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## Apoptosis Induction and Cytolytic Effects of Newcastle Disease Virus Strain Af2240 on DBTRG.05 mg Brain Tumor Cell Line

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### ABSTRACT

Newcastle Disease Virus (NDV) is a member of the new genus *Avulavirus* within the family Paramyxoviridae. Interest in the use of NDV as an anticancer agent has arisen from the ability of the virus to selectively kill human tumor cells with limited toxicity to normal cells. The aim of this study was to examine the potential of local strain NDV AF2240 as an anticancer agent against brain tumor cell line, glioblastoma multiforme (DBTRG.05 mg) *in vitro*. The IC<sub>50</sub> values for cytolytic effects of NDV strain AF2240 on DBTRG.05MG cell lines was determined using assay MTT assay. IC<sub>50</sub> values was 460 HAU mL<sup>-1</sup> and no significant cytolytic effect was observed on normal cell lines at the same titre. Further study using TUNEL assay showed that the mode of cell death in response to infection by NDV strain AF2240 on brain tumor cell lines was by apoptosis. However, analysis of the cellular DNA content using PI showed that the virus caused an increase in sub-G1 region (apoptosis peaks). Early apoptosis was observed 6 h post-inoculation by annexin V flow-cytometry method. From this study, it was concluded that NDV strain AF2240 is an effective anti-cancer agent in brain tumor cell lines *in vitro*. It has a great potential to be an effective anticancer due to the ability to kill cancer cells and not normal cells. The mode of cell death induced by this virus is apoptosis and its cytotoxicity increasing while increasing the titer of the virus and increasing of time.

**Key words:** NDV, glioblastoma multiforme, apoptosis, cytotoxicity, DNA damage, flow-cytometry

### INTRODUCTION

Brain tumors can be dangerous because of the importance of the brain and the limited amount of space inside the skull. Glial cells are non-neuronal cells, they are supportive cells of the central nervous system and most of the brain is made up of these cells. The majority of these cells are called astrocytes. Primary tumors are tumors that originated in the brain. They can be further divided into astrocytomas, glioblastoma, etc., based on the cell type involved. The treatment of primary brain tumors is difficult because of polyclonicity, the blood brain barrier, the diffuse infiltrative nature of these tumors and the perilous location of some tumors. So, to cure brain tumors some consideration must be taken to kill all cells within the tumor and spare the remaining normal brain cells. There are three standard types of treatment for patients with primary brain tumors: surgery, radiation therapy and chemotherapy (Henson, 1999; Mangiardi and Kane, 2003; Stupp *et al.*, 2005). Viral therapy for cancer (virotherapy) has significantly been identified to show some promise

in cancer therapy. Virotherapy involves the treatment of cancer by using a virus specifically to infect cancer cells while leaving normal cells unharmed (You *et al.*, 2004). These viruses infect, replicate in and kill human cells through diverse mechanisms (Everts and van der Poel, 2005). The Newcastle Disease Virus (NDV) is a member of the new genus *Avulavirus* within the family Paramyxoviridae. The virus causes a highly contagious disease in poultry. Exposure to humans however, results in mild conjunctivitis, laryngitis and influenza-like symptoms (Othman *et al.*, 2010). A very virulent strain of the virus known as strain AF2240 has been shown to be responsible for a very high mortality and morbidity among poultry flocks in Malaysia (Othman *et al.*, 2010). Interest in the use of NDV as an anticancer agent has arisen from the ability of the virus to selectively kill human tumor cells with limited toxicity to normal cells. It has oncolytic activity that can destroy tumor cells and stimulate the immune system. Strains 73-T, MH68, Italian, Ulester, Rokin, PV701 (MK107) and HUI strains of NDV have been shown to exhibit an oncolytic activity. In addition, the oncolytic effects of six Malaysian strains of NDV, AF2240, 01/C, Ijuk, S, F and V4, have also been studied on several tumor cell lines (Omar *et al.*, 2003; Freeman *et al.*, 2006; Niederhuber, 2006). However, no studies have yet been made on NDV strain AF2240 oncolytic activity on brain tumor cells. Therefore, in this study the oncolytic effects of NDV strain AF2240 was tested *in vitro* against brain tumor cell line DBTRG.05MG.

