Chapter 9: In-vitro cell line studies

9.0 In-vitro cell line studies

9.1 Introduction

Cancer is characterized by heterogenous proliferation of cells having intrinsic potential to metastasize to major organs. Currently, cocktail of chemotherapeutic drugs acting through multiple mechanistic pathways on tumor cells have been explored as preferable option in chemotherapy (1). Triple negative breast cancer (TNBC) and Non-small cell lung cancer (NSCLC) are aggressive forms of breast and lung cancer whose treatment efficiency is often affected by the metastasis associated with them (2, 3). Additionally, TNBC has been clinically observed to metastasize to secondary NSCLC (4). 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 (5).

The drug doxorubicin in solution dosage form has been widely used in combination therapy of various cancers including NSCLC and TNBC (6). However, treatment efficacy of the chemotherapeutic agent in these two cancers has been limited due to drug resistance and toxicity (7, 8). Poly-ethylene glycol (PEG)ylated liposomal doxorubicin (Doxil™) as a clinical standard with naïve chemotherapeutic agents has been previously tested against the aforementioned cancers but with limited success (9). Reports suggest that microtubule destabilizing agents have significant tumor cell killing properties in both TNBC and NSCLC. These cell cycle specific mitotic agents have been found to potentiate the action of non-cell cycle specific anthracyclines (10, 11). Combining the drugs doxorubicin hydrochloride (DOX) and vincristine sulphate (VCR) allows action on multiple targets during different cell cycle phases, presenting improved chances of reduction in tumor cell load and requiring lower doses when in combination (12). While DOX arrests the cell cycle majorly at G_2/M phases, VCR has been reported as a mitotic inhibitor of the cell cycle (13, 14). Additionally, patients suffering from TNBC have been clinically found to have increased risk of developing secondary NSCLC (4). Consequently, suitable drug combinations effective against both of these carcinomas may prove to be effective in increasing the overall survival rates and improving quality of life of the patients.

Various attempts have been made to include both DOX and VCR in a nanoliposomal carrier, these attempts include the use of transition metal based complexation (15), surface functionalization (16) and that of thermosensitive components (17). However, these approaches have presented various short comings which include lack of efficacy, lack of physicochemical stability, unfavorable drug release profiles and non-scalability in manufacturing. Importantly, none of these approaches have been tested favorably in NSCLC and TNBC.

Clinically, nanoliposomes have been tested for the delivery of chemotherapeutics, providing altered pharmacokinetic and pharmacodynamic profiles resulting in enhanced permeation and retention (EPR)-mediated specific controlled drug delivery to the desired loci of action with reduced toxicity profiles (18). The administration of synergistic molar ratios of two agents as a combination of two single drug liposomes for delivery to tumor cells may not guarantee the temporospatial presence of both agents. Additionally, delivery of this combination may result in higher load of lipid excipients to the human system which may result in immunological responses (19). Consequently, effective delivery of a synergistic combination of drugs in a single multidrug loaded liposomes may ensure the simultaneous drug release and presence at the tumor site [7]. Since PEGylated liposomal doxorubicin has been used in chemotherapeutic treatment regimen, PEGylated liposomes for synergistic DOX combinatorial delivery may be a clinically effective approach. PEGylated combinatorial liposomes would increase the drug retention times while exhibiting reduced uptake by components of reticuloendothelial system (RES) ensuring temporospatial presence of the drug combination in tumor (20).

The initial assessment of level of optimization and subsequent efficacy of the drug loaded liposomes need to be done using suitable in-vitro studies prior to initiation of the pre-clinical studies. Since two drugs were co-loaded on the basis of the combinatorial index determined using the Chou-Talalay method, it was important to assess whether the optimal ratio translated to same efficacy when encapsulated in the PEGylated liposomes (19). Consequently, comparative *in-vitro* biological activity of the optimised formulations was evaluated using cellular uptake studies, cell proliferation assay along with its mechanistic evaluation using cell cycle analysis, apoptosis assay and wound healing.

The cell lines used for the in-vitro studies for non-small cell lung cancer and triple negative breast cancer were A549 and MD MBA-231 respectively.

9.1.1 A549 cell line

The human non-small adenocarcinoma cell line A549 represents the alveolar basal epithelium of lungs which was first generated by Dr. DJ Giard in 1972 from white Caucasian male patient (aged 58 years). Physiologically these squamous cells have been associated with the transport of electrolytes through the alveoli and traditionally associated with monolayer formation during cell culture. Morphologically, these cells have been found to present increased amounts of unsaturated phospholipids owing to their ability of lecithin synthesis. Additionally, this hypotriploid cells exhibit modal chromosome 66 in 24% cells as karyotype, with population-doubling time of approximately 22 hours and mean diameter of 10.59-14.93 µm morphologically (21).

9.1.2 MDA MB-231 cell line

The aggressive basal breast cancer subtype MDA MB-231 has been used for the development of triple negative breast cancer model having absence of ER, PR, E-cadherin, HER-2 receptors along with the mutated p53 protein. The epithelial adenocarcinoma was established from the Caucasian female (aged 51 years) having metastatic mammary carcinoma at MD Anderson Cancer centre (22). These cells have shown proteolytic extracellular matrix dissemination mediated invasiveness and spontaneous metastatic potential to lymph nodes, brain, lungs and bone marrow when developed as orthotropic or xenograft models. This cell line presents chromosomal counts of triploid range having modal number 64 (aneuploid female) while having absence of N8, N15 chromosomes as karyotype. The population doubling time is approximately 38 hours with 18.9± 0.4 µm (23)

9.2 Cell lines and materials

The cell lines MDA MB-231 (breast cancer) and A549 (non-small cell lung cancer) were procured from NCCS (National Centre for Cell Science), Pune, India Thiazolyl Blue Tetrazolium Bromide (MTT); Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) were purchased from HiMedia (India); Cell cycle analysis kit and Annexin V-FITC Apoptosis Detection kit were purchased from Thermofischer Scientific (USA) and

Invitrogen (USA); McCoy's 5A medium and Penicillin Streptomycin-Amphotericin-B antibiotic concentrate solution were procured from Sigma Aldrich (India). All other chemicals used in the study were of analytical grade.

9.3 Cell culture

The procured cell lines were cultured for the various in-vitro studies using methods as previously described (24). Briefly. the cell lines were grown and perpetuated in supplemented McCoy's 5A medium. The medium was added with 10% FBS; 2 mM L-glutamine and 100 µg/ml antibiotic concentrate solution to facilitate the maintenance. The cell cultures were incubated in incubator (Jouan Ltd. IGO150, Thermofischer scientific, USA) under optimal atmospheric conditions of humidified 5% CO2 at 37°C temperature. Since, the doubling time of A549 cell line is nearly 22 hours, the confluency of the culture was split every 4-5 days (1:4-1:9) to maintain the concentration of viable cells between 2 x 10^3 to 1 x 10^4 per square centimetre. Similarly, for MDA-MB 231, the cell population doubling time is approximately 38 hours and the confluency was split on the same duration (1:4-1:5) for maintaining the concentration of viable cells between 4 x 10^4 to 5 x 10^4 per square centimetre.

9.4 Methodology

9.4.1 Cellular uptake study by confocal microscopy

The FITC tagged VCR liposomes by adding 100 µl FITC solution (0.4%w/v FITC dissolved in ethanol) was added to the alcoholic lipid solution and all other steps including formation of preformed liposomes, active drug loading was followed as mentioned in section 10.3.1. These FITC tagged liposomes were used for the cellular uptake studies. Liposomal formulations were analysed for the cellular uptake by confocal microscopy using previously reported methods (25). Briefly, A549 and MDA-MB 231 cells (5 X 10^4 cells/well) were grown overnight on circular cover slips in complete medium in 12-well tissue culture plate. Post seeding, cells were incubated with DOX solution, DOX liposomes, Dual drug liposomes (at DOX concentration of 6 µg/mL) and FITC tagged VCR liposomes (at VCR concentration of 3 µg/mL) in serum free media for 1 hour and 4 hours. The concentration of these drugs was taken as the one which presented the optimal synergistic ratio (DOX: VCR- 1:2 weight ratio). The media was removed and the cells were subjected to 5 µg/mL DAPI treatment for 5 minutes followed by thorough washing using sterile PBS and fixed to cover slips using paraformaldehyde (4% w/v) for 10 minutes at room temperature. The cover slips were then thoroughly washed using PBS, mounted on microscopic slides using fluorescence free Flouromount-G and viewed using Confocal laser microscope (Leica DMi8, Leica Microsystems, Germany) with images being captured at excitation of DAPI, DOX and FITC (358nm, 470 nm and 495 nm repectively). The experiments were performed in triplicate and processed using Image J software (1.47V, NIH, USA).

9.4.2 Cellular uptake studies using flow cytometry

Flow cytometer was used for the quantification of the cellular uptake of the various liposomal formulations as previously described (26). Briefly, seeding of MDA-MB231 and A549 cells (4 X 10^5cells/well) was done in 6-well tissue culture plates and left for overnight incubation. The formulations DOX solution, DOX liposomes, Dual drug liposomes (at DOX concentration of 6 µg/mL) and FITC tagged VCR liposomes (at VCR concentration of 3 µg/mL) was incubated with the cells in serum-free medium for 1 hour and 4 hours. The concentration of these drugs was taken as the one which presented the optimal synergistic ratio (DOX: VCR- 1:2 weight ratio). These cells were centrifuged (5 minutes/ 4˚C) to cell pellets post washing with PBS and collected using trypsinization for further processing. These pellets were suspended in 200 µl sterile PBS and analysed for uptake using flow cytometer (Amnis FlowSight, Millipore, USA) having argon laser (excitation for DOX and FITC at 470 nm and 495 nm respectively). The data was collected in triplicate, analysed using Ideas analysis software (version 6.0) with 10000 gated events for each run.

