Chapter 6: Determination of synergism

6.0 Determination of Synergism

6.1 Introduction

6.1.1 The integrative approach to use of cocktail of drugs

The current strategy for treatment of solid tumors such as NSCLC and TNBC involve the usage of cocktail of drugs when using conventional and non-personalized therapy (1). The complexity, metastasis and resistance are main factorial determinants which decide upon the agents to be combined in such therapies. The recent trend of the drug product approvals by various regulatory agencies entails the targeting of newer novel pathways which helps in attaining a better control over the barriers exhibited to the efficient treatment of both solid tumors (2). The major focus has now shifted from usage of single agents acting through single molecular pathways to usage of agents (single or multiple) which act through multiple non-overlapping pathways which entails a better control over the disease conditions of resistance, relapse and progressing despite the heterogeneity of the tumors (3). Usage of such chemotherapies activating multiple signaling pathways can lead to different cell death outcomes. However, a detailed understanding of how usage of such multiple agents affecting multiple pathways shall cooperate to produce optimal therapeutic effect and may ensure interference with tumor dynamics is essential for the design of rationally-based chemotherapeutic combinations (4). Care should be taken to ensure that such rationally combined usage of drugs shall not result in the generation of antagonistic pathways which shall invariably aid the tumor resistance and growth (5). The current therapeutic regimens entail the usage of maximum tolerated dose of any drug in these cancers as the drug to be used in the cancer treatment (3). The usage of this dose for every drug in the combination therapy is based on the "Maximum-Maximum" theory which hypothesizes that the dosing of the maximum tolerated dose of any drug is associated with the maximum therapeutic benefit from the drug in the specific cancer being treated. However, this assumption fails to take into consideration the combined effects of the presence of two or more drugs being used at MTD in the treatment, their associated toxicities, whether they are present at the tumor site at the same concentration as administered to the patient and the last but not the least whether they are present at the similar time frame to ensure the effective static or cidal effects of the drugs being used (6). Since, the conventional therapy encompasses the higher usage of cell cycle specific drugs, it should be

noted that along with the heterogeneity exhibited by the tumors, the phase of the cell cycle which the cells are transitioning along with the presence of the drug efflux transporters at the time of the drug presentation at the site of tumor shall hold key to the effective management of TNBC and NSCLC (4). Thus, an integrative approach is required while selecting the appropriate chemotherapeutic agents for the treatment of such cancers.

6.1.2 Need for Synergism and Chou Talalay Method

The conventional drug delivery of chemotherapeutic agents against solid tumors exhibit various challenges to the treatment of cancer: administration of high drug loads, inefficient bio-distribution, lack of specificity in delivery, presentation of sub-optimal concentrations of the drug at the tumor sites besides the incidence of high levels of toxicities (7). Further, the delivered dose of the anticancer drugs in combination are administered as a thumb rule based on the maximum tolerated dose of the individual drugs individually combined together. However, it is important to determine whether these drug combinations are synergistic, additive or antagonistic when used together depending on the dose of the drugs used with the ratio of at the time of administration and at the site of action being primary determinants of their activity (8). Such determination of activity of these drug ratios in in-vitro conditions are feasible and controllable, but the translation of the in-vitro to in-vivo biological efficacy is complicated by the independent dissimilar pharmacokinetics exhibited by the drugs when presented as naked drugs in cocktail conventional delivery systems. The lack of effective control over the ratio of drugs being presented at the tumor site may result in the drug combinations becoming therapeutically ineffective (antagonistic) than when administered initially (9). Such conditions shall then limit the effectiveness of the therapeutic agents when used in combination and may increase the propensity of development of drug resistance in the tumor cells (10). Thus, besides the evaluation of combinatorial index to ensure therapeutically effective synergism among the drugs of choice, another important aspect which currently affects the available therapies is the lack of therapeutic carriers which can ensure that the drugs can be shipped across to the tumor sites in the aforementioned ratios while ensuring that they are able to offload the required payload efficiently (11). These two aspects of the unmet clinical need can be met by first ascertaining the effectiveness of the drugs based on the Chou-Talalay Method (Figure 23) and second usage of nanocarriers to ensure efficient delivery (12). The attainment of both these

objectives can ensure the effective in-vitro in-vivo transition and clinical effectiveness of such drug combinations in treatment of solid malignancies (13).

