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# Synergistic Effects of Oncolytic Newcastle Disease Virus AMHA1 and Chemotherapy in Treating Breast Cancer cells

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## Abstract

Breast cancer is a significant health concern, necessitating innovative therapeutic approaches beyond traditional treatments. This study investigates the synergistic effects of combining virotherapy and chemotherapy using the mouse mammary adenocarcinoma model. Combination cytotoxicity assays were performed using NDV at different hemagglutination units (HAU) and chemotherapy drugs, including Cisplatin, Methotrexate, Etoposide, Mitomycin C, Vindesine, and Cyclophosphamide, at different concentrations. Cell viability was assessed using the MTT assay, and synergistic interactions were determined through isobologram analysis. The AMN3 cell line, representing mammary adenocarcinoma, was cultured under standard conditions. NDV was propagated, purified, and quantified using embryonated chicken eggs and a hemagglutination assay. Our findings demonstrate that the combined application of NDV and chemotherapy significantly enhances the therapeutic outcomes compared to either modality alone. Mitochondrial permeability transition assays confirmed enhanced apoptosis rates in combination treatments, particularly with NDV plus Cisplatin, Methotrexate, Vindesine, and Cyclophosphamide. Additionally, NDV replication remained stable across all treatment conditions, suggesting that chemotherapy agents did not significantly impact NDV's oncolytic activity. In conclusion, the findings highlight the potential of NDV as an adjunct to chemotherapy, enhancing cytotoxicity and apoptosis in mammary adenocarcinoma. The observed dose-dependent synergy suggests that optimal dosing strategies should be considered to maximize therapeutic efficacy while minimizing antagonistic effects. Further in vivo studies are warranted to explore the translational potential of NDV-chemotherapy combinations in breast cancer treatment.

***Keywords:*** *Breast cancer, Chemotherapy, cisplatin, methotrexate, Oncolytics*

## Introduction

Breast cancer remains one of the most prevalent and deadly cancers affecting women worldwide (1). Despite advancements in early detection and treatment, there is still a pressing need for novel therapeutic strategies to improve patient outcomes and reduce mortality rates (2). Traditional treatments such as surgery, radiation, and chemotherapy have limitations, including resistance and adverse side effects Therefore, exploring innovative approaches like combination therapies is crucial in the fight against breast cancer (3). The combination of virotherapy and chemotherapy represents a promising strategy for enhancing the efficacy of cancer treatment (4). Virotherapy involves the use of oncolytic viruses that selectively infect and kill cancer cells while sparing normal tissues. These viruses can also stimulate the immune system to recognize and attack tumor cells (5). Chemotherapy, on the other hand, uses cytotoxic drugs to target rapidly dividing cancer cells. By combining these two modalities, it is possible to achieve a synergistic effect, where the virotherapy enhances the sensitivity of cancer cells to chemotherapy, leading to improved therapeutic outcomes (6). Mouse mammary adenocarcinoma is a well-established animal model for studying breast cancer. This model closely mimics the human disease in terms of tumor biology and response to treatment (7). Using this model, researchers can evaluate the efficacy and safety of combination therapies in a controlled environment before translating the findings to clinical settings. The insights gained from these studies can provide valuable information on the potential benefits and mechanisms of action of combining virotherapy and chemotherapy for breast cancer treatment.

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The aim of our study was to investigate the potential synergistic effects of combining virotherapy with chemotherapy in the treatment of breast cancer using the mouse mammary adenocarcinoma model. Specifically, we sought to evaluate the efficacy of oncolytic Newcastle Disease Virus (NDV) in combination with various chemotherapeutic agents (Cisplatin, Methotrexate, and Etoposide) in inhibiting tumor growth. By analyzing the interactions between these treatments, we aimed to identify the most effective combinations and understand the underlying mechanisms of action.

## Methods

**AMN3 Cell Line Culture and Maintenance**

The AMN3 cell line was cultured and maintained under standard conditions suitable for mammary adenocarcinoma cells (8). The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. They were incubated at 37°C in a humidified atmosphere containing 5% CO2. The medium was changed every 2-3 days, and the cells were passaged when they reached approximately 80% confluence.

**Newcastle Disease virus Propagation**

Virus samples were obtained from the liver, lung, and trachea of suspected birds and supplied by the Poultry Department, College of Veterinary Medicine, Baghdad University. These samples were transported to the laboratory in a cooled container, where they were cut, crushed, and mixed with a measured amount of PBS containing a ten-fold concentration of antibiotics. The mixture was then centrifuged at 3000 rpm for 30 minutes. The supernatant (0.1 ml) was injected into the allantoic fluid of 10-day embryonated chicken eggs. The eggs were monitored daily for embryo mortality. Upon embryo death, the eggs were stored at 4°C for 12-24 hours. The allantoic fluid was then collected using a sterile syringe, purified by centrifugation at 3000 rpm for 30 minutes at 4°C to remove debris, and stored in small tubes at -20°C.

For virus purification, the collected virus was ultracentrifuged at 50000 x g for 60 minutes at 4°C using a Sorval ultracentrifuge (USA). The resulting sediment was resuspended in PBS and further purified using a density gradient cushion with 35% sucrose (BDH, England) at 97000 x g for 60 minutes at 4°C. This purification step was performed twice. The purified virus was resuspended in PBS and stored at -196°C (9).

The NDV was quantified using a hemagglutination assay. A 0.05 ml amount of a 5% RBC solution was added to each well of a microplate, shaken gently, and observed at room temperature until hemagglutination occurred. The result was recorded, with one hemagglutination unit (HAU) defined as the smallest virus concentration that led to visible chicken erythrocyte agglutination.

**MTT Assay**

The MTT assay was performed using the AMN3 cell line. Methyl thiazolyl tetrazolium (0.2g) (Sigma-Aldrich, USA) was dissolved in 100 ml of PBS to prepare a 2 mg/ml concentration of the dye. The solution was filtered through a 0.2 µm syringe filter to remove any blue formazan product and then stored in sterile, dark, screw-capped bottles at 4°C. The solution was used within no longer than 2 weeks of preparation.

**In Vitro study for the Combination Therapy of NDV and Chemotherapy on AMN3 Cells**

The combination therapy in vitro for NDV (Newcastle Disease Virus) and chemotherapy on AMN3 cells involved the use of various chemotherapeutic agents were studied using MTT viability assay. All chemotherapeutic agents were diluted with medium without calf bovine serum just before use for in vitro studies. The following drugs were used throughout this study: Cyclophosphamide 1g (Baxter Oncology GmbH, Germany), Etoposide 5ml vial contains 100mg (MEHECO, China), Mitomycin-C 10mg (Kyowa, Japan), Vindesine Sulfate 5mg (LILLY France), Methotrexate 10mg (Ebewe, Austria), and Cisplatin 50mg (Laboratorios Filaxis, Argentina).