## **MATERIALS AND METHODS**

This project carried out at Faculty of Biotechnology and Molecular Biology, Universiti Putra Malaysia since 2003 to 2006.

**Propagation and purification of NDV strain AF2240:** NDV strain was propagated in allantoic fluid of 9-11 days-old embryonated chicken eggs at 37°C for 48 h. The allantoic fluid was harvested and the presence of virus was confirmed by the haemagglutination test (Alexander, 1988). NDV strains AF2240 purified as previously described by Chambers and Samson (1980) and Yusoff *et al.* (1996).

**Cells and cell culture:** A human brain tumor cell line was used in this study; DBTRG .05MG and a normal cell lines, HCN-2 and 3T3, were used as control.

All cell lines were grown as a monolayer in 25 cm<sup>2</sup> tissue culture flasks (Nunclon™, Denmark) 37°C in an atmosphere of 5% CO<sub>2</sub> in RPMI-1640 medium for DBTRG .05MG supplemented with 10% fetal calf serum and 1% antibiotics. Three additional supplements were needed, 2 mM Glutamine, 1% HT and 1 mM Sodium Pyruvate. Cells were maintained at 37°C in 5% CO<sub>2</sub> atmosphere. Further maintenance and subculturing of cells were done according to supplier's protocol.

**MTT cytotoxicity assay:** About of 5×10<sup>5</sup> cells were seeded into of flat-bottom 96-well plate (Nunclon™, Denmark) and were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. After 24 h, the medium was discarded and 50 µL of the 2-folded serial virus dilution were added into the wells. In the last well, 50 µL of PBS were added to instead of the virus which represented as control. Then one 150 µL of complete medium were added to top up the final volume to 200 µL and the plate was incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. Seventy-two hours later, 20 µL of MTT (5 mg mL<sup>-1</sup>) in PBS solution was added to each well and then the plate was further incubated for 4 h. Most of the medium then was removed and 100 µL of DMSO (dimethyl sulfoxide) was added into the wells

to soluble the crystals. Finally the absorbance was measured by Enzyme-linked Immunosorbent Assay (ELISA) reader at wavelength of 570 nm. Then graphs of percentage of viable cells versus virus titer (HAU mL<sup>-1</sup>) were plotted. The value of IC<sub>50</sub> was determined from the graphs obtained at the concentration that cause 50% cell reduction as compared with controls.

**TUNEL assay:** The cells treated with concentration of IC<sub>50</sub> value of virus value were grown on Lab-Tek Chamber slides and the slides were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub>. The slides were washed with PBS after 24, 48 and 72 h and processed in the apoptosis detection assay. The TUNEL Assay was carried out using a kit for Apoptosis Detection from Promega, USA.

**Analysis of cellular DNA content using propidium iodide:** Cells at a concentration of 5×10<sup>6</sup> cells mL<sup>-1</sup> of DBTRG.05MG cell line were seeded into 6-well plate in 2 mL culture medium with a concentration of IC<sub>50</sub> value of virus and were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 72 h. Some wells were left with no virus to be used as a control. After the incubation period, the cultured cells were harvested using trypsin and centrifuged for 10 min at 1000 rpm at room temperature. Cell pellets were fixed by adding 500 µL of 80% cold ethanol and kept for at least 2 h at 20°C. Cells were pelleted at 1000 rpm for 10 min and the ethanol was discarded. The cell pellet was washed with 1 mL (PBS/sodium azide) twice. The pellet was resuspended with 1 mL of (PBS + 0.1% triton X-100 +10 mM EDTA + 50 µg mL<sup>-1</sup> RNase + 2 µg mL<sup>-1</sup> Propidium iodide) followed by incubated for ½ to 1 h at 4°C. Finally, samples were placed in 12×75 Falcon tubes and the cell cycle was analyzed by flow cytometer (Beckman Coulter, USA).

