THE EFFECTS OF CURCUMIN AND FLAVOKAVAIN B ON THE ONCOLYTIC ACTIVITY OF VESICULAR STOMATITIS VIRUS

A Thesis
by
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Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

May 2013
Department of Biology

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Abstract

THE EFFECTS OF FLAVOKAVAIN B AND CURCUMIN ON THE ONCOLYTIC ACTIVITY OF VESICULAR STOMATITIS VIRUS

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A promising cancer treatment modality is the use of oncolytic viruses to kill cancer cells. One virus that is currently being studied as an oncolytic agent is vesicular stomatitis virus (VSV). We are interested in developing the rM51R-M virus, a matrix (M) protein mutant of VSV, as a candidate oncolytic agent due to its capacity to effectively kill cancer cells and its low virulence in vivo; however, prostate cancer cell lines like PC-3 are relatively resistant to infection with rM51R-M virus. Studies have shown that the transcription factor NF-κB is constitutively active in PC-3, suggesting a role for NF-κB in induction of the antiviral immune response and invasiveness in PC-3 cells. We hypothesize that the inhibition of NF-κB in cancer cells will render them susceptible to oncolytic VSV. Our goal is to determine the ability of natural compounds known to inhibit NF-κB (flavokavain B and curcumin) to suppress the antiviral immune response that may promote VSV induced cell killing of cancer cells. Our results show that pretreatment of PC3 cells with flavokavain and curcumin inhibited the phosphorylation and activation of STAT1, a key player in the antiviral immune response. Furthermore, curcumin potentiated VSV-induced oncolysis after 24 hours post-infection; however, flavokavain B did not augment PC-3 cell death by VSV. Neither

curcumin nor flavokavain B decreased viral protein expression in vitro, as measured by ^{35}S-Methionine metabolic labeling. Additionally, pretreatment of cells with curcumin prior to infection led to a cumulative decrease in expression of the proapoptotic protein, BCL-XL as determined by Western blot analysis. These results suggest that natural products have the potential to synergize with oncolytic VSV therapies. Ongoing studies will determine the mechanisms by which these products promote anti-cancer effects and aid in oncolytic therapies.

Acknowledgments

The Office of Student Research and Graduate Student Association Senate provided partial funding for the research outlined in this thesis. I would like to thank Dr. Douglas Lyles for generously providing the virus and cell lines used in this study and for future collaborative efforts with Dr. Maryam Ahmed, of which I will have the distinct honor to be a part. I would also like to thank the incomparable Biology department at Appalachian State University and my fellow biology graduate students.

Dedication

This thesis is dedicated to my mother, whose fight against multiple myeloma has been one of the instrumental turning points in my life. I would like to dedicate this work to the rest of my loving family, whose support and guidance has turned me into the man I am today. I would like to take this opportunity to thank my thesis to my advisor, Dr. Maryam Ahmed, who took a chance on me and whose brilliance, unending patience, and support are greatly appreciated. Thank you all. You are wonderful people.

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Foreword

The research outlined in this thesis will be submitted to the *Journal of Virology*, an international peer reviewed journal. This thesis has been prepared according to the citation requirements and formatting for said journal publication.

INTRODUCTION

Current treatment options for a variety of different types of cancers are relegated to surgical intervention, radiation administration, and chemotherapeutics; however, patients undergoing these therapies suffer from poor survival rates in the case of many cancers due to multiple factors, including the development of resistance to drugs and the presence of undetectable micrometastases. Researchers have sought alternative treatments to these varied cancers. Alternative options currently available to patients include immunotherapies as well as the delivery of anti-cancer genes to the tumor site using different gene therapy protocols, including bacterial and viral delivery systems (19). In the last 20 years, there has been great interest in developing oncolytic viral agents such as modified recombinant adenovirus, for the treatment of cancer (17, 19, 33) due to the natural ability of viruses to replicate in tumors, spread to distal metastatic sites, and kill tumor cells.

Oncolytic viruses are viruses that kill cancer cells, either directly by inducing apoptosis or indirectly by eliciting an immune response (10, 19, 36, 37). Generally, viruses that are considered attractive oncolytic agents have to be able to effectively replicate in cancer cells, to have the ability to spread to surrounding susceptible tissues like metastases, and to preferentially kill cancer cells while leaving normal, healthy cells intact. Some of the first lab adapted oncolytic viruses to be studied were the recombinant adenoviruses. The major drawback of using adenovirus and other human viruses as tools for killing cancer cells is the heavy genetic recombination required to render them non-pathogenic (33). Additionally, these vectors require local administration, as intravenous administration has

shown to be less efficacious due to the liver tropism of these adenoviral vectors which results in sequestration (33). Alternative viruses which are not normally pathogenic to humans and are not hepatotropic, such as vesicular stomatitis virus (VSV), are currently being investigated for their ability to preferentially infect and kill cancer cells (2, 32, 33).

VSV is a negative-stranded neurotropic RNA virus that is a member of the family *Rhabdoviridae*, which includes the human rabies virus; however, unlike rabies virus, VSV is a pathogen of livestock, such as cattle, pigs and horses. Although VSV rarely infects humans, infection of humans with field strains of VSV is occasionally associated with acute febrile illness. Recent studies have demonstrated that less pathogenic, lab-adapted strains of VSV preferentially kill cancer cells *in vitro* and *in vivo* (2, 4). This is due to the fact that many cancer cells have defects in their antiviral response, such as the type I interferon (IFN) response, thus rendering them susceptible to the oncolytic effects of VSV (2, 4, 36, 37). In addition to this feature, there are several advantages of using VSV as an oncolytic agent. Because VSV is a pathogen of livestock, there is a low probability of acquiring immunity to this vector in the general human population. With the advent of reverse genetics techniques, the virus genome may be altered readily; and foreign genes, such as immunomodulatory genes, may be incorporated into the genome of VSV. Furthermore, little recombinant modification is required to render this virus avirulent *in vivo* (2). Currently, in our lab, two strains of VSV are being tested in preclinical trials as potential oncolytic vectors, rwt and r-M51R-M viruses. These strains are isogeneic to one another with the exception of the matrix (M) protein. The M protein of VSV has two main functions in infected cells. It is critical for

virus assembly and is responsible for the inhibition of host gene expression. The rwt virus is a recombinant wild type (wt) virus derived from several wt strains of VSV. The wt M protein in rwt virus potently shuts off host gene expression including expression of genes in the antiviral immune response. r-M51R-M virus expresses a mutant M protein containing a methionine (M) to arginine (R) substitution at position 51 of the amino acid sequence (M51R). This M51R M protein mutation renders this virus defective at inhibiting host gene expression, including expression of genes in the antiviral response (3); therefore, rM51R-M virus has been shown to be a more selective anti-cancer agent due to its ability to effectively kill cancer cells, while sparing normal cells due to enhancement of antiviral responses in these cells. A major drawback with VSV and other viral vectors is that not all cancer cells are susceptible to infection and killing by oncolytic viruses (2, 4). This is due to the fact that some cancer cells acquire mutations that allow them to maintain antiviral responses and/or develop resistance to apoptotic signals; therefore, in order to effectively treat cancer cells, it is important to understand and target these specific pathways.

My project involves the investigation of oncolytic VSV therapies for prostate cancers. Prostate cancer is a leading cause of cancer-related morbidity and mortality in men in the Western world (5, 29). Most prostate cancer-related deaths are due to advanced disease resulting from any combination of lymphatic, blood, or contiguous local spread. Management of the metastatic disease involves combination therapeutic regiments including cytotoxic, hormonal, and immunotherapeutic treatments (39). Although the expanding array of treatment options offer promise, many are constrained in terms of tolerance, efficacy, and

cross-resistance. Therefore, second-line treatment options are limited and many patients with metastatic prostate cancer die within five years of diagnosis. Because of this, there is great need for the development of non-conventional therapeutic approaches.

Previous studies by Ahmed, Cramer, and Lyles (2004) have demonstrated that prostate carcinoma cell lines exhibit different sensitivities to VSV infection. PC-3 prostate cancer cells were shown to be highly resistant to infection whereas LNCaP cells were shown to be permissive to VSV infection and subsequent oncolytic activity (2). This differential sensitivity to the virus may be due to the constitutive activation of antiviral genes detected in the PC-3 cells as compared to LNCaP cells (2, 6). Furthermore, studies have indicated that proliferative signaling pathways are enhanced in PC3 cells as compared to LNCaP cells. We hypothesize that the attenuation of antiviral and proliferative cell signaling pathways may sensitize resistant cells to oncolytic viral activity. The goal of my project is to promote the oncolytic ability of VSV by suppressing antiviral, anti-apoptotic, or proliferative pathways in prostate cancer cells using natural products that are known to inhibit these pathways. During oncogenesis multiple pathways involved in proliferation, apoptotic responses, cytokine secretion, and cell adhesion can become disregulated resulting in overproliferation and resistance to apoptotic signals. Of these signaling pathways, two relatively important signal cascades have been implicated in tumorigenesis and tumor progression, NF-κB and Akt (11, 12, 14, 24, 34, 35, 42).

NF-κB (nuclear factor kappa B) is a transcription factor that has various functions in cells, including the control of cancer development, inflammation, apoptosis, and antiviral responses (11, 14). Studies have shown that NF-κB is upregulated or constitutively active in many cancer cell lines, including PC-3 cells. A consequence of this upregulated or constitutive activity is that cancer cells develop resistance to being killed by common therapeutic agents due, in part, to upregulation of anti-apoptotic factors. Furthermore, NF-κB activity may enhance antiviral responses due to the increased expression of genes whose products regulate antiviral activity, including cytokines such as type I IFN (2, 4, 6, 14). This has generated interest in the effects of downregulating cytokines and modulating intracellular signals in the NF-κB pathway in certain cancers to make them more susceptible to chemotherapy (9, 13, 20, 24, 32, 42, 43).

Akt is a proliferative signal associated with mitogen activated protein kinases (MAPK) signaling pathways, which activate the CREB transcription factor by phosphorylation. This activation leads to increased proliferative signals within the cell in addition to upregulation of growth factor receptors (35). An overactivation of the Akt signaling pathway in cancer cells is thus associated with uncontrolled growth and tumorigenesis in some instances; therefore, the Akt signaling pathway has also become an attractive target for both chemotherapeutics and adjunctive therapies.

The PC-3 prostate carcinoma cell line has been shown to have constitutive activation of NF-κB in addition to upregulated Akt phosphorylation (3, 14, 18, 34). Constitutive or

overactivation of these pathways leads to an increase in proliferative signaling, upregulation of anti-apoptotic factors, and a decrease in pro-apoptotic factors. This disregulation results in an increase in cell division and a decrease in sensitivity to apoptosis-inducing agents, including various chemotherapeutic drugs. NF-κB has previously been shown to regulate the expression of apoptotic factors such as Bax, a pro-apoptotic factor, and the anti-apoptotic factors BCL-2 and BCL-XL (11, 32). The ratio of these factors determines the apoptotic fate of the cells (8, 31). By attenuating these pathways in these cancer cells, it may be possible to sensitize them to apoptotic responses and decrease proliferative signaling.

Recent interest in targeting NF-κB and Akt in conventional chemotherapeutics has yielded lists of bioactive pharmaceutical compounds and naturally derived plant compounds shown to impact cancer cell lines (9, 13, 20, 24, 42, 43). Of these, curcumin and flavokavain B are of particular interest given their ability to block the activity of NF-κB and Akt. Curcumin is a phenolic coloring agent found in turmeric that has been shown to exhibit strong anti-inflammatory and anti-cancer properties (9). Flavokavain B is a chalcone derived from the root of *kava kava*, a common natural remedy for anxiety that also attenuates inflammatory and proliferative signal cascades like NF-κB (42).