**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 was detected by fluorescence microscopy using a band-pass filter (detects FITC and rhodamine). AMN3 cells were exposed for 24hrs to 1 dose of each chemotherapy tested (2µg/ml) and NDV at 1 HAU (256) and combination treatment which was done on fixed ratio (2:256) to induce apoptosis. Repeated measures multivariate ANOVA was used to demonstrate statically significant differences between groups. Apoptosis induced in cells by infection with NDV and or chemotherapeutic drugs, and a control culture was incubated without induction. AMN3 cells were cultured as described previously in tissue culture slides (labtech – Denmark). After confluency (18-24 hrs) they were treated as described and treated cells further incubated in 37C for another 24hrs. Prior to use: 1µl MitoCapture diluted to 1 ml pre-warmed Incubation Buffer for each assay. 0.4 ml of the diluted MitoCapture solution was added to cultured cells and incubated at 37C° for 15-20 min. Cells washed with pre-warmed Incubation Buffer three times and 0.4 ml of the pre-warmed Incubation Buffer was added. 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 mitochondria, it remains as monomers in the cytoplasm and fluoresces green.

**Study the interaction between NDV replication and chemotherapeutic agents in infected cells:**

Tissue culture supernatant was collected from the previous cytotoxicity experiment and tested for the presence of NDV by using Antigen capture sandwich ELISA (Tropbio, Australia), for the detection of Newcastle disease virus antigens. This assay is particularly useful for monitoring the presence of Newcastle disease viral antigens in fluids. Depending on the isolation of virus the sensitivity of the assay may be in excess of 100 times that observed with hemagglutination. The Kit contains reactive Antigen - a mixture of three inactivated, NDV antigens (V4, APl and 3245). Non-Reactive Antigen-allantoic fluid (AF) from uninfected SPF eggs. Monoclonal antibodies are a mixture of three NDV monoclonal antibod-

ies. The plates were read at the single wavelength at 405 nm. Samples recording an OD reading of greater than 0.5 test positive for the NDV group antigen. Samples recording an OD reading of less than 0.2 test negative.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism version 6.07 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± standard error of the mean (SEM). One-way ANOVA (Analysis of Variance) was used to determine the statistical significance of differences among multiple groups. Following ANOVA, post hoc comparisons were conducted using Tukey's multiple comparison test to identify significant differences between specific groups. A p-value of less than 0.05 was considered statistically significant.

## Results

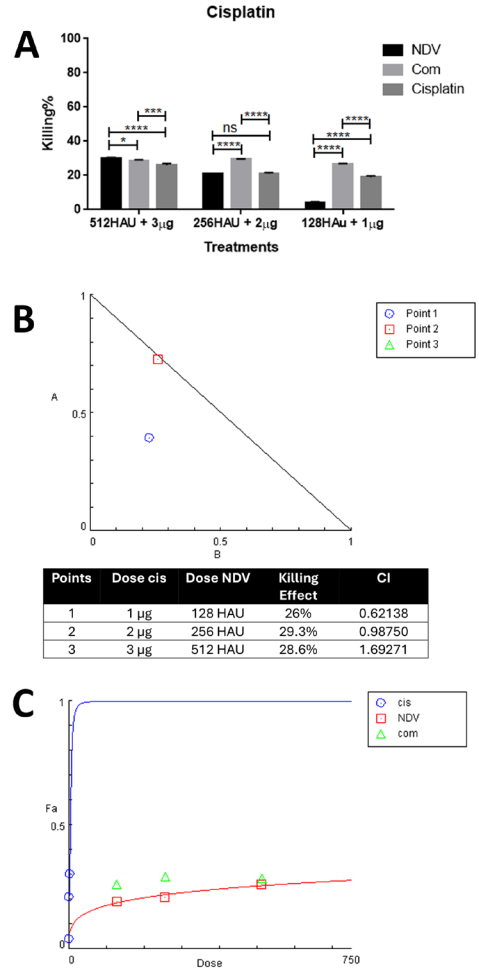
**Study the synergistic effect of NDV and chemotherapy by combination cytotoxicity assay:**

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 AMN3 mouse mammary adenocarcinoma cell line. Cells were treated with NDV, chemotherapy alone or with combination of NDV and chemotherapy, and the cell viability was determined after 72 h by MTT assay by Isobologram analysis.

**Cisplatin and NDV**

Results demonstrated that the combination of NDV and Cisplatin exhibited a more significant killing effect compared to either NDV or Cisplatin (cis) alone. The killing effect of NDV at 512HAU is higher significantly than the combination of cis and NDV at 3 µg and 512HAU (Figure-1A). Isobologram analysis showed synergism at both low doses (2 µg + 256HAU and 1 µg + 128 HAU), while at high dose of 3 µg and 512HAU showed antagonism between NDV and Cis at 50% growth inhibition dose (Figure-1B).

The dose-response curve graph in Figure-C illustrates the relationship between the dose and the fraction affected (Fa) for cis, NDV, and com. The "cis" group curve exhibits a steep increase in the fraction affected (Fa) with an increasing dose of cisplatin, reaching a maximum Fa of 1 at a relatively low dose of 3 µg/ml. This demonstrates a potent cytotoxic effect of cisplatin on cultured cancer cells at these doses. The curve for the "NDV" group shows a gradual increase in the fraction affected (Fa) with increasing doses of NDV. However, it does not reach the maximum Fa of 1 within the dose range (up to 512 HAU), suggesting a less potent effect compared to the cisplatin treatment when considering their respective measurement units. The combination treatment of NDV and cisplatin at similar doses used alone, is generally shown to induce a higher fraction affected (Fa) compared to each therapy alone at the corresponding doses. This suggests a synergistic effect as shown by Isobologram analysis where the cytotoxic impact of the combined NDV and cisplatin exceeds the effect observed when each is used individually. Overall, the data indicate that the combination treatment is more potent, achieving a higher killing effect on cancer cells at combined doses compared to either NDV or cisplatin alone. The synergy between NDV and cisplatin enhances the oncolytic and chemotherapeutic efficacy, making the combination therapy a more effective approach for inducing the desired biological response in esophageal carcinoma cells.



