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**FACULTY OF GRADUATE AND
POSTDOCTORAL STUDIES**

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**Investigating the role of histone deacetylase inhibitors in enhancing the
anti-tumor activity of oncolytic vesicular stomatitis virus**

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enhancing the anti-tumor activity of oncolytic vesicular
stomatitis virus**

M.Sc. Thesis

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ABSTRACT

Oncolytic virotherapy (OV) is an innovative alternative to conventional cancer therapies based on the concept of selecting or engineering viruses to preferentially replicate in and kill tumor cells by exploiting their genetic defects. Intra-tumoural innate immunity plays a significant role in blocking the effective therapeutic spread of OV. Histone deacetylase inhibitors (HDIs) are known to blunt cellular anti-viral response. This research demonstrates that HDIs enhance the sensitivity of various cancer cell lines to the replication and spread of three different OV platforms – vesicular stomatitis virus (VSV), semliki forest virus (SFV) and vaccinia. The increased oncolytic activity of VSV correlated with a dampening of cellular interferon responses and augmentation of virus replication. Enhancement of virus replication post HDI treatment was also observed in multiple primary human tumor explants as well as in tumor bearing *in vivo* models. These results illustrate the general utility of HDIs as chemical switches to regulate cellular innate antiviral responses and to provide controlled growth of therapeutic viruses within malignancies.

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List of Abbreviations

CI	Combination Indices
CPA	Cyclophosphamide
FACS	Fluorescence Activated Cell Sorter
GFP	Green Fluorescent Protein
HDAC	Histone Deacetylase
HDI	Histone Deacetylase Inhibitor
HSV	Herpes Simplex Virus
IFN	Interferon
IHC	Immunohistochemistry
IRF	Interferon Regulating Factor
IVIS	In Vivo Imaging System
ISG	Interferon Stimulated Gene
JAK	Janus Kinase
LUC	Luciferase
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
OV	Oncolytic Virus
PBMC	Peripheral Blood Mononuclear Cell
RIG	Retinoic acid Inducible Gene
SFV	Semliki Forest Virus
SN	Supernatant
STAT	Signal Transducers and Activators of Transcription
TLR	Toll-Like Receptor
VSV	Vesicular Stomatitis Virus
VSVA51	Vesicular Stomatitis Virus mutant with a deleted amino acid (methionine) at position 51 of the virus's matrix protein

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1. INTRODUCTION

Cancer continues to rank the second highest cause of mortality among Canadians [Canadian Cancer Society 2007 statistics], and despite advances in anticancer therapies, long-term control of advanced metastatic cancer remains a difficult goal. The root of this problem lies in the complexity of cancer biology, a reflection of many genetic and epigenetic changes between a cancer cell and its normal counterpart that give cancer cells a growth and survival advantage. At the molecular level, these changes inhibit programmed cell death within the neoplastic cells, encourage autonomous cell growth as well as promote the production of factors that enhance tumor vascularization and spread. Cancer cells have also adopted mechanisms to downregulate inflammatory responses suppress innate immunity and evade adaptive immune surveillance directed against it. The plasticity of cancer cells allow small subsets of cells to evade attempts at therapy, resulting in the development of recurrent, refractory disease. The clinical effectiveness of current anticancer therapies may be constrained by the ability of the tumor to evolve and bypass the targeted process. Resistance develops to chemotherapy and even to new, highly effective targeting agents such as Imatinib [47]. More robust tumor responses may result from therapies that disrupt or target more than one of the tumor's adaptive processes.

Oncolytic virotherapy (OV) is an innovative alternative to conventional cancer therapies based on the concept of engineering or selecting viruses to preferentially replicate in and kill tumor cells by exploiting their unique genetic defects [8, 16, 32, 49, 59]. Thus, OV can be thought of as a tumor specific self_amplifying dosing regimen. Viruses hold the potential of being highly effective anticancer therapeutics because of their multifaceted interactions with the cancer cell. In addition to killing cancer cells by direct lysis and induction of cytotoxicity, OV passively target tumor parenchyma by extravasating through its unique leaky vasculature [10, 32, 49]. The direct infection

and killing of cancer cells stimulates an acute inflammatory reaction within the tumor bed. This process attracts neutrophils and other immune cells to penetrate the tumor mass and cause thrombosis of its microvascular network [10]. This damaging effect significantly compromises blood flow to the tumor, and subsequently kills uninfected cancer cells by depriving them from oxygen and other essential nutrients resulting in a phenomenon known as “bystander tumor killing effect” [10].

Acute inflammation induced by OV therapy also breaks down tumor immune tolerance by exposing its proteins to antigen presenting cells (APCs) of the immune system. These immune cells mark cancerous tissues as foreign entities and present their antigens to B and T-cells of the adaptive immune system, which then mount a cell-mediated antitumor immune response and seek cancer cells for destruction [8, 32, 66]. Oncolytic viruses can be armed with immunostimulatory genes, such as Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) and interleukin 12 (IL 12), to amplify this anti-tumor immune response [32, 59]. Anticancer immunity post OV therapy can potentially remain as long-term protection against tumor recurrence [29]. The important role of antitumor immunity was demonstrated using *in vivo* immunocompetent mice bearing mammary tumors. Treating these mice with herpes simplex virus (HSV) caused direct tumor lysis and induced a strong T-cell response against primary and metastatic breast lesions. Transferring T-cells from tumor bearing mice that have been treated with HSV prevented the formation of metastatic lesions in HSV-nontreated mice bearing primary breast tumors [37]. In another *in vivo* experiment, HSV treatment has led to 90% survival of mice bearing intraperitoneal fibrosarcoma. Rechallenging the treated mice with fibrosarcoma cells at 90 days after their cure failed to re-establish tumors and all rechallenged mice survived. These *in vivo* models highlight the ability of OV therapy to induce an antitumor immune response and to modulate the arsenals of the immune system against cancer cells [63].

The response of the immune system to OV therapy is a double edged sword. On one side it plays an important role in eradicating malignant cells, and on the other it protects normal tissues from viral pathogenic effects [21, 24, 51]. One of the first line defenses triggered by the host cell to antagonize virus infection and to alarm neighboring cells is the production of several antiviral cytokines and interleukins, most importantly type 1 interferons (IFN α and β) [21, 24, 25]. The activation of type I IFN signaling cascade involves complex and redundant pathways which are divided into early and delayed events [24]. These events engage a network of intra and extra-cellular factors in order to amplify the warning signal and ensure the protection of the host. Although, detailed review of these pathways is beyond the scope of this thesis, key concepts are worth mentioning. Each cell has developed sensors associated with either its membrane or the cytoplasm in order to recognize various viral proteins and genetic material [21]. Examples of these sensors include the Toll-like receptor (TLR) family as well as the retinoic acid-inducible gene I (RIG I) [21, 24]. Each of these sensors is programmed to recognize a specific pathogenic entity. For example, TLR7 and TLR8 recognize single stranded RNA, while RIG I identifies double stranded RNA. Once these sensors are stimulated, they lead to activating interferon regulatory factor 3 (IRF3) through phosphorylation [21, 24, 50, 62]. IRF3 then binds to other transcription factors in the cytoplasm, such as NF- κ B, and subsequently translocates to the nucleus and initiates the transcription of IFN β along with other interferon stimulated genes (ISG) [21, 24, 62]. These primary response genes are induced within 3-6 hrs of virus infection [61]. They are exported from the nucleus and induce the production of secondary and tertiary response genes through the autocrine activation of the Janus kinase (JAK)/Signal transducers and activators of transcription (STAT) signaling pathway [61]. Important secondary response genes include IRF7 and MxA, while tertiary response genes include IFN α [61]. These factors restrict virus propagation by inhibiting viral protein synthesis as well as

inducing programmed death of infected cells to prevent them from being used as factories for virus production [21, 24]. Almost all normal cells possess this innate ability to mount type 1 IFN antiviral response [21].

One acquired mutation which frequently arises during tumorigenesis is defects in the innate antiviral IFN signaling pathway. This aberrant development is perhaps due to strong selective pressures which allow tumors to evade immune surveillance and to maintain their growth advantage [17]. Fortunately, this genetic abnormality makes cancer cells more susceptible targets for OV treatment [17]. Over the past decade, preclinical investigation has highlighted the anticancer therapeutic potential of a group of viruses whose replication is strongly inhibited by IFN, and thus has an inherent preference to grow in tumor but not normal cells [8, 55, 66]. Vesicular stomatitis virus (VSV) is a prototypical oncolytic agent that belongs to this group of viruses, and will be the focus of this research project. Other features that also characterize VSV as a potent anticancer agent are its ability to infect a wide spectrum of cancer tissue types as well as its short replicative cycle and robust growth kinetics which facilitates its production as a virus therapeutic [36, 44, 55, 60, 61].

VSV is a single stranded RNA virus which belongs to the rhabdoviridae family [36]. It replicates entirely in the cytoplasm without integrating into the host's genome, and thus doesn't pose a high risk of inducing harmful mutations [36]. It has a relatively small genome of 11 kilobase pairs which encodes for five essential viral proteins [36, 5]. These proteins are nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and polymerase protein (L). The matrix protein is one of the important virulent factors that VSV uses to antagonize the host's innate antiviral defenses [24, 36, 61]. It interrupts cellular transcription and expression of antiviral gene products, such as IFN β , by blocking nucleocytoplasmic mRNA transport as well as interfering with Jak/STAT signaling pathway [24, 36, 61, 66]. Although wild type (WT) VSV has the preference of infecting

cancer cells that are deficient in their IFN response, it is also able to infect normal tissues [49]. In humans, VSV infection is usually asymptomatic but can cause flu-like symptoms as well as vesicular ulcerations of the skin [36]. There has also been one reported case of encephalitis in a 3-year old boy associated with WT VSV-Indiana strain infection [36]. WT VSV infection can also be fatal to immunocompromised mice and can cause them significant neurotoxicity such as hind limb paralysis [61]. Therefore, in order to further harness the therapeutic benefits of WT VSV it needed to be modified to enhance its safety and anticancer selectivity [61].

Using tissue culture selection techniques, small sized VSV plaques that grew on IFN-responsive cells were isolated and purified for analysis [61]. Sequencing of these plaques revealed a mutant VSV strain that harbored a single amino acid substitution in the M protein; where methionine at position 51 was replaced by arginine [61]. Further *in vitro* testing demonstrated the ability of this mutant strain to induce over twenty times more IFN α than the parental WT strain after infecting 'normal epithelial cell lines [61]. Stojdl *et al.* created a similar mutant by completely deleting methionine 51 and called it VSV Δ 51 [10, 61, 66]. This mutation in the M protein rendered VSV Δ 51 incapable of mitigating the host's antiviral response because of its inability to block nuclear transport of mRNAs required for IFN production [36, 61]. RT-PCR analysis used to compare nuclear fractions of cells after infection with either WT or Δ 51 strains revealed similar amounts of IFN β mRNA. However, IFN β was undetectable in cytoplasmic fractions or culture supernatants of cells infected with WT strain while abundant amounts were revealed after VSV Δ 51 infection [61]. Since IFN β is an important precursor for the induction of IFN signaling cascade, levels of down stream factors, such as IRF-7 and IFN α , were also elevated in cells infected with VSV Δ 51 compared to WT VSV [61]. *In vivo* systemic administration of VSV Δ 51 has demonstrated potent oncolytic activity against

cancer cells with defective IFN response, while being highly attenuated in normal tissues [10, 61]. In fact, the LD50 of VSVΔ51 is 10,000 times greater than WT parental strain [61].

Although impaired IFN signaling has been identified in approximately 80% of tested human cancer cell lines, the magnitude of this defect is quite variable and can be a barrier to the effective spread of an attenuated OV through a malignancy [12, 55, 61, 66]. Preclinical investigation has noted the poor intratumoral spread of VSVΔ51 in various IFN responsive tumor models, such as melanoma (B16), breast carcinoma (4T1) as well as prostate carcinoma (PC3) (data not published). Similarly, this phenomenon has been noted with other attenuated OV agents that have proven to be safe in human trials but have had disappointing clinical efficacy due to limited spread and replication within human tumors [55, 66]. Indeed more potent OVs are being developed that express viral gene products to combat cellular innate immune responses [4, 23]; however this genetic approach may increase toxicity.

Another approach reported in the literature to circumvent intratumoral antiviral immunity is combining OV therapy with immunosuppressive agents, such as cyclophosphamide (CPA) [26, 35]. CPA is a chemotherapeutic agent that is used to treat forms of leukemia and lymphoma, as well as autoimmune diseases such as lupus erythematosus and rheumatoid arthritis. The literature has reported several preclinical studies which have used CPA treatment to enhance intratumoral spread of OV therapy. Ikeda *et al*, combined CPA with HSV to treat intracerebral glioma tumors in rats [26]. Indeed, this combination therapy enhanced intratumoral virus propagation as well as survival rates. CPA played an important role in attenuating innate and adaptive immune response by suppressing inflammatory cytokines, complement proteins as well as non-specific immunoglobulins (IgM). Another study has combined CPA with intratumoral injections of adenovirus to treat glioma-bearing mice [35]. This experiment also demonstrated enhancement of virus replication within the

tumor post CPA treatment, which correlated with decreased infiltration of leukocyte and macrophages. However, there was no enhancement in antitumor efficacy. Results from these studies are encouraging and provide a proof-of-principle to the proposition that intratumoral immune environment formulates a significant barrier to OV spread and replication. However, the limited efficacy data reported in these studies as well as the use of a single tumor model to demonstrate such efficacy has prompted us to explore the potential of other chemotherapeutic agents that can enhance OV therapy. After detailed literature review, we chose to investigate the ability of histone deacetylase inhibitors (HDIs) to enhance OV therapy, especially since they haven't been used in the context of OV therapy before.

Over the past decade, researchers have been exploring HDIs as a novel therapeutic platform for the targeted treatment of cancer. This concept has been built on growing evidence that the evolution of tumorigenesis is closely linked to aberrant epigenetic regulation of the chromatin structure [9, 69]. Post translational modifications of proteins bound to DNA, such as histones, play a crucial role in remodeling chromatin and in controlling gene expression that regulates important cellular programs such as growth and apoptosis [2, 9, 69]. One of the important post-translational modifications includes acetylation of lysine residues on amino-terminal tails of histones. This process is tightly regulated by a dynamic equilibrium between histone acetyltransferases (HATs) and histone deacetylase enzymes (HDACs) [9]. Defects in this equilibrium have been recently recognized as a hallmark of certain cancers, such as promyelocytic leukemia, where hyperactivity of HDACs is a dominant feature [9, 31]. Thus, HDACs have constituted promising targets for therapeutic interventions intended to reverse aberrant epigenetic states associated with cancer. There are 18 structurally distinct HDAC enzymes which have been identified in humans, and that have been classified into four main classes [9, 69]. Although the function of some of these HDACs has

not been well characterized, each class has a specific subcellular localization and dominance in certain tissues [2, 9, 69]. For example, class I is generally detected in the nucleus, while class II can shuttle between the nucleus and cytoplasm. HDIs have been designed to target certain HDAC enzymes. For example, MS-275 targets class I while SAHA and PXD-101 target classes I, II and IV. HDIs have been found to have potent anticancer activities with remarkable tumor specificity [2, 9, 31, 69]. HDIs are able to kill tumor cells through various mechanisms including apoptosis, autophagy or necrosis. It has been noted that the overexpression of certain HDACs in tumor cells repress growth-regulatory and apoptotic genes, and that the knockdown of these HDACs via *siRNA* reversed these transcriptional repressions and induced apoptosis, cell cycle inhibition and differentiation [9]. HDIs can also inhibit DNA repair responses by disrupting cell-cycle checkpoints. Normal cells were found to be much more resistant to this disruption in comparison to tumor cells. The disruption of these checkpoints result in the premature exit of tumor cells from an abortive mitosis and the increased accumulation of DNA damage which eventually induce apoptosis and cell death [43]. Many of these HDIs agents are currently in early phases of clinical development and SAHA has been approved as first line treatment for cutaneous T-cell lymphoma [18].

In addition to their potent antitumor activity, HDIs have also shown the ability to modulate cellular immune responses [9]. Numerous groups have proven that HDAC activity is essential for inducing the transcription of various IFN-stimulated genes [13, 14, 22, 28, 30, 42, 45, 61]. Levy *et al* has also shown that inhibition of HDAC activity prevented the transcriptional activation of antiviral gene products following IFN stimulation or virus infection [13]. These experiments were performed in IFN responsive cancer cell lines, such as HeLa and U2OS, using trichostatin A as the HDAC inhibiting agent.

Research Rational

Since intra-tumoral innate immunity can play a significant role in blocking the effective therapeutic spread of OV_s, we reasoned that combining a viral therapeutic, such as VSV Δ 51 with a compound that reversibly compromises cellular antiviral genetic programs, such as an HDI, could provide a means to enhance OV growth in tumor cells. Thus, it has been proposed that HDIs can be used as tumor specific viral sensitizers that have the potential to increase the spectrum of malignancies amenable to OV therapy.

Hypothesis

HDI_s can enhance the intra-neoplastic spread and anti-tumor killing of OV therapy.

