

# **A NOVEL ORAL FORMULATION FOR CANCER THERAPY- LOADED IN A SLOW RELEASE MATRIX FOR TARGETED DELIVERY**

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# INTRODUCTION

- **Cancer summit (2015)** wakes us up to the truth about how modern medicine's toxic cancer treatments actually **CAUSE** cancer to keep coming back! (**One of the most common side effects of chemotherapy is more cancer...**).

(Source: Natural News Date 30.03.2015)

Isn't it time we stopped treating disease with poison?

Nerve gas theory of WWII by Dr. Nicholas Gonzalez broadcasted (?) on 26.07.2015 (Source Natural News 26/07/2015).

Free yourself from the cycle of disease and medical interventions, that just don't work. (Source: Natural News Date 30.03.2015)

- Chemo & Radio therapeutic agents for cancer therapy, cause nonspecific killing of cells, whereas **natural products offer holistic, protective and therapeutic** action on all cells with low or no cytotoxicity and are beneficial in producing nutrient repletion to compromised people [Reddy et al 2003].

# Objectives

- A formulation comprising of aqueous extracts of plants and an avian virus (non-pathogenic for human).
- Selectively targeting cancer cells and directing them for apoptosis.
- A hydrogel for oral delivery of the above non-toxic, non-immunogenic formulation, for slow release directly in the intestine.

# Materials and Methods

Aqueous extract of fresh leaves of

1. Belada mara (*Aegle marmelos*) – Indian *Bael*
2. *Oxalis corniculata*
3. Ginseng from the plant *Panax ginseng*
4. Cotyledons of Custard apple (*Annona reticulate*) - *Sitaphal*
5. Seeds of Fenugreek (*Trigonella foenum-graecum*) - *Methi*
6. Newcastle disease virus (Lytic strain)

# Belada mara (*Aegle marmelos*)- (Indian Bael)

- This plant (the favorite of Lord Shiva) has over 20 anticancer constituents.
- It has properties like anti-cancer, anti-diarrhoeal, anti-microbial, anti-viral, anti-pyretic, ulcer healing, anti-genotoxic, diuretic, anti-fertility and anti-inflammatory properties (Rahman & Parvin, 2014). It has high nutritional and environmental values.
- Beside its anti-neoplastic, radio-protective, chemo-protective, chemo-preventive, cardio-protective, anti-spermatogenic, analgesic properties, the leaf has properties par excellence, in the treatment and prevention of cancer (Sharma *et al* 2006).
- It belongs to the family Rutaceae which has a prominent position in both Indian medicine and culture.

# Phytoconstituents in Belada mara (Indian *Bael*) with anti- cancer properties

<u>Marmenol</u> (induce TNF- $\alpha$ , TNFR1, and TRADD mRNA and protein expression, G1 cell cycle arrest and apoptosis)	<u>Marmin</u> (activation of caspase-8 and Bid, release of cytochrome C, suggesting the existence of a cross-talk between death receptor and the mitochondrial pathways. Inhibit AKT and extracellular signal regulated kinase phosphorylation.
Marmelosin	Marmelide
Psoralen	Alloimperatorin
Rutaretin	Scopoletin
Aegelin	Marmelin
Fagarine	Anhydromarmelin
Limonene	$\alpha$ -Phellandrene
Betulinic acid	Marmesin
Imperatorin	Marmelosin
Luvangentin	Auroptene

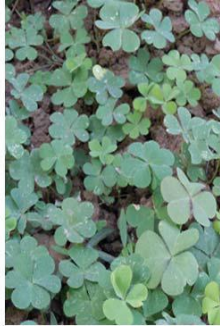




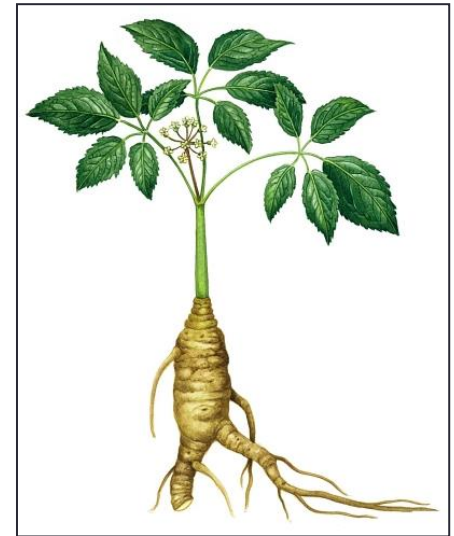
*Belada mara (Aegle marmelos) – Indian Bael*







*Oxalis corniculata*



*Panax ginseng*



**Custard apple (*Annona reticulata*) –Sitaphal**



# Fenugreek (*Trigonella foenum-graecum*) - Methi



### **Oxalis corniculata :**

Phytochemical constituents like flavanoids, tannins, phytosterols, phenol, glycosides, fatty acids, galacto-glycero-lipid and volatile oil. The leaves contain flavonoids, iso vitexine and vitexine-2"- O- beta – D- glucopyrunoside. (Badwaik *et al* 2011). It is rich source of essential fatty acids like palmitic, oleic, linoleic, linolenic and stearic acids and it possesses important activities like Anticancer, Antioxidant, anthelmintic, Anti-inflammatory, Analgesic, Steroidogenic, Antimicrobial, Anti-amoebic, Antifungal, Astringent, Depurative, Diuretic, Emmenagogue, Febrifuge, Cardio relaxant, stomachic and Styptic have also been reported (Kathiriya *et al* 2010).

### **Panax ginseng:**

Ginsenoside Rp1, a component of ginseng, reduces cancer cell proliferation through inhibition of the insulin-like growth factor 1 receptor (IGF-1R)/Akt pathway .

### **Custard apple (*Annona reticulate*):**

Anti-cancer properties of custard apple appear to be mainly due to a class of compounds called acetogenins which are specific to Annonaceae species (Alai *et al.*, 1999; McLaughlin, 2008). Acetogenins are very long chain fatty acids (McLaughlin, 2008).

### **Fenugreek (*Trigonella foenum-graecum*) :**

Several compounds with anticancer properties, including gingerol, cedrene, zingerone, vanillin and eugenol [Al-Daghri *et al* 2012]

# Methods

The present work is divided into four segments:

1. Development of an anti-cancer formulation for selective killing of cancer cells.
2. Development of an inert hydrogel for oral delivery and slow & targeted release in the intestine.
3. Testing the formulation in MCF7 (**Breast cancer**) & NCI60 (**Cervical cancer**) cell lines.
4. Testing the loaded hydrogel (oral & i.p.) *in-vivo* in strain 'A' cancer induced **mice**.

# Development of Phytochemical formulation

The anti-cancer formulation incorporated in the hydrogel for selective apoptosis of cancer cells, comprising AQUEOUS EXTRACTS of

Leaves of Belada mara (*Aegle marmelos*) - 50 mg/ml fc

Ginseng from the plant *Panax ginseng* - 35 mg/ml fc

Fresh leaves of the plant *Oxalis corniculata* - 50 mg/ml fc

Custard apple (*Annona reticulate*) - 25 mg/ml fc

Seeds of Fenugreek (*Trigonella foenum-graecum*) - 30 mg/ml fc

Newcastle disease (lytic strain) virus (5 $\mu$ g/ml) suspended in 1X Phosphate- saline buffer (pH= 7.5) - 0.1 $\mu$ g/ml fc



# Hydrogel development (Cont.)

- The hydrogel was prepared by dissolving

Agarose: 0.4 – 1.4% fc

Casein hydrolysate: 0.003-0.005 g/ml fc

PEG (4000): 0.1-0.3% fc

Glycerol: 0.1-0.8% fc

PBS (1X) : qs

Heat at 80°C to dissolve

- Addition of anti-cancer formulation is made during solidification.
- The liquid suspension was poured in a 360 $\mu$ L well of an ELISA plate and cooled.