NF-κB regulates the innate antiviral response by controlling the transcription of the antiviral cytokines, type 1 IFN (38). NF-κB associates with an inhibitory factor, IκB, in the cytoplasm preventing NF-κB's translocation to the nucleus and subsequent transcriptional activity; however, upon phosphorylation of IkBα by IKK, NF-κB is released and is able to

translocate to the nucleus to promote transcription of a variety of genes. Studies have indicated that curcumin and flavokavain B regulate NF-κB by inhibiting the phosphorylation of IκBα by IKK, thereby inhibiting the translocation of NF-κB to the nucleus (9, 42). This block prevents the transcriptional activity of this protein resulting in the reduction of anti-apoptotic factors and inflammatory cytokines produced within these cells (9, 13, 20, 24, 32, 42, 43). What is not known is whether these compounds affect the antiviral responses in cancer cells. Studies have shown that resveratrol, another natural modulator of NF-κB activity, increases the replication of hepatitis C virus in cells in culture due to suppression of the host antiviral response (27), thus demonstrating a link between NF-κB inhibition and increased permissivity of cells to virus infection. Blocking the antiviral response in cancer cells by commonly known bioactive compounds may sensitize them to replication and killing by oncolytic viruses. In addition, by inhibiting NF-κB activity in cancer cells, one may also have the potential to decrease the ability of cells to proliferate and survive. Due to the low success rate of current treatments, it is important to test alternative agents and synergistic combination therapies in order to treat cancers that are resistant to common therapeutics.

Curcumin and flavokavain B have also been implicated in attenuating the Akt signaling pathway (13, 24). Flavokavain B has previously been shown to downregulate Akt phosphorylation sensitizing HSC-3 cells to G2/M cell cycle arrest and apoptosis via the differential expression of BCL-2/Bax (16). Curcumin inhibits Akt through the dephosphylation of downstream factors in the Akt pathway by the PTEN phosphatase (13,

24). Downregulating overactivation of proliferative signaling pathways like Akt and others are also promising targets for both conventional and adjunctive cancer therapies.

The purpose of this project is to determine if downregulating signaling pathways controlling apoptosis and antiviral responses in VSV-resistant cancer cells (PC-3) will render these cells more susceptible to infection. Naturally derived plant compounds flavokavain B and curcumin have previously been shown to decrease NF-κB and Akt activation, resulting in a decrease in anti-apoptotic factors and an increase in cell death in several cancer systems (9, 16, 24, 30, 42); however, data on combination therapies is sparse. Previous studies have shown that curcumin potentiates the action of the oncolytic action of the bacterium *Bacillus calmette-guerin* by upregulating apoptotic factors and downregulating NF-κB, demonstrating that combination therapies may be efficacious (17). The link between downregulating NF-κB and VSV mediated oncolysis has yet to be established.

We hypothesize that downregulating NF-κB and Akt activation by using curcumin and flavokavain B in PC-3 cells will render them more susceptible to VSV oncolytic activity and increase apoptotic signaling in these cells. This project will determine the ability of flavokavain B and curcumin to down-regulate the antiviral immune response in prostate cancer cells with constitutive NF-κB activity. In addition, we will determine if these compounds synergize with VSV to promote killing of prostate cancer cells that are normally resistant to VSV. Lastly, we will elucidate if these compounds enhance virus replication in the VSV-resistant prostate cancer cell line, PC-3. Upon completion of these studies, we hope

to be in a position to evaluate the utility of using such compounds to promote VSV oncolytic therapies.

MATERIALS AND METHODS

Cell Lines and Virus:

PC-3 prostate carcinoma cells were propagated in RPMI media supplemented with 10% fetal bovine serum (FBS) in 100mm tissue culture plates and incubated at 37°C + 5% CO_2. Recombinant wild-type virus and the matrix protein mutant (rM51R-M) virus were a generous gift from Dr. Doulas Lyles from Wake Forest University Baptist Medical Center (Winston-Salem, NC). Viral titers for the rwt-VSV, rwt-VSV-GFP, rM51R-VSV, and rM51R-VSV-GFP were determined by the standard VSV plaque assay analysis. Specifically, rwt-VSV and rM51R-VSV stock samples were grown in BHK cells at 75-85% confluency in DMEM plus 10% FBS. 24h post-infection the media containing progeny virus was collected and centrifuged at 1200 rpm for five minutes to remove cellular debris. Virus supernatant was aliquoted and stored at -80°C. For the plaque assay, two 6-well dishes were seeded with BHK cells and allowed to grow to 75-85% confluence. The virus stocks were subjected to 10 step serial dilutions and added to the cells in 60 well dishes in duplicate. After allowing viruses to attach to cells for 1h, cells were overlayed with oxoid agar containing DMEM plus 2% FBS. Cells were incubated at 37°C for 24-48h until plaques were visible, at which time cells were stained with Crystal Violet to visualize the plaques. Viral titer was calculated by counting the number of plaques at each dilution and multiplying this number by the dilution factor.

MTT Cell Viability Assay:

Viability of VSV-infected and flavokavain B or curcumin-treated cells was measured by MTT assay (as described in 26). PC-3 cells grown in 96-well plates were treated with 25µmol, 60µmol, and 100µmol curcumin or 1µg/mL, 5µg/mL, and 25µg/mL flavokavain B and infected with rwt-VSV and rM51R-VSV at MOIs of 10 and 50 pfu/ml. At 24h, 48h, and 72h post-infection the cells were incubated with the MTT solution provided by Roche Applied Science (Penzberg, Germany) for 4h and incubated overnight at 37°C and 5% CO_2 in solubilization solution. The optical density (OD) at 550nm was then determined by a 96-well spectrophotometer plate reader. The reference wavelength was set to 650 nm. The OD is an arbitrary measure of the number of metabolically active cells in culture. All data was normalized as a ratio to mock-infected cells. Experiments were run in triplicate and were carried out 3-5 times. Error was determined by standard deviation from the mean.

Western Blot analysis:

Relative intracellular concentrations of Bax, BCL-2, and BCL-XL were determined by Western blot analysis. B-actin was used as an internal loading control. PC-3 cells grown in 6-well plates were treated with 25µmol and 60µmol curcumin or 1µg/mL and 5µg/mL flavokavain B 6h prior to infection with rwt-VSV or rM51R-VSV at an MOI of 10. As controls, cells were mock infected, infected with virus alone, or treated with the natural compounds alone. At 18h post infection, the media was removed and cells were washed twice with 1X phosphate buffered saline (PBS). Cells were directly lysed in RIPA buffer

containing aprotinin and stored at -80°C until use. A BCA assay was conducted using the manufacturer's (Thermo Scientific) recommended protocol to determine the total protein concentration in the cell lysates. 30µg total protein for each lysate was subjected to SDS-polyacrylamide gel electrophoresis using 10% polyacrylamide gels. The proteins were transferred onto a nitrocellulose membrane. The membrane was then blocked using 5% dry milk and 0.1% tris-buffered saline-tween 20 (TBS-T) pH 7.6 20 mM Tris at room temperature (RT) for one hour and incubated with the primary antibody of interest (Cell Signaling Technology) overnight at 4°C in 5%BSA+0.1% TBS-T. The blot was then incubated with goat-anti-rabbit horseradish peroxidase (HRP) linked secondary antibody. Pico or Dura (Thermo Scientific) luminol and stable peroxide substrate were used to visualize the signal on Gememate Blue Lite Autorad film. Imagequant TL v 2005 software was used to quantitate relative band intensities. Experiments were carried out three times. Data was normalized to a ratio of β-actin expression in each lane.

^{35}S Metabolic Labeling Assay:

The rates of host and viral protein synthesis were determined by ^{35}S metabolic labeling assay. PC-3 cells grown in 6-well plates were treated with 25µmol and 60µmol curcumin or 1µg/mL and 5µg/mL flavokavain B 6h prior to infection with rwt-VSV or rM51R-VSV at an MOI of 10. Mock-infected and virus infected cells served as controls The media was removed at 24h post-infection and methionine-deficient RPMI media supplemented with L-glutamine and 2% FBS was added to the wells for methionine

starvation for 20 minutes. The medium was then removed, and the cells were pulse labeled with 200µL of methionine deficient RPMI media supplemented with 2% FBS and 20µCi of ^{35}S-Methionine. The media were removed after 15 minutes, and the cells were washed twice with PBS and lysed in 150µL RIPA buffer containing the protease inhibitor, aprotinin. Protein concentrations were determined by BCA assay (Thermo Scientific). A standard curve was generated using a serial dilution of BSA, which was used to determine the protein concentration of the cell lysates. Twenty micrograms (µg) of total protein for each lysate was then subjected to SDS-PAGE using a 12% polyacrylamide gel. The gels were stained using the Coomassie Blue staining method. Specifically, gels were fixed in a 50% methanol, 10% acetic acid solution for 1h and stained in a 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 10% acetic acid solution for 45 min with gentle agitation. The gels were later destained for 2h using a 40% methanol and 10% acetic acid solution and dried at 80°C for 2h under 26"Hg vacuum using a Bio-Rad model 583 gel dryer and Bio-Rad HydroTech vacuum pump. After drying, the gels were exposed to phosphor screen overnight and imaged using a Typhoon variable mode scanner. Host and viral protein bands were quantified using ImageQuant 5.1 software. Host protein bands were detected in the regions of the gels that were devoid of viral proteins, and band intensities were normalized as a ratio to mock. Viral M and N protein band intensities were normalized as a ratio to M and N protein band intensities in cells infected with rwt virus for 8h. Experiments were carried out three times.

Flow Cytometry Analysis:

PC-3 cells were grown in 6 and 12 well dishes and treated with 5µg/mL flavokavain B and 60µmol curcumin 6h prior to infection with GFP expressing rwt and M51R viruses at an MOI of 10. At 6, 12, 18, 24, and 48h postinfection, the cells were washed with sterile PBS, trypsinized, and resuspended in 400µL ice-cold PBS. Cells were then treated with 5µL 7-AAD for 20 minutes in the dark prior to flow cytometry analysis. A Beckman-Coulter Cytometrics FC 500 MPL was used to determine GFP expression and 7-AAD fluorescence.

RESULTS

Six hour pretreatment with Curcumin, but not Flavokavain B, augments the ability of VSV to kill PC-3 after 24 hours.