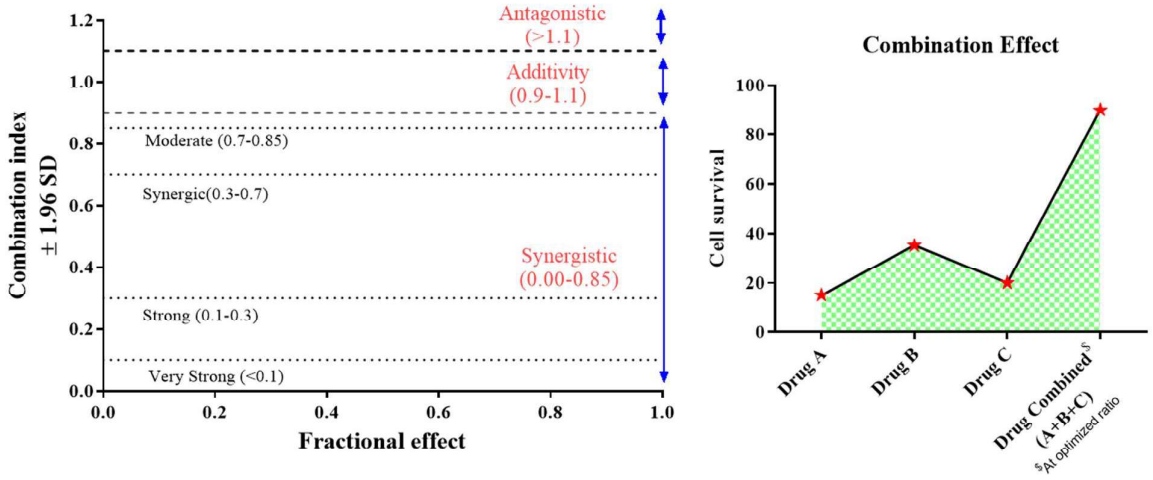

Figure 23: Chou-Talalay diagram and the combination effect diagram. (A) Schematic diagram of Chou-Talalay method to determine the combination index of two drugs in combination used in the treatment of cancer. Combination index values of <0.85 are synergistic, 0.9–1.1 are nearly additive and >1.1 are antagonistic. The synergistic combinatorial index values can be further subdivided into very strong (<0.1), strong (0.1-0.3), synergistic (0.3-0.7) and moderate (0.7-0.85); (B) Schematic diagram representing hypothetical tumor inhibition (cell killing) by synergistically acting drug combinations targeting multiple key pathways (Adopted with permission from (14))

6.1.3 Rationale based selection of combination drugs and synergism

Prior to the usage of the nanotherapeutic platforms, one needs to make a rationale selection of suitable drug combinations to utilize the potential offered by these carriers in tumor cell death. While looking into the current unmet clinical needs of these particular solid tumors, the choice of such combinations would be based on two aspects: Tumor characteristics and clinically effective therapeutics (15). Probing in the tumor characteristics would necessitate the identification of the disease resistance mechanisms, careful evaluation of the tumor macro- and micro-environments and identifying the possible molecular/cellular/surface targets which can