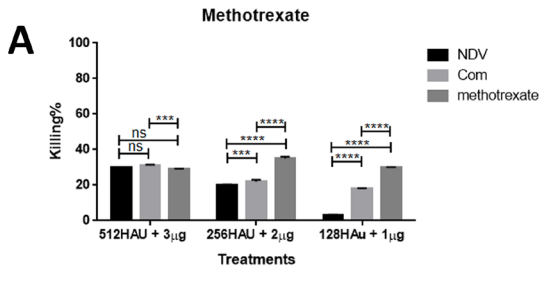

| Points | Dose cis | Dose NDV | Killing Effect | CI |
|---|---|---|---|---|
| 1 | 1 µg | 128 HAU | 26% | 0.62138 |
| 2 | 2 µg | 256 HAU | 29.3% | 0.98750 |
| 3 | 3 µg | 512 HAU | 28.6% | 1.69271 |

**Figure-1:** The combination of Newcastle disease virus (NDV) and cisplatin (cis) demonstrated a more significant killing effect on mouse mammary adenocarcinoma cells compared to either agent alone. A) Combination of cisplatin and NDV exhibited a higher killing effect than each monotreatment alone specially at the lower doses. B) Isobologram analysis showed synergism at lower doses (2 µg + 256 HAU and 1 µg + 128 HAU), whereas antagonistic effects were observed at a higher dose of 3 µg and 512 HAU. C) The dose-response curve illustrated the relationship between dose and fraction affected (Fa) for cisplatin, NDV, and their combination. Cisplatin alone showed a potent cytotoxic effect with a steep increase in Fa, reaching 1 at 3 µg/ml. NDV alone resulted in a gradual increase in Fa, not reaching 1 within the dose range tested. The combination treatment achieved a higher fraction affected compared to individual therapies, indicating synergistic effects enhancing the overall oncolytic and chemotherapeutic efficacy, making the combination therapy more effective in killing cancer cells.

**Methotrexate and NDV**

Treatments revealed statistically significant differences between Methotrexate (MTX) and NDV, and combination at lower doses of MTX at 1 and 2 µg. While at higher combination doses of 3 µg and 512HAU were significantly higher than MTX (Figure-2A). An Isobologram analysis showed antagonism between NDV and MTX combination at higher dose, however, its showing synergism but in reducing the MTX killing effect (Figure-2B).

The dose-response curve for the combination of Newcastle Disease Virus (NDV) and methotrexate (MTX) against the AMN3 mouse mammary adenocarcinoma cell line (Figure-2C). The MTX alone curve demonstrates a significant cytotoxic effect with a nearly linear increase in Fa as the dose increases, reaching a maximum Fa at a relatively low dose of 3 µg/ml, indicating potent efficacy in reducing cell viability. The NDV treatment curve shows a gradual dose-dependent increase in Fa, reaching a peak without hitting the maximum effect (Fa of 1) within the tested dose range, suggesting lower potency compared to MTX when examined independently. The combined treatment curve indicates an enhanced cytotoxic effect at higher doses, specifically at 3 µg MTX + 512 HAU NDV, where Fa is higher than either agent alone. This points to a synergistic interaction where the combined agents are more effective at higher doses. However, at lower doses, such as 1 µg MTX + 128 HAU NDV and 2 µg MTX + 256 HAU NDV, the anticipated synergistic effect is less evident as MTX alone shows a higher Fa. Overall, these observations highlight a dose-dependent synergy between NDV and MTX at higher concentrations, enhancing their combined cytotoxic impact on AMN3 cells. In contrast, the lower dose combinations may exhibit antagonistic effects, not maintaining the expected synergistic interaction in vitro.




| Points | Dose MTX | Dose NDV | Killing Effect | CI |
|---|---|---|---|---|
| 1 | 3 µg | 512 | 31% | 1.16383 |
| 2 | 2 µg | 256 | 22% | 0.71447 |
| 3 | 1 µg | 128 | 18% | 0.40772 |

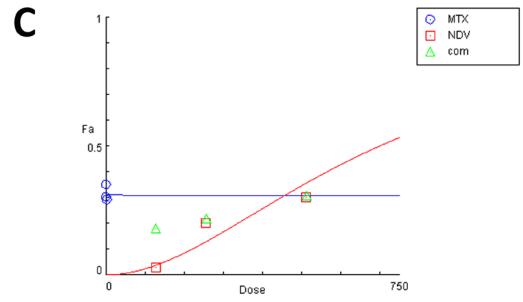
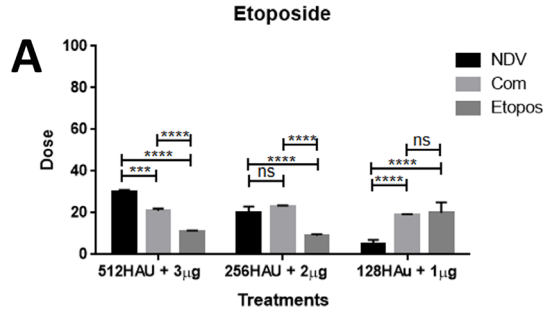


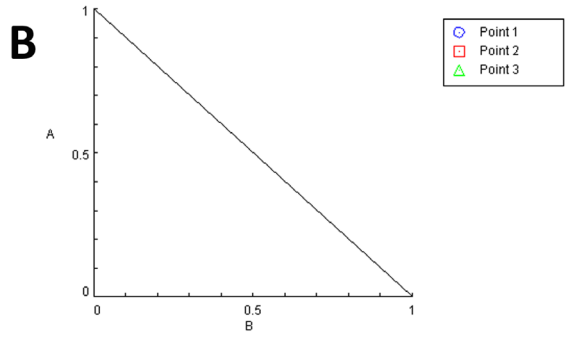

**Figure-2:** The combination of Newcastle disease virus (NDV) and Methotrexate (MTX) against mouse mammary adenocarcinoma cells compared to either agent alone. A) Results indicated statistically significant differences between Methotrexate (MTX) and NDV, with combination treatments at lower doses of MTX (1 and 2 µg). Higher combination doses (3 µg MTX and 512 HAU NDV) were significantly more effective than MTX alone. B) Isobologram analysis showed antagonism at higher doses between NDV and MTX, but synergy in reducing MTX's killing effect. C) Dose-response curve for the combination of NDV and MTX against the AMN3 mouse mammary adenocarcinoma cell line. MTX alone demonstrated a significant cytotoxic effect with a nearly linear increase in Fa, reaching a maximum effect at 3 µg/ml. NDV alone showed a gradual dose-dependent increase in Fa but did not reach maximum effect within the tested dose range. The combined treatment exhibited enhanced cytotoxicity at higher doses (3 µg MTX + 512 HAU NDV), suggesting a synergistic interaction, while lower dose combinations (1 µg MTX + 128 HAU NDV and 2 µg MTX + 256 HAU NDV) showed less evident synergy and potential antagonism.