Previous data in our laboratory indicate that flavokavain B and curcumin reduce the activation of STAT1, an upstream transcription factor in the type I IFN response, in PC-3 cells upon infection with VSV (data not shown). These results suggest that these natural products may reduce the constitutive activation of the type 1 IFN response in PC-3 cells and render these cells more susceptible to infection and killing by VSV. To assess the ability of VSV to kill PC-3 cells upon treatment with curcumin, cell viability was determined by MTT cell viability assays. These assays determine the metabolic activity of cells by the action of mitochondrial reductase in live cells reducing the yellow MTT tetrazolium salt into a purple fomazan product, the OD of which can be read to derive a relative measure of live cells in culture. For data shown in Figures 1A and 1B, cells were pretreated with curcumin (25, 60 and 100µmol) for 6h, followed by infection with wt or M protein mutant VSV at MOI of 10pfu/cell. At 24h (Fig. 1A) and 48h (Fig. 1B) post infection, cell viability was determined, and data were expressed as a percentage of mock cells (cells that were not subjected to treatment with drugs and infection with viruses). Data in Figure 1 revealed that curcumin or virus-infection alone was ineffective at killing PC3 cells by 24h (Fig. 1A; untreated cells); however, 6h pretreatment of PC-3 cells with 25µmol and 60µmol curcumin significantly promoted the ability of VSV to kill PC-3 cells as indicated by a reduction in cell viability to 40% to 60% of mock cells by 24h (Fig. 1A). This result was apparent upon infection of cells

with both strains of virus and was statistically different from results seen in cells treated with curcumin or infected with virus alone ($p<0.05$, Fig. 1A). By 48h postinfection this effect was not statistically significant, yet there was an increasing trend in VSV induced cell killing ($p=0.09$, Fig. 1B). In contrast, co-treatment of cells with curcumin and virus did not lead to a decrease in cell viability as compared to cells singly treated with curcumin or infected with virus at 24h (Fig. 1C) or 48h (Fig. 1D). These data indicate that pretreatment with curcumin may sensitize PC-3 cells to VSV mediated oncolytic activity.

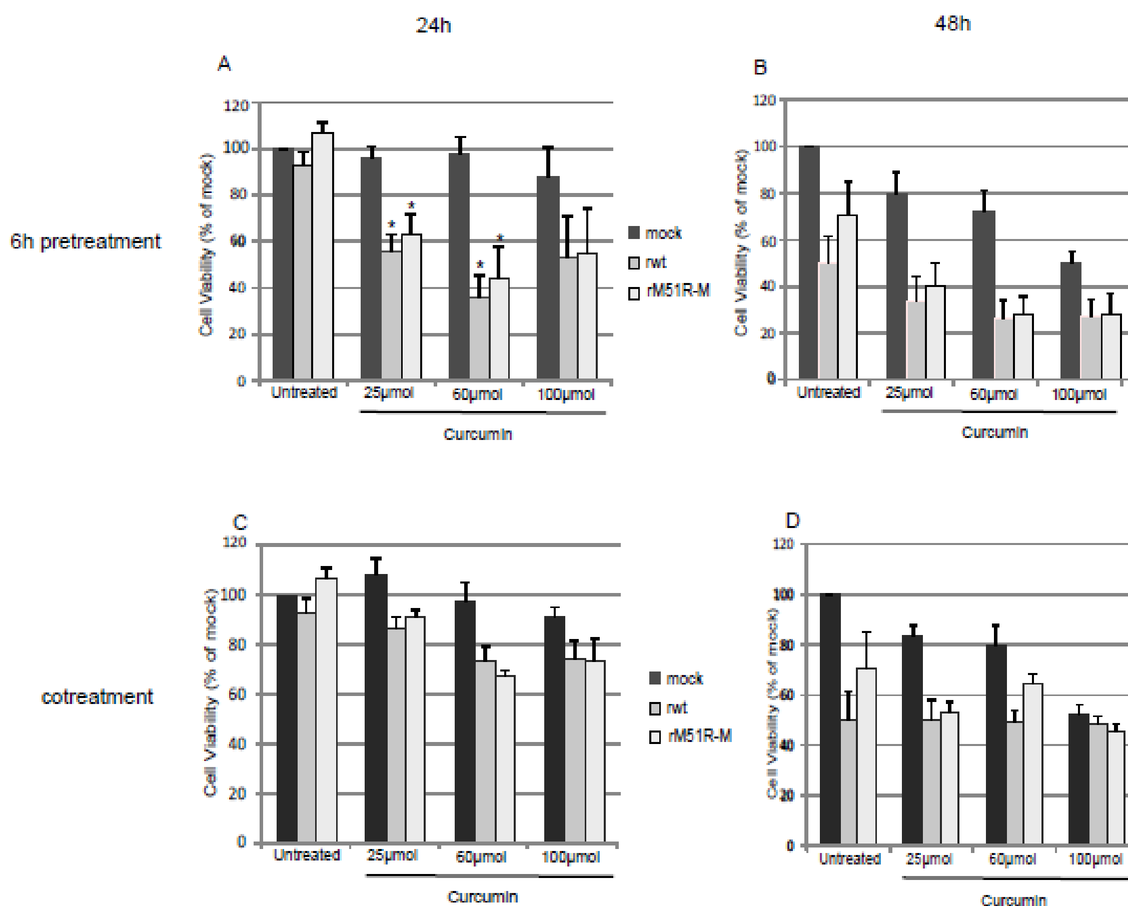

Figure 1: Pretreatment of PC3 cells with curcumin augments the oncolytic activity of VSV when infected at an MOI of 10pfu/cell. PC3 cells were pretreated with curcumin at concentrations of 25, 60, or 100µmol for 6h prior to infection with rwt and r-M51R-M viruses (A, B) or co-treated with curcumin and viruses (C, D). Cells were infected for 24h (A, C) or 48h (B, D) and subjected to MTT assay to determine cell viability. Data was normalized as a percentage of mock cells (untreated and uninfected) and is shown as the averages ± standard error for 3-5 experiments. Statistical analysis was carried out using unpaired Student's t-test.

Previous studies have shown that PC-3 cells are not synchronously infected by VSV at an MOI of 10, but rather at an MOI of 50 (6). Data in Figure 1 indicates that pretreatment with 25 and 60μmol curcumin augments VSV mediated oncolysis at an MOI of 10pfu/mL, suggesting a potential synergistic effect between pretreatment with curcumin and the virus. Synchronous infection of PC-3 cells by VSV may further augment oncolytic activity. MTT cell viability assays were used to determine if there was an increase in VSV mediated oncolysis in PC-3 cells treated with 25, 60, and 100μmol curcumin and synchronously infected for 24h and 48h. For data shown in Figure 2A and 2B, PC-3 cells were pretreated for 6h with 25, 60 and 100μmol curcumin and infected with the wt and M protein mutant virus at an MOI of 50pfu/mL. At 24h (Fig. 2A), infection with wt virus alone reduced cell viability by approximately 40% while the M protein mutant reduced cell viability by 10%; however, pretreatment with curcumin alone did not reduce cell viability in uninfected cells at 24h (Fig. 2A). Treatment with curcumin and infection by both strains of virus did not significantly reduce cell viability compared to drug alone; however, there was a trend in an increase in cell death with curcumin treatment compared to virus alone (Figure 2A). By 48h postinfection, this trend was not as apparent (Fig. 2B). Similarly to results shown in Figure 1, co-treatment with curcumin and infection by the virus did not reduce cell viability compared to virus alone (Fig. 2C and 2D). There was a slight increase in death in cells treated with 60 and 100μmol curcumin and infected with the M protein mutant virus compared to rM51R-M infection alone; however, these trends were not statistically significant (Fig. 2C). By 48h postinfection, cells pretreated with curcumin did not show any increase in cell death compared to virus

alone; although, there was a trend towards a reduction in cell viability in cells treated alone that was concentration dependent (Fig. 2D). These data suggest that treatment with curcumin can reduce PC-3 cell viability when synchronously infected with VSV, but these reductions are not significant compared to virus infection alone.

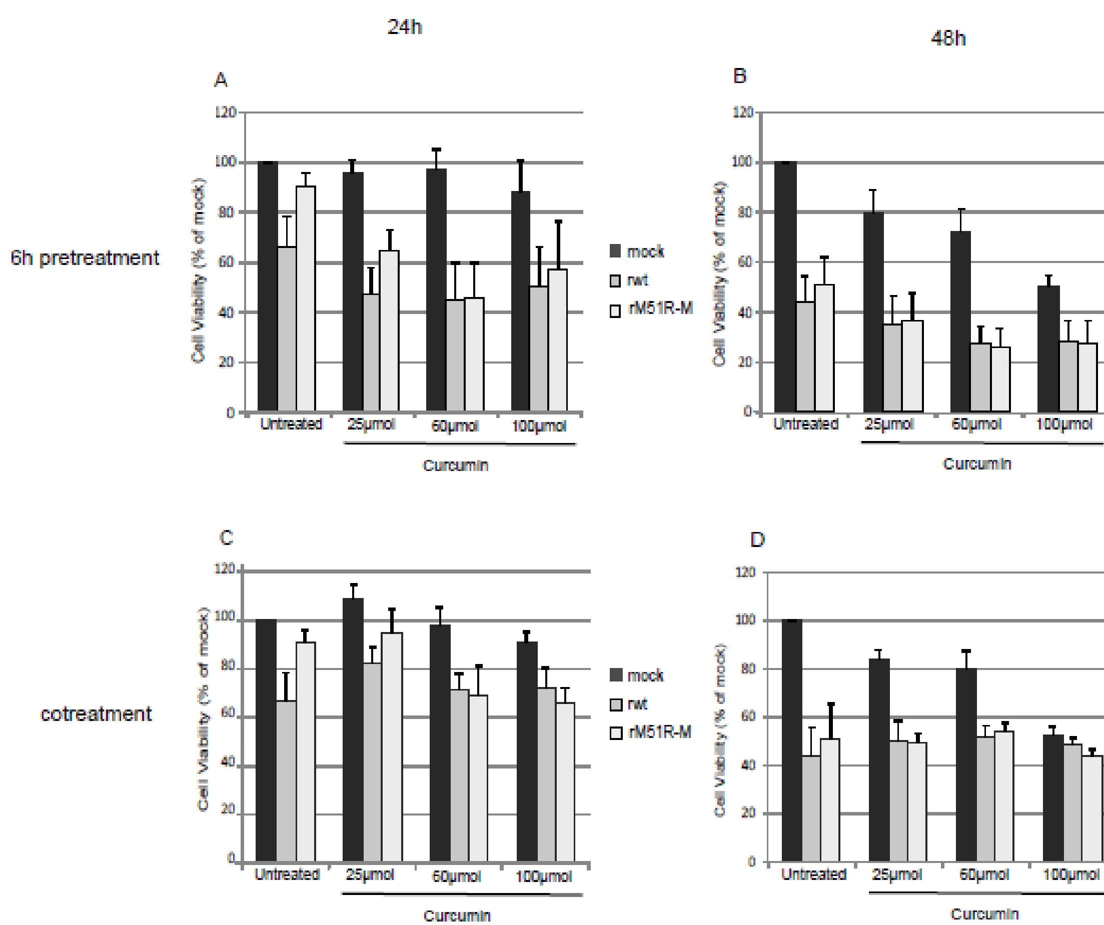

Figure 2: Pretreatment of PC3 cells with curcumin does not augment the oncolytic activity of VSV when infected synchronously at an MOI of 50pfu/cell. PC3 cells were pretreated with curcumin at concentrations of 25, 60, or 100µmol for 6h prior to infection with rwt and r-M51R-M viruses (A, B) or co-treated with curcumin and viruses (C, D). Cells were infected for 24h (A, C) or 48h (B, D) and subjected to MTT assay to determine cell viability. Data was normalized as a percentage of mock cells (untreated and uninfected) and is shown as the averages + standard error for 3-5 experiments. Statistical analysis was carried out using unpaired Student's t-test.

