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Enhancement of Vaccinia Virus Based Oncolysis with Histone Deacetylase Inhibitors

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the School of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the degree of

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ABSTRACT

Histone deacetylase inhibitors (HDI) dampen cellular immune response by decreasing interferon production and have been shown to increase the replication of Vesicular Stomatitis Virus and HSV. As attenuated tumour-selective oncolytic vaccinia viruses (VV) are already undergoing clinical evaluation, the goal of this study is to determine whether HDI can also enhance the potency of these poxviruses in infection-resistant cancer cell lines. Multiple HDIs were tested and Trichostatin A (TSA) was found to potently enhance the spread and replication of a tumour selective VV in several infection-resistant cancer cell lines. TSA significantly decreased the number of lung metastases in a syngeneic B16F10LacZ lung metastasis model yet TSA treatment did not increase the replication of VV in normal tissues. We conclude that TSA can selectively and effectively enhance the replication and spread of oncolytic vaccinia virus in cancer cells.

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ABBREVIATIONS

bFGF	Basic Fibroblast Growth Factor
CDKN1A	Cyclin-Dependent Kinase Inhibitor 1A
CEV	Cell-Associated Enveloped Virus
CTCL	Cutaneous T-Cell Lymphoma
DMEM	Dulbecco's Modified Eagles Medium
EEV	Extracellular Enveloped Virus
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
GFP	Green Fluorescent Protein
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDI	Histone Deacetylase Inhibitor
HIF1α	Hypoxia-Inducible Factor 1-α
HSV	Herpes Simplex Virus
IEV	Intracellular Enveloped Virus
IFN	Interferon
IMV	Intracellular Mature Virus
IRF3	Interferon Regulatory Factor 3
ISG	Interferon Stimulated Genes
ISGF3	Interferon Stimulated Gene Factor 3
IV	Immature Virus
Jak/STAT	Janus Kinase-Signal Transducer and Activator of Transcription

MOI	Multiplicity Of Infection
NF-κB	Nuclear Factor Kappa-B
OV	Oncolytic Virus
PFU	Plaque Forming Unit
SAHA	Suberoylanilide Hydroxamic Acid
SBHA	Suberoyl Bis-Hydroxamic Acid
TK	Thymidine Kinase
TTP	Thymidine Triphosphate
TRAIL	Tumour Necrosis Factor Related Apoptosis Induced Ligand
TSA	Trichostatin A
VEGF	Vascular Endothelial Growth Factor
VGF	Vaccinia Growth Factor
VSV	Vesicular Stomatitis Virus
VV	Vaccinia Virus
XIAP	X-Linked Inhibitor of Apoptosis Protein

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1. INTRODUCTION

1.1 General Introduction

Replicating oncolytic viruses are powerful biological tumour killing machines that exploit cellular defects inherent in cancer cells and can be engineered to specifically target and destroy tumours. As an anti-cancer treatment they encompass many of the positive aspects of existing cancer therapies while adding numerous benefits such as targeted tumour cell lysis and the ability to stimulate the immune system against the tumour cell. Vaccinia virus is a major player in the field of oncolytic viruses as it naturally has many properties that make up an ideal oncolytic virus. Attenuating oncolytic viruses by removing virulence genes increases the safety profile but can diminish the cancer cell killing ability of the virus. Combining oncolytic viruses with other anti-cancer agents for the purpose of further sensitizing cancer cells to virus infection can help to increase the efficacy of attenuated oncolytic viruses. Increasing the oncolytic effect of viruses that have been rendered safer and more specific to cancer cells would result in a more potent and targeted therapeutic agent.

1.2 Oncolytic Viruses

Oncolytic viruses (OV) are viruses that specifically target and kill cancer cells and are increasingly explored as a targeted alternative to standard cancer therapy. These viruses naturally have or can be engineered to have anti-cancer activities and are effective at destroying tumours by directly killing tumour cells, recruiting immune cells to the tumour and by their anti-vascular activity (21, 37). Using viruses to fight cancer has many advantages over existing therapeutics. First they can be attenuated by removing virulence

genes and genetically modified to better target cancer cells. Secondly many OVs have the capacity to accommodate large genes and can deliver a toxic payload to hasten the killing of the cancer cell (21, 37). A third benefit is the possibility for systemic delivery which enables distant metastases to be reached. Because viruses are self-replicating, they have the unique ability to subsequently amplify the therapeutic dose in the tumour once the virus has reached the cancer cells. Lastly many OVs have immune modulating genes and the ability to stimulate the immune system against tumour cells (4, 21, 37). OVs can exploit the very same defects in cancer cells that give the cell its tumourgenic properties. They can naturally target or be engineered to take advantage of various tumour cell characteristics such as: antigens over-expressed on the tumour cell surface, signaling pathways constitutively activated or tumour-specific mutations in the cell's anti-viral defence pathway (37). While many wild-type virus strains can be cytopathic in normal tissues, tumour selectivity can be improved using genetically attenuated virions through the removal of important virulence genes. Once certain virulence genes have been removed, there is a reduced ability to replicate in normal cells that have functional defence mechanisms. Cancer cells have defects in many anti-viral pathways and aberrant cell signaling which enables OVs to replicate to a greater extent in the tumours. Tumour cells can complement the genetically engineered viral mutations and remain uniquely sensitive to OV infection.

Despite the many advantages of OV therapy, there exist barriers to the use of OVs in the clinic. Systemic delivery can be challenging when the innate immune system recognizes the virus as a foreign pathogen and marks it for destruction before the targeted tumour site is reached. Tumour complexity and heterogeneity can become a challenge for the specific targeting of tumour cell receptors by OVs. In a solid tumour model, most

viruses are unable to completely clear the entire tumour mass as a single agent therapeutic (60). Studies show that broad distribution of the virus throughout the entire tumour may be more important to successful tumour destruction than high local concentrations of the virus (60). Importantly OVs are showing promising results in clinical trials (37), however due to several barriers that limit efficacy the possibility of combination treatments is warranted.

1.3 Vaccinia Virus

1.3.1 Vaccinia Overview

Vaccinia virus (VV) is a large complex enveloped virus with a linear double stranded DNA genome. There are approximately 200 genes along the 200 kilobase pairs of DNA (15). The virion shape is of a rounded rectangle with dimensions of 350 x 270 nm (11). It is a member of the *Orthopoxvirus* genus and the *Poxviridae* family. Vaccinia was the virus used in the successful worldwide vaccination campaign to eradicate smallpox (13) and thus has been extensively studied for its use in humans. Several key features of VV make it an excellent candidate for oncolytic virus therapy (21). VV replicates solely in the cytoplasm and never enters the nucleus of the host cell. This is an important safety consideration because this cytoplasmic replication eliminates the possibility for integration of or recombination with host DNA. Vaccinia is capable of infecting a wide range of host cells and enters cells via membrane fusion without the requirement of a specific cell surface receptor (29). A greater variety of cancer cells can be targeted by VV compared to an OV that requires a specific tumour cell antigen or marker to enter a cell. Though VV can enter a wide variety of cells, it has natural tumour tropism which is partially attributed to the leaky vasculature in and around tumour tissues (27, 40). Another useful trait of VV is that

due to its size, VV can accommodate large amounts of foreign DNA. The virus can consequently be genetically engineered to carry toxic payloads or immune stimulatory molecules directly to the cancer cells (22, 47). Because of these numerous features, VV is being explored and tested as a clinical agent in numerous different models and is showing tumour responses in several trials (21). A Phase I trial of an oncolytic vaccinia virus JX-594 demonstrated acceptable safety and promising anti-cancer activity in patients with advanced liver tumours (38). Other important benefits of VV are its efficient replication, as VV releases the first infectious particles as early as 8 hours after entry into the cell. The virus can then lyse the infected cell releasing thousands of newly synthesized virions after only 48-72h (21). Vaccinia has another feature that enables long-range spreading and helps to increases its oncolytic efficiency. VV produces three forms of infectious particles throughout morphogenesis that have structural and antigenic differences. The extracellular enveloped virus (EEV) form has an additional host-derived membrane which helps the virus evade neutralizing antibodies and complement when spreading to distant tumour sites (41, 48). VV has multiple means for reaching and quickly destroying tumour cells yet does not recombine with host DNA and has natural tumour tropism. This makes VV an excellent candidate for OV therapy and it is showing promising anti-cancer activity in multiple clinical trials (37). Though vaccinia is effective in many models, some cancer cells remain resistant to vaccinia infection and would benefit from treatment with viral sensitizers to enhance replication and spread of the virus.

1.3.2 Lifecycle of Vaccinia

Vaccinia has evolved to have a high level of independence from the host cell. It is a large virus containing 200 genes which encode most of the proteins necessary for efficient

replication (6). When the virus enters the cell its external membrane is removed and the virus core is transported into the cytoplasm. Virus proteins interact with microtubules and the virion is transported along the microtubules to a juxtanuclear region that becomes the virus factory from which cellular organelles are mostly excluded (41). Transcription of early mRNA occurs as early as 20min after infection by a DNA-dependent RNA polymerase transcription system already present in core of the virion (2). Approximately half of vaccinia's 200 genes are transcribed early and maximum levels of early gene mRNA are reached 2 hours after infection (2). Early genes code for transcription factors for intermediate genes which subsequently code for transcription factors for late genes (6). Early genes are involved in virus replication and evasion of the host defence, intermediate genes encode regulatory proteins and late genes encode proteins to make up the new virus particle, including proteins needed to initiate early transcription (41). DNA replication takes place in the cytoplasm following transcription. Assembly begins in the virus factories and the first identifiable structures are crescents consisting of lipid and virus encoded proteins. This becomes the non-infectious precursor form: immature virus (IV) (41). The IVs mature and condense into intracellular mature virus (IMV) which is the most abundant form and remains inside the cell until lysis. The IMV travels to the trans-Golgi network and the early endosome for wrapping by intracellular membranes to form the intracellular enveloped virus (IEV) (41). IEVs travel on microtubules to the cell periphery where there is a membrane fusion of the outer envelope with the plasma membrane as the virion travels to the extracellular environment. This particle is termed cell-associated enveloped virus (CEV) and when it is released from the cell surface it becomes the extracellular enveloped virus (41). CEV mediates cell to cell spread while EEV can be released before lysis of the host cell and mediate long-range spreading of infection. IMV and EEV are structurally and

antigenically distinct and each has its own function throughout vaccinia infection. Because the EEV is wrapped by a membrane derived from the host cell, it is capable of evading detection by the immune system and is useful for long-range dissemination of the virus (48).

1.3.3 Tumour Selective Vaccinia Virus Mutant: VVdd

Vaccinia contains close to two hundred genes, some of which are redundant for growth in tumours (21, 27, 46). These can be targeted to attenuate the virus in normal cells while retaining its oncolytic potential. VV mutants exhibiting deletions in the thymidine kinase gene (TK) and/or the vaccinia growth factor gene (VGF) grow selectively in cancer cells and are well advanced in pre-clinical and clinical studies (21, 24, 38, 56). The lack of viral TK and/or VGF is complemented in rapidly dividing cancer cells that express cellular TK or over-express growth factors and/or growth factor receptors (27). Thymidine kinase catalyzes the phosphorylation of thymidine to thymidine 5'monophosphate, an important step in the formation of thymidine triphosphate (TTP) one of the four nucleotides required for DNA synthesis. The viral TK gene is encoded by an early promoter and once translated can quickly begin to increase the pool of precursors for DNA replication. A deletion of the TK gene eliminates the rapid accumulation of DNA precursors necessary for the production of the thousands of new virions. Vaccinia with a TK deletion is restricted to rapidly dividing cells (where there exists a large pool of nucleotides) or cells that endogenously express high levels of cellular TK (27). Since many cancer cells constitutively express TK (19), the ideal environment for a VV with a TK deletion is a quickly dividing cancer cell. Vaccinia growth factor protein is secreted early after infection and binds to the cells epidermal growth factor receptor (EGFR) stimulating cell growth and priming surrounding

cells for vaccinia infection (27, 56). The EGFR pathway is activated in more than 80% of cancers (37, 45) and a VV with a deleted VGF is restricted to growth in cells with an active EGFR pathway (45). The combination of these two deletions termed VVdd makes the virus more specific to actively dividing cancer cells and diminishes the virus' ability to replicate in normal cells (27).

