In Vitro Synergistic enhancement of Newcastle Disease Virus to Methotrexate cytotoxicity against tumor cells

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Abstract

Combination chemotherapy is a common treatment for cancer patient. In the recent years there is a new type of combination had been introduced to the clinical field, that by combine chemotherapy with virotherapy, Using oncolytic viruses. Newcastle disease virus is one of the most important oncolytic viruses and has been introduced to clinical field, and showed no serious side effects. In the present study, Newcastle disease virus combined with one of the most common chemotherapy used extensively in cancer patients, Methotrexate (MTX). Study results showed synergistic effect of NDV with MTX in the four cell line tested, Hep-2, RD, AMN3 and Vero cell lines. Combination therapy was always more effective than chemotherapy alone or virotherapy alone. This study suggests presence of synergistic action between MTX and NDV against tumor cells in vitro, which may give a new hope to stand against cancer.

الزيادة المتجانسة في السمية الخلوية على الخلايا السرطانية بفعل فايروس النيوكاسل بالتعاضد مع الميثوتركسيت

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الخلاصة

طورت العديد من العوامل المضادة للسرطان للتغلب عليه، والعلاج الكيميائي واحد من أكثر العوامل المضادة للسرطان استعمالا على الرغم من العوارض الجانبية الخطيرة والنتائج غير المرضية. أجري العديد من الأبحاث لزيادة فعاليته ولتقليل سميته، والميثوتركسيت واحد من العلاجات الكيميائية الواسعة الانتشار والذي مازال يستعمل بكثرة وبخليط مع الأنواع الأخرى من العلاجات الكيميائية. العلاج الفايروسي واحد من العوامل الواعدة المضادة للسرطان وذلك لسلامته وانتقائيته باتجاه الخلايا السرطانية. ثبت أن فيروس النيوكاسل آمن ويستهدف الخلايا السرطانية بانتقائيته. وقد أثبتنا أنه يمكن استخدامه لتقوية تأثير الميثوتركسيت، حيث يقلل ذلك من سميته عن طريق خفض الجرعة المعطاة إلى النصف، وتستبدل بفايروس النيوكاسل مع احتفاظها بنفس الفعل المضاد للأورام. هذه الدراسة تسحب الأنظار باتجاه جانب غير مكتشف لفايروس النيوكاسل، حيث تشير النتائج إلى أن فايروس النيوكاسل فعال بشكل خاص بالاشتراك مع العلاجات الكيميائية التقليدية.

Introduction

Methotrexate (MTX) continues to play an important role in the treatment of a variety of malignancies and is well understood with respect to its mechanism at the molecular level. Antitumor drugs such as methotrexate (MTX) are known to cause damage to the small intestine, leading to its dysfunction (1, 2). Nausea, vomiting, diarrhea, stomatitis and gastrointestinal ulceration are reported to occur after the use of this chemotherapeutic agent (3). However, its toxic dose related to side effects and lacks of selectivity limit the clinical application of this drug (4). Successful anticancer strategies require a differential response between tumor and normal tissue (*i.e.*,