9.4.3 Cell viability assay

Cellular viability of MDA-MB 231 and A549 in presence of various treatment conditions was evaluated using MTT assay. The cells were seeded at 5 X 10^3 cells/well density in 96 well plate (Corning, USA) with optimum conditions being maintained for growth and adherence as previously described (24). Free drug treatments (VCR solution, DOX solution) and formulation treatments (VCR liposomes, DOX liposomes, mixture of DOX liposomes and VCR liposomes as well as dual drug liposomes) at concentration of 3.125, 6.25, 12.5, 25, 50, 100 µg/mL post dilution in serum free DMEM media were added to the seeded cells upto 72 hours in triplicate. The treatment groups of combination of the single liposomes and dual drug loaded liposomes were treated with concentration of the drugs equivalent to VCR: DOX weight ratio of 1:2 being diluted to aforementioned concentrations. The drug treatment free cells were incubated with PBS and further processed as negative control. The positive control for the experiment was generated by treating the cells of the control group with Triton X 100. All the treatment and control group cells were further processed with yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide resulting in the formation of purple insoluble formazan crystals. The insoluble crystals solubilized using acidified ethanol solution while the color intensity (optical density) of dissolved formazan measured using microtiter plate reader (Biorad, California, USA) at 570 nm and the cellular viability was expressed as ratio of affected cells to the untreated control cells using previously described equations (24).

$$\text{Cell viability (\%)} = \frac{(\text{Absorbance}_{570nm}\ \text{Sample} - \text{Absorbance}_{570nm}\ \text{Positive control})}{(\text{Absorbance}_{570nm}\ \text{Negative control} - \text{Absorbance}_{570nm}\ \text{Positive control})} \times 100$$

The IC_{50} (inhibitory concentration) were obtained from the cellular viability vs concentration plots with the optical density of untreated cells taken as 100%.

9.4.4 Cell cycle analysis

The cell cycle analysis was carried out as per the manufacturer's protocol (Thermofisher Scientific). The MDA-MB 231 and A549 cells were seeded at a concentration of 0.8 million/ well and incubated with formulations (DOX solution, VCR solution, liposomal DOX, liposomal VCR, dual) at 50 µg/ml concentration in 6 well plates at 37°C for 24 hours under 5% CO_2. Treatment free cells were incubated as control. The cells were harvested by centrifugation at 2000 rpm for 5 minutes at 5°C. The cells were then fixed for 30 minutes at 4°C in 70% cold ethanol. These were then washed twice with PBS, further treated with RNase (50 µl of 100 µg/ml stock solution) and Propidium Iodide (5 µl of 1 mg/ml stock solution) and thereafter incubated at 37°C for 1 hour prior to FACS analysis (Becton Dickinson, FACS Aria III, USA).

9.4.5 Apoptosis study by Annexin V assay

The free drug solution and liposomal formulations were evaluated for the apoptosis induction using Annexin V/PI cellular binding assays as per manufacturer's protocol (Invitrogen, USA). Drug solutions (DOX, VCR) and liposomal formulations (DOX, VCR, dual) at 50 µg/ml concentration were incubated with MDA-MB 231 and A549 cells seeded at 0.8 million cells/well in 6-well plates for 24 hours (37°C/ 5% CO_2). The treated cells were harvested post cold PBS washing while cells receiving no drug treatment were taken as control. FITC Annexin V and PI were added to the suspended cells as recommended in the protocol and incubated in dark for 15 minutes. The treatment samples were then analysed for percentage of early apoptotic, late apoptotic, necrotic and live cellular levels using dot plot from FITC/PI (Excitation/Emission: FITC: 494nm/518 nm, PI: 535nm/617nm) channels of flow cytometer (Amnis FlowSight, Millipore, USA) derived using Ideas analysis software (version 6.0).

9.4.6 Wound Scratch Study

Potential of cellular migration in presence of the drug treatments were evaluated using the wound healing study as described earlier (24). The MDA-MB 231 and A549 cells were seeded to grow in 6 well plates till 80% confluency was reached. Wound creation was performed for a mean of 300 µm (±5% SD) with sterile pipette tip and washed thrice with sterile PBS ensuring the complete removal of the dislodged cellular monolayer. DOX and VCR drug solutions were diluted with incomplete medium to attain treatments of 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml for incubation with the wound cells under 5% CO2 condition for 72 hours at 37°C. Further, the free drugs, single liposomes, dual drug liposomes, drug free liposomes were evaluated at 50 µg/ml concentration in comparison with the untreated wound cells incubated to similar conditions as mentioned earlier. Post incubation, the aforementioned treatment conditions were removed, followed by the PBS washing and fixation using 70% ethanol while wound width was calculated using Nikon Eclipse TS100 inverted microscope (NIS elements imaging software). The experiment was performed in triplicate with % relative values expressed as mean± SD as compared to untreated cells.

9.4.7 Statistics

All experiments were performed in triplicate with results being presented as values in mean ± SD (standard deviation) unless mentioned otherwise. Statistical significance of the data

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

9.5 Results and Discussion

9.5.1 Cellular uptake study by confocal microscopy

Confocal microscopy was used to visualize the cellular uptake of the drug solution and the liposomal drug treatments. FITC was used for the tagging of the VCR liposomes as VCR molecule lacks molecular fluorescence while it is observed in case of DOX which exhibits auto-fluorescence. The results of the cellular uptake in MDA-MB231 and A549 at 1 hour and 4 hours post treatment are shown in Figure 49 & 50. The micrographs of different treatments in MDA-MB 231 (Figure 49) and A549 (Figure 50) indicate towards a time dependent increase in the cellular uptake of the DOX liposomes and the dual drug liposomes with non-significant increase in the uptake of the DOX free drug and the liposomal VCR. Further, the nuclear localization of the dual drug formulation was observed from the purple merged fluorescent signals which was found to increase significantly as compared to other treatments with time. The higher cellular uptake of the liposomal formulations as compared to drug solutions may be attributed to the drugs being presented at the cell surface encapsulated in the nanoliposomes and their subsequent endocytosis (27). The lower uptake of the free drug may be due to the efflux of the naïve DOX due to the presence of drug efflux proteins present on surface of MDA-MB 231 and A549 cells (28). The VCR liposomal formulation had high content of free drug as compared to liposomal DOX and same may be responsible for the reduced uptake of the liposomal formulation as comparted to liposomal DOX formulations (29). The increased purple fluorescence in the cells treated with dual drug liposomes may be due to improved cellular uptake and simultaneous nuclear localization of the drugs (30).

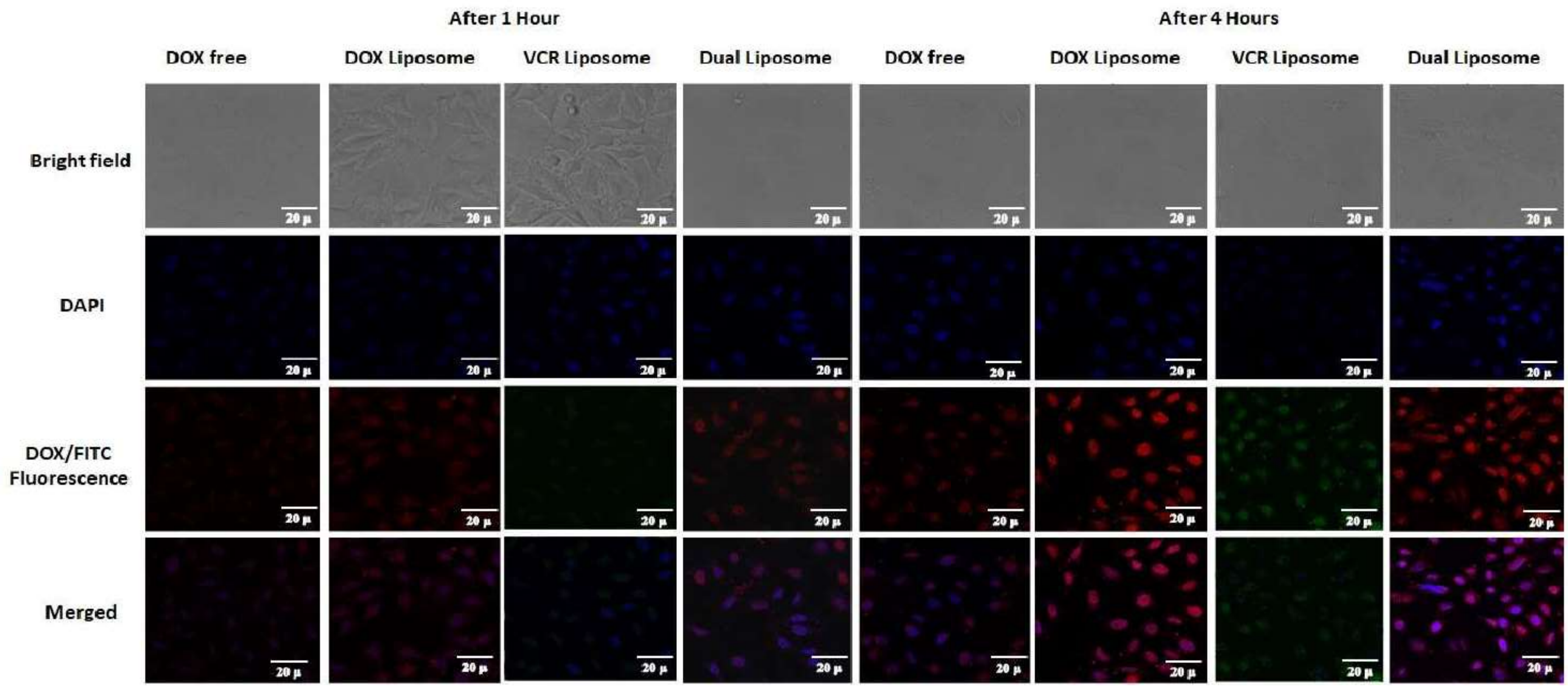


Figure 49: Confocal microscopic fluorescent images of MDA-MB 231 with free DOX, Liposomal DOX, FITC liposomal VCR and dual drug liposomes. For each panel, the images from top to bottom showed the bright field image of cells, cell nuclei stained by using DAPI solution(blue), Dox fluorescence in cells (red)/ FITC fluorescence in cells (green) and merged image.