- Liquid FTIR was done to identify the active functional groups that were responsible for interaction.
- MTT assay was done to check cytotoxicity of the formulation with NDV and with/without gel on MCF7.
- Medication was administered orally or intra-peritoneally, 2 times a week with 200µl of the formulation.
- Medication continued for 45 days and then discontinued.

N.B. Average life of cancer induced, untreated mice was 19 days.

# Hydrogel Characterization

FTIR measurements were done on a FTIR-ATR model Alpha-P, Bruker by using dry agarose-casein hydrolysate hydrogels. The content was chilled in liquid nitrogen and then crushed in a mortar pestle to get a fine powder.

Powdered samples were stored in a vacuum desiccator until use.

SEM and TEM micrographs of the cylindrical surface and cross-section of the swollen hydrogels were taken.

# Slow release behavior

- Studies on **Time dependent release** was performed after incorporation of the phytochemical combination.
- Release was checked by UV-Vis spectroscopy for increased absorbance.



## Results & Discussion:

### Treatment of MCF7, NCI60 and DF1 cell lines with Phytochemicals

Cancer cells 'MCF7' (**Breast cancer**) and 'NCI60' (**Cervical cancer**), grown *in vitro* using DMEM media at 37°C temperature, in the presence of 10% fetal bovine serum (FBS) and 10% CO<sub>2</sub> and treated with phytochemical combination.

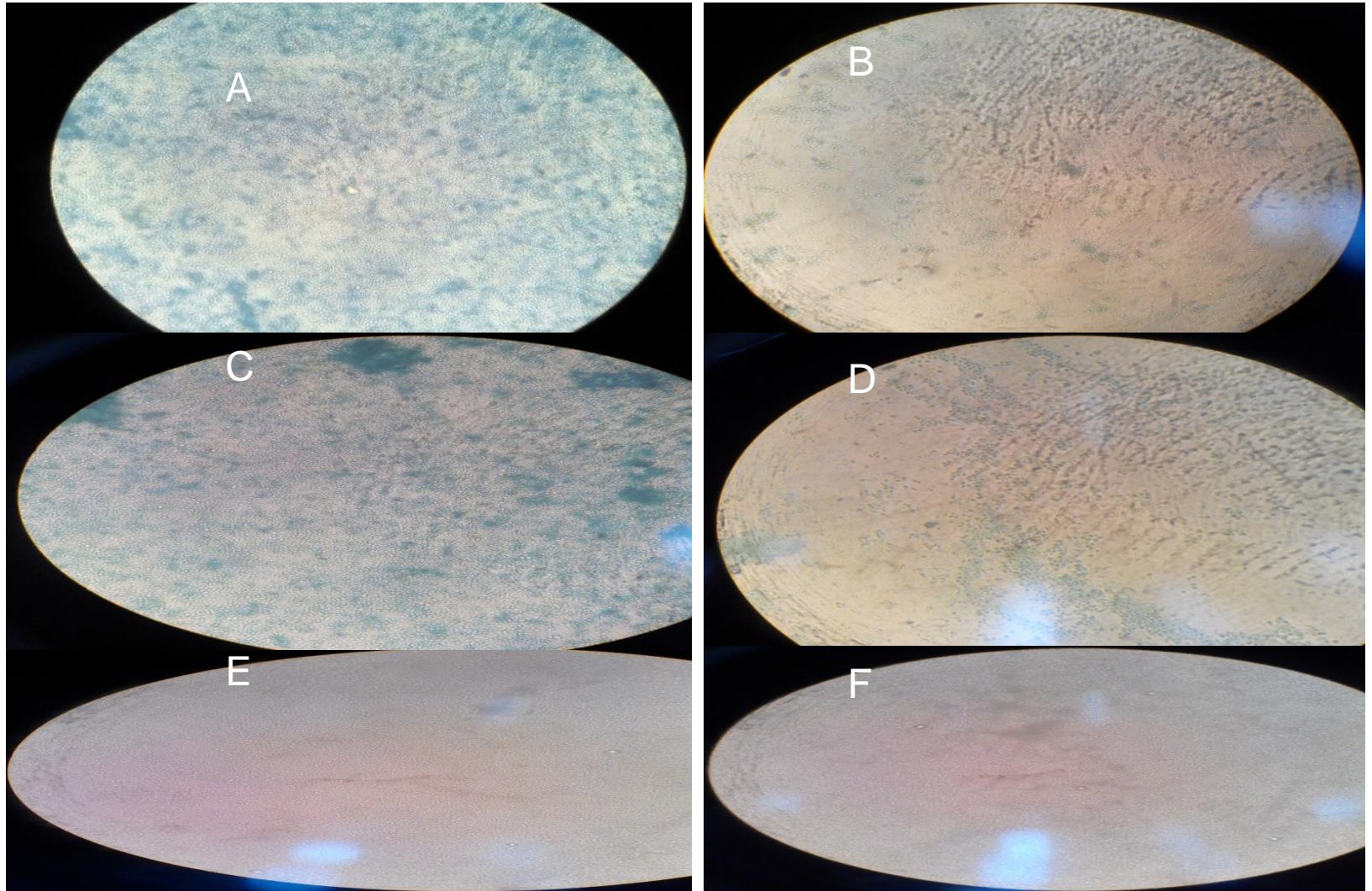
**As a control**, healthy avian fibroblast cells 'DF1' were grown as recommended and then treated with the same formulation.

**Apoptosis of both cancer cell lines were observed.**

**The control did not show any sign of apoptosis nor a drastic fall in cell number when counted in a hemocytometer, unlike the case with both 'MCF7' and NCI60 cell lines.**

All Images were taken under inverted microscope and cell counting done using a hemocytometer.

## Treatment on different cancer & healthy cell lines



A: MCF7 prior to exposure. B: MCF7 post exposure. C: NCI60 prior to exposure. D: NCI60 post exposure. E: DF1 prior to exposure. F: DF1 post exposure.

# Animal Experimentation

## EARLY MEDICATION

1. Tumor regressed and all the animals survived (100%) when medication was started as soon as tumor was visible after administration of a lethal dose of ionizing radiation or injection of cancer cells. (Medication started approximately 10 days after tumor induction or when tumor was clearly visible).

**Conclusion:** All animals survived & Tumor regressed when drug was administered at an early stage.

## DELAYED MEDICATION

2. Over 67% animals survived even after delayed start of medication (when large tumors were distinctly visible). After 3 months, animals were sacrificed on humanitarian ground, as tumors were neither regressing nor enlarging.

**Conclusion:** Tumor progression stopped, maintained its current state and animals survived for an extended period of time when drug was administered at a later stage.