1.3.4 Anti-Viral Interferon Response in Cancer Cells

The host cell's primary defence against invading foreign viruses is the anti-viral type-I Interferon (IFN) response (12, 51, 61-63). Interferons are cytokines that are secreted upon viral infection. They bind to specific cell surface IFN receptors. This initiates a signaling cascade leading to the transcription of many genes. These genes encode proteins involved in establishing an intracellular environment that is unfavorable for virus replication (16) (signaling pathway for the induction of the anti-viral state is described in section 1.4.4). Among other aspects of the innate immune system, the anti-viral response can be a barrier to effective OV delivery and entry into target cancer cells. However a normal cell's ability to protect itself from invading foreign viruses with a robust IFN response is important when systemically delivering OVs.

The interferon pathway is defective in a large proportion of cancer cells (~70-75%) (51, 61, 62). Viruses that are naturally sensitive or engineered to be sensitive to IFN therefore replicate poorly in normal cells which exhibit a robust IFN response yet still replicate well in cancer cells in which this response is abrogated. As with many defects in cancer cells, the extent of the IFN defect can vary extensively. In some tumor cell lines the IFN response is only partially defective and some capacity to launch an anti-viral response is retained, greatly limiting OV effectiveness. Because OVs are often engineered to

replicate in cells with deficient IFN signaling, treatments that could impair or dampen the anti-viral response in cancer cells would augment the efficacy of OVs in these more resistant cell lines and broaden the range of OV sensitive tumours.

1.3.5 Vaccinia Soluble Interferon Receptor B18R

While several genes including TK and VGF are important for viral growth, vaccinia also expresses proteins that inhibit innate anti-viral immune response in order to maximize its spread. The B18R gene encodes a soluble type-I IFN receptor (B18 protein), which allows VV infection to continue even if there is cellular secretion of type-I IFN (1, 9, 53). IFN activation leads to the creation of an intracellular environment that antagonizes viral replication. The B18 protein mimics the host cell type-I IFN receptor and locally blunts the cellular IFN response. Type-I IFNs bind the B18 protein with high affinity. B18 is secreted from infected cells but can also bind to the cell surface to prevent the IFN from reaching its host cell receptors. This accordingly prevents the induction of an anti-viral state and allows infection to continue (1). Akin to the IFN-sensitive mutants of VSV (50), adenovirus (44), and herpes simplex virus (HSV) (17, 31, 39), it has recently been shown that a VV strain in which the B18R gene has been deleted is more sensitive to type I-IFN and more rapidly cleared from normal tissues than the parental strain while remaining active within tumours (22). This is attractive from a clinical standpoint because this B18R-deleted virus is further attenuated in normal cells, yet remains active in cancer cells in which the IFN is disrupted.

1.4 Histone Deacetylase Inhibitors

1.4.1 Histone Deacetylase Inhibitor Overview

Eukaryotic DNA is tightly packaged and condensed in the nucleus into chromatin which consists of DNA wrapped around histone proteins to form repeating units called the nucleosome (20). A nucleosome is made up of two copies of each of the four histone subunits: H2A, H2B, H3 and H4 and DNA is wrapped around this octomer of histone proteins with a fifth subunit H1 serving as a linker between successive nucleosomes (30). Gene expression is a tightly regulated process and can be controlled by remodelling of the chromatin. When chromatin is fully condensed in its most compacted form, DNA is not transcribed; however when chromatin is open, the DNA is unwrapped and transcription can occur. The amino-terminal tails of the histone proteins extend out of the nucleosomes and undergo post-translational epigenetic modifications (30). Acetylation, phosphorylation and methylation are the most common of these epigenetic modifications on the histone tails and occur primarily on histone H3 and H4 (20). Lysine residues are frequently acetylated, bringing a negatively charged acetyl group to partially neutralize the attraction of the negatively charged phosphate group in the DNA to the positively charged lysine (57). Depending on the degree of acetylation, the weaker contacts between the histones and DNA cause the chromatin to adopt a more open conformation which in turn allows transcription factors to gain access to the DNA for gene transcription. Histone deacetylases (HDAC) and histone acetyltransferases (HAT) are enzymes that regulate gene expression by de-acetylating and acetylating histones respectively. This leads to chromatin remodelling and subsequent changes in the expression levels of many genes (42, 55). HDAC and HAT activity must be carefully balanced in the cell and shift in this balance could lead to drastic

changes in the cell's phenotype (30). Abnormal expression and aberrant recruitment of HDACs has been linked to cancer progression and thus HDACs are attractive targets for therapeutic intervention (5, 20, 30). Consequently there has been a lot of effort invested in developing histone deacetylase inhibitors (HDI) as an anti-cancer treatment (5, 18, 30).

Histone deacetylase inhibitors block histone deacetylase action leading to increased acetylation of histones and other proteins (5, 26, 30, 63). Acetylation levels affect the expression of numerous genes and upon acetylation there can be both up and down regulation of transcription. Potentially because HDACs are up-regulated in tumours (5, 30), treatment with HDIs typically leads to tumour cell apoptosis, differentiation and cell-cycle arrest (5, 20, 26, 30). HDIs can be subdivided into six different classes based on their chemical structure: short-chain fatty acids, hydroxamates, benzamides, cyclic tetrapeptides, electrophilic ketones and miscellaneous structues. There is variation among classes regarding efficiency of inhibition and which classes of HDACs become inhibited. For example certain HDIs such as Trichostatin A and Suberoylanilide hydroxamic acid (SAHA) can inhibit most HDACS and others such as tubacin can discriminate between HDACs and have a more specific action (5).

1.4.2 Histone Deacetylase Inhibitor Biological Activities

Histone deacetylase inhibitors can affect tumour cells in various ways. The biological outcome of HDI treatment can be dependent on cell type (transformed versus normal), the cell line or the class of HDI used. Histone deacetylase inhibitors are being used in research generally for anti-cancer treatments because the most profound effects of treatment are seen in cancer cells (20). Importantly cancer cells are sensitive to HDI treatment at concentrations at least ten fold lower than normal cells which creates a good

therapeutic window (5). The most common effects observed from HDI treatment are the induction of cell cycle arrest, cancer cell apoptosis and differentiation. Tumour cell apoptosis is induced by an up-regulation of pro-apoptotic genes involved in the extrinsic death-receptor pathway (FAS and TRAIL) and the intrinsic mitochondrial death pathway (bax and bak) or by a down-regulation of pro-survival genes (XIAP and bcl-2) (5, 28). Treatment with HDI causes down-regulation of cyclins A and D and up-regulation of CDKN1A gene encoding the protein $p21^{WAFI}$ which inhibits cell-cycle progression causing a block at G1 (5, 26). Another biological effect of HDI treatment is their anti-angiogenic properties caused by a downregulation of certain pro-angiogenic genes such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hypoxia-inducible factor-1α (HIF1α) (5, 26). Reducing angiogenesis starves the tumour of nutrients and can help to diminish the continued growth and spreading of tumour cells. The immunogenicity of tumour cells can be augmented by HDIs by upregulating transcription of major histocompatibility complex class I and II proteins and co-stimulatory molecules to alert the immune system (25). Immune cell activity and cytokine production can also be altered by HDI treatment (5, 20). The host cell's anti-viral interferon response can be obstructed by HDI treatment by inhibition of signaling in the interferon stimulated gene pathways (See section 1.4.4 below) (7, 14, 16, 36).

1.4.3 Histone Deacetylase Inhibitor Clinical Trials

Several HDIs are in various stages of clinical trials for use as anti-cancer agents. Vorinostat is the first HDI to be approved by the Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL). There are approximately 80 different clinical trials underway testing a dozen different HDIs in an assortment of cancer models

(18, 54). Hydroxamates such as SAHA (Vorinostat), LBH589 (Panobinostat) and PXD101 (Belinostat) are in phase I and II trials for CTCL, leukemias, ovarian tumours or advanced solid tumours. The benzamide MS-275 is in phase I for refractory solid tumours, lymphoid and leukemias. Valproic Acid is in phase I and II for non-small cell lung cancer, leukemias and cervical cancer (54). As single agent therapies, HDIs are effective against a subset of haematological malignancies such as CTCL, however many of the HDIs in clinical trials are being tested as combination therapies with chemotherapeutics (Nguyen T-A, et al. Article in Press 2010). An HDI mono-therapy has not yet been proven effective in destroying a solid tumour (54) and HDIs could benefit from a combination with potent tumour killing oncolytic viruses.

1.4.4 Histone Deacetylase Inhibitors and the Anti-Viral Interferon Response

It has been previously shown that HDIs blunt the interferon-mediated anti-viral response (7, 14, 16, 30, 36). The anti-viral state is initiated when a foreign viral particle is detected by its specific receptor on the host cell, or with a non-specific host cell receptor (16). Infection can trigger the post-translational activation of transcription factors NF-κB, ATF-2/c-Jun and interferon regulatory factor 3 (IRF3) which are present in the cytoplasm and when activated are translocated into the nucleus (12, 32, 35). This begins the early anti-viral response and type-I IFNs are produced (32). IFNs circulate and bind to specific cell surface cytokine receptors on neighbouring cells leading to the activation of the Janus kinase-signal transducer and activator of transcription (Jak-STAT) signaling cascade and the transcription of the interferon stimulated genes (ISG) important in the late anti-viral response (7, 35). Expression of ISGs is regulated by IFN-stimulated gene factor 3 (ISGF3) which is made up of STAT1, STAT2 and IRF9. An essential co-activator of ISGF3 is

HDACs (35). Given that HDAC activity is required for the induction of the ISGs, production of IFNs is directly affected by HDAC activity (34). Since altered expression of HDAC has been linked to tumour onset and progression, HDI action is most effective and specific in cancer cells (30). The use of HDIs can dampen the cell's anti-viral interferon response thus rendering cells more susceptible to viral infection.

Interferons are important for protecting cells from harmful viruses and pathogens. Normal non-transformed cells are capable of carrying out the innate immune cascade resulting in the creation of the anti-viral state in an effort to fight off viral replication and infection. Conversely many cancer cells have defects in the interferon response pathway. These defects are heterogeneous in cancer cells where some cells have completely lost the ability to respond to IFN and others have retained some, albeit reduced, ability to respond to IFN (32). This trait, common to cancer cells, is exploited by many oncolytic viruses that are engineered to or are able to naturally replicate only in cells where the IFN response is abrogated (50). Because of the variability of the IFN pathway defect in certain cancer cells, OV therapy could benefit from reduced IFN responses in cancer cells. If HDIs could dampen the IFN response specifically in cancer cells, rendering them more permissive to virus infection, even the IFN-sensitive virus strains, such as vaccinia carrying the B18R deletion for example, could effectively replicate in these cells while retaining their inability to spread in normal tissues. Indeed we have recently demonstrated that HDIs can be used to enhance the replication and spread of VSVΔ51 in resistant tumours but not normal tissues (33) . We also observed that in addition to VSVΔ51, HDIs could enhance the spread of a TK/VGF-deleted VV strain *in vitro* (VVdd) (33).

