Study the synergistic effect of virotherapy and chemotherapy on tumor cells in vitro

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Abstract

Virotherapy, by using Newcastle disease virus, is a successful anti-tumor agent for in vitro studies. As with other modalities of treatment, oncolytic virotherapy, by itself, has not been effective in complete tumor eradication in both preclinical animal models and clinical studies. It appears that the best chance for complete tumor eradication lies with combining its mechanism of action with current treatment strategies of chemo- and radiation therapies and the emerging field of clinical gene therapy. The theory, based on combination chemotherapy, is that attacking tumor cells through different mechanisms of action will prevent tumor cells from having time to develop resistance to treatment. Oncolytic viruses can engender an increased sensitivity of tumor cells to chemotherapy and radiotherapy, so we investigate the possibility of synergistic effect between Newcastle disease virus and chemotherapy in vitro. In the current study we used cytotoxicity assay and combination index to determine the most synergistic chemotherapeutic agent with Newcastle disease virus Iraqi strain, we used panel of different chemotherapies, and tested this combination for apoptosis induction. This combination tested for safety on normal cells. The results showed that Cyclophosphamide work in synergism with NDV as shown in cytotoxicity and apoptosis assays also it was safer when tested on normal cells. This study encourage to move for in vivo study to determine if Cyclophosphamide and NDV are powerful if used to treat transplanted tumors in mice.

Introduction

Finding efficient and less side effect cancer treatment is the major concern of scientists and researchers in the field of oncology. Many types of cancer treatment regimes were used in different hospitals and most of them depended on combination of two or more cytotoxic drugs. Combination chemotherapy has been found to be more effective than single-drug regimens and was documented as the basis of the major success in the treatment of various cancer types (1). This type of therapy has been found as critical in the development of curative regimens for hematologic malignancies and solid tumors. Such chemotherapy trials derive, in part, from studies of the development of drug resistance, the therapeutic challenge will be not only to maximize the effectiveness of individual agents, but particularly, to integrate these drugs into optimal combination therapy (2).

Virotherapy, which uses replication competent oncolytic viruses to kill tumor cells, is a promising biological approach for more efficient tumor destruction (3). The transformed phenotype of tumor cells provides a permissive environment for some viruses or functions to complement viral mutations. Oncolytic viruses are able to selectively replicate in tumor cells and kill them. A major advantage of such replication-competent viruses is this in situ amplification and subsequent spread within the tumor (4). Many viral-based therapies are under investigation as treatment for cancer (5), including 'suicide" pro-drug gene therapy, tumor suppressor-gene replacement therapy, immunomodulatory therapy, and oncolytic viral therapy. Although promising, active investigation needs to evaluate multimodality therapy in order to increase efficacy of treatments. Such combination therapy should be strategically chosen to facilitate a synergistic interaction between therapies that would permit lower doses of each agent to be used to minimize both cost and toxicity (6). The aim of this work to investigate the most synergistic chemotherapy when used in combination with NDV Iraqi strain. Newcastle disease is a highly contagious and fatal viral disease that affects all species of birds. NDV is a member of the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae and genus Avulavirus (7). NDV has been categorized into three main pathotypes depending on the severity of the disease in chickens: the lentogenic, mesogenic and velogenic strains. Newcastle Disease Virus has a single stranded negative-sense RNA genome, has a molecular weight of 5.2 to 5.6 x 106 Daltons. The genomic RNA consists of 15, 186 nucleotides (8). The helical nucleocapsid, rather than the free genome RNA, is the

template for all RNA synthesis. The genomic RNA contains six genes encoding at least eight proteins (9). Three proteins which constitute the nucleocapsid are the nucleoprotein (NP), the phospoprotein (P), and the large polymerase protein (L). The two external envelope proteins are the fusion protein (F) and the hemagglutinin neuraminidase protein (HN). The inner layer of the virion is formed by the matrix protein (M). The two additional proteins formed by the editing process during P gene transcription; are the V and W proteins. The V protein of NDV functions as an alpha interferon antagonist (10). Function of the W protein is unknown (11). In previous work by the authors they provide that Iraqi strain of NDV is oncolytic (12) and hence we want to increase the antitumor effect by combining it with classical chemotherapies.

Materials and methods

Virus propagation:

The virus sample injected (0.1ml) into 10-day olds embryonted chicken eggs allantoic fluid. The eggs observed daily for mortality, immediately after the death of embryo, it transferred to the refrigerator (4C˚). After 12-24hrs the allantoic fluid was collected by sterile syringe purified from debris by centrifugation (3000 rpm, 30 minute, 4C˚). Then it dispensed into small tubes and stored at -20C˚.

Virus Purification:

Virus samples ultracentrifuged (50000xg, 60min, 4C˚) by using (Ssorval ultracentrifuge, USA) the sediment resuspended in Phosphate buffer saline (PBS) and purified over cushin Density gradient with 35% sucrose (British Drug House , England) with 97000 xg at 60min, 4C˚. This repeated twice. The virus re-suspended in PBS and stored at -196 C˚.

Hemagglutination test:

The chicken red blood cells (RBC) prepared after collection from healthy birds in heparinized tubes and washed three times in PBS by centrifugation at 1000rpm 4C˚, the supernatant discarded and we token 0.5ml of washed RBCs brought to 10ml with PBS to achieve 0.5% RBC solution were it used in hemagglutination test. Newcastle disease virus quantified in which one hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination.

Cell lines:

Cell lines subcultured when the monolayers became confluent. Cells were maintained in RPMI 1640 growth medium with 10% FCS, 100ug penicillin, streptomycin and 2.5ug amphotericin B and incubated at 37°C.

