# *Chapter 10: In-vivo animal studies*

# 10.0 In-vivo animal studies

## 10.1 Introduction

Cancer may be defined as the abnormal, uncontrolled growth of cells with the inherent ability to spread to other tissues of the human body, facilitated majorly through the components of the hematic system. One of the current treatment options for cancer involves the use of a cocktail of chemotherapeutic drugs, this allows multiple agents to act through multiple mechanistic pathways on tumor cells (1).

Triple negative breast cancer (TNBC) is an aggressive form of breast cancer which is more difficult to treat than the hormone sensitive forms of breast cancer and often necessitates the use of combination chemotherapy (2). Similarly, the adenocarcinoma form of non-small cell lung cancer (NSCLC) is amongst the most aggressive forms of lung cancer and requires combination therapy for effective treatment (3). Two aspects critical for the efficacy of such combination therapies include synergism between the drugs and the use of a suitable carrier system which can ferry the synergistic drug load to the tumor site (4).

The drug combination Doxorubicin hydrochloride (DOX) and Vincristine sulphate (VCR) have been indicated together for treatment of both these aggressive adrenocarcinomas (5). These drugs acting on multiple targets during different cell cycle phases ensuring improved reduction in tumor cell load (1). Nano-carrier mediated controlled delivery of agents may help in effective translation of combination drug therapy to have tumor temporospatial presence (6). Combinatorial nanocarrier approaches such as passive targeting and surface engineering for active targeting may be used for tumor specific delivery (7-9). Clinically approved pegylated liposomal DOX has been used as a component of established combination chemotherapies against TNBC and NSCLC in place of conventional DOX solutions. However, using this approach neither synergistic co-presence of liposomal DOX along with another chemotherapeutic agent nor the desired efficacy may be ascertained (10). Strategies to improve efficacy of liposomal DOX including active targeting, active/inactive agents co-encapsulation and alteration of drug release using external stimuli have failed in eliciting favourable response in TNBC and NSCLC (11-13). Synergistic combinatorial incorporation of another active agent

(VCR) into clinically used liposomal DOX may present another approach for improvement in efficacy without altering physicochemical, toxicological and safety profiles (14). However, a rationalistic approach considering the clinical risk-to-benefit ratio needs to be adopted while building a pegylated combinatorial liposomal formulation of DOX and VCR (15). This would include bridging physicochemical properties with detailed biological studies to ascertain the efficacy and toxicity potential of such formulations (16). The aim of the current section was to evaluate effect of synergistic VCR co-loading onto *in-vivo* characteristics of pegylated liposomal DOX. The *in-vivo* evaluation of co-encapsulation approach included determining maximum tolerated dose, tumor regression studies, pharmacokinetic and bio-distribution profiling.

Importantly, the efficiency of pre-clinical to clinical translation of these co-loaded formulations are often affected by the choice of the preclinical model and its predictiveness. While the in-vitro cell line studies in 2D and 3D spheroid culture models present assessment of initial efficacy, in-vivo studies are important to establish the effect of tumor microenvironment, the presence of growth factors, cytokines among others on the efficiency of the nanocarrier (17). The two widely used human cancer cell line based tumor predictive models include the xenograft and orthotropic models. The xenograft cancer models are generated by subcutaneous injection of the cancer cell line in immunocompromised animals (18). This model leads to generation of homogenous tumor mass at sites other than its natural occurrence and are preferable for the tumors have a faster cell doubling time. The orthotropic models are generated by similar injection except that they are intended for the development of the tumor mass at the natural orthotropic site of the cancer. Both the models generated by subcutaneous injection present suitable opportunities for the development of the tumor and subsequent evaluation of the effects of the formulation (19). In the current study, the xenograft models of non-small cell lung cancer and triple negative breast cancer was established using A549 and MDA MB-231 cell lines respectively. The cell doubling time of both cell lines (22 hours for A549 & 38 hours for MDA MB-231) presented suitable opportunities for the development of the xenograft models. These tumor models were developed in immunocompromised nude athymic mice which exhibited lack of T-lymphocytes and thymus gland leading to absence of T cell-mediated adaptive immunity and antibody formation. These mice have been characterized to been hairless, forkhead box N1 gene abnormality with the presence of B-cells and natural killer cells

(NK cells) (20). The dose response studies were performed in the tumor free mice while the efficacy studies were done in the tumor induced models. 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 method was used as they provide consistent correlations based on algebraic equations without organ/tissue compartment assumptions. The bio-distribution studies were done by collecting samples at pre-determined time points from tissues (Plasma, Spleen, Liver, Kidney, lungs, heart, tumor).

### 10.2 Animals

Female nude athymic mice (weighing 30± 5 g) were used for acute toxicity, efficacy and tissue distribution studies while Sprague Dawley female rats (weighing 160± 20 g) were used for pharmacokinetics study. All the animals were housed in propylene cages for acclimatization under controlled room temperature (25 ± 2°C), humidity (60 ± 10%) and alternative light (12-h light/12-h dark cycle) prior to experimentation for 10 days. The animals were fed pellet diet and water ad libitum.

### 10.3 Ethical Disclosure

Care was taken that all experimental conditions and all animals were maintained as per requirements of IAEC (Institutional Animal Ethics Committee) of SPIL (Sun Pharmaceutical Industries Limited) in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines.

### 10.4 Methodology

#### 10.4.1 Acute toxicity study

The maximum tolerated dose (MTD) of the liposomal formulations was analysed by conducting acute toxicity study in healthy female nude athymic mice. Liposomal DOX (clinically approved formulation) has been previously reported to have MTD of 6 mg/Kg and 9 mg/Kg (21, 22). Briefly, 10-week-old nude athymic mice were taken and randomly grouped (n=6) to receive liposomal treatments of liposomal DOX (6 mg/kg and 9 mg/Kg), liposomal VCR (treatment at ratio-mimetic doses of 3 mg/Kg and 4.5 mg/Kg, considering VCR: DOX 1:2 synergistic weight ratio) with one group receiving normal saline as control. The formulations were intravenously administered at q7d*4 dosage regimen and MTD was

determined based on the absence of mortality and less than 20% change in body weight over the study period (35 days). Further, based on the determined MTD, dual drug liposomes were evaluated for similar MTD determination (n=6 mice) using the same dose regimen (6 mg/Kg DOX and 3 mg/Kg VCR) and study period (35 days).