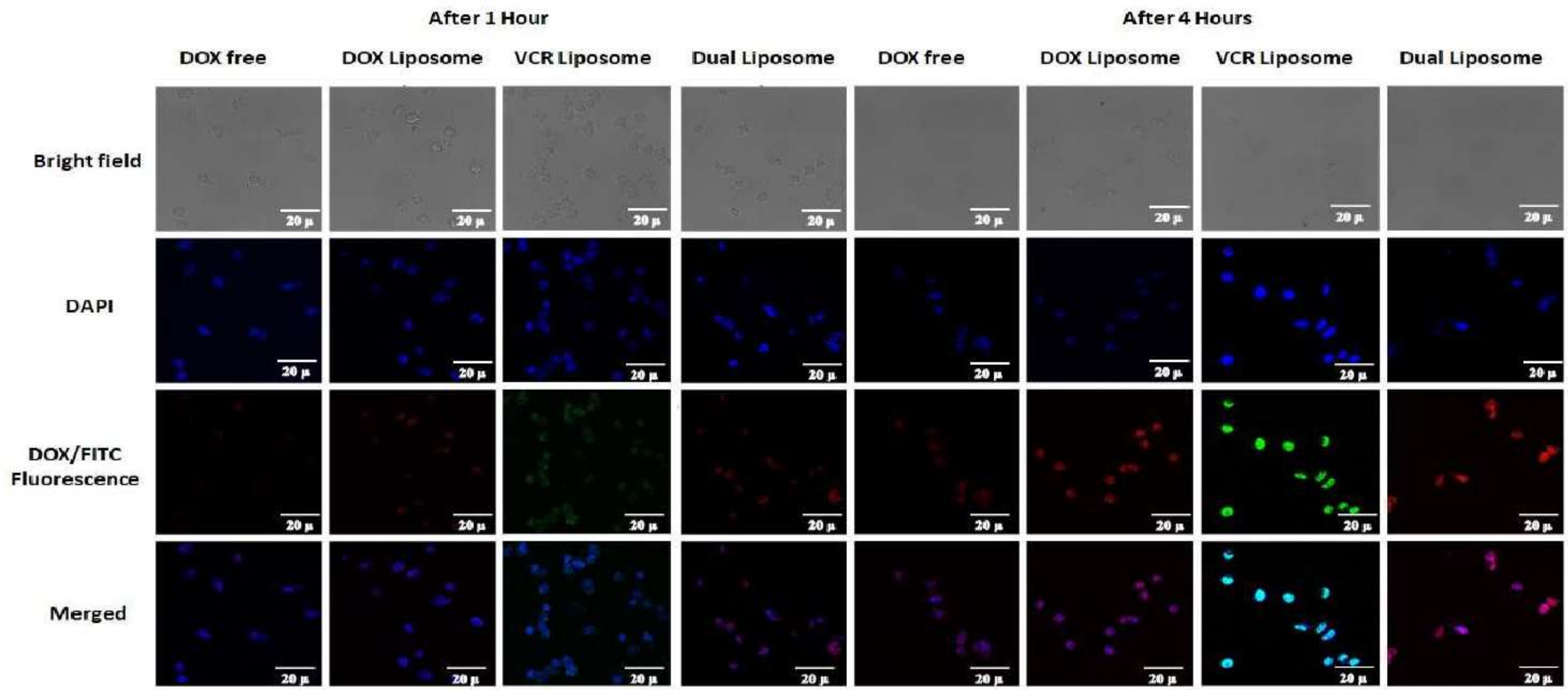


Figure 50: Confocal microscopic fluorescent images of A549 with free DOX, Liposomal DOX, FITC liposomal VCR and dual drug liposomes. For each panel, the images from top to bottom showed the bright field image of cells, cell nuclei stained by using DAPI solution (blue), Dox fluorescence in cells (red)/ FITC fluorescence in cells (green) and merged image.

11.5.2 Cellular uptake studies using flow cytometry

The quantitative estimation of the cellular uptake was ascertained using flow cytometry. Since VCR molecule lacks chromophore groups and does not present any molecular fluorescence, the cellular uptake studies involving single drug VCR groups were performed only for the liposomes encapsulating the drug and tagged with fluorescent marker Fluorescein isothiocyanate (FITC). The tagging of the VCR molecules (VCR solution) was not performed as it would not exhibit the true characteristics of the presentation of VCR and such changes may alter the in-vitro properties of the drug when presented as solution. DOX molecule presents suitable chromophore groups while exhibiting auto-fluorescence and cellular uptake studies for single drug DOX groups included both DOX solution and DOX liposomes. The micrographs of the cellular uptake in MDA-MB 231 (Figure 51) and A549 (Figure 52) indicate towards significant increase in active uptake into the cells in time dependant manner. Dual drug liposomes exhibited maximum uptake while least uptake was observed for free DOX formulation in both the cell lines among DOX formulations. The fluorescent intensity of uptake in MDA-MB 231 (Figure 51) for dual drug liposomes increased significantly from 35000±150 at 1 hour to 55000±205 at 4 hours while for A549 cell line (Figure 52), it increased from 25000±200 (1 hour) to 35000±140 (4 hours). Conversely, quantitative estimation of uptake in both the cell lines for VCR liposomes was found to be less as compared to that of DOX liposomes and Dual drug liposomes. Further, the uptake into MDA-MB 231 was higher than that observed in A549 which may be due to presentation of the formulations to the cell lines undergoing different stages of cell cycle (which shall be confirmed during cell cycle analysis) (31).

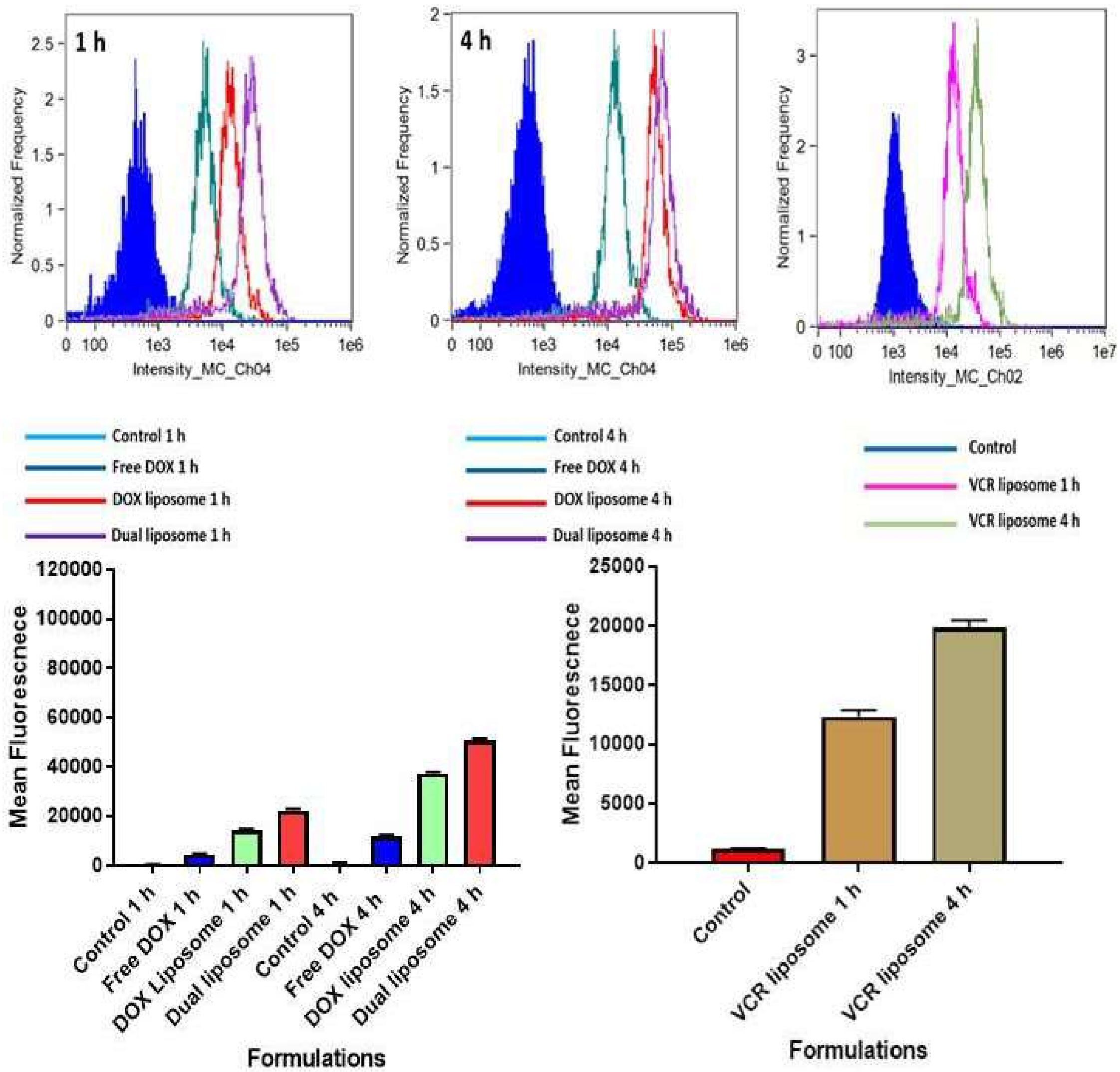


Figure 51: Cellular uptake by flow cytometry of MDA-MB 231 incubated with free DOX, Liposomal DOX, FITC liposomal VCR and dual drug liposomes. The histogram and Geometric mean fluorescence intensity data plotted as bar graph for the cellular uptake quantification. (The data were represented as Mean ± SD, n=3).