Viable Cell Count:

An amount of 0.2 ml of cell suspensions will be mixed with 0.2 ml of trypan blue solution and 1.6 ml PBS and a sample of cells will be counted using an Improved Double Naubauer Ruling Counting Chamber. Magnification powers of 100X and 400X will be used to count the cells. Viable cells do not stain, dead cells stain blue as seen under the light microscope. The following formula will then be used to calculate the number of cells per unit volume (cells/ml):

$$c = n \, X \, d \, X \, 104$$

Where c is the number of viable cells per milliliter, n is the number of viable cells counted, and d is the dilution factor (= 10)

Chemotherapeutic agents:

All chemotherapeutic agents diluted with basic RPMI medium without calf bovine serum just before use for in vitro studies.

Study the synergistic effect of NDV and chemotherapy by combination cytotoxicity assay:

To determine the synergistic effect of NDV and chemotherapy in combination treatment, MTT cell viability assay was conducted on 96-well plates (Nunc, Denmark). Tumor and normal cells were seeded at 3-4x104 cells/well and 200 µl of cells in growth medium added to each well of a sterile 96-well microtitration plate. The plates were sealed with a self-adhesive film, lid placed on, and incubated at 37°C.

After 24hr, or when the confluent monolayer is achieved, when the cells are in exponential growth, the medium removed and serial dilutions of; virus alone (infected with NDV at 28 HU with two fold serial dilutions); drug alone (the chemotherapeutic agent in two fold serial dilutions; or in combination (virus + chemotherapeutic agent in two fold serial dilutions) will be added to the wells. Triplicates were used for each concentration of each treatment modality. Control cells treatment made with Serum Free Media only. Afterwards, the plates re-incubated at 37°C for 72 hrs.

The procedure of adding these therapeutic agents was carried out by adding the virus at first for 2 hrs at room temperature to allow virus attachment and penetration. After that, cells washed with PBS and three concentrations of the drug were added (1, 2 and 3µg) on the non-infected cells, and on the infected cells. Cell viability measured after 72 hrs of infection by removing the medium, adding 28 µl of 2 mg/ml solution of MTT (Sigma-Aldrich, Germany) and incubating for 1.5 hrs at 37°C. After removing the MTT solution, the crystals remaining in the wells solubilised by the addition of 130 µl of DMSO (Dimethyl Sulphoxide) (USbio, USA) followed by 37°C incubation for 15 min with shaking.

The absorbency determined on a microplate reader (expert plus reader Asyshitech, Austria) at 492 nm (test wavelength); the assay performed in triplicate (13).

Endpoint parameters that are calculated for each individual cell line includes:

1 – Percentage of cell growth or percentage of cell proliferation (PR) = mean of treatment / mean of control (14).

2 – The inhibiting rate of cell growth (the percentage of cytotoxicity) will be calculated as (G.I) = (A-B)/Ax100, Where A is the mean optical density of untreated wells and B is the optical density of treated wells (14).

Synergism determination:

Data analyzed by the dose oriented Isobologram technique. The axis on an isobologram represents the doses of each drug. Two points on the x and y axes are chosen that correspond to the doses of each drug necessary to generate that particular combination index (CI) effect, that is the fraction affected (Fa) value. The straight line or hypotenuse drawn between these 2 points on the x and y axes corresponds to a strictly additive interaction between 2 therapeutic agents. If the point lies to the lower left of the hypotenuse, the effect is synergistic, and if the point lies to the upper right of the hypotenuse, the effect is antagonistic (15). This represented in Figure-1.

Mitochondrial permeability transition apoptosis test:

The BioAssay™ Apoptosis Detection Kit (USbiological, USA) provides a simple, fluorescent-based method for distinguishing between healthy and apoptotic cells by detecting the changes in the mitochondrial transmembrane potential. The kit utilizes a cationic dye that fluoresces differently in healthy cells and in apoptotic cells. In healthy cells, the dye accumulates and aggregates in the mitochondria, giving off a bright red fluorescence. In apoptotic cells, the dye cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescing green. The fluorescent signals can be easily detected by fluorescence microscopy using

