ST6Gal 1 Targeted and New Castle Disease Virus Mediated Combinational Therapy for Cancer

Submitted by

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In

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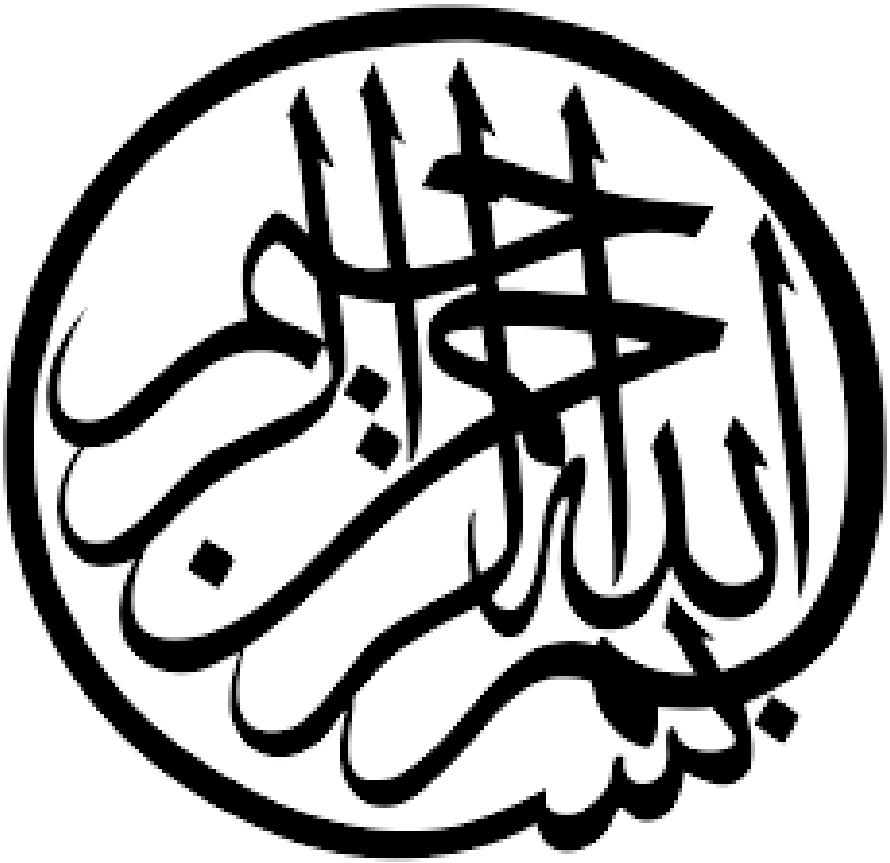

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Tahira Khan

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List of Acronyms

AAF	Amino Allantoinic Fluid
APAF-1	Apoptotic protease activating factor 1
APC	Antigen-presenting Cells
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl Sulfoxide
dNTP	Deoxynucleotide
DR-4	Death Receptor 4
FBS	Fetal Bovine Serum
GAPDH	Glycerol-3-Phosphate Dehydrogenase
HA	Hemagglutination Assay
HBA	Hepatitis A Virus
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus

HN	Hemagglutinin-neuraminidase protein
IFN-1	Interferon-1
IRF-3	Interferon Regulatory Factor 3
LymphoSep	Lymphocyte Separation Media
MDA-5	Melanoma Differentiation-Associated protein 5
MHC	Major Histocompatibility Complex
NDV	Newcastle Disease Virus
NF-kβ	Nuclear Factor kappa light chain enhancer of activated B cells
NP	Nucleocapsid protein
PBMCs	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PEL	Percutaneous Ethanol Injection
RBCs	Red Blood Cells
RIG-1	Retinoic acid-inducible Gene 1
RNA	Ribonucleic acid

RT	Reverse Transcriptase
TAA	Tumor-Associated Antigens
TACE	Transcatheter Arterial Chemoembolization
Tm	Melting Temperature

ABSTRACT

Cancer is a global problem with a high mortality rate and morbidity rate affecting 8.2 million cases annually). The current treatment regimens for cancer include chemotherapy, radiotherapy and anticancer drugs. Apart from being cancer therapeutics they have a lot of side effects and cause problems like chemoresistance, mutagenesis, radiation induced mutations, secondary tumorgenesis, limiting patient quality of life and survival. Therefore we need to focus on molecular targeted therapies for cancer boosting the immune system. The current project focusses on the combinational therapy for cancer involving in silico drug design(ST6Gal I inhibitors) and biological therapy (Newcastle disease mediated virotherapy) to effectively combat cancer.

Aberrant glycosylation is the hallmark of cancer cells regulated by sialyltransferases enzymes in the cells. Out of these enzymes ST6Gal 1 belonging to the ST family is overexpressed in more than 20 cancer types having roles in invaseness, chemoresistance and malignancy. The insilico part of the study focuses on the use of aromatic and flavonoid compounds with potent biological activity as ST6Gal I inhibitors. Structure based approach was used in the study and the compounds were docked in the binding cavity of the enzyme and ligand-protein interactions were computed to understand the binding affinity and interactions of these compounds with the target site so that target hit molecules and lead compounds could be isolated from this approach and use as potent inhibitors against ST6Gal .

The second therapeutic regimen investigates the role of New castle disease virus Pakistani strain with the cancer cells and its interactions with the innate immunity; interferon and apoptosis pathway was studied. NDV effectively replicated in Hela cells effectively utilizing the RIG-

1/MDA5 pathway. The type 1 interferon was induced leading to the activation of JAK /STAT pathway. NDVPv uses the extrinsic pathway of apoptosis using the Fas/L death receptor pathway using caspase 8 leading to intrinsic pathway of activation independent of NF-KB pathway.

Introduction

Cancer is global challenge humanity is facing with a high morbidity and mortality rate globally. 8.2 million People die each from cancer is estimated to be the cause of 13% deaths annually. There is a 70% expected increase in the number of cancer cases by 2030 (WHO, 2016). Cancer is basically the uncontrolled proliferation of cells leading to the inflammation, angiogenesis and metastasis across all parts of the body. There are many types of cancers of different organs of the body parts and tissues. Some of the most common cancers with highest mortality rates are lung, liver, breast, brain, stomach, colon, pancreatic, cervical cancers including others. There are numerous risk factors associated with cancers including aging, smoking, bacterial infections, parasites, viruses, dietary factors, obesity, Nitric oxide, free radicals, hormonal imbalance, produced in tissues including others (Ohshima *et al*., 1994 Brawley *et al.*, 1998; Kelsey *et al.*, 1993; Moller *et al*., 1994; Pike *et al*., 1983). The risk factors damage the DNA inside the cells leading to mutations, chromosomal aberrations, activation of oncogenes, changes in epigenetic material of cells ultimately leading to dysfunction in the normal cell cycle clock causing abnormal proliferation of cells. The aberrations in the genetic material cause the cells to escape apoptosis, alteration in signaling pathways and encompassing molecular mechanisms undergoing immune escape (Gibbs et al., 1994; Hinds et al., 1994). There are different sizes, grades and location of tumors. The type of therapy is specifically dependent on the cancer types and above mentioned factors. There has been advancement in cancer therapeutics for the past twenty years and diagnosis and prognosis of cancer has improved remarkably. The current therapies include chemotherapy which has been observed as one of the most effective approaches to deal with cancer depending on the patient condition, tumor type, survival rate and drug delivery system used for targeting the drug to the target tissue(Moller et al., 1994; Pike et al., 1983).

Chemotherapy being the main treatment regime has a number of side effects including chemoresistance as one of the chief ones. Chemoresistance being a complex problem could be innate, acquired, from a single class of drugs or multiple drugs altogether with a wide array of clinical manifestations. Chemoresistance mechanisms in cells include mutations in cell cycle check points, immune system check points, changes in chromatin material and DNA repair mechanisms, tumor stroma modification, efflux of the chemotherapeutic agents into the tumor stroma alongside other physiological processes (Chien *et al*., 2008; Gatti *et al*.,2005; D'Amato *et al*, 2006; Coley *et al*.,2008). Keeping the complications arising due to chemotherapeutic agents that include chemoresistance, secondary tumorgenesis, cancer relapse in patients, limited survival rate damages to the cell cytology and physiology. There has been focus on development of target specific therapies that has high specificity and involves one single target molecule or a specific receptor (Fauvel *et al.,* 2014). In the present decade a lot of focus has been on immunotherapy and therapeutic agents that strengthen the immune system to fight cancer effectively it involves the use of monoclonal antibodies, targeting immune checkpoint inhibitors including anti PD-1 and CTLA -4 inhibitors (Fauvel *et al.,* 2014; Stern *et al.,* 2006; Kahl *et al.,* 2008; Nadler *et al.,* 1980). Antibodies targeting growth receptors, cell surface glycoproteins and tumor associated antigens (Kahl *et al.,* 2008; Nadler *et al.,* 1980). These therapies are still under clinical trials and data obtained from them is insufficient and doesn't provide efficacy in clinical settings when compared to histological controls. Therefore we need to make thoughtful changes in cancer treatment regimens and therapeutics focusing more on the combination to target cancer in a better way (Baeuerele *et al*., 2009; Rosenberg *et al*., 2004; Sharkley *et al*., 2006). The present study focusses on the use of two strategies which might be helpful in prognosis and diagnosis of cancer can be combined together for future cancer treatment regimens. The first

strategy is in silico drug inhibitors against enzymes contributing in aberrant glycosylation a hallmark of cancer cells. Cell surface glycoproteins are covered with negatively charged molecules known as sialic acids that are over 50 naturally occurring sialic acid derivatives containing a nine carbon atoms of neuramic acid. N-glycolneuramic acid (Neu5Gc) and i-acetylneruamic acid (Neu5Ac) being the most commonly occurring ones (Varki 1992; Chou *et al.,* 1998). Sialic acids have variety of functions in cell physiological and metabolic processes including growth, fertilization, apoptosis, transmembrane signaling and variety of other cellular processes (Schultz *et al*., 2012; Schauer *et al.,* 2009) Altered sialyation is the principal characteristic of tumor cells but the molecular mechanisms encompassing this are not well understood. In order to deepen our understanding about the molecular mechanisms of sialyation on tumor cells we need to discuss the role of enzymes regulating the sialic acid levels in cells. The level of sialylconjugates on the cells is controlled by a diverse class of enzymes known as *sialyltransferases* (ST) s that are encoded by more than 20 human genes. These enzymes transfer the sialic acid moiety from activated cytidine 5'-monophosphate N- acetyl neuramic acid (CMP –Neu5Ac) to a sialic acid or a glycan with attached galactose moiety. The ST enzymes are classified into three groups as ST3, ST6 and ST8 transferases on the type of alpha glycosidic bond they have with the C2 atom of the sialic acid residues (Takashima *et al.,* 2002; Kuhn *et al.,* 2013).Having defined the role of hypersiaylation in cancer progression which is regulated by a number of ST enzymes undergoing mutations and changes in gene expression. The role of human ST6GAL1 is substantial in tumor progression, malignancy, invasiveness and metastasis (Azab *et al.,* 2008; Hedlund *et al*., 2008.). There is an overexpression of the α2-6 sialic acid residues on the cell surface regulated by the ST6GALI enzyme which has been observed in many cancer types including breast, liver, colon, cervix, brain, acute myeloid leukemia and a number

of other different cancer types (Dall'Olio *et al*., 2001; Recchi,*et al.*,1998; Dall'Olio *et al*., 1992; Wang *et al.*,2001; Zhao *et al*., 2014; Sata *et al*.,1991; Julien *et al.,* 2006).. Tumor cells escape apoptosis which can be correlated with the selective enrichment of the α2-6 sialic acid residues on the tumor cells. Hypersiaylation of the sialic acid of cell surface galectin ligands block the apoptosis pathways. Fas and TNFR signaling pathways are downregulated in cancer cells promoting immune escape (He & Baum 2006; Ochieng *et al*., 2002; Elola *et al.,* 2007; Zhou *et al.,* 2011; Swindall *et al*.,2011; Liu *et al*.,2011). It has been observed in colon cancer and acute myeloid leukemia (Ma *et al*., 2015). ST6GAL I can be correlated with the activation of the epithelial mesenchymal state in cancer cells that leads to invasion and metastasis. ST6GALI downregulation leads to the activation of TGF-β signaling pathway promoting tumor cell migration (Lu *et al.,* 2015). α2-6 sialyation alters a number of physiological processes including EGFR, PECAM, Fas receptor activating signaling pathways, alteration of β1 integrin. In summary promote tumorgenesis, invasiveness, metastasis, inflammation, and angiogenesis leading to immune escape and cancer spread eventually (Swindall *et al*., 2011; Liu *et al*., 2011; Möller *et al.,* 1994). In the present study we aim to focus on targeting the inhibition of ST6GAL I protein by investigating its molecular interactions with the natural compounds including aromatic and flavonoid compounds which can be developed into lead compounds and used as inhibitors against ST6GALI.

Alternative to cancer therapeutics and drug molecules is another approach more biological in nature the use of oncolytic viruses. They selectively replicate inside the tumor cells. The cancer immunosurvillence and immunoediting requires T cell infiltration in the tumors to elicit antitumor responses causing a decrease in angiogenesis and metastasis which is diminished in cancers because of the defects in the immunosurvillence pathways Interferon signaling and NF-

ҚB pathway which produce a microenvironment efficient enough to recruit T cells and chemokines in the tumors to cause effective killing of the tumor hence preventing malignancy and metastasis. Interferons have antiviral and antitumor responses with classical role in innate and adaptive immunity. Viruses activate the immune system and as a result produce a robust immune response and activate a cascade of antiviral genes which leads to the production of a plethora of chemokines and cytokines to produce an efficient T cell response. Oncolytic viruses can be used as super therapeutic tools to cure a wide variety of cancers and some of the viruses are New Castle Disease virus (NDV), Reovirus, VSV(Vesicular stomatitis virus) are proficient target for metastatic cancers (Lorenc et al., 1994; Peng et al., 2001; Stojdl et al., 2000). New castle disease virus a paramyxovirus is an excellent tool to be used against a variety of cancers is under clinical trials for glioblastomas, lung adenocarcinoma and HCC. NDV is a non-segmented, negative-strand RNA , filamentous , pleomorphic in shape virus of the Paramyxoviridae family with a 15kb long genome, enter cells by binding to sialic acid residues present on a wide range of human and rodent cancer cells (Adam et al., 2011). NDV has been shown to selectively replicate in and destroy tumor cells, while sparing normal cells, a property believed to stem from the defective antiviral responses in tumor cells. Normal cells, which are competent in launching an efficient antiviral response quickly after infection, are able to inhibit viral replication before cell damage can be initiated. The sensitivity of NDV to interferon (IFN), coupled with the defective IFN signaling pathways in tumor cells, provides a mechanism whereby NDV can replicate exclusively within neoplastic tissue. NDV is a safe and effective therapeutic agent; with no reports of pathologic effects in patients beyond conjunctivitis or mild flu-like symptoms (Ravindra et al., 2008).The above facts elucidate the usage of NDV as an ideal agent for virotherapy against hepatocellular carcinoma and Horvath (1993). Cancer cells have a defective

apoptotic system, NDV after entry in the cells activate the innate anti-viral genes up regulating Cells to induce apoptosis inducing pathways. NDV mediates apoptosis by intrinsic pathways and the activation and opening of mitochondrial permeability transition pore and loss of mitochondrial potential leading to the release of cytochrome c, SMAC/DIABLO. The Complex Causes suppression of inhibitory apoptosis proteins, where as cytochrome c binds to the apoptosis protease activator APAF-1 and pro Caspase 9 forming apoptosome which activates apoptosis (Ravindra et al., 2008; Elankumaran et al., 2006; Kumar et al., 2012; Elmore ,2007; Molouki and Yusoff 2012; Ch'ng et al., 2013). NDV is a promising oncolytic agent with its details of oncolysis being discussed in the section of literature review will help us elucidate and understand the virology, immunological and molecular mechanisms of how virus effectively kills the tumor cell and in the present scenario serve as an effective therapy to cure cancers in the years to come. The study shall be based upon the following basic objectives mentioned in the next chapter of aims and objectives and review of literature

Literature Review:

The literature review of the thesis is divided into two parts based on the approaches used in the study.

1) Computational approach (ST6GAL I based drug inhibitors)
2) Virotherapy approach (Newcastle disease virus mediated virotherapy).

In silico studies: **ST6 beta-galactoside alpha-2,6-sialyltransferase 1 mediated drug inhibitors design for cancer**

Literature Review:

2.1Aberrant Glycosylation sweet and sour of cancer:

Tumor cells by virtue of their fate have a distinct biochemistry that differs from the normal cells of the body. They undergo immune escape and proliferate endlessly. It is because of the aberrations in the genetic material, mutations in the tumor suppressor genes, proto-oncogenes and cell cycle checkpoints that give rise to tumor cells. Besides this dysfunctional apoptosis and alterations in the cell signaling pathways are all the hallmark of tumor cells. Alongside all of these factors aberrant glycosylation is one of the most significant factors that plays a role in tumor development and metastasis. It has been long since known the role of cell surface glycans that play a role in tumor progression and enhanced malignancy.(Hanahan et al.,2011; Hakomori, et al.,1985; Kannagi et al,,1997; Kim et al., 1997). Tumor cells have invasive characterstics that enable them to detach from the cell surfaces which involve the degradation of the basal membrane, extracellular matrix, and cell-cell junctions. This allows the tumor cells to spread across the body causing metastasis (Hakomori, et al.,1985; Kannagi et al,,1997; Kim et al., 1997).

Cell surface glycans play an important role in a variety of physiological processes such as cell-cell communication, cell growth, fertilization, immune responses and number of other processes (Moremen et al.,2012).Glycans are oligosaccharide structures covalently attached to the proteins through Ser or The (O-linked glycan) or through Asn (N-linked glycan) residues (Moremen et al.,2012). Many studies have discussed the role of cell surface glycan in tumor progression but the focus of this study is on special type of cell surface glycans known as sialic acids that have a role in cancer progression and metastasis.

2.2 Sialic acids and their role in Tumor progression

Sialic acids are a diverse group of negatively charged monosaccharide molecules comprising of a nine –carbon backbone (C1-9) atoms of neuramic acid. There are more than 50 types of sialic acid residues and most common derivatives of N-Acetylneruaminic acid found in mammals. (1,2) N-glycolneuramic acid (Neu5Gc) and i-acetylneruamic acid (Neu5Ac) being the most commonly occurring ones (Varki 1992; Chou *et al.,* 1998). Sialic acids have variety of functions in cell physiological and metabolic processes including growth, fertilization, apoptosis, transmembrane signaling and variety of other cellular processes (Schultz *et al*., 2012; Schauer *et al.,* 2009) Altered sialyation is the principal characteristic of tumor cells but the molecular mechanisms encompassing this are not well understood. In order to deepen our understanding about the molecular mechanisms of sialyation on tumor cells we need to discuss the role of enzymes regulating the sialic acid levels in cells. The level of sialylconjugates on the cells is controlled by a diverse class of enzymes known as *sialyltransferases* (ST) s that are encoded by more than 20 human genes. These enzymes transfer the sialic acid moiety from activated cytidine 5'-monophosphate N- acetyl neuramic acid (CMP –Neu5Ac) to a sialic acid or a glycan with attached galactose moiety. The ST enzymes are classified into three groups as ST3, ST6 and ST8 transferases on the type of alpha glycosidic bond they have with the C2 atom of the sialic acid residues (Takashima *et al.,* 2002; Kuhn *et al.,* 2013).Having defined the role of hypersiaylation in cancer progression which is regulated by a number of ST enzymes undergoing mutations and changes in gene expression (Varki et al.,2009; Schneider et al., 2001; Cohen et al.,2001).

2.3 Mechanisms of Aberrant Sialyation in cancer

Hypersiaylation in cells promotes invasiveness, tumor cell proliferation and malignancy and hence is regulated by the changes in the activity of sialyltransferases enzymes that regulate sialylation in the cells. There have been different mechanisms proposed that lead to the defects in these enzymes (Astronomo, *et al.*,2010; Häuselmann, *et al*.,2014). The different genes including proto-oncogenes such as c-myc and ras have been shown to regulate the expression of sialyltransferases including ST6GAL I and ST3Gal I, II and IV respectively (10,11). Therefore alterations in these genes, hypoxia and hormonal imbalance have been reported to cause hypersiaylation. ST3GAL I was overexpressed in colon cancer cells in state of hypoxia (Sakuma *et al*.,2012; Hatano *et a*l.,2012).

The second mechanism encompassing hypersiaylation is the upregulation and overexpression of the genes involved in sialic acid synthesis pathway. It was proposed that there is a metabolic flux through the sialic acid synthesis pathway (Almaraz et al.,2012; Miyagi et al., 2012). Increased sialyation is caused by the increased expression of the sialidases that cleave sialic acid residues from glycan substrates were dound to be altered with low expression leading to the accumulation of sialic acid residues on the cell surface. Some of the examples include NEU 1, NEU 2 and others (Almaraz et al.,2012; Miyagi et al., 2012). Apart from the role of different sialyltransferases and their role in tumor progression we are focused on the role of ST6Gal I enzyme which has been reported in a variety of cancer types and is linked to the cancer stem cells maintenance and hypersiaylation (Miyagi et al., 2012 Picco, et al.,2010; Swindall et al., 2013).

2.4 ST6GAL I resistance to chemotherapeutics:

The role of human ST6GAL1 is substantial in tumor progression, malignancy, invasiveness and metastasis (Azab *et al.,* 2008; Hedlund *et al*., 2008.). There is an overexpression of the α2-6 sialic acid residues on the cell surface regulated by the ST6GALI enzyme which has been observed in many cancer types including breast, liver, colon, cervix, brain, acute myeloid leukemia and a number of other different cancer types (Dall'Olio *et al*., 2001; Recchi,*et al*.,1998; Dall'Olio *et al*., 1992; Wang *et al.*,2001; Zhao *et al*., 2014; Sata *et al*.,1991; Julien *et al.,* 2006).. Tumor cells escape apoptosis which can be correlated with the selective enrichment of the α2-6 sialic acid residues on the tumor cells. Hypersiaylation of the sialic acid of cell surface galectin ligands block the apoptosis pathways. Fas and TNFR signaling pathways are downregulated in cancer cells promoting immune escape (He & Baum 2006; Ochieng *et al*., 2002; Elola *et al.,* 2007; Zhou *et al.,* 2011; Swindall *et al.*,2011; Liu *et al*.,2011). It has been observed in colon cancer and acute myeloid leukemia (Ma *et al*., 2015). ST6GAL I can be correlated with the activation of the epithelial mesenchymal state in cancer cells that leads to invasion and metastasis. ST6GALI downregulation leads to the activation of TGF-β signaling pathway promoting tumor cell migration (Lu *et al.,* 2015). α2-6 sialyation alters a number of physiological processes including EGFR, PECAM, Fas receptor activating signaling pathways, alteration of β1 integrin. In summary promote tumorgenesis, invasiveness, metastasis, inflammation, and angiogenesis leading to immune escape and cancer spread eventually (Swindall *et al*., 2011; Liu *et al*., 2011; Möller *et al.,* 1994).

Besides all these effects of ST6GAL I on tumor cells there have been numerous studies reporting the overexpression of ST6GAL I and its role in chemoresistance and radiotherapy. It has been

reported the ST6GAL I overexpression interacts and confers resistance against anticancer drugs such as cisplatin and other platinum based drugs (Schultz et al., 2013). ST6GAL I also mediates resistance against the chemotherapeutic agent irinocetan regulating cancer stem cell chemoresistance Liu *et al*., 2011; Möller *et al.,* 1994). There have been studies indicating the resistance in cancer cells undergoing radiotherapy. It has been shown that radiotherapy in both healthy cells and cancer cells induces the level of ST6GAL I which is linked to cell migration and tumor cell adhesion (Lee et al.,2008; Lee et al.,2010).Having discussed the role of ST6GAL I enzyme in tumor progression, invaseness and metastasis we need to develop targeted approaches interfering with the sialic acid expression on cancer cells. There are so far very few compounds available interfering with the sialic acid residues .In the present study we aim to focus on targeting the inhibition of ST6GAL I protein by investigating its molecular interactions with the natural compounds including aromatic and flavonoid compounds which can be developed into lead compounds and used as inhibitors against ST6GALI.