**Etoposide**

There were statistically significant differences between the combination of Etoposide (Eto) and NDV, at 2 µg + 256 HAU , and Eto alone and the NDV. While the other doses not showing effective increase in cancer cells killing (Figure-3A). Isobologram analysis demonstrated antagonism between the combination of NDV and Eto at a 50% growth inhibition dose (Figure 3B). The dose-response curve showed that NDV alone curve exhibits a dose-dependent increase in Fa, reaching a peak effect without hitting the maximum Fa (expected at Fa=1), indicating a moderate cytotoxic impact. While the etoposide alone demonstrates a relatively flat response, which implies that its cytotoxic effect is less potent within the tested dose range compared to NDV. The combination - NDV + Etoposide treatment curve shows a more pronounced dose-dependent cytotoxic effect, particularly noticeable at the higher doses (e.g., 3 µg etoposide + 512 HAU NDV), suggesting a potential interaction where the combined therapy yields a higher Fa than individual treatments alone. At lower doses, such as (1 µg etoposide + 128 HAU NDV and 2 µg etoposide + 256 HAU NDV), the combination exhibited a higher Fa than NDV or etoposide alone, but not significantly different, indicating either additive or slightly enhancement effects. Overall, these observations suggest that the combination of

NDV and etoposide results in an enhanced cytotoxic effect on the AMN3 cell line, particularly at 2 µg etoposide + 256 HAU NDV doses, which may indicate beneficial therapeutic interactions in vitro.




| Points | Dose NDV | Dose Etopos | Killing Effect | CI |
|---|---|---|---|---|
| 1 | 512 | 3 | 21% | 5.37805 |
| 2 | 256 | 2 | 23% | 3.72587 |
| 3 | 128 | 1 | 19% | 1.49258 |

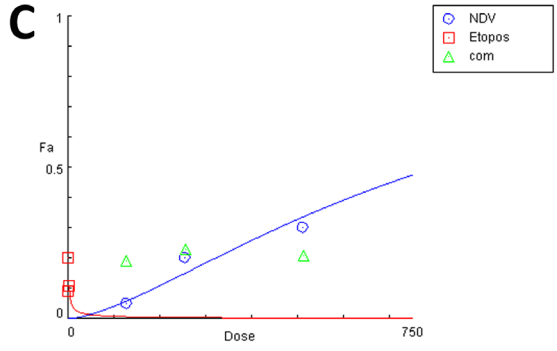



**Figure 3,** The cytotoxic effects of methotrexate (MTX) and Newcastle Disease Virus (NDV) and their combination, on the AMN3 mouse mammary adenocarcinoma cell line, highlighting dose-dependent interactions and synergistic potential. A) Statistically significant differences were observed between the combination of Etoposide (Eto) and NDV at 2 µg + 256 HAU and Eto or NDV alone, while other doses did not show a significant increase in cancer cell killing effect. **B**) Isobologram analysis demonstrated antagonistic effects between the combination of NDV and Eto at a 50% growth inhibition dose. **C**) The dose-response curve showed that NDV alone exhibited a dose-dependent increase in Fa, reaching a peak effect without hitting the maximum Fa (expected at Fa=1), indicating a moderate cytotoxic impact. Etoposide alone showed a relatively flat response, implying a less potent cytotoxic effect compared to NDV. The combination of NDV and Etoposide exhibited a more pronounced dose-dependent cytotoxic effect, especially at higher doses (3 µg etoposide + 512 HAU NDV), suggesting a potential synergistic interaction where combined therapy is more effective than individual treatments. At lower doses, such as 1 µg etoposide + 128 HAU NDV and 2 µg etoposide + 256 HAU NDV, the combination showed a higher Fa than NDV or etoposide alone, but the difference was not statistically significant. Overall, these observations highlight the enhanced cytotoxic effect of NDV and Etoposide combination on AMN3 cells, particularly at the higher dose combinations, indicating potential therapeutic benefits in vitro.

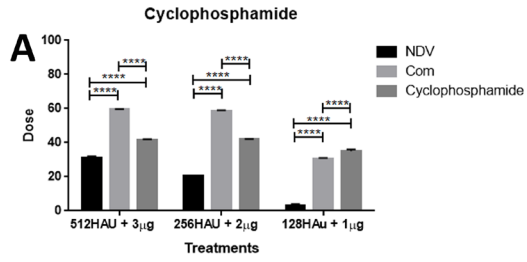
**Mitomycin C**

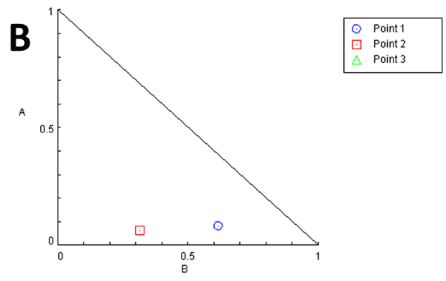
Statistically insignificant differences were observed between Mitomycin C (mmc) and control, mmc and NDV, virus and control, virus and combination, mmc and combination, and combination and control. Isobologram analyses revealed antagonism between NDV and mmc at 50% growth inhibition doses.

Here, we evaluated the combined cytotoxic effects of oncolytic Newcastle Disease Virus (NDV) and Mitomycin C on mouse mammary adenocarcinoma cells using different analytical assays. The cytotoxicity of the combination treatment was assessed at various doses, showing that the combined treatment significantly increased cell death compared to individual treatments. Notably, higher doses such as 512 HAU NDV + 3 µg Mitomycin C, 256 HAU NDV + 2 µg Mitomycin C, and 128 HAU NDV + 1 µg Mitomycin C, exhibited a more pronounced cytotoxic effect, indicating the enhanced effectiveness of the combination therapy in reducing cell viability (Figure-4A). The isobologram analysis further elucidated the nature of the interaction between NDV and Mitomycin C. At lower doses (128 HAU NDV + 1 µg Mitomycin C), the combination demonstrated a synergistic interaction (Combination Index CI<1), suggesting that the drugs work better together than individually. However, at higher doses (256 HAU NDV + 2 µg Mitomycin C and 512 HAU NDV + 3 µg Mitomycin C), the analysis revealed additive or slightly antagonistic effects, implying that the benefits of the combination might not be as pronounced at higher concentrations (Figure-4B).