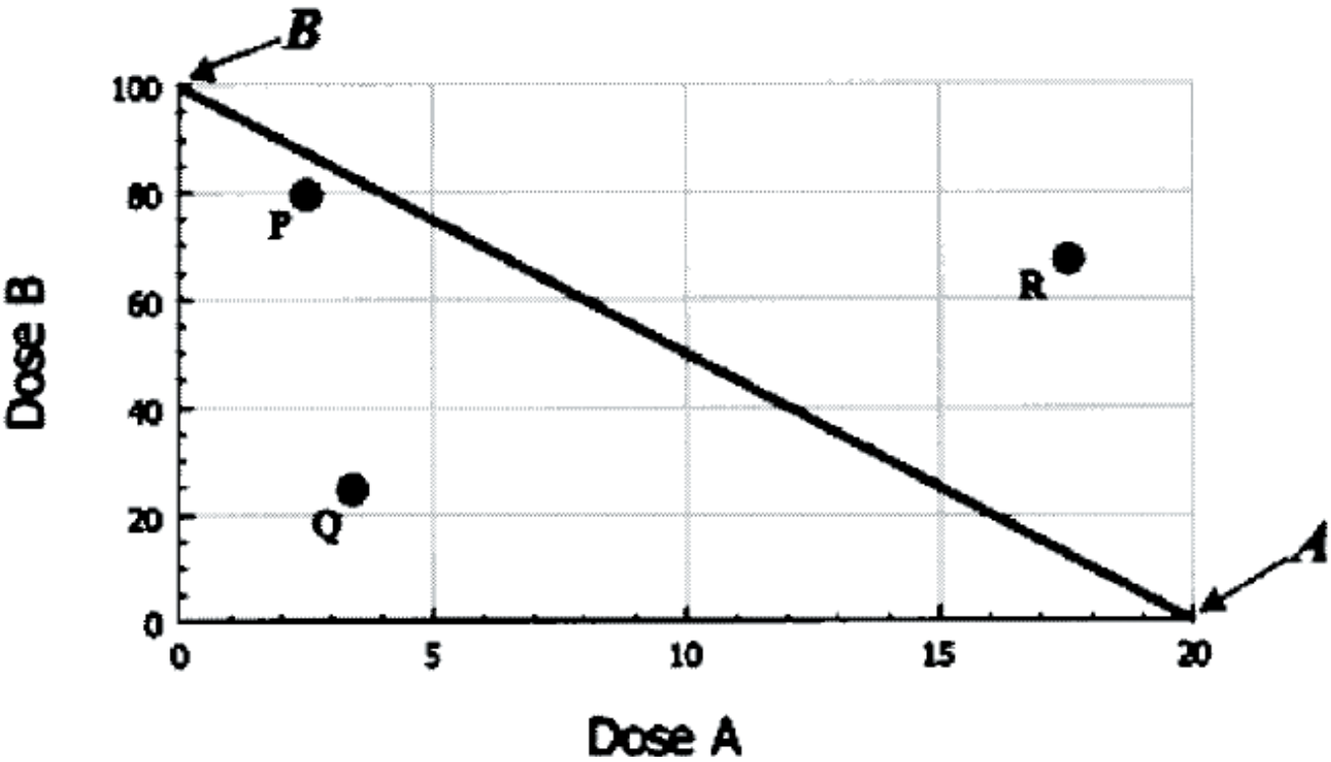


Figure-1: Illustration of isobologram analysis of combined drug effects. Given the combination of drugs A and B at equipotent concentrations, possible responses from the drug mixture are shown. Point 'Q" represents a synergistic effect, point 'P" an additive effect and point 'R" represents antagonism ( ).

a band-pass filter (detects Fluorescein isothiocyanate (FITC) and rhodamine). The cells exposed for 24hrs to 1 dose of each chemotherapy tested (2µg/ml) and Newcastle disease virus (NDV) at 1 hemagglutination unite (HAU) (256) and combination treatment which is on fixed ratio (2:256) to induce apoptosis. Repeated measures multivariate ANOVA were used to demonstrate statically significant differences between groups.

Apoptosis induction in vitro:

1. Apoptosis induced in cells by infection with NDV and or chemotherapeutic drugs, and a control culture incubated without induction. The cells cultured in tissue culture slides (Labtech – Denmark). After confluency (18-24 hrs) they were treated as described above, the treated cells further incubated in 37C for another 24hrs. 0.4 ml of the diluted MitoCapture solution then added to cultured cells and Incubated at 37C° for 15-20 min. Cells observed immediately under a fluorescence microscope using a band-pass filter (detects fluorescein and rhodamine). MitoCapture that has aggregated in the mitochondria of healthy cells fluoresces red. In apoptotic cells, MitoCapture cannot accumulate in the mitochondria, it remains as monomers in the cytoplasm, and fluoresces green.

The Results

Propagation of the Virus in embryonted chicken eggs and titration of the virus by hemagglutination test:

Isolated virus kills all embryos during 48hrs with marked hemorrhage in the infected embryos in compare to control uninfected embryos. Isolated virus was tittered by hemagglutination test which showed titer of 256HAU, further passaging increased the titer and the purification increased it further.

Study the synergistic effect of NDV and chemotherapy by combination cytotoxicity assay:

To study the potential interaction between NDV and chemotherapy in vitro, the effectiveness of the combined treatment of three concentrations of a panel of chemotherapy drugs (1,2 and 3µg) with NDV at three HAU (128,256 and 512) was evaluated, in the Hep-2, Glioblastoma tumor cells, AMN3, Rat embryo fibroblast and Vero cell lines. The cell viability was determined after 72 hrs by MTT assay and combination synergism determined by Isobologram analysis. Repeated measures multivariate ANOVA demonstrated statistically insignificant differences between groups.

AMN3 Mammary adenocarcinoma tumor cell line:

Only Cyclophosphamide showed synergism according to isobologram analyses at 50% growth inhibition doses as shown in (Figure-7). Other chemotherapies were antagonistic except vindesine which had additive effect.

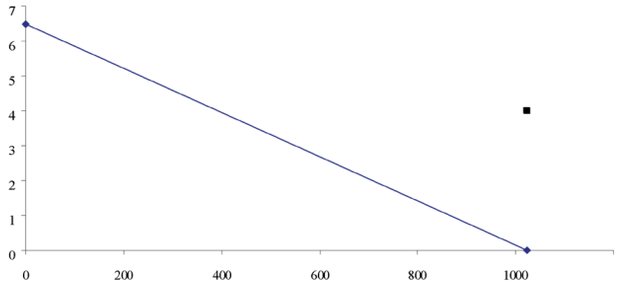


Figure-2 Isobologram analyses show antagonism between NDV and Cis at 50% growth inhibition doses on AMN3 cell line.

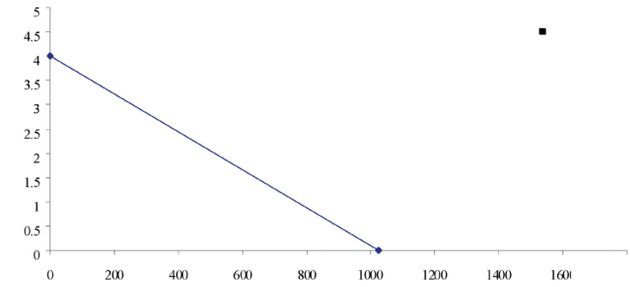


Figure-3 Isobologram blot show antagonism between NDV and MTX at 50% growth inhibition dose on AMN3 cell line.