be leveraged to attain a better cell killing ratio (16). The choice of the combination agents may be made based on whether they have proven clinically effective or are have been in used in established therapeutic regimens in the disease or predicament that their non-overlapping distinct loci of action (different molecular pathways or molecular targets) may ensure low cell viability with lower potential of additive toxicities or alteration of basic metabolism properties (17). Such combinations may include the use of multiple active agents or even the use of modulators of various drug resistance mechanisms (e.g. P-gp modulation etc.) with chemotherapeutics or addition of immunotherapeutic agents/ targeted therapy agents/gene (i.e. DNA- and RNA-based) therapy/ epigenetic therapeutic agents with active components (18). Traditionally the use of the drugs in combination have been based on the maximum tolerated dose of the individual drugs. This approach may however not be efficient to ensure that the full potential of the combination as usage of the drugs at that concentrations result in them being synergistic, additive of antagonistic (19). Hence, for the effectiveness one needs to first assess the inhibitory concentration (IC_{50}) of the individual drugs when used together in the in-vitro cell lines representative of the neoplasm, then using the median effect analysis to calculate the combinatorial index (CI) of the drugs (20). Fundamentally to ensure that the combination has potential to eradicate the tumor, the CI values should be synergistic (CI<1) and not additive (CI=1) or Antagonistic (CI>1) (see figure 8.1) (21). Along with the requirement of the optimal ratio of the drugs, the in-vitro drug administration schedule plays an important role in the ensuring the synergism being maintained and effectiveness being achieved particularly when the site of action remains the same tumor cell (22).

6.2 Materials and Cell lines

6.2.1 Materials

Vincristine sulphate (VCR) and Doxorubicin hydrochloride (DOX) were procured from Minakem (France) and Synbias Pharma (Ukraine) respectively. Thiazolyl Blue Tetrazolium Bromide (MTT); Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) were purchased from HiMedia (India). 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.

6.2.2 Cell culture

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 and cultured for the cell line studies using methods as previously described (23). 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 ºC 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.

6.3 Methodology

The combinatorial index for determination of optimal synergism of the two drugs DOX and VCR was determined using the Chou-Talalay method. The drugs individually and together (in weight ratios of 10:1 to 1:10), were evaluated for the MTT cell viability assay for 72 hours. The A549 and the MDA-MB 231 cells were seeded in sterile 96 well plate (Corning, USA) at the concentration of 5 X 10^3 cells per well in 200 µl of 10% FBS supplemented DMEM media with incubation at 37°C under 5% CO_2 atmosphere for 24 hours to facilitate the growth as well as adherence of the cells. Post this period, the medium was removed and the cells were exposed in triplicate to various weight ratios of VCR and DOX prepared by diluting the mixed drug solution in serum free DMEM media to attain appropriate concentrations for 72 hours (24). The control cells were exposed to only the serum free DMEM media. Further the medium was removed and 100 µl of MTT reagent solution was added to each well while keeping it for 4 hours under the incubation conditions. This reagent solution was then replaced with 100 µl of dimethyl sulfoxide (Himedia, India) with the measurement of the intensity of dissolved formazan crystals at 570 nm wavelength using microtiter plate reader (Biorad, Calfornia). The cell viability of the treated cells was then expressed relative to the untreated control cells using previously established equation (23). All experiments were performed in triplicate. The

fraction of the affected cells (f_a) was determined for each treatment ratio using the median effect equation:

$$\frac{f_a}{(1-f_a)} = \frac{(D)^m}{(D_m)^m}$$

where, f_a is the fraction of cells affected, $f_u = (1-f_a)$ is the fraction of cells unaffected, D is the dose of the drug ratio, D_m is the median effect dose of drugs, m is kinetic order or the slope of the plot of % cell viability vs concentration of the drugs. These f_a values were then analysed for the median-effect analysis for determination of the combinatorial index (CI) using CalcuSyn software (version 2.0, Biosoft, UK) (25).

Based on the combination index theorem (CIT), median effect plot and equations, the CI values for drug combination were calculated using the Chou-Talalay method equation:

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = \frac{(D)_{1,2}[P/(P+Q)]}{(D_m)_1\,[f_a/(1-f_a)]^{1/m1}} + \frac{(D)_{1,2}[Q/(P+Q)]}{(D_m)_2\,[f_a/(1-f_a)]^{1/m2}}$$

where, $(D_x)_1$, $(D_x)_2$ were concentrations of drugs (for current study DOX and VCR) when used alone to get x% cellular killing; $(D)_1$, $(D)_2$ were concentrations of the drugs when used in combination to get the same response; m1,m2 are slopes of median effect plot for the drugs; $(D_m)_1$,$(D_m)_2$, $(D)_{1,2}$ were the median effect dose (IC_{50}) of the drugs when used alone and in combination; P,Q: the ratio of the drugs used (25). The CI values were then classified in heat map according to antagonistic (CI>1.1), additive (CI=0.9-1.1), Moderate synergy (CI=0.7-0.85), synergistic (0.3-0.7), strong synergy (CI=0.1-0.3) and very strong synergy (CI<0.1).