In addition to curcumin, other phytochemicals have been shown to differentially regulate cell proliferation and survival pathways. One of these phytochemicals, flavokavain B, also downregulates the NF-κB pathway by reducing IKK activity thereby sensitizing cells to apoptotic stressors (43). Treatment with flavokavain B could potentially render PC-3 cells susceptible to VSV infection by attenuating the antiviral and proliferative response pathways regulated by NF-κB. To determine whether flavokavain B also augments VSV's oncolytic capacity, PC-3 cells were treated in a manner similar to the curcumin treatments previously outlined and subjected to MTT cell viability assays. Cells were pretreated for 6h or co-treated with 1µg/mL, 5µg/mL, or 25µg/mL flavokavain B and infected with the wt and M protein mutant strains of VSV or mock treated. Cell viability was then assessed 24h and 48h postinfection by MTT analysis. Data was normalized to mock infected cells and subjected to an unpaired Student's t-test, comparing treated and infected cells to viral infection alone. Data shown in Figure 3 represents PC-3 cells infected by both strains of virus at an MOI of 10pfu/cell. Unlike the results for curcumin shown in Figure 1, a 6h pretreatment with flavokavain B did not significantly reduce cell viability by 24h or 48h postinfection compared to virus alone (Fig. 3A and 3B, respectively). Pretreatment with 25µg/mL flavokavian B alone did reduce cell viability 30% by 48h as compared to mock cells, but this trend was not significant (p=0.1, Fig. 3B). Similarly, cotreatment with flavokavain B and infection by the virus did not reduce cell viability 24h or 48h postinfection compared to virus alone (Fig. 3C and D, respectively). Treatment with flavokavain B alone did not reduce cell viability compared to mock infected cells (Fig. 3A-3D, black bars). The synergistic effect

between increased cell killing and sub-synchronous infection by VSV observed with curcumin pretreatment was not evident with flavokavain B treatment.

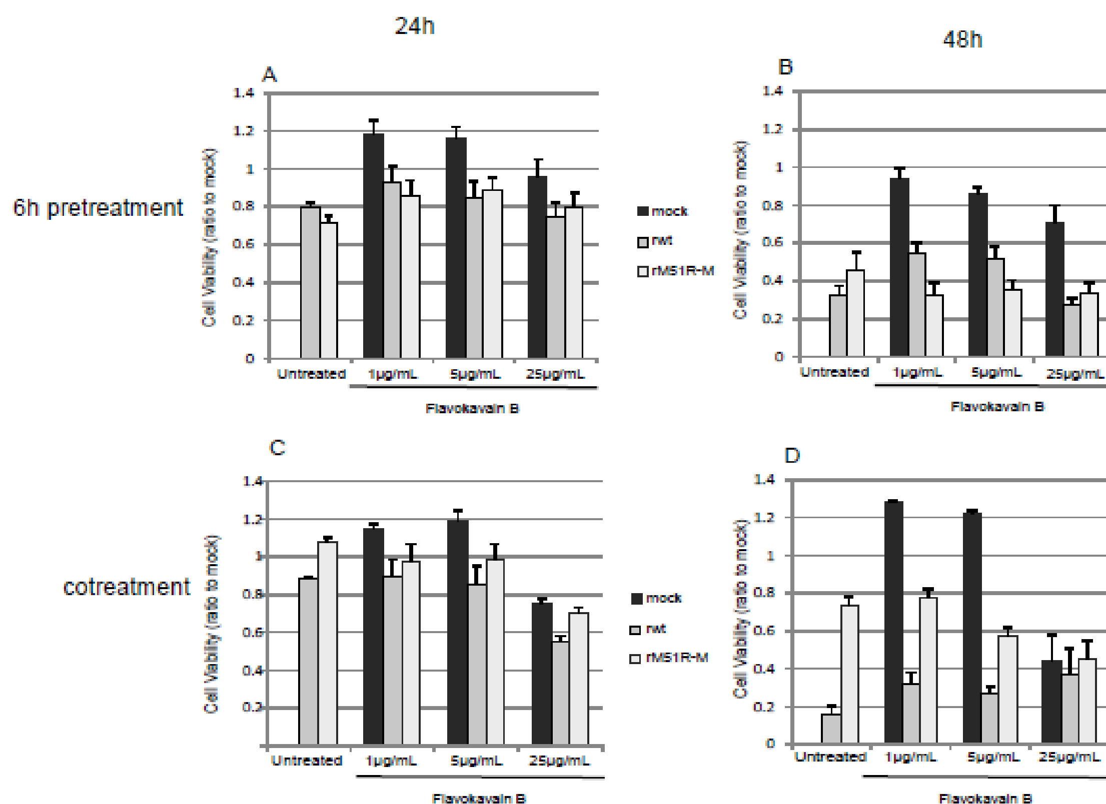

Figure 3: Cotreament or pretreatment with flavokavain B does not potentiate the oncolytic activity VSV at an MOI of 10pfu/mL. (A) PC-3 cells pretreated 6h with 1µg/mL, 5µg/mL, and 25µg/mL flavokavain B prior to infection. Data indicates 24h postinfection. (B) PC-3 cells 48h postinfection with a 6h pretreatment of flavokavain B at the indicated concentrations. (C) PC-3 cells 24h postinfection cotreated with flavokavain B at the indicated concentrations. (D) Cell viability of PC-3 cells cotreated with flavokavain B at the indicated. Data indicates 48h postinfection. Data indicates average cell viability as a percentage of mock ± standard error for 3 experiments.

To determine whether flavokavain B augments VSV induced cell killing upon synchronous infection, cells were infected with wt and M protein mutant VSV at an MOI of 50pfu/cell and treated with flavokavain B in the manner previously described. These cells were then subjected to MTT cell viability assays 24h and 48h post-infection. Data shown in Figure 4 represents cell viability as a ratio to mock versus treatment. As shown in Figure 4A, a 6h pretreatment at the indicated concentrations of flavokavain B does not augment cell killing compared to virus infection alone by 24h post-infection. This trend persists by 48h post-infection; however, there is a reduction of cell viability of 30% in cells treated with 25µg/mL flavokavain B alone (Fig. 4B, black bars). Co-treatment with flavokavain B at each concentration and infection by the virus did not reduce cell viability compared to virus infection alone at both 24h and 48h post-infection (Fig. 4C and 4D). There was an approximately 50% reduction in cell viability with cells treated with 25µg/mL alone. These data suggest that flavokavain B does not enhance VSV's ability to kill PC-3 cells even under conditions of synchronous infection. Flavokavain B does exhibit some cell killing activity in PC3 cells in the absence of virus infection.

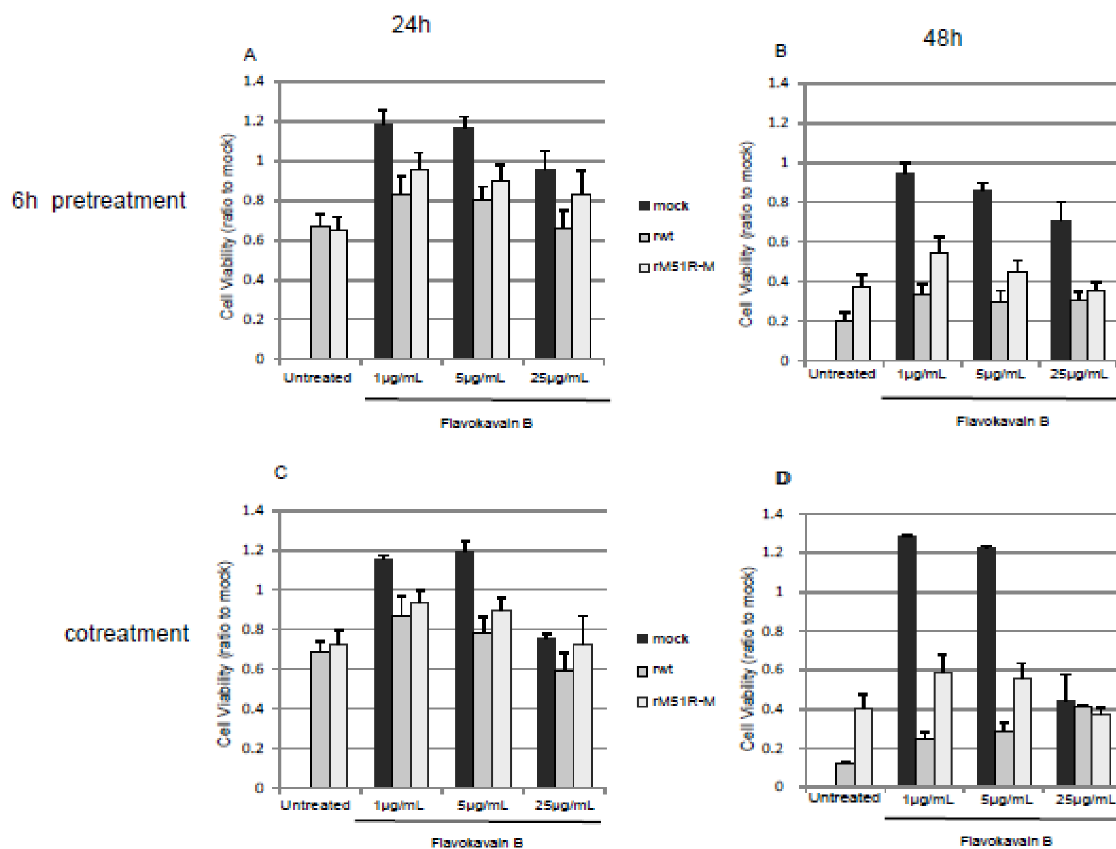

Figure 4: Cotreament or pretreatment with flavokavain B does not potentiate the oncolytic activity VSV when PC-3 cells are synchromously infected at an MOI of 50pfu/mL. (A) PC-3 cells pretreated 6h with 1µg/mL, 5µg/mL, and 25µg/mL flavokavain B prior to infection. Data indicates 24h postinfection. (B) PC-3 cells 48h postinfection with a 6h pretreatment of flavokavain B at the indicated concentrations. (C) PC-3 cells 24h postinfection cotreated with flavokavain B at the indicated concentrations. (D) Cell viability of PC-3 cells cotreated with flavokavain B at the indicated. Data indicates 48h postinfection. Data indicates average cell viability as a percentage of mock $\pm$ standard error for 3 experiments.

Curcumin and flavokavain B do not significantly alter expression of anti-apoptotic and pro-apoptotic factors in conjunction with VSV

Previous studies have shown that VSV preferentially induces the intrinsic apoptotic pathway by inactivating BCL-XL and increasing Bax expression in permissive HeLa cells (28). The increased sensitivity of PC-3 cells pretreated with curcumin to VSV oncolytic activity may be due to the differential regulation of factors in the intrinsic apoptotic pathway. Western blot analysis was used to determine whether the reduction of cell viability correlated to a reduction in the expression of the anti-apoptotic factors BCL-2 and BCL-XL or an increase in the expression of the pro-apoptotic factor Bax. Cell viability data shown in Figure 1 indicate that 6h pretreatment with curcumin correlated to the greatest increase in VSV mediated oncolysis after 24h, suggesting that the differential regulation of cell signal cascades promoting a synergistic effect between the drugs and the virus may take place early in treatment and infection. To test this, PC-3 cells were pretreated with 1 and 5μg/mL flavokavain B and 25 and 60μmol curcumin for 6h prior to infection by the wt and M protein mutant VSV at an MOI of 10pfu/cell. Cell lysates were then collected 24h post-infection and subjected to SDS-PAGE and immunoblot analysis for BCL-XL, BCL-2, and Bax concentrations. Data shown in Figure 5 are the relative band intensity normalized as a ratio to β-actin. Interestingly, there was a decrease trend in BCL-XL expression in cells treated with curcumin and infected with VSV (Fig. 5A) even though results were not significant ($p=0.08$). This trend reflects the synergistic cell killing effect seen in Figure 1 with this treatment combination. Furthermore, BCL-XL expression was not consistently down-regulated in cells

treated with flavokavain B and infected with VSV, except when cells were treated with flavokavain at 25µg/ml and infected with rM51R-M virus (Fig. 5B). BCL-2 expression was not altered by treatment with curcumin and flavokavain B and infection by the virus compared to mock (Fig. 5C and 5D). Interestingly, treatment with drug alone and infection by the virus alone did not reduce BCL-XL or BCL-2 expression (Fig. 5A-D). Additionally, Bax expression was not altered by flavokavian B, curcumin, or infection by the virus (Fig. 5E and 5F). These results suggest that the increase in cell killing by VSV in PC-3 cells treated with curcumin may be due to a decrease in expression of the antiapoptotic factor, BCL-XL; however, the antiapoptotic factor BCL-2 and the proapoptotic factor, Bax were minimally affected by this treatment combination. Furthermore, we cannot rule out the possibility that cells treated with curcumin and infected by the virus may not be regulated through the intrinsic apoptotic pathway controlled by Bax but rather another mechanism.