### 10.4.2 In-vivo efficacy

The in-vivo efficacy of VCR co-loading into the liposomal DOX was evaluated using tumor regression studies in xenograft model in female nude athymic mice. Briefly, 10-week-old mice were subcutaneously injected with 5 X $10^7$ tumor cells of A549 (non-small cell lung cancer) and MDA-MB 231 (metastatic triple negative breast cancer) to reach 100-160 $mm^3$ tumor volume. The animals were then divided randomly into 5 groups for each tumor model (with each of 6 animals) based on tumor volume as well as body weight (> 28 grams) to receive formulation treatments (drug free liposome, liposomal DOX, liposomal VCR, combination of liposomal DOX and liposomal VCR, dual drug liposome). The formulations were intravenously administered q7d*4 dosage regimen at the determined MTD (6 mg/Kg DOX and 3 mg/Kg VCR) while the disease progression was monitored till 35 days as a function of tumor volume and body weight determined twice/week while mortality was measured daily. Tumor volume was calculated based on the two perpendicular diameters (D1 and D2) using the formula for a sphere $[(D_1 + D_2)/ 3 \times 0.5236\ mm^3$ (23). All measurements were made in triplicate and efficiency of the various treatments were evaluated statistically using ANOVA, logrank test and percentile ratio of test with control (%T/C).

### 10.4.3 Pharmacokinetics

Comparative pharmacokinetic profile of the formulations was evaluated in 5-8-week-old female Sprague-Dawley rats as per method described earlier (22). Briefly, overnight fasted rats were subdivided into five groups (n=6) and administered with DOX solution, VCR solution, liposomal DOX, liposomal VCR and dual drug liposome with single intravenous injection (through tail vein) at dose equivalent to 4 mg/Kg DOX and 2mg/Kg VCR. Blood samples were collected into heparinised tubes at 0, 0.5, 1, 2, 4, 6, 12 hours post injection through retro-orbital puncture. Plasma was separated from the blood samples by centrifugation at 3000 rpm for 5 minutes at 5°C and stored at -20°C until analysis. The plasma samples were analysed for DOX and VCR content using RP-HPLC with some modifications while the data was analysed for

pharmacokinetic parameters using Kinetica software 5.1 assuming non-compartmental modelling (22). The statistical significance of the results was determined using paired t-test with $p<0.05$ considered significant.

### 10.4.4 Tissue Distribution studies

The tissue distribution studies were carried out in 10-week-old female athmic nude mice bearing xenograft model of subcutaneously injected 5 X $10^7$ tumor cells of MDA-MB 231 after it reached tumor volume of 100-160 $mm^3$. The animals were then divided randomly into 5 groups (with each of 6 animals) with each group to receive a single intravenous injection of formulation treatments (DOX solution, VCR solution, liposomal DOX, liposomal VCR, dual drug liposome) with dose equivalent to 6 mg/kg DOX ad 3 mg/Kg VCR (23). The animals of each group were euthanised after 4 and 24 hours of treatment (with three animals at each time point). Blood samples were collected into EDTA containing centrifuge tubes while organs (liver, spleen, kidney, heart, lung, tumor) were removed and blotted on Whatman filter paper at each time point. Plasma was separated from blood by centrifugation at 3000 rpm for 5 minutes at 5°C and analysed for drug content using RP-HPLC (as mentioned in 10.3.2). The tissues were homogenized with minimum volume of Triton X-100 (0.25% solution), de-proteinized using acetronitrile, centrifuged and evaluated for drug content as mentioned in section 10.3.2 (24). The statistical significance of the results was determined using paired t-test with $p<0.05$ considered significant.

### 10.4.5 Statistics

Statistical significance of the data ($p<0.05$) was determined using ANOVA and Student's t-test using GraphPad Prism (version 6.0 USA).

## 10.5 Results and Discussion

### 10.5.1 Acute toxicity study

Maximum tolerated dose of single drug and dual drug liposome was evaluated post q7d*4 dosage regimen till 35 days. The single drug liposomes were evaluated at different concentrations (Table 28, Figure 59) with mortality being observed in case of higher doses (9mg/Kg for DOX and 4.5 mg/Kg for VCR) while no mortality (with % change in body weight

<10 %) was observed at the lower doses. The dual drug liposome was then evaluated at MTD of 6 mg/Kg DOX (3 mg/Kg VCR) with no mortality being observed at this dose.

The MTD of the new formulation in nude athymic mice was found to be 6 mg/Kg DOX (with 3 mg/Kg VCR) and the results were found to be in good correlation with the clinically used formulation, PEGylated Liposomal Doxorubicin (25).

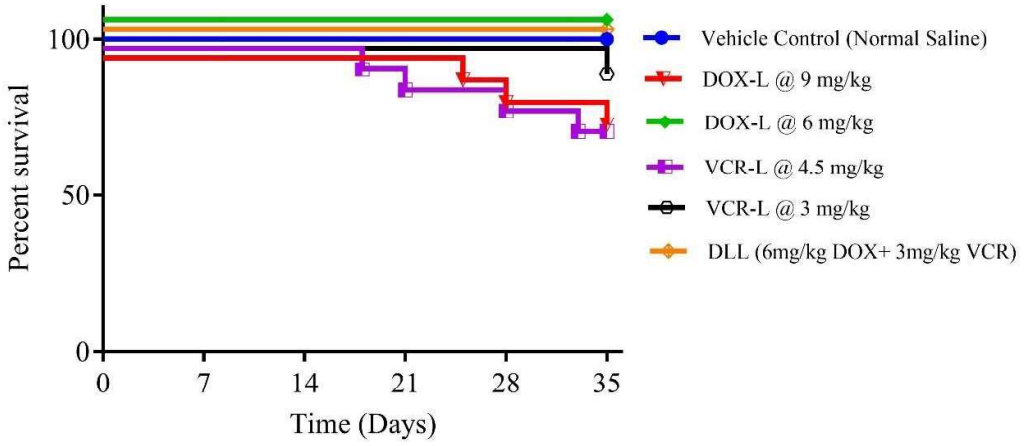


**Figure 59:** Results of acute toxicity study of Liposomal Doxorubicin (DOX-L), Liposomal Vincristine (VCR-L) and dual drug liposome at various concentrations (graphs are offset for clarity)