**Flow cytometry (Annexin V/PI double staining):** Cells at a concentration of 5×10<sup>6</sup> cells mL<sup>-1</sup> of DBTRG.05 mg cell line were seeded into 6-well plate in 2 mL culture medium with a concentration of IC<sub>50</sub> value of virus and were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 72 h. Some wells were left with no virus to be used as a control. After the incubation period, the cultured cells were harvested using trypsin and centrifuged for 10 min at 1000 rpm at room temperature. The early apoptosis for treated and untreated cells were carried out using a kit for Annexin V and Apo 2.7-PE Clontech Laboratories, Inc. USA.

**Statistical analysis:** MTT assay data for brain tumor cell lines were reported as mean values of three independent observations. Error bars represent standard deviations. Numerical analysis was performed via independent sample t-test (assuming equal variance) with SPSS version 13.0 for Windows. Differences were considered significant at p<0.0001.

## RESULTS

**Cytotoxic effects of NDV strain AF2240 on brain tumor cell lines:** MTT assay is a standard colorimetric assay; it was done for both brain tumor cell lines as described previously. The graphs were obtained by plotting the percentage of viable cells versus virus titer (HAU mL<sup>-1</sup>). The value of IC<sub>50</sub> was determined from the graphs obtained which are the concentration that reduce 50% cell population as compared with untreated control.

Inoculation of NDV strain AF2240 showed a decrease in cell viability of DBTRG.05MG cell line. The IC<sub>50</sub> value for cytolytic effect of NDV strain AF2240 on DBTRG.05MG was 460 HAU mL<sup>-1</sup> (Fig. 1).

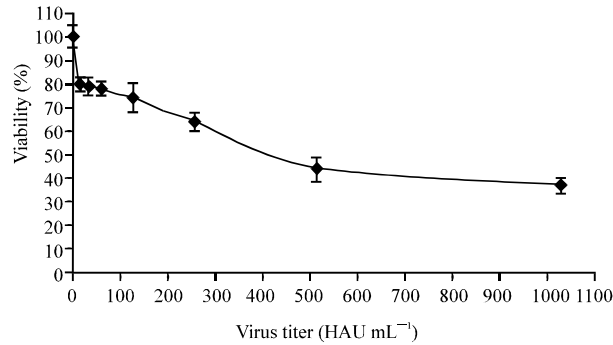


Fig. 1: Cytolytic effects of NDV strain AF2240 on DBTRG.05MG cell line. Fifty percent of cell viability ( $IC_{50}$ ) was obtained at virus titer of 460  $HAU mL^{-1}$

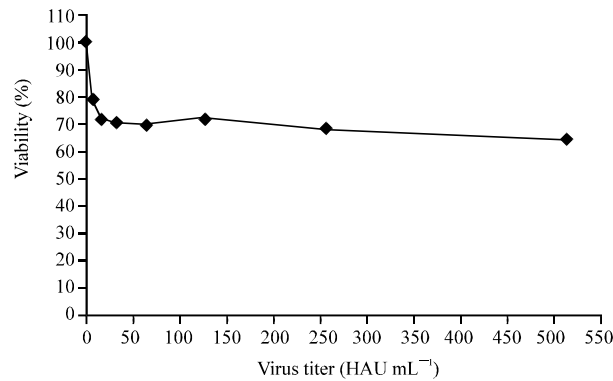


Fig. 2: Cytolytic effect of NDV AF2240 strain on HCN-2 cell line. The  $IC_{50}$  value was not obtained because the cell reduction was not effected by same virus titer

**Effects of NDV strain AF2240 on normal human brain cells, cerebral cortex neurons (HCN-2 cell line) and normal mouse fibroblast cells (3T3 Cell Line):** In this study, we used human brain cells, cerebral cortex neurons (HCN-2 cell line) (Fig. 2) and 3T3 fibroblast cell line as normal cells (Fig. 3). Inoculation of NDV strain AF2240 on this cell lines showed no significant cytolytic effect at the same titre used in the brain cell lines.

