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The Potential Selective Oncolytic Effects of Newcastle Disease Virus (NDV) on Cervical and Colon Cancer Cell Lines

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ABSTRACT

The mesogenic and velogenic strains of the avian Newcastle Disease Viruses (NDV) have been investigated for use in cancer virotherapy. However, despite its promising results, these strains have the potential to cause outbreaks leading to morbidity and mortality in large numbers of avians that can devastate the poultry industry. Therefore, there is a growing interest towards the use of lentogenic strains such as NDV LaSota vaccine strain for virotherapy. This study investigated the oncolytic potential of the NDV LaSota strain on colorectal cancer (HT29) and cervical cancer (HeLa) cell lines. The NDV LaSota stain was propagated in pathogen free embryonated chicken eggs and quantified using a hemagglutination assay. The oncolytic activity was evaluated by comparison of HT29 and HeLa cell lines to the non-cancerous human embryonic kidney (HEK293) cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. The highest NDV concentration of 16 HAU was found to reduce the viability of HT29, HeLa, and HEK293 cell lines to 42±3% (p = 0.0001), 49±12% (p = 0.0009), and 77±5% (p = 0.007), respectively, indicating its potential selective cytotoxicity, as supported by the disturbed cell morphology. The NDV LaSota strain exhibits a potential selective oncolytic activity towards cancer cell lines, thus may serve as an interesting candidate for virotherapy against colorectal and cervical cancer.

1. Introduction

According to a 2018 World Health Organization (WHO) report, cancer is the second major cause of global deaths, with an estimated mortality of 9.6 million people in 2018 and causing a large economical burden of US$1.15 trillion in 2010 (World Health Organization 2018). According to the Ministry of Health in Indonesia, around 347,792 people in Indonesia have been estimated to have cancer in 2013 (Pusat Data dan Informasi Kementrian Kesehatan RI 2015).

Colorectal cancer (CRC) is among the top three most commonly diagnosed malignancies worldwide. In fact, the projected global burden of CRC in 2030 is expected to reach more than 2.2 million in cases with an approximate 1.1 million deaths, with a 14% increase in its incidence over the last few years, partially due to improved early diagnosis (Dekker *et al.* 2019; Lee *et al.* 2019). Meanwhile, according to 2018 Global Cancer Observatory (GLOBOCAN), cervical cancer ranks the second and fourth most prevalent and deadliest cancer in women, respectively (Bray *et al.* 2018). Cervical cancer, usually caused by infection with the human papillomavirus, is the most prevalent gynecological cancer (Okunade 2020). The number of deaths caused by cancer is due to a combination of inefficient prevention and the limitation of conventional treatments such as surgery, chemotherapy and radiation (Miri *et al.* 2020).

To overcome the limitation of conventional treatments, new approaches need to be explored. One such approach is the utilization of oncolytic viruses. An early observation in which certain viral infections, such as measles and chickenpox, improve the status of leukemia and lymphoma patients, marked the start of oncolytic virotherapy. Later, it was demonstrated that the antitumor activity of

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certain viruses is associated with defective type I interferon (IFN-α and IFN-β) signaling pathways in cancer cells, either through mutations or downregulation (Gopisankar and Surendiran 2018). These oncolytic viruses offer one of the simplest and more direct approaches to target cancer through the infection and lysis of these cells (Lee *et al.* 2019; Tilgase *et al.* 2018). There are a few viruses that have been found to possess an oncolytic potential including the vaccinia virus, herpes simplex virus, adenovirus, and Newcastle disease virus (NDV).

NDV is an avian paramyxovirus with a 15,186-nucleotide, negative-sense, non-segmented, single-stranded RNA genome. NDV was first mentioned around 1926 in Java, Indonesia (Etriwati *et al.* 2017; Ganar *et al.* 2014). NDV is classified based on the range of severity it causes on poultry: lentogenic (low virulence), mesogenic (intermediate virulence), and velogenic (high virulence) (Omar *et al.* 2003; Putri *et al.* 2016). This avian pathogen does not naturally infect humans and has only been found to cause mild flu-like symptoms and conjunctivitis, with no reported human-to-human transmission (Charan *et al.* 1981; Cheng *et al.* 2016; Lippmann 1952; Miller and Yates 1971; Nelson *et al.* 1952; Shirvani and Samal 2020). Importantly, NDV RNA genome is relatively conserved with the absence of genetic recombination and antigenic drift, and seronegative percentage of 96% in humans, suggesting its low immunogenicity compared to other viruses (Al-Shammari and Yaseen 2012; Charan *et al.* 1981; Miller and Yates 1971; Zamarin and Palese 2012). NDV has already been used as an oncolytic agent in several clinical trials of particular human cancers, such as melanoma, CRC, and glioblastoma (Chu *et al.* 2019; Song *et al.* 2019).