| Days | Vehicle control (Normal Saline) | | Liposomal DOX @ 9 mg/Kg | | Liposomal DOX @ 6 mg/Kg | | Liposomal VCR @ 4.5 mg/Kg | | Liposomal VCR @ 3 mg/Kg | | Dual drug Liposome @ 6 mg/Kg DOX+3 mg/kg VCR | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | % Survival | n | % Survival | n | % Survival | n | % Survival | n | % Survival | n | % Survival |
| **0** | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 |
| **4** | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 |
| **7** | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 |
| **10** | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 100 |
| **14** | 10 | 100 | 10 | 100 | 10 | 100 | 10 | 90 | 10 | 100 | 10 | 100 |
| **18** | 10 | 100 | 10 | 100 | 10 | 100 | 9 | 90 | 10 | 100 | 10 | 100 |
| **21** | 10 | 100 | 10 | 100 | 10 | 100 | 8 | 80 | 10 | 100 | 10 | 100 |
| **25** | 10 | 100 | 9 | 90 | 10 | 100 | 8 | 80 | 10 | 100 | 10 | 100 |
| **28** | 10 | 100 | 8 | 80 | 10 | 100 | 7 | 70 | 10 | 100 | 10 | 100 |
| **33** | 10 | 100 | 8 | 80 | 10 | 100 | 6 | 60 | 10 | 100 | 10 | 100 |
| **35** | 10 | 100 | 7 | 70 | 10 | 100 | 6 | 60 | 9 | 90 | 10 | 100 |

DOX: Doxorubicin Hydrochloride; VCR: Vincristine Sulphate

**Table 28:** Results of acute toxicity study of DOX-L, VCR-L and dual drug liposome at various concentrations: Survival (%) with varying doses and time [ The presented values of "n" indicate the number of living animals at each time point (Days)].

### 10.5.2 In-vivo efficacy

The in-vivo efficacy of the optimized formulation was evaluated using the A549 and MDA-MB 231 tumor regression study. The animals were sacrificed when the tumor volume in the drug free liposomal treatment group reached 2500 $mm^3$ (MDA-MB 231) and 2200 $mm^3$ (A549) was considered as the end point of the study with results of A549 xenograft model (Table 29, 31 and Figure 60) and MDA-MB 231 (Table 30, 31 and Figure 61) representing the data.

The results of treatment of A549 tumor with drug loaded liposomes exhibited significant reduction in the tumor volume as compared to the drug free liposomal treatment (%T/C values: liposomal DOX-38.46; liposomal VCR- 48.06; liposomal DOX+ liposomal VCR- 35.35; Dual drug liposomes- 17.58). Further, the dual drug treated group exhibited significantly higher ($p<0.05$) tumor volume reduction as compared to the other treatment groups (>50%tumor volume reduction). The tumor regression curves of the drug treated groups indicate towards moderate tumoricidal activity in the single drug liposome and combination of single drug liposome treated groups while indicating towards high tumoricidal activity for dual drug loaded liposomes.

The treatment of MDA-MB 231 tumor with dual drug liposome resulted in significant reduction in the tumor volume as compared to that of the drug free liposomal treatment group (%T/C= 2.08). All the other drug treatment groups (liposomal DOX, liposomal VCR and liposomal DOX+ liposomal VCR) showed significant reduction in the tumor volume as compared to the drug free liposomal treated group (%T/C values of 4.98, 5.77, 4.37 respectively). Additionally, the tumor regression was found to be significantly higher ($p<0.05$) in dual drug liposome treated group as compared to single drug loaded and combination of single drug loaded liposomes (approximately 50% reduction in the tumor volume). Further, the tumor regression curves in all the drug treatment groups indicate towards the high tumoricidal potential of the drugs encapsulated in the lipid nanocarrier.

Importantly, increase in the tumor volume of animals in drug free liposomal treatment group in case of both the studies indicate towards the absence of efficacy of drug free liposomal carrier. In both the studies no significant loss in body weight was observed and no mortality was observed in all the animal groups (Table 29 and 30).

| **Mean Tumor volume ($mm^3$)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (Days)** | **Placebo** | | | **Liposomal doxorubicin** | | | **Liposomal Vincristine** | | | **Lip DOX+ Lip VCR** | | | **Dual drug Liposome** | | |
| | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** |
| **0** | 150.70 | 14.51 | 6 | 152.80 | 18.95 | 6 | 158.10 | 19.63 | 6 | 154.30 | 19.63 | 6 | 156.30 | 16.19 | 6 |
| **7** | 410.80 | 51.23 | 6 | 363.60 | 38.75 | 6 | 365.10 | 45.17 | 6 | 370.50 | 35.46 | 6 | 370.80 | 24.71 | 6 |
| **14** | 830.90 | 70.81 | 6 | 524.50 | 41.83 | 6 | 624.30 | 78.85 | 6 | 576.20 | 44.31 | 6 | 360.60 | 21.15 | 6 |
| **21** | 1350.10 | 105.17 | 6 | 681.90 | 85.56 | 6 | 771.20 | 86.34 | 6 | 670.40 | 75.49 | 6 | 365.53 | 23.19 | 6 |
| **28** | 1745.42 | 138.92 | 6 | 749.20 | 78.54 | 6 | 900.10 | 105.62 | 6 | 683.20 | 80.22 | 6 | 370.70 | 34.21 | 6 |
| 35 | 2210.70 | 159.47 | 6 | 850.30 | 69.21 | 6 | 1062.40 | 150.11 | 6 | 781.50 | 55.71 | 6 | 388.70 | 40.53 | 6 |
| **Body weight (gm) and survival** | | | | | | | | | | | | | | | |
| **Time (Days)** | **Placebo** | | | **Liposomal doxorubicin** | | | **Liposomal Vincristine** | | | **Lip DOX+ Lip VCR** | | | **Dual drug Liposome** | | |
| | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** |
| **0** | 30.40 | 1.53 | 6 | 31.20 | 1.82 | 6 | 32.30 | 1.51 | 6 | 31.80 | 1.07 | 6 | 30.70 | 0.81 | 6 |
| **7** | 32.80 | 2.31 | 6 | 30.40 | 1.62 | 6 | 30.80 | 0.63 | 6 | 28.60 | 0.87 | 6 | 28.30 | 1.12 | 6 |
| **14** | 33.50 | 1.89 | 6 | 28.50 | 1.32 | 6 | 28.40 | 0.69 | 6 | 26.50 | 0.79 | 6 | 27.30 | 1.18 | 6 |
| **21** | 34.10 | 2.56 | 6 | 30.60 | 1.12 | 6 | 29.30 | 0.52 | 6 | 28.40 | 0.63 | 6 | 30.80 | 0.95 | 6 |
| **28** | 36.20 | 2.31 | 6 | 31.50 | 0.89 | 6 | 30.20 | 0.57 | 6 | 29.40 | 0.74 | 6 | 30.70 | 0.66 | 6 |
| **35** | 38.10 | 3.12 | 6 | 32.50 | 0.81 | 6 | 31.90 | 0.70 | 6 | 30.50 | 0.78 | 6 | 30.70 | 0.78 | 6 |