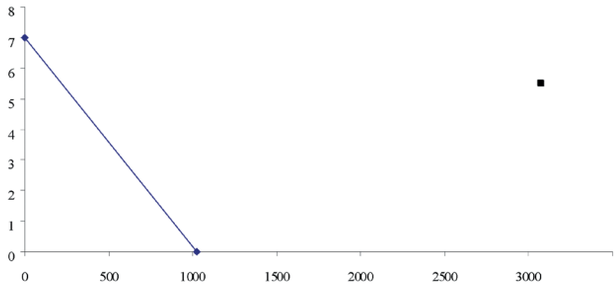


Figure-4 Isobologram analyses show antagonism between NDV and Eto at 50% growth inhibition doses on AMN3 cell line.

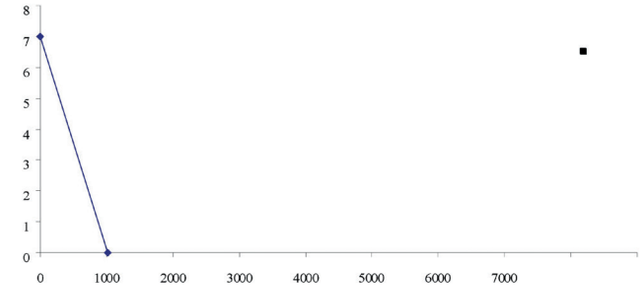


Figure-5 Isobologram analyses show antagonism between NDV and mmc at 50% growth inhibition doses on AMN3 tumor cell line.

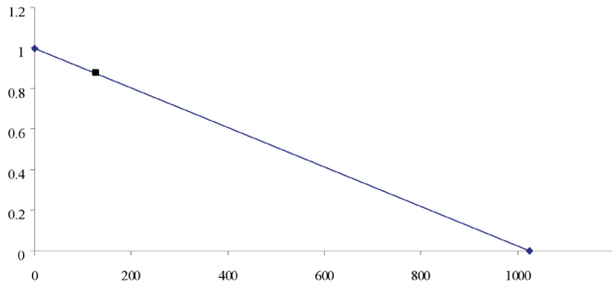


Figure-6 Isobologram analyses show additive effect between NDV and Vin at 50% growth inhibition dose.

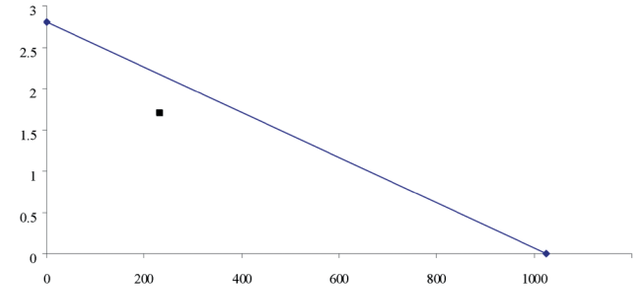
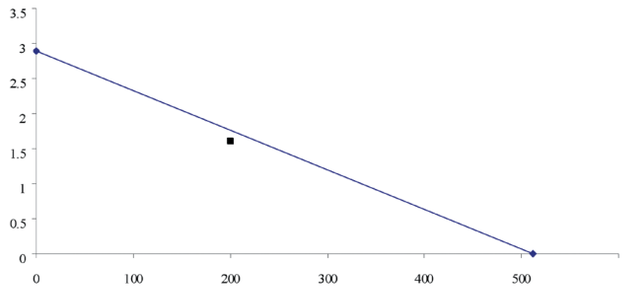


Figure-7 Isobologram analyses revealed that there is synergism between NDV and CPA as the point lies to the lower left of the hypotenuse, indicating the effect is synergistic

Glioblastoma tumor cell line:

Isobologram analyses revealed synergism between NDV and Cisplatin and Etoposide at 50% growth inhibition doses as shown in (Figures-8 and 10). There was additive effect between NDV with methotrexate and NDV with vindesine and NDV with Cyclophosphamide combinations at 50% growth inhibition dose as shown in isobologram analyses (Figure-9, 12 and 13). While NDV and Mitomycin C combination showed antagonism at 50% growth inhibition doses as shown in (Figure-11).



(Figure-8). Isobologram analyses showed synergism between NDV and Cis.

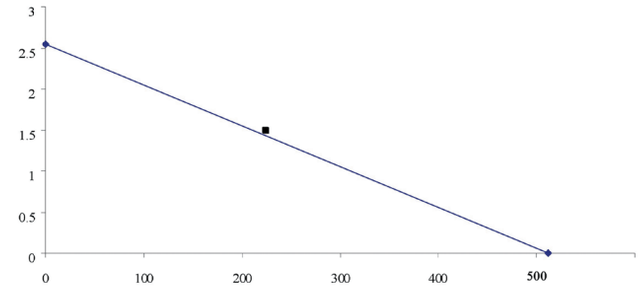


Figure-9, Isobologram analyses show additivity between NDV and MTX combination at 50% growth inhibition dose on Glioblastoma tumor cells.

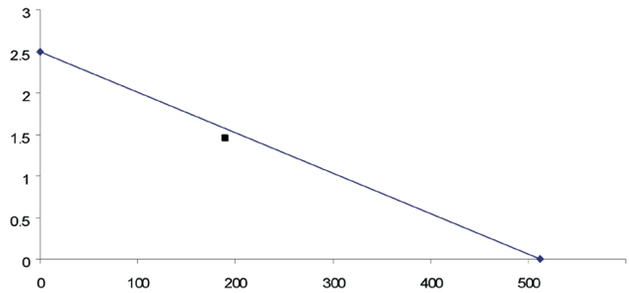


Figure-10 Isobologram analyses show synergism between NDV and Eto combination at 50% growth inhibition doses on glioblastoma tumor cells.