While mesogenic and velogenic strains of NDV can efficiently replicate in and lyse human cancer cells, lentogenic strains do not demonstrate the same activity (Zamarin and Palese 2012). Despite its potential oncolytic activity, mesogenic and velogenic strains may not be suitable for large-scale human therapeutic use as they can lead to major outbreaks in the poultry industry if not handled properly. (Ganar *et al.* 2014; Hammad *et al.* 2022). Therefore, the use of lentogenic strains for cancer treatment needs to be explored. NDV LaSota is a lentogenic strain that has been widely used as an avian vaccine, demonstrating a potential for oncolytic virotherapy due to its high safety profile. In this study, the oncolytic activity of NDV LaSota strain was investigated against human colorectal adenocarcinoma cell line HT-29 and cervical cancer cell line HeLa, and compared with human embryonic kidney-293 (HEK-293) cell line to represent a non-cancerous cell line. While NDV LaSota strain has been tested against HeLa cells (Chu *et al.* 2019), to the best of our knowledge, this is the first study to demonstrate the oncolytic activity of LaSota strain on HT-29 cells.

2. Materials and Methods

2.1. Virus Propagation and Harvest

Lentogenic NDV LaSota strain and specific-pathogen-free (SPF) embryonated eggs were obtained from Institut Pertanian Bogor, Indonesia. Virus propagation was done by injecting 100 µl of 1280 HAU (hemagglutination unit) NDV suspension, supplemented by 10% penicillin/streptomycin, into the allantoic cavity of 9-day-old SPF embryonated eggs. The inoculated eggs were incubated at 37°C in an egg incubator and observed daily for embryo development and viability. Once the embryos showed no signs of viability, as seen by the absence of movement upon egg candling, roughly 4 days after incubation, they were chilled at 4°C for at least 4 hours to ethically euthanize the embryo and obtain a better viral yield. The allantoic fluid was obtained by opening the eggshell above the air sac using a sterile tweezer and breaching the chorioallantoic membrane. The allantoic fluid was then centrifuged to remove debris at 12,000 rpm for 10 minutes at 4°C pre-chilled centrifuge. The NDV-containing supernatants were then aliquoted and transferred to -80°C for long-term storage (McGinnes *et al.* 2006; Yurchenko *et al.* 2018).

2.2. Hemagglutination Assay

Hemagglutination assay (HA) is a way to measure the titer of the virus based on the ability of the virus to bind sialic acid (such as N-Acetylneuraminic acid or glycolylneuraminic acid) on the surface of the red blood cells (RBCs). Virus binding to the RBC causes the RBCs to form a lattice-like structure coating the bottom of a well. However, in absence of the virus, the RBCs fall to the bottom creating a round button. Viral titer is measured in a hemagglutination unit (HAU) where 1 HAU was determined by which highest dilution factor shows a complete agglutination of the RBCs. Using the HAU value, one

can roughly estimate the amount of virus based on 1 HAU ~ 5-6 logs of a virus (Killian 2014). The HA titer of the sample is therefore corresponded to the dilution factor that produces one HAU.

The NDV suspension was assayed in V-bottom 96-well microtiter plate according to adapted protocol (Wu *et al.* 2016). The NDV sample was diluted in 11 wells of row A-F from the second well to the twelfth well, which has been filled with 20 µl PBS. A 40 µl of NDV supernatant was added into first wells for each row A-E, and 20 µl of the supernatant in the first well was transferred into the second wells. A 20, 60, 100, 140 µl of PBS was added into well B2, C2, D2, E2, respectively, and was serially diluted until the twelfth wells. The last 20 µl in the twelfth wells were discarded from the wells of each row. Then, 20 µl of PBS was added into every well. A 20 µl of 1% RBC was added into every well and was stroked for 10 seconds and incubated for 60 min at 4°C. At the end of the incubation period, plates were then assessed for hemagglutination and photographed.