The dose-response curve (Figure-4C) illustrates the relationship between the dose and the fraction affected (Fa) for NDV, Mitomycin C, and their combination. NDV alone exhibited a dose-dependent increase in Fa, reaching a peak effect without hitting the maximum (Fa=1), suggesting moderate cytotoxic impact. Mitomycin C alone showed a relatively flat response, indicating a less potent cytotoxic effect within the tested dose range compared to NDV. The combination treatment, however, showed a more pronounced dose-dependent cytotoxic effect, particularly at the higher doses (e.g., 3 µg Mitomycin C + 512 HAU NDV), suggesting a potential enhanced interaction where the combined agents yield a higher Fa than individual treatments

alone. Even at lower doses (1 µg Mitomycin C + 128 HAU NDV and 2 µg Mitomycin C + 256 HAU NDV), the combination exhibited a higher Fa than NDV or Mitomycin C alone, indicating a possible additive or slightly synergistic effect. Overall, these observations highlight the enhanced cytotoxic effect of the combination of NDV and Mitomycin C on mouse mammary adenocarcinoma cells, particularly at lower doses. The synergy between NDV and Mitomycin C enhances their combined therapeutic efficacy, indicating potential benefits of using this combinatorial approach in breast cancer treatment.




| Points | Dose CP | Dose NDV | Effect | CI |
|---|---|---|---|---|
| 1 | 3 µg | 512 | 59.5% | 0.70088 |
| 2 | 2 µg | 256 | 58.6% | 0.37802 |
| 3 | 1 µg | 128 | 30.5% | 2.66881 |

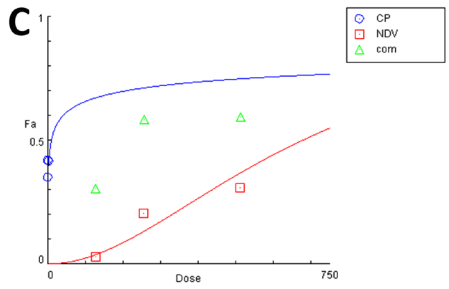
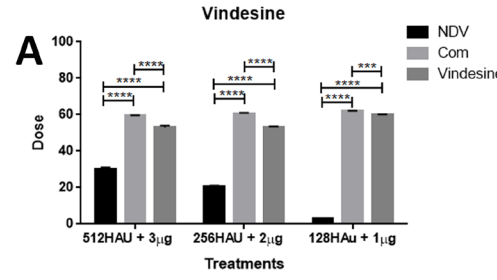


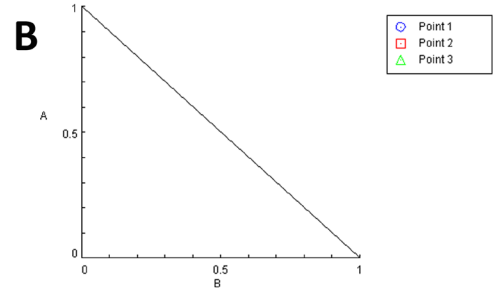

**Figure-4,** demonstrates the combined effects of oncolytic Newcastle Disease Virus (NDV) and Mitomycin C on mouse mammary adenocarcinoma cells through different experimental analyses. A) The cytotoxicity assay reveals that the combination treatment significantly increased cell death compared to individual treatments, especially at 128 HAU + 1 µg doses. B) The isobologram analysis indicates a synergistic interaction at lower doses (CI<1), particularly at 128 HAU NDV + 1 µg Mitomycin C, while higher doses show additive or slightly antagonistic effects. C) The dose-response curve depicts a higher fraction affected (Fa) for the combination compared to NDV or Mitomycin C alone, suggesting enhanced cytotoxic efficacy of the combinatorial therapy.

**Vindesine**

The results of combined cytotoxic effects of oncolytic Newcastle Disease Virus (NDV) and Vindesine against mouse mammary adenocarcinoma cells, showing that combination treatments at higher doses (512 HAU NDV + 3 µg Vindesine, 256 HAU NDV + 2 µg Vindesine, and 128 HAU NDV + 1 µg Vindesine) demonstrated a significantly higher cell killing effect compared to individual treatments, as indicated by the statistical significance (for $p<0.0001$, for $p<0.001$). This result highlights the enhanced efficacy of combination therapy in reducing cell viability (Figure-5A). This result shows the improved effectiveness of the combination therapy in reducing cell viability. The isobologram analysis revealed antagonistic or non-synergistic effects, as shown by the CI values greater than 1. Here, the combined drugs' effects did not exceed their individual impacts (Figure-5B).

The dose-response curve (Figure-4C) illustrates the relationship between the dose and the fraction affected (Fa) for Vindesine, NDV, and their combination. Vindesine alone showed a relatively flat dose-response relationship, indicating steady cytotoxic effect within the tested dose range. NDV alone exhibited a dose-dependent increase in Fa, reaching a peak effect but not attaining the maximum effect (Fa of 1), suggesting moderate potency. The combination treatment demonstrated a more pronounced dose-dependent cytotoxic effect, especially at higher doses (e.g., 3 µg Vindesine + 512 HAU NDV), suggesting a enhanced interaction as the combined therapy yielded a higher Fa than individual treatments alone. Even at lower doses (1 µg Vindesine + 128 HAU NDV and 2 µg Vindesine + 256 HAU NDV), the combination exhibited a higher Fa compared to NDV or Vindesine alone, although the difference was not statistically significant, indicating additive or slightly enhanced effects.




| Points | Dose VDS | Dose NDV | Killing Effect | CI |
|---|---|---|---|---|
| 1 | 3 µg | 512.0 | 59.5% | 3.70082 |
| 2 | 2 µg | 256.0 | 60.6% | 2.72950 |
| 3 | 1 µg | 128.0 | 62% | 1.64716 |