2.5 ST6GAL I Enzyme Structure:

The human α2-6 siayltransferase1 (ST6Gal I) (EC 2.4.99.1; P15907) belongs to the GT29 family of GTs that comprises family of transferases including mammals and viruses. The enzyme is a type II membrane protein that catalyzes the transfer of sialic acid from CMP-sialic acid to galactose-containing substrates. It is localized in the Golgi apparatus and is involved in a variety of cell physiological processes involving generation of antigens including CD 76, CD75, HB-6 and others.

Structure based approach:

There are few studies available on ST6GAL I enzyme structure explaining its confirmations, interactions with various inhibitors and substrates. There are only two available structures in the protein data bank of the enzyme ST6GAL I. Conserved motifs are discussed below in detail.

Enzyme	Origin	PDB ID	Ligand bound	Chain	Resolution	Reference
ST6GAL1	Homo Sapiens	4JS1	CTN CMP	A	2.09°A	Kuhn et al.,2013
ST6GAL 1	Homo Sapiens	4JS2	Cytidine-monophosphate N-Acetyl-D-Glucosamine	A	2.3 A	Kuhn et al.,2013

The crystal structure of ST6GAL I with ligands bound structures are available. The structure comparison of 4JS1 and 4JS2 reveal an RMSD value of 1.25 and 1.31 respectively. The binding cavity and ST6GAL I structure is explained in detail below.

Binding cavity of ST6GAL I

ST6GAL I enzyme is a six stranded β-Sheet linked to two pairs of alpha helices. There are Rossmann folds as well. The structure reported by Kuhn et al., 2013 depicts ST6GAL I bound to cytidine 5'monophosphate (CMP) adapting a globular GT-A fold (Audry et al., 2011; Lairson et

al.,2008).. The fold forms the boundary of the catalytic domain. The structure of the enzyme comprises of four distinct characteristic regions. The first part is the N –terminal nine residues that are cytoplasmic in nature followed by the transmembrane sequence that extends and form a stem like structure extending to C-terminal which forms the catalytic domain (Takashina et al., 2002; Weijers et al., 2008; Audry et al.,2011).

The conserved residues exist in the catalytic domains that receive localization signals from the golgi apparatus. There have been some conserved domains that exist in the all the eukaryotes STs Known as (VS) very small, S (small), III and Large (L). These motifs have variety of functions. Motif S(Pro321-Phe 343) is involved in donor and substrate binding. Motif VS (His 370-Glu 375) contributes in catalytic reaction. Motif III (Tyr354-Gln 357) is involved in binding of sugar donor CMP-Neu5Ac. Motif L(Trp181-Gly 324) in ST6GAL I numbering (Datta et al.,2001; Datta, 2009). The exact size of the active site of ST6GAL I enzyme is mapped by some N-terminal deletions, not known exactly (Legaigneur et al.,2001). The first 80 residues of human ST6GAL I include the transmembrane and cytoplasmic domain. Catalytic domain of the enzyme has six cysteine residues involved in disulfide bonds. There is a glycan binding residue at Asn 149 that mediates glycan binding with the ST6GAL-I enzyme. The N-teriminal domain (89-136 residues) of ST6GAL I is involved in substrate binding. The catalytic domain of the enzyme starts at Phe 93. There are 11 residues that form and cover the active site include **Asn** 212, **Ser** 323, **Ser** 322, **Gly** 324, **Lys** 376, **Thr** 365, **Tyr** 354, **Asn** 233, **His** 370, **Cys** 353 and **Cys** 364 respectively. Cys 353 and Cys 364 forms unique disulfide bind that gives cytosine specificity to the enzyme. There is an additional glycosylation site at Asn 161 in ST6GAL-I. His 370 is the part of the catalytic domain in Human ST6GAL I. Human ST6Gal I exhibits three disulphide bonds Cys142—Cys406, Cys184—Cys335 and Cys353—Cys364. The hST6Gal I crystal

structure revealed that CMP binds with the ribose in a 3′-endo conformation created via interactions with the first and last residues of a SerSerGly sequence within the sialyl motif S

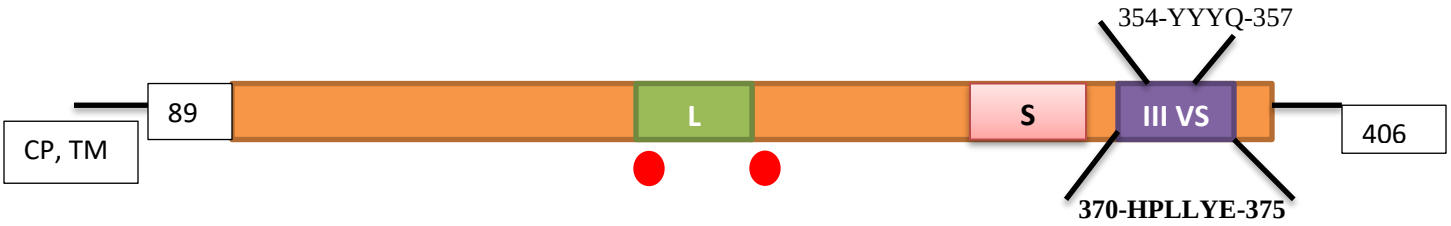

Figure 2.1 ST6Gal I enzyme detailed structure

2.6 Docking studies:

There are very few molecular docking studies being conducted on human ST6GAL I enzyme by authors. These studies are carried out to classify and improve the drug candidates and novel inhibitor designs against ST6GAL I. It is doing by investigating the molecular interactions between the ligands and the target macromolecules. The catalytic site and domain of the enzyme have been investigated using different methods of Nuclear magnetic resonance (NMR), X-ray diffraction, site directed mutagenesis, docking studies, protein modeling and X-ray crystallography. The studies reported in the literature illustrate the following important amino acids interacting with ST6GAL I include **Asn** 212, **Ser** 323, **Ser** 322, **Gly** 324, **Lys** 376, **Thr** 365, **Tyr** 354, **Asn** 233, **His** 370 respectively (Andrew et al.,2015; Kuhn et al.,2013).

In order to understand the 3D- ligand protein interactions there were docking studies performed using different classes of compounds. In a study conducted by Andrew et al., 2015 used ST6GAL I X-ray crystal structure PDB ID 4JS2 respectively was investigated. In this study phosphodiester SI inhibitors were used as ligands and they were placed with the potential neutral isostere such as a carbamate or a 1,2,3-triazole instead of the phosphodiester bond and

docking results were validated using MD simulations and interactions were studied accordingly. The carbamate linker was reported to be in proximity of the following amino acid residues including Asn212, His370, and Tyr354 similar to the position of phosphodiester linker in the phosphodiester linker based ST inhibitors. Tyr 354 showed hydrogen bonding in all the compounds. In the study M-phenoxy substituent in all the replaced ST inhibitors were reported to have hydrophobic interactions with the following amino acids including Pro259, Gln235, Asn233, Tyr369 ,Tyr275 and Trp257 that are proposed to be present in the sialic acid binding site of the enzyme.

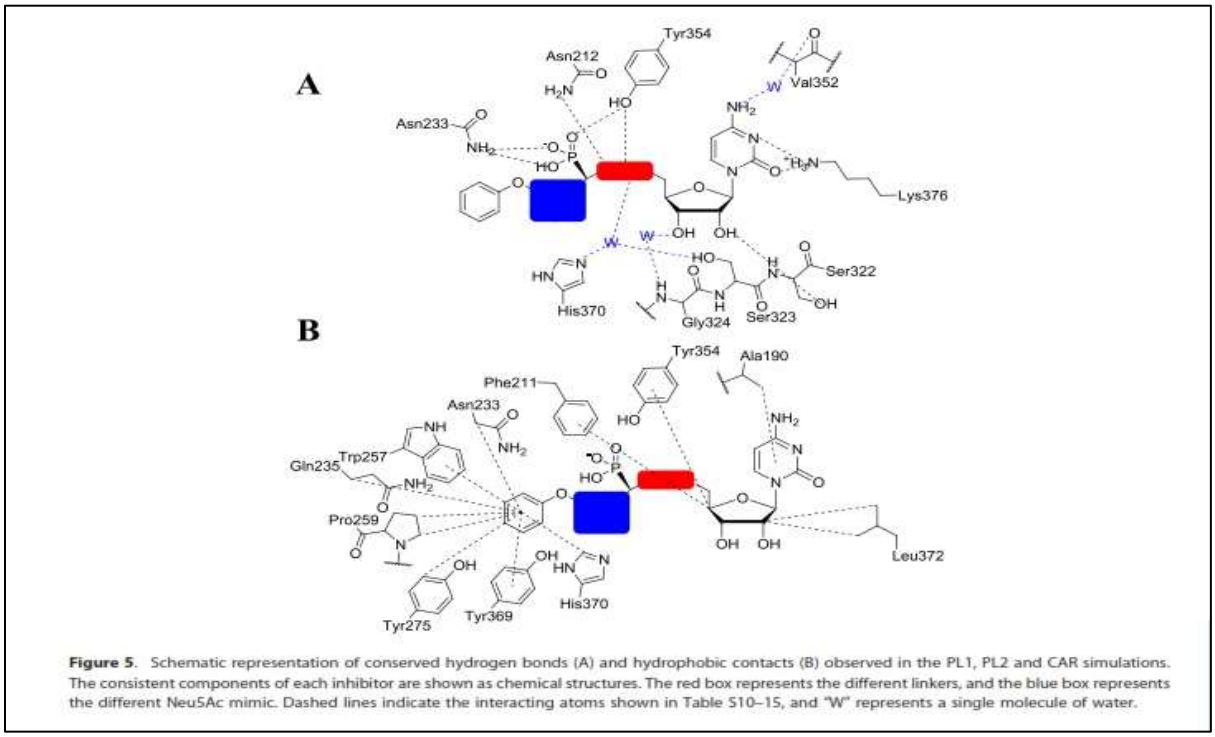

Figure 5. Schematic representation of conserved hydrogen bonds (A) and hydrophobic contacts (B) observed in the PL1, PL2 and CAR simulations. The consistent components of each inhibitor are shown as chemical structures. The red box represents the different linkers, and the blue box represents the different Neu5Ac mimic. Dashed lines indicate the interacting atoms shown in Table S10–15, and "W" represents a single molecule of water.

Figure 2.2 illustrating the ligand protein interactions of ST6GAL I bound inhibitors reported in literature(Andrew et al.,2015).

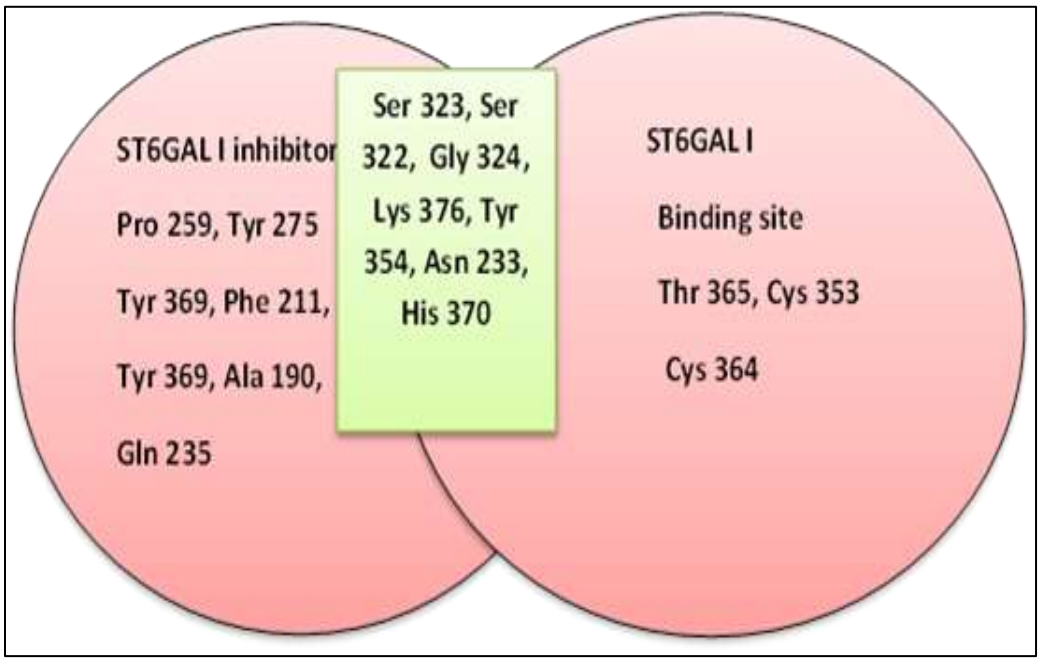

Figure 2.3 Conserved amino acids of ST6Gal I enzyme from literature

The figure illustrates the conserved amino acid residues extracted from literature of ST6GAL I binding site and interacting residues of ST inhibitors against (CM-Neu5Ac). The conserved residues are illustrated in green bound to 4JS1 PDB structure of HST6Gal I

The work flow of the ST6Gal I enzyme inhibitors included in the study are aromatic and flavonoid compounds designed using structure based approach will be discussed in the methodology section

Literature Review: Newcastle disease virus mediated virotherapy as cancer therapeutics.

LITERATURE REVIEW:

2.1 HISTORY OF NEW CASTLE DISEASE

New Castle disease a pathogenic virus was first reported in 1926 in two different geographical regions of Java and Tyne in Newcastle England. There were other reports from different parts of the world regions including Western Scotland and central Europe back in 1896 (Macpherson, 1956; Doyle, 1927; Kranveld, 1926; Alexander 2001; Halasz 1912). Doyle coined the name of "New castle disease" momentarily as it was first reported in New Castle in Tyne but name is still used to date (Doyle 1935; Alexander *et al.,* 2004). Then after its discovery in Europe there cases with similar symptoms included mild respiratory tract infections and nervous system disorders were reported in United States collectively referred to as pneumoencephalitis. The virus was found to be similar to NDV, based upon serological testing (Beach, 1942; Alexander *et al.,* 2004). Since then mildly virulent and a virulent strains of NDV have been isolated and it is established that wild birds and water fowls serve as reservoir for New Castle disease virus (Alexander *et al.,* 2004).

2.2 ETILOGY:

New castle disease is caused by new castle disease virus (NDV) also known as Avian paramyxovirus type 1(APMV-1). It has been placed with other eight APMV serotypes (APMV-2-APMV-9) in genus *Avulavirus,* sub family *Parmamyxovirdae* and order *Mononegavirales* (Lamb *et al.*, 2002*;* Mayo 2002).

NDV diagram

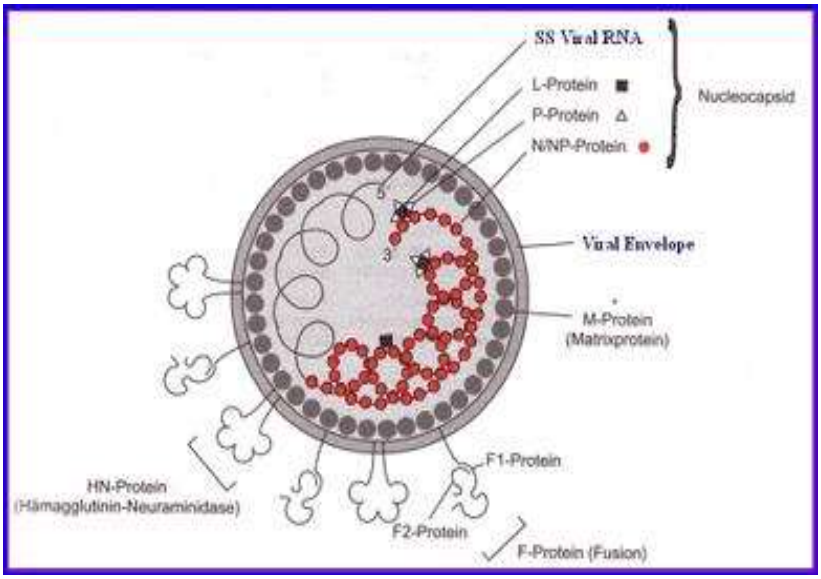

Figure 2.4 Newcastle disease virus diagram.
New castle disease virus genome adapted from Wangh Laboratory (http://www.brandeis.edu/wanghlab/technologies/apps/newcastle.html).

2.3 GENOME:

New castle disease virus is an enveloped, pleomorphic (filamentous and as well as spherical form) virus ranging in diameter from 15-30 nm, covered with glycoprotein spikes of 8-14nm in length (MacLachlan and Dubovi, 2010). It has herring bone shaped nucleocapsid having helical symmetry and is 18nm in diameter. NDV genome consists of non-segmented, negative sense single stranded linear RNA (ssRNA) molecule with 15 kilo base pair genome (Kolakofsky *et al*., 1974).

Virus particle has 6% carbohydrate and 20-25% lipid content .NDV genome consists of six genes containing total 15,198 nucleotides, with the following gene order 3‟N-P-M-F-HN-L-5‟(Krishnamurthy and Sanal ,1998; Philips *et al*., 1974).These genes encode for six polypeptides Nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), Fusion protein (F), Hemagglutinin – neuraminidase protein (HN) and large polymerase protein (L) (Millar and

Emmerson ,1988).

Three of the polypeptides including Nucleocapsid protein (NP), phosphoprotein (P), and large polymerase protein (L) are associated with the genomic RNA, making transcription complex.

HN and F protein are the two surface glycoproteins which are embedded in the lipid bilayer surrounding the genome and act synergistically in a complex manner (Stone and Morrison, 1997; Markwell and Fox, 1980). Matrix protein is found beneath the lipid bilayer and interacts with the transcriptive complex (Cathomen *et al.,* 1998).

2.4 MOLECULAR BASIS OF VIRAL ONCOLYSIS:

Normal cells of the body are altruistic in nature with an aim of programmed cell death with a proper regulated cell cycle. They have a normal mechanism of cell death or apoptosis. They have mechanisms to inhibit viral infections, replication and other infections by preferring cell death to prevent infection to other cells of the body. Tumor cells are on their quest to immortality, have established ways to overcome and alter their apoptosis programs which makes them susceptible to cell death. They continue to grow invariably to tumors that metastasize the normal tissue leading to the neoplastic abnormal cells in the body creating a disease with genetic, epigenetic and immunological defects. Oncolytic viruses are genetically modified smart viruses that prey upon the entire malignant cells of a particular phenotype. There are different mechanisms of oncolytic viruses the replication of oncolytic viruses is strictly confined to the cancer cells are based on the malignant activation of mutations in the cancer cells and some oncolytic virus derived mutations. Oncolytic viruses are self replicating, intricate biological machines that replicate their proteins and genetic information, self assemble generating virus particles in situ packed with mutations that alter the anti-apoptotic functions of the tumor cell. They are capable of replicating in the cancer cells sparing the normal tissues of the body creating a

microenvironment and an army of chemokines, cytokines that activate the B and T cells to produce neutralizing antibodies that causes tumor regression (Tollefson *et al.,* 1996; Shtrichman and Kleinberger, 1998).

The second mechanism of oncolytic virus replication is the generation of proteins by their virus during their replication cycle that is cytotoxic to the tumor cells and tissues. Adenoviruses encode a protein E3 with 11.6 kD size is a death protein, and E40RF4 protein which are expressed in late cycle of the cell cycle and both of these proteins are cytotoxic to the cells (Tollefson et *al.,* 1996; Shtrichman and Kleinberger, 1998).

The third mechanism of oncolytic viruses mediated tumor cell destruction is done by induction of specific and nonspecific antitumor immunity. Tumor cells are intrinsically weakly immunogenic because they display decreased expression of major histocompatibility complex (MHC) antigens and stimulatory signals. These signals include the chemokines and cytokines that activate local innate immune responses. For example the Infection of tumor cells by adenovirus which expresses EIA protein leads to the increased sensitivity of the tumor cell to release tumor necrosis factor (TNF) and activates the TNF mediated killing mechanisms (Gooding 1994). The E1A protein is expressed early in the tumor cell by the virus thereby hijacks the host cell and prevents the host cell from shutting down and allows the continuation of protein synthesis so that the replication can proceed which leads to the induction of specific antitumor immunity and gives long-term protection against tumor recurrence.

Virus infection in the tumor cells leads to the production of an army of cytokines and chemokines that lead to the infiltration of antigen presenting cells and lymphocytes in the tumor Viral antigens are presented to the cell via the Major histocompatibility complex (MHC-1) protein complex that activates the cytotoxic T lymphocytes(CTLs). They are attracted to the

virally infected tumor. CTLs now acquire the specificity for tumor specific antigens leading to tumor cell killing and viral antigen recognition (Toda *et al.,* 1999; Savage *et al.,* 1986).

The final and fourth mechanism of oncolytic viruses mediated tumor regression and anti-neoplastic activity is by the expression of therapeutic Transgenes by the virus which are inserted in the viral genome. Such mechanisms offer a good advantage to viruses over the replication incompetent viruses. The virus replicates it amplifies itself through special rounds of replication and there is a differential expression of the Transgene leading to the production of an amplified tumor effect (Wildner *et al.,*1999; Freytag *et al.,* 1998; Andreansky *et al.,* 1998).

2.5 NEW CASTLE DISEASE VIRUS AS AN ONCOLYTIC AGENT:

New castle disease virus an enveloped, pleomorphic (filamentous and as well as spherical form) is a single stranded RNA virus with size ranging in diameter from 15-30 nm, covered with glycoprotein spikes of 8-14nm in length (MacLachlan and Dubovi, 2010). NDV genome consists of six genes containing total 15,198 nucleotides .with the following gene order 3‟N-P-M-F-HN-L-5‟(Krishnamurthy and Sanal ,1998; Philips *et al*., 1974). NDV like all RNA viruses activates the wide array of cellular defense mechanisms including the release of interferons alpha and beta. New castle disease virus divides invariably in tumors because they provide relatively permissive environment because of their defective IFN system and in ability to provide resistance to apoptosis. NDV like other viruses has developed escape mechanisms in the host cell and possess a special V protein for that purpose that interferes with innate immune system Signal Transducer and Activator of Transcription (STAT) mediated type I IFN signaling JAK /STAT pathway .this response is confined to tumor cells only as normal mammalian cells have a proper functional interferon system that is capable of inhibiting viral replication (Russell, 2002; Fiola *et*

al., 2006; Reichard *et al*., 1992). Integral to the life cycle of all RNA viruses is the formation of double-stranded RNA (dsRNA), which activates a spectrum of cellular defense mechanisms involving interferon (IFN)-α and -β. 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. The property of permissiveness of NDV in tumor cells is the defect and weaker IFNR signaling and weaker sensitivity towards the interferon receptor 1 signaling. This basic impairment of the innate immune system is a common attribute of tumorigenesis ensuring the oncolytic activity of the virus in tumor cells (Russell, 2002). 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. In normal cells NDV with a functional IFN system NDV establishes an early antiviral state which leads to the production of such transcriptional factors that inhibit NDV replication in the normal cells (Reichard *et al.,* 1992; Krishnamurthy *et al.,* 2006). NDV enters the cell via receptor mediated endocytosis, selectively replicating in the cytoplasm where it activates the four most significant genes of innate antiviral immunity, i) Interferon Regulatory Factor (IRF)-3, (ii) IRF-7, iii) Retinoic acid-Inducible Gene (RIG)-I and (iv) IFN-β (Wilden *et al.*,2009; Fournier *et al.,* 2012).In normal cells NDV activates the innate immune system through its dsRNA by specific pathogen recognition receptors (PRR) of two types RIG-like receptors (RLRs) and Toll-like receptors (TLRs), especially TLR3 (Yoneyama *et al.*,2004; Schmidt *et al*., 2012). NDV enters the tumor cells and activates the interferon response which occurs in two steps early and late phase via binding through RIG-I pathway specifically binds to the RNA containing 5'-phosphate such as viral RNA, while mammalian mRNA is either capped or comprises of nucleotide base modifications (Gitlin *et al*.,

2006). RIG-I pathway of the innate immune system is able to differentiate between non-self (viral) RNA and self components (Bowie *et al*., 2007). RIG-I after activation binds to the CARD containing adaptor protein IPS-1 that initiates the downstream signal cascade and turn on the early phase expression of IRF-3. This transcription factor is then phosphorylated and is thus translocated to the nucleus to initiate IFN responses (Taniguchi *et al*., 2002). In the late phase of the IFN response, the type 1 IFN molecules secreted in the early phase interact with cell surface expressed type 1 IFN-receptors and initiate an amplification loop of the IFN response, which involves STAT proteins and IRF-7 (Gitlin *et al.,* 2006; Hornung *et al.*,2006; Bowie *et al.,* 2007;Taniguchi *et al*.,2002).