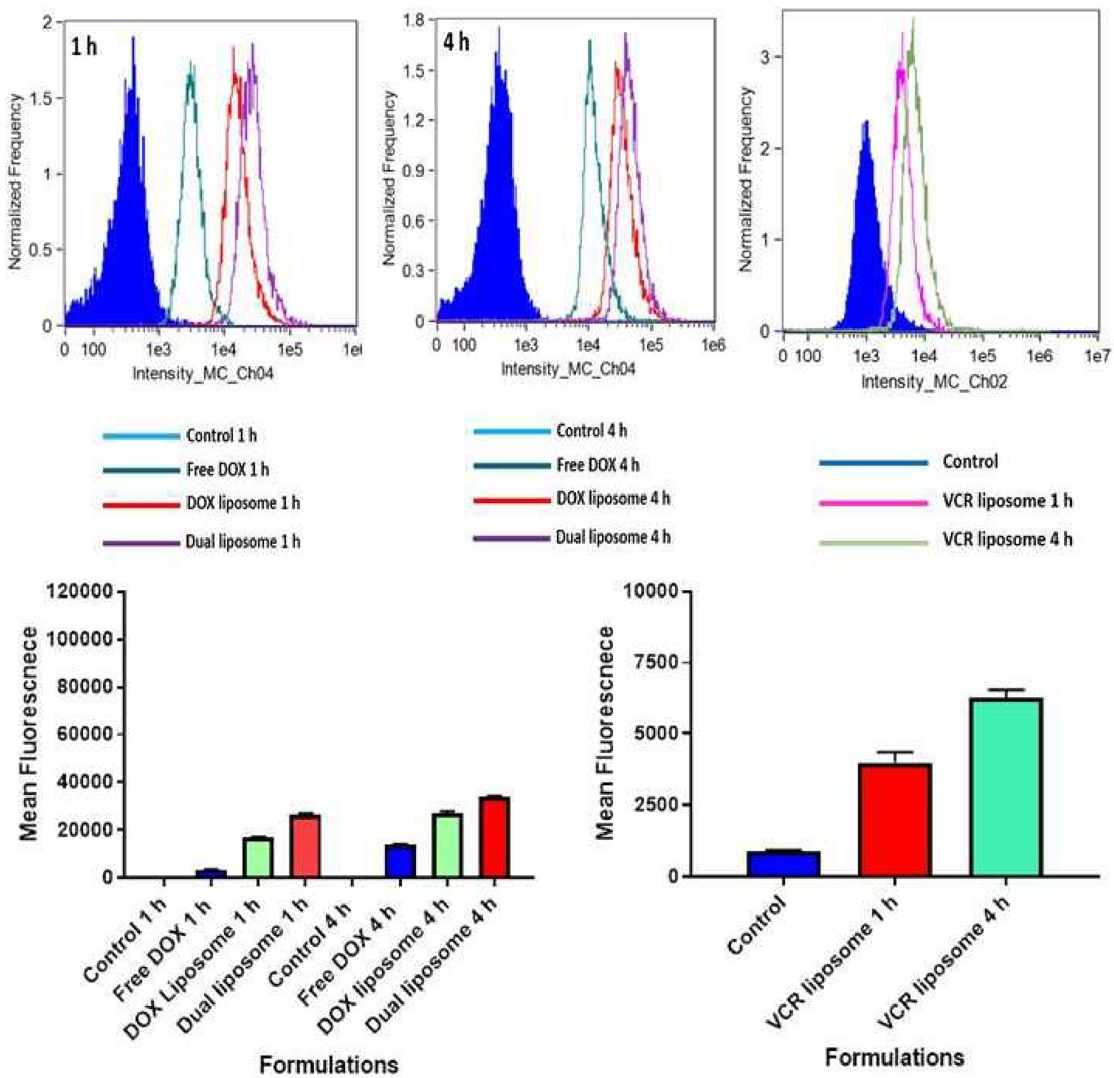


Figure 52: Cellular uptake by flow cytometry of A549 incubated with free DOX, Liposomal DOX, FITC liposomal VCR and dual drug liposomes. The histogram and Geometric mean fluorescence intensity data plotted as bar graph for the cellular uptake quantification. (The data were represented as Mean ± SD, n=3).

The cellular uptake of the formulations was ascertained using confocal microscopy and flow cytometry. Results in both studies (Figure 49 to 52) indicate towards time dependent active uptake with dual drug liposomes exhibiting the highest uptake among all treatments. Importantly, this treatment presented significant increase in the nuclear localization which may have resulted in the higher cell killing potential of the formulation as indicated by the MTT assay.

9.5.3 Cell viability assay

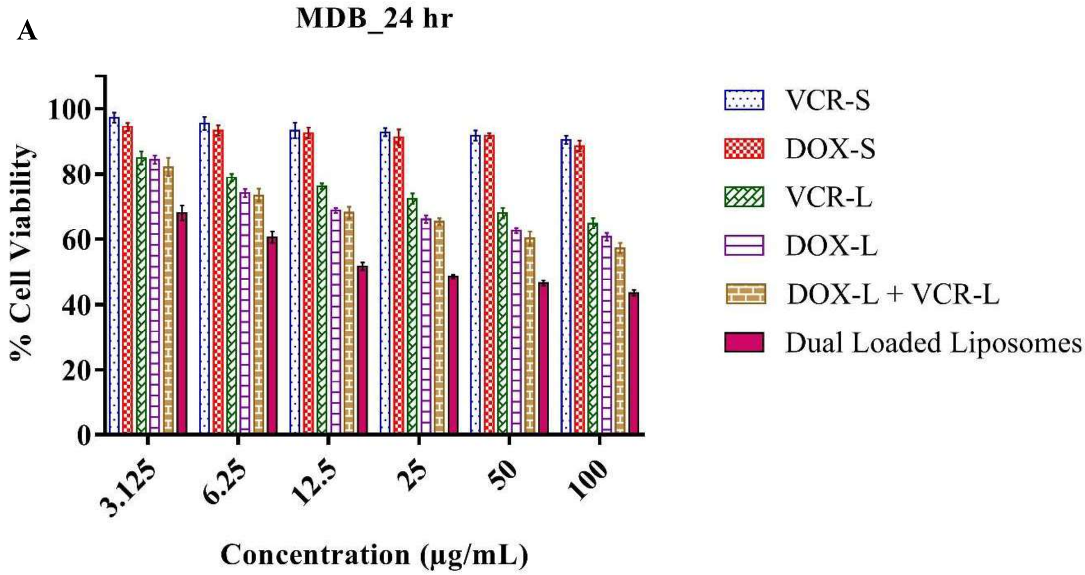
The cytotoxicity of the optimized formulations encompassing the predetermined synergistic ratio of DOX and VCR (2:1 weight ratio co-loaded in dual drug liposomes as prepared in 10.3.1) was evaluated against A549 lung cancer cell line and MDA-MB 231 breast cancer cell line using MTT assay. In this study, nanocarrier free drug solutions, single drug liposomes, mixture of single drug liposomes and dual drug loaded liposomes were evaluated for the cell viability over 24, 48, 72 hours. The effect of treatment of the cell lines with mixture of two single drug loaded liposomes was evaluated to ascertain whether the presence of the synergistic ratio of drugs in single combinatorial liposomes was needed to elicit the similar response. The results of the MTT assay over 48 hours and 72 hours are shown in figure 53 & 54. The results of cytotoxicity assay in MDA-MB-231 cell line (Figure 53 A-D) indicate towards decrease in the cell viability in concentration and time dependent manner with inhibitory concentration with 50% reduction in cell viability (IC_{50}) values after 72 hours of treatment were found to be 49.80 µg/ml, 50 µg/ml, 50 µg/ml and 5.83 µg/ml respectively for DOX liposomes, VCR liposomes, DOX liposomes + VCR liposomes and DOX+VCR liposomes. Additionally, IC_{50} values of the dual drug liposomes reduced 4 folds with time from 24.31 µg/ml (24 hours) to 5.83 µg/ml (72 hours). Further, the cell viability on treatment with the dual drug carrier was significantly reduced with 1.5 times at 48 hours and 2.06 times at 72 hours as compared to single drug liposomes and their mixture. Since, the free drug solutions presented high cellular viability after 72 hours when tested till 100 µg/ml of the agents, they were further tested till 400 µg/ml to evaluate the effect of increased concentration of the drugs (Figure 53 D). The results showed reduced cellular viability after 72 hours for both drugs with approximately 50% viability post treatment.

The results of cytotoxicity study in A549 cell line (Figure 54 A-D) indicate towards similar concentration and time dependent decrease in the cellular viability with all the treatments including the carrier free drug solutions. The IC_{50} values after 72 hours of treatment were found to be 100 µg/ml, 93.42 µg/ml, 100 µg/ml, 25 µg/ml, 25 µg/ml, and 3 µg/ml respectively for VCR solution, DOX solution, VCR liposomes, DOX liposomes, DOX liposomes+ VCR liposomes and DOX+VCR liposomes. Treatment with dual drug liposomal formulation exhibited 8-fold reduction in the IC_{50} values from 25 µg/ml (24 hours) to 3 µg/ml (72 hours) with significant decrease (>2 fold) in the cell viability as compared to the other drug treatments during the same duration. Importantly, contrary to the significant decrease in the cell viability of DOX+VCR liposomes, VCR solution and VCR liposomal formulation exhibited much higher viability. The VCR treated cells exhibited similar viability over 72 hours of study and this may be due to cellular drug resistance offered to VCR (32). Similar to the results of the MDA-MB 231 cell line, the dual drug liposomal formulation exhibited much lower IC_{50} values and cellular viability as compared to individual drug liposomes and their mixture. Consequently, the presentation of both drugs in synergistic ratio in the same liposomal carrier presented significant reduction ($p<0.05$) in IC_{50} and cellular viability in both the cell lines as compared to other treatments (33). Additionally, the free drug solutions were tested till 400 µg/ml and results showed reduced cellular viability 30% for DOX and 50% for VCR after 72 hours of treatment (Figure 54D).