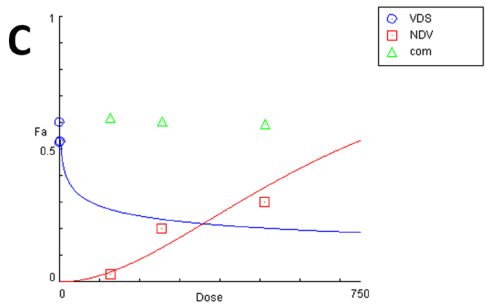


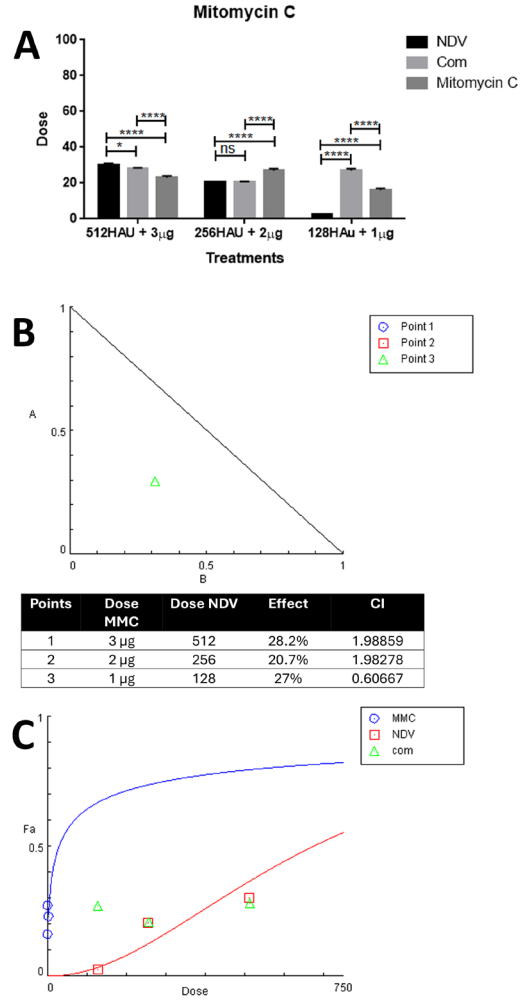

**Figure-5,** This figure illustrates the combined cytotoxic effects of Newcastle Disease Virus (NDV) and Vindesine (VDS) on mouse mammary adenocarcinoma cells. A) Cytotoxicity assay demonstrated that the combination of NDV and Vindesine at all doses (512 HAU NDV + 3 µg Vindesine, 256 HAU NDV + 2 µg Vindesine, and 128 HAU NDV + 1 µg Vindesine) resulted in a significantly higher cell killing effect compared to individual treatments, indicated by statistical significance (**** for p<0.0001, *** for p<0.001). B) Isobologram analysis indicated non-synergistic effects (CI>1). C) Dose-response curve illustrated that Vindesine alone showed a flat dose-response, NDV alone exhibited a dose-dependent increase in Fa but did not reach maximum Fa, and their combination demonstrated a more pronounced cytotoxic effect, especially at higher doses, suggesting potential synergistic interaction. Lower dose combinations showed additive or slightly enhanced effects but were not significantly different from individual treatments.

## Cyclophosphamide

The cytotoxicity assay was performed to evaluate the effectiveness of different treatments: NDV alone, Cyclophosphamide (CP) alone, and their combination (Figure-6A). At higher doses (512 HAU NDV + 3 µg CP, 256 HAU NDV + 2 µg CP, and 128 HAU NDV + 1 µg CP), the combination treatments showed a statistically significant increase in cell killing effect compared to each treatment alone. This significant increase is indicated by asterisks (**** for p<0.0001, *** for p<0.001), highlighting the enhanced cytotoxic efficacy of the combination therapy.

The isobologram analysis (Figure-6B) was conducted to understand the interaction between NDV and CP. The analysis indicated a synergistic interaction at lower doses, specifically at 1 µg CP + 128 HAU NDV (Combination Index CI=0.70088 and CI=0.37802), suggesting that the combinations work better together than individually. In contrast, at higher doses, such as 3 µg CP + 512 HAU NDV, the analysis revealed non-synergistic or antagonistic effects (CI=2.66881), implying that the combination might not be as effective at high concentrations.

The dose-response curve results (Figure-6C) describes the relationship between the dose and the fraction affected (Fa) for NDV, CP, and their combination. The NDV treatment alone exhibited a dose-dependent increase in Fa, reaching a peak effect without achieving the maximum Fa (expected at Fa=1), indicating moderate cytotoxic impact. CP alone showed a relatively flat dose-response curve, indicating its lower cytotoxic effect within the tested dose range. The combination treatment demonstrated a more pronounced dose-dependent cytotoxic effect, particularly at higher doses (e.g., 3 µg CP + 512 HAU NDV), indicating potential synergistic interaction where the combined therapy was more effective than individual treatments alone. At lower doses (1 µg CP + 128 HAU NDV and 2 µg CP + 256 HAU NDV), the combination exhibited a higher Fa than NDV or CP alone but exhibited either additive or slightly synergistic effects, not significantly different from individual treatments.




| Points | Dose MMC | Dose NDV | Effect | CI |
|---|---|---|---|---|
| 1 | 3 µg | 512 | 28.2% | 1.98859 |
| 2 | 2 µg | 256 | 20.7% | 1.98278 |
| 3 | 1 µg | 128 | 27% | 0.60667 |

**Figure 6:** illustrates the combined cytotoxic effects of Newcastle Disease Virus (NDV) and Cyclophosphamide (CP) on mouse mammary adenocarcinoma cells. A) Cytotoxicity assay showed that the combination treatments at higher doses (512 HAU NDV + 3 µg CP, 256 HAU NDV + 2 µg CP, and 128 HAU NDV + 1 µg CP) resulted in a significantly higher cell killing effect compared to NDV or CP alone, denoted by statistical significance (**** for p<0.0001, *** for p<0.001). B) Isobologram analysis indicated synergistic interaction at lower doses (1 µg CP + 128 HAU NDV

with CI=0.70088 and CI=0.37802), while higher doses (3 µg CP + 512 HAU NDV with CI=2.66881) exhibited non-synergistic effects. C) Dose-response curve demonstrated NDV alone exhibited a dose-dependent increase in Fa without reaching maximum Fa, and CP alone had a relatively flat response. The combination showed a more pronounced dose-dependent cytotoxic effect, suggesting potential synergistic interaction.

**Mitochondrial Permeability Transition Apoptosis Test**

Mitochondrial Permeability Transition is an early event of apoptosis, so considered at good indicator for apoptosis induction by the various treatments, including Newcastle Disease Virus (NDV) and several chemotherapeutic agents (Cisplatin (Cis), Methotrexate (MTX), Etoposide (ETO), Mitomycin C (MMC), Vindesine (VDS), and Cyclophosphamide (CPA)), as well as their combinations with NDV. The control group exhibited a baseline apoptosis percentage of 53.3%. Treatment with NDV alone resulted in a higher apoptosis rate of 66.7% (normalized to 125.1% of control), indicating a notable cytotoxic impact. Cisplatin alone induced apoptosis in 68.3% of cells (128.2% of control), while MTX alone led to apoptosis in 66.7% of cells (125.0% of control). Etoposide alone caused apoptosis in 62.0% of cells (115.7% of control), and MMC alone resulted in a 68.3% apoptosis rate (128.2% of control). Vindesine treatment showed a 73.3% apoptosis rate (137.5% of control), while CPA alone caused apoptosis in 66.7% of cells (125.1% of control).

