaction between drug or drugs and sensitizer
- Both follow the distribution pattern of carrier irrespective of individual differences in metabolism and distribution in absence of carrier
- Mask the pharmacokinetic and physico-chemical differences between selected agents
- Targeted features can be enriched for site specific delivery

Challenges of combinational nano-formulation

- Differences in physicochemical properties make it challenging to incorporate in to single nano-formulation.
- Stabilization of both the agents during preparation steps.
- Obtaining proper EE to get desired dosing ration of the combination
- Control of release characters i.e. ideally the sensitizer is desired to be released first followed by chemotherapeutic agent
- Accumulation at the tumor site and un-compromised therapeutic after encapsulations is essential for successful delivery of co-loaded delivery system

1.5.2. Nano-formulations used to deliver combination of therapeutic agent

Different nano-formulations experimentally prepared (figure 1.8) those addressed different challenges mentioned above. In this perspective, Hu et al presented a detailed review of various nano-formulations prepared containing combination of therapeutic agents for reversal of chemo-resistance. (101, 102) Table 1.5 enlists different combinational nano-formulations prepared for experimentation so far and their implications. Among several formulations, nano-particles and liposomes have been experimented widely with significant number of successful outcomes. Both formulations have different pros and cons. (102) For instance, nano-particles provide advantages in terms of controlled release and stability due to solid core that was most suited for lipophilic drugs. On the other hand, liposomes have as large aqueous core that can be prepared with high degree of encapsulations of several hydrophilic drugs.

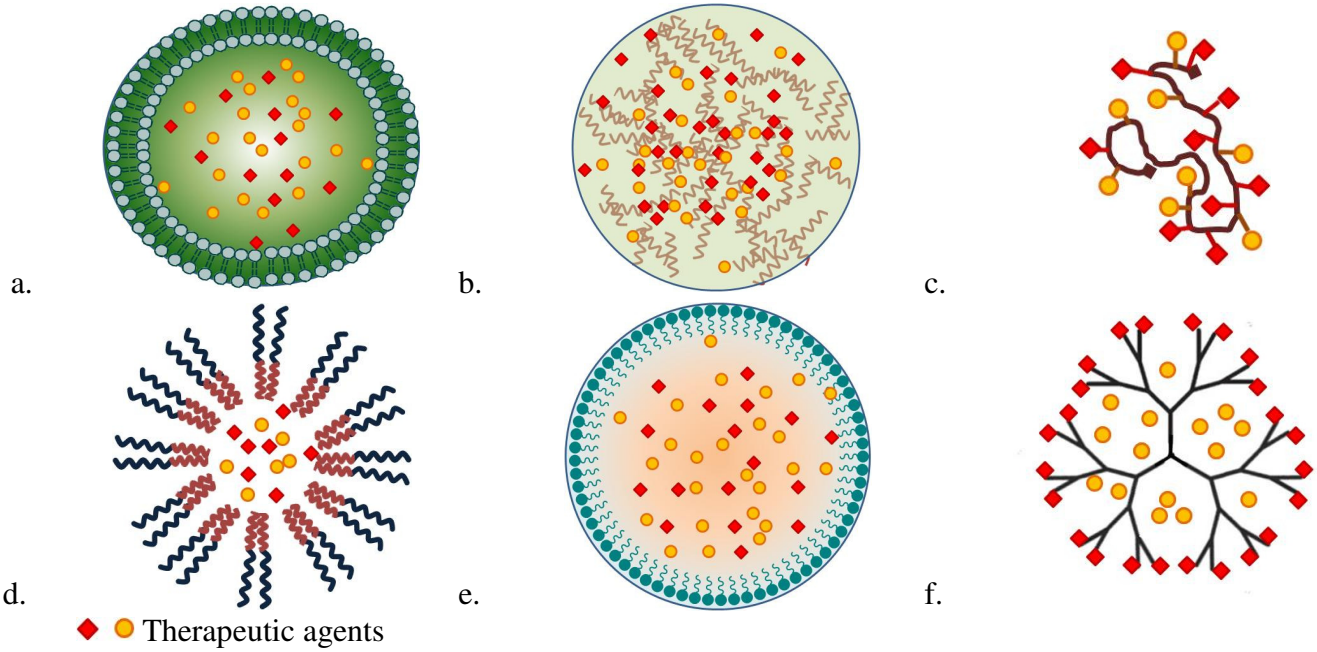


Figure 1.8. Nano-formulations used for combinational drug delivery; liposomes (a), polymeric nano-particles (b), polymer drug conjugates (c), micellar nano-particles (d), nano-emulsion (e) and dendrimers (f).

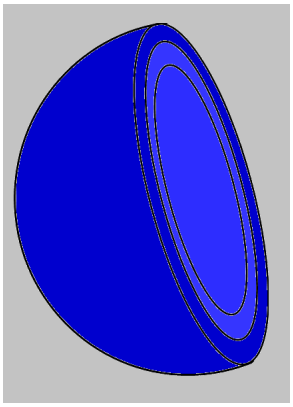
Table 1.5. Combinations of chemotherapeutic agent and sensitizer in nano-formulations.

Drug	Chemo-sensitizer	Formulation	Implication in cancer	Reference
DOX	Cur	Lipid nano-particle, micellar nano-particle, liposomes	Liver, breast, lungs, tumor vasculature	(89, 123-126)
DOX	BioA	Liposomes	Colon	(127)
DOX	Verp	Liposomes	Leukemia	(128)
DOX	siRNA for MRP1 and BCL2 mRNA	Liposomes	Lungs	(129)
Vincristine	Quercetin	Liposomes	Breast	(128)
Vincristine	Verp	Nanoparticles (PLGA)	Breast	(130)
DOX	Cyclosporin A	Nanoparticles (PACA)	Lymphoma	(131)
DOX	Combretastatin	Nanoparticles (PLGA core and lipid envelope)	Melanoma	(132)
Paclitaxel	Interleukin-12-encoded plasmid	Liposomes	Breast	(133)
Methotrexate	all-trans retinoic acid	Dendrimer (4 polyamidoamine)	Leukemia	(134)
Vincristine	Quinacrine	Liposome	Leukemia	(135)
Paclitaxel	Tariqidar	Liposome	Ovarian	(136)

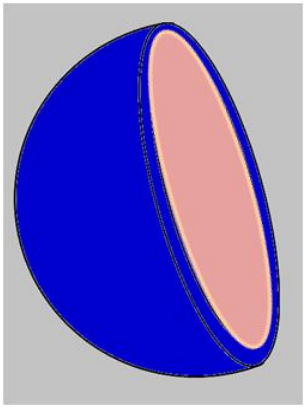
Additionally, liposomes provide an opportunity for encapsulation of lipophilic agents in the bilayer, thus allowing encapsulation of both hydrophilic and lipophilic drugs without any major change in the processing steps. Furthermore, ease of functionalization and technology transfer of liposomal delivery paved its way to market and clinical trials for delivery of single and combinational agents respectively. (103-105) Here, two nano-formulations are dealt in detail with components and preparation methods.

1.5.2.1. Polymeric or micellar nano-particles

These are self-aggregated or arranged particles having in nanometer size range often containing therapeutic agent in the polymeric core. However, in some instance surface coating containing drugs were also adopted to alter release of drugs. Based on the drug distribution pattern in the nano-particle core, nano-particles can be matrix type, nano-spheres or reservoir type nano-capsules (figure 1.9). (106) The polymers used for preparation of nano-particle are diverse and commonly used polymers are enlisted in table 1.4. For preparation of polymeric nanoparticles, different methods adopted are shown in the figure 1.10. Among the methods presented, emulsification and solvent diffusion or evaporation and dialysis are the methods adopted widely. The procedures adopted from the enlisted ones are described in the materials and method section.



Nano-sphere



Nano-capsule

Figure 1.9. Schematic presentation of nano-sphere and nano-capsules used in drug delivery.

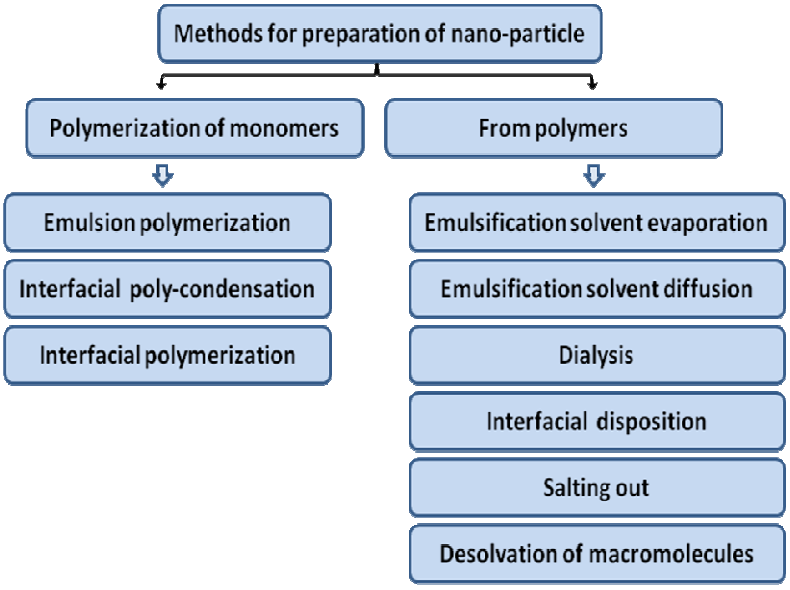


Figure 1.10. Methods adopted for preparation of drug loaded nanoparticles.

1.5.2.2. Liposomes

Lipid based vesicles of micro or nano meter size range with aqueous core are named liposomes. The vesicle formed may contain one or more bilayer made up of phospholipids and cholesterol. Use of liposomes for drug delivery is well demonstrated and several novel forms and functionalities are being explored. These experimentations showed its suitability of diversified applications such as drug, vaccine or gene delivery, diagnosis, bioengineering, cosmetic and agro-food industry.(107-109)

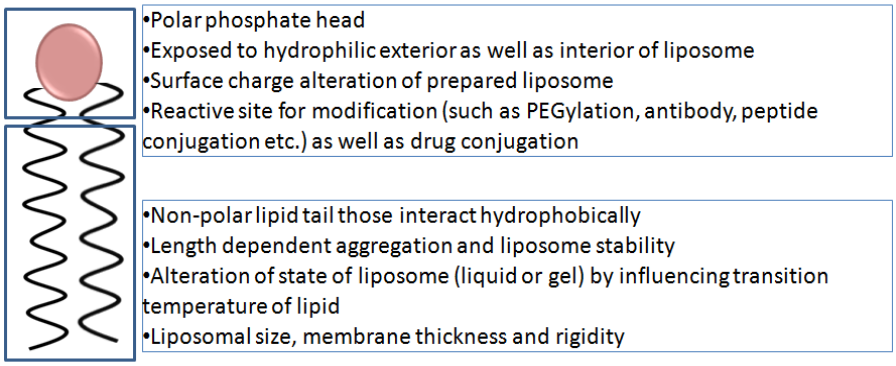


Figure 1.11. Components of phospholipids, their properties and modification aspects.

Phospholipids are composed of a hydrophilic head (phosphate) and hydrophobic chain (lipid) (figure 1.11) that in aqueous environment self-arrange in a sphere to decrease interaction of hydrophobic chains with aqueous phase. (100) Self-arranged lipid vesicles classified conventionally as SUV, LUV and MLV based on size and lamellarity. Both polar and non-polar regions of lipids can be modified to attenuate nature of membrane formed by them. In addition, phosphate group can be PEGylated to overcome short half-life and to enrich with targeting features by attaching targeting moieties. Post these developments, liposomes are further categorized as immune-liposomes, long circulating stealth liposome, responsive liposomes, targeted liposomes, cationic liposomes etc. (105) To optimize several aspects, lipids are modified to have different chemical structure. Table 1.6 enlists different properties of lipid that are varied to get advantages on various challenges of liposomal development.

Table 1.6. Challenges of liposomal formulation and lipid variants developed to overcome.

Challenges	Properties to be altered	Lipids to be incorporated
Mechanical stability	Membrane fluidity, permeability and phase transition temperature	Saturated and hydrogenated phospholipids
In vivo stability	Opsonization	PEGylated phospholipids
Bio-distribution	Passive diffusion	Lipid composition and surface charge
Accumulation	Passive targeting, active targeting	Size of liposomes and use of functional lipids
Cellular internalization	Endocytosis	Fusogenic liposomes
Encapsulation of drugs	Liposome size, membrane permeability, ionic properties of lipid and drug	Formulation of SUV and reduction of permeability by saturated lipids and optimal cholesterol concentration.
Release of drugs	Membrane stability and phase transition temperature of lipids	Use of different degree of hydrogenated lipids

For preparation of drug loaded liposomes there are several approaches adopted (figure 1.12). Out of these mechanical dispersion methods such as lipid film rehydration, sonication and

extrusion are used widely. In the present work, lipid film rehydration technique was used which is described in section 3.2.5.

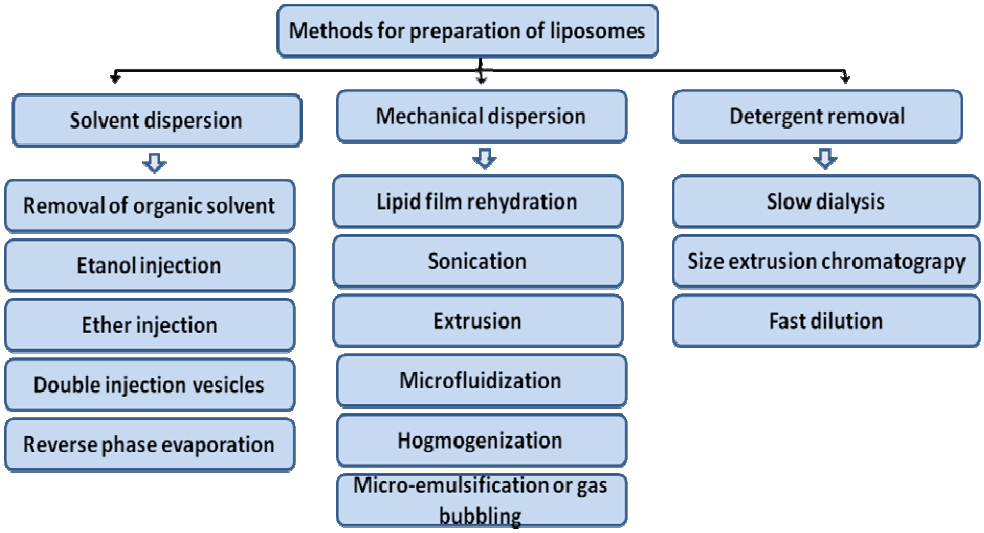


Figure 1.12. Methods adopted for preparation of liposomes.

Chapter 2. Aim and objective

Drug resistance is the major obstacle in the treatment of cancer. Various pharmacological and pharmaceutical approaches have been adopted for circumvention of chemo-resistance in cancer. The aim of my PhD work is to optimize and formulate combinational nano-formulations for circumvention of chemo-resistance in different cancers. To achieve above aim following objectives were framed.

1. Development of DOX resistance cell line as a model system.
2. Validation of DOX resistance by analyzing of growth pattern.
3. Evaluation of expression of efflux pumps and validation of reversal of developed resistance by standard P-gp inhibitor.
4. Screening of P-gp inhibitors from natural sources for P-gp inhibitory activity and reversal of DOX resistance in developed resistance cell lines.
5. Optimization of nano-formulation of the P-gp inhibitor along with DOX in terms of size, zeta potential and EE.
6. In vitro evaluation of reversal of DOX resistance in developed resistance cell lines by combinational nano-formulation.

Figure 2.1 presents work flow for achieving above objectives and various techniques those will be adopted.

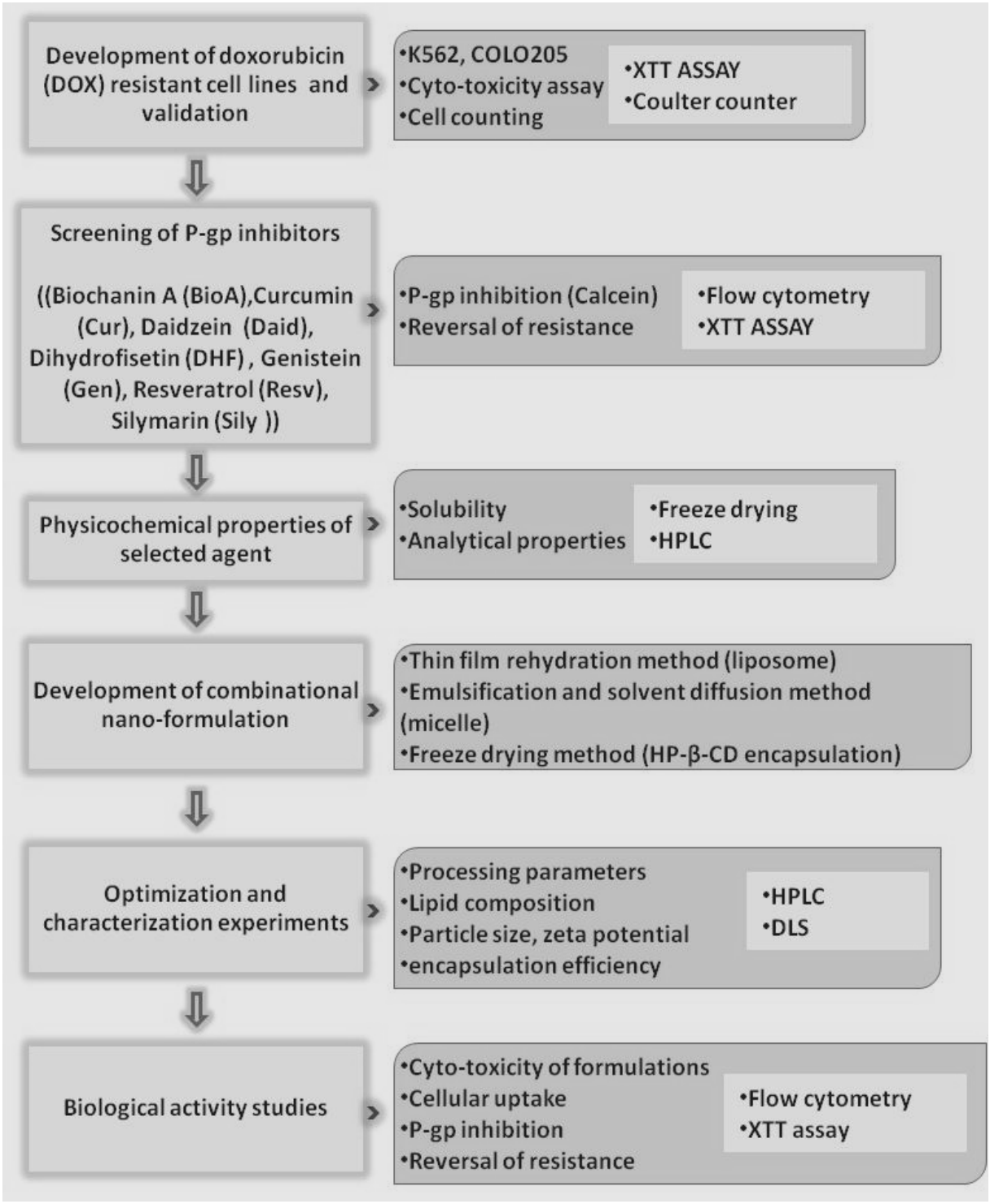


Figure 2.1. Flow chart presenting work plan and methods to be adopted.

Chapter 3. Materials and methods

3.1. Materials and equipments

Chemical used	Company (catalogue no.)
Acetonitrile	RANKEM (A0745)
Acetone	RANKEM (A0740)
Amphotericin-B solution	HI MEDIA (A011)
Penicillin streptomycin solution 100X	HI MEDIA (A001)
BioA	MP Biomedicals (157745)
Calcein AM	Sigma (17783)
Chloroform	RANKEM (C0580)
Cholesterol	Bio world (40330036-1)
Cur	MP Biomedicals (190313)
Cyclosporin A	MP Biomedicals (199276)
Daid	MP Biomedicals (158812)
Dialysis bag	HI MEDIA (LA390)
Dichloromethane	RANKEM (D0320)
DHF	MP Biomedicals (210015)
DMEM	HI MEDIA (AL007A)
DOPE	Avanti polar lipids (850725P)
DOX	MP Biomedicals (215910105)
DSPE-PEG(2000) maleimide	Avanti polar lipids (880126P)
Egg phosphatidyl glycerol	Avanti polar lipids (841138P)
Ethanol	SD Fine chemicals (58051)
FBS	HI MEDIA (RM9970)
Genistein	MP Biomedicals (152355)
HP-β-Cyclodextrin	HI MEDIA (RM2256)
Hydroxypropyl B-cyclodextrin	MP Biomedicals (0215354005)
IMDM	HI MEDIA (AL070S)
Hydrogenated- L-α-phosphatidylcholine	Avanti polar lipids (840059P)
Methanol	RANKEM (M0279)
MPEG-b-PCL	Sigma (570303)
PFA	HI MEDIA (GRM3660)
PVA	HI MEDIA (GRM6170)
Resv	MP Biomedicals (196052)
RPMI -1640	HI MEDIA (AL162S)
RPMI with out Phenol red	MP Biomedicals 091646754
Sily	MP Biomedicals (198792)
Soy phosphatidyl choline	Avanti polar lipids (441601G)
Sterile cell Scraper	HI MEDIA (TCP004)
Trypsin-EDTA solution 1X	HI MEDIA (TCL007)
Verp	MP Biomedicals (195545)

XTT	MP Biomedicals (158788)
Equipments used	
Microplate reader	Biorad, USA
Coulter Counter	Beckman Counter, USA
Flow cytometer	BD Biosciences, USA
Biosafety-II cabinet	Nuaire, USA
HPLC	Shimadzu, Japan
Probe sonicator	Cole-parmer, India
Bath sonicator	Wadegati, India
Extruder	Avanti polar lipids, USA
Dynamic light scattering	Malvern, UK
Freeze dryer	Labconco, USA
Rotary evaporator	Heidolph, Germany.
Centrifuge	Eppendorf, Germany, Thermo Scientific, USA
Magnetic stirrer	Tarsons, India
Electronic balance	Ohaus, China
Inverted microscope	Olympus Corporation, Japan
Fluorescence microscope	Olympus Corporation, Japan
pH meter	Thermo Scientific, USA
Thermo shaker incubator	MSC 100, China

3.2. Literature information of drug and excipient used

3.2.1. Drug and therapeutic agents used

The chemotherapeutic agent used for our study is DOX (figure 3.1). It was produce by *Streptomyces peucetius*.and named due to its color (ruby-red). DOX is used for different cancers such as breast cancer, bladder cancer, Kaposi's sarcoma, neuroblastoma, gastric cancer, lymphoma, leukemia etc. The mechanism of action is documented as cell cycle specific Topoisomerase-II inhibitor but recent demonstrations also report its DNA cross linking rendering cell cycle non-specific.(110) Physically, it is red colored, weakly basic compound having poor water solubility in its native form. Therefore it is converted in to its hydrochloride salt that increases its water solubility and makes it suitable for parentral infusion. The major limiting factor for its therapeutics is cardio toxicity. Thus in current practice liposomal DOX having lower cardio toxicity and improved bioavailability has been adopted widely. (103)

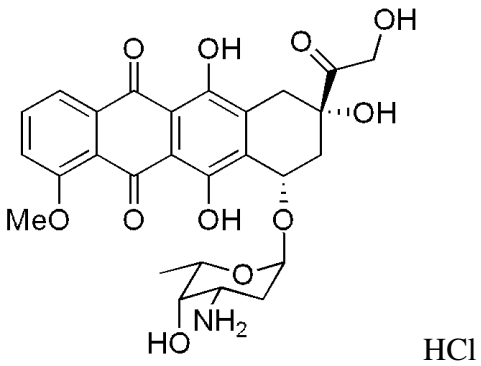


Figure 3:1. Chemical structure of doxorubicin hydrochloride (DOX).