therapeutic index) (5). Replication component, oncolytic viruses represent a mean of achieving a therapeutic index by selectively destroying tumor cells with minimal toxicity to normal cell (6). Oncolytic viruses can increase sensitivity of tumor cells to chemotherapy and radiotherapy. For example, adenoviruses used in combination with cisplatin (7), 5-FU and leucovrin (8), paclitaxol and cisplatin (9), 5-FU alone (10, 11), 5-FU and cisplatin (12), doxorubicin (13). Adenoviruses also used in combination with radiotherapy (14). In addition, oncolytic virus vector expressing Herpes Simplex virus-tk was used in combination with paclitaxol and carboplatin (15), and used with radiation therapy for treatment of prostate cancer (16). Herpes Simplex viruses used in combination with chemotherapy (17), oncolytic HSV combined with cyclophosphamide on glioma and gliosarcoma cells (18). HSV combined with radiation therapy also (19, 20). Newcastle disease virus is an important disease of poultry, it is a member of the order *mononegalovirales* in the family *paramyxoviridae* and has been designated an *Avulavirus*. The virus is a non-segmented, single-strand; negative-sense enveloped RNA virus (21). Many researches (22, 23) proved that NDV to be effective oncolytic virus against tumor cells, in previous study we proved the effectiveness of using the Iraqi local strain of NDV as anti-tumor agent (24) also we proved that NDV Iraqi strain in combination with MTX is of some effectiveness against tumor cell lines *in vitro* (25). Avki *et al*, (26) used LaSota strain of NDV in treatment of bovine papillomatosis. Iraqi strain of NDV also tested for treatment of bovine papillomatosis with good results (27). In the present study, we examined the efficacy of Newcastle disease virus (LaSota) in combination with methotrexate as possible anti-tumor therapy. We compared the effect of combination of NDV and methotrexate (MTX) against tumor cell lines with NDV or MTX alone. Moreover, investigate the possible synergistic effect that may enhance the potency of methotrexate antitumor effect with lowest dose available to achieve dose reduction, which may reduce the toxic effects that may combine the high dose of methotrexate without decreasing the antitumor activity.

Material and Methods

- **Experimental Design:** The primary objective of this study was to augment the effect of cancer chemotherapies (methotrexate) using virotherapy (Newcastle disease virus). In addition, another objective was to reduce toxicity of cancer chemotherapeutic agents by reducing the administered dose while still have the same effect on tumor cells by combining with virotherapy and compare the effect of this combined therapy with chemotherapeutic agent alone and virotherapy alone.
- **Cell lines and Culture:** The human Hep-2 (larynx carcinoma), and RD (Rhabdomyosarcoma), murine AMN3 (mammary adenocarcinoma) and Vero (transformed monkey kidney) cell lines were obtained from the Iraqi center for cancer and medical genetic research (ICCMGR) and maintained in RPMI 1640 (Sigma-Oldrich-Germany) supplemented with 5% calf bovine serum (ICCMGR), 100 I.U/ml penicillin, and 100 µg/ml streptomycin. While Vero cell line was maintained on MEM (Sigma-Aldrich-Germany) supplemented with 5% calf bovine serum (ICCMGR), 100 units/ml penicillin, and 100 µg/ml streptomycin.
- **Virus:** The lentogenic strain of NDV (LaSota) was obtained from Al-Kindy Company for veterinarian vaccines (Baghdad, Iraq). A stock of infectious virus was propagated in embryonated chicken eggs, harvested from allantoic fluid, purified from debris by centrifugation (3000 rpm, 30 minute, 4C°). NDV was quantified by a hemagglutination test in which one hemagglutination unit (HAU) is defined as the smallest virus concentration leading to visible chicken erythrocyte agglutination (28).

- **Chemotherapeutic agent:** Methotrexate (MTX) Pharmaceuticals (Albuquerque, NM) was purchased from radiation and atomic medicine hospital (Baghdad-Iraq) this agent was diluted with medium without calf bovine serum just before use for *invitro* studies.
- **Cell viability and cytotoxicity:** To determine the cell killing effect of NDV and MTX in combination treatment, MTT cell viability assay was conducted as on 96-well plates (Falcon), Hep-2, RD, AMN3, and Vero cells were seeded at 3-4x10^4 cells/well after 24hr or confluent monolayer is achieved. Cells were treated with virus alone (infected with NDV at 512 HAU with two fold serial dilution). Drug alone (the chemotherapeutic agent - MTX at 10 µg in two fold serial dilution reaching to 0.078µg/ml. Or in combination (virus+MTX). The procedure of adding these therapeutic agents was by adding the virus at first for 2 hrs at room temperature to allow virus attachment and penetration. After that, cells were washed with PBS and serial dilution of the drug was added on the non-infected and infected cells. Cell viability was measured after 72 hrs of infection by removing the medium, adding 28 µl of 2 mg/ml solution of MTT (Sigma Alderch-co) and incubating for 1.5 hrs at 37°C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 130 µl of DMSO (Dimethyl Sulphoxide) (BDH, England) followed by 37°C incubation for 15 min with shaking. The absorbency was determined on a microplate reader (organon Teknika Reader 230S, Austria) at 492 nm (test wavelength); the assay was performed in triplicate (29, 30). Endpoint parameters that are calculated for each individual cell line include: cell proliferation rate (PR) which is the percentage of control absorbance (31, 32). The inhibiting rate of cell growth (GI) (the percentage of cytotoxicity) was calculated as (A-B)/Ax100, Where A is the mean optical density of untreated wells and B is the optical density of treated wells (33, 34). The LC_{50}, which is the lowest concentration that kills 50% of cells was estimated according to (35).