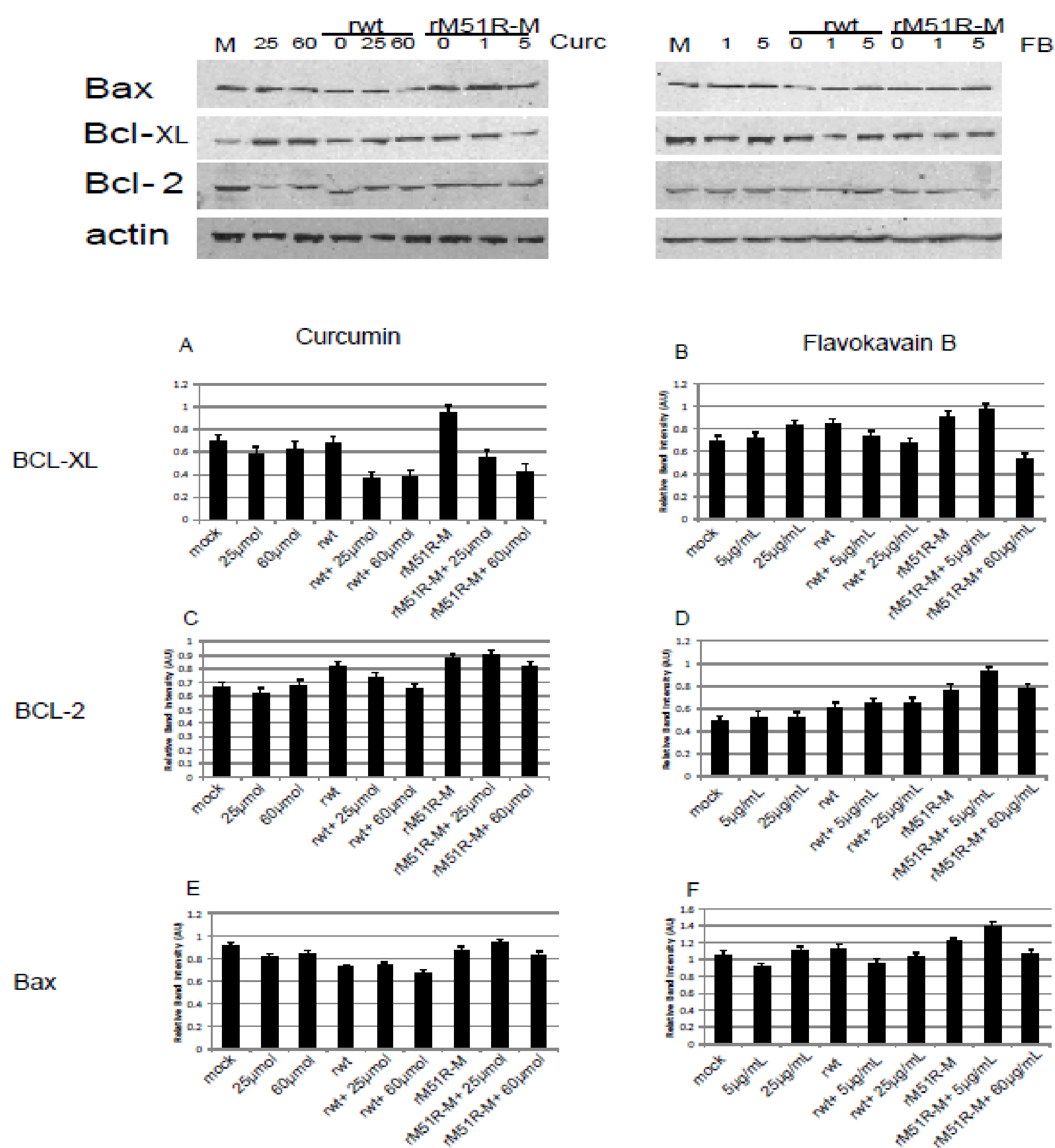

FIGURE 5: Expression of apoptotic factors in response to a 6h pretreatment of flavokavain or curcumin and infection of both strains of virus at an MOI of 10pfu/mL. Lysates were collected 24h postinfection and subjected to Western blot analysis with antibodies to BCL-XL (A, B), BCL-2 (C, D) and Bax (E, F). Data represents average band intensity normalized to β-actin ±standard error for 3 experiments. Top panels indicate images from bands subjected to development on photographic film.

Flavokavain B and Curcumin do not reduce viral or host protein expression

Due to data shown in Figure 5, it is possible that the increase in cell death may not be due to the Bax regulated apoptotic pathway, which is the pathway wild-type VSV uses to induce apoptosis in permissive cells, but rather another mechanism. Previous studies have shown that naturally derived plant compounds have the potential to augment viral gene and protein expression (27). We hypothesized that treatment with curcumin or flavokavain B may enhance viral replication and increase potential cytopathic effects exerted by the virus. One indication of virus replication is the expression of viral proteins in infected cells. To determine if these compounds augmented or reduced viral protein replication, ^{35}S metabolic labeling was used to assay the relative rates of viral and host protein expression in infected cells. Data in Figure 6 show results in cells treated with curcumin and infected VSV. PC-3 cells were co-treated (Fig. 6 A,C) with 25 and 60µmol curcumin, or pretreated 6h prior to infection (Fig. 6B,D) by wt and rM51M mutant VSV at an MOI of 10, or mock treated. At 24h postinfection, cells were starved of methionine using methionine deficient media and pulse-labeled with ^{35}S-methionine for 15min. Cells were lysed, subjected to SDS-PAGE, and phosphorimaged. Representative images of SDS-PAGE gels are shown in the top of Figure 6. Bands corresponding to viral proteins are shown on the left. Viral M protein synthesis in each lane was quantified as a ratio of the M protein expression in cells infected with rwt VSV for 6 h. Host protein synthesis was determined by measuring the rates of host proteins in bands devoid of viral proteins and is expressed as a ratio of host protein expression in mock-infected cells. As shown in Figure 6A, rates of viral protein expression were greater in cells

infected with rwt virus as compared to rM51R-M virus at 6h post-infection. By 12h post-infection, the expression of viral proteins in PC3 cells infected with each virus was similar. Pretreatment of cells with curcumin did not significantly alter viral protein expression upon infection with rwt or rM51R-M virus, indicating that curcumin does not affect virus replication in PC-3 cells. Host protein synthesis in cells infected with rwt virus decreased to 50% of mock by 12 h post-infection (Fig. 6B). In contrast, host protein synthesis was not inhibited by rM51R-M virus by 12 h postinfection due to enhancement of antiviral responses in cells infected with this virus (2). Upon pretreatment of cells with curcumin, host protein synthesis did not significantly change as compared to cells infected with each virus alone. These data show that curcumin pretreatment does not affect rates of host protein synthesis in cells infected with either virus. This is in contrast to cell viability results shown in Figure 1. Similarly to results in Figures 6A and 6B, co-treatment with curcumin did not impact viral and host protein expression compared to that in cells infected with virus alone Figure 6B and Figure 6D, respectively.

To determine the rates of viral and host protein synthesis in cells treated with flavokavain B in conjunction with VSV, experiments similar to those shown in Figure 6 were conducted. Cells pretreated with 25µg/mL flavokavain B and infected with the rwt virus showed a 0.4 fold increase in viral protein expression at 6h post-infection, but this increase was not significant compared to infection by the wt virus alone (Fig. 7A). A similar trend was seen in cells pretreated with flavokavain B and infected with rM51R-M virus for 12h. Viral protein expression in cells cotreated with flavokavain B and infected by both wt and the

M protein mutant virus decreased slightly as compared to that in cells infected with virus as well, but again, results were not significant (Fig. 7B). Host protein synthesis at 6h and 12h in treated and infected cells was not altered compared to cells infected by the virus alone in cells pretreated or cotreated with curcumin (Fig. 7C and D, respectively). Overall, host protein expression in cells infected with the M protein mutant was higher than cells infected with rwt, which correlates to previous studies showing that the M protein mutant is defective in inhibiting host protein synthesis in cells with intact antiviral responses (3). In total, these data indicate that treatment of PC-3 cells with curcumin and flavokavain B does not significantly alter viral or host protein synthesis in infected cells, implying that these compounds to not negatively impact viral replication in infected cells