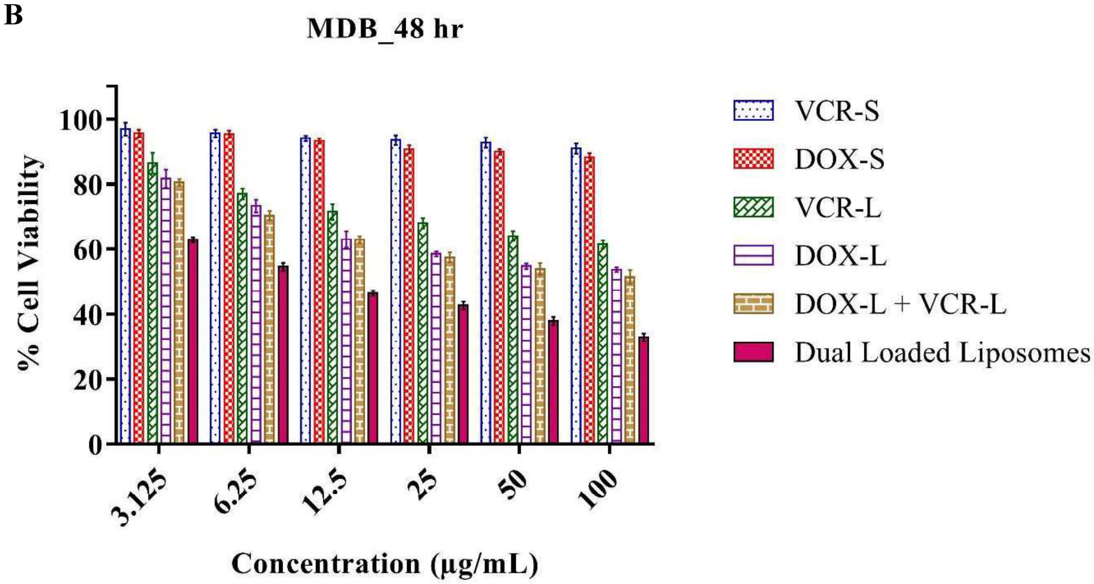
The free drug solutions exhibited high cellular viability potential as compared to liposomal formulations which may be due to the presence of efflux proteins and subsequent resistance offered by the cell lines to the drug uptake (34). The increased cellular killing presented by the liposomal formulations may be attributed to their endocytic uptake with subsequent drug release in the cytoplasm and translocation into the nucleus (as confirmed by cellular uptake studies). The effect of presentation of combination of DOX and VCR (2:1 weight ratio) in the same nanocarrier was evaluated by measuring the cytotoxicity afforded by mixture of two single drug liposomes (DOX-L and VCR-L) as additional test group with the other treatments. Such presentation resulted in cellular viability levels similar to that of liposomal DOX, which may be attributed to the high load of liposomal formulation being presented at the cell membrane for endocytic uptake leading to reduced uptake in the cells. VCR presentation to both the cell lines resulted in higher cellular viability as compared to DOX which may be due to their tendency of cellular arrest potential at mitotic phase while DOX has been previously reported for both cellular arrest and apoptosis. Importantly, the addition of the ratio-mimetic concentration of VCR to DOX (1:2 weight ratio) in single dual drug loaded liposomes resulted in significant decrease in the cell viability and IC_{50} values as compared to other formulations indicating towards superior in-vitro effectiveness (33).

Further, the MTT assay results indicate towards the in-vitro effectiveness of the determined synergistic combination when presented in a single liposomal carrier as compared to carrier free systems as well as single drug liposomes.

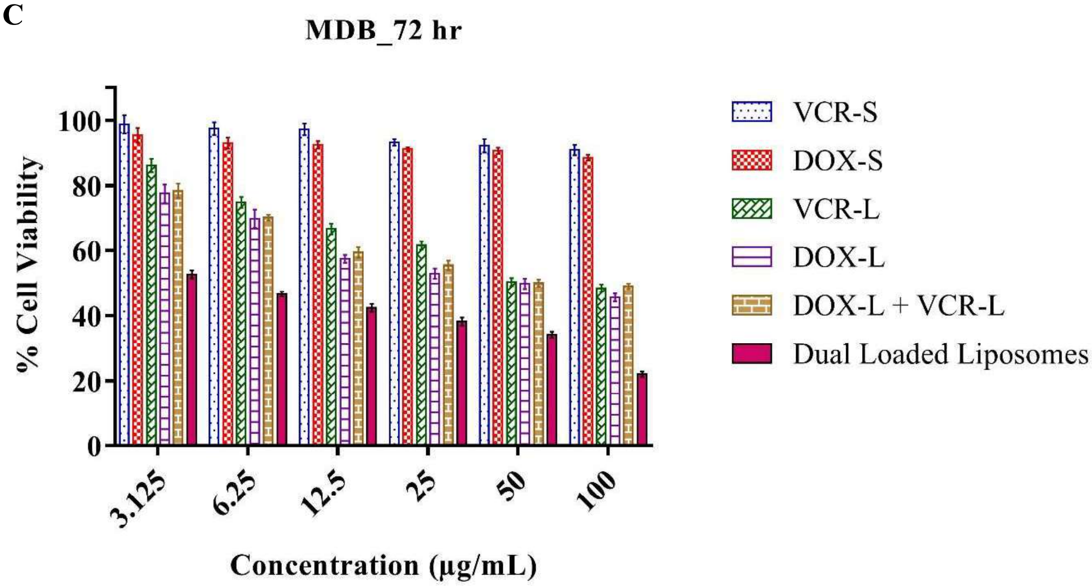
A


MDB_24 hr
% Cell Viability
0
20
40
60
80
100
3.125
6.25
12.5
25
50
100
Concentration (µg/mL)
VCR-S
DOX-S
VCR-L
DOX-L
DOX-L + VCR-L
Dual Loaded Liposomes

B


MDB_48 hr
% Cell Viability
0
20
40
60
80
100
3.125
6.25
12.5
25
50
100
Concentration (µg/mL)
VCR-S
DOX-S
VCR-L
DOX-L
DOX-L + VCR-L
Dual Loaded Liposomes