1.5 RATIONALE

Combining anti-cancer agents with oncolytic viruses can be an effective way of enhancing oncolysis and better targeting to cancer cells. In the present study, the goal was to assess whether HDIs could be used to enhance the efficacy of VVdd and other attenuated VV strains *in vitro* and *in vivo*, including IFN-sensitive B18R-deleted strains. Any attenuation of an OV increases its safety profile as it would be less able to replicate in normal tissues. However cancer cell killing must be retained to preserve the efficacy of this therapy. Combining OVs with HDIs to enhance the virus preferentially in cancer cells could lead to a more potent but still targeted tumour killing treatment regimen.

1.6 HYPOTHESIS

Histone deacetylase inhibitors can be used to enhance oncolytic vaccinia virus replication and spread in infection resistant cancer cells *in vitro* and *in vivo*.

1.7 OBJECTIVES

1) Determine if HDIs can enhance vaccinia virus replication and spread and determine which HDI is the most potent enhancer
2) Test cell lines *in vitro* for enhanced virus production and cancer cell killing
3) Test *in vivo* animal models for efficacy and safety
4) Test the interferon sensitive VVdd with B18R deletion for HDI enhancement

2. MATERIALS AND METHODS

2.1 Cells and Viruses

The cell lines: 4T1 murine breast cancer cells, HCT116 human colorectal cancer, MCF7 human breast cancer cells, OVCAR4 human ovarian cancer, 786O human renal cell carcinoma, MRC5 secondary human lung fibroblast and MEF mouse embryonic fibroblasts were obtained from ATCC. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% 3:1 calf serum: fetal bovine serum and grown at 37°C with 5% CO_2. B16F10-LacZ murine melanoma cells were obtained from Dr. Ann Chambers at the LRCP, London Ontario and maintained in αMEM supplemented with 10% 3:1 calf serum: fetal bovine serum. GM38 normal human fibroblasts were kindly provided by Dr. Bruce McKay (Ottawa Hospital Research Institute, Ottawa, ON) and maintained in DMEM supplemented with 15% 3:1 calf serum: fetal bovine serum. Experiments done by Stephen Thorne and Baocheng Huang at the University of Pittsburgh (Hillman Cancer Center, Pittsburgh, PA) for Figure 11 were done using human cell lines MRC-5, UCI-101 and HeLa. VVdd was derived from the wild type strain Western Reserve with a deletion of the genes thymidine kinase and vaccinia growth factor (27). Green fluorescent protein (GFP) was inserted at the TK locus. Virus was propagated in U2OS cells and purified by centrifuge at 11.5K for 90min at 4 degrees Celcius on a 36% sucrose cushion. VVddB18R- was made by further deleting the B18R gene from the VVdd strain by Dr. Fabrice LeBoeuf. The wild type Western Reserve (WR) and WR B18R-TK-Luc+ were also used by Stephen Thorne and Baocheng Huang from the University of Pittsburgh in Figure 11.

2.2 Fluorescence Microscopy and Fluorescence Quantification

A fluorescent microscope (Zeiss Axiovert S 100) was used to photograph the cells in both phase contrast and fluorescent conditions. Cells infected by virus encoding the GFP transgene can be visualized with a fluorescence microscope. Infected cells with viral encoded green fluorescent protein expression appear green under the fluorescent microscope. Images were quantified for green fluorescent positive pixels using the image analysis software Image J (NIH).

2.3 In Vitro Assays and Cell Staining

The HDI screening was done in 96-well plates with 20,000 cells per well. Cells were plated and 24 hours later were pre-treated for 3 hours with indicated HDI. The drugs: Suberoyl Bis-Hydroxamic Acid (SBHA) from Enzo Life Sciences International Inc. Plymouth Meeting, PA, USA, Trichostatin A (TSA), Scriptaid, M344, and CHAHA were purchased from Sigma; Oxamflatin and Apicidin from Alexis Biochemicals, Plymouth Meeting, PA, USA, MS-275 from Selleck Chemicals, Houston, TX, USA and Suberoylanilide Hydroxamic Acid (SAHA) from Exclusive Chemistry, Obninsk, Russia. The drugs were added to wells at indicated concentrations and cells were infected with VV at a multiplicity of infection (MOI) of 0.1 pfu/cell. Images spanning well surface were quantified as described above. For further TSA testing *in vitro* cells were plated in 12 well plates with 2.5 X 10^5 cells per well. Once approximately 90-95% confluence was reached after 24 hours, the cells were pre-treated with TSA (0.0375μM) or DMSO as the drug vehicle control. After 3 hours of pre-treatment, the virus was added at the indicated MOI. Fluorescence images were taken after 72 hours. Cells were collected after 72 hours and frozen at -80°C for titration on U2OS cells. In other wells the cells were stained by first

rinsing each well with PBS, then fixing the cells for 10 minutes using 3:1 ratio of methanol to acetic acid. After the cells were fixed, they were stained with Coomassie Blue (0.4g Brilliant Blue from Sigma in 280ml ddH20, 80ml methanol and 40ml acetic acid) to visualize viral plaques. Cell lines GM38, MRC5, HCT116, MCF7, 786O and OVCAR4 were pre-treated for 3hrs with TSA and then treated with 200 IU/ml of IFN (Intron A from Schering, IFN-alpha 2b recombinant) overnight (16hrs) and then infected with VVdd at indicated MOIs. For combination index 20,000 4T1 cells were plated in 96-well plates. Cells were treated with serial dilutions of a fixed ratio combination mixture of VVdd and TSA (1562 PFU: 1μM VVdd:TSA). Cytotoxicity was assessed using 5 μL per well of alamar blue reagent after 96h. Combination indices (CI) were calculated according to the method of Chou and Talalay (8) using Calcusyn (Biosoft). Plots represent the algebraic estimate of the CI in function of the fraction of cells affected (Fa). Error bars indicate the estimated standard error.

2.4 Titration of virus samples

Each sample (cells and supernatants) was collected from the sample well and cells were lysed by freezing and thawing (-80°C to 37°C) three times. Samples were diluted serially by a factor of 10 and then 500μL of each dilution were put on confluent U2OS cells in a 12 well plate (5 X 10^5 cells per well plated 24 hours prior). The samples are placed in an incubator for 2 hours at 37°C. After the 2 hour incubation, the virus sample was removed from the U2OS cells and an 1mL of an overlay solution was put on the cells (1:1 ratio of 3% CMC : 2XDMEM + 20% FBS). The plates were placed in the incubator at 37°C for 72 hours. After 72 hours, the overlay was removed and each well was stained with Commassie Blue to visualize the plaques (as described in section 2.3).

2.5 Western Blot Analysis

4T1 cells were plated in 10cm dishes and treated with HDI at indicated concentrations. The following day cells were lysed with RIPA lysis buffer containing protease inhibitor (Sigma, P2714). Equal amounts of proteins (50μg of protein per sample quantified using Biomate 3 Spectrophotometer from Thermo Scientific) collected from samples were separated on a SDS-polyacrylamide gel at 15% acrylamide by electrophoresis. The fractionated proteins were transferred from the gell to the nitrocellulose membrane and detected by probing with the antibody Anti-hyperacetylated Histone H4 (Penta, 06-946) diluted 1:5000 in 5% milk. Actin was used as the loading control and was detected by mouse anti-human actin antibody (Sigma) diluted 1:10,000.

2.6 Lung metastasis model

B16F10-LacZ cells were injected intravenously into female C57BL/6 mice from Charles River Laboratories (Wilmington, MA). Each mouse was given 3 X 10^5 cells into the tail vein on day 0. On days 1-4 mice were given intra-peritoneal injections of TSA (Sigma) at a dose of 0.05mg/mouse. On days 1 and 3 mice were administered 1 X 10^7 pfu of VVdd intravenously through the tail vein. On day 14 mice were sacrificed and lungs were dissected. Lung tumours were stained with the substrate X-gal and each metastasis was counted (23, 49).

2.7 Biodistribution

Balb/C mice were pre-treated with 0.05mg of TSA per mouse or a PBS control by intra-peritoneal injections on day 0. Mice were given the same dose of TSA or control each day for days 0 through 3. Mice were given 1 X 10^8 pfu of VVdd-luciferase intravenously into

the tail vein on day 1 after a 3 hour pre-treatment with TSA. One mouse from each treatment condition was sacrificed at various timepoints: 3 hours after virus injection and on day 1, 4, 7 and 10. The following organs were collected and virus infection was quantified in: lymph nodes (brachial and inguinal), ovaries, spleen, kidney, liver, lungs, heart and brain. Organs were homogenized in PBS using a tissue homogenizer (IKA T8.01 Netzgerat) and the viral titer was measured using the plaque assay on U2OS cells (as described in section 2.4).

2.8 Colon Cancer Survival Model

This work was done by Stephen Thorne and Baocheng Huang at the University of Pittsburgh. Athymic nu-/nu- mice were implanted subcutaneously with HCT-116 cells ($5x10^6$ cells/ mouse). Once palpable tumours had formed ($50\text{-}100mm^3$), mice were treated with either (i) intraperitoneal PBS; (ii) intraperitoneal TSA (6μg/mouse) on days -1, 0 and 2; (iii) intravenous injection of WR B18R- TK- Luc+ ($1x10^8$ pfu/mouse) on day 0; or (iv) both TSA and WR B18R- TK- Luc+ (n=10 mice/group). Subsequent tumour burden was followed by caliper measurement and mice sacrificed when their tumours reached $1400mm^3$.

3. RESULTS

3.1 TSA is a potent enhancer of vaccinia virus spread.

The question of which HDI was most effective at enhancing VV spread *in vitro* was addressed. A panel of HDIs was screened on 4T1 murine breast cancer cells by pre-treating the cells with the HDI over a range of concentrations then challenging with a GFP-expressing VVdd using a multiplicity of infection (MOI) of 0.1 plaque forming units (pfu)/cell at which 4T1 cells are fairly resistant to VV infection. GFP positive cells indicate active virus replication and fluorescence microscopy images were taken spanning the entire well surface after 120h of incubation. Images were quantified for GFP positive pixels using image analysis software (Image J, NIH). Each condition was related to the percent GFP-positive area in the vehicle control. Table 1 shows HDIs are able to enhance the spread of VVdd to varying extents. Overall it was found that SBHA, M344 and Trichostatin A (TSA) elicited the greatest response, increasing GFP-positive area over 3 fold (Table 1). At the most effective concentrations tested, TSA also elicited the least toxicity of the top three drugs (4%, 7% and 15% cell death respectively for TSA, M344 and SBHA). Western blot analysis was used to confirm that all the HDIs were able to affect histone acetylation in treated cells at the most effective concentration. The extent of histone H4 acetylation corresponded with the ability of the HDI to enhance VVdd growth (Table 1).