Objectives

There are five main objectives that have been set to support the proposed hypothesis. **First**, select a panel of cancer cell lines that are poorly permissive to VSV Δ 51 infection, and determine whether IFN treatment further protects these cells against viral infection. **Second**, investigate the ability of HDI treatment to enhance the replication and spread of VSV Δ 51 in the chosen cancer cell panel. **Third**, identify whether this enhancement in viral replication post HDI treatment correlates with blunting of cellular IFN response. **Fourth**, determine whether the combination treatment of HDI_s and VSV Δ 51 can be safely administered to tumor-bearing mice without increasing viral toxicity in normal tissues. **Fifth**, investigate if the combination treatment is more effective in controlling the tumor burden in these mice compared to the individual treatments.

2. METHODS AND MATERIALS

Drugs and Chemicals

For *in vitro* use, MS-275 (Calbiochem) and SAHA (Alexis Biochemicals) were dissolved in DMSO to a stock concentration of 15 mM and stored at -20°C. IFN α treatment was performed using Intron A (Schering), stored at 4°C at stock concentration 10x10⁶ IU/ml. For *in vivo* use, MS-275 was dissolved in PBS, 0.05 N HCl, 0.1% Tween or DMSO and stored at -20°C. The pan-caspase inhibitor Z-VAD-fmk was purchased from Calbiochem. For *in vivo* imaging, mice were anesthetized under 3% isofluorane (Baxter Corp., Deerfield, IL).

Cell lines

The following cell lines were purchased from the American Type Culture Collection: PC3 (human prostate Cancer), SW620 and HT29 (human colon carcinoma), SN12C (human renal cell carcinoma), SF268 (human glioblastoma), M14 (human melanoma), BHK (baby hamster kidney cells), 4T1 (mouse breast adenocarcinoma) and B16 (mouse melanoma). All cell lines, except PC3, were cultured in HyQ Dulbecco's modified Eagle medium (High glucose) (HyClone) supplemented with 10% fetal calf serum (CanSera, Etobicoke, Canada). PC3 was cultured in RPMI (Wisent) supplemented with 10% fetal bovine serum (Wisent). All cell lines were tested negative for mycoplasma using Hoechst staining.

Viruses

The Indiana serotype of VSV was used throughout this study and was propagated in Vero cells (American Type Culture Collection). VSV Δ 51 is a naturally occurring interferon-inducing mutant of the heat-resistant strain of wild-type VSV Indiana, while VSV Δ 51 expressing GFP and GFP-firefly

luciferase fusion are recombinant derivatives of VSV Δ 51 {Stojdl, 2003 #41}. Virions were purified from cell culture supernatants by passage through a 0.2 μ m Steritop filter (Millipore, Billerica, MA) and centrifugation at 30,000 g before resuspension in PBS (HyClone, Logan, UT). Doubled deleted vaccinia virus expressing GFP {McCart, 2001 #36} was propagated in Vero cells, while Semliki Forest Virus expressing GFP was grown and titered using baby hamster kidney cells.

Viral titers

Supernatants from each treatment condition were collected at the specified time point. A serial dilution was then performed in DMEM and 200 μ l of each dilution was applied to a confluent monolayer of Vero cells, 5×10^5 cells/well, for 45 minutes. Subsequently, the plates were overlaid with 0.5% agarose in DMEM-10% FBS and the plaques were grown for 24h. Carnoy fixative (Methanol:Acetic Acid is 3:1) was then applied directly on top of the overlay for 5 minutes. The overlay was gently lifted off using a spatula and the fixed monolayer was stained via 0.5% crystal violet for 5 minutes, after which the plaques were counted. VVDD samples were titered on U2OS monolayer using 1.5% carboxyl methyl-cellulose in DMEM-10% FBS for 48h. The overlay was removed and the monolayer stained via 0.5% crystal violet for 5 minutes, after which the plaques were counted.

Titration of VSV from whole tissue specimens.

Primary tissue specimens were obtained from consented patients who underwent tumor resection. All tissue specimens were processed within 48h post surgical excision. Samples were manually divided using a 15 mm scalpel blade into equal portions under sterile techniques. After the indicated treatment condition, samples were weighed and homogenized in 1 ml of PBS using a homogenizer

(Kinematica AG-PCU-11). Serial dilutions of tissue preparations were prepared in serum free media and viral titers were quantified by standard plaque assay.

Primary *ex-vivo* prostate cancer cell cultures

Radical prostatectomy specimens were obtained from the Jewish General Hospital (McGill University) from untreated, consenting patients diagnosed with prostate cancer, with Institutional Review Board approval. Prostate cancer tissues and their adjacent normal tissues were histologically defined by the pathologist. Specimens were washed immediately in cold, sterile phosphate-buffered saline (PBS). After removing excess, damaged epithelium and stromal tissue, specimens were cut into small pieces and incubated for 10 min at 37°C in 0.05% trypsin/0.53mM EDTA (Wisent). Surface epithelium was mechanically separated to dissociate cells into a single cell suspension. Prostate cells were collected after centrifugation and resuspended in mammary epithelial growth medium (MEGM, Clonetics). Cells were seeded on plastic dishes (Falcon) and fed every 48 h. After 1 week, MEGM medium was replaced with KSF medium supplemented with 5 mg/100 ml of bovine pituitary extract (BPE) (Gibco/BRL).

PBMC isolation

Blood Mononuclear cells were isolated by blood centrifugation (400 g at 20°C for 25 min) on a Ficoll-Hypaque density gradient (GE Healthcare Bio-Sciences Inc - Oakville, ON, Canada). PBMCs were cultured in RPMI 1640 supplemented with 15% heat-inactivated Fetal Bovine Serum (FBS, Wisent INC - St-Bruno, Qc, Canada) and 100 U/ml penicillin–streptomycin. PBMCs were cultured at 37°C in a humidified, 5% CO₂ incubator.

Flow cytometry

To measure apoptosis, cells were trypsinized, washed in cold PBS and stained on ice with allophycocyanin (APC)-conjugated Annexin V for 15 min in Annexin V binding buffer (BD biosciences, Mississauga, ON, Canada). For measurement of mitochondrial membrane depolarization ($\Delta\Psi_m$) cells were trypsinized, washed in PBS and resuspended in media containing JC-1 stain (JC-1; CBIC2(3) (5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolyl-carbocyanine iodide - Molecular Probes-Invitrogen Canada Inc., Ontario, CA) at final concentration of 1mM and incubated at 37°C for 15 min. After incubation cells were subjected to flow cytometry analysis (10^4 events/measurement) on a FACS Calibur (Becton-Dickinson, Mountain View, CA) and analyzed with FCS Express V3 software.

IFN ELISA

IFN α levels were measured using a Human Interferon ELISA kit (PBL Biomedical). PC3 cells were treated or not with MS-275 (2 μ M) or SAHA (5 μ M) for 24h and then infected with VSV- Δ 51-GFP at 0.1 MOI. Culture medium (100 μ L) was collected at different times post-infection and processed as per manufacturer's instructions. Samples were read on a DYNEX plate reader at primary wavelength of 450 nm (with a reference wavelength of 630 nm).

Western blotting

Cells were washed in cold PBS and lysed in standard NP-40 lysis buffer (50 mM Tris, pH7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 30 mM β -glycerophosphate, 1% NP-40 substitute (IGEPAL), 50 mM NaF, 0.1 M NaVO₄, 0.1 M PMSF, 0.001% protease inhibitor cocktail (Sigma-Aldrich)). Whole-cell extracts (50 μ g) were separated on a SDS-polyacrylamide gel and transferred to PVDF membrane, then probed with the following antibodies as indicated: anti-IRF-7 (sc-9083;

Santa Cruz), anti-IRF-3 (sc-9082; Santa Cruz), anti-ISG56 (a gift from Dr. Ganes Sen, Cleveland Clinic, Department of Molecular Genetics, Cleveland OH), anti-IKK ϵ (BD Biosciences, [50]), anti-RIG-I (in-house,[50]), anti-VSV (Polyclonal antiserum to VSV described in [4]), anti-acetylated histone 3 (Ac-H3), anti-total H3 (all from Cell Signaling Inc.) , and anti-Actin (sc-8432; Santa Cruz).

Reverse transcription and polymerase chain reaction.

Total RNA from infected or mock-infected and either HDI-treated or non-treated PC3 cells was isolated as per manufacturer's instruction (RNeasy; Qiagen). RNA (400 ng) was reverse transcribed with Oligo dT primers and Superscript II reverse-transcriptase (Invitrogen). Five percent of RT reaction was used as template for PCR using Taq polymerase (Amersham, GE Healthcare), amplification was carried for 25-30 cycles. Primers used were as follows: IFN- α forward primer 5'-TTG CTC TCC TGT TGT GCT TC -3' and reverse primer 5'-GGC CTT CAG GTA ATG CAG AA -3'; IRF7 forward primer 5'-CTT CGT GAT GCT GCG AGA TA R -3' and reverse primer 5'-AAG CCC TTC TTG TCC CTC TC -3'; MxA 5'-CTG ATC TCC AGG GTG ATT AGC TCA T-3' and reverse primer 5'-ATG GTT GTT TCC GAA GTG GAC A - 3' and GAPDH forward primer 5'-AAT CCC ATC ACC ATC TTC CA-3' and reverse primer 5'-TGA GTC CTT CCA CGA TAC CA - 3'.

***In vivo* tumor models**

All mice used were obtained from Charles River Laboratories. HT29 and M14 xenograft models were established in 6-8 week old female nu/nu mice by injecting 1×10^6 cells in 100 μ l PBS subcutaneously in the hind flanks. PC3 xenograft models were established in male nu/nu mice by injecting 5×10^6 cells in 100 μ l PBS subcutaneously in hind flanks. After tumors became palpable

and of an approximate size of 4 X 4 mm, the double treated group received MS-275 intraperitoneally at a concentration of 23mg/kg/day. Four hours post administering the second HDI dose, all mice were injected intratumorally with 5×10^6 pfu of VSV-Luc suspended in 50 μ l of PBS. The double treated and MS-275 treated groups continued to receive 23mg/kg of MS-275 intraperitoneally every 24 h until sacrificed.

The ovarian transgenic tumor model tgMISIIRTA_g564 is based on the transgenic model from [15], in which the SV40 early region, including the large T antigen is under the control of the mullerian inhibiting substance type II receptor (MISIIR). Female mice reproducibly developed bilateral ovarian tumors leading to loss of wellness at approx. 15 weeks of age. Thirteen week old mice were administered MS-275 IP every 12h at 6mg/kg, while VSV was administered IP at 1×10^8 pfu at 4h after the initial HDI dose. Viral replication in live animals was assessed 48h following viral infection by IVIS imaging.

Human xenograft colon carcinoma SW620 tumors were established subcutaneously in 6 week old CD1 female nude mice (n=4) by injecting 5×10^6 cells suspended in 100 μ l PBS. Tumor development was attained after 2 weeks. At this time point, all tumors were visible subcutaneously as well as easily palpable. MS-275 was administered intraperitoneally at a concentration of 7mg/kg every 12 h for 10 consecutive doses. VSV-Luc (1×10^7 pfu) was administered intravenously 4 h after the second MS-275 dose. This treatment cycle was repeated once more 72 h after the last MS-275 dose.

Syngeneic breast carcinoma tumors were established subcutaneously in the hind flanks of 6 week old female Balb/c mice 5 days after injecting 3×10^5 of 4T1 cells suspended in 100 μ l PBS (n=5). Three doses of MS-275 were administered intraperitoneally at a concentration of 6mg/kg every 24 hrs. VSV-Luc (2×10^8 pfu) was introduced intravenously 4 h following the second MS-275 dose. This

treatment cycle was repeated every 3 days for five cycles. All mice were hydrated by administering 1 ml of 0.9% normal saline subcutaneously once or twice a day for 10 days. Tumor sizes were measured every 3 – 4 days using an electronic caliper. The average tumor size from each treatment group was calculated for each time point and standard error was calculated to generate error bars.

IVIS imaging

Mice were injected with D-luciferin (Molecular Imaging Products Company, Ann Arbor, MI) (200ul intraperitoneally at 10 mg/ml in PBS) for Firefly luciferase imaging. Mice were anesthetized under 3% isoflurane (Baxter Corp., Deerfield, IL) and imaged with the In Vivo Imaging System 200 Series (Xenogen Corporation, Hopkinton, MA). Data acquisition and analysis was performed using Living Image v2.5 software. For each experiment, images were captured under identical exposure, aperture and pixel binning settings, and bioluminescence is plotted on identical color scales.

Immunohistochemistry (IHC)

Tissues were harvested, placed in OCT mounting media (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) then placed directly on dry ice until the sample freezes. These frozen samples were then cut in 4 μ m sections with a microtome cryostat (Microm HM500 OM Cryostat). Sectioned tissues were fixed in 4% paraformaldehyde for 20 minutes and used for hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC). IHC was performed using a Vectastain ABC kit for rabbit primary antibodies (Vector Labs, Burlingame, CA), according to manufacturer's instructions. Primary antibodies used were rabbit polyclonal antibodies against VSV (gift of Earl Brown), active caspase 3 ((BD Pharmingen, Rockville, MD) and acetylated histone H3 (Upstate, Cedarlane Lab). Briefly, endogenous peroxidase activity was inhibited by incubating with 3% H₂O₂ followed by blocking of non-specific epitopes with 1.5% normal goat serum. Subsequently, all sections were incubated with

avidin then biotin blocking agents, each for a period of 15 min. PBS washes were performed between all blocking and incubating steps. Sections were then incubated with primary antibodies, either anti-VSV antibody (1:5000, 30 minutes), anti-active caspase 3 antibody (1:200, 60 minutes) or anti-acetylated H3 antibody (5:2000, 120 minutes), followed by anti-rabbit biotinylated secondary antibody. The avidin: biotinylated enzyme complex obtained from the ABC Vectastain Kit was added to each slide for 30 min. After three consecutive PBS washes, the antigen was localized by incubation with 3,3-diaminobenzidine reagent. Sections were counterstained with hematoxylin. For assessment of cell morphology, sections were stained with hematoxylin and eosin according to standard protocols. Whole tumor images were obtained with an Epson Perfection 2450 Photo Scanner while magnifications were captured using a Zeiss Axiophot HBO 50 microscope.

Analysis of tumor perfusion

Mice were injected intravenously with 100 μ l of a 50% solution of 100 nm diameter orange fluorescent microspheres (Molecular Probes, Burlington, Canada). Five minutes later, animals were sacrificed by cervical dislocation and the tumors were immediately snap frozen. Tumor perfusion was analyzed by visualizing fluorescent microspheres in the vasculature of 10 μ m unfixed frozen sections using a ScanArray Express microarray scanner with a standard Cy3 laser (Packard Bioscience).

Serum-neutralizing antibody assay

Blood samples were obtained from the saphenous veins of mice at specified time points, and collected in heparinized vials. Samples were then centrifuged at 3000 rpm to isolate the plasma. Five ul of the plasma sample was diluted in 50ul of medial and then serially diluted in a 96-well plate. Each dilution was incubated with 300 PFU of VSV for 90 min at 37⁰C and then applied to Vero cell monolayers cultured in a 96-well plate. Wells were examined for cytopathic effects 48 h post inoculation. Neutralizing titer was taken as the highest dilution factor of serum that prevented the appearance of cytopathic effects.