2.3. Cell Culture and Passaging

Human colon carcinoma HT29 and human cervical carcinoma HeLa were used as cancer cell lines in this study, while HEK293 was selected as the healthy cell comparison. All cell lines were cultured in complete media containing Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated in a humidified 5% CO_2 incubator at 37°C. The cells were routinely sub-cultured when they reached 80-90% confluency. Viable cells were enumerated using trypan blue exclusion assay, which can differentiate dead cells (blue) from live cells (translucent) under the light microscope, with the aid of a hemocytometer.

2.4. Cell Viability Assay

Cells (2 x 10^4 cells/well) were seeded into 96-well flat-bottom plates in the aforementioned complete media. The cells were incubated in a humidified 5% CO_2 incubator at 37°C until 80% confluency or around 4 x 10^4 cells per well was reached. The virus inoculation was adapted from Yurchenko *et al.* (2018) and modified. Cells were infected with NDV-inoculated medium (2, 8, and 16 HAU) and inoculated medium without NDV (0 HAU) was used as negative control. The cells were incubated at 37°C for 60 minutes and stroked for 10 seconds with an interval of 15 minutes. After the incubation, the virus was removed and the media was replaced with DMEM supplemented with 1% FBS and 1% penicillin/streptomycin. The inoculated cells were incubated for 48 hours and the cell morphology was recorded using an inverted light microscope. Cell viability was determined by MTT assay. Briefly, the medium was replaced with 100 µl of DMEM, followed by addition of 20 µl of MTT solution and incubated for 4 hours at 37°C in an incubator with 5% CO_2. After violet formazan crystals have formed, 200 µl of DMSO solubilization solution was added to each well to dissolve the crystals. The plate was then read using an plate reader at 570 nm. The experiment was conducted in triplicate for each cell line used and NDV concentration. The relative cell viability was calculated using Equation (1).

The cell viability was calculated with the formula:

$$\%\ \text{cell viability} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}}\right) \text{x } 100 \text{.....(1)}$$

2.5. Observation of Cell Morphology

The inoculated cells were incubated for 96 hours and were observed every 24 hours under the inverted light microscope and photographed to capture changes in cell morphology.

2.6. Statistical Analysis

All statistical analyses were performed with IBM SPSS. Statistical significance analysis was determined using one-way ANOVA followed by Tukey's post-hoc test. The data were presented as mean % cell viability ± standard deviation with $p<0.05$ considered as statistically significant.

3. Results

3.1. Virus Propagation and Quantification by HA Assay

The NDV is propagated in the allantoic cavity, typically the area that produces the highest yield of this virus, of the SPF embryonated chicken eggs in order to evaluate the ability of the virus to infect the cells and to obtain enough viral titer to conduct the study.

Following the harvest of the virus, it was put through a rapid HA assay and then passed to a full HA assay for quantification of the virus (Figure 1). Figure 1 shows the number of HAUs/20 µl of the sample. In this study, the virus concentration was found to be between 2,400-4,000 HAU/ml, indicating its ability