**Table 29:** Results of in-vivo efficacy study in A549 xenograft model in nude athymic mice (n=6 animals/ group). [ The presented values of "N" indicate the number of living animals at each time point (Days)].

| **Mean Tumor volume ($mm^3$)** | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Time (Days)** | **Placebo** | | | **Liposomal doxorubicin** | | | **Liposomal Vincristine** | | | **Lip DOX+ Lip VCR** | | | **Dual drug Liposome** | | |
| | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** |
| **0** | 105.60 | 15.29 | 6 | 105.30 | 14.95 | 6 | 100.30 | 13.59 | 6 | 104.80 | 14.53 | 6 | 106.80 | 18.17 | 6 |
| **7** | 310.50 | 58.56 | 6 | 230.40 | 34.75 | 6 | 229.70 | 24.42 | 6 | 225.80 | 26.95 | 6 | 172.90 | 24.71 | 6 |
| **14** | 765.20 | 81.94 | 6 | 177.90 | 20.83 | 6 | 221.50 | 25.95 | 6 | 180.50 | 21.68 | 6 | 132.10 | 11.15 | 6 |
| **21** | 1423.00 | 156.15 | 6 | 138.30 | 17.56 | 6 | 153.60 | 28.13 | 6 | 128.10 | 19.32 | 6 | 105.80 | 12.19 | 6 |
| **28** | 1712.70 | 202.35 | 6 | 131.30 | 18.54 | 6 | 157.10 | 15.17 | 6 | 120.30 | 16.17 | 6 | 61.10 | 8.31 | 6 |
| **35** | 2526.70 | 250.13 | 6 | 125.80 | 10.21 | 6 | 145.70 | 17.25 | 6 | 110.50 | 11.33 | 6 | 52.50 | 5.25 | 6 |
| **Body weight (gm) and survival** | | | | | | | | | | | | | | | |
| **Time (Days)** | **Placebo** | | | **Liposomal doxorubicin** | | | **Liposomal Vincristine** | | | **Lip DOX+ Lip VCR** | | | **Dual drug Liposome** | | |
| | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** | **Mean** | **SD** | **N** |
| **0** | 29.50 | 0.67 | 6 | 29.00 | 1.82 | 6 | 32.90 | 0.99 | 6 | 31.40 | 1.23 | 6 | 32.80 | 0.67 | 6 |
| **7** | 29.00 | 0.86 | 6 | 27.40 | 1.4 | 6 | 32.10 | 0.63 | 6 | 30.10 | 1.31 | 6 | 30.60 | 0.51 | 6 |
| **14** | 28.20 | 1.44 | 6 | 26.90 | 1.06 | 6 | 31.50 | 0.69 | 6 | 29.80 | 1.09 | 6 | 30.10 | 0.53 | 6 |
| **21** | 29.60 | 0.89 | 6 | 26.50 | 0.89 | 6 | 31.10 | 0.52 | 6 | 29.60 | 1.11 | 6 | 30.80 | 0.58 | 6 |
| **28** | 31.90 | 0.99 | 6 | 27.00 | 0.72 | 6 | 32.50 | 0.57 | 6 | 29.40 | 0.95 | 6 | 30.70 | 1.16 | 6 |
| **35** | 33.00 | 1.24 | 6 | 27.20 | 0.79 | 6 | 32.70 | 0.70 | 6 | 29.00 | 0.87 | 6 | 30.70 | 0.69 | 6 |

**Table 30:** Results of in-vivo efficacy study in MDA-MB 231 xenograft model in nude athymic mice (n=6 animals/ group). [ The presented values of "N" indicate the number of living animals at each time point (Days)].

| %Test/Control values: A549 xenograft model in nude athymic mice (n=6) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (Days) | Lip DOX | Lip VCR | Lip DOX+ Lip VCR | | | Dual drug Liposome | | | |
| | %TC-Placebo | %TC-Placebo | %TC-Placebo | %TC-LD | %TC-LV | %TC-Placebo | %TC-LD | %TC-LV | %TC-LV+LD |
| 0 | 101.39 | 104.91 | 102.39 | 100.98 | 97.60 | 103.72 | 102.29 | 98.86 | 101.30 |
| 7 | 88.51 | 88.88 | 90.19 | 101.90 | 101.48 | 90.26 | 101.98 | 101.56 | 100.08 |
| 14 | 63.12 | 75.14 | 69.35 | 109.86 | 92.30 | 43.40 | 68.75 | 57.76 | 62.58 |
| 21 | 50.51 | 57.12 | 49.66 | 98.31 | 86.93 | 27.07 | 53.60 | 47.40 | 54.52 |
| 28 | 42.92 | 51.57 | 39.14 | 91.19 | 75.90 | 21.24 | 49.48 | 41.18 | 54.26 |
| 35 | 38.46 | 48.06 | 35.35 | 91.91 | 73.56 | 17.58 | 45.71 | 36.59 | 49.74 |
| %Test/Control values: MDA MB 231 xenograft model in nude athymic mice (n=6) | | | | | | | | | |
| Time (Days) | Lip DOX | Lip VCR | Lip DOX+ Lip VCR | | | Dual drug Liposome | | | |
| | %TC-Placebo | %TC-Placebo | %TC-Placebo | %TC-LD | %TC-LV | %TC-Placebo | %TC-LD | %TC-LV | %TC-LV+LD |
| 0 | 99.72 | 94.98 | 99.24 | 99.53 | 104.49 | 101.14 | 101.42 | 106.48 | 101.91 |
| 7 | 74.20 | 73.98 | 72.72 | 98.00 | 98.30 | 55.68 | 75.04 | 75.27 | 76.57 |
| 14 | 23.25 | 28.95 | 23.59 | 101.46 | 81.49 | 17.26 | 74.26 | 59.64 | 73.19 |
| 21 | 9.72 | 10.79 | 9.00 | 92.62 | 83.40 | 7.43 | 76.50 | 68.88 | 82.59 |
| 28 | 7.67 | 9.17 | 7.02 | 91.62 | 76.58 | 3.57 | 46.53 | 38.89 | 50.79 |
| 35 | 4.98 | 5.77 | 4.37 | 87.84 | 75.84 | 2.08 | 41.73 | 36.03 | 47.51 |