**TUNEL assay:** The apoptosis detection fluorescein system (Promega, USA) was used according to the supplier's recommended protocol. Apoptotic cells were detected and localization by green fluorescence (FITC-12-dUTP) on a red background [propidium iodide (PI)] as observed by fluorescence microscopy, as described above. Untreated DBTRG.05MG cells were shown in red because of the staining with propidium iodide. Red staining is indicating viable cells which contain intact genomic DNA. Whereas treated DBTRG.05MG cells were shown the TUNEL<sup>+</sup> nuclei appeared as yellow to green fluorescence. The treated cells containing multiple DNA breaks at 24, 48 and 72 h post-inoculation were stained yellow to green fluorescence indicating apoptosis (Fig. 4). The analysis of many different slides revealed that the green staining was generally more pronounced in apoptotic cells treated with the NDV strain AF2240 according to time (Fig. 5).

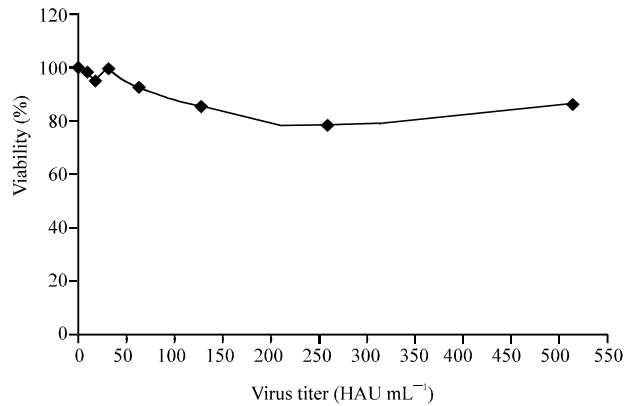


Fig. 3: Cytolytic effect of NDV AF2240 strain on 3T3 cell line. The  $IC_{50}$  value was not obtained because the cell reduction was not effected by same virus titer

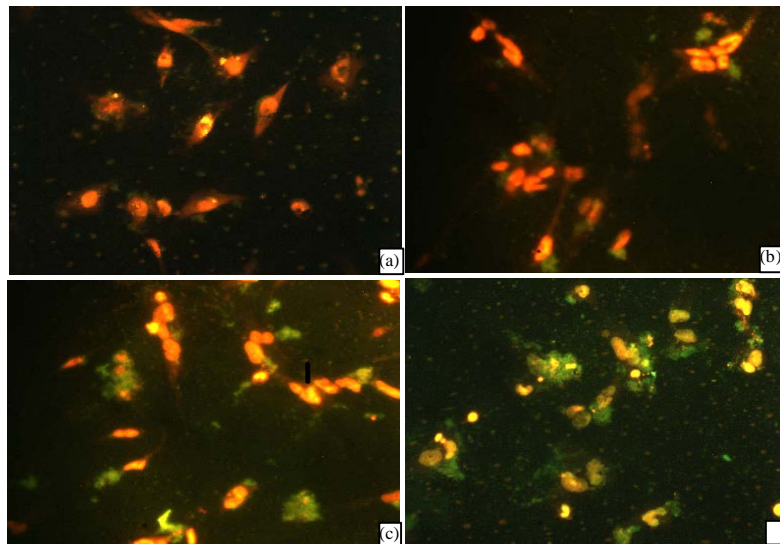


Fig. 4: TUNEL-stained DBTRG.05MG cells. ((Magnification 400X); (a) Untreated cells, (b) Cells at 24 post-inoculation, (c) Cells at 48 post-inoculation and (d) Cells at 72 post-inoculation. Cells were double-stained with fluorescein-12-dUTP and propidium iodide. Normal cells show red nuclei, whereas apoptotic cells show yellow to green nuclei (arrows)

**Analysis of cellular DNA content using propidium iodide:** DBTRG.05MG cells were treated with  $IC_{50}$  value of NDV strain AF2240 for 24 and 48 h and the DNA content was analyzed by flow cytometry after staining with propidium iodide. In untreated DBTRG.05MG cells, the  $G_1$ , S and  $G_2/M$  populations represented 54.34, 18.66 and 22.83% of the cells, respectively (Fig. 6). With both cell lines, treatment with NDV strain AF2240 did not induce cell cycle arrest in any specific phase. The treatment resulted in a loss of DBTRG.05MG cells in all three phases of the cycle accompanied with a large increase in the sub- $G_1$  region (Table 1).