Other component of combinational drug delivery is P-gp inhibitors. In the current thesis, the primary aim is to screen some of the natural P-gp inhibitors. Most of them considered in the study are flavonoids as shown in figure 3.2. Table 3.1 describes their properties and implications studied so far in combination with chemotherapeutic agents.

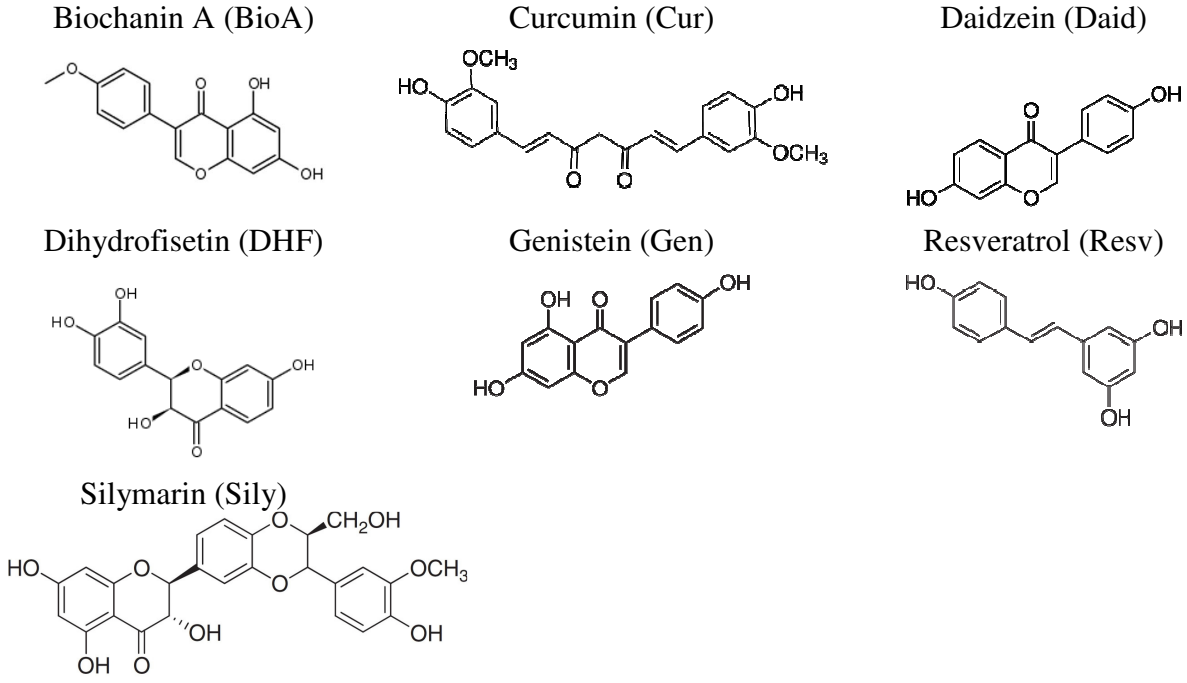


Figure 3:2. Structure of P-gp inhibitors used for our study.

Table 3.1. P-gp inhibitors and their combinations studied in different cancer.

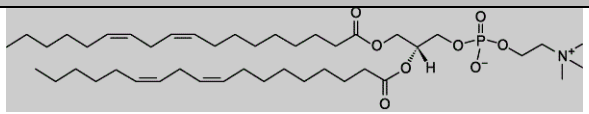
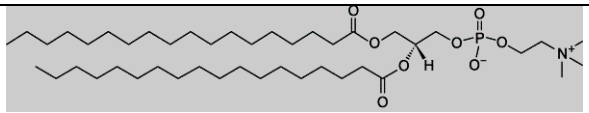
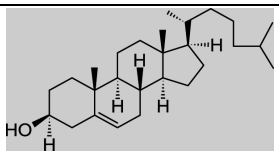
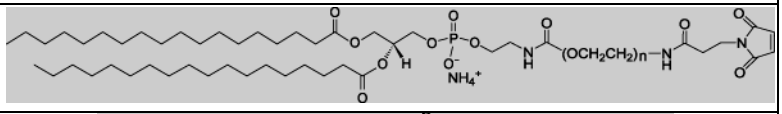
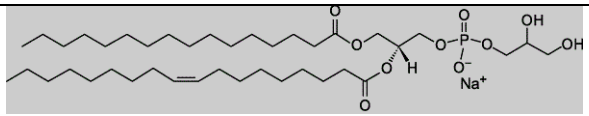
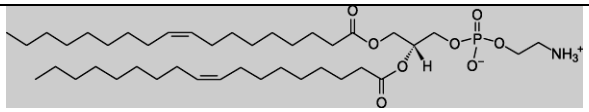
Name	Limitations	Other agent combined	Cancer models system	Reference
Biochanin A	Aqueous insoluble; lower bioavailability;	Vincristine, sorafenib, DOX, radiotherapy, gemcitabine;	Liver (HepG2, SNU449, Huh-7), breast (MCF-7, LCC6), colon, pancreas (MiaPaCa2, AsPC1)	(111-115)
Curcumin	Aqueous insoluble; lower bioavailability; rapid clearance; degradation in basic pH	DOX, 5-fluoro uracil, cyclophosphamide, dasatinib, cisplatin, cytarabine, caclitaxel	Lungs (LL/2 and MS1); pancreas (PANC-1, MiaPaCa-2), lymphoma (HT/CTX), colon (HCT-116, HT-29), cervical (SiHa), leukemia, ovary (A2780)	(48, 116-120)
Daidzein	Insolubility, inconsistent effect in combination,	Nitrofurantoin, tamoxifen, DOX, radiotherapy,	Pharmacokinetic interaction, breast (MCF-7), prostate (PC-3),	(121-125)
Genistein	Insolubility, inconstant effect in combination,	Paclitaxel, imatinib, 5-fluorouracil, nitrofurantoin, DOX, cisplatin, radiotherapy,	Pharmacokinetic interaction, colon (HT 29), breast (MCF-7), ovary (A2780), pancreas (BxPC-3), prostate (PC-3)	(121, 122, 125-131)
Resveratrol	Insolubility, limited bioavailability	Gefitinib, DOX, temozolomide, ProstaCaid, paclitaxel, cisplatin, oxaloplatin,	Mouse models of mammary cancer, melanoma, glioma, prostate, lungs (A549, EBC-1, Lu65), breast (MCF-7), ovary (A2780),	(132-135)
Silymarin	Mixture of components, different in solubility	DOX, paclitaxel, taurine, baicalein	Colon (LoVo), liver (HepG2),	(136-139)

3.2.2. Excipients and biomaterials used

In the experiments in this thesis excipients used can be categorized in to three classes such as lipids, biodegradable polymers and stabilizers.

Lipids used in the study are SPC, HSPC, cholesterol, DSPE-PEG, EPG and DOPE. The physicochemical properties and features of the lipids are presented in table 3.2. From the table, SPC, HSPC and cholesterols were used as most frequent constituent of liposomes. HSPC improves the stability due to improved packing of saturated hydrocarbon chain. EPG and DOPE were employed to incorporate charge in to liposome. PEGylated liposomes improve the stability under *in vivo* conditions. Thus we used DSPE-PEG for preparation of stearic stabilized liposomes.

Table 3.2. Properties and structure of lipids used.

Lipid	Saturation and Tm	Structure
SPC	Unsaturated, -57˚C	
HSPC	Saturated, 53˚C	
Cholesterol	NA, mp-148˚C	
DSPE-PEG	Saturated, NA	
EPG	Saturated, -2˚C	
DOPE	Unsaturated, -16˚C	

Other biodegradable polymer used is mPEG-PCL. It is an amphiphilic copolymer of PCL reported to have P-gp inhibitory activity.(140) However, for drug delivery the concentration employed is lesser than its P-gp inhibitory concentration. The hydrophobicity of PCL renders formation of micelles at very low concentration and makes it suitable candidate for encapsulation of hydrophobic moieties. (141) PVA and Pluronics are the stabilizers employed for stabilization of biphasic systems. These are most commonly used polymers for stabilization of biphasic systems in pharmaceutical formulation. The incorporation, advantages and suitability of these in nano-formulations is elaborated in our review on PCL. (94)

3.3. Methods

3.3.1. Cell lines and development of DOX-resistant cell lines

Chronic myelogenous leukemia (K562) and colorectal adenocarcinoma cell line (COLO205) were used to develop their DOX resistant counterparts. K562N was a kind gift from Dr Soumen Chakraborty, Institute of Life Sciences, Bhubaneswar and ColoN was procured from NCCS (Pune, India). K562N and ColoN cells were maintained in IMDM and RPMI-1640 respectively supplemented with 10% fetal bovine serum, 1% penicillin streptomycin and amphotericin-B. Incubation was done in a humidified atmosphere with continuous supply of 5% CO_2. Typically sub-culturing was done 2-3 times cell per week.

K562N and ColoN were treated with gradually increasing concentration of DOX to develop resistant counter parts K562R and ColoR respectively. The concentration was increased from 0.01 µM to 1 µM and from 0.01 µM to 0.1 µM to get ColoR and K562R cells respectively. Moderate increment of concentration was done in every 2-3 passages ensuring sufficient cell density. DOX resistance developed (in ColoR and K562R cells) was evaluated for change in

sensitivity comparison to similarly treated ColoN and K562N cells using XTT cell viability assay. The procedure of development of acquired drug resistance took about 8 months and 5 months in ColoN and K562N cells respectively. After the selection, resistant cells ColoR and K562R were maintained in similar conditions like non-resistant cells with intermittent DOX stress. However prior to an experimental setup, the resistant cells were maintained in DOX free media for a week.

3.3.2. Evaluation of resistance developed

The resistance developed was analyzed by XTT based cell viability study. Direct cell counting in coulter counter was adopted to analyze the alteration of growth pattern after development of resistance. For growth pattern studies, both cell counting and XTT assay were performed simultaneously to correlate the results.

3.3.2.1. XTT cell viability assay

This assay is based reduction of slightly yellow colored XTT to produce a brightly orange colored formazan by mitochondrial enzymes. Reduction of XTT occurs outside the cell (as negatively charged XTT cannot enter in to the cell) by NADH produced by mitochondria in electron transport chain. Since the formazan is water soluble, it eliminates need for solubilization and can be adopted for real time analysis.

Here the general procedure adopted for XTT based cell viability assay is described. (82) At individual sections of the thesis different cell lines were used and treatment was done different agents singly or in combination.

- About $10x10^3$ cells were sown in to individual well of a 96 multi-well plate.
- Treatment was done singly or in combination of agents at various concentrations.

Note: When treatment was done with combination, P-gp inhibitor was treated 2h prior to treatment of DOX. Here, treatment with DOX and P-gp inhibitors was done after reconstitution of DMSO dissolved stock with cell culture media. In all cases the final concentration of DMSO was restricted to a non-toxic level of ≤0.5%.

- The treated cells and untreated controls were allowed to grow for 72 h or specified time period at cell culture conditions.
- After the incubation period, 50 µl of XTT and PMS at a final concentration of 330 µg/ml and 2.6 µg/ml respectively were added in to each well.
- The plates were further incubated for reduction of XTT into a colored product and the intensity was measured at of 490 nm in a micro-plate reader (iMark, BioRad) at different time points.
    - Viability of cells at different treatment points were calculated setting absorbance from the untreated cell to 100%.

$$Percentage\ viability\ = \frac{absorbance\ obtained\ from\ treated\ cells}{absorbance\ obtained\ from\ untreated\ cells} \times 100$$

    - IC_{50} of agents K562N and K562R cells was determined from the dose response curve.

3.3.2.2. Cell counting by coulter counter

Alteration in growth pattern upon development of resistance in K562R and ColoR was compared to non-resistant K562N and ColoN respectively. The cell numbers were analyzed directly in coulter counter and viability was determined indirectly by XTT based cell viability assay simultaneously. $10x10^4$ and 10^4 cells were sown in to each well of 24- and 96-well plates respectively and immediately treated with DOX in different concentrations. At specified time

points post treatment, i.e.24 h, 48 h and 72 h, absolute cell numbers were counted using coulter counter after suitable dilution and percentage cell viability was calculated in comparison to untreated cells. Similarly XTT assay was performed to analyze comparative cell viability from 96-well plate as described in previous section and percentage viability was calculated.

3.3.3. Screening of P-gp inhibitors for P-gp inhibition and reversal of DOX resistance

We screened some of the natural sourced P-gp inhibitors to combine with DOX for reversal of acquired chemo-resistance. The list of inhibitors we used for screening is presented in figure 3.2. Their P-pg inhibitory activity was analyzed by calcein accumulation assay in flow-cytometry and their reversal of DOX resistance activity was analyzed by XTT assay.

3.3.3.1. Evaluation of P-gp inhibition by natural P-gp inhibitors

Calcein AM is a non-fluorescent compound which permeates into the cell and gets hydrolyzed by intracellular esterases to result a fluorescent anion calcein. Resistance cells over-expressing P-gp rapidly efflux non-fluorescent calcein AM from the plasma membrane and thus decrease the cyto-plasmic accumulation of calcein. Inhibition of P-gp activity by various P-gp inhibitors results in increased calcein accumulation. Intracellular calcein fluorescence can be measured with excitation and emission wavelengths of 485 nm and 535 nm respectively. Thus increased calcein accumulation directly correlates with P-gp inhibitory activity of substrates.

To analyze accumulation of calcein-AM as a measure of P-gp activity, $10x10^4$ numbers of cells were sown in to each well of 24 well plates. Cell either treated with specified concentration of P-gp inhibitors or left untreated for the treatment period. Following the treatment 0.25-0.5 μM of calcein-AM was treated and allowed intracellular accumulation and de-esterification to calcein for 15 mins in culture condition. Then the cells were washed once

with ice cold PBS and re-suspended in sheath fluid containing 1% PFA and kept at 4˚C until acquisition in flow cytometry. Accumulation of calcein (fluorescent P-gp substrate) in $10x10^3$ gated cells was measured in terms of MFI in FL-1 and presented comparatively considering MFI obtained from untreated cells as 100%.

3.3.3.2. Cellular uptake of DOX or P-gp inhibitors

$10x10^4$ cells were sown in to each well of a 24-multiwell plate and treated with specific concentration DOX or P-gp inhibitor in DMSO assisted dispersion or nano-formulation. The treated cells then incubated for 2-4 h at culture conditions. Following accumulation, cells were harvested and washed once with ice cold PBS. The cells then re-suspended in sheath fluid containing 1% PFA and kept at 4˚C until acquisition in flow-cytometry. Data acquired from $10x10^3$ gated cells was plotted based on the MFI obtained from FL-1 and presented as absolute values.

3.3.4. HPLC methods for detection and quantification

Instrumental set up: HPLC used was a binary gradient system equipped with C18 column, column oven and PDA detector. Solvent degassing was done by bath sonication for 30 min and application of vacuum. The selected agents were quantified by individual methods but when combination of agent had a closer maximal absorbance wavelength (DOX; 232 nm, BioA; 262 nm), method for simultaneous detection and quantification for the combination was developed.

HPLC method for quantification of DOX

HPLC method for detection and quantification of DOX was performed using a binary mobile phase of ACN and 0.5% OPA (pH 3) in water at ratio of 70:30. C18 column was used as a stationary phase and base line was stabilized with mobile phase at a flow rate of 1.5 mL/min.

DOX was detected at 232 nm and quantified using correlation function obtained from a 5-point calibration curve of Cur diluted in a solvent mixture consisting ACN and water 50:50.

HPLC method for quantification of Cur

HPLC method for detection and quantification of Cur was performed using a binary mobile phase of acetonitrile and 0.1%OPA in water at ratio of 70:30. C18 column maintained at 40˚C was used as a stationary phase and base line was stabilized with mobile phase at a flow rate of 1 mL/min. Cur was detected at 420 nm and quantified using correlation function obtained from a 5-point calibration curve of Cur diluted in a solvent mixture consisting ACN and water 50:50.

HPLC method for quantification of DOX and BioA

HPLC method for detecting DOX and BioA as individually were adopted from Dharmalingam et al. and Chen et al. respectively. (142, 143) Since they have a closer maximal absorbance wavelength, HPLC method was suitably modified and optimized to detect and quantify DOX and BioA simultaneously. Briefly, separation was done using solvent system of water containing 0.5% OPA as solvent-A and ACN as solvent-B that was run in a binary gradient system. PDA detector was used for detection of DOX and BioA by measuring absorbance at 232 nm and 262 nm respectively. For determining the conditions for proper separation, solvent B composition was varied between 30-80% in isocratic or gradient system. The optimal condition was evaluated based on optimal separation and area under the peak and used for further studies.

3.3.5. Preparation of drug encapsulated liposomal nano-dispersion

Liposomes can be formulated and drugs can be loaded in different methods possess advantage of precise size control by adaptation of various size reduction methods. When hydrophobic drug is mixed in the lipid mixture before formulating them in to liposome, it remains in the lipid bilayer (figure 3.3 (a)). After rehydration of lipid and liposome formation, the drug incorporated remains in molecular dispersion along with carrier thus increases drug dispersibility.

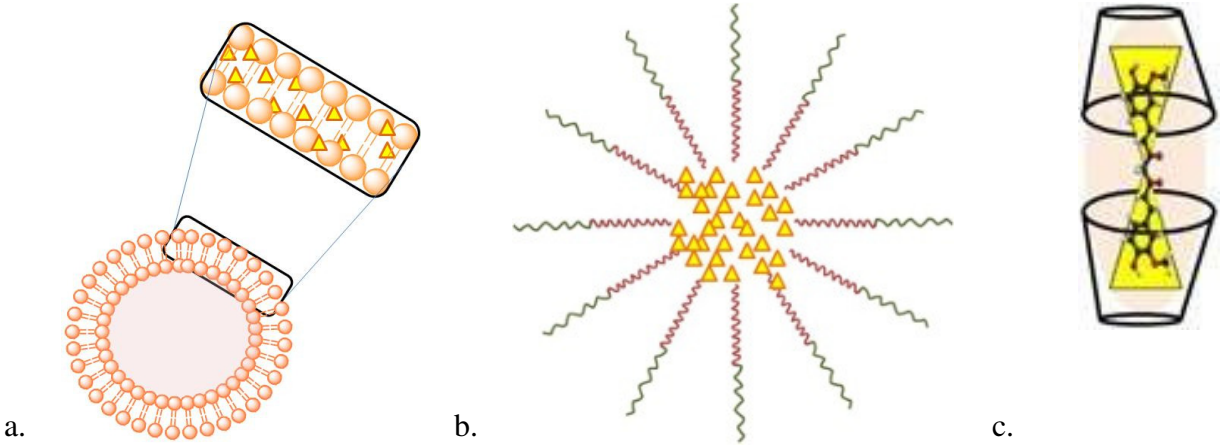


Figure 3.3. Solubility improvement of hydrophobic compounds by cyclodextrin encapsulation (a), micellar solubilization (b) and liposomal encapsulation (c).

Liposomes were prepared by thin film rehydration technique as described in the literature. (144, 145) Procedure for preparation of liposomes with drugs containing in the liposomal membrane is presented in figure 3.4. Briefly, all the lipids in the desired ratio and the hydrophobic drug were dissolved in a round bottom flask in a solvent mixture consisting acetone: methanol (2:1). If needed complete dissolution was achieved by sonication of prepared mixture. Then the lipid-drug mixture was evaporated to dryness as a thin layer in a rotary evaporator. The thin film formed around walls of the flask was further kept overnight vacuum in a lyophilizer to remove out traces of organic solvent. The film was then rehydrated with PBS or 250 mM ammonium sulfate by vortexing and/or sonicating.

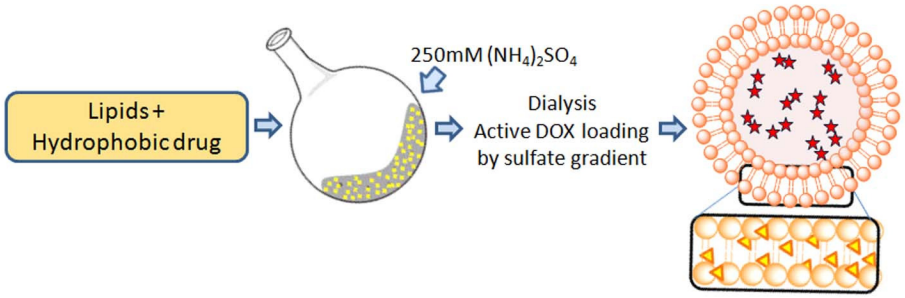


Figure 3.4. Preparation of liposomes with drug loaded in the bilayer.

The liposomes formed were then extruded through 100 nm polycarbonate membrane 21 times to get uniformly sized liposomes. Liposomes formed kept at 4˚C until pharmaceutical characterization and biological evaluation. The drug loading was determined by quantification in HPLC after breaking the liposome by addition of equi-volume of ACN.

For preparation of DOX loaded liposomes, a sulfate gradient mediated active loading was adopted (figure 3.5). It is considered as most effective that can load up to 1/3 of the total lipid with encapsulation efficiency of almost 100%. Initially lipids were redispersed with 250 mM ammonium sulfate. Sulfate gradient was created by dialysis of these liposomes after extrusion. Dialysis was done against 150 mM NaCl for 4 h to get sulfate gradient across the liposomal bilayer. For DOX loading pre-measured quantity of DOX solution was added and allowed for active encapsulation for 1 h at 37˚C with moderate stirring.