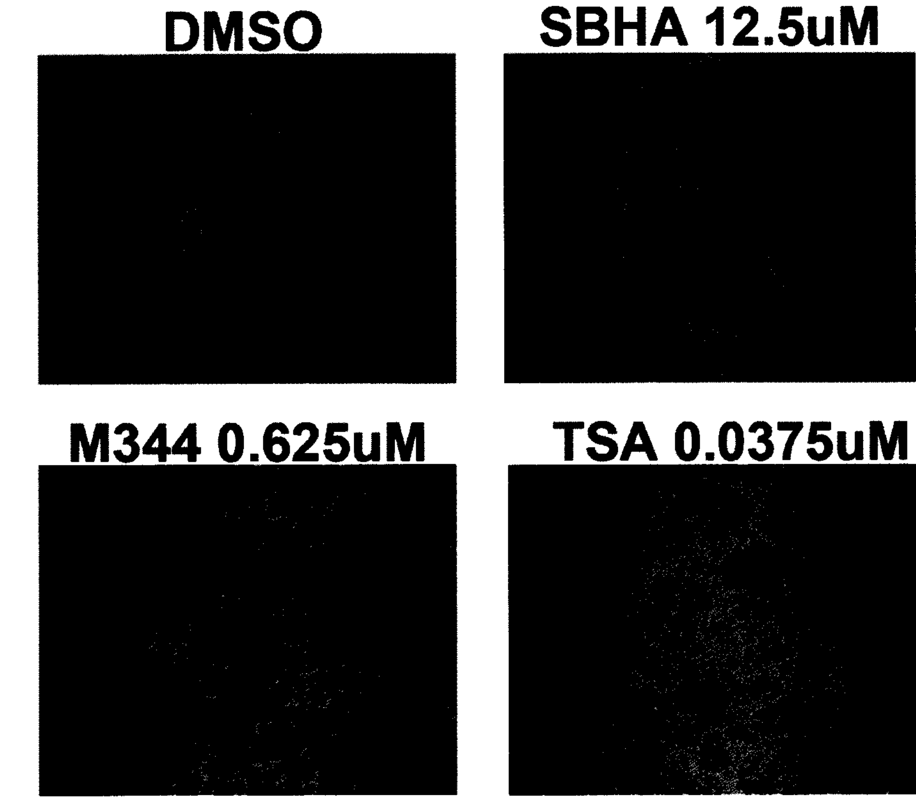
To further investigate the three compounds that produced a greater than 3 fold increase in spread a similar experiment was repeated testing a wider range of concentrations. Fluorescence microscopy images were taken (Figure 1a) and quantified

Table 1. TSA is a potent enhancer of vaccinia virus spread. 4T1 cells were plated in 96-well plates then pre-treated with a concentration gradient of the indicated drugs. DMSO was used as a control. Following pre-treatment with drugs, cells were challenged with VVdd-GFP at an MOI of 0.1. After 120h incubation period, fluorescence pictures were taken of each well, spanning the entire well-surface. Green fluorescence, indicating vaccinia spread, was measured using an image analysis software (Image J, NIH) and reported as a fold change in GFP-positive comparison to control (average of triplicate). + indicates increase in spread < 2-fold, ++ indicates between 2 and 3-fold increase, +++ indicates >3-fold increase. TSA was the most active compound at the lowest dose tested. 10cm plates of confluent 4T1 cells were treated with indicated HDI and samples were probed for hyper-acetylated histone H4 by Western Blot and reported as level of acetylated H4 relative to the untreated control.

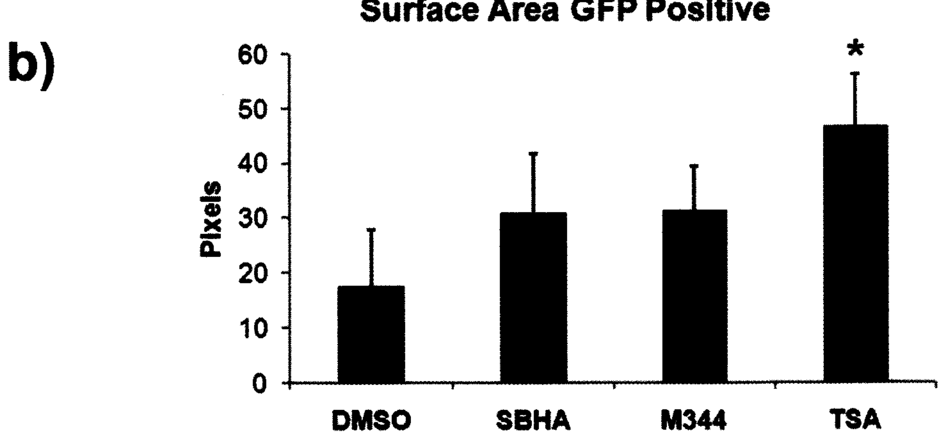
HDI	Maximum effect	Effective dose (μM)	Level of Acetylated H4
SAHA	++	0.8	33.7
MS-275	+	1.6	26.6
Oxamflatin	++	2.5	28.0
Apicidin	+	0.25	26.7
SBHA	+++	25	50.8
Scriptaid	++	0.5	16.0
CHAHA	+	0.6	4.7
M344	+++	0.6	66.2
TSA	**+++**	**0.08**	**52.7**

Figure 1. 4T1 cells were plated in 96-well plates then pre-treated for 3h with a concentration gradient of M344, SBHA, TSA or DMSO as the vehicle control. Following pre-treatment, cells were challenged with VVdd-GFP at an MOI of 1 (done in 6 replicates). (A) After 72h of incubation, fluorescence pictures were taken of each well, spanning the entire well-surface. Images are one of the 6 replicates at the most effective drug concentration. (B) Green fluorescence, indicating vaccinia spread, was measured using image analysis software (Image J, NIH) to quantify the number of fluorescent pixels. The most effective concentration for SBHA (12.5μM), M344 (0.625μM) and TSA (0.0375μM) are displayed with * indicating significant increase over the DMSO control ($p=0.0029$).

a)



b)

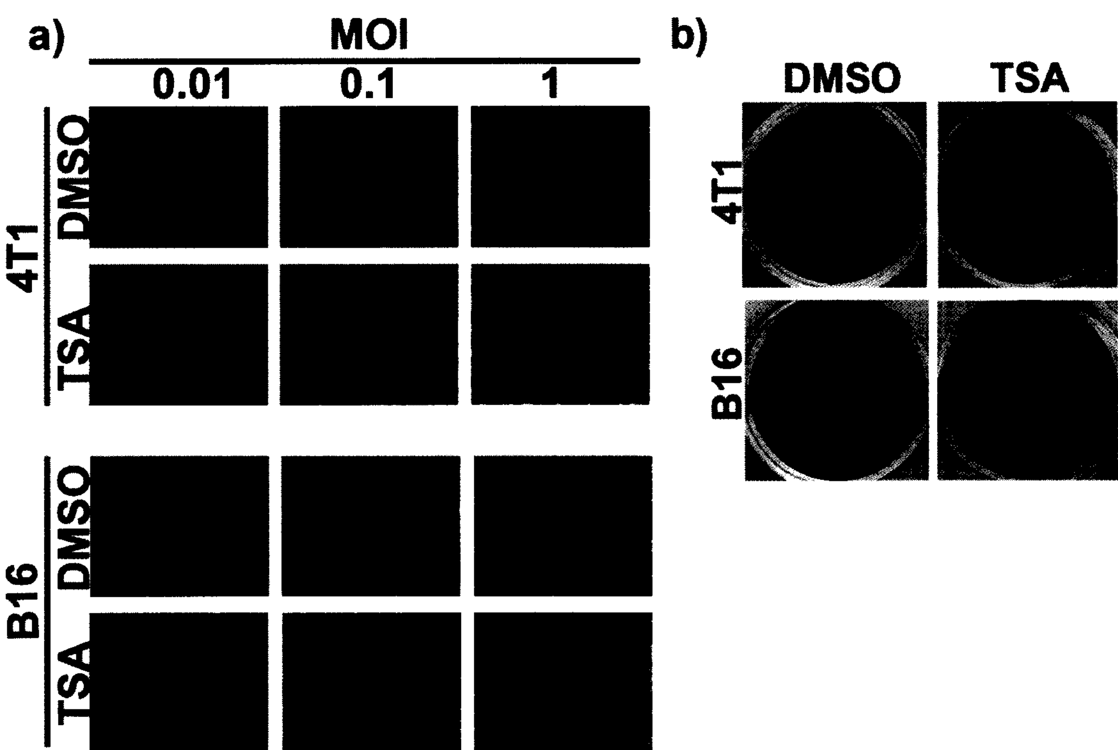


using an image analysis software (Image J, NIH) to determine which drug at which concentration was most effective at enhancing VVdd spread compared to the vehicle control (Figure 1b). Notably TSA effectively enhanced VVdd-associated GFP-positive area at lower doses than M344 or SBHA (as low as 37.5nM in contrast to 625nM and 12.5μM respectively). TSA significantly ($P < 0.05$) increased the number of GFP positive pixels relative to the control. Because of its increased efficacy in augmenting the VVdd-associated GFP positive pixels, low toxicity and histone H4 hyper-acetylation at such a low dose, TSA was chosen for further study.

3.2 TSA increases the spread and replication of VV in cancer cells.

TSA was further tested for its ability to enhance oncolytic VV in cancer cells *in vitro*. Confluent murine 4T1 breast cancer cells and B16 melanoma cells were pre-treated with low-dose TSA (37.5nM) for 3hrs. Subsequently, cells were infected with VVdd-GFP at MOIs of 0.01, 0.1 and 1 pfu/cell. Fluorescent images taken 72 hours post-infection reveal that TSA could increase VVdd-associated GFP expression in both cell lines (Figure 2a). Cell monolayers stained with Coomassie Blue 72h post-infection reveal visibly more plaques and plaques that appear larger in size in the TSA treated groups (Figure 2b). The cells and supernatants were collected and titered for virus content and the viral titer was increased up to 100-fold upon TSA pre-treatment (Figure 2c). A multi-step growth curve was done on B16 cells to track the increased virus output over time relative to the control. Samples collected every 12 hours for 3 days show that the virus titer is increased by 10 fold after only 36 hours with the largest increase peaking at 48 hours (Figure 3).

Figure 2. TSA enhances the spread and replication of VVdd *in vitro*. (A) 4T1 breast cancer and B16 melanoma cells were pretreated with TSA (0.0375μM) for 3hr and infected at an MOI of 0.01, 0.1 or 1. Fluorescence microscopy pictures were taken at 72 hours post infection. (B) 4T1 and B16 cells were infected with VVdd at an MOI of 0.1 and 0.01 respectively and plaques were stained for visualization with Coomassie Blue 72 hours post-infection. (C) The samples were collected after 72 hours. Viral titers were measured by standard plaque assay on U2OS cells. The experiment was done in triplicate. Star indicates P value < 0.05.



c)

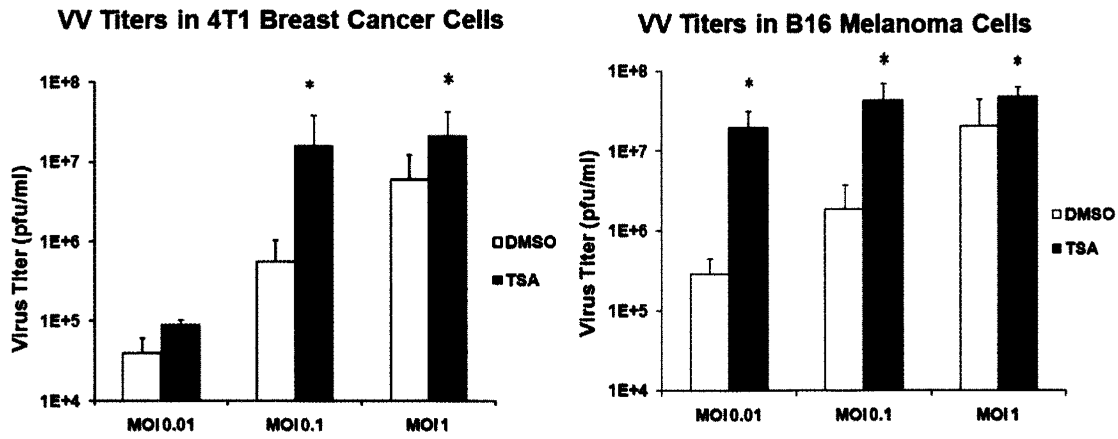
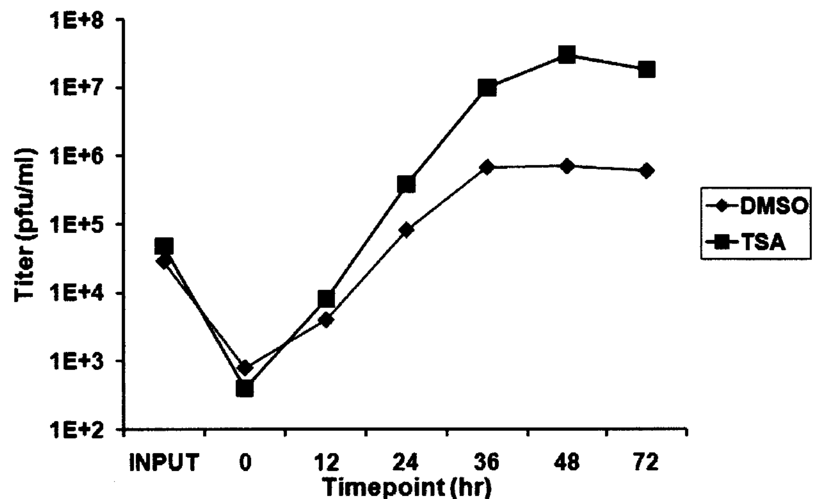


Figure 3. Growth curve demonstrates TSA enhancement of virus production. B16 Cells were pre-treated with TSA (0.0375μM) for 3h and infected with VVdd-GFP at an MOI of 0.1 for 1h. Virus input was removed and samples were collected at indicated timepoints and titered for virus content.