C



D

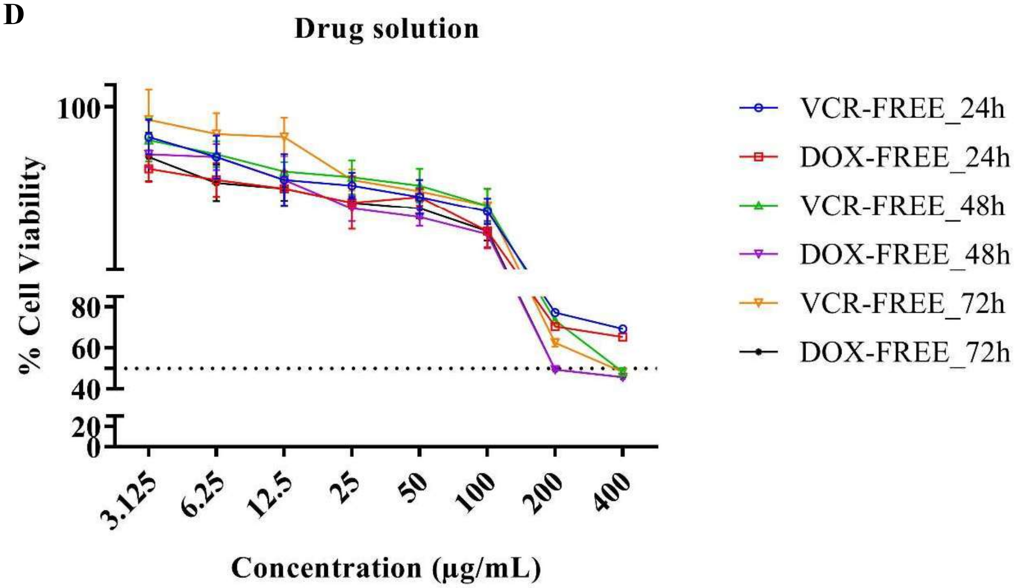
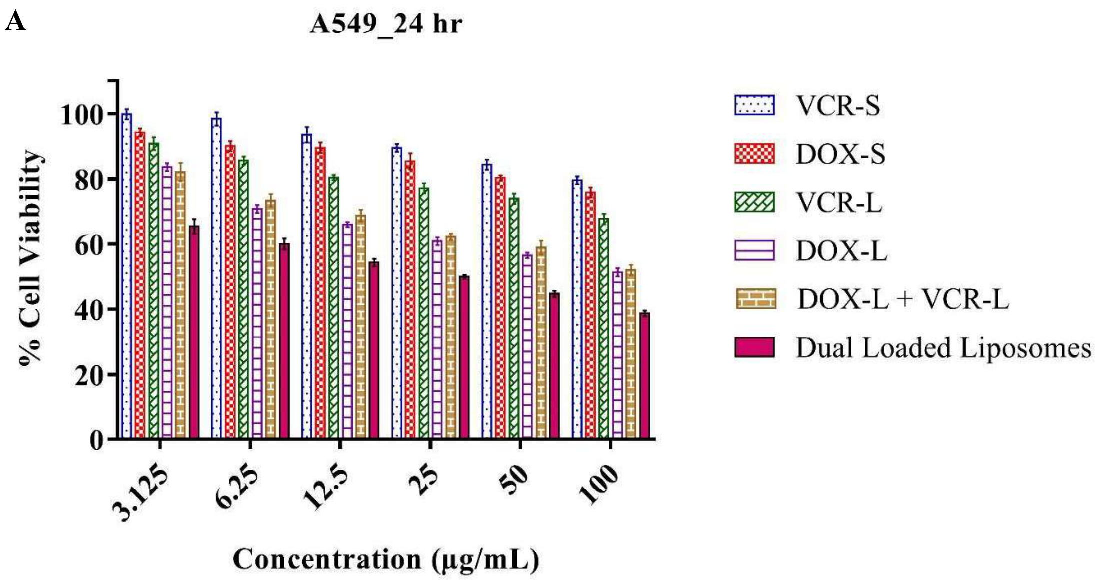
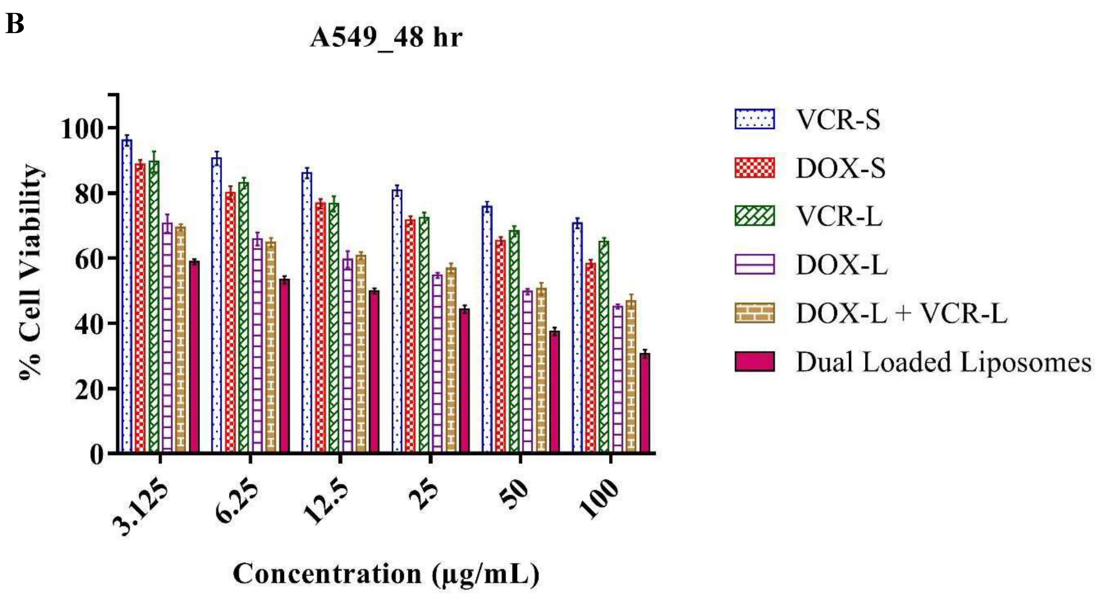
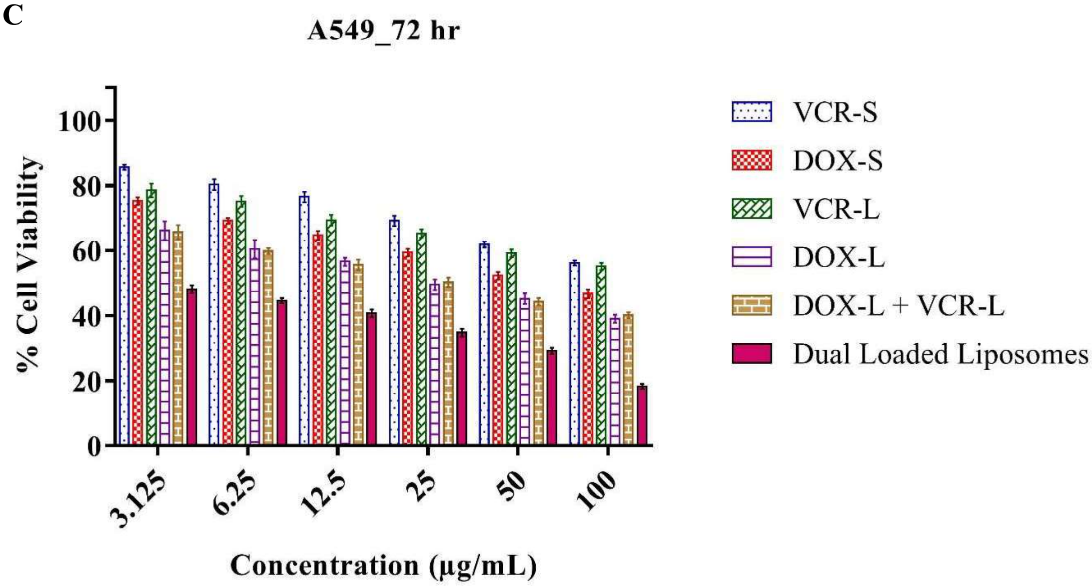


Figure 53: Results of Cell viability (%) at various concentrations of DOX solution, VCR solution, DOX liposomes, VCR liposomes, DOX liposomes+ VCR liposomes and DOX+VCR liposomes after 24 hours (53 A), 48 hours (53 B), and 72 hours (53 C) of treatment in MDA-MB 231 cell line. Cell viability of free drug solutions after 24, 48, 72 hours is indicated (53 D)


A
A549_24 hr
% Cell Viability
Concentration (µg/mL)
3.125
6.25
12.5
25
50
100
VCR-S
DOX-S
VCR-L
DOX-L
DOX-L + VCR-L
Dual Loaded Liposomes


B
A549_48 hr
% Cell Viability
Concentration (µg/mL)
3.125
6.25
12.5
25
50
100
VCR-S
DOX-S
VCR-L
DOX-L
DOX-L + VCR-L
Dual Loaded Liposomes

C



D

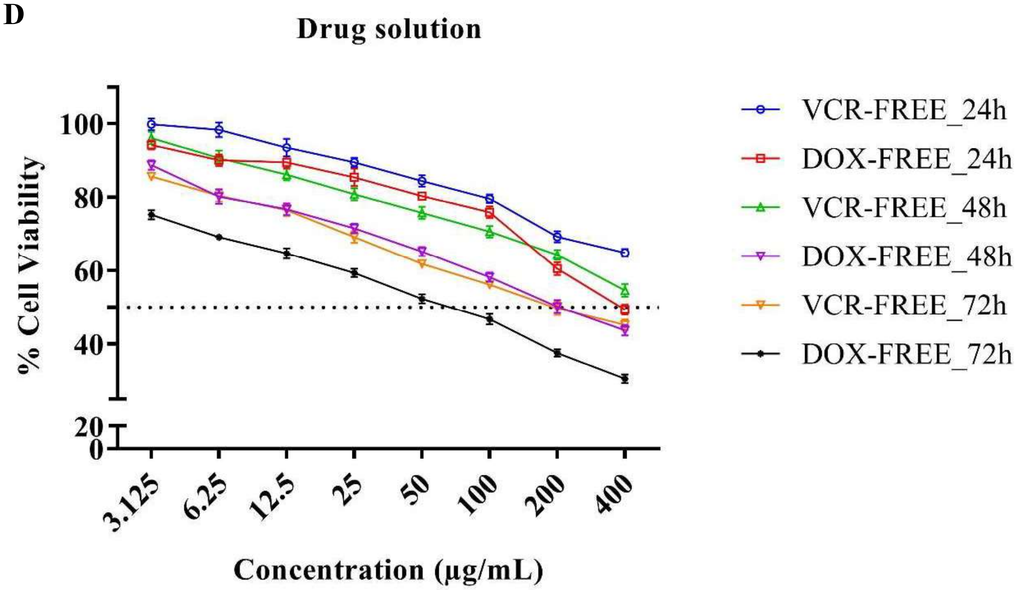
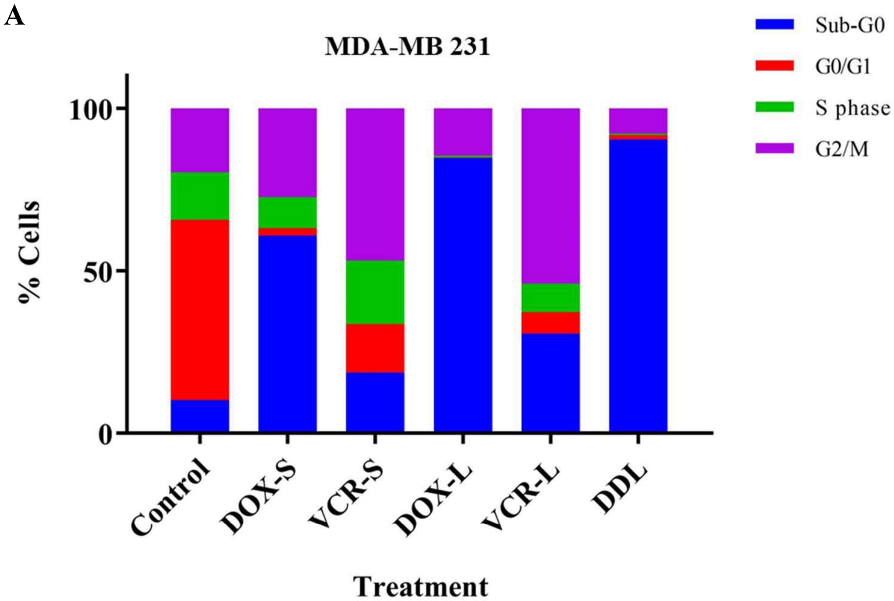


Figure 54: Results of Cell viability (%) at various concentrations of DOX solution, VCR solution, DOX liposomes, VCR liposomes, DOX liposomes+ VCR liposomes and DOX+VCR liposomes after 24 hours (54 A), 48 hours (54 B), and 72 hours (54 C) of treatment in A549 cell line. Cell viability of free drug solutions after 24, 48, 72 hours is indicated (54 D)

9.5.4 Cell cycle analysis

The effect of the formulations on the cell cycle of MDA-MB 231 and A549 cell lines was evaluated using FACS analysis. The results were presented as cells (%) at each phase of the cell cycle (sub G_0, G_0/G_1, S, G_2/M) in Figure 55 A (MDA-MB 231), B (A549). In both cell lines, VCR solution treatment resulted in cell cycle arrest at the G_2/M phase while DOX solution treatment resulted in cell cycle arrest at G_2/M phase and subsequently at sub G_0 phase. Presentation of DOX in form of liposomes resulted in significantly high levels of cells in sub G_0 phase as compared to G_2/M than DOX solution in both the cell lines. While VCR liposomes in MDA-MB 231 (Figure 55 A), showed in the increase in cell accumulation in sub G_0 phase as compared to VCR solution, whereas the results of both the treatments were found to be similar in case of A549 cells (Figure 55 B). Dual drug liposomes treatment in both the cell lines resulted in higher accumulation of the cells in sub G_0 phase which was significantly higher than all other drug treatments including liposomal DOX.