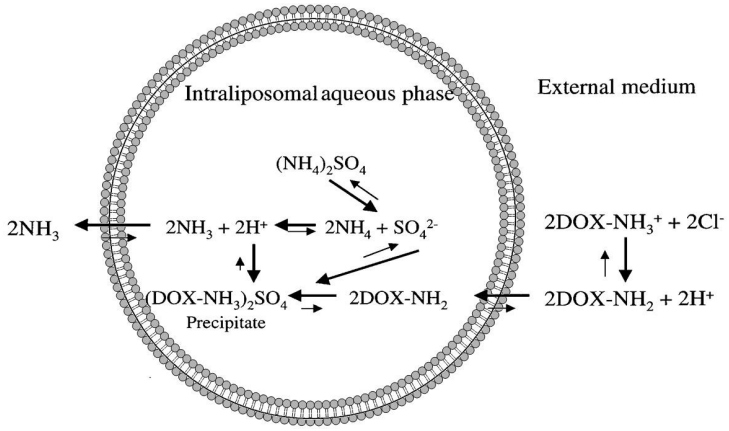


Figure 3.5. DOX loading in to liposomes by ammonium sulfate gradient.(146)

3.3.6. Micellar solubilization in MPEG-PCL micelles

Micellar solubilization is based on self-arrangement of amphiphilic molecules. When amphiphilic molecules placed in aqueous medium, they self arrange themselves to minimize the interaction of hydrophobic segments with external aqueous environment (figure 3.3 (b)). The arrangement results a micelles with hydrophilic exterior and a hydrophobic core where in lipophilic drugs can be partitioned and placed. Lipophilic drug in the micellar core remains in the dispersion and possess physicochemical properties same aqueous soluble ones.

MPEG-PCL micelles containing hydrophobic agent was prepared using emulsification and solvent evaporation method as shown in figure 3.6. (94) Briefly, hydrophobic drug and MPEG-PCL stocks were prepared using acetone. Drug and polymers were mixed at a ratio of 1:20 and dispersed in aqueous phase. The dispersion formed was soincated for 30 sec in probe sonicator with 50% amplitude of 130 W ultra sonic processor and emulsion formed than evaporated for 8 h on a magnetic stirrer with moderate stirring. The micelles formed than centrifuged at 2000 x g for 10 min at 4˚C to remove any un-encapsulated drug. The supernatant containing micellar hydrophobic drug loaded micelles were stored at 4˚C.

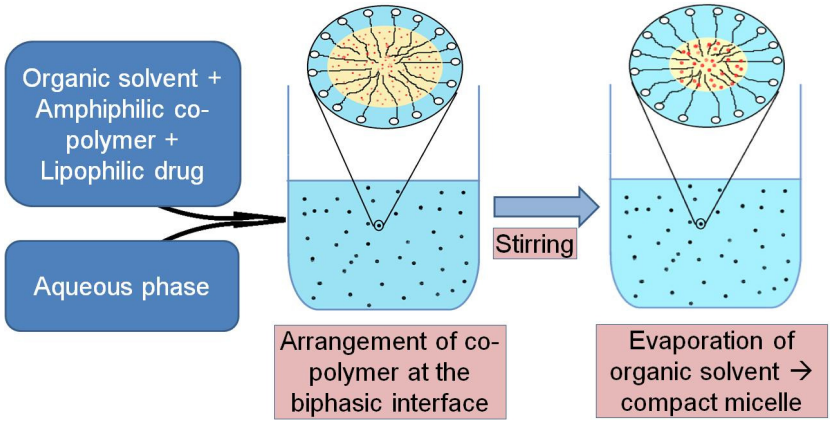


Figure 3.6. Preparation of PCL co-polymeric micelle.(94)

3.3.7. Encapsulation of hydrophobic compounds in HP-β-CD

As shown in the figure 3.3(c), cyclodextrin possesses a hydrophilic exterior and hydrophobic cavity. Any hydrophobic drug fits in to its cavity can be encapsulated. However, the stoichiometry of encapsulation varies and it may be 1:1, 1:2, 1:4 or it can be complexes having encapsulation ration of 1:8 or 1:16. (147, 148) Further it has been modified differently for improvement of encapsulation and formulation processing characters. (149) Since cyclodextrin are water soluble, encapsulated drug also remain in solubilized form. Thus, encapsulation in to cyclodextrin increases the water solubility of hydrophobic drugs.

At different instances we evaluated; optimal pH for encapsulation, presence of different stabilizer and use of different organic solvent for effective encapsulation. Following are the method used for encapsulation of hydrophobic agents in to hydrophobic cavity of cyclodextrin.

3.3.7.1. Emulsification and solvent evaporation

Encapsulation of hydrophobic compounds in to cyclodextrin was carried out in a modified protocol by Rachmawati et al.(150) Briefly cyclodextrin solution of pH range of 5-8 was prepared using citric acid or NaOH. 0.5 ml from each pH range were taken in a 1.5 ml micro-centrifuge tube and weighed to get the initial weight of the tube along with the solution. The hydrophobic agent solubilized in organic solvent was added to each of tube specific concentration to maintain its ration 1:2 with cyclodextrin. A maximum of 150 µl of organic stock was added to each tube and the weight was noted. Further the tubes were placed on a thermo shaker to allow evaporation of organic solvent at 37˚C under mild stirring. When the organic solvent is evaporated completely (based on weight difference), tubes were centrifuged at 2000 rpm and supernatant was analyzed in HPLC to quantify encapsulated hydrophobic drug.

3.3.7.2. Freeze drying

An alternative way of encapsulation of hydrophobic drugs in to cyclodextrin is freeze drying. Here we removed organic solvents selectively from frozen emulsion of hydrophobic agent in organic solvent in cyclodextrin solution at about -80˚C. The organic solvents used i.e. acetone and DCM has freezing points of -78˚C and -95˚C respectively. Thus in the frozen condition the aqueous phase will be frozen but the organic phase will remain in liquid state. Thus upon application of vacuum the organic phase will be evaporated faster with in short period of time. Then the thawed solution was evaporated under vacuum for 12 h to remove traces of organic solvent and centrifuged to precipitate un-entrapped aggregates of Cur. The cyclodextrin solubilized Cur remains in the supernatant.

Chapter 4. Combinational nano-formulation for reversal of DOX resistance in K562 cells

4.1. Background information

Chronic myeloid leukemia cell line K562 has been studied in various studies as a model system for drug resistance. DOX resistance in the models system was mainly mediated by over-expression of P-gp. (151, 152) In similar study, we aim to develop a combinational nano-formulation of P-gp inhibitor and doxorubicin (DOX) for reversal of acquired DOX resistance in K562 cells. The P-gp inhibitory activity of different agents varies in different model systems. In addition different anti-cancer agents acquire resistance by different cellular pathways. Thus for each chemotherapeutic agent and model system, P-gp inhibitors has to be screened for case to case basis for their efficacy for reversal of resistance. After development of DOX resistant K562 (K562R) cells from non-resistant cells (K562N), few selected natural P-gp inhibitors such as BioA, Cur, Daid, DHF, Gen, Resv, Sily etc. were screened for their P-gp inhibitory activity and efficacy to re-sensitize the K562R cells to DOX. Cur was found to be most suitable agent. Cur is a known hydrophobic agent. Thus we formulated different nano-formulations of Cur for improving its water dispersibility. Those nano-formulations were studied for their uptake, cyto-toxicity and efficacy to reverse DOX resistance in K562 cells. From the above studies, we deduced liposomal Cur as most suitable nano-formulation. Thus we prepared liposomes co-loaded with Cur and DOX and evaluated for its formulation as well as therapeutic properties.

4.2. Methods

4.2.1. Evaluation of resistance development and alteration of growth pattern

Resistance developed was evaluated by XTT assay as described in section 3.2.2.1. Briefly, about $10x10^3$ number of K562N and K562R cells were sown in to individual well of a 96 multi-well in

triplicate. Treatment with DOX in a concentration range of 0.01-10 µM was done. After the incubation period XTT based cell viability assay was performed and percentage viability was calculated as described earlier. After resistance developed, the growth pattern of non-resistance and resistance were studied as described in section 3.2.2.2. The growths of non-resistant and resistant cells were quantified in presence or absence of 0.1-10 µM of DOX. The percentage viability in each treatment range was calculated considering viability (number in case of coulter counter and absorbance in case of XTT) of untreated cells as 100%.

4.2.2. Selection of P-gp inhibitor for reversal of DOX resistance in K562 cells

Prior to screening of P-pg inhibitors, validation of reversal of DOX resistance by standard P-gp inhibitor verapamil in this model system was performed. Thus K562R cells were co-treated with verapamil (10-40 µM) and DOX (0.01-10 µM) and XTT based cell viability assay was performed as described previously.

To determine appropriate dose of P-gp inhibitors, we determined the safe concentration of P-gp inhibitors by treating them in a concentration range of 0.1-100 µM in both K562N and K562R cells. Their dose response curve in both the cell lines were plotted from the XTT cell viability assay as described in section 3.2.2.1. The P-gp inhibitors were studied for their effect on calcein accumulation and sensitivity of DOX in K562R cells. P-gp inhibitory activity in K562R cells was performed as described in section 3.2.3.1. Here various P-gp inhibitors were treated at different concentration as per their sub-toxic dose (indicated in table 4.1) followed calcein assay after 4 h. Enhancement in accumulation was presented comparatively to that of untreated cells.

In a further step to validate the functional significance of P-gp inhibition, we analyzed sensitization of resistant cells to DOX by co-treatment of P-gp inhibitor with DOX. For this

purpose we treated K562R cells at different concentration of P-gp inhibitor (indicated in table 4.1) and 2 h post-incubation, DOX was treated at different concentrations ranging 0.1-10 µM. After 72 h of incubation XTT assay was performed as described in section 3.2.2.1.

Table 4.1. Doses of P-gp inhibitors used for biological activity study.

P-gp inhibitor	Dose for calcein accumulation assay	Dose for reversal of DOX resistance
BioA	100 µM	20 µM, 40 µM
Cur	40 µM	20 µM, 40 µM
Daid	100 µM	50 µM, 100 µM
DHF	100 µM	50 µM, 100 µM
Gen	50 µM	50 µM, 100 µM
Resv	50 µM	20 µM, 40 µM
Sily	100 µM	50µM, 100µM
Verp	40 µM	10-40 µM

4.2.3. Determination of optimal dose of Cur for reversal of DOX resistance

P-gp inhibitor must inhibit efflux of drug at a dose below its cyto-toxic. Thus to find out suitable dose of Cur, we analyzed dose of Cur needed for P-pg inhibition and reversal of DOX resistance. For P-gp inhibition, calcein accumulation in K562N and K562R cells were analyzed after treatment of Cur at 20-60 µM to K562R cells. Calcein assay was performed as described previously (section 3.2.3.1) and accumulation in K562R cells were compared to accumulation in K562N cells after pretreatment with Cur.

In a second study, K562N and K562R cells were sown into each well of 96 multi-well plates for XTT assay as described in section 3.2.2.1. Cur was pretreated to K562R cells in a concentration range of 10-40 µM and following 2 h of incubation both K562N and K562R cells were treated with DOX in a concentration range of 0.01-10 µM. The treated cells along with untreated controls were processed with XTT assay and percentage viability was determined as described previously (section 3.2.2.1).

4.2.4. Preparation of Cur nano-formulations to improve solubility

In different model system, Cur faced number of physicochemical and pharmacokinetic problems. Its hydrophobic nature makes Cur difficult to deliver and after oral administration and rapid elimination in feces render its oral bioavailability very low. Upon parentral administration, it is rapidly eliminated from plasma.(153, 154) Thus a nano-formulation that enhances its solubility and enrich delivery system with controlled release feature much desired to overcome above mentioned problems. (155) Till date Cur has been evaluated for its activity in different carrier system and nano-formulations. Here we prepared, different nano-dispersion of Cur such as liposomal Cur (CurL), micellar Cur (CurM) and nano-sized Cur (CurN) and evaluated their activity for drug resistance in comparison to DMSO assisted nano-dispersion of Cur.

4.2.4.1. Preparation of DMSO assisted Cur nano-dispersion (CurD)

For preparation of DMSO assisted nano-dispersion of Cur, 50 mM of Cur stock was prepared in DMSO. Further it was diluted in cell culture media to a working stock of 500 µM. Dilutions were prepared from it and treated in a final concentration of 10-40 µM. Under these preparative conditions, the translucent dispersed Cur was in nano-sized range and contained non-toxic concentration of DMSO.

4.2.4.2. Preparation of liposomal Cur (CurL)

Liposomes encapsulating Cur in the bilayer was formulated by lipid film rehydration technique. For preparation of liposomes, HSPC, cholesterol and DSPE-PEG of 10 mM stock were mixed in a round bottom flask in a molar ratio of 56.3:38.4:5.3 respectively. The formulation procedure of liposomes and size reduction procedure adopted were described in section 3.2.5.

4.2.4.3. Preparation of micellar Cur (CurM)

For preparation of micelles, 3.6 mg/ml (10mM) and 50 mg/ml stock of Cur and MPEG-PCL respectively were prepared using of acetone. 0.4 ml of MPEG-PCL and Cur from the above stocks was dispersed in 10 ml of distilled water and soincated for 30 sec in probe sonicator. Detailed procedure of evaporation of organic solvent and separation of micellar Cur were followed as described in section 3.2.6.

4.2.4.4. Preparation of HP-β-CD encapsulated Cur (CurN)

Here 2.5 mM of Cur solubilized in acetone was emulsified in 5 mM HP-β-CD at different pH adjusted by using citric acid. Cur was encapsulated in to HP-β-CD by emulsification and solvent evaporation method as described in section 3.2.7.1. Encapsulation in basic pH was excluded as Cur was not stable at basic pH. However, following this process the EE resulted was very low ranging between 2-3% at different pH as described in table 4.2. This lower EE was correlated well with available literature. (156-158) Thus in order to get effective encapsulation we followed a modified freeze drying method and optimized as described in following section.

Table 4.2. Encapsulation of Cur in to HP-β-CD at different pH.

Citric acid	pH	EE (%)
50 µM	3	2.58
20 µM	4	2.66
10 µM	5	2.84
5 µM	5	2.92
1 µM	6	3.08
Nil	7	2.2

4.2.5. Optimization of Cur encapsulation in to HP-β-CD using freeze drying method

Freeze drying method (figure 4.1) was adopted to encapsulate Cur HP-β-CD. Briefly, DCM or acetone was used as organic solvent to solubilize Cur and Pluronic F68 or PVA as stabilizer. Cur

was solubilized in the organic solvent and HP-β-CD, Pluronic as well as PVA were solubilized in distilled water. 0.2 mM Cur (in organic phase) was emulsified by gradually adding in to aqueous phase containing 0.5 mM HP-β-CD and 5 µM citric acid under bath sonication (ultra sonication was used in case of DCM for initial emulsification).

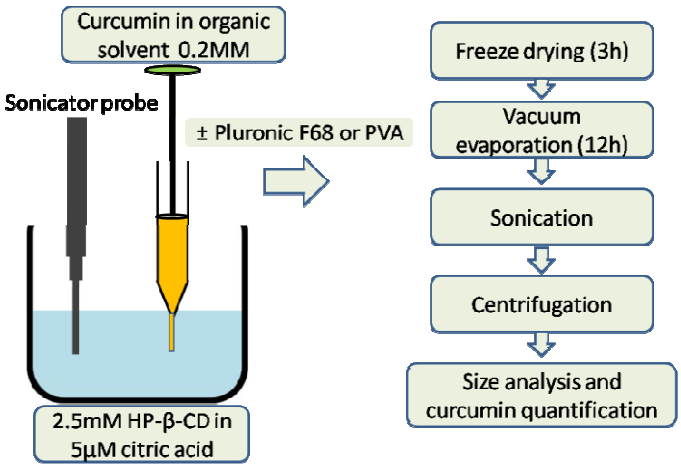


Figure 4.1. Procedure for encapsulation of Cur in HP-β-CD by freeze drying method.

The formed emulsion was stabilized by addition of 0.1% PVA or Pluronic-F68 and sonicated further. The various encapsulation conditions i.e. presence or absence of HP-β-CD and stabilized by different stabilizer are presented in table 4.3. The biphasic system was then frozen in -80˚C for 30 minutes prior to lyophilization for 3 h. Any trace of reminiscent organic solvent was removed by leaving the preparation overnight in vacuum desiccator. The nano-particulate suspension was ultra sonicated (70% amplitude, 30:10 pulse on-off cycle, 5 min) followed by centrifugation for 10 min at 2000xg to obtain non-aggregated nano-Cur in the supernatant. Optimal conditions were selected based on as particle size and encapsulation efficiency of nano-Cur.

Table 4.3. Formulations prepared to optimize effect of stabilizer and organic solvent.

DCM/ Acetone	Cur (0.2 mM)	HP-β-CD (0.5 mM)	PVA (0.1%)	Pluronic F68 (0.1%)
F1	+	-	-	-
F2	+	+	-	-
F3	+	-	+	-
F4	+	+	+	-
F5	+	-	-	+
F6	+	+	-	+

4.2.6. Evaluation of pharmaceutical propertries of Cur nano-formulations

Particle size and zeta potential were determined by using Zeta-sizer. Particle size was measured after suitable dilution to render the formulations nearly colorless solutions in a glass cuvette with recommended settings. Zeta potential of the same sample was measured using zeta dip-cell electrode.

Further EE of formulations were calculated after analyzing dissolved fraction in HPLC. Initially Cur from the formulations was brought in to free from by adding equi-proportional volume of ACN. Then the mixture was diluted in a 1:1 mixture of water and ACN to restrict the concentration to quantifiable range. Cur was quantified in HPLC conditions as described in section 3.2.4. After quantification the EE was calculated using following formula.

$$EE = \frac{Actual\ amount\ of\ drug\ present\ in\ the\ formulation}{Amount\ of\ drug\ taken\ initially\ for\ preparation\ of\ formulation} \times 100$$

4.2.7. Biological activity of Cur nano-formulations

4.2.7.1. Cyto-toxic activity

XTT based cell viability assay was performed to access the safety of Cur nano-formulations. Treatment of about $10x10^3$ K562R cells sown in to each well of a 96 well plate was done with

different nano-formulations equivalent of 5-40 µM Cur concentration. After 72 h of incubation cyto-toxicity assay was performed as described in section 3.2.2.1.

4.2.7.2. Cellular uptake study

Cellular uptake of Cur nano-formulations in K562R were analyzed after treating cells with 20 µM or 40 µM of Cur or formulation equivalent for 2 h. Harvesting, washing and acquisition was done as described in section 3.2.3.2.

4.2.7.3. P-gp inhibition study by calcein uptake

$10x10^4$ K562R cells were sown in to each well of a 24-multiwell plate and treated with 20 µM of Cur or equivalent formulation. After 2 h of incubation, cells were treated with 0.25 µM of calcein-AM and allowed to accumulate for 15 mins. The reaction was stopped by bringing the cells to ice cold temperature. Analysis of calcein accumulation was performed as described in section 3.2.3.1. The increase in calcein MFI is presented as percentage accumulation under different Cur nano-formulation treated conditions, considering calcein accumulation in untreated conditions as 100%.

4.2.7.4. Reversal of DOX resistance in K562R cells

Cur nano-formulations were co-treated with DOX to about $10x10^3$ K562N or K562R cells sown in to each well of a 96 well plate. Cur equivalent of formulation at a previously determined dose of 20 µM were 2 h pretreated to K562R cells. Then, DOX treatment was done in both non-resistant and resistant cells at a concentration range of 0.1-5 µM. The treated cells were then incubated for 72 h in culture conditions and XTT assay was performed as described previously in section 2.2.2.1.

4.2.8. Preparation and characterization of DOX and Cur co-loaded liposomes

Cur loaded PEGylated liposomes were prepared as described in section 4.2.4.2. Then DOX was then loaded at various dosing rations to Cur by active loading by sulfate gradient as described in section 3.2.5. Its particle size and zeta potential were measured as described in section 4.2.6.

Evaluation of release pattern of Cur from prepared liposomes was done by dialysis. 0.5mL of liposomal suspension was enclosed in a dialysis bag with MWCO 14KDa and placed in 10 ml of PBS containing 20% ethanol to maintain sink conditions. 40 µl samples collected at different time intervals and equal volume of release medium was replaced. The release medium was replaced completely after 24 h. The samples collected were analyzed in HPLC to determine Cur concentration. The released amounts obtained were converted to percentage of total Cur and release pattern was presented as cumulative release vs. time.

4.2.9. Biological activity study of co-loaded liposomes

Cellular uptake of liposomal Cur after DOX loading was analyzed as described in section 3.2.3.2 and section 4.2.7.2.Coloaeded liposomes were treated to K562R cells at concentration ranging from 125-500 nm and 10-20 µM of DOX and Cur respectively. The XTT cell viability assay was performed as described previously in section 3.2.2.1

4.3. Results and discussion

4.3.1. Development of resistance and characterization of resistant cells

From the cyto-toxicity studies on non-resistant (K562N) and resistant counterpart (K562R), the IC_{50} for K562R was determined to be 0.45 µM (Figure 4.2) which is almost a 300% increase, in comparison to K562N with an IC_{50} of 0.15 µM. Further studies were performed using developed the model system to screen P-gp inhibitors for their efficacy to reverse DOX resistance.

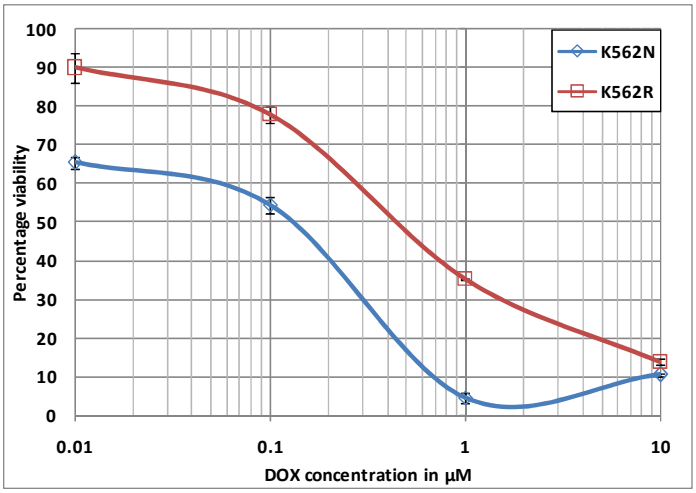


Figure 4.2. Dose response curves of DOX in K562N and K562R cells.

4.3.1.1. Resistant cells grows slower in comparison to non-resistant cells

Since resistant cells are often exposed to chemotherapeutic stress, they develop mechanisms to tolerate the stress. In this process cellular growth is generally compromised. To evaluate this, the growth of non-resistant and non-resistant cells in presence and absence of DOX stress was studied. As indicated in figure 4.3, non-resistant counter part of K562N had almost twice cell number in 72 h indicating a slow growth phenotype due to acquired drug resistance.

