Epigenetic modifiers enhance Vesicular Stomatitis Virus-mediated oncolysis in the refractory PC3 cell line

Marnie Goodwin Wilson¹, ², Thi Lien-Anh Nguyen², Laura Shulak², Peyman Nakhaei¹, ² and John Hiscott¹, ²

¹Department of Microbiology and Immunology, McGill University, Duff Medical Building, 3775 University Street, Montreal, Quebec, Canada, H3A 2B4
²Lady Davis Institute for Medical Research, Sir Mortimer B. Davis - Jewish General Hospital, 3755 chemin Côte Ste Catherine, Montréal, Québec, Canada, H3T 1E2.

ABSTRACT

Introduction: Vesicular Stomatitis Virus (VSV) is an oncolytic virus that preferentially replicates in and kills cancerous cells. However, many cancer cell lines are resistant to VSV treatment alone. Previous work has shown that treating cancerous cells with histone deacetylase inhibitors makes them more susceptible to VSV infection and oncolysis. We hypothesize that treatment with a histone deacetylase inhibitor, Suberoylanilide hydroxamic acid (SAHA or Voronistat), and a methyltransferase inhibitor, 5-aza-2’-deoxycytidine (5-AZA or Decitabine), will result in an increase in VSV replication and virus-induced oncolysis in vitro. Methods: PC3 prostate cancer cells were treated with 1 μM SAHA, 1 μM 5-AZA or both. Of these samples, half were infected with oncolytic Vesicular Stomatitis Virus expressing green fluorescent protein VSV AV1 – GFP at a multiplicity of infection of 1 × 10⁻², 24 hours after treatment. Cells were then collected and subjected to either FACS analysis or protein extraction at 12, 24, 48 and 72 hours post-infection. We confirmed increases in cell death by western blotting for cleavage of Poly A Riboprotein, an important downstream effector of the Caspase pathway, as well as Caspases 8 and 9, hallmarks for the extrinsic and intrinsic apoptotic pathways respectively. Results: Treatment with SAHA, 5-AZA or a combination of both resulted in increases in VSV replication and cell death. These observations were consistent over four time points spanning 72 hours. Discussion: Treatment with histone deacetylase inhibitor/methyltransferase inhibitor combination increases VSV replication and cell death in tumour cell lines resistant to VSV infection. In combination with previous work, this data suggests that modulation of the antiviral response and apoptotic pathways increases susceptibility to VSV.

KEYWORDS
Epigenetics, Cellular Transformation, Oncolytic Virus, Apoptosis

*Corresponding author: marnie.gwilson@gmail.com
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INTRODUCTION

Cancer is an extremely prevalent disease, causing approximately 500 000 deaths and nearly 1.5 million new cases annually in the United States (16). Many current cancer therapies lack efficacy, and can even induce therapeutic resistance in cancer cells (19). Further, since current cancer treatments often have low specificity for cancerous cells, they are often toxic and highly detrimental to the health of the patient (17).
Transformation occurs when a cell acquires a cancerous or malignant phenotype, characterized by uncontrolled cell proliferation (4). Clinical manifestations of cancer result if the body does not recognize and eliminate these cells. Oncolytic viral (OV) therapy uses non-pathogenic, replication-competent viruses selected or engineered to grow in and destroy tumour cells (5). During the evolution of malignancies, genetic abnormalities accumulate and provide cancer cells with growth and survival advantages, but compromise host antiviral defences (20). Most prominently, the interferon system, which is responsible for cell-cell communication during infection, is down-regulated or absent in many transformed cells (23, 34). Although the interferon pathway is most commonly associated with defence against infection, it has also been shown to play a role in cancer immunology (28, 34, 37). Infected cells secrete interferon molecules, which bind to receptors on neighbouring cells and trigger the production of molecules that protect against further virus infection (14, 18).

Vesicular Stomatitis Virus (VSV) is a negative-stranded RNA virus and a well-characterized oncolytic virus (4, 5). It replicates preferentially in cells that have acquired defects of the interferon pathway. In normal cells, interferon is produced, which protects surrounding cells from VSV infection, resulting in rapid clearance of the virus and recovery (23). Conversely, transformed cells are unable to mount a response against the mutated virus and are killed (2). This results in selective replication in transformed cells, increasing the specificity of the treatment and decreasing the potential for adverse side effects, illness or systemic infection (13). Side effects due to oncolytic virus treatment are generally limited to flu-like symptoms, a vast improvement over the side effects of current chemotherapies (4, 3). Unfortunately, many transformed cell lines, including most primary tumours, are resistant to VSV-mediated oncolysis (35). Resistance often occurs when cell lines have not lost their ability to mount an interferon response. Several studies have combined OVs with other cancer therapeutics to combat problems related to tumour cell resistance (6, 11, 12, 15, 26, 37, 39). Small molecule inhibitors of various cellular processes are candidates for such combinations.