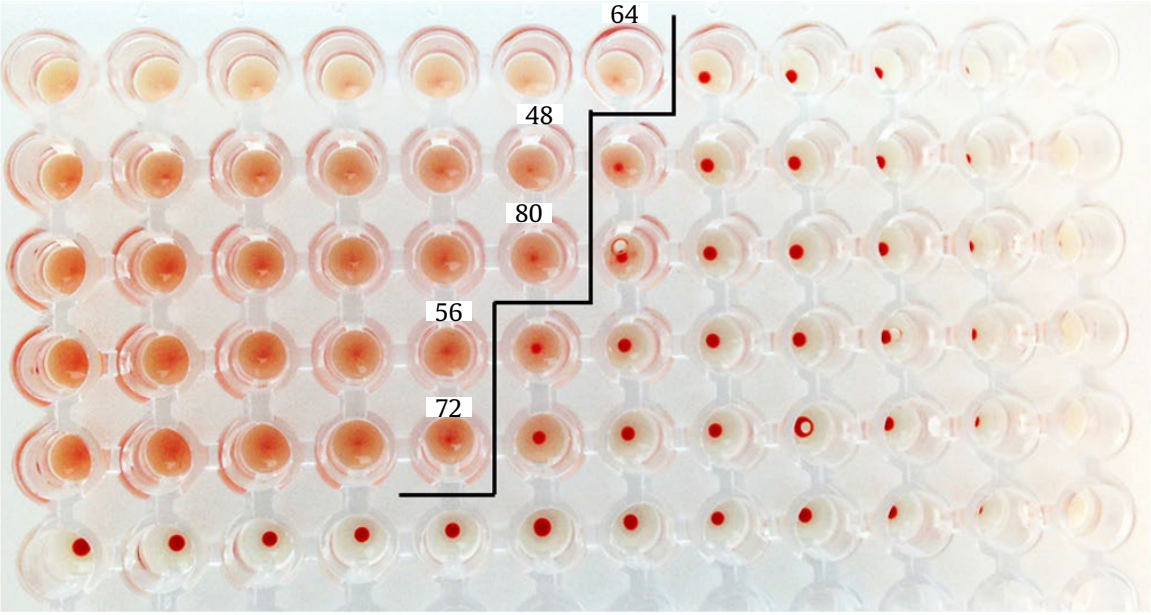

Figure 1. Hemagglutination assay. The results obtained from the HA assay to quantify the virus in the allantoic fluid of eggs on day 4 after being inoculated with NDV. The greatest dilution in which agglutination still occurred was between 1/48–1/80, which represents 2,400 HAU/ml–4,000 HAU/ml

to infect cells and proliferate. Using the HAU value, one can roughly estimate the amount of virus based on 1 HAU ~ 5-6 logs of a virus.

3.2. Effect of NDV on Cell Morphology and Viability

Following incubation, the morphology of the cancer cell lines (HT-29 and HeLa) changed, as shown by the formation of apoptotic bodies and syncytium, which is more apparent at doses of 8 HAU and higher, as well as a qualitative decline in cell number (Figure 2). In contrast, the morphology of HEK-293 cell line was less aberrated, implying that HEK-293 cells are less susceptible to cell lysis upon NDV infection. MTT assay was also performed to determine the cell viability (Figure 3). Both HT-29 and HeLa cell lines are susceptible and permissive towards NDV LaSota strain infection, as shown by a dose-dependent reduction in the relative viability, reaching a minimum of 43.9±3.3% ($P<0.0001$) and 49.5±12.5% ($P<0.001$), respectively, at 48 hours post-infection. Based on the regression analysis, the IC_{50} values of 14.43 ($R^2 = 0.9452$) and 15.22 HAU ($R^2 = 0.8489$) were obtained for HT-29 and HeLa cell lines, respectively (Table 1). HEK-293 cell line was used to determine the effect of using NDV on highly proliferating, non-cancerous cells. Interestingly, the relative viability of HEK293 cells were only reduced to 77±5% ($P<0.01$), suggesting the potential selective toxicity of NDV against cancer cell lines.

4. Discussion

The present study has demonstrated the potential oncolytic activity of NDV LaSota strain on HT-29 and HeLa cells. The selective cytotoxicity of NDV LaSota strain on cancer cell lines is consistent with previous studies using other NDV strains. The specificity of the virus against tumor cells has been tested, in an experiment involving several cancer cell lines (HCT116, HeLa, A549) the wild-type NDV has been reported to infect all the cancerous cells while leaving the normal Peripheral Blood Mononuclear Cell (PBMC) uninfected (Yurchenko *et al.* 2018). Similarly, NDV exhibited an oncolytic effect on human fibrosarcoma HT-1080 but not on normal human fibroblast CCD-1122Sk (Krishnamurthy *et al.* 2006). Furthermore, Ginting *et al.* (2017) also demonstrated that HEK-293 cell viability was only reduced to 84% at 72 hours post-NDV infection. The selective cytotoxicity of NDV on cancer cells is postulated to come from the fact that cancer cells have defective interferon (IFN) production and/or IFN signaling pathways. Since interferons induce an antiviral state and inhibit cell growth and proliferation, cancer cells are more susceptible to viral infection and spread to