Combination treatments showed significant enhancements in apoptosis rates. The combination of Cisplatin and NDV produced an increased apoptosis rate of 71.7% (134.5% of control), indicating a synergistic effect. The combination of MTX and NDV markedly enhanced apoptosis to 86.7% (162.7% of control), demonstrating significant synergy. Etoposide and NDV combination resulted in a slight increase in apoptosis to 62.3% (116.3% of control), indicating a modest improvement over Etoposide alone. The combination of MMC and NDV maintained the apoptosis rate at 76.7% (143.9% of control), without a further increase compared to MMC alone. Vindesine and NDV treatment showed a substantial increase in apoptosis to 86.7% (162.7% of control), suggesting a strong synergistic effect. The combination of CPA and NDV resulted in a significant increase in apoptosis to 86.7% (162.7% of control), indicating a potent synergistic interaction. The combination of NDV with chemotherapeutic agents such as Cisplatin, MTX, Vindesine, and CPA significantly enhanced apoptosis in AMN3 cells (Figure-7A).

**Study the interaction between NDV replication and chemotherapeutic agents in infected cells:**

The results presented in Figure 7B illustrate the impact of various chemotherapeutic agents on Newcastle Disease Virus (NDV) replication in AMN3 cells. NDV replication was assessed in cell culture supernatants collected at 24- and 48-hour post-infection, utilizing an antigen capture ELISA to quantify viral antigen levels. The threshold for a positive result was set at an optical density (OD) reading of 0.2, according to the manufacturer's guidelines.

The Figure 7B compares NDV replication across different treatment conditions, including NDV alone and NDV in combination with chemotherapeutic agents: cisplatin (CIS), cyclophosphamide (CPA), mitomycin C (MMC), methotrexate (MTX), vindesine (VDS), and etoposide (Etopos). It compares viral replication at 24 hours post-infection, in corresponding to 48-hour post-infection measurements.

Overall, the results indicate that there were no significant differences in NDV replication between treated and untreated conditions. This suggests that none of the tested chemotherapeutic agents exerted a notable inhibitory or enhancing effect on NDV replication within the tested timeframe. The statistical analysis confirms this observation, as indicated by the absence of significant differences between groups, except for a statistically significant overall increase in NDV replication at 48 hours compared to 24 hours, as denoted by the "***" symbol above the bars. These findings suggest that NDV replication remains stable regardless of the presence of these chemotherapeutic agents, highlighting its potential as an oncolytic virus that retains its replication ability even in chemotherapy-treated environments. Further studies may be needed to explore the biological implications and potential therapeutic synergy between NDV and chemotherapeutic agents.

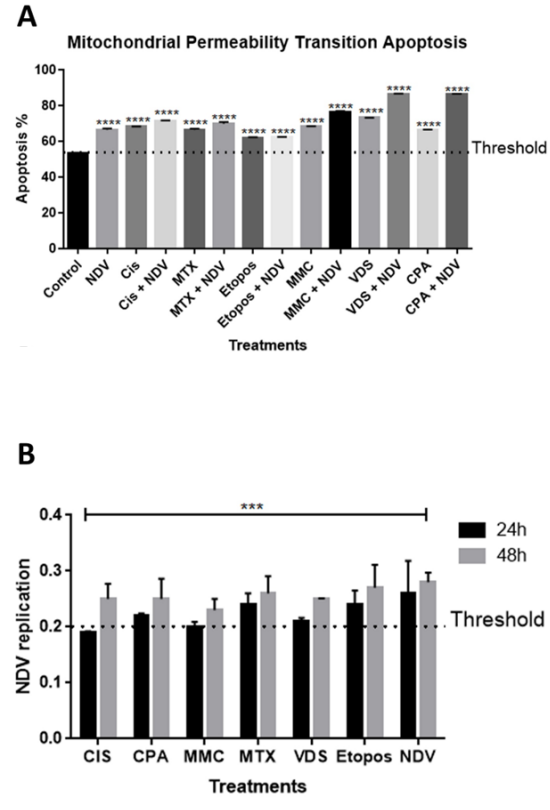



**Figure-7,** A) Mitochondrial Permeability Transition, an early marker of apoptosis, was assessed to evaluate apoptosis induction by Newcastle Disease Virus (NDV) and various chemotherapeutic agents (Cisplatin (Cis), Methotrexate (MTX), Etoposide (ETO), Mitomycin C (MMC), Vindesine (VDS), and

Cyclophosphamide (CPA), as well as their combinations with NDV. The control group showed a baseline apoptosis of 53.3%, while NDV alone increased apoptosis to 66.7% (125.1% of control). Chemotherapeutic agents alone induced varying levels of apoptosis, with Vindesine (73.3%) showing the highest effect. Combination treatments significantly enhanced apoptosis, particularly NDV with MTX, VDS, and CPA (86.7%, 162.7% of control), indicating strong synergistic effects. These findings suggest that NDV enhances chemotherapy-induced apoptosis in AMN3 cells.

B) illustrate the impact of various chemotherapeutic agents on Newcastle Disease Virus (NDV) replication in AMN3 cells. The black bars represent viral replication at 24 hours post-infection, while the gray bars correspond to 48-hour post-infection measurements. Utilizing an antigen capture ELISA to quantify viral antigen levels. The threshold for a positive result was set at an optical density (OD) reading of 0.2. The bar graph compares NDV replication across different treatment conditions, including NDV alone and NDV in combination with chemotherapeutic agents: cisplatin (CIS), cyclophosphamide (CPA), mitomycin C (MMC), methotrexate (MTX), vindesine (VDS), and etoposide (Etopos). Overall, the results indicate that there were no significant differences in NDV replication between treated and untreated conditions. This suggests that none of the tested chemotherapeutic agents exerted a notable inhibitory or enhancing effect on NDV replication within the tested timeframe. The statistical analysis confirms this observation, as indicated by the absence of significant differences between groups, except for a statistically significant overall increase in NDV replication at 48 hours compared to 24 hours, as denoted by the "***" symbol above the bars.

## Discussion

To investigate the potential interaction between Newcastle Disease Virus (NDV) and chemotherapy, a combination cytotoxicity assay was performed using the AMN3 mouse mammary adenocarcinoma cell line. The study assessed the effects of NDV and six chemotherapy agents—Cisplatin, Methotrexate, Etoposide, Mitomycin C, Vindesine, and Cyclophosphamide—alone and in combination at varying concentrations. Cytotoxicity was evaluated using MTT assay and analyzed through isobologram and dose-response curve analysis. To assess synergy the Isobologram method was used. This type of analysis determines synergy, and it is an acceptable method of assessing the efficacy of chemotherapy combination therapies as well as combination chemotherapy and viral therapy (10).