Apoptosis peak (sub- $G_1$ ) was found in untreated cell line with small percentage for about 4.09%. The sub- $G_1$  population in DBTRG.05MG cells treated with NDV strain AF2240 was 18.40% and

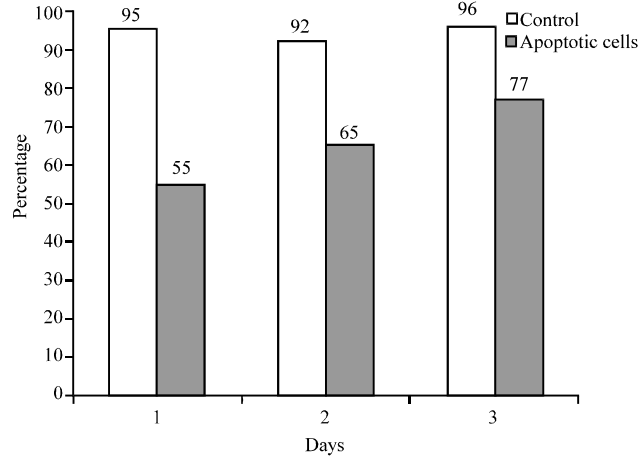


Fig. 5: TUNEL-stained DBTRG.05MG.cells revealed that apoptotic cells generally increasing after treatment with the NDV strain AF2240 according to time compare with control