B16 Multi-Step Growth Curve
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
1E+2
Titer (pfu/ml)
INPUT
0
12
24
36
48
72
Timepoint (hr)
DMSO
TSA

3.3 TSA and vaccinia exhibit synergistic activity

To better understand if TSA and VV are working synergistically to kill cells rather than the observed enhancement in cell killing being attributed to additive effects from the TSA and VV independently, the combination index was calculated for the two treatments on 4T1 breast cancer cells. The combination index graphed versus the factor of cells affected was calculated as described by Chou and Talalay (8). Values with a combination index smaller than 1 are considered synergistic and for TSA and VVdd the combination index was found to be below 1 at various fractions of affected cells or Fa (in other words at various combined treatment efficacies) suggesting truly synergistic killing in 4T1 cells (Figure 4).

3.4 TSA results in the hyper-acetylation of histone H4

To investigate the effect of TSA on the hyperacetylation of histone H4 at the low dose used for treatment alone or in combination with VVdd, a Western Blot was performed. Bands indicating H4 acetylation appear in the TSA treatment group and the TSA and virus combination group but not in the untreated control or virus alone group (Figure 5). The level of acetylation by TSA does not appear to decrease when the virus and TSA are combined and VVdd alone did not increase acetylation levels. Since the level of acetylation seemed to correlate with the enhancing effect of the HDI in Table 1, the clear increase in acetylation upon low-dose TSA treatment with and without virus is consistent with the initial screen and shows that the addition of VVdd doesn't change the HDI effect on histones.

Figure 4. TSA and VVdd exhibit synergistic cell killing activity. 4T1 cells were treated with serial dilutions of a fixed ratio combination mixture of VVdd and TSA (1562 PFU: 1uM VVdd:TSA). Cytotoxicity was assessed using alamar blue reagent after 96h. Combination indices (CI) were calculated according to the method of Chou and Talalay using Calcusyn. Plots represent the algebraic estimate of the CI in function of the fraction of cells affected (Fa). Error bars indicate the estimated standard error.

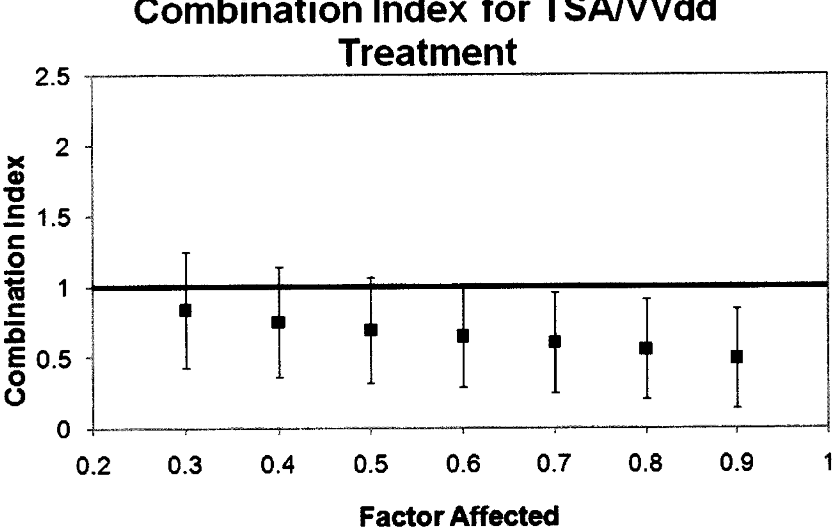
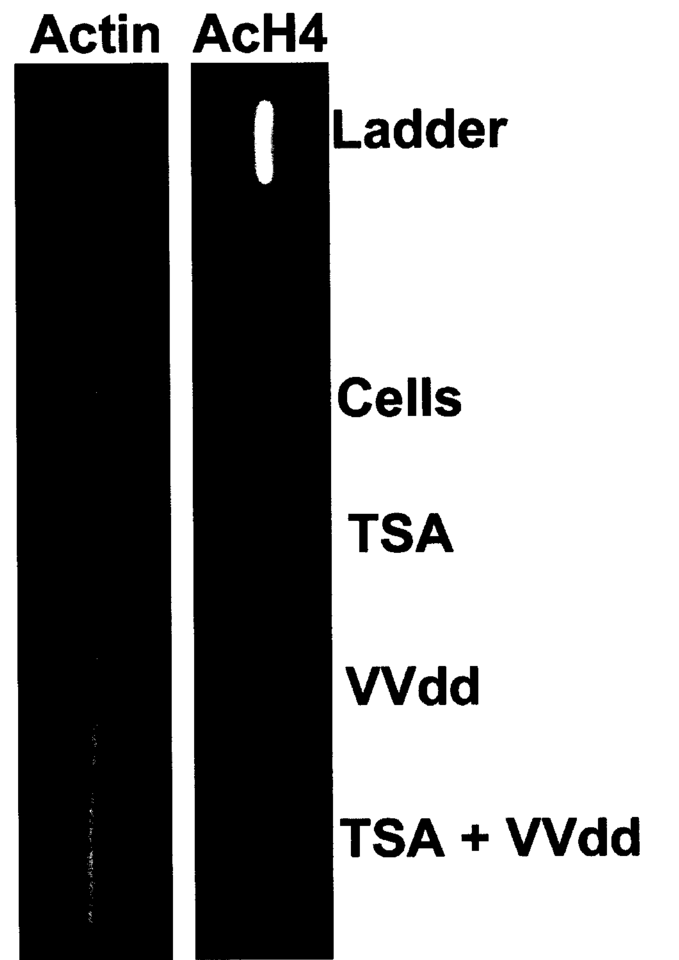

Combination Index for TSA/VVdd Treatment
Combination Index
2.5
2
1.5
1
0.5
0
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Factor Affected

Figure 5. Hyperacetylation of histone H4 by TSA. Equal amounts (50µg) of protein from 4T1 cells treated with TSA (0.04µM) or DMSO control and VVdd at an MOI of 0.1 or not were transferred to a nitrocellulose membrane and detected with the antibody specific to hyperacetylated histone H4.


Actin
AcH4
Ladder
Cells
TSA
VVdd
TSA + VVdd

3.5 TSA does not enhance vaccinia in normal cells

Oncolytic virus therapy is ideally given systematically to ensure that it can reach all metastases throughout the body. The oncolytic VV targets cancer cells due to the natural biology of the virus and the introduced deletions. To address the question of whether TSA would increase VV replication in normal cells the same experiments were conducted in mouse embryonic fibroblasts (MEF), GM38 normal human fibroblasts and MRC-5 secondary human lung fibroblasts. The viral titer in these normal cell samples was not increased by TSA. Consistent with Figure 2c, there is a large increase in titer upon TSA treatment in the cancer cell lines 4T1 and B16F10-LacZ. This suggests that *in vitro,* the enhancing effect of TSA on vaccinia production is specific to cancer cells (Figure 6) which is encouraging for the safety of this combination.

3.6 TSA enhances VVdd efficacy in a syngeneic lung metastasis model

The ability of TSA to enhance VV spread in cancer cells but not in normal cells raised the idea of using TSA to enhance the oncolytic ability of VV *in vivo*. Although other HDIs such a SAHA are already clinically available, we rationalized that because the concentration of drugs within tumors can be low and/or decrease rapidly, TSA is likely a better candidate because of its relative ability to enhance VVdd spread and function at very low doses (compare SAHA to TSA in Table 1) To test the ability of TSA to enhance vaccinia oncolysis in vivo the syngeneic B16F10LacZ lung metastasis model was chosen. This is an aggressive model where B16 cells are injected intravenously and form metastases on the lungs. C57/Bl6 mice were injected with 3 x 10^5 B16F10-LacZ melanoma cells and

Figure 6. TSA does not enhance vaccinia in normal cells. MEF, GM38 and MRC-5 normal cells along with 4T1 and B16 cancer cells were pre-treated for 3h with TSA (0.04μM) and subsequently infected with VVdd. Samples were collected after 72h and titered for virus content and displayed as fold increase (over the DMSO vehicle control) in plaque forming units per cell upon TSA treatment.

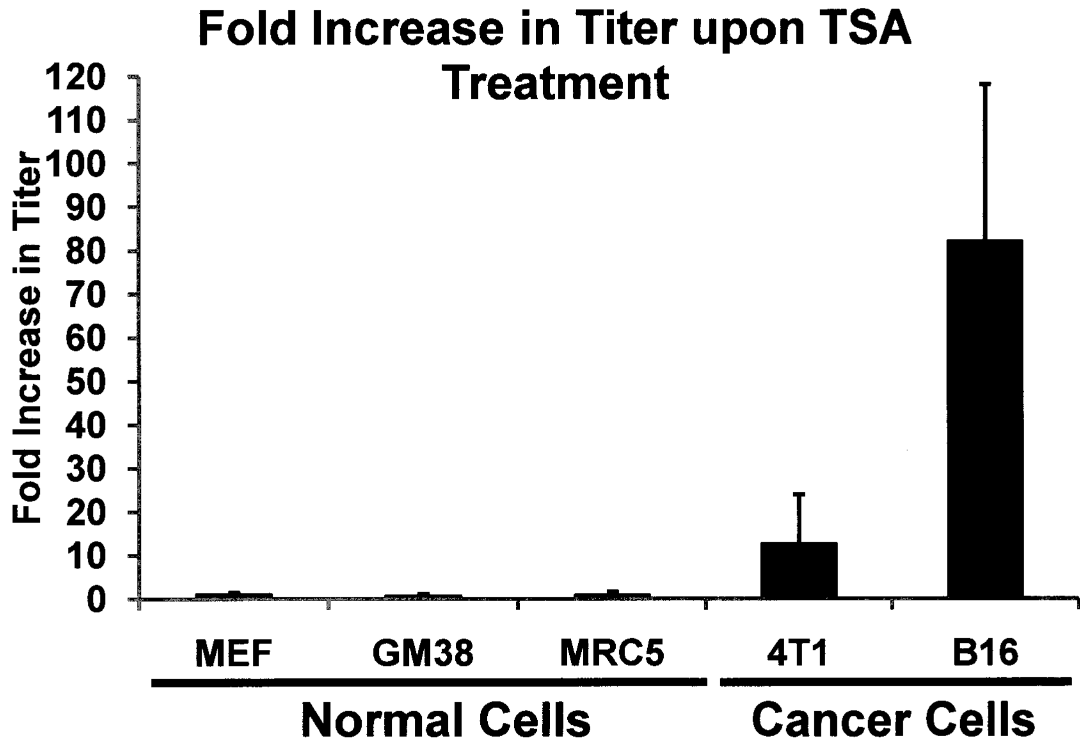
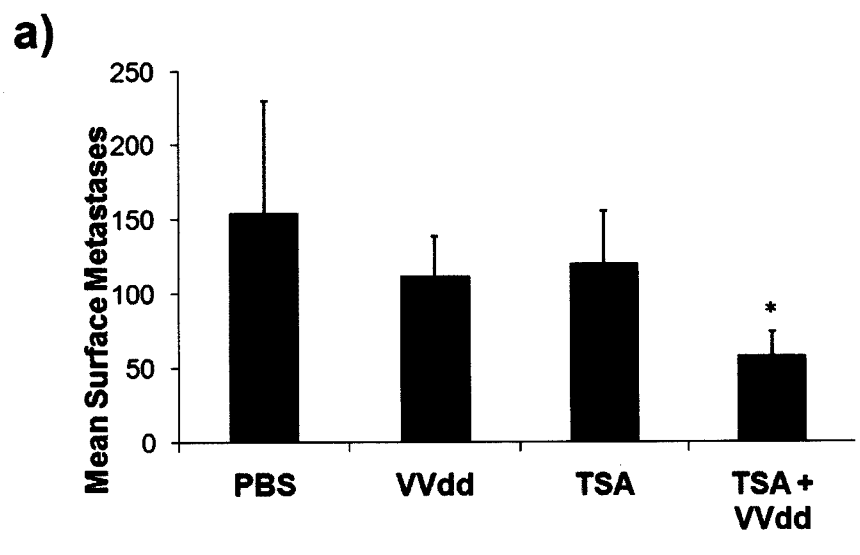