A



B

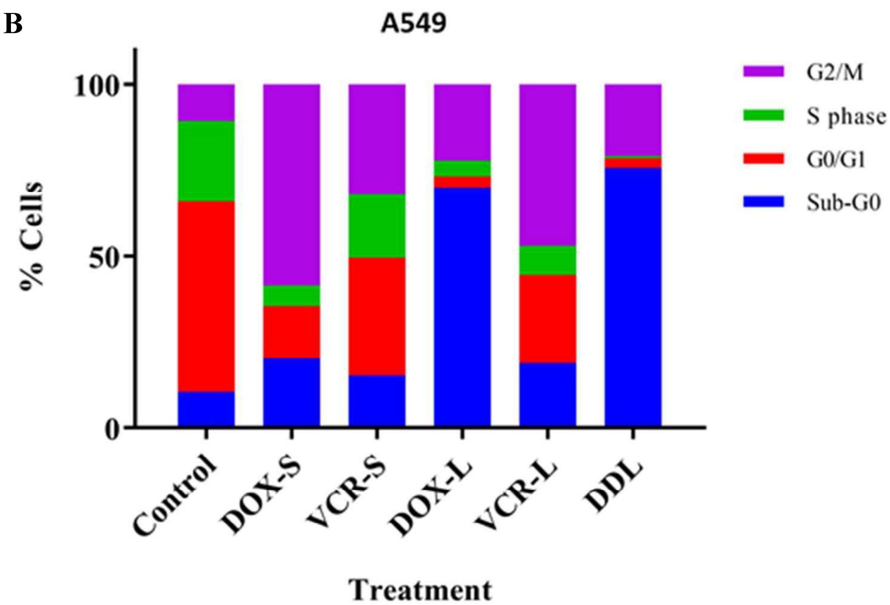


Figure 55: Results of cell cycle analysis in MDA-MB 231 (A) and A549 (B) cell lines.

The microtubule depolymerizing agent VCR has been reported to cause cell cycle arrest in G_2/M while anthracycline DOX has shown cell cycle arrest at G_2/M and subsequent cell death through apoptosis (sub G_0 phase) (35). The cell cycle profile of DOX treatments in MDA-MD 231 and A549 indicate towards high apoptotic potential of the drug while VCR treatments present cell cycle arrest. Liposomal encapsulation of the drugs resulted in improved outcomes which may be due to the increased uptake of the carriers as indicated by the cellular uptake studies (36). The dual drug liposomes exhibited significantly increased concentrations of cells in sub G_0 phase than all other treatments in both the cell lines which is indicative of its apoptotic potential and in good correlation with its cellular viability results.

9.4.5 Apoptosis study by Annexin V assay

The comparative apoptotic potential of the various forms of drug treatments in MDA-MB 231 and A549 was evaluated using the Annexin V binding assay to phosphatidyl serine (PS) present on the cellular surface. Induction of apoptosis is associated with the translocation of cellular marker PS from the inner plasma membrane leaflet (normal cells) to the cellular surface (apoptotic cells). The results of FACS analysis of PS upregulation for the free drug, single drug liposomes and dual drug liposomes are presented in Figure 56 (A549) and 57 (MDA MB-231).

The cell population dot plot represents necrotic cells (R5), late apoptosis (R4), early apoptosis (R3), Live cells (R2) by red, orange, yellow and green color respectively in the four quadrants of the treatments in both the cell lines. In both the cell lines, the free drug VCR was found to have lesser apoptotic potential as compared to DOX with results being comparable to live cell population of control panel. The DOX loading into liposomes resulted in significant improvement in the percentage of A549 cell (Figure 56) population undergoing apoptosis from 30.59% (free DOX) to 85.67% (dual drug liposomes) and 80.02% (DOX liposomes). Similar results were obtained in MDA-MB 231 cells (Figure 57) with cellular apoptotic levels at 67.87%, 90.71% and 95.75% respectively for free DOX, DOX liposomes and Dual drug liposomes. Although, incorporation of VCR into the liposomes resulted in improvement in the levels of apoptotic cells (32.75%) as compared to free VCR (22.84%) in MDA-MB 231 cell line, the levels were significantly lower as compared to that of free DOX, liposomal DOX and dual liposomes. However, this improvement in apoptotic levels of VCR treatments was not observed in case of A549 cells.

The decrease in the viability of the cells of MDA-MB 231 and A549 was further evaluated using Annexin V apoptotic assay. The apoptotic potential of the formulations indicated by the increased presentation of the cells in sub G_0 phase was evaluated using the Annexin V assay. The results of this assay were found to be in good correlation with cell viability and cell cycle analysis. While treatment with DOX formulations presented very high apoptotic potential, VCR formulations showed high concentration of live cells. DOX VCR co-loaded liposomes exhibited improved apoptotic potential as compared to liposomal DOX.

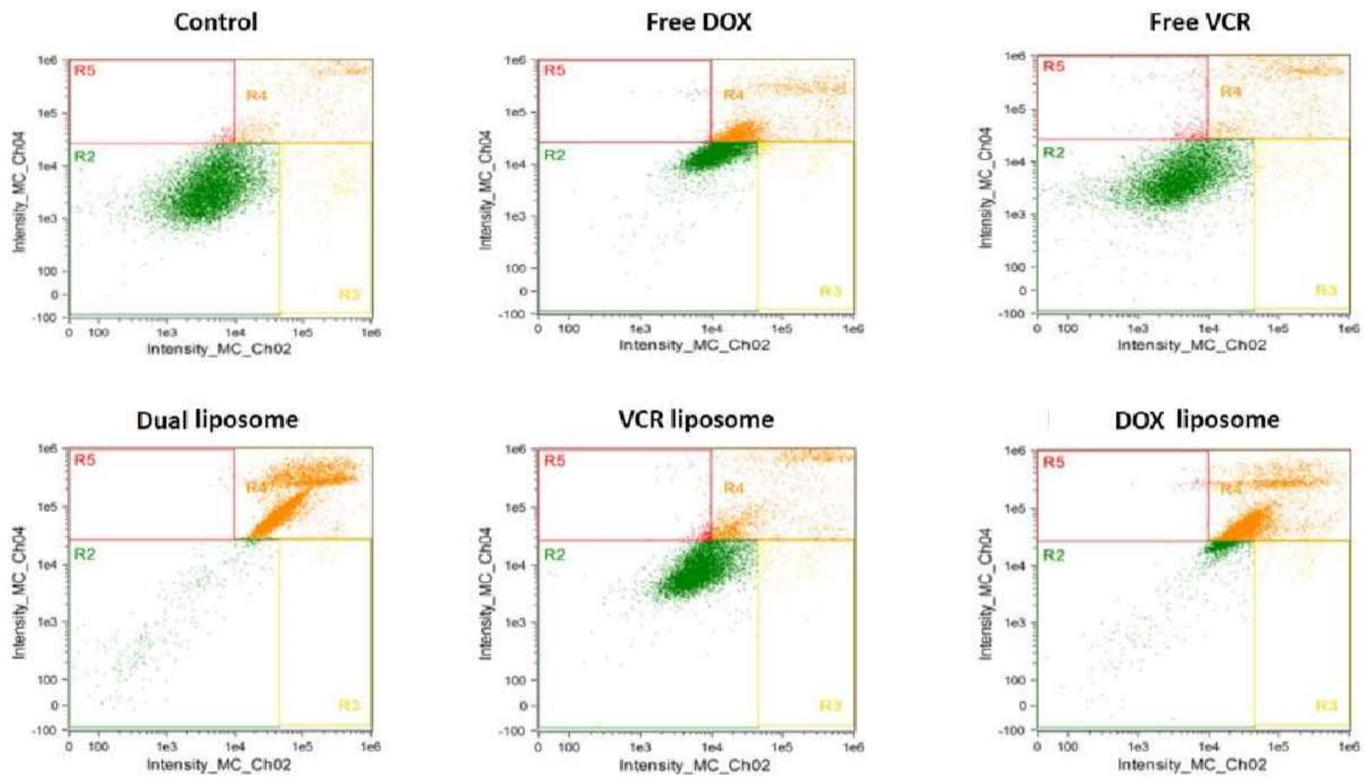


Figure 56: Quantitative assessment of apoptosis in A549 cells induced by formulations using Annexin V assay (R5 – Necrotic cells (red), R4 - late apoptosis (orange), R3 – early apoptotic cells (yellow), R2 – Live cells (green)).