**N.B.** Mice usually dies in 18 to 20 days after lethal dose of ionizing radiation (tumor induction).

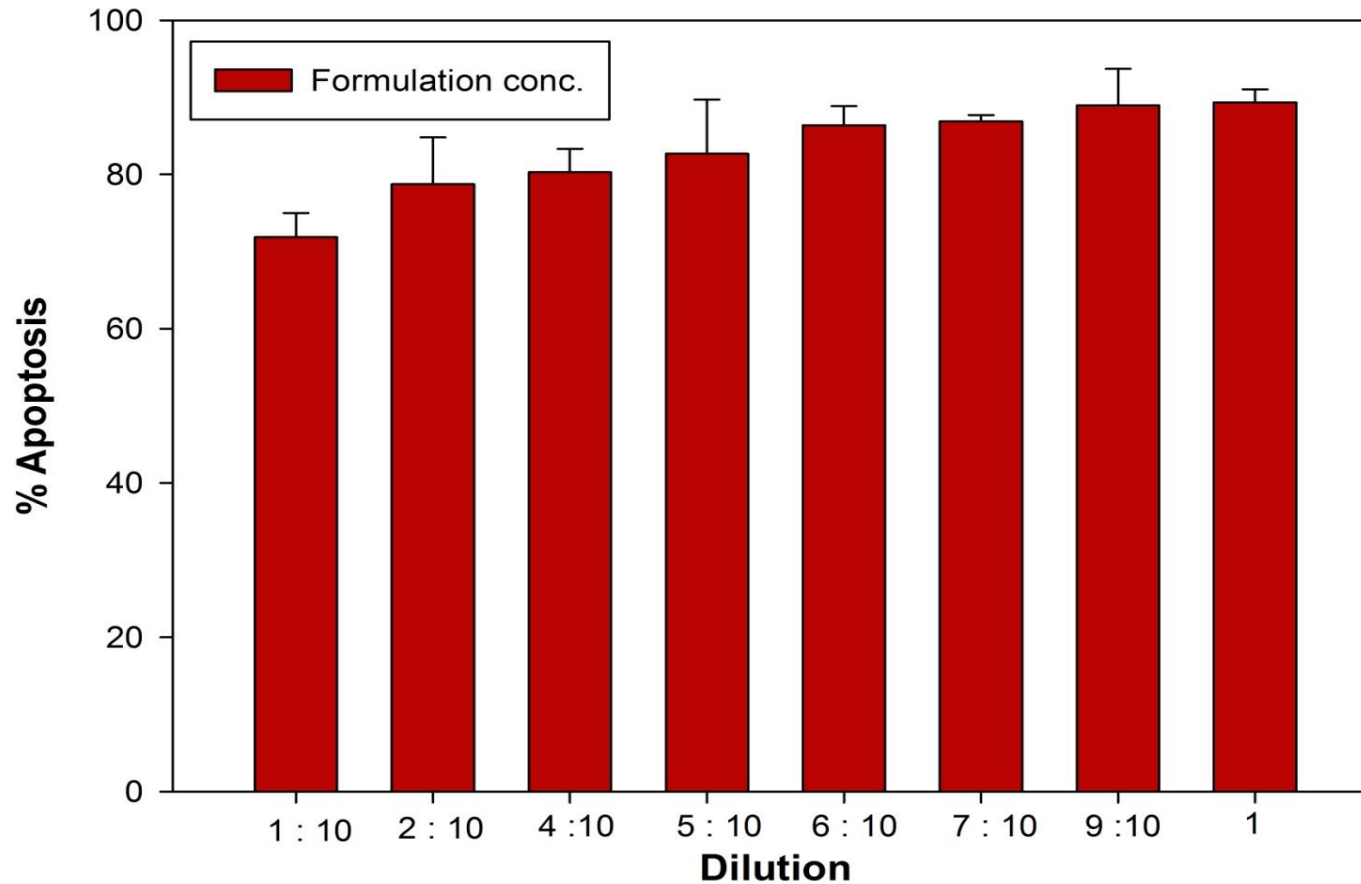


○ Tumor induced by injecting cancer cells.



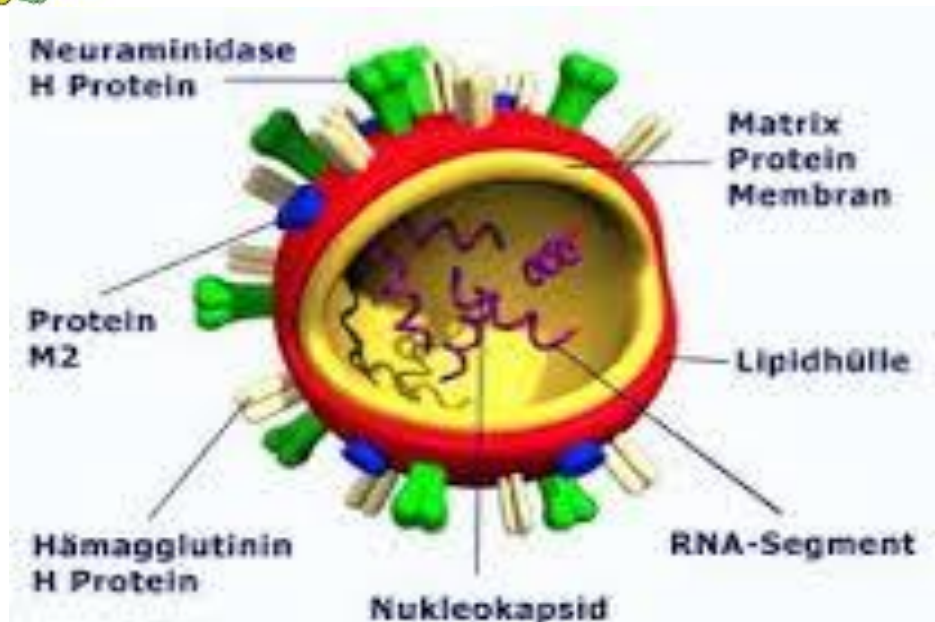
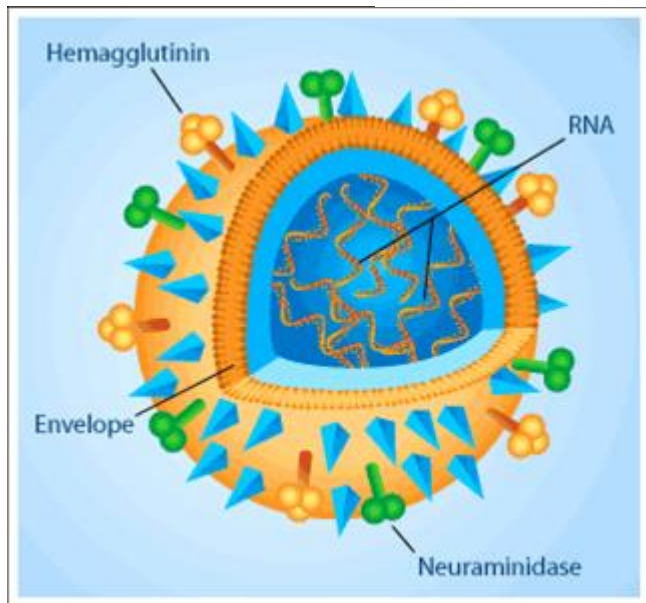
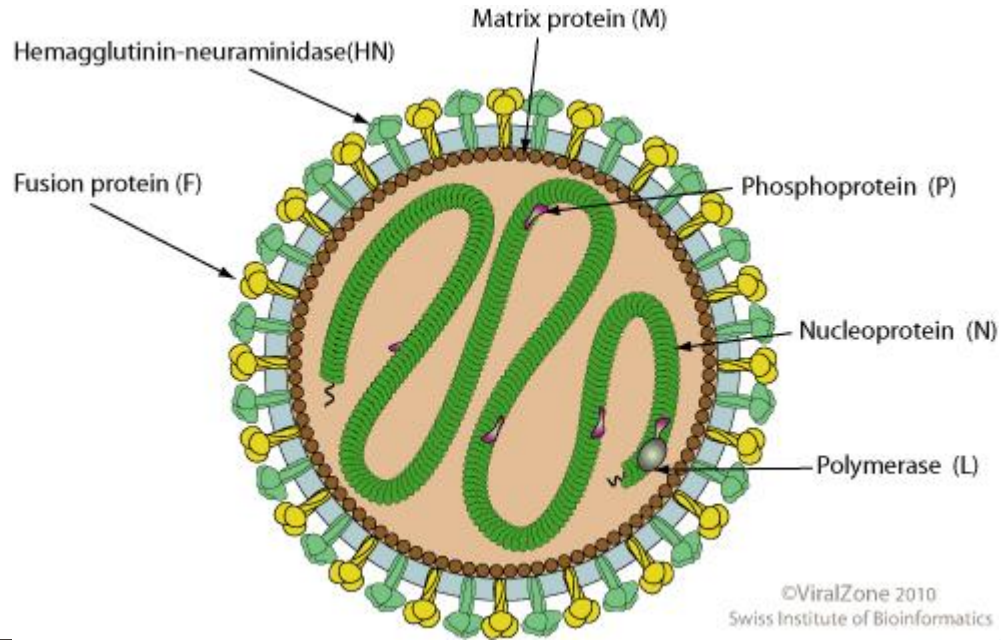