Results

To study the potential interaction between NDV and chemotherapy *in vitro*, the effectiveness of combined treatment of several concentrations of MTX with NDV at various HAU was evaluated, in the Hep-2, RD, AMN3, and Vero cell lines. Cells were treated with NDV, MTX or with combination of NDV and MTX, and the cell viability was determined after 72 hrs by MTT assay. Enhanced cytotoxicity observed in the combination treatment of NDV and MTX, which refer to synergistic effect, the results of all cell lines tested are summarized in Table 1.

Table (1) Growth inhibition by combination of NDV+MTX compared with MTX and NDV, Cells were exposed to the indicated drug for 72 h (four cell lines); LC50 values are given as the least concentration of drug at which growth is inhibited by 50% compared with controls. LC Values are given as the least concentration of drug induces the highest growth inhibition

Cell line	Combination concentration	%of G.I	Chemotherapy concentration	%of G.I	NDV concentration	%of G.I
Hep-2	4HAU+0.0725 µg(LC50)	63.6	0.6 µg (LC50)	51.4	8HAU (LC50)	55.6
RD	256HAU+5µg(LC)	45.9	10µg(LC)	35.7	256HAU(LC)	2.3
AMN-3	64HAU+1.25µg(LC)	44	2.5µg(LC)	46	64HAU(LC)	27
Vero	64HAU+1.25µg(LC)	32.2	2.5 µg(LC)	24	64HAU(LC)	31

In Hep-2, larynx carcinoma cell line, combination treatment showed significant growth inhibition 63.6% (G.I) (P= 0.0001) at concentration of 4 HAU of NDV+0.0725 µg of MTX. Which was the least concentration inhibit 50% of cell proliferation (LC50). Whereas MTX

alone was 51.4% GI (P= 0.0001) at 0.6µg/ml (LC50), NDV alone at 8HAU/ml (LC_{50}) produced 55.6% G.I (p= 0.0001) (Fig. 1). In RD cell line, combination treatment showed 45.9% growth inhibition (G.I) (P= 0.0001) at concentration of 256 HAU of NDV+5µg of MTX (LC), whereas MTX alone was 35.7% GI (P= 0.0001) at 10µg/ml (LC), NDV alone at 256HAU/ml (LC) produced 2.3% G.I (p= 0.0001) (Fig. 2). In AMN3 cell line, combination treatment showed 44% growth inhibition (G.I) (P= 0.0001) at concentration of 64HAU of NDV+1.25µg of MTX (LC), whereas MTX alone was 46% GI (P= 0.0001) at 2.5µg/ml (LC), NDV alone at 64HAU/ml (LC) produced 27% G.I (p= 0.0001) (Fig. 3). In Vero cell line, combination treatment showed 32.2% growth inhibition (G.I) (P= 0.0001) at concentration of 64 HAU of NDV+1.25µg of MTX (LC), whereas MTX alone was 24% GI (P= 0.0001) at 2.5µg/ml (LC50), NDV alone at 64HAU/ml (LC) produced 31% G.I (p= 0.0001) (Fig. 4).