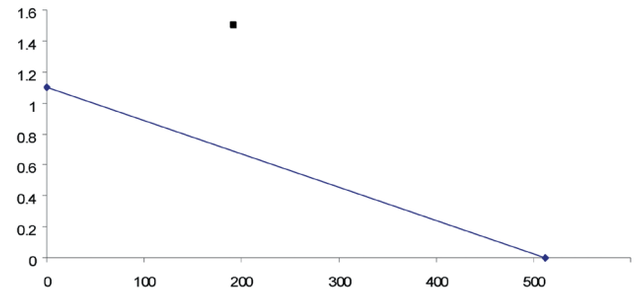


Figure-11 Antagonism between NDV and mmc as the point lies to the upper right of the hypotenuse, indicating that the effect is antagonistic.

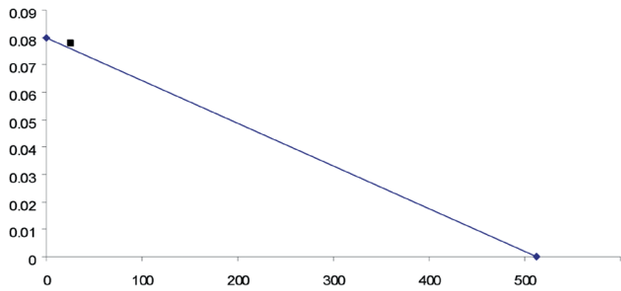


Figure-12 Isobologram analyses show additive effect between NDV and Vin at 50% growth inhibition dose on glioblastoma tumor cells.

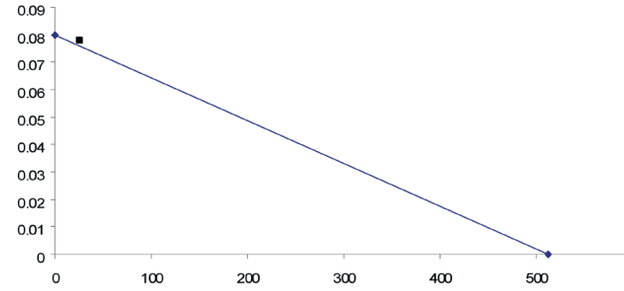


Figure-12 Isobologram analyses show additive effect between NDV and Vin at 50% growth inhibition dose on glioblastoma tumor cells.

Hep-2 Larynx carcinoma tumor cell line:

The synergism between NDV with Cyclophosphamide and NDV with Cisplatine at 50% growth inhibition dose is shown in (Figure-14, 19). While Isobologram analyses show antagonism between NDV and methotrexate and etoposide and mitomycine c and vindesine combinations at 50% growth inhibition doses as shown in (Figure-15,16,17 and18).

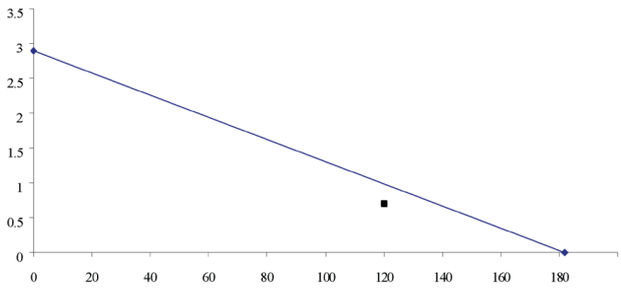


Figure-14 Isobologram curve show synergism between NDV and Cis as the point of combination lies to the lower left of the hypotenuse, indicating the effect is synergistic.

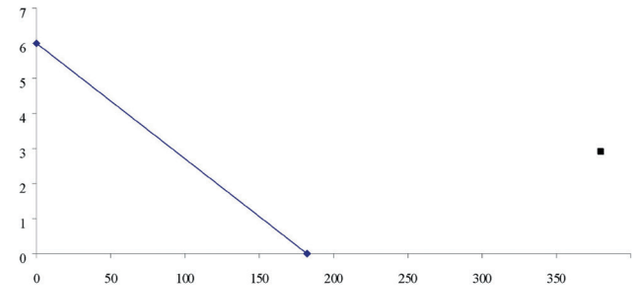


Figure-15 NDV and methotrexate combination at 50% growth inhibition dose on Hep-2 Larynx carcinoma tumor cell line as the point lies to the upper right of the hypotenuse, indicating that the effect is antagonistic.

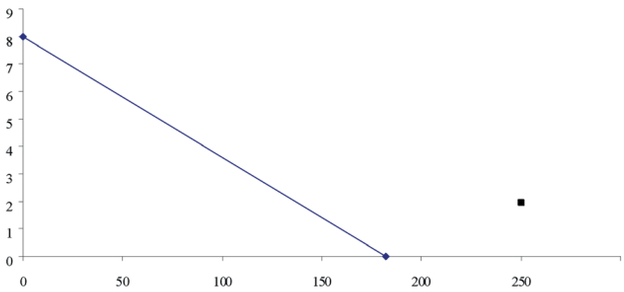


Figure-16 The point of combination lies to the upper right of the hypotenuse, indicating that the effect is antagonistic.

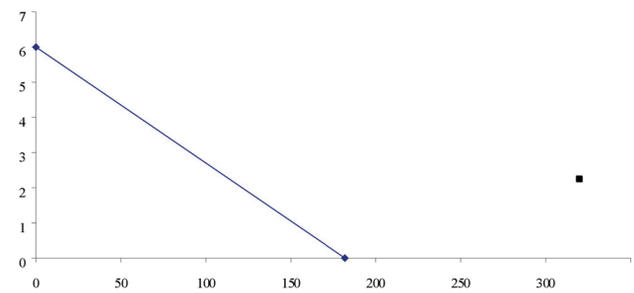


Figure-17 Isobologram analyses show antagonism because the point of combination lies to the upper right of the hypotenuse.