Fold Increase in Titer upon TSA Treatment
Fold Increase in Titer
120
110
100
90
80
70
60
50
40
30
20
10
0
MEF
GM38
MRC5
4T1
B16
Normal Cells
Cancer Cells

Figure 7. TSA is an effective enhancer of vaccinia *in vivo*. (A) C57BL/6 mice were injected intravenously with B16F10LacZ melanoma cells (3 X 10^5 cells/mouse) and TSA (4 daily intraperitoneal doses of 0.05 mg/mouse) alone or in combination with VVdd (1 x10^7 pfu.) The mice were sacrificed after 14 days and the lung metastases were counted after staining with X-gal. Asterix * indicates a P value < 0.05 and is significantly different than PBS group and each of the single treatment groups. (B) Lung lobes from control or VVdd and TSA treated mice. B16F10LacZ cells stained with X-Gal.



b)

Control VVdd TSA TSA+VVdd

were treated with TSA (4 intra-peritoneal doses of 0.05mg/mouse) or the vehicle and VVdd (2 intravenous injections of 1 x 10^7 PFU of VVdd) or PBS. The mice appeared to tolerate each of the treatments well and did not show adverse events in any of the treatment groups. Two weeks after tumour implantation the mice were sacrificed and lungs were stained with the substrate X-gal to allow for counting of individual surface metastases on each of the five lobes of the lungs (23, 49). The combination of TSA and VVdd resulted in significantly fewer lung metastases than any of the other single treatment groups (Figure 7a, $p<0.05$). Representative pictures of the lungs demonstrate visibly fewer metastases in the combination treatment group (Figure 7b).

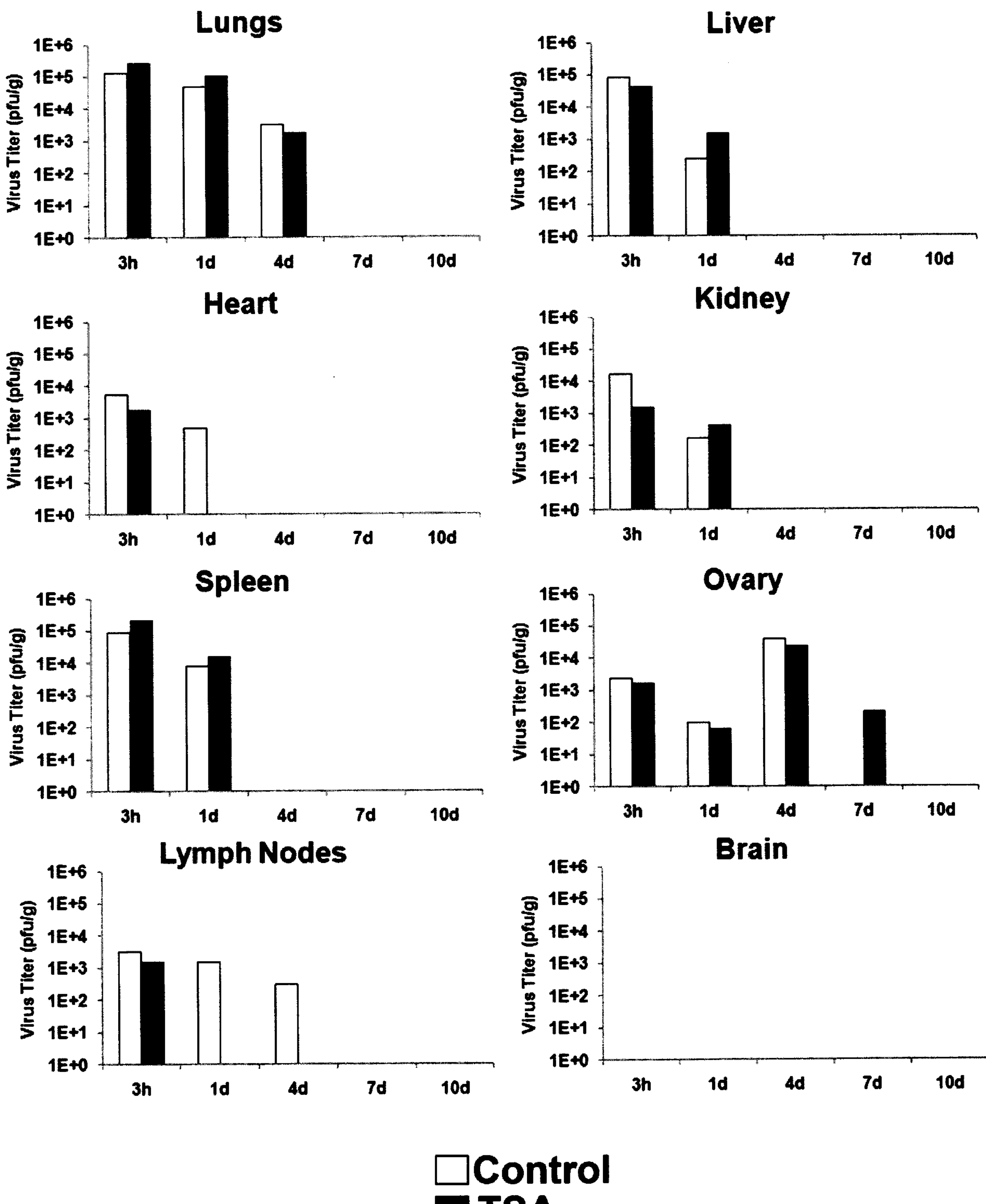
3.7 TSA does not alter the biodistribution of VVdd *in vivo*

To further complement the *in vitro* data where TSA did not enhance vaccinia in normal cell lines, a biodistribution experiment was done to investigate the *in vivo* effects of the combination treatment. Balb/C mice were treated or not with 4 intra-peritoneal doses of TSA and infected with 1 x 10^8 PFU of VVdd intravenously. Mice were sacrificed at various time points and organs were titered for virus content. TSA did not change the overall virus biodistribution in normal tissues and all virus was cleared after 10 days with most of it gone as early as 4 days (Figure 8). This result highlights the safety of the combination treatment as vaccinia is not enhanced in normal tissues *in vivo* and TSA treatment did not prolong the presence of VVdd in the body.

3.8 In the presence of IFN TSA enhances vaccinia in cancer cells and not normal cells

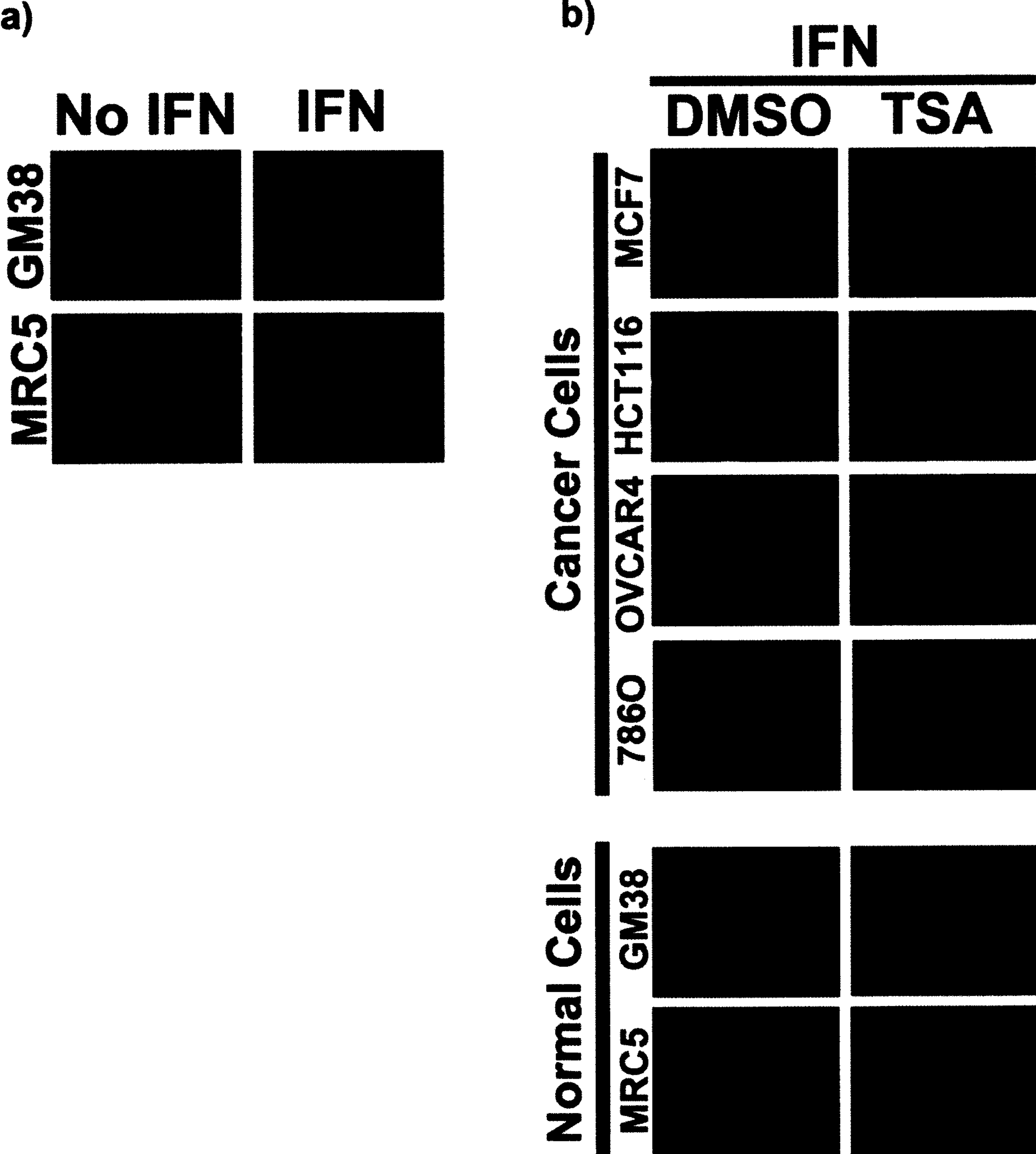
Interferon is the host cells primary anti-viral defence and pre-treatment with IFN leads to strong inhibition of infection in both normal human fibroblast cell lines GM38 and

Figure 8. TSA does not change the biodistribution of vaccinia. Balb/C mice were pre-treated or not with TSA (0.05mg/mouse) on days 0 through 3. After 3h pre-treatment on day 0, mice were given an intra-venous dose of VVdd-luciferase of 1 x 10^8 pfu/mouse. Mice were sacrificed at various timepoints and organs were titered for virus content by standard plaque assay on U2OS cells.