6.4 Results and discussion

The combination therapy potential of the two drugs DOX and VCR when used together was assessed for dose dependent synergistic therapeutic improvements. The in-vitro cytotoxicity of the two drugs individually and in combination was evaluated for synergism, antagonism and additive effects using the median effect analysis wherein the measure of such pharmacological activity (combinatorial index) of the drugs in combination was assessed as a function of the affected cells and the drug concentrations (fixed drug-drug weight ratios) (25). The combinatorial index was determined for the drug combination, DOX and VCR by evaluating

the results of MTT assay using the median effect analysis. An exploratory cytotoxicity study in triple negative breast cancer (MDA-MB-231) and non-small cell lung cancer (A549) cell lines was conducted by varying the weight ratios of the two drugs (VCR and DOX) from 1:10 to 10:1 (VCR: DOX) to assess the combinatorial potency of the drugs. The results of the CI determination are presented in Figure 24 and as a heat map (Table 15).

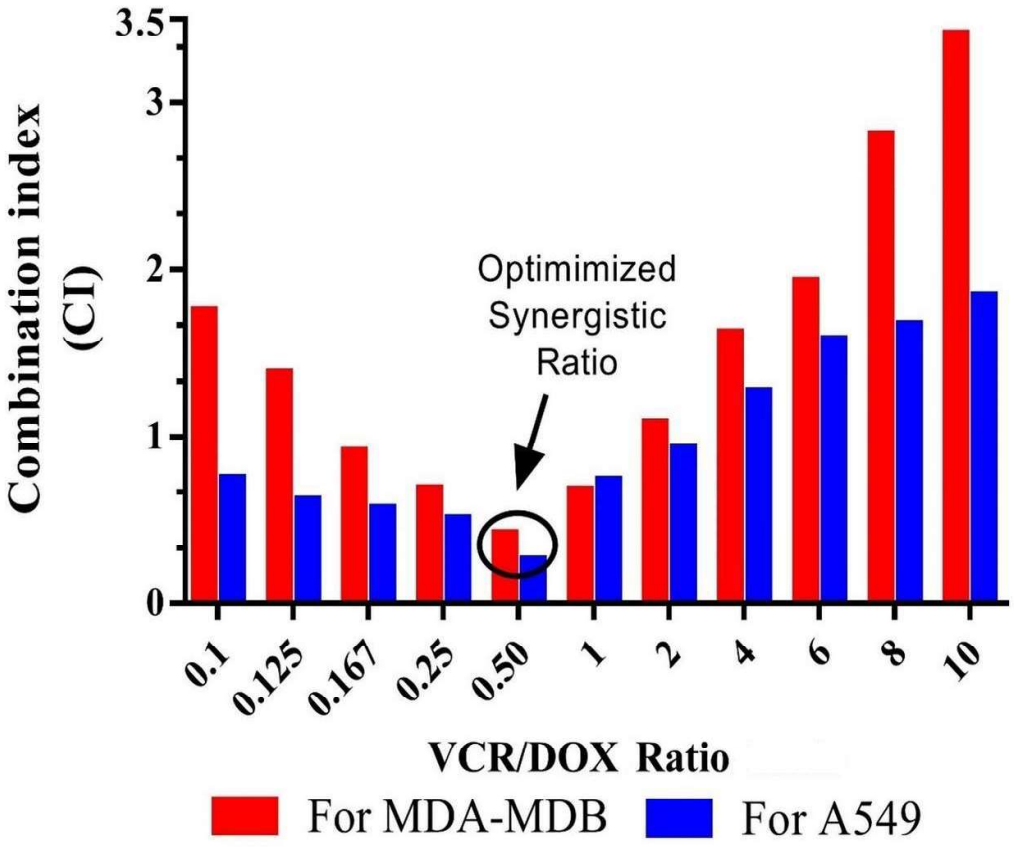

Figure 24: Combinatorial index of varying weight ratios of free drugs VCR and DOX in MDA-MB-231 and A549 cell lines (figure reproduced with permission from (26)). For better presentation in the figure, the various VCR:DOX ratios were indicated as 0.1 (1:10), 0.125 (1:8), 0.167 (1:6), 0.25 (1:4), 0.50 (1:2), 1 (1:1), 2 (2:1), 4 (4:1), 6 (6:1), 8 (8:1) and 10 (10:1).