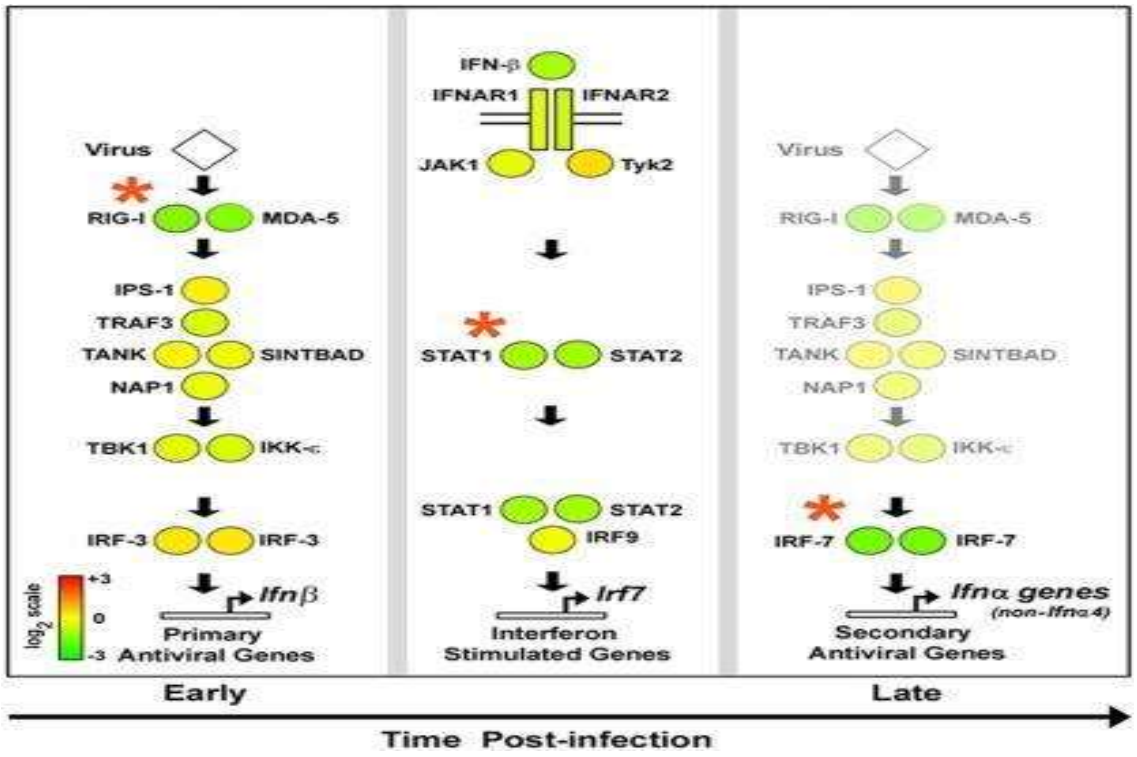

Figure 2.5 NDV virus Molecular mechanisms of tumor cell immunity.
The figure illustrates Newcastle disease virus entry in the cell followed by innate immunity and antiviral response activation The Figure is adapted from Suresh et al (2011).*Distinct Roles for the NF-κB RelA Subunit Antiviral Innate Immune Responses J Virol.; 85(6): 2599–2610.*

NDV causes Newcastle disease in birds with severe mortality rate but the same virus has an excellent oncolytic potential. NDV infecting humans causes mild fever and conjunctivitis; no adverse effects have been reported and are a safe potent agent to be used as a therapeutic agent, because of the following reasons. NDV does not breach the immune response and IFN system of the normal mammalian cells. NDV is an ssRNA virus selectively replicates in the cytoplasm, it lacks a DNA phase in its life cycle making it a safe, suitable vector for cancer therapy. It has additional in vivo mechanisms such as syncytium formation and cell fusion that prevent it from the neutralizing antibodies and the normal human population is seronegative for NDV. The virus exhibit a high tolerability in humans, with the maximal dose of 3×10^9 infectious particles injected when the virus is applied by the intravenous route and 4.3×10^{12} infectious particles by the intra-tumoral route. There exist a complete safety database for NDV and it exhibits as an oncolytic agent because of its tolerability and safety in cancer patients (Lorence *et al.,* 2001; Russell 2002).All of the above features make NDV an excellent candidate against cancer (Lamb and Parks , 2007;Hornung *et al.*,2006;Bowie *et al.,* 2007).

New castle disease virus plays an important role as an oncolytic agent which is capable of inducing tumor lysis and regression after it is infected in the animal models and body. NDV has shown strong capacity as an oncolytic agent both in vivo and in vitro (Apostolidis *et al.*,2007).The oncolytic effects on cell models lead to complete cell lysis and death where as they appear to become reduced because of the interferon response as it blocks NDV virus replication after its administration in the body. (Sinkovics and Horvath 2000; Schirrmacher *et al.,* 2001; Apostolidis *et al*., 2007).

2.6 NDV APOPTOSIS ACTIVATING MECHANISMS:

Programmed cell death is the altruistic property of normal cells of the body to maintain a homeostatic balance for the survival of body tissues, to control the normal physiological functions of the body such as aging etc. NDV like other viruses activate apoptosis mechanisms through the intrinsic or mitochondrial pathway to initiate its oncolytic activities. NDV is an RNA virus it selectively replicates inside the cytoplasm creating ER stress and mitochondrial stress and thus causes the opening of the mitochondrial permeability transition pores which activates SMAC/DIABLO causes suppression of inhibitory apoptosis proteins(IAP) and leads to the release of cytochrome c which Binds to the APAF-1 (Apoptosis protease activator factor 1) and procaspase 9 release that activates caspase 3 leading to the loss of mitochondrial membrane potential causes that activates a complex mechanism forming at the end the apoptosome, which activates the apoptosis process. Tumor cells as a result of NDV replication under goes suicide which is the primary mechanism of virus protection against infection spreading to neighboring cells (Sinkovics *et al*., 2000). NDV matrix (M) protein binds to Bax anti-apoptotic protein which aids to an accelerated cell death. Some pro-apoptotic proteins (Bax, Bak and Bok) upstream of mitochondria are involved in the process as well. NDV also activates apoptosis via the of TNF-related apoptosis-inducing ligand (TRAIL) pathway that leads to the activation of caspases and causes cell death by cytotoxicity (Ravindra *et al*., 2008; Elankumaran *et al.,* 2006; Kumar *et al,* 2012;Elmore ,2007; Molouki and Yusoff 2012; Ch'ng *et al*., 2013).

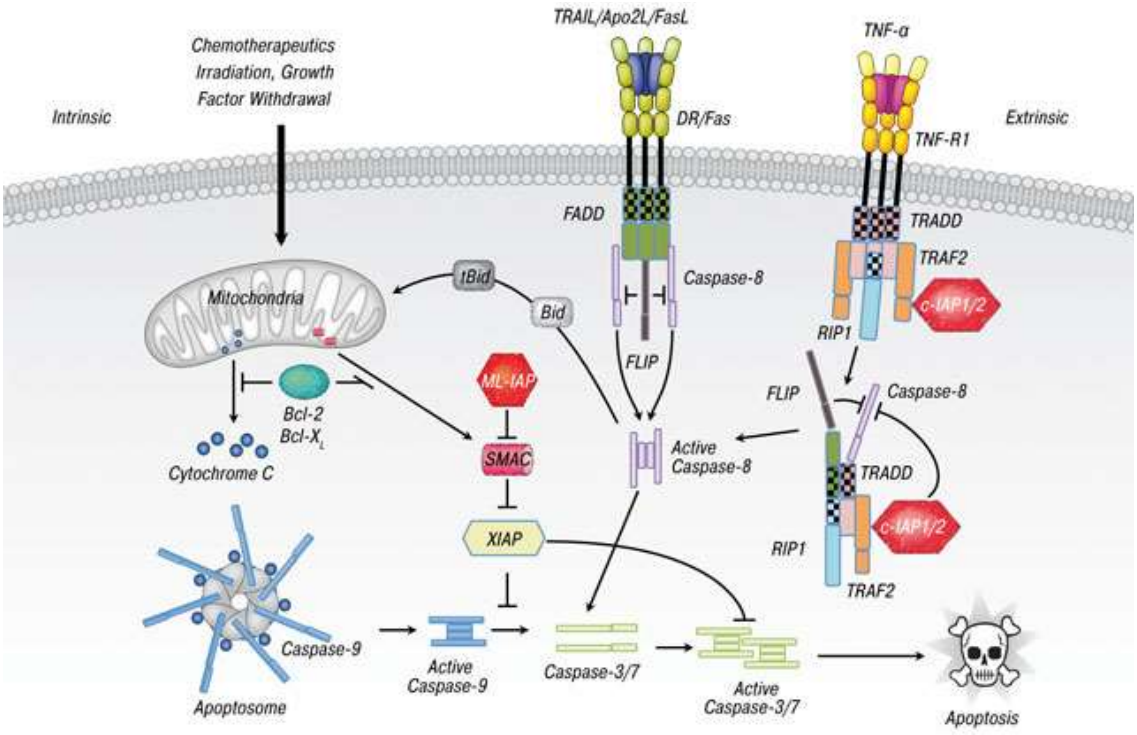

Figure 2.6 Apoptosis mechanisms activated by NDV in the cancer cells.
The figure illustrates the types of apoptotic pathways present inside the cell which can be activated by Newcastle disease virus. The figure is adapted from De Almagro et al.,2012.

NDV was discovered as an oncolytic agent back in the 1950s when the series of classical experiments were done by *Sato* and *Miyadera* on Yoshida sarcoma in rats and the first application of human trials dates back to 1964 when Wheelock and Dingle published their studies on observations on a patient who was by repeated injections doses of NDV in an endeavor to cure his acute leukemia. Cassel and Garrett had determined the anti neoplastic potential of NDV in 1965 and they performed their studies on a woman with inoperable cervix carcinoma which resulted in the William Cassel, who described already in 1965 the anti-neoplastic properties of NDV(Cassel and Garrett 1965).The NDV 73-T strain is used to treat cancer patients was the first oncolytic strain is a field isolate which is neurovirulent in young birds but has the ability to lyse ascites tumor in vivo in the Ehrlich tumor bearing mice and increased the life span of the mice bearing tumor after having a intratumoral

injection (Jacotot ,1967; Cassel and Garrett 1965).Laszlo K csatary passaged a strain with remarkable genetic stability in the eggs is an attenuated lytic variant designated as *Herz'33* .Csatary"s work on the attenuated vaccine strain led to the development of an attenuated variant designated as *MTH-68/H* which is an excellent oncolytic agent and is used in clinical trials for a variety of cancers (Csatary ,1971, 1993, 1999). NDV 73T and MTH-68 strains were used for the treatment of cancer in depth and animal trials were done to evaluate the anticancer properties of these agents (Lorence *et al.,* 1994). Lorence and colleagues executed clinical trials using a Mesogenic NDV isolate called *PV701,* which was derived via plaque purification from the naturally attenuated vaccine strain *MK107* (Lorence *et al.,* 2007; Pecora *et al*.,2002).

Phase 1 clinical trial was carried out to investigate the safety aspects of the strain *PV701* and 79 patients with advanced solid tumors were administered with multiple Intravenous doses of from 1.2×10^{10} to 1.2×10^{11} pfu were well tolerated with only grade 1 and 2 toxicity observed that included (fatigue, flu-like and gastrointestinal syndromes as most frequent side-effects). The results were quite promising out of the 79 patients one suffered from partial remission. 14 patients showed no tumor progression between 4 and 30 months after therapy. The most common adverse events were flu like symptoms and fever (Pecora *et al.,* 2002; Laurie *et al.*,2006; Freeman *et al.,* 2006). Some clinical trials based on systemic administration of the Lentogenic NDV strain *HUJ* were performed at the Hadassah Medical Center (Jerusalem, Israel). From the involved patients suffering from glioblastomamultiforme (GBM) and receiving systemic application of the *HUJ* virus strain, one patient has been reported to have achieved a complete response.(Freeman *et al.*,2006).

A Hungarian research group studied *MTH-68/H* as an effective oncolytic agent for a variety of cancers and is in clinical trial phase II for the glioblastomas (Csatary *et al.,* 1999; Lorence *et al,*

2003).

2.7 RIG 1/MDA5 pathway

RIG-1 and MDA-5 are DEx D/H RNA helicases combine to form two CARD domains at their amino terminus. Along with LGP2, they make up the RIG-1-Like Receptors (RLRs) family. RIG-1 receptors share 40% homology within the Helicase domain and ~25% similarity with MDA-5 in the CARD domain (Takeuchi & Akira, 2008; He et al., 2002). The Signaling cascade is activated when RIG-1 and MD-5 recognize a 5'-triphophate and dsRNA from RNA viruses and interact with IPS-1 (IFN-β Promoter Stimulator 1). TRAF-3 is required for activation of IPS-1 signalling pathways and results in the phosphorylation of IRF-3. Along with that, NF-kβ is activated via a FADD/RIP-1 and caspase8/caspase10 dependant pathway. IRF-3 and NF-kβ migrate towards the nucleus where they promote the transcription of genes leading to the production of type 1 interferons and inflammatory cytokines (Figure 5) (Takeuchi & Akira, 2008).

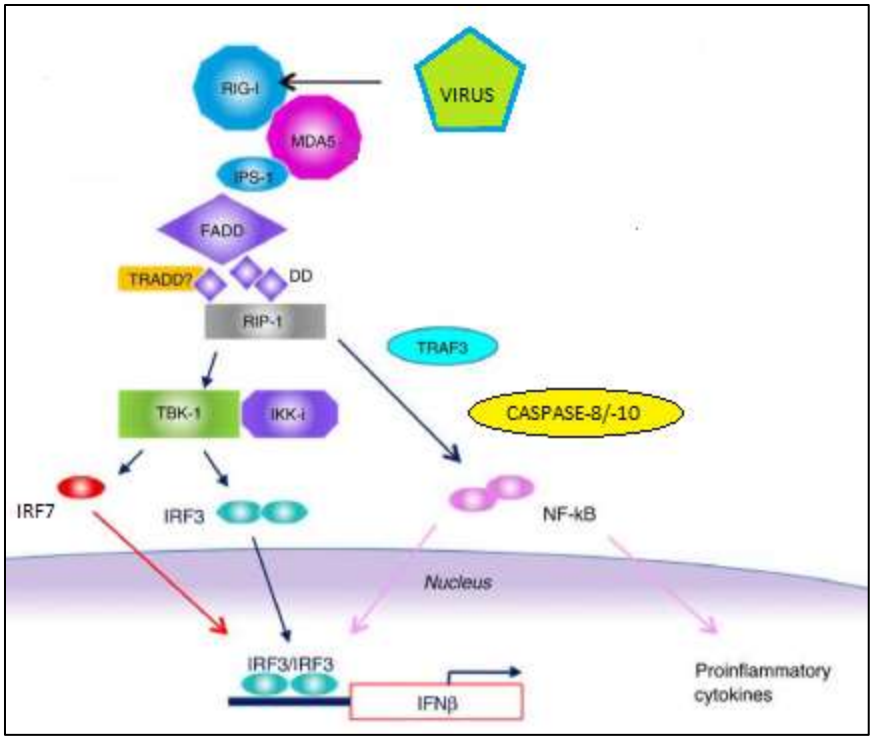

Figure 2.7 RIG-1/MDA5 pathway interactions with the virus

The RIG-1/MDA-5 signaling pathway is depicted. Virus activates RIG-1 and MDA-5 molecules to initiate a signaling cascade that ultimately leads to the production of pro-inflammatory cytokines and type 1 interferons. Reproduced from Barral et al., 2009 Pharmacology & Therapeutics, 124(2), pp.219-234.

Significance of important genes of RIG-1/MDA5 pathway

The melanoma differentiation-associated gene-5 (MDA-5) is induced during apoptosis, differentiation and cancer reversion. It is included in the category of an early response gene and its function in induced by IFN and TNF-α with a pre-dominant response to IFN-β. The gene displays a strict RNA-dependant ATPase activity. This gene has confirmed growth-suppressive properties and plays a significant role in IFN-induced growth suppression as well as apoptosis (Kang et al., 2002).

IRF-3 is a member of the IFN-inducible family of IRF transcription factors. IFN is involved in antiviral defence, immune activation and cell growth regulation and it carries out this function by inducing expression of various genes including IRF-3. It is proposed that any virus that has the capacity to generate dsRNA can signal the upregulation of IRF-3 and the IFN genes. An overexpression of IRF-3 leads to the generation of antiviral state in the cell with upregulated IFN genes that also restrict further replication of the virus (Hiscott et al., 1999).

The active elimination of cells during development, differentiation, homeostasis or even a state of threat is termed as apoptosis and it mainly occurs through two major pathways: mitochondrial and death receptor pathway. Both pathways rely on the activation of caspases for proper functioning. Caspase-8 is a protein involved in apoptosis induced by Death receptors: CD95, TNFR-1 and DRs 3, 4 and 5. Caspase-8 triggers apoptosis by activating down-stream caspases 2, 3, 6 and 7. Furthermore, it induces Bid to translocate to the mitochondrial membrane ad trigger the release of cytochrome *c*, thus activating the mitochondrial apoptotic pathway (Salmena, 2003).

INTERACTION OF NDV WITH THE RIG-1/MDA-5 PATHWAY AND ONSET OF APOPTOSIS

Tumors cells by virtue of fate have defects in the IFN signaling pathways and dysfunctional apoptosis that make them as ideal substrates for virus infection. This deficiency of the innate immune system, allows the virus to replicate and survive. NDV enters the tumor cell via receptor mediated endocytosis and activates the interferon signaling via binding to RIG-1. The early and late phase of viral infection leads to the formation of a signaling cascade that amplifies the IFN response (Taniguchi et al., 2002).

Paramyxoviruses, like NDV, target the production of type 1 interferons for effective replication and it has been suggested that the V protein of the virus inhibits the activation of both IRF-3 and NF-kβ (He et al., 2002; Poole, He, Lamb, Randall & Goodbourn, 2002). By this blockage of pathway, NDV replicates efficiently and activates apoptotic pathways. NDV does so by activating the intrinsic pathways as well as increasing the mitochondrial pore permeability which leads to the release of cytochrome c (binds with APAF-1 which activates Procaspase-9 which leads to the induction of apoptosis) and SMAC/DIABLO which is responsible for inhibiting anti-apoptotic proteins. NDV also activates apoptosis via the manipulation of the TNF-Related Apoptosis-Inducing Ligand (TRAIL) pathway that is followed by the activation of caspases and causes cellular death by cytotoxicity (Ravindra et al.,2008; Elankumaran et al.,2006).

Apart from activating apoptosis mechanisms we need to investigate the interaction of NDV with the FAM26F gene an enigmatic protein also known as INAM (IRF-3–dependent NK-activating molecule (INAM) which is known to be activated in case of viral infections (Takashi et al., 2002).It is basically a 315 amino acid 34KD protein with four transmembrane domains as motifs predicted by TMHMM server (version 2.0).

FAM26F is known to be involved in the activation of innate immunity pathways, viral infections, bacterial infections and others. The role of FAM26F on a study conducted in SIV infected Rhesus Macaques investigated the role of FAM26F in immune system and it was found to be correlated with the SIV viral titer quantified for IP-10,MX-1 and tetherin gene altogether alongside CD4+ T-cells (Javed ,2012).In another study conducted to investigate the efficacy of MVA85A and T cell immune responses. The results elucidated an induction of a strong immune response followed by activation of FAM26F alongside IP-10,CASP3 GBP5, PARP9 and other genes (Matsumiya et al.,2014).

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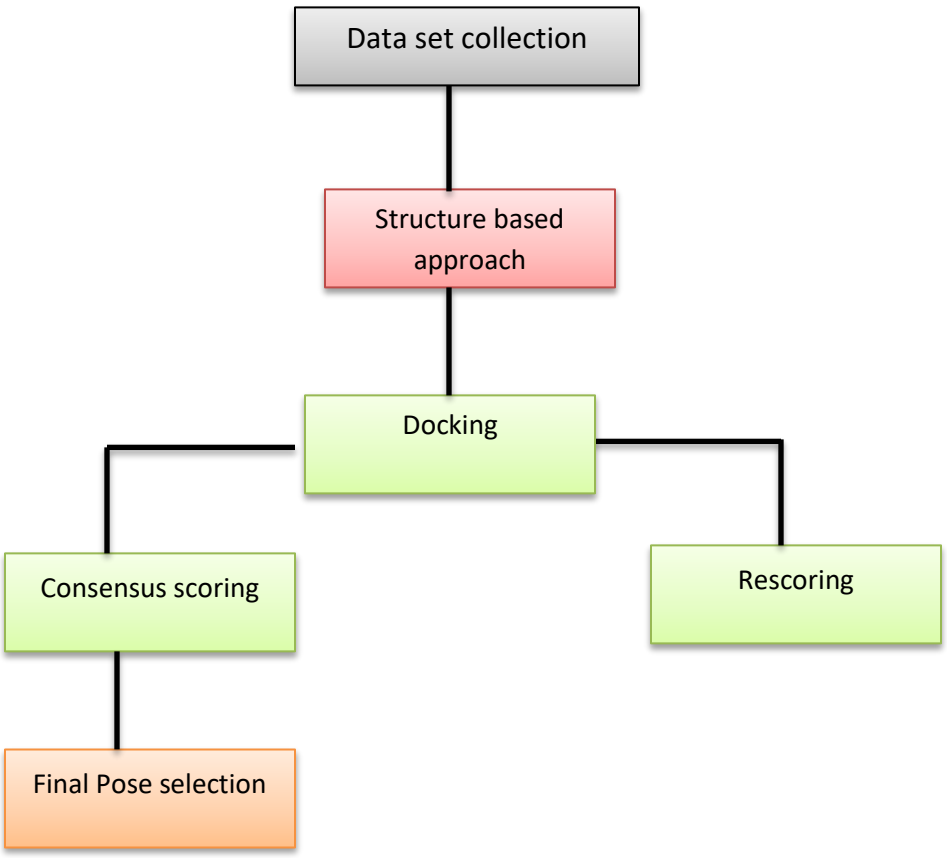
Materials and Methods

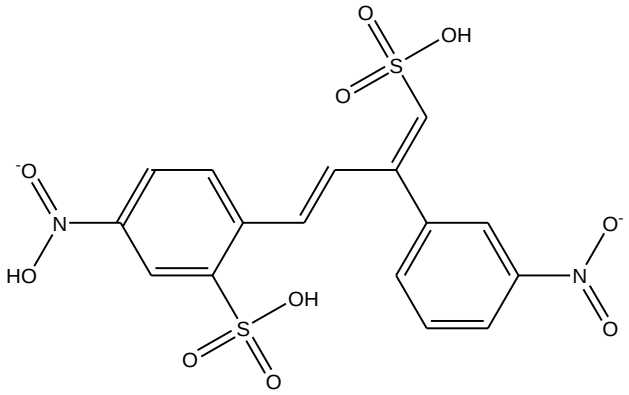
Structure based approach was used to identify the ligand protein interactions of ST6GAL I enzyme against the Aromatic and Flavonoid compound based inhibitors of the enzyme following workflow was adopted in the methodology illustrated in the flow chart below.



3.1 Databases:

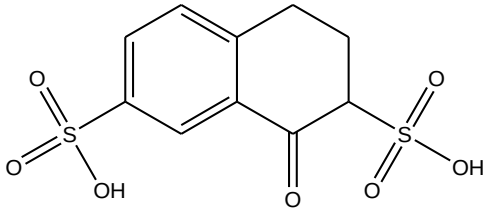
Different classes of ST6GAL I inhibitors were taken from literature. A total of 9 inhibitors were selected from two classes of compounds Aromatic and Flavonoid compounds on the basis of their Inhibitory concentration (IC_{50}) values exhibiting strong activity values. All the molecules

were extracted from studies reported in the literature for (IC_{50}) values for both aromatic and flavonoid compounds. The SMILEs of all compounds were constructed using Chemoffice Ultra (Chemdraw 8.0) software. The spatial orientation of atoms and structures were kept intact. Final 3D structures were constructed using SMILES string in the Molecular operating environment (MOE) 2007 Software.