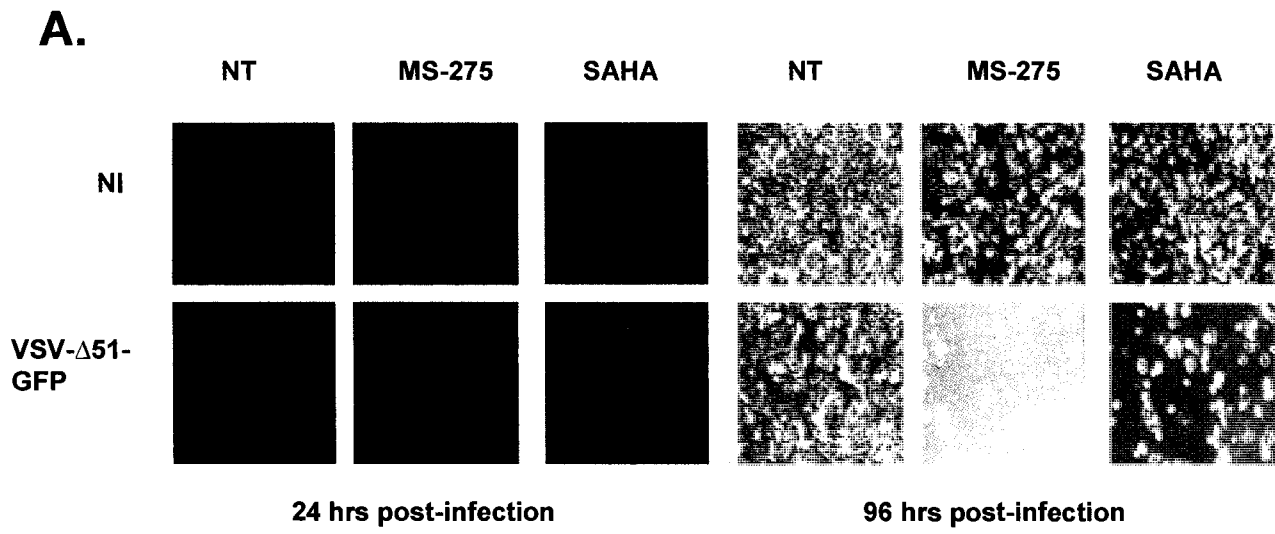
3. RESULTS

3.1 HDI treatment enhances VSVΔ51 replication and synergistically induce cell killing in partially VSVΔ51-resistant PC3 prostate cancer cells

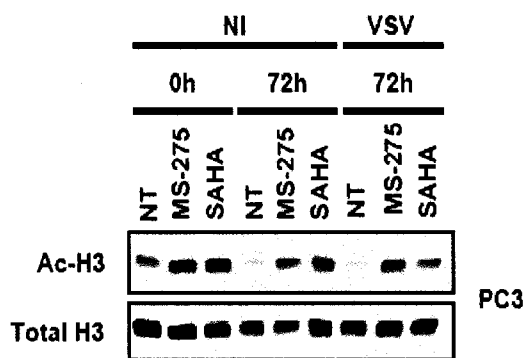
As described earlier, VSVΔ51 is a rhabdovirus mutant strain that grows poorly in normal tissues but replicates well in cells lacking an intact IFN response [36], a property that prompted the development of VSVΔ51 as an oncolytic agent for tumor cells with acquired defects in IFN signaling. Previous preclinical experiments showed that approximately 80% of tumor cell lines tested lack a normal IFN response [61]; however, the extent of this defect is variable and, at low virus concentrations, IFN production may be sufficient to blunt VSVΔ51 spread [3, 61, 66]. As an example, the androgen independent prostate cancer cell line PC3 is partially responsive to IFN and at low multiplicities of infection (MOI 0.1 pfu/cell), is refractory to VSVΔ51 infection ([3] and **Figure 1a**). Since HDIs are known to interfere with the ability of cell lines to mount an IFN response, we tested whether pretreatment of PC3 cells with the HDIs would sensitize them to VSVΔ51 infection and subsequent virus induced apoptosis. For these experiments, the attenuated strain of VSV encoding the GFP gene (VSV-Δ51-GFP) has been used in combination with two distinct HDIs, MS-275 and SAHA (Vorinostat), which have shown promising anti-cancer activity in pre-clinical (MS-275) or clinical (SAHA/Vorinostat) models [7, 18, 20, 25, 39, 40, 48, 53, 54]. As shown by the western blot analysis in **Figure 1b**, the *in vitro* concentrations for MS-275 (4μM) and SAHA (4μM) were adequate to induce hyperacetylation of chromatin bound histone 3. Also, both HDIs dramatically increased VSVΔ51 replication in PC3 cells as early as 24h post-infection, at which time robust GFP expression was detected by fluorescence microscopy and fluorescence activated cell sorter (FACS) analysis. Increased GFP expression correlated well with virus production from HDI treated cells (**Figures 1a, c, d**). The enhancement of virus production post HDIs ranged from 10 up

to 100 fold. In addition to augmenting viral replication and spread, HDI-VSV Δ 51 combination treatment ultimately destroyed the cultured monolayer (**Figure 1a**).

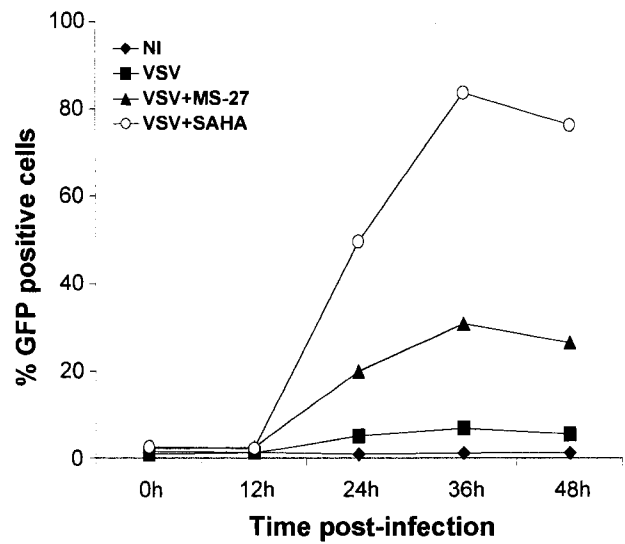
The striking increase in oncolysis suggested the possibility that our virus pharmacophore and the HDIs interacted in a synergistic fashion. To test this idea, *in vitro* cytotoxicity of PC3 cells was assessed at varying concentrations of VSV Δ 51 and HDI in a fixed ratio design. Combination indices (CI) calculated using the equations of Chou and Talalay [14] were then used to qualify the interaction between VSV Δ 51 and MS-275 or SAHA. It is generally considered that CI values below 0.7 indicate *bone fide* synergy. Both HDIs interacted with VSV in a highly synergistic fashion (CI<0.4 at ED50) (**Figure 2**).



B.



C.



D.

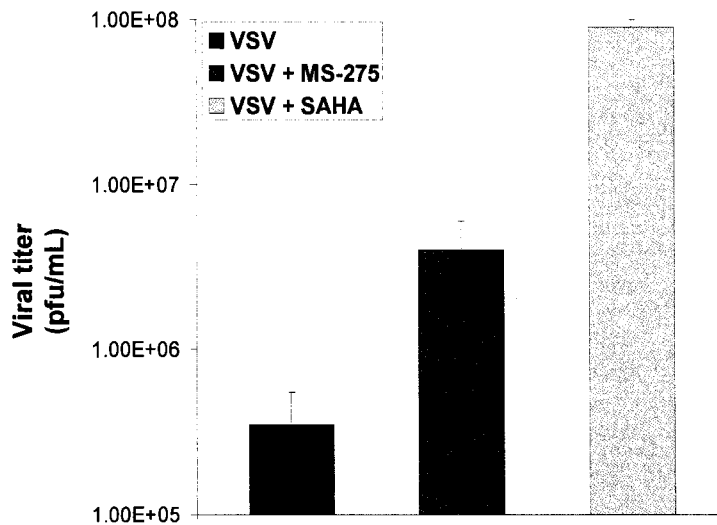


Figure 1

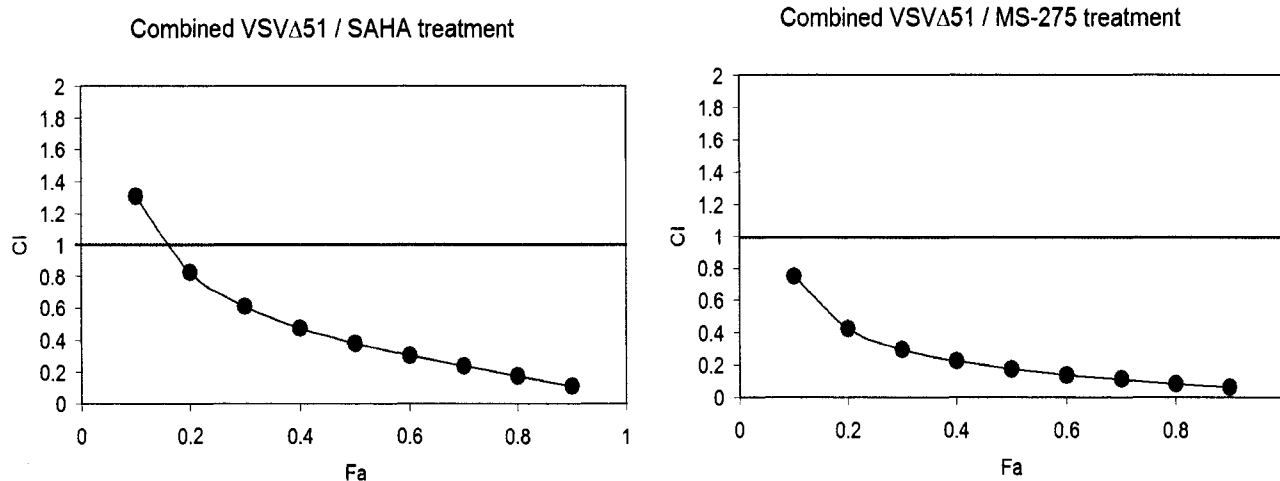
Figure 1 - HDIs enhance VSV Δ 51 replication in partially VSV Δ 51-resistant PC3 cancer cell line.

a) Subconfluent PC3 cells (75% confluence) were either pre-treated or not with MS-275 or SAHA 7hrs prior to VSV Δ 51 inoculation at 0.1 MOI. Viral replication was assessed after 24h of infection by fluorescent microscopy for GFP expression. Phase-contrast microscopy at 96h demonstrated massive cell death in combination-treated cells. **b)** PC3 cells were treated with either MS-275 (4 μ M) or SAHA (4 μ M) for the specified times, in the presence or absence of VSV Δ 51 infection. Following acid extraction of histones, hyperacetylation was assessed by immunoblot using an antibody against total or acetylated histone H3. **c)** Replication of VSV Δ 51 was quantified by FACS analysis for GFP expression at 12, 24, 36, and 48h post-infection in the presence or absence of HDIs. **d)** Standard plaque assays were used to determine viral titers at 48 hrs post VSV Δ 51 infection of PC3 cells at 0.0001 MOI, with or without HDI treatment. Supernatants were collected for standard plaque assay.

NT - No Treatment

NI - No Infection

Figures a,b&c by Dr. Thi Lien-Anh Nguyen



Drug : Virus (ratio)	CI Values at		
	ED50	ED75	ED90
VSV:SAHA (31250 PFU:1 μ M)	0.37835 +++	0.20614 ++++	0.11331 ++++
VSV:MS-275 (25000 PFU:1 μ M)	0.17471 ++++	0.09734 +++++	0.06088 +++++

Range of CI	Symbol	Description
<0.1	+++++	Very strong synergism
0.1-0.3	++++	Strong synergism
0.3-0.7	+++	Synergism
0.7-0.85	++	Moderate synergism
0.85-0.90	+	Slight synergism
0.90-1.10	\pm	Nearly additive
1.10-1.20	-	Slight antagonism
1.20-1.45	--	Moderate antagonism
1.45-3.3	---	Antagonism
3.3-10	----	Strong antagonism
>10	-----	Very strong antagonism

Figure 2

Figure 2 - VSV Δ 51 exhibits synergic in vitro cytotoxicity against PC3 cells when combined with the HDIs, MS-275 and SAHA.

PC3 cells were treated with serial dilutions of a fixed ratio combination mixture of VSV Δ 51 and either MS-275 or SAHA (5x10⁵ PFU to 1 μ M) in 96-well plates. Cytotoxicity was assessed using MTS reagent after 60 hrs. DMSO-treated cells were used as control. Combination indices (CI) were calculated according to the method of Chou and Talalay. Plots represent the algebraic estimate of the CI in function of the fraction of cells affected (Fa)

3.2 HDIs enhance VSVΔ51 replication in PC3 cells by dampening the IFN response

To gain an understanding of how MS-275 and SAHA enhanced VSVΔ51 infection, virus activation of the IFN cascade in PC3 cells was examined in the presence or absence of HDIs, using a combination of assays including ELISA, RT-PCR and immunoblot analysis. It has been found that VSVΔ51 infection of PC3 cells induced expression of a variety of gene products from the IFN cascade, including RIG-I, IFN alpha and beta, IRF-7, ISG56 as well as Mxa, an IFN-inducible GTPase that is directly involved in the inhibition of VSVΔ51 replication (**Figures 3a-c**). As expected, HDI treatment led to the blunting of the cellular IFN response and robust virus protein production (**Figure 3c**). RT-PCR revealed that infected PC3 cells treated with HDIs expressed significantly less IFN-β mRNA at 12h and essentially undetectable levels at 24 hrs post-infection, in stark contrast to the infected untreated cultures. Similarly, the levels of Mxa and IRF-7 mRNA were strongly inhibited in HDI treated PC3 cells. Taken together, these results suggest that HDIs predominantly impact the IFN signaling cascade downstream of IRF-3, while minimally affecting upstream factors such as IKKε and RIG-I. Indeed, virus induced IRF-3 phosphorylation and activation was easily detected by immunoblot in samples pretreated with HDIs but not in samples infected with VSVΔ51 alone (**Figure 3c, first row**). This result is in agreement with previous studies demonstrating that at low MOI, IRF-3 phosphorylation was not detected by immunoblot, yet was sufficient to induce expression of downstream ISGs that inhibited virus multiplication ([64] and unpublished results). Treatment of PC3 cells with HDIs in the absence of virus infection did not affect IFN levels or IFN-inducible gene expression.

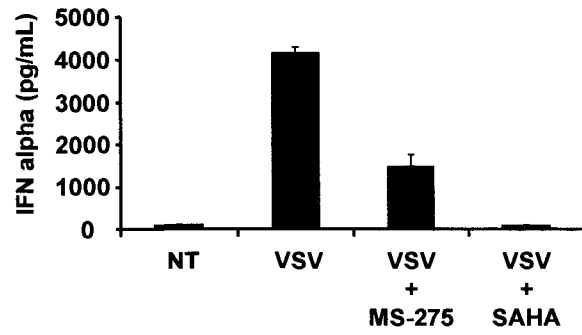
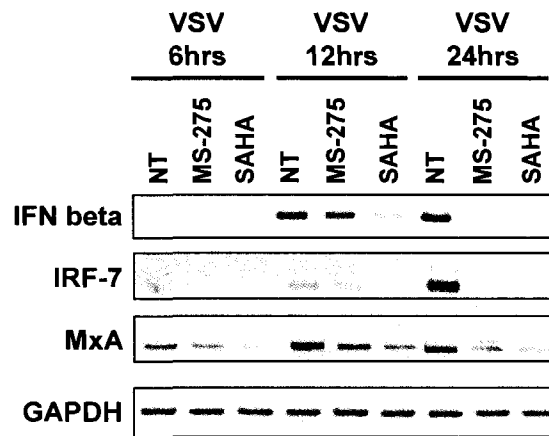
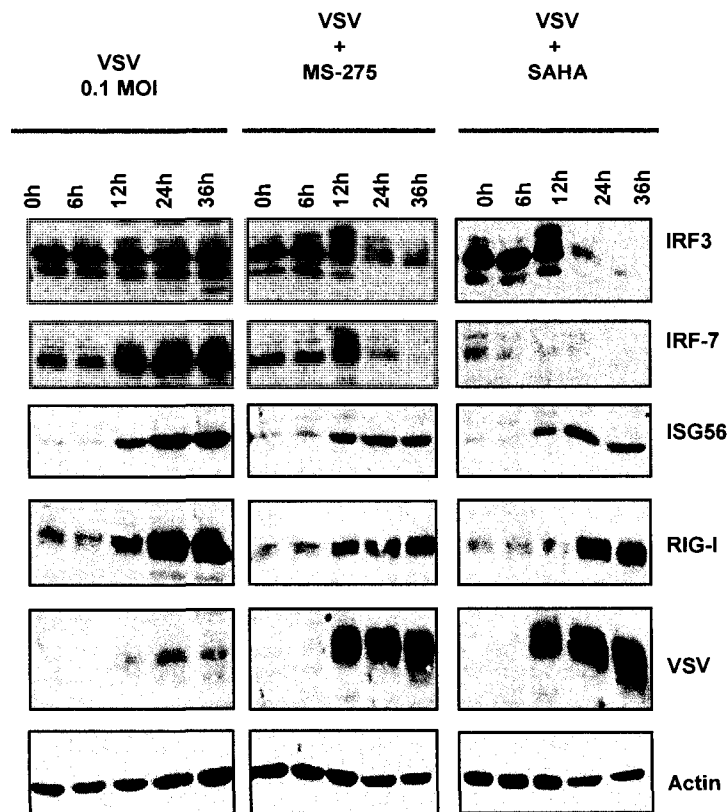
A.**B.****C.****Figure 3**

Figure 3 - MS-275 and SAHA augmentation of VSVΔ51 replication in PC3 cells correlates with dampening cellular interferon antiviral response.

a) PC3 cells were pre-treated with MS-275 or SAHA for 24h and then infected with VSV-Δ51-GFP at 0.1 MOI. IFN-α production in culture media was assayed by ELISA at 24h post-infection. **b)** Induction of antiviral genes *IFN-beta*, *IRF-7* and *MxA* was assessed at different times post-infection by RT-PCR. **c)** IFN-stimulated antiviral genes in PC3 cells were analyzed by immunoblot at different times after VSVΔ51 alone or VSVΔ51 plus HDI treatment.

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3.3 HDIs enhance the replication and oncolytic activity of VSVΔ51 in multiple cancer cell lines which are partially resistant to VSVΔ51 infection.

As discussed above, many tumor cell lines maintain a partial response to IFN that is sufficient to interfere with oncolytic virus spread in cultures [3]. A panel of cancer cell lines with this phenotype has been screened using three different HDIs (MS-275, SAHA and PXD 101) to investigate how generalized these enhancing effects of HDIs towards VSVΔ51. This collection of cancer cell lines was infected with VSVΔ51 in the presence or absence of IFN α and used GFP expression as well as quantification of viral titers to measure enhancement in virus infection and spread. As predicted, virus infection was severely impaired by addition of IFN α to the culture media, but this protective effect was reversed by pretreatment of cells with HDIs (**Figure 4a, b**). These data demonstrate that HDIs are able to blunt secondary waves of antiviral response stimulated by IFN alpha treatment, and thus further supporting the notion that HDIs may not affect the early activation of IRF-3 by upstream factors such as TBK-1/IKK ϵ and RIG-I, but rather act at the transcriptional level to inhibit expression of IFN and IFN-inducible genes. Note that SW620 cell line did not respond to IFN treatment and was permissive to VSVΔ51 infection.