Furthermore, the percentage viability was calculated from the absolute numbers obtained from cell counting in coulter counter. The viability at different time points at different concentration was plotted for percentage viability Vs time post-incubation (figure 4.4 (a)). On the other hand similar percentage viability data was also obtained from the XTT assay. A similar graph was plotted (figure 4.4 (b)) to compare the observations from both techniques to determine cell viability. Here a good correlation was observed in the trends except for K562N treated at 0.1 µM concentration.

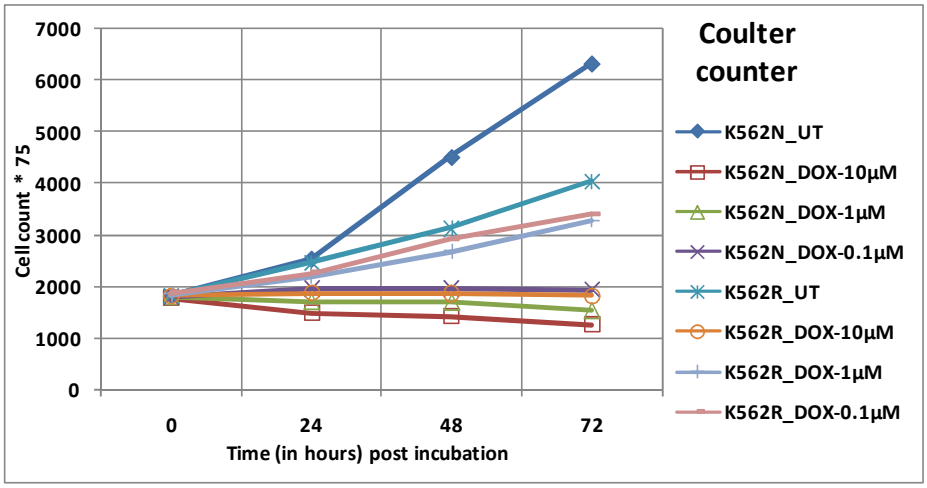
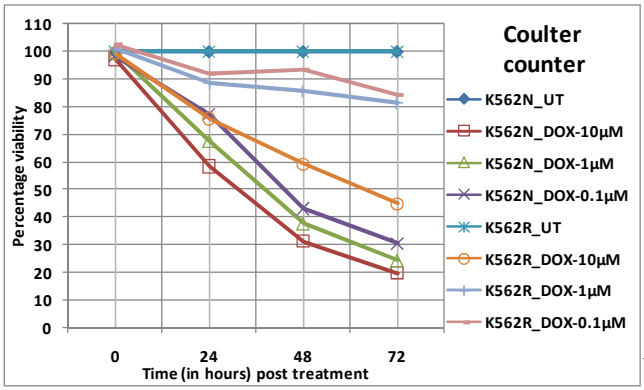


Figure 4.3. Growth pattern of K562N and K562R cell in presence and absence of DOX.

a.



b.

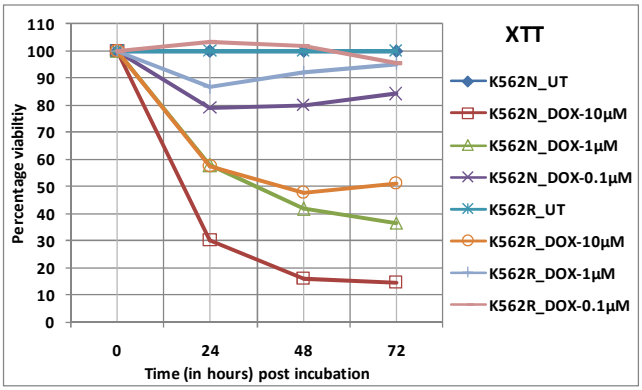


Figure 4.4. Percentage viability of K562 cell at different concentration of DOX determined by coulter counter (a) or XTT assay (b).

When DOX was treated at 0.1 µM, there was higher cell viability in coulter counter but viability was low in XTT assay. At this treatment point there was no growth of the cells observed (figure 4.3). Conceptually, in XTT cellular metabolic activity is being considered but in case of coulter counter cellular integrity of shape is taken into account. Thus 0.1 µm of DOX was treated, possibly the cells were metabolically inert but maintained their shape.

4.3.1.2. Verapamil reversed DOX resistance in K562R cells dose dependently

Verapamil a first generation P-gp inhibitor is able to inhibit efflux mediated by ABC transporters. It was observed to be active in vitro and in vivo conditions for reversal of drug

resistance by P-gp inhibitions and effective in cancer therapy. However in clinical trial it was found unsuitable for its application in circumvention of acquired drug resistance due the toxicity associated with it. (81) Still several study reports its use as a standard P-gp inhibitor in experimental conditions and thus we studied effect of verapamil on reversal of DOX resistance. When verapamil was been treated along with DOX, in developed resistance cell line K56R, it reversed DOX resistance dose dependently (figure 4.5).

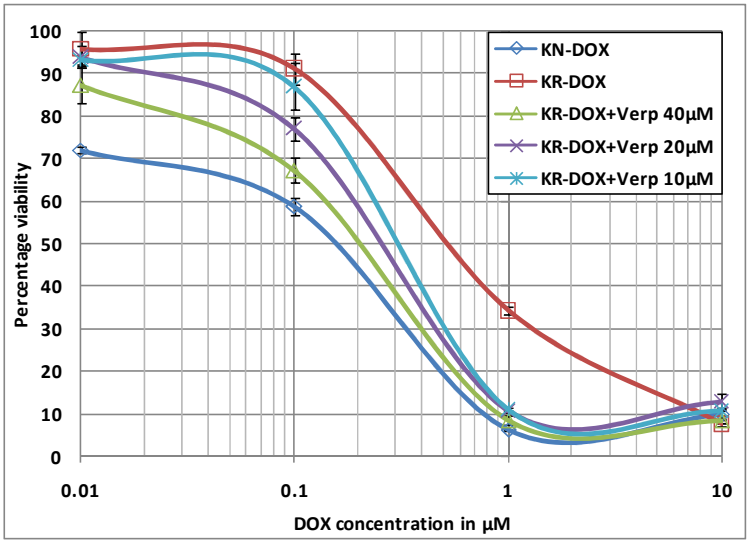


Figure 4.5. Reversal of DOX resistance by verapamil in K562R cells.

4.3.2. Selection of P-gp inhibitor for reversal DOX resistance in K562R cells

In recent practice, beneficial pharmacokinetic or pharmacological interaction of combination of P-gp inhibitors with chemotherapeutic agents in nano-formulation is widely studied. This approach has its application in reversal of drug resistance, reduction of toxicity, improvement of pharmacological activity, bioavailability improvement, etc.(159, 160) But to get interaction with pharmacologically significant and having minimal toxicity which are deliverable in a single formulation is indeed a challenge. In the search for suitable agents researchers explored numerous agents that included natural or novel synthetic entities and currently used therapeutic

agents or polymers. (81, 87, 88, 161) In this study, we aim to screen some of selected natural sourced P-gp inhibitors (figure 3.2) to combine them with DOX in a nano-formulation for reversal of resistance.

4.3.2.1. Determination of safe concentration of P-gp inhibitors

From the XTT assay after treatment with P-gp inhibitors, it was inferred there was very less difference in sensitivity of K562N and K562R cells. From the dose response curve it is marked that, P-gp inhibitors like BioA, Cur, Gen and Resv affected cell proliferation beyond 10 µM (figure 4.6). Their IC_{50} were varied between 40 µM-60 µM and therefore were studied their P-gp inhibitory activity in both safe concentration as well as sub-toxic concentration.

4.3.2.2. P-gp inhibitory activity of studied inhibitor in K562R cells

Enhanced calcein accumulation in K562R cells upon treatment with P-gp inhibitors is presented in figure 4.7. The change in accumulation was calculated considering, calcein accumulation in untreated resistance cells to be 100%. K562R cells accumulated only about 40% calcein to that of K562N cells. The accumulation in various treated conditions was calculated comparatively. As shown in the figure, out of screened agents, calcein accumulation in BioA and Cur treated cells increased by 149% and 184% respectively which was comparable to accumulation to that of verapamil (167%). In cancer cells over-expression of P-gp, in addition with other mechanisms may act together to result resistance. (162, 163) Thus their efficacy to reverse drug resistance must be evaluated to validate re-sensitization by any P-gp inhibitor.

a. b.

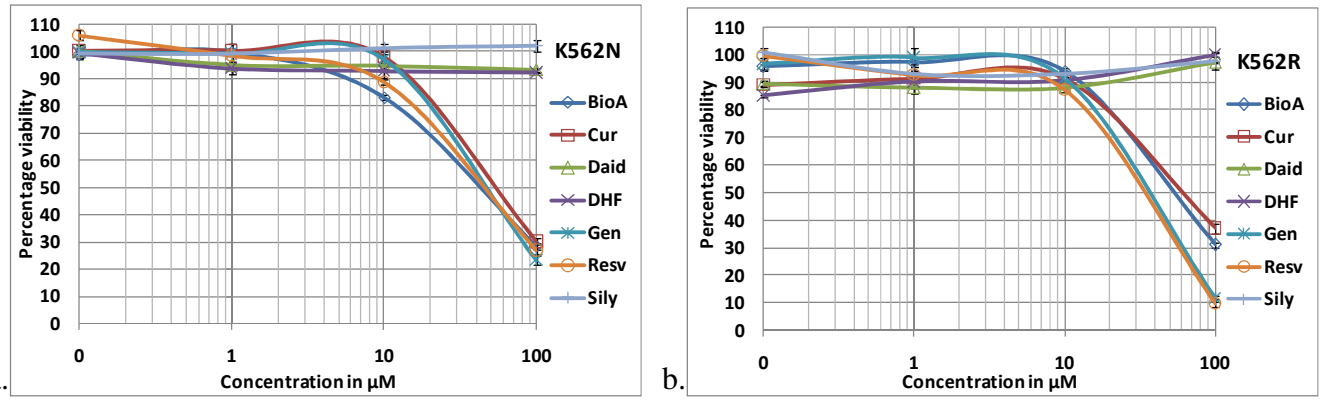


Figure 4.6. Cyto-toxicity of different P-gp inhibitors in K562N (a) and K562R (b) cells.

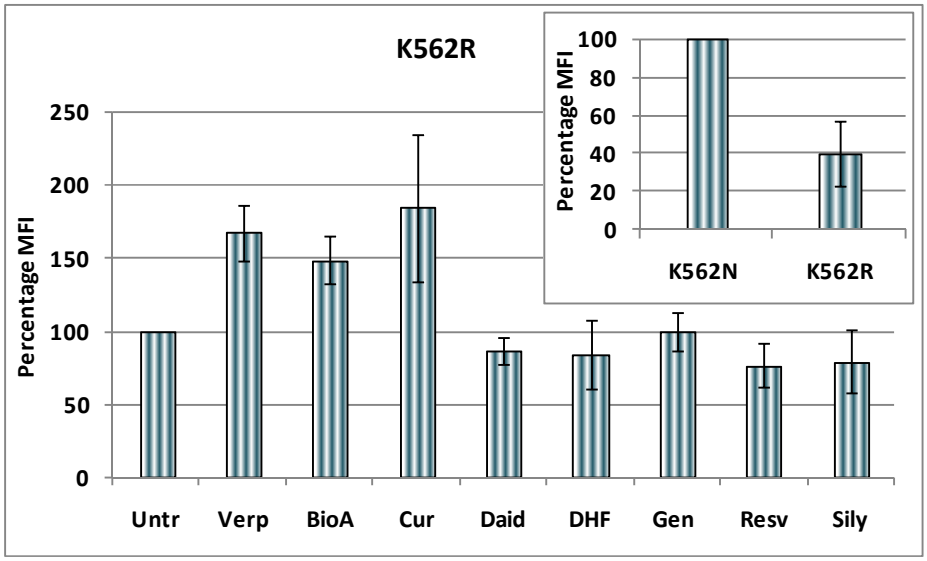


Figure 4.7. Enhanced calcein accumulation up on pretreatment with different P-gp inhibitors.

4.3.2.3. Reversal of DOX resistance by P-gp inhibitors

To validate the results from calcein accumulation assay, functional validation for reversal of DOX resistance was performed. Here, DOX was co-treated with P-gp inhibitors and the sensitivity was analyzed by XTT based cell viability assay. Results from XTT assay is presented in figure 4.8 (8a, 8b, 8c, 8d, 8e, 8f and 8g). BioA, Cur, Gen, Resv were able to sensitize K562R cells at lower doses of DOX (0.1 µM). However at DOX dose beyond 1 µM, only Cur was able to reverse drug resistance at concentration of 20 µM. Sensitization of K562R cells by other agents may be due to their anti-proliferative activity rather than P-gp inhibitory activity. Thus

Cur was found suitable for reversal DOX resistance in K562R cells and was considered for further studies to develop combinational nano-formulation along with DOX.

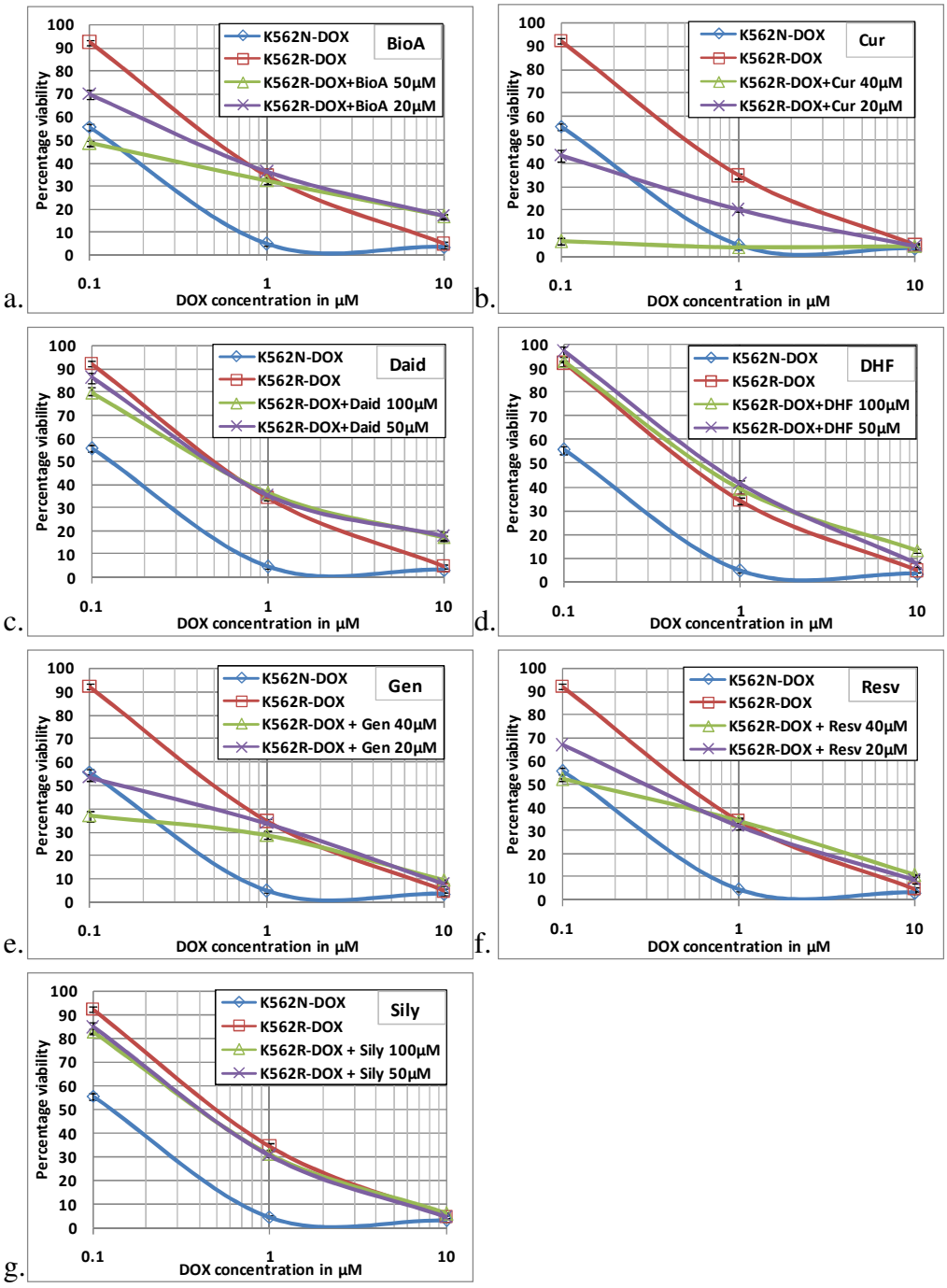


Figure 4.8. Effect of co-treatment of DOX with P-gp inhibitors such as BioA (a), Cur (b), Daid (c), DHF (d), Gen (e), Resv (f) and Sily (g).

4.3.3. Determination of dosing ration of DOX and Cur

The strategy of combinational delivery starts from selection of drugs for combination and finding an appropriate dosing ratio for the combination. Therefore, dose for both P-gp inhibitory activity as well as reversal of DOX resistance were determined. From the results of calcein accumulation assay, it was observed that resistant cells accumulated only 40% of calcein to that is accumulated in K562N cells (figure 4.9 (a). This indicates involvement of P-gp in conferring resistance. Upon co-treatment with Cur, accumulation of calcein increased dose-dependently. At co-treatment dose of 20 µM of Cur resistant K562R accumulated equivalent amount of calcein to that of K562N cells. Finally, 20 µM dose of Cur determined to be suitable dose for P-gp inhibition.

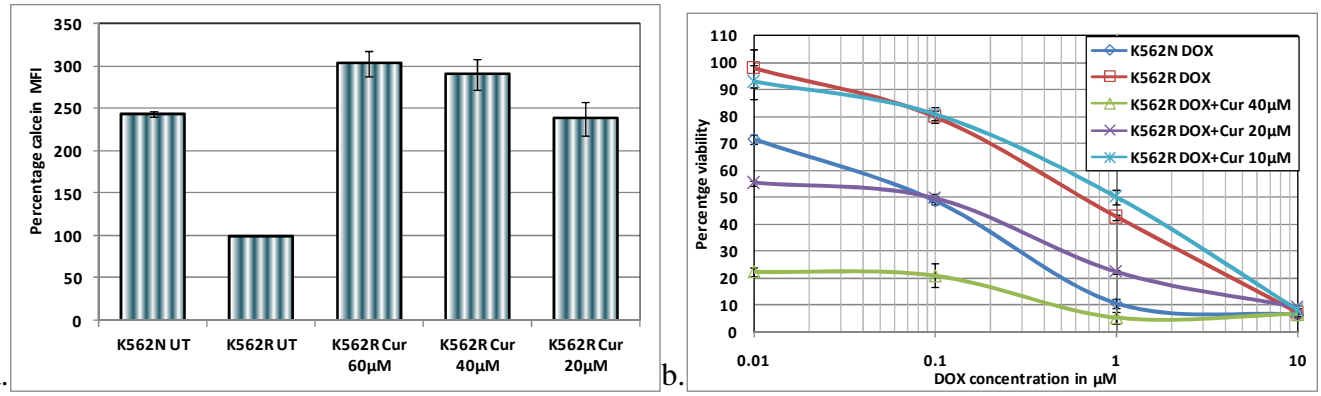


Figure 4.9. Dose of Cur needed for P-gp inhibition (a) and reversal of DOX resistance (b).

Furthermore, validation of reversal by Cur at 20µM was performed using cyto-toxicity studies in co-treated conditions and dose-response curves were plotted. As shown in figure 4.9(b), co-treatment with 20 µM of Cur along with DOX re-sensitized K562R cells were on par with K562N cells. Cur was observed to be insensitive at lower concentration (10 µM) and increased cyto-toxicity observed at 40 µM was due to inherent toxicity of Cur. Thus 20 µM of Cur was considered to be most appropriate for reversal of DOX resistance in K562R cells.

4.3.3.1. Approaches for solubilization of Cur

Chemo-sensitizing activity of Cur has been explored where in use of Cur along with DOX, gemcitabine, paclitaxel, cisplatin, vincristine etc. for potentiation and reversal of acquired resistance is suggested. (164-167) However, under in vivo conditions and clinical studies, it had lower bioavailability resulted from aqueous insolubility and rapidly cleared from systemic circulation. In addition hydrophobic nature of Cur makes it difficult to deliver. Delivery of Cur through nano-formulations looked promising that solved solubility problem by increasing dispersibility and bioavailability problem by controlled release. Nano-formulations containing Cur singly as chemotherapeutic, chemo-preventive and chemo-sensitizing agent or in combination with a chemotherapeutic agent has been studies widely. Different nano-formulations studied possessed different features of solubility enhancement, carrier retention and therapeutic activity. (155, 168)

There are several nano-formulation based approaches adopted so far for improvement of Cur solubility. (155) Encapsulation in to hydrophobic cavity of cyclodextrin was the most simple and effective. Cur encapsulation into cyclodextrins improved the activity in prostate and lungs cancer by increasing its water solubility. (147, 169) In another method micellar solubilization was adopted to increase aqueous solubility of Cur. Different amphiphilic co-polymers such as MPEG-P (CL-co-PDO), MPEG-PCL, Pluronic F-127, MPEG-PLA etc. were used to formulate Cur loaded micelles for treatment of cancers. Micellar solubilization increased the solubility, but biological activity of encapsulated form varied in different cell lines. (119, 170-172) Polymeric nano-particles of PLGA, vinyl acryl amide, polybutylcyanoacrylate etc. encapsulating Cur also increased the dispersibility of Cur and released Cur for a longer period of time. (173-175)

Furthermore, lipid based formulations of Cur as a primary or secondary carrier has been demonstrated in various studies to increase their water solubility or dispersibility. (156, 176)

4.3.4. Optimization of Cur encapsulation in to HP-β-CD

This is the simplest approach to increase Cur solubility where it is encapsulated in to cyclodextrins in a molecular stoichiometry of 1:1, 1:2 or 1:4. (177, 178) In various studies, the complexation improved solubility of Cur. Further improved Cur solubility led to improvement of its activity in ovarian cancer, squamous cell carcinoma etc and other inflammatory conditions. (156, 158, 178) But lower EE (1-5%) was the major obstacle resulted from the conventional method of emulsification and solvent evaporation as described in section 3.2.7.1. (157, 158, 179). The possible reasons for this lower encapsulation are as follows.

- Firstly the equilibrium between encapsulated and unencapsulated state is facilitated for the later. This may be a result of several factors such as cavity size, pH, ionic concentration etc.
- Secondly reason is possibly the cohesive hydrophobic interaction between Cur molecules are of higher strength in comparison to that of HP-β-CD and Cur. Resultantly during the dispersion of organic solvent in aqueous phase containing HP-β-CD Cur remains in the cavity as long as organic solvent is present (figure 4.10, case A). However up on evaporation of organic solvent Cur possibly forms intra-molecular large sized aggregates which settle down up on centrifugation.