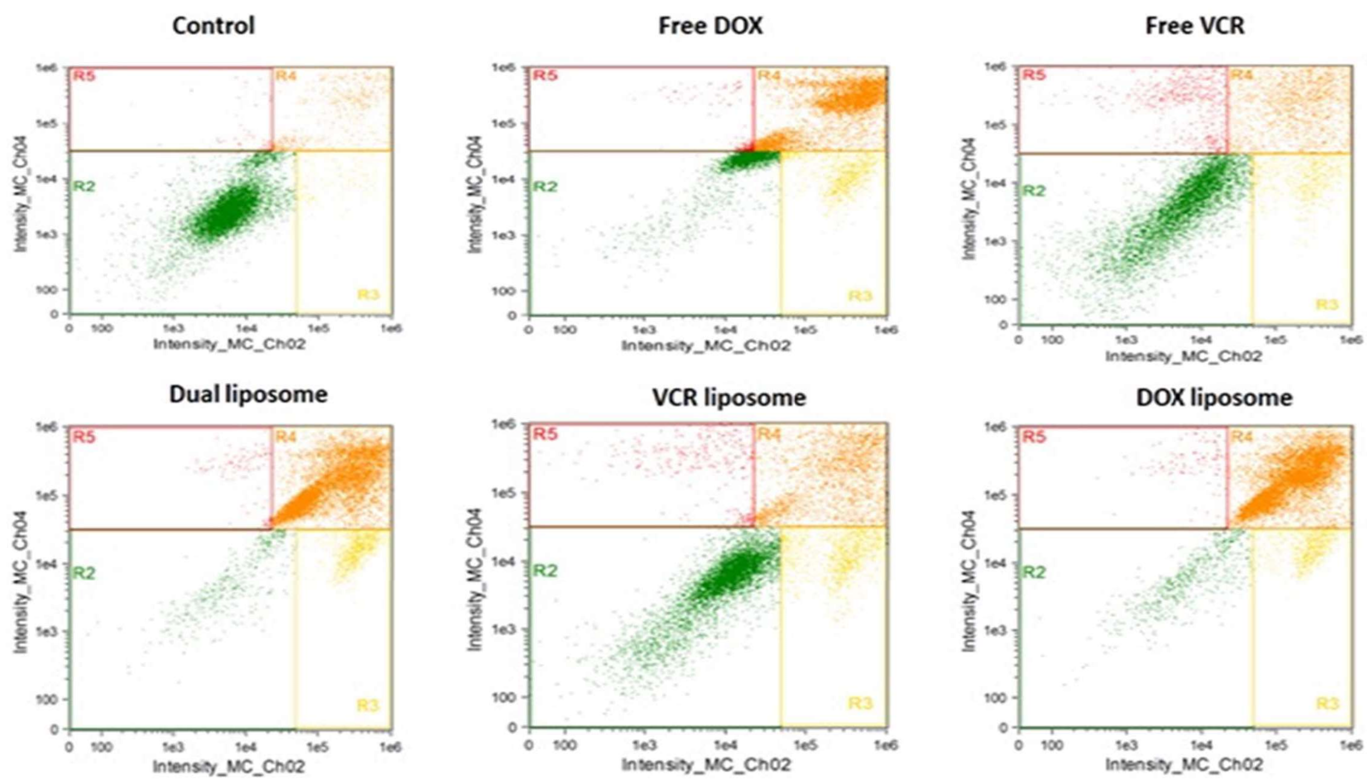
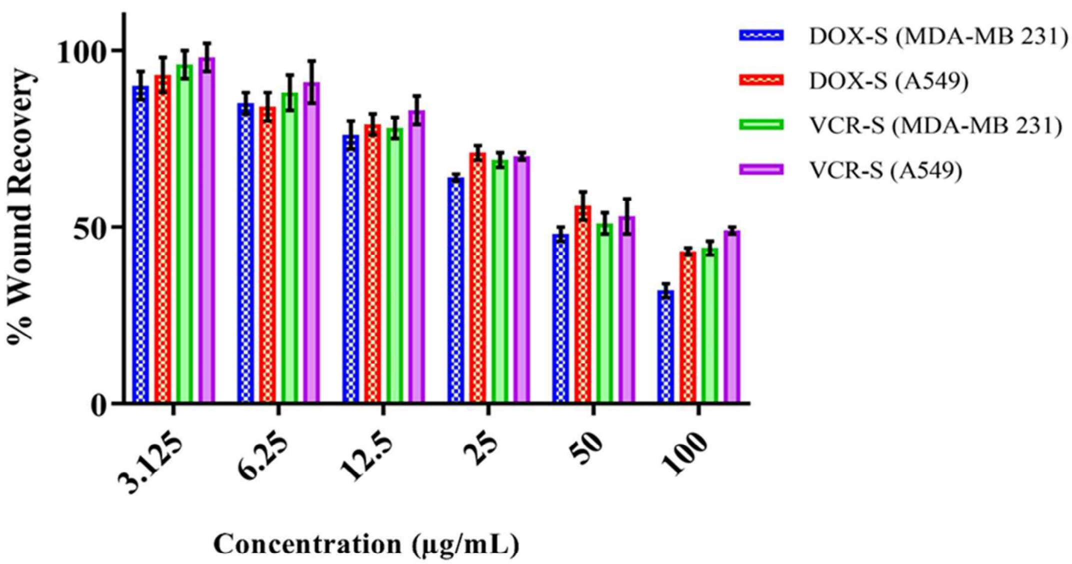


Figure 57: Quantitative assessment of apoptosis in MDA-MB 231 cells induced by formulations using Annexin V assay (R5 – Necrotic cells (red), R4 - late apoptosis (orange), R3 – early apoptotic cells (yellow), R2 – Live cells (green)).

9.4.6 Wound Scratch Study

The migration potential and in-vitro assessment of the effect of the formulations on the tumor cells was done using wound healing study. The recovery of formulation exposed wound is inversely proportional to the efficacy. Untreated cells (control) and drug free liposomes treated cells showed 100% recovery in both the cell lines. Both DOX and VCR free drugs showed concentration dependent inhibition of the wound recovery ($p<0.01$) (Figure 58 A). The recovery potential on equimolar treatment of all the formulations showed 1.82- and 1.36-fold decrease in recovery of MDA- MB 231 and A549 cell lines respectively post treatment with dual drug liposomes as compared to Liposomal DOX indicating stronger cellular growth inhibition potential (Figure 58 B).

A



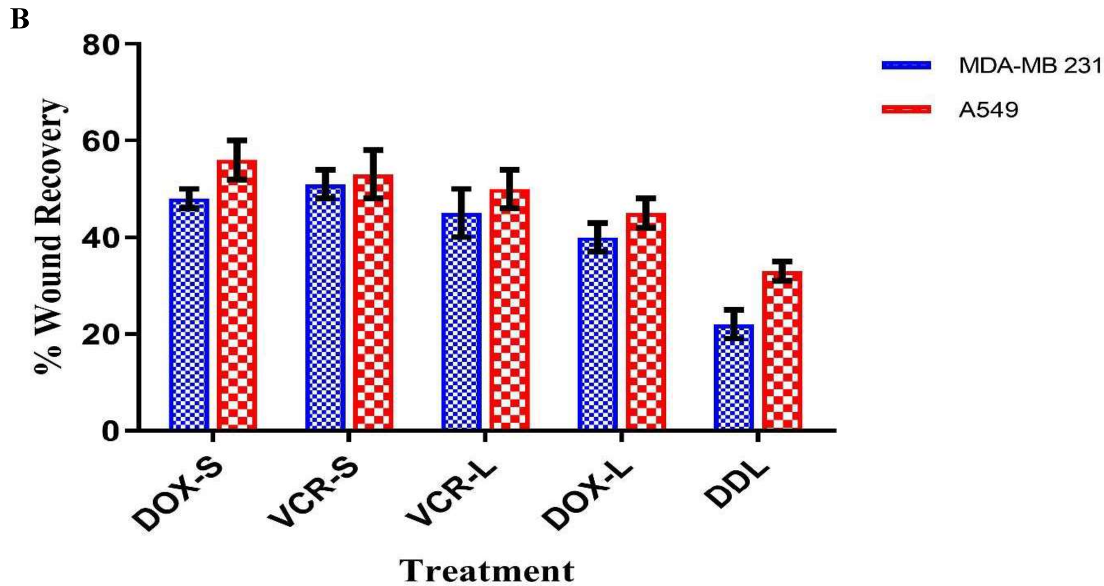


Figure 58: Wound recovery study results as a function of A) various concentrations of free drugs in MDA-MB 231 and A549 (B) effect of various formulations at 50 µM effective concentration.

The wound recovery with drug free liposomes treatment indicated the absence of carrier therapeutic efficacy. The dual liposomes exhibited the highest inhibition of the cellular recovery indicating towards significant reduction in cellular viability as well as presenting improved chances of in-vivo anti-angiogenic properties as compared to liposomal DOX. The results of in-vitro cell line studies in MDA-MB 231 and A549 cells were in good correlation with each other (37).

9.6 Conclusion

The cellular uptake studies in A549 and MDA-MB 231 using confocal microscopy and flowcytometry show significantly increased uptake of the dual drug formulation as against the liposomal DOX (as well as all other formulations). This resulted in significantly increased cell cycle arrest in G2/M phase with subsequently increased apoptosis and reduced cell viability in both tumor cell lines when presented with co-loaded formulation than with the single drug liposomes. These in-vitro cell viability studies of this formulation in both tumors 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. Thus, ratio-mimetic co-encapsulation of the drugs in combinatorial 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. Further, these in-vitro cell line study results warrant further evaluation of the in-vivo preclinical toxicity as well as efficacy studies.

The above-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 (38); 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 (39).

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