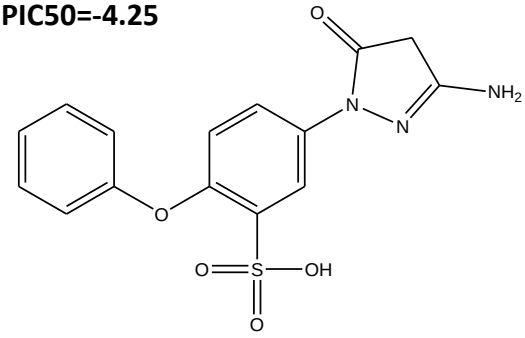
Ligand 1

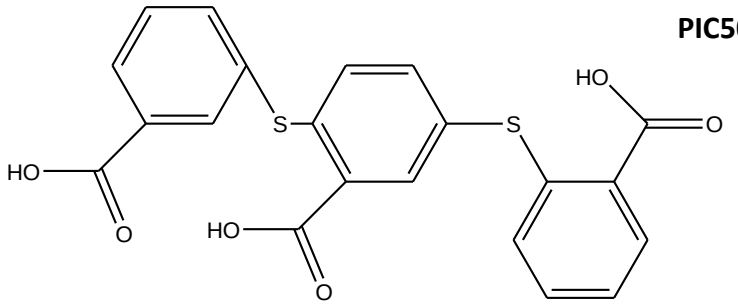
IC50=56µM
PIC50=-4.25



Ligand 9

IC50=106.4µM
PIC50=-3.97



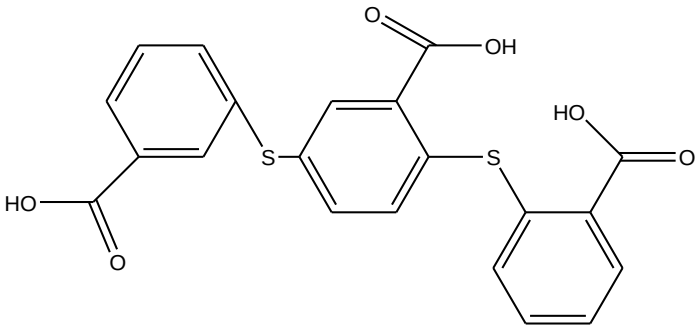


Ligand 2
IC50=10.8µM
PIC50= -4.96657

Ligand 3

IC50=133.5M,

PIC50=-3.8745

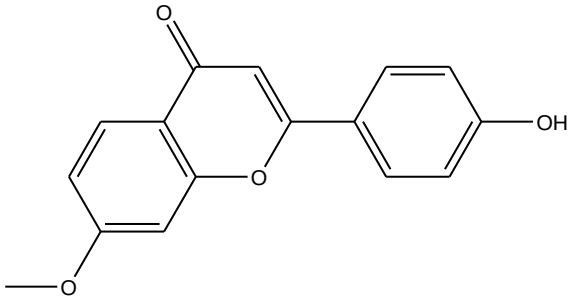


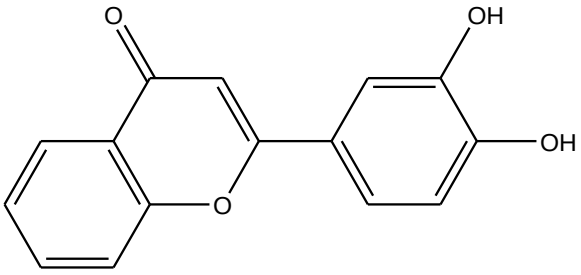
Ligand 4

IC50=10.8µM
PIC50= -4.96657

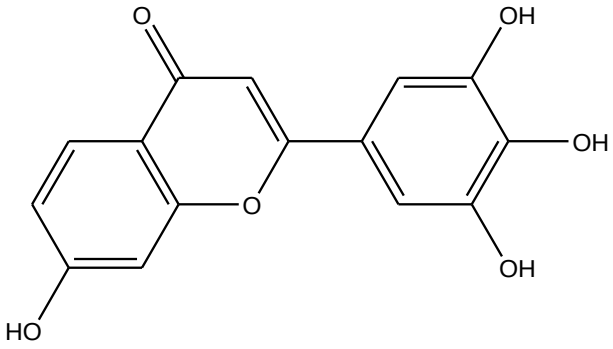
Ligand 5

IC50=57.5µM
PIC50=-3.240339



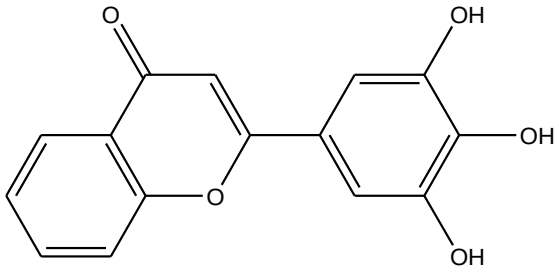


Ligand 6
IC50=81.7µM
PIC50=-4.0877



Ligand 8

IC50=7.5µM
PIC50=-5124996



Ligand 7

IC50=7.1µM
PIC50=-5.14880

Docking studies and Pose Analysis:

3.2 Docking by MOE:

Ligand Preparation:

The chemical structures were drawn using chemdraw ultra 8.0 and were saved in the MOE file format. MOE-2007 by Chemical Computing Group Inc. was used. The MOE file containing the aromatic and flavonoid compounds were opened in MOE .The minimization of the model and structure total energy was doing utilizing the current MOE default force field that is MMFF94s in the MOE 2007 program package. The file was saved in .Moe format. The database of the ligands drawn was built in MOE software using SMILE strings from the structures drawn in

chemdraw ultra 8.0.The prepared ligands were subjected to docking by using the dock option under simulations option using variety of scoring functions and placement methods to optimize the docking protocol.

Protein Preparation:

The 4JS1 structure of the enzyme ST6GLI was downloaded from Protein data bank and imported in MOE 2007 software for docking purpose. 4JS1 structure was subjected to Protonation for adding hydrogen molecules to the protein structure. The binding cavity was isolated by computing the ligand protein interactions of the ST6GALI protein and co crystalized ligand cytidine. Docking protocol was performed after the binding cavity isolation of a total area of 5.0A°. The binding cavity and surrounding area of the molecule was selected. ST6GALI inhibitors database was constructed in MOE software and it was subjected to protein docking to study the ligand protein interactions between the ST6GAL I inhibitors and ST6GAL I protein. Different scoring functions and placement methods and maximum number of poses were generated to optimize the docking protocol.

Docking was performed to analyze and compute the ligand protein interaction of the aromatic and flavonoid compounds inhibitors against the ST6GAL I active site and obtain the best possible confirmation of each ligand for further analysis. The structure used in the study for docking of ST6GAL I was that of 4JS1 (Kuhn *et al.,* 2013). The resolution of the structure was 2.09 °A and a polymer of 318 amino acids was taken into account. Different combination of scoring functions and placement methods were carried out to find the best pose for the ligand closest to the co –crystalized ligand with lowest RMSD value. Different placement methods including Alpha PMI, Alpha triangle, Triangular matcher with scoring functions including

London dG, Affinity HB, Alpha HB were used. 100 poses per ligand were generated for 9 ligands with 532 poses retained. Alpha PMI Placement method and London DG, was selected after docking protocol optimization. Best poses were further investigated for ligand protein interactions. Best scoring poses were further rescored and validated using Affinity DG, London DG and alpha HB scoring functions. Top ten poses were selected in each category after rescoring and the poses with best score of 3/3 and 2/3 were selected on the basis of consensus scoring and the ligand protein interactions of the aromatic and flavonoid compounds were computed. The ligand protein interactions shall be discussed in depth in the results and discussion section.

Table 3.1: List of laboratory equipment, consumables and reagents.

Name	Manufacturer
25mM $MgCl_2$	Thermoscientific, USA
Nanodrop Biophotometer Plus	Eppendorf, USA
Gel documentation Dolphin Doc (S/N470883)	Wealtec, USA
TRIZOL reagent (Cat#15596-018)	Invitrogen, USA
Thermocycler GeneAmp® PCR System (9700)	Thermoscientific, USA
-80°c upright ultralow freezer	Thermoscientific, USA
Pipette tips rnase/dnase free (10-1000 µl),	Biologix, USA
12 well petri plates	Corning, USA
-Refrigerated Centrifuge (5810R)	Eppendorf, USA
10ml sterilized syringes	Bio west, France
Fetal bovine serum	Bio west, France
Penicillin/ streptomycin	Bio west, France
Real Time PCR system (7300)	Applied Biosystems, USA
Maxima SYBR Green/ROX qPCR Master Mix	Thermoscientific, USA
Taq polymerase (EP0402)	Fermentas, USA
Reverse Transcriptase	Fermentas, USA
Dnase/Rnase free ultrapure water (RO582)	Fermentas, USA
Oligo Dt	Thermoscientific, USA
50bp and 100 bp ladder (Catalog No: SM0373)	Fermentas, USA
10mM dNTPs	Thermoscientific, USA

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Materials and Methods:

3.3 Virus Propagation and Harvestation:

The Velogenic strains of the Newcastle disease virus were provided by the kind courtesy of Pakistan Poultry research institute, Rawalpindi, Pakistan. A stock of the infectious virus was propagated in the 9 day old chicken embryonated Specific Pathogen free (SPF) eggs. The virus was harvested from the chorioallantoic fluid 48 hours post infection. The harvested virus from the chorio-allantoic fluid was then purified by centrifugation (3000 rpm for 30 min at 4°C) later passed through a 0.2μm filter and stored at -80°C. The presence of virus and calculation of viral titer was done by Hemagglutination assay.

3.4 Hemagglutination Assay:

A 96 well round bottom microtitration plate was taken and 50μL of PBS with PH 7.2 was dispensed in all wells. In the first well, 50μL of allantoinic-amniotc fluid (prepared form NDV containing samples) was poured by micropipette and mixed thoroughly. From the first well, 50 μL was transferred to the next well and this twofold serial dilution was done up till the last 11th well to dilute the AAF to 1:2048. 50 μL of chicken RBC 1% suspension was dispensed from 1 to 12 wells of the plate and the 12th well served as negative control because it only had PBS and washed chicken RBC suspension. The plate was shaken smoothly for few seconds and was kept in the incubator at 37 ℃ for 20 minutes. The RBC in the 12th well settled down as a bead or button and interpretation of results was done as follows:

Hemagglutination Positive: Uniform reddish beads throughout the well indicating uniformly distributed RBCs indicated haemagglutination between the virus and the RBCs.

Hemagglutination Negative: RBCs settled at the bottom of the well in the form of a sharp button.

HA titer is defined as the reciprocal of the highest dilution and minimum amount of virus that gives the complete agglutination of the virus suspension per unit of RBCs. The highest dilution of the AAF which gave the hemagglutination was the 1 HA unit and its reciprocal indicated the HA titer of the sample.

Hemagglutination (HA) and Hemagglutination Inhibition (HI) Assays:

HA testing and HI test of the infected virus on Huh 7 cell line were performed using the 1% chicken RBC solution as described earlier [30]. Briefly stating, two fold serial dilution of serum was prepared in the 50µl volume and 4HA unit of the adapted NDV Velogenic strain in 50µl was added to each well. The plate was incubated at room temperature for 30 minutes and then 50 µL of the 1%(v/v) of chicken RBC were added to each well; mixed by gentle shaking and incubated for 30 minutes at 37°C.

Cells:

HeLa cell line was maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% Fetal bovine serum and 1 % Penicillin/Streptomycin antibiotic). Hela cells were maintained in a humidified incubator with 5% CO_2 at 37°C. The cells were grown to a density of 60-70% in T-25cm^2 flasks prior to passaging.

3.5. Infection of HeLa cells with NDVPv

Hela cells were grown to 70-80% confluence in a 25cm^2 tissue culture flask (Corning USA) and infected with 6 Hemagglutinating units (1:64) of the virus particles of the Newcastle disease virus Velogenic strain. NDV parental stock diluted in 1ml of DMEM media) and incubated at 37°C for 1 hour 5% CO_2. Non- infected Huh7 cells were used as controls. A monolayer of infected cells was observed under an inverted microscope at 6h and 24hr interval for cytopathic effects. The control cells remained unaffected and morphological changes were observed in the experimental (infected cells with virus) like cell shrinkage, cell rounding, ,detachment of cells from culture flask and syncytium formation were observed. The cells were harvested at 72 post infection and supernatants were collected for further second round infection of Huh 7 cells. At 72 hours of infection the passage 1 of the cells containing the virus was harvested and frozen at -20°C and thawed later to infected the fresh 60-70% confluent Huh 7 cells again as described earlier.

Molecular detection of NDV F protein by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA extraction was done from the Newcastle disease virus infected Huh 7 cells by the INSTANT virus RNA/DNA kit RoboGene® Detection Kit. The protocol was carried out using the manufacturer's instructions. Complimentary DNA synthesis for the NDV Velogenic F protein was carried out using the universal primers for NDV F-Protein hypervariable regions with sense-strand 3'TTGATGGCAGGCCTCTTGC-5'and antisense 5'-TTGATGGCAGGCCTCTTGC 3'. It was carried out using MMLV 5X Buffer, 10mM dNTPs, NDV antisense primer (0.5µM), MMLV reverse transcriptase 200U/µL, RNase inhibitor 40U/ µL, RNA template and PCR volume to make up the final concentration up to 20 µL. The reaction

was carried out on Applied Biosystems Thermocycler at 33°C for 55 minutes followed by incubation at 95°C for 5minutes.

Detection of F protein

The cDNA synthesis was done by using the 1.25mM $MgCl_2$, 10X Taq Buffer (Thermoscientific), 2mM DNTPs Forward and Reverse Primer (0.5µM) each, cDNA 7 µL (1µg concentration), 2 units of Taq Polymerase and PCR water to make up the final volume to 25 µL using the following reaction conditions. The reaction mixture was incubated at 94°C for 5minutes. Then, 94°C for 1minute, 58°C for 1 minute and 72°C for 1 minute for 35 cycles. A last step of 72°C for one minute was added. The amplified F-protein was fractionated on 2% Tris acetate EDTA agarose gel, stained with ethidium bromide solution (50ng/ml) and analyzed on Chemi-Doc MP imaging system (BioRad).The image was analyzed using Image Lab software.

3.6 Cell viability assays

Trypan Blue exclusion Assays

For cell growth and viability studies, a uniform suspension of Hela cells were seeded in T25cm^2 flasks maintained in DMEM medium in a humidified chamber at 37°C with 5%CO_2. The cell viability was calculated using trypan blue exclusion assay as described by (Steven *et al*., 1993). Cell viability for NDV infected Huh 7 cells at 24, 48 hpi was determined by the protocol as described above.

Trypan blue exclusion assay and Cell Counting

Hela cells were counted using a hemocytometer. The hemocytometer is a glass microscope slide that is engraved with perpendicular lines forming a grid. 10µl of uniform suspension of cells was separated in an eppendrof tube and same amount of trypan blue stain was added in it, thus preparing a 1:1 dilution of cells in trypan blue. The mixture was pipette to ensure a homogeneous cell suspension. A cover slip was affixed to the hemocytometer and approximately 10µl of the trypan blue added cell suspension was loaded to the chamber of the hemocytometer by cautiously placing the cover slip at the edge, hence allowing the chamber to fill by capillary action. The slide was then placed under a microscope and cells were counted using 100X magnification.

3.7 SEMI QUANTITATIVE POLYMERASE CHAIN REACTION

RNA extraction and Quantification

Treated cells were pelleted down and washed with PBS. The pellet was resuspended in 200µL of Phosphate buffer saline (PBS). The pellet was re-suspended in 1200µl of TRIZOL reagent (Invitrogen Cat#15596-018) and 5N of 25µl of glacial acetic acid was added and incubated on ice for five-ten minutes. 500µl of chilled chloroform was added to the mixture and centrifuged at 14000rpm in (Model 1-15K, Sigma Germany) for 20 minutes. The RNA was separated from the supernatant in a clear aqueous phase by pipetting and chilled 500µL of isopropanol was added to it and incubated on ice for ten minutes for effective precipitation of RNA. RNA was subjected to centrifugation at 14,000rpm for twenty minutes. The supernatant was removed; RNA was washed with 85% ethanol by centrifugation again at 14,000rpm and was then re-suspended in 25µL of Nuclease free ultrapure water. RNA quantification was done using the

spectrophotometer (Eppendorf BioPhotometer plus™).The quantified RNA was subjected to cDNA synthesis.

cDNA synthesis

The extracted RNA was converted to complementary DNA using the reverse transcription process. Oligo(dT) primers were used in the following reaction mixture

- RNA_____2000ng
- DNase/RNase free water (volume upto) 16µL
- Oligo dT_____1.5µL
- 10mMdNTPs_____2µL

The reaction mixture was incubated at 70^{o} C for 5 min in a thermocycler later chilled on ice. The other reagents were added and mixture was incubated again

- 5X RT buffer_____4 µL
- RNase inhibitor (Ferment)_____0.5µL

The reagents were incubated at 37^{o} C for 5 min and then reverse transcriptase was added keeping the reagents on ice.

- Reverse Transcriptase _____1µL
- Final volume_____ upto 20 µL

The prepared reaction mixture was then profiled on a thermocycler GeneAmp® PCR System The PCR primers were extracted from the review of literature from the following studies(Rodriguez-Jimenez et al. 2003; Abel et al 2001 Nasirudeen et al., 2011; Yang et al.,2007; Buß et al.,2010) and some were obtained from primer bank of ASAB, NUST, Islamabad. The primers

were optimized at the following annealing temperatures at 58°C, 60°C and 62 °C annealing temperature with conventional PCR followed by agarose gel electrophoresis of the PCR products. The master mix containing following reagents was prepared as follows: 9700 at 42°C for 60min followed by extension at 70°C for 10min. The synthesized cDNA was stored at -20°C until further use.

3.7.1 Primer designing and Optimization

- 25mM $MgCl_2$_____1.2µl
- 10mM dNTPs_____0.5µl
- Forward primer_____ 1µl
- Reverse primer_____ 1µl
- Taq buffer 10X_____1.5µl
- cDNA_____ 2µl
- Taq DNA polymerase_____0.4µl
- Nuclease Free water (volume upto)__15µl

The reaction mixture was placed in a thermocycler GeneAmp® PCR System 9700 at following profile:

- Initial denaturation_____95℃ for 5 min
- Denaturation _____95℃ for 45 sec
- Anealing_____60℃ for 45 sec

} 35 cycles

- Extension _____72℃ for 45 sec
- Final extension_____72℃ for 10 min

7µl of PCR product was mixed with 2µl of 6X loading dye. 2.5uL of 50 and 100bp DNA ladder were loaded in 2% gel for electrophoresis. The bands were then visualized in UV light.

The primer sequences along with their amplicon length and annealing temperature are summarized in following table.

Table 3.2: List of genes along with their primers sequence and amplicon size.

Serial No.	Gene	Primer Sequence (5'-3')	Annealing temperature (^{o}C)	Amplicon size (bp)
1	GAPDH-F	CCTGCACCACCAACTGCTTA	60	74bp
	GAPDH-R	CATGAGTCCTTCCACGATACCA	60	
	FAM26F-R	TTTGCTGCCACTCTTTCATGC	60	
4	IP10-F	GTGGCATTCAAGGAGTACCTC	60	198bp
	IP-10-R	TGATGGCCTTCGATTCTGGATT	60	
5	CCL5 F	CAATGTAGGAGCAGCAG	58	
	CCL5 R	CTGCTGCTTTGCCTACATTG		
7	MDA-5 F	GGCACCATGGGAAGTGATT	62	86bp
	MDA-5 R	ATTTGGTAAGGCCTGAGCTG		
8	IRF-3 F	TACGTGAGGCATGTGCTGA	60	425bp
	IRF-3 R	AGTGGGTGGCTGTTGGAAAT		
9	JAK1-F	GAACCAACGACAATGAGCAG	60	132bp
	JAK1-R	CCTGAGCAAACAGATACTCCAG	60	
10	STAT1-F	CGGAACCCAGGAATCTGTC	60	85bp
	STAT1-R	GAAAACTGCCAACTCAGCAC	60	
11	CASP3-F	GAAGCGAATCAATGGACTCTGG	60	152bp
	CASP3-R	GTTTGCTGCATCGACATCTGTAC	60	

12	NFκB-F	ACATTGCCTGGATGAAGTTTG	60	192bp
	NFκB-R	CATCAATGCCTTCCCAGTAAA	60	
13				113bp
	Caspase-8 F	CTGCTGGGGATGGCCACTGTG	70	
14	Caspase-8 R	TCGCCTCGAGGACATCGCTCTC		
	CASP3-F	GAAGCGAATCAATGGACTCTGG	60	
15	CASP3-R	GTTTGCTGCATCGACATCTGTAC	60	152bp
16	NDV F protein (F)	TTGATGGCAGGCCTCTTGC	58	362bp
	NDV F Protein (R)	TTGATGGCAGGCCTCTTGC	58	

3.8 QUANTITATIVE POLYMERASE CHAIN REACTION

Real time PCR optimization

The primer efficiency and specificity was calculated by amplifying serial dilutions of cDNA (dilution factor=5) on Applied Biosystems 7300 Real Time PCR by analyzing the melting curve. The reaction mixture was prepared in real time pcr tubes.

- NF water (volume upto)_____12.5µl
- cDNA_____ 20ng

- Forward primer_____0.5µl
- Reverse primer_____0.5µl
- SYBR green_____ 6.25µl

All samples were run in duplicates and as one cycle at 95 ºC for 10 minutes, followed by 40 cycles at 94 ºC (denaturation) for 15 seconds and 60 ºC (annealing) for 60 seconds and 72 ºC for 45 seconds followed by addition of diss. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene and reference for calculating the relative expression level of the genes in question.

Real time PCR results calculation

The fold change in the expression of targeted markers was determined by comparing Ct values of the target genes with reference housekeeping gene GAPDH as follows:

Ct Value of GAPDH in control sample= X1

Ct Value of GAPDH in target sample= X2

Ct Value of Target Gene in control sample = Y1

Ct Value of Target Gene in treated sample = Y2

ΔCt Control = (Ct of Target in Control) - (Ct of GAPDH in Control) = Y1- X1= ΔCt1

ΔCt Treated = (Ct of Target in treated) - (Ct of GAPDH in treated) = Y2- X2= ΔCt2

ΔΔCt = (ΔCt treated) – (ΔCt control) = ΔCt2- ΔCt1

Copy number change = 2 (-ΔΔCt) and **Copy Number /100 copies of GAPDH= 2 (-ΔCt)*100**

3.9 STATISTICAL ANALYSIS

Statistical tests were carried out in Graph pad prism, version 5.01. The hypothesis was tested with two way ANOVA checking for bonferroni's multiple comparison test.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ was considered significant.

Results and Discussion

4.1. Protein structure selection:

There are two crystals of human ST6GAL I available in literature reported by (Kuhn et al., 2013). Both of these structures are in ligand bound form. Human ST6GAL I 4JS1 PDB id was selected in ligand bound form shown in the figure below. The carbon alpha chain RMSD (root mean square deviation) was calculated from literature was 2.03 °A. When both the structures were superimposed it was 0.4685°A**.** All the residues were superimposed exactly with a slight variation in position of oxygen atoms. Therefore 4JS1 structure was selected for docking analysis on the basis of high resolution and almost exact superimposition of the ligands.