For each of the aforementioned cell lines, the ability of various HDIs to enhance VSVΔ51 spread in the absence of exogenous IFN treatment has been compared. A confluent monolayer of cells was infected with a low concentration of VSVΔ51 (MOI 0.0001) after HDI treatment. Fluorescent microscopy showed poor viral spread represented by a punctate pattern of GFP expression in the non-HDI treated controls compared to a more uniform GFP distribution after drug treatment (**figure 5a**). In addition, higher yields of viral titers were produced in the supernatants of HDI-treated cells compared to the controls. HDI-VSVΔ51 combination therapy also induced superior cell killing than each of the individual treatments (**figure 5b, c**).

To further demonstrate that HDIs enhance the spreading of virus particles across the cultured monolayer high viral concentrations, MOI 10, were used for infection to enhance the probability of having all cultured cells infected at the time of inoculation close to 100%. This technique allowed for determining the effects of HDIs on single step growth kinetics of VSV Δ 51 while limiting the confounding factor of viral progenies produced from secondary replication cycles if low virus concentrations were used for infection. As seen in **figure 6**, MS-275 treatment didn't enhance single step growth kinetics of VSV Δ 51 in either of 4T1, PC3 or HT29 cancer cell lines, although MS-275 did enhance virus production in these cell lines when lower virus concentrations were used. This experiment elucidates the idea that HDI treatment enhances the spread of virus particles across the cultured monolayer rather than augmenting the absolute virus replication kinetics per cell. Interestingly, this potentiation of virus spread was not restricted to oncolytic VSV Δ 51, as we found that both vaccinia virus [41] and Semliki Forest virus also rapidly spread through tumor cell cultures that had been exposed to HDIs (**Figure 7a, b**).

A.

	VSVA51	IFN Alpha VSVA51	MS-275 + IFN A VSVA51	SAHA + IFN A VSVA51	PXD 101 + IFN A VSVA51
4T1 (Breast)					
PC3 (Prostate)					
HT29 (Colon)					
M14 (Melanoma)					
SF268 (Glioblastoma)					
SN12C (Renal)					
SW620 (Colon)					

B.

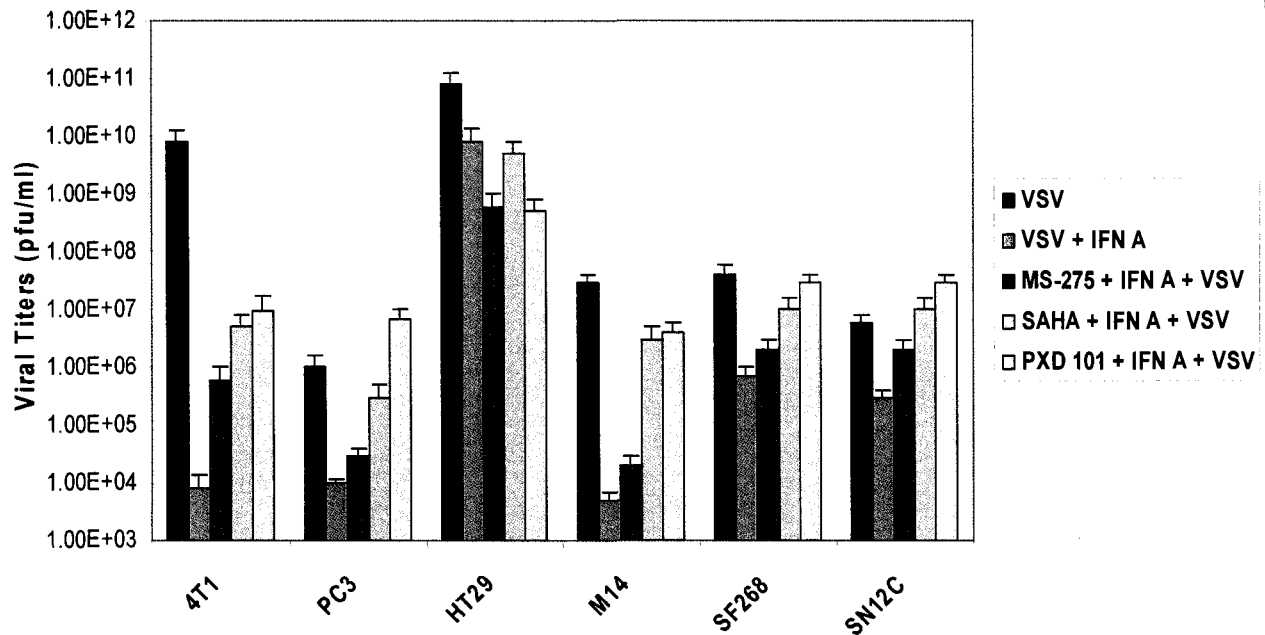
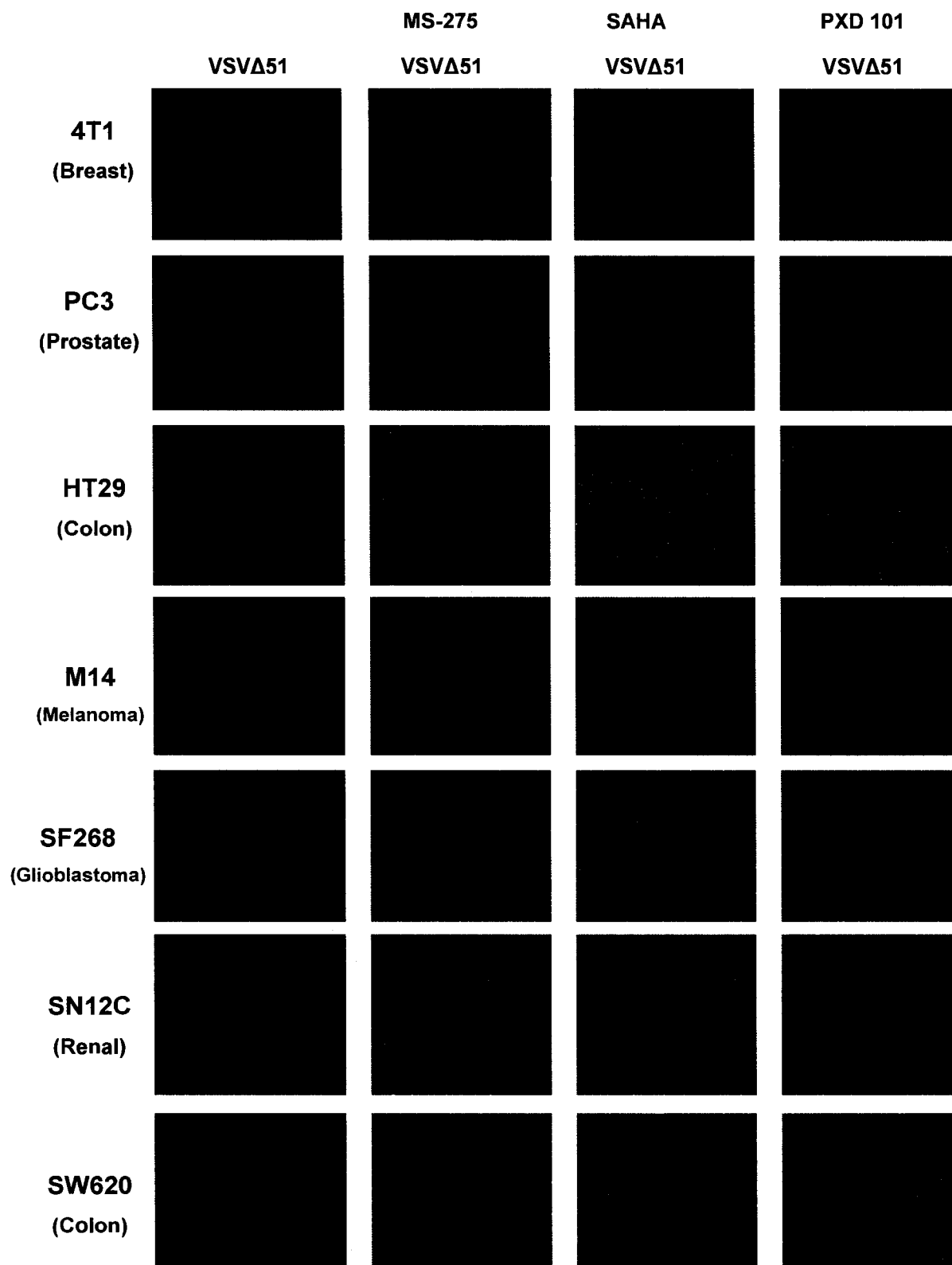


Figure 4

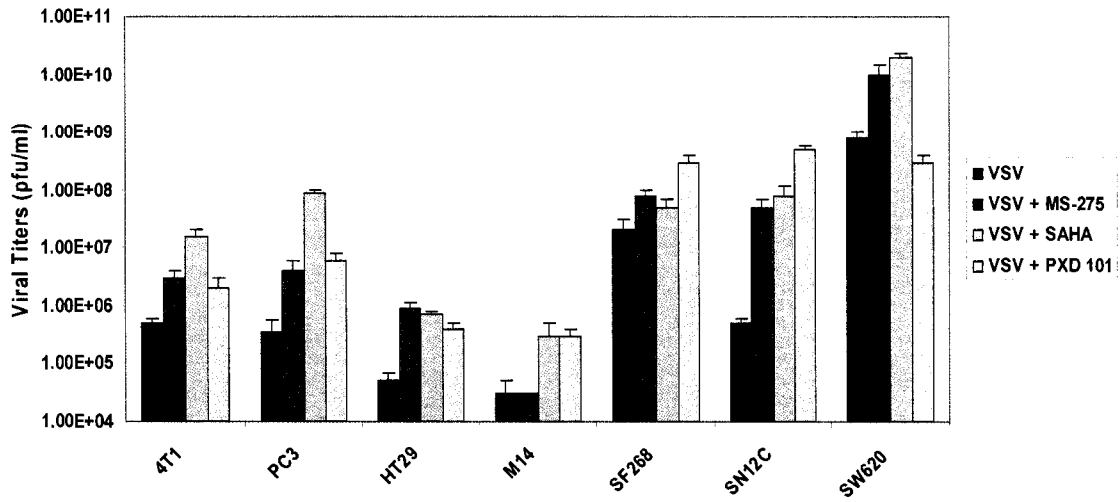
Figure 4 - HDIs can blunt the inhibitory antiviral effect of exogenous IFN α and rescue VSV Δ 51 replication.

a) Different IFN α sensitive cancer cell lines were pre-treated with HDIs for 7h and then infected with VSV at 0.1 MOI in the presence or absence of IFN α treatment (50 IU). GFP expression was monitored 24h post VSV inoculation. **b)** Supernatants were collected 24h later for standard plaque assay. Pre-treatment with HDIs blunted the antiviral effects of IFN and rescued VSV replication. IFN treatment did not inhibit VSV infection in SW620 cell line.

A.



A.



B.

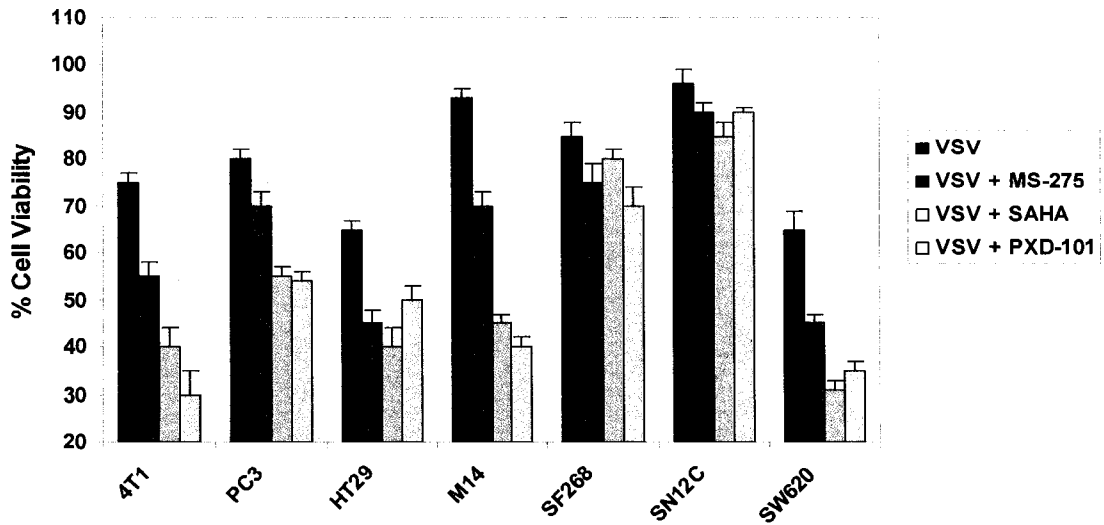
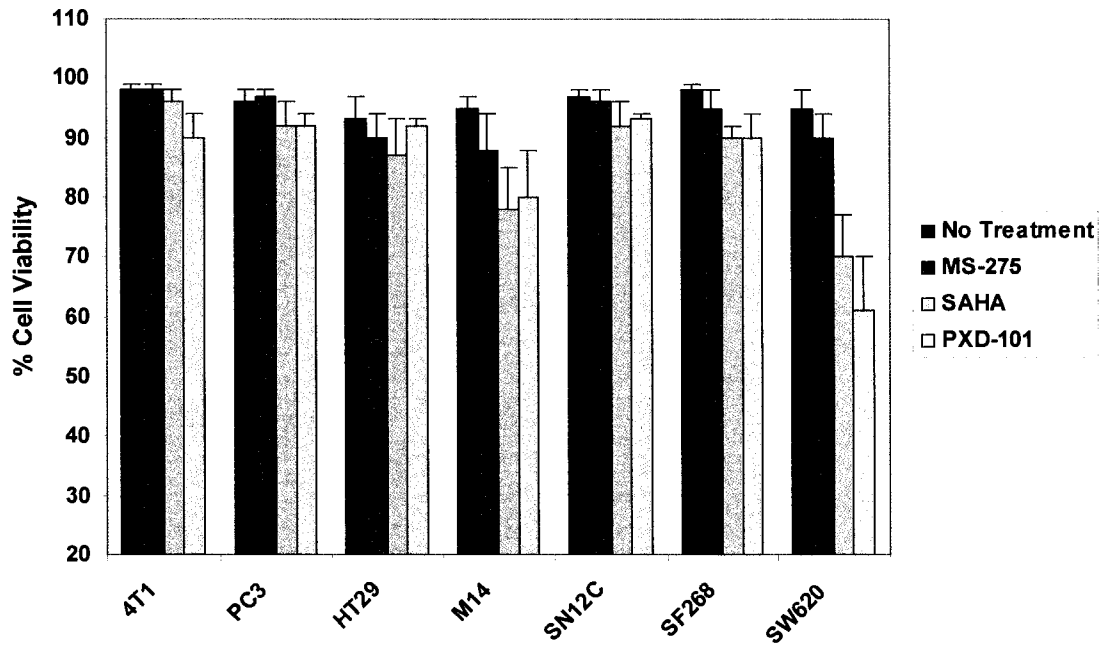


Figure 5 – HDI treatment enhances VSVΔ51 replication and oncolytic activity in multiple cancer cell lines which are partially resistant to VSVΔ51. This enhancement is noted when considerably low virus concentrations are used for infection.

a) A panel of cancer cell lines that are partially resistant to VSVΔ51 were inoculated at low MOI 0.0001, with or without HDI pre-treatment (MS-275 or SAHA at 4 μM). Viral replication was monitored 36h post VSVΔ51 infection using fluorescence microscopy to assess GFP expression.

b) Supernatants from the various treatment conditions were collected 24h following viral infection and titered on Vero cells to quantify VSVΔ51 titers. **c)** Cell viability was assessed using trypan blue assay at 48 hrs post VSVΔ51 infection, at MOI 0.1, in the absence or presence of HDI treatment.