**Table 31:** Results of % Test/control of drug treatments in A549 and MDA-MB 231 xenograft model in nude athymic mice (n=6 animals/group)

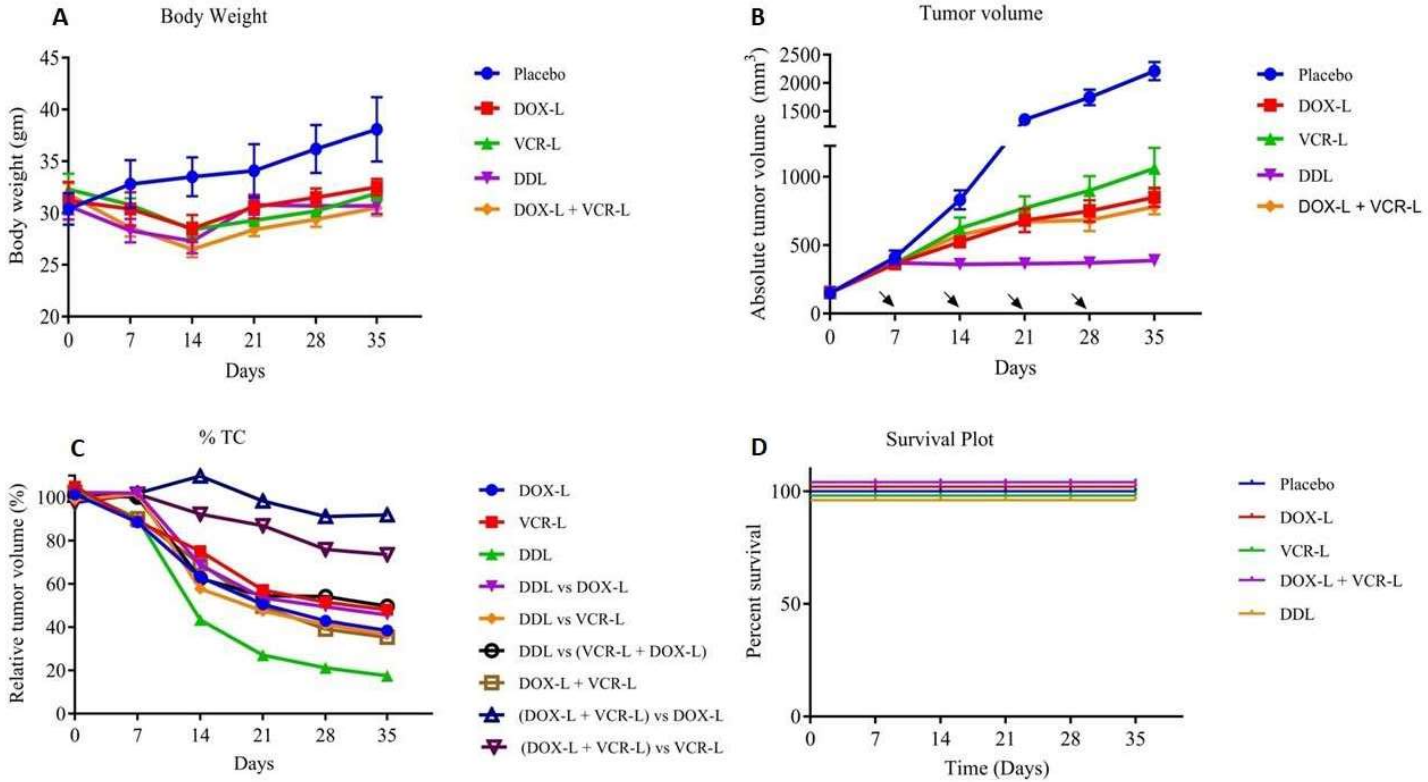


**Figure 60:** In-vivo efficacy study in A549 xenograft model in nude athymic mice (A) Body weights (B) Mean tumor volumes (C) % Test/Control (D) Kaplan–Meier percent survival (graphs are offset for clarity) for various liposomal formulations (Arrows in figure 10.2 B indicates dosing days; values displayed as mean ±SD, n=6).