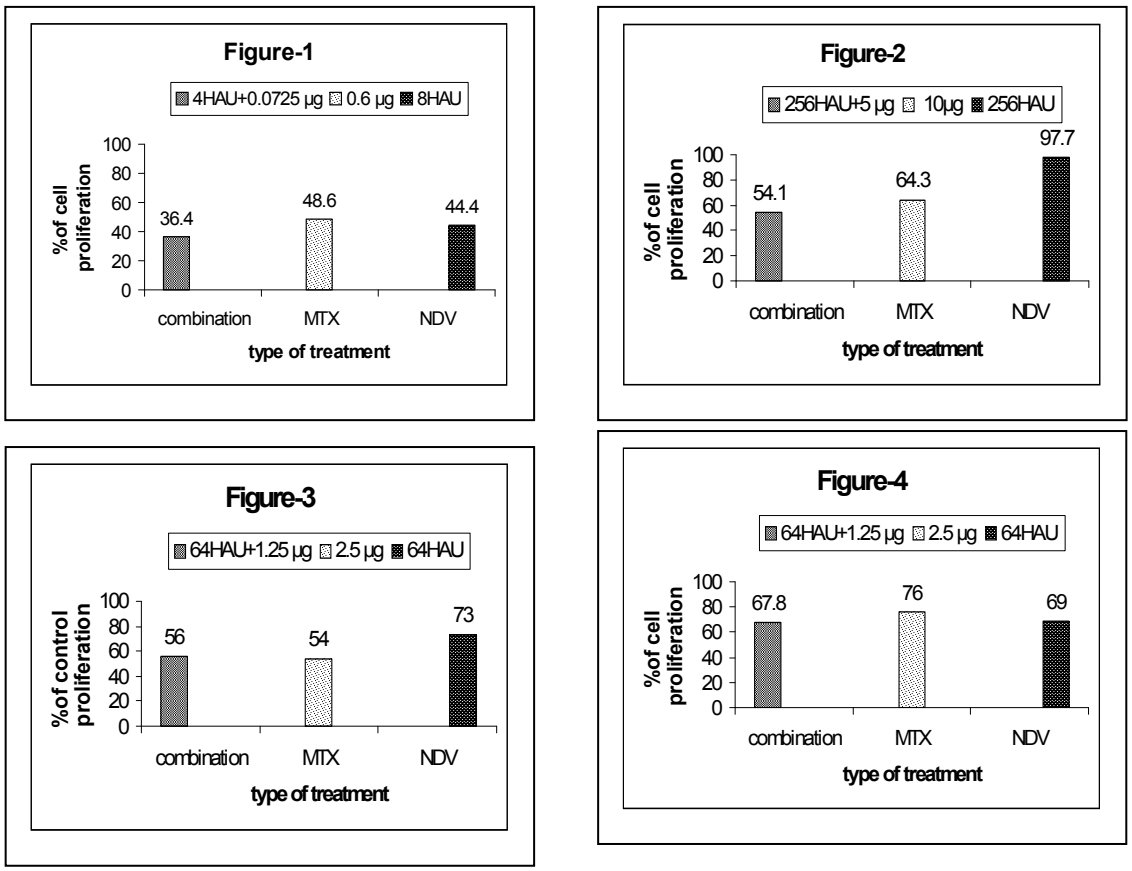

Fig. (1) Showed that combination of NDV and MTX induce significant decrease in cell proliferation in Hep-2 cell line more than MTX alone even with 3 fold concentration (0.0725µg in combination therapy compare with 0.6µg in MTX treatment alone) Data presented as proliferation rate which is opposite of growth inhibition rate. Fig. (2) Combination treatment on RD cell line showed proliferation rate less than MTX alone (54.1%, 64.3% respectively) GI for combination treatment is more then for MTX alone even with 2 fold concentration (5µg for combination in compare with 10µg for MTX alone, also combination therapy is more effective than NDV alone. Fig. (3) Showing the effect of different rout of treatment on AMN3 cell line. no significant difference between combination therapy (64HAU+1.25µg of MTX) and MTX alone (2.5µg), (44%, 46% GI respectively) even in the fact of MTX alone concentration is at 2 fold than in combination treatment. Combination treatment is more effective then virotherapy alone. Fig. (4) showed that combination of NDV and MTX induce significant decrease in cell proliferation in Vero cell line more than MTX alone even with 2 fold concentration (1.25µg in combination therapy compare with 2.5µg in MTX treatment alone)