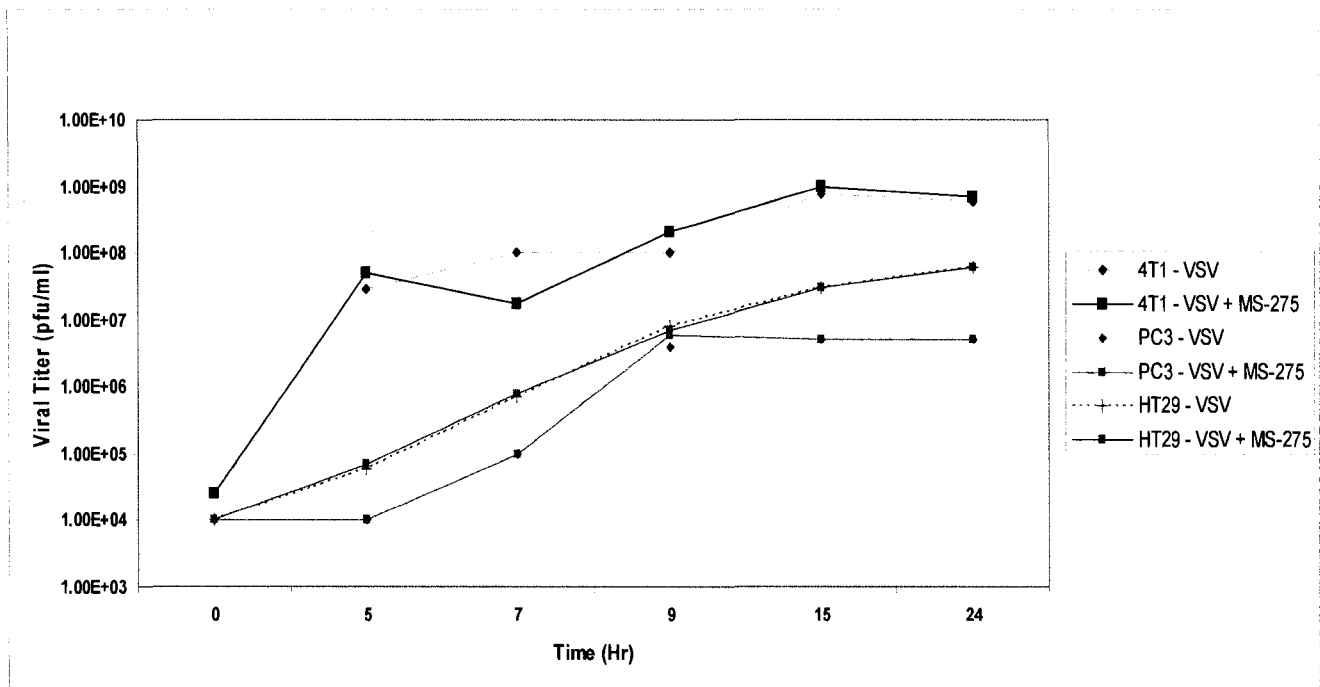
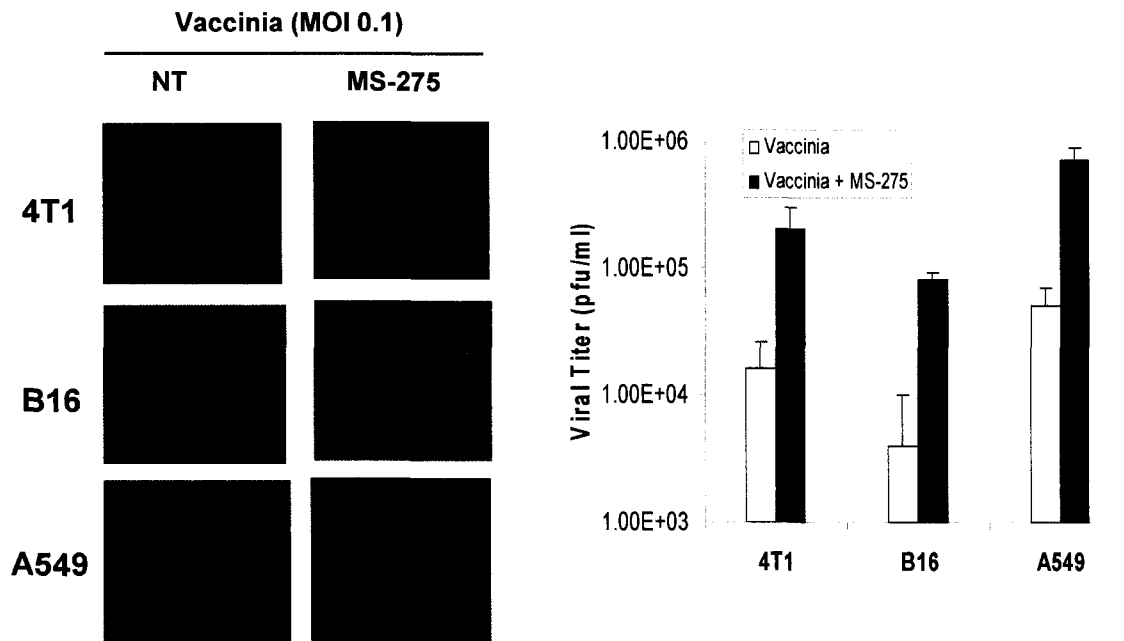


Figure 6

Figure 6 – HDI treatment did not alter or enhance the single step growth kinetics of VSVΔ51 in either of 4T1, HT29 or PC3 cancer cell lines.

Each cell line was cultured at a sub-confluent level of 80%. 24 hrs following plating, the culture was inoculated with high concentrations of VSVΔ51 at MOI 10. Samples of supernatants were collected at different time points post viral infection and used to quantify amounts of virus production. The sample used for each time point has been obtained from a separate infected well.

A.



B.

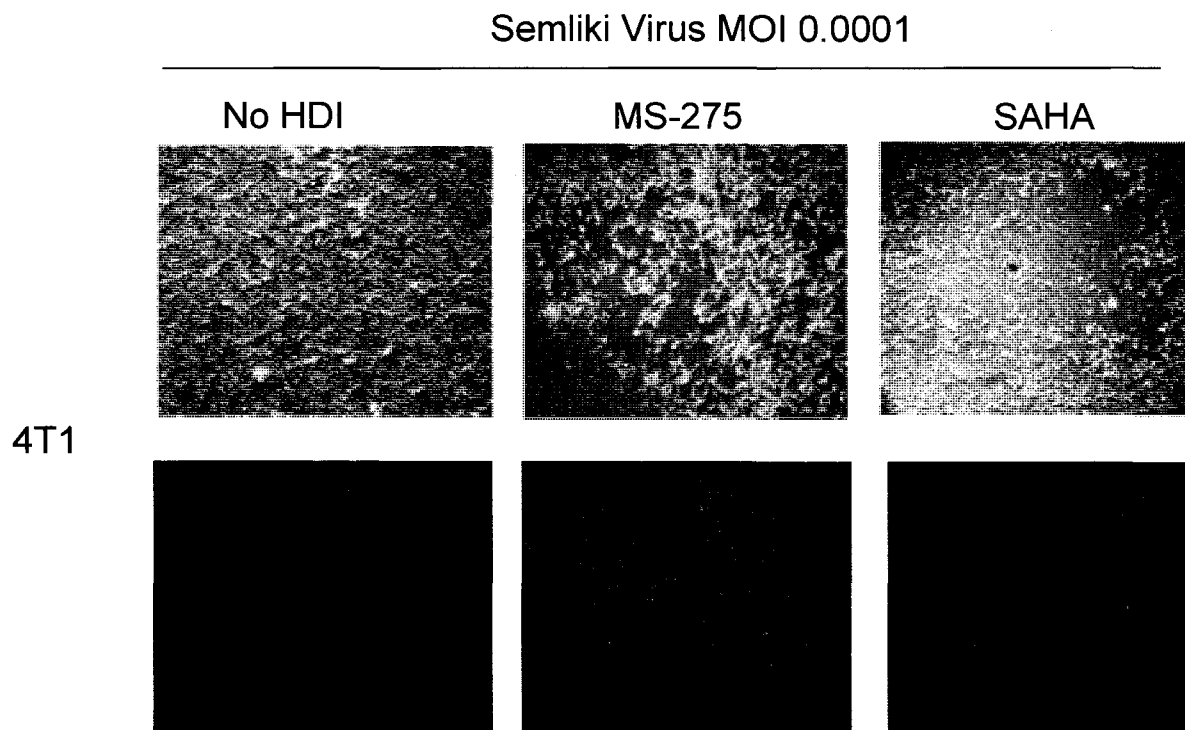


Figure 7 - HDIs enhance the oncolytic effects of other viruses such as vaccinia and Semliki Forest (SFV)

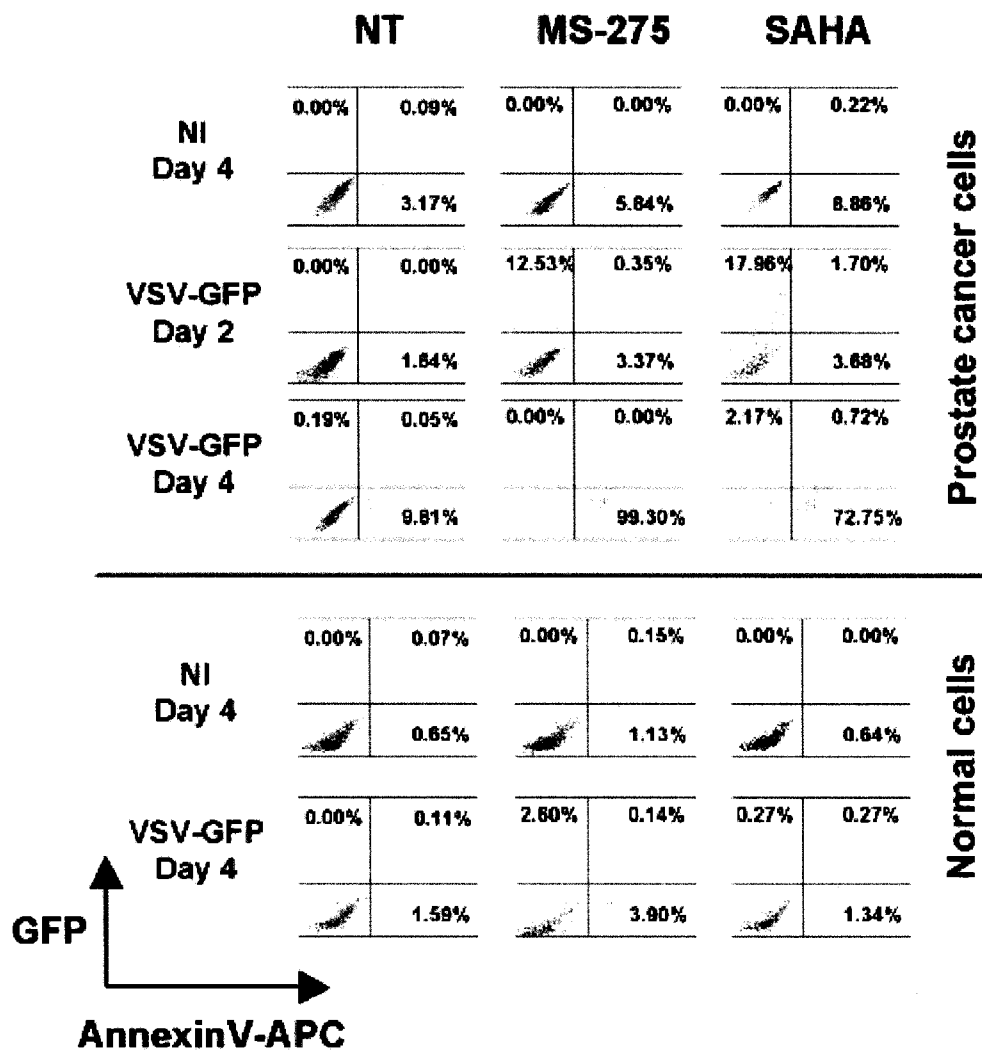
a) Fluorescent micrographs obtained 72h after the inoculation of 4T1, B16 and A549 cancer cells with VVDD-GFP at MOI 0.01. MS-275 (4 μ M) was added at the time of viral inoculation and left in the media for 72h, after which supernatants were collected and titered on U2OS cells. **b)** 4T1 cell were inoculated with SFV at MOI 10⁻⁴ together with either MS-275 (4 μ M) or SAHA (4 μ M). Micrographs were obtained at 36h after virus inoculation.

3.4 HDIs specifically enhance VSVΔ51 spread in primary human tumor specimens

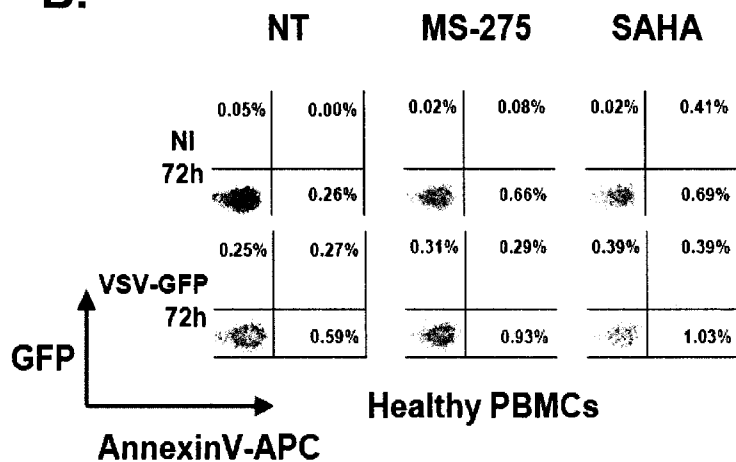
To determine whether HDI enhancement of VSVΔ51 replication and tumor cell killing was effectively translated to primary human samples, malignant and adjacent normal cell cultures were established from prostates obtained following radical prostatectomy. Dissociated cultures infected in the presence or absence of HDIs were analysed for virally expressed GFP and Annexin-V staining by flow cytometry. No evidence of VSVΔ51 replication or virus-induced apoptosis was observed in either normal or tumor cultures in the absence of HDIs (**Figure 8a**). However, prostate cancer cells became GFP positive and were ultimately killed following VSVΔ51 infection in the presence of either MS-275 or SAHA (**Figure 8a, upper panels**). In contrast, normal prostate cells from the same patient remained refractory to VSVΔ51 infection, even when exposed to HDIs (**Figure 8a, lower panels**). In both, normal and tumor tissue, the efficacy of HDIs treatment was confirmed by monitoring acetylation of histone H3 by immunoblot in PC3 cells and normal peripheral blood mononuclear cells (PBMCs) (**Figure 8c**).

Next, the ability of HDIs to stimulate virus growth in intact primary tumor samples was evaluated in human explants obtained from patients undergoing surgical resection. Tissue slices were incubated with VSVΔ51 in the presence or absence of drug; similar to the dispersed primary cultures, HDIs specifically enhanced VSVΔ51 replication and spread only in primary tumor explants but not in normal tissue slices. For example, in both ovarian cancer and sarcoma samples, intense GFP fluorescence and virus replication were detected in the combination treated samples (**Figures 8d, e**), whereas slices of normal colon, muscle and lung tissues remained refractory to virus infection even in the presence of HDIs. Furthermore, PBMCs isolated from healthy donors (**Figure 8b**) remained resistant to VSVΔ51 infection and killing at high MOI and HDIs pretreatment. Taken together, these results demonstrate the ability of HDIs to specifically enhance virus replication in primary tumor tissues but not in normal human samples.

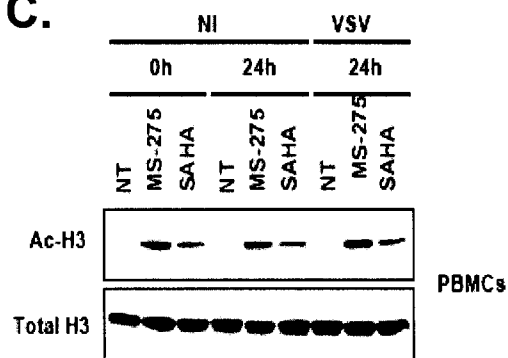
A.



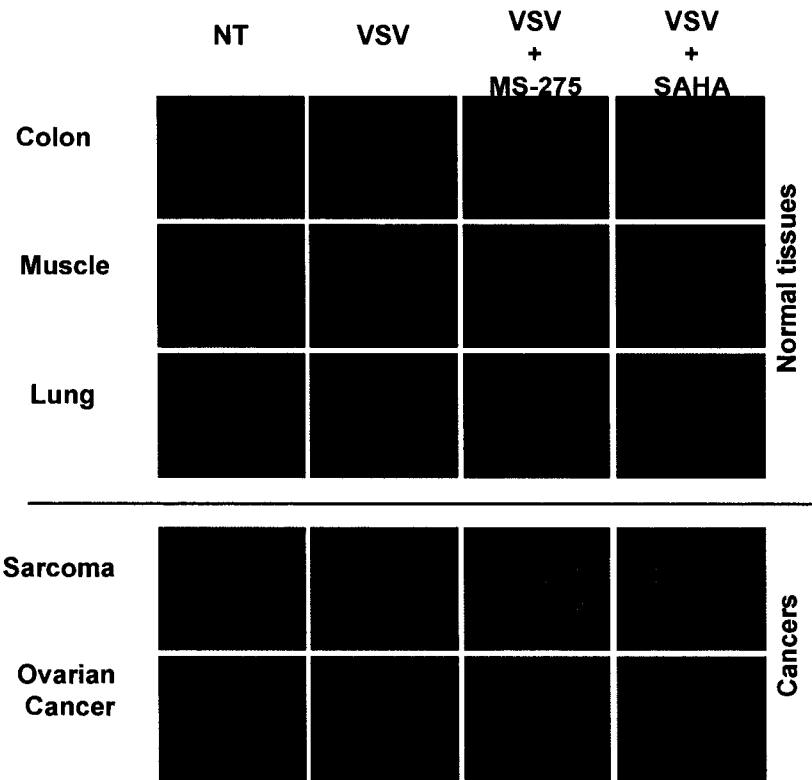
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C.



D.