Out of factors those effect encapsulation, effect of pH was studied and the results of which is presented in section 2.4.4.4. From the results, it can be inferred that, alteration of pH did not changed the encapsulation significantly (table 4.2). Therefore, for further attempt to reduce the cohesive interaction, incorporation of stabilizer in the aqueous phase to restrict formation of

intra-molecular aggregation of Cur was considered. During evaporation of organic solvent, stabilizer will restrict Cur in the hydrophobic cavity of HP-β-CD enforcing equilibrium towards encapsulated state (figure 4.10, case B). For optimization we used two most common stabilizers like PVA and Pluronic (94). Emulsification process was optimized using two different solvent such as acetone and dichloromethane.

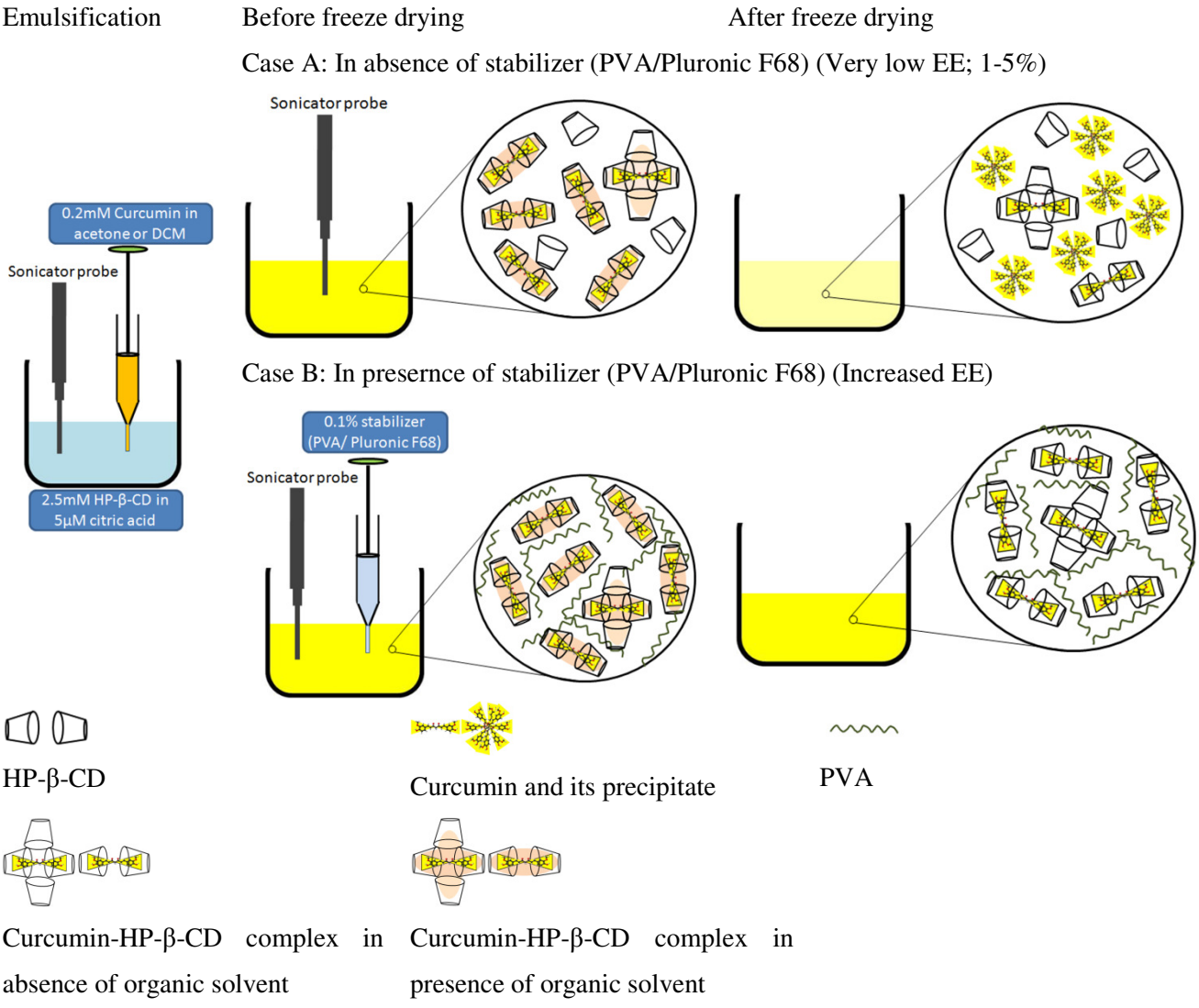


Figure 4.10. Encapsulation of Cur in HP-β-CD in absence and presence of stabilizer.

In a further step to get effective encapsulation, the difference in freezing temperature of water and organic solvent was adopted for selective evaporation of organic solvent. The freezing

point of water is 0˚C while, DCM and acetone freezes at -96.5˚C and -95˚C respectively. Therefore, freeze drying of the biphasic mixture frozen at -80˚C is expected to remove the non-frozen organic solvent at very faster rate in comparison to aqueous phase.

Other approaches adopted for preparation of nano-Cur are size reduction, flash evaporation, anti-solvent precipitation, complete freeze drying method etc (180-182). These methods use high shearing homogenization or increased temperature which may affect the stability of Cur. A method by Cheng et al., indeed yielded nano-Cur with higher EE, but requires specialized instrument setup with multiple processing parameters. Emulsification solvent diffusion resulted in higher nano-Cur size. Complete freeze drying resulted in smaller sized nano-Cur but re-dispersed nano particles were aggregated with in 48h to give larger sized particles (data not shown). Thus we followed a method as depicted in figure 4.1. Cur was encapsulated in to HP-β-CD using PVA and Pluronic F68 as stabilizer in two different emulsions consisting DCM-water and acetone-water. Formulations are prepared in various combinations of solvent and stabilizer (F1-F6) as indicated in table 4.4. Solubilized Cur in the supernatant was undertaken size analysis using DLS and Cur content was measured in HPLC. Table 4.4 presents average diameter and Cur content of different formulations prepared using different solvents.

Table 4.4. Characterization of nano-Cur prepared using DCM as organic solvent.

	Particle size (nm)		**Concentration of Cur in supernatant (µM)**	
	DCM	**Acetone**	**DCM**	**Acetone**
F1	349.4 ± 35.6	376.2 ± 24.8	22.7 ± 0.5	BDL*
F2	132.3 ± 26.7	237.4 ± 22.5	26.5 ± 0.9	8.5 ± 3.8
F3	171.8 ± 29.7	48.8 ± 11.4	82.9 ± 3.5	78.4 ± 5.1
F4	217.1 ± 35.1	39.2 ± 6.9	81.2 ± 9.0	117.6 ± 3.1
F5	177.3 ± 26.6	200.3 ± 17.2	30.9 ± 2.4	41.1 ± 7.9
F6	116.8 ± 30.3	269.7 ± 21.9	31.5 ± 1.7	91.6 ± 2.8

*Below detection limit

The comparative trend of alteration of nano-Cur size and solubilized quantities prepared in different condition is shown in figure 4.11. As shown in the figure irrespective of solvent used, presence of stabilizer decreased the size of Cur nanoparticles and increased the solubilized fraction in the supernatant. Presence of HP-β-CD alone in the specified concentration decreased the particle size but there was marginal increase in solubilized Cur. Effect of different stabilizers was prominently observed with acetone but not with DCM. There was not much difference in size of different formulations prepared using DCM (varied between 116-217 nm). On the other hand, acetone produced particles that ranged between 39-299 nm in different formulations. Furthermore, the comparative curve for acetone (figure 4.11) indicates, in F3 and F4 where in PVA was used as stabilizer, produced much lower particle size (40-50 nm and higher transparency; sample 3A and 4A) and resulted in maximum amount of Cur in the solubilized fraction (117 μM) (formulation F4). This lower size and higher solubilized fraction is possibly mediated by synergistic interaction of HP-β-CD and PVA. Therefore, synergistic combination of F4 prepared using acetone with average particle diameter of 39.2 nm was further concentrated and used for further studies. In the figure 4.12, the number corresponded to formulation F1-F4 mentioned in the table 4.4. Figure shows transparency initial emulsion of Cur in acetone and varying aqueous phase that was increased in presence of PVA. The PVA and HP-β-CD containing formulation was clear and transparent indicating formation of nano-emulsion.

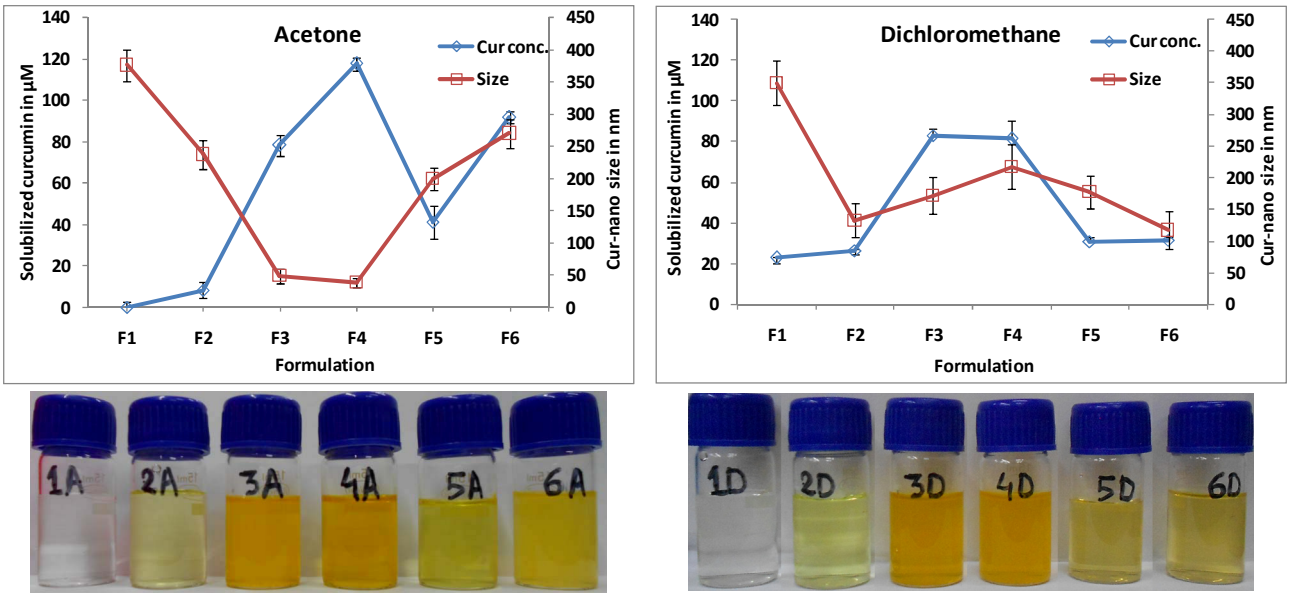


Figure 4:11. Size of Cur nano-particles and concentration of Cur solubilized in different formulation.

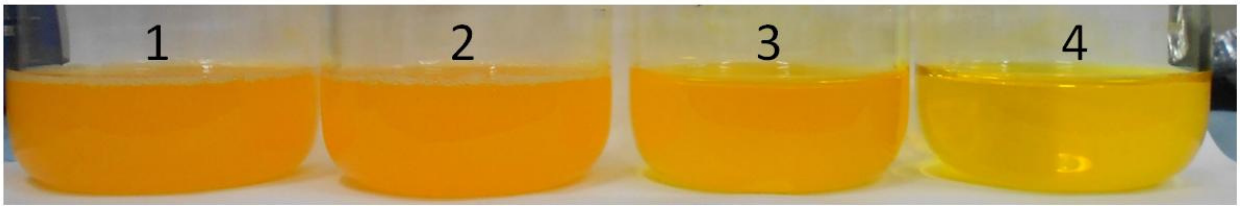


Figure 4.12. Initial emulsion of Cur in acetone in varying aqueous phase.

4.3.5. Preparation and characterization of Cur nano-formulations for improvement of aqueous solubility

Different approaches adopted for improving solubility of Cur is presented in section 4.3.4. These approaches were employed in different studies to evaluate specific activity of Cur. But to find an optimal formulation for a specific application, a comparative study of different nano-formulation is desired. In recent studies comparative study of Cur in different lipid formulation were studied for Cur retention and solubility improvement. (183) Beloqui et al. studied different lipid based nano-carriers containing Cur for their effect as an anti-inflammatory agent in inflammatory bowel disease. (184) Superior activity of nano-structured lipid carriers of Cur in comparison to self-nano-emulsifying drug delivery systems and lipid core-shell protamine nanocapsules was

reported. Thus for our current study, we prepared different Cur nano-formulations for reversal of DOX resistance. Nano-formulations of Cur such as liposomal Cur (CurL), micellar Cur (CurM) and nano-sized Cur (CurN) were prepared and evaluated in comparison to DMSO assisted nano-dispersion of Cur (CurD).

Figure 4.13 shows stock of different forms of nano-dispersed Cur. CurD (500 µM) and CurL (500 µM) were translucent where as CurM (400 µM) and CurN (366 µM) were transparent. Other pharmaceutical properties such as size, PDI, zeta potential and encapsulation efficiency are shown in table 4.5. CurM and CurN formulations had low particle sizes but were poly-disperse as no size control procedure was adopted. But, DMSO assisted nano-dispersed Cur and extruded liposomes were mono-disperse. Most of the formulations possessed negative surface potential. Presence of DMSO in CurD and presence of negatively charged phosphate group in DSPE-PEG resulted in negative zeta potential of CurD and CurL respectively. (185) The negative zeta potential of MPEG-PCL micelles was mainly due to the presence of ionizable carboxyl group on the surface of PCL. (186) In terms of EE, CurL (85%) and CurM (100%) possessed advantage indicating improved carrier retention and stability.

Table 4.5. Pharmaceutical properties of Cur nano-formulations.

Formulation	Size (nm) ±SD	PDI ±SD	Zeta potential (mV) ±SD	EE (%) ±SD
CurD	260.8±20.8	0.279±0.033	-24.3±1.24	NA
CurL	165.6±1.882	0.095±0.035	-16.4±1.02	85.7±0.264
CurM	18.4±3.2	0.644±0.169	-7.58±1.88	100±2.45
CurN	37.7±8.2	0.523±0.176	0.298±0.182	58.8±1.55

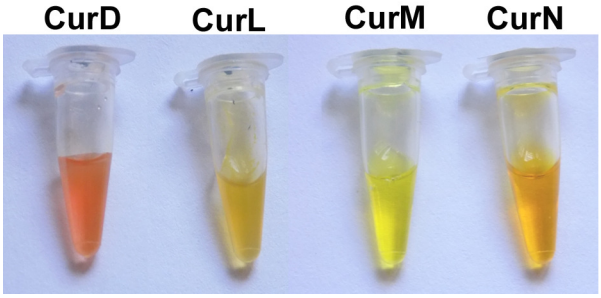


Figure 4.13. Physical appearance of Cur nano-formulations.

4.3.6. Biological activity of Cur nano-formulations

In numerous studies, Cur in combination was reported to sensitize of various chemotherapeutic agents in different model systems that may be efflux pump dependent and independent. For example, Cur reported to increase the effectiveness of DOX in breast cancer (cell lines; MDA-MB-468, MDA-MB-231, BT-549, and BT-20), ovarian sarcoma (cell line; M5076), liver cancer (cell line: HA22T/VGH) etc. (187-189) In addition, Cur has its implication in Ehrlich ascites carcinoma, breast cancer (cell line: MCF7), liver cancer (HepG2), osteosarcoma (cell line: KHOS) etc. for reversal of DOX resistance in free form or nano-encapsulated form. (190-193)

4.3.6.1. Liposomal and micellar Cur decreases the cyto-toxicity of Cur

The dose response curves of different nano-formulation in K562R cells are plotted in figure 4.14.

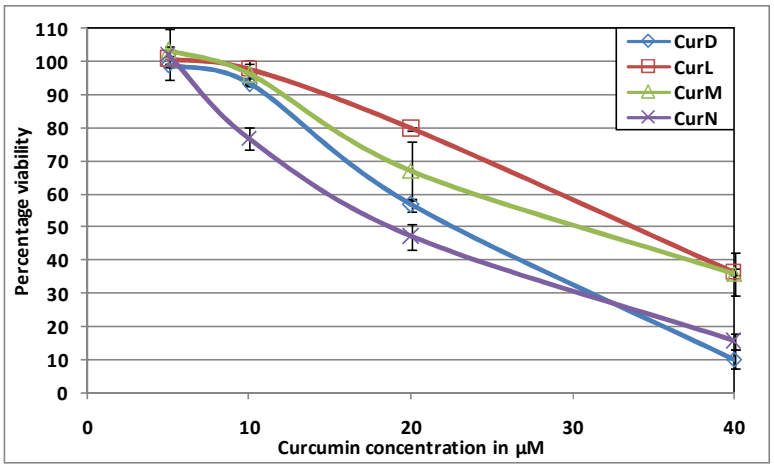


Figure 4.14. Cyto-toxic activity of Cur nano-formulations.

Nano-carrier based systems, CurL and CurM increased the safety levels of Cur. In contrast nano-dispersed (DMSO assisted; CurD or cyclodextrin assisted; CurN) had comparatively higher cyto-toxic effect. Cyto-toxicity difference of prepared Cur nano-formulations (Figure 4.14) would have attributed to lower uptake or hindered interaction after uptake. So in order to find out, uptake of different Cur nano-formulation in both K562N and K562R cells were studied.

4.3.6.2. Uptake of Cur was mildly reduced up on encapsulation in to micelles and liposomes

The absolute values indicating uptake of different nano-formulation is comparatively presented in figure 4.15.

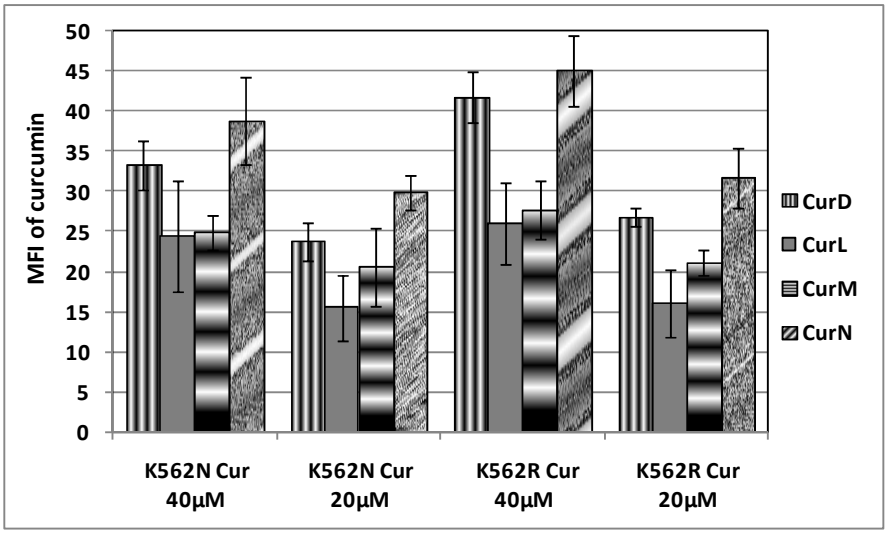


Figure 4.15. Uptake of Cur nano-formulations in to K562N and K562R cells.

As results indicate, at all treatment levels and in both K562N and K562R cells there was moderate decrease of Cur uptake up on encapsulation in to liposome (CurL) and micelle (CurM). Cur in CurN penetrated in to cells to a higher extent to that of CurD. This may be attributed to higher dispersibility of CurN in comparison to CurD due to lower particle size. (194, 195) In other ways there is probable hindrance of permeation by nano-carriers in comparison to that of

when Cur was nano-dispersed. In Cur nano-dispersions, Cur uptake was mediated by direct interaction Cur with cells, but on the other hand in carrier systems, uptake was mediated by interaction of cell membrane with the carrier. From cyto-toxicity and uptake assay it can be noted that Cur loaded nano-formulations exerted cyto-toxic activity corresponding to cellular uptake.

4.3.6.3. P-gp inhibitory activity of Cur nano-formulations

P-gp inhibitory activity of Cur nano-formulations was analyzed by their effect on calcein accumulation. As described previously, K562R cells accumulate only 40% of calcein to that of K562N cells. Upon treatment with Cur nano-formulations, calcein uptake in K562R cells increased for CurD, CurL and CurN, but CurM caused no significant improvement of calcein accumulation after treatment for 2 h (figure 4.16). From collective observation of Cur uptake (figure 4.15) and quantitative analysis of calcein (figure 16 (b), it can be inferred that increase of calcein accumulation by CurD, CurL, CurN was dependent on Cur accumulation in the cell from these carriers. CurD and CurN treated cells noted highest increase in calcein accumulation (increased about 250%) followed by CurL that increased accumulation to 180%. In contrast to these, calcein accumulation by micellar Cur was highly compromised. The inactivity of CurM may be attributed to unavailability of free Cur at site of action. This possibility was supported by a study by Binkhathlan et al. where in decreased activity of P-gp inhibitor valspodar upon encapsulation in to micelles and liposome was reported. (196, 197) In a similar experimentation, cyclosporin A encapsulated PEO-PCL micelles resulted in higher AUC in plasma due to stabilized micellar encapsulation. But, free cyclosporine-A after release from the micelle was lower in comparison to marketed formulation (Sandimmune). (198)

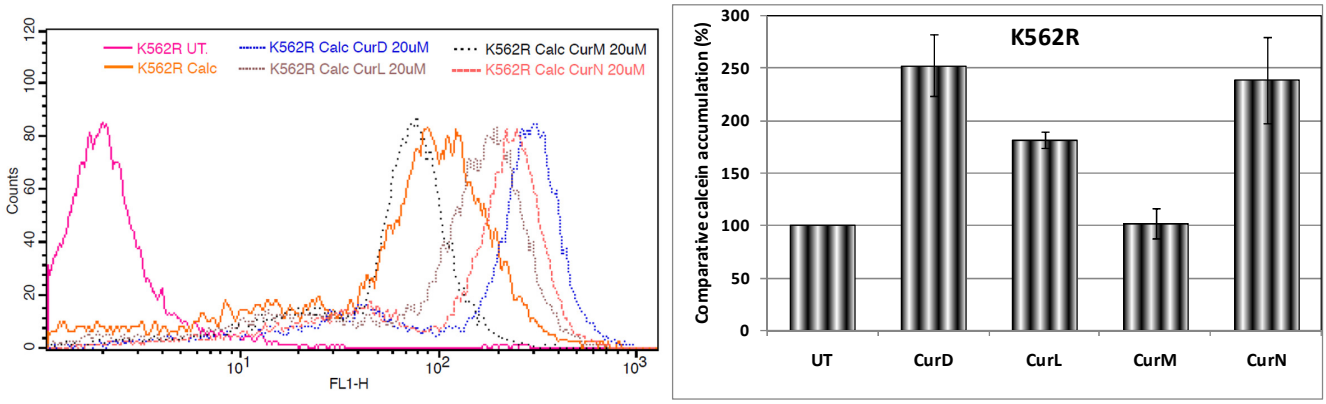


Figure 4.16. Effect of Cur nano-formulations on calcein accumulation.