Histone deacetylase inhibitors are one such candidate (7). Epigenetic alterations of chromatin, the scaffold of histone proteins that bind DNA in the nucleus result in abnormal gene expression of cancer cells (25). Acetylation occurs on lysine residues of histone tails and thereby alters their electrostatic interactions with DNA (26). Acetylation and deacetylation of histones is a dynamic and tightly regulated process, resulting in transcriptional control and alterations in gene expression (25, 31). Acetylation is mediated by protein histone acetyltransferases (HATs) and is generally associated with chromatid decondensation and gene expression. Meanwhile, histone deacetylation, which is mediated by histone deacetylases (HDACs), results in chromatin condensation and silencing of gene expression (27). Epigenetic modifications also play an important role in cell division, both through the modulation of gene products involved in the progression of the cell cycle and because near-complete histone deacetylation and condensation of chromatin are required to complete mitosis (7). In transformed cells, histone modifications are often dysregulated, resulting in aberrant transcription of genes and the loss of cell cycle control (26, 30). HDI treatment has been repeatedly shown to blunt the cellular antiviral response, which in turn makes OV treatment more effective (29, 30).

Methyltransferase inhibitors (MTIs) are another epigenetic-based cancer therapeutic (10). Methylation of gene promoters is a common means of gene silencing, and is used in the methylation of certain tumour suppressors, such as p53 and proapoptotic and anti-metastatic genes (22, 36). Unlike acetylation, which affects the histone scaffold, DNA methylation transfers a methyl group directly to the 5’ position of specific cytosine residues in DNA (10, 24). Most MTIs do not directly inhibit the methyltransferase enzyme; rather, they are derivatives of cytosine with different chemical groups (for instance, a nitrogen-based group) substituted in the 5’ position (10). These cytosine derivatives intercalate into the DNA of rapidly dividing cells, thus altering the target for methyltransferase and inhibiting its activity (10). The requirement that cells be rapidly dividing is the basis for the tumour-selective mechanism of MTI therapy (24). MTI treatment is often used in combination with HDI treatment in preclinical experiments as well as clinical trials (32, 33). The two have been shown to produce a synergistic effect both in vitro and in vivo.

HDI and MTI have been shown to act synergistically in a number of ways, indicating that their combined effect could further sensitize transformed cells to VSV-mediated oncolysis. This project investigates whether the addition of MTI to the established combination of VSV and HDI can further enhance the therapeutic benefits of OV use in refractory cell lines.

**METHODS**

**VIRUS**

VSVΔ51 expressing Green Fluorescent Protein (GFP) is a recombinant derivative of VSVΔ51, a naturally occurring interferon (IFN) inducing mutant of the VSV Indiana serotype. Viruses were propagated and purified, as described in (35), in Vero cells.

**CELL TREATMENT AND INFECTION**

PC3 prostate cancer cells were plated at a 1:4 dilution and left overnight in RPMI media (Wisent) supplemented with 10% foetal bovine serum (FBS) and 1% Penicillin/ Streptomycin mixture. Cells were then either left untreated or treated with
1 μM concentrations of either the histone deacetylase inhibitor suberoylanilide hydroxamic acid (Vorinostat, denoted SAHA), the methyltransferase inhibitor 5-aza-2’-deoxycytidine (Decitabine, denoted 5-AZA), or both. Of these samples, half were infected with VSV AV1 – GFP at a multiplicity of infection (MOI) of $1 \times 10^{-2}$ 24 hours after treatment. Cells were then collected for flow cytometry (FACS) analysis, protein extraction or RNA extraction at 12, 24, 48 and 72 hours post-infection. Cells were either immediately used (in the case of FACS analysis) or kept at −80 °C until needed (in the case of protein extraction).