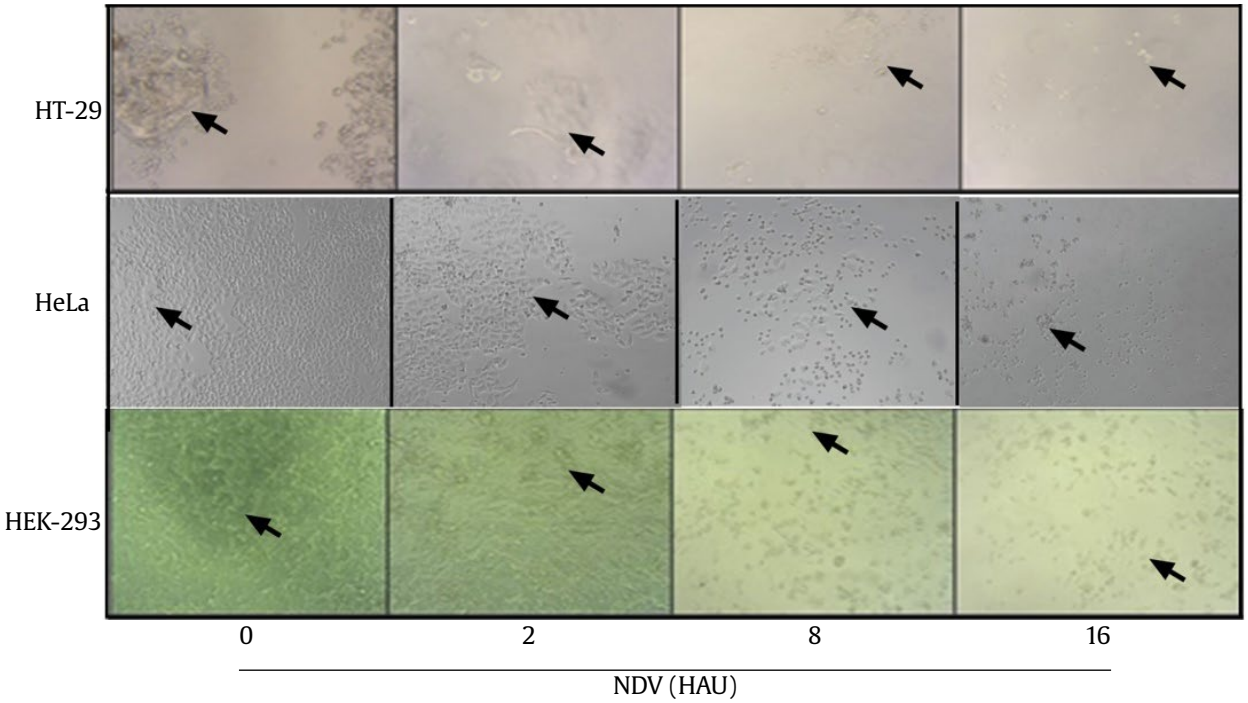

Figure 2. Morphology of HT-29, HeLa, and HEK-293 cells post-treatment with various concentrations of NDV LaSota strain. The morphology of cancer cell lines remained relatively preserved at 2 HAU of NDV, although visible decline in the cell number was observed compared to the negative control. The presence of syncytium and apoptotic bodies were observed in higher NDV concentrations (8 and 16 HAU). Meanwhile, the cell number and morphology in NDV-treated HEK-293 cells were qualitatively more preserved

Table 1. Relative cell viability after treatment with various concentrations of NDV LaSota

NDV (HAU)	HT-29			HeLa			HEK-293		
	Viability (%)	p value	Notation	Viability (%)	p value	Notation	Viability (%)	p value	Notation
0	100±0	-	-	100±10.5	-	-	100±5.05	-	-
2	83.5±5.78	0.008	**	83.1±0.80	0.182	ns	99.4±2.41	0.999	ns
8	70.7±4.21	0.0002	***	58.1±4.21	0.003	**	81.9±7.34	0.023	*
16	43.9±3.30	<0.0001	****	49.5±12.5	0.0009	***	77.7±5.17	0.007	**

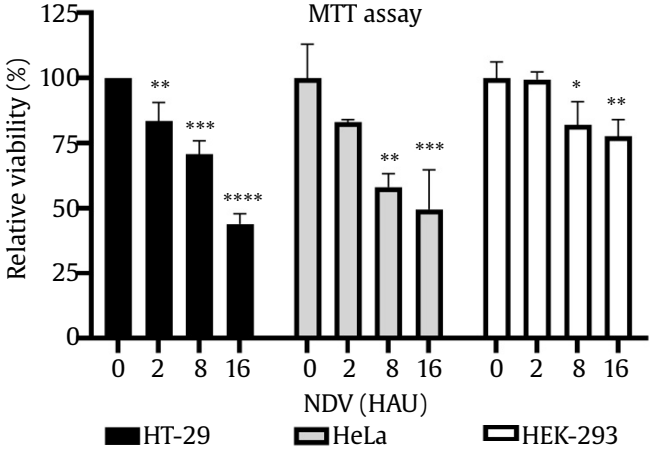

Figure 3. Relative viability of HT-29, HEK-293, and HeLa cell lines (n = 3) 48 hours post treatment with different concentrations of NDV. Asterisks represent statistically significant differences in viability compared to negative control. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001

neighboring cells, eventually leading to cell death (Ginting *et al.* 2017; Schreiber 2017).