Results demonstrated that NDV enhances the cytotoxic effects of certain chemotherapy agents in a dose-dependent manner. The combination of NDV and Cisplatin exhibited significant synergy at lower doses, whereas higher doses showed antagonism. Methotrexate and NDV displayed synergistic effects at specific concentrations, although some combinations reduced the killing effect of MTX. Etoposide combined with NDV showed moderate cytotoxic enhancement, particularly at intermediate doses, but overall exhibited antagonistic interactions. Mitomycin C and NDV demonstrated synergism at lower doses, with higher doses resulting in additive or antagonistic effects. Vindesine and NDV showed increased cytotoxicity at higher doses but lacked significant synergy. Cyclophosphamide and NDV exhibited a strong synergistic effect at lower doses, while higher doses led to antagonism. Overall, the findings highlight the potential of NDV to enhance chemotherapy efficacy, particularly at optimized dose combinations. These results provide valuable insights into the application of NDV as an oncolytic agent in combination cancer therapies.

NDV was reported to work well in combination with many other chemotherapeutic agents such as temozolomide against resistant glioblastoma cells (11). Although it is found that oncolytic activity of Newcastle disease virus well enhanced through combination with retinoic acid against group of digestive system tumors (12). Furthermore, 5-Fluorouracil enhance NDV cytotoxicity against panel of tumor cells as well as mouse mammary adenocarcinoma (13). In another study by our team, we found that NDV synergized with doxorubicin and rituximab, enhancing their cytotoxic effects. The synergy was p53-independent in plasmacytoma cells but involved p53 activation in non-Hodgkin lymphoma (14).

To further study the possible mechanism of combined antitumor activity, we studied the ability for apoptosis induction. detection of the mitochondrial permeability transition event (PT) provides an early indication of the initiation of cellular apoptosis. This process is 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 pro-apoptotic 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 (15). The results of the present experiment suggested that all combination treartments caused mitochondrial permeability transition event (PT) higher than each treatment alone, which lead finally to apoptosis. Most chemotherapeutic agents induce apoptosis in cancer cells in different mechanisms by inducing release of cytochrome c from mitochondria (16). The results showed that NDV alone induce apoptosis in AMN3 cells by induction the mitochondrial permeability transition event (PT) which is an early event of apoptosis led to a drop of ($\Delta\Psi$m), Following a drop of ($\Delta\Psi$m), cytochrome c can be released from mitochondria through the opened mitochondrial pores(17). Elankumaran et al. 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 (18).

Fabian et al. suggest that NDV-induced apoptosis involves inactivation of eIF2α and activation of caspase-12 and caspase-3 but is independent of caspase-8 and caspase-9. In order to determine the apoptotic pathways involved in NDV-induced apoptosis, they observed Strong accumulation of cleaved caspase-3 after 10 h of incubation in NDV-infected PC12 cells, but proteolytic activation of the initiator caspases caspase-8 and caspase-9 were not detected before the activation of effector caspase-3. In contrast, activation and nuclear translocation of the endoplasmic reticulum enzyme

caspase-12 correlated well with the accumulation of cleaved caspase-3. Activation of caspase-12 suggests that NDV disturbs endoplasmic reticulum functions in virus-infected cells (19).

From in vitro combination study and apoptosis experiment finding 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. Thus, we may consider that chemo-virotherapy-induced cell death proceeds through three distinct general phases. (1) Phase I: an insult-generating mechanism. In this phase, each class of therapeutic agents interact with a specific target (DNA in case of chemotherapy and metabolic exudative stress with endoplasmic reticulum disruption in case of NDV) (20) and the action of these agents on their respective targets causes target injury or dysfunction. 2) Phase II: signal transduction. In this phase, the cell is able to decipher and assess the specific injury to the chemo-virotherapy target. DNA-damaging agent (chemotherapy) may induce expression of the mitochondrial proapoptotic factor Bax, while NDV induce ER damage which increases efflux of calcium from the ER into the mitochondria (21). (3) Phase III: induction of apoptosis. In the third and final phase, a decision point may exist such that susceptible cells react to the signals generated in response to chemotherapy-induced injury as a go-ahead for the execution phase of apoptosis, while the cells with resistance to chemotherapy may be susceptible to virotherapy-induced injury to undergo apoptosis as we described earlier, and the occurrences' of both ways of apoptosis in the same tumor cell is not excluded but moreover may explain the synergistic effect.

To confirm that combination therapy doesn't affect NDV replication we studied NDV HN antigen quantifications after 24h and 48h post infection in presence and absence of the chemotherapeutic agents. Results of viral replication demonstrated that chemotherapies do not interfere with viral replication and proliferation in AMN3 tumor cells tested. Absence of interference with virus replication supports previous results of enhancement and supports the positive interaction between chemotherapy and NDV inside tumor cells. Yu and his team suggested a hypothesis that chemotherapy at synergistic dose may be augmenting viral replication (22). Moreover, it is suggested that chemotherapy may increase expression of a gene that may increase virus tropism and replication in the tumor cell (23). One of the most novel strategies for tumor eradication is to combine chemotherapy and virotherapy, some viruses induce in vitro growth inhibition and apoptosis when used with chemotherapy (24). There is a possibility that each agent can work independently on different cell populations (25).

In conclusion, this study explored the synergistic effects of Newcastle Disease Virus (NDV) and various chemotherapeutic agents in vitro using a combination cytotoxicity assay on AMN3 mouse mammary adenocarcinoma cells. The results demonstrated variable interactions between NDV and different chemotherapy drugs, with some combinations exhibiting synergistic effects while others showed antagonism at higher doses. The mitochondrial permeability transition apoptosis test confirmed that NDV significantly enhanced apoptosis induction when combined with chemotherapy, particularly with cisplatin, methotrexate, vindesine, and cyclophosphamide, reinforcing the potential of NDV as an effective adjunct to chemotherapy. Furthermore, NDV replication remained stable in the presence of chemotherapy, indicating that these agents do not interfere with the virus's oncolytic activity.

In conclusion, these findings highlight the potential of NDV as a complementary therapy to traditional chemotherapy, particularly in carefully optimized dosing regimens. Further investigations, including in vivo studies and mechanistic analyses, are warranted to explore the clinical applicability of these findings and refine combination strategies for enhanced cancer treatment efficacy.

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Ethical Approval:** The work approved by the scientific committee of the Iraqi center for cancer and medical genetic research, Mustansiriyah university, Baghdad, Iraq.

**Author's Contribution:** All authors contributed equally to the work.

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