## MTT assay showing Apoptosis of MCF7



Dilution was made using sterile ultra pure water  
( formulation even in 1:10 dilution, is >70% effective)

# Newcastle Disease virus



# Advantage of NDV

1. The avian virus is not pathogenic to human.
2. Selective killing of tumor cells.
3. NDV mediated oncolysis to be efficient while treating chemo-resistant tumors associated with apoptosis resistance.
4. Ability of virus to bind to tumor cell surface via its hemagglutinin-neuraminidase (HN) and fusion (F) glycoprotein. Although, NDV enters healthy & tumor cells alike, they can't replicate in healthy cells. This is due to action of PKR, Mx and OAS1a, also prolific Type-1 IFN response, RIG-1, IRF-3, IRF-7 and IFN- $\beta$ . In cancer cells, these genes are deregulated, so virus produces proteins required for replication.
5. Virus replicating rapidly in tumor cells, also modifies the cell surface by increasing expression of the viral HN and F proteins after about 10 hours). This lead to an enhanced expression of viral antigen on tumor cell surfaces.



# Advantage of NDV (cont.)

6. Ability of virus to induce synthesis of cytokines, like IFN, TNF, NK cells, NFkB etc. in normal cells.
7. Pleiotropic immuno-stimulatory effect of NDV, can augment the effect of T helper ( $T_H$ ) cells, cytotoxic T lymphocyte (CTL), NK cells, and macrophages, in normal cells.

# Challenges of NDV

First report of NDV application dates back to 1950s, when NDV and adenovirus were used to treat uterine carcinoma resulting in partial necrosis.

However this was soon followed by re-growth, possibly due to production of neutralizing antibodies against the virus.

## Possible solutions:

\*Use of Lytic strains like *Italien*, *MTH68/H*, *PV-701*, *73-T* etc.

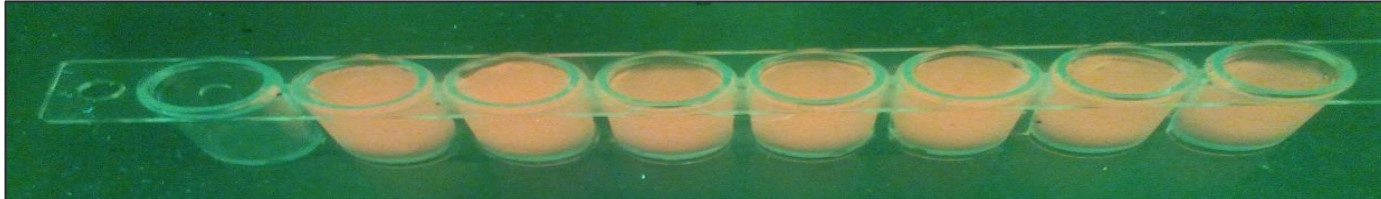
\*Herbal rescue (Herbs & NDV)

Enhanced inhibition of PKR, Mx, OAS1a and down/ deregulation of RIG1, IRF3, IRF7 and IFN- $\beta$