Lungs
Liver
Heart
Kidney
Spleen
Ovary
Lymph Nodes
Brain
Virus Titer (pfu/g)
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
1E+0
3h
1d
4d
7d
10d
Control
TSA

Figure 9. TSA enhances vaccinia in presence of IFN in cancer cells. (A) Normal cells GM38 and MRC-5 were pre-treated or not with TSA (0.04μM) for 3hr and subsequently treated or not with 200 IU/ml of IFN for 16hrs. Cells were then infected with VVdd at MOI 0.001 and fluorescent pictures were taken after 72h. B) Indicated cancer or normal cells were pre-treated for 3hr with TSA (0.04μM) and subsequently with 200 IU/ml of IFN for 16hrs. Cells were then infected with VVdd at MOI 0.001 and fluorescent pictures were taken after 72h.


a)
No IFN
IFN
GM38
MRC5
b)
IFN
DMSO
TSA
Cancer Cells
MCF7
HCT116
OVCAR4
786O
Normal Cells
GM38
MRC5

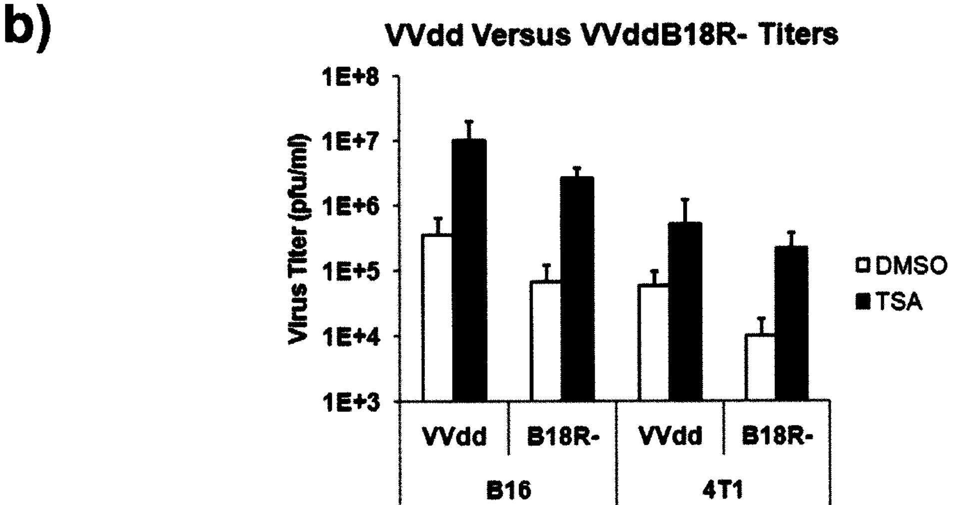
MRC-5 (Figure 9a). The protective effect of IFN was not overcome by TSA in normal cells; however there was enhancement of vaccinia spread in several cancer cell lines even in the presence of IFN (Figure 9b). Hence, TSA is able to enhance vaccinia spread in cancer cells independently of IFN while normal cells remain protected by IFN, even in presence of TSA.

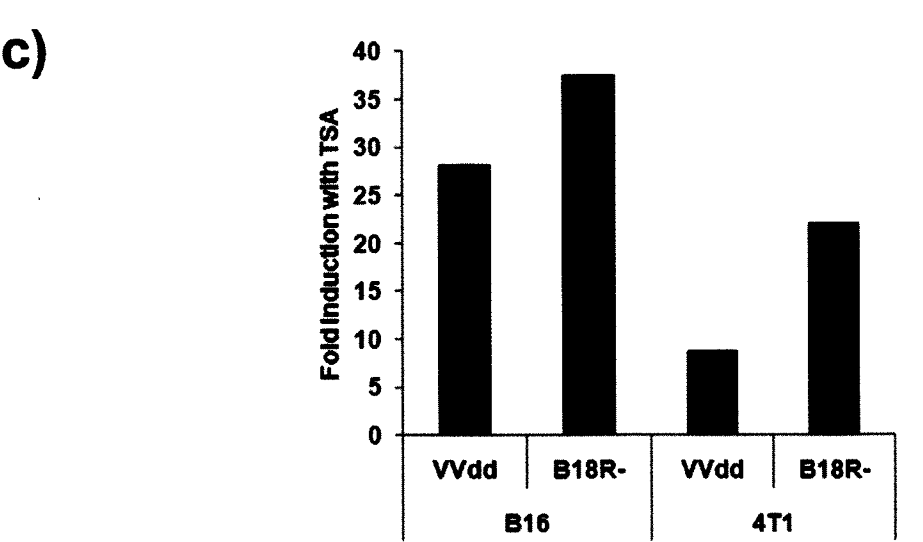
3.9 TSA enhances further attenuated B18R-deleted vaccinia strain

Because the VV B18 protein (encoded by the B18R gene) plays a major role in scavenging secreted IFN, it was rationalized that TSA could be particularly useful in enhancing VV strains in which this gene has been deleted. The use of a B18R deleted vaccinia leads to further tumour selectivity (22) and we hypothesized that HDIs could significantly enhance B18R-deleted VV replication specifically in tumour cells with defective interferon signaling. To investigate this possibility, cells were pre-treated with TSA and then infected with VVdd B18R-. Fluorescence microscopy images reveal the B18R deleted virus is also enhanced by TSA (Figure 10a). As expected, the B18R deleted strain is further attenuated and the titers are slightly lower than that obtained with VVdd. However upon TSA pre-treatment, the titers are increased to a greater extent in the B18R deleted strain as compared to the VVdd strain (Figure 10b). This suggests that the B18R deleted strain is even further enhanced than the VVdd strain by TSA (Figure 10c). To complement these results, and to further investigate the effects of using the B18R deleted vaccinia on human cell lines, the fold induction in plaque forming units produced upon pre-treatment with TSA was compared between TK-deleted and TK/B18R-deleted WR strains in several cell lines. Whereas TSA did not lead to any increase in viral production in

Figure 10. B18R Deleted Vaccinia is enhanced by TSA. (A) 4T1 and B16 cells were pre-treated for 3hr with TSA (0.04 μM) and infected at MOIs 0.01 and 0.1 with VVddB18R-. Fluorescent pictures were taken 72 hours post-infection. (B) 4T1 and B16 samples were collected after 72hrs and titered by standard plaque assay on U2OS cells for virus titer in plaque forming units/cell (C) Titers are displayed as fold induction (over the DMSO vehicle control) in plaque forming units/cell upon TSA pre-treatment for VVdd versus VVddB18R- strains.

a)
B16
4T1
0.01
0.1
0.01
0.1
DMSO
TSA


b)
VVdd Versus VVddB18R- Titers
1E+8
1E+7
1E+6
1E+5
1E+4
1E+3
Virus Titer (pfu/ml)
DMSO
TSA
VVdd
B18R-
VVdd
B18R-
B16
4T1


c)
40
35
30
25
20
15
10
5
0
Fold Induction with TSA
VVdd
B18R-
VVdd
B18R-
B16
4T1

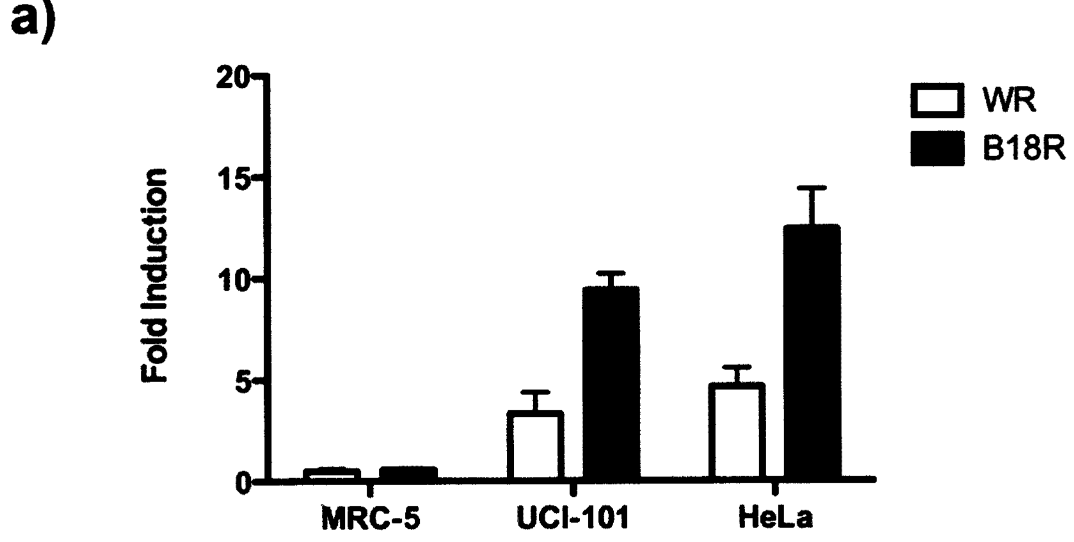
normal MRC-5 cells for either strain, it increased viral titers for both strains in human UCI-101 ovarian cancer cells and HeLa cervical cancer cells (Figure 11a). This result is consistent with the B16 and 4T1 data and indicates that the IFN-sensitive B18R deleted strain of VV can be further enhanced by TSA.

3.10 TSA in combination with B18R-deleted vaccinia increases survival in a xenograft model

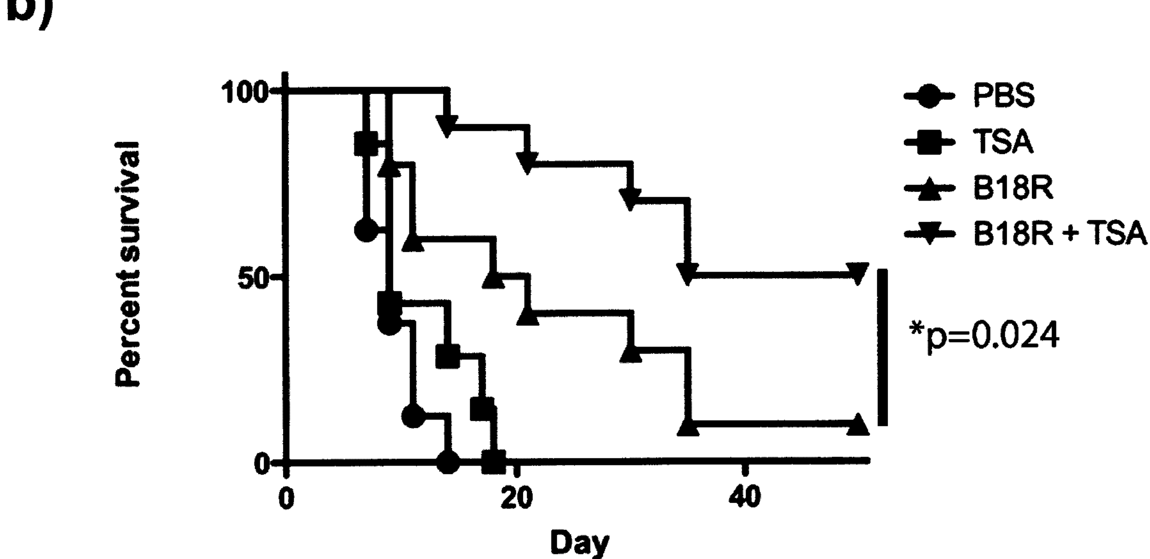
These data in combination with the observation that TSA could enhance VVdd efficacy *in vivo* suggested that TSA may be useful to enhance the oncolytic activity of the B18R/TK-deleted WR strain in a mouse tumour model. Immunocompromised mice with palpable HCT-116 colon cancer tumours were treated with TSA (or vehicle) and a luciferase-expressing B18R/TK-deleted WR (or control). Consistent with this observation and the results obtained in the lung metastasis model (Figure 7a), mice treated with the combination of TSA and TK/B18R-deleted WR survived significantly longer than any of the control groups (Figure 11b, p=0.024).