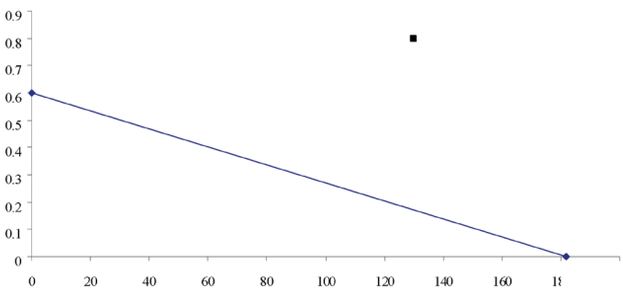


Figure-18 Isobologram analyses show antagonism between NDV and Vin at 50% growth inhibition doses on Hep-2 Larynx carcinoma tumor cell line.

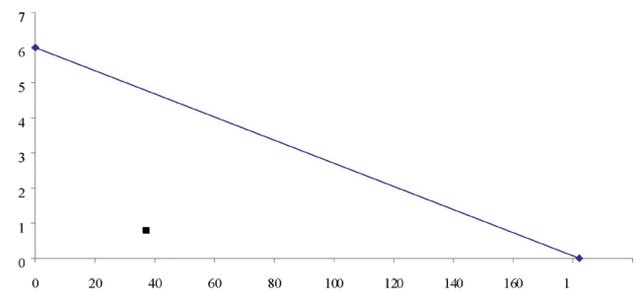


Figure-19 Isobologram analyses show synergism effect between NDV and CPA at 50% growth inhibition doses on Hep-2 Larynx carcinoma tumor cell line.

Rat embryo fibroblast cells:

The results showed synergism between NDV and Cisplatin and etoposide at 50% growth inhibition doses as represented in (Figure-20 and 22). Additive effect between NDV and methotrexate combination was shown at 50% growth inhibition doses (Figure-21). Isobologram analyses show antagonism between NDV and mitomycin c and vindesine and Cyclophosphamide at 50% growth inhibition doses as shown in (Figure-23, 24 and 25).

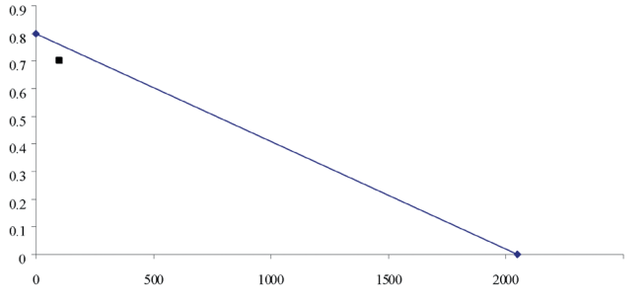


Figure-20 Isobologram analyses show synergism between NDV and Cisplatin at 50% growth inhibition doses on Rat embryo fibroblast cells.

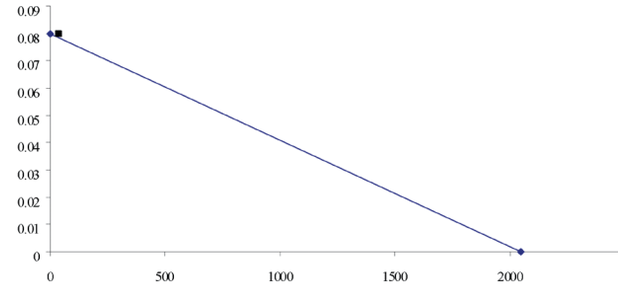


Figure-21 Isobologram analyses show additive effect between NDV and MTX combination as the point of combination lies on the hypotenuse.

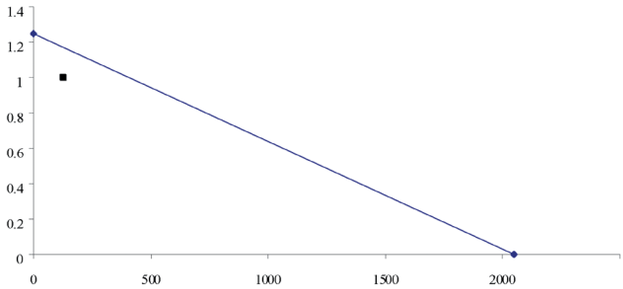


Figure-22 Isobologram analyses show synergism between NDV and Eto combination as the point of combination lies on the lower left of hypotenuse.

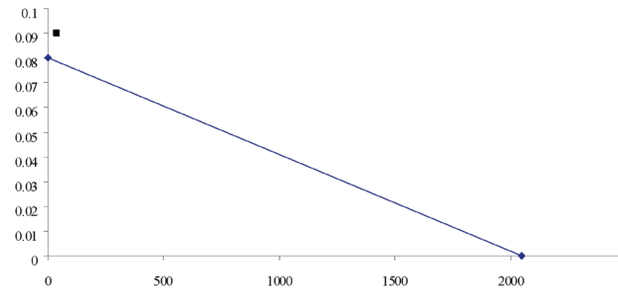


Figure-23 Isobologram analyses show antagonism between NDV and mmc at 50% growth inhibition doses on Rat embryo fibroblast cells

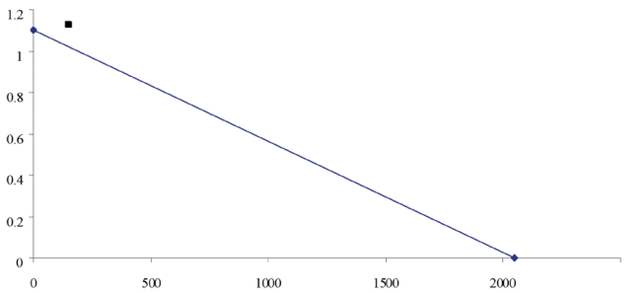


Figure-24 Isobologram analyses show antagonism between NDV and Vin at 50% growth inhibition doses on Rat embryo fibroblast cells.