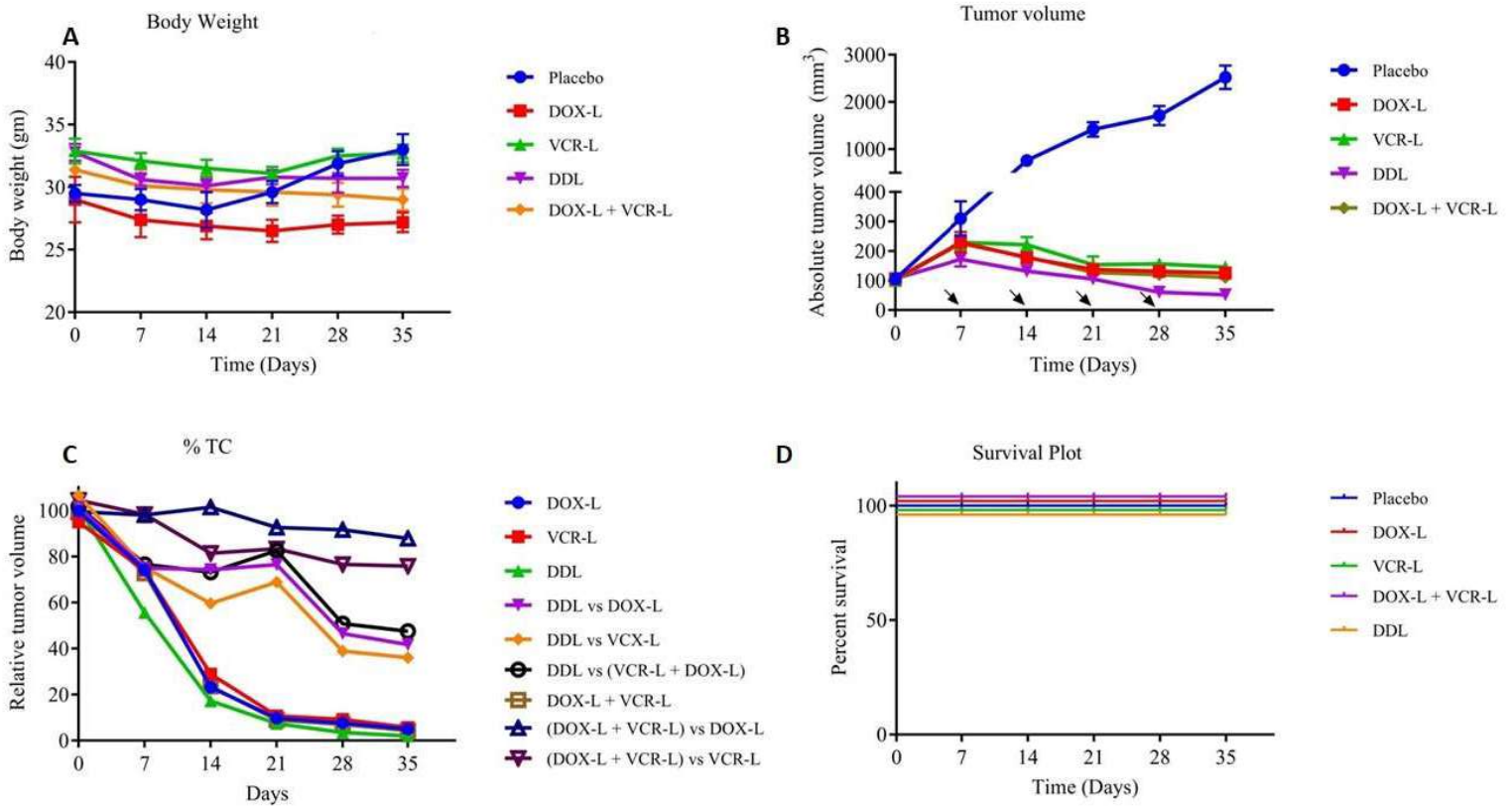


**Figure 61:** In-vivo efficacy study in MDA-MB 231 xenograft model in nude athymic mice (A) Body weights (B) Mean tumor volumes (C) % Test/Control (D) Kaplan–Meier percent survival (graphs are offset for clarity) for various liposomal formulations (Arrow in figure 10.3 B indicates dosing days; values displayed as mean ±SD, n=6).

The %Test/Control and ANOVA results amongst the treatment groups in both the studies indicate towards significant tumor reduction in case of the presentation of the synergistic combination of the drugs (DOX, VCR) in a single lipid carrier as compared to single drug liposomes and combination of single drug liposomes. These results are in good correlation with the in-vitro cell viability study and indicate towards the significantly improved efficacy of the DOX and VCR combination against the tested solid tumors.

Tumor regression curves in both studies indicate high tumoricidal activity of dual drug liposome with significant improvement ($p<0.05$) over liposomal DOX which may be attributed to the presence of the VCR in addition to DOX when the liposomes were presented to the tumor cells. These results present good correlation with cell viability study while indicating towards improved efficacy of liposomal DOX in NSCLC and TNBC on co-loading with VCR.

### 10.5.3 Pharmacokinetics

The pharmacokinetic profile of DOX and VCR post single bolus intravenous injection of the formulations (equivalent to 4 mg/Kg liposomal DOX and 2 mg/Kg Liposomal VCR) was evaluated for the total quantity of drugs over 24 hours in female Sprague-Dawley rats. The plasma concentration vs time profile of DOX and VCR are presented in Table 32, Figure 62 A and 62 B respectively while the pharmacokinetic parameters of the drugs considering non-compartmental kinetic modelling from the formulations are presented in Table 33. Since, total drug content (drug entrapped in liposomes+ free drug) were determined during this PK evaluation, the concentrations of the drugs DOX and VCR at 0-hour time point was found to be high post intravenous injection. While the non-liposomal drug solutions exhibited time dependent decrease in the concentration of the drugs over 24 hours study period, the liposomal formulations showed gradual removal of the formulation from the blood and probable assimilation in organs (indicative of the long circulation property of PEGylated liposomes). The carrier free drugs showed quick elimination profiles with observed mean residence time (MRT) of 9.24±0.31 h ($t_{1/2}$=1.25±0.25 h) and 2.94±0.18 h ($t_{1/2}$=0.34±0.15 h) for DOX and VCR respectively. The liposomal formulations of both drugs showed non-significant difference in pharmacokinetic parameters when tested from single drug liposomes and dual drug liposomes while having significant difference with the free drug (Table 33). For DOX containing formulations, liposomal DOX and dual drug liposomes presented 24-fold and 15-fold increase

in $t_{1/2}$ and AUC respectively with 35-fold decrease in clearance as compared to free drug. Comparative plasma concentration-time profiling of DOX and VCR from the liposomes indicate towards more rapid elimination of VCR than DOX which may be attributed to the higher permeability of VCR sulphate as compared to DOX sulphate (Figure 27 and 62). Interestingly, VCR plasma concentration profiles indicate an initial burst release similar to that presented in the in-vitro release profile.

| Time (Hr) | Concentration of DOX (ng/ml) | | | Concentration of VCR (ng/ml) | | |
|---|---|---|---|---|---|---|
| | DOX solution | Liposomal DOX | DDL-DOX | VCR solution | Liposomal VCR | DDL-VCR |
| **0** | 7561±139 | 9541±155 | 9517±147 | 3315±116 | 5489±169 | 5512±136 |
| **0.5** | 850±77 | 8869±160 | 8845±139 | 471±28 | 2568±118 | 2593±105 |
| **1** | 737 ±61 | 8253±108 | 8234±151 | 315±31 | 2216±135 | 2244±109 |
| **2** | 668 ±52 | 7752±121 | 7730±119 | 258±20 | 1853±109 | 1886±121 |
| **4** | 541±45 | 7139±114 | 7120±161 | 117±11 | 1459±69 | 1490±77 |
| **6** | 430±35 | 6547±126 | 6516±133 | 61±6.8 | 1136±29 | 1170±55 |
| **12** | 225±21 | 5269±158 | 5238±144 | 15±2.5 | 818±47 | 852±37 |
| **24** | 100±12 | 4850±139 | 4855±154 | 6±1.1 | 562±33 | 587±29 |

Values presented as Mean±SD (n=6)

**Table 32:** Concentration (total drug content) vs Time profile of DOX and VCR on single IV dose of formulations at concentrations equivalent to 6 mg/Kg DOX (3 mg/Kg VCR)