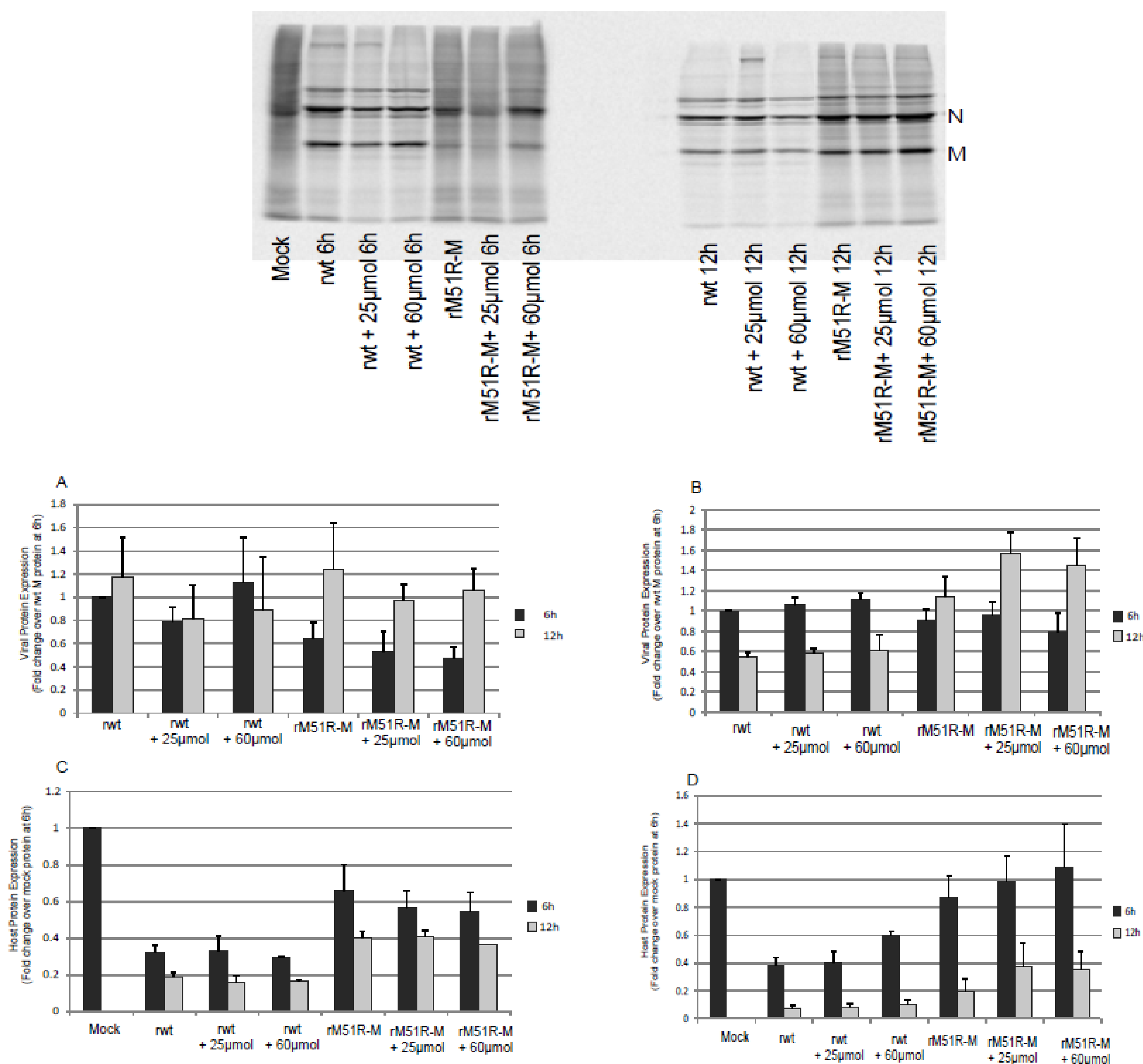

Figure 6: Host and viral protein synthesis upon treatment of cells with curcumin and infection with virus for 6h and 12h as determined by ^{35}S metabolic labeling. PC-3 cells were treated (B,D) or pretreated 6h (A,C) with curcumin at the indicated concentrations and infected with both strains of virus at an MOI of 10. Viral protein expression (A, B) is shown as fold change over rwt M protein at 6h postinfection. Host protein expression is shown as a percentage of mock (C, D). Data shown is an average of three experiments ± standard error.

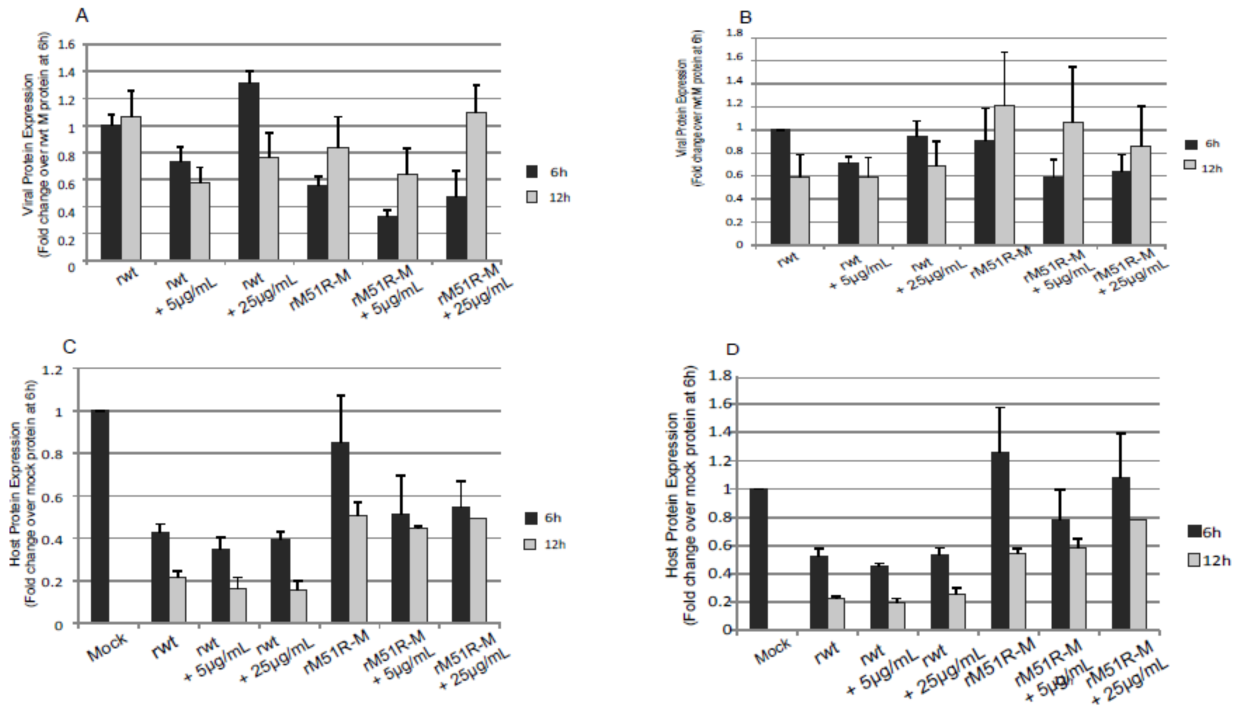

Figure 7: Host and viral protein synthesis upon treatment of PC3 cells with flavokavain B and infection with VSV for 6h and 12h as determined by ^{35}S metabolic labeling. PC-3 cells were treated (B,D) or pretreated 6h (A,C) with flavokavain B at the indicated concentrations and infected with both strains of virus at an MOI of 10. Viral protein expression (A, B) is shown as fold change over rwt M protein at 6h postinfection. Host protein expression is shown as a percentage of mock (C, D). Data shown is an average of three experiments ± standard error.

To further measure virus replication in infected and treated cells, the fluorescence of green fluorescence protein (GFP) was measured in rwt virus engineered to express GFP (rwt-GFP). The gene for GFP was inserted into an extra transcriptional unit between the M and G genes of rwt virus such that GFP expression is a measure of expression of viral genes in infected cells. Cells were pretreated with curcumin or flavokavain B for 6h and infected

with rwt-GFP at an MOI of 10pfu/cell. At 6 and 12h postinfection, cells were harvested and the percentage of cells expressing GFP was determined by flow cytometry. Mock infected cells and cells treated with 25µg/mL flavokavain B or 60µmol curcumin were negative for GFP expression at both 6 and 12h (Fig. 8). By 6h post-infection, there was a slight reduction in GFP expression in cells treated with either drug and infected with both strains of virus. Specifically, there was an approximately 10% reduction in the percentage of GFP-positive cells upon drug treatment and infection with rwt virus and a 30% reduction for the M protein mutant virus (data not statistically significant). By 12h postinfection, these reductions were no longer evident, as nearly 100% of all infected cells were positive for GFP expression. These data suggest that curcumin and flavokavain B may slightly reduce early viral replication; but by 12h, the effects of these drugs on virus replication are not apparent. These natural products do not inhibit viral genome replication which may benefit combination therapeutic approaches with oncolytic viruses like VSV by being innocuous to viral replication processes.

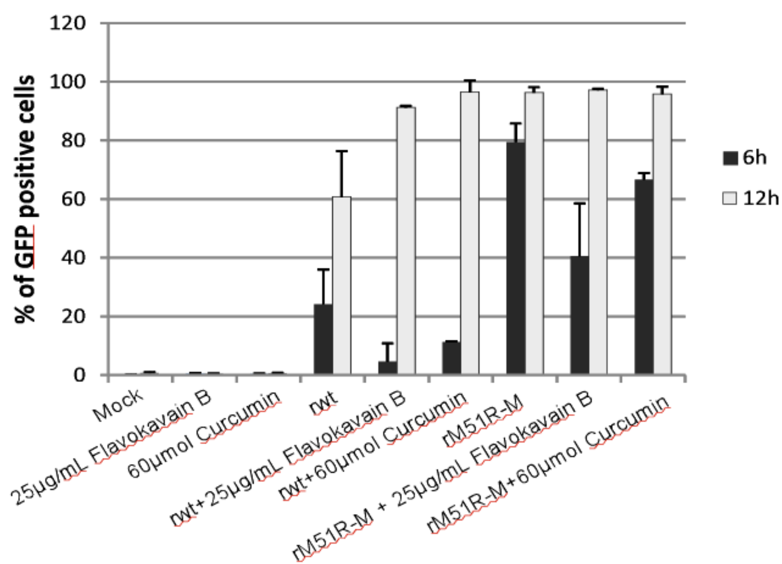

Figure 8: Flow cytometry analysis of PC-3 cells treated with flavokavain B (25µg/mL) or curcumin (60µmol) and infected with GFP expressing virus for 6 and 12 hours. Treatment with either drug did not significantly reduce or enhance GFP expression in infected cells. Data shown is the average of three experiments + standard error.

DISCUSSION

Numerous studies have shown that many cancer cells are resistant to the oncolytic effects of virus infections (2, 3). Studies have suggested that this may be due, in part, to the ability of cancer cells to retain antiviral responses and to prevent virus replication and cell death (6). The data presented here suggests that naturally derived plant compounds can augment the ability of an oncolytic viral vector to kill a prostate cancer cell line naturally resistant to the cytopathic effects of VSV. Furthermore, data collected in our lab indicate that the natural compounds, curcumin and flavokavain B, reduce the activation of an essential transcription factor in the antiviral response, STAT1 (data not shown). Recent studies have shown that equol and resveratrol effectively potentiate the action of a mutant adenoviral oncolytic vector, AdΔΔ, in PC-3 and DU145 cells by increasing apoptotic signals from PARP and caspase-3 cleavage (1). Thus, there is increasing evidence that plant compounds can effectively synergize with oncolytic viral vectors perhaps by increasing sensitivity of cells to apoptosis and by decreasing production of antiviral genes.

Data shown here indicates that a 6h pretreatment of curcumin reduces PC-3 cell viability in a concentration dependent manner upon infection with both the wt and M protein mutant strains of VSV (Fig. 1A). The synergistic effect of curcumin and virus on killing of cancer cells was observed at 24h postinfection; but by 48h, results were not significant. In addition, co-treatment of curcumin and virus did not promote killing of PC3 cells as compared to virus or curcumin alone; therefore, these results indicate that pretreatment of cells with curcumin is required to enhance the ability of VSV to kill cancer cells. This may

be due to attenuation of proliferative and/or immune signaling pathways by curcumin allowing virus to effectively enter, replicate, and induce cytopathology in these cells. This also reinforces the data by Carey et al. (6) that the early steps of the virus replication cycle are delayed in PC3 cells. They show that PC-3 cells constitutively express antiviral factors that reduce primary transcription upon infection reducing genomic expression. Additionally, early protein expression is reduced in PC-3 cells upon asynchronous infection with VSV. The reduction of viral protein and RNA expression in the early phases of infection is due to the over-expression and constitutive activation of antiviral effector proteins like OAS, ISG15, and Mx proteins (6). Carey et al. were able to demonstrate that PC-3 cells are more resistant to VSV infection by the activation of antiviral factors in these cells. Curcumin may be decreasing the expression of these factors thus rendering PC-3 cells more susceptible to VSV infection and subsequent oncolysis.