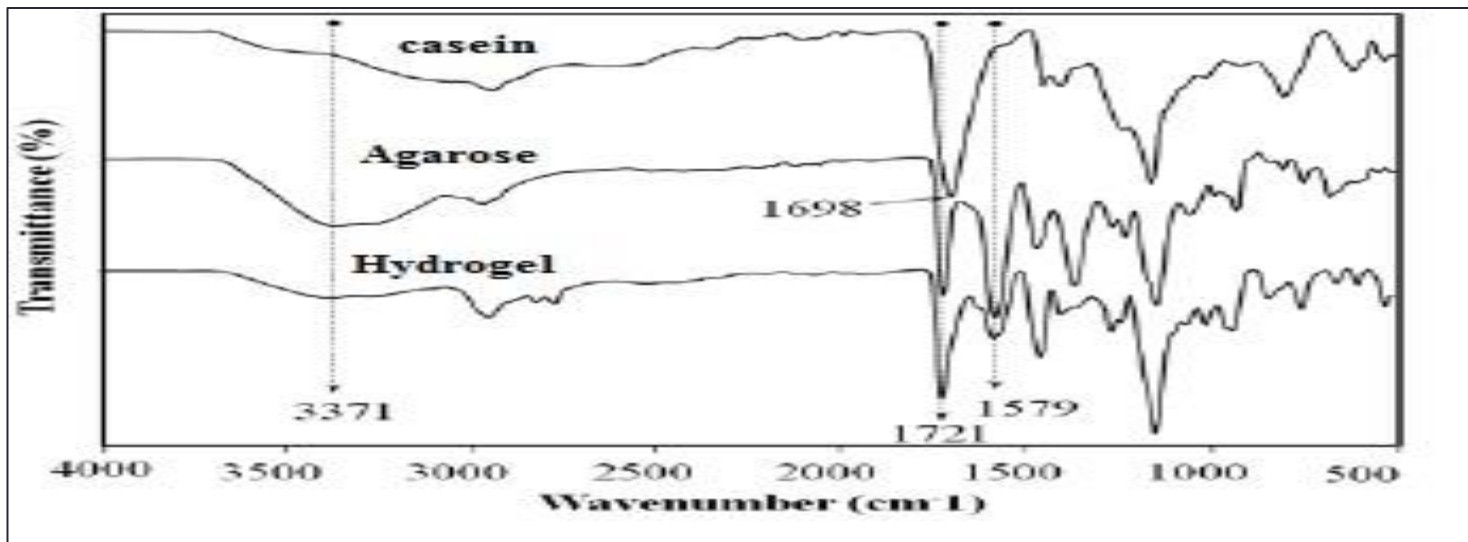
\* our approach

# Conceptual applications of NDV in treatment of cancer and other diseases

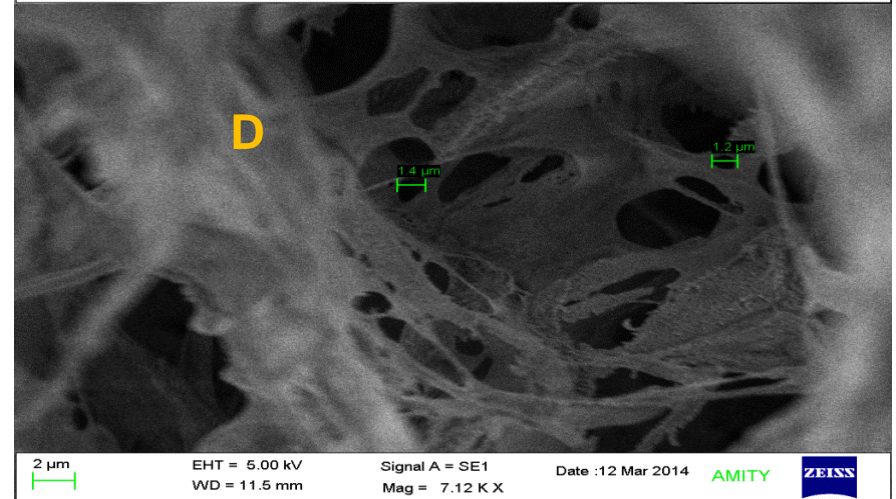
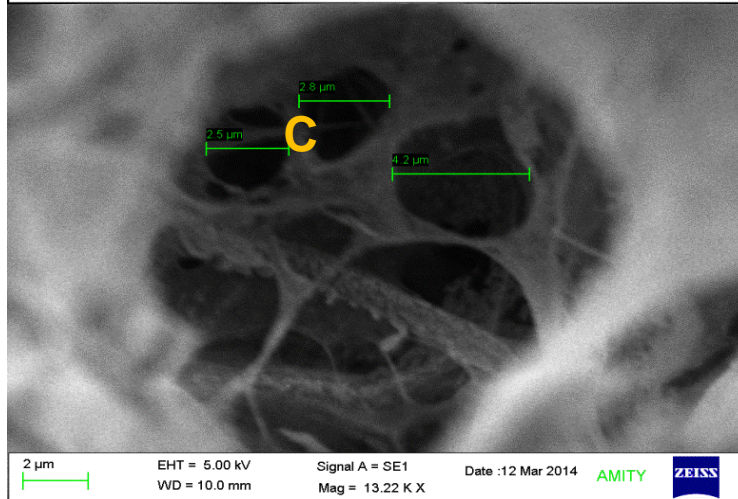
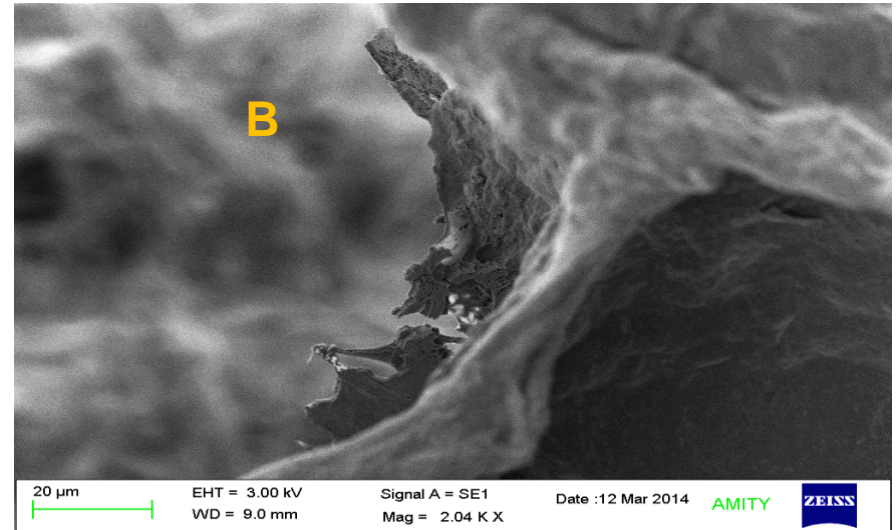
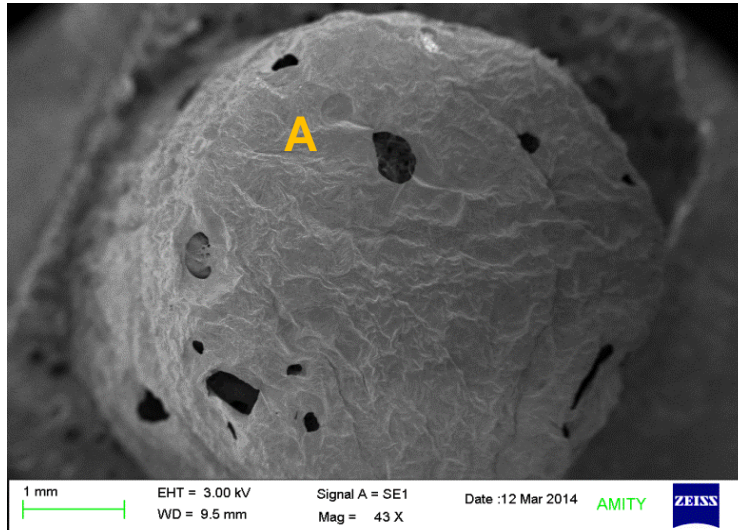
1. Use for tumor selective cytolysis (oncolysis).
2. Use of NDV as an adjuvant in a tumor vaccine for stimulation of T lymphocytes (CTL) and delayed-type hypersensitivity (DTH) responses after antitumor vaccination.
3. Use of NDV for nonspecific immune stimulation and induction of cytokines, like IFNs, TNF etc.
4. Use of NDV as viral vector for delivering therapeutic genes.
5. Use of NDV as a vaccine vector for immunization.



Hydrogel with the formulation casted in 360  $\mu\text{L}$  well of a flat bottom Thermo Scientific ELISA plate