Non-Compartmental pharmacokinetic profiling was done to access the differences in plasma concentration-time profiles when presented as various formulations in Sprague Dawley rats. Incorporation of the drugs into pegylated liposomal formulations resulted in longer plasma circulation with slow elimination profile which resulted in significant increase ($p<0.05$) in AUC, $t_{1/2}$ and MRT while having significant reduction ($p<0.05$) in clearance and steady state volume of distribution as compared to the free drug. Additionally, presentation of both drugs together in single liposome did not change the PK profile of the individual drugs as compared to the profile presented from the single liposome. The DOX pharmacokinetic profile from dual drug liposome exhibited similar characteristics as compared clinically used pegylated liposomal DOX while presenting significant difference from free DOX and the results are in good correlation with earlier reports on the same (26, 27).

| Pharmacokinetic Parameter | Values for DOX | | | Values for VCR | | |
|---|---|---|---|---|---|---|
| | Free DOX | DOX-L | Dual | Free VCR | VCR-L | Dual |
| $C_0$ (µg/ml) | 7.56± 1.38 | 9.54± 1.06 | 9.49± 1.56 | 3.32± 1.04 | 5.49± 1.23 | 5.51± 1.39 |
| $t_{1/2}$ (hr) | 1.25± 0.25 | 30.21± 0.36* | 30.39± 0.44* | 0.34± 0.15 | 18.25± 0.16* | 18.59± 0.18* |
| $AUC_{0-t}$ (hr* µg/ml) | 9.29± 2.58 | 141.62± 15.62* | 141.95± 16.33* | 2.37± 1.09 | 25.29± 4.29* | 26.04± 3.81* |
| MRT (hr) | 9.24± 0.31 | 44.76± 0.55* | 45.05± 0.63* | 2.94± 0.18 | 23.97± 0.31* | 24.53± 0.38* |
| Cl (ml*Kg/hr) | 574.96± 76.31 | 16.19± 2.15* | 15.76± 2.29* | 1266.36 ±82.34 | 74.83± 10.23* | 71.80± 12.62* |
| $V_{ss}$ (L/Kg) | 5.311± 0.26 | 0.761± 0.29* | 0.766± 0.25* | 3.724 ±0.24 | 1.79± 0.19* | 1.76± 0.23* |

Values presented as Mean±SD (n=6); Abbreviations: $C_0$- Concnetration at time t=0; $t_{1/2}$- Half-life; $AUC_{0-t}$-Area under the curve for time =0 to t; MRT-Mean residence time, Cl- Clearence observed, $V_{ss}$ – Steady state volume of distribution; * indicates significant difference ($p<0.05$) of values of liposomal DOX and dual drug liposome with that of free DOX; # indicates significant difference ($p<0.05$) of values of liposomal VCR and dual drug liposome with that of free VCR

**Table 33:** Pharmacokinetic parameters of DOX and VCR on single IV dose of formulations at concentrations equivalent to 6 mg/Kg DOX (3 mg/Kg VCR)

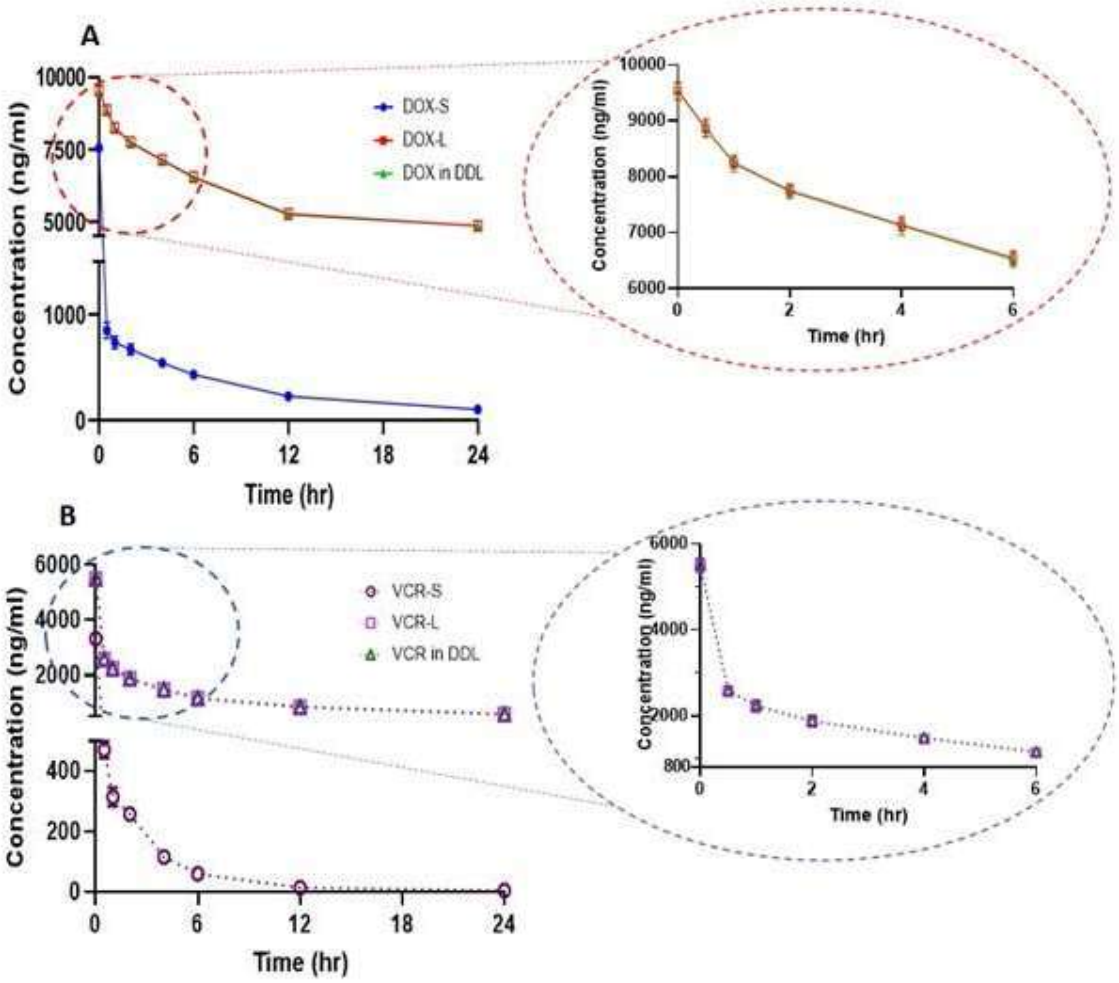


**Figure 62:** Pharmacokinetic profile of DOX (A) and VCR (B) in Sprague Dawley rats from various formulations.

### 10.5.4 Tissue Distribution studies

Tissue distribution studies post intravenous injection of the formulations was evaluated using tumor bearing nude athymic mice. The distribution profiles were analysed 4 h and 24 h post the injection of the formulations and results were presented in Table 34. The free drugs showed time dependent increased accumulation in the kidney and liver which is consistent with the elimination profile of the drugs. Pegylated liposomal formulations of both drugs exhibited time dependent significant increase in plasma concentrations and accumulation in spleen and liver