Figure 11. TSA enhances B18R-deleted vaccinia *in vitro* and increases survival in a xenograft model (A) Human cell lines (MRC-5; UCI-101 or HeLa) treated with PBS or TSA (1uM) for 6h. At the end of the pre-treatment, cells were infected at an MOI or 1.0 by vaccinia strains Western Reserve or Western Reserve with a deletion in the B18R gene. Cells and media were collected after 72h and viral PFU titered by plaque assay on BSC-1 cells. Fold induction of titer relative to control is reported. (B) Athymic nu-/nu- mice were implanted subcutaneously with HCT-116 cells ($5x10^6$ cells/ mouse). Once palpable tumours had formed ($50-100mm^3$), mice were treated with either (i) intraperitoneal PBS; (ii) intraperitoneal TSA (6μg/mouse) on days -1, 0 and 2; (ii) intravenous injection of WR B18R- TK- Luc+ ($1x10^8$ PFU/mouse) on day 0; or (iv) both TSA and WR B18R- TK- Luc+ (n=10 mice/group). Subsequent tumour burden was followed by caliper measurement and mice sacrificed when their tumours reached $1400mm^3$. Percent survival of mice is graphed (p=0.024).

a)



b)



4. DISCUSSION

In this study, it was found that when compared with other HDIs TSA was the most effective drug for enhancing VV oncolytic activity *in vitro*. Though other HDI showed some efficacy, TSA was the most potent and could be used at very low concentrations (as low as 30 nM) to elicit increases in VVdd titers of up to 100-fold (Figure 2c). Although TSA is not approved for use as an anti-cancer agent in humans, TSA was chosen for further testing in animals as an enhancer of VV oncolysis due to the fact that it was highly effective even at low concentrations where little to no toxicity was observed using the drug alone *in vitro*. Plaque number and size increased with TSA pre-treatment (Figure 2a and b) and the greatest increase in viral output was found at 48-72 hours post infection (Figure 3) (21).

Histone deacetylase inhibitors are being tested in the clinic as therapeutic anti-cancer agents and many oncolytic viruses including VV are in clinical trials for cancer treatment (37, 54). One might wonder if the TSA enhancement of VV oncolysis could be attributed to the additive cancer cell killing effects of TSA and VV independently. However as seen in Figure 4 the cell killing effect by TSA and VVdd are in fact synergistic based on calculations of combination index. This result is important because it promotes the idea of using oncolytic viruses with a combination drug for maximum therapeutic benefit beyond what either agent could provide on its own.

HDIs are known for having primarily tumour cell specific action; however enhancing the efficacy and cell killing ability of any oncolytic virus does raise safety concerns. Both the *in vitro* cell data and *in vivo* biodistribution data demonstrate that TSA

does not enhance VV in normal cells (Figure 6, 8, 9a, 9b and 11a). This is significant because if a drug can produce cancer cell specific enhancement of an OV then the virus could be further attenuated to augment the safety in normal cells, yet be enhanced to potent levels in the tumour. The increased safety of this system is an attractive feature of the combination drug/therapy approach to OV therapeutics. This approach has also been demonstrated using other agents to enhance oncolytic VSV (10, 33). Importantly in this approach, HDIs have their enhancing effect exclusively in cancer cells. The reason for this selectivity may involve several components. For example, TSA may affect gene expression more prominently in cancer cells where HDACs are thought to be frequently up-regulated (28). In addition, partially defective antiviral signaling pathways in cancer cells may be more sensitive to inhibition of HDACs than normal cells in which anti-viral signaling is robust. A normal cell may have a much tougher anti-viral barrier to break through; whereas in a cancer cell, the anti-viral defence may be already weakened to the point where HDI treatment could easily break down the remaining barrier and allow access to the OV.

Supporting the idea that TSA can indeed be useful *in vivo*, the drug enhanced the activity of VVdd in a metastatic lung cancer model by reducing tumour burden and could prolong the survival of mice challenged with subcutaneous tumours when used in combination with the TK/B18R-deleted WR strain. These results along with the biodistribution studies are encouraging for further use of this combination in *in vivo* studies and future clinical trials.

Trichostatin A (TSA) was one of the first HDAC inhibitors to be discovered (63). It is a hydroxamic acid and inhibits all class I and II HDACs. Although its anti-cancer properties are well documented, its sub-optimal *in vivo* stability has rendered it less

attractive for use as an anti-cancer drug (28, 43, 57, 58). In response, there has been considerable effort in the HDI field to develop more stable TSA derivatives such as Vorinistat ® (SAHA), which was recently approved for limited applications such as treatment of CTCL (3, 35, 36, 52). In this study TSA was significantly more effective than SAHA at enhancing VV spread. Notably, the sub-optimal characteristics of TSA with regard to its use as an anti-cancer agent do not exclude its use for the purpose of enhancing oncolytic viruses, where the necessary dose for significant enhancement is extremely low because of the potency of the drug. In fact, one could argue that because TSA is rapidly cleared (43), it is even more suitable for use as an OV enhancer *in vivo* since the drug can quickly disappear once treatment is interrupted as would be desirable if unbridled viral replication were to occur in presence of drug.

The effect of TSA on the IFN response is well documented (14, 59) and the enhancing effect of HDIs such as TSA on IFN-sensitive strains including VSV and HSV has been previously reported (33). It is therefore not surprising that TSA can increase the activity of B18R-deleted VV strains (Figure 10, 11). However, it remains unclear why VV strains that do express the B18 protein are also significantly enhanced by TSA, since IFN can be sequestered and neutralized by secreted B18. A possible explanation would be that the B18 protein alone is not powerful enough to eliminate the IFN response and even in the presence of a fully active B18 protein, TSA can still enhance the virus by dampening the enduring IFN response. Another possibility is that TSA has multiple effects on the cell and is assisting the virus in several ways. HDI action is complex and upon treatment many genes are either up or down regulated causing major changes in cancer cells. TSA works on vaccinia to varying extents in different cell lines and future investigation into molecular differences in cells and responsiveness could give more clues as to other means of HDI

enhancement. Microarrays reveal many changes in gene transcription levels upon HDI treatment (7, 10) and it is probable that numerous pathways are being affected by TSA treatment and that the combination of these changes leads to the robust enhancement.

In previous studies with VSVΔ51, HDIs were found to increase virus-induced apoptosis in addition to increasing the viral spread (33). Because most of the VV produced within a cell following infection is retained within the cell until lysis, perhaps the increase in viral spread is due in part to increased virus-induced apoptosis and quicker release of virions that can subsequently infect neighbouring cells. This may notably explain why VV plaques are both larger and more numerous upon TSA treatment (Figure 2b).

Using small molecule OV-enhancers is attractive from a clinical standpoint. In principle, this strategy allows for severe attenuation of OVs while treatment with an enhancing compound can restore oncolytic activity equivalent or superior to wild-type virus. This is well illustrated in Figure 10a and b. The IFN-sensitive VVdd B18R-deleted strain is more attenuated compared VVdd, however TSA is still able to enhance this strain. It is interesting that in both Figure 10c and 11a the B18R deleted virus is enhanced to a greater extent by TSA compared to VVdd and the TK/B18R deleted strain respectively. The TK/B18R deleted WR strain is not enhanced by TSA in normal cells where TSA has no effect and the IFN-sensitive strain can be easily neutralized. In further support of this, Figure 9 reveals that VVdd is strongly inhibited by IFN in normal cells. In cancer cells, TSA can increase VVdd replication independently of IFN. Depending on the cell line TSA may decrease the IFN response from a nearly functional to less functional to an insufficient level or completely blocking the IFN response and fully preventing the onset of the anti-viral state. Both outcomes could lead to enhanced viral replication though the level of

resulting enhancement of VV may be different depending on the cell type. Even in the presence of IFN, the addition of TSA continues to enhance the virus in cancer cells but does not overcome the inhibitory effect of IFN in normal cells. To this end, the idea of combining OVs with complementary OV enhancers such as TSA in the case of IFN-sensitive mutants provides an additional layer of safety, since replication of virus can be controlled on demand simply by providing the drug or interrupting its administration.

Overall, the data presented here suggests that several different VV strains can be enhanced by TSA *in vivo*. This is of considerable interest since VV strains such as JX-594 (TK-deleted, expressing GM-CSF), JX-963 (TK/VGF-deleted, GM-CSF expressing), and JX-969 (TK/VGF-deleted) are currently undergoing Phase I/II clinical trials. It would therefore be relevant to determine whether efficacy of these OVs can be further enhanced by TSA. Using lower doses than what has been previously attempted for direct anti-cancer applications, TSA could be reconsidered for a new use as an OV enhancer. With respect to this idea, perhaps beginning with Vorinostat®, though potentially sub-optimal, may be more realistic since it is already approved by the FDA for recurrent CTCL.

5. CONCLUSION

We conclude that TSA is a potent enhancer of VV *in vitro* and *in vivo*. TSA was effective at enhancing replication of attenuated strains of VV including those in which the B18R gene product has been deleted, for which TSA elicited an even greater enhancement compared to the wild-type strain. We propose that HDIs such as TSA could be used to enhance the effectiveness of OVs *in vivo* and that further clinical evaluation of this possibility is warranted.

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CONTRIBUTION OF COLLABORATORS

Dr. Jean-Simon Diallo was responsible for the maximum effect and effective dose portions of Table 1. He did the calculations of the data using Calcusyn for the combination index in Figure 4 and helped perform the lung metastasis experiment in Figure 7. Importantly he trained me on most techniques used in this thesis.

Dr. Marianne Stanford is most experienced in the lung metastasis model and was an important part of designing and performing the experiment in Figure 7.

Tanya Guimond helped with the Western Blots in Table 1 and Figure 5.

Baocheng Huong and Stephen Thorne did the experiments in Figures 11a and 11b.

Theresa Falls did all intra-venous injections in mice for experiments in Figure 7 and 8 and taught me dissection techniques and how to do intra-peritoneal injections.

Dr. Fabrice LeBoeuf made the B18R deleted virus.

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PUBLICATIONS

Diallo, J. S., F. L. Boeuf, F. Lai, J. Cox, M. Vaha-Koskela, H. Abdelbary, **H. MacTavish**, K.Waite, T. Falls, J. Wang, R. Brown, J. E. Blanchard, E. D. Brown, D. H. Kirn, J. Hiscott, H. Atkins, B. D. Lichty, and J. C. Bell. 2010. A High-throughput Pharmacoviral Approach Identifies Novel Oncolytic Virus Sensitizers. Mol. Ther.doi: 10.1038/mt.2010.67.