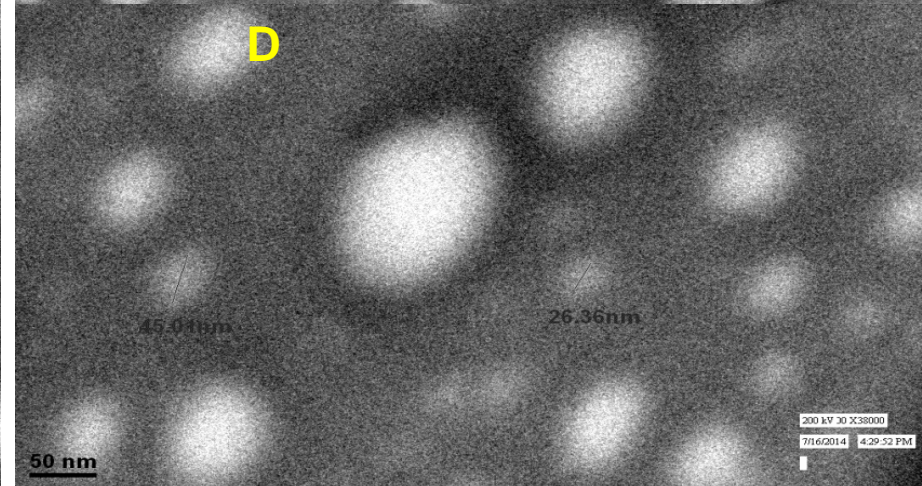
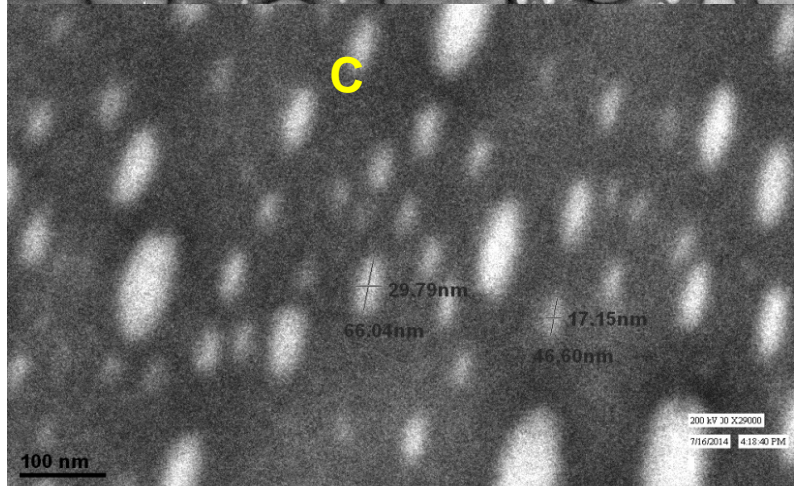
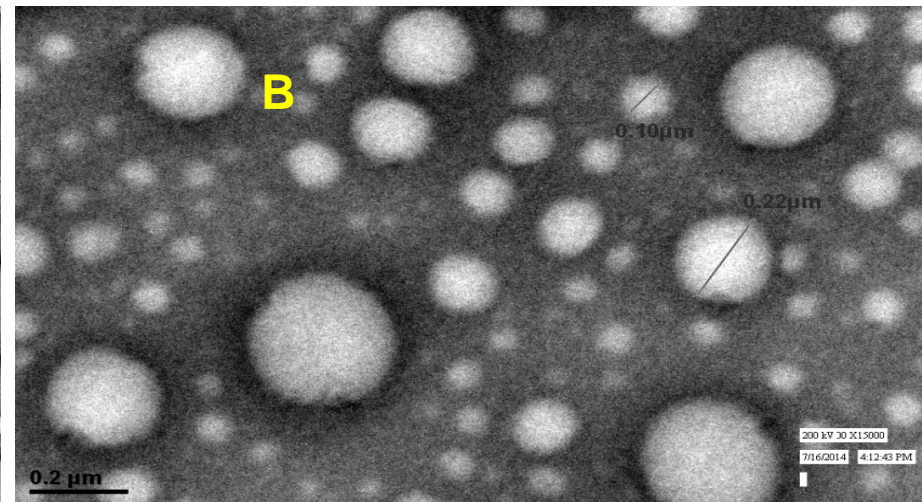
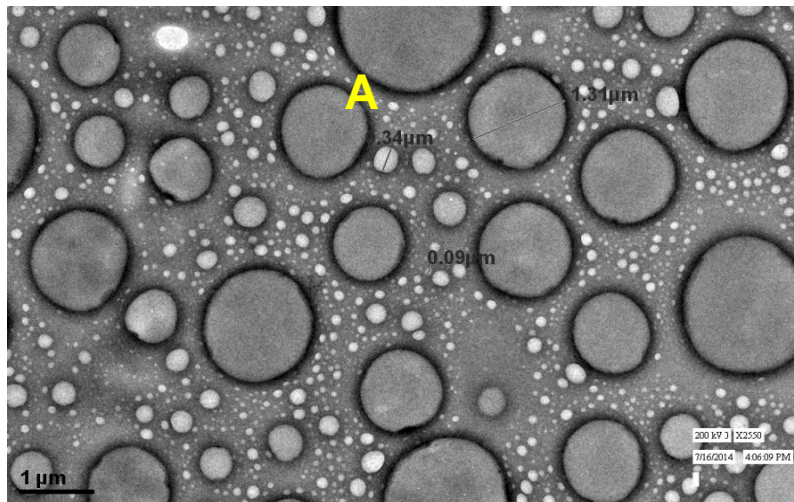


FTIR-ATR spectra of casein, agarose and the hydrogel



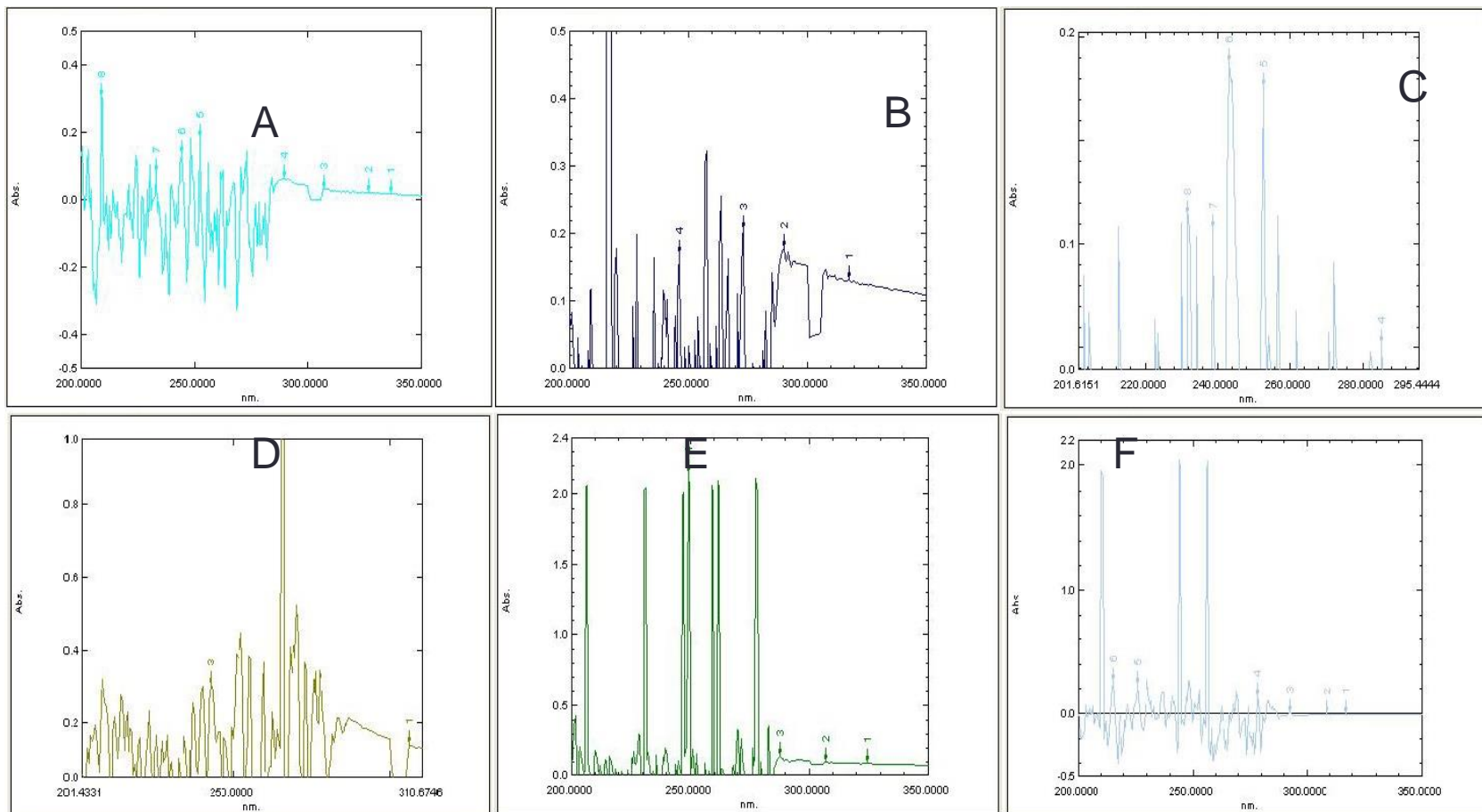
*SEM micrograph of hydrogel. A: top surface B: Cylindrical surface C: cross section with large pores. D: cross section with small pores and granular structure.*





TEM micrograph of the hydrogel. A: 1 μm resolution showing pores and Micelles B: Micelles visible at 0.2 μm resolution C: Cross-section area of Nano-Pores D: Nano-pores at a resolution of 50 nm.