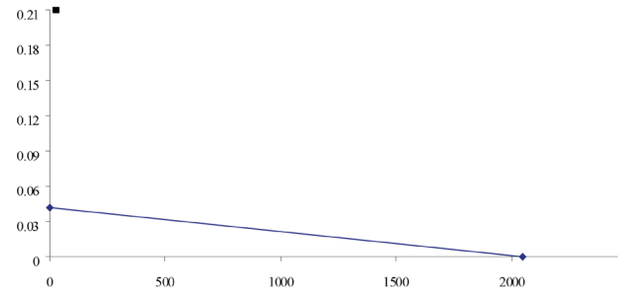


Figure-25 Isobologram analyses show antagonism effect between NDV and CPA at 50% growth inhibition doses on Rat embryo fibroblast cells

table-1 summarize all combination treatment results on all cell line tested.

	AMN3	Glioblastoma	Hep-2	REF
Cis	antagonism	Synergism	Synergism	Synergism
MTX	antagonism	additive	antagonism	Additive
Eto	antagonism	Synergism	antagonism	Synergism
mmc	antagonism	antagonism	antagonism	Antagonism
Vin	additive	additive	antagonism	Antagonism
CPA	Synergism	additive	Synergism	Antagonism

Mitochondrial Permeability Transition Apoptosis Test:

1. AMN3, Mammary adenocarcinoma tumor cell line:

Cyclophosphamide in combination with NDV was the most significant combination to induce apoptosis when compared to control group and other chemotherapies (etoposide, methotrexate, mitomycine c and cisplatine. apoptotic green cells and live red cells can be seen in (figure-26 and 27)

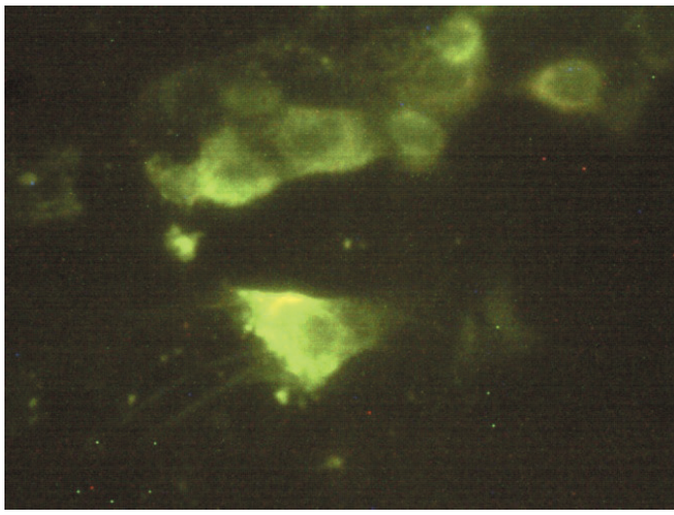


Figure-26 AMN3 cells were stained with MitoPT and viewed through a fluorescence microscope. Apoptotic cells at varying stages of mitochondrial ΔΨ appear green after treatment with Newcastle disease virus for 24hrs.

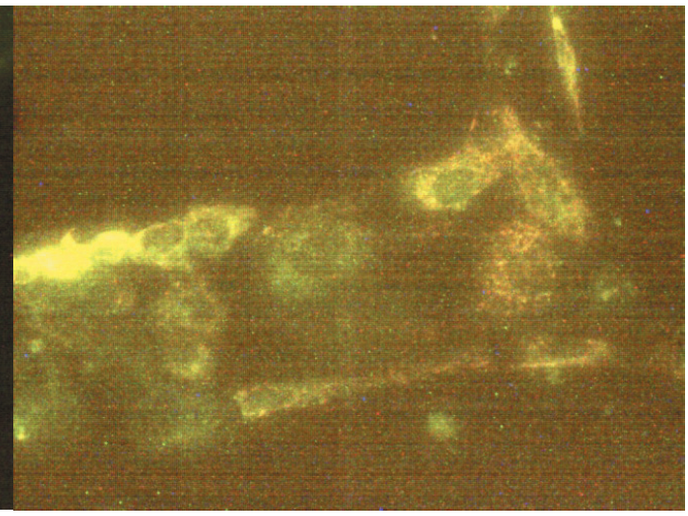


Figure-27 AMN3 cells were Non-apoptotic cells exhibit red stained mitochondria, non treated cells

Glioblastoma tumor cells:

Mitomycin C, Vindesine and Cyclophosphamide and their combination significantly induce apoptosis when compared to control and cisplatine, methotrexate and vindisine. Apoptotic green cells and nonapoptotic red cell showed in figures-28 and 29.

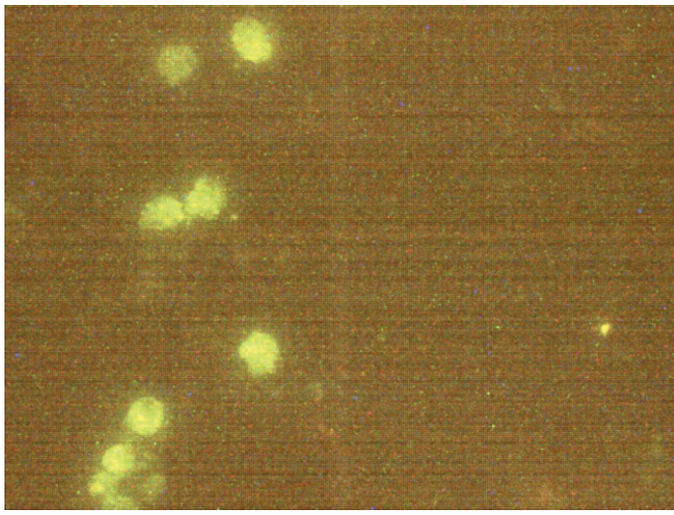


Figure-28 Glioblastoma cells treated with NDV and Cyclophosphamide where apoptotic cells fluorescent green.