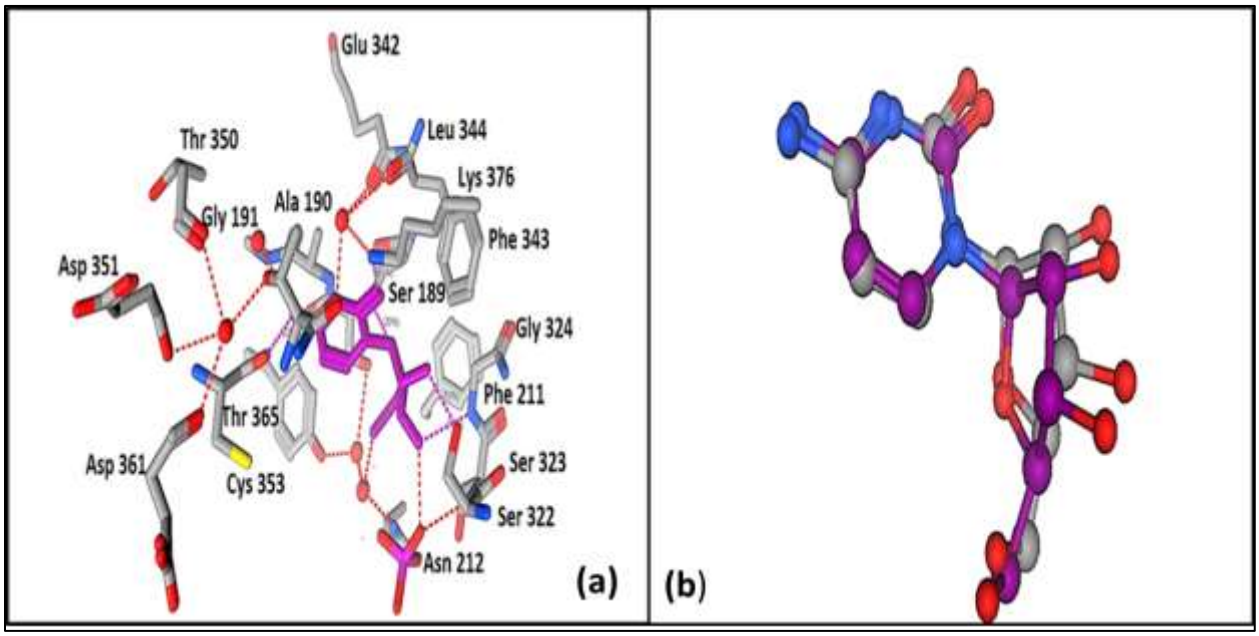


Figure 4.1 Co-crystalized ligand binding with ST6GAL I enzyme binding cavity.

(a) The figure illustrates the binding cavity of the Protein structure 4JS1 with CTN ligand bound followed shown in purple. (b) CTN original co-crystalized ligand shown in purple and gray is the superimposed ligand after docking protocol optimization.

4.2 Pose generation and Binding cavity:

The dataset of ligands discussed in the methodology section containing aromatic and flavonoid compounds were selected and used for docking in the ST6Gal I enzyme. The binding cavity of the enzyme was selected from review of literature and mutagenesis studies reported. The most significant features of the binding cavity of the enzyme were its shape, size , catalytic domain and active site discussed above . The exact size of the binding site of ST6Gal I enzyme is not known though exactly in literature but the active site is made up of 11 conserved residues that differentiates it from the porcine and other ST6Gal I enzymes. These residues include Asn 212, Ser 323, Ser 322, Gly 324, Lys 376, Thr 365, Tyr 354, Asn 233, His 370 respectively (Andrew et al.,2015; Kuhn et al.,2013). The binding cavity of the enzyme and active site were identified in MOE 2007 software using an area of 5.0°A. Docking protocol was optimized with a scoring function and placement method of Alpha PMI and London DG used respectively. There were multiple poses generated for each ligand. The ligand and protein interactions were computed and analyzed for the interacting residues and types of interactions between the docked ligands and ST6Gal I enzyme binding site. About 100 poses were ligands were generated to certify and validate the precision and reproducibility in the data.

4.3 Pose evaluation

The method used to analyze the poses was that of consensus scoring. All the poses were subjected to rescoring for validation of the docking protocol using the following scoring functions including London DG, Affinity DG and Alpha HB using MOE 2007 software. 10 best ranked poses for each ligand were selected and used to compute the ligand protein interactions.

The ligand protein interactions of the two classes of aromatic compounds and flavonoid compounds were done separately. The following amino acid residues that were common amongst the aromatic compounds and binding cavity Phe 343,Cys 364, Asp 361, Asp 351,Ser 189,Glu 342,Tyr 356, Ser 323Gly 324,Ser 322, Asn 212(Kuhn et al.,2013; Andrew et al., 2015). Illustrated in the figure below

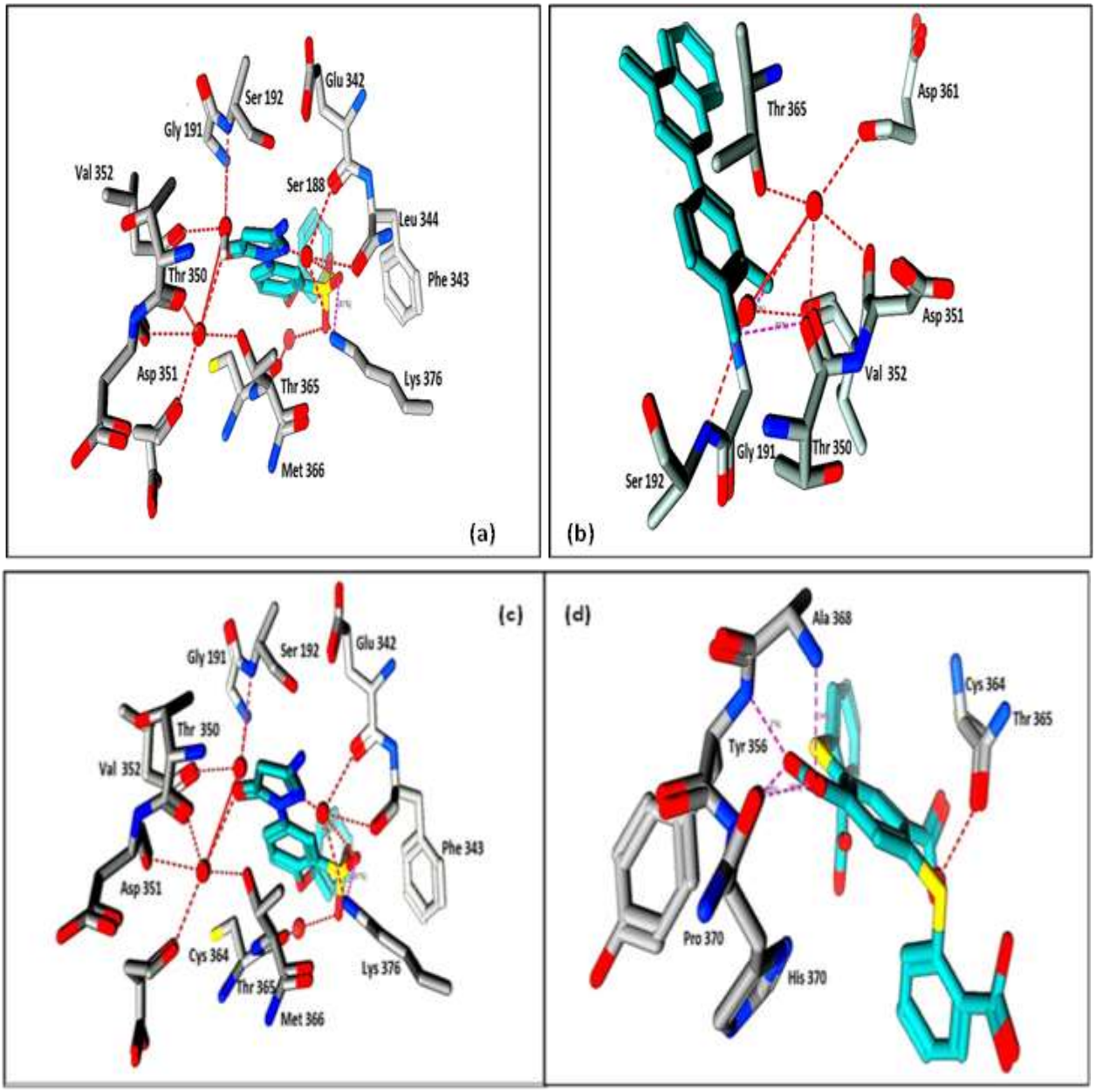
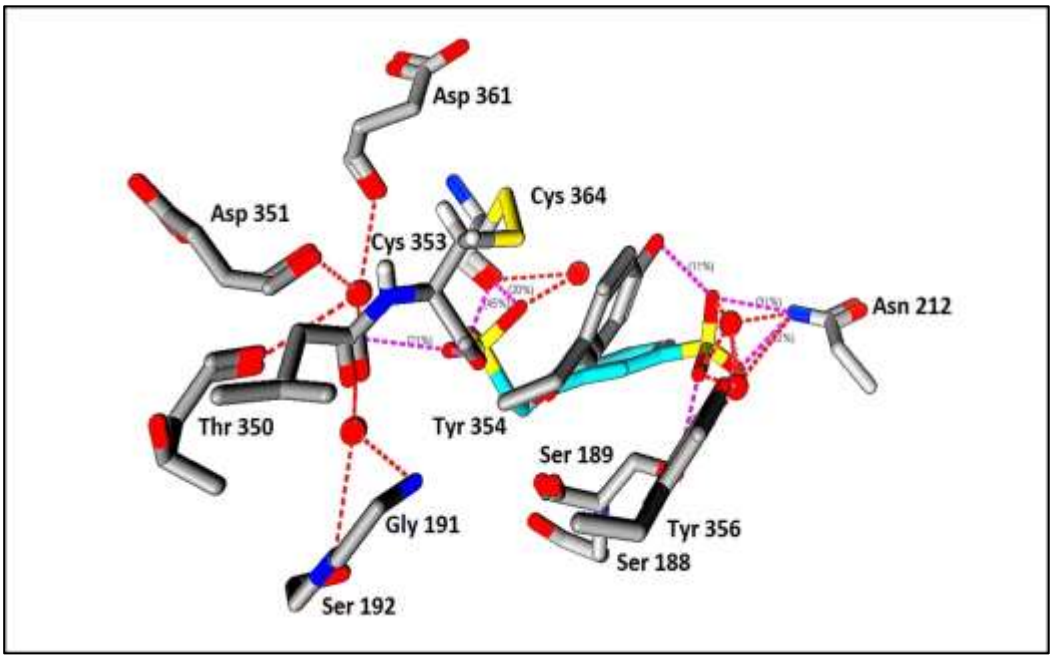


Figure 4.2 Ligand Protein interaction of Aromatic compounds and ST6GAL I binding site

The figure illustrates the ligand protein interaction between the aromatic compounds and the interacting amino acid residues. (a) ligand 1 (b) ligand 2 (c) ligand 3(d) ligand 4 (e) ligand 9 interaction with 4JS1 structure. The Ligands 1-4 & 9 are shown in cyan interacting with binding cavity.

The residues common amongst flavonoid compounds were Asn 212, Thr 365, Thr 365, Ser 322, Ser323, Ser 189, Ser 188, Met 366, Cys 353, Phe 21, Phe 343 Cys 364, Glu 342 illustrated in figure below.

Figure 4.3 Ligand protein interactions of ST6Gal I and aromatic compounds



The figure illustrates the ligand protein interactions between co-crystalized ligands and aromatic compounds.

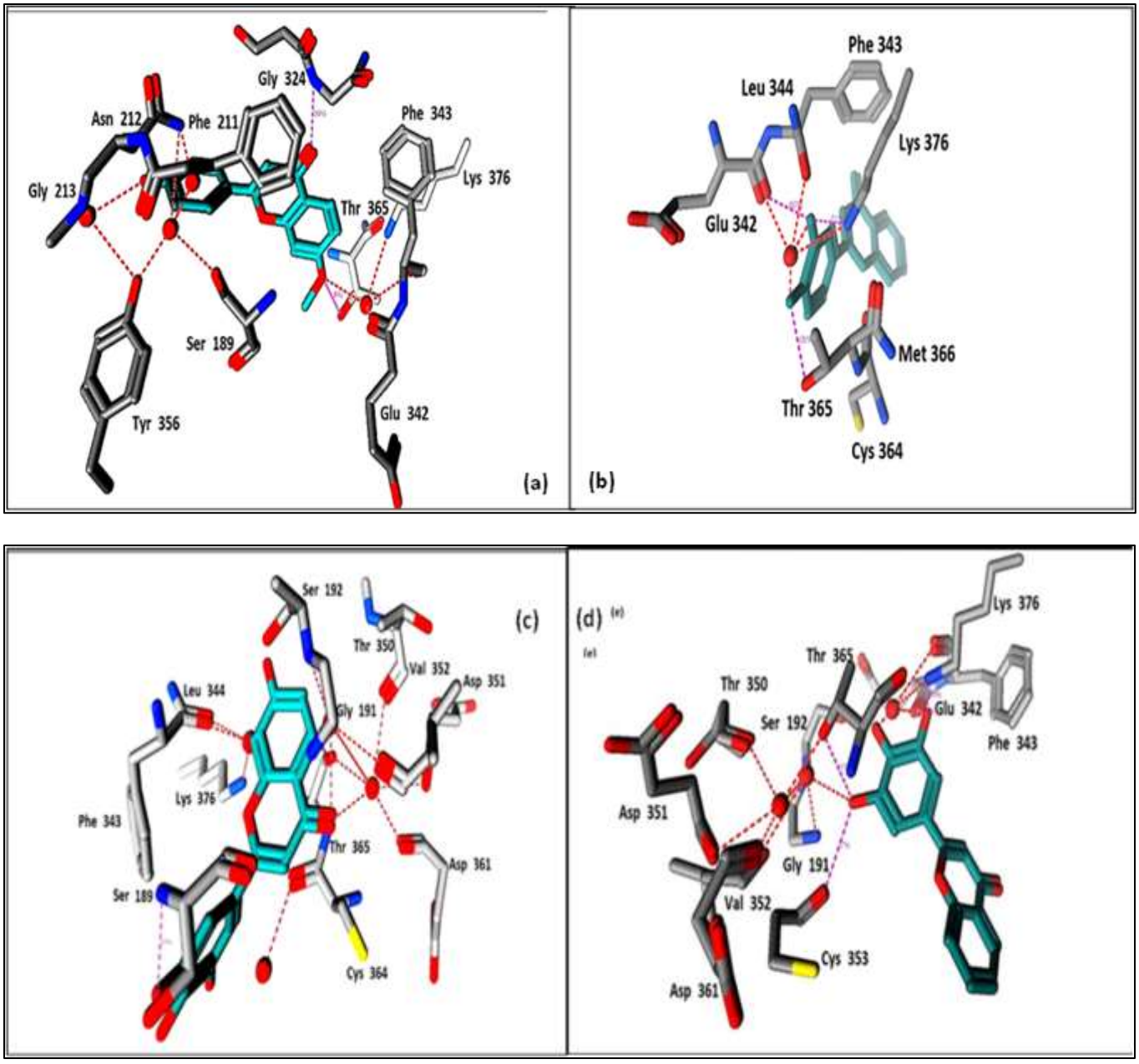


Figure 4.3 Ligand protein interactions of ST6Gal I and Flavonoid compounds

The figure illustrates the flavonoid compounds binding with the ST6Gal 1 active site with (a) ligand 5 (b) ligand 6 (c) ligand 8 (d) ligand 9 shown in cyan interacting with ST6Gal I binding site.

Asn 212, Ala 368,Ser 189, Ser 322, Ser 323, Glu 342, Cys 353, Cys 364,Gly 191, Tyr 354, Lys 376, Ser 192 are the common amino acids residues interacting between the Aromatic and flavonoid compounds interacting with the ST6GAL1 protein. His 370 is the common amino acid residue part of the catalytic domain interacts with aromatic compounds. Ala 363 in flavonoid compounds and His 370 in the aromatic compounds are the different residues interacting with the ST6GAL 1 protein.

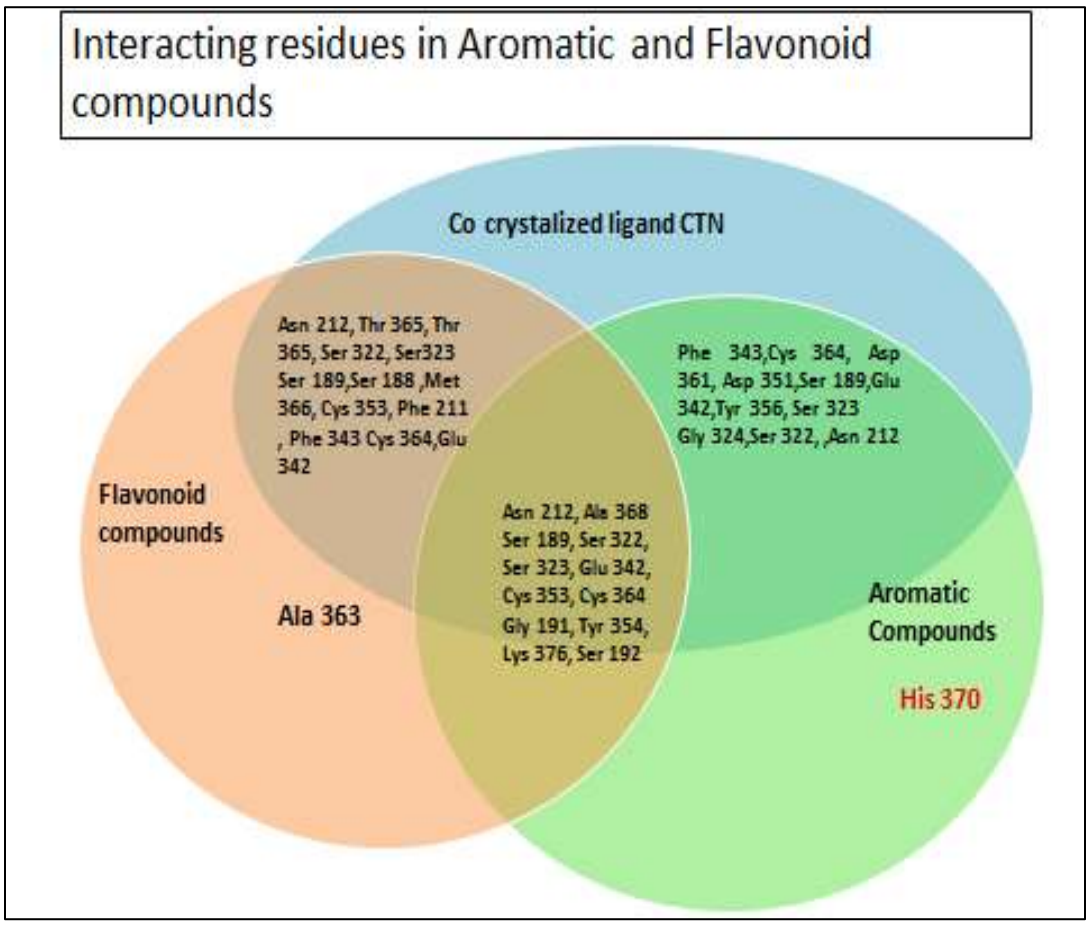


Figure 4.3.1 Conserved amino acid residues of ST6Gal1 with aromatic and Flavonoid compounds

The figure illustrates the overlapping of important amino acid residues in the literature review blue circle compared with aromatic compounds in green circle and flavonoid compounds in light orange. (Kuhn *et al.*, 2013; Andrew *et al*.,2015).

Sulphur and oxygen residues in Aromatic compounds interact with the amino acids in ST6GAL I protein via the hydrogen bonding and water mediated interactions Hydroxyl groups in the Flavonoid compounds interacts with the amino acids in ST6GAL I protein via the hydrogen bonding and water mediated interactions and strong hydrophobic interactions.

Conclusion:

The current study focusses on the development of novel drug inhibitor molecules for the ST6Gal 1 enzyme. The enzyme is overexpressed in more than twenty cancer types and is involved in malignancy, invaseness and metastasis. In the present study we aim to focus on targeting the inhibition of ST6GAL I protein by investigating its molecular interactions with the natural compounds including aromatic and flavonoids compounds. Ligand protein interactions of 9 compounds belonging to aromatic and flavonoid compounds were docked and ligand-protein interactions were computed and the interaction pattern revealed both the classes of compounds interact with the active site of the ST6Gal I enzyme and the ligand protein interaction pattern shows strong binding affinity with active site. The inhibitor with a high IC50 value shows strong binding affinity with the active site.

Results:

4.4. Virus Inoculation and Harvesting:

Figure 4.4.Virus inoculation and harvestation in embryonated eggs-

The figure illustrate the following events of virus inoculation and harvestation.(A) Innoculation of virus in the 9 day old chicken embryonated eggs through the chorio –Allantoic Route. (B) (C) (D) show collection and harvestation of the Amino Allantoic fluid (AAF) from the allantoic cavity of the 9 day old chicken embryonated egg containing the virus.

4.4.1 Hemagglutination Assay:

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Figure 4.4.2 virus titer determination by Hemagglutination Assay.

The figure illustrates the HA assay results for virus titer determination. The well with agglutination show positive result and with button formation shows negative result. The above plate shows NDV Velogenic strain dilution up to the 10^{th} well (1:1024) dilution. 11^{TH} well has clumped RBCs and the 12th well was taken as a negative control with no agglutination.

According to the formula 10% RBC suspension contains =8x108 RBCs per ml
Since 0.5% RBC suspension is used so, 0.5% RBC suspension
= 4x107 RBCs per ml
Hence each well will contain=2x105 RBCs per 50 μL

At a dilution of 1:1024 there were 4.096 x 10^{10} hemagglutinating virus particles per ml of the Chorio-allantoic fluid harvested for NDV Velogenic strain.

SAMPLE	Dilution showing complete agglutination	HA Titer * dilution factor	Approximation of virus particles per ml
NDV VELOGENIC STRAIN	1:1024	$4.7x10^{10}$ x 1024	$4.096x\ 10^{10}$

Table 4.1 The table illustrates the viral titer of NDV present in the chorio-allantoic fluid after Hemagglutination assay.

4.5 Cell culturing and Virus infection in Hela cells:

Hela cells were cultured in DMEM media supplemented with 10%FBS AND 1% pencillin streptomycin antibiotic. The cells were kept in a humidified chamber at 37°C till 70-80% confluence was retained. The cells were grown in T25cm^2 flasks. The cells were washed with PBS and trypsinized using 0.25% Trypsin EDTA to detach the monolayer of cells prior to counting and were centrifuged. The pellet was washed and trypsin was removed and was resuspended in the media.

The cells were counted with hemocytometer, based on trypan blue exclusion method. Cell suspension was prepared by adding 10µl of cells and 20µl of trypan blue [dilution factor=total volume/dilute volume (20/10=2)]. The mixture was loaded onto the hemocytometer grid and was focused by 20X objective of the microscope. The cells were counted in four 16 corner squares (eliminating the dead cells, stained by trypan blue) as,

Cell count = Average cell count * dilution factor * 10^4 cell/ml

95% cells viability was determined by dividing the live cell count (unstained by trypan blue) from the total cell count (including the stained dead cell).

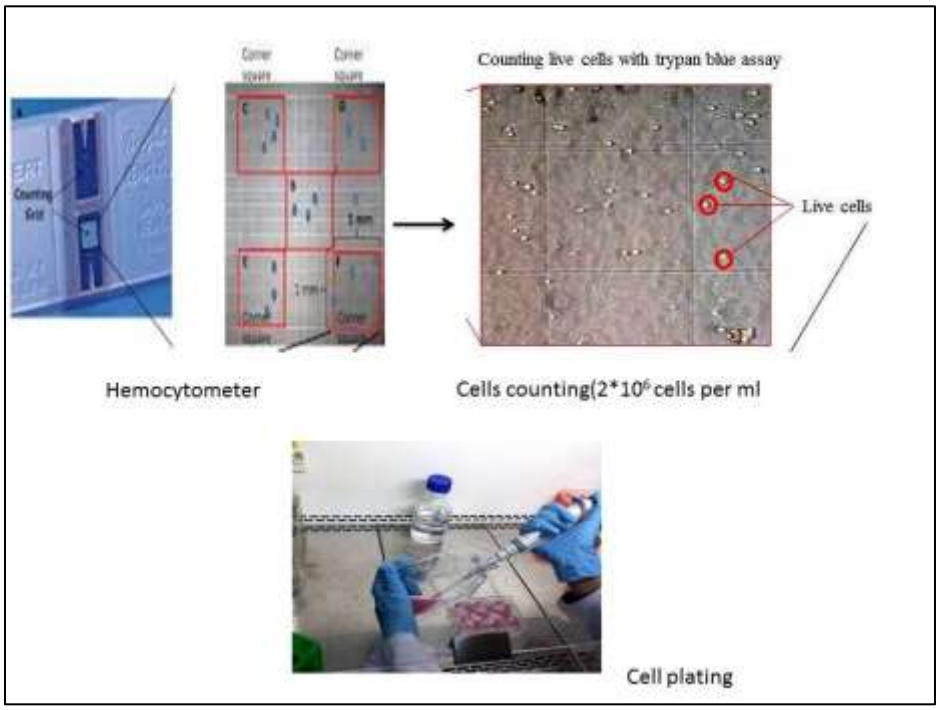


Figure 4.5: Trypan blue exclusion assay. Cell counting on hemocytometer grid using trypan blue staining solution under microscope followed by culturing of Hela cells in DMEM medium.