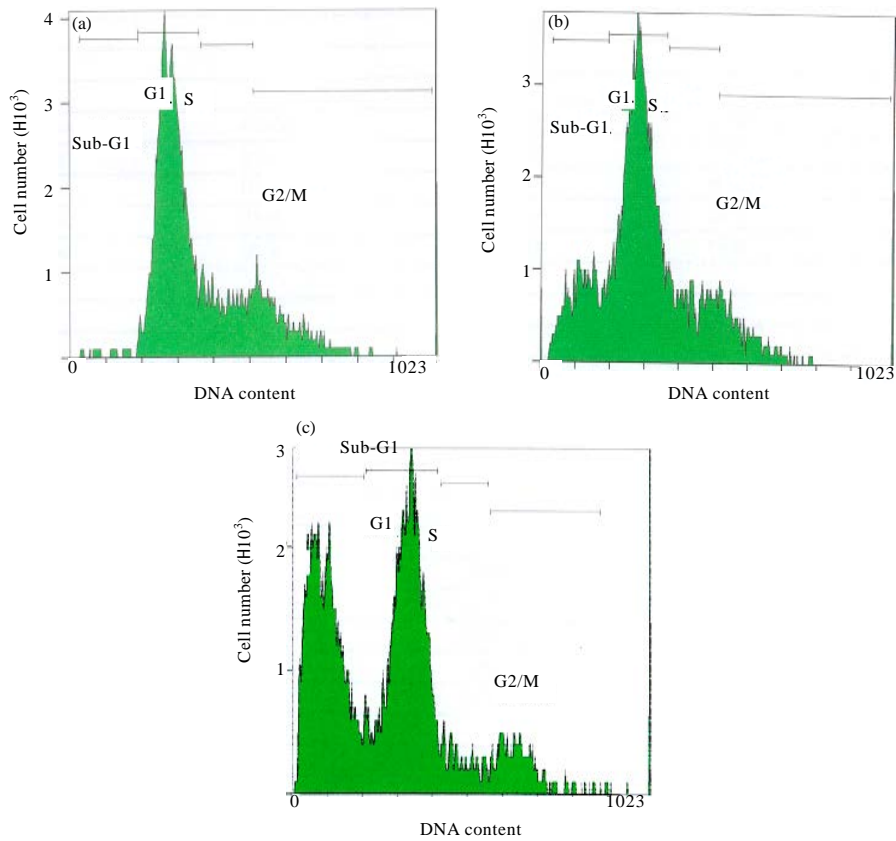


Fig. 6: Cell cycle (DNA content)flow cytometer histograms of DBTRG.05MG brain tumor cell line treated with IC<sub>50</sub> value of NDV strain AF2240, (a) untreated cells (control), (b) cells treated with NDV after 24 h and (c) cells treated with NDV after 48 h. Shows increasing in percentage of sub-G<sub>1</sub> (apoptotic) cell population (broken DNA)

Table 1: Percentage of DBTRG.05MG cells in different cell cycle phase after treatment with virus

DBTRG.05MG cells	Cell cycle phases			
	Apoptosis (%) (Sub-G1)	G1 (%)	Synthesis (%)	G <sub>2</sub> /M phase (%)
Untreated cells	4.09	54.34	18.66	22.83
Cells treated after 24 h	18.4	49.57	16.43	15.59
Cells treated after 48 h	37.4	41.66	7.85	13.07

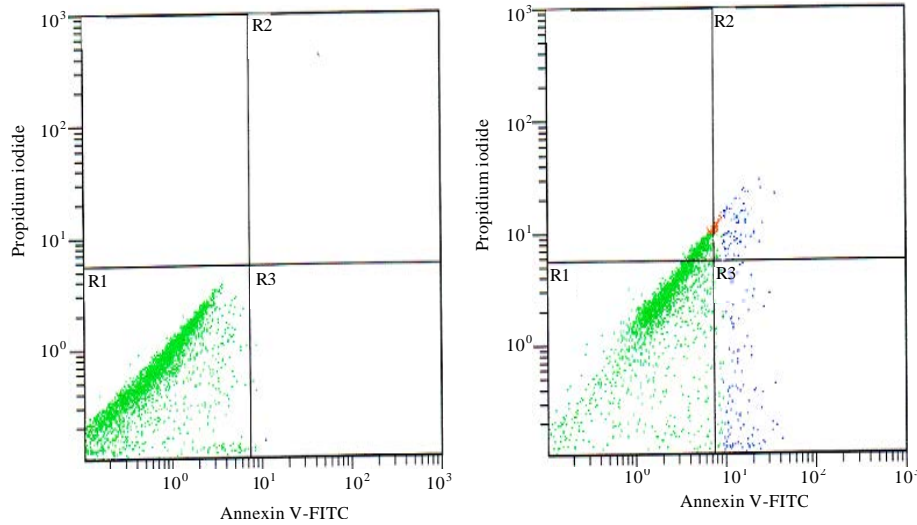


Fig. 7: Contour diagram of Annexin V/PI flow cytometry (1) Untreated DBTRG.05MG cells (2) DBTRG.05MG cells at 6 hours post-inoculation of IC<sub>50</sub> value of NDV strain AF 2240. The lower left quadrants of each panel (R1) show the viable cells which exclude PI and are negative for Annexin V binding. The upper right quadrants (R2) contain the non-viable, necrotic cells, positive for Annexin V binding and for PI uptake. The lower right quadrants (R3) represent the apoptotic cells, Annexin V positive and PI negative

37.40% for 24 and 48 h post-inoculation, respectively. This virus-induced cell cycle perturbation concurred with the results of the TUNEL assay to suggest that brain tumor DBTRG.05MG cell line undergo apoptosis more extensively with increasing in time.