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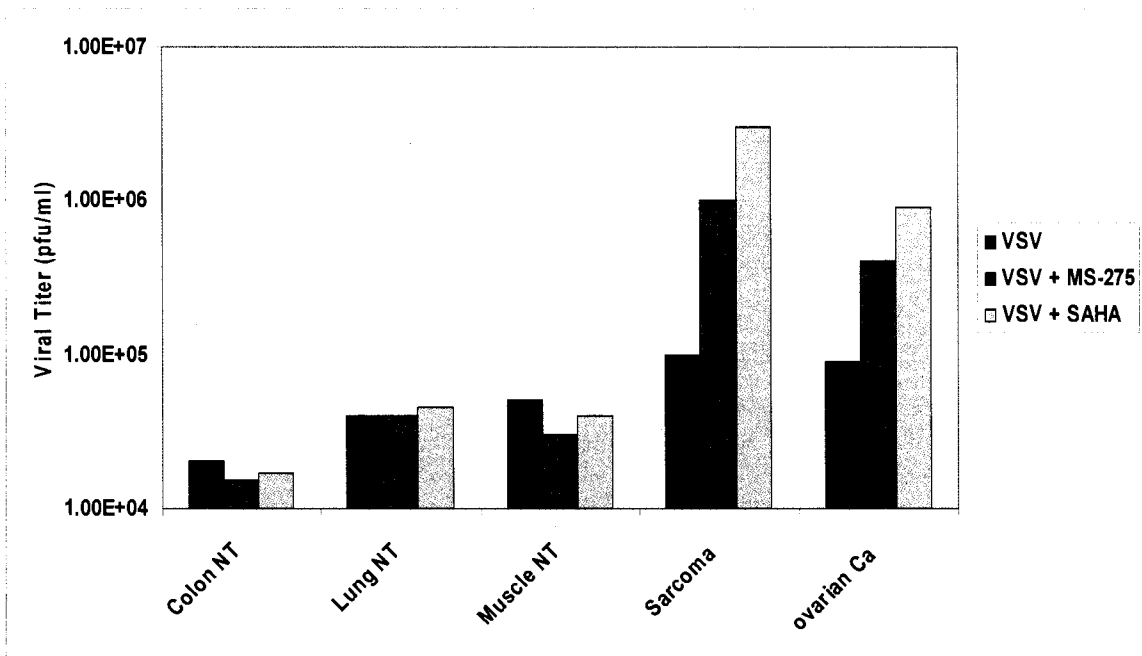


Figure 8

Figure 9 – HDI treatment enhances permissiveness of primary tumor specimens to VSVΔ51 oncolytic activity while sparing normal tissues.

- a) *Ex-vivo* primary cancer or normal prostate cells from radical prostatectomy samples were placed in culture and subjected to 24h HDI pre-treatment followed by VSVΔ51 infection (5 MOI). VSV replication (GFP, y axis) and apoptosis induction (AnnexinV-APC staining, x axis) were determined at 2 and 4 days post infection by FACS. **B)** Normal PBMCs isolated from a healthy donor were subjected to the HDI/VSVΔ51 combination and FACS analysis as described in panel A. **c)** PBMCs were treated with MS-275 (4 μM) or SAHA (4 μM) for 24 hrs prior to the presence or absence of VSVΔ51 inoculation. Following acid extraction of histones, hyperacetylation was assessed by immunoblot using an antibody against total or acetylated histone H3. **d)** Human *ex-vivo* cancer or normal tissue specimens were inoculated with 5×10^6 pfu/ml of VSVΔ51 in the absence or presence of HDI pre-treatment: MS-275 (4 μM) or SAHA (4 μM) for 7 h. GFP expression was monitored 48h after viral inoculation by fluorescence microscopy. **e)** Specimens in (d) were homogenized and virus titers were determined using standard plaque assay to quantify extent of enhancement in virus replication.

NT – No Treatment

Figures a,b&c by Dr. Thi Lien-Anh Nguyen

3.5 *In vivo*, systemic co-administration of MS-275 with VSVΔ51 augments viral oncolytic activity strictly at the tumor site.

Six different *in vivo* cancer models were used to investigate the safety and anti-tumor efficacy profiles of HDI plus VSVΔ51 combination therapy. These models included mice bearing PC3 (prostate), M14 (melanoma), HT29 (colon), 4T1 (breast), and SW620 (colon) subcutaneous tumors, as well as a spontaneous bilateral transgenic ovarian cancer model [15]. MS-275 was chosen for the treatment of all models because of its noticeable enhancing effects demonstrated *in vitro*, as well as the safety profile demonstrated in multiple phase I clinical trials [25, 34, 56]. Also, unlike SAHA and PXD-101, MS-275 was commercially available in quantities necessary to perform the outlined *in vivo* experiments. In all cases, the drug was administered intraperitoneally (IP), while an assortment of virus delivery routes was tested, including intratumoral (IT), intravenous (IV) as well as IP. For these experiments, VSVΔ51 was engineered to express firefly luciferase so that virus replication could be monitored *in vivo* using an ‘In Vivo Imaging System’ (IVIS). In initial studies, mice bearing subcutaneous PC3, M14 or HT29 tumors were treated by direct intratumoural (IT) injection, along with daily drug (or vehicle) administration. In each tumor model, MS-275 therapy was able to promote VSVΔ51 replication *in vivo*, as measured by firefly luciferase activity and by immunohistochemical (IHC) staining for VSV antigens (**Figure 9a**). Hyperacetylation of chromatin bound H3 was furthermore confirmed in the PC3 tumors by immunohistochemistry at 6 and 24h post-treatment (**Figure 10**). Notably in these and subsequent experiments, the luciferase signal was restricted to the tumor mass and replication within normal tissues in any of the mice receiving combination therapy was not detected. Recently, our lab has shown that OV infection of tumors initiates a rapid and profound loss of blood flow to the tumor that can be measured by decreased uptake of intravenously administered fluorescent microspheres [10]. This “vascular shutdown”

phenomenon was clearly replicated in perfusion studies performed following co-administration of VSV Δ 51 and MS-275 in these three models (**Figure 9a**).

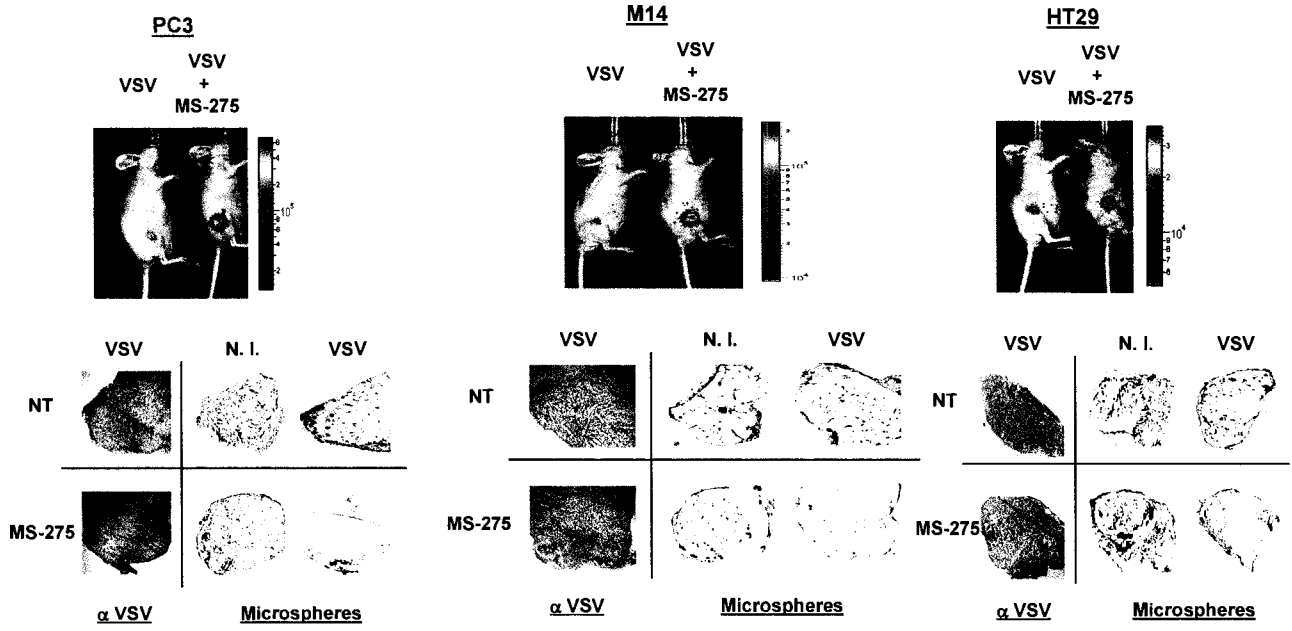
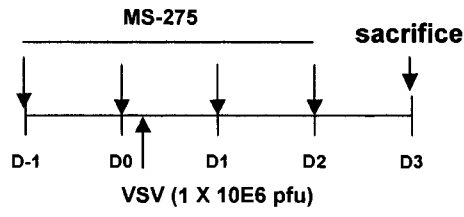
A spontaneous transgenic tumour model of ovarian cancer was also tested with the combination of MS-275 and VSV Δ 51, both delivered by intra-peritoneal injection. In **figure 9b**, IVIS imaging clearly demonstrated that VSV Δ 51 replication was augmented by HDI treatment and restricted to the bilateral tumors arising on the ovaries of these mice. In both tumor bearing and tumor free mice treated with VSV Δ 51 alone, some luciferase activity was occasionally observed in the spleen but this activity was transient and was not significantly enhanced by HDI treatment.

The 4T1 breast cancer model is highly metastatic when implanted subcutaneously and is refractory to VSV Δ 51 therapy (see **Figure 11** and [19]). In mice infused IV with VSV-luciferase alone, a weak signal emanating from infected metastatic nodules was observed between 24-48h but ultimately VSV Δ 51 was cleared (**Figure 11a**) and treatment had no effect on disease progression. However, when combined with HDI therapy, virus replication is clearly evident both in the primary subcutaneous lesion, as well as at multiple metastatic sites; replication persisted past 80h and had a dramatic impact on tumor size (**Figures 11a, b**).

A more marked effect on tumor size was observed in the SW620 model of colon cancer, where intravenous VSV Δ 51 infection of subcutaneous SW620 tumors concomitant with daily MS-275 therapy resulted in a steady increase in luminescence at the tumor site and virtually no tumor growth in the combination treatment group (**Figures 12b**). Immunohistochemistry of frozen tissue sections also reflect the abundant presence of VSV antigen in double treated tumors compared to the single treated VSV Δ 51 (**Figure 12c**)

Not only did MS-275 show tumor specific enhancement of VSV Δ 51 replication, but the HDI also behaved as a regulatory chemical switch that can be used to control virus replication within the tumor. This unique characteristic of the combination therapy was demonstrated in the SW620 model, where MS-275 therapy was halted on day 4 and the luciferase signal was lost from the tumor by day 7. Re-initiation of HDI therapy at day 7.5 resulted in a re-emergence of the luciferase signal at day 9 and continued regression of the tumor (**Figures 12a**). This observation demonstrates that VSV Δ 51 replication closely correlated with the pharmacokinetics of the HDI used and the combination strategy requires effective protein hyperacetylation within the tumor in order to reach maximal effective oncolytic activity.

A.



B.

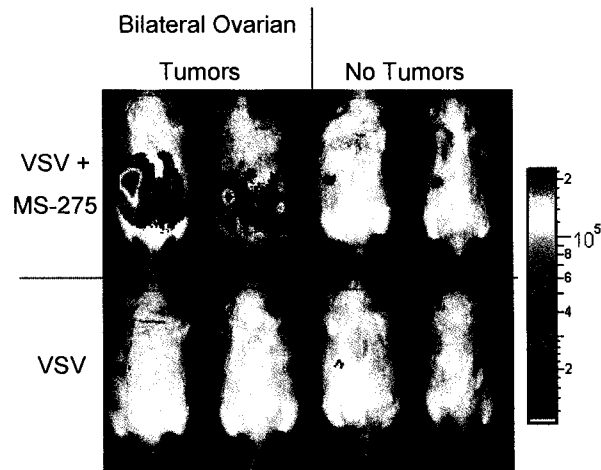


Figure 9 - MS-275 treatment enhances VSV-Δ51Luc replication at the tumor site after intratumoral or intraperitoneal administration.

a) Schematic representation of the treatment schedule for *in vivo* analysis. PC3, M14 and HT29 subcutaneous xenograft tumor models were established in nude mice. After tumor growth, the combination treatment group received MS-275 intraperitoneally every 24h until sacrificed (23mg/kg/day). At 4h following administration of the second HDI dose, all tumors were injected with VSV-Δ51Luc (1×10^6 pfu). Viral replication at the tumor site was imaged using the In Vivo Imaging System (IVIS). At 5 min. prior to sacrifice, mice were perfused with fluorescent orange microspheres to outline the tumor microvasculature. Frozen tumor sections were obtained for anti-VSV immunohistochemistry. **b)** Transgenic mice bearing bilateral ovarian tumors received IP injections of MS-275 every 12h at 6mg/kg, while VSV was administered IP at 1×10^8 pfu at 4h after the initial HDI dose. Viral replication in live animals was assessed 48h following viral infection by IVIS.

NT – No Treatment
NI – No Infection

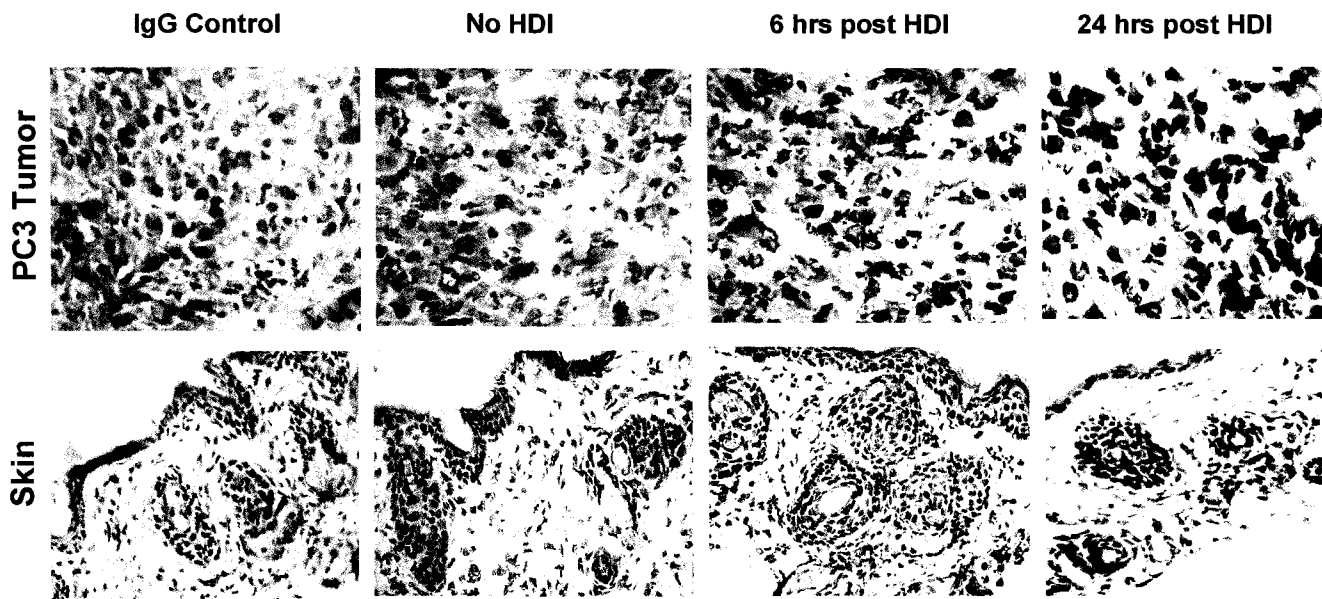


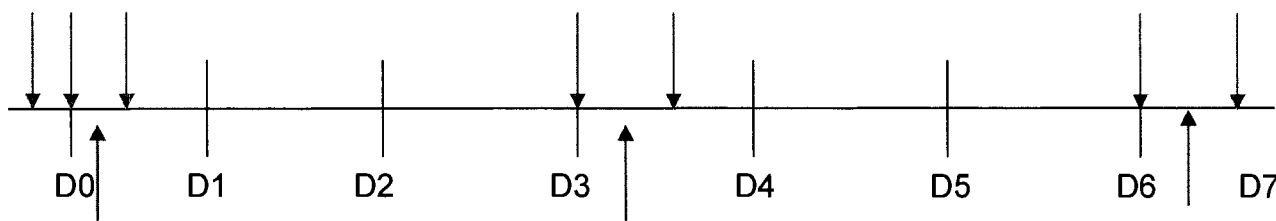
Figure 10

Figure 10 – The MS-275 dosing regimen used to treat *in vivo* PC3 tumors causes hyperacetylation of chromatin bound histones lasting for 24 hrs.