UV-Vis Spectra of the release of the phytochemical formulation from the gel.  
 A: 1h, B: 2h, C: 3h, D: 4h, E: 24h, F: Control: 0h,

THANK YOU

# Mode of action of NDV

- NDV induces cytotoxicity via 2 mechanisms:

- (1) HN viral protein interact with APCs & also tumor cell surface, facilitating interaction between immune cells and tumor cells.

- (2) Local induction of cytokines (type I IFN), which function for cell migration, activation and differentiation. [NDV is a strong type-I interferon inducer in normal cells]

- Based on the mechanism by which oncolysis is achieved, NDV has been classified as **lytic or non-lytic strains**: Lytic strains cause syncytia (multinuclear) formation to induce changes in the plasma membrane (HN?) resulting in lysis of target cells. Non lytic strains cause tumor regression slowly by disrupting normal host cell metabolism, preventing further tumor growth without causing lysis.

- Depending on their properties such as cytotoxic effects, replication rates etc. both lytic and non lytic strains of NDV have been evaluated. Non lytic strain such as *Ulster*, produces non-infectious viral particles with superior cytotoxic effects when it infects monolayer colon carcinoma cells. Lytic NDV strains such as *Italien*, *MTH68/H*, *PV-701*, *73-T* etc., cause production of infectious particles that can infect other tumor cells, thus leading to an amplification of the viral load resulting in syncytium promotion and plaque degeneration.

• Upon infection, NDV undergoes replication rapidly, releases from the host cell and infects neighbouring tumor cells. These have different effects on tumor cell, contributing to its therapeutic properties:

1. The lytic strains of the virus induces lysis and prevents further tumor spread
2. The non lytic strains causes enhanced immunostimulatory response, post infection.
3. The virus itself stimulates the production of effector cytokines such as interferons (IFN- $\alpha$ ) or TNFs which activate NK cells, macrophages and sensitized T-cells to kill the tumor cells.
4. Apoptosis
  - a. NDV induces expression of various cytokines which can activate extrinsic death pathway (involves transmembrane death receptors) causing oncolysis.
  - b. NDV also augments the expression of induced nitric oxide synthase (iNOS) in infected cells inducing apoptosis also through intrinsic death pathway (non-receptor mediated intracellular signals).
5. NDV induces NF $\kappa$ B and upregulates the expression of MHC I genes inducing oncolysis through NF $\kappa$ B pathway.

# Tumor selectivity

- The first step of infection by NDV takes place in all cell types whereas the second step (which corresponds to viral replication) occurs only in tumor cells since it is stopped very rapidly in normal cells.
- Tumors provide a relatively permissive substrate for the propagation of RNA viruses such as NDV because mutations in tumor cells often cripple the IFN system to allow un-inhibited proliferation and to provide resistance to apoptosis
- Tumor cells have a weaker type 1 IFN response and a weaker sensitivity to type 1 IFN-receptor (IFNR) signalling. The impairment of interferon pathways appears to be a rather common feature during tumorigenesis. That makes tumor cells highly susceptible to NDV infection and to the ensuing oncolytic events. Stimulates the production of cytokine response, like IFN and TNF, and that of heat shock proteins, adrenocorticotrophic hormone, and tissue inhibitor of metalloproteases.
- Pleiotropic immunostimulatory effects of virus as it can augment the effects of T helper ( $T_H$ ) cells, cytotoxic T lymphocyte (CTL), NK cells, and macrophages
- With defects in the anti viral protein PKR (protein kinase dsRNA activator) signaling and IFN system, however, there is an added advantage for the use of viruses to destroy tumor cells.
- dsRNA dependent PKR is present in a form that results in a defective signalling pathway in tumor cells, thereby enabling the virus to synthesize its proteins. Also the virus itself produces proteins to avoid any existing effect of the PKR and associated antiviral defense.

•Upon infection, NDV has been found to induce apoptosis via mitochondrial pathway activation. this results in opening of mitochondrial permeability transition pores and loss of mitochondrial membrane potential, leading to release of SMAC/DIABLO and cytochrome c causing suppression of inhibitory apoptosis proteins (IAP) and binding to apoptotic protease activator factor-1 (APAF-1) and procaspase 9 respectively, forming the apoptosome.

•This cause activation of apoptosis. However, if this form of defense mechanism occur prior to viral assembly, it could prevent further viral infection thereby lowering the net oncolytic effect.



- A recombinant NDV strain NDV-NS1, comprising of an anti apoptotic protein NS1 used in a study showed enhanced oncolytic activity demonstrating enhanced NDV replication and enhanced fusogenicity allowing neighbouring cell fusion and thus enhancing syncytium formation.

- Similarly, the anti apoptotic protein Bcl-xL is responsible for **showing resistance to anti cancer agents**. In a study using A549–Bcl-xL cells it was found that NDV-mediated cytolysis and fusogenicity are enhanced compared to those in A549-neo cells. Presence of significantly higher viral titers and viral proteins in A549-Bcl-xL cells than in A549-neo cells indicated that viral replication was enhanced in apoptosis resistant cells. A549–Bcl-xL cells showed an overexpression of Bcl-xL, allowing early NDV replication and production of type 1 IFN thereby inducing activation of apoptosis by antiviral signalling. This resulted in more cells infected by viral replication.

- Type I IFN has many tumor suppressor effects some of which are mediated through the enhancement of apoptosis induction in cancer cells and cell cycle arrest mediated by many IFN stimulated genes (ISG) or through host-dependent mechanisms including upregulated expression of major histocompatibility complex class I and II molecules, antigen presentation, memory T cell survival, and CD8<sup>+</sup> T cell recruitment many of which occur during NDV infection.

•All these factors prove NDV mediated oncolysis to be efficient while treating chemo-resistant tumors associated with apoptosis resistance. This was proven in a study wherein melanoma showing chemo-resistance to IAP Livin was found to undergo oncolysis on administration of NDV-HUJ.

•NDV could be used as a form of **monotherapy** or could be used in conjugation with conventional therapies so as to open the apoptotic pathways and sensitize the cells to the chemotherapeutic agents resulting in a synergic effect.

•This subsequently led to higher number of cells being infected during the following **viral replication cycles allowing deeper tumor penetration and oncolysis** with a low initial viral inoculum.

• Like many other viruses, NDV had developed some mechanisms to delay apoptosis in the infected cells in order to facilitate the viral replication such as the antiapoptotic v protein blocking the IFN.