**Flow cytometry (Annexin V/PI double staining):** Apoptotic cells exclude all those dyes which are in use for cell viability assays, such as PI, while necrotic cells do not. In cells with a damaged cell membrane PI induces a red fluorescence on the DNA, while it is excluded by cells with a preserved cytoplasm membrane. Hence during the initial phase of apoptosis, the cells are still able to exclude PI and therefore, do not show any red fluorescence signal, similar to that of living cells. Figure 7 showed the results of Annexin V/PI flow cytometry of DBTRG.05MG cells after treatment with IC<sub>50</sub> value of NDV strain AF2240 at 6 h post-infection. The lower left quadrant of the cytograms shows the viable cells which excluded PI and were negative for Annexin V binding. The upper right quadrant represents the non-viable, necrotic cells, positive for Annexin V binding and showing PI uptake. The lower right quadrant represents the apoptotic cells, Annexin V positive and



PI negative, demonstrating Annexin V binding and cytoplasmic membrane integrity (Fig. 7). The Annexin V+/PI- apoptotic cell population for DBTRG.05MG cell line increased from 0.2% in untreated cells to 3.7% in treated cells at 6 h post-infection.

## DISCUSSION

Oncolytic viruses are viruses that infect and replicate in cancer cells, destroying these harmful cells and leaving normal cells largely unaffected. Like all viruses, oncolytic viruses seek to penetrate a host cell and "trick" it into replicating more of the virus until ultimately, it bursts. NDV is an oncolytic virus with the ability to induce tumor lysis through different mechanisms (Szeberenyi *et al.*, 2003). In this study the ability of NDV strain AF2240 to induce tumor cytolysis was assessed towards brain tumor cell lines. MTT assay was carried out to determine the titer of the virus that cause 50% cell reduction. The  $IC_{50}$  values for cytolytic effect of NDV strain AF2240 on DBTRG.05MG cell lines was 460 HAU mL<sup>-1</sup>. This study also showed that no significant reduction in cell viability was observed in treated HCN-2 and 3T3 normal cell lines treated with NDV strain AF2240. This result complies with the previous studies, a study reported by Lorence *et al.* (1994) found that NDV strain 73-T killed human and rat neuroblastoma but not normal fibroblast. Another study showed that the NDV appears to replicate and kill tumor cells selectively better than normal human cells (Reichard *et al.*, 1992).

Furthermore, a study by Meyyappan (2003) reported that NDV strain AF2240 induced cytolytic effect on the MCF-7 and MDA-231 breast cancer cell lines, with  $IC_{50}$  values of 64 and 4 HAU mL<sup>-1</sup>, respectively and  $IC_{50}$  values of NDV strain V4-UPM on the MCF-7 and MDA-231 breast cancer cell lines were 128 and 96 HAU mL<sup>-1</sup>, respectively. Wali (2003) stated that the  $IC_{50}$  values of NDV strains F on the MCF-7 and MDA-231 breast cancer cell lines were 2048 and 8 on the MCF-7 and MDA-231 breast cancer cell lines whereas, the  $IC_{50}$  values of NDV strains Ijuk on MDA-231 breast cancer cell lines was 8.6 HAU mL<sup>-1</sup>. Another study by Zawawi (2006) stated that  $IC_{50}$  values of NDV strain V4-UPM on HL60 and CEM-SS leukemia cell lines were 150.9 and 110.6 HAU mL<sup>-1</sup>, respectively. In conclusion, the NDV AF2240 is an effective anti-cancer agent in brain tumor cell lines *in vitro* ( $IC_{50}$  values on DBTRG.05MG cell line is 460 HAU mL<sup>-1</sup> and on U-87MG cell line is 52 HAU mL<sup>-1</sup>). It has a great potential to be an effective anticancer due to the ability to kill cancer cells and not normal cells.

On the other hand, the Terminal Deoxynucleotidyl Transferase (TDT)-mediated dUTP-fluorescein nick-end labeling (TUNEL) staining is the standard technique for detection of apoptosis because it permits visualization of DNA cleavage which can be visualized and quantities directly by fluorescence microscopy (Gavrieli *et al.*, 1992; Stahelin *et al.*, 1998; Gao *et al.*, 2001; Yasuhara *et al.*, 2003). TUNEL assay showed that NDV strain AF2240 caused a significant increase in the percentage of apoptotic cells on DBTRG.05MG brain cell line and the percentage of apoptotic cells increased with increasing of time, in which apoptotic cells were more abundant at longer durations of treatment. In the cell populations of control in both cell lines, cells were having intact DNA or low undetectable levels of fragmentation but treated cells were TUNEL positive and apoptotic cells increased correlated with the time of treatment.