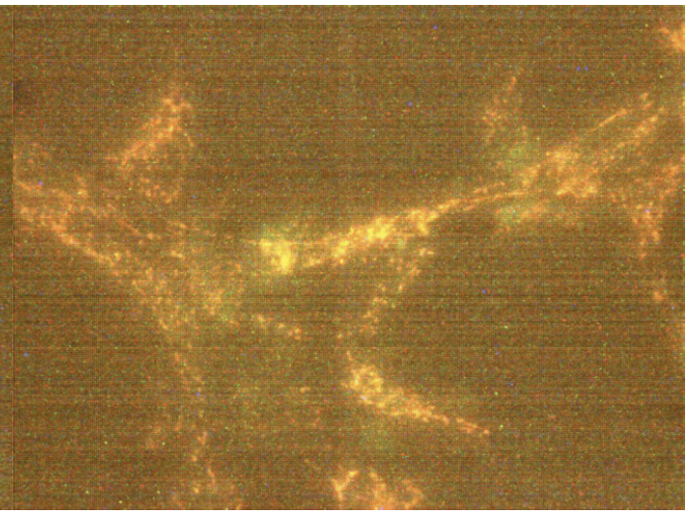


Figure-29 control Glioblastoma cells untreated where nonapoptotic cells fluorescent red.

Rat Embryo fibroblast cells:

In Rat Embryo Fibroblast cells, all treatments were without significant differences between groups, NDV treated cells and control cells.

Discussion:

In the present study the virus was isolated in embryonted chicken eggs allantoic fluid as routinely done (17). The virus killed the embryonated chicken eggs within less than 48hrs which refer to the virus as virulent as described by Alexander (18). The virus was purified and quantified by hemagglutination assay and characterized by infection the Chicken embryo fibroblast in vitro, the use of in vitro cell lines and cell cultures offers the opportunity to study mechanisms of infection and the immediate host responses under control. Most studies on NDV have been carried out using chicken embryo cell lines. Primary cell culture systems provide a valuable intermediate between animal studies and the use of cell lines to study mechanisms of infection and the immediate host responses under highly controlled conditions as described by Zaffuto et al. (19).

The results showed that only cyclophosphamide in its inactive form have the most optimum synergistic properties as it has synergistic effect with NDV on murine mammary adenocarcinoma AMN3 cell line and with additive effect on human glioblastoma cell line, and has antagonism effect with NDV when used on normal rat embryo cells. Cisplatin have the good results but with synergism on human glioblastoma tumor cells only with antagonism effect on AMN3 cells, while it has synergistic effect on normal rat embryo fibroblast cell (REF). This led to identifying cyclophosphamide as the most synergistic chemotherapeutic agent when combine with NDV and appear to be safe when used on normal cells. Decker and Sausville (1) described in vitro results in more than one tumor type is important in the evaluation of potential combination regimens. In Rat embryo fibroblast cells, cisplatin and etoposide were synergistic with NDV action and methotrexate had additive effect when combine with NDV. However mitomycin C, vindesine and cyclophosphamide were antagonized to NDV effect. The results of synergism of CPA and NDV have not reported before. This may suggest that NDV infection to tumor cells may help to activate CPA to be more toxic even in absence of the CYP2B which is needed for CPA activation. Recent study showd that NDV can make resistant tumor cells more sensitive to cisplatine treatment and induce apoptosis (20).

Moreover, cyclophosphamide and NDV had the most powerful effect on inducing apoptosis in AMN3 mammary adenocarcinoma, Glioblastoma tumor cells with less effect on REF normal cells which was in correlation with in vitro cytotoxicity combination experiment results. Detection of the mitochondrial permeability transition event (PT) provides an early indication of the initiation of cellular apoptosis. Narita et al., (21) described this process which typically defined as a collapse in the electrochemical gradient across the mitochondrial membrane, as measured by the change in the membrane potential ($\Delta\Psi$). Changes in the mitochondrial $\Delta\Psi$ lead to the insertion of proapoptotic proteins into the membrane and possible oligomerization of BID, BAK, BAX or BAD. This could create pores, which dissipate the transmembrane potential, thereby releasing cytochrome c into the cytoplasm (22).

The results of the present experiment may be suggested that CPA caused mitochondrial permeability transition event (PT) which lead finally to apoptosis confirmed by results obtained from Schwartz and Waxman (23) who found Cyclophosphamide Induces Caspase 9-Dependent Apoptosis in tumor Cells, where CPA alkylate DNA and proteins induce expression of the mitochondrial proapoptotic factor Bax which in turn enhanced caspase 9 activation, plasma membrane blebbing, and drug-induced cytotoxicity.

The results showed that NDV alone induce apoptosis in AMN3 tumor cell line by induction the mitochondrial permeability transition event (PT) which is an early event of apoptosis led to release of cytochrome c from mitochondria through the opened mitochondrial pores. Elankumaran et al. (24) investigated the localization of cytochrome c after NDV infection, and they found the level of cytochrome c in cytosol increased twofold after NDV infection to tumor cells. These results indicate that the intrinsic mitochondrial pathway is initiated after infection with NDV.

From in vitro combination cytotoxicity assay study and apoptosis experiment findings we can proposes that each therapeutic agent interacts with a specific target causing dysfunction and injury, which is then interpreted by susceptible cancer cells as an instruction to undergo apoptosis. This enhances the oncolytic activity and explains the synergistic effect.

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