2x 10^6Hela cells per ml after counting are resuspended in the DMEM media and cultured after 80% confluence is retained they are infected with 6HAU (1:64) NDV Velogenic virus at different time points 6h, 24h and 48h respectively. After virus infection in the cells cytopathic effects were observed in the form of syncytium formation, membrane blebbing, cell scarring and cell death. RNA was extracted from the samples followed by c DNA synthesis, PCR and real time analysis and gene expression analysis of the innate immune system.

4.6 Semi Quantitative PCR

RNA samples were extracted from the NDV infected Hela cells at different time points at 6h, 24h, and 48hours post infection . RNA samples were quantified using Nanodrop (Biophotometer plus) followed by complimentary DNA (c DNA) synthesis**.**

Confirmation of c DNA synthesis:

The control and experimental RNA samples were subjected to complimentary DNA synthesis using reverse transcriptase. Verification of cDNA was done using PCR with GAPDH illustrated in the figure below**.**

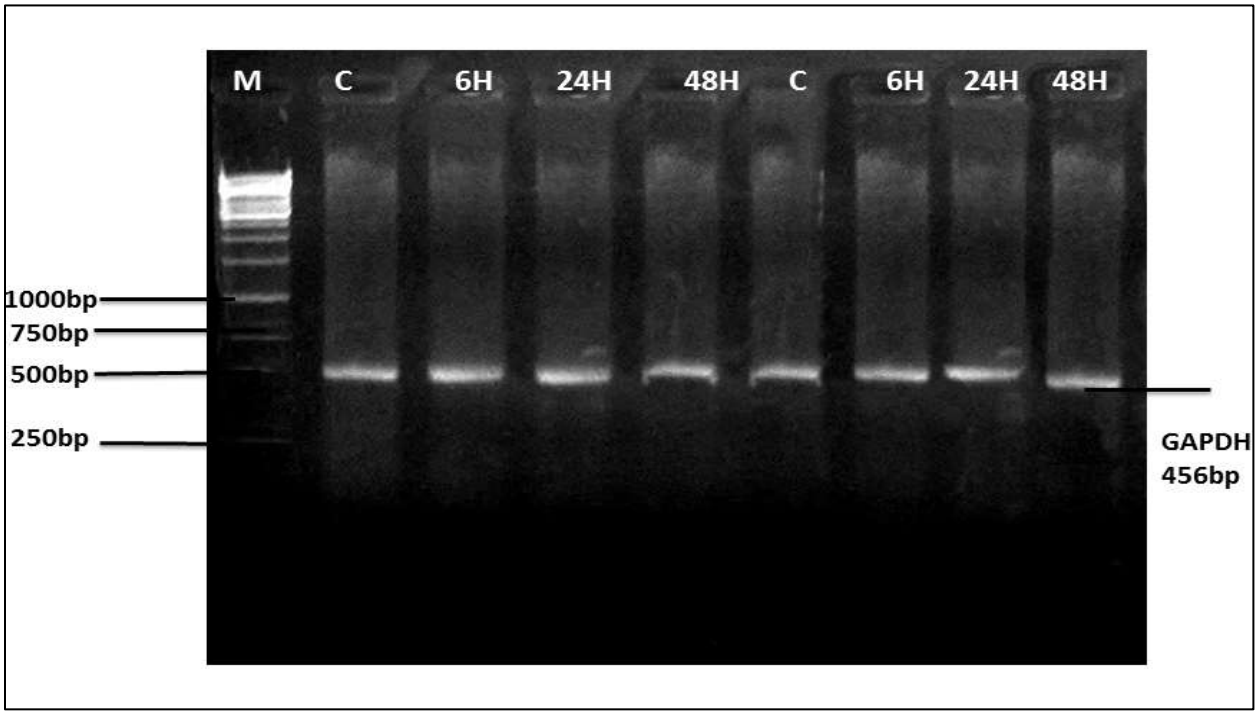
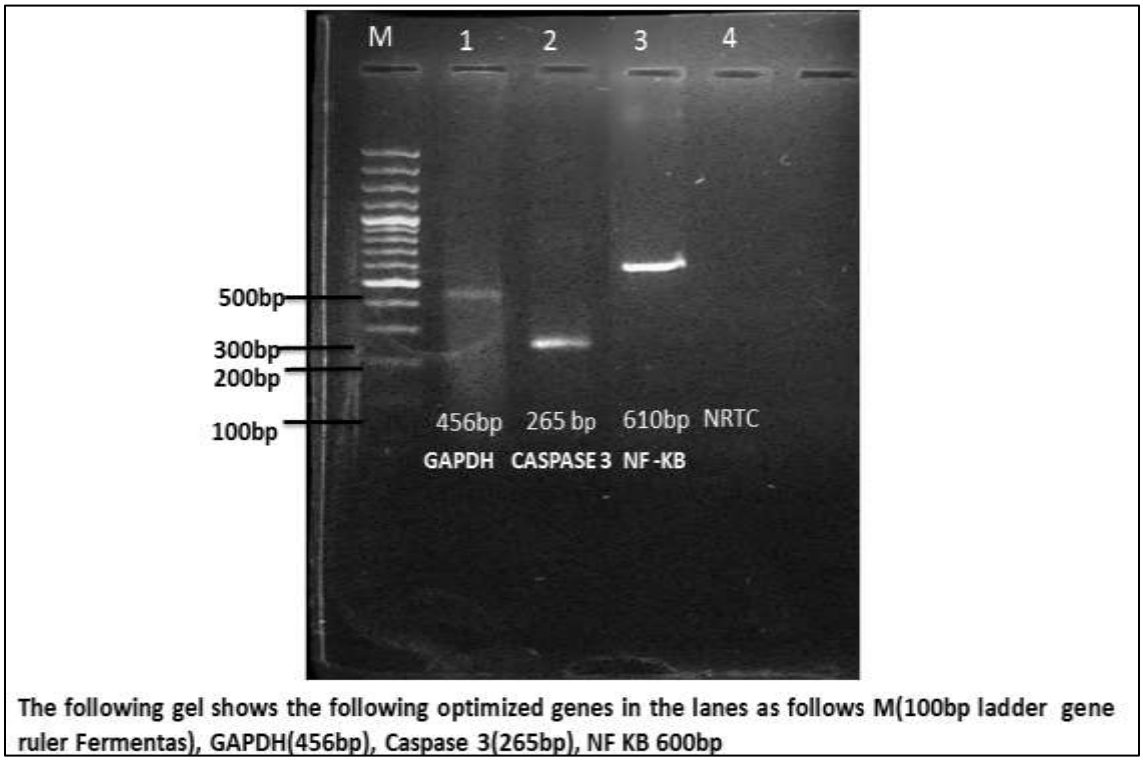


Figure 4.6 Verification of c DNA synthesis by Semi quantitative PCR

The figure illustrates the verification of cDNA synthesis in the following samples. Lane M shows 1000bp ladder, Lane C is the control cell sample followed by 6H,24H and 48H samples for GAPDH with 456bp size sharp bands resolved on 2% agarose gel.

4.7 Gene optimization by Semi Quantitative PCR

The desired genes used in the study were as follows IP-10, GAPDH, FAM26F, MDA5, CASP 8, IRF-3, JAK 1, STAT -1, CCL5, NF-Kβ, Caspase 3, Caspase 8 and NDV F protein respectively. They were optimized on the conditions described above in the materials and methods section . The PCR products were analyzed on 2% agarose gel electrophoresis. 50, 100 and 1000bp Ladders were used as a reference. The primers with optimum temperature and suitable product size were subjected for q PCR analysis.



The following gel shows the following optimized genes in the lanes as follows M(100bp ladder gene ruler Fermentas), GAPDH(456bp), Caspase 3(265bp), NF KB 600bp

Figure 4.7.1 Optimization of Genes by semi quantitative PCR.

The figure illustrates the optimized PCR products of GADPH, Caspase 3 and NF –KB genes respectively.

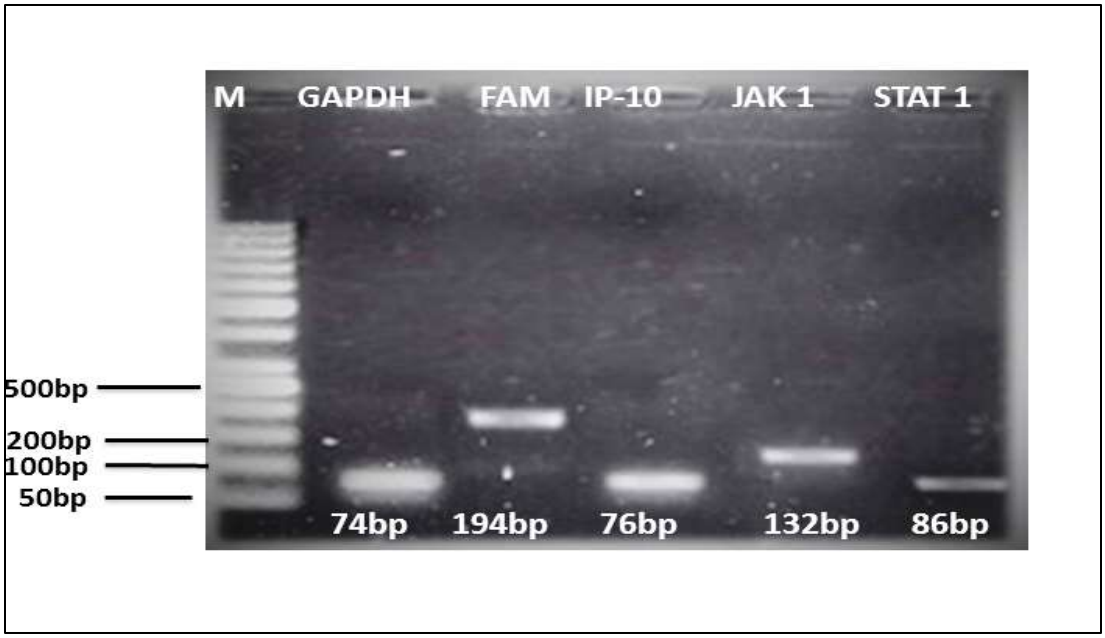


Figure 4.7.2 the figure illustrates the optimization of following genes from rows (1-5) including GAPDH, FAM26F, IP-10, JAK-1 and STAT-1 respectively.

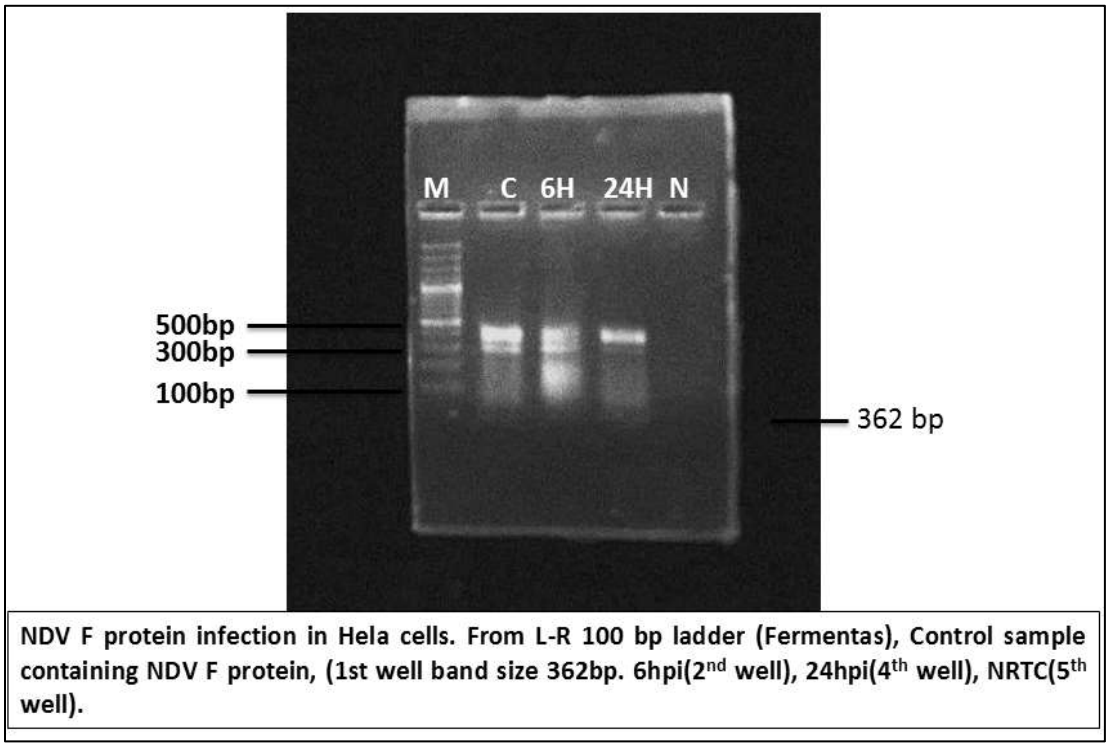


Figure 4.7.3 The figure illustrates NDV F protein optimization in NDVPv infected cells at 6h and 24h respectively a band size of 362 bp for F protein cleavage region was observed.

4.8 NDVPv Infection experiment in Hela cells

Newcastle disease virus Pakistani Velogenic strain was infected in Hela cells at different time points in T-25cm^2 flasks. Cells were grown to confluence about 2x10^6 cells per ml were infected with the 6 HAU (1:64) serial dilution of NDV parental stock diluted in 1ml of DMEM media. The virus was infected in cells at 6h, 24h and 48 hours respectively. Supernatants for each time pointswas collected and cells were pelleted down as well for further processing. The samples containing the supernatants were subjected to viral RNA extraction for the presence of NDV in the culture followed by RNA extraction using Trizol LS method for further analysis.

NDV infection confirmation by F-protein PCR:

NDV F –protein plays a significant role in virus infection, fusion and cell tropism. F- protein gene expression confirms NDV infection in the Hela cells at different hours post infection including 6h ,24h and 48h post infection confirmed by semi quantitative PCR with a band size of 362 base pair was observed (Dortmans et al., 2011: Chang and Dutch et al., 2012)

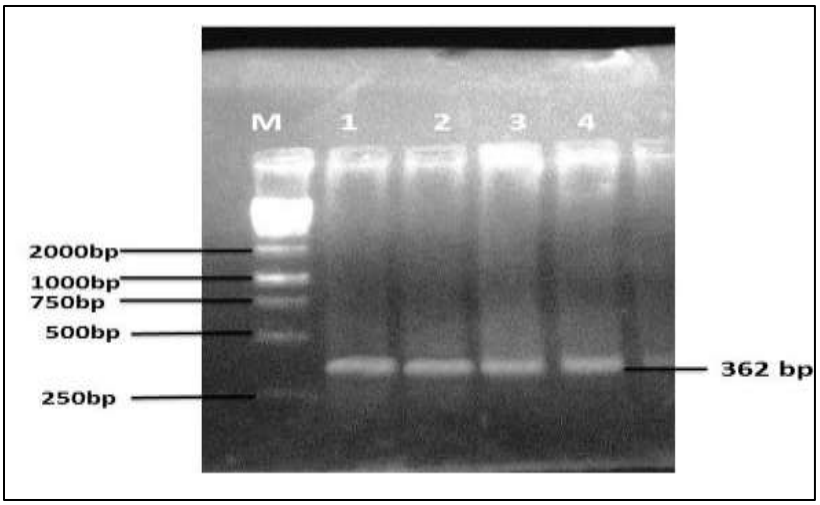
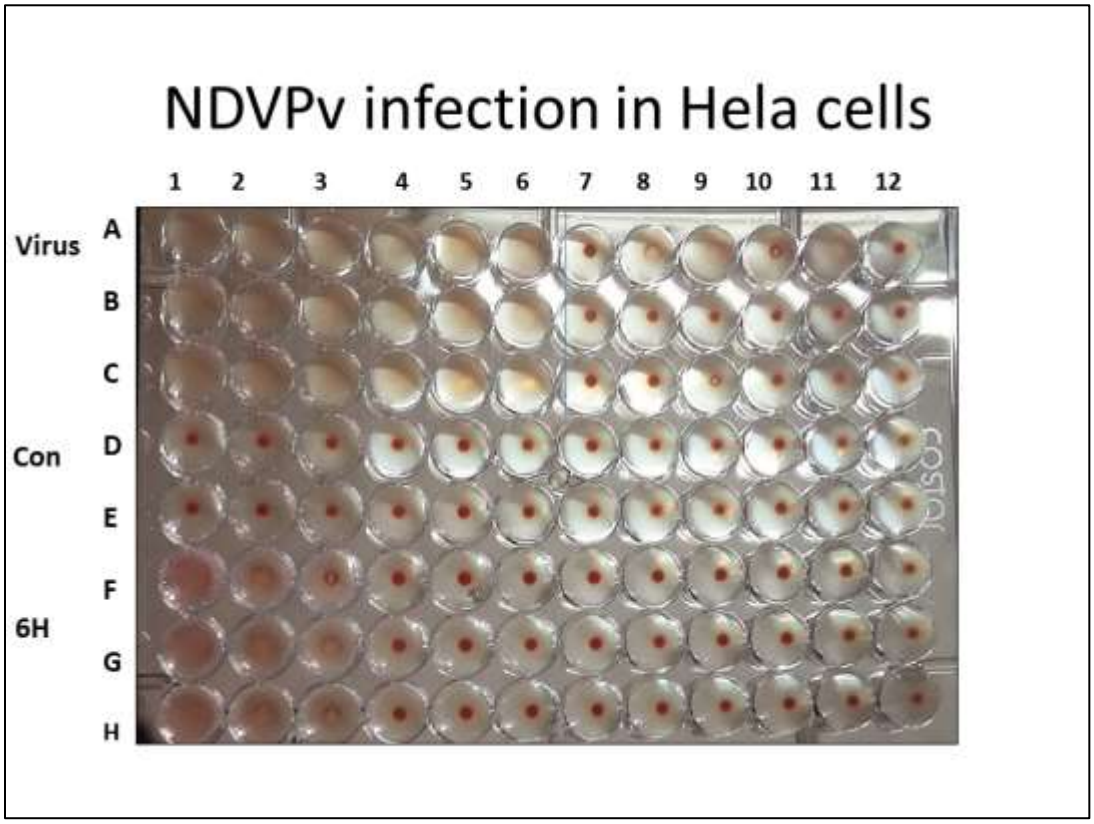


Figure 4.8.1: NDV infection at 6h,24h and 48hpi in Hela cells

The figure illustrates NDVPv infection in Hela cells and presence of virus in control and experimental conditions. Lane (M) is the reference gene ruler 1KB ladder by fermentas, Lane 1 is NDV F protein from pure virus culture Lane 2, NDVPv infection at 6h in Hela cells followed by 24h and 48h respectively in Lane 3 and Lane 4 respectively.

4.8.2 NDV Viral titer determination post infection

NDV virus infection was confirmed in the cells by Hemagglutination assay to confirm the number of virus particles infecting Hela cells at different times post infection and the exact number of virus particles can be calculated infecting the cancer cells. It is illustrated in the figures below.



S

Figure 4.8.2(a) Virus infection confirmation in Hela cells at 6hpi

the figure illustrates the Hemagglutination assay measuring the number of virus particles used to infect Hela cells. 6 HAU (1:64) dilution of the virus particles were used to infect Hela cells Rows (A-C) illustrate pure virus from chorioallantoic fluid. (C-D) shows control cells supernatant with no virus infection was taken as a negative control. (F-H) shows NDV viral titer from 6h post infection Hela cells with a viral titer of 3 HAU (1:8) dilution.

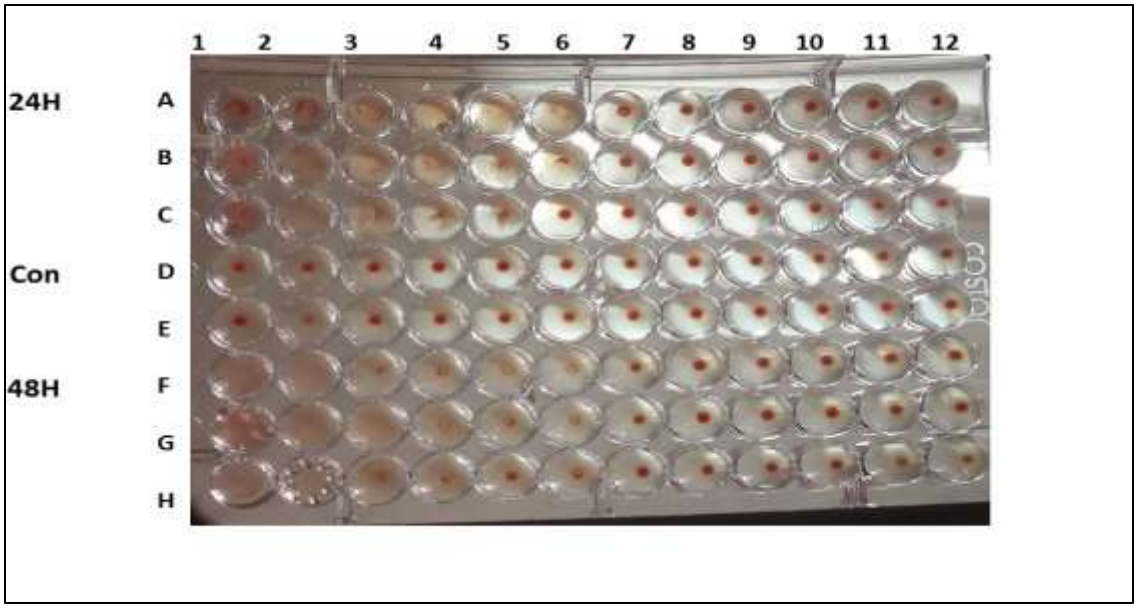


Figure 4.8.2(b) Virus infection confirmation in Hela cells at 24hpi and 48hpi

The figure illustrates the Hemagglutination assay measuring the number of virus particles used to infect Hela cells. 6 HAU (1:64) dilution of the virus particles were used to infect Hela cells Rows (A-C) illustrate Virus titer in 24H supernatant to be 5HAU(1:32). (C-D) shows control cells supernatant with no virus infection was taken as a negative control. (F-H) shows NDV viral titer from 48h post infection Hela cells with a viral titer of 6 HAU (1:64) dilution.

4.9 NDV Growth kinetics:

Newcastle disease virus was adapted in Hela cells and supernatants were collected at different time points followed by viral RNA extraction, cDNA synthesis and detection of infection by F – protein semi quantitative PCR at different time points. Having defined the role of NDV F – protein in virus infection in the cells. We investigated the expression of F –protein using real time PCR at different hours post infection and its role in virus increasing infection in the cells. It is illustrated in the figure below.

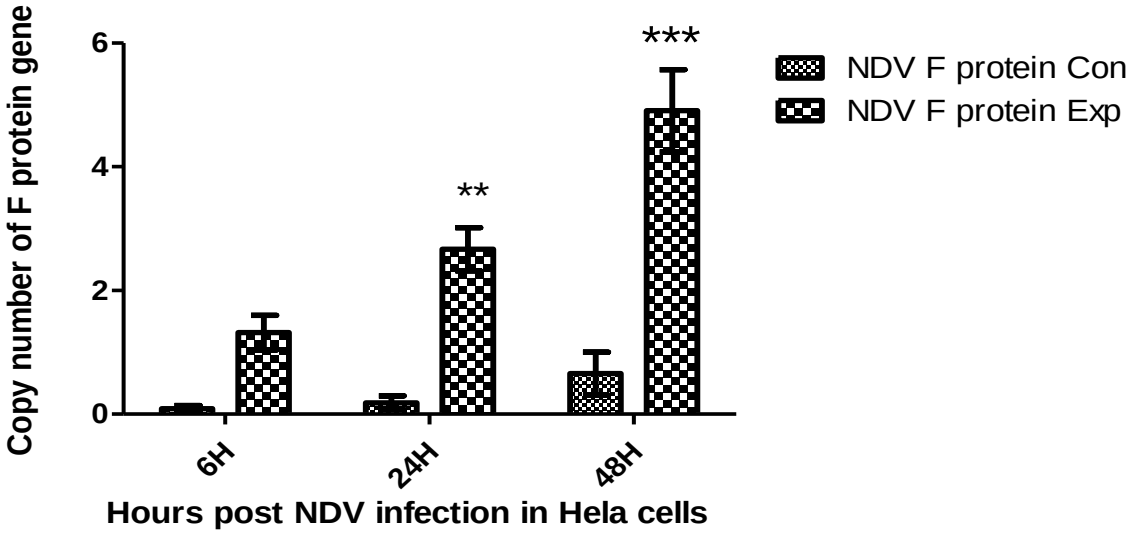

Copy number of F protein gene
6
4
2
0
***
**
6H
24H
48H
Hours post NDV infection in Hela cells
NDV F protein Con
NDV F protein Exp

Figure 4.9 copy number change of NDV F protein

The illustrates the increase in the gene expression of NDV F- protein in Hela cells at different time points 6h, 24h and 48h respectively. The significant increase in gene expression is observed at 24H ** with ($p < 0.01$ and $p<0.001$) for 48H *** using Bonferroni Multiple comparison test.