Further confirmation of the mode of cell death was carried by Flow cytometric analysis of cell cycle. It is rapid and quantitatively measures on apoptotic cells. It measures apoptotic changes in cells by staining with DNA dyes (Telford *et al.*, 1994). This method is useful for quantitative estimates of the fractions of cells in the different phases of the cell cycle (Fried *et al.*, 1976). Untreated and treated DBTRG.05MG brain cells were evaluated for apoptosis by measuring the

amount of apoptotic cells using of DNA flow cytometry (FCM). NDV strain AF2240 caused an increasing in the sub-G<sub>1</sub> region which increased with increasing of time and did not induce specific cell cycle arrest in specific phase. Apoptotic cells, due to a change in membrane permeability, showed an increased up-take of the vital dye compared to live cells. The apoptotic cells with degraded DNA were represented in so-called "sub-G<sub>1</sub>" peaks on DNA histograms. The percentage of sub-G<sub>1</sub> cells was measured. PI is added to discriminate late apoptotic or necrotic cells which had lost membrane integrity from early apoptotic cells which still had intact membranes by dye exclusion (Nicoletti *et al.*, 1991).

In this study we carried out a flow cytometry quantitative method for measuring cellular cytotoxicity using propidium iodide staining. Analysis of cellular DNA content using propidium iodide devised for estimating the fractions of cells in all phases of the mitotic cycle. This method has important implications as a more convenient and more accurate means of cell cycle analysis of populations in culture, also for monitoring the status and kinetic response to therapy of patients with neoplasm (Fried *et al.*, 1976). It measures apoptotic changes in cells by staining with DNA dyes (Telford *et al.*, 1994). This method is useful for quantitative estimates of the fractions of cells in the different phases of the cell cycle (Fried *et al.*, 1976). The results of this study reveal that there was a loss of treated DBTRG.05MG cells in all three phases of the cycle (G<sub>1</sub>, S and G<sub>2</sub>/M) accompanied with a large increase in the sub-G<sub>1</sub> region (apoptosis peak) in the fluorescence histograms. The apoptotic cells with degraded DNA were represented in so-called "sub-G<sub>1</sub>" peaks on DNA histograms. Apoptotic cells, due to a change in membrane permeability, showed an increased up-take of the vital dye compared to live cells.

The novel Annexin binding assay, using annexin V together with propidium iodide in which the visualization of cells dying by membrane damage and lysis are identified. This method appears to be sensitive, correlates with the other tests, easy to perform and has been extensively utilized for the quantification of apoptotic cells by flow cytometry (Aubry *et al.*, 1999). The method permits the detection of the early phases of apoptosis before the loss of cell membrane integrity (Vermees *et al.*, 1995). This is based on the phenomenon that phosphatidylserine (PS) is exposed at the outer membrane of the cell during apoptosis and on the ability of annexin V to bind to PS with high affinity (Van Engeland *et al.*, 1998). Phosphatidylserine (PS) only exists in the cytoplasm side of cell plasma membrane and externalization of PS occurs in the early stage of apoptosis. Annexin-V could specifically conjugate to the PS to detect the apoptotic cells (Lin *et al.*, 2004). The analysis of the treated DBTRG.05MG cells by annexin V versus PI, revealed four populations: live cells, apoptotic cells, late apoptotic cells and permeabilized cells. In this experiment, we observed increasing in the percentage of target cells in the annexin positive-PI negative (early apoptosis) quadrant at 6 hours post-inoculation. These results complies with the other studies such as Meyyappan (2003), Wali (2003) and Zawawi (2006) reported that NDV induced cytolytic effect on the breast cancer cell lines (MCF-7 and MDA-231) and leukemia cell lines (HL60 and CEM-SS) (Omar *et al.*, 2003).

In conclusion all the previous results show that NDV strain AF2240 was capable to induce apoptosis on brain tumor cells, glioblastoma multiforme GBM (DBTRG.05MG), *in vitro*.

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