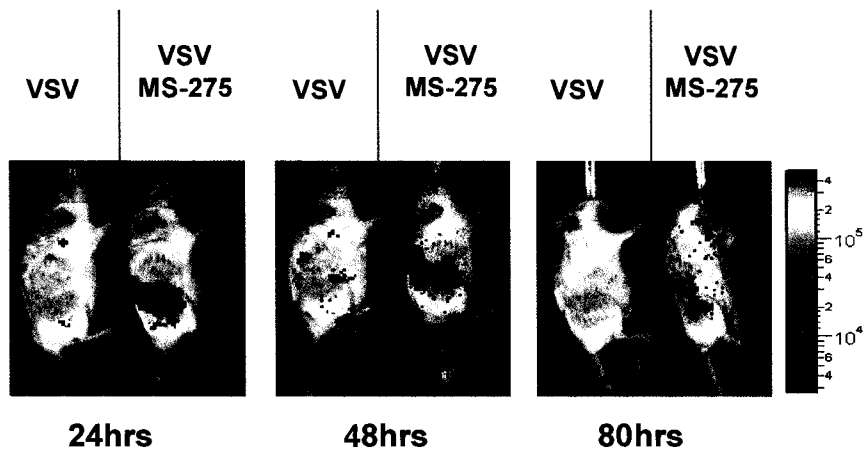
Using immunohistochemistry, acetylation of histone H3 proteins was assessed in PC3 tumors at 6h and 24h following single intraperitoneal delivery of a 23 mg/kg dose of MS-275. Skin sections were used as normal control. Histones in PC3 tumors are hyperacetylated at 6h and 24h post-HDI treatment. In skin sections, hyperacetylation of H3 returns to normal levels 24h after drug administration.

A.

4T1 Tumor Model



VSVΔ51 1x10⁸ pfu I.V.



B.

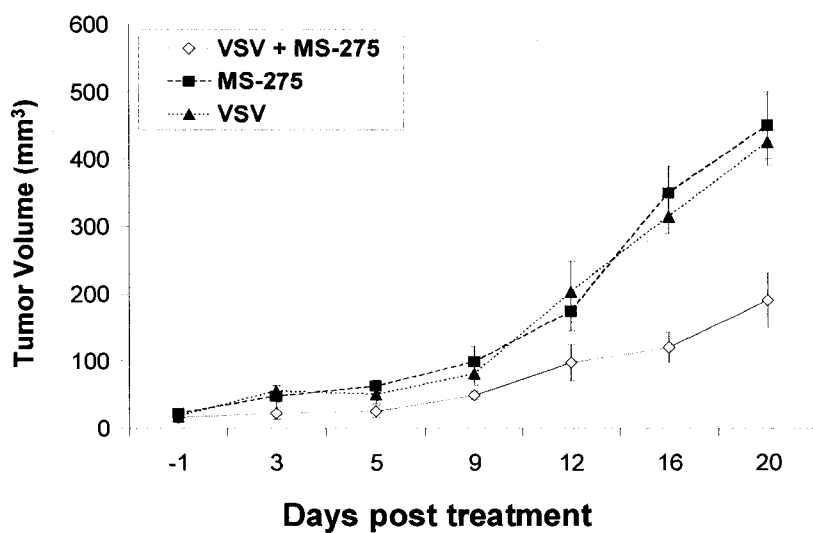


Figure 11

Figure 11 – Systemic combination treatment of MS-275 plus VSV Δ 51 augments tumor specific viral replication and results in a significant reduction of tumor size in an immunocompetent mouse model bearing syngeneic 4T1 subcutaneous tumors.

a) Subcutaneous 4T1 tumors were established in the hind flanks of Balb/c mice. The combination treatment group received MS-275 intraperitoneally, 6 mg/kg every 12h. At 4h after administration of second HDI dose, VSV- Δ 51-Luc (1×10^8 pfu) was injected intravenously. IVIS images were captured at 24, 48 and 80h post-VSV injection. b) The efficacy of MS-275, VSV Δ 51 and VSV Δ 51/MS-275 combination treatment in reducing tumor growth was assessed by tumor volume measurement over time.

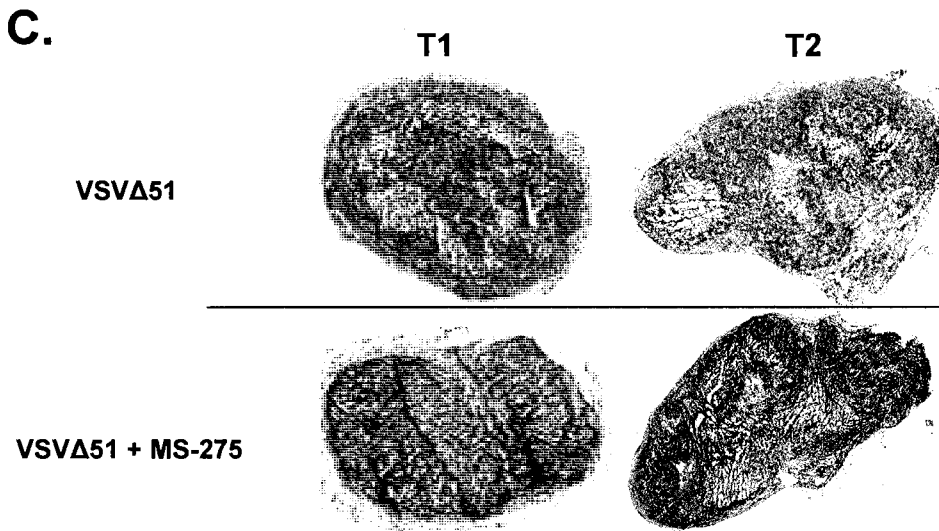
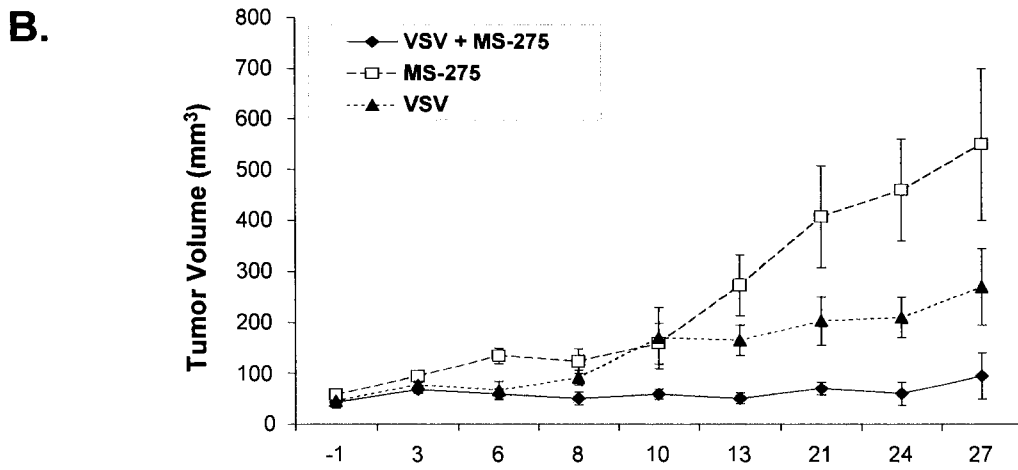
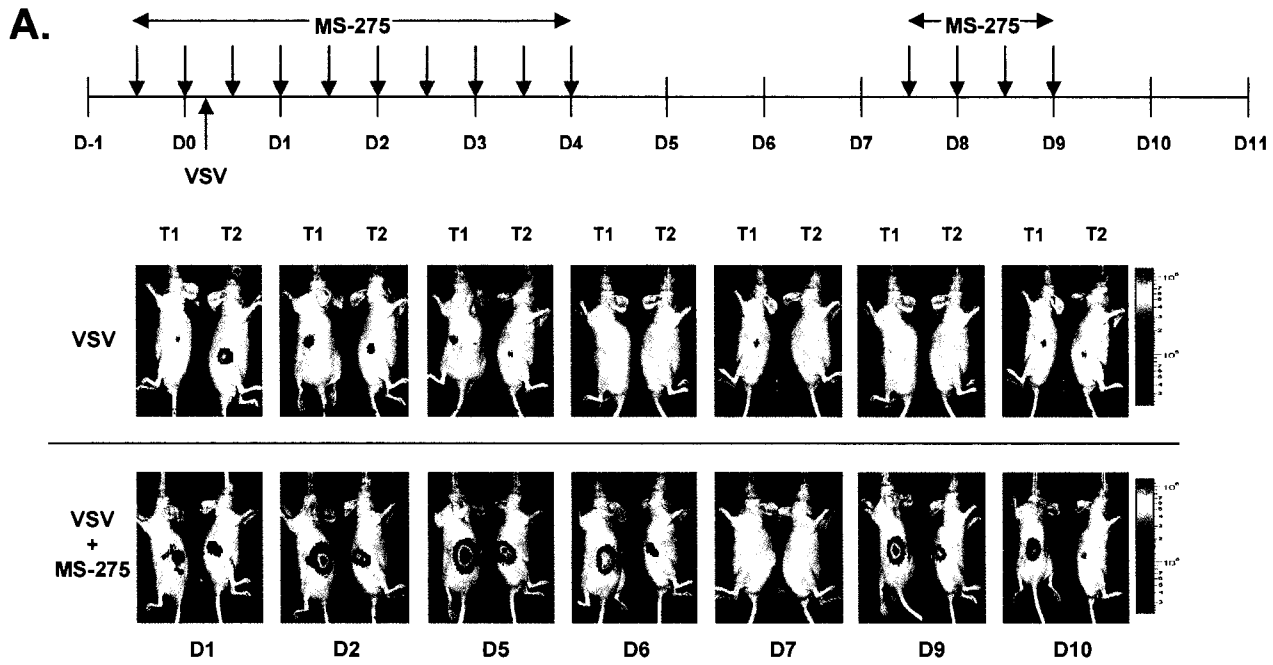


Figure 12

Figure 12 – Systemic combination treatment of MS-275 plus VSVΔ51 augments tumor specific viral replication and results in a significant reduction of tumor size in an immunocompromised mouse model bearing SW620 subcutaneous tumors. VSVΔ51 replication at the tumor site is also quite sensitive to the administration of MS-275.

a) SW620 tumors were established in hind flanks of CD1 nude mice. A schematic representation of the treatment schedule is shown. The combination treatment group received 7 mg/kg MS-275 intraperitoneally every 12h. Four hours following administration of second HDI dose, VSV-Δ51-Luc (1×10^7 pfu) was injected intravenously. MS-275 treatment was stopped between days 4-7 and days 9-11 to assess effect on viral replication. Virus replication at the tumor site was revealed by IVIS imaging, as well as by anti-VSV immunohistochemistry post-sacrifice. **b)** The efficacy of MS-275, VSVΔ51 and VSVΔ51/MS-275 combination treatment in reducing tumor growth was assessed by tumor volume measurement over time. **c)** At day 11 post viral treatment, all mice in figure (a) were sacrificed and their tumors harvested. IHC was performed on tumor frozen section using anti-VSV antibody to identify the extent and distribution of viral replication.

4. DISCUSSION

Oncolytic viruses are often selected or engineered to exploit tumor specific genetic deficiencies or constitutively activated signaling pathways [8, 16, 32, 49, 59]. Regardless of the mechanism of selective targeting, all OV's also need to overcome the arsenal of antiviral programs that the individual cell has at its disposal to resist infection and/or the spread of viruses. One strategy to generate a more potent OV is to arm the virus with genes that express products to circumvent or blunt the innate antiviral response of the individual cell [4, 23]. Our research group and others [33, 60] have taken a different approach by engineering attenuated virus strains that are unable to resist cellular antiviral programs, thus increasing their therapeutic index since they cannot replicate in normal tissues but retain the ability to grow in malignant tissues. During the evolution of malignancies, genetic abnormalities accumulate that provide cancer cells with growth and survival advantages but at the same time compromise the ability of individual tumor cells to mount a robust antiviral response [5, 6, 44, 60, 61]. It seems likely that the inability of a tumor cell to secrete or respond to IFN may facilitate tumor escape from immune surveillance [60]. While defects in cellular innate immunity are commonly found in tumor cells, the extent of the defect is quite variable [Appendix I 3, 19, 61]. This research was set out to find a means other than genetic arming, to broaden the spectrum of tumors that OV's could infect and destroy. Since HDACs have been implicated in regulating the IFN response in cell lines [13, 22, 28, 30, 42, 43, 45, 64, 69], the hypothesis was that HDIs could complement OV's and facilitate the infection and killing of tumors that had an impaired but not completely deficient cellular innate antiviral response. Our study design was to use relatively low multiplicities of infection (MOI) *in vitro*, to closely approximate the situation *in vivo*; furthermore we expected the effects of HDIs on virus spread to be most pronounced at low MOI. Using a variety of cell lines established from human malignancies, we

demonstrated that three HDIs – MS-275, SAHA and PXD-101, currently in clinical development - enhance replication and spread of three different oncolytic virus platforms (VSV, vaccinia and SFV). As demonstrated in the PC3 model, the effect of the combination on cell death was synergistic and increased at higher effective doses of combined treatment, supporting a clinically relevant interaction between HDIs and VSVΔ51.

This research also presents evidence that the combination approach is effective in human tumor xenografts in nude mice, in transplantable syngeneic tumor models and in a spontaneous mouse model of ovarian cancer. In addition to the direct virus-mediated induction of tumor cell death, oncolysis can also be mediated by indirect mechanisms triggering apoptosis, through the release of inflammatory cytokines and mediators from infected and dying tumor cells. In vivo, the effective dose of an OV that reaches a tumor is further compromised by the limitations imposed by tumor architecture and micro-environment. However, despite poor virus delivery, a significant portion of cells within OV-treated tumors undergo apoptosis. Our lab has recently demonstrated that loss of blood flow to the interior of the tumor causes massive cellular apoptosis. Although the mechanisms remain to be defined, the absence of vascular perfusion within infected tumors was induced by neutrophil recruitment to the tumor bed [10]. The current studies reinforce the involvement of vascular shutdown as an important mechanism of tumor cell killing, and further illustrate that the combination of OV and HDI augments ‘vascular shutdown’ in the tumor bed.

Importantly, the combination of VSVΔ51 and HDIs enhances tumor killing but does not significantly increase VSVΔ51 replication in normal human or mouse tissues, even in immunocompromised mouse model. While the exact basis for this exquisite selectivity remains to be determined, the results are consistent with the idea that HDIs blunt the cellular IFN response in tumor cells. In combination treated PC3 cells, key antiviral genes downstream of *IRF3*, such as

IRF7, *ISG56* and *MXA* were expressed at low levels and were poorly inducible by virus infection. These results and those of others are consistent with the effects of HDIs occurring at the level of gene transcription [13, 22, 28, 30, 42, 43, 45, 64, 69], but clearly HDAC activity is found in other cellular compartments besides the nucleus and has been implicated in the cellular response to a variety of stresses [57, 68]. While this research has demonstrated a key role for HDIs in dampening IFN activity in tumor cells, the existence of additional stress responses affected by HDI that impact on virus replication cannot be excluded.

A potential clinical advantage of this combination therapy approach was highlighted by the observation that continuous systemic administration of HDI was required to maintain robust virus replication within the tumor; therefore, it may thus be possible to regulate the magnitude of OV therapy by withdrawing or applying HDI. These results illustrate the general utility of HDIs as chemical switches to regulate cellular innate antiviral responses and to provide controlled growth of therapeutic viruses within malignancies. This strategy could be particularly advantageous when the OV therapeutic harbors a gene that facilitates imaging of the infection, or if the malignancy is located in an area (eg brain) where tumor swelling - as a consequence of virus infection - could lead to unwanted complications. HDI induced sensitization of tumor cells to viral oncolysis was not restricted to VSVA51, as both Semliki Forest virus and vaccinia virus also displayed increased oncolytic activity in the presence of HDIs. This observation is immediately relevant as vaccinia virus has entered into clinical trials [31] and the effects of HDIs on the magnitude of oncolysis may be applicable to a wide spectrum of OVs under development. Thus, the diverse and safe applicability of HDI plus oncolytic virus combination therapy will make it a valuable addition to the armamentarium used to fight cancer and should facilitate translation towards clinical application.

Although this research has identified the ability of HDIs to enhance the replication and spread of OV_s within malignancies, numerous questions still need to be investigated in order to obtain a better understanding of mechanisms and limitations of this combination therapeutic approach. As noted from the presented data, HDI enhancement of OV_s correlated with dampening of cellular IFN α response in the tested cancer cell lines. However, SW620 cells didn't fit this paradigm. These cells were sensitized to VSV Δ 51 infection, both *in vitro* and *in vivo*, after HDI treatment despite the fact that they were non-responsive and non-productive to IFN α (**Figure 5, Appendix I**). This model raises the possibility of other antiviral cytokines that HDIs might modulate to augment virus replication. **Appendix II** outlines a preliminary experiment designed to test for this hypothesis. In this experiment cancer cells were treated with the replication incompetent G-Less VSV Δ 51. This mutant virus lacks the genome encoding for the G-protein and therefore was grown in a G-complementing cell line, which allows the virus to perform only one round of replication. Thus, G-Less VSV Δ 51 can trigger an antiviral response without undergoing multiple rounds of replication. Indeed, the supernatant collected 24 hours after G-less VSV Δ 51 infection was able to protect the IFN α insensitive SW620 cells from infection of the replication competent VSV Δ 51. These results elucidate the possibility that other antiviral cytokines besides IFN α might have been produced by SW620 cells in the supernatants after their challenge with G-Less VSV Δ 51. Further investigations are needed to carefully analyze the cytokine composition of these supernatants and determine how they might be modulated by HDI treatment.