My results also showed that the synergistic effect of curcumin and virus was only observed when cells were infected asynchronously (MOI 10pfu/cell) such that not all cells were initially infected. When cells were infected synchronously at a MOI of 50pfu/cell, curcumin was unable to augment VSV-induced cell killing. A synchronous infection of cells measures the cytopathic effect of virus on all cells in the environment, whereas asynchronous infections measure the ability of the virus to spread throughout the culture where it is influenced by factors in the environment. These results indicate that VSV spread was promoted by curcumin pre-treatment and suggest that the cytopathic effect being exerted by the virus may not solely be responsible for the increase in PC-3 cell death. Attenuation of the

immune response by curcumin may be enhancing the ability of virus to spread to surrounding cells and to induce their effects. In our lab, we are currently investigating the antiviral response in prostate cancer cells treated with curcumin by measuring the type I IFN secreted in the culture supernatant.

It is a possibility that the attenuation of proliferative and immune processes in PC3 cells by curcumin may be transient. Previous studies have shown that curcumin reduces NF-κB activity and several other proliferative signal pathways in a time and concentration dependent manner (9, 13, 20, 24, 32). The increase in cell death by VSV upon pretreatment with curcumin may be attributed to the reduction in crosstalk between proliferative pathways and an increase in apoptotic signaling. Future work in our lab will determine whether the activity of the transcription factor NF-κB, which controls the transcription of cytokines responsible for the production of antiviral factors, and Akt, a proliferative signal upregulated in many cancer cell lines, are altered upon combination treatments with flavokavain B and VSV .

Similar to curcumin, studies have shown flavokavain B also exerts inhibitory effects on the activity of NF-κB. NF-κB regulates genes responsible for prosurvival factors and apoptotic resistance, like Bax and the inhibitory BCL protein family in addition to the type I IFNs necessary for inducing antiviral responses. Type 1 IFNs stimulate the production of the antiviral factors by the induction of the JAK/STAT cell signaling pathway. By interacting with their receptors on cells, IFNs activate the STAT complexes. Jaks are recruited to the

receptor and phosphorylate the STATs (STAT1 and STAT2) resulting in the formation of the IFN-stimulated gene factor 3 (ISGF3) complex. This complex contains a STAT1-STAT2 heterodimer and interferon regulatory factor 9 (IRF9) that, when phosphorylated, translocates into the nucleus as a transcription factor that binds to the interferon stimulated response element (ISRE) promoter region. The STAT transcription factors regulate genes in the IFN-stimulated response element (ISRE) that produce mRNAs expressing antiviral response factors. Antiviral factors include MX proteins, which reduce viral mRNA synthesis, ISG proteins, which shut off global protein expression, and cytoplasmic viral sensory molecule like RIG-I (23). Constitutive activation of NF-κB correlates to an increased antiviral response. An attractive treatment strategy in downregulating NF-κB is the use of modulatory plant compounds like curcumin.

In addition to its effects on the antiviral response, NF-κB is also responsible for the production of survival responses like the upregulation of antiapoptotic factors and the production of inflammatory cytokines (i.e. interleukins and type 1 IFNs), and mediates crosstalk with proliferative pathways. For example, NF-κB mediates crosstalk with the Akt pathway through cyclooxygenase-2 (COX-2) (13, 24). In normal physiological responses, upstream signals cause IKK (IκB kinase) to phoslphorylate IκB, the inhibitory factor of the inactive NF-κB complex, which targets IκB for proteolytic degradation allowing the p50 and p65 subunits of NF-κB to translocate to the nucleus and begin its transcriptional activity. In some cancers NF-κB is constitutively active thus upregulating proliferative responses mediated by MAPK and Akt, anti-apoptotic factors, proinflammatory cytokines and antiviral

factors (24). NF-κB is only one facet of proliferative signaling pathways, though. Others, like the Akt pathway, are vital to normal and pathological proliferative signaling processes; therefore, it is possible that the synergistic effects of curcumin and virus on PC-3 cells may be mediated by multiple pathways.

Studies have implicated the Akt signaling pathway is in the proliferative, survival, and the invasive nature of PC-3 cells. Therefore, the suppression of this pathway by curcumin may be contributing to the ability of VSV to promote killing of PC-3 cells. Akt controls the transcription of mRNAs responsible for resistance to apoptotic signaling and the progression of the cell cycle. Both flavokavain B and curcumin have previously been shown to downregulate Akt activation in cancer cells. Specifically, Hseu et al. (16) demonstrated that flavokavain B reduces BCL-2 and Bax expression in oral carcinoma cells HSC-3 by reducing Akt and MAPK signaling in addition to increasing reactive oxygen species (ROS), which induces mitochondrial outer membrane permeabilization (MOMP) and apoptosis in treated cells (16). Similarly, Gunadharini et al. (12) demonstrated that the total extract of neem leaves induced apoptosis in LNCaP and PC-3 cells and reduced Akt signaling. They demonstrated that this plant extract reduced BCL-2 mRNAs in treated cells and increased the expression of the pro-apoptotic factor Bad while decreasing Akt phosphorylation (12). The reduction of Akt activation by other naturally derived plant compounds has been shown to induce apoptosis and inhibit cell cycle progression in cancer cells suggesting a potential pathway by which curcumin and VSV may be synergistically acting.

Normally, the secretion of growth factors stimulates Akt activity. When these growth factors bind to receptors on the plasma membrane Ras and SOS are phosphorylated, initiating a signal cascade that ultimately leads to the upregulation of additional growth factor receptors in addition to anti-apoptotic proteins. Overstimulation or constitutive expression of growth factor receptors can lead to oncogenesis (35). An important mediator of this process is PTEN, a tumor suppressor that inhibits the phosphorylation of Ras by the growth receptor. Specifically, Mahimainathan and Choudry (25) showed that PTEN inactivates Platlet-derived Growth Factor (PDGF) and elucidated an unobserved mechanism not demonstrated in PTEN previously. PTEN was previously shown to inhibit PIP2 kinase activity, thereby resulting in a decrease of PIP3 and subsequently Akt activity (25). Akt is responsible for expressing proliferative and antiapoptotic factors; thus overexpression results in greater proliferative signaling and an increased resistance to apoptotic signals. In a study by Shulka et al. (34), prostate carcinomas differentially expressed lower levels of PTEN and higher phosphorylated Akt expression, particularly in LNCaP and PC-3 cells (34). By increasing Akt proliferative responses and decreasing PTEN tumor suppressor activity, prostate carcinomas like the PC-3 cells used in this study more robustly express proliferative and apoptotic factors. By attenuating these responses in PC-3 cells through the use of phytochemicals like curcumin and flavokavain B, these cells may be rendered more susceptible to apoptotic signals from therapeutics or an oncolytic vector like VSV.

In a review by Steelman et al., mutations in the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways are implicated in the apoptotic resistance and increased

proliferative signal exhibited by a multiplicity of cancer cell lines (35). The hyperactivation of these pathways results in crosstalk with other inflammatory pathways, like the NF-κB pathway and JAK/STAT pathway, which induces the transcription of antiapoptotic factors like the BCL-2 family, the overactivation of which increases BCL-2 antiapoptotic proteins in the cell. An upregulation in growth receptors upstream of the Akt pathway and mutations within the signal transduction molecules and inhibitory factors are required to overactivate Akt and increase proliferative signaling, specifically through MAPK activation. Additionally, mutations occur more frequently in upstream activators of Akt like Ras, Raf, and ERK, and rarely are associated with Akt mutations in human cancers. Hyperactivation of Akt usually results from overactivation of upstream regulators and the inhibition of tumor suppressor inhibitors like PTEN and p53 leading to a loss of sensitivity to apopotic signals. In some instances, weak mRNA expression regulated by Akt and ERK is overactive, leading to an increase in apoptotic resistance as well (35).

The activation of Akt has been shown to promote the invasiveness of PC-3 and DU145 prostate cancer carcinomas. Shulka et al. (34) demonstrated that PC-3 and DU-145 cells treated with PI3K and PTEN inhibitors, both of which are upstream activators of Akt, decreased the expression of matrix metalloproteinase (MMP)-9 and urokinase-type plasminogen activator (uPA) which are established markers for cellular invasion. They also show that these inhibitors decreased Akt phosphorylation but did not appreciably affect the total levels of endogenous Akt (34). These results suggest that Akt plays an important role not only in cell survival and proliferative responses, but also are important in the progression

of prostate cancer metastasis. Targeting Akt using natural therapeutics may render PC-3 cells more susceptible to apoptotic signals from VSV infection and reducing proliferative responses in these cells.

Several studies have detailed some of the cell signaling pathways affected in cancer cells by curcumin, which may partially be responsible for the synergistic action of curcumin and VSV in Figure 1. Curcumin has been reported to inhibit proliferative pathways either directly or indirectly. Curcumin has been shown to reduce pro-inflammatory factors like IL-1, IL-6, TNFα, and COX-2, which are controlled by the NF-κB transcription factor (13, 24). COX-2 mediates crosstalk between the NF-κB pathway and the Akt and MAPK cell proliferative pathways. Furthermore, curcumin has been shown to reduce Akt and mTOR activation leading to the inhibition of downstream factors like PI3K and PDK1 (7, 9, 18). Curcumin not only serves to inhibit proliferative pathways through attenuation of NF-κB activity, but also by directly inhibiting activation of Akt, mTOR and MAPKs. These pathways control proliferative responses upon induction by upstream signaling events, such as the binding of a mitogen to a growth receptor. Aberrant expression during oncogenesis decouples these responses from the appropriate receptors leading to overactivation of proliferative signaling in the absence of external signals.

Previous studies have shown that curcumin upregulates TRAIL receptors in LNCaP prostate cancer cells and promotes apoptosis by upregulating the pro-apoptotic factors Bax and Bak and by reducing expression of the anti-apoptotic factor, BCL-2, in treated LNCaP

and PC-3 cells (32). My results did not show alterations in several pro- and anti-apoptotic factors upon treatment with curcumin. There was only a measurable decrease in BCL-XL expression in PC-3 cells upon infection with VSV. We are unsure why our results differ from previous studies and are currently investigating the possibility that other apoptotic pathways may be involved.

Similar to curcumin, carnosic acid, another natural product that chemosensitizes cancer cells, reduces p50 and p65 NF-κB subunit translocation into the nucleus of treated PC-3 cells and promotes apoptosis by the intrinsic pathway by increasing the proapoptotic factor Bax and reducing the expression of the antiapoptotic factor BCL-2. In addition, carnosic acid promotes chemosensitation through the extrinsic pathways that caspase 8 and 9 inhibitors are unable to reverse (18). This study suggests that natural compounds can modulate several apoptotic and proliferative pathways thereby rendering cancer cells more sensitive to killing. By reducing the crosstalk between different survival pathways, curcumin may be sensitizing PC-3 cells to VSV oncolytic activity; however, the exact pathways and mechanisms have yet to be revealed.