The evaluation of various weight ratios of the drug combination presented three ratios (4:1, 2:1 and 1:1) which presented synergism with 2:1 DOX: VCR presenting the most optimum synergism. The results of fraction affected (f_a) of tumor cells indicate that combinations having higher DOX content shows more cytotoxicity in MDA-MB 231 cell line while increased

cytotoxicity in A549 was observed when the combination is used in weight ratios of 4:1, 2:1 and 1:1. DOX was found to be more potent than VCR as evidenced from the increase in the CI values with the increase in the weight concentration of VCR in the tested drug combination. The response in terms of the fraction of the cells (f_a) affected on exposure to the varying concentrations of drugs was found to be more in A549 cells than in MDA-MB-231. In-vitro synergistic interactions were observed at VCR: DOX weight ratios of 1:10 to 1:1 in A549 cells and at 1:4 to 1:1 in MDA-MB-231 cells while antagonistic or additive effects were observed at the other evaluated weight ratios.

VCR: DOX weight ratio	MDA-MB 231 (Triple negative breast cancer)		A549 (Non-small cell lung cancer)	
	Fraction affected	Combinatorial Index	Fraction affected	Combinatorial Index
1:10	0.49	0.75	0.22	1.75
1:8	0.52	0.62	0.30	1.38
1:6	0.55	0.57	0.34	0.91
1:4	0.56	0.51	0.42	0.69
1:2	0.70	0.26	0.55	0.42
1:1	0.49	0.74	0.42	0.68
2:1	0.34	0.93	0.32	1.08
4:1	0.29	1.27	0.25	1.62
6:1	0.25	1.58	0.16	1.93
8:1	0.22	1.67	0.11	2.81
10:1	0.19	1.84	0.09	3.41

Results of the in-vitro synergy were performed in triplicate; Red indicates antagonistic effect, orange indicates additive effect, yellow indicates moderate synergy; light green indicates synergistic; green indicates strong synergism

Table 15: Combinatorial index determination heat map- in-vitro synergy with varying mole ratios of doxorubicin and vincristine (table reproduced with permission from (27))

The most optimum drug ratio exhibiting highest degree of synergism in both the cell lines was found to be 1:2 VCR/DOX (CI= 0.26 for A549 cell line and 0.42 for MDA-MB-231 cell line). Increase in the concentration of VCR in the combinatorial treatment resulted in strong antagonism in MDA-MB-231 cells and moderate antagonism in A549 cells. The observed in-

vitro synergistic and antagonistic results are similar to the ones previously reported for this drug combination when tested under appropriate conditions (28, 29). The difference in the values of CI and effect of the presentation of different weight ratios of drugs may be explained based on mass-law action explained by Chou-Talalay method for the drug combination studies. The method indicates that the effects of drug combinations are often mutually non-exclusive, cellular killing of each drug being independent of the presence of the other drug. The effect (synergistic, additive, antagonistic) of simultaneous presence of both drugs being affected by the multiple mechanistic pathways in cellular killing and the cell cycle presentation of such tumor cells to the drug load.

6.5 Conclusion

The optimal quantitative degree of synergism for treatment using both the drug combination in both the cell lines was chosen as the synergistic ratio to be encapsulated in the liposomal nano-carrier. The synergistic ratio of 1:2 VCR/DOX was further used for encapsulation in the nanocarriers and other studies.

The above-mentioned sections have been published in the review article, Ghosh et al. (2019) "Combinatorial nanocarriers against drug resistance in hematological cancers: Opportunities and emerging strategies" in Journal of Controlled Release, Volume 296, Pages 114-139 (14), 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 (27); 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 (26).

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