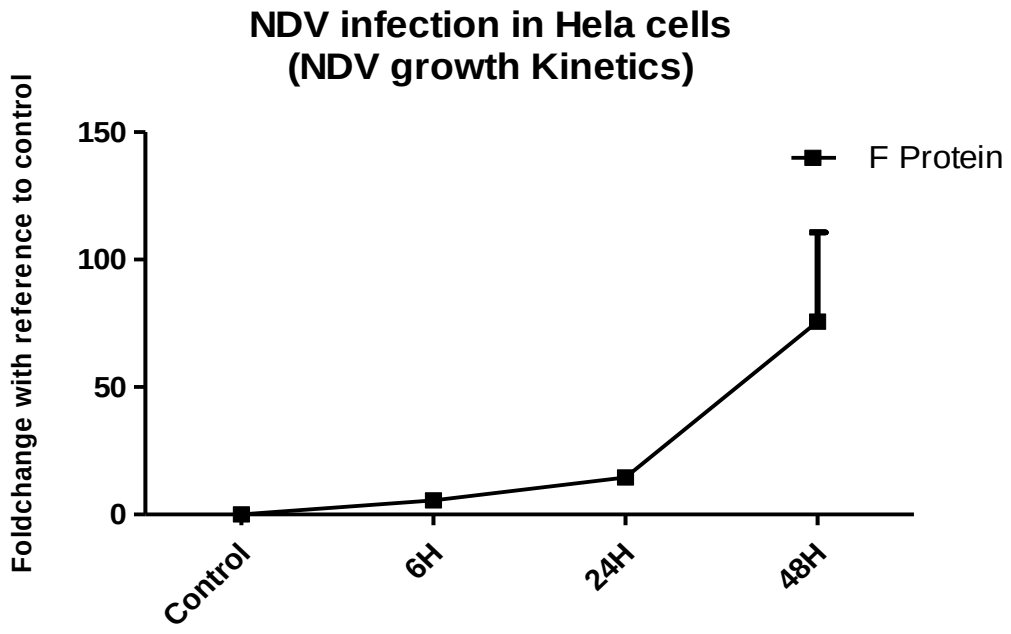


Figure 4.9(b) NDV Growth Kinetics in Hela cells

The illustrates the fold change increase in the gene expression of NDV F- protein in Hela cells at different time points 6h, 24h and 48h respectively. The significant increase in gene expression is observed at 24H ** with ($p < 0.01$ and $p<0.001$) for 48H *** using Bonferroni Multiple comparison test.

The results show significant increase in the NDV F protein gene expression (in terms of fold change and copy number change)at 24H and 48H respectively correlates with the viral titer

illustrated in the Hemagglutination assay. There is a gradual increase in the virus particles at increasing hours post infection indicating NDV successful replication in the cancer cells. Thus in the upcoming figures we shall now discuss in detail NDV interaction with the innate immunity pathway and apoptosis pathway in the Hela cells.

4.10 Cytopathic effect post NDV infection

Morphological changes were observed in Hela cells post NDV infection. Newcastle disease being the oncolytic virus induces cell death by apoptosis and cytopathic effect induction including syncytium formation and cell rounding are observed (Ravindra et al., 2008; Freeman, et al., 2006)**.**

The images shown below depict the cytopathic effect post NDV infection in HeLa cells in moc cells (without NDVPv), 6hpi, 24hpi and 48hpi. The characteristic features of syncytium formation, cell rounding, and peripheral scarring are observed.

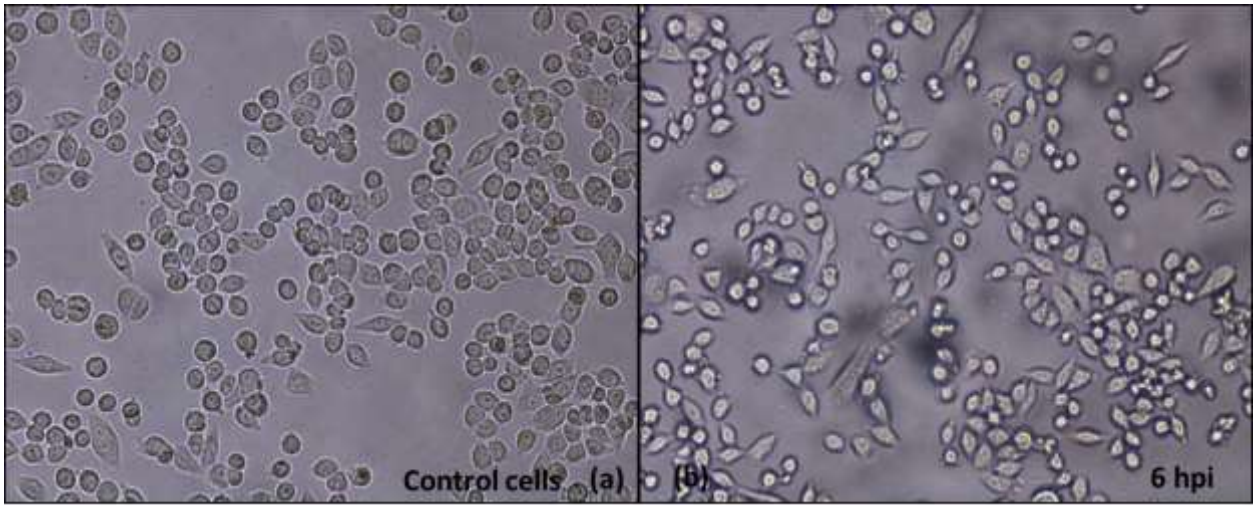


Figure 4.10.1 NDV infection in Hela cells at 6hpi

The figure shows (a) Mock cells without NDV infection exhibit normal morphology. (b) NDV infected cells at 6hpi cytopathic changes are observed including scarring of cells and cell rounding.

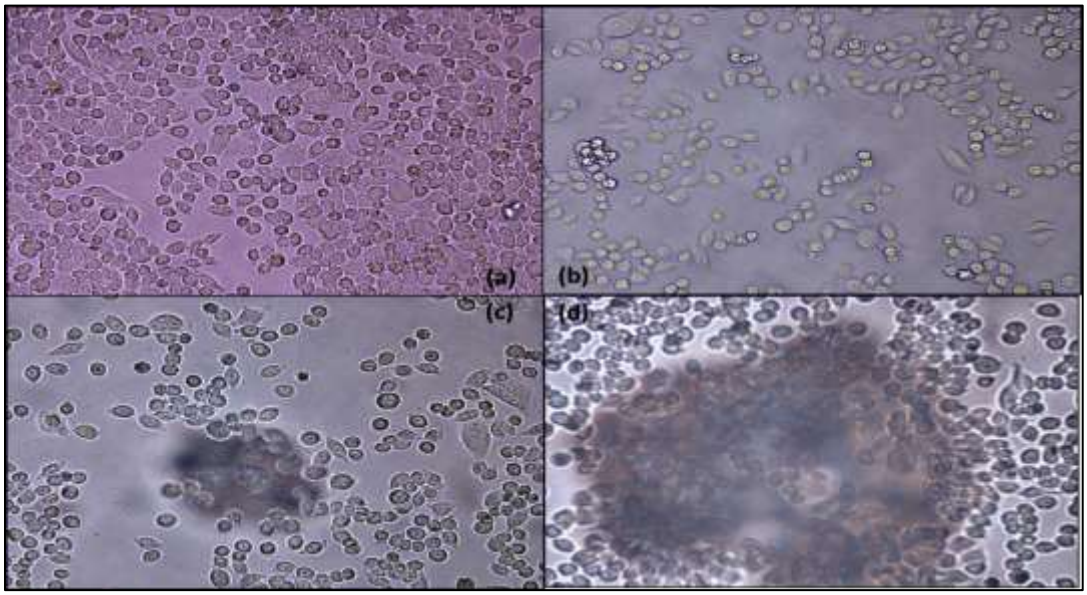


Figure 4.10.2 NDV infection in Hela cells at 24 hpi.

The figure illustrates cytopathic effect changes post NDV infection in Hela cells. (a) 24hour mock cells (b) cell rounding and cell death is observed. (c) cell scarring, cell death and cell rounding (d) mass of floating dead cells 24hpi

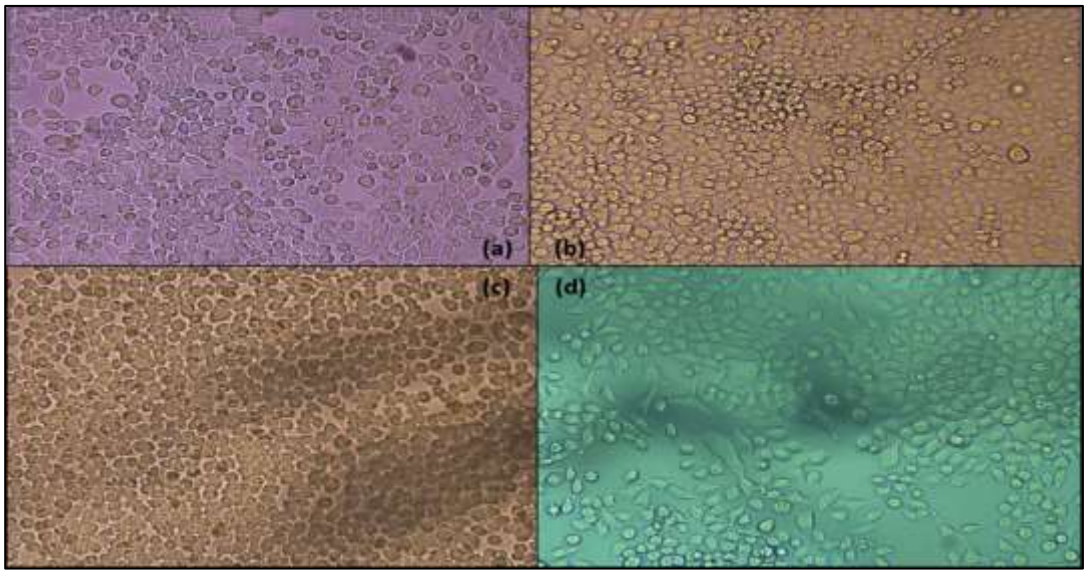


Figure 4.10.3 NDV infection in Hela cells at 48hpi.

The figure illustrates cytopathic effect changes post 48h NDV infection in Hela cells. (a) 48 hour mock cells (b) syncytium formation. (c) Cell scarring (d) fusogenic ring formation by NDVPv at 48hpi.

4.11 Quantitative PCR

The relative gene expression analysis of Newcastle disease virus interaction with innate and adaptive immunity pathway was analyzed using real time qPCR. The genes were optimized for primer specificity illustrated in the form of amplification plot and melt curves (dissociation curves) were analyzed to avoid primer dimer formation and secondary structures presence. The following genes were analyzed NDV post infection in Hela cells (IP-10, IRF-3, STAT 1, JAK 1, FAM, NF KB, CCL5, IP-10, MDA5, Caspase3, Caspase 8 and NDV F protein respectively.

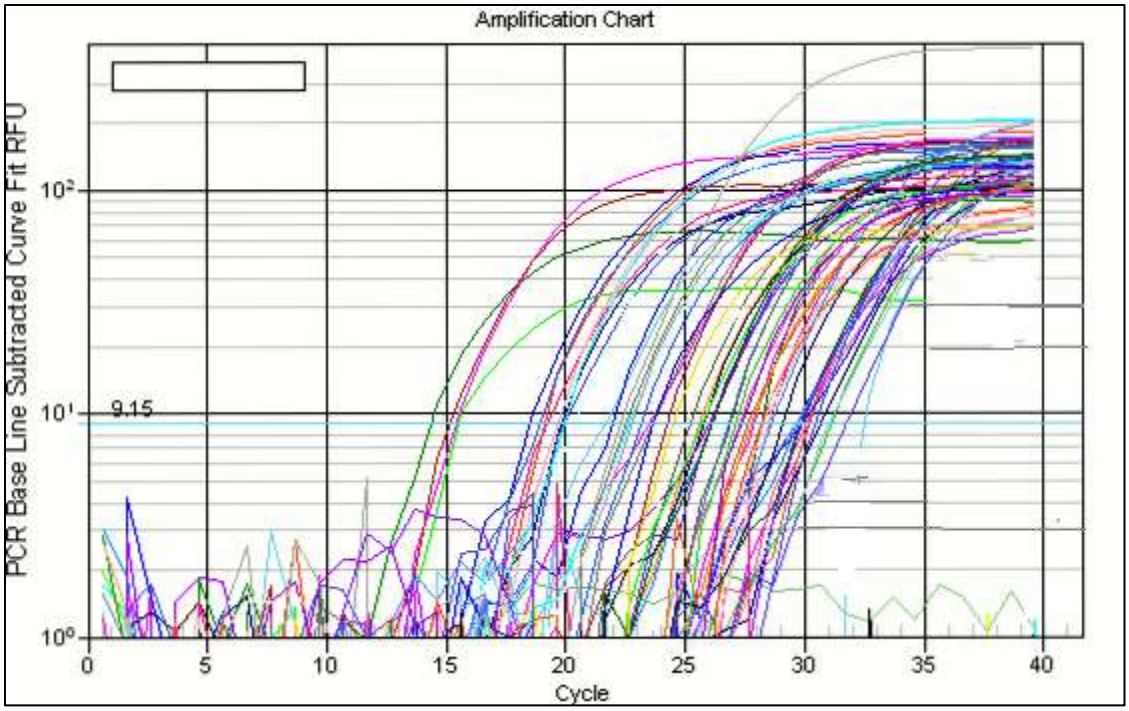


Figure 4.11: Representative amplification plot of qPCR reaction. The figure illustrates the presence of smooth plot indicated that reaction was run successfully.

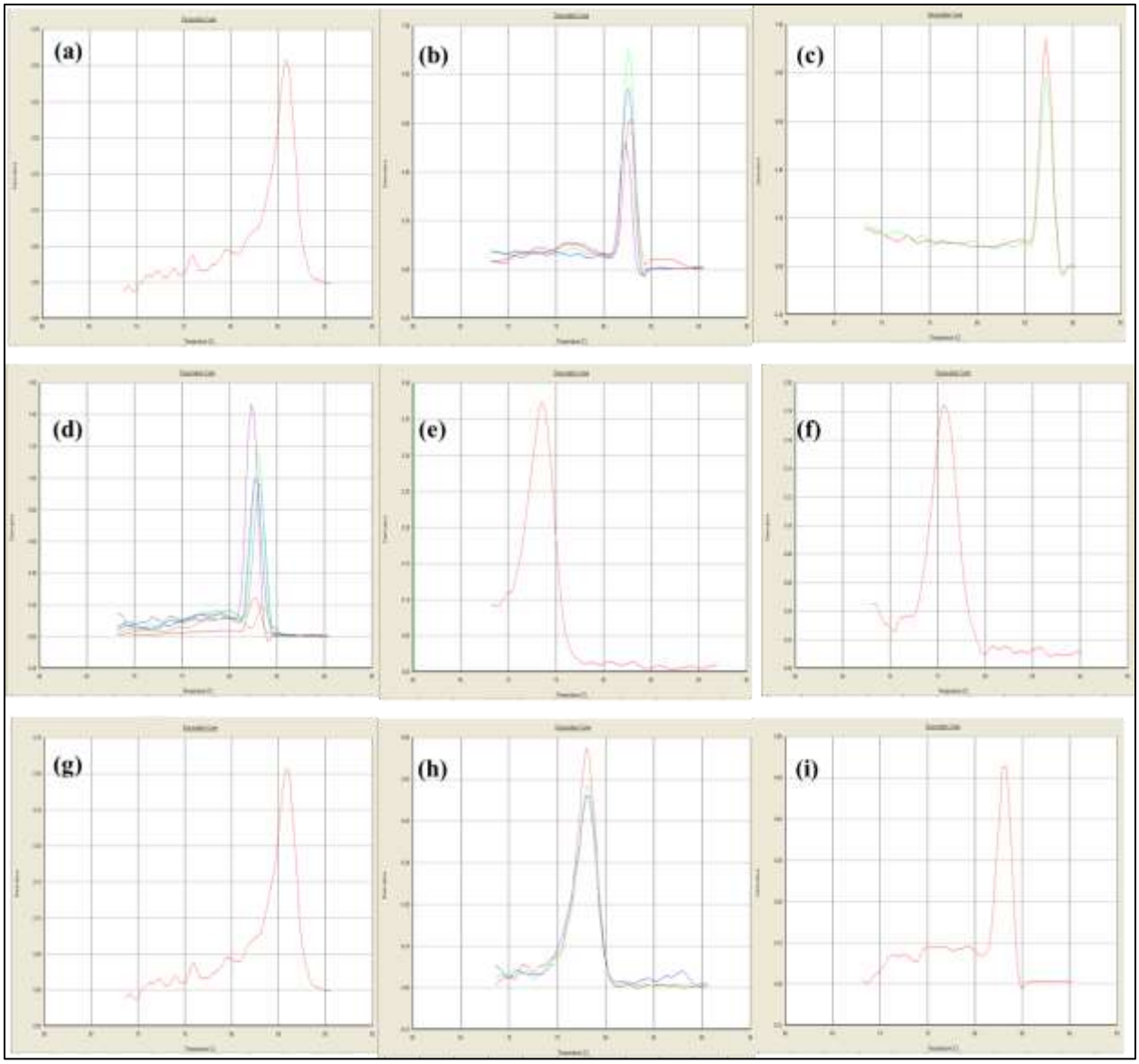


Figure 4.11 a: Representative melt curve of optimized genes (I). A single peak dissociation curve indicated the specificity of primers and predicted that no secondary structures are present in amplification of genes (a) GAPDH, (b), FAM26F, (c) IP-10, (d) NFKB, (I) CASP3(e)IFNAR, (f) JAK, (g)STAT, (h) CASP 8.

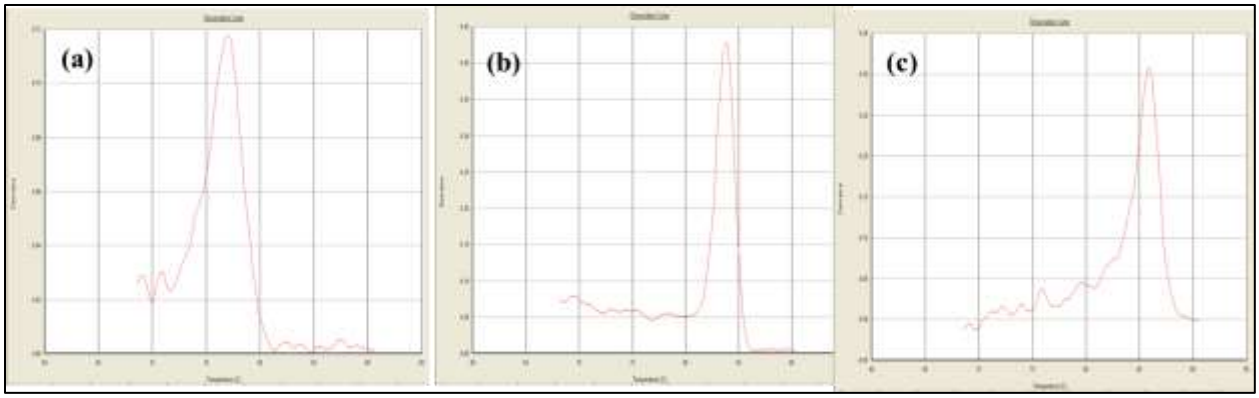
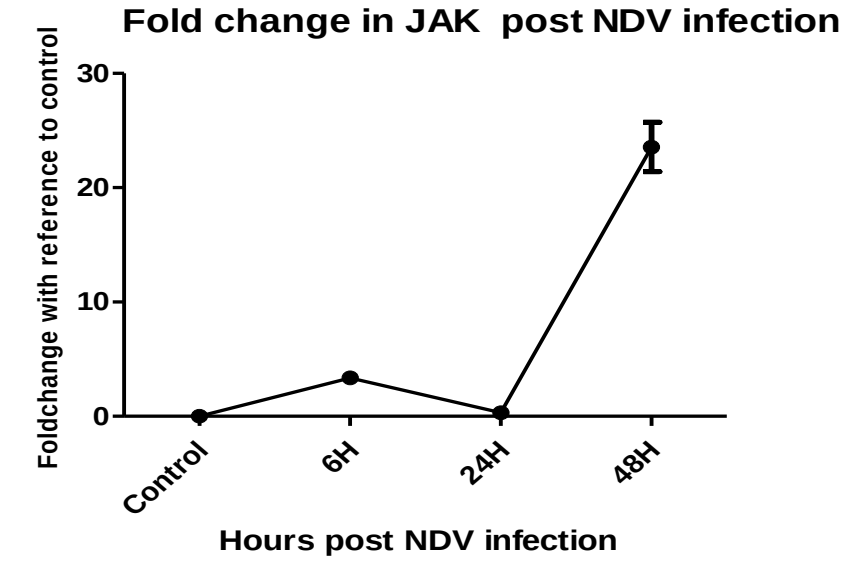


Figure 4.11(b) : Representative melt curve of optimized genes (I). A single peak dissociation curve indicated the specificity of primers and predicted that no secondary structures are present in amplification of genes (a) CCL5, (b), NDV F-Protein, (c) CCL5.

4.12 Analysis of JAK and STAT gene Expression upon NDV virus infection in Hela cells

Newcastle disease virus interacts with the tumor cells and induces a potent immune and interferon specific response in Hela cells. In order to investigate the expression of JAK and STAT genes involved in the activation and localization of type 1 interferon production were studied using Real time PCR. The optimized primers of JAK-1 and STAT -1 were used.



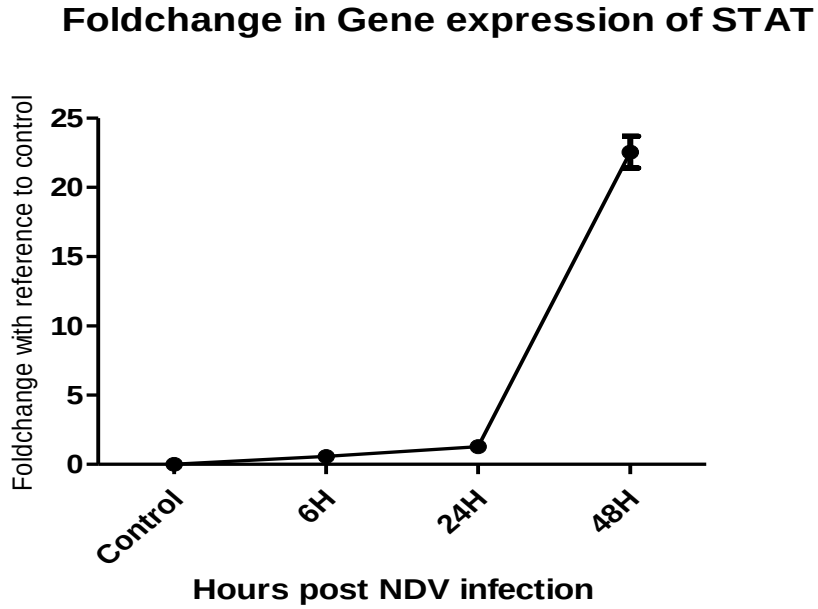


Figure 4.11.1 Fold change in JAK1 and STAT 1 Gene expression post NDV infection

The figure illustrates relative gene expression of JAK and STAT post NDV infection in NDV infected Hela cells.

There is an increase in the gene expression of JAK at 6H post infection followed by STAT following the same trend at 6H. There is no expression of JAK at 24H.At 48H both JAK and STAT have highest fold change in gene expression.