In contrast to curcumin, data presented in Figures 3 and 4 suggest that flavokavain B does not augment the ability of VSV to kill PC-3 cells compared to virus infection alone. This may be due to the differences in which flavokavain B affects signaling pathways in cancer cells as compared to curcumin. Studies sugggest that flavokavain B modulates NF-κB and other pathways similarly to curcumin except the interacting proliferative pathways it

attenuates are slightly different. Flavokavain B upregulates the proapoptotic factor Bax alone and does not attenuate COX-2, a mediator for crosstalk between Akt and NF-κB proliferative signaling (42). Both compounds reduce IKK activity which reduces NF-κB and MAPK activity (24). Flavokavain B exerts similar effects on NF-κB transcriptional activity in that flavokavain B inhibits IKK activity, thus leaving NF-κB bound to its inhibitor factor IKB in the cytoplasm**.** Flavokavain B differs from curcumin in that it preferentially regulates the death receptor apoptotic pathway as compared to the mitochondrial apoptotic pathway (41). Flavokavain B has been shown to increase caspase-8 activity and promote caspase-3 cleavage which results in DNA fragmentation and apoptosis (16). Caspase-8 is activated upon the binding of a ligand, such as Fas, to a death receptor, which, in turn, activates the Fas associated death domain (FADD). In contrast, the intrinsic apoptotic pathway is activated once the ratio of proapoptotic factors, like Bax and Bad, to antiapoptotic factors, like BCL-XL and BCL-2, increases, resulting in the leakage of cytochrome c from mitochondria initiating caspase-9 and caspase-3 cleavage; however, there can be crosstalk between these pathways mediated by caspase-8, which can also initiate caspase-3 cleavage (16). The potential synergistic action of curcumin and VSV infection may be attributed to how each strain induces apoptosis.

The wt and r-M51R-M mutant strains of VSV have previously been shown by Gaddy and Lyles (2004) to induce apoptosis through distinct pathways. The rM51R-M virus induces apoptosis through the cleavage of caspase-9 and the death receptor pathway and the wt induces caspase-8 activation and mitochondrial outer membrane permeabilization (10). The

wt virus directly induces apoptosis in infected cells by the inhibition of host protein synthesis resulting in a reduction of mRNAs encoding antiapoptotic factors like BCL-2 and BCL-XL, which degrade quickly in the cytoplasm. The M protein mutant, rM51R-M, lacks the ability to shut off effectively host gene expression and relies on other viral components to induce apoptosis via the activation of caspase-8, caspase-9, and caspase-12, all of which are upstream activators of caspase-3 (10). These results indicate that the induction of apoptosis in M protein mutant VSV infected cells is dependent on crosstalk between the death receptor and mitochondrial pathways by caspase-8 activation. The data shown in Figure 1 indicates that curcumin potentiates VSV induced oncolysis more than flavokavain B perhaps by altering different pathways that increase cell death. The data presented in Figures 1-4 suggest that curcumin may downregulate pathways that render PC-3 cells sensitive to VSV induced oncolysis more effectively than flavokavain B. The exact nature of this synergistic action remains to be elucidated.

The dysregulation of apoptotic factors has been shown in increase the chemoresistance of several cancer cell lines, including the PC-3 prostate carcinomas (9). Of these factors, the proapoptotic factor Bax and its ratio to the antiapoptotic factors BCL-2 and BCL-XL have been promising targets in sensitizing cancer cells to apoptotic signaling and subsequent death (32). In particular, the macronutrients Vitamin E and selenium were shown to increase the Bax/BCL-2 ratio in PC-3 and DU145 cells inducing apoptosis (31). Data shown in Figure 5 indicate that in PC-3 cells treated with curcumin and flavokavian B and infected with wt and m protein mutant VSV do not show significant differential expression of

the BCL-2 antiapoptotic; however, as mentioned before, there was a slight decrease in BCL-XL in cells treated with curcumin and infected by both wt and M protein mutant virus (Fig. 5A). This trend was not statistically significant given the number of replicates; however, it does implicate the involvement of the Bax regulated intrinsic apoptotic pathway. Given the significant reduction of cell viability in treated and infected cells shown in Fig. 1, it is plausible that other apoptotic signals may be involved in this process. Other mechanisms of apoptosis may be altered with pretreatment with curcumin. A study by Hilchie et al. (2010) showed that curcumin can affect lipid signaling pathways to induce apoptosis. Ceramide, an intracellular signaling lipid, accumulated in the mitochondrial outer membrane in PC-3 cells causing a subsequent release of cytochrome C and DNA fragmentation leading to cell death (14). The increase in cell killing in Figure 1 may be due to the induction of apoptosis involving one or more pathways acting in concert with the Bax regulated intrinsic apoptotic pathway.

One potential drawback of attempting to use phytochemicals in conjunction with oncolytic viruses is the potential of inhibitory effects on viral replication processes. In particular, curcumin has been shown to inhibit hepatitis C virus (HCV) by activating proflierative pathways like heme oxygenase-1, Akt, and NF-κB (7). Crude leaf extracts of *Curcumin longa*, *Cratoxylum formosum*, *Momordica charantia*, and *Moringa oleifera* have also been shown to inhibit hepatitis B virus (HBV) replication in human hepatocellular carcinoma cells and reduce HBV induced cell killing (40). Here I show that both curcumin and flavokavain B do not reduce VSV protein expression (Figs 6, 7, and 8). Specifically,

neither curcumin nor flavokavain B significantly reduced the synthesis of VSVM or N proteins (Figs 6 and 7), both of which are required for budding and the release of viral progeny. Additionally, these compounds did not reduce viral gene expression as determined by the fluorescence of GFP engineered in the genomes of wt and M protein mutant VSV (Fig. 8). Curcumin has previously been shown to inhibit herpes simplex virus replication by blocking the expression of immediate-early genes soon after infection (21). Curcumin does not inhibit VSV gene expression 6h or 12h postinfection compared to virus alone (Fig. 8). Thus curcumin does not impede the expression of viral proteins in infected cells. This effect makes it an attractive candidate complementary therapeutic agent in VSV induced oncolytic activity. Similarly, flavokavain B does not inhibit VSV gene expression (Fig. 8) thereby increasing its potential as a complementary agent for potential oncolytic viral vectors.

Vesicular Stomatitis Virus is currently being investigated as a potential oncolytic agent for use in cancer therapeutics. I used two strains, rwt and rM51R-M, to reduce cell viability in infected PC-3 prostate carcinomas. What makes the rM51R-M virus strain an attractive candidate for oncolytic virotherapy is the fact that this M protein mutant is replication competent, enabling it to spread to surrounding susceptible tissue including cancer metastases, while being avirulent. As compared to the wt strain of VSV (rwt virus), rM51R-M is effective, but safe in vivo (2, 3, 37, 38). In addition to VSV, other oncolytic viruses, such as recombinant adenoviruses, are also being developed for the treatment of cancers (1, 33). One advantage to using adenoviruses is the ubiquity of the adenovirus receptor on human cells, allowing a greater range of potential oncolytic targets. These

viruses, like VSV, can effectively kill cancer cells and are currently in clinical trials (1, 33). One potential drawback in the efficacy of oncolytic virotherapy is the fact that some cancers are resistant to infection due to the differential expression of proliferative and antiviral factors. Combination therapeutic approaches, such as the use of natural plant compounds to downregulate these pathways, are currently being investigated to determine if these compounds can augment an oncolytic viruses' ability to kill cancer cells.

My results indicated that 6h pretreatment of PC3 cells with curcumin increased VSV induced oncolysis at 24h postinfection (Fig. 1A). This increase in cell death was not due to the Bax mediated intrinsic apoptotic pathway (Fig. 5) suggesting apoptosis is being induced by another mediator of cell death. Lastly, both curcumin and flavokavian B do not decrease expression of viral genes in treated cells (Figs 5, 6 and 7), suggesting that they may be attractive candidates for other oncolytic vectors. In particular, curcumin potentiates the oncolytic activity of the rM51R-M virus, which is the strain being investigated as a potential oncolytic vector. Curcumin synergizes effectively with VSV by increasing VSV induced oncolysis and not inhibiting viral protein synthesis. This is the first study to determine if natural compounds promote VSV induced cancer cell killing. Future studies will determine the mechanisms by which these compounds promote VSV-induced oncolysis. Specifically, studies will determine if these compounds affect the Akt proliferative pathway and if they modulate NF-κB activation in VSV infected cells. Whether the downregulation of NF-κB is responsible for the increased oncolytic activity of VSV in cells treated with curcumin still needs to be investigated. Future work in our laboratory will determine if flavokavian B or

curcumin reduces NF-κB activation in infected cells as well as determine the relative gene expression of IFN stimulated genes by quantitative RT-PCR and microarray analysis. Additionally, the exact apoptotic mechanism by which VSV and curcumin potentiate cell death needs to be determined. The results from these studies will provide a platform for testing of additional natural compounds with the potential to augment VSV oncolytic therapies.

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LIST OF ABBREVIATIONS

7-AAD- 7-Aminoactinomycin D

Akt- Protein Kinase B

BCA- Bicinchoninic Acid Assay

BHK- Baby Hamster Kidney cells

BSA- Bovine Serum Albumin

COX-2 cyclooxygenase-2

CREB- cAMP Response Element Binding Protein

ERK- Extra-cellular Related Kinase

FADD- Fas Associated Death Domain

Fas- Tumor Necrosis Factor- Alpha Receptor Member 6

FBS- Fetal Bovine Serum

GFP- Green Fluorescence Protein

HBV- Hepatitis B Virus

HCV- Hepatitis C Virus

HRP- Horseradish Peroxidase

IFN- Interferon

IκB- Inhibitors of κB

IKK- Inhibitor of κB kinase

IRF-9- Interferon Regulatory Factor 9

ISGF3- Interferon-stimulated gene factor 3

ISRE- Interferon Stimulated Response Element

Jak- Janus Kinase

LNCaP- LN Prostate Carcinoma

M- Matrix Protein

MAPK- Mitogen Activated Protein Kinase

MMP-9- matrix metalloproteinase-9

MOI- Multiplicity of Infection

mRNA- Messenger Ribonucleic Acid

mTOR- Mammalian Target of Rapamycin

MTT- (3-(4,5-Dimethlythiazol-2-yl)-2,5-diphenyltetralozium bromide

N-Nucleocapsid Protein

NF-κB- Nuclear Factor Kappa B

OD- Optical Density

p50- protein 50

p53- Protein 53

p65- protein 65

PAGE- Polyacrylamide Gel Electrophoresis

PBS- Phosphate Buffered Saline

PC-3- Prostate Carcinoma Line 3

PDGF- Platlet Derive Growth Factor

PI3K- Phophoinositide 3-kinase

PIP2- Phosphatidylinositol 4,5-bisphosphate

PIP3- Phosphotidylinositol 3,4,5-triphosphate

PKR- Protein Kinase R

PTEN-Phosphatase and Tensin Homolog

Ras- Rat Sarcoma

RIPA- Modified Radioimmunoprecipitation Buffer

r-M51R-M- Recombinant Methionine to Arginine Substitution on Matrix Protein

RPMI-Roswell Park Memorial Institute Medium

rwt- Recombinant Wild Type

SDS- Sodium Dodecyl-Sulfate

SDS-PAGE- Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis

SOS- Son of Sevenless

STAT- Signal Transducer and Activator of Transcription

TBS- Tris Buffered Saline

TBS-T- Tris-Buffered Saline- Tween 20

TNF-α- Tumor Necrosis Factor- Alpha

uPA- urokinase-type plasminogen activator

VSV- Vesicular Stomatitis Virus

Vita

Dylan John Fehl was born in 1983 in Atlanta, Georgia, to Teri Ann Fehl and Thomas Micheal Fehl as the youngest of three children. Dylan has an older sister, Holly Bernice Fehl, and an older brother, Barry Raymond Fehl. Dylan was awarded a Bachelor of Science in Biology with a minor in Chemistry in 2006. He is currently a teaching assistant at Appalachian State University with aspirations of going into cancer research.