4.3.6.4. Reversal of resistance by Cur nano-formulations

In the functional study to evaluate activity of Cur nano-formulations to re-sensitize K562R cells, cyto-toxicity assay was performed after co-treatment of Cur nano-formulations with DOX. Equivalent sensitization by CurD and CurN followed by CurL was noted (figure 4.17). At lower DOX concentration there was marginal sensitization by CurM to DOX but IC_{50} level of treatment, CurM was inactive. This result is in accordance with its inactivity to enhance calcein accumulation. Thus, the re-sensitization corresponded to calcein accumulation indicated P-gp associated inhibitory activity. Different nano-formulations improved the activity of encapsulated agent to different extent. Here CurL or CurM was compared with CurD. All the formulations are nano-dispersed state and thus possibly will have common uptake mechanisms i.e. endocytic uptake. But there no carrier involved in CurD and thus, it can be considered as standard form of nano-dispersed Cur. Therefore enhancement in activity beyond to that of CurD is not expected by any other formulation.

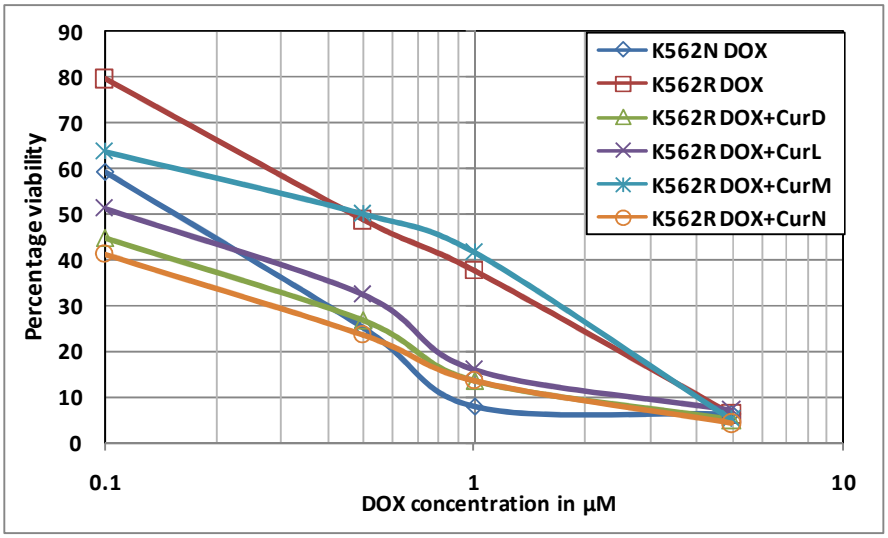


Figure 4.17. Reversal of DOX resistance by Cur nano-formulations in K562 cells.

4.3.6.5. Comparison of different Cur nano-formulations

Comparative features of Cur nano-formulation based on their carrier retention, stability, scale-up, biocompatibility and biological activity are presented in table 4.6. All the formulations (CurL, CurM, and CurN) increased the solubility and maintained their stability at 4˚C up to several weeks. CurM was the most amiable formulation for scaling up owing to its minimal preparatory steps and efficient encapsulation. However, EE of CurN was limited to 58% and thus further optimization of process was desired for scaling up. When evaluation was done in terms of uptake, liposomes and micelles compromised cellular uptake to certain extent but they can provide stability in systemic circulation which was much desired for clearance problem of Cur. (155, 199, 200) Encapsulation in to HP-β-CD (CurN) is able to increase the solubility but in the *in vivo* system, it needed a secondary carrier to avoid rapid clearance. This reasons the need of secondary carrier for CurN to deliver it under *in vivo* conditions.

Table 4.6. Comparative pharmaceutical and scale-up feature of Cur nano-formulations.

	Solubility improvement	**Aggregation on storage at 4˚C for 2 weeks**	**Scaling up**	**Biocompatibility**	**Cellular uptake**	**Biological activity (reversal of resistance**	**In-vivo delivery**
CurD	Moderately improved	Reversible	NA	No	Not affected	Not compromised	NA
CurL	Improved	No	Yes	Yes	Mildly compromised	Mildly compromised	Possible
CurM	Highly improved	No	Yes	Yes	Mildly compromised	Highly compromised	Possible
CurN	Highly improved	No	Further optimization needed	Yes	Not affected	Not compromised	Secondary carrier needed

It is generally expected that cellular uptake corresponds to biological activity. However to our surprise, CurM in spite of its uptake in to the cell unable to inhibit P-gp mediated efflux of calcein and remain inactive for reversal drug resistance as well. Therefore its implication in circumvention resistance in this model system is not recommended. The mechanistic reasons will be an open question to be addressed. Based on above results liposomal Cur (CurL) was found to be most suitable for reversal of DOX resistance hence used for development of combinational nano-formulation.

4.3.7. Preparation and characterization of DOX and Cur co-loaded liposomes

Due to amiable multimodality nano-formulations such as liposomes, nano-particles, micelles etc. has gained essential focus. For pharmaceutical application, liposomal formulation progressed to significant extent and moved ahead of other formulations in several expects.(104, 201) In the current study, DOX and Cur has to be combined but, both are having distinct physicochemical properties. Thus to load drugs in combination hydrophobic Cur was encapsulated in to hydrophobic regions of liposomal bilayer by incorporation in the lipid mixture. Hydrophilic DOX was loaded in to the core *via* active loading through ammonium sulfate gradient.(202)

Alternatively, Cur first solubilized in water by using different water soluble carrier and then encapsulated in to hydrophilic cavity of liposomes. Cur was either solubilized using cyclodextrins or nano-dispersed by encapsulating in to polymeric nano-particles. Furthermore, Cur was conjugated to lipid to increase dispersibility and liposome was prepared where in Cur was positioned at the surface of bilayer. (176, 203, 204)

Liposomes with Cur loaded in the bilayer were prepared as mentioned in the section 4.2.8 to improve its water dispersibility. Rehydration was done by sonication after addition of appropriate volume of 250 mM ammonium sulfate. Sulfate gradient was created post dialysis for active loading of DOX. Following extrusion, the prepared liposomes possessed an uniform size distribution with a z-average diameter of 165.1±1.37 nm, poly-dispersity index of 0.095± 0.035 and possessed zeta potential of -16.4±1.33 mV. Post dialysis, the Cur content in the liposomes was measured in HPLC and subsequent calculations yielded an EE of about 85%. Cur loaded in to the bilayer was advantageous as, higher EE can be obtained without involving numerous processing steps. Encapsulation further controlled the release of Cur to an extended period of time that was much desired under in-vivo condition to decrease its clearance. (204, 205) As shown in figure 4.18, the release was time dependent and followed zero order kinetic

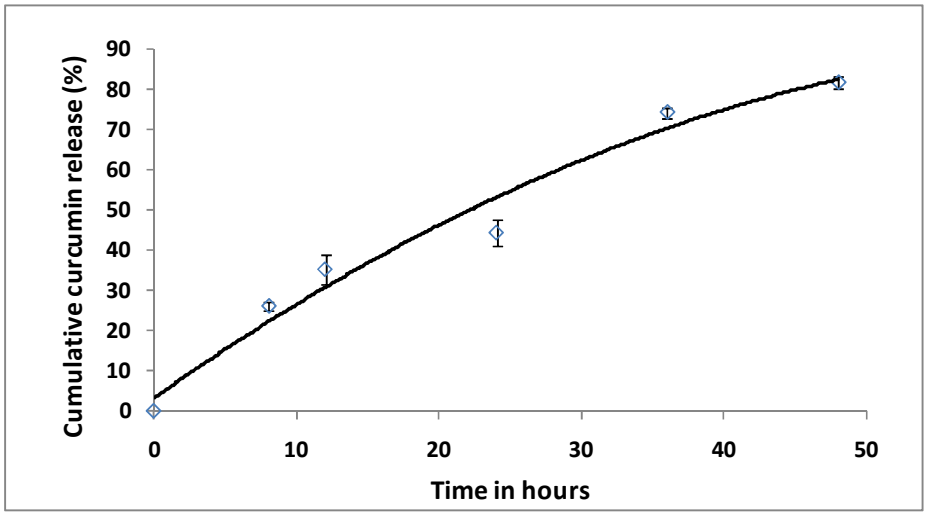


Figure 4.18. Prolonged release of Cur from liposomal Cur.

4.3.8. Reversal of DOX resistance by liposomal DOX and Cur

Evaluation of liposomes co-loaded with DOX and Cur was performed using XTT based cell viability assay. Dose dependent reversal of DOX resistance by Cur loaded liposomes was observed (Figure 4.19). From the dose response curve, it was observed that Cur at concentration

of 20 µM, re-sensitized K562R cells to an extent equivalent to that of K562N cells. When liposomal Cur was compared to that of DMSO nano-dispersed Cur, a marginal increase of activity was inferred.

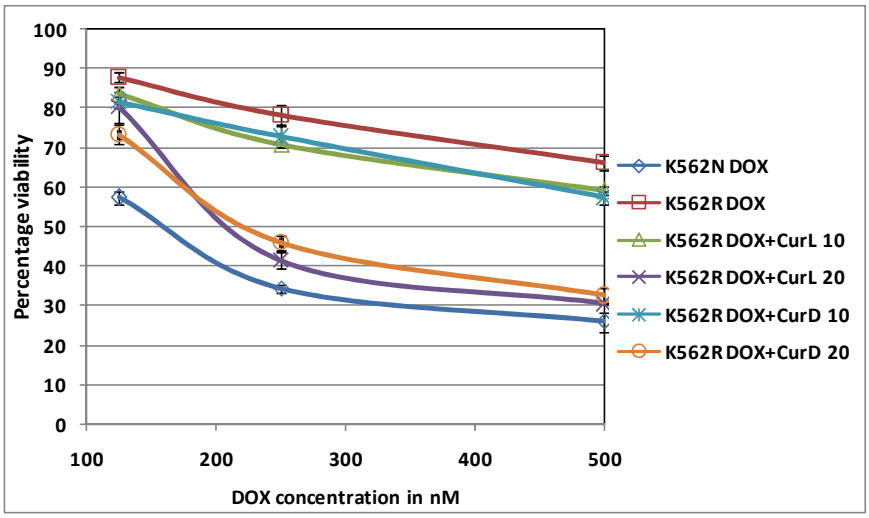


Figure 4.19. Reversal of DOX resistance in K562 cell by DOX and Cur co-loaded liposomes.

4.4. Summary and observation

The presented work involved development of a resistant counter part of leukemic cell line K562. The resistance in cell lines was validated by growth pattern and a prior insight of involvement of P-gp was obtained by calcein uptake assay. The reversal of P-gp mediated resistance by P-gp inhibitor was established by using standard P-gp inhibitor verapamil. From the study of P-gp inhibitors for reversal of DOX resistance, Cur was selected as a suitable agent. However, Cur had problem of aqueous insolubility. To increase its solubility different nano-formulations of Cur were prepared and evaluated for their pharmaceutical properties and pharmacological activity. Form these studies, liposomal Cur was found to be most suitable comparison to micellar and nano-Cur. Thus, DOX was loaded to liposomal Cur by sulfate gradient and the co-loaded liposomes resensitized K562 resistant cells to DOX.

Chapter 5. Combinational nano-formulation for reversal of DOX resistance in COLO205 cells

5.1. Background information

Epithelial colon carcinoma is the third largest cancer as reported and its model was considered for the current study for reversal of DOX resistance. (206) For study purpose, COLO205, a colon cancer cell line was used. There are several mechanisms proposed for acquired drug resistance in colon cancer but transport related mechanisms, alteration of target and over-expression of CYP-P450 are most significant. (207-209) However over-expression of efflux pumps plays a significant role in progression, therapeutic response and clinical outcome in patients with colon cancer.(206) For model system colon cancer cell lines such HT29 and COLO205 are used widely for study of mechanism as well as reversal of acquired chemo-resistance. (209-212) Similar to our previous study with K562, we aim to develop a combinational nano-formulation of P-gp inhibitor and doxorubicin (DOX) for reversal of acquired DOX resistance in COLO205 cells. After development of DOX resistant COLO205 (ColoR) cells from non-resistant cells (ColoN), few selected natural P-gp inhibitors such as BioA, Cur, Daid, DHF, Gen, Resv, Sily etc. were screened for efficacy to re-sensitize ColoR cells to DOX. BioA and Cur were was found to be most suitable agent exerting an additive effect where as none of the agent was able to inhibit the efflux of pumps in the model system. Both the agents were hydrophobic in nature. Solubility analysis was performed as very minimal solubility data was available for BioA. Furthermore, BioA and DOX had a close overlapping maximal UV absorbance wavelength of 262 nm and 232 nm respectively. Thus, to avoid any interference a binary gradient system in HPLC was developed to separate, detect and quantify BioA and DOX simultaneously. To develop a combinational nano-formulation, liposomes containing DOX and

BioA were then optimized and prepared. The optimal liposomes were studied for their efficacy to reverse DOX resistance in ColoR cells. For Cur, optimized Cur nano-particles were prepared by its encapsulation in to HP-β-CD as described previously (section 4.3.4). Cur nano-particles were used for reversal of DOX resistance along with DOX.

5.2. Methods

5.2.1. Evaluation of resistance development and alteration of growth pattern

Resistance developed was evaluated by XTT assay as described in section 3.2.2.1. Briefly, about $10x10^3$ number of ColoN and ColoR cells were sown in to individual well of a 96 multi-well in triplicate. Treatment with DOX in a concentration range of 0.01-100 µM was done. After the incubation period XTT based cell viability assay was performed and percentage viability was calculated as described earlier. After resistance developed, the growth pattern of non-resistant and resistant cells were studied as described in section 3.2.2.2 in presence or absence of 0.1-10 µM of DOX. The percentage viability in each treatment range was calculated considering viability (number in case of coulter counter and absorbance in case of XTT) of untreated cells as 100%.

5.2.2. Selection of P-gp inhibitor for reversal of DOX resistance in ColoR cells

Prior to screening of P-gp inhibitor, validation of reversal of DOX resistance in ColoR cells by standard P-gp inhibitor verapamil was undertaken. Thus ColoR cells were co-treated with verapamil (1-40 µM) and DOX (0.01-10 µM) and XTT based cell viability assay was performed as described previously.

To determine appropriate dose of P-gp inhibitors, we determined the safe concentration of P-gp inhibitors by treating them in a concentration range of 0.1-100 µM in both ColoN and

ColoR cells. After 72 h of incubation, XTT cell viability assay as described in section 3.2.2.1 and their dose response curve in both the cell lines were plotted. Reversal of developed resistance was evaluated by XTT assay after co-treatment DOX and P-gp inhibitor in ColoR cells. Here various P-gp inhibitors were treated at sub-toxic doses followed by DOX treatment at concentrations ranging 0.1-10 µM after 2 h. After 72 h of incubation, XTT assay was performed as described in section 3.2.2.1.

5.2.3. Determination of optimal dose of BioA and Cur for reversal of DOX resistance

The objective of the experiment was to find out suitable dose of BioA and Cur needed for reversal of DOX resistance. Both of these agents were treated to $10x10^3$ ColoR cells sown in to each well of a 96 multi well-plate at safe to cyto-toxic concentration range. BioA was treated at 1-50 µM and Cur was treated at concentration ranging 1-60 µM. Then DOX treatment was done after 2 h and post 72 h if treatment cell viability assay was performed as described in section 3.2.2.1.

5.2.4. Development of HPLC method to detect and quantify DOX and BioA simultaneously

The method for combinational detection and quantification was performed as described in section 3.2.4.

5.2.5. Solubility improvement of BioA

BioA is aqueous insoluble at its unmodified form. Therefore, in the present work, attempts were made to improve its solubility by encapsulation in to HP-β-CD. For encapsulation, emulsification and solvent evaporation method was adopted as described in section 3.2.7.1.

Briefly, solutions of pH range 5-8 were prepared using citric acid or NaOH. Similar solutions of different pH were also prepared containing 2.5 mM of HP-β-CD. 0.5 ml from each pH range with or without HP-β-CD were taken in a 1.5 ml micro-centrifuge tube and weighed to get the initial weight of the solvent. BioA was aqueous insoluble, therefore a stock of 5.5 mM BioA was prepared in acetone and 150 µl of stock was added to each tube to maintain final concentration after evaporation as 1 mM and weights of the tubes were noted. Evaporation and processing of individual experimental sets were done as described in section 3.2.7.1. The supernatant containing solubilized BioA was analyzed in HPLC in a method as described in section 3.2.4. The solubilized fraction was calculated using following formula.

$$Solubilized\ fraction = \frac{Conc.of\ BioA\ in\ the\ supernatant}{Conc.of\ BioA\ added\ for\ encapsulation} \times 100$$

5.2.6. Optimization of lipid composition for preparation of liposomes containing BioA

To optimize liposomal formulation, lipid film rehydration technique as described in 3.2.5 was adopted. Here, the lipids (SPC, cholesterol, EPG or DOPE) were dissolved in chloroform and BioA in methanol. For preparation of liposomes, specific ratios of lipids and BioA (6% molar ratio of total lipid) were mixed in a round bottom flask for preparation of thin film around the wall of the flask. Rehydration and size reduction was done as described in section 3.2.5. The liposomal mixture was dialyzed (145KDa MWCO dialysis bag) in 400 mL of 150 mM sodium chloride for 3 h to create an ammonium sulfate gradient across the liposomal membrane and to remove any un-encapsulated BioA. (29). The size, PDI and zeta potential of liposomes prepared was measured using a Zeta-sizer, where as EE was calculated after quantification of BioA in HPLC after solubilizing the lipids by addition of equivalent amount of ACN.

5.2.6.1. Optimization of cholesterol concentration

To evaluate the effect of cholesterol content on encapsulation of BioA liposomes, liposomes with varying amounts of cholesterol concentration were prepared as per table 5.1(a) in triplicate. The liposomes were further characterized for size, PDI, zeta potential and EE. Standard deviation (SD) was calculated from triplicate readings.

5.2.6.2. Optimization of surface charge

To modulate surface charge of liposomes, positively (DOPE) or negatively charged lipids (EPG) were incorporated into the liposomes as per the composition illustrated in table 5.1(b) and processed as described in section 3.2.5. The prepared liposomes were analyzed for their formulation properties and encapsulation of BioA.

Table 5.1. Lipid composition of liposomes formulated to optimize cholesterol concentration and (a) surface charge (b).

a.

Formulation	SPC:Chol	BioA conc.
F0	60:40	Nil
F1	70:30	300
F2	65:35	300
F3	60:40	300
F4	55:45	300

b.

Formulation	SPC:Chol:DOPE/EPG	BioA conc.
F0	60:40:0	300
F1-ve	40:40:20 (EPG)	300
F2-ve	20:40:40 (EPG)	300
F1+ve	40:40:20 (DOPE)	300
F2+ve	20:40:40 (DOPE)	300

5.2.7. Preparation and evaluation of pharmaceutical propertries of DOX and BioA coloaded liposomes

Optimized liposomes from pervious section were loaded with DOX by sulfate gradient mediated active loading. The amount of DOX to be loaded was decided based on the results from dosing ratio study done in section 5.2.2. For better control over liposomal size the process of extrusion was done through a double stacked 100nm polycarbonate membrane.

Particle size and zeta potential were determined using DLS after suitable dilution in a glass cuvette with recommended settings. Zeta potential of the same sample was measured using

zeta dip-cell electrode. Further EE of formulations were calculated after analyzing dissolved fraction in HPLC.

5.2.8. Biological activity of co-loaded liposomes of DOX and BioA

5.2.8.1. Cellular uptake study

To analyze cellular uptake of co-loaded liposomes in ColoR cells, $10x10^4$ cells were sown in to each well of a 24 well plate. 5 µM of DOX in combination with 55 µm BioA either in free form or in co-encapsulated liposomal form were treated. The treated cells were incubated for 2 h. Harvesting, washing and acquisition was done as described in section 3.2.3.2.

DOX fluorescence was measured from about 10000 gated cells in a flow-cytometer (FACS, BD Calibur) and mean fluorescence intensity (MFI) obtained was presented considering MFI from untreated ColoR cells as 100%.

5.2.8.2. Reversal of DOX resistance in ColoR cells

Optimized liposomes were prepared and after co-loading DOX and BioA, those were adopted for studies to evaluate their efficacy to reverse DOX resistance. Cyto-toxic effect of DOX and BioA encapsulated liposomes was evaluated by using XTT assay in a narrow concentration range of DOX 1-10 µM. The procedure was followed as described earlier in section 3.2.2.1 along with other liposomal controls.

5.2.9. Preparation and characterization of DOX co-loaded liposomes and nano-Cur

PEGylated liposomes were prepared as described in section 4.2.4.2. DOX was actively loaded by a sulfate gradient as described in section 3.2.5. Its particle size and zeta potential were measured in Zetasizer as described in section 4.2.6.

5.2.10. Biological activity study of DOX liposomes in combination with nano-Cur

Reversal of DOX resistance was evaluated by treating nano-combination of PEGylated liposomal DOX (DOX-L) and nano-Cur in ColoN and ColoR cells. Nano-Cur was added to $10x10^3$ ColoR cells sown in to each well of a 96 well plate in a concentration equivalent 20 µM, 40 µM and 60 µM of Cur and incubated for two hours. Liposomal DOX was then treated in concentration varying 0.1-10 µM. Cells treated with the combination were allowed to grow for 72 h in normal culture conditions followed by XTT assay as described in section 3.2.2.1. The sensitivity ColoR cells to liposomal DOX and nano-Cur in cells were compared to ColoN.

5.3. Results and discussion

5.3.1. Development of resistance and evaluation of growth pattern

Dose response curves were plotted from the results of XTT assay performed as described in section 5.2.1. From the cyto-toxicity assay (figure 5.1) the IC_{50} of ColoN and ColoR cells were determined to be 0.2 µM and 15 µM respectively. There is an increased possibility of alteration of growth pattern of ColoR cells in comparison to ColoN cells as those were developed by maintaining cells in continuous DOX stress. Previous reports indicates cellular stress slows down the growth and in a population, slow growing cells are considered to be stress resistant (213, 214). Further characterization will be done to study the growth of resistant cells in comparison to non-resistant ones.