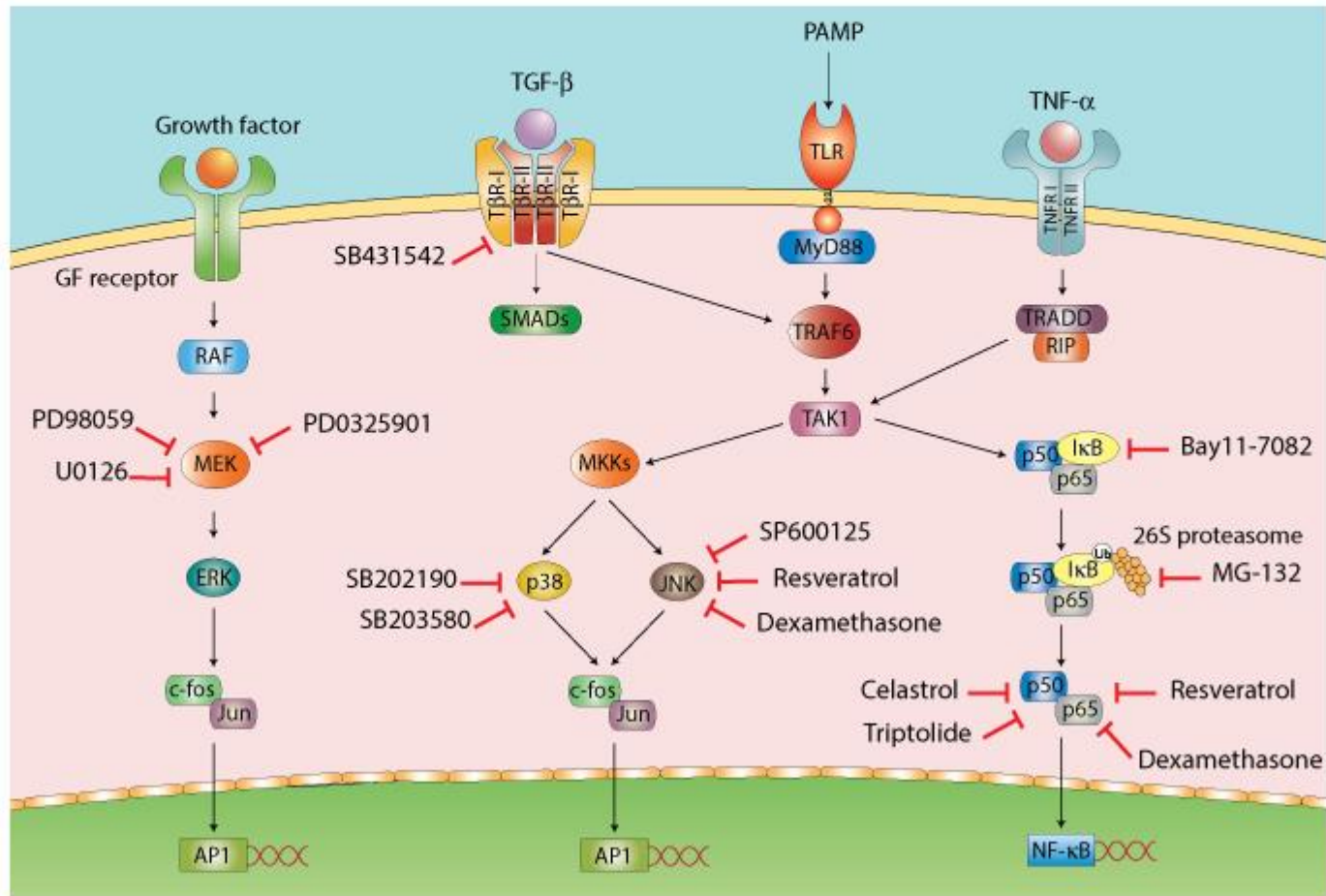
•After initial viral entry, **non-cancerous cells quickly undergo apoptosis**, resulting in non-productive infection, while apoptosis-resistant cancer cells survive longer, producing more virus particles and initiating new rounds of infection.

Sl. No.	Dil. Ratio	Absorbance 1	Absorbance 2	Absorbance 3	Avg. Test Sample Absorbance	Control Absorbance	Avg. Control Absorbance	% Apoptosis of MCF7	% apoptosis lower limit	% apoptosis higher	Range
1	1:10	0.1732	0.1802	0.1975	0.2169	0.769	0.771	71.87	74.38	77.54	3.15
2	2:10	0.1467	0.1527	0.1936	0.164	0.835		78.73	74.89	80.97	6.08
3	4:10	0.1408	0.1643	0.1506	0.1519	0.685		80.30	78.69	81.74	3.05
4	5:10	0.1129	0.1295	0.0586	0.1336	0.845		82.67	85.36	92.40	7.04
5	6:10	0.117	0.1026	0.0979	0.105	0.723		86.38	84.82	87.30	2.48
6	7:10	0.0987	0.1005	0.1049	0.101			86.90	86.39	87.20	0.80
7	9:10	0.0931	0.0994	0.063	0.0851			88.96	87.11	91.83	4.72
8	1	0.0747	0.0841	0.0878	0.0822			89.34	88.61	90.31	1.70

# NF-κB & MAPK Activation Inhibitors

In vivoGen provides a choice of inhibitors of the nuclear transcription factor **NF-κB** (nuclear factor kappa-light-chain-enhancer of activated B cells) and of the **MAPKs** (mitogen-activated protein kinases).

NF-κB and MAPK pathways regulate the expression of many genes involved in a variety of processes including inflammation, apoptosis, tumorigenesis, and auto-immune diseases. Several of the **PRR** (pattern recognition receptors) signaling cascades culminate in the activation of NF-κB transcription factor and /or MAPK. The inhibitors can be used to dissect these signaling pathways.



- PRR and related shRNAs
- Antibodies for Neutralization
- TLR Antagonists

### Literature



- Newsletters
- Catalogs
- Reviews
- Posters

## NF-κB & MAPK Activation Inhibitors

<a href="#">AZD6244</a> <b>NEW!</b>	MEK1/2 Inhibitor
<a href="#">Bay11-7082</a>	NLRP3 Inflammasome Inhibitor - IκB-α Inhibitor
<a href="#">Celastrol</a>	NF-κB inhibitor
<a href="#">Dexamethasone</a>	NF-κB Inhibitor - MAPK Inhibitor
<a href="#">MG-132</a>	26S proteasome inhibitor
<a href="#">PD98059</a>	MAP Kinase Inhibitor - MAP Kinase Kinase Inhibitor
<a href="#">PD0325901</a>	Reprogramming Enhancer - MAP Kinase Inhibitor - MEK Inhibitor
<a href="#">PLX4032</a> <b>NEW!</b>	B-Raf Inhibitor
<a href="#">PLX4720</a> <b>NEW!</b>	B-Raf Inhibitor
<a href="#">Resveratrol</a>	NF-κB inhibitor - mTOR inhibitor - Autophagy inducer
<a href="#">SB202190</a>	MAP Kinase Inhibitor - p38/RK MAP Kinase Inhibitor - Autophagy inducer
<a href="#">SB203580</a>	MAP Kinase Inhibitor - p38/RK MAP Kinase Inhibitor - Autophagy inducer
<a href="#">SB431542</a>	Reprogramming Enhancer - TGF-β receptor inhibitor
<a href="#">SP600125</a>	MAP Kinase Inhibitor - Autophagy Inhibitor - JNK inhibitor
<a href="#">Triptolide</a>	NF-κB Inhibitor
<a href="#">U0126</a>	MAP Kinase Inhibitor - Autophagy Inhibitor - MEK1 and MEK2 Inhibitor
<a href="#">ZM 336372</a>	RAF-1 Inhibitor