In addition to identifying other potential antiviral cytokines that can be influenced by HDIs, it is also critical to determine which of the different HDAC enzymes are important in modulating such an effect. Careful analysis of the data reveals that the hydroxamate class of HDIs, such as SAHA and PXD101, are relatively more effective in enhancing OV activity compared to the

benzamide HDI, MS-275 (**Figure 5**). These differences in response should be critically analyzed, especially since it is known that benzamides target class I HDAC enzymes, while hydroxamates target classes I, II and IV. Microarray analysis and systemic knockdown of different HDAC enzymes are possible techniques that can be used to identify which of the HDAC enzymes are important targets to achieve the desired enhancement in viral replication post HDI treatment.

Other investigations in our lab are also under way to delineate how HDIs may modulate the tumor microenvironment and possibly influence the host adaptive immune response to further enhance the anti-tumor effects of OV. Preliminary *in vitro* experiments have demonstrated the ability of MS-275 to enhance MHC I expression on the surface of multiple cancer cells (**Appendix III**). This data correlates well with results presented in the literature [9]. Since down regulation of MHC I is a strategy used by cancer cells to evade immune surveillance, enhancement of MHC I expression by HDI treatment can increase the chances of cytotoxic T-cells to recognize cancer cells as foreign entities and seek them out for destruction. MS-275 has also shown the ability to modulate the adaptive immune response *in vivo* by dampening anti-VSV antibody production post systemic administration of VSV Δ 51 (**Appendix IV**). Harnessing this feature can allow the effective systemic delivery of multiple viral doses to the tumor site while avoiding antibody neutralization. Therefore, achieving the desired *in vivo* anticancer therapeutic effects of HDI_OV combination treatment will greatly depend on dosing regimens and treatment kinetics of each therapeutic agent.

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6. Contributions of Collaborators

This research project has been performed in collaboration with **Dr. John Hiscott Laboratory**.

Dr. Thi Lien Nguyen generated the data presented in:

Figure 1 a-c

Figure 3

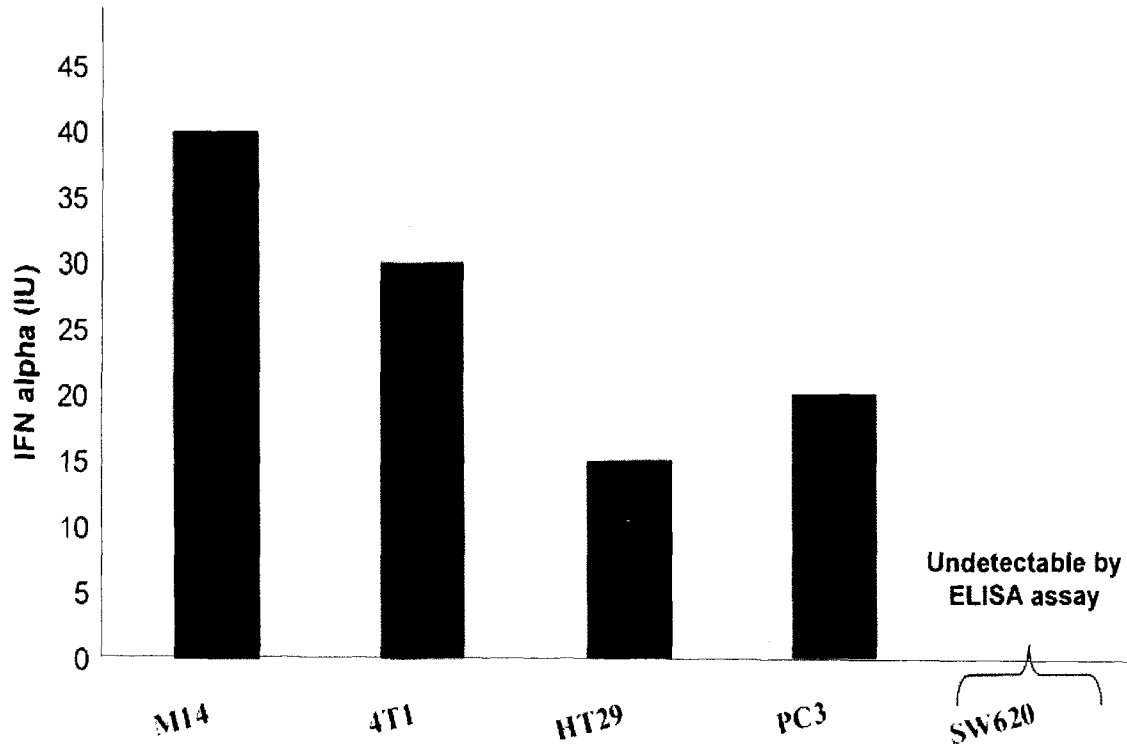
Figure 8 a-c

Dr. Jean- Simon Diallo generated data presented in Figure 2

Theresa Falls – performed *in vivo* intravenous and subcutaneous injections

Valerie Snoulten – assisted with treatment of transgenic ovarian tumor model presented in figure 9b

**IFN Alpha levels produced 24 hrs Post VSVΔ51
Inoculation at MOI 1.0 - ELISA**

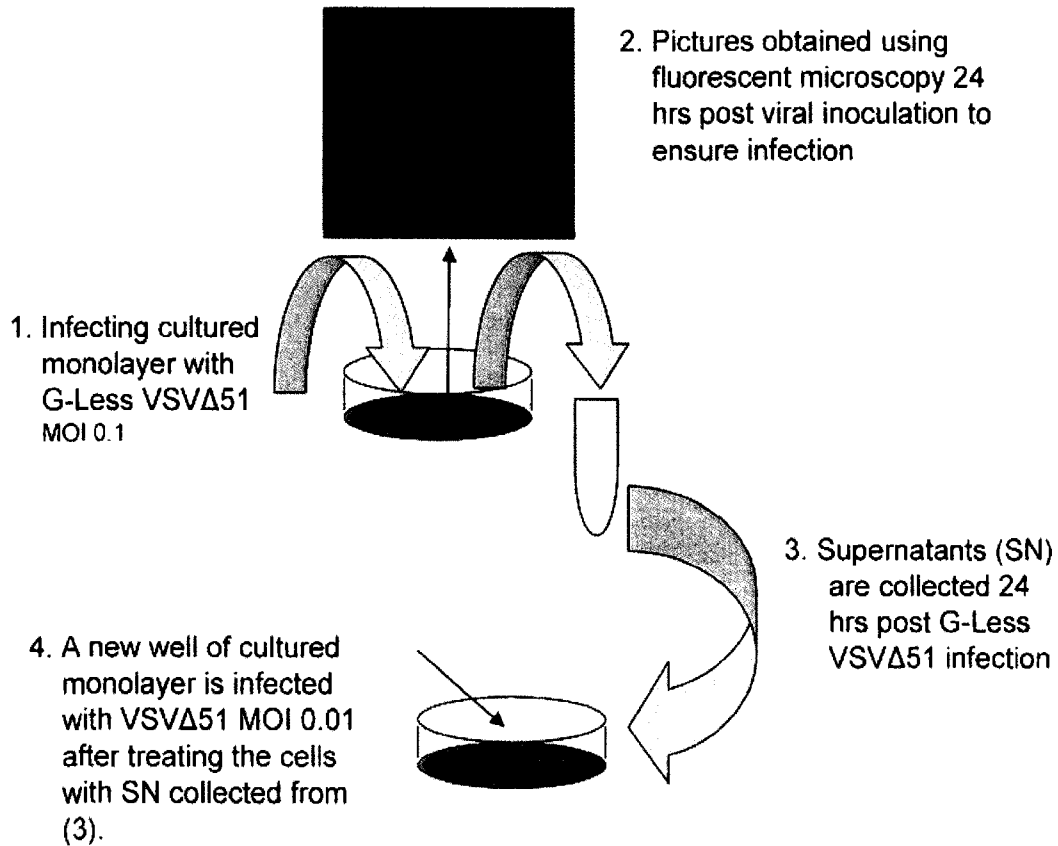


Appendix I

Appendix I - There are notable differences of IFN α levels produced by various cancer cell lines after infection with VSV Δ 51. Levels have been detected using ELISA assay.

Each cell line was infected with VSV Δ 51 at MOI 1.0. IFN α levels in the supernatants were measured using ELISA at 24 hrs post infection.

A.



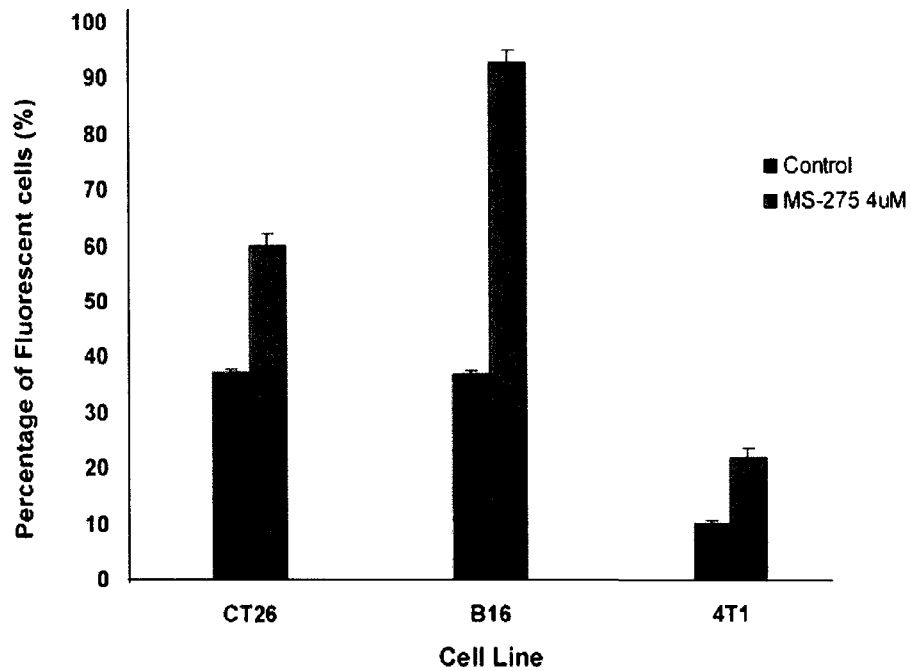
B.

	G-Less SN	VSV Δ 51	VSV Δ 51 + G-Less SN
M14 (Melanoma)			
4T1 (Breast)			
HT29 (Colon)			
PC3 (Prostate)			
SW620 (Colon)			

Appendix II - Supernatants collected from cancer cell lines after challenging them with replication deficient G-Less VSVΔ51-GFP strain is able to protect the same cancer cell lines from infection of the replication competent VSVΔ51 strain

G-Less VSVΔ51-GFP is a replication incompetent strain because it lacks the genetic material encoding for the G protein. However, this virus strain has been pseudotyped with the G protein which allows it to infect once without being able to bud out of the infected cell. Thus, supernatants collected after G-Less VSV infection don't contain virus infecting particles. **A)** An outline of the protocol used to collect supernatants after G-less infection. This protocol was used for each of the cancer cell lines presented in (b). **B)** Each cancer cell line has been treated with its respective supernatant collected from (A) before infecting it with the replication competent VSVΔ51 MOI 0.1. Infection was detected by fluorescent microscopy at 24 hrs post VSVΔ51 inoculation.

Flow Analysis - MHC I Expression post MS-275 Treatment

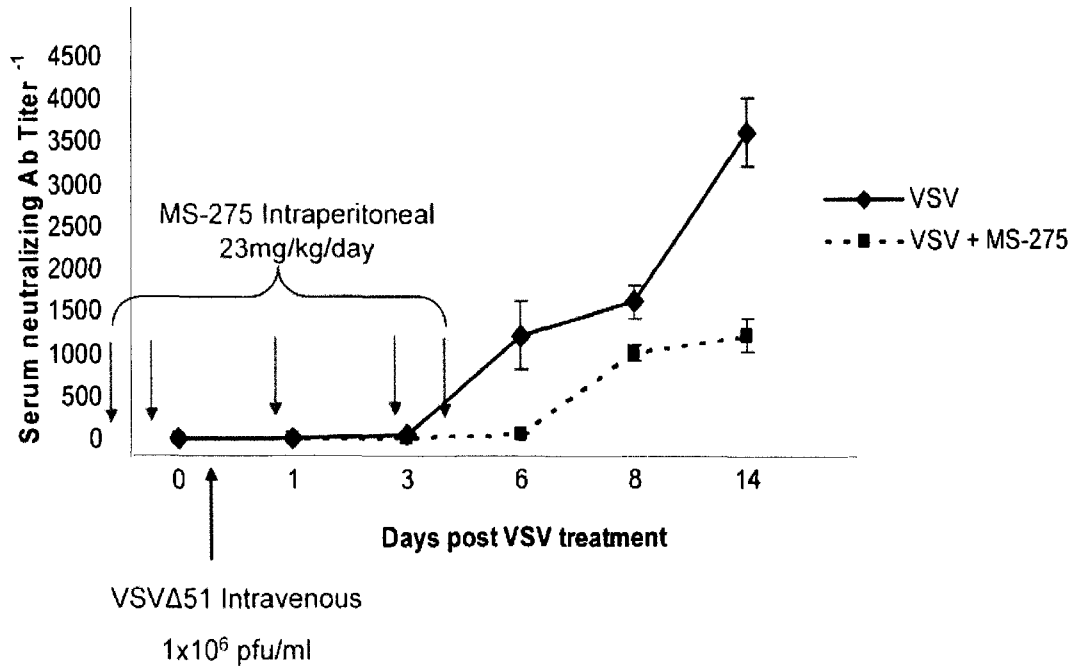


Appendix III

Appendix III – HDI treatment enhances MHC I expression on the surface of cancer cells.

Each of the CT26 (colon), B16 (melanoma) and 4T1 (Breast) mouse cancer cell lines were treated with MS-275 (4uM) for 24 hrs. Cells were then trypsinized and treated with 5ul of fluorescent anti-MHC I antibody per 1 ml of cell suspension for 1 hr. Flow analysis was then used to measure the enhancement in fluorescence.

Serum titers of anti-VSV antibodies post VSV and MS-275 treatment



Appendix IV

Appendix IV –MS-275 dampens antibody production against VSVΔ51 after its systemic administration to an immunocompetent mouse model.

Balb/C immunocompetent mice were treated with MS-275 and VSVΔ51 as indicated in the figure. Each group had a total of 3 mice. Blood samples were obtained from the saphenous veins of each mouse at the specified time points and processed as indicated in the materials and methods section.

7. Curriculum Vitae

Personal Details

Name: Hesham A. Abdelbary

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Education

2006 – 2008 Masters of Science in Biochemistry, University of Ottawa, Canada
Supervisor: Dr. John Bell, Ottawa Health Research Institute

2006 – 2008 Clinical Investigator Program, The Royal College of Physicians
and Surgeons in Canada
Supervisors: Dr. Joel Werier, Ottawa General Hospital

2003 – 2006 Orthopedic Surgical Residency Training, University of Ottawa
PGY1, PGY2 and PGY3

2000 – 2003 Faculty of Medicine, McMaster University

1998 – 2000 Faculty of Pharmacy, University of Toronto

1996 - 1998 Undergraduate Human Biology Program, University of Toronto

Abstract Presentations

June 2008 Canadian Orthopedic Association – Quebec City
“Oncolytic viral therapy: A novel biotherapeutic approach in treating
Ewing’s Sarcoma”

Jan. 2008 Canadian Prostate Cancer Research Initiative – Vancouver
“The role of epigenetic modulators in enhancing oncolytic virus therapy”

Nov. 2007 In Vivo Barriers to Gene Therapy – Cold Spring Harbor Laboratories, NY
“Histone Deacetylase Inhibitors and Oncolytic viruses: A combination
tumor targeting therapy”

Nov. 2007 Connective Tissue Oncology Society 13th Annual Meeting - Seattle
“Histone Deacetylase Inhibitors and Oncolytic viruses: A combination
tumor targeting therapy”

Awards

2008	Department of Surgery Award
2007	Hans K. Uthhoff Orthopedic Research Award
2006 – 2008	University of Ottawa Graduate School Admission Scholarship
2006	Physician Services Incorporated Foundation research award

Publications

Thi Lien-Anh Nguyen*, Hesham Abdelbary*, Meztli Arguello, Caroline Breitbach, Simon Leveille, Jean-Simon Diallo, Amber Yasmeeen, Tarek Bismar, David Kirn, Theresa Falls, Valerie Snoultten, Barbara Vanderhyden, Joel Werier, Harold Atkins, John Hiscott and John Bell. **Chemical targeting of the innate antiviral response by histone deacetylase inhibitors renders refractory cancers sensitive to viral oncolysis.** Proceedings of the National Academy of Sciences USA (PNAS) –Vol 105, no. 39, September 30, 2008.

Hesham Abdelbary, John Bell, Harold Atkins. **Harnessing nature's biodiversity to kill cancer: The renaissance of virus biotherapeutics.** Canadian Medical Association Journal (CMAJ) – In Press

Bednar D., Toorani B., Denkers M., Abdelbary H. (2004), **Assessment of stability of the cervical spine in blunt trauma patients: review of the literature, with presentation and preliminary results of a modified traction test protocol.** Canadian Journal of Surgery Oct. 2004 volume 47 Number 5: 338-342.

Research Interests

- Exploring the role of oncolytic virus therapy in enhancing long term survival of high grade sarcoma patients post limb salvage procedures.
- The role of functional imaging modalities such as PET and SPECT scans in improving operative and non-operative management of sarcoma patients.

Interests

Soccer
Tennis
Travel
History & Astronomy