FLOW CYTOMETRY
Cells were washed once with PBS, then trypsinized in 0.25% trypsin (Wisent), diluted to 1:4 in PBS and centrifuged at 1200 rpm for five minutes. Supernatant was discarded and cells were stained with Annexin-V APC (BD Biosciences) as per the manufacturer’s instructions. Cells were subjected to FACS analysis (10⁶ events per measurement) on a FACS Calibur (Beckton-Dickson) and analyzed using FCS express V3 Software. FACS analysis showed expression of GFP (indicative of viral replication) and the binding of Annexin-V (indicative of cell death) for each sample.

PROTEIN EXTRACTION AND WESTERN BLOTTING
Cells were treated with protease inhibitor cocktail (Sigma Aldrich) and lysed using Triton-X lysis buffer. Protein samples were stored at −20 °C until needed. Samples were run on 14% polyacrylamide gels, transferred to nitrocellulose membranes, blocked for one hour with milk and stained with antibodies to PARP, Caspase-3, Caspase-9 and Caspase-8, as well as VSV and actin (all from Cell Signaling Technology).

RESULTS
ENHANCEMENT OF VSV REPLICATION AND ONCOLYSIS IN PRETREATED CELLS
Treated and infected cells were harvested and analyzed by FACS at 24 and 48 hours post-infection. A GFP-expressing virus was used to indicate virus replication while Annexin-V staining was used to indicate cell death. An increase in viral replication (most prominent at 24 hours post-infection) and cell death (most prominent at 48 hours post-infection) was observed when cells were pre-treated with both HDI SAHA and MTI 5-AZA.

Consistent Increases in Virus Replication and Cell Death

Fig. 1. Use of SAHA/5-AZA combination treatment results in increased VSV replication and cell death. FACS analysis showed viral replication (indicated by GFP) and cell death (indicated by Annexin-V) at 24 and 48 hours after VSV infection under various treatment conditions. Increases in both VSV replication and cell death in cells subjected to both treatments were observed.

Fig. 2. Decreases in uninfected, viable cell populations are seen after combination treatment. Percentages of uninfected, viable cells were determined using FACS analysis. Marked decreases in this population are observed when both treatments are used (VSV+SAHA+5-AZA), while single treatments also enhance VSV replication and cell death. Treatment with SAHA, 5-AZA or the combination without VSV infection results in only a small decrease in viability.

Proportions of non-treated, non-infected cells were determined via FACS analysis by taking percentages from the bottom left quadrant, as indicated (no Annexin-V binding or GFP expression), at 12, 24, 48 and 72 hours after VSV infection. Amounts
of viable uninfected cells decreased when cells were treated with either 5-AZA or SAHA, while a more marked decrease resulted from treatment by both compounds together. Treatment with either SAHA, 5-AZA or the combination without VSV infection resulted in little cell death, indicating that increased activity was due to an increase in VSV replication and virally-induced oncolysis.

INCREASES DEMONSTRATED BY WESTERN BLOTTING

Increases in the VSV component proteins Polymerase, G Glycoprotein and Phosphoprotein/Nucleoprotein were seen after treatment. The most marked increase was seen in cells subjected to both treatments, while cells treated with either only Decitabine or only SAHA also showed increased virus replication. This pattern was reproducible both 24 and 48 hours following virus infection.

Fig. 3. VSV replication is increased following single treatment of either SAHA or 5-AZA. Notably, a more marked increase was observed following treatment with both inhibitors. These patterns of changes in VSV replication were consistent at 24 and 48 hours post-infection.

Fig. 4. Treatment with SAHA and 5-AZA at 48 hours post-infection increases activity of the intrinsic apoptotic pathway, but not the extrinsic pathway. Cleavage of Caspase-9, a hallmark of the intrinsic apoptotic pathway, is increased when cells are pre-treated with SAHA or 5-AZA, and shows a more marked increase in the case of double treatment. Meanwhile, these treatments do not affect the activity of Caspase-8, a hallmark of extrinsic apoptosis.

DISCUSSION

The PC3 cell line is generally considered to be resistant to VSV-mediated oncolysis. As such, this cell line is a valuable tool for the study of treatments to enhance virus replication in resistant cell lines. The antiviral response of this cell line is well documented, aiding the detection of variances resulting from pre-treatment. This cell line could therefore prove useful in future research to assess the potential utility of OV combination treatment.