Interestingly, this study found that with the highest concentration of 16 HAU, the cell viability of the cancerous cell lines was only reduced to slightly below 50%, supporting the lower antiproliferative activity of lentogenic NDV strains compared to velogenic counterparts. For instance, NDV velogenic strain AF2240 has been reported to induce intrinsic apoptosis in HT-29 cell line (Assayaghi *et al.* 2016; Chia *et al.* 2012; Molouki and Yusoff 2012). Similarly, several velogenic NDV strains have also been reported to induce apoptosis in cervical cancer cell lines, including murine (TC-1) and human (HeLa). For instance, NDV Bareilly strain reduces viability of HeLa cells to around 45% after 48 hours post-infection (Morla *et al.* 2019). Nevertheless, apoptosis of CRC cell lines HT-29 and CT26 upon infection by NDV

lentogenic strains V4-UPM and Ulster, respectively, have also been reported, suggesting that there are still prospective potentials of lentogenic NDV strains for cancer treatment (Assayaghi *et al.* 2016; Schirrmacher *et al.* 2001). The study supports the idea that NDV LaSota strain, being a lentogenic strain, might also have similar antitumor and oncolytic activity, with lower risk of outbreaks compared to its more virulent counterparts. In fact, lentogenic NDV strains may serve more as an immunostimulant, which has drawn more interest, since it has been used in several clinical trials, either as single or combination therapy (Fournier and Schirrmacher 2013; Lam *et al.* 2011; Liang 2003; Mansour *et al.* 2011; Matveeva *et al.* 2018).

One of the main anti-cancer mechanisms of NDV is through the formation of syncytium which indicates a successful viral spread among neighboring cells. This syncytium can be induced through modification of the F protein of NDV, which is one of the main components for viral attachment to cell membrane and fusion, as proven in previous experiments involving LaSota strains (Zamarin and Palese 2012). Prolonged incubation time may also allow increase in viral replication, thus inducing more cytopathic effects, such as formation of syncytium, apoptotic bodies, and plaques (Cuadrado-Castano *et al.* 2015; Keshavarz *et al.* 2020; Mansour *et al.* 2011; Zamarin and Palese 2012). However, since the present study did not demonstrate the mode of cell death on HT-29 or HeLa cells upon infection, future studies regarding such assays may be warranted. As mentioned earlier, the antitumor properties of NDV may also be further enhanced by combination with other treatment modalities, such as chemotherapy and immunotherapy (Al-Shammari and Yaseen 2012; Al-Shammari *et al.* 2016; Miri *et al.* 2020; Mozaffari Nejad *et al.* 2020). The rationale being NDV able to induce immunogenicity of the tumor cell, and with combination of immune checkpoint inhibitors to improve host immune response it would synergistically increase the chance of tumor clearing, as proven by the success recent experiments which utilize a modified NDV that also express checkpoint inhibitors anti-PDL1 (Vijayakumar *et al.* 2020).

In this study, we tested the ability of the NDV LaSota strain on two cancer cell lines (HeLa and HT-29) and compared it to HEK293 cells, which represented the cell line of a non-cancerous origin, in order to test its specificity. As shown in Figure 3, cell viability of HT29, HeLa and HEK293 decreases consistently as the viral concentration increases. Incubation of the virus with the three cell lines revealed the selective nature of the virus with a more significant amount of HeLa and HT29 cells being reduced after infection as compared to the HEK293 cells, confirming the original hypothesis.

There were several limitations in our study, such as the usage of highly proliferating, immortalized cell line HEK-293 instead of a primary cell line or PBMC as a control of non-cancerous cell lines. The cellular assay is also restricted to cell viability assay. Regardless, we managed to demonstrate the oncolytic potential and specificity of NDV LaSota strain against colorectal adenocarcinoma and cervical carcinoma cell line. Further studies should be geared towards investigating the effect of NDV LaSota strain on other indicators, such as cell migration and survival. Combinatorial assays with chemotherapy drugs may also be conducted to assess which drugs are potentially synergistic with the oncolytic virotherapy.

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