SYNOPSIS

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

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

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

Post determination of the optimum combinatorial index, the most effective ratio of the drugs was encapsulated in a single nanoliposome. The dual drug loaded nanoliposome was optimised using an array of OFAT studies to determine the factors responsible optimal CQA for encapsulation efficiency of both drugs, particle size, zeta potential and drug release. The independent variables evaluated for the active co-loading of the drugs included transmembrane

salt gradient, concentration of cholesterol (lipid molar ratio), drug loading temperature, phosphatidylcholine chain length, sequence of addition of drugs, pH of drug loading, external medium, drug to lipid molar ratio and concentration of Ammonium sulphate. Three dependent variables- concentration of ammonium sulphate, pH of drug loading and lipid molar ratio was found to have significant effect on the tested parameters. These parameters were then evaluated using 2^3 full factorial design to understand the effect of the variation of the factors individually and together on the tested CQAs. The significance of combination of three individual causal factors and corresponding interactions on the dependent outcomes were explored using regression analysis and ANOVA. The results of the DOE studies and regression equation indices indicate that 350 mM ammonium sulphate, drug loading at pH 5.5 and lipid ratio of 56.55:38.19:5.26 (HSPC: Cholesterol: mPEG-2k-DSPE) were found optimal for suitable particle size, zeta potential and entrapment efficiency of both drugs.

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

The ATR-FTIR and microcalorimetric characterization of the formulation showed the lack of any physicochemical interactions between the active agents and excipients. Further, these studies indicated the presence of the drugs within the aqueous core of the liposomes while exhibiting the characteristic thermal melting of the formed doxorubicin sulphate crystals present in the aqueous compartment at 68.75±1.07°C in doxorubicin containing formulations. The in-vitro characteristics and stability of the formulation was further evaluated using studies

of fixed aqueous layer thickness (FALT), electrolyte-induced flocculation/aggregation, interaction with Serum proteins and protein adsorption, plasma stability and liposome membrane integrity. These tested parameters indicated the presence of sufficient in-vitro stability at the storage conditions, stability and lack of interaction potential with blood components. These results of morphology, interaction potential, localization of the drugs, the absence of aggregation and other characteristic properties of the dual drug liposomes were confirmed by small angle X-ray analysis (SAXS) evaluation of the formulation.

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

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

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

significantly improved cytotoxicity potential of the drugs when co-encapsulated in a single carrier as compared to neat drugs, individual liposomal carriers and combination of individual liposomal components. The enhanced cytotoxicity potential of the optimized formulation was explained by the increased cellular uptake which resulted in significantly increased cell cycle arrest in G_2/M phase. Further, higher presentation of the cells in the G_0 phase resulted in the significantly improved apoptotic potential which were in good correlation with the reduced cell viability in both tumor cell lines when presented with co-loaded formulation than with the single drug liposome. The dual liposomes exhibited the highest inhibition of the cellular recovery (in wound scratch study) which indicated significant reduction in cellular viability as well as presenting improved chances of in-vivo anti-angiogenic properties as compared to liposomal DOX.

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

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