Discussion

The aim of this study was to determine the possibility of augmentation cancer chemotherapy (MTX) by virotherapy (NDV) and to reduce toxicity of cancer chemotherapeutic agents by decreasing the administered dose and enhanced with NDV

therapy. Lentogenic NDV strain (LaSota- which is used as live vaccines against NDV) have an oncolytic activity on three tumor cell line studied, Hep-2, RD, AMN3 and in less degree on transformed cell line (Vero). Previous studies showed that NDV is oncolytic (24). Schirrmacher *et al.* (23) used non-virulent lentogenic strain Ulster and obtained results that infection of tumor cells by non-lytic NDV Ulster (30 HU/10^7 cells) eventually leads to tumor cell death in vitro and have selectivity in replication in tumor cell (36). This study proved that combination of NDV and MTX had greater antitumor efficacy than NDV alone or 2-3 fold dose of MTX alone. This clearly appear in Hep-2 cell line, in which we need only 0.0725µg of MTX in combination with 4HAU of NDV to achieve 63.3% growth inhibition while we need 0.6µg of MTX alone to achieve 51.4% GI and this concentration is 3 fold of that used in combination therapy. Similar results were noticed in the other three cell lines with less degree of effectiveness. The effect appears to be synergistic because minimal or similar cytotoxicity was observed when MTX was used alone at 2-fold concentration. The mechanisms of synergistic activity in the combination of MTX with NDV is thought to be that NDV may be augmenting the antitumor activity of MTX by increase cellular sensitivity to chemotherapeutic agents and this enhanced sensitivity is partial caused by the induction of apoptosis which is previously proved to be induced by NDV (37, 38). The possibility that each agent works independently on different cell populations can not be ruled out. Another mechanism can explain the synergistic effect which resemble the prodrug theory for virotherapy and chemotherapy, in which the virus is taken up by tumor cells, then expresses an enzyme (naturally-as we thought), the substrate specifity of the enzyme allows it to interact and the chemotherapeutic agent may metabolized by the expressed enzyme in the tumor environment to create a toxic metabolite (39). Thus, virotherapy with NDV may complement the clinical utility of MTX effectively targeting tumor cell populations that are, for one reason or another, resistant to chemotherapy. This may be important because many human tumors are composed of a mixture of cells having varying genetic makeup's, intratumoral heterogeneity may be a major reason why most monotherapies fail to achieve a cure, thus NDV may augment the efficacy of standard cancer modalities and such novel therapeutic combinations may prove valuable in the clinic. In addition, one of the important objectives of this study was to reduce toxicity of chemotherapeutic agents on tumor patients by reducing the dose while still having the same antitumor effect and this achieved as we showed above by combined it with Newcastle disease virotherapy, and not only we get the same effect but 2-3 fold effectiveness. To explain the safety of NDV there is some characteristics of NDV that made it favorable for human trails; include the genetic stability of vaccine strains, the absence of genetic recombination, lack of antigenic drift, human-to-human transmission has not been observed (21, 40 and 41). The virus has been safely given to humans in tumors vaccine studies and accidental exposure has been reported to cause only self-limiting conjunctivitis (21, 42, 43). NDV-LaSota strain because its safety and selectivity used for efficient selective gene transfer to tumor cells (44). While NDV is safe and have no toxicity, MTX have toxic dose related side effects and lack of selectivity (1). We conclude that MTX combined with NDV have synergistic effect *in vitro*, which suggests an important possible new adjuvant therapy for the treatment of cancer.

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