as compared to free drugs. Tumor accumulation was found to increase in time dependent manner with the liposomal formulations of DOX showing higher accumulation than that of VCR formulations with both having presenting significant difference from the free drugs. The higher accumulation of DOX than VCR in the tumor cells may be attributed to the significantly lower clearance of the drug post intravenous administration (Table 34). Further, the DOX accumulation in heart from the liposomal DOX and dual drug liposomes were similar and significantly reduced as compared with free DOX solution. The reduced accumulation of DOX in heart from the liposomal formulations may help in the reduction of the cardiotoxicity associated with the drug and allow for more effective delivery of the drug to the tumor site (25).

Consistent with the pharmacokinetic profiles, the tissue distribution profile of free drugs was found to more in organs of elimination (kidney and liver). Long circulation afforded by pegylated liposomal formulations were found to have higher plasma concentrations and accumulation in spleen and kidney as compared to the free drugs which is in good correlation with their pharmacokinetic profiles (28). Importantly, these formulations showed time dependent increase in accumulation and reduced elimination from the tumor cells as compared to the free drugs and this may have led to the increased tumor regression of the liposomal formulations. The drugs DOX and VCR presented non-significant ($p>0.05$) differences in tissue distribution profile when presented as dual drug liposomes as compared to single drug liposomes. The incorporation of VCR into pegylated liposomal DOX did not significantly affect the tissue distribution profile of the later and the results are in good correlation with the results of DOXIL™ (29). Additionally, the improved tumor regression profile in NSCLC and TNBC tumor models of the dual drug liposomal formulation as compared to the current liposomal DOX may be attributed to simultaneous temporospatial tumoral presence of both the drugs (30).

| Tissue | Time (hrs) | Concentration of DOX | | | Concentration of VCR | | |
|---|---|---|---|---|---|---|---|
| | | Free DOX | DOX-L | Dual | Free VCR | VCR-L | Dual |
| **Plasma** | 4 | 48.33±2.15 | 144.52±12.53* | 139.54±19.28* | 18.12±0.25 | 24.52±0.31 | 28.33±0.51 |
| | 24 | 12.73 ±0.35 | 118.52±18.62* | 126.59±10.44* | 6.15±0.39 | 10.68±0.14# | 11.55±0.46# |
| **Spleen** | 4 | 9.96 ±1.52 | 7.81 ±1.34 | 6.95 ±1.29 | 3.67±0.11 | 7.42±0.19# | 8.03±0.11# |
| | 24 | 6.81 ±2.81 | 15.67±1.45* | 15.80±4.59* | 10.05±0.55 | 21.55±0.25# | 24.91±0.29# |
| **Liver** | 4 | 7.16 ±0.65 | 14.96 ±4.52* | 11.78 ±1.52* | 3.81 ±1.15 | 1.31±0.22 | 1.89±0.44 |
| | 24 | 9.54 ±3.71 | 15.67 ±0.35* | 14.95±0.35* | 8.76 ±1.71 | 14.29±0.19# | 13.65±0.26# |
| **Kidney** | 4 | 8.65 ±0.20 | 4.23 ±0.20* | 4.65 ±0.20* | 4.58±0.18 | 1.28±0.16 | 1.37±0.23 |
| | 24 | 16.27±1.31 | 11.28± 0.78* | 10.69± 0.67* | 21.96±0.27 | 8.91±0.09# | 10.02±0.09# |
| **Lungs** | 4 | 8.44 ±3.18 | 10.52 ±0.46 | 9.64 ±0.75 | 1.83±0.17 | 2.35±0.41 | 1.68±0.39 |
| | 24 | 4.59 ±2.12 | 15.89 ±0.85* | 14.49±0.85* | 1.95±0.27 | 5.89±0.26# | 4.62±0.18# |
| **Heart** | 4 | 9.19 ±1.54 | 3.42 ±0.46* | 3.87 ±0.33* | 1.15±0.19 | 1.23±0.18 | 1.39±0.16 |
| | 24 | 4.73 ±0.85 | 1.49 ±0.61* | 1.63 ±0.41* | 0.78±0.15 | 0.36±0.20 | 0.58±0.18 |
| **Tumor** | 4 | 0.15 ±0.13 | 0.56 ±0.29 | 0.74 ±0.33 | 0.10±0.09 | 0.11±0.05 | 0.19±0.14 |
| | 24 | 0.09±0.03 | 1.98 ±0.23* | 2.09 ±0.15* | 0.08±0.05 | 0.78±0.29# | 0.84±0.31# |

Values presented as Mean±SD (n=3); Data presented as mean tissue cocentrations of DOX/VCR in µg/gm of tissue or µg/ml of plasma; * indicates significant difference ($p<0.05$) of values of liposomal DOX and dual drug liposome with that of free DOX; # indicates significant difference ($p<0.05$) of values of liposomal VCR and dual drug liposome with that of free VCR

**Table 34:** Tissue distribution profile of the DOX and VCR on single IV dose of formulations at concentrations equivalent to 6 mg/Kg DOX (3 mg/Kg VCR)

## 10.6 Conclusion

These in-vivo studies indicate towards improved efficacy of pegylated liposomal DOX upon incorporation of VCR (in 1:2 VCR: DOX weight ratio) against NSCLC and TNBC without altering the toxicity, pharmacokinetic and tissue distribution profiles of the clinically used standard. However, further studies related to therapeutic potential of such formulation is warranted before it can be tested on the clinical set up.

The below-mentioned sections have been published in the research articles, Ghosh et al. (2021), "Synergistic co-loading of vincristine improved chemotherapeutic potential of pegylated liposomal doxorubicin against triple negative breast cancer and non-small cell lung cancer" in Nanomedicine: Nanotechnology, Biology and Medicine, Volume 31, 102320 (31); and Ghosh et al. (2020), "Optimization and efficacy study of synergistic vincristine coloaded liposomal doxorubicin against breast and lung cancer" in Nanomedicine (London), Volume 15, Issue 26, Pages: 2585–2607 (32).

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