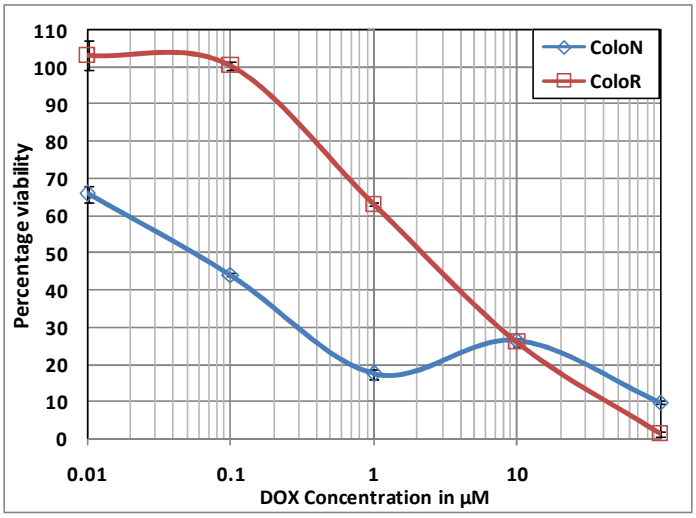


Figure 5.1. Dose response curves of DOX in ColoN and ColoR cells.

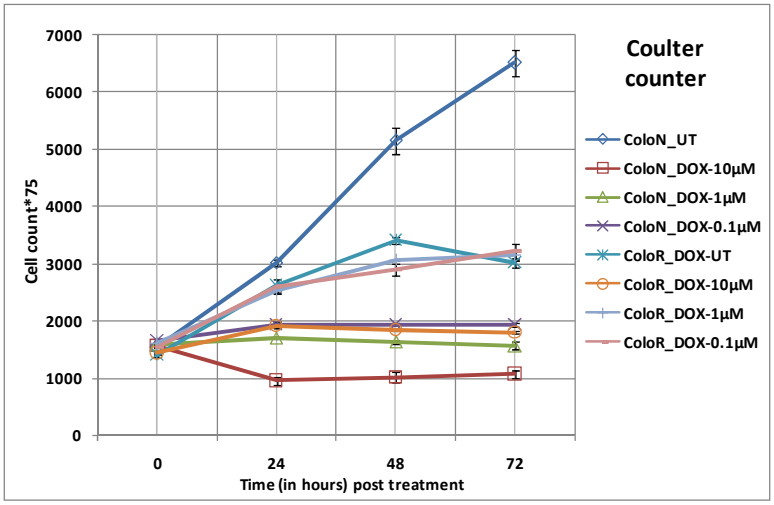
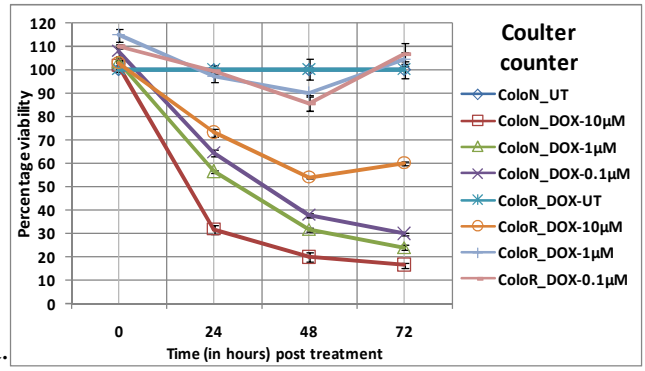


Figure 5.2. Growth pattern of ColoN and ColoR cells in presence and absence of DOX.

a.



b.

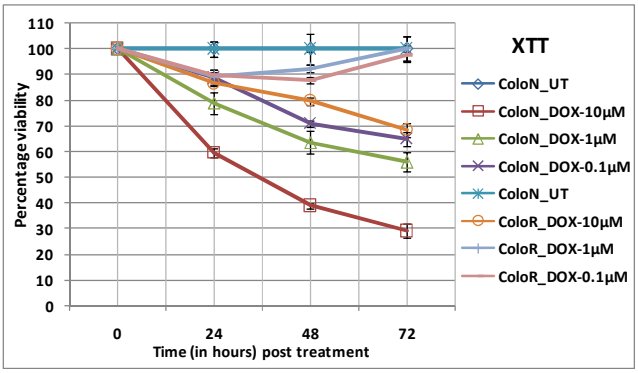
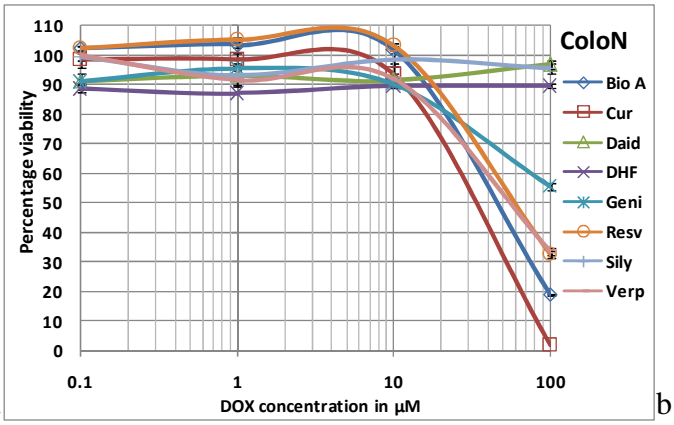


Figure 5:3. Growth of ColoN and ColoR cells at different DOX concentrations measured by coulter counter (a) or XTT assay (b).

To analyze any alteration in growth pattern of ColoR cells absolute measurements from coulter counter are presented in figure 5.2 and the respective percentage viabilities in figure 5.3 (b). From the cell count it can be inferred that unlike non-resistant cells, resistant ColoR cell undergo slower division i.e. it takes longer than 24 h to divide. In spite of slower growth, percentage viability trend was consistent with observation in dose response curve. Further the trend of percentage viability obtained from coulter counter (figure 5.3 (a)) and XTT cell viability (figure 5.3 (b)) assay were identical. Thus XTT reduction based cell viability assay can be adopted for further experimentations.

5.3.2. Determination of cyto-toxic effect of P-gp inhibitors

Cyto-toxic effects of P-gp inhibitors were been studied for determination of safe and toxic concentration in the developed resistant colon cancer model system. The dose response curves of different P-gp inhibitors in ColoN and ColoR cells are presented in figure 5.4 (a) and figure 5.4 (b) respectively. As shown in the figure, Daid, DHF and Sily possessed no cyto-toxic effect. On the other hand BioA, Cur, Gen and Resv caused induction of cell death beyond 30 µM having IC_{50} of 35 µM, 30 µM, 70 µM and 55 µM respectively. Thus their effect for reversal will be evaluated both above and below the above mentioned concentrations.

a. 

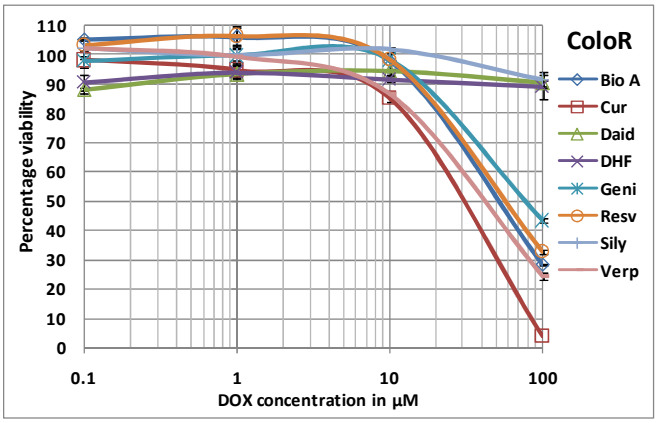
b. 

Figure 5.4. Cyto-toxic activity of P-gp inhibitors in ColoN (a) and ColoR (b) cells.

5.3.3. Screening of P-gp inhibitors for reversal of DOX resistance in ColoR cells

Prior to analysis of P-gp inhibitors for their efficacy to reverse DOX resistance, a study was performed to validate reversal of DOX resistance using standard P-gp inhibitor. For the above purpose, verapamil was pretreated at different concentrations followed by DOX treatment. From the XTT assay dose response curves were plotted and presented in figure 5.5 (a).

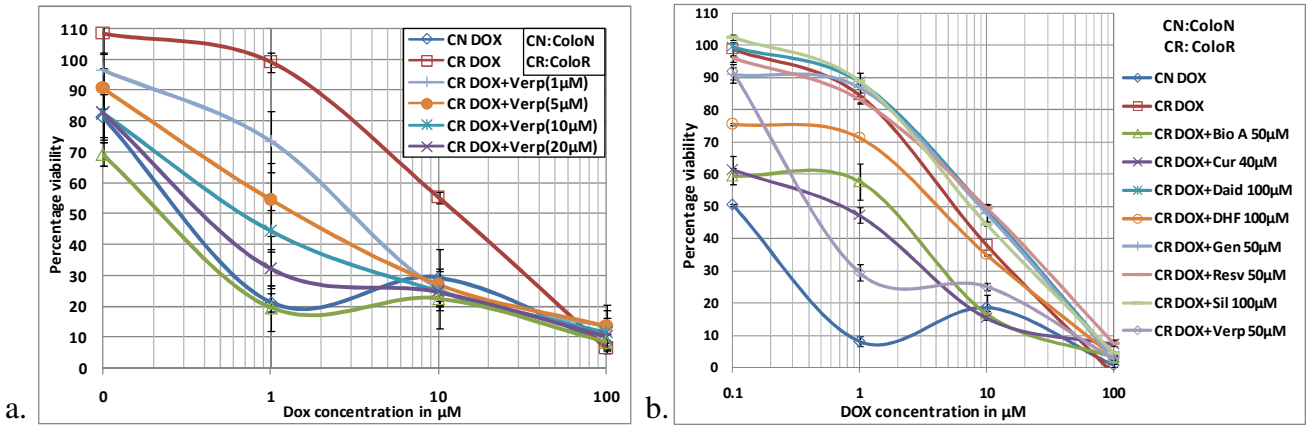
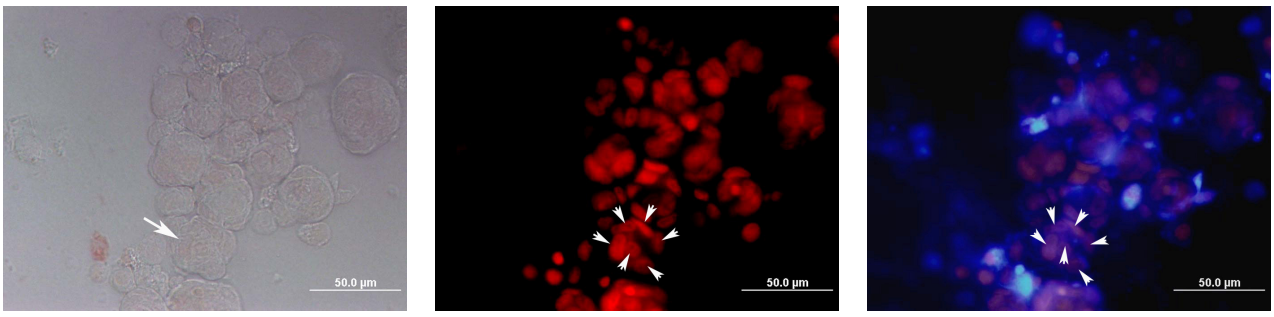


Figure 5.5. Sensitization of ColoR cells to DOX by verapamil (a) and effect of pretreatment with P-gp inhibitors on sensitivity of DOX in ColoR cells.

As shown in the figure, verapamil was able to reverse the acquired resistance in ColoR cells dose dependently. A spontaneous formation of spheroids was observed in ColoN cells at 10µM of DOX and ColoR cells at 10µM of DOX in combination with Verp 40 µM (figure 5.6).



Phase contrast DOX DOX+DAPI

Figure 5.6. Spontaneous and random formation of spheroids.

In the figure phase contrast denotes a single spheroid (arrow). The single spheroid contained of 5-6 cells which is evidenced by nuclear DOX and DAPI uptake in the subsequent

images. There is an overlap of DAPI and DOX fluorescence in co-treated image. Thus formed spheroids are more resistant to DOX; hence the cell viability was increased at those particular concentrations. Following this successful validation step, further screening of reported P-gp inhibitors can be studied for reversal.

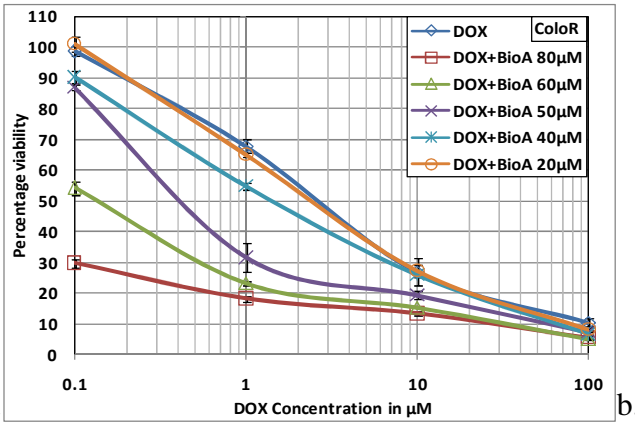
To analyze the effect of P-gp inhibitors on calcein accumulation, similar set of experiments were performed as described in section 4.3.2. However none of the agent was able to increase the calcein accumulation in ColoR cells (data not shown) and hence possibly will not be able to reverse P-gp mediated efflux of DOX. In this scenario, the effect from co-treatment for reversal of DOX resistance is mostly expected to be an additive effect. We studied the sub-toxic concentration or 100 µM of P-gp inhibitor which ever was lower. The dose response curves for the co-treated ColoR cells are presented in figure 5.5 (b). As shown in the figure, combination of BioA, Cur and DHF with DOX significantly increased the sensitivity of ColoR cells. The agent that is able to reverse resistance at a lower concentration preferentially selected as increased concentration would be unsuitable for processing in to nano-formulation. Thus out of these Cur and BioA were used for nano-formulation development.

5.3.4. Determination of dose of BioA and Cur for reversal of DOX resistance

Before processing the nano-formulation, an estimate of optimal dose of both P-gp inhibitor and DOX is desirable. Thus, dose response curve of the combination in the specified concentration range was plotted after a cell viability assay. Figure 5.7 (a), demonstrates the dose response curves for DOX and BioA combination where as figure 5.7 (b) represents the curves for DOX and Cur combination in ColoR cells. From the figures, a concentration of 50 µM and 40 µM of

BioA and Cur respectively were optimal to combine with DOX for reversal of DOX resistance in ColoR cells. ColoR cells beyond 40 µM of Cur.

a.



b.

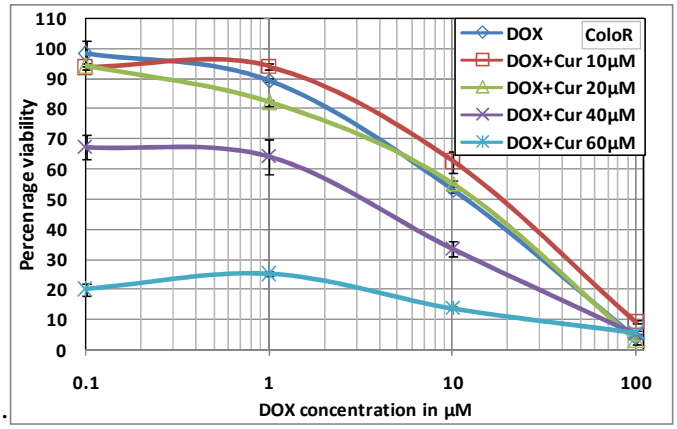


Figure 5.7. Dose-response curves of DOX upon pretreatment with different doses of BioA (a) and Cur (b) in ColoR cells.

5.3.5. Physicochemical and pharmacokinetic properties of selected P-gp inhibitors

Based on the results described in section 5.3.3, BioA and Cur were selected to combine them with DOX for reversal in ColoR cells. BioA belongs to chemical class flavonoids that has gained much significance in reversal drug resistance and increased effectiveness of anticancer drugs. (78). Reports suggest BioA block the functionality efflux pumps such as p-gp and BCRP, those lowers intracellular availability of chemotherapeutic agent and render resistance. (115, 215, 216). In addition when used singly BioA prevented and minimized tumor progression in various cancers such as colon, pancreas, prostate, breast, brain etc in cell lines or murine xenograft tumor model. (217-221)

However solubility of BioA in water is very low which limits its oral bioavailability in rats despite entero-hepatic recirculation. Hence, its parentral administration is a viable option (112) and which its encapsulation in nano-formulation is desired. Additionally, nano-formulations increase the solubility and optimize physicochemical as well as pharmacokinetic related properties. Furthermore, nano-formulations as a carrier of BioA along with chemotherapeutic

agent will be advantageous since it follows uniform distribution and retention pattern to that of nano-formulations resulting in increased accumulation in the tumor site due to EPR effect (97, 102). Here, the formulation and selected combination complement each other for successful reversal of chemo-resistance. Subsequent sections describe optimization of analytical properties and solubility which are essentially to be considered prior to formulation development.

5.3.6. Simultaneous detection and quantification of DOX and BioA in HPLC

For initial reference, reports from literature were applied to detect and quantify these agents. Selected methods for DOX and BioA consisted common mobile phase (0.5% OPA and ACN) in binary system but the composition was different (142, 143). To optimize the solvent composition for separation and quantification, a trial and error method with different isocratic and gradient flow rates were adopted as illustrated in table 5.2. Initially, both the agents were analyzed in solvent composition of BioA, where DOX elutes first (RT ≈2.5 min). Therefore from the subsequent time point, gradient modifications (method 1-4) were done to get optimum individual peak profile (difference in RT, height and AUC) of DOX. From the results, it was observed that that method M2 is the most suitable method for separation and detection with optimum peak height (415 mAU for DOX and 310 mAU for BioA) and AUC (1772241 for DOX and 13585663 for BioA) (figure 5.8) with RT difference about 1.2 min.

Table 5.2. Chromatographic conditions and analytical method development for the combination DOX and BioA

	Sample	Solvent A:B/ Flow rate (mL/min)	Solvent ratio - Isocratic /Gradient (I/G)	Details B Concentration	Peak 1 (DOX) RT/h/AUC	Peak 2 (BioA) RT/h/AUC
DOX method	DOX 20 µl of 100 µM	0.5%OPA: CAN/1.5	70:30 (I)	NA	2.8/542.7/3314494	NA
BioA method	BioA 10 µl of 100 µM	0.5%OPA: ACN/1.0	30:70 (I)	NA	NA	4.3/260.2/1459601
BioA method	DOX 10 µl of 100 µM	0.5%OPA: ACN/1.0	30:70 (I)	NA	2.5/118.8/1073347	NA
BioA method	BioA 10µl of 100 µM DOX 10µl of 100 µM	0.5%OPA: ACN/1.0	30:70 (I)	NA	2.4/167.1/883282	4.3/211.9/1110755
Method 1 (M1)	BioA 10µl of 100 µM DOX 10µl of 100 µM	0.5%OPA: ACN/1.0	20:80 (G)	0.1-3.0 min 80%, 3.0-6.0 min; 80-50%, 6.0-10.0 min; 50-80%	2.5/252.5/2397778 (splitted peak)	3.6/150.6/662765
Method 2 (M2)	BioA 10µl of 100 µM DOX 10µl of 100 µM	0.5%OPA: ACN/1.0	20:80 (G)	0.1-2.6 min; 80% 2.6-3.0 min; 80-70%, 3.0-6 min; 70%, 6.0-9.0 min; 70-80%, 9.0-10.0 min; 80%	2.4/415.9/1772241	3.6/310.1/13585663
Method 3 (M3)	BioA 10µl of 100 µM DOX 10µl of 100 µM	0.5%OPA: ACN/1.0	20:80 (G)	0.1-2.6 min; 80-60%, 2.6-8.0 min; 60%, 8.0-10.0 min; 60-80%	2.4/352.9/1594528	3.6/287.1/1269083
Method 4 (M4)	BioA 10µl of 100 µM DOX 10µl of 100 µM	0.5%OPA: ACN/1.0	20:80 (I)	NA	2.5/403.8/1331643	3.6/250.3/1089451

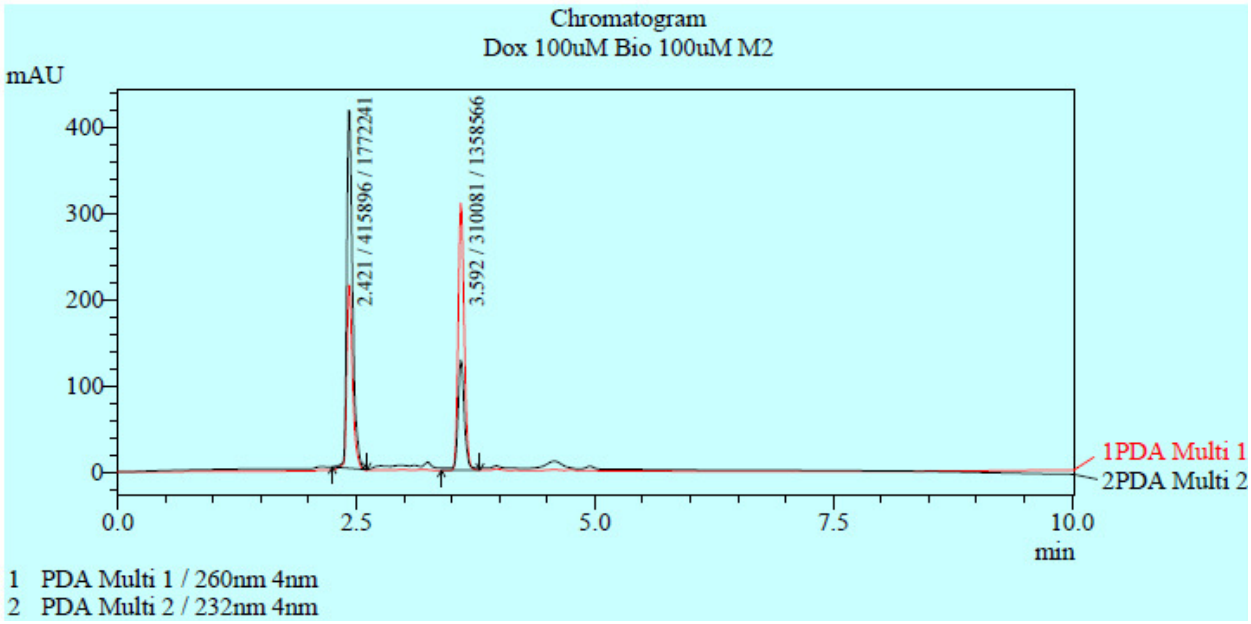
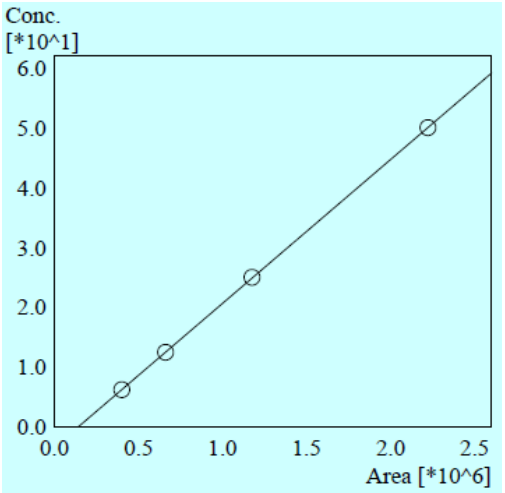


Figure 5.8. Chromatograms of BioA (red) and DOX (black) in the optimized method for simultaneous detection.