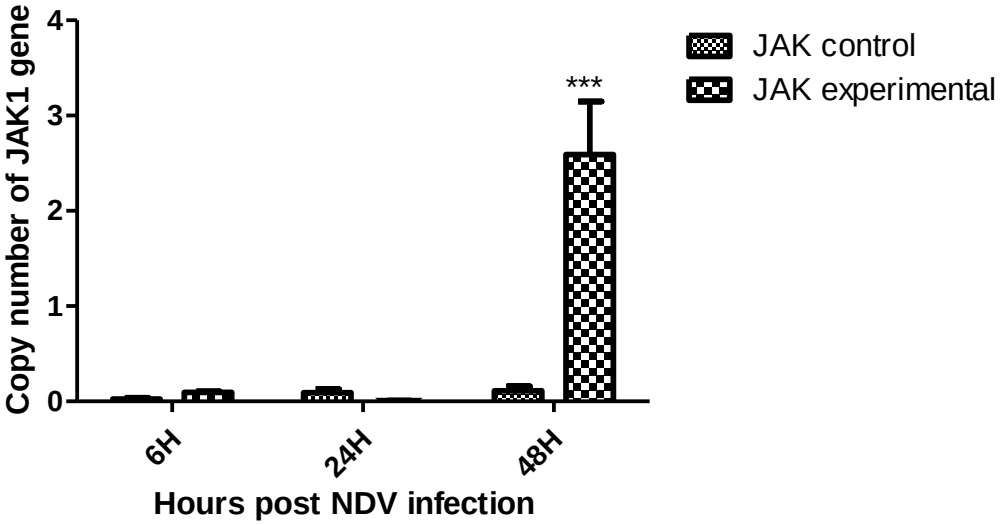


Figure 4.11.2 (b) Copy number change in JAK1 1 Gene expression post NDV infection

The figure illustrates the copy number increase in the gene expression of JAK 1 gene protein in Hela cells at different time points 6h, 24h and 48h respectively. The significant increase in gene expression is observed at for 48H *** ($p<0.001$) using Bonferroni Multiple comparison test.

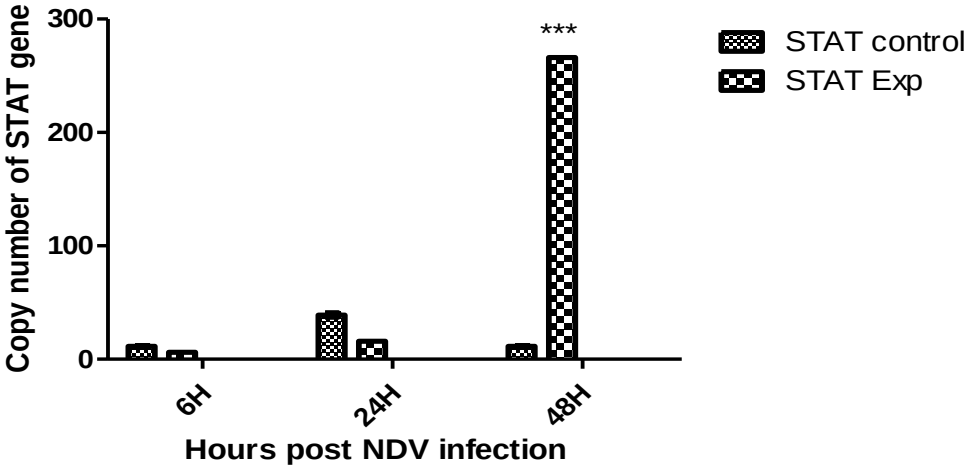


Figure 4.11.3 (b) Copy number change in STAT 1 Gene expression post NDV infection

The Figure illustrates the copy number increase in the gene expression of JAK 1 gene protein in Hela cells at different time points 6h, 24h and 48h respectively. The significant increase in gene expression is observed at for 48H *** ($p<0.001$) using Bonferroni Multiple comparison test.

4.12 Analysis of Caspase 3 and Caspase 8 gene Expression upon NDV virus infection in Hela cells

New castle disease virus infection in the cancer cells activates the apoptosis cascades in the tumor cells downregulated in case of cancers normally. NDV infection in Hela cells led to the activation of caspase 3 (intrinsic apoptosis pathway) and caspase 8 (extrinsic pathway) cascades of apoptosis post NDVPv infection in Hela cells (Elankumaran et al., 2006; Igney et al., 2002).

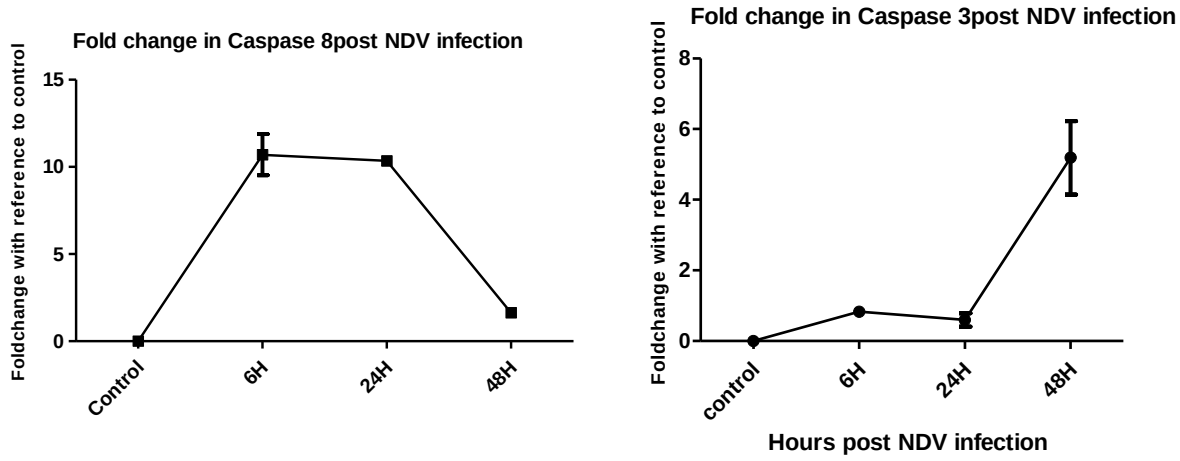


Figure 4.12 Fold change in Caspase 3and Caspase8 Gene expression post NDV infection

The figure illustrates the fold change in gene expression of the caspase 3 and caspase 8 enzymes NDV post infection in Hela cells at 6h, 24h and 48h respectively.

There is about 10 fold increase in caspase 8 expression at 6hour post infection in NDV infected Hela cells. This shows the activation of apoptosis by extrinsic pathway using the caspase 8-death receptor pathway. Then there is a gradual decrease in gene expression at 24 hour post infection and at 48hours there is a decrease in the gene expression of caspase 8 followed by a gradual increase in caspase 3 gene. No significant change in gene expression of caspase 3 gene is observed at 6h and 24h respectively corresponding to the fact that NDVPv activates extrinsic apoptosis pathway in the cell and at 48hours post infection there is an increase in caspase 3 gene

expression whereas caspase 8 expression is downregulated. This shows a striking pattern of NDVPv utilizing the intrinsic and extrinsic apoptotic pathways at different time points in the infected Hela cells.

4.13 Analysis of FAM26F Expression upon NDV virus infection in Hela cells

The expression of the FAM26F gene has been reported in many studies being the regulator of innate and adaptive immunity, its role in cancers, viral infections and bacterial infections (Pankla et al., 2009; Waddell et al.,2010, Zhang et al.,2010). Its interaction in TLR-3 dependent pathways but here we investigate the role of FAM in NDV infection in Hela cancer cells. There is a significant increase in the gene expression of FAM at 6 hours post infection where the genes of innate immunity including IP-10 and CCL5 are upregulated as well having roles in innate immune system activation.

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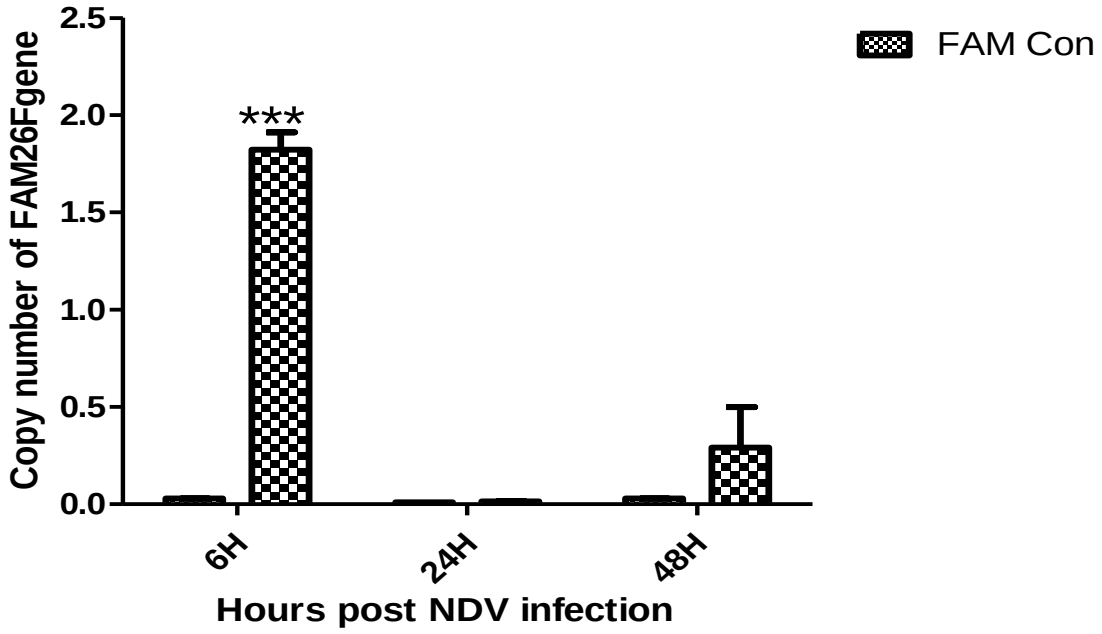

Figure 4.13 Copy number changes in FAM26F Gene expression post NDV infection

The figure illustrates FAM26F expression in Hela cells post NDV infection at 6h, 24h and 48h respectively. The significant increase in gene expression is observed at for 6H *** ($p<0.001$) using Bonferroni Multiple comparison test.

4.14 Analysis of IP-10 and CCL5 gene expression upon NDV virus infection in Hela cells

This section deals with the expression of the CXCL10/ IP-10 and CCL5 chemokine both upregulated in cancer cells promoting tumor cell growth and angiogenesis (Lo et al., 2010; Zhang et al., 2013). Thus both of these chemokines play a significant role in the tumor microenvironment and modulate the signaling pathways promoting tumor growth. The role of CXCL10 chemokine is of dual nature lead to the activation of T –cells that modulate the immunomodulatory pathways causing the activation of interferon mediated responses in tumor cells as well. In the present study NDV is infected in Hela cells and both CXCL10 and CCL5 are upregulated at different time points indicating their role in activation of immunity.

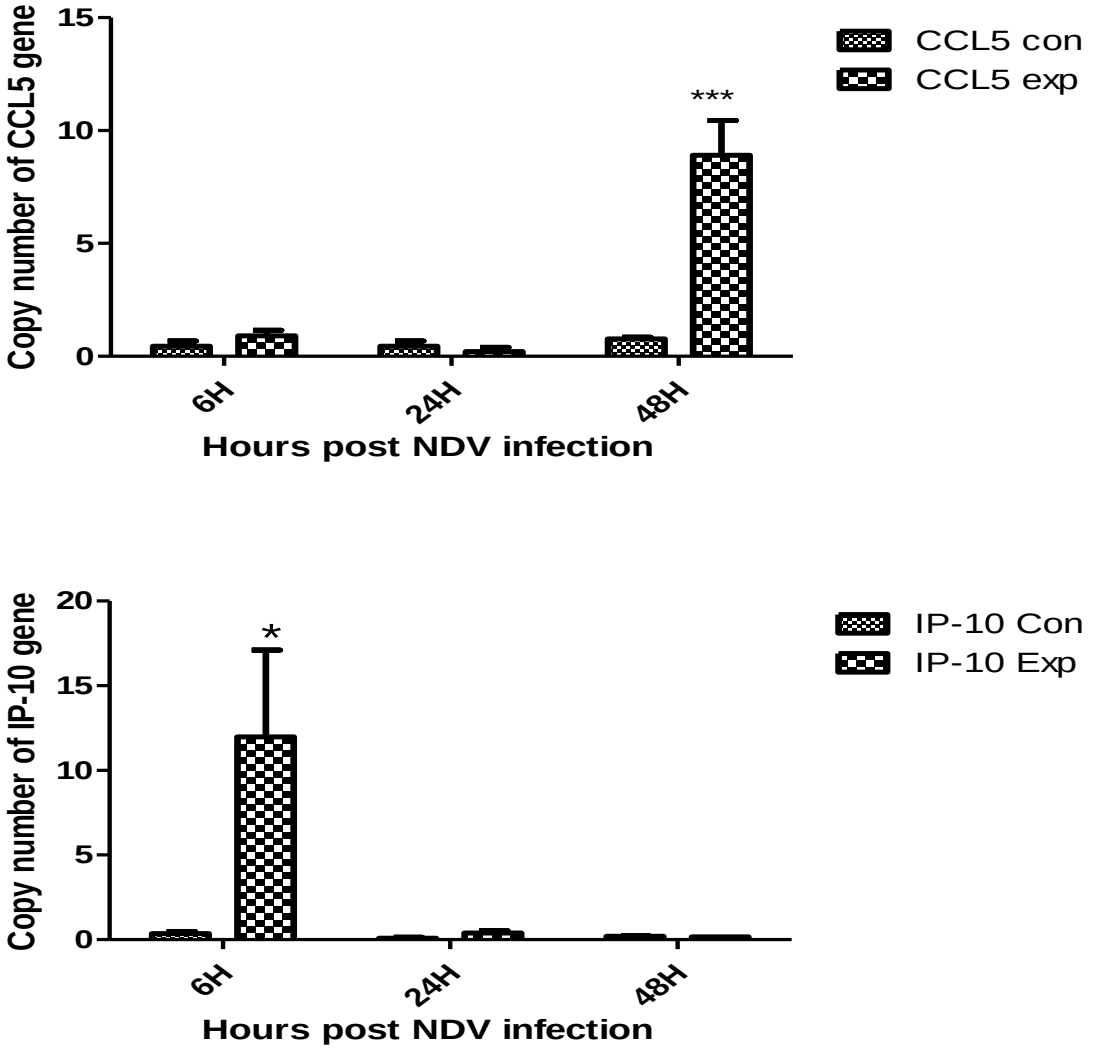

Figure 4.14 Copy number changes in CCL5 and IP-10 Gene expression post NDV infection

The figure illustrates CCL5 and IP-10 gene expression in Hela cells post NDV infection at 6h, 24h and 48h respectively. The significant increase in gene expression is observed for CCL5 is at for 48H *** ($p<0.001$) and for IP-10 at 6H*($p<0.01$) using Bonferroni Multiple comparison test.

4.15 Analysis of MDA5 gene expression upon NDV virus infection in Hela cells

The melanoma differentiation-associated gene-5 (MDA-5) is expressed in cells during differentiation and apoptosis and virus infections. It is included in the category of an early response gene and its function in induced by IFN and TNF-α with a pre-dominant response to IFN-β. The gene displays a strict RNA-dependant ATPase activity (Kang et al., 2002). NDV is a dsRNA virus enters the tumor cells via receptor mediated endocytosis using the RIG-1 /MDA5 pathway.

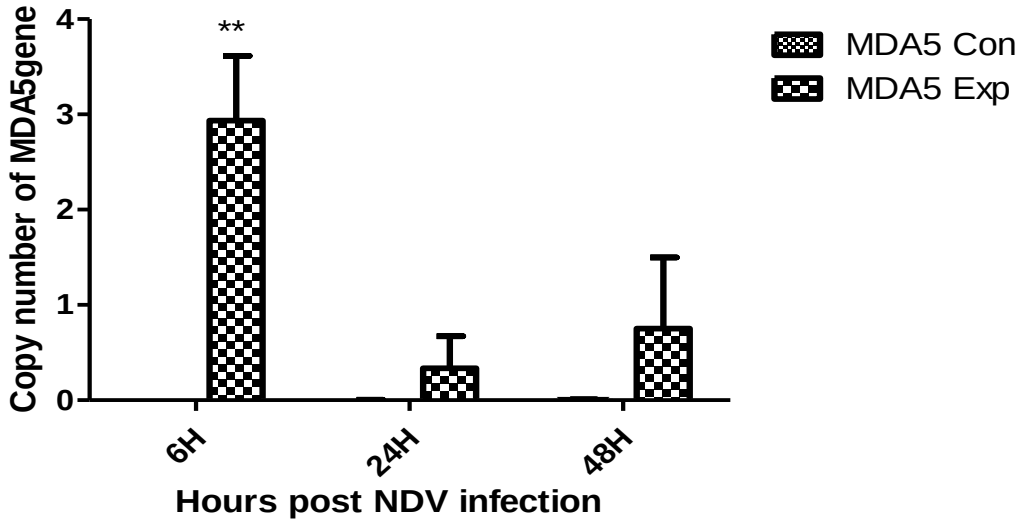

Figure 4.15 Copy number change in MDA-5 Gene expression post NDV infection

The graph illustrates the role of MDA5 pathway receptor gene expression in NDV infected cells at 6h, 24h and 48h virus post infection respectively. There was significant increase in MDA5 gene expression is observed at for 6H *** ($p<0.001$) using Bonferroni Multiple comparison test.

The MDA5 gene expression is induced highest at 6hours post infection of NDV in the tumor cells validates its interaction with the MDA5 pathway involved in virus entry and infection induction in the cancer cells.

Discussion

Cancer is a global problem with a high mortality rate and morbidity rate with 8.2 million cases annually. There is a 70% expected increase in the number of cancer cases by 2030 (WHO, 2016). There are numerous types of cancers reported and multiple are risk factors associated with cancers including aging, smoking, bacterial infections, parasites, viruses, dietary factors, obesity, Nitric oxide, free radicals, hormonal imbalance, produced in tissues including others (Ohshima *et al*., 1994 Brawley *et al.,* 1998; Kelsey *et al.,* 1993; Moller *et al*., 1994; Pike *et al*., 1983). The current treatment regimens for cancer include chemotherapy, radiotherapy and anticancer drugs apart from being cancer therapeutics have a lot of side effects cause problems like chemoresistance, mutagenesis, radiation induced mutations, secondary tumorgenesis, limiting patient quality of life and survival. In the current scenario there has been advancement on molecular targeted drug therapies that working on boosting the immune system enhancing the body immune system to fight of cancer. The use of it involves the use of monoclonal antibodies, targeting immune checkpoint inhibitors including anti PD-1 and CTLA -4 inhibitors (Fauvel *et al.,* 2014; Stern *et al.,* 2006; Kahl *et al.,* 2008; Nadler *et al.,* 1980). Antibodies targeting growth receptors, cell surface glycoproteins and tumor associated antigens (Kahl *et al.,* 2008; Nadler *et al.,* 1980). These therapies are still under clinical trials and data obtained from them is insufficient and doesn't provide efficacy in clinical settings when compared to historical controls. Therefore we need to make thoughtful changes in cancer treatment regimens and therapeutics focusing more on the combination to target cancer in a better way (Baeuerele *et al*., 2009; Rosenberg *et al*., 2004; Sharkley *et al*., 2006).

Alternative to these therapies is the virotherapy using oncolytic viruses to eradicate cancer in an efficacious manner that inherently replicates in the tumor cells of the body sparring normal cells of the body. Newcastle disease virus is a known oncolytic virus that triggers the interferon activation and apoptosis pathways activation in the tumor cells by both intrinsic and extrinsic pathways of apoptosis (Adkins et al., 2014; Bian et al.,2011; Yaacov et al.,2008). In the present study Newcastle disease virus Pakistani Velogenic strain is used in the study and its interaction with RIG-1/MDA5 pathway, interferon and apoptosis pathways is investigated.

The virus enters the cell via receptor mediated endocytosis in cells preferably uses the melanoma differentiation-associated gene-5 (MDA-5) receptor pathway is expressed in cells during differentiation and apoptosis and virus infections. It is included in the category of an early response gene and its function in induced by IFN and TNF-α with a pre-dominant response to IFN-β. The gene displays a strict RNA-dependant ATPase activity (Kang et al., 2002) to gain entry inside the cell. This data correlates with our findings in the Figure 4.15 where there is a significant change in gene expression of MDA5 at six hours post infection of NDV infection in the Hela cells. It further leads to the activation of IRF-3 gene (interferon regulatory factor 3) involved in the transcription of type 1 interferons and a modulator of innate and early antiviral immunity (Ngyugen et al.,1997; Mamane et al.,1999). It is further activated in the cells post NDV infection in the Hela cells that further activates its innate ant viral set of genes to effectively activate the production of Type 1 interferon pathways in Hela cells. Highest fold change in gene expression was observed at 6hours post NDV infection in the cells further elucidating the activation of Type 1 interferons in the cells. It can further confirmed by the activation of IP-10 gene expression observed highest at 6hours post infection coupled with the high gene expression of FAM 26F both known to be involved in activation of innate immunity in

case of viral infections and other infections in the cells (Pankla et al., 2009; Waddell et al.,2010, Zhang et al.,2010; Nakamichi et al.,2004; Jiang et al., 2009). Both of these genes were upregulated in case of NDVPv infection in the Hela cells with highest expression observed at 6 hours post infection illustrated in Figure 4.13 and Figure 4.14 respectively.

Cancer cells are sensitive to interferon production when a virus enters inside the cells it produces a potent immune response and production of type 1 interferons. NDVPv produces a potent type 1 interferon response in cell leading to the activation of JAK 1 and STAT 1 involved in type 1 interferon pathway activation in case of NDV infection in tumor cells (Goodbourn et al.,2008).There was a significant fold change in JAK1 and STAT 1 gene expression at 48hpi at late stages of virus infection in the cancer cells (Suresh et al 2011). It can be correlated with the highest NDV F –protein gene expression at 48hpi in Hela cells and maximal number of virus particles released by the cancer cells illustrated in figure 4.11 and Figure 4.9 respectively. NDVPv infection in the cells was correlated with the cytopathic effect observed in Hela cells at different hours post infection in the form of cell rounding, membrane blebbing, cell scarring and syncytium formation and fusogenic ring formation all characteristic features of NDVPv strain in cancer cells as shown in Figure 4.10. It was correlated by the increase in the Viral titer at different time points of NDVPv growth kinetics Figure 4.9 and Hemagglutination Assay figure 4.8 respectively.

CCL5 is an important chemokine of the CC family of chemokines is expressed on the tumor cells has its role in malignancy and increases the migratory and invasion related properties of the tumor cells (Zhang et al.,2013). It plays a significant role in inflammation and provides a chemotactic environment for the recruitment of T cells and macrophages to infiltrate in the tumor to modulate immune responses (Wong et al., 2003). CCL5 showed a strong fold change at 48

hours post infection of NDVPv in Hela cells might be contributing its role in the later stages of antiviral immunity shown in figure 4.14.

NDV activates the mechanisms of apoptosis in the cell by both the intrinsic and extrinsic pathways (Elankumaran et al., 2006; Igney et al., 2002). NDVPv interacts with Hela cells and activates the apoptosis in the cells as early as 6hours post infection in the cells via the extrinsic apoptosis pathway. There is an increase in gene expression of caspase 8 at 6hpi in Hela cells that persists till 24hpi and is drastically reduced at 48hpi. This could be correlated with the increase in NDV F-protein increase in infection in the tumor cells that is a modulator of infection confirmation of NDV in cancer cells. The intrinsic pathway of apoptosis using the caspase 3 cascade or the mitochondrial pathway gets activated at 48hours post infection when maximum virus particles are present in the cell and cell death is maximal at 48hpi illustrated in figure 4.12. We can assume from the investigation that apoptosis induction in these cells is due to the extrinsic apoptosis pathway activation that leads to the activation of caspase 3 activation causing cell death.

Future prospects:

NDVPv strain has shown promising results in the in vitro cancer cell model produced a robust immune response by activating the innate immunity and extrinsic and intrinsic apoptotic pathways in Hela cells. There are more studies of interaction of NDVPv with other immunomodulatory pathways, TNF alpha, type 2 interferon and other signaling pathways to understand its interaction with the tumor microenvironment. NDVPv can be checked in mouse models for its interaction with the tumor cells, its safety and efficacy so that it can be developed into a future anti cancer vaccine.

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