Previous experiments have assessed the utility of VSV/HDI combination in PC3 cells. In untreated cells infected with VSV, a number of gene products relating to the IFN cascade were induced. Treatment with HDIs blunted the induction of this response (28). As expected, a down-regulation of the antiviral response was accompanied by a dramatic increase in VSV replication and oncolysis. PC3 prostate cancer cells, which are normally minimally sensitive to VSV oncolysis, were quickly killed by VSV replication when infected in the presence of HDIs (28). Little to no VSV replication and almost no cell death was observed in normal cells or in infected PC3 cells that had not undergone HDI treatment (28). This effect was also observed in mouse models. VSV replicated rapidly and showed enhanced therapeutic benefit in mice treated with HDIs.
FACS ANALYSIS SHOWS INCREASES IN VSV REPLICATION AND CELL DEATH IN TREATED CELLS.

VSV replication was assessed using FACS analysis. A dramatic increase in viral replication can be seen at 24 hours after infection in cells treated with both SAHA and 5-AZA (Fig. 1). A similarly dramatic increase in Annexin-V positive cells (indicative of cell death) is seen at 48 hours after infection (Fig. 1). This indicates some modulation of the host-cell mechanisms that increase susceptibility to VSV infection. Importantly, VSV infection alone resulted in very little virus replication, which confirms the PC3 cell line’s high level of resistance to OV treatment. Treating with SAHA, 5-AZA or the HDI/MTI combination without VSV infection also resulted in very little cell death.

TREATMENT RESULTS IN CONSISTENT INCREASE IN VSV REPLICATION AND CELL DEATH OVER 72 HOURS

The proportions of uninfected, viable cells at 12, 24, 48 and 72 hours post-infection were determined using FACS analysis (Fig. 2). A steady decline in viability was observed when infected cells were subjected to pretreatment by both 5-AZA and SAHA. A less prominent decline was visible when cells were subjected to only one type of pretreatment. Very little decline was seen in untreated or uninfected cells. Statistical analysis is required to determine whether these results indicate an additive or synergistic effect. These changes in the replication of VSV were consistent with data obtained by western blot (Fig. 3).

ENHANCED ACTIVITY IN THE INTRINSIC APOPTOTIC PATHWAY INCREASES CELL DEATH

Increases in cell death were further investigated using western blotting for components of the Caspase pathway. An increase in the cleavage of Poly-ADP Ribose Polymerase (PARP), a cellular protein that assists in the repair of DNA cleavage, was observed (Fig. 3). Cleavage of PARP results in an arrest in DNA repair, the depletion of ATP, and ultimately cell death. Treatment also resulted in an increase in the cleavage of Caspase-9, indicating an increase in the activity of the intrinsic apoptotic pathway (Fig. 3). This is consistent with past results, which indicate that HDI and MTI treatment both act via this intrinsic pathway alone (8, 9, 10). No increase in Caspase-8 was seen, which indicates that the extrinsic pathway is unaffected by these treatments, VSV and actin were observed as controls. An increase in VSV replication similar to that seen using FACS analysis was detected by western blotting. Actin expression remained consistent, an indication of similar protein loading concentrations.

Several interesting avenues exist for further research. Previous results from our laboratory indicate that treatment with HDIs regulates components of the IFN response, including the adapter molecule RIG-I, the cytokines IFNα and IFNβ, the intracellular messenger protein IRF7, ISG56 (induced by interferon signaling and having antiviral activity) and MxA (a GTPase directly involved in the inhibition of VSV replication). However, how these components will respond to low-dose SAHA treatment or to the SAHA/5-AZA combination is not yet known. Since the doses of both treatments used in this study are significantly lower than most previously reported, it is reasonable to postulate that there will be some modification of the effects of treatment on cellular processes. RT-PCR will be used to demonstrate changes in RNA levels of various proteins that mediate interferon response. Although in vitro studies have shown promising potential therapeutic benefits of this treatment, the use of animal models is an essential step in the evaluation of potential cancer therapeutics. Experiments evaluating the efficacy of these treatments in immunocompetent tumour xenograft animal models are currently underway.

Oncolytic viruses provide a novel approach to cancer therapy and may greatly improve patient care. There is already evidence that epigenetic therapeutics, including HDIs, may increase susceptibility to VSV treatment. The results of this study indicate that treatment with MTIs alone, or in combination with HDIs, can also increase susceptibility to VSV infection, virus-induced oncolysis and cell death.

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