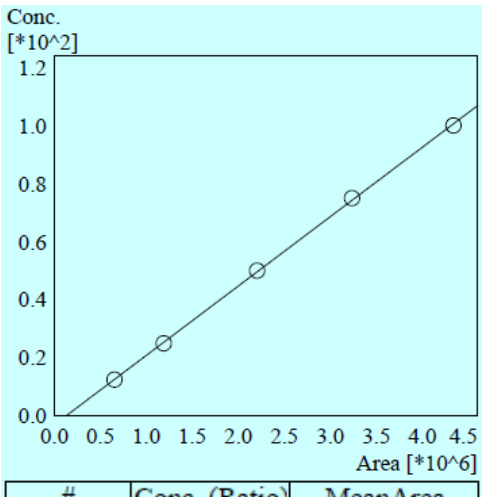
Therefore, M2 was adopted for further analysis of BioA and DOX separately as well as in combination. 4-point calibration curve was plotted for BioA and a 5-point calibration curve was plotted for DOX in the optimized method (figure 5.9).

a.



#	Conc. (Ratio)	MeanArea
1	50.0	2222455.4
2	25.0	1176260.2
3	12.5	662409.0
4	6.3	401651.0

b.



#	Conc. (Ratio)	MeanArea
1	100.0	4339647.9
2	75.0	3236046.9
3	50.0	2204224.3
4	25.0	1188886.9
5	12.5	653910.2

Figure 5.9. Calibration curve of BioA (a) and DOX (b) with measurement points.

5.3.7. Solubility of BioA at different pH

The objective of the following experiment was to increase water dispersibility of BioA by encapsulation in to HP-β-CD. To optimize the pH suitable for encapsulation, we performed the encapsulation at pH ranging 3-8 (data not shown). However, it was observed that irrespective of presence or absence of HP-β-CD, the solubility of BioA increased at basic pH. Table 5.3 summarizes the solubilized fraction of BioA in the supernatant obtained from the procedure as described in section 5.2.5.This was evidenced by the nature of dispersion formed at different pH. In neutral and acidic pH the nature of dispersion was biphasic where as basic pH produced a monophasic clear dispersion.

Table 5.3. Solubility of BioA at different pH.

Serial no.	Volume of NaOH added in µl (stock 50 mM)	pH ≈	Nature of system after evaporation/ Ppt or Clr	BioA in the supernatant or solubilized fraction (in %)
1	-	6.0-7.0	Biphasic/Ppt	6.76
2	2	6.0-7.0	Biphasic/Ppt	16.40
3	8	7.0-8.0	Monophasic/Clr	66.48
4	10	7.0-8.0	Monophasic/Clr	70.88
5	15	8.0-9.0	Monophasic/Clr	76.28

Ppt; Precipitate, Clr; Clear.

5.3.8. Optimization of liposomes for its cholesterol content and surface charge

Pharmaceutical properties of liposomes are determined by lipid composition and formulation processing. To optimize the lipid composition, the effect of cholesterol and other lipids on EE, size and zeta potential was studied. Previous studies report cholesterol incorporation render the membrane more rigid by increasing packing, hence increase the stability and hinders permeability. However at high concentrations, it may restrict the hydrophobic interaction of lipids by increasing water permeation into polar regions of the membrane. (222-224). Furthermore, drug encapsulation and release can also be modified by cholesterol concentration.

In a similar study, Deniz et al. reported decreased release of celecoxib by increasing cholesterol concentration. (222, 225, 226). The effect of cholesterol on concentration on size distribution, PDI, zeta potential and encapsulation efficiency of liposomes are presented in table 5.4. Cholesterol concentration had lesser effect on different formulation properties other than PDI. PDI of formulations prepared increased proportionately with increase in cholesterol in a concentration dependent manner. Increased resistance to break and form a spherical lipid globule during the process of rehydration and extrusion may be the major cause of this increased size. In addition extrusion was done through 100nm polycarbonate membrane, but the liposomal size was significantly larger. This may be resulted by coalescence of liposomes after extrusion. If so, incorporation charge may stabilize the extruded liposomes and prevent formation of larger liposomes. Therefore, we included a charged lipid into liposomes and studied its effects on pharmaceutical properties which are described further.

Surface charge facilitates formation of small unilamellar liposomes and further modulates encapsulation/release of drugs (223, 227). Therefore, we prepared charged liposomes using positively charged DOPE or negatively charged EPG. The properties of liposomes prepared by incorporation of charged lipid is presented in table 5.5. DOPE containing lipid layer was not rehydrated to form liposomes. The possible reason may be; to presence of negatively sulfate ions from ammonium sulfate in rehydrating medium possibly interact with positively charged DOPE resulting inter-linking. But later steps sulfate gradient is essential for DOX loading and thus we excluded cationic liposomes for further experimentation (excluded from the table). In other case, EPG containing formulations (F1 -ve and F2 -ve) noted a concentration dependent increase in surface charge and an inverse correlation with liposomal size was observed. Thus incorporation charge prevented formation of larger sized liposome. Among the charged formulations both

possessed equivalent BioA EE of but "F1 –ve" had zeta potential closer to reported formulations (228), therefore was used further.

Table 5.4. Optimization of cholesterol concentration for BioA loaded liposomes.

Formulation	Liposome size (nm) ± SD	PDI ± SD	Zeta potential (mV) ± SD	EE (%) ± SD
F0	189.8 ± 5.1	0.164 ± 0.038	-3.6 ± 0.6	0.9 ± 0.8
F1	189.3 ± 2.9	0.158 ± 0.033	-2.7 ± 1	81.6 ± 8.5
F2	187.8 ± 7.3	0.193 ± 0.032	-4.1 ± 1.7	89.6 ± 5.2
F3	189.9 ± 13.1	0.235 ± 0.031	-3.3 ± 1.1	86.8 ± 4.8
F4	192.9 ± 10.7	0.254 ± 0.019	-2.7 ± 1.2	80.2 ± 8.3

Table 5.5. Optimization of surface charge for stability and optimal encapsulation.

Formulation	Liposome size (nm) ± SD	PDI ± SD	Zeta potential (mV) ± SD	EE (%) ± SD
F0	194.4 ± 11.9	0.157 ± 0.001	-3.88 ± 1.6	78.3 ± 1.7
F 1 –ve	148.9 ± 4.6	0.235 ± 0.034	-23.16 ± 0.79	80.3 ± 1.7
F 2 –ve	125.1 ± 5.8	0.196 ± 0.055	-35.56 ± 0.76	79.7 ± 9.7

5.3.9. Preparation and characterization of optimized liposomes

From the co-treatment studies to determine dosing ratio, it was inferred that combinational liposomes should contain DOX and BioA in concentration range of 1-10 µM and 50-60 µM respectively for optimal activity. From the solubility studies lowered solubility of BioA was inferred. This prevented us from loading BioA in to the liposomal core because rehydration of lipid film with BioA solubilized in basic medium becomes incompatible with proposed sulfate gradient mediated DOX active loading. On the other hand when BioA was encapsulated in to the bilayer, it was advantageous since, leaching of BioA into external acidic medium would be limited. Thus BioA was directly mixed with the lipid solution having composition of "F1 –ve" (table 5.5) and processed for preparation BioA encapsulated liposomes. Thus, liposomes of optimal composition F1-ve was prepared and extrusion was done with double stacked membrane

to get increased control over liposomal size. DOX was loaded by sulfate gradient as described earlier. Prepared DOX and BioA containing liposomes were of size 125 nm (figure 5.10) and had zeta potential -19.5 mV with BioA EE about 70%. 25 µM of DOX incorporation was done to maintain the final ratio of 8:1 (BioA:DOX).

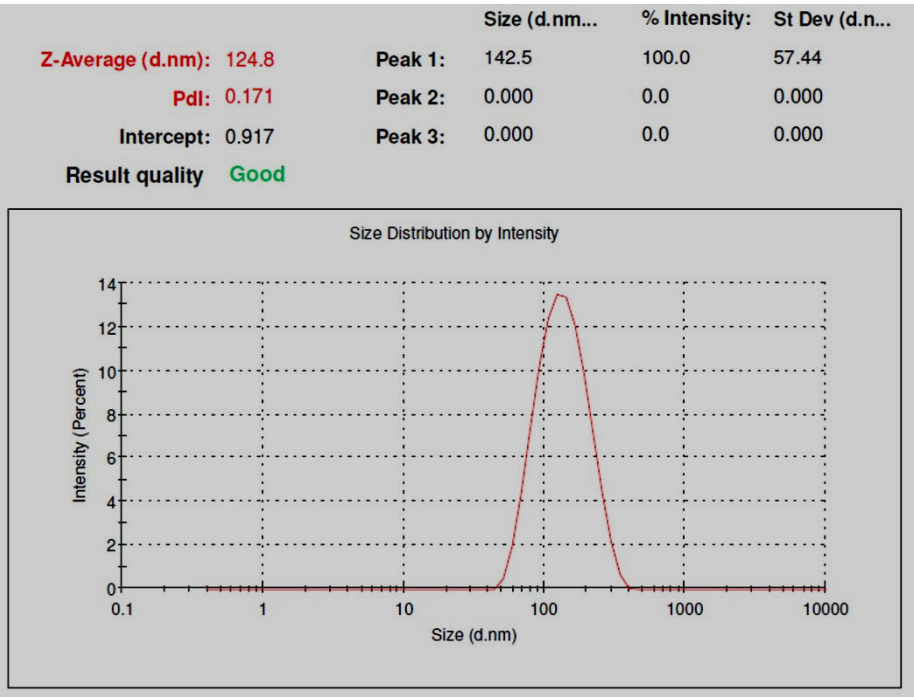


Figure 5.10. Size and PDI of optimized liposomes.

5.3.10. Biological activity of prepared liposomes

5.3.10.1. Cellular DOX uptake from combinational liposomes

The uptake of DOX was performed in ColoR cells, singly or co-treated with BioA in free or liposomal encapsulated form. Results of uptake study were shown in figure 5.11, where in moderate increase in DOX accumulation by liposomal encapsulation was observed. In addition co-treatment further increases DOX uptake. About 30% increase in DOX uptake was observed by DOX and BioA co-encapsulation in to liposomes.

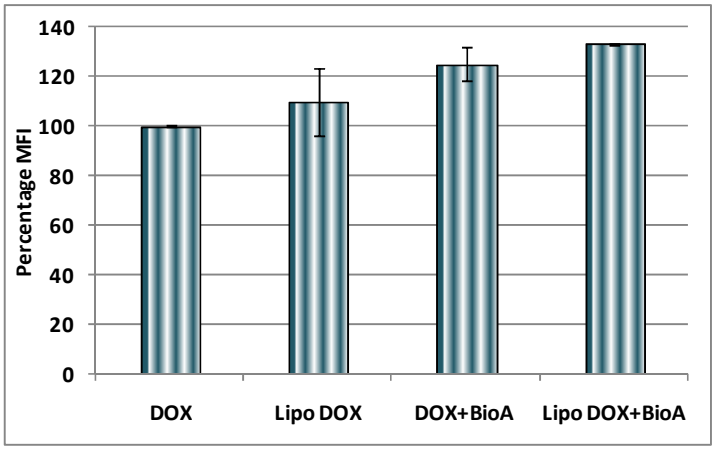


Figure 5.11. Uptake of DOX in ColoR cells at different treatment conditions.

5.3.10.2. Reversal of DOX resistance by liposomes co-loaded with DOX and BioA

The co-loaded liposomes were evaluated for their efficacy to reverse DOX resistance in ColoR cells. Figure 5.12 presents the percentage viability of combinations in encapsulated forms. As shown in the figure, at concentration below 5 µM of DOX combined dose of BioA remains ineffective for sensitizing ColoR cells. However at dose of 40 µM of BioA and 5 µM DOX, both them relatively inactive when treated individually, but upon treatment in combination sensitivity of ColoR cells increased significantly. IC_{50} of ColoR cells upon co-treatment confirmed about 2 fold sensitization by combinational liposomes. Effectiveness of liposomal BioA was as par with DMSO solubilized BioA, therefore liposomal encapsulation improved the solubility issues associated with BioA. Similar to that Cur, since DMSO assisted dispersion remains in nano-particulate form, thus can be considered as positive control. Therefore further increase in activity is not expected. Modulation of DOX resistance by liposomal formulation was reported about two decades earlier but still the problems persists. Similar combinational nano-formulations of DOX along with verapamil, Cur, nitric oxide donors, lapatinib, siRNA etc. have been combined in nano-formulations. Results positively supports the use these formulations for circumvention of DOX resistance in various tumor models. (104, 229-233).

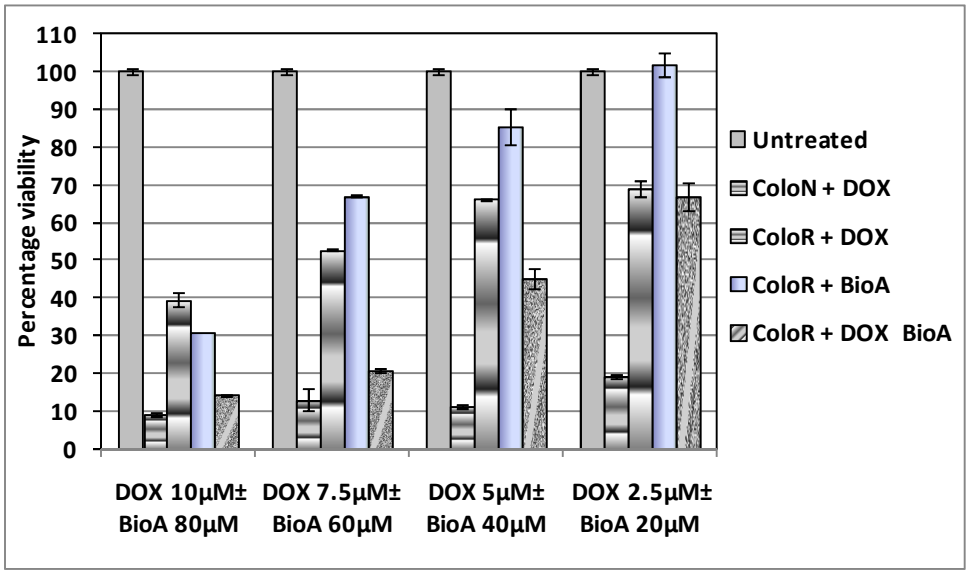
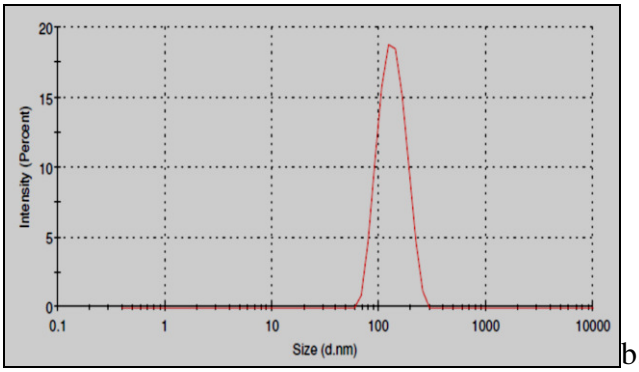


Figure 5.12. Reversal of DOX resistance in ColoR cells by liposomes co-loaded with DOX and BioA,

5.3.11. Characterization of liposomal DOX and nano-Cur

The PEGylated liposomes used for this study were prepared in a lipid composition identical to that of Doxil®. DOX was loaded by most efficient ammonium sulfate gradient that was able to encapsulate more than 95% of added DOX (data not shown). The liposomes were found to have a Z-average of size of 126.7 nm and had a narrow size distribution with poly dispersity index 0.068 (figure 5.13 (a)). The DOX loaded liposomes had a zeta potential of -15.6 ± 3.05 mV. The properties of nano-Cur (CurN) were same as that of descried in section 4.3.5 i.e. had size; 37.7 (figure 5.13 (b)), PDI; 0.523 with an encapsulation efficiency of 58.8%.

a. 

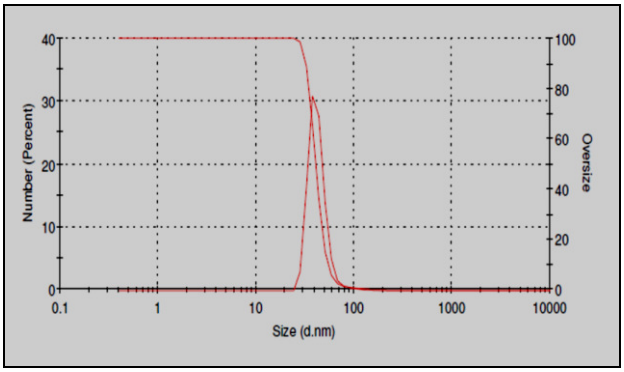
b. 

Figure 5.13. Particle size distribution of PEGylated liposomes (a) and nano-Cur (b).

5.3.12. Reversal of DOX resistance by liposomal DOX and nano-Cur in ColoR cells.

DOX loaded liposomes and CurN were co-treated at different concentration to ColoR cells. The results from cyto-toxicity assay in combination of nano-formulation are presented in figure 5.14. At all treated condition of DOX; ColoR cells sustain DOX stress in comparison to ColoN cells. However upon co-treatment with nano-Cur, resistant was reversed dose dependently but notable sensitization was observed beyond 40 µM. The effect of nano-Cur was found identical to that of solubilized Cur (DMSO as carrier) and thus it can be suitable water soluble alternative for reversal of DOX resistance.

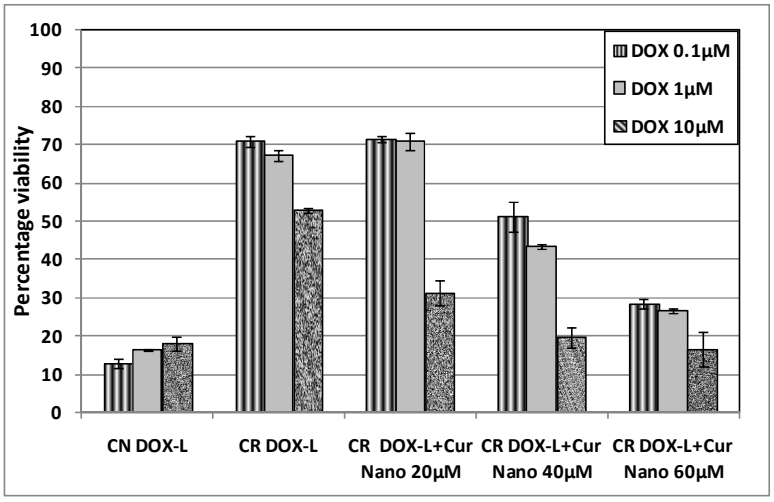


Figure 5.14. Reversal of DOX resistance by liposomal DOX and nano-Cur.

5.4. Summary and observation

The models system, resistant counterpart of ColoN was developed and termed as ColoR. ColoR possessed slower growth in comparison to non-resistant ColoN. Dose dependent reversal of DOX resistance up on treatment by standard P-gp inhibitor verapamil was noted. In contrast, none of the screened P-gp inhibitors were able resensitize ColoR cells in a P-gp dependent manner. However to combine agents for reversal of DOX resistance by additive interaction we selected agents those were able to reverse DOX resistance at lowest dose. BioA and Cur were the

agents selected for further formulation development. Prior to formulation analytical and physicochemical parameters were studied and optimized for BioA. Then for formulation of combinational liposomes, lipid composition was optimized for efficient encapsulation of BioA and liposomal characteristics. DOX loading was done in the optimized liposomes to get combinational liposomes containing BioA and DOX. These combinational liposomes were able to reverse DOX resistant in ColoR cells on par to that of standard nano-dispersed BioA. Furthermore liposomal DOX was evaluated along with nano-Cur for reversal of DOX resistant and from the results nano-Cur was observed to be a suitable water soluble alternative to that of nano-dispersed Cur.

Chapter 6. Conclusion and future perspective

Aim of the work was to develop combinational nano-formulations for circumvention of chemo-resistance in different cancer model system. Choosing a suitable P-gp inhibitor and developing it in to a single nano-formulation with DOX in proper dosing ratio and EE are the major challenges addressed here. Finding a suitable P-gp inhibitor for reversal of chemo-resistance is indeed a challenge because mode of action of different drug and mechanisms of acquire resistance varies in different case. Hence for circumvention selection has to be done on case-to-case basis for different anticancer drugs and model systems. Thus for experimentation we developed DOX resistant models of leukemia and colon cancer where over-expression of P-gp majorly contributed to resistance. P-gp inhibitors from natural sources were screened in terms of P-gp inhibitory activity and reversal of drug resistance. From the screening, Cur was chosen for K562 while BioA and Cur were selected for COLO205. The combination was subjected for optimization of solubility and analytical parameters. For K562, various formulations of Cur were also adopted for finding suitability as a carrier. The formulations were compared in terms of pharmaceutical features such as size, zeta potential, PDI and EE. Furthermore, their biological activity to inhibit P-gp and reverse DOX resistance was evaluated. Combining the pharmaceutical and biological features, liposomal Cur was observed to be optimal and thus co-loaded liposomes containing DOX and Cur were formulated and evaluated for their efficacy to reverse drug resistance. For COLO205, liposomal composition was optimized for pharmaceutical properties and encapsulation of BioA. The optimized BioA containing liposomes were co-loaded with DOX and analyzed for reversal of DOX resistance. In addition nano-Cur in combination with DOX was studied for their efficacy to resensitize resistant COLO205 cells.

Key findings of this work are as follows.

- Suitable combination for reversal of DOX resistance in K562 cells were found out.
- Among Cur nano-formulations CurL was found to be most suitable.
- Presence of PVA as stabilizer during processing significantly increased the EE of Cur in HP-β-CD.
- MPEG-PCL micelles of Cur abolished the P-gp inhibitory activity of Cur in this model system.
- A combinational HPLC method was developed to detect and quantify DOX and BioA simultaneously.
- Combinational liposomes containing Cur or BioA along with DOX were effective in sensitizing resistant K562 and COLO205 cells respectively,

Future perspectives of this work are as follows.

- Study of pharmaceutical featurs of the formulations such as lyophilization, stability, morphology and other properties.
- Validation of selected combination in other P-gp over-expressed *in vitro* models will be supportive evidence to present finding. In addition, *in vivo* screening of optimal co-loaded formulations in leukemia is much desired.
- In one of our studies Cur loaded micelles of MPEG-PCL was found inactive in terms of biological activity in K562 cell line. This report is in contrast to available literatures. Thus mechanistic insights to find out the possible reasons will be interesting to study.

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